BALB/c 3T3 FIBROBLASTS: A STUDY OF THE SENSITIVITY TO MELPHALAN AS A FUNCTION OF THE PROLIFERATIVE RATE

by

RITA ERNA BLOSMANIS

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A B S T R A C T

Melphalan transport by Mouse Balb/c 3T3 fibroblasts was investigated. Balb/c 3T3 fibroblasts, which demonstrate different states of growth: exponential and stationary phase or resting monolayers in culture were used in these studies. The sub-confluent dividing cultures synthesize DNA at a rate 2-fold higher than the corresponding resting monolayers. This was confirmed by two independent sets of experiments; the rate of incorporation of [^{3}H] - thymidine, and fluorescent activated cell sorting to determine the percentage of cells in S phase of the cell cycle. Melphalan transport studies over a range of 1 to 100 μM demonstrated a 3-fold greater drug uptake in exponentially dividing cultures than stationary monolayer cultures. Balb/c 3T3 cells have two major amino acid transport systems involved in melphalan transport, as have been found in previously studied mammalian cell lines. As the melphalan ranges from low to high concentrations a shift in the contribution of each amino acid system to uptake was noted. At low concentrations of 1μM, 71% of the melphalan transport was by the ASC-like system which is sodium dependent. Increasing concentrations to 100 μM, the major contributor to melphalan uptake was decreased and the system L increased from 15 to 28%. The system L is sodium independent. These finding confirmed previous data with other cell lines. What was interesting to note, as the Balb/c 3T3 cells were maintained in prolonged monolayer both the ASC-like and L-systems were less active. In general, research with several other cell

lines showed reduced transport activity in non-dividing cells. The Balb/c 3T3 exponentially dividing and resting monolayers were exposed to various concentrations of melphalan and dose-survival curves were determined. The stationary cultures were less sensitive to the cytocidal activity of melphalan which could largely be accounted for by reduced drug transport. The proliferative activity of the Balb/c 3T3 cells was altered by deprivation of serum. Exponentially dividing cultures were incubated in serum depleted medium for 24 hours prior to uptake experiments; melphalan transport was reduced 40%. Stationary cultures showed no difference in the rate of melphalan uptake after serum depletion. The role of uptake in exponential cultures approximated the rate of uptake in the stationary cultures. Both proliferative rate and transport activity affect the sensitivity of Balb/c 3T3 cells to melphalan. The greater sensitivity of exponentially dividing cells can be accounted for by a more efficient transport system.

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HISTORICAL

History of the Alkylating Agents

Sulfur mustard, known as mustard gas was used in chemical warfare during World War I. Although originally synthesized in 1884, it was not until 1887 that the vesicant effects of the gas were first noted (Calabresi and Parks, 1975). Initial observations focused on the vesicant action directed at the respiratory tract and skin. At first the serious intoxication following exposure was overlooked. Krumbhaar and Krumbhaar, in 1919 observed the leukopenia, bone marrow suppression, and dissolution of the lymphoid tissue (Calabresi and Parks, 1975). These were the pertinent observations which led to extensive biological and chemical studies of the nitrogen mustards during the interval between the wars. Sulfur mustard was far too reactive to use, but it was found that substitution of a nitrogen atom for the sulfur atom increased the molecule's stability. The cytotoxicity of the lymphoid tissues led to studies of the effects nitrogen mustards had on lymphosarcoma in mice. After the second World War, the nitrogen mustards were declassified. Early investigations had been restricted because these were chemical-warfare agents. In 1942, Gilman, Goodman, and Dougherty were able to launch the first clinical trials and open the field of chemotherapy (Gilman, 1963).

The Military and post war studies led to the synthesis of thousands of variations of the basic mustard structure.

Desired were new and "active site-directed" compounds. Unfortunately, the rational design approach has not been too successful. As a whole, the alkylating agents have a similar action, and of the thousands synthesized, only a few agents have proven useful clinically. Five major groups of alkylating agents are being used clinically: (1) the nitrogen mustards, (2) the ethylenimines, (3) the alkyl sulfonates, (4) the nitrosoureas, and (5) the triazenes. Although the alkylating agents in clinical use are structurally diverse, they share the capability under physiological conditions to alkylate vital cellular macromolecules. In chemotherapy, these agents have been applied in a wide variety of malignancies. By the early 1960's, the era of cancer chemotherapy had been established - the first chemical treatment for cancer patients. The structure-activity relationship of the alkylating agents is not well understood. The original, rational premise of synthesis usually did not correlate with the actual biological reactions. Still today, physical and chemical parameters are not completely understood. Unfortunately, all the alkylating agents have undesirable side effects and exhibit host toxicity. Because of the non-specificity of action they are cytotoxic to healthy tissues. Especially vulnerable are rapidly dividing cells such as the bone marrow, and hair follicles. The general aim of cancer chemotherapy is to destroy selectively malignant cells without seriously affecting the proliferation of normal

cells.

Mechanisms of Action

The alkylating agents are electrophiles, or they generate electrophiles in vivo; they are highly reactive and attack electron rich regions of the cell. Most macromolecules within the cell are potential targets as the actions of the agents are indiscriminate. The alkylating reactions can disturb cell growth, mitosis, differentiation, and many metabolic processes (Wheeler and Alexander, 1969). The therapeutic application lies within the cytotoxic actions observed, and the interference with cell division. In contrast to many chemotherapeutic drugs, the alkylating agents are not cell cycle specific yet the cytotoxicity appears to be expressed when the cell enters S phase and its progression becomes blocked at G_2 of the cell cycle (Meyn and Murray, 1984). The lethality is also expressed by inhibition of DNA synthesis and by damage due to interaction with the DNA (Lawley and Brooks, 1967). Within the nitrogen mustard group, different substitutions of the amine result in marked differences in chemical reactivity and clinical applications, despite the similarity of the actual alkylating arm. Between compounds there are differences in rates and mechanisms of nucleophilic attack. Lethality after exposure to an agent has been attributed to some form of DNA damage.

Most evidence indicates that the primary target of alkylation is the DNA. Cytotoxic action is predominant

with the bifunctional agents, whereas with the monofunction agents, mutagenesis and carcinogenesis are mostly observed. This suggests that cross linking of two nucleic acid chains causes major disruptions in nuclear function (Ludlum, 1975). Unless the crosslink is excised and repaired, DNA synthesis is affected. The primary site of alkylation is the nitrogen-seven position of guanine; this accounts for ninety percent of the base substitutions. Other positions of guanine are affected, as well the other nucleic acid bases are susceptible. An alkylated guanine can then be crosslined to another guanine; secondary reactions include intrastrand and interstrand crosslinking (Ludlum, 1975). Minor reactions include depurination, base shift changes, and single or double strand scissions. These effects are evidenced by the agent's mutagenic and carcinogenic effects. The structural alterations of the DNA interfere with its proper functioning. Secondary structural changes to the nuclear material express the sensitivity to alkylation when the cells divide. Cross-links block replication and transcription, therefore resulting in an inactive product or incomplete product. Lesions will interfere with base pairing. Because alkylating agents express their cytotoxicity by damaging the cellular DNA, the damage must be expressed upon mitosis and cell division. At the molecular level the alkylating agents have been useful in genetic studies of mutagenesis, carcinogenesis, and repair mechanisms of structurally altered DNA.

Melphalan

Originally, melphalan was synthesized in 1954 with the aim to increase the specificity of action of the alkylating moiety (Bergel and Stock, 1954). As phenylalanine is a biosynthetic precursor of melanin, the goal was for this new derivative to be used in the treatment of malignant melanoma. There is no direct evidence of selective action. In addition, it was hoped that since tumors are actively engaged in protein synthesis, melphalan would be recognized by tRNA and incorporated into proteins. In particular, it is used in the treatment of multiple myeloma (Alexanian et al., 1968) and in breast cancer (Fisher et al., 1975).

Melphalan is a bifunctional agent: that is, it has two highly reactive side arms [bis-(2-chloroethyl)] groups which in aqueous solution undergo an internal SN_1 cyclization forming aziridinium ions. It is these aziridinium ions which attack electron rich regions within the cell forming covalent bonds with them (Ross, 1962; Ludlum, 1977).

In chemotherapy the bifunctional nitrogen mustards have been found to be the most effective. Because melphalan has two side arms, two reactive centers are formed and the agent has the ability to cross-link DNA, RNA, and proteins. Most cellular macromolecules are vulnerable, thus there is a wide potential of critical functions which can be impaired. The bifunctional agents have been found to be more cytotoxic than other nitrogen mustards that have only one arm (Ross 1962; Goldenberg and Alexander, 1965). The lethal lesion

following exposure to the bifunctional agents is believed to be the formation of DNA cross-links, which ultimately interfere with DNA synthesis, mitosis, and cell division (Calabresi and Parks, 1975; Gilman, 1963).

Mechanisms of Resistance to Alkylating Agents

Several mechanisms have been suggested which may play a role in development of resistance. There may exist intracellularly a high concentration of protective groups in the cell such as thiols which spare the critical target macromolecules from alkylation (Calcutt and Connors, 1963). Fisher et al. (1983) detected increased levels of glutathione, a free sulfhydryl in cells exhibiting resistance to alkylating agents. It has been suggested that reduced drug permeability may explain increased resistance (Rutman et al., 1968). Much speculation exists that the resistant cells may be more efficient at repair of damaged DNA (Roberts et al., 1968). The technique of alkaline elution has proved useful in studies of DNA cross-link damage and repair (Ross, 1978).

Often partial cross-resistance to other alkylating agents can be demonstrated both clinically and experimentally (Goldenberg and Begleiter, 1978). Ling and Thompson (1974) isolated colchicine resistant Chinese Hamster Ovary (CHO) cell mutants which exhibited cross resistances to a variety of unrelated drugs including melphalan. Their findings suggested that colchicine resistance was at the plasma membrane level, and that

resistance was due to reduced drug permeability. Further work by several laboratories has shown that the multidrug resistance is due to enhanced expression of a membrane P-glycoprotein (Riordan et al, 1987; Foko et al, 1987). Experiments of drug resistance in microorganisms by Moyed (1964) indicated that single-step mutations may be responsible for resistance. The same may apply to mammalian cells, however Goldenberg (1969) demonstrated that prolonged drug exposure was required in the case of nitrogen mustard (HN2) indicating that resistance may involve several mutations.

Membrane Transport of Alkylating Agents

Drug uptake may occur by at last three different mechanisms (Goldenberg and Begleiter, 1978); all of which are applicable to the alkylating agents. The first mechanism is passive diffusion; by this process influx is proportional to concentration of the substrate and the drug may freely enter and exit through the plasma membrane. There is no interaction between the substrate and receptor sites on the cell membrane; the reaction is essentially independent of temperature and energy. A second mechanism is facilitated diffusion. The process involves a specific recognition site on the membrane, the drug must interact with this receptor. Chemical specificity is demonstrated and saturation kinetics observed, however the internal concentration of drug may not exceed the external concentration. The third mechanism is active transport. As

in facilitated diffusion, a specific receptor molecule recognizes the drug. Chemical specificity, saturation kinetics, and temperature dependence are noted. An important characteristic is that drug transport will proceed against a concentration gradient.

The mechanisms of membrane transport of the alkylating agents has been an important field of study. Many of the agents and their metabolites are charged, so that the drugs are not permeable to the cell membrane. The alkylating agents of a hydrophilic nature, such as melphalan were found to be actively transported whereas those agents of a lipophilic nature tend to be transported by passive diffusion (Goldenberg et al., 1970). Vistica et al. (1976) presented evidence that the nitrogen-mustards are transported by carrier-mediated mechanisms. A variety of mammalian cell lines have been utilized to elucidate uptake of the various agents (Goldenberg and Begleiter, 1979). Identification of the major transport systems revealed that several of the drugs were found to share a transport system with a natural substrate (Goldenberg et al., 1979; Begleiter, 1979). These were revealed through competitive studies. Since some of the transport systems were found to be shared with those of common substrates, it was critical to use buffer not containing those competitive substrates (Begleiter, 1979; Goldenberg et al., 1977). Competition for the carrier by substrate and drug confuses the identification of the system and actual mechanism of transport. In kinetic studies the Km value may be artifically elevated.

The alkylating agents are highly reactive and may alkylate the carrier molecule at the level of the plasma membrane. Evidence for alkylation of the carrier was found by preincubation of the cells with the agent, then comparison of drug uptake results with that observed in untreated cells. In order to study uptake without the complication of alkylation reactions, drug transport may be studied by two different methods (Goldenberg et al., 1971a). Firstly, thin layer chromatography (TLC) of cell constituents was used so that uptake of free intracellular drug may be studied (Goldenberg et al., 1977, 1979). Secondly, uptake of hydrolyzed drug may be employed since hydrolyzed drug is inactive as an alkylating agent.

The intact alkylating agent and its decomposition product however, may be transported differently, and by entirely independent systems. This problem was encountered with melphalan transport (Goldenberg et al., 1977, 1979; Begleiter et al., 1979); the parent compound and hydrolyzed derivative were transported by independent mechanisms. Intact melphalan therefore had to be used in studies of drug transport. Within the period of the uptake study thin layer chromatography revealed that eighty-five percent of radioactivity was in the form of free intracellular drug. Melphalan uptake was not inhibited by nitrogen mustard nor cyclophosphamide, suggesting that these drugs use separate transport systems.

Work with L1210 murine leukemia cells by Vistica et al. (1976) and by Goldenberg et al. (1987,1979) indicated that the amino acids in the medium conferred protection against melphalan toxicity. In the study by Vistica et al. (1976) the specificity of melphalan transport was analyzed in some detail. The L1210 cells in culture were preincubated with a variety of amino acids in order to saturate carrier molecule sites prior to treatment with melphalan. Dose survival curves were determined by cloning in low percentage agar. The protection conferred by each respective amino acid could thus be evaluated. Significant protection of L1210 cells was observed with L-leucine and L-glutamine at low concentrations. Increased concentrations of some other amino acids also resulted in varying degrees of protection. The D-isomers of the amino acids offered virtually no protection; however they are known to be poorly transported in mammalian cells. These results by Vistica's group (1976) provided preliminary evidence that melphalan transport is shared by the amino acid transport systems for leucine and glutamine.

Further studies with murine LPC-1 plasmacytoma cells were initiated by Goldenberg et al. (1979). Evidence indicated with melphalan transport was shared by two separate and distinct amino acid transport systems. The effects of several amino acids and their inhibition of melphalan uptake were compared and evaluated. At excessive concentrations all the amino acids exhibited inhibition, but

to varying degrees. Those amino acids which significantly inhibited melphalan uptake included some transported by amino acid transport system L and some by system ASC. Detailed classifications had been made of the amino acid transport systems (Christensen and Liang, 1966, Christensen, 1975). On the basis of this classification Goldenberg et al. (1979) attempted to determine the chemical specificity of melphalan transport. First, the applicability of the L-A-ASC classification to the LPC-1 plasmacytoma cells was established. Next melphalan uptake was examined in the presence of saturation levels of the specific inhibitor of the amino acid transport systems.

Other studies by Goldenberg et al. (1979) revealed biphasic Lineweaver-Burk plots indicating at least two transport systems must be involved in melphalan transport. One component was identified as system L due to its Na^+ independence, and inhibition by BCH. The other component was identified as system ASC because of its Na^+ dependence, and BCH- independence (Christensen et al., 1967). Comparison of drug uptake at low versus high concentration range of melphalan showed there was a shift in contribution of the two systems. The contribution of system L increased at higher concentrations whereas system ASC predominated at lower concentrations.

Evidence from studies of several cell lines (Goldenberg and Begleiter, 1979) indicated that melphalan transport is an active carrier mediated process. Drug uptake proceeded

"uphill" against a high concentration gradient. Time course experiments showed rapid initial uptake to be linear for a short time interval up to two minutes, and plateaued (Goldenberg et al., 1977). Time course of uptake at 4 C showed no evidence of drug uptake, indicating that uptake was temperature-dependent.

Amino Acid Transport Systems

Several distinct amino acid transport systems have been defined; each differs with respect to substrate specificity and conditions required for activity. The kinetics of the amino acid transport systems have been studied in a wide variety of cell types. Transport activity can be affected by many factors including cell density (Foster and Pardee, 1969; Otsuka and Moskavitz, 1975), position in the cell cycle (Tupper, et al., 1976) and viral transformation (Parmes and Isselbacher, 1978). The major neutral amino acid transport systems have overlapping substrate specificities. Consequently an amino acid may be transported by two or three independent systems (Guidotti et al., 1978). The availability of non-metabolizable amino acid analogues has helped elucidate and characterize the distinct features of these transport systems. The most extensive characterization of amino acid transport has been with the Ehrlich ascites tumor cell line Oxender and Christensen, 1963), which led to the designation of four distinct amino acid transport systems: A, ASC, L, and Ly^+. Studies of neutral amino acid transport in the Ehrlich cells

indicated that there cannot be separate systems for each individual amino acid, but that they can be divided into groups. However, there is extensive overlap of transport specificity. Several amino acids will inhibit the uptake of one another. These competitive interactions complicated elucidation of the true nature of the systems. Both uptake and efflux out of the Ehrlich cells could be demonstrated (Oxender and Christensen, 1963); thus, in order to study unidirectional uptake a short time interval must be observed to avoid exchange. Almost every neutral amino acid is inhibitory to the uptake of another neutral amino acid. However these original experiments by Oxender and Christensen revealed that the inhibition of glycine uptake was not correlated with the inhibition of leucine uptake. A correlation was observed with glycine inhibition of alanine uptake and phenylalanine inhibited leucine uptake (Oxender, et al., 1976; Oxender and Christensen, 1963). These correlations were strengthened through the use of leucine and phenylalanine analogues (Christensen et al. 1965). Two sets of relationships were observed, and these were interpreted as most neutral amino acids are transported by at least two transport systems. These were then designated as system L and system A for leucine and phenylalanine preferring, and alanine and glycine preferring respectively.

It is very significant however, that almost every naturally occurring amino acid has some affinity for both systems (Christensen, 1975). Although leucine inhibits

alanine transport, and alanine will inhibit leucine transport, these interactions are small. Leucine uptake is less subject to alanine competition and vice versa. The development of synthetic analogues to specifically inhibit a system was important in providing a more precise characterization. MeAIB, (2-(methylamino) isobutyric acid) was utilized because it in part inhibits phenylalanine transport (Christensen et al., 1965). That portion was presumed to be mediated by system A; the residual uptake observed was believed to be transport by the L system (Christensen, 1969). Because MeAIB inhibits a small portion of phenylalanine uptake, it was concluded that system A is a small component of the uptake, the major component being system L (Christensen et al., 1965). These types of experiments confirmed a parallel operation of the systems A and L.

A major difference in operation of the two systems was that the Na^+ concentration of the medium primarily influenced system A. It was interesting to discover the different sensitivity to Na^+ and its effect on the uptake rate between leucine and alanine. Depletion of the extracellular Na^+ concentration slows the entry rate of alanine: on the other hand leucine uptake was relatively independent of Na^+. In order to differentiate between the amino acid uptake by these two amino acid transport systems the Na^+ dependence of system A can be utilized. In addition, there was a pronounced sensitivity of alanine

uptake to changes in pH whereas little effect was observed on the uptake of leucine and phenylalanine. The "alanine preferring" system was designated as system A, and the "leucine preferring" system as system L. N-methylation of amino acids was further utilized to determine whether the amino acids actually share several transport systems or not (Christensen, et al., 1965). These substrates aided in inhibition analysis and countertransport studies. N-methylated analogues were then further utilized to define the specificities of the systems already identified in the Ehrlich tumor cell system and to strengthen the earlier observations (Christensen and Liang, 1965).

System A would not accept branched hydrocarbon side chain, and its activity was greatly reduced at lower pH. This system preferentially transports amino acids with short, polar, or linear side chains. Alanine's non-metabolizable analogue AIB (2-aminoisobutyric acid) and the methylated derivative MeAIB (2-(methyl amino)isobutyric acid) were also substrates. Although system A occurs widely, its activity was absent in various erythrocytes examined (Winter and Christensen, 1965). Independent of system A, a second sodium dependent system designated ASC was revealed. Residual alanine uptake was observed even though all transport was believed to be inhibited by a combination of MeAIB and phenylalanine (Christensen, et al., 1967). The ASC system transports alanine, serine, and cysteine, however it was found to be relatively insensitive

to pH and not inhibited by MeAIB. Although formally defined in the Ehrlich cell and observed in the rabbit and pigeon reticulocyte, the contribution of system ASC to alanine transport appears to be a minor component.

System L in contrast to A is sodium independent and pH insensitive. This system transports branched chain, and aromatic amino acids, as well the non-metabolizable analogue BCH (2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid) which is a specific antagonist (Christensen, 1964). As with the other transport systems, the L system was formally defined for the Ehrlich cell and appears to be ubiquitous and includes mature erythrocytes. Further examination of the system L led to the designation of system Ly^+. As with the discovery of system ASC, at extreme concentrations of phenylalanine, lysine, and BCH, residual lysine uptake could be observed. BCH transport appeared to be limited to system L and has a weak interaction for transport with Lysine. Both amino acid transport systems L and Ly^+ are in close operation.

Transport Systems In Other Cell Types

It is interesting that the various amino acid transport systems were classified in the Ehrlich Ascites cell. Originally these classifications were assumed ubiquitous in other mammalian cell lines. These mammalian systems were in contrast to studies of microorganisms. Research identified individual transport systems for each amino acid (Halpern and Lipo, 1965). However, unique systems were found to

exist in the red blood cells. Changes in amino acid transport were observed during the maturation process (Winter and Christensen, 1965). Winter and Christensen (1965) studied transport of the same amino acids in rabbit reticulocytes and erythrocytes.

In operation at the immature reticulocyte stage, system A is strongly active (Winter and Christensen, 1965). Upon maturation the reticulocyte loses uptake of glycine and alanine; it appears that in the mature erythrocyte system L is predominant in the transport of amino acids. As the red blood cell matures, changes in the transport systems are observed (Winter and Christensen, 1965). The reticulocyte no longer uptakes glycine and alanine; which indicates a loss of system A (Winter and Christensen, 1964). This is an interesting system to study because during differentiation, transport systems undergo changes. The transport is very similar to that observed in human erythrocytes. In contrast the transport of leucine showed little difference between erythrocytes and reticulocytes.

The rate of uptake of alanine and glycine was significantly greater in reticulocytes, yet in erythrocytes, apparently these amino acids were not accumulated (Winter and Christensen, 1965). The reticulocyte transport systems resembled those previously described for the Ehrlich Ascites cell when studied for cation requirement and pH effects (Oxender and Christensen, 1963). Michaelis-Menten kinetics were observed, thus indicating a saturable component of

transport. The reticulocyte shows countertransport of alanine by both systems A and L, but this property is lost upon maturation. This is in contrast to the Ehrlich cell which shows little countertransport.

Cell Cycle Effects On Amino Acid Transport

The original work of amino acid transport in Ehrlich tumor cells had been studied on asychronous populations (Oxender and Christensen, 1963). Tupper et al. (1975) were successful in synchronizing the Ehrlich cells using the double thymidine block technique. They then studied amino acid transport in these cultures, and the relationship of amino acid transport to the position of the cell cycle. Using AIB to follow uptake by system A, it was found that uptake occurs throughout the cell cycle with a maximum observed in late S, early G_2. Using mouse L cells and Chinese hamster ovary cells (CHO), Sander and Pardee (1972) observed a reduction in AIB uptake during the M phase. As in the Ehrlich cells maximal leucine uptake was observed during the S phase.

Cell Density and Viral Transformation on Amino Acid Transport

Studies of pre- and post-confluent 3T3 and Polyoma Virus transformed 3T3 cells (Py3T3) showed differences in the transport of amino acids (Foster and Pardee, 1969). The 3T3 cell line is strongly "contact inhibited" whereas the Py3T3 are not sensitive to density-dependent cell growth. Foster and Pardee (1969) demonstrated confluent 3T3 cells accumulated AIB and cycloleucine about 30% less rapidly than

non-confluent cells. No differences were observed in the Py3T3 cells between confluent and non-confluent cultures. But comparing Py3T3 to 3T3, the virally transformed lines accumulated some amino acids more rapidly. Foster and Pardee (1969) studied initial uptake kinetics to determine if these could expalin the slower and lower accumulation of AIB and cycloleucine. On the basis of Km and Vmax, no major differences between confluent and non-confluent cells were observed. An entire range of cell densities of non-confluent through to confluent monolayer demonstrated essentially the same values. Kinetically system A was not affected in contact-inhibited 3T3 cells and the reduced transport could not be explained on the basis of kinetic difference. Thus, Foster and Pardee (1969) concluded from their work that growth regulation by density-dependent inhibition was not due to membrane transport. It has been suggested that a correlation exists between membrane permeability and growth regulation (Eagle et al., 1961). Foster and Pardee concluded, however, that there are growth dependent and density dependent decreases in the transport of normal non-transformed cells.

In sub-confluent cultures, 3T3 demonstrate relatively high affinity for leucine. When these sub-confluent cultures become confluent this high affinity system is shut down and the transport is shifted to another lower affinity system. Transport of amino acids by 3T3 cells versus virally transformed 3T3 cells indicated that there are

growth dependent and density dependent decreases in transport in the non-transformed cultures (Foster and Pardee, 1969; Otsuka and Moskowitz, 1975). These conclusions were drawn from kinetic studies. When a methocell suspension culture was initiated from a confluent 3T3 culture, the former non-confluent high affinity system apparently was not reactivated. The methocell suspension prevents the 3T3 cultures from attaining contact inhibition. Once shut down the lower affinity leucine transport system remained in operation. Sub-confluent 3T3 in suspension culture continued to operate at a high level of several hours, then this leucine high affinity system was gradually shut down. These studies demonstrated differences existed in leucine transport between attached and suspended cells. Although previous work by Foster and Pardee (1969) did not show decreased leucine uptake by 3T3 cells grown at different densities, these experiments by Otsuka and Moskowitz (1975) indicated changes in the two leucine transport systems. Thus transport by the two major amino acid systems is affected at different cell densities.

A time course of uptake of leucine, alanine, and glycine by confluent 3T3 and confluent SV3T3 lines showed that the transformed cells have a higher uptake (Cecchini et al., 1976). Initial uptake rates were observed at various amino acid concentrations. The 3T3 cells showed biphasic kinetics for leucine uptake yet the plot for SV3T3 cells was not biphasic. By utilizing a large number of amino acids and

their analogues it was confirmed that the 3T3 and SV3T3 lines possessed both systems A and L. Quantitatively the data was similar to that for the Ehrlich cell (Christensen, 1963). Between 3T3 and its virally transformed line there did not appear to be differences in the amino acid transport. Cecchini et al. (1976) found cell density effects on amino acid transport between system L to be different from that of system A. Their results showed an increase in the activity of system L upon confluence, and showed that the activity of system A decreased.

Oncogenic transformation by DNA and RNA tumor viruses has been shown to increase the uptake and accumulation of amino acids (Parmes and Isselbacher, 1978). This has been questionable because much disagreement exists whether or not the transformed cells have increased uptake due to the actual transformation or due to changes in proliferation, or a combination of both. Discrepencies arise because much heterogeneity is observed in transport.

Although there is much heterogeneity, groups of amino acids are preferentially transported by distinct systems. Activity of the various systems can be discriminated in the presence of or absence of sodium in combination with amino acid analogues which are specific system inhibitors (Shotwell et al., 1981). The BALB/c 3T3 and its virally transformed SV3T3 were preincubated in buffer to deplete intracellular amino acid pools (Borghetti et al., 1980). These preincubations revealed that there was a marked

decrease in the transport activity of systems A and ASC with an increase in cell density, whereas activities of systems L and Ly^+ remained unchanged in the untransformed cell lines. Their explanation was that density dependent changes in the activity of system A could be explained by a change in Vmax, the transport maximum. Measuring the initial rate of uptake of several amino acids in SV3T3 cells over a wide range of densities, from sparse to confluent cultures showed that there were differences. Transport of MeAIB and proline were significantly affected by density; the increase in cell density was associated with a decrease in their transport. These substrates are preferentially transported by system A; the same results were observed with AIB. Thus in the virally transformed lines systems A and ASC were affected. The normal BALB/c 3T3 line also exhibited a density-dependent regulation of the activity of transport system A and ASC, but at a reduced uptake rate compared to SV3T3 cultures. Even the revertant SV3T3 line will show this effect. Under all experimental conditions they showed a linear uptake for all cell densities. The results of this study indicate that of the four neutral amino acid systems studied, two systems are affected by cell density in BALB/c 3T3, SV3T3, and revertant SV3T3 lines. These are the sodium dependent systems A and ASC. When assayed under appropriate conditions favoring transport systems L and Ly^+, no differences were observed between different cell densities. Borghetti *et al.* (1980), speculated that the density-

dependent regulation of amino acid transport may be linked to the rate of proliferation.

The uptake of neutral amino acids in BALB/c 3T3 and Chinese hamster ovary (CHO) cells was studied by Oxender et al. (1977). The original designation of amino acid transport systems A and L were further examined and investigations led to designations of the systems ASC and Ly^+. These experiments established the framework for all subsequent amino acid transport studies. The time course of uptake of glycine, alanine, leucine, and phenylalanine by BALB/c 3T3 cells were similar to those originally described by Oxender and Christensen (1963) in theEhrlich cells. Uptake was demonstrated as linear and rapidly reached a plateau value, very high distribution ratios were observed in logarithmically growing cells. As in previous studies the systems A and L could be distinguished on the basis of Na^+ dependence and independence respectively. Oxender et al. (1977) showed a time course uptake of radio-labelled amino acids; amino acids were transported rapidly by BALB/c 3T3 cells and reached a steady state within two minutes. In addition they studied the effects of sodium by preparing a sodium depleted uptake buffer. In order to obtain kinetic parameters initial rate measurements were made over a wide substrate concentration range. The Michaelis-Menten plot of L-leucine showed a biphasic curve. Alanine and Glycine uptake demonstrated a strong Na^+ requirement. Similar results were obtained for the CHO cell line. Kinetic

parameters were examined over a wide range of substrate concentrations; several amino acids revealed a biphasic plot and two Km values were obtained by Neal's analysis (1972). Results from inhibition analysis studies on the uptake of leucine and alanine confirmed the presence of the defined systems. The branched chain amino acids were more restrictive to leucine uptake than that of alanine. Glycine was more inhibitory to the uptake of alanine rather than leucine. In the reciprocal experiments however, alanine did not complete for glycine uptake; this indicates the presence of another separate transport system for glycine. Their results demonstrated that in general the BALB/c 3T3 cell line possesses the same distinct neutral amino acid transport system as characterized in the Ehrlich cell.

Transport activity of both systems A and L changed as the growth conditions of BALB/c 3T3 were altered (Oxender et al., 1977). When cell division ceased upon attainment of confluency by serum removal, the activity of system A decreased and system L increased significantly. Intracellularly the amino acid concentrations were elevated when growth decreased. This corresponds with the decrease in activity of transport system A. Each of the amino acid transport systems appear to be under independent regulation of each other. External factors primarily alter one sytem but not the other. Glycine uptake dropped off markedly; the uptake activity of alanine was similar. However, under the same conditions lysine transport increased several fold. In

this study similar results were obtained as previous studies which found that the amino acid transport systems A and L responded differently to cell growth rate changes.

Serum Deprivation

When cells in culture are deprived of serum they cease to grow, internal amino acid pools rise, and the cells enter a quiescent state, designated as G_o (Brooks, 1976). Changes in amino acid uptake are observed (Kram et al., 1873). in the BALB/c 3T3 cells Oxender et al. (1977) found decreased activity of system A, and increased activity of system L. Readdition of serum stimulated growth and cell division. Activity of system A increased while that of the other systems did not change significantly. Normally dividing fibroblasts, when deprived of serum, enter the G_o state of the cell cycle (Brooks, 1976). Readdition of serum restimulated growth and cell division after a lag period characteristic of the cell line. Only a pulse of serum exposure of a few hours was required, for once initiated a cell will continue its round of the cell cycle and complete cell division. The length of the mitotic stimulation required for reinitiation of the G_1 cycle is characteristic of the cell line in question. However, the DNA synthesis is independent of the serum concentration.

Establishment of the 3T3 Cell Line

Todaro and Green (1963) followed a very strict subculturing regimen of whole mouse embryos. Within three months of culturing mouse cells the 3T3 cell line was

established. This cell line displayed some interesting properties. Cultures of 17 to 19 day old Swiss mouse embryos were minced and disaggregated with trypsin. Careful cell counts were taken, resuspensions at 3 x 10^5 cells per plate were inoculated and grown to confluence by 2 to 3 period days. Therefore a strict subculturing regimen was followed, cell transfer occurred every three days and cells were reseeded at the same 3 x 10^5 cells per plate density. The growth characteristics were carefully monitored as well as chromosomal studies. Cells from proliferating subconfluent cultures were allowed to grow for 3 day intervals. The cells of this 3T3 line were never allowed to become confluent because of the routine transfer, and replating at a relatively low cell density. During the development of this 3T3 cell line, the cultures had little or no cell-to-cell contact; the cell line was considered as established once a constant growth rate could be maintained without cell death.

This newly established 3T3 cell line demonstrated a greater ability to grow at low cell densities. This reduced cell dependence was one of the criteria used to distinguish the 3T3 line from normal or primary cells. Established cell lines usually show progressive increases in saturation densities. The striking characteristic demonstrated by the 3T3 line was its ability to terminate cell division once the confluent state was achieved. These confluent monolayers could be maintained up to one month providing medium was

changed frequently. No increase in cell number was observed, yet on replating at low cell densities the cells were fully viable. The 3T3 cell line demonstrated several unique characteristics. By microscopic observation they appeared different from other cell lines. In subconfluent cultures the cells were indistinguishable from fibroblasts, however, in confluent cultures cell borders were no longer distinct. Instead they appeared more epithelial-like. The cell borders interlaced and formed a thin syncytium-like sheet. As well the cultures became more difficult to trysinize from the plate surface. Prior to establishment of this 3T3 cell line it was believed that only normal cells and primary-cultures exhibited contact inhibition (Hayflick and Moorhead, 1961) and that established cell lines had lost this control of cell-cell interaction. The 3T3 cell karyotypes showed an increase in chromosome number. First, chromosomal number increased to tetraploidy, and eventually gross chromosomal alternations were observed.

Green and Kehinde (1974) described the isolation of clones of the 3T3 cell line which accumulated triglycerides upon entering the quiescent state. This suggested that these 3T3-L1 cells were related to adipose cells; follow up studies by Green and Meuth (1974) showed that in culture these 3T3-L1 cells can differentiate into adipose cells. Various clones of the 3T3 cell line show differing degrees of adipose conversion, both high and low frequency lines have been isolated. Among the subclones, all cells have the

potential to convert but the intensity of lipid accumulation differs. When the cultures are kept subconfluent adipose conversion is never observed. The cells in logarithmic growth are fibroblasts since they synthesize fibrous collagen and hyaluronic acid (Green and Goldenberg, 1965). Differentiation is spontaneous upon entering the resting state; kinetic and biochemical evidence indicates a distinct G_o.

MATERIALS AND METHODS

Cell Culture Conditions

Wild type BALB/c 3T3 mouse fibroblasts,kindly supplied by Dr. Rose Sheinin, Department of Microbiology, University of Toronto (Sheinin and Lewis, 1980) were routinely cultivated on 150mm^2 tissue culture flasks (Corning Ware Ltd.) in alpha minimal essential medium (αMEM), nucleoside free (Flow Laboratories) supplemented with 10% fetal bovine serum (Gibco Canada, Ltd.), penicillin (100 units/ml), and streptomycin sulfate (68 μg/ml), and incubated at 37^{0}C in incubators flushed with 95% humidified air and 5% CO_2.

BALB/c 3T3 cells were subcultured from the preconfluent state on the growth surface; cells were released from attachment using 0.1% trypsin (Bacto) in Dulbecco's phosphate buffered saline (Gibco). Cell concentrations were measured using an electronic cell counter (Coutler Electronics).

Freshly plated cells were incubated at least 24 hours prior to any experiment to permit cell adhesion to occur and allow re-entry of cells into logarithmic growth. For further experiments logarithmic cells were cultured until a confluent monolayer formed and confirmed by microscopic examination; this was designated Day 0, the next 24 hour period as Day 1, and so on. To maintain monolayer cultures and allow cells to enter the stationary phase, the complete growth medium supplemented with 10% fetal bovine serum was aspirated and replaced with αMEM containing the above

described antibiotics with 1% fetal bovine serum. This αMEM with 1% serum was replaced every other day to ensure cells remained viable and remained attached to the growing surface.

Drugs and Buffers

[^{14}C]-Melphalan (L-p(di-2-chloroethylamino)-L-phenylalanine) was prepared by M. Leaffer of the Stanford Research Institute (Menlo Park, CA). [^{14}C]-Melphalan was available ring labelled (specific activity 14.2 mCi/mmol); radiochemical purity of thering labelled was 97%, as was determined by thin layer chromatography on silica gel in n-butyl alcohol;acetonitrile:water (7:2:1). Unlabelled melphalan (alkeran) was provided by Dr. J.R. MacDougall (Burroughs Wellcome and Co., Ltd., Lachine, Quebec), DL-β-2-amino-bicyclo[2,2,1]-heptane-2-carboxylic acid (BCH) purchased from Calbiochem-Behring Corporation, and [^{3}H-CH_3]-thymidine (specific activity 6.7 mCi/mmol) was purchased from New England Nuclear. [^{14}C]-Melphalan powder was dissolved in acidified absolute ethanol (0.1M HCl) to prevent hydrolysis which would occur in aqueous solution. A stock of 10^{-2} M was made in small volumes and stored at -20^{0}C; all dilutions were made from this stock.

Dulbecco's phosphate buffered saline (DPBS) was purchased as X10 concentrated solution from Gibco Canada Ltd. Sodium depleted Hank's balanced salt solution ((-Na)HBSS) was prepared by replacing isomolar amounts of

Tris for NaCl (Goldenberg, et al, 1974). Scintillation cocktail was prepared as follows: one part Scintanalyzed Xylenes (Fisher Scientific) to one part Phase Combining Systems (Amersham), acidified with glacial acetic acid (9.0 ml per litre cocktail).

Cell Volume Determination (M.C.V.)

The cell volume of the BALB/c 3T3 cells was determined in order to calculate the cell/medium distribution ratios of drug. Cell volume was determined using the Coulter electronic particle counter model Z_{B1} (Coulter Electronics, Inc.), calibration done with paper mulberry spores (mean cell diameter 12.5 μm; Coulter Diagnostics, Inc.). BALB/c 3T3 cells were subcultured under regular culture conditions as described in Materials and Methods page 29. Confluent cultures were maintained by reducing the serum in the medium as described on page 29 of this thesis and by Todaro and Green (1963). Cell volumes were determined for cells in logarithmic growth, and at Day 0 confluence, Day 2, and Day 4 of stationary phase.

Rapid Association Gradient (R.A.G.)

The R.A.G. is a routine correction factor that accounts for rapid non-specific association of drug which binds to the cell membrane. The BALB/c 3T3 cells in logarithmic phase were grown in Linbro multiwell plates (Flow Laboratories). The medium was aspired and the cell monolayer washed twice with 1 ml aliquots of DPBS at 4^{0}C.

Laboratories). The medium was aspired and the cell monolayer washed twice with 1 ml aliquots of DPBS at 4^0C. The multiwell plate was kept on ice; to each well was simultaneously added 100 µM [^{14}C]-melphalan in DPBS at 4^0C. The incubation time was less than 5 seconds; the supernatant removed and the cell monolayer washed four times with 1 ml DPBS at 4^0C. To each well was added 500 µl cold 10% TCA and the plate incubated a minimum of one hour; 400 µl of the TCA extract and 100 µl supernatant were sampled for scintillation counting. The cell/medium distribution ratio represents radioactivity calculated per cell volume relative to that of an equivalent extracellular medium volume. The ratio is calculated as follows.

DPM = Disintegrations per minute
ICF = Intracellular Fluid Count
ECF = Extracellular Fluid Count
MCV = Mean Cell Volume

$$\frac{DPM}{Cell} = \frac{DPM\ (TCA\ soluble\ extract)}{\left(\frac{Number\ of\ cells\ counted}{TCA\ extract\ sampled}\right)}$$

$$\frac{Moles}{Cell} = \frac{(DPM/cell)}{(2.22 \times 10^{12}\ DPM/mole) \times (Specific\ Activity)}$$

$$ICF = \left(\frac{DPM}{cell}\right) \times MCV$$

ECF = DPM of Supernatant

$$\frac{Cell}{Medium} = \frac{ICF}{ECF}$$

Inhibition of 10 µM [C]-Melphalan Uptake by BCH

The Balb/c 3T3 cells were maintained as described under cell culture conditions on page 29. A stock of 25 mM BCH was prepared in DPBS; serial dilutions were made with DPBS

yielding the desired drug concentrations. A 6.0 ml aliquot of each dilution was dispensed into capped test tubes and kept in a 37^{0}C water bath. Prior to the uptake, the [^{14}C]-melphalan stock (10^{-2} M) was diluted 10^{-1} in acidified absolute ethanol and kept on ice. Immediately before the uptake experiment was performed, 60 µl of the 10^{-3} M radio-labelled melphalan was added to the BCH dilution tube. The uptake experiment and washing procedures were performed as described under Initial Uptake Velocity, however all plates were incubated for one minute.

Fluorescence Activated Cell Sorting

BALB/c 3T3 cells were maintained under cell culture conditions described on page 29. At the various stages of growth, cells were removed from monolayer cultures by trypsinization (0.1% Bacto Trypsin in DPBS), centrifuged, and the cell pellet was washed twice in DPBS. The cells were fixed in 70% ethanol for 30 minutes at room temperature, centrifuged, the pellet washed in DPBS, and the cells resuspended in an RNase solution (1mg/ml RNase (Sigma)). The RNase solution prepared using DPBS at PH 7.0 was boiled 10 minutes to destroy DNase activity. The resuspended cells were incubated in a 37^{0}C waterbath for 30 minutes to digest cellular RNA. The cells were centrifuged and washed with DPBS to remove digested RNA. The cellular DNA was stained with ethidium bromide (1 µg/ml in DPBS (Sigma)) by incubation at room temperature for 30 minutes. The stained cells were washed in DPBS. An aliquot was

analyzed using an EPICS Flow Cytometer (Coulter Electronics) for fluorescence measurements. The percentage of the cell population in different phases of the cell cycle (G_1, S, and G_2 + M) was determined from the area of the histograms assuming a Gaussian function of the G_1 and G +M maxima. The remaining part of the histogram was attributed to cells in S phase (Eriksson et al, 1984; Albert and Gudas, 1985).

Time Course of Drug Uptake

The BALB/c 3T3 cells were maintained in culture as described under cell culture conditions, page 31. A minimum of 24 hours prior to a series of experiments the sub-confluent cultures on 150 mm^2 flasks were trypsinized, and a cell suspension in complete growth medium was prepared (5-10 x 10^4 cells/ml). Six-well plates were inoculated with 2 ml of this suspension per well (Linbro multiwell plates, Flow Laboratories). A sufficient number of multiwell plates were inoculated to compare directly the logarithmic phase cells and cells at several days in confluent monolayer from the original cell suspension. To maintain the confluent monolayer the serum in the medium was reduced to 1% fetal bovine serum and was replenished every other day as described under Cell Culture Conditions pages 31 and 32. The BALB/c 3T3 cells were incubated at 37^0C; plates were removed each day as required for experiments.

The [^{14}C]-melphalan stock (10^{-2} M) was in acidified absolute ethanol; all drug dilutions were made in the same ethanol solution. To ensure that the drug remained intact,

immediately prior to the uptake experiment, a 100^{-1} dilution of [^{14}C]-melphalan into the prewarmed (37^{0}C) uptake buffer was made. Melphalan has a short half-life and its subsequent hydrolysis product is not transported by the same carrier system (Goldenberg et al., 1977, 1979; Begleiter et al., 1979). All time course experiments were performed in DPBS buffer. By diluting the radio-labelled drug the ethanol concentration was thus diluted to at least 1% in the buffer solution.

BALB/c 3T3 cultures growing on multiwell plates were used for all drug uptake studies. The DPBS buffer was prewarmed to 37^{0}C. Plates were kept on a 37^{0}C water bath during the entire procedure. The growth medium was aspirated and the cell monolayer washed twice with 1 ml aliquots of buffer to remove residual amino acids which inhibit melphalan transport (Goldenberg) et al, 1979).

At time zero, 1 ml of [^{14}C]-melphalan containing buffer at 37^{0}C was added simultaneously to each well. The plate was incubated on a 37^{0} bath for short time intervals (less than 5 minutes). Longer time point plates were incubated in the 37^{0}C incubator. At the appropriate time, to terminate the uptake reaction the multiwell plates were placed on ice to cool and the radio-labelled drug containing supernatant removed and saved. The cell monolayer was then washed four times with 1 ml aliquots of buffer at 4^{0}C to remove excess [^{14}C]- melphalan. In order to precipitate radio-labelled drug taken up by the cells, 0.5 ml of 10% trichloroacetic

acid (TCA) was added to each well. To ensure complete precipitation of proteins the plates were incubated at $4^{0}C$ for a minimum of 60 minutes. A 400 µl aliquot from each well removed for scintillation. One plate was used as the control to determine the number of BALB/c 3T3 cells well. This control plate was treated by the identical procedure but was not exposed to drug. Cells were removed from the multiwell plate surface by adding 0.5 ml of 0.1% trypsin and 0.01% EDTA, and cell number per well determined by Coulter counter. The EDTA was added to the regular trypsin in order to prepare a single cell suspension. BALB/c 3T3 cells maintained under confluent monolayer conditions become more difficult to remove from the plate surface than those cells maintained under the routine subculturing procedure.

Initial Uptake Velocity

The BALB/c 3T3 cells were maintained under cell culture conditions (page 29). Cells were inoculated into multiwell plates as in time course experiments. The monolayer washing procedure was outlined under time course of uptake. The time points chosen for initial uptake velocity were less than two minutes. Incubations were performed on the $37^{0}C$ water bath. Prewash, incubation, postwash, and sampling for scintillation counting was described previously under time course of uptake (pages 34-36).

Special attention was given to the Na^{+} concentration of the uptake buffers. The buffers were either DPBS or (-Na)HBSS (prepared as described under Drugs and Buffers,

page 30). The appropriate buffer was used for both pre- and post-incubation washes and for the drug incubation. Immediately prior to the experiments the [^{14}C]-melphalan was diluted 100^{-1} into the 37^{0}C prewarmed buffer. In the initial uptake velocity containing BCH, the BCH was dissolved in DPBS buffer to a concentration of 5 mM before 100^{-1} dilution of [^{14}C]-melphalan.

The moles [^{14}C]-melphalan per cell for each point were calculated as described on page 43.

Temperature Effect

The stock [^{14}C]-melphalan was diluted in acidified absolute ethanol. DPBS was chilled to 4^{0}C or warmed to 37^{0}C. The radio-labelled drug was diluted 100^{-1} into the buffer to yield 0.1 μM as the final concentration. BALB/c 3T3 cells in logarithmic phase growth were used to study temperature effect on initial uptake velocity. The 37^{0}C uptake procedure was followed as described under time course of uptake (pages 34-36). However, in the 4^{0}C experiments, all prewashing and uptake incubations were preformed on ice. The pre- and post-wash buffer was kept at 4^{0}C. At the appropriate time of incubation the supernatant from each well was removed and saved, then the monolayer was washed as outlined with the 4^{0}C buffer. The number of cells per well for the control plates of both 37^{0}C and 4^{0}C experiments were determined.

The calculations of moles [^{14}C]-melphalan/cell were described under Initial Uptake Velocity.

1) Serum Deprivation - Initial uptake velocity

BALB/c 3T3 cells were inoculated into multiwell plates as described under time course of drug uptake. To study the effects of serum deprivation comparison was made between BALB/c 3T3 cells in logarithmic phase growth and those cells of Day 4 of confluence. Normally, logarithmic phase cells are grown in αMEM containing 10% fetal bovine serum, and upon reaching confluence the cultures were maintained in αMEM containing 1% serum. In these experiments, 24 hours prior to the uptake study, both logarithmic and stationary phase cells were transferred from the above described mediam to αMEM containing 0.1% serum. The cultures in this serum deprived medium were returned to the $37^{0}C$ incubator. Twenty-four fours after medium starvation an initial uptake velocity experiment was performed at $37^{0}C$ as outlined pages 36-38. The number of cells per well for the control plates were determined with an electronic cell counter (Coulter Electronics).

Dose Response Curves

BALB/c 3T3 cell were maintained in 150 mm^2 flasks as described under regular culture conditions (page 29). Logarithmic growth cells were trypinized, counted, and a cell suspension ((5-8) x 10^4 cells/ml) in complete growth medium was prepared. This cell suspension was used to inoculate 25 mm^2 flasks (Corning Ware) at least 24 hours prior to the first experiment. A sufficient number of flasks were inoculated to compare directly the logarithmic

phase cells and cells at several days in confluent monolayer from the original cell suspension. Logarithmic phase and their subsequent Day 4 confluent cells were incubated with varying concentrations of melphalan for 30 minutes under the same experimental conditions. The entire experiment was performed under sterile conditions in a Class II, type A Biological Safety Cabinet (The Baker Company). As in time course experiments, when the monolayer reached confluence, the complete growth medium was replaced with MEM containing 1.0% serum and replenished every other day.

The growth medium was aspirated from the flasks and the cell monolayer washed once with DPBS. Preheated DPBS was added to the flasks. At time zero various concentrations of melphalan were added to give a 100^{-1} dilution thus yielding the final desired concentration. In all experiments a stock of 10^{-2} M melphalan was prepared in acidified absolute ethanol, and serial dilutions of the stock made immediately prior to the experiment. This was to ensure that melphalan was intact. The drug containing flasks were returned to the 37^{0}C incubator for 30 minutes. At the end of the incubation time the drug containing solution was aspirated. The cell monolayer was washed once with 5 ml of αMEM at 37 C, and then washed once with 5 ml of DPBS. The cells were removed from the flask surface by trypsinization, centrifuged, and

resuspended in complete growth medium. Cell counts were determined from each resuspended cell pellet and appropriate dilutions made with complete growth medium. Various cell numbers were plated in duplicate or triplicate on 100 mm^2 petri plates (Lux plasticware, Miles Scientific). The drug treated and control plates were incubated at 37^0C for 7-10 days and subsequently fixed and stained with 1% methylene blue in 50% methanol. the colonies were counted and corrected for the relative plating efficiency in the control plates.

Incorporation of [^3H-CH_3] Thymidine into DNA

The rate of [^3H-CH_3] thymidine incorporation by BALB/c 3T3 cells in logarithmic phase growth and confluent cultures was studied (Hards and Wright, 1982). Cells were inoculated in multiwell plates and maintained as in previous experiments. Serum contains free nucleosides which may interfere with thymidine incorporation. The complete growth medium was aspirated from logarithmic phase cultures. At time zero, 1.0 ml of αMEM (nucleoside free) containing 0.1 μCi [^3H-CH_3]-thymidine (New England Nuclear, S.A. = 6.7 mCi/mmol) was simultaneously added to each well. These plates were incubated on a 37^0C water bath for the short time interval. Time permitting, the longer incubation plates were retured to the 37^0C incubator. To terminate the incorporation of thymidine the plate was put on ice, and the radio-labelled medium aspirated. Keeping the plate on ice, to each well was added 0.5 ml of trypsin at 4^0C (0.1%) with

EDTA (0.01%) to remove the cells from the surface of the plate. Then to each well was added 0.5 ml of cold 20% trichloroacetic acid, resulting in a final concentration of 10% TCA. All plates were kept in the cold for a minimum of one hour to precipitate the [^{3}H-CH_3]-thymidine incorporated by the cells.

Glass fibre filters (Whatman Laboratory Products Inc.) were presoaked in ice cold 10% trichloroacetic acid and placed onto a filtration manifold (Millipore Corp. Ltd.). The entire contents of each well were filtered and each well washed with 2 x 10 ml aliquots each of 5% TCA, then DPBS, and followed by a 10 ml aliquot of 70% ethanol to dry the filter. All washing solutions were ice cold. After drying, the filters were placed in scintillation vials, 1.0 ml of NCS tissue solubilizer (Amersham) was added. The vials capped and heated at 60^{0}C for one hour. The vials were cooled to room temperature before scintillation cocktail was added.

The BALB/c 3T3 cells at Day 4 confluent monolayer were maintained in αMEM containing 1% serum, the medium was replenished on Day 2 as described page 31. To avoid growth stimulation by addition of fresh nutrients and serum of the Day 4 cultures, medium was not removed. To each medium containing well, [^{3}H-CH_3] thymidine was added to yield the desired 0.1 μCi/ml. The same [^{3}H-CH_3] thymidine incorporation procedure was followed for logarithmic cells.

Kinetics of Melphalan Uptake

The BALB/c 3T3 cells maintained under routine subculturing conditions were trypsinized, and a cell suspension in complete growth medium prepared (5-8 x 10^4 cells/ml). From this suspension, multiwell plates for kinetic experiments were inoculated a minimum of 24 hours prior to the beginning of the experiment. The medium changes to establish the confluent monolayer were followed as described for all previous experiments conducted. For these kinetic studies, one plate was utilized for each drug concentration.

Prior to the kinetic experiment the [^{14}C]-melphalan stock (10^{-2}M) was serially diluted in the acidified absolute ethanol, yielding a drug concentration range from 0.1 - 10.0 mM. These dilutions were kept on ice. The DPBS uptake buffer was prewarmed to 37^{0}C, and 6 ml aliquots were dispensed into capped test tubes which are kept in a 37^{0}C water bath. Immediately prior to the incubation of each drug concentration point, 60 µl of the respective [^{14}C]-melphalan dilution was added to the 6 ml of DPBS. This 100^{-1} dilution yields the required drug concentration and dilutes the ethanol in solution. The kinetic uptake experiments were carried out over a concentration range of 1.0 to 100.0 µM [^{14}C]-melphalan.

The procedure was followed as described under the time course of uptake (pages 34-36). The growth medium was aspirated and the monolayer washed twice with a 1 ml aliquot of DPBS. At time zero, 1 ml of the radio-labelled melphalan

in DPBS simultaneously added to each well. The plates were incubated on a 37^{0}C water bath for 2 minutes. To terminate the uptake reaction the plate was put on ice, and the supernatant removed and saved. The cell monolayer was washed four times with 1 ml aliquot of DPBS at 4^{0}C. To precipitate the radio-labelled drug taken up by the cells, 0.5 ml of 10% ice cold TCA was added to each well. Plates were kept in the cold a minimum of one hour to ensure complete precipitation. A 400 µl aliguot of the TCA extract was sampled for scintillation counting. A 100 µl sample of the supernatant was also counted. A control plate was used to determine the number of cells per well.

Kinetics Calculations

ICF = Intracellular Fluid Count
ECF = Extracellular Fluid Count
DPM = Disintegration per minute
MCV = Mean Cell Volume
RAG = Rapid Association Gradient

1) $$\frac{DPM}{Cell} = \frac{DPM\ (TCA\ Soluble\ Extract)}{(Number\ of\ cells\ counted/TCA\ extract\ Sampled)}$$

2) $$ICF = \left(\frac{DPM}{Cell}\right) X\ MCV$$

3) $$\frac{Cell}{Medium} = \frac{ICF}{ECF}$$

4) $$Corrected\ \frac{Cell}{Medium} = \left(\frac{Cell}{Medium}\right) - RAG$$

5) $$\frac{Moles}{Cell} = \frac{DPM/Cell}{(2.22 \times 10^{12}\ DPM/mole) \times (Specific\ Activity)}$$

6) $$Velocity\ (moles/cell/min) = \left(\frac{Moles}{Cell}\right) - Incubation\ Time$$

7) $$Corrected\ Velocity = \left(\frac{(Cell/Medium) - RAG}{(Cell/Medium)}\right) x\ Velocity$$

Neal Correction of Kinetics

Use slope and y-intercept for each set of points as determined by linear regress analysis.

M_1 = Slope 1

M_2 = Slope 2

I_1 = y-intercept 1

I_2 = y-intercept 2

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

$$V_1 = \left[\frac{K_1}{I_1} - \left[\frac{(M_1 - M_2)}{(I_2 - I_1)} \times \frac{1}{I_1} \right] \right] \times (K_1 - K_2)$$

$$V_2 = (I_1)^{-1} - V_1$$

RESULTS

Growth Curve of BALB/c 3T3 Fibroblasts

The semi-logarithmic growth curve plot of the BALB/c 3T3 line is shown in Figure 1, and revealed an initial region of exponential growth for approximately 60 hours before entering stationary phase. As determined from several such experiments the doubling time was approximately 20 hours during logarithmic phase (a). The BALB/c 3T3 cells doubled until they reach a monolayer; this was judged by microscopic examination and designated as Day 0 confluence. After this point, the cells entered stationary phase where the cell counts per plate reached their maximum value. Subsequent measurements of cell number in stationary phase did not demonstrate any increase in cell number. The points (d) and (e) represented two days and four days of stationary phase respectively. These were stationary phase cells, and were considered to be quiescent. Although non-dividing, the population was metabolically active and viable but at a reduced level as compared to logarithmic phase cells (Todaro and Green, 1963).

Thymidine Incorporation into BALB/c 3T3 Fibroblasts in Logarithmic and Stationary Phase of Cell Growth

The incorporation of radio-labelled thymidine was undertaken as an indication of the rate of the DNA synthesis (Figure 2). As illustrated in Figure 2, an exponential region of cell division was observed until BALB/c 3T3 cells reached a monolayer. These logarithmic phase cells

demonstrated linear incorporation of [^{3}H-CH_3]-thymidine up to one hour with an incorporation rate of 1.778 x 10^{-16} moles per minute. Thereafter a plateau was maintained over the subsequent 4 hours. In comparison, the incorporation study was undertaken with day 4 stationary phase cells which did not demonstrate any increase in cell number when maintained in monolayer (page 48). The day 4 stationary phase cells also demonstrated a linear incorporation of [^{3}H-CH_3]-thymidine up to one hour, with an incorporation rate of 6.538 x 10^{-17} moles per minute. After this a plateau phase was maintained. The ratio of the initial uptake slopes comparing logarithmic phase cells to those after 4 days of stationary phase was 2.7 fold; the ratio of the steady state values was 5.5 fold. These results indicated that the logarithmic phase BALB/c 3T3 cells were synthesizing DNA. The lower level of incorporation by day 4 stationary cultures indicated a reduced level of DNA synthesis. This may represent a baseline of DNA repair.

Cell Cycle Distribution Analysis of Balb/c 3T3 Fibroblasts in Logarithmic and Stationary Phase by Fluorescent Activated Cell Sorting (FACS)

A study to determine the percentage of the BALB/c 3T3 cell population actively engaged in DNA synthesis was undertaken by Fluorescent Activated Cell Sorting (FACS) analysis with the help of Dr. E. Rector, Dept. of Immunology, University of Manitoba. Data is presented in

Table 1. This experiment was undertaken to confirm the [^{3}H-CH_3]-thymidine incorporation studies (page 40). Cell cultures at different stages in the growth cycle were fixed and stained with ethidium bromide for 30 minutes then analyzed by the flow cytometer for fluorescence measurements (pages 33-34). The two parameter cell cycle distribution pattern was used to determine the percentage of the cell population in each stage of the cell cycle (Albert and Gudas, 1985; and Eriksson et. al, 1984). The computer plotted distribution curves in Figure 3 demonstrated two peaks. The first peak represents G_oG_1, and the second peak represents G_2M (Figure 3, Table 1). The remaining area between the two peaks is attributed to cells in the S phase, or the phase of DNA synthesis. Progression from logarithmic phase, to day 0 confluence, and then prolonged maintenance at day 4 stationary phase clearly demonstrated an accumulation of the cell population in the G_oG_1 peak. This G_oG_1 phase represents a resting or non-dividing phase in the cell cycle. A computer generated percentage distribution from the area of the histogram assuming a Gaussian function of the cell population in each phase of the cell cycle is presented in Table 1 (Eriksson, et al., 1984). The BALB/c 3T3 cells in logarithmic phase displayed a maximum value of 44.62% of the population in DNA synthesis. In contrast, the day 4 stationary cultures revealed 17.11% of their population engaged in S phase. Comparing the percentage of the population in logarithmic to day 4 stationary phase

cultures in S phase a ratio of 2.6 was obtained. This value was in close agreement to the ratio of the initial incorporation rates of [^{3}H-CH_3]-thymidine comparing logarithmic to day 4 stationary phase cultures presented page 46 (ratio of 2.7). The percentage of the cell population in S phase was found to decrease steadily when comparing logarithmic phase cells with those of day 0 confluence, or those maintained in stationary phase for 4 days. By contrast, the G_0G_1 population was observed to be increasing in a reciprocal manner.

FACS analysis of the day 2 and day 4 stationary phase cultures revealed 62% of the population were not synthesizing DNA (G_0G_1). A value of 24.84% was calculated for the exponentially dividing cultures, giving a ratio of 2.5. This value was in close agreement to the ratio of 2.6 for S phase cells determined by [^{3}H]-thymidine incorporation. Thus FACS analysis confirmed the thymidine incorporation study, Balb/c 3T3 cells maintained in stationary monolayer cultures were relatively non-dividing population.

Time Course of Melphalan Uptake by Balb/c 3T3 Fibroblasts in Logarithmic and Stationary Phase of Cell Growth

The purpose of this series of experiments was to determine if a difference in melphalan uptake by BALB/c 3T3 cells could be observed between logarithmic phase as compared to cells in day 4 stationary phase cultures. A time course of drug uptake was undertaken at three melphalan

concentrations; 1, 10, and 100 μM concentrations at which different transport systems have been known to operate (Begleiter, et al., 1979). In all transport studies of alkylating agents the design of the incubation medium is an important consideration (Goldenberg and Begleiter, 1979). The complete growth medium used under routine culture conditions is rich in amino acids. Most amino acids compete for the melphalan carrier sites thus inhibiting maximal drug uptake (Vistica et al., 1976). To eliminate this problem the time course studies were incubated in DPBS. At all three melphalan doses, the maximum uptake was demonstrated by logarithmic phase cells (Figure 4). A ratio of uptake at the steady state was calculated at all drug concentrations comparing logarithmic phase cultures to day 4 stationary phase cultures. A constant ratio was observed over all concentration ranges studied. These ratios were 3 at 1 μM, 3.4 at 10 μM, and 3 at 100 μM melphalan. The steady state values of radioactive melphalan per cell were dependent upon the drug concentration. Both logarithmic and day 4 stationary phase cultures demonstrated linear uptake to a maximum of 2 minutes. Thereafter a plateau was reached by 10 to 15 minutes which was maintained over the course of the experiment. Because linear uptake was observed for approximately 2 minutes, this time interval was chosen for all subsequent kinetic studies of melphalan transport in order to insure that initial uptake velocity was being measured.

Temperature Dependence of Melphalan Transport

In the previously described time course experiments, linear uptake was observed for up to 2 minutes. To study initial uptake velocity without the complication of drug efflux short incubation times up to 2 minutes were chosen. Such experiments with BALB/c 3T3 cells in logarithmic phase growth were conducted by incubation of cells with 0.1μM [^{14}C]-melphalan at 37^{0}C and 4^{0}C (Figure 5). Cells incubated at 37^{0}C demonstrated linear uptake of the radio-labelled drug over the 90 second time period. In contrast, cells incubated at 4^{0}C did not demonstrate as significant an increase in drug uptake as cells incubated at 37^{0}C. The ratio of the slopes of the uptake curves between 37^{0}C and 4^{0}C was 20 fold. Statistical analysis by a t-test comparing the difference of these slopes was highly significant (p value <0.001). These results demonstrated a marked temperature sensitivity for the uptake of melphalan by BALB/c 3T3 cells.

BCH - Dependence of Melphalan Transport

Melphalan uptake is known to be strongly inhibited by BCH, a synthetic amino acid which is a specific inhibitor of the L amino acid transport system (Christensen *et al.*, 1965). The effect of various concentrations of BCH on melphalan influx by BALB/c 3T3 cells in logarithmic phase was undertaken to determine the minimal BCH concentration required to approach near maximal inhibition of melphalan transport. Uptake results of 10 μM melphalan in the

presence of various concentrations of BCH is shown in Figure 6.

As the concentration of BCH increased melphalan transport decreased and a plateau was reached. These results indicated a near saturation of the L system. This reduction in uptake was approximately 16% of the control uptake where no BCH was present. Addition of BCH up to 25 mM did not appear to further reduce the plateau value reached at a concentration of 5 mM BCH. This, maximum inhibition by BCH reached approximately 84% of the transport observed in the absence of BCH. In all subsequent studies, in order to eliminate the BCH-sensitive component of melphalan transport, 5 mM BCH was used to inhibit drug transport by system L.

Sodium Dependent and BCH-Sensitive Components of Melphalan Transport by Balb/c 3T3 Fibroblasts

BALB/c 3T3 cells in logarithmic phase were studied to determine the contribution of each amino acid transport system to melphalan influx at various concentrations. Incubation of the cells in DPBS allowed the study of the intact drug transport system. All incubations were terminated at 1 minute to ensure unidirectional influx. This incubation interval was chosen because in the time course of uptake experiments (pages 48-49) it was determined linear uptake occurs within 2 minutes. The cell/medium distribution ratio was calculated and corrected for rapid association gradient. All calculations are described in the

"Materials and Methods" (page 32). The cell/medium values for the intact system determined at melphalan concentrations of 1, 10 and 100 μM and were 34.41, 27.09, and 11.35 respectively (Table 2). These indicate a high concentration gradient was obtained. The high gradient is evidence of active drug transport. As the melphalan concentration increased, a downward shift in the cell/medium ratios was noted. At each melphalan concentration, a break-down of the two major transport components was made. By utilizing the strict Na^+ dependence of amino acid transport system ASC, a sodium depleted buffer was utilized to study the melphalan influx by amino acid transport system L. The specific inhibitor of system L, BCH, was included in DPBS buffer at a concentration of 5 mM in order to study the melphalan influx by system ASC.

Studies of melphalan transport in DPBS buffer allowed all amino acid systems to contribute to uptake. Experiments in sodium depleted buffer and/or in the presence of 5 mM BCH allowed the contribution of the two major amino acid transport systems to be compared to the intact system. Melphalan uptake at concentrations of 1, 10, and 100 μM was determined in Na^+ depleted buffer (Table 2). The cell/medium ratios observed for 1, 10, and 100 μM melphalan were 28.7, 26.2, and 39.5 percent of uptake by the intact system respectively. These values represent melphalan transport by system L. A difference between the high to the low drug dose was observed. The ratio of 100 μM to 1 μM

melphalan for system L was 1.4 fold.

From the previous BCH experiment, 5 mM was the concentration chosen which indicated near saturation of system L. Thus, addition of 5 mM BCH to the Na^+ rich DPBS buffer selects for the preferential study of melphalan transport by system ASC. Melphalan uptake by system ASC at drug concentrations of 1, 10, and 100 μM revealed cell/medium ratios of 85.1, 73.2, and 72.2 percent respectively (Table 2). This indicated that at low melphalan concentrations, system ASC predominated.

These experiments demonstrated the different contribution to melphalan uptake of the major amino acid transport systems, and the shift that occurs as drug concentration rises from 1 to 100 μM. Each of the two major systems L and ASC contributed to melphalan influx to a different degree depending upon the external drug concentration. System L was more pronounced at high drug doses whereas at the low drug dose transport by system ASC was the major component.

Unidirectional Melphalan Influx by Balb/c 3T3 Fibroblasts in Logarithmic and Stationary Phase of Cell Growth

In this series of experiments melphalan influx by both amino acid transport system was evaluated as well as the individual contributions by the Na^+- dependent system ASC and the BCH sensitive system L. The initial uptake velocity of malphalan by BALB/c 3T3 cells during different stages in the growth cycle were compared under various conditions

(Figure 7). The initial uptake velocity was studied in DPBS buffer (panel A), in low Na^+ buffer (panel B), and in the presence of 5 mM BCH (panel C). The slopes of the initial uptake rates are presented in Table 3.

Influx of 10 μM melphalan was measured in BALB/c 3T3 cells during logarithmic growth, in cells at day 0 confluence, and after 2 and 4 days of stationary phase growth. The greatest uptake velocity was observed in logarithmic phase cells (Table 3). Incubation in DPBS buffer allowed study of the intact transport system. Using logarithmic phase cells as the control the ratio of the rate of melphalan uptake under different growth conditions was calculated. The values obtained for day 0, day 2, day 4, and stationary phase were 0.59, 0.43 and 0.40 respectively. A t-test comparing the significance of the difference of the slopes with that of logarithmic phase cells serving as control was highly significant (p value of <0.001 for all stages of stationary growth). This represents a gradual decrease of melphalan uptake by BALB/c 3T3 cells upon entering stationary phase of growth.

To study selectively the contribution to melphalan uptake by the individual amino acid transport systems, the transport conditions were optimized to select for either system L or system ASC. System ASC is strictly Na^+ dependent, thus that component of melphalan influx mediated by system L can be studied by measuring drug uptake in Na^+ depleted medium.

The highest initial uptake velocity was observed with cells in logarithmic phase growth. As previously described these valves were set as the control. The ratio of the slopes for day 0, day 2, and day 4 stationary phase cells were 0.68, 0.53, and 0.26 respectively. A t-test comparing the significance of difference of slopes was statistically significant for day 4 stationary phase cells ($p<0.001$).

To study the contribution of melphalan uptake by system ASC, system L can be eliminated by the addition of 5 mM BCH. As observed under other conditions, there is a progressive decrease in the initial uptake rate in logarithmic phase cells to those in stationary phase. Using the logarithmic value as the control, the ratio of the slopes for day 0, day 2, and day 4 stationary phase cells was 0.55, 0.22, and 0.02 respectively. These differences in slope were all statistically significant by t-test analysis ($p<0.001$).

Kinetic Analysis of Melphalan Transport by 3T3 Fibroblasts in Logarithmic and Stationary Phase of Cell Growth

Kinetic analysis of [^{14}C]-melphalan uptake by BALB/c 3T3 cells during logarithmic phase and day 4 stationary phase over the concentration range of 1 to 100 μM was undertaken. At each drug concentration, 4-6 determinations were obtained. Both analyses (Figures 8 & 9) demonstrated biphasic Michaelis-Menten kinetics suggesting that at least two transport systems may be involved. A Km and Vmax value (mean ± S.E.) were derived for each component after correction was made for rapid binding. The Neal (1979)

correction for the interaction of two-component transport was calculated; the contribution of both amino acid transport systems was determined (Table 4). A comparison was made between cells in logarithmic phase and after 4 days of stationary phase and the linear regression analysis presented in Table 4. The kinetic parameters at the low melphalan concentration were Km of 3 μM and Vmax of 12 attomoles $cell^{-1}$ min^{-1} for logarithmic phase, and Km of 2 μM and Vmax of 15 attomoles.$cell^{-1}$.min^{-1} for day 4 stationary phase cells (Table 5). These values were very close to the results of kinetics for the Na^+-sensitive component of melphalan transport. The ASC system was the main component operating at low drug concentrations. The kinetic parameters at the high melphalan concentration, for the BCH sensitive system L, were a Km of 55 μM and a Vmax of 1138 attomoles $cell^{-1}$ min^{-1} for logarithmic phase cells, and Km of 31 μM and Vmax of 926 attomoles $cell^{-1}$ for stationary phase cells (Table 5). As at the low dose range, both values were very close.

Dose Survival Curves of Logarithmic and Stationary Phase 3T3 Fibroblasts Following Treatment with Melphalan

The dose survival curves of BALB/c 3T3 cells in logarithmic growth and after 4 days of stationary phase are shown in Figure 10. The data from four separate experiments were pooled, each point represents the means (± S.E. of 8-12 determinations). The surviving cell fraction was determined by the clonogenic assay. The relative plating efficiency of

the logarithmic phase cells was approximately 0.20 whereas the relative plating efficiency of day 4 stationary phase cells was approximately 0.10. The linear regression analysis of the slopes was calculated using the values between melphalan concentrations of 5 to 25 μM where a linear relationship was observed. Linear regression analysis of the curve for logarithmic phase cells revealed a slope of -0.0152, and that of day 4 stationary phase cells was -0.0569. This indicated that stationary phase BALB/c 3T3 cells were 3.7 fold more resistant to melphalan than were logarithmic phase cells.

Effect of Serum Deprivation on Melphalan Transport by Logarithmic and Stationary Phase 3T3 Fibroblasts

BALB/c 3T3 cells in logarithmic phase and after 4 days of stationary phase were maintained in culture under regular conditions as described in "Materials and Methods". An identical set of cells were deprived of the normal concentration of fetal bovine serum in the growth medium 24 hours prior to the uptake experiment. The medium was supplemented with 0.1% fetal bovine serum (FBS). The initial uptake velocity rate was calculated for cells in complete medium (10% FBS) and compared to the uptake rate in cells deprived of serum (0.1% FBS), as presented in Figure 11. All linear regression calculations are presented in the text of Figure 11. The uptake rate in logarithmic phase cells deprived of serum was 0.61 that of control logarithmic phase cells in medium with 10% FBS (1.073 versus 1.767

attomoles^{-1}. cells^{-1}. sec^{-1}), and the difference in rate was statistically significant ($p<0.01$). The ratio of the uptake rate in day 4 stationary phase cells between cells treated in 0.1% or 10% FBS was 0.91, however the difference was not statistically significant. The logarithmic phase cells deprived of serum displayed an initial uptake velocity almost equal to that of day 4 stationary phase cells cultured in either 0.1 or 10% FBS; the ratio was approximately 1.0 (Table 6).

FIGURE 1

Growth curve of BALB/c 3T3 cells in culture. The arrows demonstrate different stages of cellular proliferation: cells in logarithmic phase growth (a) and stationary phasee growth (b). The initial day of stationary phase is designated Day 0, two days and 4 days of monolayer culture as Day 2 and Day 4 respectively. The cells were grown in monolayer culture as described in "Materials and Methods" under Cell Culture Conditions. The generation time was approximately 20 hours during logarithmic phase growth. Each point represents the mean $\pm$ S.E. of six determinations, the confidence intervals were too small to be illustrated.

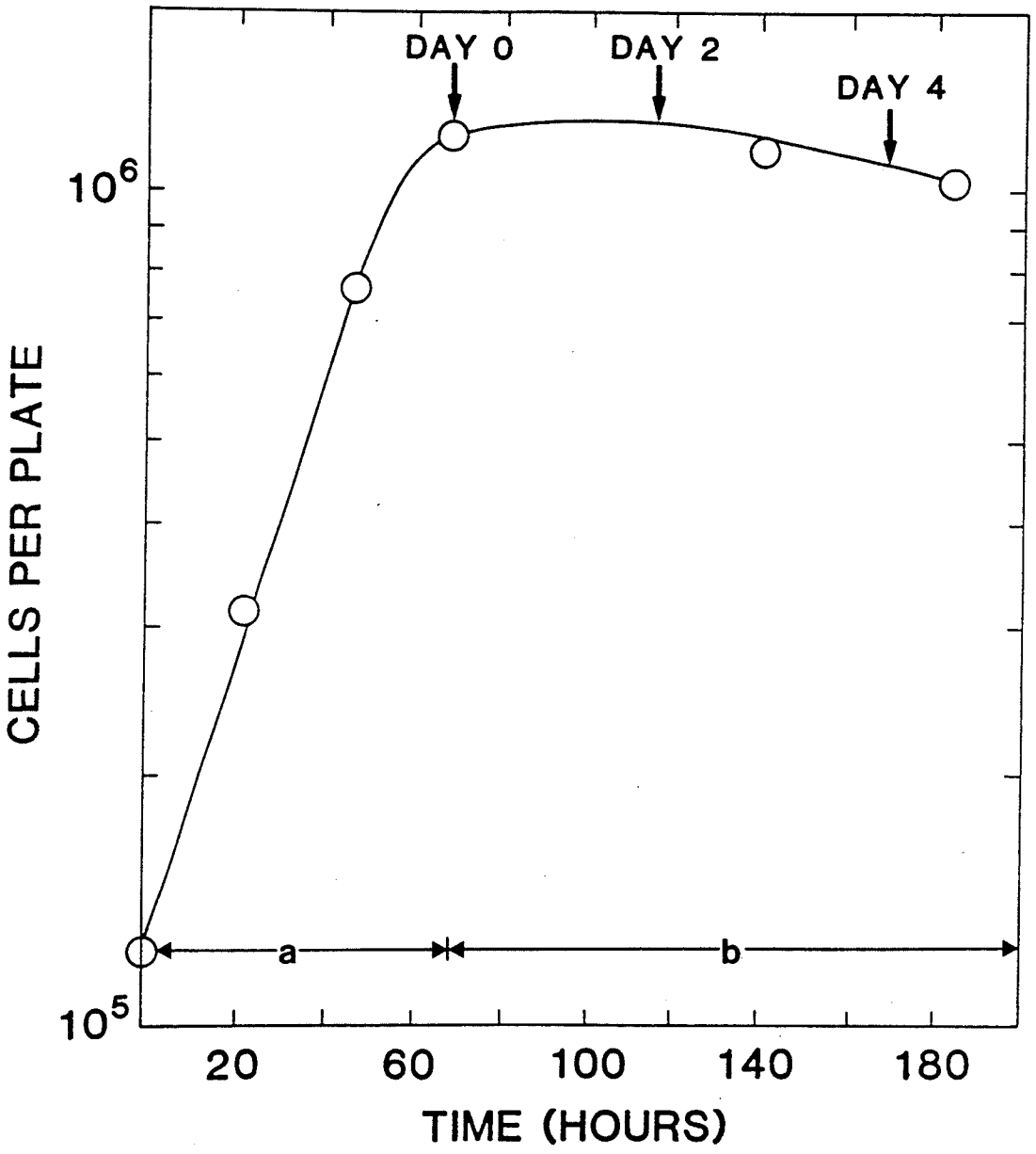
DAY 0
DAY 2
DAY 4
10^6
10^5
CELLS PER PLATE
a
b
20
60
100
140
180
TIME (HOURS)

FIGURE 2

[^{3}H-CH_3]-Thymidine incorporation by BALB/c 3T3 at 37C; comparison at different stages of cellular proliferation: cells in logarithmic phase (O) and after 4 days of stationary phase (□). The values were determined as described under "Materials and Methods". The uptake was approximately linear for one hour (see inset). Linear regression analysis of the time course over 60 minutes for logarithmic phase cells was:

$$y = (1.78 \times 10^{-16})x + (4.72 \times 10^{-17}),$$

with a correlation coefficient of 0.9855. Analysis of the plot for cells 4 days after stationary phase growth was:

$$y = (6.5 \times 10^{-17})x + (2.23 \times 10^{-17}),$$

with a correlation coefficient of 0.9978. Each point represents the mean ± S.E. of six determinations.

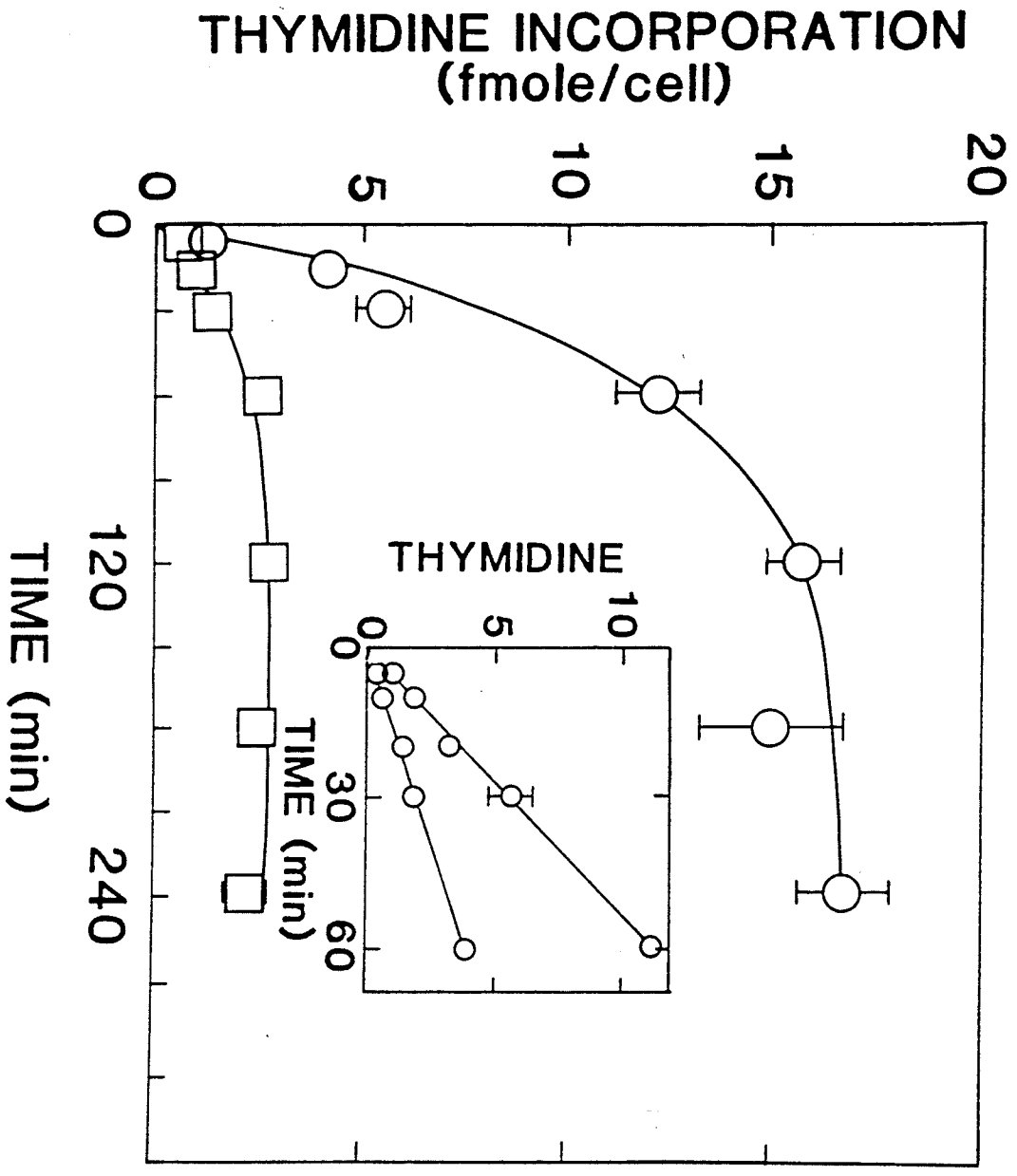
THYMIDINE INCORPORATION
(fmole/cell)
0
5
10
15
20
0
120
240
TIME (min)
THYMIDINE
0
5
10
0
30
60
TIME (min)

FIGURE 3

Cell cycle distribution analysis of BALB/c 3T3 cells by FACS (Fluorescence Activated Cell Sorter). Computer generated cell cycle distribution of cells in logarithmic phase growth (A), initial stage of stationary phase day 0 (B), and day 4 stationary phase (C). The percentage distribution of the cell population in each position of the cell cycle of logarithmic and stationary phase cells (as illustrated in Figure 1) is given in Table 1. I thank Dr. E. Rector, Dept. of Immunology for his help in the FACS analysis.

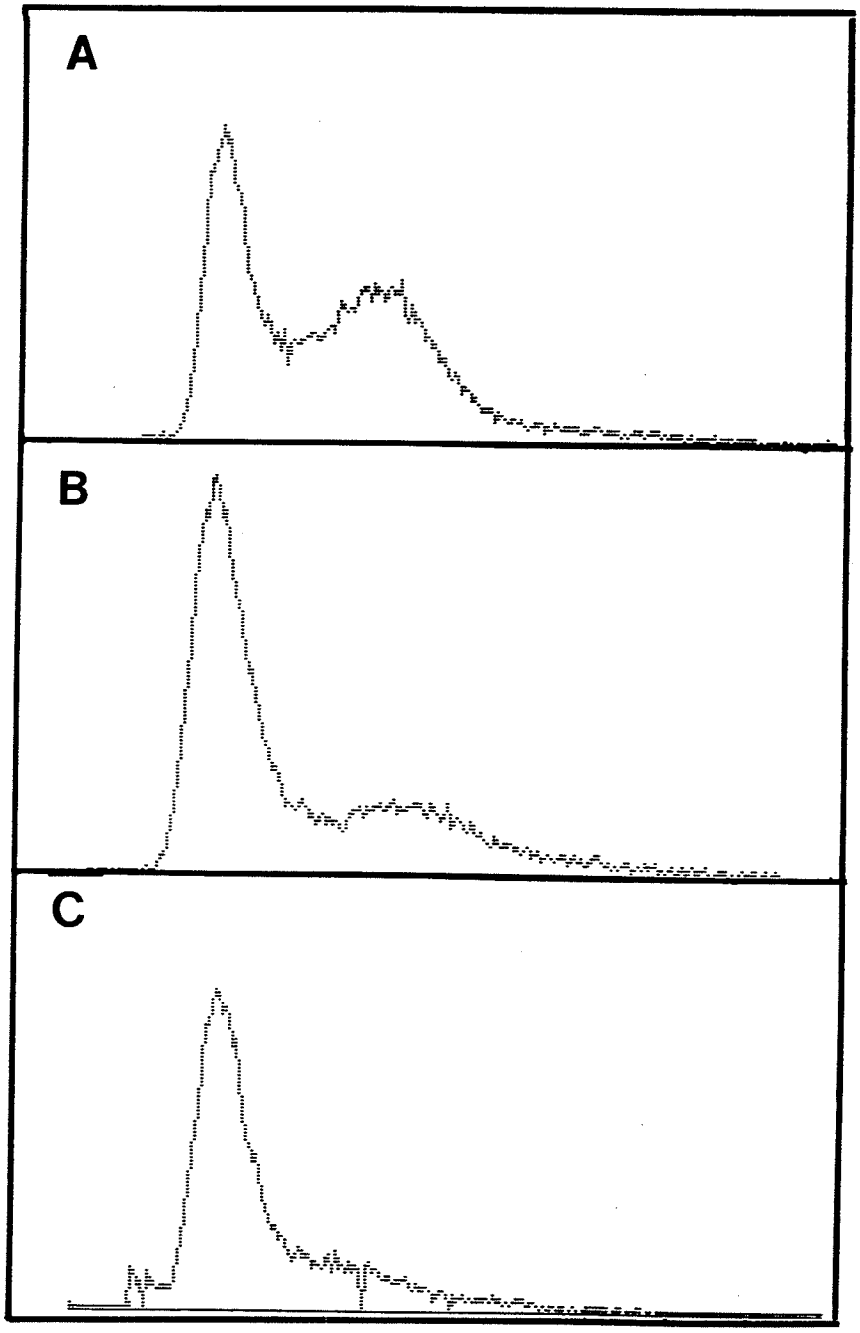
A
B
C

FIGURE 4

Time course of [^{14}C]-melphalan uptake by BALB/c 3T3 cells at 37 C during logarithmic phase (O) and day 4 stationary phase (□) growth. Comparison is made at various [^{14}C]-melphalan concentrations, 1 μM (A), 10 μM (B), and 100 μM(C). The uptake procedure was described in "Materials and Methods". Each point is the mean ± S.E. of 6-12 determinations; the confidence intervals were too small to illustrate.

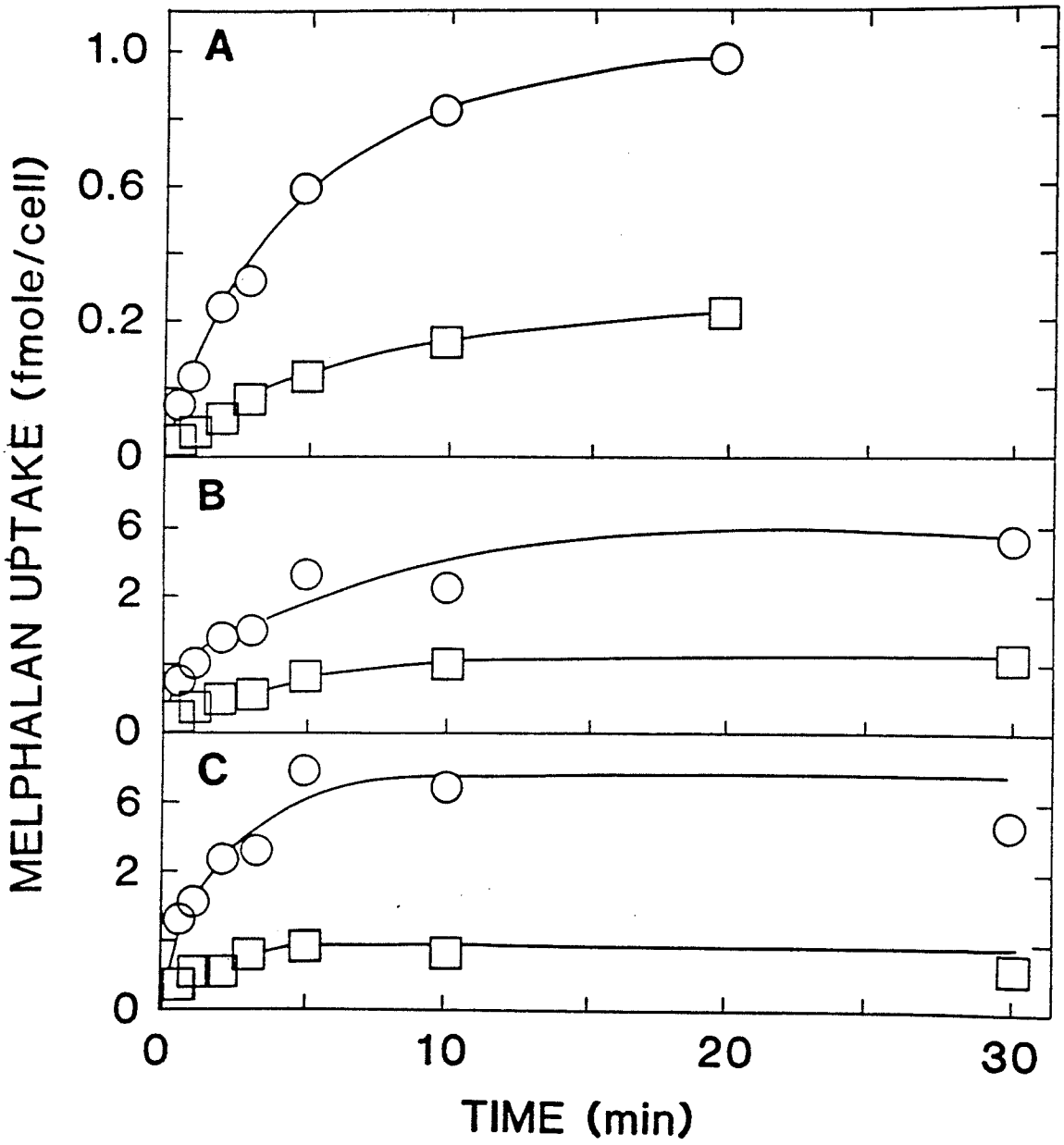
A
B
C
1.0
0.6
0.2
0
6
2
0
6
2
0
0
10
20
30
MELPHALAN UPTAKE (fmole/cell)
TIME (min)

FIGURE 5

Initial uptake velocity of 0.1 μM [^{14}C]-melphalan by BALB/c 3T3 cells in logarithmic phase growth at 37^{0}C (○) and 4^{0}C (□). Linear regression analysis of influx at 37 C was:

$$y = (1.120 \times 10^{-19})x + (3.039 \times 10^{-18})$$

with a correlation coefficient of 0.9841. Analysis at 4^{0}C was:

$$y = (5.619 \times 10^{-21})x + (2.797 \times 10^{-18})$$

with a correlation coefficient of 0.5306. A t-test comparing the significance of the difference of the slopes was highly significant ($p<0.001$). Each point represents the mean ± S.E. of 6 determinations.

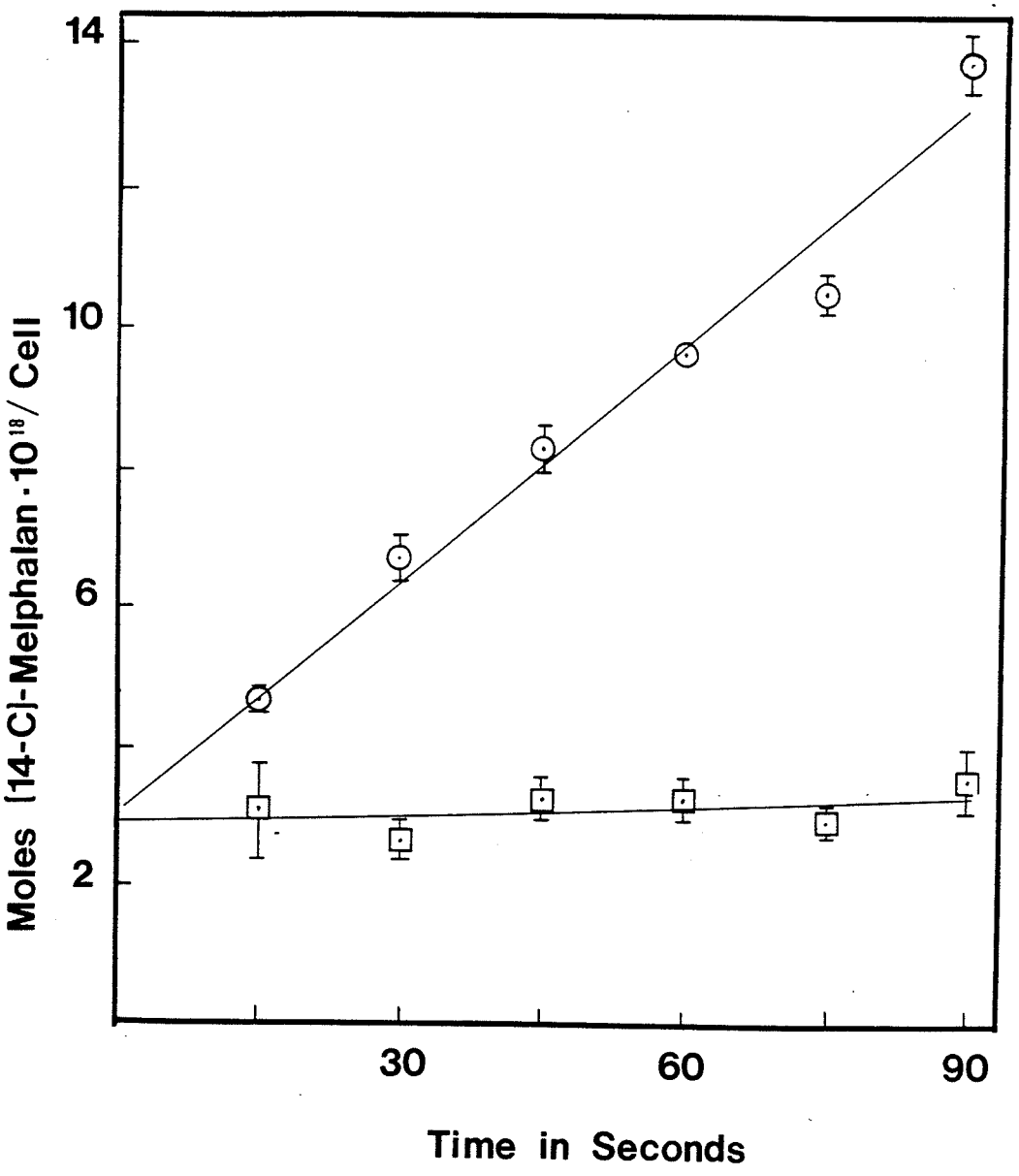
14
10
6
2
Moles [14-C]-Melphalan · 10¹⁸/ Cell
30
60
90
Time in Seconds

FIGURE 6

The effect of BCH on melphalan influx by BALB/c 3T3 cells in logarithmic phase growth at $37^{0}C$. Cells were incubated with various concentrations of BCH and 10 μM [^{14}C]-melphalan as described in "Materials and Methods". The cell/medium drug distribution ratio was determined, the data were corrected for rapid association and are presented as a percentage of the melphalan uptake observed in the absence of BCH. Each point is the mean ± S.E. of six determinations.

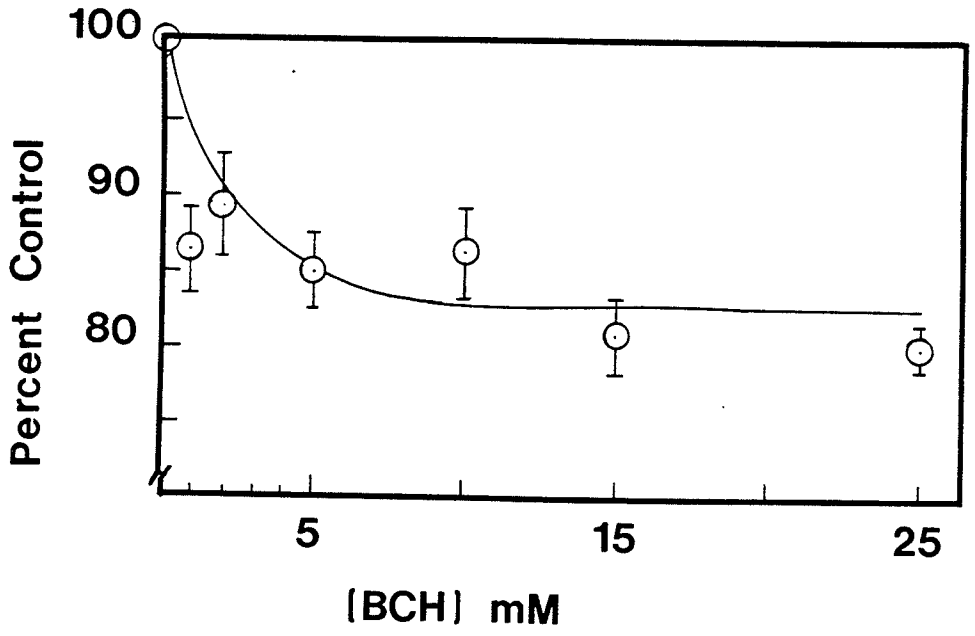
100
90
80
Percent Control
5
15
25
[BCH] mM

FIGURE 7

Initial uptake velocity of 100 μM [^{14}C]-melphalan by BALB/c 3T3 cells at 37^{0}C. Drug influx was observed under different conditions including DPBS (A), sodium depleted medium (B), or DPBS containing 5 mM BCH (C). Melphalan influx was studied under the different stages of the growth cycle; cells in logarithmic phase growth (O), in the initial stage of stationary phase designated day 0 (△), after 2 days in stationary phase (◇) and after 4 days in stationary phase (□). Each point represents the mean ± S.E. of 4-6 determinations. Confidence intervals were too small to be illustrated. The initial uptake rates are shown in Table 3.

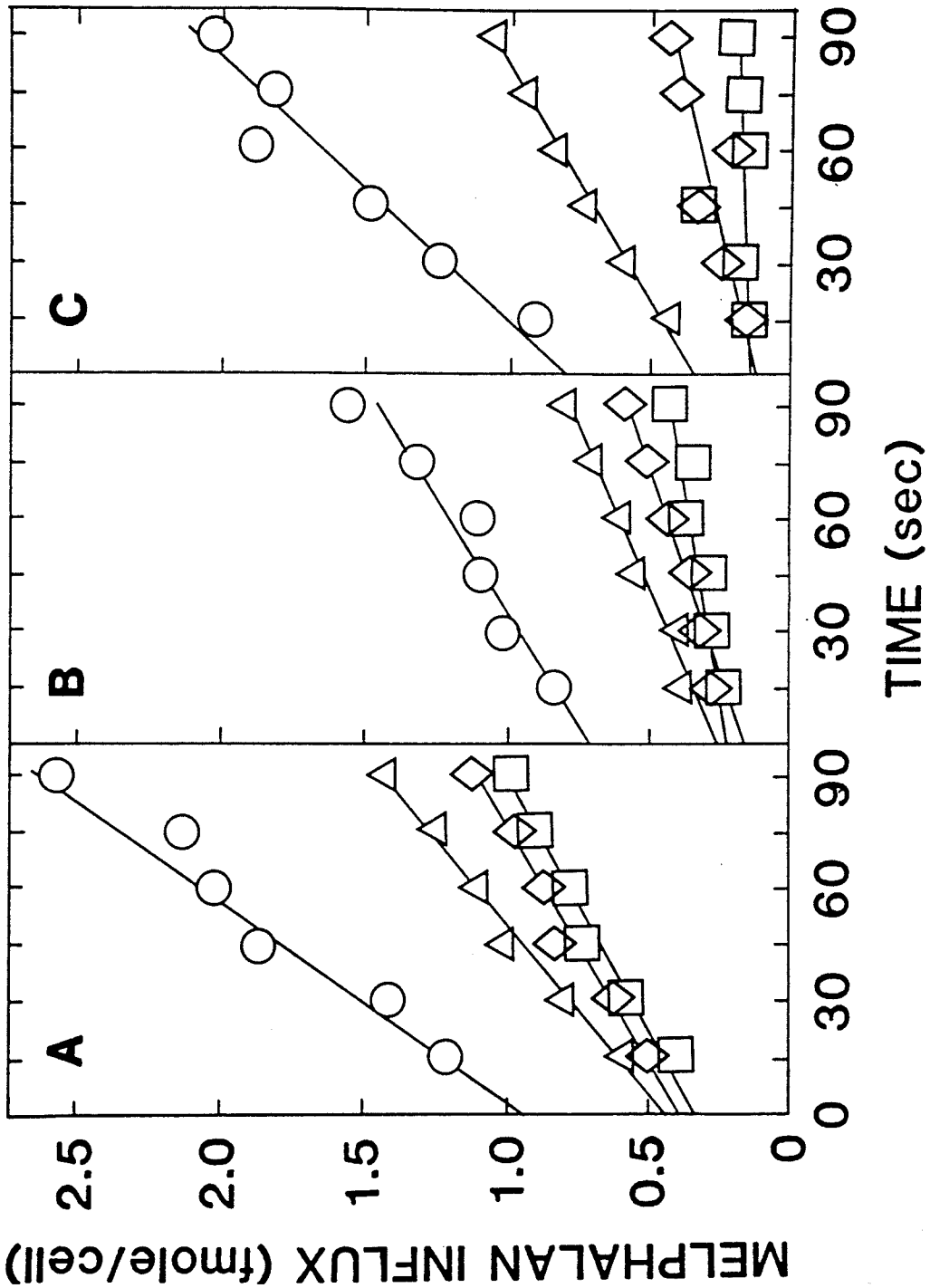
A
B
C
MELPHALAN INFLUX (fmole/cell)
2.5
2.0
1.5
1.0
0.5
0
0
30
60
90
TIME (sec)

FIGURE 8

Kinetic analysis of [^{14}C]-melphalan uptake by BALB/c 3T3 cells in logarithmic growth, in DPBS at 37^{0}C. Linear regression analysis of the Lineweaver-Burk plot of the data revealed a biphasic reaction. The data which represents the mean ± S.E. of 5-6 determinations was corrected for rapid drug association. Kinetic parameters were derived from the linear regression equations: where the y-intercept = 1/Vmax, slope = Km/Vmax, and x-intercept = -1/Km. . The kinetic parameters were also correct for the interaction of 2 components by the Neal Correction. Kinetic data is represented in Table 4.

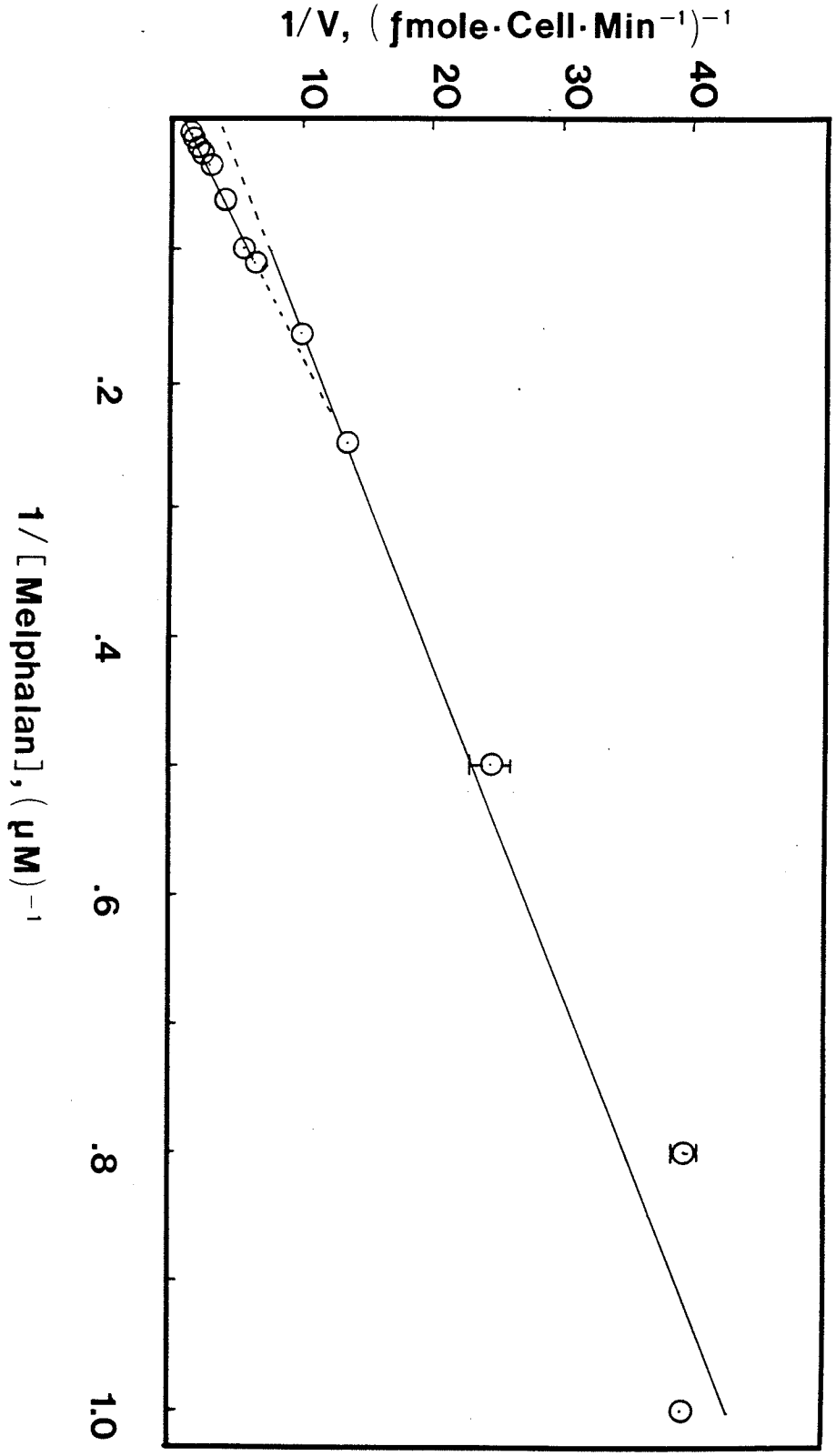
1/V, (fmole·Cell·Min⁻¹)⁻¹
10
20
30
40
.2
.4
.6
.8
1.0
1/[Melphalan], (μM)⁻¹

FIGURE 9

Kinetic analysis of [^{14}C]-melphalan uptake by BALB/c 3T3 cells in day 4 stationary phase, in DPBS at 37^{0}C. Linear regression analysis of the Lineweaver-Burk plot of the data revealed biphasic reaction. The data which represents the mean ± S.E. of 5-6 determinations was corrected for rapid drug association. Kinetic parameters were derived from the linear regression equations: where the

y-intercept = 1/Vmax, slope = Km/Vmax,

and x-intercept = 1/Km

The kinetic parameters were also correct for the interaction of 2 components by the Neal Correction. Kinetic data is represented in Table 4.

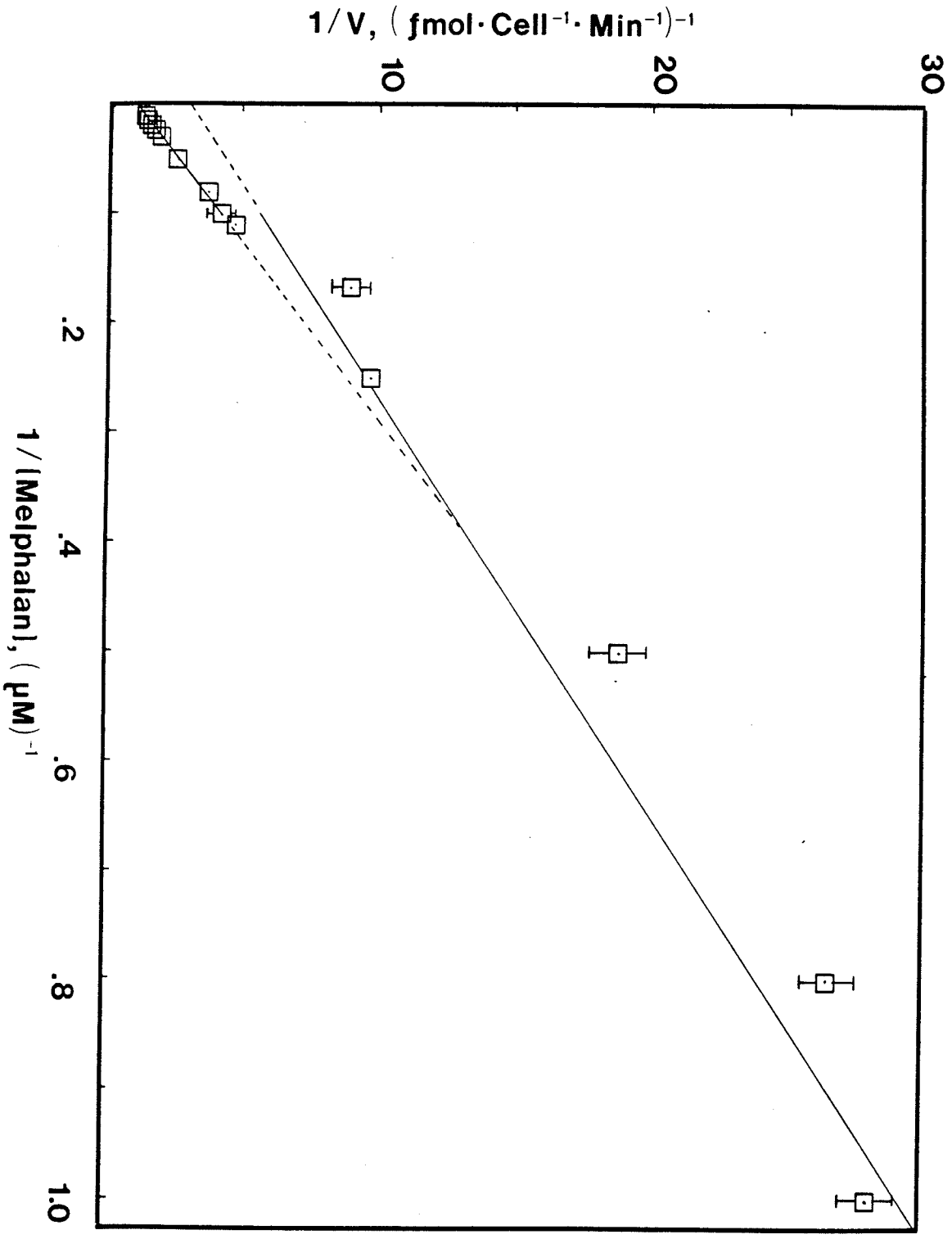
1/V, (fmol·Cell⁻¹·Min⁻¹)⁻¹
10
20
30
1/(Melphalan), (μM)⁻¹
.2
.4
.6
.8
1.0

FIGURE 10

Dose survival curves of BALB/c 3T3 cells in logarithmic phase growth (○) and day 4 stationary phase (□). Surviving cell fraction is determined by colony forming ability as described in "Materials and Methods". The linear regression equation for logarithmic phase cells was:

$$\log_e y = 0.095x - 0.783$$

with a correlation coefficient of 0.9999, and that for day 4 stationary phase cell was:

$$\log_e y = 0.0445x - 0.828$$

with a correlation coefficient of 0.997. The regression equation was calculated using the three points of the linear portion of the dose-survival curves.

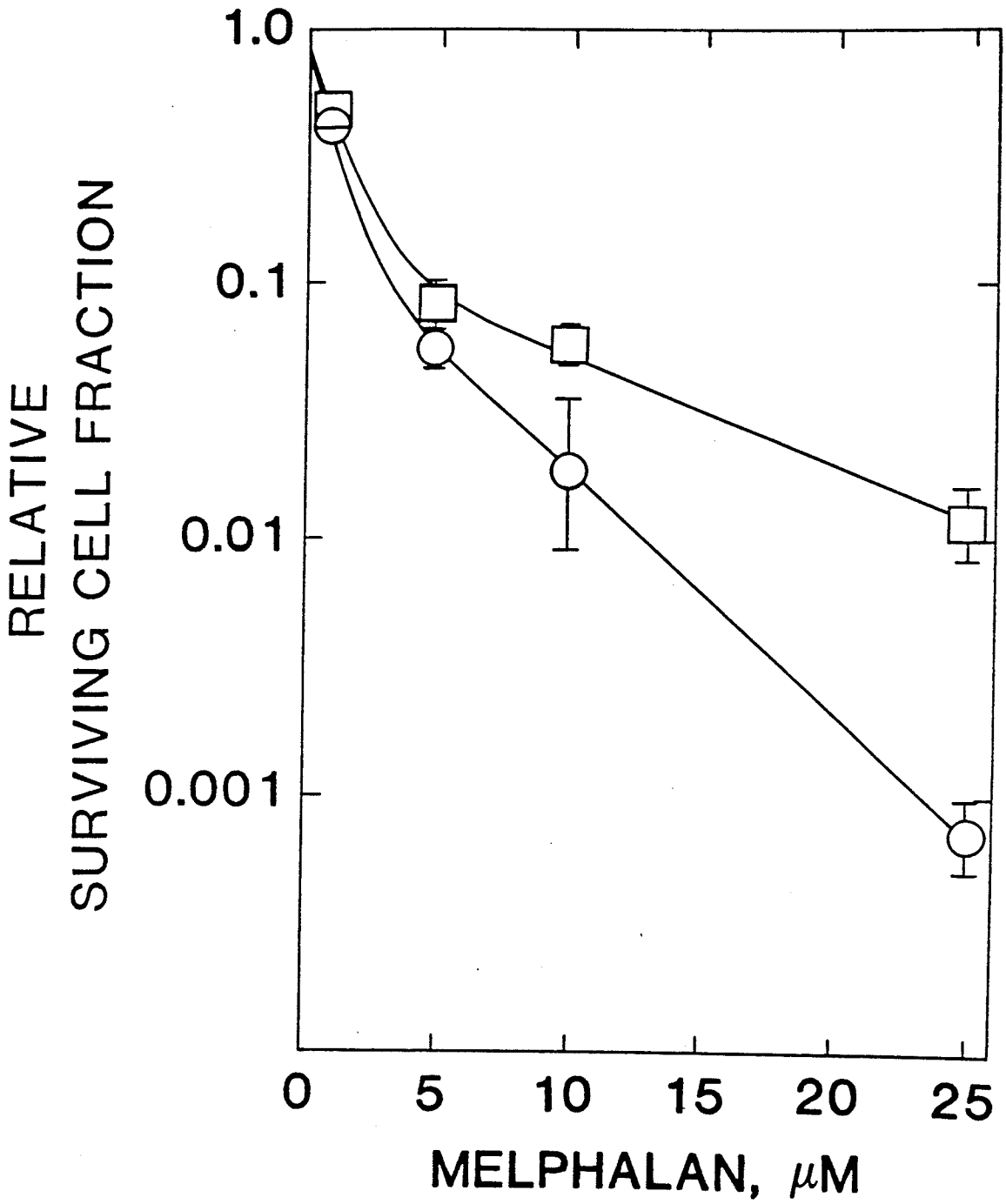
1.0
0.1
0.01
0.001
RELATIVE
SURVIVING CELL FRACTION
0
5
10
15
20
25
MELPHALAN, μM

FIGURE 11

Effect of serum deprivation on initial uptake velocity of 1 μM [^{14}C]-melphalan by BALB/c 3T3 cells. A comparison was made between cells in logarithmic phase growth (○) and day 4 stationary phase (□) cells. Linear regression analysis of logarithmic phase cells was:

$$y = (1.767 \times 10^{-18})x + (4.884 \times 10^{-17})$$

with a correlation coefficient of 0.9978, and that for stationary phase cells was:

$$y = (1.068 \times 10^{-18})x + (3.083 \times 10^{-17})$$

with a correlation coefficient of 0.9972. Cultures maintained in serum deprived medium (0.1%) for 24 hours prior to the uptake study, logarithmic phase cells (●) and day 4 stationary phase cells (■). Linear regression analysis of logarithmic phase cells was:

$$y = (1.073 \times 10^{18})x + (2.814 \times 10^{-17})$$

with a correlation coefficient of 0.9980, and that for stationary phase cells was:

$$y = (9.675 \times 10^{-19})x + (1.671 \times 10^{-17})$$

with a correlation coefficient of 0.9950.

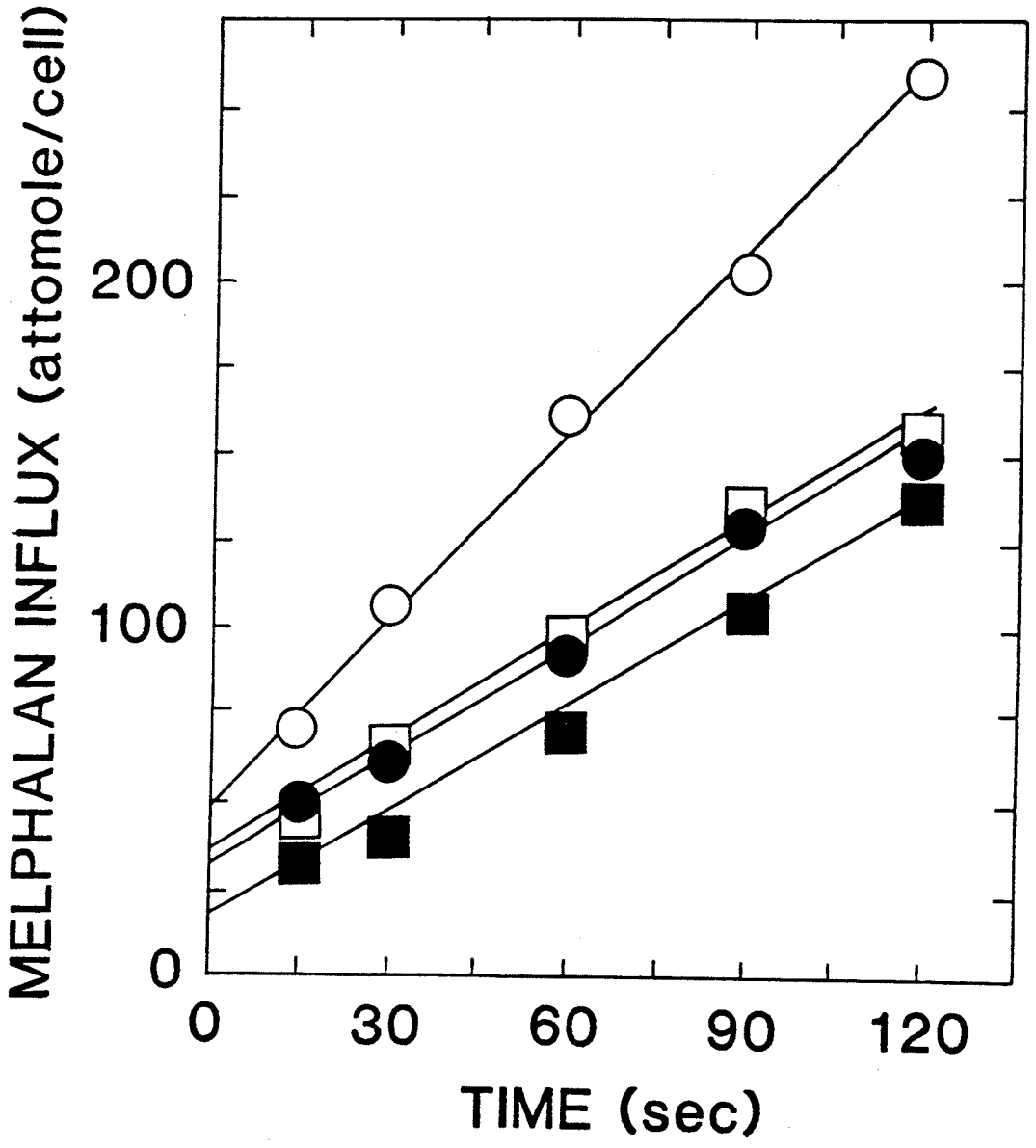
MELPHALAN INFLUX (attomole/cell)
200
100
0
0
30
60
90
120
TIME (sec)

TABLE 1

FACS ANALYSIS OF BALB/C 3T3 FIBROBLASTS PERCENT DISTRIBUTION OF CELL POPULATION IN VARIOUS STAGES OF THE CELL CYCLE

STATIONARY PHASE (DAYS)	CELL CYCLE G_0G_1	S	G_2M
LOG	24.84	44.62	30.54
DAY 0	42.23	38.01	18.76
DAY 2	62.35	28.99	8.66
DAY 4	62.85	17.11	19.22

The percentage distribution of Balb/c 3T3 cells in each position of the cell cycle for logarithmic phase and stationary phases as presented in Figure 3. Cell Cycle is shown as gap one or gap zero (G_0G_1), synthesis (S), and gap two and mitosis (G_2M).

TABLE 2

SODIUM-DEPENDENT AND BCH-SENSITIVE COMPONENTS OF MELPHALAN INFLUX IN LOG-PHASE BALB/C 3TC CELLS

[MELPHALAN] μM	CONDITION	CELL/MEDIUM RATIO MEAN ± S.E. (1)	% CONTROL
1	DPBS	34.41 ± 0.88	100.00
1	DPBS + 5 mM BCH	29.27 ± 1.57	85.10
1	Low Na^+ (2)	9.89 ± 0.22	28.70
10	DPBS	27.09 ± 0.32	100.00
10	DPBS + 5 mM BCH	19.83 ± 0.19	73.20
10	Low Na^+ (2)	7.10 ± 0.12	26.20
100	DPBS	11.35 ± 0.39	100.00
100	DPBS + 5 mM BCH	8.19 ± 0.12	72.20
100	Low Na^+ (2)	4.48 ± 0.19	39.50

(1) The data represent the mean ± S.E of 6 determinations.

(2) Sodium depleted medium consisted of modified Hank's Balanced Salt Solution; NaCl was replaced by Tris, to yield a final Na^+ concentration of 4 mEQ/L.

TABLE 3

INITIAL UPTAKE RATES OF 100 μM [14C] - MELPHALAN BY BALB/C 3T3 CELLS

PHASE IN GROWTH CURVE	CONDITIONS	SLOPE + S.E. (ATTOMOLES. CELL-1.SEC^{-1})	CORRELATION COEFFICIENT	P
LOG	DPBS	18.270 ± 1.060	0.9933	P<0.001
Day 0	DPBS	10.730 ± 0.620	0.9934	P<0.001
Day 2	DPBS	7.798 ± 0.681	0.9850	P<0.001
Log	Low Na^+	8.934 ± 1.218	0.9603	---
Day 0	Low Na^+	5.728 ± 0.470	0.9868	P<0.100
Day 2	Low Na^+	4.466 ± 0.670	0.9578	P<0.100
Day 4	Low Na^+	2.199 ± 0.430	0.9314	P<0.001
Log	DPBS + 5 mM BCH	14.610 ± 1.270	0.9888	---
Day 0	DPBS + 5 mM BCH	7.986 ± 0.354	0.9960	P<0.001
Day 2	DPBS + 5 mM BCH	3.225 ± 1.017	0.9457	P<0.001
Day 4	DPBS + 5 mM BCH	0.311 ± 0.022	0.6294	P<0.001

TABLE 4

KINETIC ANALYSIS OF [14C] - MELPHALAN UPTAKE BY BALB/C 3T3 FIBROBLASTS AND LINEAR REGRESSION ANALYSIS OF THE LINEWEAVER-BURKE PLOT

LOGARITHMIC PHASE	R	Slope ± S.E.	Int. ± S.E
(Figure 8)			
$10^{-6} - 10^{-5}$ µM	0.9843	$(4.040 \pm 0.324) \times 10^{10}$	$(2.700 \pm 1.732) \times 10^{15}$
$1.25 \times 10^{-5} - 10^{-4}$ µM	0.9944	$(4.725 \pm 0.289) \times 10^{10}$	$(8.697 \pm 0.965) \times 10^{15}$

DAY 4 STATIONARY	R	Slope ± S.E.	Int. ± S.E.
(Figure 9)			
$10^{-6} - 10^{-5}$ µM	0.9833	$(2.716 \pm 0.225) \times 10^{10}$	$(2.987 \pm 1.201) \times 10^{15}$
$1.25 \times 10^{-5} - 10^{-4}$ µM	0.9857	$(3.219 \pm 0.245) \times 10^{10}$	$(1.063 \pm 0.099) \times 10^{15}$

where R = Correlation Coefficient

TABLE 5

TABULATION OF Km AND Vmax VALUES

		Km μM	Vmax (attomol.cell^{-1}.min^{-1})
BALB/c 3T3			
Log Phase	BCH^s sensitive[1]	55	1138
	Na^{+s} sensitive[2]	3	12
Day 4	BCH^s	31	926
	Na^{+s}	2	15
MCF-7[3]	BCH^s	54 ± 13	700 ± 170
	Na^{+s}	6 ± 2	50 ± 20
L5178Y[4]	BCH^s	80 ± 30	110 ± 40
	Na^{+s}	10 ± 7	22 ± 24
LPC-1[5]	BCH^s	95 ± 24	210 ± 40
	Na^{+s}	25 ± 6	200 ± 60

1 System L
2 System ASC
3 Begleiter, et al.; 1980
4 Begleiter, et al.; 1979
5 Goldenberg, et at., 1979

TABLE 6

INITIAL UPTAKE RATES OF 1μM [^{14}C] - MELPHALAN BY BALB/C 3T3 FIBROBLASTS

Phase in Growth Curve	Slope ± S.E. (attomoles. $cells^{-1}$. sec^{-1}) Control, Complete Medium	24 Hour Serum Depleted Medium	+ serum / - serum P
Logarithmic	1.767 ± 0.067	1.073 ± 0.039	P<0.01
Day 4	1.068 ± 0.046	0.968 ± 0.056	NS

The control plates were maintained under Cell Culture Conditions as described in "Materials and Methods". The serum depleted plates were cultured 24 hours in serum deprived medium (0.1% FBS) as described in "Materials and Method" .

Discussion

The BALB/c 3T3 cell line used in this study demonstrates characteristics which differ from many other transformed cell lines (Todaro and Green, 1963). The typical growth cycle of the BALB/c 3T3 cell line more closely resembles the properties of a primary cell culture. Although the Balb/c 3T3 cells have many non-transformed properties they exhibit a non-senescent phenotype. Transformed cell lines have lost contact-inhibition and upon prolonged culturing will continue to divide forming multi-layered sheets. Growth ceases when the supply of nutrients has been depleted and/or toxic wastes inhibit proliferation of the population. A primary or non-transformed cell line has a finite life span in culture. The doubling time and number of passages are characteristic of the cell type (Pollock et al., 1968; Todaro et al., 1965). Primary cultures demonstrate a fixed generation time. In culture they exhibit 'contact-inhibition'; that is the cells in monolayer grow until the plate surface is covered then they cease division. In many aspects such as this criteria the BALB/c 3T3 cell line appears non-transformed. In sub-confluent culturing the 3T3 cells behave like normal fibroblasts (Goldenberg et al., 1963; Green and Goldberg, 1965). Upon reaching a monolayer they fill in all available space on the culturing vessel then cease cell division. Prolonged maintenance in monolayer cuture does not result in an increase in cell number. These fibroblasts however,

undergo considerable metabolic changes (Green and Kehinde, 1974). Upon prolonged maintenance under monolayer conditions of two to four weeks, the fibroblasts will develop into adipocytes (Green and Kehinde, 1976). Thus during the growth cycle the BALB/c 3T3 cell line has the genetic potential to undergo various stages of growth, quiescence, development, and terminal differentiation (Green and Meuth, 1974).

The BALb/c 3T3 system provided the opportunity to study melphalan transport under a variety of growth conditions. By utilizing one cell line active transport could be compared in actively dividing and quiescent cells under the same cell culture conditions. Further, differences in drug uptake between different stages in the growth cycle could be compared directly. Until these studies, the uptake of melphalan by cells in culture had never been approached in this fashion. It has been well documented by many researchers that melphalan and drug uptake in general is most efficient in rapidly proliferating tissues and cells in culture (Barranco and Novack, 1974; Barranco et al., 1973). This however, is not a true representation of drug uptake as related to the whole organism. Within a tumor cell population, the cells are heterogeneous (Heppner, 1974). The BALB/c 3T3 line was used as an in vitro tumor model. During the course of a malignancy, a tumor mass can undergo various stages of growth (Levan and Havschka, 1953, Klern and Lern, 1956). Cell division may be rapid or the tumor

mass may enter a non-dividing stage for extended periods. It has been speculated that within the tumor mass the cells could be divided between various stages of the cell cycle of cell growth (Heppner, 1984). New chemotherapy approaches are desired for the dormant or non-dividing malignancies. Even a rapidly proliferating malignancy undergoes cycles of reduced cell division. By understanding drug transport by these cell populations a more rational approach to tumor chemotherapy may be developed. A better understanding of transport changes that occur as the cell undergoes metabolic slow down and cycle changes was the objective of the investigation described in this thesis. These experiments allowed the comparison of melphalan cytotoxicity with the same cell line under optimum cell division versus non-dividing conditions. The BALB/c 3T3 cell line provided the opportunity to study one line either in active division or in contact inhibited quiescence. Thus it was utilized as a model for these two situations. Under normal culturing conditions a comparison was made between logarithmic phase and stationary phase growth. An additional model of serum deprivation to reach an induced quiescent state yielded another approach. Within the tumor mass, cells may be deprived of nutrients thereby entering a metabolically quiescent state (Heppner, 1984). Clinically, melphalan has proved useful in the treatment of multiple myeloma, ovarian carcinoma, breast cancer, and malignant melanoma (Bosanquet and Gilby, 1982; Alexian et al., 1968; Bergsage et al.,

1967). The studies presented in this thesis have indicated that a significantly reduced rate of proliferation is directly related to a significantly reduced rate of melphalan transport.

The BALB/c 3T3 studies, in part, consisted of time course experiments. A dramatic reduction of melphalan uptake was demonstrated as the cells entered the non-dividing, contact inhibited monolayer and remained stationary. A high proliferative rate represents exponentially dividing cells which corresponded to both the maximum melphalan transport and initial uptake velocity. Drug transport involves binding to the cell surface, influx, efflux, and metabolism. These studies were mainly concerned with the unidirectional transport incubation of short time intervals thus ensuring true membrane transport without the complications of efflux, drug binding, inactivation, or metabolism. To ensure these criteria initial uptake velocities were studies at short incubation intervals. Melphalan transport has been attributed to two main amino acid transport systems, the one preferential for low drug concentrations, the other for high drug concentrations. Both systems demonstrated reduced influx as the proliferative rate was reduced and the cultures remained stationary. Although melphalan transport occurs by two separate systems, the reduction in drug uptake was proportional to the reduced proliferative rate. These studies with BALB/c 3T3 cells supported previous findings

that rapidly growing tumors are more sensitive to chemotherapeutic agents (DeWys, 1972). Even over several days in the resting state where the metabolic rate was reduced, melphalan influx was still observed. To determine the reduction in melphalan sensitivity dose response experiments were conducted. The experimental data presented agreed with previous findings (Goldenberg et al., 1971) that confluent cultures exhibit reduced melphalan uptake. This and other studies suggest drug uptake is generally reduced for chemotherapeutic agents transported by active processes. Similar situations could exist for other drugs. It is interesting to note that the two major amino acid transport systems A and L both demonstrated reduced activity as the cultures remained in confluence. The presence of system A and L in BALB/c 3T3 cells has also been reported by Oxender et al. (1977). Researchers such as Guidotti et al. (1978) found that when cultured cells approach confluency and growth rate was reduced, activity of system A was decreased, yet increased activity was observed in system L. This phenomena was not observed however in the BALB/c 3T3 line by Oxender et al. (1977). Their work was confirmed by the experiments presented here that transport by system A and L have reduced activity, in stationary phase cells. This supports the data showing that decreased melphalan transport occured by both systems as the BALB/c 3T3 cells were maintained in confluent cultures. Cecchini et al. (1976) studied amino acid uptake in 3T3 and SV3T3 transformed

cultures. Their kinetic experiments showed biphasic plots for the 3T3 cells but linear plots for the virally transformed line. These results support the BALB/c 3T3 work presented in this thesis. Both logarithmic and stationary cultures demonstrated biphasic kinetics.

Foster and Pardee (1969) studied transport of amino acids by pre- and post-confluent 3T3 and Polyoma virus transformed 3T3 cells. The confluent 3T3 cultures accumulated non-metabolizable amino acids less rapidly than non-confluent cultures. It is interesting to note their observations of the virally transformed Py3T3 line, no differences in uptake and accumulation could be found between confluent versus non-confluent cultures. This indicates the viral transformation has affected the characteristics of the cell line. In addition these cultures do not show contact inhibition. Their kinetic studies did not reveal any major differences between confluent and non-confluent 3T3 cells on the basis of Km and Vmax values. Studies with our BALB/c 3T3 cells confirm this observation that no apparent kinetic changes can explain the reduced melphalan transport.

The growth curve represented in Figure 1 indicated that when the BALB/c 3T3 cells reached a confluent monolayer, cell division ceased as indicated by no increase in cell number per plate. In order to ascertain a true quiescent state [^{3}H-CH_3]-thymidine uptake was used as an indication of the rate of DNA synthesis which in turn is related to cell

division (Figure 2). These experiments both agree with experimental data that the BALB/c 3T3 cell line achieves a non-dividing state in monolayer culture. Further confirmation of reduced DNA synthesis was carried out by Fluorescence Activated Cell Sorting (FACS) analysis, as represented in Table 1. The logarithmically dividing cultures produced a typical cell cycle distribution pattern (Figure 3). As the cultures remained in confluent monolayer, a shift into the G_1 state was clearly demonstrated. Thus it was confirmed the BALB/c 3T3 cells used in the experiments described in this study behave as previously described by Green and Todaro (1963), and our studies on melphalan transport were performed on dividing and non-dividing populations. Clearly, melphalan transport is reduced when the BALB/c 3T3 cell population enters the quiescent, non-dividing state. Over the entire 1 to 100 μM drug range studied the general trend of reduced uptake was observed (Figure 4). This agrees with amino acid transport studies showing reduced uptake for all systems. The steadily decreasing trend corresponds and confirms previous experiments performed on a wide variety of cell lines (Foster and Pardee, 1969). These BALB/c 3T3 experiments were in agreement with the accepted findings of reduced transport in non-dividing cultures.

A second model was employed in which the cells are rendered quiescent in culture in serum-depleted medium. Melphalan influx was measured in logarithmic and stationary

phase cells maintained in complete medium and in serum-depleted medium (Figure 11). The transport findings agreed with those of the first model, in that a reduction in melphalan transport was observed in quiescent cells.

It is generally accepted that rapidly growing tumors clinically are more sensitive to chemotherapeutic agents (DeWys, 1972). In the dose response experiments presented, the logarithmic phase cultures were approximately 2.1 fold more sensitive to melphalan than the stationary, non-dividing cultures (Figure 10). Within the tumor population some cells may be cycling at different stages of the cycle, or may be non-dividing (Dexter and Calabresi, 1982; Heppner and Miller, 1983). The in vitro model, however, is not so complicated. Cells in culture have provided much insight into the transport of chemotherapeutic drugs. Because carrier mediated transport requires initial interaction of the drug with the carrier molecule, under controlled culture conditions the competition between competing substrates can be minimized. Melphalan uptake by BALB/c 3T3 cells in amino acid free buffer helped reveal the transport reactions. By understanding the common substrates which compete for the melphalan transport carriers, medium was designed to study the kinetics of drug transport. In general, drug uptake at the clinical level can be better understood by cell culture studies. The mechanisms of membrane transport of the alkylating agents has been an important field of study, establishing different mechanisms between different agents

(e.g. Goldenberg and Beleiler, 1978). By understanding the mechanisms involved under controlled culture conditions, a clearer understanding of the *in vivo* setting may be developed leading perhaps to better application of known treatments. The findings presented in these experiments suggest that the activity of the transport systems for melphalan by both the L and ASC-like systems are more active in rapidly dividing cells. Thus regulatory factors that modulate transport activity may be important determinants of chemotherapeutic sensitivity. In summary this study shows that the activity of the transport systems to a large extent account for melphalan sensitivity.

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