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KINETICS OF DRUG TRANSPORT AND CONCURRENT
METABOLISM IN CELL CULTURES: A PHYSICAL
MODEL APPROACH.

The University of Michigan, Ph.D., 1974
Chemistry, pharmaceutical

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KINETICS OF DRUG TRANSPORT AND CONCURRENT METABOLISM
IN CELL CULTURES: A PHYSICAL MODEL APPROACH

by
Howard Yoshihisa Ando

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmaceutical Chemistry)
in The University of Michigan
1974

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To
My Parents
Yo and Florance Ando
and to Shi

ACKNOWLEDGEMENTS

The author gratefully acknowledges the contributions of many persons who have enabled the completion of this investigation.

The guidance of my co-chairmen, Drs. William I. Higuchi and Norman F.H. Ho, was especially helpful. Their wide range of experience and scientific curiosity serve as guiding models for their students. The other members of the doctoral committee have shown an encouraging and active interest in this study. Dr. Joseph S. Turi and Dr. Charles Shipman, Jr. began the cell culture work upon which this study was based.

The help of many students in the College of Pharmacy was gratefully appreciated. The many discussions with David Dresbeck and the technical assistance of Greg Amidon in the laboratory were especially helpful.

The financial support received in part from the National Institute of Health General Research Support Grant (5S01-RR-05571-10) were significant contributions to this effort.

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GLOSSARY OF SYMBOLS

- A = total amount of substrate for an enzyme E in the cell suspension system of volume V.
- A_i = total amount of A inside the cell fraction.
- A_o = total amount of A in the external fluid.
- $(A_i/A_o)_\beta$ = the β -phase (quasi) steady state asymptote for A_i/A_o .
- A_{ie} = total amount of endogenous A inside the cells.
- A_{iT} = represents the area under the curve of A_i versus time at time t.
- $A(Cl4)$ = DPM of channel A due to ^{14}C .
- ALA = aluminum oxide-silver nitrate thin layer chromatography system.
- a = CPM in channel A for liquid scintillation counting in a double label experiment.
- a = specific maintenance rate.
- a' = CPM in channel A after ^{14}C internal standard has been added.
- a'' = CPM in channel A after ^{14}C and ^3H internal standards have been added.
- α = composite rate constant defined in Appendix A for the simultaneous transport and biosynthesis experiments.
- $\alpha(ipf)$ = the experimentally determined α defined above which could depend on the initial conditions, the phase sampled or the fetal bovine serum concentration.
- $\alpha(f)$ = the theoretically expected α defined above which is only a function of the fetal bovine serum concentration.
- α_o = the α defined above when the rate of enzyme activity is slow or zero compared to the transport rate.

- α = C/A internal standard constant for calculation of the extraction loss parameter, f.
- B = a second substrate that can be acted upon by the enzyme in the cells which also acts on A.
- B_{ie} = amount of the second substrate B endogenous to the cells at steady state.
- β = the second composite rate constant defined in Appendix A for the simultaneous transport and biosynthesis experiments.
- $\beta(ipf)$ = the experimentally determined α defined above which could depend on the initial conditions, the phase sampled or the fetal bovine serum concentration.
- β = C/A internal standard constant for calculation of the extraction loss parameter, f.
- β = area x (membrane permeability) when there are no external or internal concentration gradients.
- C = concentration of the substrate.
- C_o = concentration of the substrate in the external media.
- $\bar{C}$ = average concentration of the substrate inside the cell.
- c = CPM in channel C for liquid scintillation counting in a double label experiment.
- c' = CPM in channel C after ^{14}C internal standard has been added.
- c'' = CPM in channel C after ^{14}C and ^3H internal standard has been added.
- CPM = counts per minute.
- C_{14} = ^{14}C radiolabel.
- $C(\text{H}3)$ = CPM in channel C due to ^3H .
- D_i = diffusion coefficient for the cell interior.
- D_e = diffusion coefficient for the external media.

D_{sp} = specific diffusion coefficient.
 CPM = disintegrations per minute.
 δ = composite rate constant defined in Appendix A for the transport of the product, P.
 E = total amount of free enzyme in the cells.
 E_o = total amount of enzyme in the cells.
 EFFa = efficiency of channel A for ^3H .
 EFFa(SGA:H3) = EFFa for SGA quench.
 EFFa(H3:100) = EFFa for 100 microliters of water quench.
 EFFc = efficiency of channel C for ^{14}C .
 EFFc(SGA:C14) = EFFc for SGA quench.
 ϵ = correction factor for Λ when Q $\neq$ constant.
 FBS = fetal bovine serum.
 f = activity recovered after extraction-elution and dilution procedures.
 f_i = fractional change in A_i (see Appendix D).
 f_o = fractional change in A_o (see Appendix D).
 γ = composite rate constant defined in Appendix A).
 Λ = total diffusional resistance of a cell for constant Q.
 HEPES = N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid.
 h = permeability coefficient of the cell membrane.
 H3 = ^3H radiolabel.
 i = subscript designating the cell interior compartment.
 K = effective rate of enzyme catalysis.
 K_p = same as K above, but emphasizes that P is being plotted against A_{iT} .

- K_A = rate constant determined from a plot of A versus A_{iT} (this parameter is a measure of all the losses of A inside the cell).
- K_T = rate constant determined from a plot of T versus A_{iT} (this parameter is a measure of all the losses of A inside the cell not due to synthesis of P).
- K_P', K_P'' = K_P as defined above but modified for a two-substrate dependence.
- κ_C = intrinsic partition coefficient for a drug C between the cell interior and the external media at time infinity.
- κ_{ce} = the effective partition coefficient of κ_C defined above to account for serum binding in the media.
- κ_a = same as κ_C defined above, but for A.
- κ_{ae} = same as κ_{ce} defined above, but for A.
- κ_P = same as κ_C defined above but for P.
- κ_{pe} = same as κ_{ce} defined above but for P.
- K_m = Michaelis-Menten constant for the enzyme.
- k = specific transfer rate of a limiting nutrient.
- k_1 = microscopic rate constant for the transport of A into the cells from the external media.
- k_2 = microscopic rate constant for the transport of A out of the cells into the external media.
- k_3 = microscopic rate constant for the formation of X.
- k_4 = microscopic rate constant for the degradation of X to A_i .
- k_5 = microscopic rate constant for the degradation of X to E and P_i .
- k_6 = microscopic rate constant for the transport of P out of the cells into the external media.
- k_7 = microscopic rate constant for the transport of P into the cells from the external media.

LSC = liquid scintillation counting.
 λ = ^{14}C quench constant for external standard method of double label counting.
 μ = specific growth rate.
 n = number of cells in the system of volume V.
 n = the dilution number for the PC-ERROR computation of pipetting and coincidence error.
 o = subscript designating the external fluid compartment.
 PC-ERROR = pipetting and coincidence error (see section VI-A).
 P = total amount of product synthesized from the substrate A by the enzyme E in the cell suspension system of volume V.
 P_i = total amount of P inside the cells.
 $P_i(\infty)$ = P_i at time infinity.
 P_o = total amount of P in the external media.
 $P_o(\infty)$ = P_o at time infinity.
 $P_i(0)$ = P_i at time zero.
 π = ^3H quench constant for the external standard method of double label liquid scintillation counting.
 Q = source or sink of a substance.
 q = the value of Q when Q = constant.
 $\bar{Q}$ = space average of Q over the spherical cell volume.
 R = resolution factor for TLC solvent systems (see section VI-C-2).
 ρ = dummy rate constant which can represent either α or β .
 ρ_b = equilibrium serum binding constant.

- r_o = radius of the Burkitt lymphoma cell (7.4×10^{-4} cm (Turi, 1972)).
- s = external standard ratio for an unknown quenched sample.
- SGA = silica gel-silver nitrate TLC system.
- S_{ao} = specific activity of A in the external media.
- σ = ^3H quench constant for the external standard method of double label liquid scintillation counting.
- T = the total amount of ^{14}C label accounted for by the sum of A and P.
- TLC = thin layer chromatography.
- θ = ^{14}C quench constant for the external standard method of double label liquid scintillation counting.
- V = sample volume of the cell suspension.
- V_i = volume of a cell of radius r_o .
- V_o = volume of the external media in a sample of volume V .
- V_{mc} = Michaelis-Menten V_m expressed on a "per cell" basis.
- V_{ie} = steady state velocity of the enzyme reaction for an endogenous substrate level A_{ie} .
- δV = a perturbation from V_{ie} caused by A_i^*/S_{ao} .
- v_c = rate of catalytic conversion of the substrate on a "per cell" basis.
- X = the total amount of intermediate complex formed by the enzyme E with the drug A.
- x = density of the cell population.
- X_n = the number of counts for the n^{th} dilution for the PC-ERROR determinations of pipetting and coincidence error.
- * = subscript denoting a radioactively labeled substance (* is implied but omitted in the sections after IV-C).

I. INTRODUCTION

The kinetic study of the behavior of drugs within the viable mammalian cell and its immediate milieu is essential for an understanding of drug action at the mechanistic level. Such an undertaking should consider not only the interactions of the substrates and enzymes in question, but also diffusion of the drug in the exterior neighborhood of the cell, drug transport through the cell membrane and among the various compartments within the cell, and evaluation of the appropriate equilibrium interactions of the drug among the relevant intracellular pools. If such an investigation is based on a strong foundation of physical/chemical principles, then we believe that the ultimate characterization of the various aspects will have a significant physical meaning for the design and evaluation of drug action.

The purpose of this study was to develop a methodology that can be used to determine the kinetic characteristics of drugs in situations that can be applied to problems of drug bioavailability. For a model system, living, metabolizing mammalian cells in suspen-

sion and a particular intracellular enzyme-substrate-product system were used to determine the necessary physical parameters. By using such well defined systems and by systematic evaluation of the experimental data with theoretical models, it is hoped that the mechanistic assessment of the bioavailability of pro-drugs and the rational selection of adjuvants can be quantified so that the design of the best delivery system for patient therapy will be facilitated.

II. REVIEW OF THE LITERATURE

This section is intended to acquaint the reader, first, with the general area of simultaneous transport and biosynthesis in spherical cells and, second, with particular systems of drugs and enzymes relevant to this study.

A. Simultaneous Transport and Biosynthesis

Many times in the past (in in vivo and in vitro studies) the metabolic rate of synthesis of a substance has been confused with the rate of incorporation of a given precursor into the substance in question (Dietschy, 1970; Plagemann and Erbe, 1972). If one is intent on determining the rate constants for the metabolism of a drug, Dietschy (1970) has pointed out the importance of recognizing such factors as (1) the rate of penetration of a drug through the plasma membrane, (2) the existence of intracellular pools of the drug, and (3) the existence of intermediate compounds between the drug and its final product. Of course, if the drug is not endogenous, the situation is greatly simplified.

A number of authors have qualitatively treated

the problem of simultaneous transport and biosynthesis. A discussion of these studies follows.

1. Qualitative Studies

Plagemann and Erbe (1972) have cited several authors (Smets, 1969; Fuchs and Kohn, 1971) who found that the rate of thymidine incorporation into deoxyribonucleic acid (DNA) does not accurately reflect the rate of DNA synthesis. One possible reason for this discrepancy is that the rate of uptake of the precursor is rate limiting for its incorporation into the macromolecule in question (Plagemann and Roth, 1969; Plagemann, 1971; Plagemann and Erbe, 1971). In the case of Novikoff hepatoma cells in suspension, both a diffusional and a Michaelis-Menten transport system appear to be possible mechanisms which govern uptake. When the concentrations of thymidine are low, the Michaelis-Menten transport system is the relevant mechanism which limits the rate of incorporation of thymidine into DNA. Plagemann and Estensen (1971) have found that cytochalasin B affects this transport system but does not affect the rate of DNA and ribonucleic acid (RNA) synthesis from thymidine and uridine respectively. Jenden and Bassham (1968) and Duckworth (1970) have examined the simultaneous transport of precursors and the biosynthesis of macromolecules in isolated chloroplasts and

E. coli B respectively in suspension systems. The authors, however, did not attempt to determine the limiting step in the incorporation of the precursors into the macromolecule. For a more quantitative evaluation of the data, the problem of simultaneous transport and biosynthesis must be treated mathematically.

2. Diffusional Resistance of a Metabolizing Cell

Any mathematical treatment of the cell which allows for the transport of the substrate and the enzymatic reaction of the substrate inside the cell must be of sufficient symmetry that the mathematical analysis of the system is tractable; a spherically symmetric model of the cell is the simplest and yet experimentally one of the most accessible geometrical configurations that has been studied thus far. The general equation

$$D\nabla^2 C + Q = \frac{\partial C}{\partial t} \quad (2.1)$$

was considered by Rashevski (1948) as the starting point for the model of a metabolizing cell where Q is a source or a sink depending on its sign, D is the diffusion coefficient, C is the concentration of the substrate, and t is time. Assuming (1) spherical symmetry, (2) constant $Q = q$, (3) continuity of the flux

across the cell membrane, and (4) steady state, Rashevski (1948) derived an expression for the total diffusional resistance of the cell:

$$\Lambda = \frac{r_o}{3h} + \frac{r_o^2}{3D_e} + \frac{r_o^2}{15D_i} \quad (2.2)$$

where r_o is the radius of the cell, h is the permeability coefficient, D_e and D_i are the diffusion coefficients in the external media and in the cell, respectively. Best (1960) remarks that Rashevski makes an implicit assumption that the partition coefficient between the cell interior and the external media is unity. In addition, the volume average concentration of the substrate inside the cell is:

$$\bar{C} = C_o + \Lambda q \quad (2.3)$$

where C_o is the concentration of the substrate in the external media at $r = \infty$. Hearon (1953a) noted that Rashevsky's treatment assumes that the rate of metabolism is the same at every point in the cell and that the average metabolism is different from zero. If the rate of metabolism is assumed to be any function of concentration, then:

$$\bar{C} = C_o + (\Lambda + \epsilon)\bar{Q} \quad (2.4)$$

where

$$\epsilon = \frac{r_o^2}{10D_i} \left[\frac{1 - \overline{r^2 Q}}{\overline{r^2 Q}} \right] \quad (2.5)$$

and

$$\overline{Q} = 3 \int_0^{r_o} \frac{r^2 Q dr}{r_o^3} \quad (2.6)$$

(Hearon, 1953a). For the particular case when

$$Q = -k C(r), \quad (2.7)$$

$$\epsilon = \frac{r_o^2}{15D_i} \mathfrak{s}(X) \quad (2.8)$$

where

$$X = r_o \sqrt{\frac{k}{D_i}} \quad (2.9)$$

and

$$\mathfrak{s}(X) = \frac{r_o^2}{15D_i} \left(\frac{5 \tanh X}{X - \tanh X} - \frac{15 + X^2}{X^2} \right) \quad (2.10)$$

(Hearon, 1953b).

Since the cases considered by Rashevski (1948) and Hearon (1953b) are the zero- and first-order situations for Q respectively, Best (1960) states that the actual Michaelis-Menten kinetics will be bound by these two cases. Blum and Jenden (1957) considered this same problem, but in order to obtain the concentration profiles in an intermediate range, they expanded the source

factor

$$Q = \frac{D_i \cdot \alpha \cdot C}{(1 + C/K_m)} \quad (2.11)$$

by a first order Taylor series and found that

$$\bar{C} = \frac{G\{\cosh X_o - (1/X_o) \sinh X_o\}}{F \cosh X_o + \{1 - (D_i/h \cdot r_o) \sinh X_o\}} - \frac{C^{*2}}{K_m} \quad (2.12)$$

where

$$\alpha = \frac{k_2 \cdot E_o}{K_m \cdot D_i} \quad (2.13)$$

$$G \equiv 3(C_o + K_m^{-1} C^{*2})/X_o \quad (2.14)$$

$$F \equiv D_i \sqrt{\alpha} / [h \cdot (1 + K_m^{-1} \cdot C^*)] \quad (2.15)$$

$$X_o \equiv r_o \cdot \sqrt{\alpha} / (1 + K_m^{-1} C^*). \quad (2.16)$$

The value of C^* is arbitrary since it is the value of the substrate about which the expansion is taken and can be chosen to suit a particular situation; h is the permeability coefficient of the membrane, E_o is the total amount of enzyme, K_m is the Michaelis-Menten constant, and k_2 is the rate constant for the decomposition of the enzyme substrate complex into product and enzyme.

In this particular case for intermediate values of C , we see that it is not possible to separate $\bar{C}$ into

factors like Λ and ϵ .

3. Evaluation of Experimental Parameters

A number of expressions have been derived to evaluate diffusional and biosynthetic parameters from experimental data. Best (1955a) has derived an equation for a spherically symmetric metabolizing cell which accounts for transport and metabolism under the "quasi-steady state" assumption (when the rate of metabolism and the rate of transport are equal):

$$V_{mc} - v_c = \frac{K_m}{C_o/v_c - 1/\beta} \quad (2.17)$$

where V_{mc} is the Michaelis V_m expressed on a "per cell" instead of a "per unit volume" basis; v_c is the rate of catalytic conversion of substrate per cell; and β is the area $\times$ (membrane permeability) when there are no external or internal concentration gradients. In this equation the partition coefficient between the cell and the external media is assumed to be unity as in the treatment of Rashevski (1948). Best (1955b), using Baker's yeast, evaluated the parameters of Eq. (2.17) for the sucrose-invertase and the glucose-hexokinase systems at different temperatures with non-linear regression analysis. Blum and Jenden (1957) have derived a similar expression for the evaluation of the model

parameters, given by:

$$\frac{C_o}{v_c} = \frac{1}{K_m \cdot (V_{mc} - v_c)} + \frac{r_o^2}{15D_i} + \frac{r_o}{3h} \quad (2.18)$$

Here, the parameters h , K_m , and V_m cannot be resolved when the experiments are done at a constant r_o unless D_i and h greatly differ from one another.

A similar problem to the simultaneous bioconversion and transport of a substrate is exponential cell growth limited by a diffusion-transferred nutrient for a spherically symmetric cell. The specific growth rate, μ , can be expressed as (van Uden, 1969):

$$\mu = y \cdot k \quad (2.19)$$

where

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt} \quad (2.20)$$

$$k = -\frac{1}{X} \cdot \frac{dC}{dt} \quad (2.21)$$

In these expressions k is the specific net transfer rate of the limiting nutrient, X is the density of the cell population, and y is the yield factor. Van Uden (1969) applied Eq. (2.18) to the case of oxygen-limited Candida utilis in the form

$$D_{sp} \cdot (C_o - C) = V_m \cdot \frac{C}{K_m + C} \quad (2.22)$$

and for xylitol-limited A. aerogenes in the form

$$\mu + a = y \cdot \frac{V_m}{K_m} \cdot (C_o - C) \quad (2.23)$$

where D_{sp} is the specific diffusion coefficient of the limiting nutrient, and a is the specific maintenance rate.

B. Cholesterol Metabolism in Tissue Culture Cells

In studying the cholesterol metabolism of tissue culture cells, one has the advantage of working with a homogeneous cell population in an environment that can be varied to meet the experimental design requirements of the investigator. Tissue culture cells can be classified into two major groups which reflect the degree to which a cell line has been removed from the parent tissue. Primary cell lines can only be serially cultured to about ten generations before they begin to dedifferentiate; this process of dedifferentiation reflects the loss of ability of the cell line to maintain the functional characteristics of the parent tissue. Established cell lines are those that can be cultured approximately one hundred generations or more.

One primary cell line that has been studied with respect to cholesterol metabolism is the diploid human fibroblast (Holmes et al, 1969). This cell line was

found to have a growth requirement for cholesterol which could only be replaced by desmosterol (one of the immediate precursors of cholesterol). Other more distant precursors such as lanosterol and 7-dehydrocholesterol could not meet this growth requirement. This apparent lack of ability of the primary cell lines to reduce the double bond at carbon 7 was attributed either to the presence of an inhibitor in the serum (or media) or to a permeability difference in the plasma membrane of primary cells compared to established cells which can synthesize cholesterol de nova from precursors like glucose, acetate and mevalonate in a sterol free growth media (Rothblat and Kritchevsky, 1968; Rothblat, 1972).

When exogenous cholesterol is available in the external milieu of established cell lines, however, de nova cholesterol biosynthesis is spared and exogenous cholesterol is utilized by the cells for growth (Sokoloff and Rothblat, 1972; Rothblat et al, 1971; Rothblat and Burns, 1971; Rothblat and Buchko, 1971). Although the complete localization of exogenous cholesterol in tissue culture cells has not been determined, there is evidence that the major portion of it is found in the plasma membrane and in the microsomal fractions (Rothblat, 1972; Rothblat and Kritchevsky, 1968). In addition, there does not seem to be any extensive catabolism of exogenous cholesterol (Rothblat and Kritchevsky, 1968).

The major cell lines that have been studied with respect to cholesterol metabolism are the mouse L-cell line (Sokoloff and Rothblat, 1972; Rothblat and Burns, 1971; Rothblat and Buchko, 1971; Rothblat et al, 1970) and WI-38, a diploid cell line established from human lungs (Rothblat et al, 1971). The mouse L-cells are of special interest since these cells seem to have lost the enzyme that is necessary to convert desmosterol to cholesterol so that desmosterol is the major sterol synthesized by these cells (Rothblat et al, 1970). Although exogenous cholesterol is utilized for growth (Rothblat and Burns, 1971) and does cause a feedback inhibition of desmosterol synthesis, desmosterol is a better inhibitor of de nova synthesis than cholesterol which is in turn more effective than δ -cholestenone and coprostanol (Rothblat and Burns, 1971; Rothblat and Buchko, 1971). The plant steroids (β -sitosterol, stigmasterol, campesterol and ergosterol along with cholestenol) do not seem to induce a feedback response (Rothblat and Burns, 1971) in L-cells. A substance that may perhaps induce an opposite response is exogenous lecithin which, in the presence of exogenous cholesterol, causes an increase in the rate of desmosterol synthesis (Sokoloff and Rothblat, 1972).

III. STATEMENT OF THE PROBLEM

The main purpose of this investigation was to develop a methodology for assessing the bioavailability of drugs utilizing cultures of living cells. In this system, the transport parameters of the drug and its metabolite can be determined in experiments in which the metabolism of the drug is not a factor. Thus, in a model which considers the simultaneous transport and metabolism of the drug, the transport parameters can be considered known quantities. If transport of the drug (into and out of the cell) and its metabolism are the only rate processes that alter the distribution of the drug, then the metabolistic factor can be determined.

The specific objectives of this study were:

- (1) To evaluate in a quantitative manner the transport parameters of the drug into and out of the metabolizing cell in suspension culture.
- (2) To determine quantitatively the effective metabolistic component of the drug inside the viable cell.
- (3) To assess the factors that affect the effective rate of metabolism.

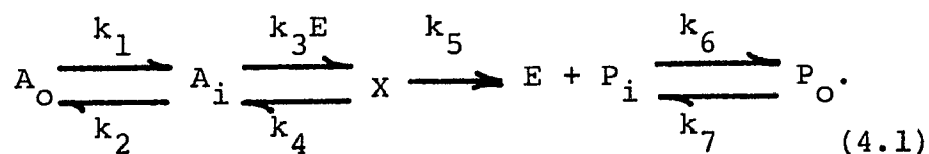
The physical parameters in this study were quantitatively evaluated by mathematical modeling. In modeling one has a tool which can abstract the essence of a complicated phenomenon, translate it into the precise language of mathematics, examine it in a rigorous manner for consistency with past evidence, and use it to predict drug behavior patterns. Initially, minimal models are chosen to provide a basis of comparison for more complicated behavior; then the experimental evidence is examined in the light of such models, and consistency is demanded. This approach has provided the means of understanding some of the most fundamental laws of nature, and we believe that it should be applied in the pharmaceutical field to obtain a fuller understanding of drug behavior. This approach would also provide the basis for the evaluation and structural design of pro-drugs.

To accomplish these objectives, the specific model system included desmesterol, cholesterol and Burkitt lymphoma cells in culture suspension. Thus, the simultaneous transport of desmesterol and cholesterol into and out of Burkitt lymphoma cells and the bioconversion of desmesterol to cholesterol within the cell was studied.

IV. THEORETICAL CONSIDERATIONS

A. Biosynthetic-Transport Model

Consider the one-step conversion of a drug, A, into a product, P, inside the spherically symmetric cell in suspension culture. Fig. 1 schematically depicts the model subsystem of an endogenous biosynthetic pathway that will be considered in this study. In this particular case, P_i is the terminal product of a biosynthetic pathway which is sensitive to feedback inhibition by P_o . If A_o and P_o are the total amount of drug and product, respectively, in the fluid compartment, A_i and P_i are the total amount of drug and product, respectively, inside the cell, and if X is the total amount of intermediate complex formed by enzyme E with drug A, then the scheme in Fig. 1 may be represented by the following catenary chain:



This is a five-compartment system with two physical and three chemical compartments. The physical compartments are due to the external fluid, denoted by the subscript o,

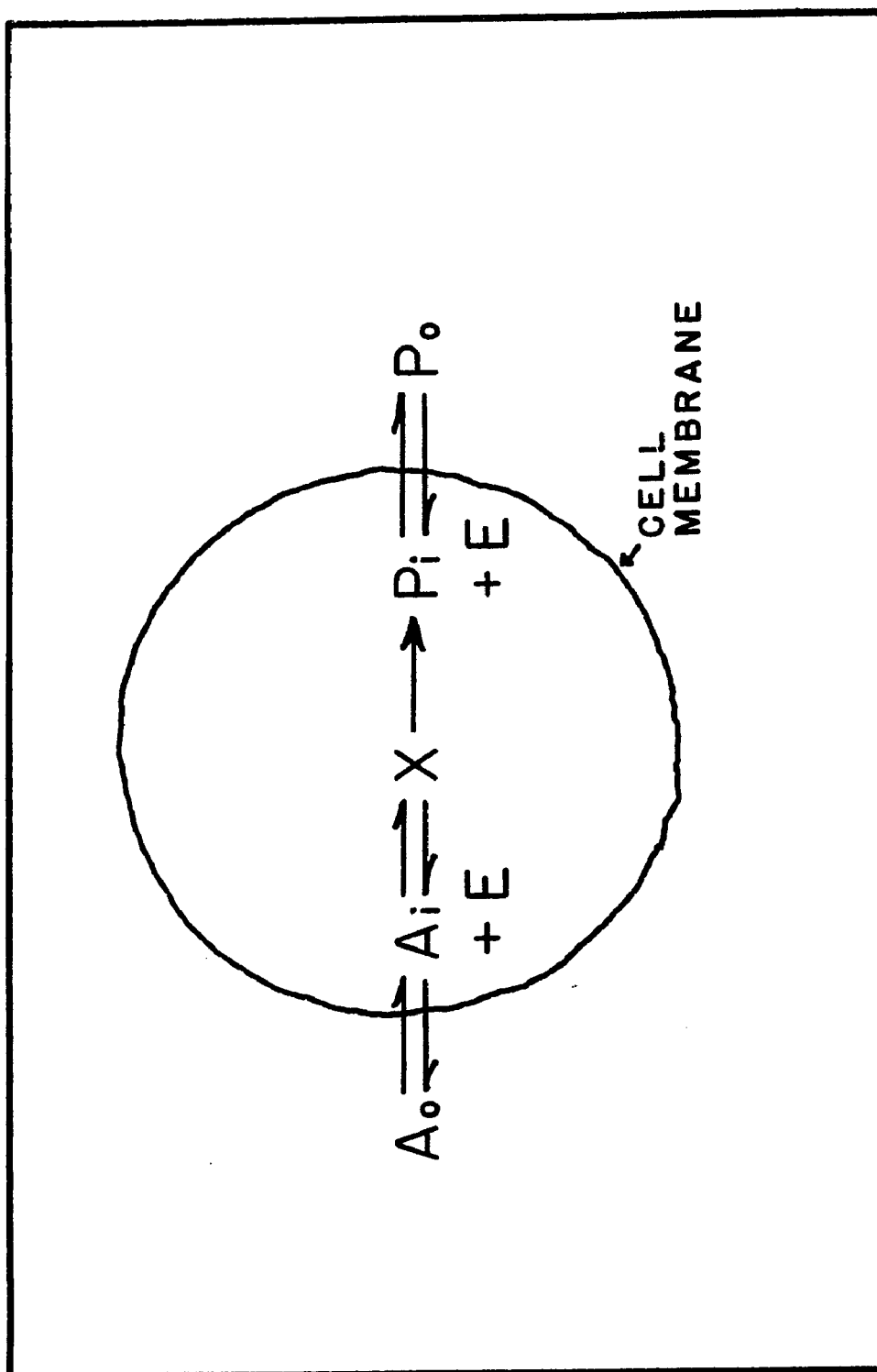


FIG.1 MODEL FOR THE TRANSPORT AND THE SYNTHESIS OF THE PRODUCT, P, FROM THE DRUG, A. THE SPECIES E AND X ARE THE ENZYME AND THE ENZYME COMPLEX, RESPECTIVELY, AND i AND o DESIGNATE THE FLUID AND CELL COMPARTMENTS RESPECTIVELY.

and the cell interior, denoted by the subscript i, of the spherically symmetric cell in suspension whereas the chemical compartments are due to the three distinct chemical species A, X and P. In this model the formation of P_i is assumed to be irreversible, and the enzyme reactions homogeneous in the cell.

The basic differential equations for this system are:

$$dA_o/dt = -k_1 A_o + k_2 A_i \quad (4.2)$$

$$dA_i/dt = -k_2 A_i - k_3 E \cdot A_i + k_1 A_o + k_4 X \quad (4.3)$$

$$dX/dt = -(k_4 + k_5)X + k_3 \cdot E \cdot A_i \quad (4.4)$$

$$dP_i/dt = -k_6 P_i + k_5 X + k_7 P_o \quad (4.5)$$

$$dP_o/dt = -k_7 P_o + k_6 P_i. \quad (4.6)$$

The transport parameters k_1 , k_2 , k_6 , and k_7 are derivable from physical considerations, and they can be determined from independent experiments in which biosynthesis is not a factor. This is important because once k_1 , k_2 , k_6 , and k_7 are determined, the only rate constant that remains to be evaluated from the simultaneous biosynthetic-transport situation is one that

involves the biosynthetic process inside the cell. Assuming that the rate of change of X is much slower than that of A_i or P_i , then one can write Eq. (4.4) after allowing for a brief but negligible induction period:

$$0 = -(k_4 + k_5)X + k_3E \cdot A_i$$

or

$$X = [k_3/(k_4 + k_5)] \cdot E \cdot A_i \quad (4.7)$$

If K_m is defined as

$$K_m \equiv (k_4 + k_5)/k_3, \quad (4.8)$$

then Eq. (4.7) becomes

$$X = K_m^{-1} E \cdot A_i \quad (4.9)$$

Upon substituting Eq. (4.9) into (4.3)

$$dA_i/dt = -k_2A_i + k_1A_o - k_3E \cdot A_i + k_4K_m^{-1}E \cdot A_i \quad (4.10)$$

or

$$dA_i/dt = -k_2A_i + k_1A_o + (k_4K_m^{-1} - k_3)E \cdot A_i \quad (4.11)$$

However, since

$$E = E_o - X$$

$$E = E_O - K_m^{-1} E \cdot A_i \quad (4.12)$$

where E_O is the total amount of enzyme in the cell,
then

$$E = E_O / (1 + A_i / K_m). \quad (4.13)$$

Using this expression for E in Eq. (4.11), we obtain

$$dA_i/dt = -k_2 A_i + k_1 A_O + \frac{(k_4 K_m^{-1} - k_3)}{(1 + K_m^{-1} A_i)} E_O A_i. \quad (4.14)$$

Since

$$\begin{aligned} k_4 K_m^{-1} - k_3 &= k_3 k_4 / (k_4 + k_5) - k_3 \\ &= -k_5 K_m^{-1}, \end{aligned} \quad (4.15)$$

and

$$V_m \equiv k_5 E_O \quad (4.16)$$

by definition Eq. (4.14) becomes

$$dA_i/dt = k_1 A_O - k_2 A_i - V_m A_i / (K_m + A_i). \quad (4.17)$$

Similar considerations applied to Eq. (4.5) give

$$dP_i/dt = -k_6 P_i + k_7 P_O + V_m A_i / (K_m + A_i). \quad (4.18)$$

B. Model Modified for Tracer Analysis

If A is the total amount of drug in the cell suspension system (the system considered here consists of the fluid and cells contained in an aliquot of volume V) and if A_i and A_o are the total amount of drug inside of the cell and in the fluid milieu of the cell, then

$$A = A_i + A_o. \quad (4.19)$$

Similarly for the total amount of product in the system,

$$P = P_i + P_o. \quad (4.20)$$

Throughout this section, all definitions for A apply equally to P. The major chemical species will usually be denoted with a capital letter, and the subscripting of this component will be denoted with small letters. The one exception to this rule is the definition of specific activity. In this case the chemical species will be denoted with a small letter.

In the external media, the ^{14}C -labeled drug has a specific activity

$$S_{ao} = A_o^*/A_o \quad (4.21)$$

where A_o^* is the total amount of labeled drug in the

fluid compartment of the system. If we assume that the flux of cold drug and the flux of labeled drug are mutually independent, then as the tracer A_o^* enters the cell and becomes A_i^* , a total amount of drug A_i^*/S_{ao} has entered the cell from the external milieu of the cell at a time t . Thus

$$A_i = A_{ie} + A_i^*/S_{ao} \quad (4.22)$$

where A_{ie} is the total amount of cold endogenous drug inside the cell under stationary conditions.

Before the labeled drug entered the cell, the enzyme which catalyzed the reaction of A_i into P_i was being driven at a rate V_{ie} by A_{ie} . The increase in A_i by an amount A_i^*/S_{ao} caused the system to be perturbed from A_{ie} . This situation is shown in Fig. 2. If we write the perturbed rate as $V_{ie} + \delta V$, then we can write

$$V_{ie} + \delta V = \frac{V_m A_i}{K_m + A_i} \quad (4.23)$$

$$= \frac{V_m (A_{ie} + A_i^*/S_{ao})}{K_m + (A_{ie} + A_i^*/S_{ao})}$$

$$= \frac{V_m A_{ie}}{K_m + (A_{ie} + A_i^*/S_{ao})}$$

$$+ \frac{V_m A_i^*/S_{ao}}{K_m + (A_{ie} + A_i^*/S_{ao})} \quad (4.24)$$

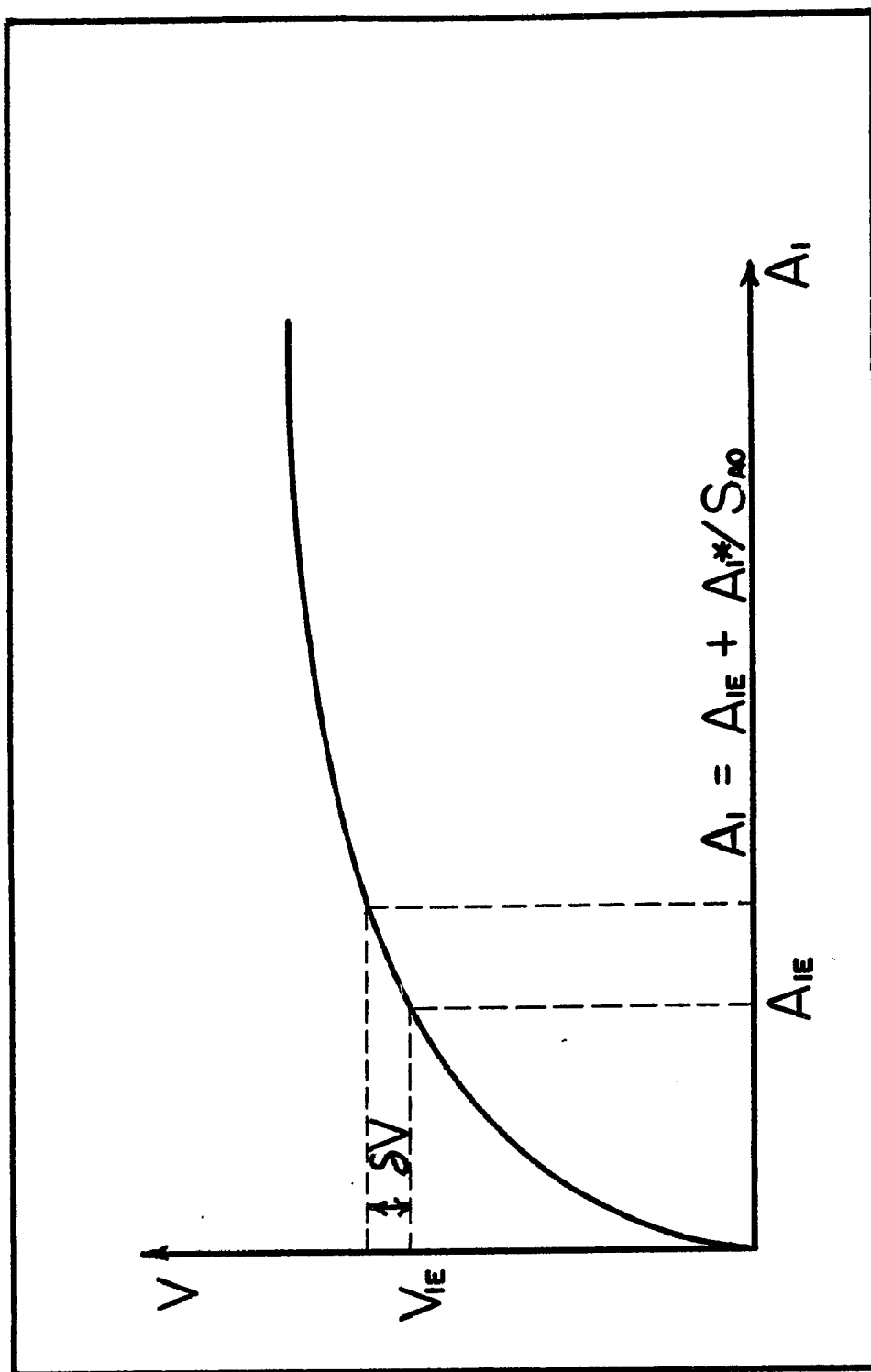


FIG. 2 PERTURBATION OF AN ENZYME SYSTEM FROM THE
OPERATING POINT (A_{IE} , V_{IE}).

Assuming that the endogenous drug level is much larger than the amount A_i^*/S_{ao} , i.e.

$$A_{ie} \gg A_i^*/S_{ao}, \quad (4.25)$$

then one can write

$$V_{ie} + \delta V \doteq V_m A_{ie} / (K_m + A_{ie}) + K \cdot A_i^* / S_{ao} \quad (4.26)$$

where

$$K \equiv V_m / (K_m + A_{ie}). \quad (4.27)$$

But

$$V_{ie} = V_m A_{ie} / (K_m + A_{ie}), \quad (4.28)$$

therefore

$$\delta V = K \cdot A_i^* / S_{ao}. \quad (4.29)$$

Applying this result to Eq. (4.17), we have

$$\begin{aligned} d(A_{ie} + A_i^*/S_{ao})/dt = \\ k_1(A_{ox} + A_o^*/S_{ao}) - k_2(A_{ie} + A_i^*/S_{ao}) - V_{ie} - \delta V \end{aligned} \quad (4.30)$$

where A_{ox} is the total amount of cold precursor in the fluid milieu of the cells in the absence of A_o^*/S_{ao} under stationary conditions. Since

$$dA_{ie}/dt = k_1 A_{ox} - k_2 A_{ie} - V_{ie} \quad (4.31)$$

then

$$dA_i^*/dt = k_1 A_o^* - k_2 A_i^* - S_{ao} \delta V \quad (4.32)$$

and using Eq. (4.29) to eliminate δV we have

$$dA_i^*/dt = k_1 A_o^* - (k_2 + K) A_i^*. \quad (4.33)$$

Similarly Eq. (4.2), (4.18) and (4.6) become

$$dA_o^*/dt = -k_1 A_o^* + k_2 A_i^* \quad (4.34)$$

$$dP_i^*/dt = -k_6 P_i^* + k_7 P_o^* + K A_i^* \quad (4.35)$$

$$dP_o^*/dt = -k_7 P_o^* + k_6 P_i^* \quad (4.36)$$

respectively.

C. Closed System Requirement

For a closed system we require

$$T^* = A^* + P^* = \text{constant} \quad (4.37)$$

where T^* is the total amount of label in the system

and

$$A^* = A_o^* + A_i^* \quad (4.38)$$

$$P^* = P_o^* + P_i^*. \quad (4.39)$$

Adding Eq. (4.33) and (4.34), we find that

$$dA^*/dt = -K \cdot A_i^* \quad (4.40)$$

and adding Eq. (4.35) and (4.36), we obtain

$$dP^*/dt = K \cdot A_i^*. \quad (4.41)$$

But since

$$dT^*/dt = dA^*/dt + dP^*/dt = 0 \quad (4.42)$$

we see that Eq. (4.40) and (4.41) are redundant and really express the same condition. For a closed system, then, we want to solve the following set of linear differential equations:

$$dA_i/dt = k_1 \cdot A_o - (k_2 + K) \cdot A_i \quad (4.43)$$

$$dP_i/dt = -k_6 \cdot P_i + k_7 \cdot P_o + K \cdot A_i \quad (4.44)$$

$$dA/dt = -K \cdot A_i \quad (4.45)$$

which are subject to the constraint that

$$T = A + P. \quad (4.46)$$

In Eq. (4.43) to (4.46) we have omitted the label * since all of the time-dependent variables refer to labelled quantities. In the remainder of this section and in those to follow this will be understood. Since:

$$P = P_o + P_i \quad (4.47)$$

$$A = A_o + A_i, \quad (4.48)$$

we can use the constraint of Eq. (4.46) in Eqs. (4.47) and (4.48) so that

$$P_o = T - A - P_i \quad (4.49)$$

$$A_o = A - A_i. \quad (4.50)$$

Substituting Eqs. (4.49) and (4.50) into Eqs. (4.44) and (4.43), respectively, we find that

$$dA_i/dt = k_1 \cdot A - (k_1 + k_2 + K) \cdot A_i \quad (4.51)$$

$$dP_i/dt = k_7 \cdot T - k_7 \cdot A + K \cdot A_i - (k_6 + k_7) \cdot P_i. \quad (4.52)$$

Eqs. (4.45), (4.51), and (4.52) form a system of linear differential equations in three variables A , A_i , and P_i .

In the next section we will examine the solutions to this system of equations, but one final remark is in order with regard to the apparent enzyme rate constant K .

Eq. (4.27) can be rewritten as

$$1/K = K_m/V_m + A_{ie}/V_m$$

so that a plot of $1/K$ versus A_{ie} is linear, and from such a plot V_m and K_m can be determined. Note that since $V_m = k_5 \cdot E_o$, we can rewrite Eq. (4.27) as

$$K = \frac{k_5}{K_m + A_{ie}} E_o$$

so that k_5 can be determined from a linear plot of K versus E_o if A_{ie} is kept constant and K_m and A_{ie} are known. It remains to be seen if one can experimentally change A_{ie} and E_o . If one can vary these parameters, then it appears that it is possible to determine the fundamental rate parameters of the enzyme catalysis within the intact cell.

D. Solutions to the Closed System Equations

In Sections IV-A to IV-C we made the following assumptions to derive the differential equations, Eqs. (4.45), (4.51), and (4.52), for the closed system.

First, it was assumed that the rate of change of the enzyme-substrate complex, X , is much slower than the rate of change of either A_i or P_i within the cell; i.e., transport of the substrate is much faster than

the bioconversion of the substrate. This assumption enables us to obtain an algebraic constraint on X such that

$$X = \frac{E}{K_m} \cdot A_i. \quad (4.9)$$

In this model, the biosynthetic reaction is taken to be homogeneous within the cell.

Next, we assumed that the endogenous levels of substrate within the cells, A_{ie} , did not change during the experiment. This means that the cells must reach a steady state with respect to the synthesis of the endogenous product. This assumption, in the light of feedback inhibition and other intracellular regulatory mechanisms, seems reasonable when the cells are not subject to sudden stress.

Using this constancy of A_{ie} as a steady state operating point and assuming that the total amount of substrate that diffuses into the cells from the fluid media, A_i^*/S_{ao} , is much less than A_{ie} , the enzymatic rate of change of the substrate reduces to the linear expression Eq. (4.26) with respect to its A_i^* dependence. The effective linear rate constant for this enzyme reaction is

$$K = \frac{k_5 \cdot E_o}{K_m + A_{ie}}. \quad (4.27)$$

In this derivation it is implicit that only one substrate is involved which can limit the rate of the enzyme reaction. However, many biological reactions need a second and sometimes a third substrate. This further complication will be discussed in detail in Section VII.

Finally, it was assumed that the substrate and the product form a closed system such that the conservation of ^{14}C -label can be assumed in the form

$$T^* = P^* + A^* \quad (4.37)$$

$$A^* = A_i^* + A_o^* \quad (4.38)$$

$$P^* = P_i^* + P_o^* \quad (4.39)$$

If there are other routes of loss of ^{14}C -label, then this conservation relationship cannot hold.

The initial and the final assumptions provided algebraic constraints which reduce the original five-compartment system to the three-compartment system expressed by Eqs. (4.45), (4.51), and (4.52). The solutions to this system of equations are derived in Appendix A and are given below.

$$A_i = A_{ib} \cdot e^{-\beta t} + A_{ia} \cdot e^{-\alpha t} \quad (4.53)$$

$$A = A_b \cdot e^{-\beta t} + A_a \cdot e^{-\alpha t} \quad (4.54)$$

$$P_i = P_i(\infty) - P_{ib} \cdot e^{-\beta t} - P_{id} \cdot e^{-\delta t} - P_{ia} \cdot e^{-\alpha t} \quad (4.55)$$

where

$$\alpha = \gamma/2 + \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (4.56)$$

$$\beta = \gamma/2 - \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (4.57)$$

$$\gamma = \alpha + \beta = k_1 + k_2 + K \quad (4.58)$$

$$\delta = k_6 + k_7 \quad (4.59)$$

$$A_{ib} = \frac{k_1 \cdot A(0) - \beta \cdot A_i(0)}{\alpha - \beta} \quad (4.60)$$

$$A_{ia} = \frac{\alpha \cdot A_i(0) - k_1 \cdot A(0)}{\alpha - \beta} \quad (4.61)$$

$$A_b = \frac{\alpha \cdot A(0) - K \cdot A_i(0)}{\alpha - \beta} \quad (4.62)$$

$$A_a = \frac{K \cdot A_i(0) - \beta \cdot A(0)}{\alpha - \beta} \quad (4.63)$$

$$P_b = \left(\frac{k_7 - \beta}{\delta - \beta} \right) \cdot A_b \quad (4.64)$$

$$P_a = \left(\frac{k_7 - \beta}{\alpha - \delta} \right) \cdot A_a \quad (4.65)$$

$$P_d = \frac{1}{(\delta-\beta)(\alpha-\delta)} \left\{ \left[1 - \frac{k_7}{k_6 + k_7} \right] \cdot \alpha \cdot \beta \cdot T \right. \quad (4.66)$$

$$\left. + (\gamma - \delta) \cdot (k_7 + \delta) \cdot P_i(o) - k_6 \cdot K A_i(o) \right\}$$

$$P_i(\infty) = \frac{k_7 \cdot T}{k_6 + k_7} \quad (4.67)$$

In these expressions $A(o)$ and $A_i(o)$ are the values of A and A_i at time 0, and $P_i(\infty)$ is the value of P_i at time ∞ . The expression for A_o can be found from Eqs. (4.38) and (4.53) and is given by

$$A_o = (A_b - A_{ib}) \cdot e^{-\beta t} + (A_a - A_{ia}) \cdot e^{-\alpha t}. \quad (4.68)$$

Similarly, P_o can be found from Eqs. (4.37), (4.38), (4.39), (4.53), (4.54), and (4.55). This expression is not needed here.

In the above formulation, all of the quantities were computed in terms of the amount of the substance in a particular compartment for a sample of suspension, containing cells and fluid, of volume V . The volume V can be expressed as

$$V = V_o + n \cdot V_i \quad (4.69)$$

where n is the number of cells in V , V_i is the volume of one cell, and V_o is the fluid volume. The above

equations can be converted to concentration units by dividing the above expressions by the product of the volumes involved in the particular expression of interest. For example, Eq. (4.60) would become in concentration units:

$$[A_{ib}] = \frac{\left(\frac{V}{n \cdot V_i}\right) \cdot k_1 [A(o)] - \beta \cdot [A_i(o)]}{\alpha - \beta} \quad (4.70)$$

where [] indicates the concentration of the quantity within, and the mass balance equations, Eqs. (4.47 and (4.48), become:

$$P = V_o \cdot [P_o] + n \cdot V_i \cdot [P_i] \quad (4.71)$$

$$A = V_o \cdot [A_o] + n \cdot V_i \cdot [A_i]. \quad (4.72)$$

Inspecting the solutions for A_i and A_o given by Eqs. (4.53) and (4.68), respectively, we see that both solutions tend to 0 as time tends to ∞ . This is the behavior we would expect for the substrate in the system when the reaction has been completed. The solutions for P_i and P_o , on the other hand, must tend to $P_i(\infty)$ and $P_o(\infty)$, respectively, as t tends to ∞ . This means that

$$T = P_i(\infty) + P_o(\infty) \quad (4.73)$$

from Eq. (4.37). Substituting Eq. (4.73) into Eq. (4.67), we have:

$$P_i(\infty) = \frac{k_7}{k_6 + k_7} [P_i(\infty) + P_o(\infty)], \quad (4.74)$$

or

$$\frac{P_i(\infty)}{P_o(\infty)} = \frac{k_7}{k_6}. \quad (4.75)$$

If we express Eq. (4.75) in terms of concentration units, we obtain:

$$\frac{[P_i(\infty)]}{[P_o(\infty)]} = \frac{V_o}{n \cdot V_i} \cdot \frac{k_7}{k_6}. \quad (4.76)$$

Turi (1972) defined the intrinsic partition coefficient κ_P of a compound P between the cell fraction of n cells, each of volume V_i , and the fluid media of volume V_o as the ratio of the concentrations expressed in Eq. (4.76), i.e.,

$$\kappa_P = \frac{[P_i(\infty)]}{[P_o(\infty)]}. \quad (4.77)$$

Since the relationship between the amounts and the concentrations of P_i and P_o at time ∞ are:

$$P_i(\infty) = n \cdot V_i \cdot [P_i(\infty)] \quad (4.78)$$

$$P_o(\infty) = V_o \cdot [P_o(\infty)] \quad (4.79)$$

we find that using Eqs. (4.73), (4.77), (4.78), and (4.79):

$$P_i(\infty) = \frac{T \cdot n \cdot V_i \cdot \kappa_P}{n \cdot V_i \cdot \kappa_P + V_O} \quad (4.80)$$

$$P_O(\infty) = \frac{T \cdot V_O}{n \cdot V_i \cdot \kappa_P + V_O} \quad (4.81)$$

$$[P_i(\infty)] = \frac{\kappa_P \cdot T}{n \cdot V_i \cdot \kappa_P + V_O} \quad (4.82)$$

$$[P_O(\infty)] = \frac{T}{n \cdot V_i \cdot \kappa_P + V_O} \quad (4.83)$$

Eqs. (4.80) to (4.83) enable one to determine these asymptotic values of P_i and P_O from data independent of the biosynthetic or the transport kinetics.

E. Determination of the Transport Parameters

The transport parameters k_1 , k_2 , k_6 , and k_7 can be derived in terms of physical constants (Ho et al, 1971; Turi, 1971). The expressions for these parameters are:

$$k_1 = \frac{3 \cdot P_a}{a} \cdot \frac{n \cdot V_i}{V_O} \quad (4.84)$$

$$k_2 = \frac{3 \cdot P_a}{a \cdot \kappa_a} \quad (4.85)$$

$$k_6 = \frac{3 \cdot P_p}{a \cdot \kappa_p} \quad (4.86)$$

$$k_7 = \frac{3 \cdot P_P}{a} \cdot \frac{n \cdot V_i}{V_o} \quad (4.87)$$

where a is the radius of the cell and P_a , P_P and κ_a , κ_P are the intrinsic permeability and partition coefficients of the drug and product, respectively. These are very important parameters because, by suitably arranging the experimental situation, they can be determined independently from the biosynthetic reaction. Thus in the simultaneous biosynthetic-transport situation, the only parameters that remain to be determined are the ones associated with the enzymatic process. Turi (1972) found that when fetal bovine serum (FBS) was used in the media of the uptake and release transport experiments, the binding of many drugs could be accounted for by assuming a linear binding model of the drug to the FBS component of the media. In this way, in the presence of FBS, the effective permeability, P_{ce} , and the effective partition coefficient, κ_{ce} , of a drug C are the parameters that are measured experimentally. These are related to the intrinsic parameters P_C and κ_C by the expressions

$$P_{ce} = \frac{P_C}{1 + \rho_b [S]} \quad (4.88)$$

$$\kappa_{ce} = \frac{\kappa_C}{1 + \rho_b [S]} \quad (4.89)$$

where ρ_b is the equilibrium serum-drug binding constant.

V. EXPERIMENTAL PROCEDURES

A. Growth and Transport Media for the Burkitt Lymphoma Cells

Burkitt lymphoma cells (P_3JT_2 , Turi, 1972), an established human cell line, was used in these studies. These cells have been characterized with respect to their growth in suspension and their transport properties for a number of sterols and cardiac glycosides (Turi, 1972; Turi et al, 1972, 1974a, 1974b).

The growth media (buffered with sodium bicarbonate) and the transport media (buffered with HEPES¹) used in these studies were modifications of the formulations of Turi (1972). The composition of these media are given in Table 1. Use of the synthetic buffer, HEPES, has been recently reviewed by Shipman (1973), and at this time it seems to be a suitable buffer for long term cell and organ culture. The concentration of fetal bovine serum² (FBS) was varied in the HEPES-buffered transport media in order to observe its effect on the

¹N-hydroxyethylpiperazine-N'-ethane-sulfonic acid, pKa = 7.55, Calbiochem, La Jolla, California 92037.

²Fetal bovine serum, Grand Island Biological Co., Grand Island, New York 14072.

Table 1. Various buffered media used in the drug transport experiments and the growth of the Burkitt lymphoma cells modified from Turi (1972)*

Ingredients	Formulations		
	0% FBS	25% FBS	Growth
McCoy's 5A Modified**	0.484 g	0.484 g	24.2 g
Basal Medium Eagle**	0.368 g	0.368 g	18.4 g
HEPES	0.4764 g	0.4764 g	0
Sodium bicarbonate	0	0	7.0 g
Fetal Bovine Serum (FBS)	0	25.0 ml	500 ml
NaOH, 0.1 N***	10.0 ml	10.0 ml	0
Balanced Salt Solution (2x)	14.0 ml	0	0
Double Distilled Water to make	1000 ml	100.0 ml	4000.0 ml

* All formulations are at 300 + 5 mosm /kg and at pH 7.2 + 0.1. The 2x balanced salt solution used to adjust the osmotic pressure of the media consists of NaCl 8.0 g, KCl 0.4 g, anhyd. Na_2HPO_4 0.1 g, hydrated dextrose 1.0 g, double distilled water to make 500 ml.

** These media are dry powders whose exact weight may vary from lot to lot.

*** This is used to adjust the pH of the formulation since HEPES¹ is in the sulfonic acid form.

transport of the drug and its metabolite. Media containing different concentrations of FBS were formed from mixtures of the 0% and the 24% FBS media given in Table 1.

Aseptic techniques were used to handle the cells throughout all stages of preparation and transport experimentation.

B. Transport Studies

The cells were harvested at the end of the exponential growth phase. At this time the final cell concentration was approximately $1.5 \cdot 10^6$ cells/ml. A 500 ml aliquot of cell suspension, approximately $7.5 \cdot 10^8$ cells, was centrifuged at $483 \times g$ for 20 minutes in a Sorvall SS-3 centrifuge. The cells were then resuspended in 100 ml of HEPES-buffered transport media and transferred to a water jacketed spinner flask.³ The final cell concentration was approximately $7.5 \cdot 10^6$ cells/ml for the following uptake and release kinetic studies.

1. Uptake Kinetics

Approximately 2 microcuries of sterile ^{14}C -desmos-terol⁴ (in 200 microliters of ethanol and 5 ml of distilled water) were added to the cells by means of a 5 ml

³Belco Glass Inc., Vineland, New Jersey 08361.

⁴New England Nuclear, Boston, Massachusetts 02118.

disposable syringe. Samples of 4 ml each were taken at various times with a sterile pipet and were centrifuged in a 10 ml conical centrifuge tube⁵ at 7987 x g for 10 minutes. The cell plugs and aliquots of the supernatant fluid were saved for the assay.

2. Release Kinetics

For the release studies approximately 4 microcuries of sterile ¹⁴C-desmosterol were added to the cells suspended in 5% FBS HEPES-buffered media. After they were equilibrated for 12 hours at room temperature, the cells were centrifuged at 483 x g and resuspended in the HEPES transport media of choice. Samples were then taken as in the uptake experiments. Fig. 3 shows the procedures for the release assay.

C. Assays

Two types of assays were used to determine the relative amounts of ¹⁴C label in the cell plug and in the fluid supernatant fractions. These were the nonspecific and the specific ¹⁴C tracer assays.

1. Nonspecific Tracer Studies

In these experiments only the total incorporation

⁵I. Sorvall, Inc., Norwalk, Connecticut 06852.

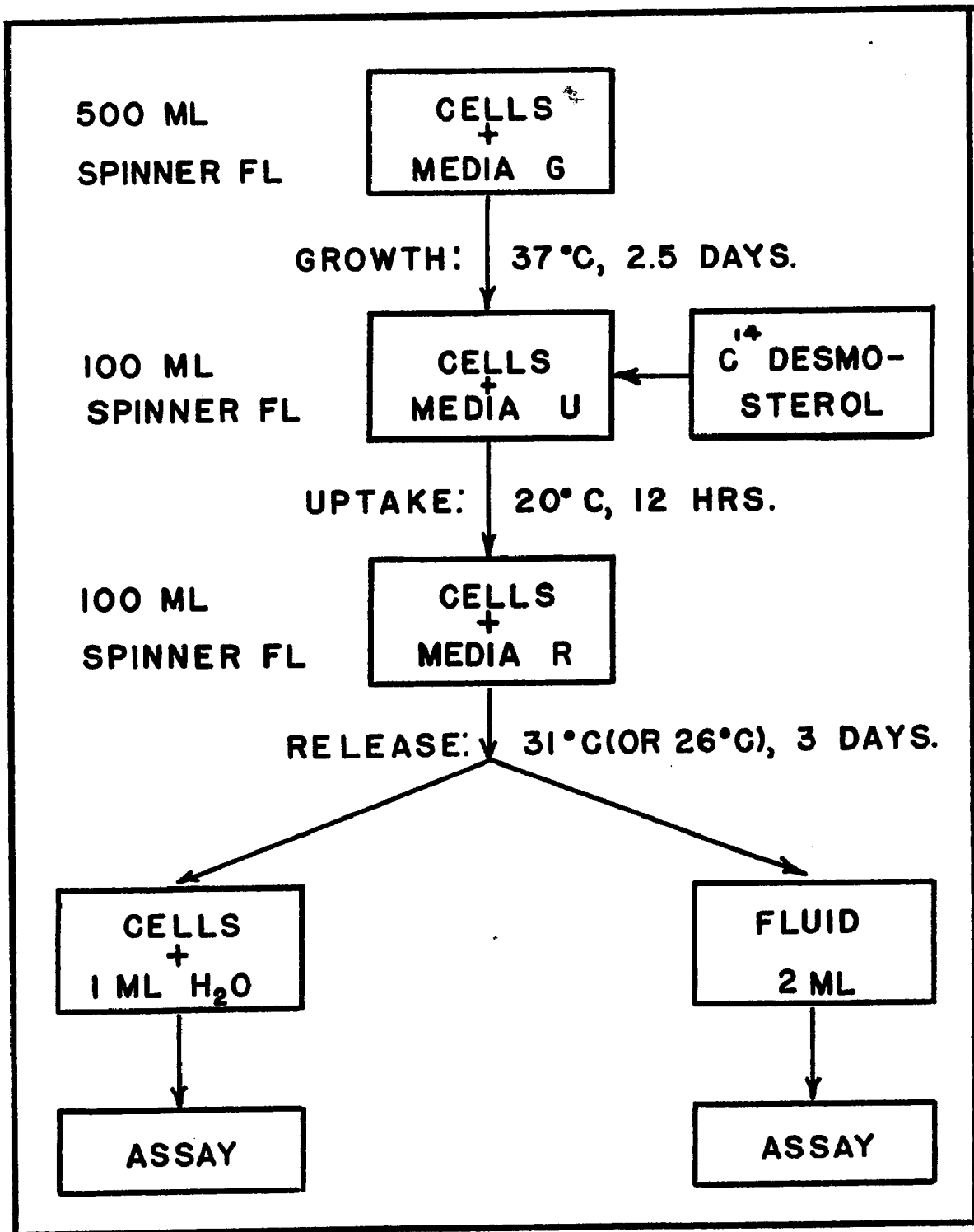


FIG.3 GROWTH AND SAMPLING OF C^{14} LABELED CELLS. MEDIA G = BICARBONATE GROWTH MEDIA, MEDIA U = HEPES BUFFERED MEDIA WITH 5% FBS, MEDIA R = HEPES BUFFERED MEDIA WITH 25% FBS.

of the ^{14}C label was accounted for without regard to chemical species. The cell plug was suspended in 1 ml of distilled water, solubilized in 10 ml of Aquasol,⁶ and counted by liquid scintillation counting (LSC) on a Beckman LS-200 counter. A 1 ml aliquot of the fluid sample was similarly counted. Quenching was corrected for by using ^{14}C -toluene⁶ as an internal standard.

2. Specific Thin Layer Chromatography (TLC) Studies

Studies by Turi (1972) using nonspecific tracer techniques for the uptake and release of ^{14}C -desmosterol in the Burkitt lymphoma cells seemed to indicate that the transport kinetics of ^{14}C -desmosterol were biexponential. These nonspecific tracer studies were repeated, and they confirmed the biexponential behavior pattern for this compound. It was speculated that if ^{14}C -desmosterol underwent bioconversion to some other metabolite in the Burkitt lymphoma cell, then the transport kinetics of the metabolite might help explain the complex transport behavior. The most likely metabolite ^{14}C -desmosterol was thought to be ^{14}C -cholesterol, since this appears to be a viable metabolite in the liver. If this hypothesis was true, then we believed we had come upon a very simple in vitro situation whereby transport and biocon-

⁶New England Nuclear, Boston, Massachusetts 02118

version could be studied simultaneously with the intact enzyme acting in its native in situ location inside the undisturbed cell and not in a cell homogenate. It follows, then, that such a situation must more closely mimic the actual physiological conditions of the intracellular enzymes than most of the in vitro systems in common use at this time.

However, desmosterol and cholesterol are a particularly difficult precursor/product pair of compounds to separate analytically because they differ from one another only by a single double bond located on the side chain of the sterol nucleus. Fig. 4 shows the structure of these two sterols. Any assay that separates these two compounds must do so on the basis of this singular difference in structure. The argentation of silica gel seems to be the most effective method of accomplishing this separation. In this particular phase of the study, the goal was to find an assay that was simple and yet quantitative.

a. Literature Review of TLC Procedures for Cholesterol and Desmosterol One system that had been used in the past to separate the free sterols of cholesterol and desmosterol was a stationary phase of silver nitrate impregnated alumina with a 95:5 solvent system of chloroform to acetone (Kammereck et al, 1967). Although this method was simple, its major disadvantage

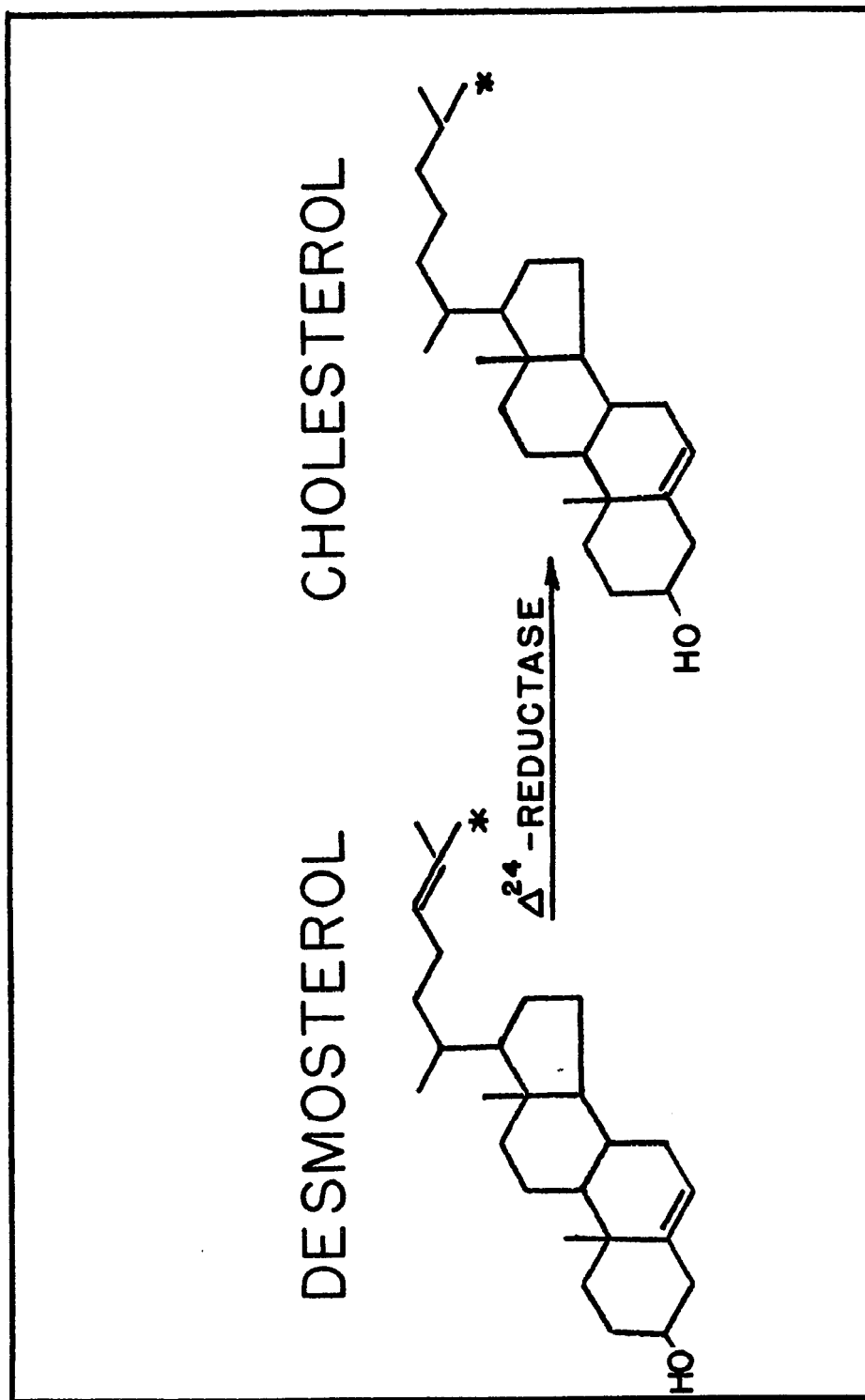


FIG. 4 BIOSYNTHETIC CONVERSION OF DESMOSTEROL INTO CHOLESTEROL BY REDUCTION OF THE C-24 CARBON-CARBON DOUBLE BOND. * INDICATES ^{14}C AT C-26.

was that the ^{14}C -labeled sterols could not be quantitatively eluted from the stationary phase. Moreover, the development time of the plates extended to more than $2\frac{1}{2}$ hours, a situation especially inconvenient when a large number of samples must be assayed.

Another system used to separate the free sterols utilized a silica gel plate developed in a 15% solution of undecane in petroleum ether. After the plates were spotted, they were redeveloped in a 1:1 solvent system of acetic acid and acetonitrile which was 70% saturated with undecane. The plates were then visualized by spraying them with a 10% phosphomolybdic acid solution and charring at 100°C for 10 minutes (Wolfman and Sachs, 1964). This assay was eliminated since it was too time consuming for a routine assay and since relatively corrosive reagents were required.

In a third TLC system (Avigan, et al, 1963), cholesterol and desmosterol were first acylated with acetic anhydride and then spotted and developed on a 40 cm silica gel plate in a 5:1 hexane/benzene solvent system. The authors also found that the cholesterol and desmosterol acetates could be separated, although not satisfactorily, on silica gel plates which had been sprayed with 90% ethanol saturated with silver nitrate. For our purposes, the requirement for acylation and its quantitation were the major disadvantages, since

the yields of these assays will never be 100%. In the next section an assay was developed which, relatively speaking, is both simple and quantitative.

b. Materials. Thin layer chromatography (TLC) plates were made from both silica gel G⁷ and from aluminum oxide GA with binder.⁸ Although the silica gel plates impregnated with silver nitrate (SGA) were used as the primary assay for the separation of cholesterol and desmosterol, the aluminum oxide plates impregnated with silver nitrate provided a confirmatory system with a different stationary phase. Slurry compositions are listed below:

SGA: 30 g silica gel G
10 g silver nitrate
60 ml distilled water

ALA: 50 g aluminum oxide
17 g silver nitrate
45 ml distilled water.

The slurries were spread on 5 x 20 cm glass plates⁹ with a corrosion resistant plexiglas¹⁰ spreader. Although the plates were usually prepared and activated for 3 hours at 100°C on the day of the assay, they could

⁷EM Laboratories Inc., Elmsford, New York 10523.

⁸Anspect Co., Inc., Ann Arbor, Michigan 48107.

⁹Brinkman Instruments Inc., Des Plaines, Illinois 60016.

¹⁰Anspect Co., Inc., Ann Arbor, Michigan 48107.

be stored in the dark without moisture for a day or two. Normal laboratory lighting conditions caused the plates to darken after about 12 hours.

The samples were spotted onto the TLC plates with 20 microliter disposable pipets¹¹ under a gentle stream of extra dry nitrogen. Each TLC plate was spotted with three samples. These pipets were especially convenient because they were disposable and because the capillary-stainless steel plunger units provided excellent control of the rate of delivery of the samples onto the plates. Small spots with minimum detectable levels of cold carriers provided the best resolution characteristics. These cold carriers¹² (desmosterol and cholesterol) were solubilized in benzene.

c. Solvent Systems and Development. Among the many different solvent systems which were investigated, the most successful systems on either SGA or ALA plates were 100% chloroform, 97.5 : 2.5 chloroform and acetone, and 95:5 chloroform and acetone.

To develop the plates, a special sandwich development chamber was constructed from a piece of rectangular U-shaped 1/16 inch thick teflon sandwiched in between a bordered TLC plate and a plain glass plate. Special sandwich clips¹³ were used to hold the chamber together.

¹¹A.H. Thomas Co., Philadelphia, Pennsylvania 19015.

¹²Anspect Co., Inc., Ann Arbor, Michigan 48107.

¹³A.H. Thomas Co., Philadelphia, Pennsylvania 19015.

These chambers were then developed by vertically placing the open 5 cm side of the chamber into a beaker containing 50 ml solvent. This chamber had the desirable characteristics that (1) the enclosed air within the chamber was rapidly saturated with the solvent vapors, (2) the edge effect was minimized, (3) the solvent volume was minimized, and (4) the development time was decreased. Because of these factors, conventional development tanks were not used in this study; cholesterol and desmosterol were separated in a simple and quantitative manner using the sandwich chambers without forming acetate esters.

d. Visualization In the earlier assays of this study, the developed plates were visualized by spraying them with concentrated phosphoric acid and charring them in an oven for 10 minutes at 180°C. It was later discovered that this procedure caused ³H-cholesterol, ¹⁴C-cholesterol, ¹⁴C-desmosterol to elute differently from the charred SGA plates by as much as 45%. For this reason the visualization technique was changed.

In the new technique, the developed plates were sprayed with a dilute solution of sodium fluorescein in methanol. The SGA plates were then observed under a long wavelength ultraviolet light. Under these conditions the cholesterol and desmosterol spots appeared fluorescent green against a dark background.

D. Determination of Cellular Bioconversion

Burkitt lymphoma cells were grown in a 500 ml spinner flask for two days. To this flask ^{14}C -desmosterol was added after the first day of growth. These pre-equilibrated cells were harvested by centrifugation at $483 \times g$ for 20 minutes, lysed by resuspending the cells in 20 ml distilled water, and digested with 20 ml KOH and 40 ml methanol. This mixture was incubated for 20 hours at 60°C and extracted with three equal volumes of n-hexane. After this extract was evaporated under a gentle stream of extra dry nitrogen, it was redissolved in a solution of 0.5 ml benzene and cold cholesterol and desmosterol. The resulting mixture was assayed by TLC, and the spots corresponding to the locations of cholesterol and desmosterol scraped into LS vials containing 10 ml Aquasol and counted.

E. Cell Plug and Fluid Analysis

If V is the volume of the sample to be assayed for ^{14}C -cholesterol and ^{14}C -desmosterol ($V = 1$ ml of cells in distilled or 2 ml of supernatant), then a $1V$ amount of 15% KOH was added to the sample and swirled until the suspension became clear. This was followed by a $2V$ volume of methanol. This mixture was shaken and incubated at 60°C for 3 hours. After the incubation, approximately 2 microcuries of ^3H -cholesterol were added

as an internal standard to the mixture which was then extracted three times with 3V volumes of n-hexane. The hexane extract was evaporated to dryness under a gentle stream of extra dry nitrogen, reconstituted with 100 microliters of cold carrier in benzene, assayed by thin layer chromatography, and counted by LSC. It was essential for good separation that the cold carrier be added to the extract residue within a few hours of spotting. If sodium fluorescein was used to visualize, then one drop of concentrated phosphoric acid had to be added to the LS vials. These vials had to be stored in the dark after the Aquasol was added. Fig. 5 summarizes the assay procedure.

F. Double Label Counting

In the assay for the determination of the bioconversion of ^{14}C -labeled desmosterol to ^{14}C -labeled cholesterol, ^3H -cholesterol was used as an internal standard so the extraction loss of these ^{14}C -labeled compounds in n-hexane could be determined for each sample. The suitability of ^3H -cholesterol as an internal standard for these ^{14}C -labeled compounds was determined by extracting known ratios of ^3H -cholesterol, ^{14}C -cholesterol, and ^{14}C -desmosterol from the cell digestants and examining the $^3\text{H}/^{14}\text{C}$ ratio. The major problem, however, in using ^3H -cholesterol as an internal standard was in

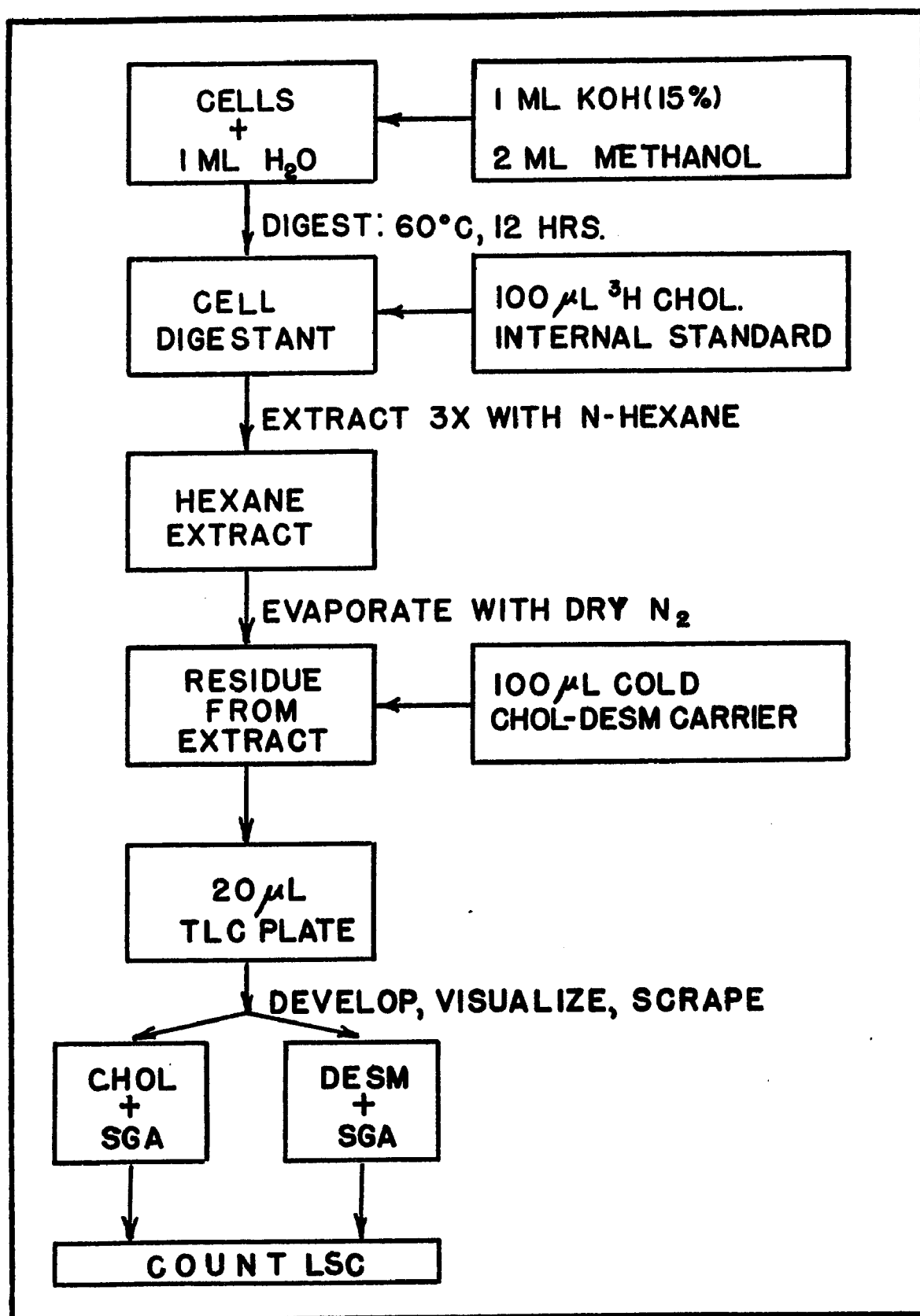


FIG.5 ASSAY OF CELLS FOR C^{14} CHOLESTEROL AND DESMOSTEROL.

resolving the ^3H counts from the ^{14}C counts.

For this reason a formula has been derived to compute the extraction loss, f , from quench curves for ^3H and ^{14}C and from the CPM of channels A, B, and C* respectively. This formula is derived in Appendix B and is:

$$f = \frac{(a - (\lambda - \pi)^{-1} \cdot (c/s - \pi \cdot a)) \cdot \text{Effa}(\text{H3:100})}{\sigma \cdot s \cdot a(\text{H3:100})}$$

where λ , π and σ are quench constants, $\text{Effa}(\text{H3:100})$ is the efficiency of channel A for ^3H with 100 microliters of water quench but with no SGA quench, $a(\text{H3:100})$ is the CPM of channel A for the ^3H -cholesterol internal standard in 100 microliters of water with no SGA quench, and a and c are the double label CPM in channels A and C, respectively, with SGA quench. The assumptions made in the derivation of the formula for f are linear quenching and independence of the external standard ratio, s , to the radioactivity in the sample.

If the internal standard method is used to determine f , then

$$f = \frac{(a - ((c - \beta \cdot a) / (\alpha - \beta))) \cdot \text{Effa}(\text{H3:100})}{\text{Effa}(\text{H3:SGA}) \cdot a(\text{H3:100})}$$

*Channel A is the ^3H channel, channel B is the ^{14}C channel, and channel C is a channel that only counts ^{14}C even in the presence of ^3H .

where $EFFa(H3:SGA)$ is the efficiency of the sample for SGA and α and β are the C/A constants defined in Appendix C.

G. Viability Studies

A number of stains have been used to measure cell viability (Hanks and Wallace, 1958; Phillips and Terryberry, 1957). Among these, erythrosin B and methylene blue represent the major types in common use. Erythrosin B is a cellular exclusion dye whereas methylene blue is a metabolic indicator dye. Both of these properties have been used as a measure of cell viability.

A study was conducted to monitor the reaction of the Burkitt lymphoma cells to these two particular stains over a time period which spanned the length of a typical uptake or release transport experiment at 26°C. A cell suspension in 25% FBS-HEPES buffered media was prepared, and samples of the cell suspension were taken at different times. An aliquot of each sample was then exposed to Erythrosin B and methylene blue for 5 minutes, the total number of cells and the number of stained cells were determined by count on a hemocytometer. Only the unstained cells were assumed to be viable. The error in counting on the hemocytometer is at least 10%.

VI. EVALUATION OF EXPERIMENTAL TECHNIQUE

A. Pipetting and Coincidence Error (PC-ERROR)

In most counting instruments there exists an upper limit on the input level such that further changes in this level do not elicit a proportional change in the output level. One reason for such nonlinear I/O behavior is error due to coincidence counting. This occurs when the finite response time of the instrument is exceeded so that two or more incidences are recorded as one. Such errors could occur both in liquid scintillation counting (LSC) where β -emissions are monitored, and in-Coulter counting, where the number of cells in a solution are counted. Another possible systematic error is due to adsorptive pipetting.

In normal pipetting, one would expect a random error. If, however, cells or radioactive compounds are adsorbed onto the pipet, then the delivered solution would give a lower count than the pipetted solution.

One method of detecting the existence of these two type of systematic errors is by sequential 1:2 dilutions. If n is the dilution number, then we can define

$$\text{PC-ERROR} = \frac{X_n - 2 \cdot X_{n+1}}{2 \cdot X_{n+1}}$$

as the error due to both pipetting and coincidence where X is the number of counts observed. The PC-ERROR would be expected to be negative for coincidence error and positive for adsorptive pipetting error.

1. PC-ERROR in Liquid Scintillation Counting

Two studies were conducted to determine the PC-ERROR in liquid scintillation counting (LSC). In the first study coincidence was considered as a possible source of error in double label counting and in efficiency determinations. When one designs a $^3\text{H}/^{14}\text{C}$ double label experiment, it is usually advisable to have the ^3H counts about ten times the ^{14}C counts. For this reason, in the double label experiments used in this study to assay cholesterol and desmosterol, the ^3H counts were usually maintained around 50,000 counts per minute (CPM). Determinations for efficiency were also measured around 50,000 CPM. If coincidence was a factor in this latter situation, this would indicate that the efficiency of the quenched liquid scintillation cocktail system was not independent of the amount of radioactivity present. Therefore, efficiency determinations measured around 50,000 CPM could not be expected to hold at 4,000 CPM, the normal assay activity.

Calibrated ^{14}C -toluene standards were diluted from 80,000 DPM and counted by LSC with Aquasol to

determine the possibility of coincidence counting error. Table 2 summarizes the results of this study. Since the efficiency of the ^{14}C channel and the PC-ERROR seem to be randomly distributed, it appears that pipetting and coincidence error are not significant for LSC up to 83,000 DPM for ^{14}C -toluene; further, in this unquenched system, the average efficiency was found to be 0.8935 ± 0.0054 . This constant efficiency over the entire range of dilutions further confirmed the absence of coincidence counting.

The second study was conducted to evaluate the PC-ERROR in pipetting the aqueous ^3H -cholesterol solution, which was used as the internal standard in the TLC assay. Since the activity levels ranged as high as 1.5×10^6 DPM, coincidence counting was also a possibility. Table 3 summarizes the results of this study. In this case, the positive PC-ERROR seems to indicate that adsorptive pipetting was greater than coincidence error, and caused a systematic underestimation of the actual activity in the solution. The maximum amount of this error was 2% of the actual activity.

2. PC-ERROR in Coulter Counting the Cell Population

The number of cells used in the transport experiments was determined by Coulter counting. After the transport experiments were begun, a 1 ml aliquot of cell

Table 2. ^{14}C -toluene pipetting and coincidence error

n	DPM	$\bar{\bar{X}}$	σ	EFF	PC-ERROR
0	83,000	73,885	828	0.890	
0	41,500	37,432	398	0.901	-0.013
1	20,750	18,647	400	0.897	+0.004
2	10,375	9,297	194	0.893	+0.003
3	5,188	4,617	119	0.885	+0.007
4	2,594	2,342	47	0.893	-0.014
5	1,297	1,176	36	0.887	-0.004
6	648	609	12	0.900	-0.034
7	324	315	12	0.895	-0.033
8	162	171	4	0.895	-0.079

n = dilution number for 1:2 dilutions

$\bar{\bar{X}}$ = grand mean of the CPM in the ^{14}C channel for three samples, each averaged over three countings

σ = standard deviation of the grand mean, $\bar{\bar{X}}$

EFF = the efficiency of the ^{14}C channel

PC-ERROR = pipetting and coincidence error

$$= \frac{\bar{\bar{X}}_n - 2 \cdot \bar{\bar{X}}_{n+1}}{2 \cdot \bar{\bar{X}}_{n+1}}$$

where $\bar{\bar{X}}_n = \bar{\bar{X}}_n - \text{Background in } ^{14}\text{C channel}$

Table 3. Aqueous ^3H -cholesterol Pipetting and Coincidence Error

n	$\bar{X}$	σ	PC-ERROR
0	1,468,489	13,874	
1	726,744	7,750	+0.010
2	361,722	6,333	+0.005
3	176,130	3,607	+0.027
4	86,975	258	+0.013
5	42,831	222	+0.015
6	20,840	207	+0.028
7	10,343	61	+0.007
8	5,130	27	+0.008
9	2,522	23	+0.017
10	1,275	11	-0.011
11	630	3	+0.012

n = dilution number for 1:2 dilutions

$\bar{X}_n$ = grand mean of the CPM in the ^3H channel minus the background for three samples, each averaged over three countings

σ = the standard deviation of the grand mean, $\bar{X}_n$

PC-ERROR = pipetting and coincidence error

$$= \frac{\bar{X}_n - 2 \cdot X_{n+1}}{2 \cdot \bar{X}_{n+1}}$$

suspension (containing cells of approximately 15 microns) was diluted with normal saline and 0.5 ml was counted with a 70 micron aperture tube on the Coulter counter.¹⁴ Dilutions of a concentrated cell suspension were made to determine the PC-ERROR. Table 4 summarizes the results of this investigation. The negative PC-ERROR indicates that coincidence counting seems to be the dominant factor. However, at the higher dilutions where the counts are less than 40,000 counts/0.5 ml, the coincidence error is less than 1%. In the actual transport experiments, most of the samples were counted in the range of 20,000 to 30,000 counts/0.5 ml.

B. Double Label Counting

A study was conducted to evaluate the accuracy of double label counting by the external and the internal standard methods. This analysis suggested the most accurate procedure for the particular assay at hand.

1. External Standard Method

Tables 5 and 6 show the effect of increasing amounts of SGA quench on the efficiencies of channels A and C and on s, the external standard number, for ¹⁴C and ³H

¹⁴Coulter Electronics, Hialeah, Florida.

Table 4. Pipetting and coincidence error in counting the cell population with the Coulter counter

n	Counts	$\bar{X}_n$	σ	PC-ERROR
0	146,378 142,684 140,075 137,469 144,221	142,185	3,473	-11.3%
1	81,357 79,955 79,320 79,867	80,125	869	-6.7%
2	44,360 43,528 42,047 41,928	42,941	1,142	-0.8%
3	21,786 22,153 21,278 21,370	21,647	403	-0.4%
4	10,962 11,505 10,337 10,299	10,776	573	

n = the dilution number for 1:2 dilutions

$\bar{X}_n$ = the mean of the counts for the n^{th} dilution

σ = the standard deviation of $\bar{X}_n$

$$\text{PC-ERROR} = \frac{\bar{X}_n - 2 \cdot \bar{X}_{n+1}}{2 \cdot \bar{X}_{n+1}}$$

Coulter counter conditions:

70 micron aperture tube

normal saline

0.5 ml of cell suspension counted

15 micron cells in diameter

Table 5. ^{14}C quench curve for silica gel-silver nitrate quench at the 41,500 DPM level

Quench (mg)	EFFa	EFFc	s
0.5	0.220	0.592	1.750
1.0	0.219	0.592	1.769
2.0	0.216	0.600	1.789
3.0	0.219	0.597	1.755
5.0	0.216	0.600	1.741
7.0	0.215	0.605	1.810
10.0	0.219	0.602	1.775
$\bar{X} =$	0.218	0.598	1.770
$\sigma =$	0.002	0.005	0.024

EFFa = the efficiency of the ^3H channel for ^{14}C

EFFc = the efficiency of a channel which only counts ^{14}C even in the presence of ^3H

s = the external standard channels ratio number for the Beckman LS-200 liquid scintillation counter

$\bar{X}$ = the mean of the column indicated

σ = the standard deviation of the column indicated

Table 6 ^3H quench curve for silica gel-silver nitrate quench at the 312,000 DPM level

Quench (mg)	EFFa	EFFc	s
0.5	0.340	0.00038	1.804
1.0	0.344	0.00038	1.822
2.0	0.342	0.00036	1.865
3.0	0.343	0.00038	1.831
5.0	0.348	0.00040	1.861
7.0	0.348	0.00040	1.888
10.0	0.345	0.00036	1.815
$\bar{X} =$	0.344	0.00038	1.841
$\sigma =$	0.003	0.000016	0.031

EFFa = the efficiency of the ^3H channel for ^3H

EFFc = the efficiency of a channel which only counts ^{14}C even in the presence of ^3H

s = the external standard channels ratio number for the Beckman LS-200 liquid scintillation counter

$\bar{X}$ = the mean of the column indicated

σ = the standard deviation of the column indicated

respectively. From these studies we observe that (1) the levels of SGA quench used in the thin layer chromatography assay do not cause any change in the efficiencies for either ^{14}C or ^3H , and (2) the external standard number, s , does seem to change with the amount of activity in the sample. This latter effect can be seen by comparing the s values for Tables 5 and 6.

2. Internal Standard Method

In order to evaluate the internal standard method, ^{14}C -toluene and ^3H -toluene were diluted and counted by LSC. The activity in these dilutions along with their variations are given in Table 7. After $^3\text{H}/^{14}\text{C}$ mixtures were made of these dilutions, three countings were made: first, of the mixtures themselves; second, of the mixtures after ^{14}C -toluene internal standard was added; and third, of the mixtures after ^{14}C -toluene and ^3H -toluene internal standard were added. In this way, the quench parameters α and β were calculated for each mixture, and $A(\text{H}3)$ and $C(\text{C}14)$, the calculated DPM of ^3H in channel A and ^{14}C in channel C, respectively, were computed by the internal standard method of Appendix C. Table 8 shows a comparison of the actual activity versus the computed activity for each mixture. From this table it is evident that when the ratio $^3\text{H}/^{14}\text{C}$ becomes very large, the internal

Table 7. ^3H and ^{14}C standard toluene dilutions for the double label evaluation of known mixtures

		Counts per minute		External Standard Ratio (S)
		Channel A	Channel C	
^3H	$\bar{X} =$	53,092	60	1.794
	$\sigma =$	612	5.9	0.021
Toluene	$\bar{X} =$	26,287	33	1.781
	$\sigma =$	482	0.92	0.011
	$\bar{X} =$	13,100	21	1.772
	$\sigma =$	233	1.6	0.018
	$\bar{X} =$	878	2294	1.765
	$\sigma =$	30.1	69.1	0.022
	$\bar{X} =$	455	1170	1.764
	$\sigma =$	20.3	6.2	0.011
C^{14}	$\bar{X} =$	245	595	1.767
	$\sigma =$	4.7	8.9	0.020
Toluene	$\bar{X} =$	141	304	1.765
	$\sigma =$	10.2	5.0	0.010
	$\bar{X} =$	81	148	1.746
	$\sigma =$	3.2	5.7	0.028
	$\bar{X} =$	51	80	1.745
	$\sigma =$	4.1	2.7	0.005

Channel A is the ^{14}C channel and channel C is a channel which only counts ^{14}C in the presence of ^3H .

Table 8. Evaluation of A(H3) and C(C14) from H3/C14 mixtures using the internal standard method for double label counting

H3/C14 Standard Mixtures	A(H3)	% dev. A(H3)	C(C14)	% dev. C(C14)
53,092/2294	53,693	1.1	2387	4.1
53,092/1170	53,774	1.3	1211	3.5
53,092/595	53,973	1.7	599	0.7
53,092/304	53,919	1.6	265	12.8
53,092/148	53,461	0.7	152	2.7
53,092/80	53,551	0.9	91	13.8
26,529/2294	26,529	0.9	2325	1.4
26,529/1170	26,647	1.4	1193	2.0
26,529/595	26,843	2.1	560	-5.9
26,529/304	26,772	1.8	276	-9.2
26,529/148	26,949	2.5	161	8.8
26,529/80	27,013	2.8	53	-33.8
13,465/2294	13,465	2.8	2340	2.0
13,465/1170	13,469	2.8	1160	-0.9
13,465/595	13,476	2.9	569	-4.4
13,465/304	13,398	2.3	290	-4.6
13,465/148	13,436	2.6	175	18.2
13,465/80	13,353	1.9	43	-46.3

A(H3) = the calculated amount of activity of ^3H in the A channel (which is a channel which counts ^3H)

C(C14) = the calculated amount of activity of ^{14}C in the channel (which is a channel which only counts ^{14}C even in the presence of ^3H)

standard method begins to give large variations. This behavior is probably due to poor counting statistics at the lower ^{14}C levels and to changes in the efficiency of the Beckman LS-200 between the three LSC countings.

3. Method Used in the Thin Layer Chromatography Assay

Since SGA quench did not change the counting efficiencies of ^{14}C and ^3H over the range of quench used in the TLC assay and since the variation from one counting to the next with the internal standard method was not satisfactory, all double label analyses were calculated from one counting. This was accomplished by determining the α and β parameters from separate samples of ^3H -toluene and ^{14}C -toluene with SGA quench. These samples were counted at the same time as the TLC samples to assure uniformity of machine function.

C. Thin Layer Chromatography (TLC) Assay

These studies were conducted to insure that the TLC assay for ^{14}C -desmosterol and ^{14}C -cholesterol was quantitative, that the level of ^{14}C -cholesterol impurities in the ^{14}C -desmosterol was low, and that both cholesterol and desmosterol extracted from the aqueous phase with n-hexane and eluted from the SGA plates with the same efficiencies.

1. Assay for ^{14}C -cholesterol Impurities

The ^{14}C -desmosterol that was used in these studies was assayed for impurities using the 100:0 and the 95:5 chloroform/acetone solvent systems on SGA plates. The results of these studies are given in Tables 9 and 10, respectively. They show that (1) both solvent systems can quantitatively separate cholesterol and desmosterol, and (2) the amount of ^{14}C -cholesterol impurity of ^{14}C -desmosterol is less than 1%.

2. Resolution of Different Solvent Systems

A number of different solvent systems were investigated for the resolution of cholesterol and desmosterol on SGA plates. If the resolution of a solvent system is expressed as

$$R = \frac{X_c - X_d}{W_c/2 + W_d/2}$$

where X_c and X_d are the distances that the cholesterol and desmosterol spots have traveled from the origin respectively, and W_c and W_d are the dimensions of their respective spots in the direction of the solvent migration, then Table 11 gives some resolution characteristics of the more successful solvent systems that were found in this study. The best resolution was obtained with the 95:5 chloroform to acetone system.

Table 9. Quantitative thin layer chromatography assay of ^{14}C -desmosterol for ^{14}C -cholesterol impurities

Desmosterol (CPM)	Cholesterol (CPM)	Blank (CPM)	CB/DB
2047	66.4	29.4	0.0183
2069	58.8	31.4	0.0134
1196	55.8	29.2	0.0228
2156	59.2	34.6	0.0116
1981	53.8	34.2	0.0101
1935	58.2	30.8	0.0144
2037	59.4	29.2	0.0150
2221	59.0	33.2	0.0118
2021	63.2	34.0	0.0147
1968	51.0	30.4	0.0105
2097	54.8	30.6	0.0117
			$\bar{X} = 0.0140$
			$\sigma = 0.0038$

100:0 chloroform:acetone solvent system

CB/DB [Cholesterol - Blank]/[Desmosterol - Blank]

Table 10. Quantitative thin layer chromatography assay of ^{14}C -desmosterol for ^{14}C -cholesterol impurities

Desmosterol (CPM)	Cholesterol (CPM)	Blank (CPM)	CB/DB
7195	107	37	0.0097
7099	82	37	0.0064
7553	68	42	0.0038
7671	93	42	0.0070
7674	96	40	0.0076
7517	91	40	0.0071
7872	86	33	0.0057
8279	90	33	0.0059
8242	83	36	0.0048
7887	89	36	0.0058
			$\bar{X} = 0.0064$
			$\sigma = 0.0016$

95:5 chloroform:acetone solvent system

$$\text{CB/DB} = [\text{Cholesterol} - \text{Blank}] / [\text{Desmosterol} - \text{Blank}]$$

Table 11. Resolution characteristics of different solvent systems

Solvent Systems	R	R _F x10		Time (min)
		C	D	
Benzene:Methanol				
98:2	1.29	1.02	0.69	40
95:5	1.06	1.52	1.21	40
Benzene:Acetone				
96:4	0.80	2.68	2.40	45
95:5	0.91	3.02	2.67	45
94:6	0.84	3.14	2.87	45
Chloroform:Acetone				
100:0	2.00	1.15	0.97	40
95:5	2.28	2.66	2.00	40

$$R = \frac{\frac{X_c}{W_c} - \frac{X_d}{W_d}}{\frac{1}{2} + \frac{1}{2}}$$

where X_c and X_d are the distances that cholesterol and desmosterol, respectively, move from the origin, and W_c and W_d are the corresponding lengths of the cholesterol and the desmosterol spots, respectively, in the direction of the solvent flow.

C = cholesterol

D = desmosterol

Silica gel-silver nitrate thin layer chromatography plates were developed in a sandwich development system (described in the text).

3. Extraction and Elution Efficiencies

A study was conducted to determine the elution efficiency of ^{14}C -cholesterol, ^{14}C -desmosterol and ^3H -cholesterol. Known DPM's of each compound were spotted onto SGA plates, sprayed with phosphoric acid, and charred in an oven at 180°C for 10 minutes. The elution efficiency of these compounds were:

$$\text{EEC14-CHOL} = 0.931 \pm 0.014$$

$$\text{EEC14-DESM} = 0.785 \pm 0.0008$$

$$\text{EEH3-CHOL} = 0.531 \pm 0.023.$$

These results were rather surprising. We expected that there might be some difference in the elution characteristics between ^{14}C -cholesterol and ^{14}C -desmosterol, but it was always assumed that ^3H -cholesterol would elute the same as ^{14}C -cholesterol. Because of these results, the TLC assay was modified to replace the acid charring step necessary for visualizing the compounds with ultraviolet detection of sodium fluorescein.

To test this new procedure, a 2 x 3 factorial designed experiment was conducted to determine whether a mixture of ^3H -cholesterol(A), ^{14}C -cholesterol(C), and ^{14}C -desmosterol(D) extracted with the same efficiency from the aqueous fluid phase (P_1) and the cell plug digestant (P_2), and whether they eluted from the SGA plates with the same efficiency. Mixtures of known activities of these compounds were solubilized in

methanol, added to the parent phase (P_1 or P_2), and extracted with an equal aliquot of n-hexane. This hexane extract was assayed on SGA plates using sodium fluorescein to visualize the compounds and double label analyzed to resolve compounds A and C. From these results the extraction-elution fraction, f , which represents the fractional recovery of a compound, was calculated for compounds A, D, and C (see Table 12). The results show that each of these compounds extracts and elutes with approximately the same efficiencies; this is in sharp contrast to the elution behavior which was found when charring was used to visualize the compounds. When A is used as an extraction standard to monitor the extraction differences from sample to sample, we would like to know of $f(C)/f(A)$ and $f(D)/f(A)$ differ from each other at their own levels and at the P_1 and P_2 levels. Table 13 gives the statistical data for these ratios and their analysis of variance.

In conclusion, these studies have shown that when sodium fluorescein is used to visualize the compounds, rather than the charring procedure, the compounds show no difference with respect to elution from SGA. In addition, all compounds were found to extract equally from the fluid supernatant and from the cell plug digestant.

Table 12. Extraction-elution fraction, f , for ^3H -cholesterol (A), ^{14}C -cholesterol (C), and ^{14}C -desmosterol (C) from the fluid phase (P_1) and the cell phase (P_2)

f	A	C	D
P_1	0.0541	0.0569	0.0552
	0.0474	0.0502	0.0465
	0.0551	0.0590	0.0560
	0.0557	0.0573	0.0550
P_2	0.0687	0.0725	0.0684
	0.0537	0.0584	0.0560
	0.0584	0.0628	0.0603
	0.0548	0.0598	0.0587

f is defined as the activity recovered after extraction from P_1 and P_2 with n -hexane and elution with Aquasol divided by the activity originally in P_1 and P_2 .

Note: There was a 1:15 dilution factor in these studies so that 100% recovery would give an f value of 0.067.

Table 13. Analysis of variance for $f(C)/f(A)$ and $f(D)/f(A)$

	$f(C)/f(A)$	$f(D)/f(A)$
P_1	1.0518	1.0203
	1.0591	0.9810
	1.0708	1.0163
	1.0287	0.9874
	$\bar{X} = 1.0526$	$\bar{X} = 1.0013$
	$\sigma = 0.0178$	$\sigma = 0.0199$
P_2	1.0553	0.9956
	1.0875	1.0428
	1.0753	1.0325
	1.0730	1.0712
	$\bar{X} = 1.0728$	$\bar{X} = 1.0355$
	$\sigma = 0.0133$	$\sigma = 0.0312$
$s_e^2 = 0.00047$		$f_e = 12$
$(s_p^2)_I = 0.00020$		$f_I = 1$
$(s_p^2)_C = 0.00784$		$f_C = 1$
$(s_p^2)_R = 0.00296$		$f_R = 1$
$(F_I)_{calc} = 0.42$		$(F_I)_{95\%} = 244$
$(F_C)_{calc} = 16.33$		$(F_C)_{95\%} = 244$
$(F_R)_{calc} = 6.17$		$(F_R)_{95\%} = 244$

where f is the extraction-elution fraction for ^3H -cholesterol(A), ^{14}C -cholesterol(B), and ^{14}C -desmosterol(C) from the fluid phase (P_1) and the cell phase (P_2). For the ANOVA: s_e^2 = error variance, s_p^2 = population variance, I = interaction level, C = column level and R = row level. Here the calculated F values are compared against the F values at the 95% confidence limits.

VII. RESULTS AND DISCUSSION

A. Nonspecific Release Experiments

Initial studies were conducted on a series of nonspecific release experiments to confirm the biexponential transport behavior that Turi (1972) found. The Burkitt lymphoma cells were prepared for the release experiments and sampled as described in section V-B. The assay was nonspecific for ^{14}C -desmosterol in that only the radioactivity of the cell plug was assayed by liquid scintillation counting (LSC) and not the activity associated with a particular isolated chemical species. Figs. 6 and 7 give the results of these experiments at 31°C and 37°C . Experiments were conducted at 0%, 5%, $2\frac{1}{2}\%$, and 30% fetal bovine serum (FBS) concentrations, and in each case the biexponential behavior was observed. The fact that the release profiles are faster with increasing FBS concentrations in the external media is attributed to the serum binding of desmosterol and cholesterol which provides better sink conditions (Turi et al, 1974). Since these release curves were biphasic, it was thought that some of the ^{14}C -desmosterol may have been enzymatically converted to some other metabolite, the

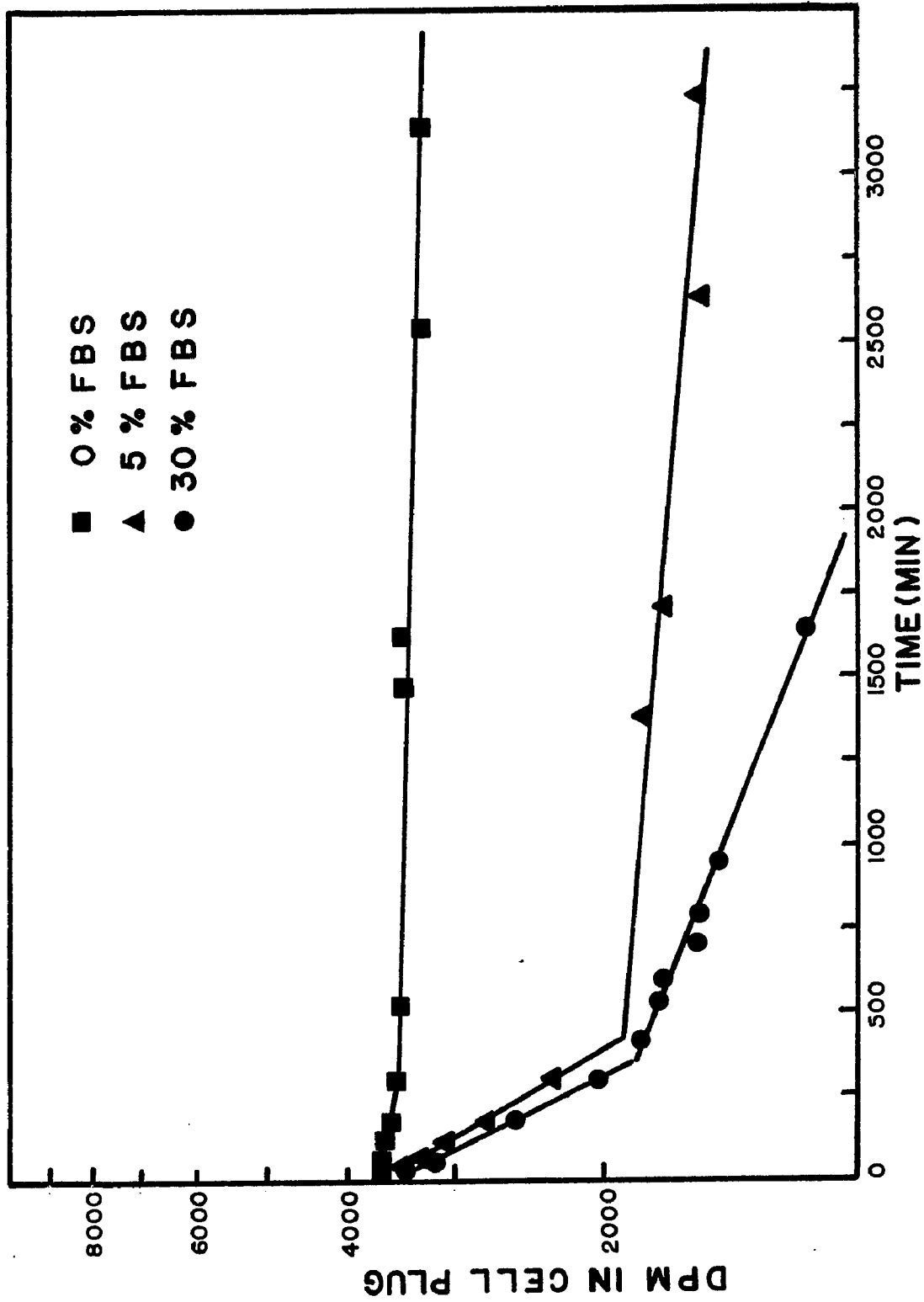


FIG. 6 NON SPECIFIC DESMOSTEROL-26-C¹⁴ RELEASE EXPERIMENTS AT 31° C.

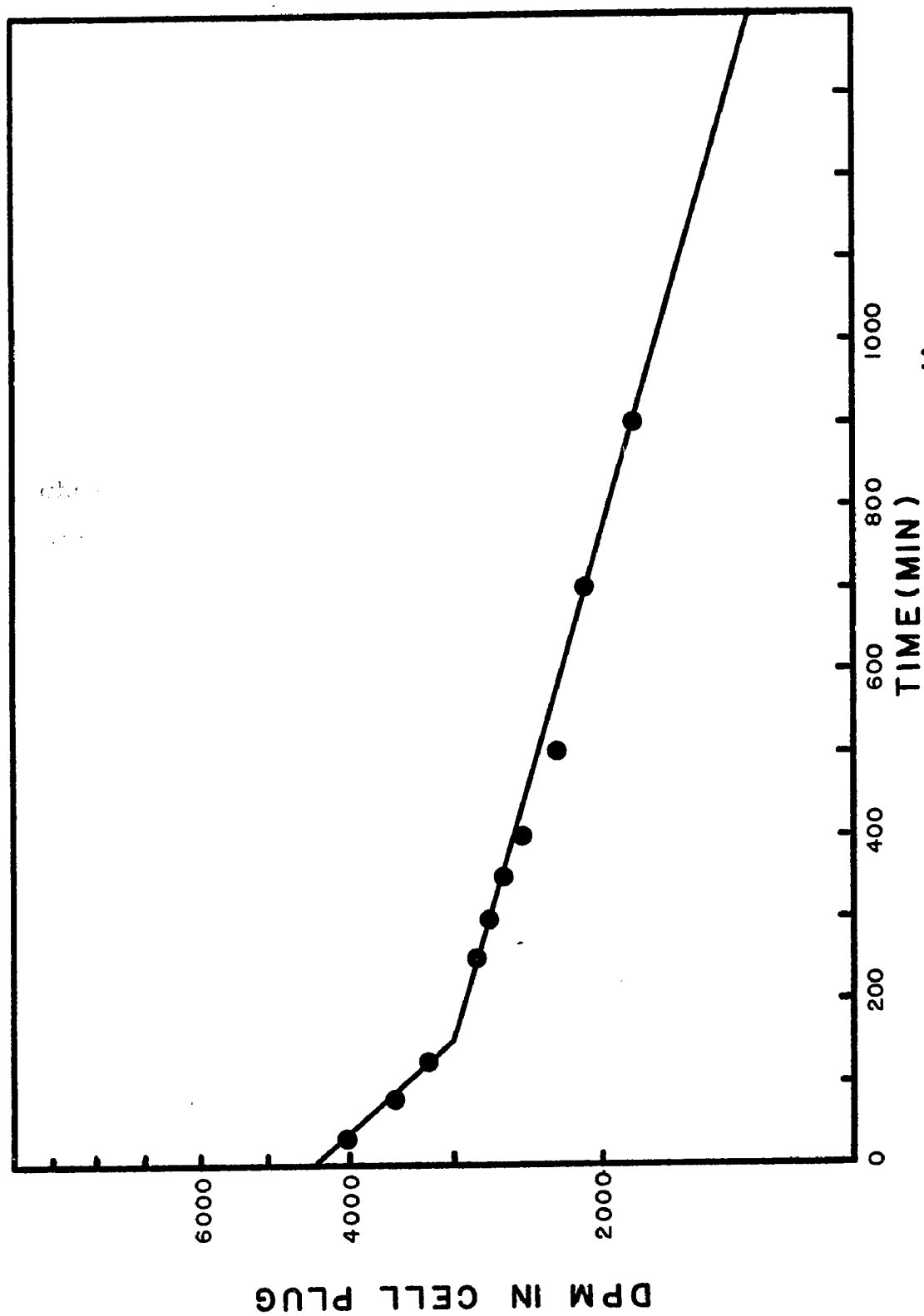


FIG. 7 NON SPECIFIC DESMOSTEROL-26- ^{14}C RELEASE EXPERIMENT AT 37° C. IN 12% FBS GROWTH MEDIA.

most likely being ^{14}C -cholesterol. If this hypothesis proved true, then it was speculated that the biphasic behavior might be explained by a "fast release" of ^{14}C -desmosterol followed by a "slower release" of ^{14}C -cholesterol. This explanation seemed reasonable since Turi (1972) has shown that the effective permeability of desmosterol in such nonspecific release experiments is approximately thirty times faster than cholesterol in this particular cell line. However, as will be shown, mathematical modeling and confirmatory experimental evidence indicate that this explanation is too simple for the simultaneous transport and biosynthetic situation. These experiments were valuable, nevertheless, because they seemed to show that the bioconversion of ^{14}C -desmosterol by the Burkitt lymphoma cells was a viable possibility. It soon became evident that an assay was needed that could isolate and quantify this conversion, if it indeed occurred. Such an assay was developed and has been discussed in section V-C. With this assay, the cellular conversion of ^{14}C -desmosterol to ^{14}C -cholesterol remained to be confirmed.

B. Cellular Biosynthesis of ^{14}C -cholesterol from ^{14}C -desmosterol

In order to determine the existence and the extent of the cellular bioconversion of ^{14}C -desmosterol,

a study was conducted on a large number of Burkitt lymphoma cells grown in suspension with ^{14}C -desmosterol. The procedure for this study has been discussed in the experimental section. Fig. 8 shows the results of a thin layer chromatography (TLC) assay of the resulting extract. Although complete separation of ^{14}C -desmosterol from ^{14}C -cholesterol was not achieved, it was evident from this study that ^{14}C -cholesterol was definitely being synthesized. The total amount of ^{14}C -impurities in the ^{14}C -desmosterol and the other metabolites that might also have been synthesized from ^{14}C -desmosterol were estimated to be about 3.6%.

C. Heat-treated and Aged Cells

Experiments were conducted to determine the effective permeability, P_{ae} , for the substrate (desmosterol) in the absence of bioconversion. Cells were first heat-treated at 55°C for periods of time ranging from 15 to 60 minutes; then uptake transport experiments were conducted according to the procedures of section V. TLC assays of the cells at the end of the experiments confirmed the fact that the heat-treatment procedure prevented the biosynthesis of cholesterol from desmosterol.

Equations (4.56), (4.57), and (4.58)

$$\alpha = \gamma/2 + \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (4.56)$$

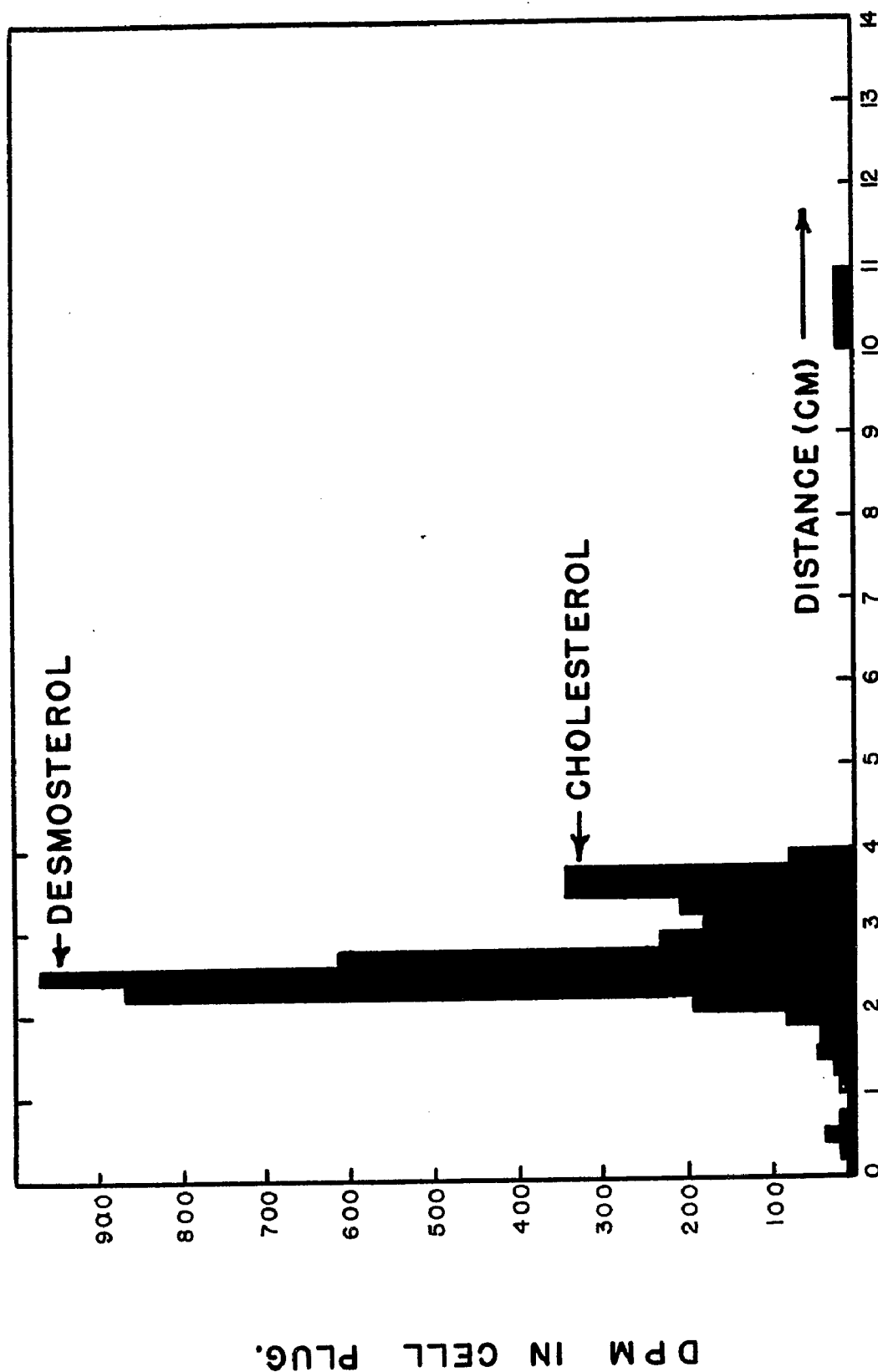


FIG. 8 CELLULAR BIOSYNTHESIS OF ^{14}C CHOLESTEROL FROM ^{14}C DESMOSTEROL IN THE BURKITT LYMPHOMA CELL. ASSAY BY TLC.

$$\beta = \gamma/2 - \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (4.57)$$

$$\gamma = \alpha + \beta = k_1 + k_2 + K \quad (4.58)$$

can be used to evaluate the effect of heat-treatment on P_{ae} . For this situation the biosynthetic rate constant, K , is equal to zero since no cholesterol is synthesized. Therefore β is equal to zero, and

$$\alpha_o \doteq k_1 \quad (7.1)$$

when κ_{ae} , the effective partition coefficient of the substrate, is large, i.e., when

$$k_2 = \frac{k_1}{\kappa_{ae}} \ll k_1 \quad (7.2)$$

(α_o is the experimentally observed first order rate constant for transport when K is equal to zero). Using Eq. (4.84) in Eq. (7.1) we have

$$\alpha_o \doteq \frac{3 P_{ae}}{a} \cdot \frac{n V_i}{V_o} \quad (7.3)$$

P_{ae} was calculated from α_o using Eq. (7.3) for heat-treatment times of 15, 30, and 60 minutes. The following permeabilities were obtained for the 5% FBS transport media:

Time cells were heat-treated at 55°C (min)	$P_{ae} \times 10^7$ (cm/sec)
15	7.3
30	11.7
60	9.9

The variation of P_{ae} with various time periods of heat-treatment of the cells is probably due to sampling variations.* The average of P_{ae} for these three determinations of P_{ae} is 9.6×10^{-7} cm/sec. This also compares favorably with the value 9.2×10^{-7} cm/sec, which can be computed from data for non heat-treated cells (Turi, 1972). Because of the comparable P_{ae} values found with nonviable and viable cells, it appears that in the viable cell system transport is the dominant factor, not biosynthesis.

Additional experiments were also designed to determine whether the permeability, P_{ae} , changes during the course of the experiment. To accomplish this, three separate spinner flasks of cell suspensions were prepared at the same time in 5% FBS media. However, ^{14}C -desmosterol was added to each flask at different

*The cells do seem to be weakened by the heat-treatment and break up with time. This causes an error to be introduced due to the inclusion of a greater amount of trapped fluid in the fragmented portion of the cell plug than normal.

times. In this way, all of the cells were aged in the transport media for the same amount of time, but the time when the uptake experiments were initiated was different for each flask.

Equation (4.58) gives the relationship between the biphasic rate constants, α and β , which are determined experimentally and the biosynthetic rate constant, K . If K is much less than k_1 , the rate constant for the transport of the substrate into the cell, and if k_1 is much greater than k_2 , the rate constant for the transport of the substrate out of the cell (which equals k_1/κ_{ae}), then Eq. (7.3) holds also for the non heat-treated situation in which the rate of biosynthesis is slow compared to the transport, and the effective partition coefficient of the substrate, κ_{ae} , is large. Experimentally, these conditions do hold for the desmosterol-cholesterol system, as we will show later, so that P_{ae} can be determined approximately from the fast rate constant, α , of the nonspecific biexponential plot. The P_{ae} values thus calculated for cells aged from 0, 1605, and 2740 minutes in 5% FBS media were:

Time of cell aging from initiation of experiment (min)	$P_{ae} \times 10^7$ (cm/sec)
0	9.7
1605	10.4
2740	15.4

From these data it appears that (1) the permeability, P_{ae} , does not change more than 50% throughout the course of the experiment; (2) the permeability seems to increase as the cells age; and (3) within the first 1600 minutes, P_{ae} does not seem to change appreciably. Points (1) and (3) are consistent with the observations of Turi (1972) who found that P_{ae} is probably determined within the first 400 minutes of the desmosterol transport experiments. For this reason, P_{ae} for the uptake and release* transport experiments was found to be about the same (Turi, 1972). Point (2), that the permeability might increase with cell age, is plausible since as the cells get older, they might be less able to exclude certain compounds from their surrounding milieu. Dye exclusion and viability studies were conducted to investigate this latter possibility. It must be borne in mind, however, that these studies are capable of distinguishing only gross change in permeability.

D. Viability Studies

If the viability of a cell is assumed by virtue of its ability to exclude certain dyes like erythrosin B

*In the release experiments the cells are aged about 720 minutes during the loading of the ^{14}C -desmosterol into the cells.

or by its ability to reduce a colored dye like methylene blue to its colorless form, then Table 14 gives the viability of the Burkitt lymphoma cells over the period of 4130 minutes at 26°C in 25% FBS-HEPES buffered media. In Table 14 the linear correlation coefficient, r , for the viability of erythrosin B and methylene blue with respect to sampling time was computed up to 2510 minutes, the time span of a normal transport experiment. The small values of r seem to indicate a high degree of noncorrelation. The probability, $P_c(r, N)$, of observing values of the correlation coefficient greater than r for a completely random sample of N observations with v degrees of freedom (Bevington, 1969) is given by

$$P_c(r, N) = \frac{1}{\sqrt{\pi}} \frac{\Gamma[(v+1)/2]}{\Gamma(v/2)} \int_{|r|}^1 (1-x^2)^{\frac{1}{2}(v-2)} dx. \quad (7.4)$$

For erythrosin B and methylene blue at 2510 minutes, $P_c(r, N)$ is greater than 0.5 and 0.4, respectively. This means that it is very probable that the viability is highly uncorrelated with the time of the experiment up to 2510 minutes.

One could also test to see if the means of the viability determined from erythrosin B and methylene blue are significantly different. If this is done, calculations show that both staining techniques are equivalent.

Table 14. Viability of the Burkitt lymphoma cells in 25% fetal bovine serum (FBS) at 26°C by the erythrosin B and the methylene blue staining tests

Sampling Time (min)	Viability (%)	
	Erythrosin B	Methylene blue
0	95	93
245	93	97
435	93	94
650	75*	95
1450	84*	94
1660	94	93
2110	93	95
2510	95	93
3220	92	97
3650	91	94
4130	92	91
$\bar{X} =$	93	94
$\sigma =$	1.3	1.7
$r(2510) =$	+0.12	-0.4

$\bar{X}$ = mean

σ = standard deviation from the mean

$r(2510)$ = linear correlation coefficient of viability
with respect to sampling time up to 2510
min

*values not included in the statistical calculations

E. Specific Transport Experiments

In order to test the mathematical model for simultaneous transport and bioconversion, a 2 x 2 set of factorial designed experiments was planned. If the temperature of the system was held constant at 26°C and the number of cells, n, in the sample of volume, V, was also held constant, then the rate constant ρ (where ρ can be either α or β) can experimentally be dependent on the following parameters: (1) I = initial condition, (2) F = FBS level, and (3) P = the particular sample phase assayed. Thus ρ can be written as $\rho(ipf)$, where i is the subscript which denotes the initial condition level, f is the subscript which denotes the FBS level, and p is the subscript denoting the phase level. Letting

$$i = \begin{cases} 1 & \text{for an uptake experiment} \\ 2 & \text{for a release experiment} \end{cases}$$

$$f = \begin{cases} 1 & \text{for the 10\% FBS level} \\ 2 & \text{for the 25\% FBS level} \end{cases}$$

$$p = \begin{cases} 1 & \text{for an assay of the fluid phase} \\ 2 & \text{for an assay of the cell phase,} \end{cases}$$

then the 2 x 2 block of experiments can be diagrammed as

		F_1	F_2
I_1	P_1	$\rho(111)$	$\rho(112)$
	P_2	$\rho(121)$	$\rho(122)$
I_2	P_1	$\rho(211)$	$\rho(212)$
	P_2	$\rho(221)$	$\rho(222)$

However, according to the proposed mathematical model, we expect degeneracies in $\rho(ipf)$ such that

$$\rho(ipf) = \rho(f) \quad (7.5)$$

so that the only dependence of ρ is on the FBS level and not on the particular initial condition or the sample phase which was assayed. In short, the I and P parameters test the model for consistency.

1. Desmosterol Time Dependence

In Table 15 the initial boundary conditions for the uptake and the release experiments are given. Figs. 9 to 12 show the semi-log time-dependent behavior of desmosterol in the cell plug and in the fluid fractions for 10% and 25% FBS uptake and release experiments at 26°C. The solid lines represent the biexponential fitting of the data. These plots illustrate two important points. First, by comparing the uptake and the release at a given FBS concentration, we note that the cell plug and fluid curves intersect in the uptake experiment at 10% FBS in such a way that the distribution of desmosterol between these two phases in the β -phases is the same as the distribution of desmosterol between these two phases in the corresponding release experiment. Similar behavior is also seen in the 25% FBS experiments,

Table 15. Initial boundary conditions for the uptake and the release of ^{14}C -desmosterol (A) and the simultaneous bioconversion into ^{14}C -cholesterol (B) in the Burkitt lymphoma cells

Compartment	Initial Boundary Condition	
	Uptake experiment	Release experiment
A_o	$A_o(0)$	0
A_i	0	$A_i(0)$
P_o	0	0
P_i	0	$P_i(0)$

A_o = amount of ^{14}C -desmosterol in the external milieu

A_i = amount of ^{14}C -desmosterol in the cells

P_o = amount of ^{14}C -cholesterol in the external milieu

P_i = amount of ^{14}C -cholesterol in the cells

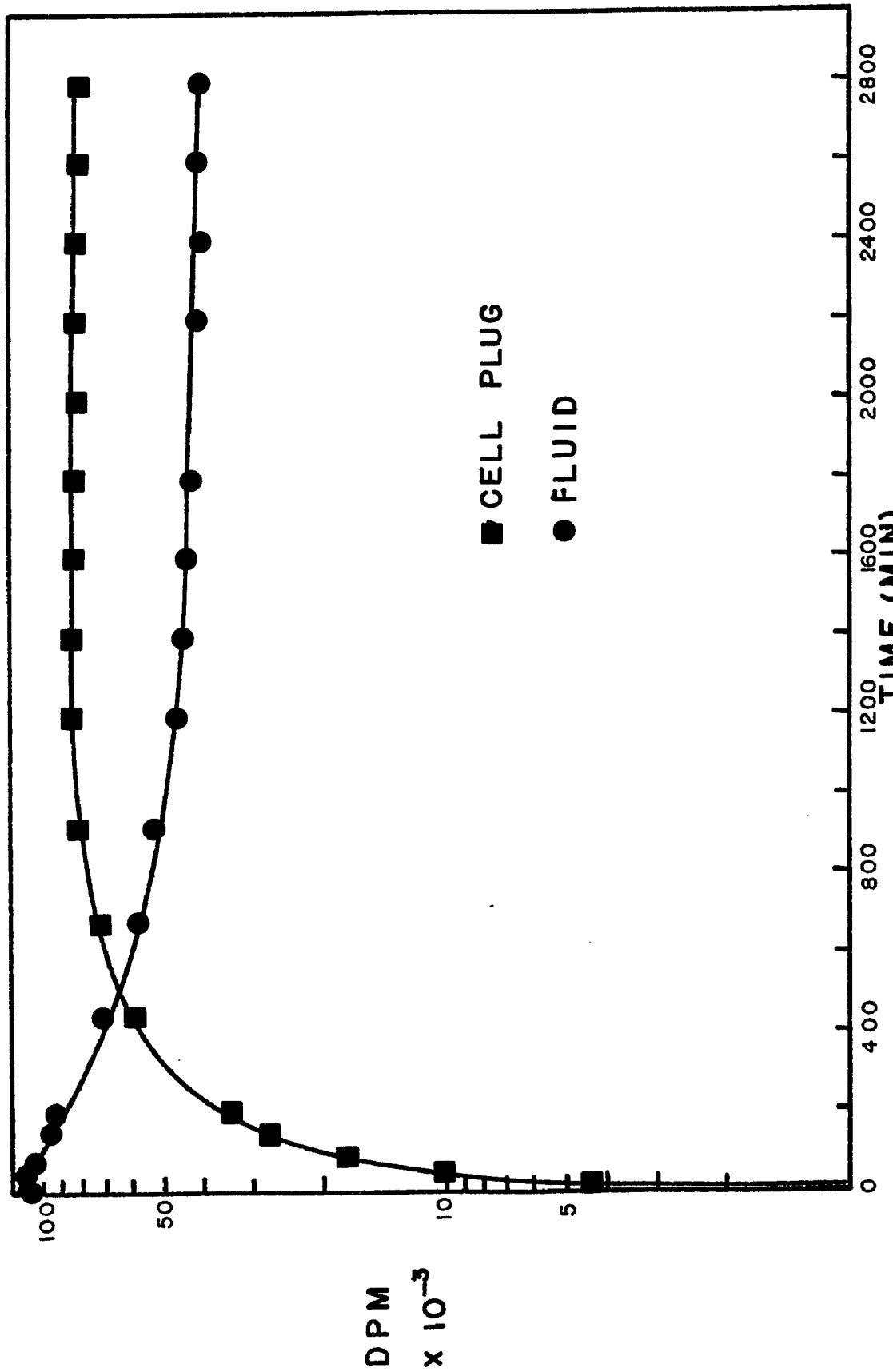


FIG. 9 DESMOSTEROL IN THE CELL PLUG AND FLUID FRACTIONS FOR A 10% FBS UPTAKE EXPERIMENT AT 26°C.

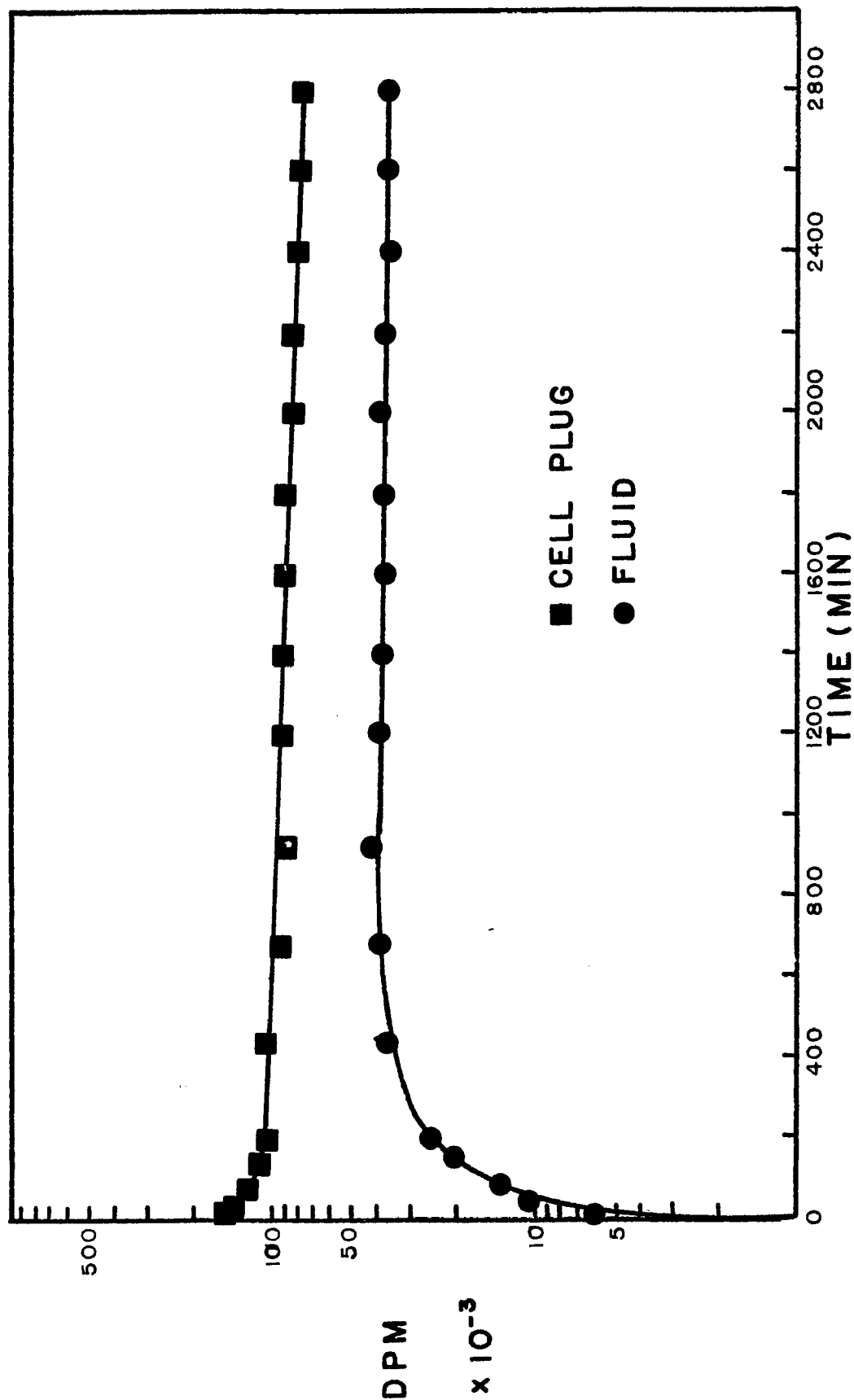


FIG.10 DESMOSTEROL IN THE CELL PLUG AND IN THE FLUID FRACTIONS FOR A 10% FBS RELEASE EXPERIMENT AT 26°C.

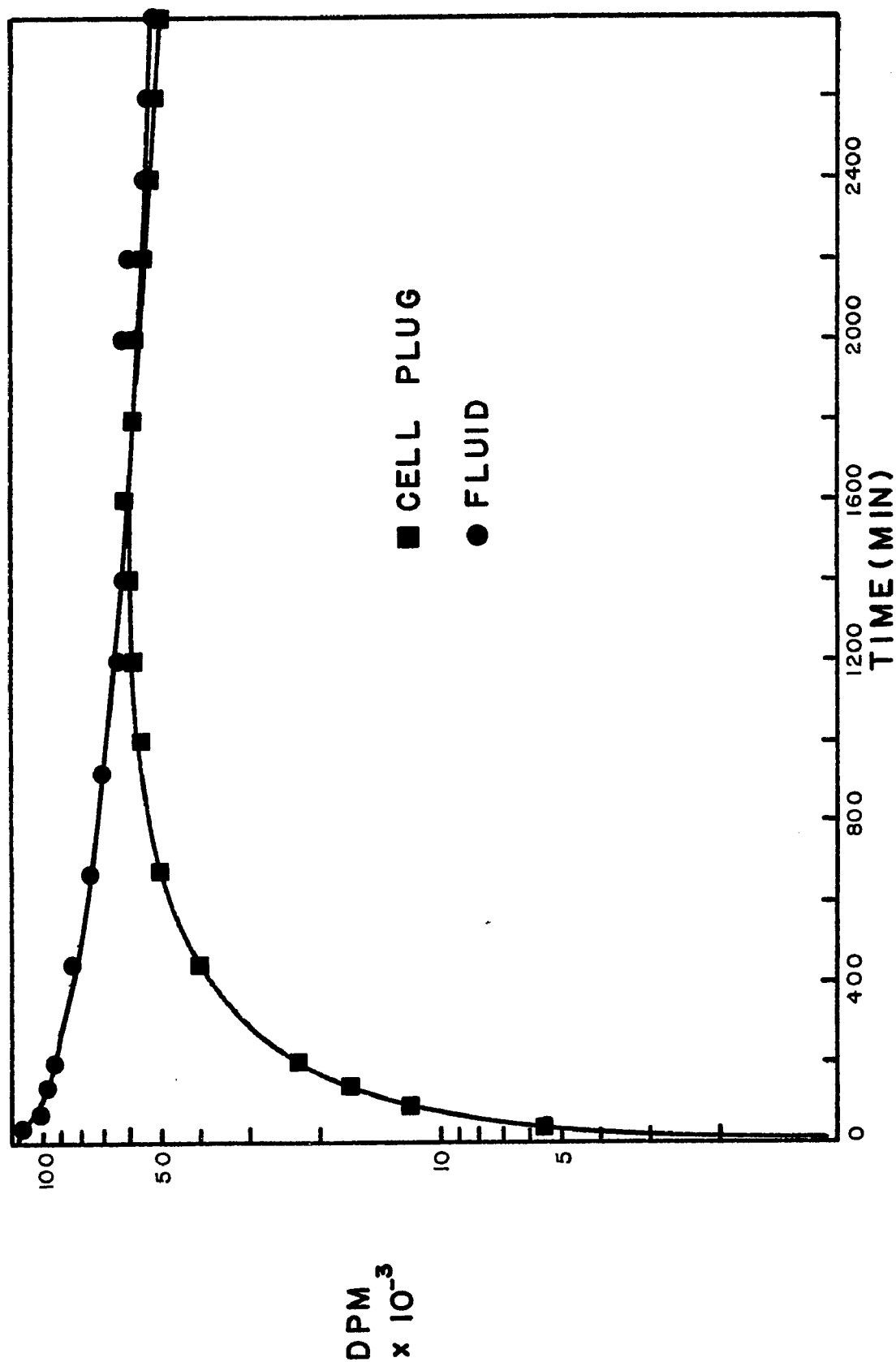


FIG.II DESMOSTEROL IN THE CELL PLUG AND FLUID FRACTION FOR A 25% FBS UPTAKE EXPERIMENT AT 26 °C.

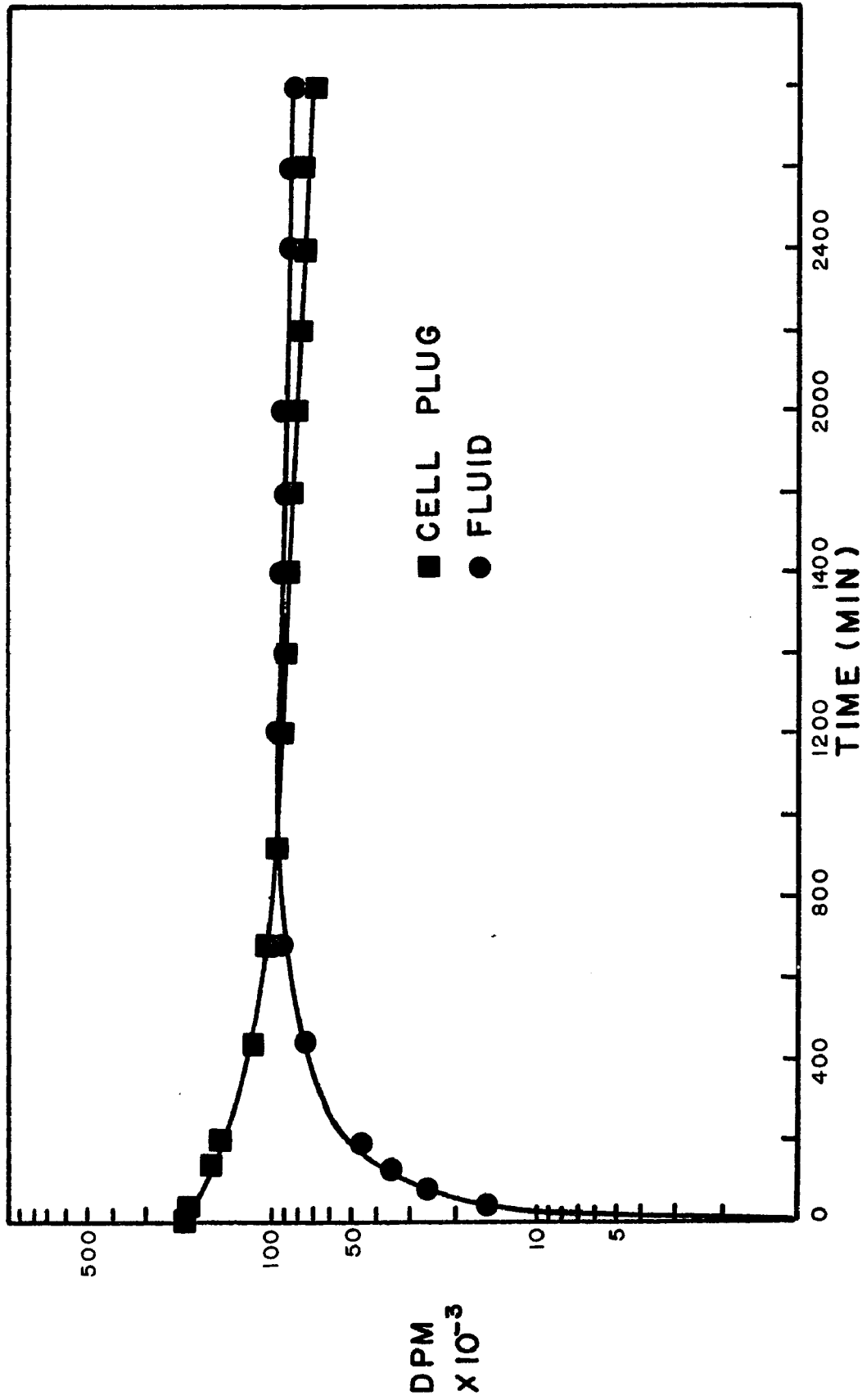


FIG.12 DESMOSTEROL IN THE CELL PLUG AND FLUID FRACTIONS FOR A 25% FBS RELEASE EXPERIMENT AT 26 °C.

except that the release curves intersect, not the uptake curves. The second point that can be noted from these plots is that as the curves approach pure β -phase, the slopes of the curves become parallel. This indicates a form of steady state has been reached, which we will call " β -phase steady state," in which both the fluid and the cell plug curves fall off at the same rate. As we will show, this condition enables the effective permeability, P_{ae} , to be calculated from the " β -phase steady state" asymptote, $(A_i/A_o)_\beta$.

2. Rate Constants, α and β , for Desmosterol

When the rate constants, α and β , were determined by fitting the curves in Figs. 9 to 12 to a bi-exponential function, it was found that the release rate constants were much faster than the corresponding uptake rate constants. It was then questioned whether a mass balance loss that was not accounted for in the assay could explain these results. Since the total amount of ^{14}C -label in the cholesterol-desmosterol system is

$$T = A_i + A_o + P_i + P_o, \quad (4.37)$$

T should be constant if the only mass balance loss of A was by the conversion to P . T was calculated for

the release experiments to check this constancy. Fig. 13 shows T plotted semi-logarithmically against time. These plots seem to show that A and P are not the only factors that determine the mass balance of the system since the decrease in T with time indicates that other factors must be causing T to decrease; e.g., possibly some physical loss or some metabolic loss of the ^{14}C -label from the system. This situation might occur very often in other experimental systems when additional paths of metabolism are available to affect the mass balance. This problem, then, is a realistic one and one of practical importance to further applications of the methodology to real systems.

Since the only metabolic step of interest here is the conversion of A to P , we can blank out the additional mass balance losses in A_i and A_o by appropriately adding on the T curves (see Appendix D). Table 16 gives the α and β rate constants for the desmosterol curves calculated in this way. As we can see, the α rate constants show the expected theoretical degeneracy, $\alpha(f)$, with respect to the F levels except for $\alpha(221)$, the release, cell phase rate constant at the 10% FBS level. However, Table 16 shows no such agreement with respect to $\beta(f)$ where it appears that the release rate constants are faster than the uptake rate constants. This is probably because the β rate constants are more

Figure 13. Total, T , for release experiments.

$T = A_o + A_i + P_o + P_i$, where i denotes the cell interior compartment, o denotes the fluid compartment, A is the amount of substrate in DPM (desmosterol) and P is the amount of product in DPM (cholesterol).

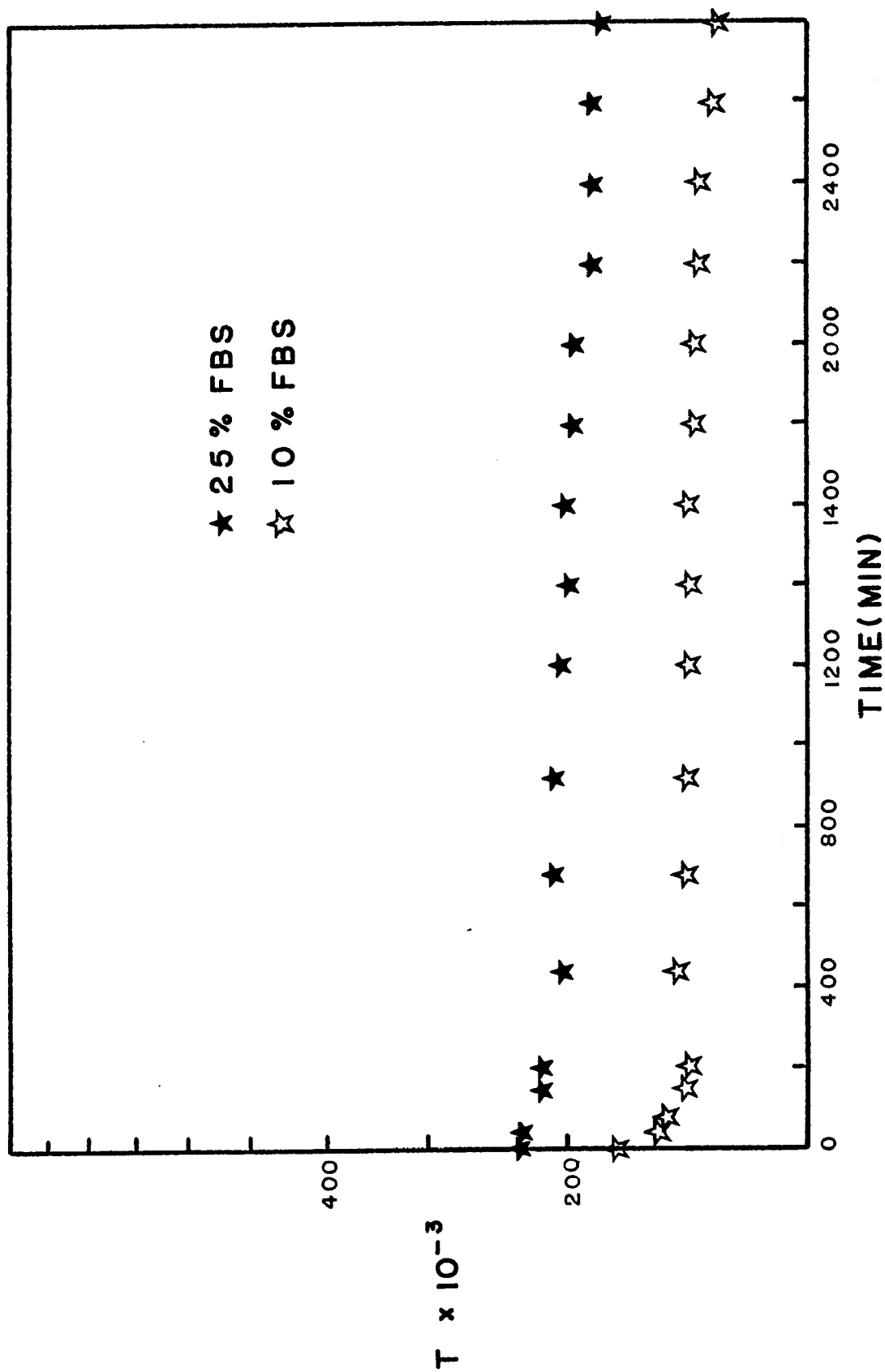


FIG.13 TOTAL, T, FOR RELEASE EXPERIMENTS.

Table 16. Desmosterol rate constants α (ipf) and β (ipf) calculated from the specific uptake and release experiments

$\alpha \times 10^3$ (min ⁻¹)		F ₁	F ₂
I ₁	P ₁	2.6	1.8
	P ₂	2.6	1.8
I ₂ *	P ₁	2.6	1.8
	P ₂	6.8	1.8
$\beta \times 10^5$ (min ⁻¹)		F ₁	F ₂
I ₁	P ₁	3.6	---
	P ₂	3.6	20.0
I ₂ *	P ₁	10.0	21.6
	P ₂	15.4	18.0

*rate constant determined after T adjustment (see Appendix D) where for

F_f f= { 1 for the 10% FBS level
 2 for the 25% FBS level

I_i i= { 1 for an uptake experiment
 2 for a release experiment

P_p p= { 1 for an assay of the fluid phase
 2 for an assay of the cell phase

Note: β (112) was not determined because of too much scatter in the data.

sensitive to changes in K , the biosynthetic rate constant, than are the α rate constants which are essentially determined by the transport parameters k_1 and k_2 when K is small. In a later section we will discuss possible reasons why K may be different for the uptake and the release situations.

3. β -Phase Steady State

It was noted earlier in Figs. 9 and 10 and in Figs. 11 and 12 that the slopes in the β -phase appeared the same. This point is more clearly illustrated in Figs. 14 and 15 where A_i/A_o is plotted against time on a semi-log scale; both the uptake and the release curves approach the same asymptote. From Eqs. (4.53) and (4.68) we have

$$A_i = A_{ib} \cdot e^{-\beta t} + A_{ia} \cdot e^{-\alpha t} \quad (4.53)$$

$$A_o = (A_b - A_{ib}) \cdot e^{-\beta t} + (A_a - A_{ia}) \cdot e^{-\alpha t} \quad (4.68)$$

where

$$A_{ib} = \frac{k_1 A(o) - \beta \cdot A_i(o)}{\alpha - \beta} \quad (4.60)$$

$$A_b = \frac{A(o) - K \cdot A_i(o)}{\alpha - \beta} \quad (4.62)$$

Using the initial conditions

$$A_i(o) = 0 \text{ for the uptake experiments}$$

$$A_o(o) = 0 \text{ for the release experiments}$$

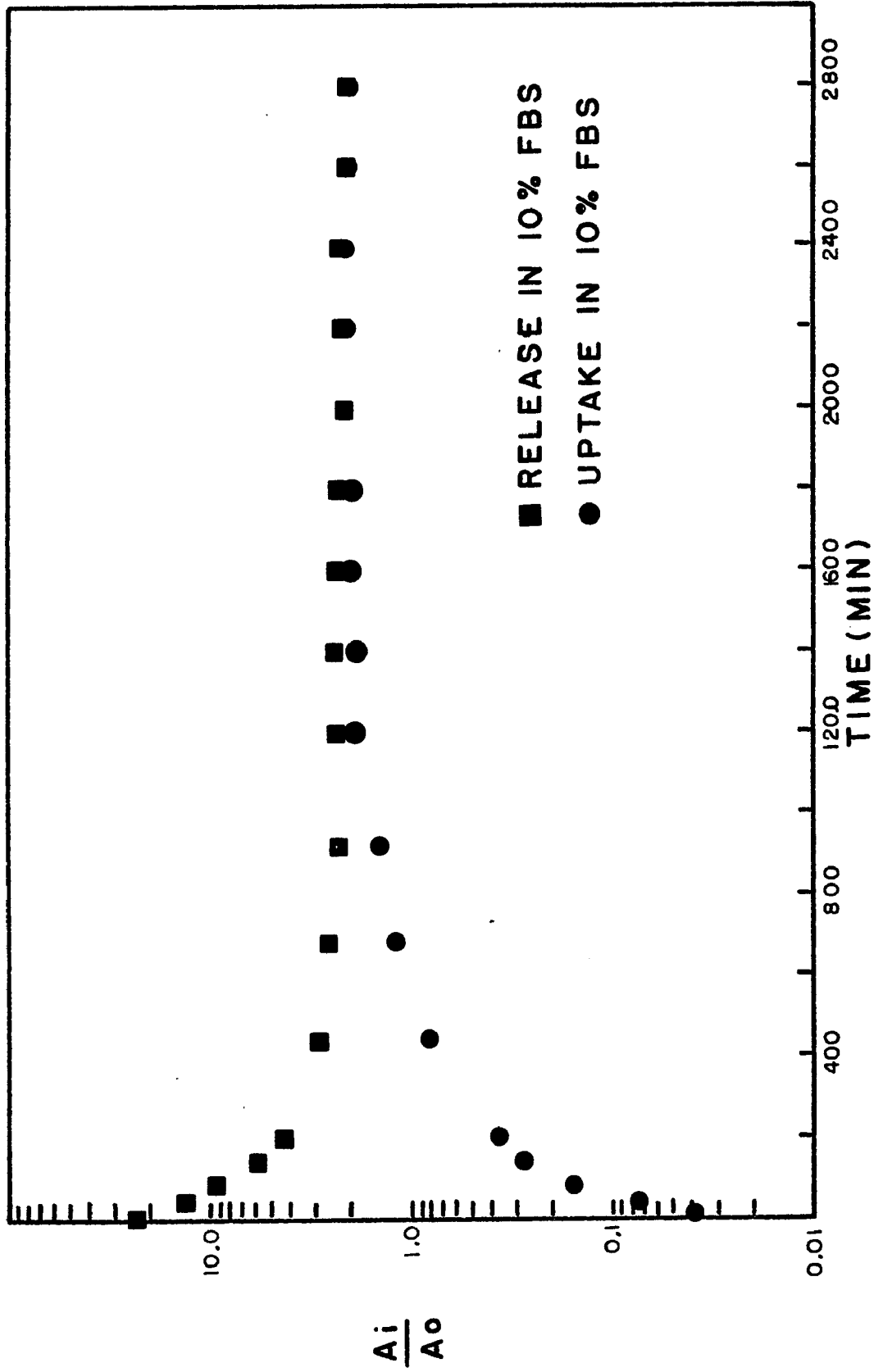


FIG. 14 DETERMINATION OF QUASI-STEADY STATE ASYMPTOTE FROM UPTAKE AND RELEASE EXPERIMENTS AT 26 °C.

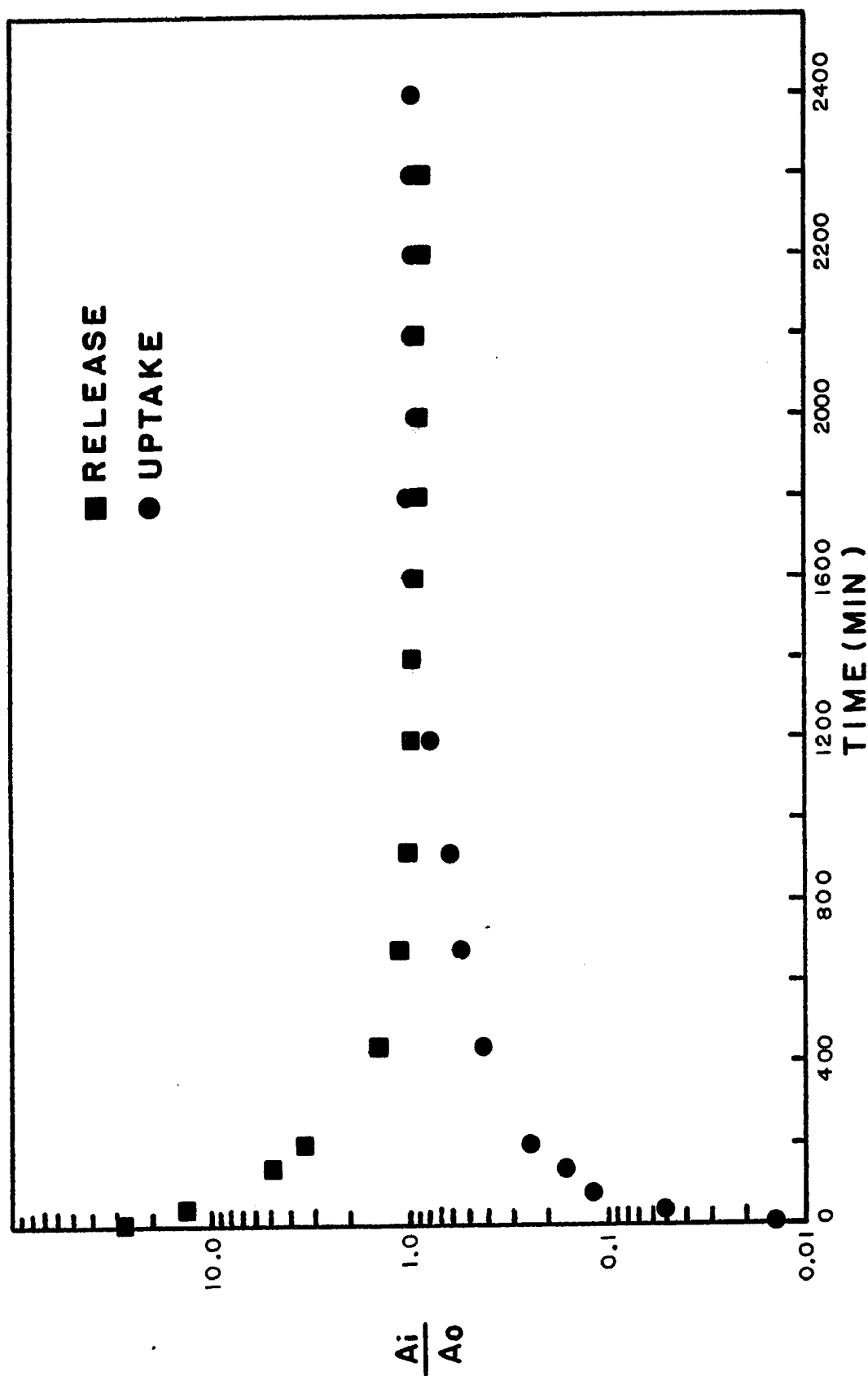


FIG.15 DETERMINATION OF QUASI-STEADY STATE ASYMPTOTE FROM 25% FBS UPTAKE AND RELEASE EXPERIMENTS AT 26°C.

we obtain

$$\left(\frac{A_i}{A_o}\right)_\beta = \frac{k_1}{\alpha - k_1} \quad (7.6)$$

for both the uptake and the release experiments when $t \gg \alpha - \beta$. Thus, we find good agreement between the mathematical model predictions of Eq. (7.6) and the experimental data given in Figs. 14 and 15. Now since

$$k_1 = \frac{3 \cdot P_{ae}}{a} \cdot \frac{n \cdot V_i}{V_o} \quad (4.68)$$

we have

$$P_{ae} = \frac{a \cdot V_o}{3 \cdot n \cdot V_i} \left[\frac{(A_i/A_o)_\beta}{1 + (A_i/A_o)_\beta} \right] \cdot \alpha \quad (7.7)$$

Eq. (7.7) allows one to calculate P_{ae} from α and the β -phase asymptote, $(A_i/A_o)_\beta$.

4. Effective Permeability Coefficient for Desmosterol, P_{ae}

In Table 17, P_{ae} is given for different FBS concentrations. P_{ae} was calculated from the $k_1 \doteq \alpha_o$ condition for the heat treated and the aged experiments and from the β -phase steady state condition for the specific transport experiments. Since the effective permeability, P_{ae} , is related to the serum concentration, S , by

Table 17. P_{ae} calculated under different conditions and different FBS concentrations

FBS (%)	Condition	$P_{ae} \times 10^7$ (cm/sec)
		7.3
5	Heat-treated cells	11.7
		9.8
		9.7
5	Aged cells	10.4
		15.4
10	QU	6.2
25	QU	2.7

QU = cells whose P_{ae} was determined using the β -phase steady state condition (see Eq. (7.6)).

Turi (1972) found the effective permeability coefficient P_{ae} , equal to:

$$9.25 \times 10^{-7} \text{ cm/sec at 5\% FBS}$$

$$4.2 \times 10^{-7} \text{ cm/sec at 15\% FBS}$$

$$3.0 \times 10^{-7} \text{ cm/sec at 30\% FBS,}$$

and the linear serum binding constant ρ_b is 15.7.

$$\frac{1}{P_{ae}} = \frac{1}{P_a} + \frac{\rho_b}{P_a} \cdot S \quad (7.8)$$

where P_a is the intrinsic permeability and ρ_b is the serum binding constant. The calculated P_{ae} from the heat-treated, the aged, and the β -phase steady state conditions were plotted in Fig. 16 and were found to agree fairly well within the linear prediction of Turi (1972).

5. Cholesterol Time Dependence

The cholesterol curves are plotted semi-logarithmically against time in Figs. 17 to 20. A number of qualitative observations can be made from these plots. First, from Figs. 17 and 19 for the uptake experiments, the amount of cholesterol in the fluid is seen to drop and then rise. The drop is probably due to the transport into the cell of the ^{14}C -cholesterol impurities present in the ^{14}C -desmosterol (see section VI-C-1); the rise is due to the synthesis of ^{14}C -cholesterol in the cells and the subsequent transport out of the cells.

Second, for release experiments, the large amount of ^{14}C -cholesterol in the cells at times close to zero seems to indicate that during the room temperature loading period of the ^{14}C -desmosterol into the cells (12 hours), some of the ^{14}C -desmosterol was converted into ^{14}C -cholesterol.

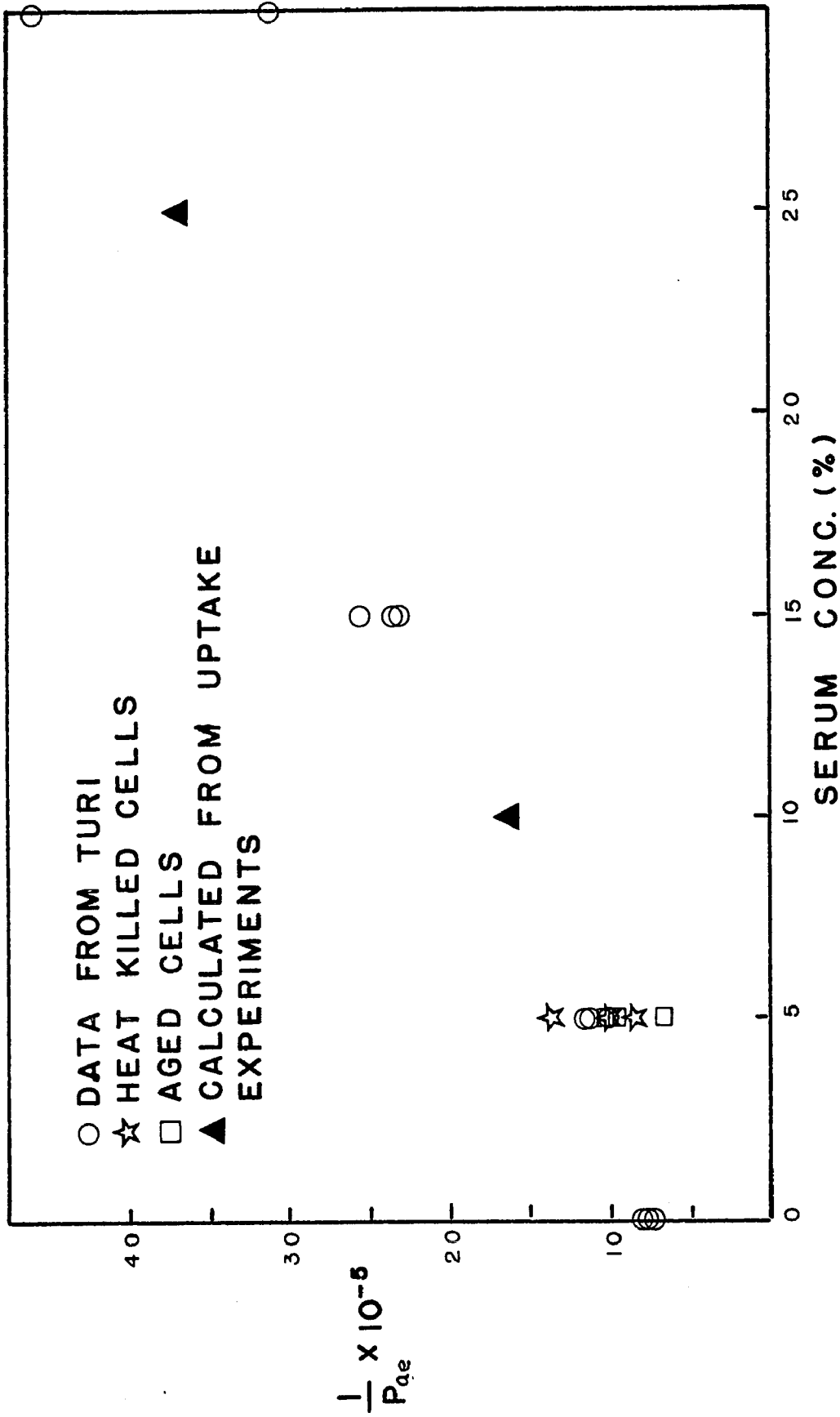


FIG. 16 EFFECTIVE PERMEABILITY, P_{ae} VERSUS SERUM CONCENTRATION (%) IN THE MEDIA.

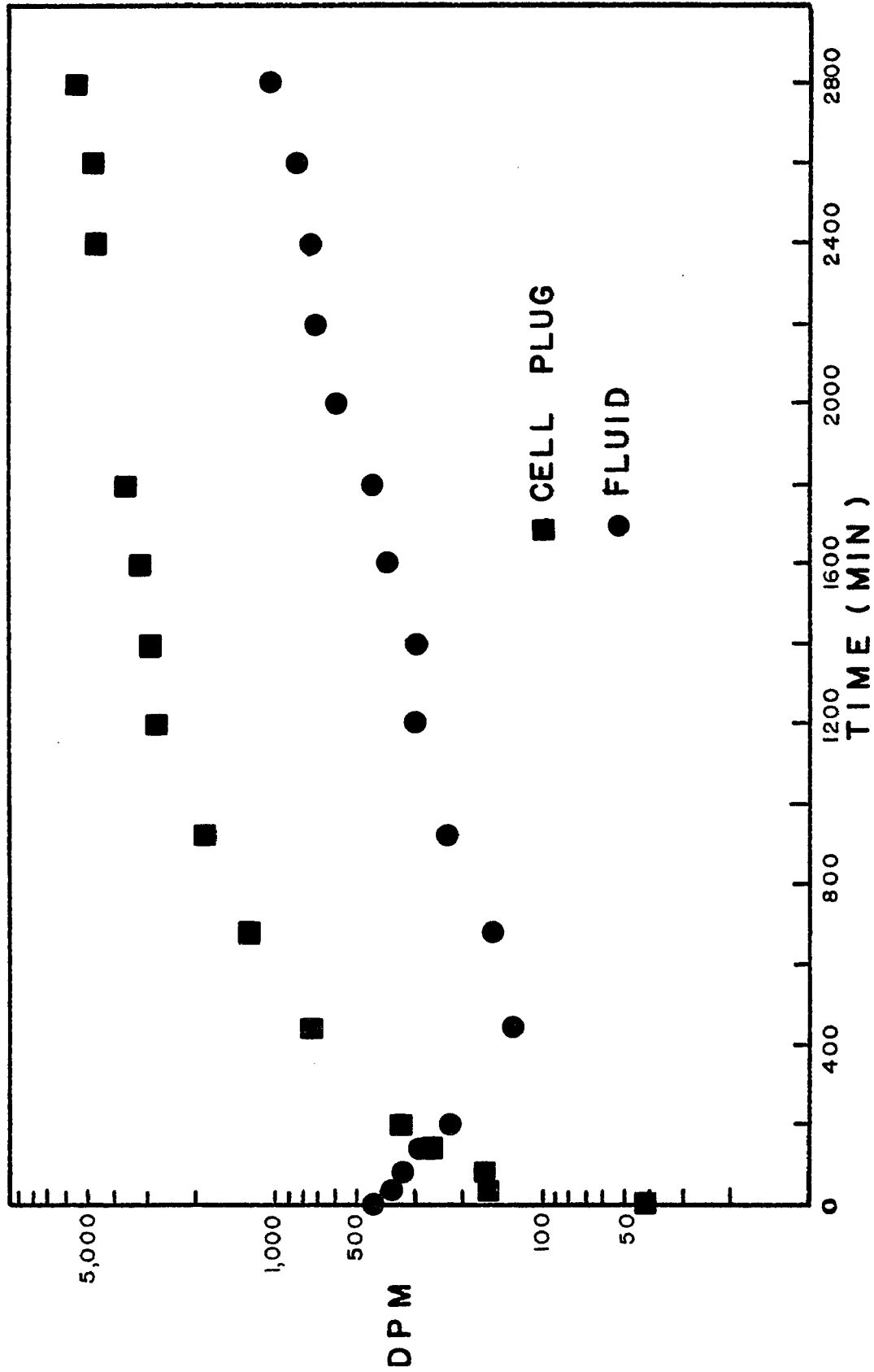


FIG.17 CHOLESTEROL IN THE CELL PLUG AND FLUID FRACTIONS
FOR A 10% UPTAKE EXPERIMENT AT 26 °C.

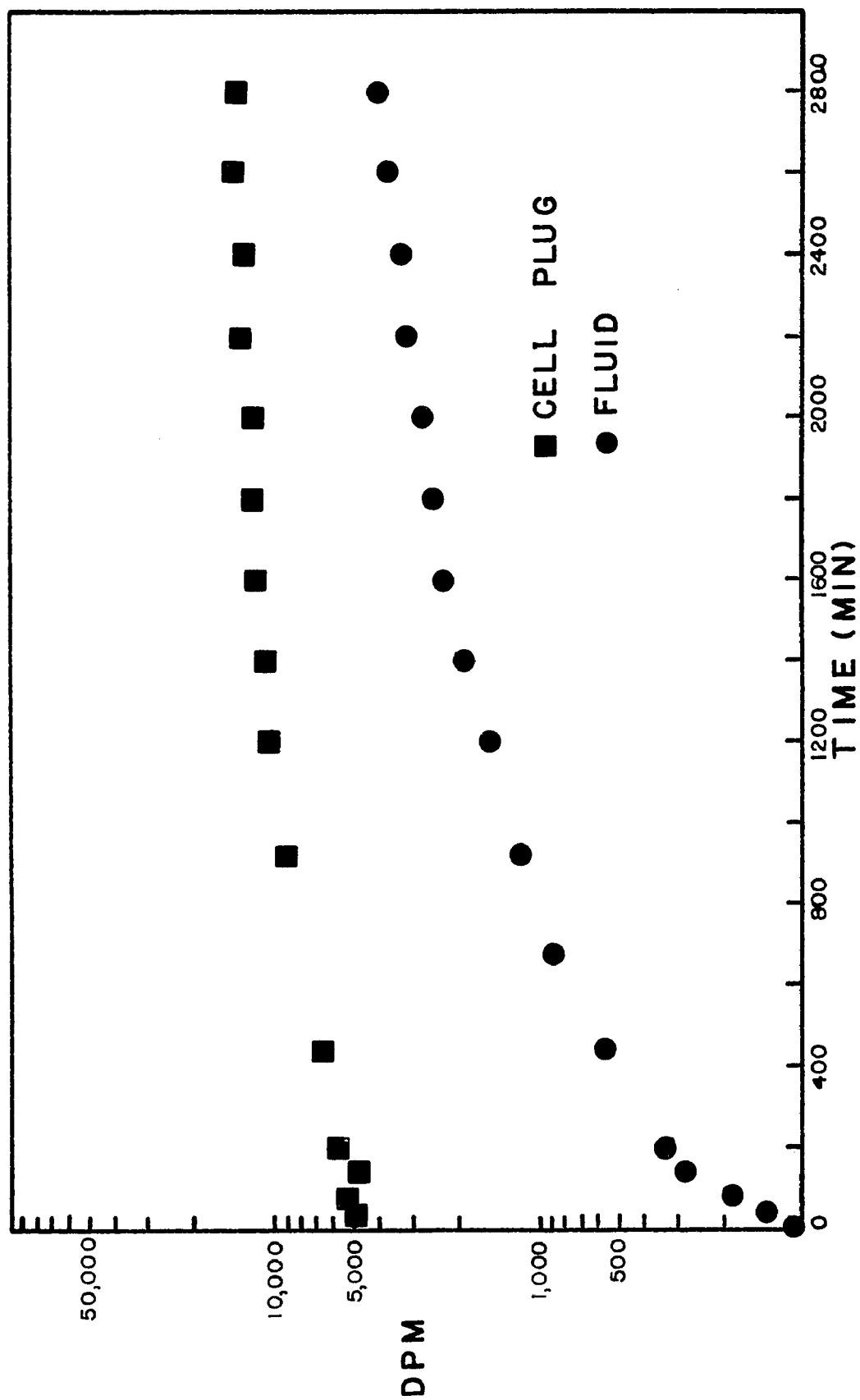


FIG.18 CHOLESTEROL IN THE CELL PLUG AND FLUID FRACTION FOR A 10% RELEASE EXPERIMENT AT 26 °C.

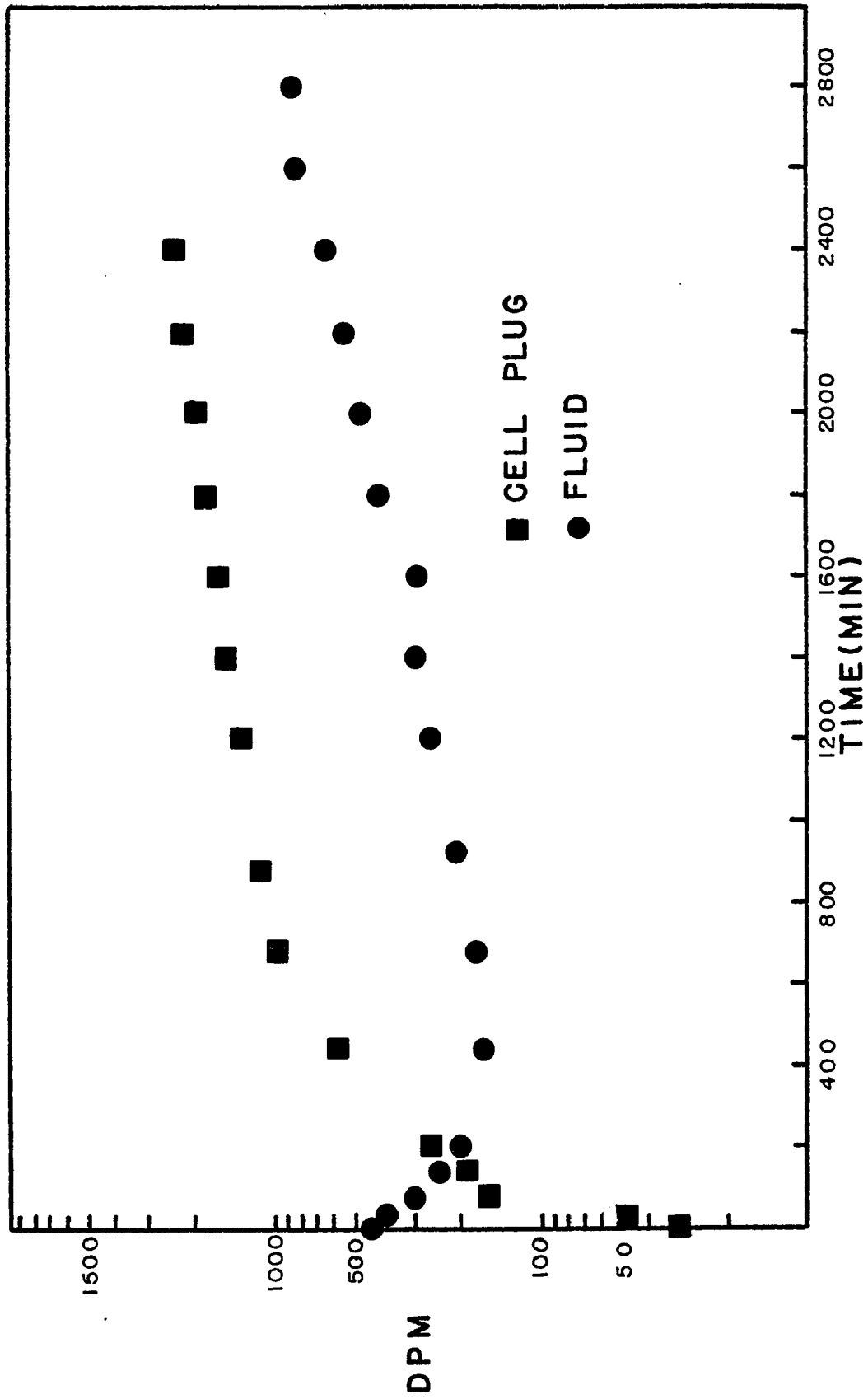


FIG.19 CHOLESTEROL IN THE CELL PLUG AND FLUID FRACTION FOR A 25% UPTAKE EXPERIMENT AT 26°C.

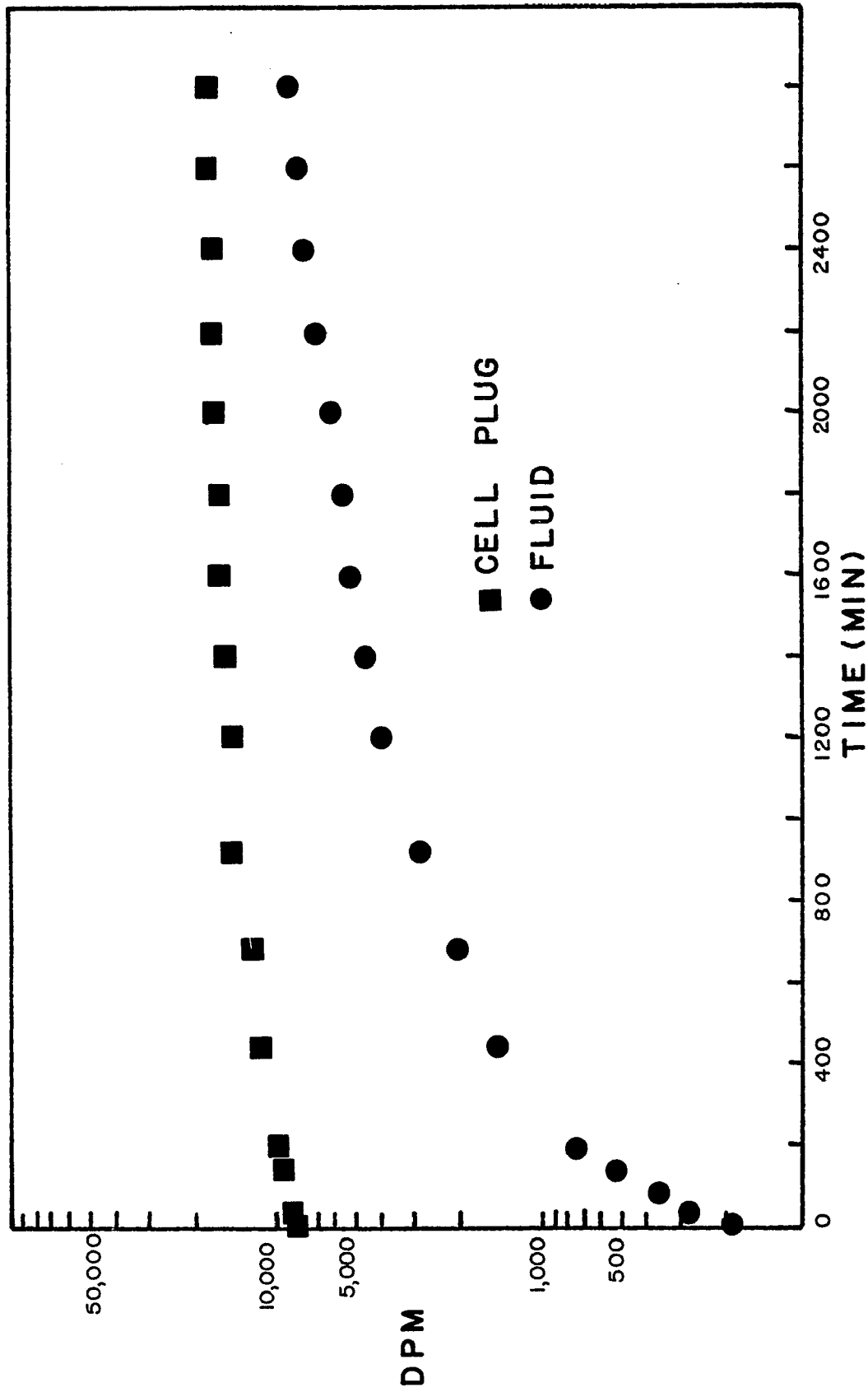


FIG.20 CHOLESTEROL IN THE CELL PLUG AND FLUID FRACTIONS
FOR A 25% RELEASE EXPERIMENT AT 26°C.

Third, while crossings of the fluid and cell curves occur as in the desmosterol plots, the cell plug and the fluid curves never quite become parallel in the case of cholesterol. This means that the β -phase steady state that was observed in the desmosterol plots is not achieved for cholesterol. This situation would be expected from the mathematical model if the δ rate constant was faster than the β rate constant but less than the α rate constant. For this reason, pure β -phase is probably never reached during the time course of the experiment for cholesterol. Since the transport data for cholesterol exists (Turi, 1972), this hypothesis can be tested. From Eq. (4.59) we know that

$$\delta = k_6 + k_7 \quad (4.59)$$

$$\dot{\delta} = k_7$$

when

$$\frac{1}{K_P} \ll 1. \quad (7.9)$$

Therefore,

$$\delta = \frac{3 \cdot P_{Pe}}{a} \frac{n \cdot V_i}{V_o} \quad (7.10)$$

using Eq. (4.87). Plotting $1/P_{Pe}$ versus serum concentration for Turi's data, one can calculate P_{Pe} at 10% and 25% FBS. Thus

$$\delta = 4.8 \times 10^{-4} \text{ min}^{-1} \text{ at } 10\% \text{ FBS}$$

$$\delta = 3.0 \times 10^{-4} \text{ min}^{-1} \text{ at } 25\% \text{ FBS.}$$

When we compare these values with the corresponding α and β values in Table 16, we see that the hypothesis is justified.

Finally, in this particular experimental system, the desmosterol-cholesterol system, accurate rate constants cannot be obtained from the cholesterol data. This is because of (1) the error in determining P_i^∞ and P_o^∞ from Eq. (4.80) and (4.81), (2) the small amount of conversion of desmosterol to cholesterol during the time course of the experiment (10% to 15%) causes $(P_i^\infty - P_i)/P_i^\infty$ and $(P_o^\infty - P_o)/P_o^\infty$ to be very nearly 1, and (3) the condition $\alpha > \delta > \beta$. These three reasons make the resolution of α , δ and β nearly impossible.

6. Biosynthetic Rate Constant, K_p

From Eq. (4.41) we have the relationship:

$$P(t) = P(o) + K_p \cdot A_{iT} \quad (7.11)$$

$$A_{iT} = \int_0^t A_i(t') dt' \quad (7.12)$$

where the subscript P emphasizes the biosynthetic rate constant K is with respect to the synthesis of P. Applying Eq. (7.12) to Figs. 9 to 12, one can calculate the area under the curve numerically. Subsequently, the

relationship in Eq. (7.11) was found to be linear as the plots in Figs. 21 to 23 show. The linear regression determinations of K_p are given in Table 18.

These results show that the biosynthetic rate constants, K_p , for the release experiments are faster than the rate constants for the uptake experiments by a factor of 2. Similar results were also observed for the β rate constants of section VII-E-2 which were determined from the biexponential plots of the experimental data. This relationship between β and K_p is similar to the strong coupling between α and k_1 which lead to Eq. (7.1). Since

$$\begin{aligned}\beta &= \frac{\gamma}{2} - \sqrt{\left(\frac{\gamma}{2}\right)^2 - k_1 \cdot K_p} \\ &= \frac{\gamma}{2} - \frac{\gamma}{2} \left(1 - \frac{k_1 \cdot K_p}{(\gamma/2)^2}\right)^{1/2},\end{aligned}\tag{4.57}$$

we can apply the binomial expansion which gives to the first order

$$\begin{aligned}\beta &\doteq \frac{\gamma}{2} - \frac{\gamma}{2} \left(1 - \frac{1}{2} \cdot \frac{4 \cdot k_1 \cdot K_p}{\gamma^2}\right) \\ \beta &\doteq \frac{k_1 \cdot K_p}{\gamma}.\end{aligned}\tag{7.13}$$

When K_p is small compared to k_1 and when κ_{ae} is large so that k_1 is much larger than k_2 , then

Table 18. Calculated "enzyme" rate constants, K

$K \times 10^5$ (min^{-1})		10% FBS		25% FBS	
			r		r
UPTAKE	K_P	2.7	.992	2.3	.993
	K_T	4.0	.822	----	----
	$K_P + K_T$	6.7	----	----	----
	K_A	6.7	.934	----	----
	K_{L3}	----	----	2.0	.983
RELEASE	K_P	5.3	.998	7.1	.999
	K_T	7.5	.954	15.7	.977
	$K_P + K_T$	12.8	----	22.8	----
	K_A	10.8	.851	22.1	.979
	K_{L3}	1.1	.909	2.2	.944

P = cholesterol, A = desmosterol, T = P + A, and L3 = synthesized impurity.

r = linear correlation coefficient.

The subscripted quantities, K_P , K_T , K_A , and K_{L3} are the linear rate constants, K, determined from a plot of P, T, A, and L3 versus A_{iT} (the accumulation of A_i in the cells given by Eq. (7.12)). The quantities K_T and K_A were not calculated for the 25% FBS uptake experiment because of scatter in the data.

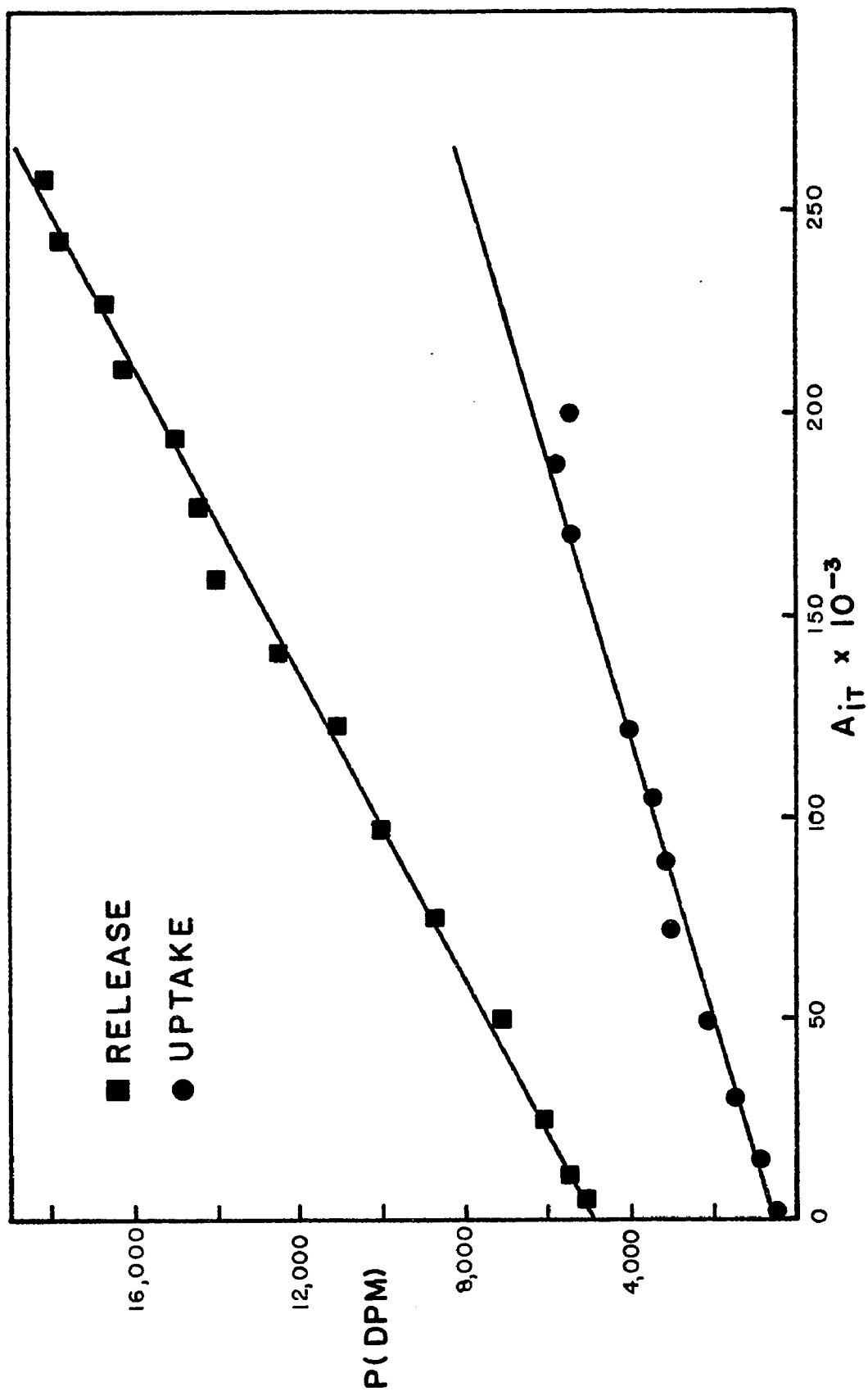


FIG.2I DETERMINATION OF RATE CONSTANT K FROM 10%
FBS UPTAKE AND RELEASE EXPERIMENTS AT 26° C.

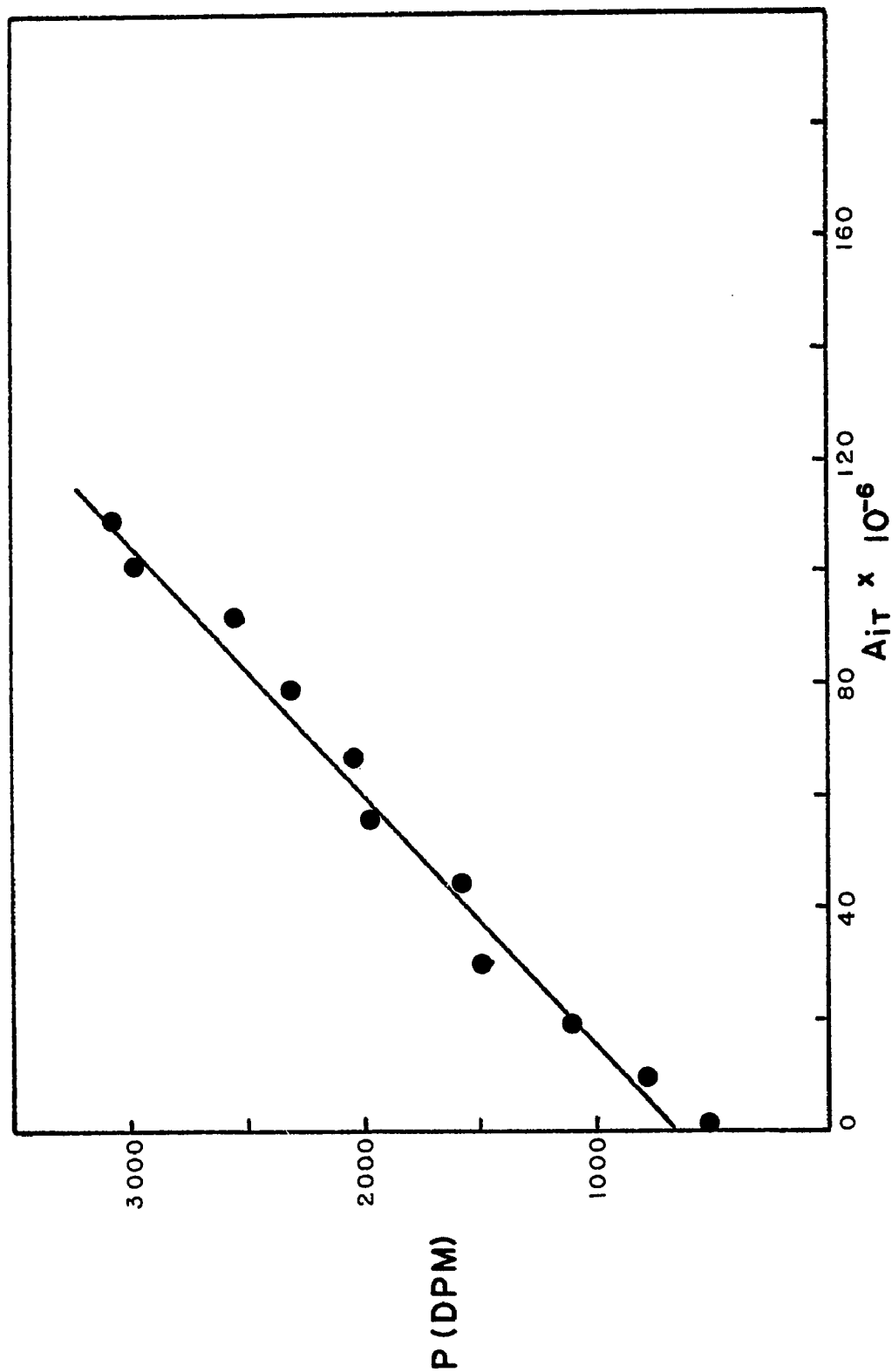


FIG.22 DETERMINATION OF RATE CONSTANT K FROM
A 25% FBS UPTAKE EXPERIMENT AT 26 °C.

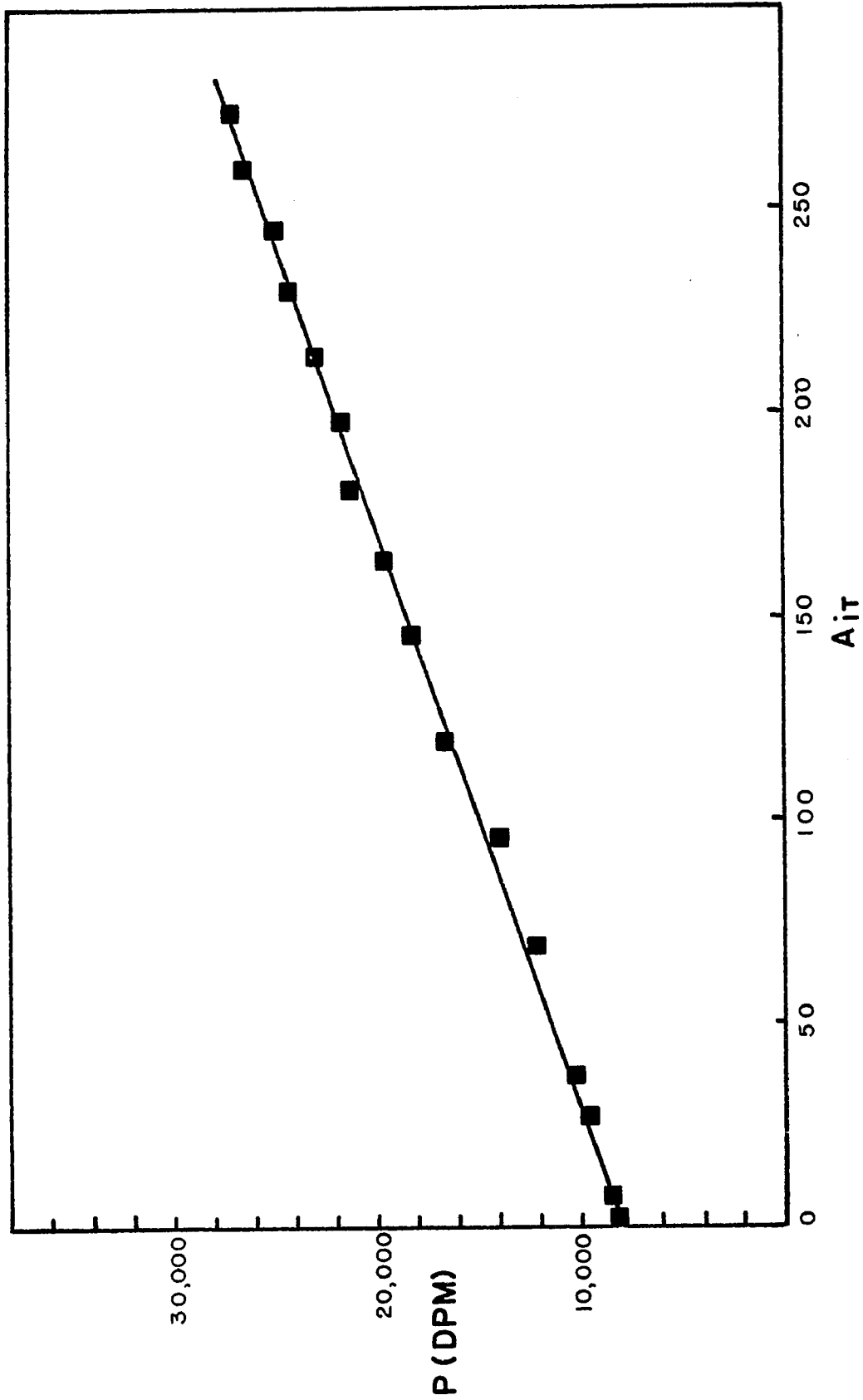


FIG.23 DETERMINATION OF RATE CONSTANT K_p FROM A
25 % RELEASE EXPERIMENT AT 26 °C.

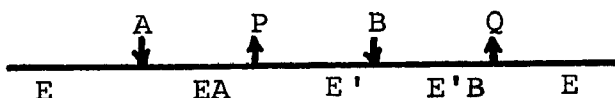
$$\dot{\gamma} = k_1 \quad (7.14)$$

so that

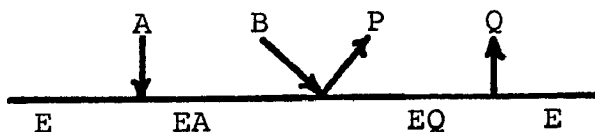
$$\dot{\beta} = K_p \cdot \quad (7.15)$$

When we examine K_p for the desmosterol-cholesterol system, the simple model presented in section IV should be generalized to a two-substrate system* since another substrate, reduced nicotinamide adenine dinucleoside phosphate (NADPH), is involved in the enzymatic reduction of the double bond at C24 to form cholesterol (Akhtar et al, 1969). The major classes of two-substrate mechanisms are (using the notation of Cleland, 1963):

(1) Ping Pong Bi Bi

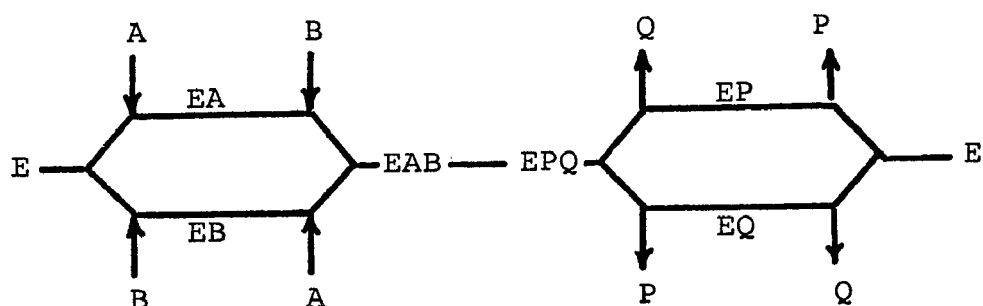


(2) Theorell-Chance Bi Bi

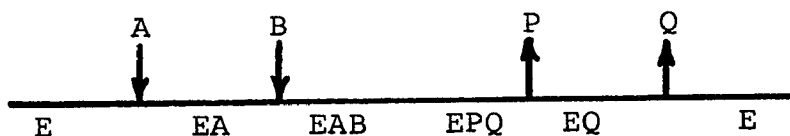


*This is really a pseudo one-substrate reaction since one hydride ion from the media is also involved. The mechanism consists of, first, a hydride ion from the media being incorporated at C24 to give an electron deficient species which is neutralized in the next step by the addition of another hydride ion from the 4 position of NADPH to C25 to give cholesterol (Watkinson et al, 1971).

(3) Iso Random Bi Bi



(4) Ordered Bi Bi



where the horizontal line represents the enzyme forms, given below the line, and the arrows indicate the addition and the loss of substrates and products, respectively. For the ping pong mechanism, the modified K_p becomes

$$K_p' = \frac{k' \cdot E_o}{\phi_a + [1 + \phi_b/B_{ie}] \cdot A_{ie}} \quad (7.16)$$

whereas for the other three mechanisms we have

$$K_p'' = \frac{k'' \cdot E_o}{\phi_a' + \phi_{ab}/B_{ie} + [1 + \phi_b'/B_{ie}] \cdot A_{ie}} \quad (7.17)$$

where k' , k'' , ϕ_a , ϕ_a' , ϕ_b , ϕ_b' , and ϕ_{ab} are constants of the microscopic rate constants for the given mechanisms,

and B_{ie} is the endogenous level of the second substrate.

By changing the initial conditions of the experiments, from the uptake to the release conditions, the cells are possibly given a metabolic shock in a number of different ways. Eqs. (7.16) and (7.17) illustrate three ways that the modified K_p can be changed. First, changes in the intracellular environment which result from changes in the extracellular milieu when the initial conditions are changed can possibly alter the total amount of enzyme, E_o , in the system by increasing or decreasing the maintenance level of E_o . These same changes could also alter the endogenous level of the second substrate, B_{ie} , and so change the modified K_p . And finally, the actual level of the endogenous substrate, A_{ie} , might be changed so as to alter the operating point given in the enzyme velocity versus substrate plot (see section IV-B). Additional changes are also possible from the effect of fetal bovine serum (FBS) on membrane transport. Such changes have been shown to influence amino acid transport in macrophage, which in turn could affect the maintenance level of many regulatory proteins and polypeptides (Strauss and Berlin, 1972). In summary, it appears that changing the initial conditions does not affect the transport parameters (see section VII-E-1) so that we have the relationship

$$\alpha(ipf) = \alpha(f) \dot{=} k_1 , \quad (7.18)$$

but that these changes do affect the biosynthetic parameters, β and K_p such that

$$\beta(ipf) = \beta(if) \dot{=} K_p . \quad (7.19)$$

7. Mass Balance Losses

Since there are alternative pathways by which the substrate, A, is lost in the desmosterol-cholesterol system, a study was conducted to see if ^{14}C -cholesterol could be further metabolized. In this experiment, ^{14}C -cholesterol was equilibrated with the Burkitt lymphoma cells for four days, then assayed for metabolites of cholesterol. The results are given in Fig. 24. They indicate that although a small amount of metabolism or degradation does occur, this amount could never account for the mass balance losses reflected in the T curves of Fig. 13. The next step was to re-examine the assay.

In the original determination of cholesterol biosynthesis from desmosterol (Fig. 8), it was noted that some activity did exist near the origin and some much further up above cholesterol on the TLC plate. In Table 19 data is given for a typical release experiment for the activity near the origin. This evidence indicates that (1) the impurity at the origin is trans-

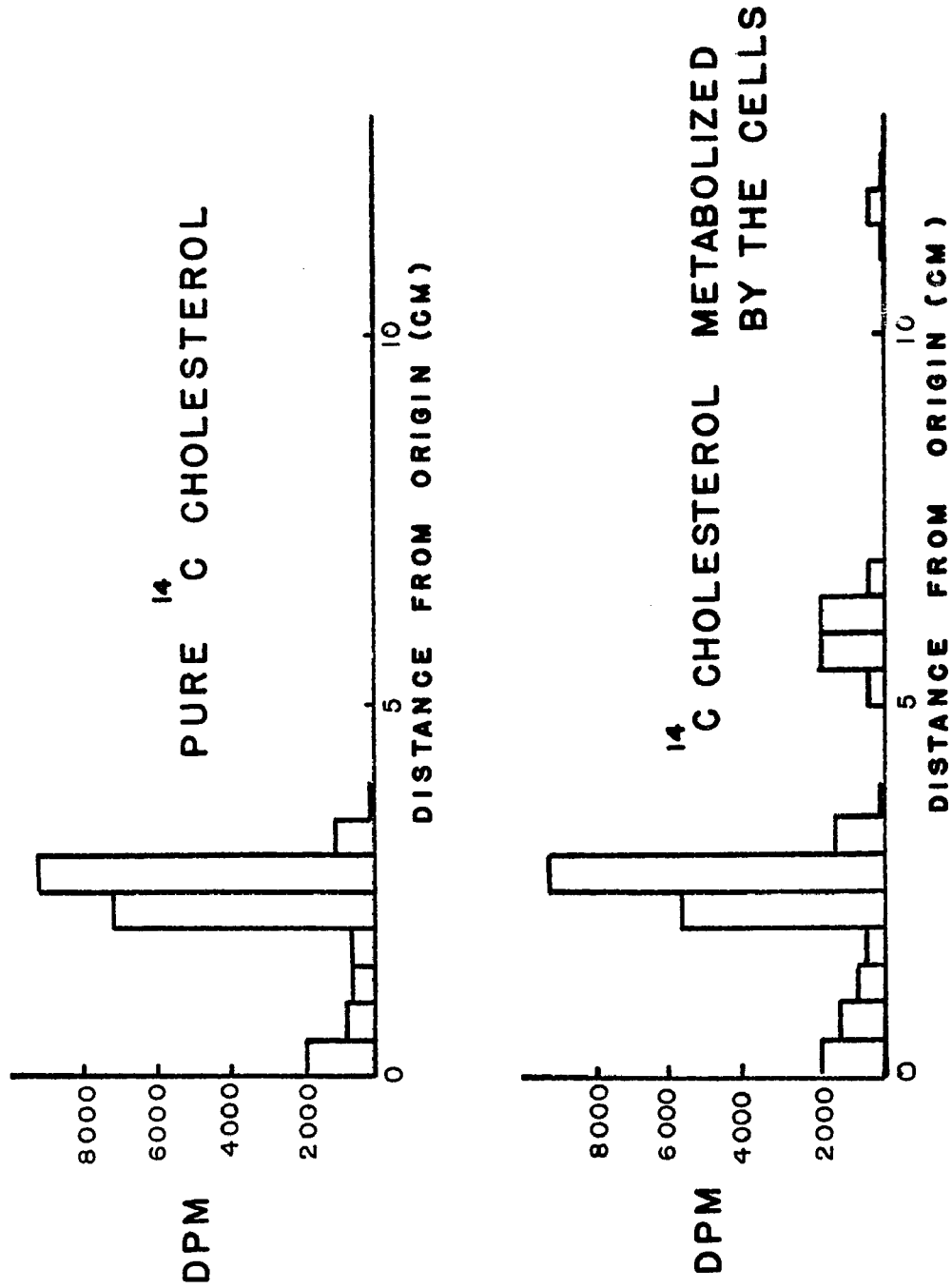


FIG.24 TLC ASSAY FOR THE EXTENT OF METABOLISM OF ^{14}C CHOLESTEROL BY THE BURKITT CELLS.

Table 19. Desmosterol impurity near the origin for a 10% FBS release experiment

Time (min)	O_c (DPM)	O_f (DPM)	$O_c + O_f$ (DPM)
10	3804	799	4603
40	3088	815	3903
80	3095	1070	4165
140	2869	1293	4162
200	2708	1506	4214
440	2938	1904	4842
680	2820	1719	4539
920	3160	1587	4547
1200	2700	1847	4547
1400	3147	1863	5010
1600	2575	1574	4089
1800	2400	1832	4232
2000	2990	1998	4988
2200	2431	1803	4234
2400	2676	2078	4754
2600	2247	1887	4136
2800	2270	2006	4276

O_c and O_f are the activities of the impurity near the origin in the cell and in the fluid fractions respectively

ported out of the cell and (2) the impurity is not synthesized by the cell from A_i since the sum of the impurity in the fluid and in the cell fractions is constant throughout the experiment.

The activity, L3, above cholesterol on the TLC plate was next investigated. In Fig. 25, the amount of the L3 metabolite in the fluid is shown in a semi-log plot against time for a 10% uptake experiment. This curve has the same characteristics as the cholesterol curves in the corresponding compartment. The initial decreasing phase is probably due to the transport loss of L3 from the fluid into the cells; the subsequent rise phase is due to the synthesis of L3 by the cells. This evidence is further confirmed by the data in Fig. 26 in which the amount of L3 in the cells is semi-logarithmically plotted for a release experiment. Since L3 increases, this also implies the synthesis of L3 in the cells from A_i . In summary, two impurities of ^{14}C -desmosterol were investigated. Only one, the L3 impurity, was found to be synthesized by the cells from A_i .

In order to determine whether the L3 metabolite could account for the mass balance loss found in T, the amount of L3 in the system was plotted against A_{iT} and the enzyme rate, K_{L3} , was calculated; similarly, K_A

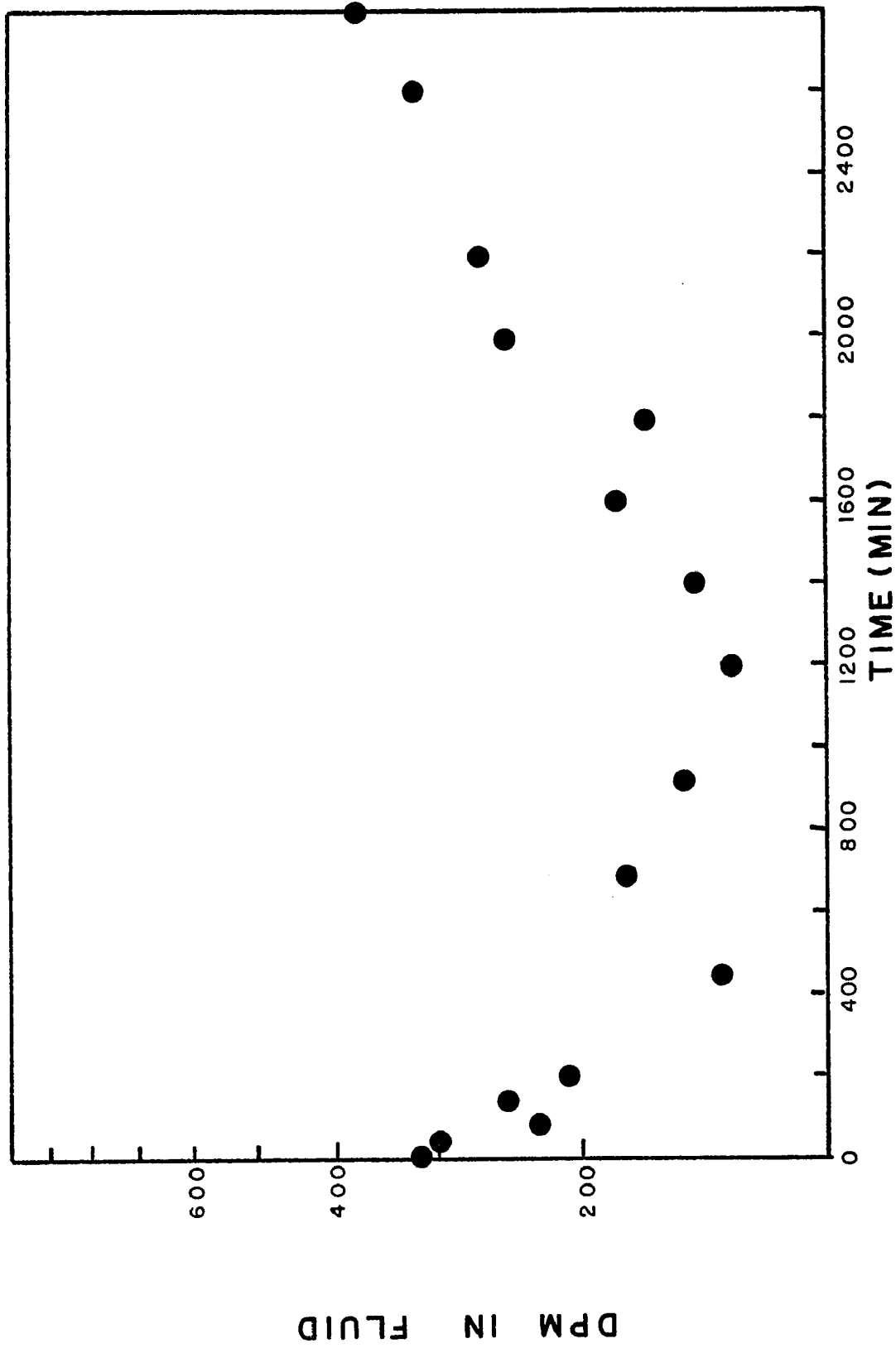


FIG.25 SIMULTANEOUS BIOSYNTHESIS AND UPTAKE OF L3 METABOLITE AT 26°C. FLUID ASSAY. 10% FBS.

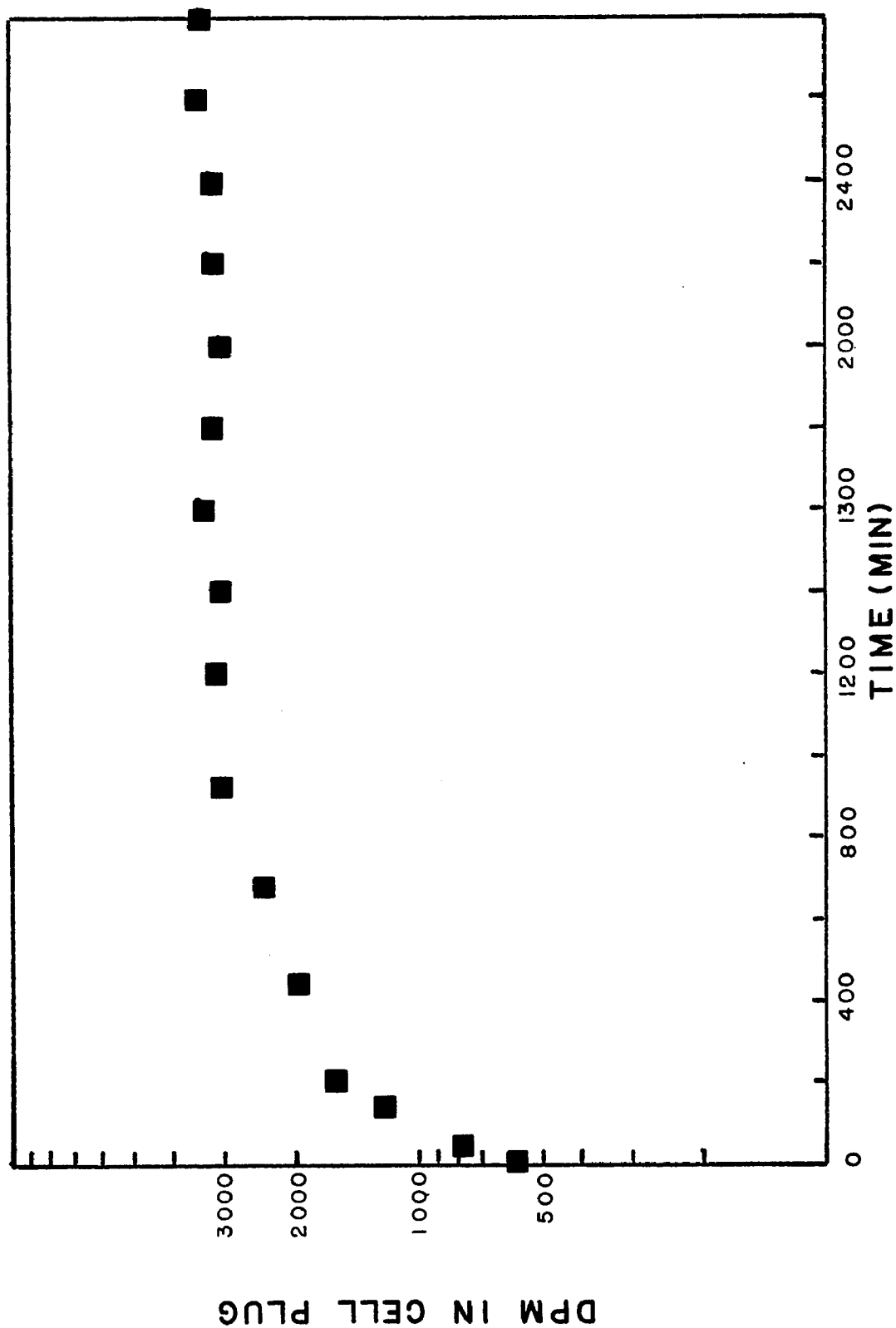


FIG.26 SIMULTANEOUS BIOSYNTHESIS AND RELEASE OF L3 METABOLITE AT 26°C. CELL PLUG ASSAY. 10% FBS.

and K_T were calculated. All of these rate constants are given in Table 18. Since K_T represents all of the rate losses of A not due to the synthesis of P, it follows that if the P loss and the T loss of A are due to parallel and not sequential reactions, then

$$K_A = K_P + K_T.$$

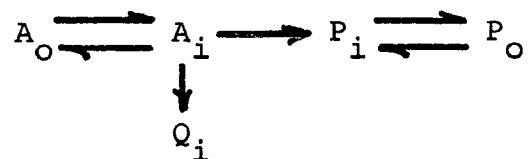
Since this appears to be true from the data in Table 18, we can conclude that P is probably not an intermediate in the T loss. Further, since

$$K_{L3} \ll K_T,$$

we have to conclude that there is some other loss, Q, such that

$$K_Q = K_T - K_{L3}$$

which has not been accounted for in the TLC assay. In conclusion, it appears that the following scheme is most likely:



Here Q_i represents some irreversible loss of A. It might be some time-dependent physical loss, but more likely it is some time-dependent chemical loss that is not accounted for in the TLC system, for example, a loss of the ^{14}C -26 carbon atom by some kind of enzyme cleavage of the side chain on the sterol nucleus. In this latter case, the TLC system might not account for the loss since the ^{14}C activity would remain in the aqueous phase. However, the exact nature of the loss is not of primary importance since it can be accounted for mathematically.

VIII. CONCLUSIONS

A methodology was developed to study, simultaneously, the transport of a substrate into tissue culture cells and the intracellular enzyme rate of reaction of that substrate into a product that could be transported out of the cells. The model system utilized Burkitt lymphoma cells in suspension culture and their ability to transport and synthesize cholesterol from desmosterol.

By using argented thin layer chromatography plates, cholesterol and desmosterol were quantitatively separated so that the biosynthesis of cholesterol from desmosterol by the Burkitt lymphoma cells was confirmed. A procedure was then established to assay the fluid and the cell fractions of the suspension, both of which were shown to extract from the aqueous phase with n-hexane and to elute from the argented silica gel plates with the same efficiency.

The experimental data were studied with respect to a simple mathematical model that was developed, the rationale being that more complex behavior than that embodied in the model would be detected as deviations from this minimal mathematical hypothesis which would

serve as a basis for comparison. Agreements and deviations were observed.

The desmosterol behavior was predicted to vary biexponentially with time according to the model; this was found to be true experimentally. The faster α phase, which was predicted and was found to be essentially determined by the transport parameters for desmosterol, agreed with the modelistic predictions with respect to its dependence on initial conditions, phased assayed and fetal bovine serum concentrations. The slower β phase, on the other hand, was found to be determined by the rate of enzyme catalysis and deviated from the model with respect to its dependence on the initial conditions.

The transport parameters for desmosterol were determined from the asymptote that was reached at times great enough that both the fluid and the cell compartment depended only on the β phase. These values agreed with previous data on the desmosterol transport (Turi, 1972).

The cholesterol data were found to be consistent with the triexponential behavior predicted by the model since the β phase steady state was never achieved during the time course of the experiment. This is consistent with the α and β values determined from the desmosterol data and independent experiments which

predicted that the third exponential would prevent a pure β phase falling off in both the cell and the fluid compartments within the time span of the experiment.

Also from the cholesterol data, the enzymic rate of reaction was computed from the linear relationship predicted by the model for plots of the total cholesterol versus the accumulation of desmosterol inside the cells.

Additional mass balance losses were detected. These were attributed mainly to enzymatic processes since the losses gave good correlation with respect to the accumulation of the substrate inside the cells. These losses, so quantified, were self-consistent with the synthesis of cholesterol by the Burkitt lymphoma cells.

APPENDIX A

Solutions to the Bioconversion-Transport Equations

The equations to be solved are equations (4.45), (4.51), and (4.52).

$$dA_i/dt = -\gamma \cdot A_i + k_1 A \quad (A.1)$$

$$dA/dt = -K \cdot A_i \quad (A.2)$$

$$dP_i/dt = K \cdot A_i - k_7 \cdot A - \delta \cdot P_i + k_7 \cdot T \quad (A.3)$$

where

$$\gamma = k_1 + k_2 + K \quad (A.4)$$

$$\delta = k_6 + k_7 \quad (A.5)$$

Taking the Laplace transform of equations (A.1), (A.2), and (A.3), we have

$$(s + \gamma) \cdot a_i - k_1 \cdot a = A_i(o) \quad (A.6)$$

$$K \cdot a_i + s \cdot a = A(o) \quad (A.7)$$

$$-K \cdot a_i + k_7 \cdot a + (s + \delta) \cdot p_i = k_7 \cdot T/s + P_i(o) \quad (A.8)$$

Since the determinant of the matrix for a_i , a , and p_i is

$$\text{Det} = (s + \beta) \cdot (s + \alpha) \cdot (s + \delta) \quad (A.9)$$

where

$$\alpha = \gamma/2 + \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (A.10)$$

$$\beta = \gamma/2 - \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (\text{A.11})$$

$$\alpha \cdot \beta = K \cdot k_1 \quad (\text{A.12})$$

$$\gamma - \alpha = \beta \quad (\text{A.13})$$

$$\gamma - \beta = \alpha \quad (\text{A.14})$$

The solutions for a_i , a , and p_i can be found from Cramer's Rule and are:

$$a_i = (s + \beta)^{-1} \cdot (s + \alpha)^{-1} \cdot [k_1 \cdot A(o) + s \cdot A_i(o)] \quad (\text{A.15})$$

$$a = (s + \beta)^{-1} \cdot (s + \alpha)^{-1} \cdot [(s + \gamma) \cdot A(o) - K \cdot A_i(o)] \quad (\text{A.16})$$

$$\begin{aligned} p_i = & (s + \beta)^{-1} \cdot (s + \alpha)^{-1} \cdot (s + \delta)^{-1} \cdot [A_i(o) \Delta_{13} - A(o) \Delta_{23} \\ & + P_i(o) \Delta_{33}] + (s + \beta)^{-1} (s + \alpha)^{-1} (s + \delta)^{-1} s^{-1} k_7 T \cdot \Delta_{33} \end{aligned} \quad (\text{A.17})$$

where the Δ_{ij} are the minors of the ij^{th} element of the matrix formed from a_i , a , and p_i . Taking the inverse transforms we have:

$$A_i = A_{ib} \cdot e^{-\beta t} + A_{ia} \cdot e^{-\alpha t} \quad (\text{A.18})$$

$$A = A_b \cdot e^{-\beta t} + A_a \cdot e^{-\alpha t} \quad (\text{A.19})$$

$$P_i = P_i(\infty) - P_{ib} \cdot e^{-\beta t} - P_{id} \cdot e^{-\delta t} - P_{ia} \cdot e^{-\alpha t} \quad (\text{A.20})$$

$$\alpha = \gamma/2 + \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (\text{A.21})$$

$$\beta = \gamma/2 - \sqrt{(\gamma/2)^2 - K \cdot k_1} \quad (\text{A.22})$$

$$\gamma = \alpha + \beta = k_1 + k_2 + K \quad (\text{A.23})$$

$$\delta = k_6 + k_7 \quad (\text{A.24})$$

$$A_{ib} = \frac{k_1 \cdot A(o) - \beta \cdot A_i(o)}{\alpha - \beta} \quad (\text{A.25})$$

$$A_{ia} = \frac{\alpha \cdot A_i(o) - k_1 A(o)}{\alpha - \beta} \quad (\text{A.26})$$

$$A_b = \frac{\alpha \cdot A(o) - K \cdot A(o)}{\alpha - \beta} \quad (\text{A.27})$$

$$A_a = \frac{K \cdot A_i(o) - \beta \cdot A(o)}{\alpha - \beta} \quad (\text{A.28})$$

$$P_b = \left(\frac{k_7 - \beta}{\delta - \beta} \right) \cdot A_b \quad (\text{A.29})$$

$$P_a = \left(\frac{k_7 - \beta}{\alpha - \delta} \right) \cdot A_a \quad (\text{A.30})$$

$$B = \frac{1}{(\delta - \beta)(\alpha - \delta)} \left\{ \left[1 - \frac{k_7}{k_6 + k_7} \right] \cdot \alpha \cdot \beta \cdot T \right. \\ \left. + (\gamma - \delta) \cdot (k_7 + \delta) \cdot P_i(o) - k_6 \cdot K \cdot A_i(o) \right\} \quad (\text{A.31})$$

$$P_i(\infty) = \frac{k_7 T}{k_6 + k_7} \quad (\text{A.32})$$

APPENDIX B

Calculation of the Extraction Loss Parameter, f

External Standard Method

A. Quench Curve for Cl4

quench: AgNO_3 and silica gel (SGA)

standard: Cl4 toluene

assumptions: linear quench

quenched samples: 2 high SGA quenched samples

To determine the efficiency and the actual DPM pipetted into the two samples to be quenched with SGA, Cl4 standard was added to three vials of pure Aquasol and counted. One vial (which had approximately ten times the number of DPM as the other two samples) of undiluted Cl4 standard was used to determine the efficiency of the machine for Cl4. Using this efficiency, the estimated DPM of the other two diluted samples was determined by back calculation. These two samples were then quenched by different amounts with SGA. Assuming a linear relationship between the external standard ratio, s , and the quench, SGA, quench constants λ and θ can be determined from the relations:

$$\lambda = \frac{c(2)/a(2) - c(1)/a(1)}{s(2) - s(1)} \quad (\text{B.1})$$

$$\theta = \frac{\text{EFFc}(2) - \text{EFFc}(1)}{s(2) - s(1)} \quad (\text{B.2})$$

where the subscripted quantities a, c, s, and EFFc denote the CPM in channels A and C, the external standard number and the efficiency of channel C for C14 with SGA quench, respectively.

Given λ and θ , we can now determine the C14 efficiency in channel C of an unknown SGA quenched sample of external standard number s from

$$\text{EFFc}(\text{SGA:C14}) = \theta \cdot s. \quad (\text{B.3})$$

In a double label experiment, however, C14 and H3 are present simultaneously. Two factors complicate the separation of the CPM due to H3 and C14. While it is true that most of the counts of H3 are restricted to channel A, some counts of H3 overflow into channel C; denote this overflow of H3 into channel C by C(H3). Similarly an even greater percentage of the C14 counts are in channel A; denote these counts by A(C14). From λ we have the following relationship between C(H3) and A(C14)

$$A(\text{C14}) = \frac{c - C(\text{H3})}{\lambda \cdot s} \quad (\text{B.4})$$

where c and s are the CPM for channel C and the external standard number, respectively, for a given unknown SGA quenched sample.

B. Quench Curve for H3

quench: SGA

standard: H3 toluene

assumptions: linear quench

quenched samples: 2 high SGA quenched samples

The determination of the efficiency of the machine for H3 and the calculation of the DPM of the quenched samples was the same as in part A of this Appendix. The following constants were determined:

$$\pi = \frac{c(4)/a(4) - c(3)/a(3)}{s(4) - s(3)} \quad (B.5)$$

$$\sigma = \frac{EFFa(4) - EFFa(3)}{s(4) - s(3)} \quad (B.6)$$

where EFFa is the efficiency of channel a for H3.

For a given unknown sample with an external standard number, s, the efficiency of channel A for H3 with SGA quench is:

$$EFFa(SGA:H3) = \sigma \cdot s \quad (B.7)$$

and the constant π gives the relation

$$C(H3) = \pi \cdot s \cdot (a - A(C14)). \quad (B.8)$$

Solving (B.4) and (B.8) simultaneously for $C(H3)$ and $A(C14)$ we have:

$$A(C14) = (\lambda - \pi)^{-1} (c/s - \pi \cdot a). \quad (B.9)$$

C. Quench for the Extraction Standard

quench: 100 microliters of water

standard: H3 toluene

After the unquenched efficiency was determined, the quenched efficiency for 100 microliters of water was determined for H3 in channel A from:

$$EFFa(H3:100) = a(H3)/A(H3) \quad (B.10)$$

where a and A denote the CPM and DPM of channel A, respectively. From this determination of $EFFa(H3:100)$ from the toluene standard, the DPM of the H3 cholesterol standard with no SGA quench but with 100 microliters water quench is

$$A(H3:100) = \frac{a(H3:100)}{EFFa(H3:100)} \quad (B.11)$$

where $a(H3:100)$ denotes the CPM in channel A for the H3 cholesterol internal standard for 100% extraction.

D. Extraction Loss Parameter, f

The extraction loss parameter, f, can be found from

$$f = \frac{\text{DPM of H3 content of cholesterol spot from SGA plate}}{\text{DPM of H3 cholesterol standard with no SGA quench}}$$

$$= \frac{(a - A(C14))/EFFa(SGA:H3)}{A(H3:100)}$$

$$= \frac{(a - (\lambda - \pi)^{-1}(c/s - \pi \cdot a))/\sigma \cdot s}{a(H3:100)/EFFa(H3:100)}$$

or

$$f = \frac{(a - (\lambda - \pi)^{-1}(c/s - \pi \cdot a)) \text{ EFFa}(H3:100)}{\sigma \cdot s \cdot a(H3:100)} \quad (B.12)$$

where a, c are the double label unknown CPM in channels A and C, respectively, with SGA quench, s is the sample external standard ratio with SGA quench, EFFa(H3:100) is the efficiency of channel A for H3 with 100 microliters of water quench, but with no SGA quench, a(H3:100) is the CPM of channel A for the H3 cholesterol internal standard in 100 microliters of water with no SGA quench, and λ , π , and σ are the C14 and H3 quench constants determined from pure toluene standards with SGA quench.

APPENDIX C

Calculation of the Extraction Loss Parameter, f Internal Standard Method

If a and c are the CPM in channels A and C when H3 and Cl4 are present simultaneously and if a' and c' are the CPM in channels A and C after Cl4 internal standard is added, then

$$\alpha = \frac{c' - c}{a' - a} \quad (C.1)$$

defines a constant which gives the C/A ratio due to pure Cl4. Similarly

$$\beta = \frac{c'' - c'}{a'' - a'} \quad (C.2)$$

gives the C/A ratio due to pure H3 where a'' and c'' are the CPM after H3 internal standard has been added.

Suppose we can resolve a and c so that

$$a = a(H3) + a(Cl4) \quad (C.3)$$

$$c = c(H3) + c(Cl4) \quad (C.4)$$

where the subscripted a and c denote the components of a and c due to pure H3 and Cl4. Solving Eq. (C.1)

to (C.4) simultaneously for $c(C14)$ and $a(H3)$ we have

$$c(C14) = \frac{\alpha \cdot (c - \pi \cdot a)}{\alpha - \beta} \quad (C.5)$$

$$a(H3) = \frac{\alpha \cdot a - c}{\alpha - \beta} \quad (C.6)$$

and solving for $a(C14)$ we obtain

$$a(C14) = \frac{c - \beta \cdot a}{\alpha - \beta} \quad (C.7)$$

Eq. (C.7) is analogous to Eq. (B.9) so that substituting (C.7) into (B.12) we have

$$f = \frac{[a - (\frac{c - \beta \cdot a}{\alpha - \beta})] \cdot \text{EFFa}(H3:100)}{\text{EFFa}(H3:SGA) \cdot a(H3:100)} \quad (C.8)$$

APPENDIX D

Method of Correcting for the Amount of Desmosterol in the System Due to Loss of Mass Balance

$$\Delta t = 10,280 \text{ min}$$

$$T = A_i + A_o + P_i + P_o$$

therefore

$$\Delta T = T(10) - T(2800)$$

$$A = A_i + A_o$$

therefore

$$\Delta A = A(10) - A(2800)$$

$$\Delta A_i = A_i(10) - A_i(2800)$$

$$\Delta A_o = A_o(10) - A_o(2800)$$

Define

$$f_i = \frac{\Delta A_i}{\Delta A}$$

$$f_o = \frac{\Delta A_o}{\Delta A}$$

therefore

$$A_i' = A_i + f_i \cdot \Delta T$$

$$A_o' = A_o + f_o \cdot \Delta T$$

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