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AMINO ACID-CONFERRED PROTECTION AGAINST MELPHALAN—

CHARACTERIZATION OF MELPHALAN TRANSPORT AND CORRELATION OF UPTAKE WITH CYTOTOXICITY IN CULTURED L1210 MURINE LEUKEMIA CELLS*

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Abstract—The uptake of melphalan by L1210 cells was reduced to one-sixth of controls by physiological concentrations of the L-isomers of leucine and glutamine, and this decrease was accompanied by a corresponding decrease in cytotoxicity. Cytotoxicity was estimated by treatment of cells with melphalan for 35 min in phosphate-buffered saline containing bovine serum albumin and glucose followed by clonal growth of the surviving cells in soft agar. It was prominent within a critical region of net melphalan uptake of 2–5 pmoles/10^5 cells. Inhibition analysis revealed that at cytotoxic concentrations melphalan is transported by a high-affinity amino acid transport system of the leucine (L) type. The values of the Michaelis constants (K_m) for L-leucine, a protective amino acid, L-valine, a minimally protective amino acid, and melphalan were 6, 58 and 19 μM respectively. These results suggest that the ability of amino acids to protect L1210 cells from melphalan cytotoxicity is related to their affinities for the leucine carrier sites.

Melphalan cytotoxicity to murine L1210 leukemia cells in culture is reduced in growth medium containing amino acids [1]. Incubation of cells first with single amino acids and then with melphalan for 35 min in phosphate-buffered saline containing bovine serum albumin indicated that the L-isomers of leucine and glutamine were primarily responsible for the observed decrease in cytotoxicity. The low concentration of melphalan required for cytotoxicity together with the stereospecific nature of amino acid protection suggested that melphalan transport may occur via a high-affinity, carrier-mediated, leucine-preferring amino acid transport system. As a result of these observations we have undertaken an analysis of the relationship between melphalan transport and cytotoxicity, and in this paper present evidence in support of our earlier suggestion.

MATERIALS AND METHODS

Bovine serum albumin† was obtained as serum fraction V from Miles Laboratories, Inc. (Elkhart, IN). Fetal calf serum was purchased from Flow Laboratories (Rockville, MD), and RPMI 1630 medium and Dulbecco's phosphate-buffered saline were supplied by the NIH Media unit. Gentamicin (50 mg/ml) and Fungizone (250 μg/ml) were purchased from Microbiological Associates (Bethesda, MD) and Grand Island Biological Co. (Grand Island, NY) respectively. Unlabeled melphalan was obtained from Burroughs Wellcome Co. (Research Triangle Park, NC). All other unlabeled amino acids were obtained from CalBiochem (San Diego, CA). The silicone oil, Versilube F-50 (specific gravity 1.045 at 25°; viscosity 70 centistrokes at 25°) was obtained from Harwick Chemical Corp. (Cambridge, MA).

L-[4,5-^{3}H]leucine (57.4 Ci/m-mole), L-[2,3-^{3}H]-valine (12.5 Ci/m-mole), and 2-aminobicyclo[2,2,1] heptane-2[^{14}C]carboxylic acid (4.78 mCi/m-mole) were purchased from New England Nuclear (Boston, MA). [^{3}H]leucine and [^{3}H]valine were diluted with the respective unlabeled amino acids and used at the concentrations indicated in the text. Analyses [2] performed by the supplier revealed that the labeled BCH was composed of 3.5% a-2-aminobicyclo[2,2,1] heptane-2-carboxylic acid and 96.5% b-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid. Since it was desirable to estimate the uptake of only the levorotatory isomer of (±) b-BCH, the racemic mixture was used at twice the recorded concentration. The structural formulas of melphalan, leucine, valine and (−)b-BCH are illustrated in Fig. 1.

Melphalan (6.4 mCi/m-mole), labeled in the chloroethyl groups with ^{14}C, was synthesized by Mr. Morris Leaffer under contract with the Stanford Research Institute (Menlo Park, CA). Radiochemical purity was 97 per cent as determined by thin-layer chromatography on silica gel in n-butanol–acetic acid–water (7:2:1). Unlabeled and labeled melphalan solutions were prepared daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid. Further dilutions were made in aqueous medium

* A preliminary report of this work has been presented [D. T. Vistica, J. N. Toal and M. Rabinovitz, *Proc. Am. Ass. Cancer Res.* **18**, 26 (1977)].

† Abbreviations used in the test are as follows: bovine serum albumin, BSA; 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid, BCH; Dulbecco's phosphate-buffered saline, PBS; and Dulbecco's phosphate-buffered saline containing 0.1 mM bovine serum albumin and 0.25% glucose, PAG.

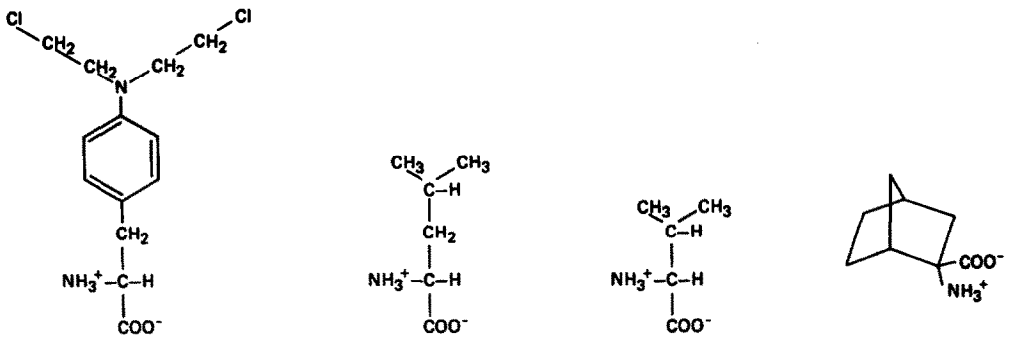

Fig. 1. Structural formulae for melphalan, leucine, valine and BCH.

immediately prior to use in order to minimize hydrolysis. Dilutions of labeled melphalan with unlabeled material resulted in a corresponding decrease in uptake of radioactivity, suggesting that the labeled and unlabeled material exhibited similar transport characteristics. Also, the cytotoxic potency of [^{14}C]-melphalan to L1210 cells as determined by clonal growth [1, 3] corresponded to that of the unlabeled material.

Non-specific surface adsorption of labeled melphalan was estimated by measuring binding at 0–4° after exposure of cells to 6.5 μM melphalan, a minimal LD_{100} (Ref. 1) for time periods up to 24 min. Since the amount of drug bound (< 0.3 pmoles/10^5 cells) was negligible with respect to the amount transported, no correction factor was applied. Intracellular radioactivity was identified as unhydrolyzed melphalan by its R_f value of approximately 0.5 after thin-layer chromatography on silica gel 60 in n-butanol–acetic acid–water (4:1:1) [4].

Cytotoxicity assays. The conditions for maintenance of cell cultures and for exposure of the cells to melphalan have been described [1]. Modifications included the use of cells in the logarithmic phase of growth (5 to 10 × 10^5 cells/ml), incorporation of fungizone (0.25 μg/ml) in the maintenance medium and 0.25% glucose in the standard incubation medium of PBS and 0.1 mM BSA.

Cells were harvested after a 35-min exposure to melphalan or the solvent, ethanol, washed twice in RPMI 1630 medium supplemented with 20% fetal calf serum and suspended in the same medium at 1.0 to 1.2 × 10^5 cells/ml. The cytotoxicity of melphalan was assessed by clonal growth of surviving cells according to the procedure of Chu and Fischer [3] with minor modifications [1]. DIFCO noble agar was suspended in distilled water at a concentration of 40 mg/ml, autoclaved and then added to growth medium kept at 45° to give an agar concentration of 0.133%. Three ml of this nutrient-agar was dispensed at 45° into Falcon 12 × 75 mm tubes and allowed to equilibrate at 37°. Ten-fold dilutions (10^5 to 10^2 cells/ml) were prepared from control and melphalan-treated cell populations, and 1 ml of the appropriate dilution was added to Falcon tubes containing 3 ml of nutrient-agar. The tubes were inverted, placed in an ice bath for 15 min and kept at room temperature for 45 min. Colonies were scored after 14 days at 37°. All data are corrected for the cloning efficiency (90–100 per cent) of L1210 cells washed and incubated in the appropriate medium.

A minimal LD_{100} concentration of melphalan was used for appropriate studies described in the text. This concentration was defined [1] as the lowest concentration of melphalan, which results in 100 per cent mortality after incubation with cells for 35 min in PAG and is 6.5 μM for a cell concentration of 10^5 cells/ml and 7.0 μM for a cell concentration of 10^6 cells/ml.

Transport of melphalan and amino acids by L1210 cells. Logarithmic phase L1210 cells (5 to 10 × 10^5 cells/ml) were harvested by centrifugation at 300 *g* for 5 min, washed three times in transport medium, composed of Dulbecco's PBS containing 0.1 mM BSA and 0.1% glucose, and were then suspended at 2.0 × 10^6 cells/ml in the same buffer system. They were added at a concentration of 2.0 × 10^6 cells/ml to the appropriate volume of medium with or without amino acid, and the uptake of melphalan and other amino acids was initiated by addition of labeled material as indicated in the individual experiments. The kinetic parameters of L-leucine, L-valine and BCH transport were estimated after a 0.4-min incubation while those for melphalan were estimated after 1.5 min. Uptake of the respective substrate was linear with time for these incubation periods. Aliquots of the incubation mixture were layered on Versilube F-50 silicone oil in a microcentrifuge tube, and transport was terminated by centrifugation of the cells through the oil at 12,000 *g* for 1 min in an Eppendorf microcentrifuge. Individual uptake estimates were performed in triplicate and cell recovery was found to be greater than 99 per cent. Tips containing the cell pellet were cut off, and the pellets solubilized in 0.2 N NaOH prior to counting on a Packard liquid scintillation counter.

Cells were maintained at 37° during all phases of the transport study and experiments were completed within 1 hr of their removal from growth medium. Control populations were found to be 90–100 per cent viable as determined by clonal growth after the transport study.

RESULTS

Conditions affecting melphalan uptake and cytotoxicity. The sharp dose–response relationship of

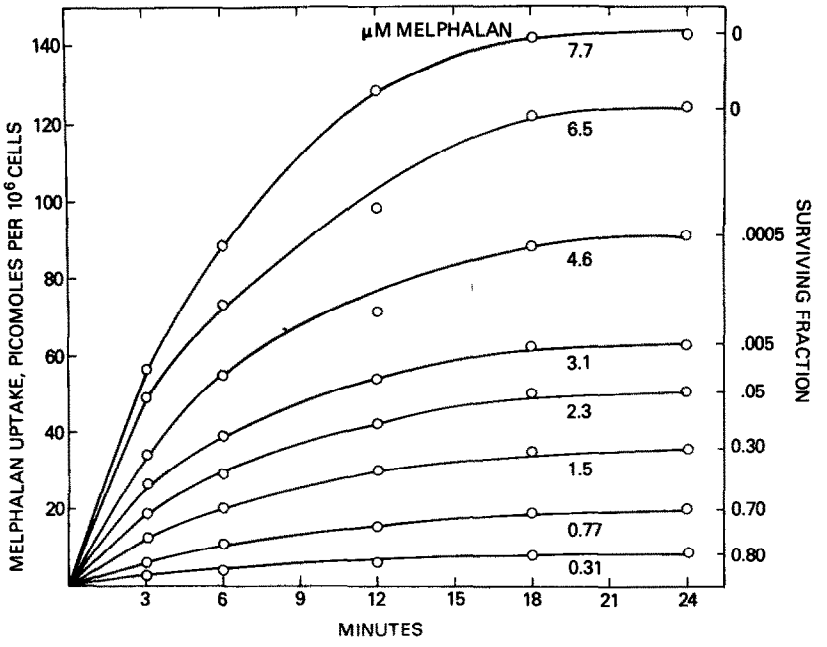

Fig. 2. Effect of melphalan concentration on its uptake and cytotoxicity. L1210 cells (1.33×10^6 cells/ml), prepared as indicated in Materials and Methods, were incubated for 15 min at 37° in transport medium with shaking. [^{14}C] Melphalan was then added to yield a cell density of 1.0×10^6 cells/ml and the indicated drug concentration. At appropriate intervals, triplicate 200-μl aliquots were removed and melphalan uptake was terminated by centrifugation of the cells through silicone oil at 12,000 *g*. Melphalan uptake at the indicated time points is shown on the left ordinate and cytotoxicity at 35 min of the same concentrations of [^{14}C] melphalan is indicated on the right ordinate. Preliminary studies indicated that melphalan uptake from transport medium containing 0.77, 3.1 and 6.5 μM melphalan was linear with time for approximately 3 min.

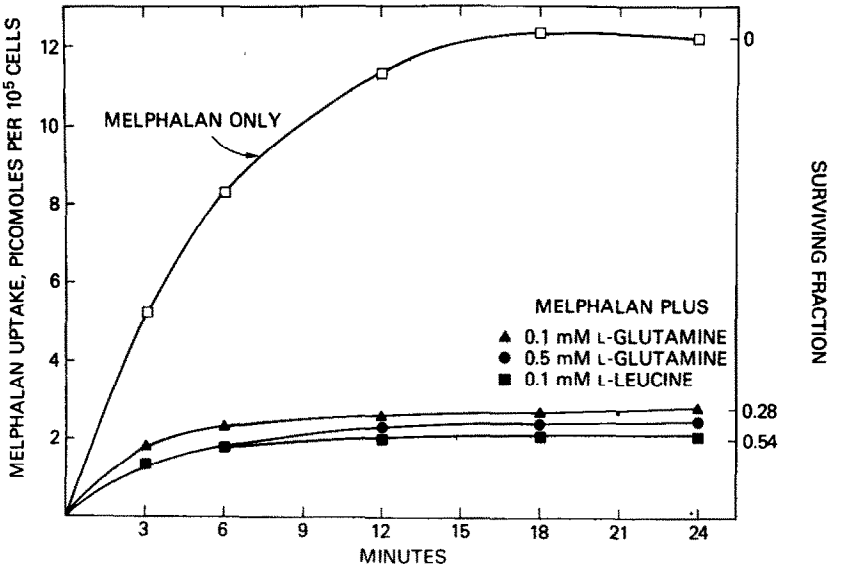

Fig. 3. Reduction in melphalan uptake by amino acids. Cells (1.33×10^6 cells/ml), prepared as indicated in Materials and Methods, were incubated for 15 min at 37° with shaking in transport medium under one of the following experimental conditions; no amino acid supplementation (□—□); 0.1 mM L-leucine (■—■); 0.1 mM L-glutamine (▲—▲); or 0.5 mM L-glutamine (●—●). [^{14}C]Melphalan was added to yield a final concentration of 6.5 μM and the experiment continued as described in the legend to Fig. 2.

Table 1. Inhibition analysis of the leucine-preferring transport system in the murine L1210 leukemia cell*

Amino acid as substrate	Amino acid as inhibitor of transport			
	L-Leucine	BCH	L-Valine	Melphalan
L-Leucine		K_i = 14.8 μM	K_i = 41.4 μM	K_i = 51 μM
	K_m = 6.1 ± 2.4 μM	r = 0.999	r = 0.992	r = 0.998
BCH	K_i = 8.0 μM		K_i = 32.5 μM	K_i = 65 μM
	r = 0.999	K_m = 8.2 ± 1.9 μM	r = 0.996	r = 0.998
L-Valine	K_i = 22.7 μM	K_i = 17.5 μM		K_i = 47.4 μM
	r = 0.978	r = 0.999	K_m = 57.7 ± 11.1 μM	r = 0.991
Melphalan	K_i = 22.4 μM	K_i = 26.6 μM	K_i = 191 μM	K_m = 19.3 ± 2.9 μM
	r = 0.999	r = 0.998	r = 0.999	

* All kinetic parameters were derived from linear regression analysis of Hunter–Downs kinetic plots; r = correlation coefficient; n = 1. Mean Michaelis constants (± S. D.) derived from three separate Hunter–Downs experiments with their respective competitors are: L-leucine (6.1 ± 2.4 μM), BCH (8.2 ± 1.9 μM), L-valine (57.7 ± 11.1 μM) and melphalan (19.3 ± 2.9 μM).

L1210 cells to melphalan described previously [1] is correlated with melphalan uptake (Fig. 2). Drug uptake increased with increasing concentration and reached a maximum in 18 min. Only 1.5 to 2.0 per cent of extracellular label was incorporated, indicating that diminished uptake was not due to exhaustion of drug from the medium. The uptake of up to 50 pmoles melphalan/10^6 cells is accompanied by a cytotoxicity of 95 per cent while additional uptake to 120 pmoles was needed to produce complete lethality. These data indicate that a critical region exists in which small increases in melphalan uptake are accompanied by large increases in cytotoxicity.

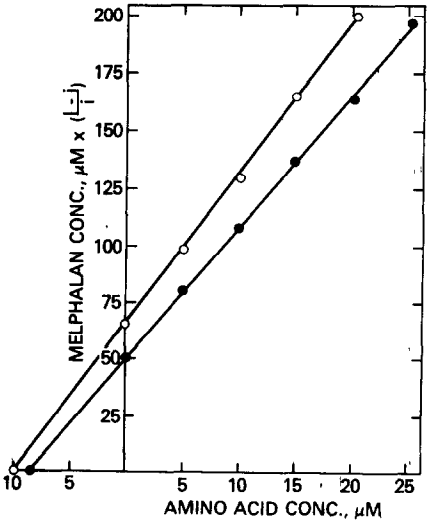

Fig. 4. Inhibition kinetics of BCH and leucine transport. In order to measure the effect of melphalan on the transport of BCH and leucine, 125 μl of cells (2.0 × 10^6 cells/ml), prepared as indicated in Materials and Methods, was incubated with 125 μl of transport medium containing the appropriate concentrations of either the labeled amino acid or the labeled amino acid and melphalan. Triplicate 200-μl aliquots were removed and amino acid transport was terminated at 0.4 min by centrifugation of the cells through silicone oil. The line of best fit for melphalan inhibition of BCH transport (○—○) was derived from the linear regression equation $y = 6.44x + 65$ while that for melphalan inhibition of L-leucine transport (●—●) was derived from the equation $y = 5.82x + 50.9$. The melphalan K_i values are 65 and 51 μM, respectively, while the amino acid K_m values are 10.1 and 8.7 μM. The data are plotted according to the method of Hunter and Downs [5, 6] where i is the fractional inhibition of substrate transport occurring in the presence of inhibitor.

L-Leucine and L-glutamine reduced melphalan cytotoxicity when L1210 cells were incubated with these amino acids before exposure to melphalan for 35 min [1]. These amino acids also caused a 6-fold reduction in melphalan uptake and a corresponding reduction in cytotoxicity (Fig. 3).

Inhibition kinetics of the leucine-preferring transport system. Although it is apparent that the protective amino acids reduced net melphalan uptake and cytotoxicity (Fig. 3), these results do not provide evidence concerning joint utilization of a specific transport system by melphalan and these amino acids. For this purpose, inhibition analysis of the initial rate of transport was employed. Since the transport of BCH has been shown to be specific for the leucine-preferring system (the L system) in the Ehrlich ascites tumor cell [2], we compared its kinetic parameters with those of L-leucine, L-valine and melphalan. These were derived from a non-inverted plot [5], originally described by Hunter and Downs [6]. Similar K_m values in the range of 6–8 μM were found for both L-leucine and BCH (Table 1), and analysis of melphalan inhibition of the rate of both BCH and L-leucine transport indicated that the inhibition was competitive with similar melphalan K_i values for inhibition of transport of both amino acids (Fig. 4).

Inhibition analysis of the transport of L-valine, an amino acid which at physiological concentrations did not protect L1210 cells from melphalan cytotoxicity [1], indicated that the affinity of L1210 cells for this amino acid is substantially lower than for L-leucine, BCH or melphalan (Table 1). These results suggest that the protective effect of individual amino acids against melphalan cytotoxicity is related to their affinities for the leucine carrier sites and that cytotoxicity is dependent upon transport by a leucine-preferring system in L1210 cells analogous to the leucine-preferring system described by Oxender and

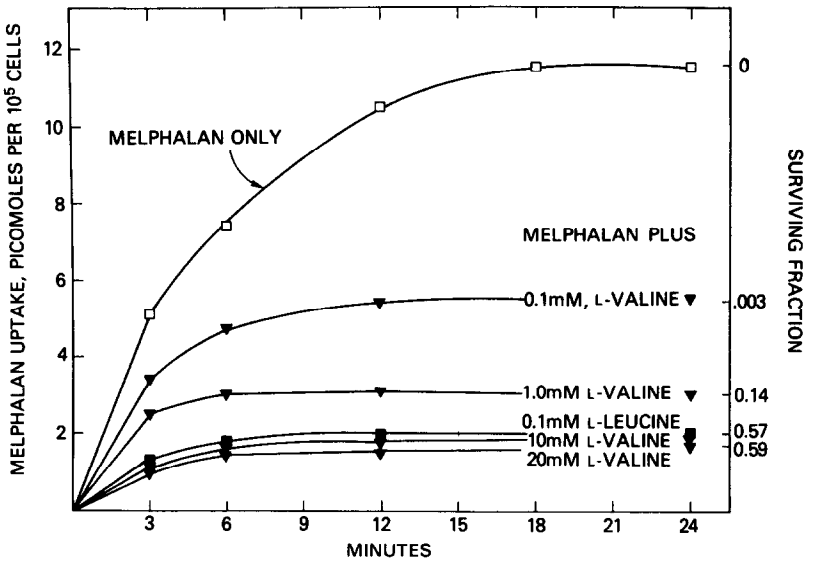

Fig. 5. Requirement for exceptionally high concentrations of valine to reduce melphalan uptake and cytotoxicity. L1210 cells (1.33×10^6 cells/ml), prepared as indicated in Materials and Methods, were incubated as described in the legend to Fig. 3 under the following conditions; no amino acid supplementation (□—□); 0.1 mM L-valine (▼—▼); 1.0 mM L-valine (▼—▼); 10 mM L-valine (▼—▼); 20 mM L-valine (▼—▼); or 0.1 mM L-leucine (■—■). [^{14}C] Melphalan was added to yield a final concentration of 6.5 μM and the experiment continued as described in the legend to Fig. 2.

Christensen [7] for the Ehrlich ascites cell. It is apparent that the amino acid K_i values for inhibition of melphalan are higher than their respective K_m values (Table 1). An explanation for this discrepancy may reside in the exchange properties of substrates of the leucine-preferring transport system [7], such as we have reported [8].

Interrelations among net uptake, kinetics of transport and melphalan cytotoxicity. L-Valine was ineffective in protecting L1210 cells from melphalan cytotoxicity at physiological concentrations, but still reduced net melphalan uptake by 50 per cent (Fig. 5). Studies were, therefore, undertaken to determine whether increasing concentrations of L-valine were accompanied by further decreases in melphalan uptake and a reduction in cytotoxicity. As can be seen in Fig. 5, a 100-fold greater concentration of L-valine (K_m = 58 μM) than of L-leucine (K_m = 6 μM) was needed to reduce both net melphalan uptake and cytotoxicity to a similar level. Melphalan uptake of from 2 to 5 pmoles/10^5 cells was accompanied by cytotoxicity ranging from 43 to essentially 100 per cent (Fig. 5). As was indicated in Fig. 2, cytotoxicity can vary from minimal to complete within a small critical range of net melphalan uptake. A protective amino acid must reduce such uptake to values below this range to be effective in reducing cytotoxicity.

DISCUSSION

Studies by Goldenberg *et al.* [9, 10] have shown that transport of the nitrogen mustard, mechlorethamine, in L5178Y lymphoblasts occurs by an active, carrier-mediated process involving the choline carrier and that it is independent of the transport of other alkylating agents including melphalan, chlorambucil, trenimon, mitomycin C, BCNU and cyclophosphamide [11]. A recent report by Goldenberg *et al.* [4] suggested that melphalan is transported via a carrier-mediated process by L5178Y cells. Phenylalanine was reported [4] to be ineffective in reducing melphalan transport, whereas we have found that it is a weak inhibitor of melphalan cytotoxicity [1]. At present it is not clear whether the differences between the observations of Goldenberg *et al.* and those reported here are due to differences in cell preparation and viability or to conditions used in the estimation of transport.

The characteristics of melphalan transport by the L1210 cell raise important considerations concerning the use of melphalan as an antitumor agent and prompted recent work in this laboratory [12] which demonstrated that injection of leucine with therapeutic doses of melphalan reduces the cure rate (60-day survivors) from 50 per cent to 0. The therapeutic efficacy of melphalan, therefore, depends upon its uptake by a leucine transport system, possibly the same one observed *in vitro*. These observations suggest that it may be possible to increase the predictability of response of certain neoplasms to melphalan by examination of its interaction with their high-affinity, leucine-preferring transport systems. Use of the drug may not be indicated where the affinity of the carrier for melphalan is low.

Although competition between specific amino acids and melphalan uptake by cells has not been previously described, an early study by Crathorn and Hunter [13] indicated that melphalan inhibited incorporation of phenylalanine into protein by 75 per cent in *Staphylococcus aureus*. The authors speculated that reaction of the nitrogen mustard with a site on the plasma membrane involved in protein synthesis may

account for the observed decrease in phenylalanine incorporation into protein. The results described in the present study indicate that this site is an amino acid transport carrier rather than a locale for protein synthesis. Thus, in retrospect, it appears that the original objective of Bergel and Stock [14, 15] and Larionov *et al.* [16] to enhance the toxicity of an alkylating agent by attaching it to an amino acid carrier was theoretically sound.

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