Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells II: Experimental Procedures and Uptake Studies with Cholesterol

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Abstract □ Following the guidelines provided by the physical models derived earlier, sensitive experimental techniques and procedures were developed. Baselines for solute transport studies were established from experiments on the uptake of cholesterol-^{14}C by Burkitt lymphoma cells in culture suspension at pH 7.3 under isoosmotic conditions. The experimental data were evaluated by the model involving the rapid equilibration of the solute in the cell interior after permeation through the plasma membrane. The time-dependent predictions of this model agreed well with the data. Effective permeability and partition coefficients were calculated and found to be inversely proportional to the serum level used. These results are in agreement with the mechanism by which only the unbound cholesterol in the external solution participates in the membrane transport process.

Keyphrases □ Membrane diffusion—cholesterol in suspension cultures of mammalian cells, permeability and partition coefficients □ Drug transport—cholesterol in suspension cultures of mammalian cells, permeability and partition coefficients □ Cholesterol, membrane transport—uptake by Burkitt lymphoma cells in suspension, baseline procedures □ Mammalian cells, suspension cultures—cholesterol transport, baseline procedures

A wide variety of experiments have been proposed for the purposes of providing insights and establishing the chemical and physical-chemical relationships that govern drug transport. At one extreme, *in vitro* studies have utilized monolayers, lipid bilayers, interfacial films of oil–water emulsion droplets, and gelatin–oil coacervates as model biological membranes (1–10). These studies are carried out on the basis that principles governing the transport of real and complicated systems may be elucidated by simple ones. At the other extreme, there have been numerous *in situ* investigations of drug transport across the gastric, intestinal, rectal, buccal, peritoneal, and cutaneous walls of animals and man (11–20). Recently, suspension cultures of mammalian cells have been utilized for transport studies (21, 22). It is believed that such systems can uniquely provide the means to interrelate *in vitro*, *in situ*, and *in vivo* experiments of drug transport, with physicochemical theories and model experiments being developed in our and other laboratories. Although a cell culture suspension is an *in vitro* system and, therefore, subject to certain biological artifacts, the use of intact cell lines in some state of metabolism and reproduction capability places one closer to biological membranes with the distinct advantage of *in vitro* controls.

The longrange goals of this research are: (*a*) to gain a physicochemical and quantitative mechanistic understanding of the factors involved in drug absorption in man through the development of meaningful baseline experiments and methods employing suspensions and multilayer systems of mammalian cells; (*b*) to investigate and, ultimately, to predict the efficiency of transport across cell membranes of drugs altered by molecular modification; (*c*) to understand the role of pharmaceutical delivery systems, including adjuvants, in drug absorption; (*d*) to study the effects of cell type, age, nutrients, and chemical additives that alter membrane permeability and the influences of irradiation, viral infection, level of metabolic activity, and other factors on the rate of drug absorption by cells.

In the first paper of this series (23), physical models for the diffusional transport of drugs across membranes of cells in suspension were introduced. Following the quasisteady-state flux of drug across the plasma membrane, the distribution of the drug in the cell interior was postulated to follow one of three principal models:

Model A—Nonsteady-state distribution in the heterogeneous cell interior.

Model B—Rapid (instantaneous) equilibration in the heterogeneous cell interior.

Model C—Rapid (instantaneous) equilibration in the aqueous environment, with slow simultaneous permeation of drug into the cytoplasmic bodies and nucleus.

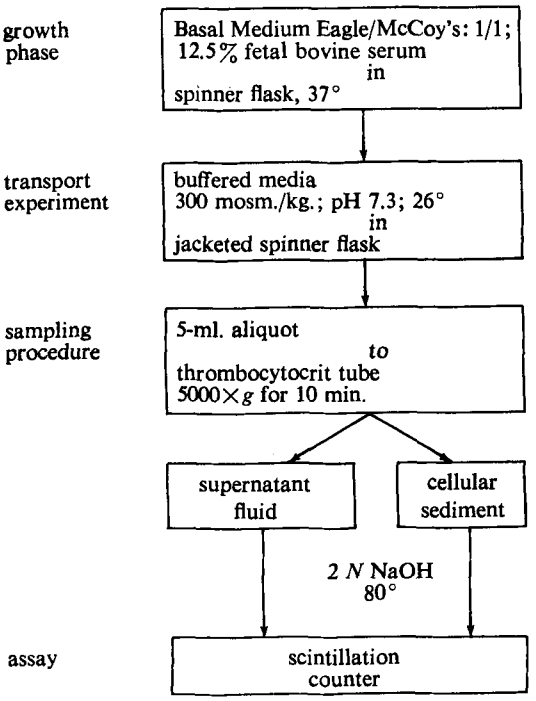
These simple models were described in such a manner that they provide the basis for the design of experiments and interpretation of data for deducing quantitative estimates of the phenomenological and geometrical parameters significant in drug transport. This approach allows one to explain the important simultaneous interactions of many variables and to make predictions of more complex situations, where empirical methods would be qualitative and of questionable value. The systematic modification of these initial models, according to well-established physicochemical concepts with the accompanying mathematics, is a continuing effort as the experimental results accumulate.

The present report describes: (*a*) the development of some baseline procedures and techniques for drug transport experiments, with emphasis on the care required with the cell culture suspensions; and (*b*) the methods of evaluating experimental data quantitatively and mechanistically by the physical model approach.

EXPERIMENTAL

General Considerations—An established line of Burkitt lymphoma cells (P_3J) were cloned according to the method of Hinuma and Grace (24), and clone P_3JT_2 was used for these studies (25–27). These cells are easily grown in suspension and are durable with respect to the desired range of *in vitro* physical and chemical environmental conditions of the transport experiments. They are geometrically spherical and uniform in size distribution.

Much effort was directed toward the assessment of the experimental techniques and the reliability of the baseline experiments.



Scheme I—Outline of the sequence of procedures for the drug transport experiments

The following variables were considered important in this regard: (*a*) maintenance of a constant cell concentration and cell volume throughout the experiment, (*b*) integrity of the cells and their viability during the experiment, and (*c*) influences of any changes made in the media or the conditions in the experiment.

Cell Propagation—By employing the basic procedures of Merchant *et al.* (28), the cells were grown in suspension at 37 ± 0.5° in spinner flasks containing sterile growth media: McCoys 5A Modified[1], 12.1 g.; Basal Medium Eagle[2], 9.2 g.; fetal bovine serum[3], 125 ml.; sodium bicarbonate, 1.8 g.; and double-distilled water to make 1 l. The medium was sterilized by filtration using a 0.22-μ Millipore membrane. The mixture was isotonic [approximately 296 milliosmoles (mosm.)/kg.] and initially at pH 7.4. Sterility was checked with thioglycollate broth. Cell viability was measured by means of erythrocin B dye exclusion (29).

The growth curve of the cells under the stated conditions was established using the Coulter counter[4] and is shown in Fig. 1. The lag time was followed by the logarithmic growth phase. The tailing of the curve at later periods is probably attributable to the effect of crowding of the cells and the accumulation of cellular waste products. At this point, fresh medium was added to sustain the cell growth.

To preserve the Burkitt lymphoma cells, aliquots were sealed in ampuls, frozen automatically at a controlled rate, and stored at −196° in liquid nitrogen.

Preparation of Cells for Drug Uptake Experiments—A schematic diagram of the sequence of procedures for the drug uptake experiments is shown in Scheme I. The maintenance of sterility throughout the experiments was essential. Aseptic procedures included the use of sterile equipment, medium, and manipulations in a laminar flow hood.

Starting with 1 l. of cell suspension, 300,000 cells/ml., in a 1 l. spinner flask containing the growth medium previously described, the cells were allowed to grow and then harvested at the end of the growth phase. The cell concentration was about 1.5 million cells/ml.

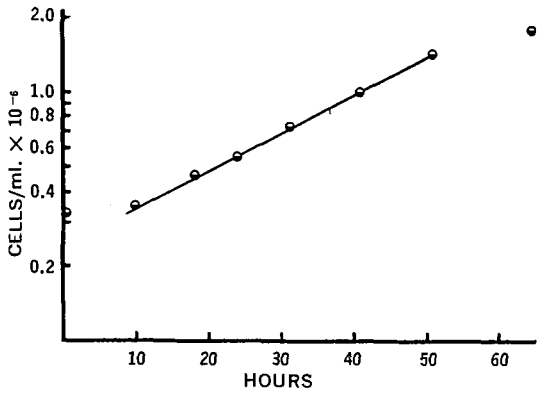


Figure 1—*Growth curve of the Burkitt lymphoma cells. The environmental conditions and culture medium used are described in the text.*

The cells were centrifuged in 150-ml. capped centrifuge tubes at 450×*g* for 5 min.

The supernatant liquid was decanted and then the cells were resuspended with buffered medium[5] (30) and centrifuged. Table I gives the formulas for the buffered medium[5] with varying concentrations of fetal bovine serum employed in the study of the effect of serum on drug transport. Finally, the cells were resuspended with 100 ml. of the fresh buffered medium[5] in a 100-ml. water-jacketed flask[6] (Fig. 2) so that the cell concentration was about 20 million cells/5 ml. The replacement of the bicarbonate buffer in the growth medium by the buffer[5] was the essential difference between the uptake medium and the growth medium.

Radioactive cholesterol (NEC-018 cholesterol-4-^{14}C)[7] was added to the cell suspension in the following manner. An aliquot of cholesterol in benzene stock solution was withdrawn. After the benzene was evaporated, the cholesterol was solubilized in ethanol (0.2 ml.); then 10 ml. of sterile water was added and the solution was introduced into the cell suspension.

The cell suspension system in the water-jacketed spinner flask (26°) was agitated with a constant-stirring motor at 150 r.p.m. The system was maintained at pH 7.3 and isotonic with an osmotic pressure of 300 mosm./kg. The final concentration of cells and their size distribution were determined with the Coulter counter and a multichannel pulse height analyzer[8] (Fig. 3). The viability of the cells was checked throughout the experiment.

Since it was desirable to maintain a constant concentration of cells and cell volume throughout the experiment, which often took several days, the temperature of 26° was chosen to prevent the multiplication of cells and yet sustain a level of cellular metabolic activity. Alternative methods to accomplish this are cell irradiation (31) and the addition of thymidine to the system (32).

Drug Uptake Experiments—To study the transport kinetics of cholesterol across the cell membrane, quantitative assay techniques were developed. Exactly 5-ml. samples of the cell suspension were taken aseptically at various times, placed into thrombocytocrit tubes[9], and immediately centrifuged at 5000×*g* for 10 min. The compacted cellular sediment was found to fill completely the narrow graduated stem of the tube, and the supernatant liquid remained in the bulb portion. One milliliter of the liquid was withdrawn and saved for assay while the rest was discarded with a syringe and needle. Next the bulb was washed with normal saline and then twice with ethanol to remove any cholesterol adhering to the walls. To facilitate the removal of the cell plug, 1 ml. of water was added, and with the cap at the tip of the stem removed, the cellular sediment was removed by exerting positive pressure at the bulb end of the tube with the aid of a syringe. The supernatant liquid and the cells were placed into separate scintillation vials. Thus, both the drug uptake by the cells and the depletion from the liquid were followed simultaneously.

[1] McCoys 5-A Medium (Modified), Catalog No. H-15, Gibco, Grand Island, N. Y.
[2] Basal Medium Eagle (Diploid), Catalog No. G-13, Gibco, Grand Island, N. Y.
[3] KAM Laboratories, Kansas City, KS 66103. Cholesterol content was 35 mg.%.
[4] Coulter Electronics, Franklin Park, Ill.
[5] The buffered media contained hydroxyethylpiperazine-*N'*-ethanesulfonic acid, pKa 7.55 at 20°, ΔpKa/°C = −0.014.
[6] Bellco Glass Inc., Vineland, NJ 08360
[7] Cholesterol-4-^{14}C has a specific activity of 59.8 mc./mmole.
[8] Radiation Instrument Development Laboratory Inc., Melrose Park, Ill.
[9] Arthur H. Thomas, Philadelphia, Pa.

Table I—Various Buffered Media Used in the Drug Transport Experiments[a]

Ingredients	Formulations			
	0% Fetal Bovine Serum	5% Fetal Bovine Serum	15% Fetal Bovine Serum	30% Fetal Bovine Serum
McCoys 5A modified	0.484 g.	0.484 g.	0.484 g.	0.484 g.
Basal Medium Eagle	0.368 g.	0.368 g.	0.368 g.	0.368 g.
Buffer[b]	0.4765 g.	0.4765 g.	0.4765 g.	0.4765 g.
Fetal bovine serum	0	5.0 ml.	15.0 ml.	30.0 ml.
NaOH, 0.1 *N*	9.5 ml.	9.5 ml.	9.5 ml.	9.5 ml.
Balanced salt solution	16.5 ml.	13.5 ml.	7.5 ml.	0
Double-distilled water to make	100.0 ml.	100.0 ml.	100.0 ml.	100.0 ml.

[a] All formulations are isotonic at 300 mosm./kg. and at pH 7.3. The balanced salt solution used to adjust the osmotic pressure of the media consists of sodium chloride (8.0 g.), potassium chloride (0.4 g.), anhydrous dibasic sodium phosphate (0.1 g.), hydrated dextrose (1.0 g.), and double-distilled water to make 500 ml. Just prior to use, 50 units of penicillin G/ml. and 1 mcg. of amphotericin B are added to prevent bacterial and mold growth. [b] See *Footnote 5*.

The samples were prepared for assay by digesting in 2 *N* NaOH at 80° for 30 min. After cooling to room temperature, a scintillation cocktail (2,5-diphenyloxazole, 5 g.; naphthalene, 100 g.; glacial acetic acid, 130 ml.; and dioxane to make 1000 ml.) was added. The samples were assayed using a liquid scintillation spectrometer[10], with quench corrections made with a calibration curve.

Cell Fractionation Procedure—This was done to determine if cholesterol-4-^{14}C was inside the cell or adsorbing to the plasma membrane. The results of this experiment showed that a considerable amount of cholesterol was inside the cell.

The cell fractionation technique, developed by Wray and Stubblefield (33), permits the removal of the plasma membrane and cytoplasm from the cells, leaving behind nuclei.

EVALUATION

The initial set of experiments was designed to obtain information about the cell. The anatomy of the cell was studied with an electron microscope (Fig. 4).

Microscopic examination indicated that the average cell diameter was 14.8 ± 0.115 (standard deviation) μ and that the average nuclear diameter was approximately 10 μ. Figure 3 confirms that the distribution of cell sizes, as measured by the Coulter counter with the multichannel particle analyzer, was rather narrow. Such determinations showed that the size distribution remained constant throughout the duration of the experiments and when experimental conditions were altered.

An immediate concern in the sampling procedure was the possibility of an appreciable amount of drug trapped between the compacted cells in the narrow stem of the thrombocytocrit tube after the centrifugation step. This was studied in two ways. First, as soon as the drug and the cell suspension were mixed, a sample of the cells was withdrawn close to "zero time," centrifuged, and assayed. If the permeability of the membrane to cholesterol was small and there was no drug trapped in the intercellular spaces or rapidly adsorbed on the plasma membrane, then the assay of cholesterol in the cell should have been negligible. Table II indicates that such entrapment of cholesterol was insignificant, not more than 2.22%.

The second method involved the following procedure. The cells were first equilibrated in a medium containing radioactive cholesterol. Then a sample was removed, and the drug content in the sedimented cells was determined as before. The remaining cells were removed from the radioactive medium by centrifugation and then resuspended in a medium containing no radioactive cholesterol. The assay was repeated with these resuspended cells. If there is no drug trapped in the cell plug, there should be no difference in the assay between the two treatments. The results in Table III support the finding of the "zero time" study that there was no entrapped drug within the experimental error.

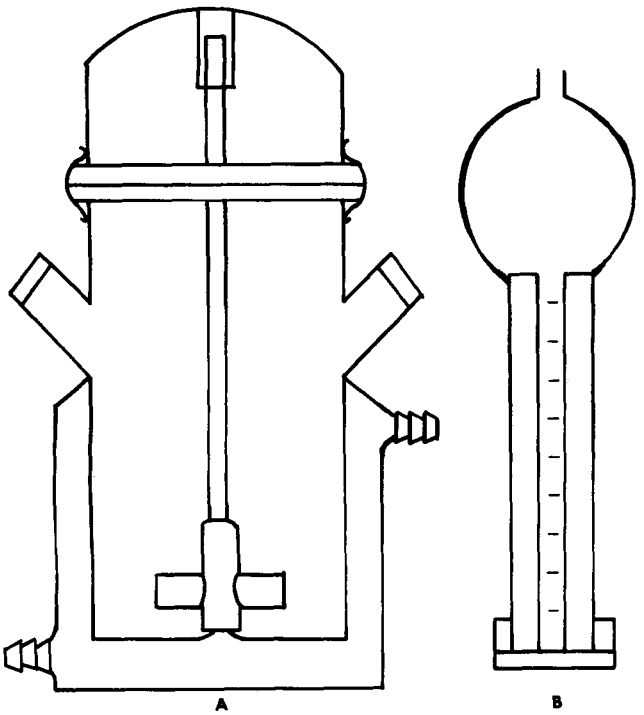


Figure 2—*Schematic diagram of: (A) the spinner flask, and (B) the thrombocytocrit tube used in the drug transport studies.*

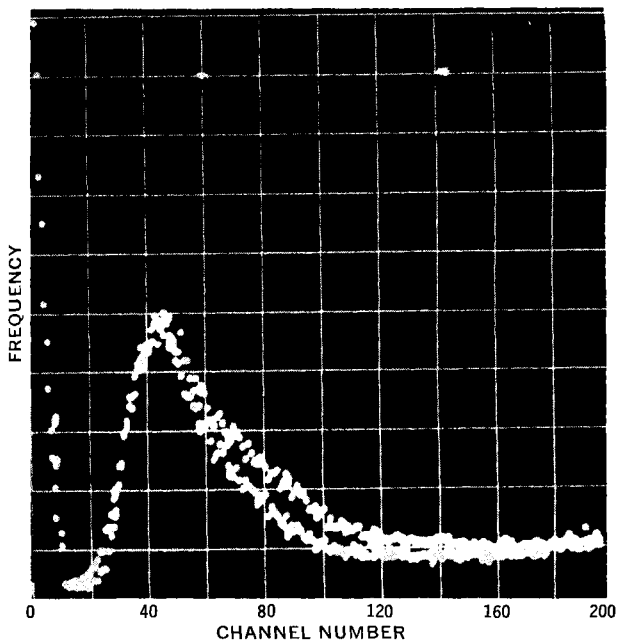


Figure 3—*Particle-size distribution of the cell suspension. The two curves, which are essentially superimposable, represent the size distributions at the beginning and at the end of an experiment.*

[10] Beckman.

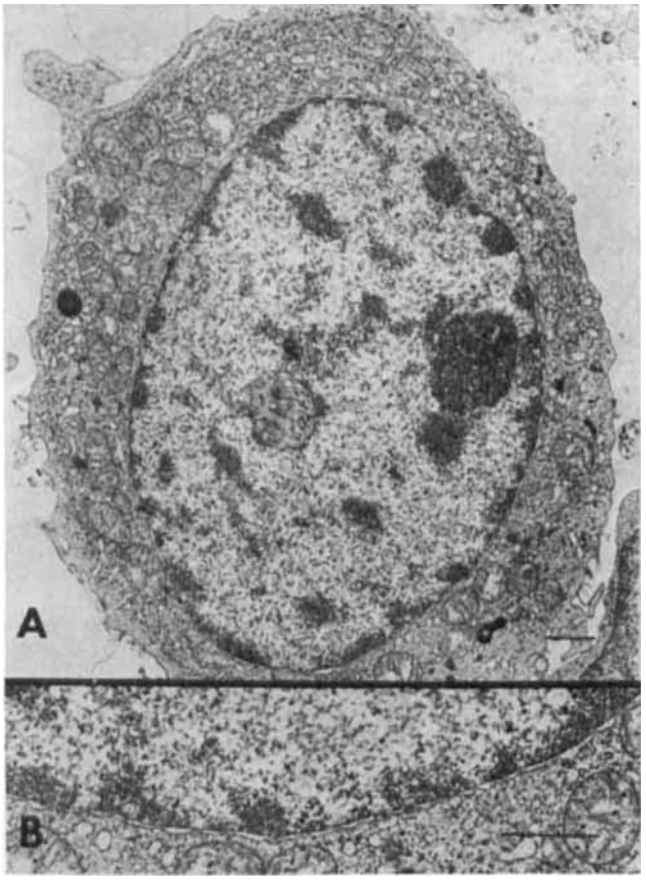


Figure 4—*(A) Electron micrograph of a Burkitt lymphoma cell. (B) Enlarged portion of the cell illustrating the nuclear membrane with pores. Each magnification marker represents 1 μ.*

Another important study was to determine if drug material balance was maintained during the entire experiment. As shown in Table IV, excellent material balance was accounted for at all fetal bovine serum concentrations. However, when no serum was added to the medium, an initial loss of cholesterol was observed. This might be attributed to adsorption to glass, but this presented no major problem to the interpretation of the uptake of drug by the cells.

RESULTS AND DISCUSSION

Theoretical Considerations—As will be seen, it was found that the kinetics of uptake of cholesterol by viable Burkitt lymphoma cells in suspension at pH 7.3, 300 mosm./kg., and 26° could best be explained by Model B, the simplest of the three models mentioned. Since this model, which describes the transport of drug into the cell as a plasma membrane process, was discussed in detail previously (23), only an outline will be given here.

Assuming that adsorption is negligible, the quasisteady-state rate of uptake by a cell can be described by:

$$\frac{dC_i}{dt} = \frac{4\pi a^2 P}{V_i}(C_o - C_i/K) \qquad \text{(Eq. 1)}$$

Table II—"Zero Time" Uptake of Cholesterol

Amount of Cholesterol in the External Media (d.p.m./5 ml.)[a]	Amount in the Cellular Sediment d.p.m.	Amount in the Cellular Sediment %
7,500	120	1.60
19,230	336	1.75
38,351	850	2.22
84,800	910	1.07
99,760	910	0.912

[a] d.p.m. = disintegrations per minute.

Table III—Amount of Cholesterol[a] in the Cellular Sediment before and after Resuspension

Amount of Cholesterol in the Cellular Sediment (d.p.m./10^6 Cells)	
Radioactive Media	Nonradioactive Media
1047	1050
1340	1288
2589	2314

[a] Cholesterol-4-^{14}C used was 98% pure determined by GC using a 2% SE-30 column with Chromosorb G support.

where P is the intrinsic permeability coefficient, C_i is the drug concentration in the cell, a is the cell radius, C_o is the drug concentration in the external medium, K is the intrinsic partition coefficient, and V_i is the volume of the cell.

With $V_i = 4/3\pi a^3$, Eq. 1 becomes:

$$\frac{dC_i}{dt} = \frac{3P}{a}(C_o - C_i/K) \qquad \text{(Eq. 2)}$$

Mass balance of the drug requires that:

$$T = C_o V_o + nC_i V_i \qquad \text{(Eq. 3)}$$

where T is the total amount of drug in the system, n is the number of cells, and V_o is the volume of the external system. This mass balance equation is only applicable to a system with a uniform or narrow cell size distribution, as with the Burkitt cells; otherwise, the cell size distribution should be considered.

By solving Eq. 3 for C_o, substituting into Eq. 2, and integrating, the solution is[11]:

$$\log\left[1 - \left(\frac{\frac{nV_i}{V_o} + \frac{1}{K}}{T/V_o}\right)C_i\right] = -\frac{3P}{2.303a}\left(\frac{nV_i}{V_o} + \frac{1}{K}\right)t \qquad \text{(Eq. 4)}$$

For initial rates, Eq. 4 becomes:

$$C_i = \frac{3PT}{aV_o} \cdot t \qquad \text{(Eq. 5)}$$

Thus, the permeability coefficient, P, can be determined by the initial slope of the C_i *versus* time plot or from the plot of Eq. 4 when K is known. The partition coefficient, K, can be experimentally obtained from the equilibrium conditions, *i.e.*, when $(dC_i/dt)_{t=\infty} = 0$; thus:

$$K = \frac{C_i}{C_o} \text{ at } t = \infty \qquad \text{(Eq. 6)}$$

The permeability coefficient may also be determined by nonlinear

Table IV—Account of Mass Balance throughout the Uptake Experiments

Minutes	Fetal Bovine Serum, %	Total Concentration in Fluid and Cellular Sediment, d.p.m./ml.
7	0	1.92×10^4
360		1.71×10^4
4240		1.72×10^4
16	5	1.72×10^4
960		1.72×10^4
2760		1.68×10^4
90	15	1.08×10^5
1759		1.06×10^5
4695		1.05×10^5
7	30	2.00×10^4
360		2.04×10^4
4240		2.03×10^4

[11] Equation 4 in this text considers the uptake of solute by the cell and is a corollary to Eq. 33 of model 2 in *Reference 23*, which considers the depletion of solute from the external solution.

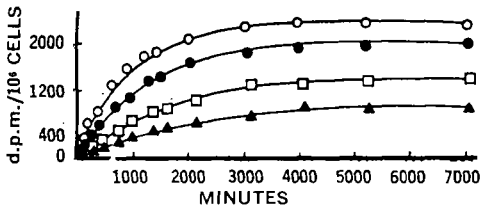


Figure 5—*Uptake of cholesterol-4-^{14}C by cells in media containing different fetal bovine serum concentrations. Key: ○, no fetal bovine serum; ●, 5% fetal bovine serum; □, 15% fetal bovine serum; and ▲, 30% fetal bovine serum.*

regression analysis[12] of the exponential form of Eq. 4 provided K is known and the model is the proper one.

In the event that there is binding of drug molecules to the serum used in the external medium, Eqs. 1-4 should be modified in the following manner. The total concentration of drug in the aqueous medium is:

$$C_{o,T} = C_o + C_o^* \qquad \text{(Eq. 7)}$$

where $C_{o,T}$ is the total drug concentration in the external phase, and C_o and C_o^* are the concentrations of unbound and bound drug in the external phase, respectively. By assuming that there is linear adsorption, *i.e.*:

$$C_o^* = k_o C_o \qquad \text{(Eq. 8)}$$

Eq. 7 becomes:

$$C_{o,T} = (1 + k_o)C_o \qquad \text{(Eq. 9)}$$

where k_o is the linear adsorption constant of the serum-bound drug.

After substituting Eq. 9 into Eq. 2, one obtains the following expression:

$$\frac{dC_i}{dt} = \frac{3P_e}{a}\left(C_{o,T} - \frac{C_i}{K_e}\right) \qquad \text{(Eq. 10)}$$

where the effective permeability coefficient, P_e, and the effective partition coefficient are defined by:

$$P_e = \frac{P}{(1 + k_o)} \qquad \text{(Eq. 11)}$$

$$K_e = \frac{K}{(1 + k_o)} = \frac{C_i}{C_{o,T}}\,(t = \infty) \qquad \text{(Eq. 12)}$$

After using the mass balance expression:

$$T = C_{o,T}V_o + nC_iV_i \qquad \text{(Eq. 13)}$$

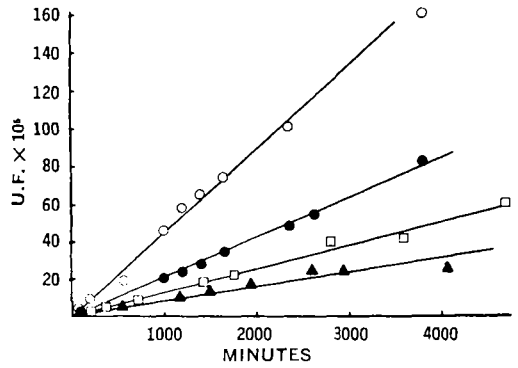


Figure 6—*First-order plots of the cholesterol-4-^{14}C uptake data from the experiments at the four fetal bovine serum levels. Key: ○, no fetal bovine serum; ●, 5% fetal bovine serum; □, 15% fetal bovine serum; and ▲, 30% fetal bovine serum. U. F. is the mathematical drug uptake function described in the text.*

[12] C. M. Metzler, in "A Users Manual for Nonlin," The Upjohn Co., Kalamazoo, Mich., 1969.

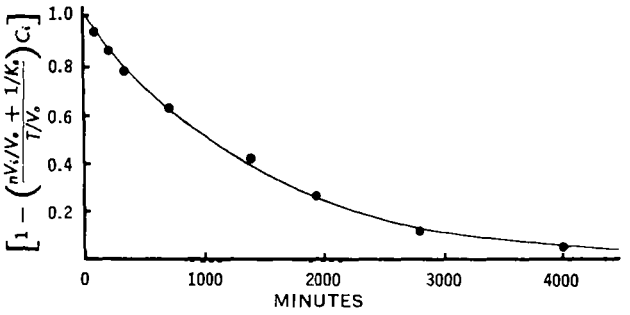


Figure 7—*Nonlinear regression analysis for a cholesterol-4-^{14}C uptake experiment at 15% fetal bovine serum. The points represent the experimental values and the curve represents the best theoretical fit.*

the solution to Eq. 10 is:

$$\log\left[1 - \left(\frac{\frac{nV_i}{V_o} + \frac{1}{K_e}}{T/V_o}\right)C_i\right] = -\frac{3P_e}{2.303a}\left(\frac{nV_i}{V_o} + \frac{1}{K_e}\right)t \qquad \text{(Eq. 14)}$$

The mathematical form of Eq. 14 is the same as Eq. 4 except that effective parameters are involved instead of intrinsic parameters due to serum binding.

Uptake of Cholesterol by Burkitt Cells at Various Serum Concentrations—Figure 5 shows the uptake data of cholesterol with four fetal bovine serum concentrations at a constant cholesterol concentration. As can be seen, it was found that when the serum concentration was increased, the uptake rate decreased.

The effective partition coefficient, K_e, of cholesterol at each serum level was calculated from the equilibrium portion of the curves by means of Eq. 6. By utilizing these values and Eq. 14, the uptake concentration function *versus* time curves (Fig. 6) show excellent agreement with Model B. The uptake function (U.F.) is defined as:

$$-\frac{2.303a}{3(60)\left(\frac{nV_i}{V_o} + \frac{1}{K_e}\right)}\log\left[1 - \left(\frac{\frac{nV_i}{V_o} + \frac{1}{K_e}}{T/V_o}\right)C_i\right]$$

where the factor 60 is necessary for determining the effective permeability coefficient, P_e, in units of centimeters per second from the slope of the linear regression line. For example, the P_e for the 15% fetal bovine serum situation is 1.27×10^{-7} cm./sec.

Furthermore, the P_e for each of the serum level cases was calculated by nonlinear regression analysis of the data according to the exponential form of Eq. 14. Figure 7 illustrates the nonlinear regression fit for the uptake of cholesterol with time at 15% serum. The coefficient of determination was found to be 0.999 for a P_e value of 1.16×10^{-7} cm./sec., with a standard deviation of 3.26×10^{-9} cm./sec. The fact that the coefficients of determination and P_e values obtained with nonlinear regression were in good agreement with those found by linear regression demonstrates simultaneously

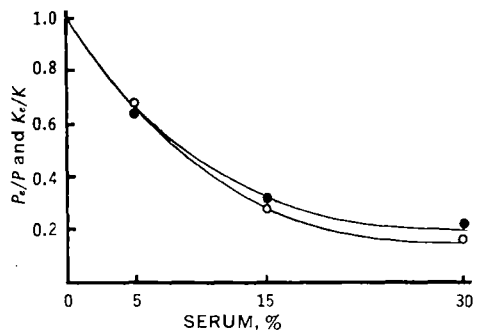


Figure 8—*Normalized plots of the effective permeability coefficient and the effective partition coefficient as a function of the fetal bovine serum concentration. Key: ●, P_e/P; and ○, K_e/K.*

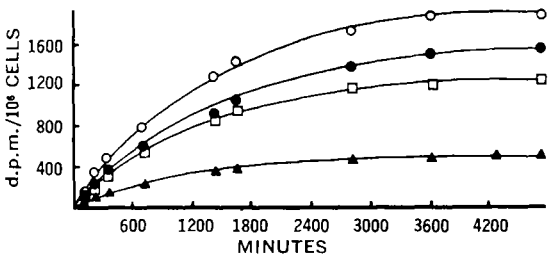


Figure 9—*Cholesterol uptake with four different cholesterol-4-^{14}C levels and 15% fetal bovine serum. Key:* ○, *145,000 d.p.m./5 ml.;* ●, *106,000 d.p.m./5 ml.;* □ *86,000 d.p.m./5 ml.; and* ▲, *40,000 d.p.m./5 ml.*

the accuracy of the experimental technique and the good fit of the model.

It is observed in Fig. 6 (and later in Fig. 10) that the scatter of the data about the linear regression lines increased as the transport of the drug proceeded closer to equilibrium. This is largely because of the nature of the uptake concentration function; a small deviation in the assay of cholesterol in the cells leads to a more pronounced deviation in these plots near equilibrium. At equilibrium the function equals zero and is, therefore, undefined. In the nonlinear plots (Fig. 7), the deviations were relatively small as expected.

Table V summarizes the values of the phenomenological constants obtained from the uptake of cholesterol at different serum concentrations. As one can observe, the small values of P_e justify the initial assumption in the model that the drug absorption is controlled by the plasma membrane. For example, the aqueous diffusion layer about a small sphere, like the Burkitt cell, is in the order of the radius. With the aqueous diffusion coefficient being $5\text{–}10 \times 10^{-6}$ cm.^{2}/sec., the permeability coefficient of the diffusion layer about the cell is about 0.1–0.01 cm./sec. Thus, the P_e values determined from the experimental data are about 10,000 times smaller than would be expected if the rate was aqueous diffusion controlled.

It was observed that the percent of viable cells decreases with time (approximately 90% viable cells after 24 hr.) as indicated by the dye exclusion technique. However, studies using nonviable cells, *i.e.*, cells heat inactivated at 50° for 30 min., gave similar permeability coefficients.

Since the data obtained in this work showed a decreased rate of uptake as bovine serum was increased, the relationship of the effective permeability coefficient with serum binding may be established in the following manner. Consider the reaction of cholesterol + serum ⇄ cholesterol − serum:

$$k_b = \frac{C_o^*}{C_o(S)} \qquad \text{(Eq. 15)}$$

where k_b is the equilibrium cholesterol–serum binding constant, (S) is the serum fraction, and C_o and C_o^* are the free and bound drug in the external medium, respectively. Using Eqs. 7 and 15, one may write:

$$C_{o,T} = C_o[1 + k_b(S)] \qquad \text{(Eq. 16)}$$

It is postulated that only the free drug is participating in the transfer across the membrane. The P_e is then proportional to the concentration of free drug. Thus:

$$P_e \propto C_o \qquad \text{(Eq. 17)}$$

and, using Eq. 16:

$$P_e \propto \frac{C_{o,T}}{1 + k_b(S)} \qquad \text{(Eq. 18)}$$

However, when there is no serum in the external medium, the effective permeability coefficient is equal to the intrinsic permeability coefficient, P. It follows that Eq. 18 may be rewritten accordingly:

$$P_e = \frac{P}{1 + k_b(S)} \qquad \text{(Eq. 19)}$$

Equation 19 is an important one, for it allows one to calculate the intrinsic permeability of the membrane from effective permeability coefficients in the presence of bovine serum, which is an important ingredient in the suspension medium to maintain the integrity of the cells for long periods of time. In turn, the intrinsic permeability coefficients can be used to relate quantitatively molecular structure and functional group substituent modifications on membrane absorptivity. Although a permeability coefficient at 0% serum (Table V) was obtained, the authors are aware that this value may not exactly be the intrinsic permeability coefficient of cholesterol for the Burkitt cell, unless there is no adsorption or reaction with the other nonserum ingredients in the medium.

In a similar manner, it can be shown that the effective partition coefficient is related to serum binding and the intrinsic partition coefficient, K, by the expression:

$$K_e = \frac{K}{1 + k_b(S)} \qquad \text{(Eq. 20)}$$

Figure 8 shows normalized plots of the effective permeability and partition coefficients as a function of fetal bovine serum concentrations. The curves are practically superimposable within the experimental error. This supports the theory that unbound drug is permeating through the membrane and demonstrates the consistency of the data.

The data presented in Figs. 5 and 6 and the above analysis support the mechanism in which cholesterol binding occurred with serum components and only free cholesterol permeated the plasma membrane. Rothblat *et al.* (21) showed that a concentrated suspension of L5178Y tissue culture cells took up free cholesterol by a nonenzymatic process which was temperature and concentration dependent. They found that cholesterol uptake increased with increasing horse serum concentration. Later, they (22) postulated that serum protein enhanced cholesterol transport, that phospholipids decreased its transport, and that uptake of free cholesterol by tissue culture cells is a physical adsorptive process. The results with the Burkitt cell system in the present study showed that increasing the fetal bovine serum concentrations decreased the cholesterol transport rates.

Robertson (34) showed that exogenous, labeled free cholesterol was incorporated into lipid vacuoles within cultured human arterial cells, suggesting that cholesterol is absorbed by the cells. These results are in good agreement with our cell fractionation results, which showed that about one-fourth to one-third of the total radioactivity of the drug resided in the nuclear fraction. Robertson (34–36) demonstrated with arterial cells in culture that the cell surface charge is important in sterol incorporation.

It is noteworthy that the uptake of cholesterol by the cells is adequately explained by Model B, which essentially describes the cell interior as a homogeneous phase like an oil droplet. Considering the electron micrograph of the cell in Fig. 4, one would initially

Table V—Permeability Coefficients Calculated from the First-Order Plots and the Partition Coefficients at Equilibrium

Fetal Bovine Serum, %	Partition Coefficient	Permeability Coefficient, cm./sec.
0	230	4.17×10^{-7}
5	155	2.66×10^{-7}
15	60	1.27×10^{-7}
30	34	8.7×10^{-8}

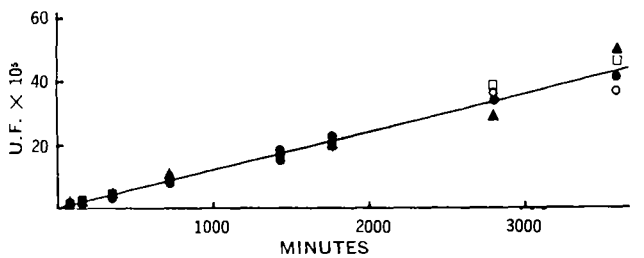


Figure 10—*First-order plot of the uptake data for the four different concentrations of cholesterol-4-^{14}C at 15% fetal bovine serum. Key:* ○, *145,000 d.p.m./5 ml.;* ●, *106,000 d.p.m./5 ml.;* □, *86,000 d.p.m./5 ml.; and* ▲, *40,000 d.p.m./5 ml.*

treat the interior of the cell as a heterogeneous phase so that the rate-determining factors include not only the permeability of the plasma membrane but also the membrane permeability of the nucleus and each kind of cytoplasmic body, weighted by their concentration. Thus, the experimental data imply that the overall permeability of the interior cellular bodies is effectively greater than that of the plasma membrane. The volume of the nucleus and cytoplasmic bodies is at least 40% of the total cell volume. Uptake studies are in progress with nuclei obtained from cell fraction techniques to estimate the permeability of the nuclear membrane.

Uptake Rate as a Function of Cholesterol Concentration—Figure 9 shows the uptake rate of cholesterol at various concentrations at 15% fetal bovine serum. The rate increases with concentration. However, the first-order plot of the data in Fig. 10 shows that the P_e (1.27×10^{-7} cm./sec.) and the K_e (60) are independent of concentration.

CONCLUSION

Experimental techniques to handle cells and to follow quantitatively and reproducibly drug transport across membranes of viable mammalian cells in suspension were developed. Initial interpretative studies on the uptake of cholesterol by Burkitt lymphoma cells should provide the basis for future studies on cholesterol and derivatives and more complex situations.

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