THE UNIVERSITY OF MANITOBA

MEMBRANE TRANSPORT OF CYCLOPHOSPHAMIDE BY L5178Y LYMPHOBLASTS

by

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ABSTRACT

Transport of the alkylating agent, ^{14}C-cyclophosphamide, was examined in L5178Y lymphoblasts *in vitro*. The time course of cyclophosphamide uptake showed a rapid, initial phase, that probably represents binding of drug to the cell surface. Subsequent uptake into the cells was carrier-mediated and consisted of two components. Uptake never exceeded a cell/medium concentration gradient of one, suggesting that transport was not an active process.

Kinetic analysis of cyclophosphamide uptake over a 40-fold range of drug concentration showed evidence of saturation kinetics only at low drug concentrations, while at high drug levels uptake occurred by a second transport system that was technically non-saturable. After correcting for binding and the interaction of two-component transport, kinetic parameters for low dose transport consisted of a Michaelis constant, K_m (mean $\pm$ S. E.) of 0.39 $\pm$ 0.03 mM and a transport capacity, V_{max} of 0.49 $\pm$ 0.07 x 10^{-17} moles/min/cell. At high dose cyclophosphamide transport, the K_m was 75 $\pm$ 29 mM, and the V_{max} was 49 $\pm$ 14 x 10^{-17} moles/min/cell.

Both low and high dose cyclophosphamide transport were temperature sensitive and partially dependent on sodium. In addition low dose transport was inhibited by oligomycin and cyanide. Other alkylating agents and several naturally-occurring substrates did not inhibit cyclophosphamide transport; thus, a native substrate was not identified for the cyclophosphamide carrier.

Evidence that low dose cyclophosphamide transport was mediated

by a facilitated diffusion process was that uptake obeyed saturation kinetics, was temperature- and sodium-dependent, was partially dependent on metabolic energy and cell/medium gradients did not exceed one. Although high dose drug uptake failed to show saturation kinetics, the demonstration of temperature- and sodium-dependence also suggested that high dose uptake was carrier-mediated.

A preliminary study of cyclophosphamide uptake by chick embryo liver cells was temperature sensitive and exhibited biphasic kinetics similar to that observed in L5178Y cells, suggesting a similar mechanism of drug transport in normal and neoplastic cells.

T O M A R Y

ACKNOWLEDGEMENTS

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I. INTRODUCTION

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A. Properties of Classical Membrane Transport

Membrane transport regulates the difference in composition between intracellular and extracellular fluids (1). The lipid component of the cell membrane is the major permeability barrier to water-soluble organic molecules (2). The selective passage of necessary materials is mediated by interaction with specialized transport systems in the cell membrane. Many important substrates such as sugars, amino acids and components of nucleic acids are transported in a highly specific and efficient manner (2 - 4).

Transport processes may exhibit saturation kinetics analogous to those described by Michaelis and Menten for enzyme kinetics (2). Christensen has pointed out that had transport phenomena been characterized first, it would have been surprising that enzyme action corresponded so well to the customary transport kinetics (5).

The use of Michaelis-Menten kinetic analysis for unidirectional transport processes requires that the initial rate of uptake be observed. The fixed time interval used for a range of substrate concentrations must be brief enough so that uptake is a linear function of time (2).

In transport phenomena, initial uptake rate of substrate approaches a maximum saturation value with increasing substrate concentration, as though there were a limited number of transport carriers (2, 6). The net rate of entry (v) of a substrate (S) which is transported across the cell membrane is given by the following equation:

$$v = \frac{S \cdot V_{max}}{K_m + S} \quad ;$$

V_{max} is the transport capacity, and K_m, the Michaelis constant, is a measure of the affinity of the transport site for the substrate (2).

Wilbrandt and Rosenberg reviewed saturable transport systems that conform to classical enzyme kinetics, and indicated that some transport systems may follow unsaturable diffusion-like uptake (7). Stein discussed the mathematical and experimental approaches of kinetic analysis used to identify and characterize transport (2). Although saturability is considered a relatively strong criterion, Stein allowed that low affinity transport systems exist which defy characterization by saturation.

B. Variations from Classical Transport

1. Rapid Association of Substrate with Cell Surface (Binding)

Deviation from initial uptake rates can occur even with the demonstration of a linear increase of uptake with time. If this linear portion does not extrapolate close to the origin of a time course, the rate of uptake per unit of time will vary for each time point considered. A time course with a positive intercept on the y-axis can result from rapid binding of substrate to the cell surface (8). This rapid association is simply a non-specific adsorption of substrate to cell surface, and is not specific like the substrate-carrier interaction involved in a mediated transport process (9).

A binding component will make up a considerable fraction of total cell uptake when studying initial uptake rates. If this process is overlooked it will yield falsely high measurements of substrate in the cells, and will mimic non-saturability (9). Correction for binding

is mandatory in order to obtain true initial uptake rates.

Goldman et al. demonstrated binding of the folic acid analogue, methotrexate, by L1210 leukemia cells (9); Fischer observed this same phenomenon in L5178Y cells (10). Folic acid and the analogue folinic acid also exhibited binding to L1210 cells (11, 12). Rapid association of drug to L1210 cells has been described for the antineoplastic drug, camptothecin, and the nucleoside analogue, arabinosyl-6-mercaptopurine (13, 14). Non-specific binding of magnesium and iron has been reported in cultured human KB cells and rabbit duodenum (15, 16). Christensen et al. have described a binding component for uptake of benzylamine, lysine and the dicarboxylic acids, glutamic acid and aminoadipic acid by Ehrlich ascites cells (17 - 19).

2. Non-saturable Uptake

True unsaturable uptake kinetics are a feature of non-mediated passive diffusion. Transfer of a substrate across the cell membrane by simple diffusion is a relatively slow, nonspecific process, depending on the permeability constant of the substrate and its concentration in the medium (2, 4).

Mediated transport systems may vary from the classical model of saturation kinetics by possessing a low affinity for the substrate. Low affinity (high K_m) transport systems result in uptake varying approximately as a linear function of concentration; such kinetics are technically non-saturable.

Several high K_m transport systems have been described. Because of apparent non-saturability, kinetic analysis alone was often inadequate

to demonstrate the presence of a low affinity transport system. The sugars, L-glucose and D-xylose, which exhibit minimal active transport by hamster small intestine had K_m values ranging from 65 to 100 mM (20 - 22). The intestinal transport of some sugars exhibited a K_m of approximately 350 mM. The K_m for sugar transport by murine pancreatic β-cells and rat lens was approximately 50 and 90 mM (23, 24). Glycine transport by rabbit renal tubules and rat kidney cortex had a K_m of 50 mM (25, 26). Schultz et al. assigned K_m values of 20 to 100 mM for the transport of six D-amino acids by rabbit ileum (27). A K_m of approximately 100 mM described the transport of β-alanine in Ehrlich ascites cells (28), α-aminoisobutyric acid (AIB) in chick embryo heart cells (29), and adenine in rabbit leukocytes (30).

The following examples demonstrate the problems of distinguishing between non-mediated and mediated unsaturable kinetics in order to identify mediated transport in low affinity uptake systems. Caspary and Crane stated that the low affinity of L-glucose, with a K_m of 65 mM, was the most likely reason why active transport of this sugar was not detected earlier (21). Christensen reported that the high K_m (at least 100 mM) and V_{max} values obtained for β-alanine transport produced nearly linear kinetics over a wide concentration range (28).

3-O-methylglucose transport by the lens, with a K_m of 90 mM, revealed identical penetration over a 50-fold range of substrate concentration (24). Similarly, 20 and 50-fold ranges in the concentration of D-alanine and two dicarboxylic acids failed to show any difference in uptake by Ehrlich ascites cells (19, 31). Uptake of these substrates, by concentration-independent kinetics, was identified as mediated

transport by non-kinetic criteria.

Matthews described the problem of identifying a transport system from a Lineweaver-Burk plot which approached the origin (32). By using hypothetical data, Matthews showed that it is difficult to distinguish between non-saturable uptake attributed to passive diffusion, and a transport system with a high K_m value. He stated that information other than kinetic data was needed to characterize such a transport system.

3. Heterogeneous Kinetics

It had been well established that two or more independent transport systems may simultaneously contribute to the transport of a single substrate across the cell membrane. Christensen described various types of transport heterogeneity and stated that kinetic variation should always be suspected (33, 34). Scriver and Hechtman regarded the availability of two or more transport systems for a single substrate as one of the characteristics of membrane transport (1). Transport heterogeneity has been demonstrated for a variety of substrates and tissues in species ranging from microorganisms to man.

Many examples of multiple transport systems have come from amino acid transport by the mammalian kidney. Scriver and co-workers (35 - 38) and Segal et al. (26) demonstrated two kinetically distinct transport systems for AIB, proline, glycine and lysine uptake by adult rat kidney cortex slices. Lysine and cysteine exhibited biphasic kinetics in studies of the human kidney *in vitro* (39, 40).

Hillman and Rosenberg separated proline and glycine transport

into three kinetically distinct systems in isolated rabbit kidney tubules (25, 41). In vivo examination of amino acid uptake by human kidney tubule has shown two modes of transport for proline, hydroxyproline and glycine (42, 43). The isolated rat kidney glomerulus revealed proline transport by two identifiable systems (44).

Christensen and his group, working with Ehrlich ascites tumour cells, described kinetic evidence for two-component transport of the amino acids lysine, diaminobutyric acid, β-alanine and leucine (18, 28, 45). Histidine accumulation by S37 ascites tumour cells also occurred by two distinguishable transport processes (32).

In addition to the above demonstrations of multi-component amino acid transport by the kidney and tumour cells, two transport systems have been reported for the following substrates in chick embryo heart cells (serine, glycine and AIB), in rabbit reticulocytes (glycine) and in rat liver slices (cysteine) (29, 46 - 48). Multiple transport systems for the uptake of amino acids have been demonstrated in several microorganisms (49 - 52).

Substrates other than amino acids have also exhibited two transport components. Glucose uptake by rabbit renal tubules and adenine uptake by rabbit leukocytes showed two distinct transport systems (30, 53). Work in this laboratory has demonstrated two components of nitrogen mustard transport by human lymphoid cells (54).

When two components of transport are involved in the uptake of a substrate, at any given concentration uptake will occur simultaneously from each system. The apparent kinetic constants obtained will be in error, but they can be separated by two-component transport analysis.

Separation of individual transport systems is achieved by representing the total uptake by the sum of two Michaelis-Menten equations (55).

Various investigators have used computer solutions of successive approximation to solve the Michaelis-Menten summation and obtain the individual kinetic parameters (25, 29, 35 - 38, 41, 44, 46, 47, 53). Neal reported an arithmetic solution to the problem of two transport systems (55). This analysis is described in the Materials and Methods section of this thesis (see page 16).

Mackenzie and Scriver generalized that when two transport systems are functioning for a substrate, transport at low substrate concentrations occurs by the low K_m system which has a low capacity and narrow substrate specificity (44). At high substrate concentrations, which often exceed the physiological range (1), transport takes place on the high K_m system with high capacity and a broad range of chemical specificity.

Kepes suggested that high capacity, shared-transport systems function in a nutritionally rich environment, while in a starved environment, low capacity systems with high substrate affinity would sustain necessary substrate accumulation (49).

4. Non-saturable Uptake Superimposed on a Mediated Transport System

There are many examples of transport systems which cannot demonstrate classical saturation kinetics because of a non-saturable uptake component. This non-saturable system is superimposed on an otherwise saturable transport system. Saturation is revealed as the rate of uptake begins to decrease with increasing concentration. However,

instead of a maximum rate of uptake as the level of substrate increases, the rate increases linearly with concentration, indicating a second uptake component with diffusion-like kinetics.

An unsaturable uptake component has been observed for neutral amino acid transport by many cells and tissues. This uptake occurs in addition to the usual amino acid transport systems (31). Amino acid transport by Ehrlich ascites cells occurred by transport systems which exhibited hyperbolic uptake and a process that at high concentrations increased linearly with concentration (18, 19, 28, 31, 45, 56, 57). In vitro examination of various preparations of the mammalian kidney has shown the occurrence of an unsaturable amino acid uptake component in addition to two (26, 35 - 38, 44) or three (25, 41) components of transport, each with distinct kinetic parameters.

The Guidotti group demonstrated a component of non-saturable amino acid uptake by isolated chick embryo heart cells, and also with intact heart preparations (29, 46, 58). The model amino acid, AIB, showed unsaturable uptake in rat diaphragm and in rabbit detrusor muscle (59, 60). Glycine accumulation in frog muscle has linear and saturable uptake processes (61).

Winter and Christensen found unsaturable neutral amino acid uptake by rabbit erythrocytes and reticulocytes (47); Vidavar demonstrated this phenomenon for glycine uptake by pigeon erythrocytes (62). A non-saturable component of AIB uptake occurred in isolated rat thymocytes (63), lymph node cells (64), and in fat cells (65). The transport of lysine by rabbit lens in vitro showed both saturable and linear components (66).

In addition to the above examples of non-saturable amino acid uptake *in vitro*, Curran reviewed data from amino acid and glucose transport *in vivo* that revealed a linear uptake component as well as a saturating component (67). Correction for linear uptake *in vivo* reduced the observed K_m so that it was comparable to that obtained *in vitro*.

Glucose and glucosamine transport *in vitro* exhibited an unsaturable uptake component in hepatoma cells (68, 69) as did the transport of glucose in white cell ghosts (70). Uridine, hypoxanthine and xanthine showed hyperbolic and linear uptake in hepatoma cells (71), human erythrocytes (72), and rabbit leukocytes (30), respectively. A non-saturating uptake process for choline has been observed in human erythrocytes (73), hepatoma cells (74), chick and hamster intestine (75, 76). Folic acid and methotrexate have a linear uptake component in L1210 cells (77, 78). Saturable and non-saturable uptake of benzylamine and 5-hydroxy-tryptamine has been reported in Ehrlich cells and in isolated nerve endings (17, 79). Potassium transport in horse and human erythrocytes (80, 81) and sodium transport in frog skin (82, 83) exhibited Michaelis-Menten and linear uptake kinetics.

i. Corrections for the Non-saturable Uptake Component

Correction for the contribution of a linear uptake component is necessary to quantitatively evaluate the kinetic parameters of a saturable transport process (67).

The Akedo-Christensen correction (59) is the most prevalent method of quantitating the non-saturable uptake component. These authors demonstrate that plotting distribution ratios against the

reciprocal of the substrate concentration gives a straight line at sufficiently high concentrations. Extrapolation of this line to the y-axis gives an intercept from which the non-saturable uptake constant is derived (59). This method was used by several investigators to correct for unsaturable amino acid uptake (18, 19, 25, 28, 29, 31, 35 - 38, 41, 44 - 48, 56, 58, 59, 66). However, Christensen et al. have pointed out that the Akedo-Christensen correction is not always applicable. It requires that cell gradients be greater than unity, and is unreliable for low affinity transport (31, 45).

Shaw (80) is credited (81) with being the first investigator to analyze saturable and unsaturable transport. He described the influx of K^+ by the sum of the Michaelis-Menten equation plus the product of a rate constant (k) times the substrate concentration (80). Shaw solved for k and the Michaelis-Menten kinetic parameters by a graphic method of obtaining the best fit of the experimental data to a series of calculated curves. Glynn (81), also studying potassium transport, corrected his data by Shaw's method (80), as did Askari (73), and Herzberg and Lerner (75) who were studying choline transport.

Another method of correcting for unsaturable transport is also based on the assumption that total observed uptake is the sum of a hyperbolic function plus a linear function whose influx is proportional to concentration. Using an uptake versus substrate concentration plot, saturable uptake is obtained by subtracting the linear part of the curve from the total uptake. This method has been described independently by several authors (62, 63, 68, 70, 72, 79). Goldfine et al. pointed out that the rate constant of the linear uptake component is given by its

slope (63).

The Michaelis-Menten and linear uptake constants describing saturable and unsaturable uptake respectively, may also be evaluated mathematically. The unknown constants of the total uptake equation have been solved simultaneously by "trial and error" (76), and by least squares computer analysis (57, 61, 82, 83).

ii. Technically Non-saturable Mediated Uptake

Many of the reports of transport plus unsaturable uptake assumed a second uptake process on the basis of linear kinetics, and did not attempt to characterize it further. There is evidence to indicate that at least some of the examples of unsaturable uptake may be mediated transport. Christensen et al. suggested that the assumption that non-saturable uptake occurs by passive diffusion was not valid (45).

Jacquez et al. reported that linear uptake of several amino acids in Ehrlich ascites cells was temperature dependent, with activation energies in the same range as those found for the V_{max} of the saturable component (57). Christensen and Liang demonstrated evidence of mediated transport for unsaturable uptake of amino acids from structural specificity, temperature and pH sensitivity in Ehrlich cells, and temperature sensitivity in rat intestinal segments (31).

Christensen and Liang speculate that chemical mediation for non-saturable amino acid transport implies that simple diffusion of hydrophilic molecules rarely occurs (31). The penetration of any organic or foreign hydrophilic molecule may have to involve chemical contact with the cell membrane.

C. Membrane Transport of Cyclophosphamide by L5178Y Lymphoblasts

Transport of the alkylating agent, cyclophosphamide, was examined in L5178Y lymphoblasts. Chemotherapeutic drugs, like physiologic substances, must first penetrate the cell membrane in order to exert an intracellular effect (84). Drugs, with a structural similarity to naturally occurring substrates, may enter cells on carrier-mediated systems intended to transport those native substrates.

Previous studies demonstrated that transport of the alkylating agent, nitrogen mustard, by L5178Y cells was an active, carrier-mediated process (85). This toxic drug entered cells on the transport carrier for choline, a structural analogue of nitrogen mustard (86). There are other examples in which drugs are transported into cells on carriers with a primary function of transporting a native substrate (9, 30, 77, 87, 88). The chemotherapeutic agent methotrexate shares the same transport system as folinic acid (12) and the clinical use of methotrexate and folinic acid in tandem has increased the therapeutic index of methotrexate in the therapy of human malignant disease (89). By analogy identification of a cyclophosphamide transport carrier may enable manipulation of drug transport so as to increase therapeutic index of cyclophosphamide.

Transport studies of alkylating agents may be obscured by binding of drug to cells through alkylation reactions. Hydrolyzed alkylating agents are inactive and therefore are the preferred drug form for transport studies (85). Cyclophosphamide, with its ring structure intact, is inactive as an alkylating agent (90 - 92), thereby providing an excellent opportunity of studying transport reactions in a pure form.

II. MATERIALS AND METHODS

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A. Cell Cultures and Transport Studies

Murine leukemic L5178Y lymphoblasts were grown in cell culture at 37° in Fischer medium containing 10% horse serum, penicillin and streptomycin. Transport studies were carried out on exponential phase cells from the same cell line at a concentration of 2.5 to 3.0 x 10^6 cells/ml in Fischer medium. Chick embryo livers were digested by 2.5% trypsin and 1% pangestin with gentle agitation for 30 minutes. This method yielded a suspension of isolated liver cells at a concentration of approximately 2 - 3 x 10^6 cells/ml.

Cyclophosphamide-5-^{14}C monohydrate with a specific activity ranging from 4 - 9.9 mCi/mmole and 3-O-methyl-^{14}C-D-glucose, specific activity 10 mCi/mmole, were obtained from New England Nuclear, Boston. Cyclophosphamide-^{14}C was mixed with appropriate amounts of unlabelled cyclophosphamide monohydrate (Mead Johnson, Evansville, Ind.) to give specific activities ranging from 0.6 to 3.4 mCi/mmole. Activated cyclophosphamide was prepared by the method of Connors et al. using a NADPH-generating system and mouse hepatic microsomes (91).

Drug uptake was determined by incubating cells with labelled drug for a fixed period of time. Incubations were done in flasks or small vials at 37° in a shaking water bath. Incubations were terminated by rapid chilling to 4° and centrifugation in Hopkins vaccine tubes through a layer of 0.25 M sucrose to remove extracellular radioactivity. The Hopkins tubes were washed above the cell pellet, and the cells were solubilized in 0.5 N NaOH. Radioactivity was determined by liquid

scintillation spectrophotometry using Aquasol (New England Nuclear, Boston) or a scintillation mixture containing 8 g of PPO, 100 mg of POPOP, 1 liter of toluene, and 500 ml of Triton X-100. Cell counts were determined manually with a hemocytometer. Cell volume was measured with a Coulter Model B electronic particle counter, calibrated with giant ragweed pollen (mean diameter, 19.5 μ) and paper mulberry spores (mean diameter, 12.5 μ).

Uptake of cyclophosphamide was expressed in moles/min/cell, and moles were derived from radioactive disintegrations using the following formula:

$$\frac{\text{dpm}}{2.22 \times 10^{12}\ \text{dpm/Ci} \times \text{Ci/mole}} = \text{moles}$$

(definition of Ci) (specific activity of drug)

Uptake was also expressed as a cell/medium ratio of radioactivity derived by dividing radioactivity in the cellular fraction by that in the supernatant medium.

B. Distribution of ^{14}C-Cyclophosphamide in Cell Sap and Membrane Fractions

The distribution of cyclophosphamide was determined by measuring the radioactivity of cell sap and membrane fractions in L5178Y lymphoblasts 10 minutes after incubation with labelled drug. The cells were washed three times in cold saline, lysed in distilled water, and separated into cell sap and membrane fractions. The cell fractions and whole cells were solubilized on 0.5 N NaOH, and radioactivity was determined.

C. Evaluation of Na^+-Dependence of ^{14}C-Cyclophosphamide Uptake

The influence of Na^+ concentration on cyclophosphamide transport was examined by comparing drug uptake in Hank's Balanced Salt Solution and in sodium-depleted medium. The Na^+ concentration in Hank's BSS was 145 mEq/l, but only 5 mEq/l in medium with Tris replacing NaCl in isotonic proportions. Cells were resuspended in the prepared medium and equilibrated for 15 minutes before 10 minute transport studies were undertaken.

D. The Effect of Metabolic Inhibitors and Structural Analogues on ^{14}C-Cyclophosphamide Uptake

L5178Y cells were incubated with varying concentrations of inhibitor for at least one hour, and viability was determined on the basis of exclusion of 1% trypan blue dye. All inhibitors were used at a concentration that did not produce cell toxicity. In transport studies, the solvent used to dissolve the inhibitors was added to the control. Cells were pre-incubated with the metabolic inhibitors for 30 minutes, and drug uptake was determined after an additional 10 minute incubation period. The structural inhibitors were added simultaneously with labelled drug.

E. Correction for the Binding Component of Uptake

Early time points at various cyclophosphamide concentrations consistently had a cell/medium ratio of 0.14. This value, which approximates the rapid binding component of uptake, was subtracted from the cell/medium ratio obtained for both control and test conditions.

Uptake was expressed as a percentage of control cell/medium ratio. Confidence intervals employed were the standard error of the mean. Statistical evaluation of data was by the two-tailed Student t-test. Analysis of both raw and corrected uptake data indicated that t values were not altered by the binding correction.

Velocity of cyclophosphamide uptake (in moles/min/cell) by L5178Y cells was corrected for binding by the following procedure. At each drug concentration the binding ratio of 0.14 was subtracted from the cell/medium gradient derived from the curve shown in Fig. 2. The resulting cell/medium ratio divided by the total observed uptake represented the fraction of uptake due to net transport, i.e. the fractional uptake rate. Corrected velocities at each drug concentration were obtained by multiplying the observed velocity by the fractional uptake rate. Similarly, the kinetic analysis with chick embryo liver cells was corrected for binding by using the cell/medium ratios from a range of cyclophosphamide concentrations (Fig. 12). A cell gradient of 0.14, representing the rapid binding component, was obtained with the same liver cell preparation.

F. Neal Correction for Analysis of Two-Component Transport

Kinetic studies were analysed using the Lineweaver-Burk plot of reciprocal uptake velocity corrected for binding and expressed as moles/min/cell x 10^{-17} plotted on the ordinate, against reciprocal mM concentration of cyclophosphamide along the abscissa (Fig. 3). The slopes and intercepts of the two linear segments of the Lineweaver-Burk plot were determined from the linear regression equations of each

component. The inflection point of the biphasic curve was determined from the combination of drug concentrations which by linear regression analysis gave the best correlation coefficient for each of the two transport components.

Total drug uptake can be described as the sum of two Michaelis-Menten equations with different kinetic parameters;

$$v = \frac{V_1 \cdot S}{K_1 + S} + \frac{V_2 \cdot S}{K_2 + S},$$

where v is total velocity and V_1 and V_2 are the maximum velocities and K_1 and K_2 are the Michaelis constants for each of the two uptake components.

The values for V_1, K_1, V_2 and K_2, which are the corrected kinetic parameters, were determined from the following formula derived by Neal (55):

$$K_1, K_2 = \frac{1}{2}\left[\frac{M_1}{I_1} + \frac{M_1 - M_2}{I_2 - I_1} \pm \sqrt{\left(\frac{M_1}{I_1} + \frac{M_1 - M_2}{I_2 - I_1}\right)^2 - 4\left(\frac{M_1 - M_2}{I_2 - I_1} \cdot \frac{M_2}{I_1}\right)}\right]$$

$$V_1 = \left(\frac{K_1}{I_1} - \frac{M_1 - M_2}{I_2 - I_1} \cdot \frac{1}{I_1}\right) \Big/ (K_1 - K_2)$$

$$V_2 = 1/I_1 - V_1 ,$$

where M_1 and M_2 are the slopes and I_1 and I_2 are the intercepts observed for each of the two components of drug transport (as illustrated in the Lineweaver-Burk plot, Fig. 3).

III. RESULTS

III. RESULTS

A. Time Course of Cyclophosphamide Uptake

The time course of uptake of 1 mM cyclophosphamide by L5178Y cells is shown in Fig. 1. This representative plot of ten observations can be resolved into three phases.

There is an initial, rapid association of cyclophosphamide with the cell membrane that presumably represents binding. This is followed by a phase of linear uptake (extrapolation of which yields a positive value on the y-intercept). Cell uptake remains linear for up to 20 minutes, following which uptake begins to enter a plateau region, the third phase of uptake. The decline in the uptake curve represents loss of drug from the cells by efflux. The final plateau region is reached at the steady state where influx and efflux are equal.

From the results of other investigators (9 - 19), the rapid association of cyclophosphamide with the cells probably represents binding to the cell surface. The value of the first time point in Fig. 1 correlates with the y-intercept obtained by extrapolation of the linear portion of the uptake plot to the y-axis. A binding correction was made from the cell gradients obtained consistently at early time points with various cyclophosphamide concentrations.

As discussed in the Introduction (see page 1), kinetic analysis of a transport process must be carried out under conditions where initial uptake is being observed. With subtraction of the binding portion of cyclophosphamide uptake, the 10 minute incubation point in Fig. 1 now provides a valid measure of the initial rate of cyclophosphamide influx,

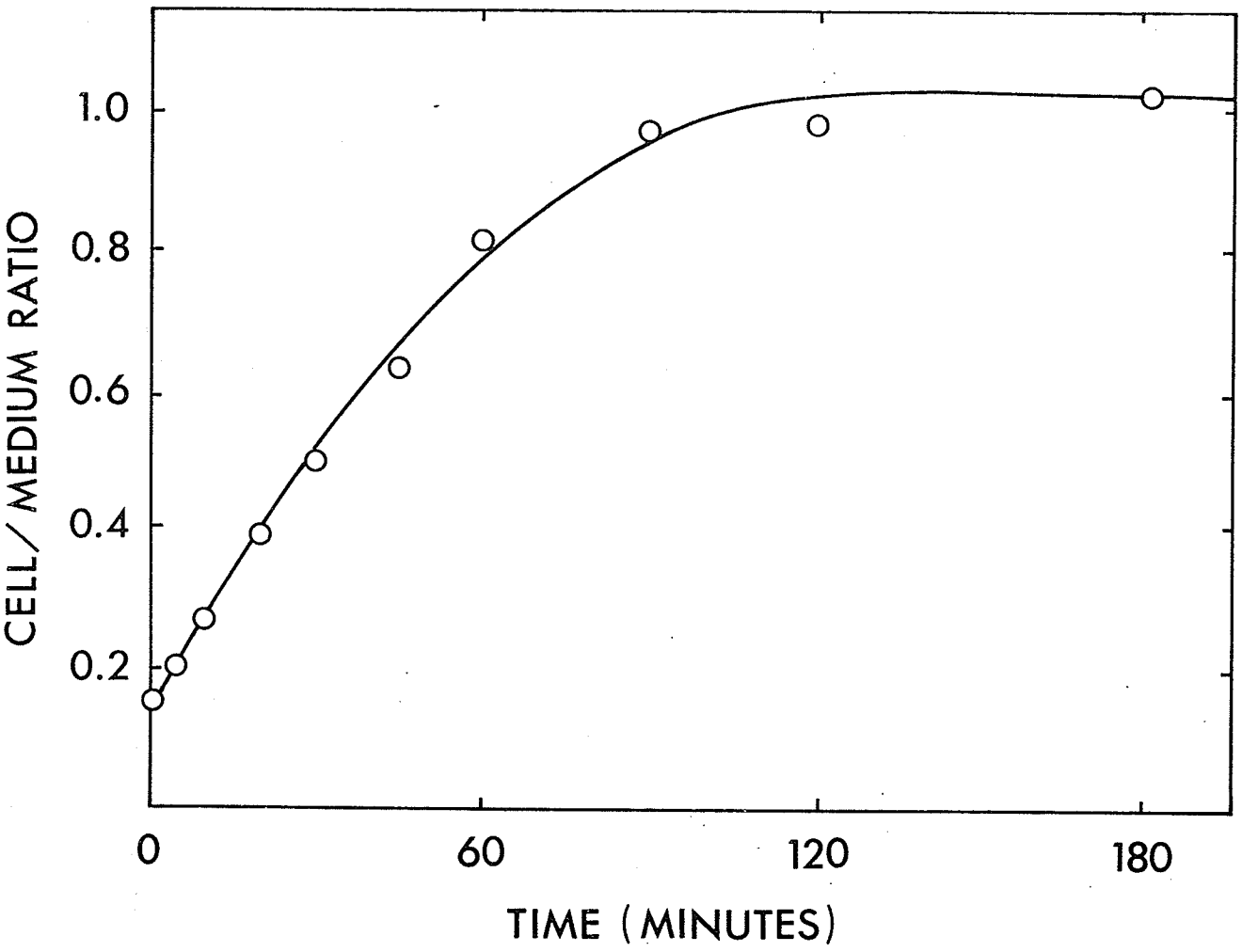


Figure 1. Time course of the uptake of 1 mM ^{14}C-cyclophosphamide by L5178Y cells at 37°. This study is representative of ten observations of the time course of cyclophosphamide uptake.

and was employed in all subsequent studies.

The time course in Fig. 1 also illustrates that cyclophosphamide uptake never exceeded a cell/medium concentration ratio of one. This suggests that cyclophosphamide transport is not an active process, in which uptake proceeds against concentration gradients greater than unity (2).

The ten minute uptake of 0.5 mM cyclophosphamide by whole cells and the distribution of drug between cell sap and membrane fractions was examined. The cell sap fraction had 89.6 $\pm$ 4.7% of the total cell radioactivity, while the membrane fraction contained only 1.31 $\pm$ 0.7% of the total radioactivity.

B. Kinetic Analysis of Cyclophosphamide Uptake

The relationship between cell/medium ratio and cyclophosphamide concentration is illustrated in Fig. 2. Each point is the mean cell/medium ratio of six experiments at cyclophosphamide concentrations ranging from 0.25 to 10 mM. The cell gradients drop as the concentration increases from 0.25 to 1 mM cyclophosphamide. In the high concentration range from 1 to 10 mM, no further drop in gradient was noted. The decrease in cellular penetration with increasing substrate concentration is due to saturation of carrier sites, and is characteristic of a carrier-mediated process (20).

The same uptake data was plotted according to the method of Lineweaver and Burk as shown in Fig. 3. Uptake is expressed as mean reciprocal uptake velocity corrected for binding, and is plotted against reciprocal cyclophosphamide concentration. This plot illustrates the

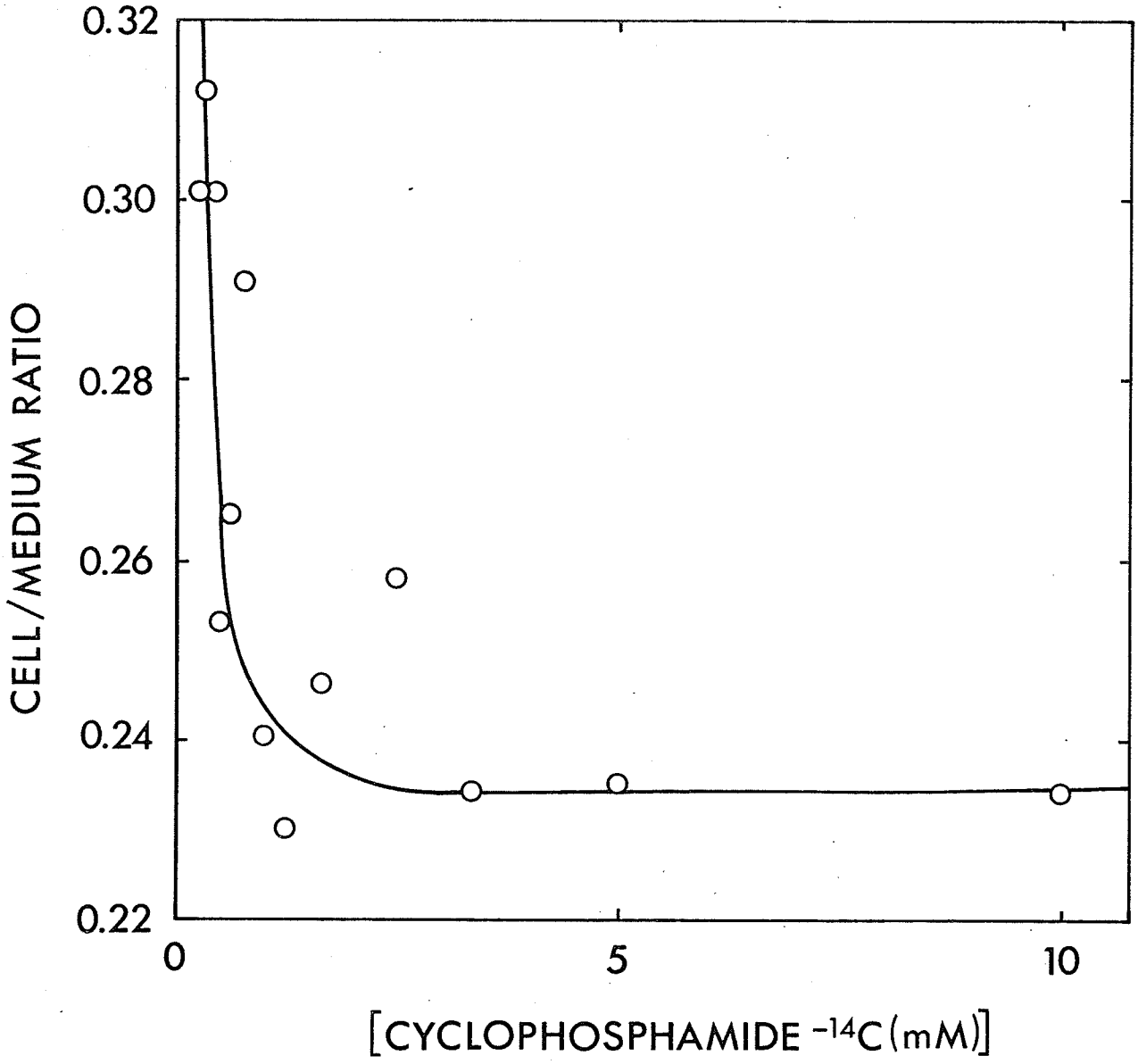


Figure 2. Uptake of ^{14}C-cyclophosphamide by L5178Y cells with cell/medium concentration gradient plotted as a function of drug concentration. L5178Y lymphoblasts at a concentration of 2 - 3 x 10^6 cells/ml were incubated for 10 minutes at 37° at drug concentrations ranging from 0.25 to 10 mM. Each point represents the mean of 6 determinations.

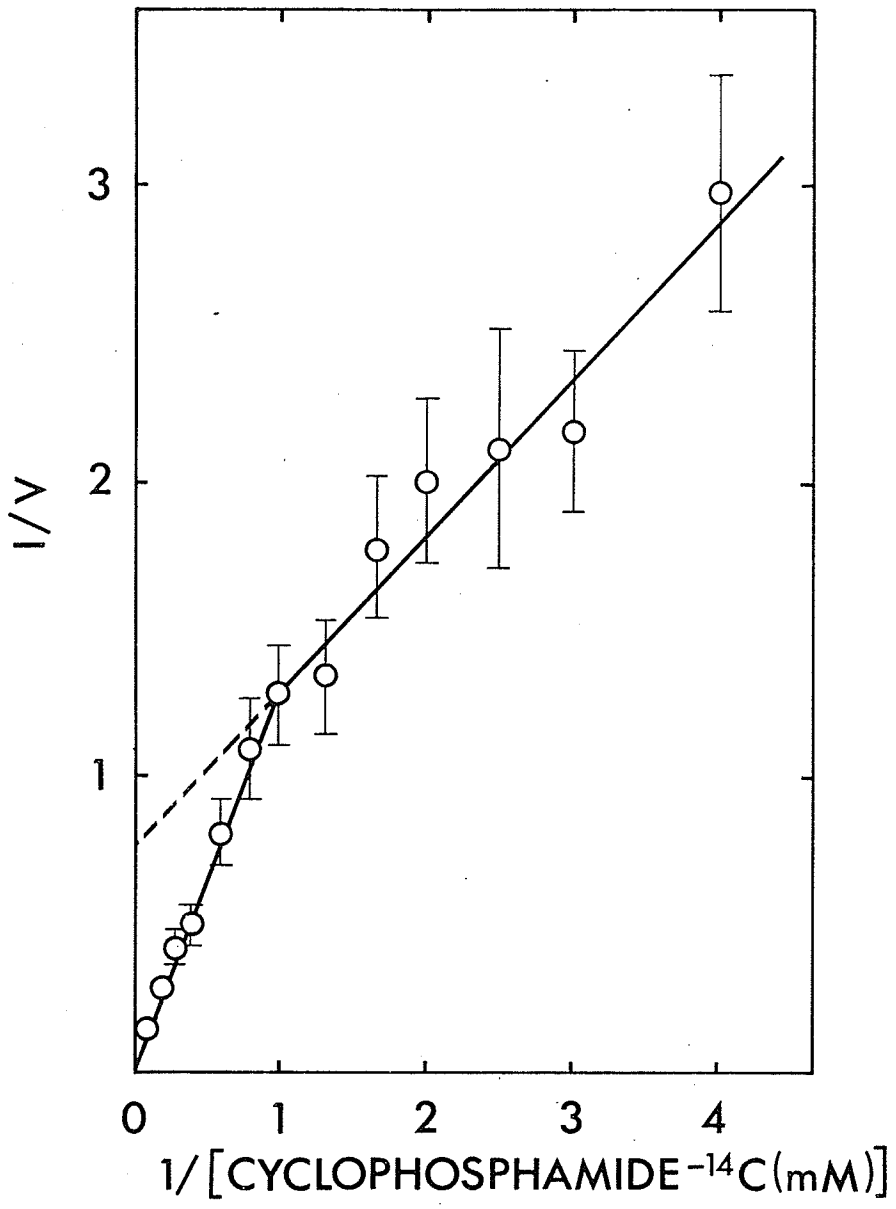


Figure 3. Lineweaver-Burk plot of the same uptake data shown in Fig. 2, after correction for binding. Reciprocal uptake velocity in moles/min/cell x 10^{-17} is plotted on the ordinate against reciprocal mM drug concentration on the abscissa. The two lines were obtained by linear regression analysis and each component included uptake data at 1 mM drug concentration. The data represent the mean of 6 determinations and the confidence intervals shown are the standard error of the mean. Standard errors are not shown where they are smaller than the circles.

biphasic nature of cyclophosphamide uptake examined over a 40-fold range in drug concentration. This represents a deviation from simple, linear Michaelis-Menten kinetics, and suggests two components of drug uptake.

Evidence for saturation of the low dose component is the finding of a positive intercept on the y-axis. The high concentration component reveals an intercept that approaches the origin, but which nevertheless has definite kinetic parameters (Table 1).

The same data was plotted according to the method of Eadie and Augustinsson (Fig. 4) in which mean velocity (V) is plotted on the ordinate against mean V divided by drug concentration (V/S) on the abscissa. The dependent variable, V, which is subject to experimental variation, appears in both axes (93). This plot clearly demonstrates the biphasic nature of cyclophosphamide uptake.

C. Resolution of Uptake Data into Two Transport Systems

The Neal analysis for two-component transport (55), outlined in the Materials and Methods section (see page 16), resolves the observed uptake pattern for cyclophosphamide into two components. Fig. 5 shows that the corrected high dose component is not appreciably changed by the Neal analysis, but the observed uptake overestimated the kinetic parameters for the low dose system.

The kinetic parameters obtained by resolution of the observed uptake into two separate transport components are displayed in Table 1. Transport at low cyclophosphamide concentrations appears to be mediated by a high affinity, low capacity system; while at high drug levels a low affinity, high capacity system predominates. The low dose system has a

TABLE I.

TRANSPORT COMPONENT	K_m (mM)	V_{max} (x 10^{-17} moles/min/cell)
LOW DOSE (0.25 - 1 mM)	0.39 $\pm$ 0.03	0.49 $\pm$ 0.07
HIGH DOSE (1 - 10 mM)	75 $\pm$ 29	49 $\pm$ 14

Kinetic parameters of cyclophosphamide transport by L5178Y lymphoblasts. Mean $\pm$ S. E. of 6 determinations from Lineweaver-Burk plots, after correction for binding and two-component transport. This Table and Figures 2 - 5 are all from the same data.

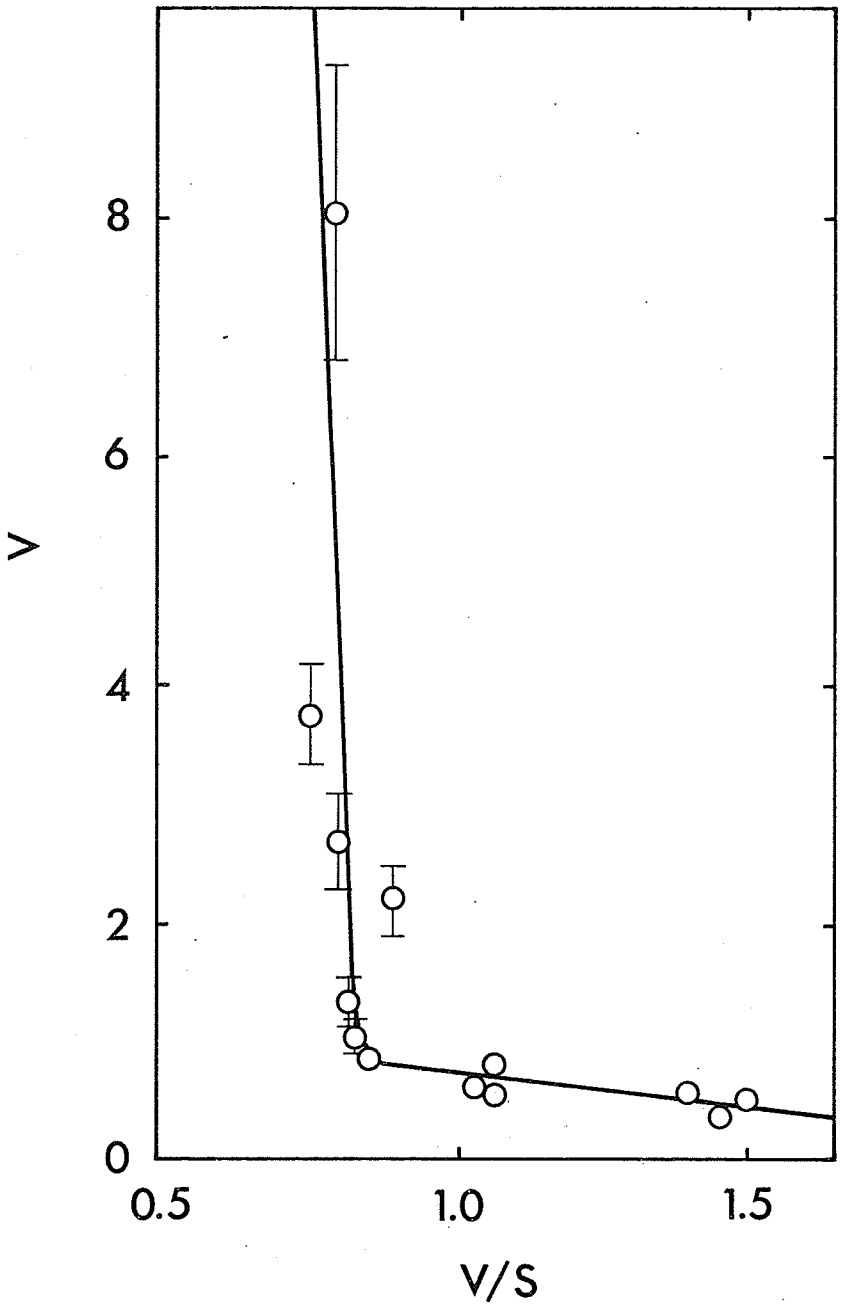


Figure 4. Eadie-Augustinsson plot of the same uptake data shown in Fig. 3. Uptake velocity in moles/min/cell x 10^{-17} is plotted on the ordinate against the ratio of uptake velocity/cyclophosphamide concentration (mM) on the abscissa. The two lines were drawn by linear regression analysis. Both components included uptake data at 1 mM drug concentration. The data represent the mean $\pm$ S.E. of 6 determinations. Standard errors are not shown where they are smaller than the circles.

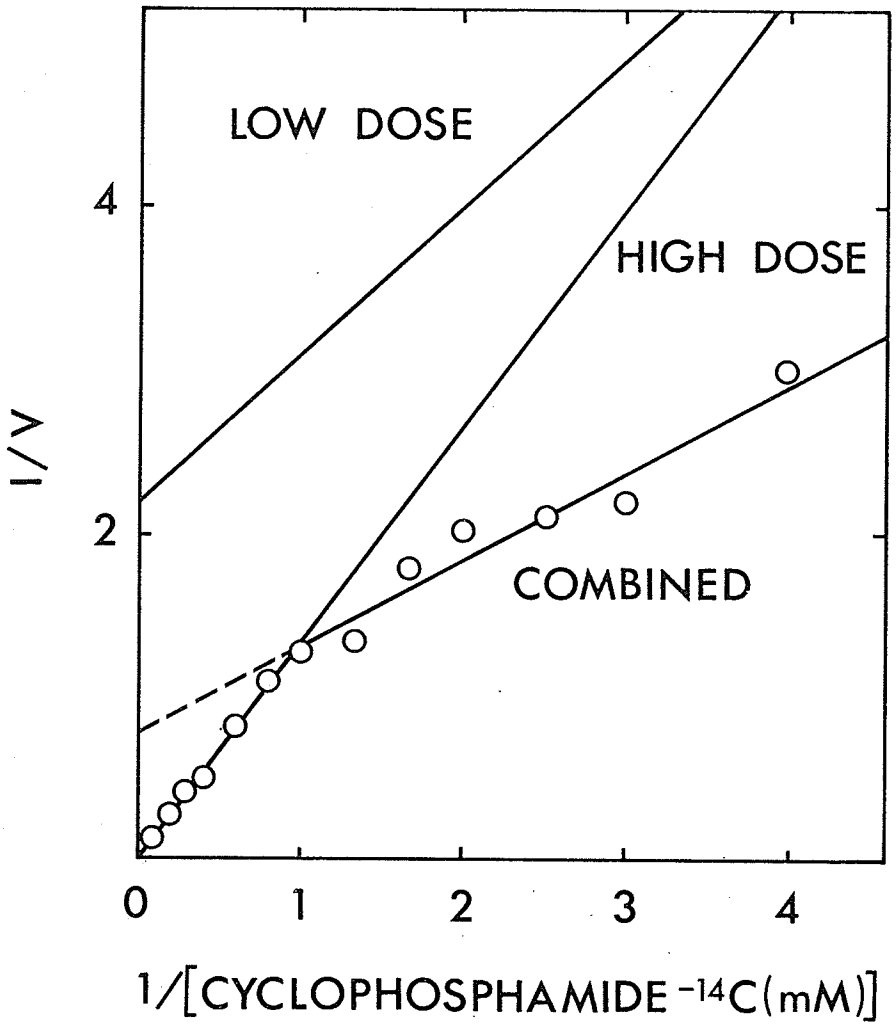


Figure 5. Neal correction for two-component transport of cyclophosphamide. The observed uptake plot is resolved into separate high- and low-dose uptake components.

K_m of 0.4 mM, which is within the range of cyclophosphamide concentrations studied.

The high dose component has a K_m value of 75 mM, well above the highest cyclophosphamide concentration examined. On the basis of kinetic analysis alone, it is not possible to determine whether this second phase of drug uptake is by simple diffusion or by a technically non-saturable, low affinity transport system.

D. Temperature-Dependence of Cyclophosphamide Uptake

Low dose cyclophosphamide uptake at 37° increased for up to two hours following an initial rapid, binding phase, while at 4° only the rapid component of drug uptake occurred (Fig. 6). High dose drug uptake also exhibited temperature dependence (Fig. 7); as demonstrated by the temperature sensitivity of drug uptake noted at 10 minutes. However, rapid binding as measured by uptake at 1 minute was temperature-independent. Temperature dependence of mediated transport of cyclophosphamide contrasts with the insensitivity of the rapid binding component.

E. The Effect of Sodium on Cyclophosphamide Transport

The effect of Na^+ on low dose cyclophosphamide transport is shown in Fig. 8. The uptake of cyclophosphamide was examined in Hank's BSS with Na^+ and in Hank's BSS with Tris replacing sodium. Drug uptake was corrected for binding and expressed as a percentage of the control uptake. The uptake in Na^+ -poor media was 46 and 40% of control, for 0.1 and 0.5 mM cyclophosphamide. Both results were statistically significant, $p < 0.001$ and $p < 0.01$, respectively.

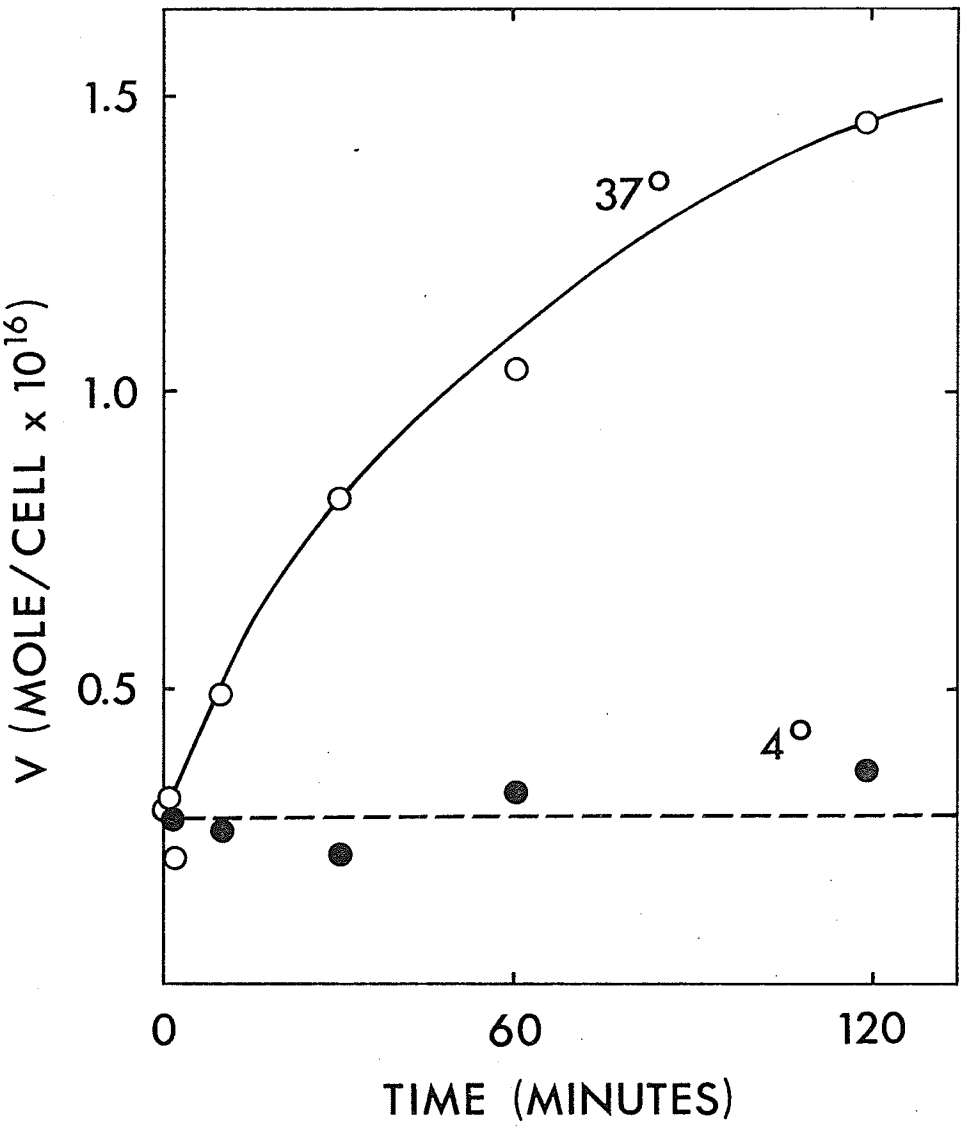


Figure 6. Time course of uptake of 0.1 mM ^{14}C-cyclophosphamide by L5178Y cells at 37° and 4°. This study is representative of three observations.

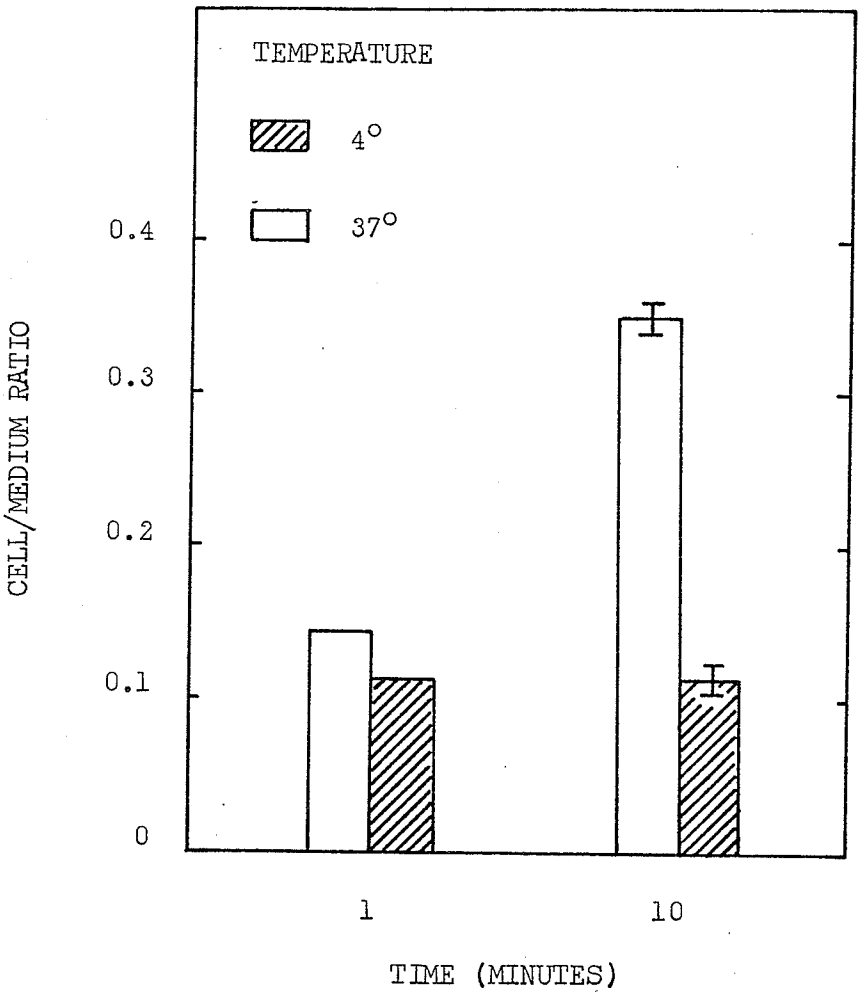


Figure 7. The effect of temperature on rapid binding of 10 mM ^{14}C-cyclophosphamide to L5178Y cells (as measured by uptake at 1 minute), and on mediated transport (as measured by uptake at 10 minutes). Duplicate samples from the same cell suspension at 1 minute and quadruplicate samples at 10 minutes were incubated at 37° and 4°. Uptake is expressed as total cell/medium concentration ratio.

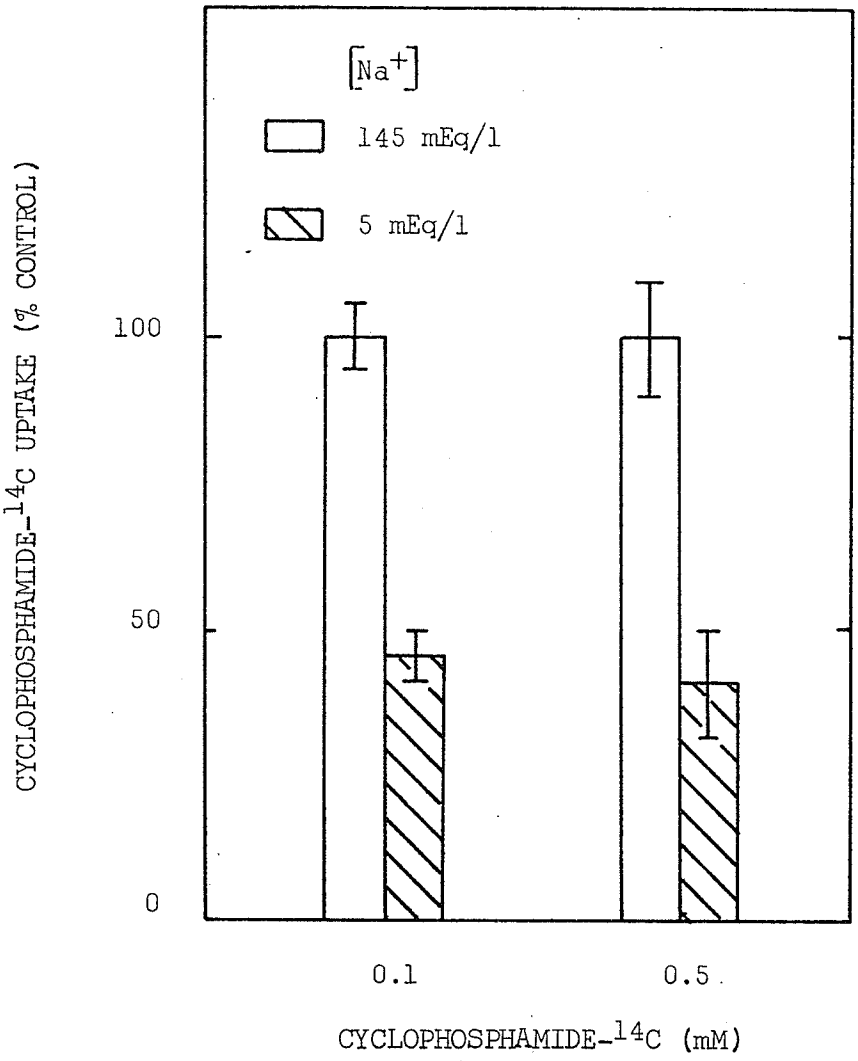


Figure 8. The effect of Na^+ on 0.1 and 0.5 mM ^{14}C-cyclophosphamide transport at 10 minutes. Uptake was compared in Hank's BSS with a Na^+ content of 145 mEq/l as control and in medium with a Na^+ content of 5 mEq/l. Uptake was corrected for binding and expressed as a percentage of the control cell/medium concentration ratio. The data are expressed as the mean $\pm$ S.E. of 4 determinations.

Ouabain at a concentration of 1 mM had no significant effect on the uptake of 0.5 mM cyclophosphamide. Drug uptake in the presence of ouabain was 94.3 $\pm$ 2.8% of control.

Sodium deprivation had no effect on the rapid binding phase of high dose cyclophosphamide uptake as illustrated by the uptake data at 1 minute (Fig. 9). However, mediated transport of 10 mM cyclophosphamide, as shown by the uptake data at 10 minutes, was 64% of control in Na^+-poor medium, and the difference was statistically significant, $p < 0.05$.

Drug binding was sodium- and temperature-independent and mediated transport of cyclophosphamide was sodium- and temperature-sensitive.

F. Effect of Metabolic Inhibitors on Cyclophosphamide Transport

The following metabolic inhibitors were examined as an investigation into the energy requirements for cyclophosphamide transport:

- Dinitrophenol and CCCP are aromatic agents which inhibit the formation of ATP;
- Oligomycin and antimycin A are antibiotics which inhibit ATP formation and electron transport;
- Sodium cyanide and sodium fluoride are anionic enzyme inhibitors;
- Iodoacetate, NEM and POMB are sulphydryl blocking reagents (94 - 98).

The effect of these metabolic inhibitors on cyclophosphamide transport is shown in Table 2. Oligomycin at a concentration of 0.1 mM

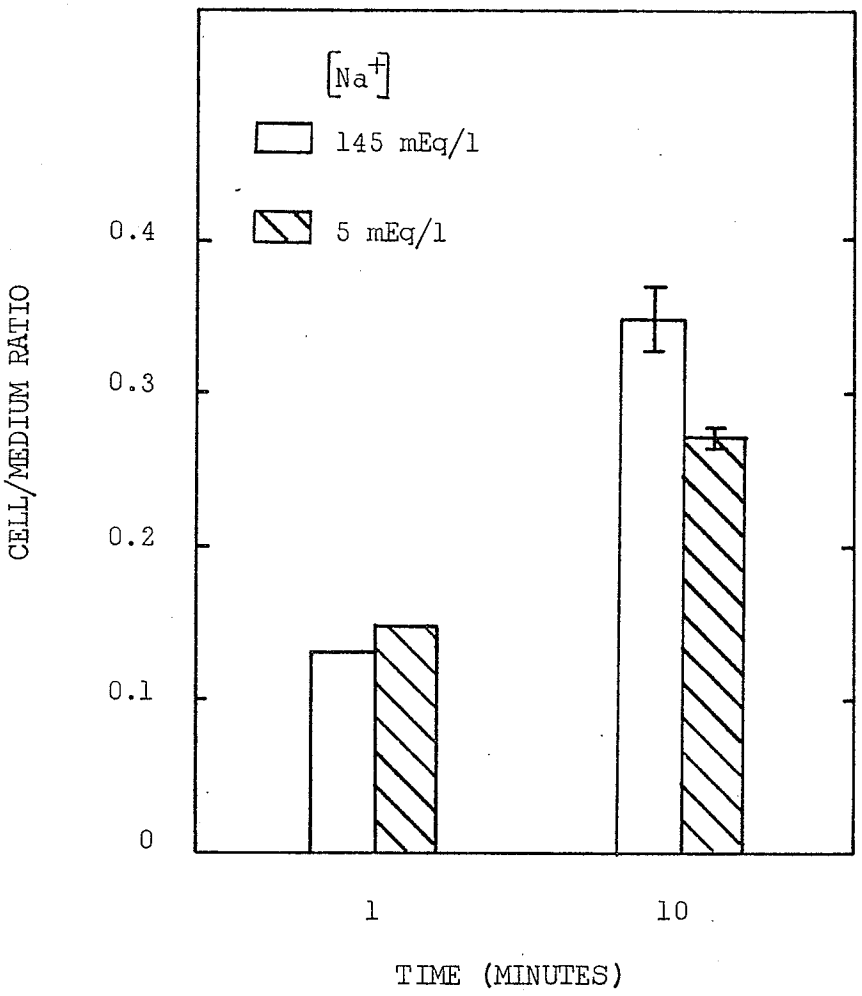


Figure 9. The effect of Na^+ on the rapid binding of 10 mM ^{14}C-cyclophosphamide to L5178Y cells (as measured by uptake at 1 minute) and on mediated transport (as measured by uptake at 10 minutes). Duplicate samples from the same cell suspension at 1 minute and quadruplicate samples at 10 minutes were incubated at 37° in Hank's BSS with Na^+ at a concentration of either 5 or 145 mEq/l. Uptake was expressed as the total cell/medium concentration ratio.

TABLE 2.

INHIBITOR	CONC. (mM)	NO. DETER-MINATIONS	% CONTROL (MEAN ± S.E.)	PROBABILITY
Oligomycin	0.1	8	63.7 ± 4.0	<0.001
Sodium cyanide	1.0	8	71.6 ± 3.4	<0.001
Sodium cyanide	0.1	4	86.2 ± 4.9	n. s.
Antimycin A	0.1	4	96.1 ± 4.2	n. s.
Dinitrophenol	1.0	4	90.8 ± 7.1	n. s.
* CCCP	0.1	4	101.3 ± 7.5	n. s.
Iodacetate	5.0	4	109.8 ± 3.9	n. s.
Sodium fluoride	20	4	94.9 ± 3.0	n. s.
* POMB	.02	8	105.6 ± 5.3	n. s.
* NEM	.0075	4	103.0 ± 0.8	n. s.

The effect of metabolic inhibitors on the transport of 0.5 mM ^{14}C-cyclophosphamide by L5178Y cells at 37° for 10 minutes. The inhibitors were preincubated for 30 minutes. Results are corrected for binding and are expressed as the percentage of mean control cell/medium concentration ratio. Simultaneous controls were obtained for each compound tested. The data represent the mean of 4 or 8 determinations. Statistical analysis was by the two-tail Student t-test. The term n. s. indicates that the data were not significantly different from the control ($p > 0.05$).

* CCCP - m-chlorophenyl carbonylcyanide hydrazone
* POMB - parahydroxymercuribenzoate
* NEM - N-ethylmaleimide

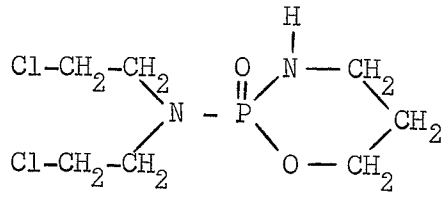
and 1 mM cyanide resulted in significant inhibition of drug uptake; all other inhibitors had no effect.

G. Evaluation of the Chemical Specificity of Cyclophosphamide Transport

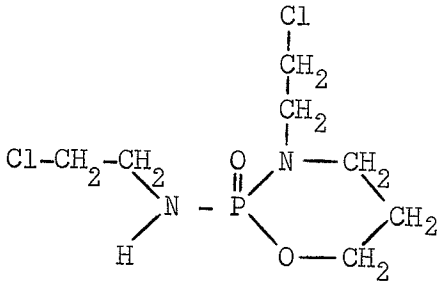
The effect of natural substrates and structural analogues on cyclophosphamide transport was examined. The following alkylating agents were tested--chlorambucil, melphalan, nitrogen mustard, isophosphamide and activated cyclophosphamide. Isophosphamide is an isomer of cyclophosphamide that has two chloroethyl groups each attached to a different nitrogen atom (Fig. 10). Activating cyclophosphamide presumably opens the ring structure of the molecule (99 - 101). The alkylating agents examined had no effect on cyclophosphamide transport (Table 3).

Various amino acids, components of nucleic acids, and the compounds, nicotine, nicotinamide and phenobarbital, were investigated for their effect on cyclophosphamide transport. None of these agents inhibited drug uptake as shown in Table 4.

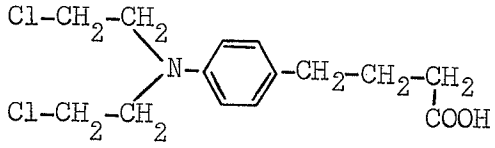
Cyclophosphamide uptake was determined in the presence of two specific inhibitors of glucose transport (97) to determine whether cyclophosphamide might be transported on a sugar carrier (Table 4). Phloretin significantly reduced drug uptake, while the difference in uptake with phlorizin fell short of statistical significance. To examine more directly the interaction of glucose and cyclophosphamide transport, uptake of 0.05 mM ^{14}C-3-O-methylglucose was examined in the presence of 0.1 and 1 mM unlabelled cyclophosphamide acting as potential inhibitor. Cyclophosphamide did not inhibit uptake of the labelled sugar.



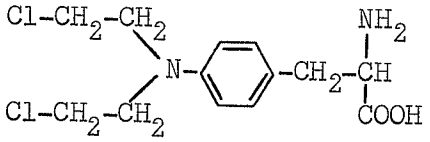
Cyclophosphamide



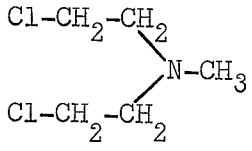
Isophosphamide



Chlorambucil



Melphalan



Nitrogen Mustard

Figure 10. Structural formulae of alkylating agents.

TABLE 3.

ALKYLATING AGENT	CONC. (mM)	NO. DETERMINATIONS	% CONTROL (MEAN ± S.E.)
Chlorambucil	1.0	3	107.4 ± 6.0
Melphalan	0.5	4	104.2 ± 6.0
Nitrogen mustard	0.5	8	100.8 ± 7.0
Isophosphamide	0.5	4	105.7 ± 4.0
Activated cyclophosphamide	0.5	4	100.5 ± 1.9

The effect of other alkylating agents on mediated transport of ^{14}C-cyclophosphamide. The agents were added simultaneously with the labelled drug and were incubated for 10 minutes at 37°. Cyclophosphamide was used at a concentration of 0.5 mM except for the study with chlorambucil where 1 mM ^{14}C-cyclophosphamide was used. Results are expressed as percentage of control cell/medium concentration ratio, as described in Table 2. All uptake values did not differ significantly from the control ($p > 0.05$).

TABLE 4.

INHIBITOR	CONC. (mM)	NO. DETERMINATIONS	% CONTROL (MEAN ± S.E.)
Amino acids			
Cycloleucine	5.0	4	86.1 ± 5.5
AIB	0.5	2	91.8
Proline	5.0	2	93.4
Phenylalanine	5.0	2	97.8
Components of nucleic acids			
Hypoxanthine	1.0	4	93.6 ± 4.1
Uracil	1.0	4	100.8 ± 2.3
6-methyladenine	0.5	4	104.3 ± 6.8
Adenine	0.5	2	96.0
Cytosine	0.5	2	97.3
Thymine	0.5	2	93.3
Adenosine	0.5	2	97.8
Compounds affecting cyclophosphamide metabolism			
Nicotine	5.0	4	106.6 ± 4.9
Nicotinamide	5.0	4	105.2 ± 5.5
Sodium phenobarbital	0.05	4	95.8 ± 2.1
Specific inhibitors of glucose transport			
Phloretin	0.1	8	87.8 ± 2.0
Phlorizin	1.0	4	85.7 ± 3.8

The effect of various compounds on transport of 0.5 mM ^{14}C-cyclophosphamide for 10 minutes at 37°. Results are expressed as a percentage of control with standard errors where applicable, as described in Table 2. The difference in uptake with phloretin was statistically significant ($p < 0.01$). All other uptake values did not differ significantly from the control ($p > 0.05$).

H. Cyclophosphamide Uptake by Chick Embryo Liver Cells

The time course of cyclophosphamide uptake by chick embryo liver cells at 37° and 4° is shown in Fig. 11. These results indicate that cyclophosphamide uptake by liver cells is temperature dependent and has a rapid binding component.

A single kinetic analysis of drug uptake by liver cells shows a decrease in uptake with increasing concentration (Fig. 12). A Lineweaver-Burk plot of cyclophosphamide uptake was biphasic (Fig. 13) with a K_m of 0.43 mM, and a V_{max} of 1.05×10^{-17} moles/min/cell for low dose transport, after a Neal-type correction for two-component transport. These values are similar to those obtained with L5178Y cells (Table 1). Cyclophosphamide transport in the high dose range was characterized by a K_m of 6.9 mM, and a V_{max} of 12.4×10^{-17} moles/min/cell. These values for high dose transport were lower than those demonstrated for L5178Y cells.

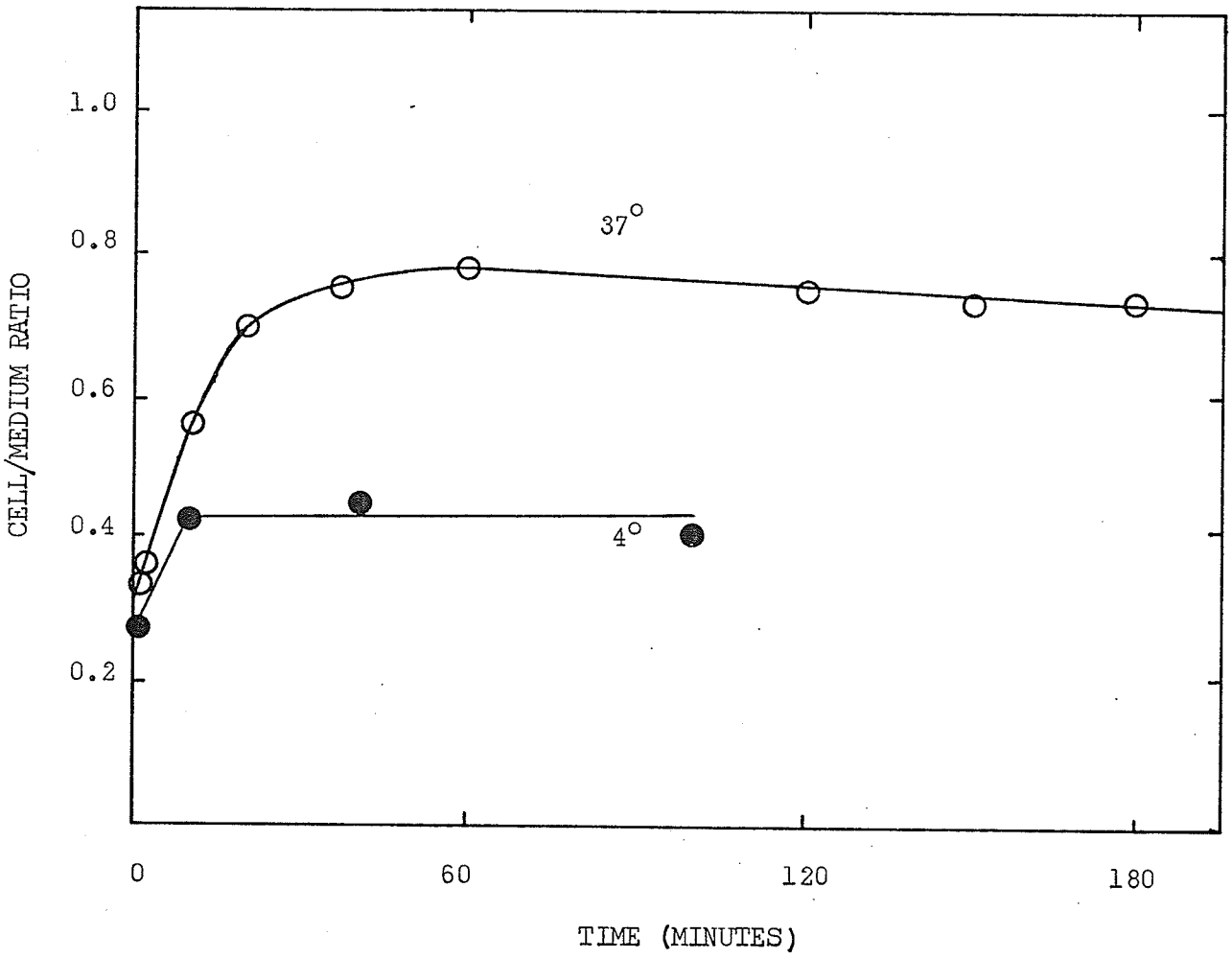


Figure 11. Time course of uptake of 1 mM ^{14}C-cyclophosphamide by chick embryo liver cells at 37° and 4°. This study is representative of two observations.

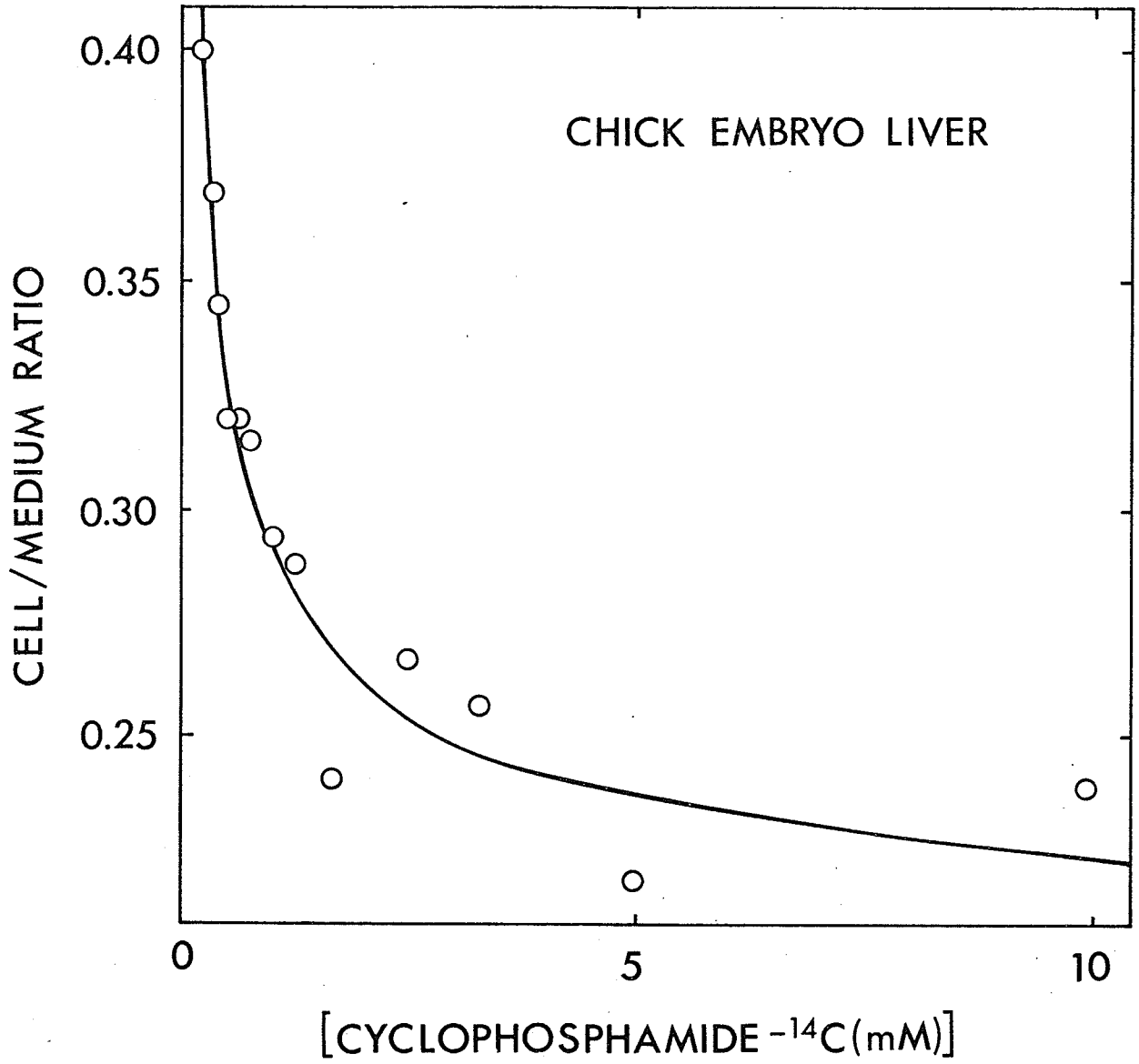


Figure 12. Uptake of ^{14}C-cyclophosphamide by chick embryo liver cells with cell/medium concentration gradients plotted as a function of drug concentration. Liver cells at a concentration of 2.6 x 10^6 cells/ml were incubated for 10 minutes at 37° at drug concentrations ranging from 0.25 to 10 mM.

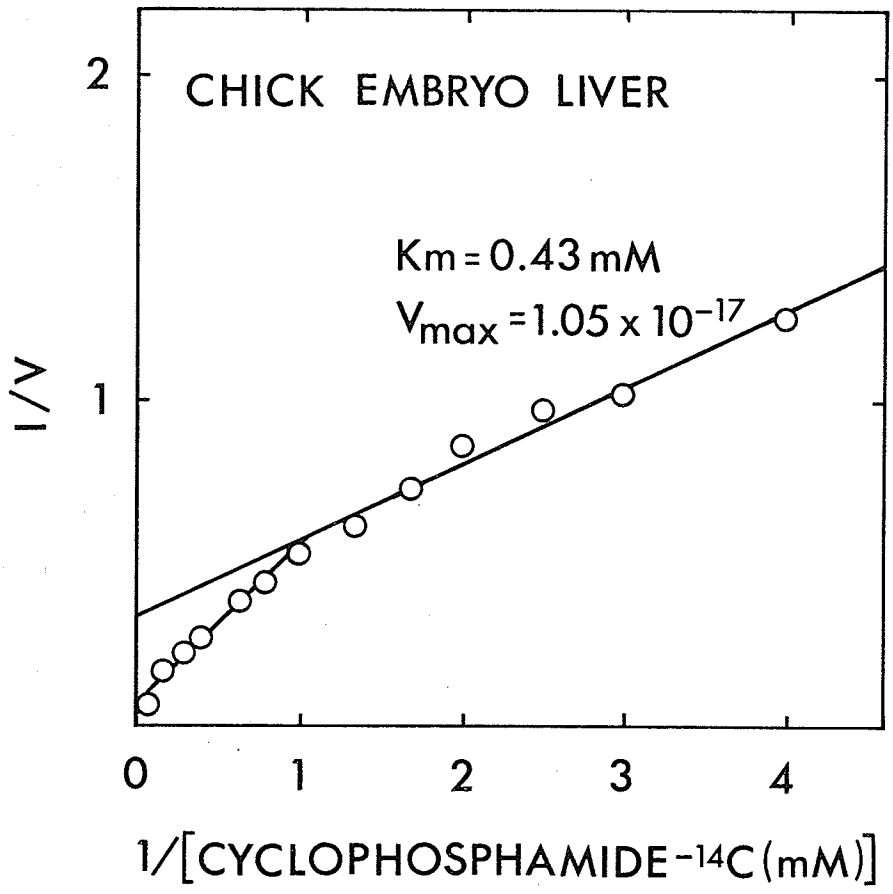


Figure 13. Lineweaver-Burk plot of the same data shown in Fig. 12, after correction for binding. Reciprocal uptake velocity in moles/min/cell x 10^{-17} is plotted on the ordinate against reciprocal mM drug concentration on the abscissa. The two lines were obtained by linear regression analysis with the 1 mM drug concentration being included in each component. The kinetic parameters shown are for the low dose component.

IV. DISCUSSION

IV. DISCUSSION

A. Binding of Cyclophosphamide to the Cell Surface

The rapid initial binding of cyclophosphamide to L5178Y lymphoblasts was temperature- and sodium-independent (the subsequent slower, mediated phase of drug uptake was temperature- and sodium-dependent). Uptake due to binding was subtracted from the total observed uptake to measure initial influx rates.

Evidence that cyclophosphamide enters cells was shown by the distribution of drug between membrane and sap fractions. The majority of cyclophosphamide appeared in the cell sap, with relatively small quantities in the membrane fraction. Furthermore, the small amount of radioactivity in the membrane fraction indicates that washing effectively removes the binding of drug to the cell surface (9, 17).

B. Kinetic Analysis of Cyclophosphamide Transport

The uptake of cyclophosphamide across the plasma membrane of L5178Y cells was mediated by two distinct processes. This was revealed most clearly by the Eadie-Augustinsson plot of drug uptake. The Lineweaver-Burk plot of cyclophosphamide transport was resolved into two independent components by the Neal analysis. This correction markedly altered the calculated kinetic parameters of low dose transport, but had little effect on the high dose component.

Kinetic analysis of low dose uptake showed evidence of saturability. Absolute saturation could not be shown because of superimposition of the high dose component. The demonstration that the rate

of uptake of a substrate approaches a limiting saturation value with increasing concentration is strong evidence of carrier-mediated transport (2).

High dose cyclophosphamide uptake cannot be characterized by saturation kinetics as this carrier system exhibits a very low affinity for the drug. The demonstration that cyclophosphamide transport by the high concentration component was temperature- and sodium-dependent provides evidence for a carrier-mediated mechanism.

The existence of two systems for transport of cyclophosphamide across the cell membrane is not unusual. As indicated in the Introduction (see page 5), there are many reports of more than one mode of mediated transport for a single organic molecule. Many transport systems have exhibited low affinity comparable to the unsaturable high dose component of cyclophosphamide transport (see page 3).

C. Temperature-Dependence of Cyclophosphamide Uptake

The uptake of cyclophosphamide at 0.1 and 10 mM concentrations was strongly temperature sensitive. Temperature sensitivity of transport systems greater than that expected for the effect of absolute temperature is generally regarded as evidence of mediated transport. According to Stein, the rate of transfer of a substrate across the cell membrane by carrier-mediated transport is more dependent upon temperature than if the substrate crossed a lipid barrier by simple diffusion (2). Christensen and Liang state that strong temperature dependence exhibited by amino acid uptake provided evidence of mediated transport and excluded simple diffusion as a mode of uptake (28, 31).

The temperature dependence of cyclophosphamide transport by L5178Y cells is strong evidence for carrier-mediated transport throughout the 100-fold concentration range examined.

D. Sodium-Dependent, Ouabain-Insensitive Transport of Cyclophosphamide

1. Na^+ -Dependence as Evidence of Carrier-Mediated Transport

A partial Na^+ requirement was demonstrated for cyclophosphamide transport. At low drug concentrations uptake in media deficient in Na^+ was 40 - 46% of control uptake. The transport of cyclophosphamide (0.5 mM) was not affected by ouabain, a drug which disrupts ion gradients by inhibiting the Na^+ - K^+ pump (102). The uptake of 10 mM cyclophosphamide in sodium-depleted media was 64% of the control. The requirement for sodium provides evidence for carrier-mediated transport of cyclophosphamide by both low and high dose systems.

The significance of sodium dependence as a criterion of mediated transport is illustrated by investigations which correlate Na^+ -dependence with carrier-mediated transport (27, 103). Sodium dependence of amino acid transport is usually considered to be linked to active accumulation (1, 67). However, Bihler has pointed out that the potential for active transport of sugars depends on the extent of sodium activation of the sugar carrier (20). Bihler et al. demonstrated a facilitated diffusion component of Na^+ -dependent accumulation of sugars in the intestine (104).

2. Mechanisms for Na^+ -Dependence--the Ternary Complex Model

The assumption that a sodium-linked substrate must be actively

transported probably comes from the wide acceptance (105) of Crane's model for the interaction of organic molecules and sodium ions (106, 107). The Na^+ gradient hypothesis suggests that Na^+ is co-transported with either an amino acid or sugar molecule. This ternary complex of sugar-carrier-sodium was postulated by Crane from observations of the flux of sugar molecules and sodium ions across the brush-border membrane of mammalian small intestine.

The ouabain-sensitive $Na^+ - K^+$ pump maintains a low internal level of sodium so extracellular Na^+ tends to move downhill into the cell, carrying the sugar with it in the form of a ternary complex. The electrochemical energy of the sodium ion gradient is the driving force which allows the accumulation of organic molecules against a concentration gradient (106, 107).

Although the above model explains the data for some transport systems (105), the results from a number of investigations have shown that Crane's ternary complex description of the mechanism of sodium-dependent, metabolite transport across mammalian intestine cannot be universally applied to other tissues.

The basis of the Na^+ requirement for transport of amino acids was demonstrated to be an optimal extracellular Na^+ concentration, not a physiological sodium gradient, in Ehrlich ascites cells (108 - 110), mouse pancreas (111), rat muscle (112), mouse brain (113) and chicken small intestine (114). Schafer and Heinz calculated that a major part of the energy necessary for active transport of amino acids in Ehrlich ascites cells could not be accounted for by energy derived from cation concentration gradients (115). Johnstone et al. showed that ATP was

the most important energy source for concentrative transport of amino acids in Ehrlich cells and in mouse pancreas (108-111).

3. Na^+ -Dependent, Ouabain-Insensitive Transport

Inhibition of metabolite transport by the cardiac glycoside ouabain is regarded as evidence that accumulation of metabolite depends upon the cation gradients produced by the $Na^+ - K^+$ pump. Thus, ouabain sensitivity and sodium dependence are usually coupled phenomena (105 - 107). However, ouabain was shown to have little or no effect on Na^+ - dependent transport in the following cases: glucose transport by rabbit renal tubule membranes (53); choline transport by chick small intestine (75); histidine uptake by S37 ascites cells (32); AIB transport by cultured heart cells and by rat muscle (112, 116). Cyclophosphamide uptake appears to be another example of Na^+ -dependent, ouabain-insensitive transport.

4. Speculation on the Mechanism of the Na^+ Requirement of Cyclophosphamide Transport

Hillman and Rosenberg suggested that Na^+ may act at more than one transport step, and that it may have different mechanisms of action in the total transport process (117). They examined both the substrate-carrier interaction and membrane translocation processes of proline uptake by renal tubules (41, 117). Interaction of proline with the carrier was partially sodium-dependent and was ouabain-insensitive, while transport across the tubule membrane was markedly sodium-dependent and ouabain-sensitive.

The partial Na^+ -dependency and ouabain-insensitivity of cyclophosphamide transport may be explained by a sodium requirement for the interaction of substrate and carrier. Evidence against a Na^+ requirement for the membrane translocation of cyclophosphamide was the finding that uptake was not inhibited by ouabain, and did not proceed against a concentration gradient, which would occur with Na^+ -linked co-transport.

Sodium ions may act on the cyclophosphamide carrier, producing allosteric changes at the active site. Bihler has speculated that Na^+ produces an allosteric change in the intestinal sugar carrier such that the affinity for certain sugars is increased (20).

E. Metabolic Energy Requirements of Cyclophosphamide Transport

1. The Effect of Metabolic Inhibitors on Transport Systems

Metabolic inhibitors, acting as enzyme poisons, may interfere with the production or utilization of energy which is necessary for the function of the cell. Facilitated diffusion transport systems (which do not accumulate substrate against a concentration gradient) do not require a large input of energy, unlike an energy-coupled active transport process. However, energy is still necessary to maintain the structure of the cell membrane, including the transport carrier. Thus, a reduction in metabolic energy can also inhibit an inactive carrier-mediated process (2, 97).

Many studies of the effects of specific metabolic inhibitors, have employed subcellular fractions obtained from disrupted cells (96). Attempts to reproduce these effects in intact cells are complicated by

the need to define precise intracellular concentrations of the inhibitors.

Hewitt and Nicholas have stated that "the effects of enzyme inhibitors *in vitro* on isolated systems may not always resemble their apparent effects on more complex metabolic systems when studied in living cells" (94).

2. Respiratory Inhibitors

Antimycin A blocks the flow of electrons from cytochrome b to cytochrome a in mitochondrial respiration. At this point, the second of three respiratory chain phosphorylations takes place (98). Cyanide is a very reactive molecule and can produce enzyme inhibition in many ways, the most common being metalloenzyme inhibition. Cytochrome oxidase, the terminal enzyme in the respiratory chain which reacts with oxygen, is the most sensitive enzyme in that 10^{-8} M cyanide yields 50% inhibition (94).

Cyclophosphamide uptake in the low concentration range was significantly inhibited by 1 mM sodium cyanide, was slightly inhibited by 0.1 mM cyanide but not to a significant degree, and was not affected by 0.1 mM antimycin A. Higher concentrations of antimycin A were not used because of cell toxicity. Relatively high cyanide concentrations (5 to 10 mM) have been used in characterizing the metabolic requirements of other transport systems (15, 25, 32, 35 - 37, 41, 44).

Metabolic inhibition of intact cells may require high cyanide concentrations because of poor membrane permeability of this anionic inhibitor. Since cyanide is a weak acid, penetration is limited to the uncharged form (94). By contrast, antimycin is lipid soluble and can

penetrate cells more readily than the hydrophilic sodium cyanide molecule (2). This precludes a direct comparison of the concentration of antimycin and cyanide in evaluating their effect on cyclophosphamide transport.

Respiratory inhibition by cyanide (without parallel inhibition from antimycin) may be due to the high sensitivity of cytochrome oxidase by cyanide, or to the wide range of enzyme inhibition produced by cyanide. Cyanide reacts with other metal enzymes besides cytochrome oxidase and also with carboxyl and disulphide groups in enzymes (94).

3. Inhibitors of ATP Synthesis

The metabolic inhibitors 2,4-dinitrophenol (DNP) and m-chlorophenyl carbonylcyanide hydrazone (CCCP) uncouple oxidative phosphorylation from mitochondrial respiration without inhibiting the respiratory process (95, 98).

Oligomycin blocks ATP formation in a different way than the uncoupling agents so that although oxidative phosphorylation is the primary locus of action, cell respiration is inhibited as well (98). Oligomycin has been postulated to inhibit energy transfer from high-energy compounds to ADP in the synthesis of ATP, in contrast to the uncouplers which cause the discharge of all high-energy compounds (95).

Oligomycin at a concentration of 0.1 mM significantly inhibited cyclophosphamide uptake; however, no inhibition was observed with either 0.1 mM CCCP or 1 mM DNP. A similar rate of cell penetration may occur for these three inhibitors as they are lipid soluble compounds. The difference in mechanism of inhibition between oligomycin and the

uncoupling agents may be related to the preferential sensitivity of cyclophosphamide transport to oligomycin. More marked inhibition with oligomycin than with dinitrophenol has also been reported for other transport systems (116, 118).

Dunand et al. studied the effect of oligomycin and DNP on AIB uptake in cultured heart cells (116). Dinitrophenol caused significant inhibition only at a concentration (1 mM) that also reduced beating activity of the heart cells. Oligomycin resulted in highly significant inhibition of AIB transport at a concentration of 0.24 to 12 μM, without inducing changes in the contractile activity of the cells.

Hempling examined the effect of 7.8 μM oligomycin, 0.75 μM antimycin A, and 0.1 mM dinitrophenol on the transport of sodium and potassium by Ehrlich ascites cells (118). The influx of potassium was reduced 2-fold more with oligomycin than with DNP when normal cell ATP levels were maintained by glucose. Under these conditions, antimycin A had no effect on ion transport. Hempling concluded that oligomycin exerts its effect closer to the transport process than do the other inhibitors (118).

4. Sulphydryl Reagents

N-ethylmaleimide (NEM) and parahydroxymercuribenzoate (POMB) inhibit transport processes directly by interacting with sulphydryl (SH) groups on carrier proteins (97). NEM and POMB show high selectivity, reacting only with accessible thiol groups such as those at active sites of enzymes and transport carriers. Iodoacetate has a broader, less specific reactive spectrum than POMB or NEM, since it reacts with

sulphydryl and amino groups on proteins (96).

SH groups apparently do not play a critical role in cyclophosphamide transport since uptake was not affected by the sulphydryl reactive reagents used in this study.

5. Inhibitors of Glycolysis

In addition to direct inhibition of membrane transport by reacting with SH groups, iodoacetic acid can inhibit transport indirectly by affecting intermediary metabolism at many points (96). A major effect of iodoacetate is to depress glycolysis by reacting with the enzyme glyceraldehyde-3-phosphate dehydrogenase. Fluoride ions inhibit metabolism by complexing with many metal-enzyme systems, enolase being particularly sensitive (94).

Cyclophosphamide transport was not inhibited by 5 mM iodoacetate or 20 mM sodium fluoride--inhibitor concentration capable of inhibiting glycolysis (20, 96). The inability to inhibit drug transport with iodoacetate or fluoride suggests that glycolysis is not an energy source for maintaining cyclophosphamide transport.

6. Sensitivity to Metabolic Inhibitors as a Criterion of Mediated Transport

Inhibition of cyclophosphamide transport by oligomycin and cyanide suggests that energy produced by oxidative phosphorylation may be required for drug transport. On the basis of this study, it is impossible to identify the mechanism of inhibition produced by oligomycin and cyanide, particularly since other metabolic inhibitors with similar

mechanisms of action were ineffective. Stein lists the reduction of the rate of substrate penetration by chemical inhibitors as a strong criterion for facilitated diffusion (2).

Inhibition of uptake by the two metabolic inhibitors, oligomycin and cyanide, together with the demonstration of temperature- and sodium-dependence, constitute additional evidence that cyclophosphamide transport is carrier-mediated.

F. Investigation of the Chemical Specificity of Cyclophosphamide Transport

1. Alkylating Agents

In an attempt to demonstrate chemical specificity for cyclophosphamide transport, other alkylating agents were examined for their effect on cyclophosphamide uptake by L5178Y cells. In preliminary studies we observed that the presence of equivalent amounts of unlabelled cyclophosphamide inhibited uptake of ^{14}C-cyclophosphamide. Activated cyclophosphamide, isophosphamide and hydrolyzed analogues were all ineffective in blocking transport of cyclophosphamide.

The inability of activated cyclophosphamide to inhibit ^{14}C-cyclophosphamide uptake indicates that the intact and activated forms of the drug are transported by different mechanisms. Isophosphamide, an isomer of cyclophosphamide, has the same basic ring structure, differing only in that one chloroethyl group is attached to the ring nitrogen (Fig. 10). This suggests a narrow range in the chemical specificity of cyclophosphamide transport since a close structural analogue had no effect on drug uptake.

The lack of inhibition by other alkylating agents suggests

that transport of cyclophosphamide is by an independent mechanism. Work in our laboratory showed that both intact and activated cyclophosphamide, melphalan and chlorambucil were transported by mechanisms independent to that observed for nitrogen mustard (85). The independence of cyclophosphamide and nitrogen mustard transport, together with the inability of other alkylating agents to inhibit transport of either drug, suggests that many mechanisms exist for transport of alkylating agents.

2. Natural Substrates

It was assumed that the cell membrane would not contain a transport carrier specific for a toxic compound such as cyclophosphamide. Accordingly, several naturally-occurring substances were investigated in order to identify a native substrate for the cyclophosphamide carrier. The amino acids, glucose-related sugars, and components of nucleic acids compromise three major transport systems, each one demonstrating chemical specificity.

i. Amino Acids

The amino acids, AIB, proline, phenylalanine and cycloleucine resulted in no significant inhibition of cyclophosphamide transport. AIB and proline are representatives of the A-system as originally described for amino acid transport in Ehrlich ascites cells, phenylalanine is mediated by the L-system, and cycloleucine uses both systems (34, 56). Cyclophosphamide, proline, phenylalanine and cycloleucine are compounds with a ring structure. Amino acids are relatively non-specific for their variable side chains and their principal transport specificity

relates to carboxylic and amino groups (84) which are lacking in the cyclophosphamide molecule.

ii. Sugars

The facilitated diffusion of sugars exhibits a relatively broad range of specificity for transport (97). Stein suggests that specificity requirements for facilitated diffusion systems are less stringent than for active transport (97).

The drugs phloretin and phlorizin are described as specific inhibitors of glucose and its analogues (97). Phlorizin is usually most effective in inhibiting active accumulation of sugars, while facilitated diffusion systems are most sensitive to phloretin (97, 119). Phloretin and phlorizin resulted in a small reduction in the transport of cyclophosphamide. ^{14}C-3-O-methylglucose, an analogue of glucose which is not metabolized (120), was used as substrate to examine more directly the interaction between cyclophosphamide and glucose transport. Sugar uptake was not affected by 2- and 20-fold higher concentrations of unlabelled cyclophosphamide. Despite the slight inhibition of cyclophosphamide transport by phloretin and phlorizin, it is unlikely that cyclophosphamide enters L5178Y cells on a sugar carrier because unlabelled drug had no effect on 3-O-methylglucose transport.

Furthermore, phlorizin and phloretin have been reported to inhibit transport of substrates other than sugars. Jacquez has reported that phlorizin inhibited the uptake of uridine into Ehrlich ascites cells (121), and Alvarado has described phloretin as an inhibitor of neutral amino acid transport by guinea pig small intestine (122).

iii. Components of Nucleic Acids

Purine and pyrimidine nucleosides apparently share a common transport carrier in rabbit leukocytes (123) and human erythrocytes (124, 125), although multiple nucleoside transport systems have been reported for Novikoff hepatoma cells (71). The purine and pyrimidine bases may have individual transport systems. Adenine transport by rabbit leukocytes exhibits transport specificity for the NH group at position 9 on the adenine imidazole ring (84). 6-methyladenine has a relatively high affinity for the adenine carrier in leukocytes.

Transport of cyclophosphamide on a carrier functioning for nucleic acid components could not be demonstrated since adenine, 6-methyladenine, hypoxanthine, uracil, cytosine, thymine and adenosine did not significantly inhibit cyclophosphamide transport.

3. Compounds Affecting Cyclophosphamide Metabolism

Activation of cyclophosphamide by hepatic microsomes has been reported to be inhibited by nicotine and nicotinamide (92, 126) and stimulated by phenobarbital (127, 128). However, nicotine, nicotinamide and phenobarbital, each of which contain a 6-membered heterocyclic ring with an NH group, had no effect on cyclophosphamide uptake.

4. Considerations on the Transport Specificity of Cyclophosphamide

The amino acid, diethylglycine (DEG), was synthesized as an analogue which would not be transported by known carrier systems (19). Although DEG exhibited minimal inhibitory action on the transport of

several amino acids, transport occurred predominantly by an independent system. Christensen and Liang emphasized that failure of a particular substance to inhibit known transport systems does not exclude a carrier-mediated transport mechanism for that substance (19).

Because of the existence of a transport system functioning only for specific, synthetic amino acids, Christensen and Liang speculate that there may be other transport systems available for non-biological substances (19). Christensen and Liang have stated that the diversity of transport systems has not been determined by biological need alone (33), and even the penetration of foreign hydrophilic molecules may involve some form of chemical recognition by functional groups within the cell membrane (31).

Good and Rose suggest that compounds which appear to have radically different structural characteristics may adopt similar conformations in solution, due to structural changes related to hydration (129).

Examination of several of the known transport systems has failed to identify a native substrate for the cyclophosphamide carrier. It has been speculated that chemical specificity may relate to allosteric conformation in solution (129), and that transport specificity may include chemical recognition of non-biological molecules (19, 31). Cyclophosphamide may exhibit a structural conformation in solution that resembles a metabolite that has not been examined, or alternatively, cyclophosphamide may utilize a transport system with specificity toward similar heterocyclic compounds.

G. Cyclophosphamide Transport in Chick Embryo Liver Cells

The demonstration of temperature dependence and heterogeneous kinetics indicates that transport of cyclophosphamide in chick embryo liver cells is also carrier-mediated. The parallel mechanism of cyclophosphamide transport in liver cells and L5178Y lymphoblasts suggests a similarity of drug transport in normal and neoplastic tissue. The liver is the principal site of cyclophosphamide activation (90, 92, 126, 130) so evidence of carrier-mediated transport in this tissue is relevant to the pharmacology of cyclophosphamide.

V. SUMMARY AND CONCLUSIONS

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Transport of the alkylating agent (^{14}C) cyclophosphamide by L5178Y lymphoblasts *in vitro* was characterized by the following properties:

1. The time course of cyclophosphamide uptake showed a rapid, initial binding phase of drug to the cell surface, following which drug uptake into the cells was linear for up to 20 minutes.
2. Cyclophosphamide uptake never exceeded a cell/medium gradient of one, suggesting that uptake was not an active process.
3. Kinetic analysis over a 40-fold range of drug concentration was biphasic, suggesting the existence of two transport systems. At low drug concentrations, uptake showed evidence of saturation, while at high cyclophosphamide levels, uptake occurred on a second transport system that was technically non-saturable.
4. After correcting for binding and simultaneous uptake by two transport systems, the low dose K_m (mean $\pm$ S. E.) was 0.39 ± 0.03 mM and V_{max} was $0.49 \pm 0.07 \times 10^{-17}$ moles/min/cell. For high dose cyclophosphamide transport, the K_m was 75 ± 29 mM, and V_{max} was $49 \pm 14 \times 10^{-17}$ moles/min/cell.
5. Both low and high dose cyclophosphamide transport were temperature sensitive and partially dependent on extracellular Na^+.
6. Low dose drug transport was partially dependent on metabolic energy, as uptake was inhibited by oligomycin and sodium cyanide.
7. Other alkylating agents and several natural substrates did not inhibit cyclophosphamide transport.

8. A preliminary study of cyclophosphamide transport by chick embryo liver cells was also temperature sensitive and showed biphasic kinetics similar to that observed in L5178Y lymphoblasts.
9. Evidence that low dose cyclophosphamide transport was mediated by a facilitated diffusion process was that uptake obeyed saturation kinetics, was temperature- and sodium-dependent, was partially dependent on metabolic energy and cell/medium gradients did not exceed one. Although high dose drug uptake failed to show saturation kinetics, the demonstration of temperature- and sodium-dependence also suggested that high dose uptake was carrier-mediated.

VI. REFERENCES

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