Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells IV: Uptake and Release Kinetics of Sterols

J. S. TURI *x, W. I. HIGUCHI ‡, N. F. H. HO ‡, and C. SHIPMAN, Jr. §

Abstract □ The kinetics of uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells at 26°, pH 7.3, and isoosmotic conditions were quantitatively and mechanistically interpreted. The data are in agreement with the model involving the passive transport of the unbound sterol across the rate-determining plasma membrane, with rapid distribution within the heterogeneous cell interior. Effective permeability (P_e) and partition (K_e) coefficients of the sterols were inversely proportional to the serum concentration in the external media due to sterol–serum binding. These results are consistent with the mechanism in which only the unbound solute in the external solution participates in the membrane transport process. At all serum levels, P_e and K_e increased with increasing sterol polarity: desmosterol > cholesterol > β-sitosterol.

Keyphrases □ Membrane diffusion—systems approach to drug transport, uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells, effective permeability and partition coefficients determined and related to sterol–serum binding □ Drug transport—uptake and release kinetics, desmosterol, cholesterol, and β-sitosterol, Burkitt lymphoma cells, effective permeability and partition coefficients determined, related to sterol–serum binding □ Mammalian cells, suspension cultures—uptake and release of sterols, systems approach to study of drug transport across membranes □ Sterols—kinetics of uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells, systems approach to drug transport

This paper represents an experimental followup of the theoretical models presented earlier (1) in which the plasma membrane of the cell is the rate-determining barrier to the transport of solutes. To demonstrate the utility of the physical model approach in the design of experiments and the quantification of the relevant transport parameters and physical factors, the kinetics of the uptake and release of cholesterol, desmosterol, and β-sitosterol using Burkitt lymphoma cells in culture suspension are reported.

EXPERIMENTAL

The methods for the cultivation of Burkitt lymphoma cells were described previously (2). Included are the details of the quantitative techniques and procedures for the baseline uptake experiments of cholesterol-^{14}C by the cells in culture suspension at pH 7.3 and 26° under isoosmotic and sterile conditions in spinner flasks. The suspending media contained modified McCoys 5A, basal medium Eagle, balanced salt solution, buffers, and varying concentrations of fetal bovine serum.

To follow the changes in concentration of solute with time, both the cell and extracellular fraction were assayed by liquid scintillation counting after centrifugation. To study the release kinetics of drugs from the cell, the following two techniques were used to load the cells with solute. With the "static" equilibration technique, the cell suspension was prepared and allowed to mix with the radioactive drug at 26° for at least 48 hr. With the "prolific" equilibration technique, the cell suspension containing the radioactive drug was incubated at 37° so that the cells could grow.

After loading the cells with the solute, the cells were washed and resuspended in fresh media. Thereafter, the release studies followed the sampling procedures as outlined previously for the uptake kinetic studies (2). All uptake and release studies were performed at 26° and used identical isoosmotic media at pH 7.3. The studies were repeated at least three or four times.

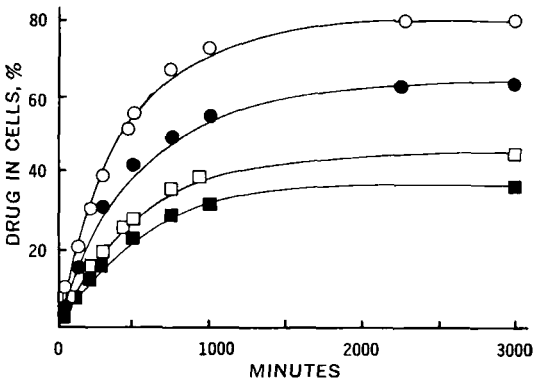

Figure 1—*Uptake of desmosterol at 26° at four fetal bovine serum concentrations. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%. Points represent the experimental data, and curves represent the best theoretical fit by nonlinear regression of Eq. 1 using the phenomenological constants in Table I.*

THEORETICAL

As will be seen, the kinetics of uptake and release of the various sterols by viable Burkitt lymphoma cells can best be explained by Model 2 (1), in which: *(a)* the interior of the cell is treated essentially as a homogeneous phase, *i.e.*, the membranes of the cytoplasmic bodies and nucleus are not the rate-determining barriers; *(b)* the plasma membrane is the rate-limiting barrier to the passive transport of the solute; and *(c)* there is binding of the solute to the serum and, perhaps, other components comprising the support media for the cell suspension. Only the essential mathematical description will be presented here.

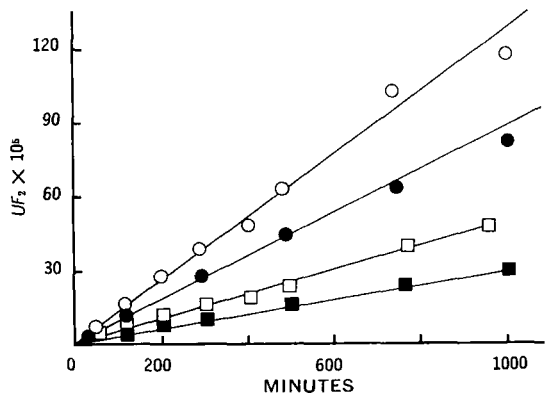

Figure 2—*Plot of uptake kinetics of desmosterol at four fetal bovine serum concentrations according to linear predictions of Eq. 1. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%. Solid curves are least-squares lines.*

Table I—Permeability Coefficients for Desmosterol Uptake and Release Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*		Effective Permeability Coefficient[b] × 10^7, Average ± *SD*	
	Uptake	Release	Uptake	Release
0	750 ± 44	—	13 ± 0.8	—
5	270 ± 24	288 ± 15	9.25 ± 0.5	8.80 ± 0.9
15	100 ± 10	107 ± 4	4.18 ± 0.3	3.94 ± 0.6
30	51 ± 7	56 ± 4	2.99 ± 0.4	2.64 ± 0.5

[a] Effective serum–desmosterol binding constant is K_b = 18.7. [b] Units of centimeters per second.

Table II—Permeability Coefficients for Cholesterol Uptake and Release Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*		Effective Permeability Coefficient[b] × 10^7, Average ± *SD*	
	Uptake	Release	Uptake	Release
0	230 ± 9	—	4.44 ± 0.6	—
5	155 ± 6	—	2.69 ± 0.4	—
15	60 ± 4	64 ± 8	1.27 ± 0.1	1.32 ± 0.2
30	34 ± 3	37 ± 2	0.88 ± 0.09	0.89 ± 0.07

[a] Effective serum–cholesterol binding constant is K_b = 15.7. [b] Units of centimeters per second.

The uptake transport function of this model, UF_2, is:

$$UF_2 = \frac{-2.303a}{180B}\log\left[1 - \frac{B}{A}C_i\right] = P_e t \qquad \text{(Eq. 1)}$$

$$A = T/V_0 \qquad \text{(Eq. 2)}$$

$$B = \frac{nV_i}{V_0} + \frac{1}{K_e} \qquad \text{(Eq. 3)}$$

where a is the radius of the cell, n is the number of cells, V_i is the volume of the cell, V_0 is the volume of the external aqueous phase, T is the total amount of drug in the system, C_i is the concentration of drug in the cell, K_e is the effective partition coefficient, P_e is the effective permeability coefficient, and t is time. Here, the initial boundary condition is $C_i(0) = 0$.

The release transport function, RF_2, is expressed by:

$$RF_2 = \frac{2.303a}{180B}\log\left[\frac{BC_i - A}{BC_i(0) - A}\right] = -P_e t \qquad \text{(Eq. 4)}$$

The initial boundary condition is $C_i = C_i(0)$. According to Eq. 1 or 4, a plot of UF_2 or RF_2 *versus* t in minutes is linear with a slope equal to P_e in units of centimeters per second.

The effective permeability coefficient is given by:

$$P_e = \frac{P}{1 + K_b(S)} \qquad \text{(Eq. 5)}$$

where K_b is the solute–serum binding constant, (S) is the serum concentration, and P is the intrinsic permeability constant of the unbound drug. It is assumed here that the serum is the only adsorbent in the external medium of the cell suspension. The effective partition coefficient is:

$$K_e = \frac{K}{1 + K_b(S)} \qquad \text{(Eq. 6)}$$

where K is the intrinsic partition coefficient.

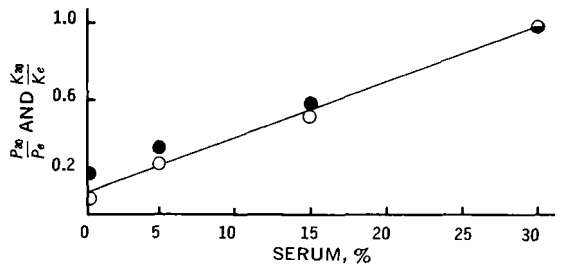

Figure 3—*Normalized plot of the effective permeability coefficients and partition coefficients of desmosterol as a function of fetal bovine serum concentration. Key:* ●, P_{30}/P_e; *and* ○, K_{30}/K_e. *The* P_{30} *and* K_{30} *values are the effective permeability and partition coefficients at the 30% fetal bovine level and are used as the references.*

RESULTS AND DISCUSSION

Uptake and Release of Desmosterol-26-^{14}C—Figure 1 shows the uptake kinetics of desmosterol at four serum concentrations. The rate of transport decreases with increasing serum concentration due to solute–serum binding. After calculating the effective partition coefficient at each serum level from the equilibrium portion of the curves and then applying the data to Eq. 1, the predicted linear relationship of the uptake function *versus* time of this

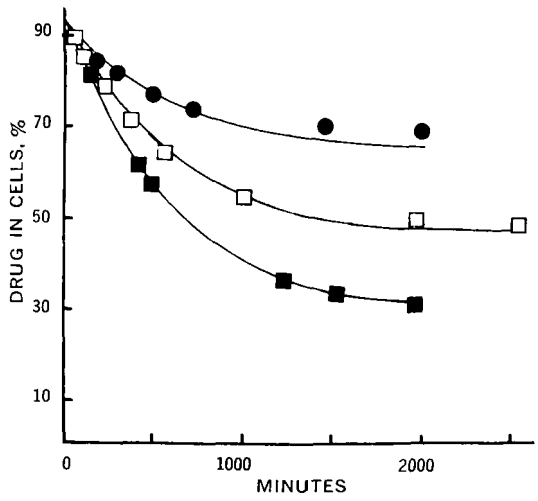

Figure 4—*Release of desmosterol at 26° at various fetal bovine serum concentrations. Key:* ●, *5% (cells equilibrated with desmosterol by the prolific method);* □, *15% (cells equilibrated by the static method); and,* ■, *30% (static equilibration method). Points represent experimental data, and solid curves are the best theoretical fit by nonlinear regression of Eq. 4 using the constants in Table I.*

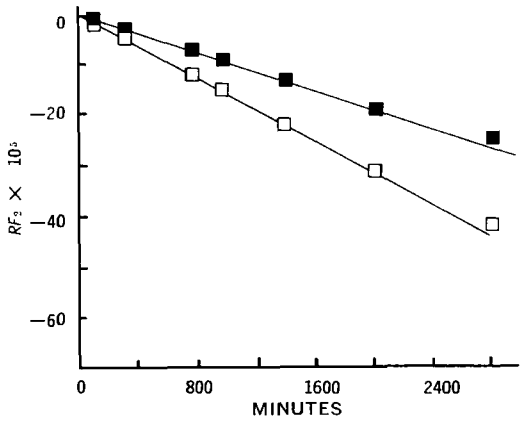

Figure 5—*Release function* versus *time of desmosterol at two fetal bovine serum concentrations. Key:* □, *15%; and* ■, *30%. The solid curve is the linear regression line.*

model is followed (Fig. 2). It is readily observed that both the effective partition and permeability coefficients decrease with increasing serum concentration.

Another indication of the fit of the theoretical model is found in Fig. 3. Here, the treatment of the experimental data followed the linear relationships of Eqs. 5 and 6, using the effective partition and permeability coefficients at the 30% serum level as reference[1]. Thus, this finding supports the theory that the unbound desmosterol is permeating through the plasma membrane.

Experiments were also done with various concentrations of desmosterol at constant serum concentration. The results showed that the desmosterol concentration did not alter the effective coefficients. This finding indicates that the concentration of serum-bound desmosterol was linearly related to the concentration of unbound drug within the experimental conditions.

Figure 4 shows the release kinetics from the cells at three fetal bovine serum levels after the cells were loaded by the static method at 26°. As expected, at higher serum levels more desmosterol diffused from the cells, reflecting a lower effective partition coefficient at higher serum levels. In Fig. 5 the profiles of the release function *versus* time show that the effective permeability coefficients decrease with increasing serum levels.

Release studies from cells loaded with desmosterol by the prolific method at 37° indicated that both radiolabeled desmosterol and cholesterol were being transported across the plasma membrane.

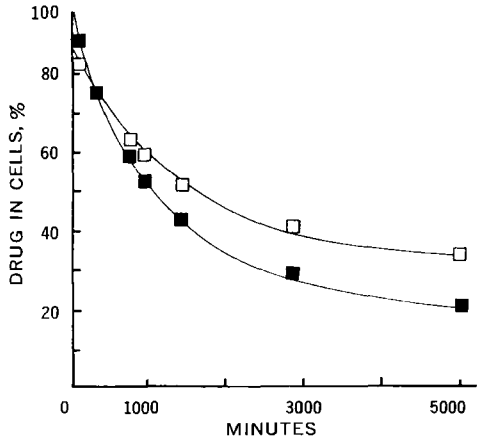

Figure 6—*Release kinetics of cholesterol at 26° and two fetal bovine serum concentrations. Cells were equilibrated with cholesterol by the static procedure. Key:* □, *15%; and* ■, *30%. Points are experimental data, and curves are the best nonlinear theoretical fit of the model using the effective permeability and partition coefficients in Table II.*

[1] In Fig. 3 the plot of P_{30}/P_e *versus* percent serum gives a slope equal to $K_b/(1 + 0.3K_b)$ and an intercept equal to $1/(1 + 0.3K_b)$.

Table III—Permeability Coefficients for β-Sitosterol Uptake Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*	Effective Permeability Coefficient[b] × 10^7, Average ± *SD*
0	150 ± 10.5	2.35 ± 0.55
5	45 ± 2.5	1.27 ± 0.23
15	23 ± 1.4	0.74 ± 0.06
30	12 ± 2.4	0.44 ± 0.04

[a] Effective serum–β-sitosterol binding constant is K_b = 18.8. [b] Units of centimeters per second.

These two solutes were subsequently identified by radiochromatographic analysis. The theoretical models[2] and quantitative experiments on the simultaneous transport of desmosterol and cholesterol and the bioconversion of desmosterol to cholesterol by Burkitt lymphoma cells will be reported later. No bioconversion of desmosterol was detected when the cells were loaded by the static method at 26° and when subsequent release studies were carried out at 26°.

Table I summarizes the phenomenological transport coefficients of desmosterol for Burkitt lymphoma cells. There is substantial agreement in the P_e and K_e values at the various serum levels between the uptake and release experiments. The reproducibility of the experiments is good.

The fact that the effective permeability coefficients determined either by linear or nonlinear regression analysis of the data were similar underscores the good precision of the experimental technique and design. The P_e of 1.3×10^{-6} cm/sec and K_e of 750 could be interpreted as intrinsic coefficients; however, it is not certain

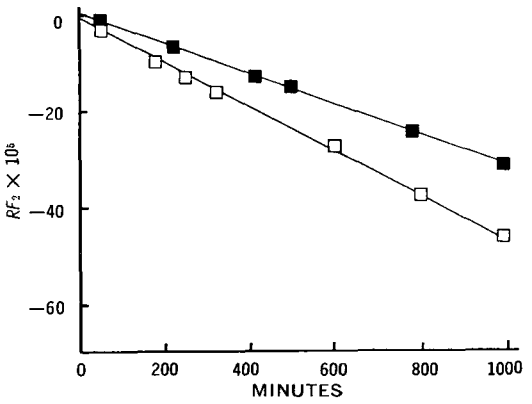

Figure 7—*Physical model treatment of release data of cholesterol in Fig. 6 by the linear Eq. 1. Key:* □, *15%; and* ■, *30%. The solid curve is the linear regression line.*

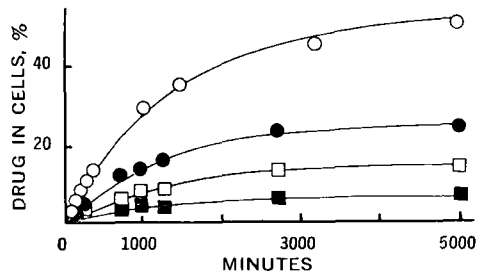

Figure 8—*Uptake of β-sitosterol at 26° and various fetal bovine serum levels. Key:* ○, *0%;* ●, *5%;* □, *15%; and* ■, *30%. The experimental data points are compared against the best theoretical fit of the model using the phenomenological constants in Table III.*

[2] H. Ando, N. F. H. Ho, W. I. Higuchi, J. S. Turi, and C. Shipman, Jr., to be published.

Table IV—Permeability Coefficients for Cholesterol, Desmosterol, and β-Sitosterol at 26° Calculated from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Permeability Coefficient, cm/sec × 10^7			Effective Partition Coefficient		
	Desmosterol	Cholesterol	β-Sitosterol	Desmosterol	Cholesterol	β-Sitosterol
0	13.00	4.44	2.35	750	230	150
5	9.25	2.69	1.27	270	155	45
15	4.18	1.27	0.74	100	60	23
30	2.99	0.88	0.44	51	34	12

[a] Intrinsic permeability and partition coefficients can be calculated from Eqs. 5 and 6.

whether there is adsorption of desmosterol to the nonserum components of the external medium.

Uptake and Release of Cholesterol-4-^{14}C—Figure 6 shows the decrease in the percent of cholesterol in the cells with time at 26° at 15 and 30% serum concentrations. These cells were loaded by the static method. The treatment of the data by the model is shown in Fig. 7.

The results of the kinetics of cholesterol uptake were thoroughly described and reported elsewhere (2). However, the effective permeability and partition coefficients from the uptake studies at various serum levels are included in Table II with these coefficients determined from the release studies for comparison. As can be seen, the results are similar to those obtained for desmosterol in that the effective permeability and partition coefficients decreased as the serum concentration increased.

Uptake of β-Sitosterol-4-^{14}C—Figure 8 shows the β-sitosterol uptake data at four fetal bovine serum levels at a constant β-sitosterol concentration. As seen previously with cholesterol and desmosterol, β-sitosterol showed a decreased rate of uptake as the serum level increased. The uptake function *versus* time curves in Fig. 9 show very good agreement with the model, while Fig. 10 illustrates the nonlinear regression fit for the uptake of β-sitosterol at 15 and 30% fetal bovine serum. Figure 11 shows the plot of P_{30}/P_e and K_{30}/K_e *versus* percent of serum and is another indication of the fit of the theoretical model. Their absolute values are found in Table III.

General Discussion of Sterols—The experimental data and their interpretative analyses on the passive transport of the three sterols into and out of Burkitt cells in culture suspension demonstrate that the experimental methods employed are quantitative and that physically meaningful results can be obtained by the physical model approach. The evidence strongly supports that the model described in the *Theoretical* section is highly appropriate.

With regard to the other physical models reported earlier (1), Model 1 is inappropriate since it does not account for sterol–serum binding in the external media. Models 3, 4, and 5 do not apply because of the lack of evidence of plasma membrane–sterol binding. The simultaneous transport of two mutually independent solutes of Models 6 and 7 is not applicable here. Model 8 cannot satisfactorily treat the data because the uptake or release function *versus* time plots did not show a biexponential pattern, which would strongly indicate that not only the plasma membrane but also the membranes of the cytoplasmic bodies are rate-determining barriers in the cell transport of the sterols.

In summarizing this study, it is interesting to note the trends in the physical transport coefficients among the sterols (Table IV). At all serum levels, the partition and permeability coefficients increased with increasing polarity of the sterols, *i.e.*, K_e (desmosterol) > K_e (cholesterol) > K_e (β-sitosterol) and P_e (desmosterol) > P_e (cholesterol) > P_e (β-sitosterol). Similar observations were qualitatively reported (3) on uptake studies of cholesterol and β-sitosterol using L-cell mouse fibroblasts. The sterol–fetal bovine serum adsorption constant is about the same among the sterols (K_b = 15.7–18.8).

To assess the differences in the molecular structure of these sterols with regard to their transport into and out of Burkitt cells, the ratio of the permeability coefficients or the partition coefficients, using cholesterol as the reference sterol and the values at the zero percent serum level, may be a useful evaluating parameter. This technique was used in obtaining the incremental partition coefficient per CH_2 group in a *n*-alkyl chain and other chemical substituents for the buccal membrane in humans (4, 5) and the rat intestinal tract (6).

Therefore, as shown in Table V, the double bond as in desmosterol resulted in a threefold increase in the partition and permeability coefficients of cholesterol, whereas the addition of an ethyl group on the 24-position of cholesterol reduced the partition and

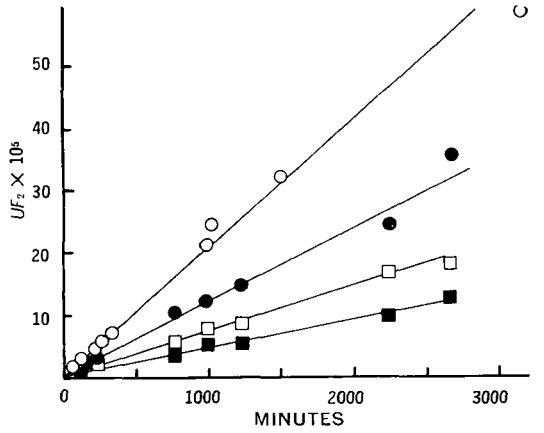

Figure 9—*Physical model treatment of uptake kinetics of β-sitosterol and various fetal bovine serum levels by the linear predictions of Eq. 1. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%.*

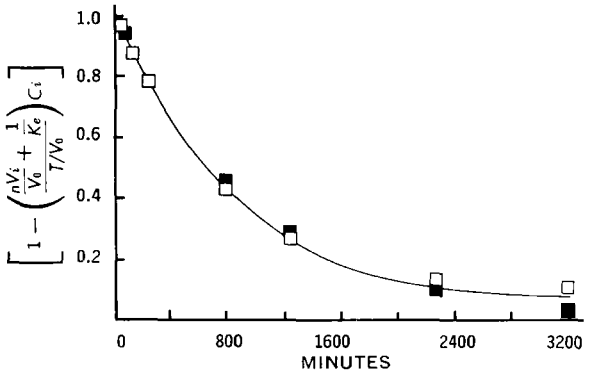

Figure 10—*Nonlinear regression analysis plot of the uptake of β-sitosterol at two fetal bovine serum levels. Key: □, 15%; and ■, 30%. Points are experimental data, and the solid curve is the theoretical fit using the constants in Table III.*

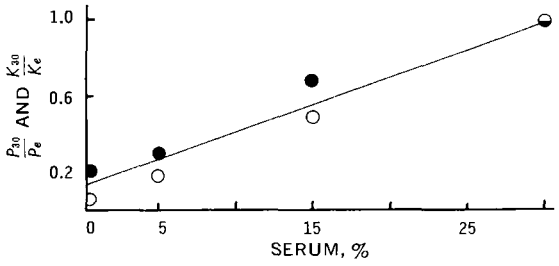

Figure 11—*Normalized plot of the effective permeability and partition coefficients of β-sitosterol as a function of fetal bovine serum concentration. Key: ●, P_{30}/P_e; and ○, K_{30}/K_e.*

Table V—Incremental Constant Evaluating Effect of Molecular Modification of Side Chain of Cholesterol on Permeability and Partition Coefficients of Desmosterol and β-Sitosterol

Parameter	Incremental Constant	
	Desmosterol	β-Sitosterol
$K_{sterol}/K_{cholesterol}$	3.26	0.65
$P_{sterol}/P_{cholesterol}$	3.0	0.53

permeability coefficients by a factor of two. The interpretation of these molecular modifications of cholesterol is unclear. It is apparent that the expectation of increased partition coefficient with decreasing polarity of the solute is not found here.

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Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells V: Uptake and Release Kinetics of Cardiac Glycosides by Burkitt Lymphoma Cells

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Abstract □ Mass transport studies with three cardiac glycosides in the Burkitt lymphoma cell system have provided significant examples of the factorization and quantification of the influences of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability upon the uptake and release kinetics of drugs in living cell systems. All of the data from the glycosides are in agreement with the general physical model involving the rapid equilibration of the solute within the cell after permeation through the rate-determining plasma membrane barrier. The transport of digitoxin was influenced by membrane and serum binding and that of digoxin was influenced by membrane binding. There was no binding of ouabain to the plasma membrane and serum. The variables in the uptake and release kinetic studies at pH 7.3 included the use of viable and heat-inactivated cells, fetal bovine serum levels, and temperature.

Keyphrases □ Membrane diffusion—systems approach to drug transport, uptake and release of cardiac glycosides by Burkitt lymphoma cells, influence of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability □ Drug transport—uptake and release kinetics, cardiac glycosides, Burkitt lymphoma cells, influence of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability □ Mammalian cells, suspension culture—uptake and release of cardiac glycosides, systems approach to study of drug transport across membranes □ Cardiac glycosides—kinetics of uptake and release by Burkitt lymphoma cells, systems approach to drug transport

The lack of a mechanistic and quantitative understanding of the kinetics of drug transport to the receptor site has impeded the quantitative approach to drug design. Nearly all previous studies in the field have been of a rather descriptive nature; it has been difficult to separate, for example, the driving force factors from the kinetic factors in describing the transport and the bioavailability of drugs at the site of action from a physical–chemical standpoint.

Existing evidences indicate that one cannot always rely solely on oil–water partition coefficients, blood levels of drugs, or rates of intestinal transport as true indications of drug availability at the site of drug action. It is believed that the developed physical models and experimental techniques will give more insight into drug availability, especially at the receptor site. The concepts put forth here can be valuable in assessing molecular modifications of drugs. This study illustrates that collaborative research among pharmacokineticists, medicinal chemists, and experts in mass transport across biological membranes can be very valuable in assessing drug availability.

This paper describes the mass transport of ouabain, digoxin, and digitoxin in suspension cultures of an established cell line. This study evolved from the development of various biophysical models (1, 2) and the accompanying experimental systems and techniques. Its significance lies not only in the important implication it may have on the understanding of the