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INHIBITION OF S37 ASCITES CELL AMINO ACID TRANSPORT SYSTEMS BY α-CHLOROMETHYLKETONE ANALOGS

RICHARD H. MATTHEWS, NEIL J. LEWIS, JAN HES, JEONG H. IM and GEORGE E. MILO

Department of Physiological Chemistry, College of Medicine, and Division of Medicinal Chemistry, College of Pharmacy, Ohio State University, Columbus, OH 43210 (U.S.A.)

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Summary

Alanine chloromethylketone and leucine chloromethylketone were synthesized and their effects on amino acid transport in sarcoma 37 murine ascites tumor (S37) cells were studied. Alanine chloromethylketone preincubation weakly inhibited system A. Leucine chloromethylketone preincubation strongly inhibited both amino acid transport systems L and A. Leucine chloromethylketone was also a competitive inhibitor of leucine transport. Labeled leucine chloromethylketone was concentrated by S37 cells. Leucine chloromethylketone preincubation inhibition was concentration dependent and partial protection of transport was afforded by leucine. Steady-state retention of amino acids was decreased more than the initial velocity of transport by leucine chloromethylketone preincubation. Glutathione was also depleted. Labeled leucine chloromethylketone was incorporated in a plasma membrane protein fraction comigrating on a DEAE-cellulose column (DE52) with γ-glutamyltranspeptidase activity. There was a modest increase in vital staining after treatment of S37 cells with leucine chloromethylketone, and glucose uptake was also inhibited. Whilst several effects occur during treatment of S37 cells with leucine chloromethylketone, it is suggested that one prominent effect is alkylation of amino acid transport system components.

Abbreviation: S37, sarcoma 37 murine ascites tumor.

Introduction

Tenenhouse and Quastel [1] noted that there were multiple amino acid transport systems in the Ehrlich cell and Oxender and Christensen [2] designated two systems as L (leucine-preferring) and A (alanine-preferring). Our studies have described the functional properties of these two transport systems for neutral amino acids as they occur in the S37 ascites tumor cell [3—6]. System L preferred amino acids having bulky apolar side chains, such as leucine. System A preferred amino acids having smaller side chains, such as alanine. The amino acid analog, *N*-methyl-α-aminoisobutyric acid, was more completely specific to system A than was alanine. Transport of labeled histidine across a broad concentration range revealed a biphasic double-reciprocal plot; system L dominated in the low concentration region and system A dominated histidine uptake in the high concentration region. System L was very active in exchange processes while system A was usually inactive in exchange. System A activity was stimulated by Na^+.

One of the probable points of attachment of an amino acid to its carrier is the carboxyl group [7]. The carboxyl group has been replaced with a methylketone moiety, e.g., histidine methylketone, and this analog has demonstrated an inhibitory interaction with transport systems L and A of the S37 cell [5]. In the present study, the above information was used as the basis for the design, synthesis, and evaluation of analogs with altered carboxyl groups capable of forming covalent bonds with transport system components. The chloromethylketone grouping proved effective for this functional group modification. This introduced a potential alkylating feature to the methylketone group previously shown to interact with transport systems of the S37 cell. Alanine chloromethylketone, and leucine chloromethylketone, the specific analogs evaluated in the present study, demonstrated inhibition of systems A and L. The actions of leucine chloromethylketone were apparently much greater than those of alanine chloromethylketone.

Materials and Methods

General. Infrared spectra were obtained with a Beckman model 4230 infrared spectrometer. Melting points were determined with a Thomas-Hoover melting-point apparatus. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Preparation of ascites cells, buffers, and the liquid scintillator have been described in detail previously. General procedures for amino acid transport experiments have also been described in detail. Incubations were initiated by adding cells to media in a shaker bath and terminated by mixing with chilled buffer. Intracellular water was determined by measuring dry weights and excluded sulfate space [3,6].

[^{3}H]Histidine was often chosen as a test substrate because it interacted with systems A and L of the S37 cell with such disparate kinetic parameters that a double-reciprocal plot of its uptake was clearly biphasic (Refs. 3—5 and Figs. 1 and 2). Depending upon the additional demands of the experimental situation, differential effects on systems A and L could be observed as differential effects on the limbs of a double-reciprocal plot as in Fig. 1, or as in percent

changes in low- or high-concentration histidine uptakes as in Fig. 3. System L typically accounts for 85% of the uptake of 0.1 mM [^{3}H]histidine, whereas sytem A accounts for 80% of the uptake of 10 mM [^{3}H]histidine [5]. *N*-[^{14}C]-Methyl-α-aminoisobutyric acid was also used as a test substrate for system A activity.

The potential alkylating agents were synthesized in improved yields via a four-step reaction sequence that paralleled the method of Birch et al. [8]. This involved conversion of either DL-leucine or DL-alanine to the respective *N*-*t*-butyloxycarbonyl derivative in yields exceeding 90% [9]. These products were converted to the reactive mixed anhydrides in yields of 90% or better which were then treated with excess diazomethane to produce their respective diazoketones in yields of 20%. Treatment of the diazoketones in anhydrous diethyl ether with anhydrous hydrogen chloride yielded the α-chloromethyl-ketones as their hydrochloride salts. The DL-amino acids were utilized, since reactions with either the D- or L-amino acids alone failed to yield crystalline products.

Preparation of 1-chloro-3-aminobutan-2-one hydrochloride (alanine chloromethylketone hydrochloride. DL-*N*-*t*-butyloxycarbonyl alanine was prepared from DL-alanine by modification of the procedure described by Grzonka et al. [9]. The resulting oil, representing a 90% yield, was of sufficient purity as judged from nuclear magnetic resonance and thin-layer chromatography (silica gel, ethyl acetate) to be used immediately in the preparation of the alanyl ethoxyformyl anhydride.

DL-*N*-*t*-butyloxycarbonyl alanyl ethoxyformyl anhydride was prepared by modification of the procedure of Birch et al. [8], utilizing a cooled mixture of 18.9 g of the *N*-*t*-butyloxycarbonyl alanine and 19.1 g of triethylamine in 500 ml diethyl ether. Ethylchloroformate (11.9 g) was added dropwise with stirring and the reaction was stirred for 1 h at room temperature. The white triethylamine hydrochloride precipitate was filtered off and ether evaporated in vacuo. The resulting 21.2 g of labile anhydride, representing a 90% yield, were used without further purification.

For the preparation of DL-3-*N*-*t*-butyloxycarbonyl amino-1-diazobutan-2-one, a 250 ml ethereal solution of the anhydride (2.37 g) was treated with a 10-fold excess of diazomethane at 0°C for 2 h and the excess diazomethane removed under reduced pressure. The ether was extracted with water (two portions of 50 ml); the organic portion was then dried over Na_2SO_4 and the solvent evaporated in vacuo. The residual oil showed characteristic infrared absorptions at 1640 cm^{-1} (C=O) and 2110 cm^{-1} (CHN_2).

Thin-layer chromatography on silica gel with chloroform showed one spot and no further purification was performed.

A solution of diazoketone (2.23 g) in 75 ml of dry ether was cooled in an ice bath and HCl gas was bubbled through for 20 min. The reaction mixture was kept at −10°C for 3 days and the white precipitate filtered off. This afforded 0.72 g (48%) of white solid with a melting point 99–102°C, infrared (KBr) 1740 cm^{-1} (-$COCH_2Cl$) (literature: 95—100°C [8]). Paper chromatography (butanol/acetic acid/water, 12 : 3 : 5, v/v) revealed one spot and purity was also established by elemental analysis:

(Calculated ($C_4H_9NOC_2$): C, 30.40; H, 5.74; N, 8.86;

found: C, 30.56; H, 5.98; N, 8.84.)

Preparation of DL-1-chloro-3-amino-5-methylhexan-2-one hydrochloride (leucine chloromethylketone hydrochloride). Synthesis of the leucine analog from 13.1 g of DL-leucine yielded 20.7 g of protected amino acid, DL-*N*-*t*-butyloxycarbonyl leucine representing a 91% yield.

The mixed anhydride, DL-*N*-*t*-butyloxycarbonyl leucyl ethoxyformyl anhydride, was prepared according to the methods described above. Reaction of 11.6 g of DL-*N*-*t*-butyloxycarbonyl leucine with ethylchloroformate gave 13.6 g, representing a 92% yield, of the anhydride as a reactive white solid which was used immediately.

Reaction of 3.1 g of the anhydride with diazomethane as previously described yielded 2.28 g of the labile diazoketone (DL-1-diazo-3-*N*-*t*-butyloxycarbonyl aminohexane-2-one) as a white solid which was not purified further. The diazoketone (2.1 g) was treated with anhydrous HCl as described above. Workup gave 1.78 g (86%) of DL-1-chloro-3-amino-5-methylhexan-2-one hydrochloride as a crystalline white solid, melting point 146—148°C, infrared (KBr) 1740 cm^{-1} (-$COCH_2Cl$) (literature: 134—140°C [8]). Paper chromatography (butanol/acetic acid/water, 12 : 3 : 5, v/v) revealed one spot and purity was also established by elemental analysis.

(Calculated ($C_7H_{15}NOCl_2$): C, 41.99; H, 7.56; N, 7.00;

found: C, 41.88; H, 7.96; N, 6.90.)

Other chemicals and solutions. L-Histidine was obtained from Sigma Chemical Co. ^{3}H-labeled L-histidine and $Na_2{}^{35}SO_4$ were obtained from New England Nuclear Co. Stored solutions were sterilized by ultrafiltration using a Swinnex filter obtained from Millipore Co. Leucine chloromethylketone was dissolved and neutralized immediately before use, as was alanine chloromethylketone. The synthesis of [2-^{14}C]leucine chloromethylketone (20.7 μCi/mmol) has previously been described by Lewis et al. [10]. The compound was purified by paper chromatography prior to experiments and assayed for radiochemical purity.

Labeling of a plasma membrane protein fraction. 80 ml of washed and packed S37 cells were incubated with 5 mM [2-^{14}C]leucine chloromethylketone for 5 min at 20°C. The S37 cells were washed three times in the modified Krebs-Ringer phosphate buffer. Membrane isolation followed the method of Turk and Milo [11]. The S37 cells were washed three times in 1 mM calcium acetate, then homogenized in 20 mM Tris buffer (pH 7) containing 10 mM EDTA. The lysed cells were centrifuged in 2 M sucrose at 750 $\times g$ for 15 min. The central portion of the supernatant was layered on top of 2 M sucrose in ultracentrifuge tubes; these were centrifuged at 10 000 rev./min for 1 h in a swinging-bucket rotor. The central supernatant from the centrifuge tubes was pooled and made 0.2% in Triton X-100. This was allowed to stand for 1 h at room temperature to solubilize membrane proteins. The material was then centrifuged (70 000 $\times g$) for 16 h in an ultracentrifuge. The central portion of the supernatants was pooled and dialyzed overnight in a cold room against 1 l of 10 mM Tris buffer, pH 8.5, containing 0.2% Triton X-100. The dialyzed protein sample was concentrated using an Amicon filtration unit with a UM20 membrane. The sample was applied to a DEAE-cellulose column (DE52, 2.5 $\times$ 45 cm) which had been pre-equilibrated with 10 mM Tris buffer, pH 8.5, also

containing 0.2% Triton X-100. 30—40 ml of the initial equilibration buffer were first used in elution, followed by a linear NaCl concentration gradient (300 ml 10 mM Tris buffer, pH 8.5, containing 80 mM NaCl and 0.2% Triton X-100/300 ml 10 mM Tris buffer, pH 8.5, containing 0.3 M NaCl and 0.2% Triton X-100). The elution of proteins was monitored using an LKB Uvicord II and strip chart recorder. Fractions were approx. 5 ml. 0.1 ml aliquots were taken for liquid scintillation counting and 0.4 ml aliquots were taken for assay of γ-glutamyltranspeptidase activity. γ-Glutamyltranspeptidase activity was measured according to the method described by Tate and Meister [12].

Results

Preincubation inhibitions with alanine chloromethylketone and leucine chloromethylketone

Fig. 1 illustrates crossover results with alanine chloromethylketone. In the low-concentration region distant from the origin of the double-reciprocal plot, uptakes into cells preincubated with alanine chloromethylketone were greater than control uptake; conversely, in the high-concentration region near the origin of the double-reciprocal plot, uptakes into cells preincubated with alanine chloromethylketone were less than control uptakes. The preincubation inhibition observed occurred in the case of the transport system displaying specificity preference for the parent compound, alanine. In this case, however, both effects noted were of modest magnitude, although the results were reproduced in a repetition of the experiment.

The preincubation inhibition elicited by leucine chloromethylketone was a much more dramatic effect (Fig. 2). Both limbs of the double-reciprocal plot were deflected, both in slope and in elevation. Preincubation inhibition of system L, dominant in the low-concentration region [5], is a phenomenon never previously observed to the best of our knowledge.

The short-term reversibility of the leucine chloromethylketone preincubation inhibition was tested in a three-incubation experiment. Cells were first incubated with or without 5 mM leucine chloromethylketone present for 1 h at 20°C. Following the first incubation, both cell preparations were washed in chilled Krebs-Ringer phosphate buffer for a second incubation at 20°C for 30 min. The purpose of this second incubation was to test for quick reversal of the leucine chloromethylketone effect as would be expected if it did not bind covalently. After washing in chilled Krebs-Ringer phosphate buffer once more, 2-min uptakes in low- or high-concentration [^{3}H]histidine were performed in triplicate to assess activity of the L and A transport systems. For this purpose, uptake of 0.1 mM [^{3}H]histidine was used as a test of system L activity and uptake of 10 mM [^{3}H]histidine was used as a test of system A activity. It has previously been shown by curve-fitting procedures that uptake of 0.1 mM [^{3}H]-histidine was 85% by system L, whereas uptake of 10 mM [^{3}H]histidine was 80% by system A [5]. The experiment was performed on three occasions. In all cases, inhibition of both transport systems persisted, although the degree of inhibition of system A was greater than the degree of inhibition of system L. Uptake through system L in the inhibited cells averaged 61% of control, whereas uptake through system A averaged 32% of control. This varies from

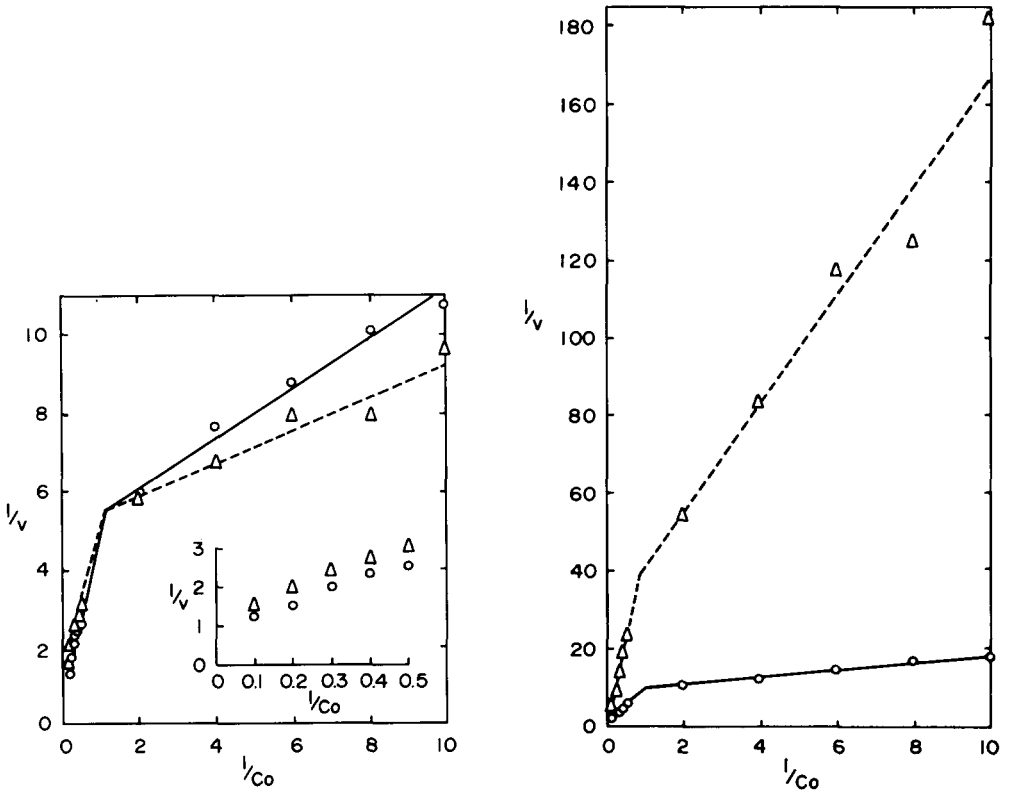


Fig. 1. The effect of alanine chloromethylketone preincubation on amino acid transport in S37 cells. S37 cells were first incubated for 1 h at 20°C with (△) or without (○) 5 mM alanine chloromethylketone present. After washing and resuspending the cells, they were incubated for 2 min at 20°C in the presence of varying concentrations, C_o, of [^{3}H]histidine (mM). The initial velocity of uptake, v (mM/min), was estimated by dividing the intracellular concentration of [^{3}H]histidine determined subsequent to the 2 min incubations by 2 min. Repetition of the experiment yielded similar results.

Fig. 2. The effect of leucine chloromethylketone preincibation on amino acid transport in S37 cells. Particulars were the same as for Fig. 1 except that 5 mM leucine chloromethylketone was employed (△) instead of alanine chloromethylketone.

the result shown in Fig. 2, in which system L was more severely inhibited.

The potential of leucine to protect S37 cells against the effects of leucine chloromethylketone was examined in a two-incubation experiment. Uptake of 0.1 and 10 mM [^{3}H]histidine was used to test transport system activities as described above. Cells were first incubated for 30 min at 20°C in one of three media: containing 5 mM leucine chloromethylketone, containing 5 mM leucine chloromethylketone plus 10 mM leucine, or containing neither. The experiment was conducted twice with samples in triplicate on each occasion. The results were expressed as percent of control activity and averaged; system L activity was 46% of control with leucine chloromethylketone alone present in the preincubation and 62% of control when leucine was also present. System A activity was 42% of control with leucine chloromethylketone alone present in the preincubation and 66% of control when leucine was also present. The possibility of chemical reaction directly between leucine and leucine chloromethyl-

ketone was ruled out on the basis of incubations of the two compounds under conditions of the preceding experiment followed by radiochromatography. [2-^{14}C]Leucine chloromethylketone was used for this purpose [10]. Paper chromatography with *n*-butanol/acetic acid/water (12 : 3 : 5) yielded R_f values of 0.986 for leucine chloromethylketone and 0.63 for leucine.

Attempts to study the time- and concentration-dependence of the preincubation inhibition of transport by leucine chloromethylketone gave variable results, but Fig. 3 was representative of these experiments in that inhibition progressed with concentration and was greater for system A than for system L.

Effect of leucine chloromethylketone on steady-state retention of amino acid

In an alternative experimental design, S37 cells were first incubated with or without 5 mM leucine chloromethylketone present for 30 min at 20°C. After washing the cells in chilled Krebs-Ringer phosphate buffer, they were incubated for 1 h at 37°C in Eagle's medium supplemented with 0.1 mM [^{3}H]histidine. This experiment was conducted twice, with measurement made in triplicate on each occasion. Previous work has indicated that these conditions would relate to the steady-state retention of amino acid without the necessity to allow for appreciable depletion due to metabolic conversion or incorporation into protein (unpublished results). The cells preincubated with leucine chloromethyl-

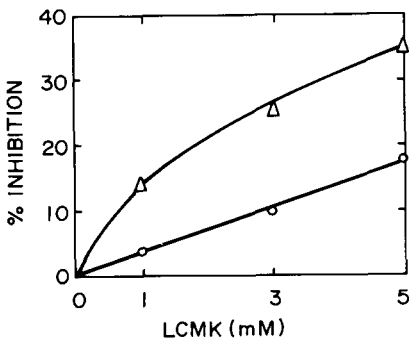


Fig. 3. Concentration dependence of leucine chloromethylketone preincubation inhibition of amino acid transport systems A and L. S37 cells were incubated for 10 min at 20°C with varying concentrations of leucine chloromethylketone (LCMK). The percent inhibition of system L (○) relative to control (no exposure to leucine chloromethylketone) was tested as percent decrease in uptake of 0.1 mM [^{3}H]histidine for 2 min at 20°C. The percent inhibition of system A (△) was tested as percent decrease in uptake of 10 mM [^{3}H]histidine for 2 min at 20°C.

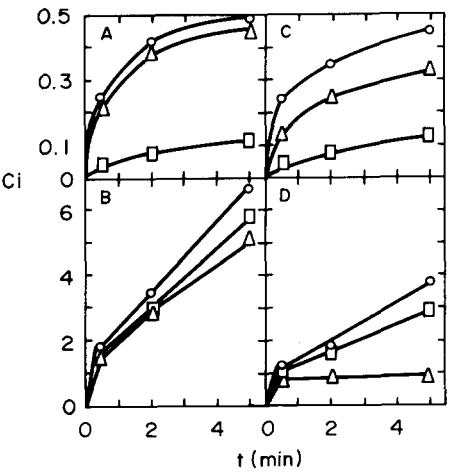


Fig. 4. Direct inhibition of transport systems A and L by leucine chloromethylketone. All incubations were at 20°C. C_i is intracellular concentration of test substrate (mM). (A) 0.1 mM [^{3}H]histidine uptake control (○); with 1 mM leucine chloromethylketone also present (△); with 1 mM leucine also present (□). (B) 10 mM [^{3}H]histidine uptake control (○); with 1 mM leucine chloromethylketone also present (△); with 1 mM leucine also present (□). (C) 0.1 mM [^{3}H]histidine uptake control (○); with 5 mM leucine chloromethylketone also present (△); with 1 mM leucine also present (□). (D) 1 mM *N*-[^{14}C]methyl-α-aminoisobutyric acid uptake control (○); with 5 mM leucine chloromethylketone also present (△); with 1 mM leucine also present (□).

ketone retained 1 and 4% of the soluble intracellular histidine retained by the control cells in this 'steady-state' experiment. Apart from the decreased influx which has been demonstrated after leucine chloromethylketone treatment, this suggested that efflux of labeled histidine was more rapid. Attempts to measure efflux showed it to be rapid to the point that it could not readily be quantified.

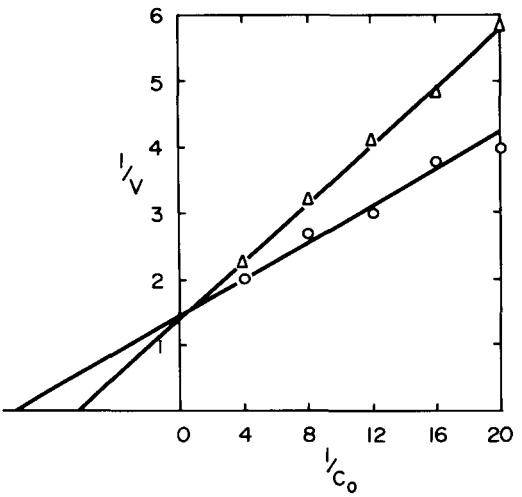
Direct effect of leucine chloromethylketone on amino acid transport

Uptake of test substrates by transport systems A and L for 30 s to 5 min was decreased by the presence of leucine chloromethylketone (Fig. 4). Transport sytem L was less affected by leucine chloromethylketone than by leucine (Fig. 4A and C). Transport system A, whether tested by high-concentration labeled histidine or by labeled *N*-methyl-α-aminoisobutyric acid, was more inhibited by leucine chloromethylketone than by leucine under the conditions of our experiments.

Transport of [^{3}H]leucine was also studied, and the direct effect of leucine chloromethylketone upon leucine transport was an inhibition-competitive type (Fig. 5). Averaging the kinetic parameters determinable from Fig. 5 with those from a duplicate experiment yielded values of 0.11 mM for the K_m of leucine transport and 3 mM for the K_i of the leucine chloromethylketone inhibition.

Uptake of labeled leucine chloromethylketone

Transport of labeled leucine chloromethylketone was studied. S37 cell pellets were lysed in 95% ethanol after varying periods of incubation, and solids



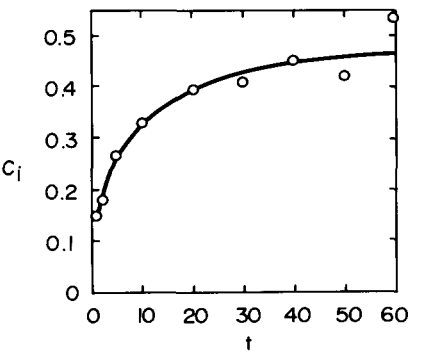


Fig. 5. Competitive inhibition of [^{3}H]leucine uptake by leucine chloromethylketone. 1 min uptake of varying concentrations of [^{3}H]leucine (C_o is in mM) at 20°C was measured in the presence (△) of 3 mM leucine chloromethylketone or its absence (○). v represents velocity of transport (mM/min). Individual points were the averages of duplicates and repetition of the experiment yielded a similar result.

Fig. 6. Time course of uptake (t in min) of 0.1 mM [2-^{14}C]leucine chloromethylketone at 20°C. C_i is the soluble intracellular concentration of the labeled analog (mM) and was determined by counting aliquots of a 95% ethanol extract of the cells after centrifugation.

removed by centrifugation. The labeled leucine chloromethylketone soluble in 95% ethanol was counted. At higher medium concentrations leucine chloromethylketone transport was non-concentrative, presumably because of the nature of the transport system inhibition occurring. At a medium concentration of 0.1 mM, however, it appeared at higher concentrations in the intracellular water in a progressively increasing manner over a period of 1–60 min (Fig. 6).

Labeling of a plasma membrane protein fraction with [^{14}C]leucine chloromethylketone

The pattern of elution of label from the DE52 column suggested that a substantial proportion of the labeled leucine chloromethylketone had been incorporated into a single protein fraction from the plasma membrane. Furthermore, this labeled material eluted at the same position as the major portion of γ-glutamyltranspeptidase activity (Fig. 7). Repetition of the experiment yielded similar results.

Interaction of leucine chloromethylketone and glutathione

Concurrently with the measurements of amino acid retention described above, measurements of cellular glutathione were carried out by the fluorimetric assay of Cohn and Lyle [13]. Various other compounds, including cysteine and oxidized glutathione, give less than 1% of the response of reduced glutathione in the assay. Cells incubated with 5 mM leucine chloromethylketone for 30 min at 20°C contained 38 and 39% control glutathione levels in triplicate determinations in the two experiments. Although the depressions in glutathione levels were not quantitatively as great as the depressions in amino acid retention on a percent basis, the effects on both amino acid retention and on glutathione levels were greater than similar corresponding effects elicited by diamide (data not shown), which has been reported to oxidize reduced glutathione in a specific manner [14]. A portion of the S37 cell's glutathione may be protected in some manner not presently understood in that EDTA in combination with diamide was not able to destroy the major part of the cell's glutathione. Responses to alanine chloromethylketone were variable, but GSH

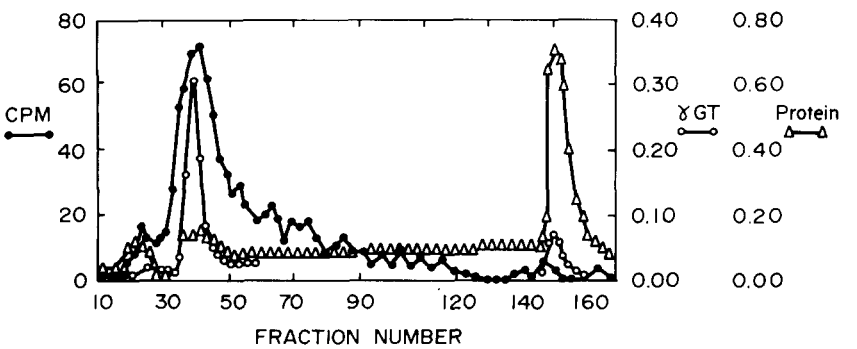


Fig. 7. Elution profile from a DE52 column of plasma membrane protein (△———△) labeled with [^{14}C]-leucine chloromethylketone (●———●) and also γ-glutamyltranspeptidase activity (○). The protein was monitored using an LKB Uvicord II and strip chart recorder set at 254 nm with no scale expansion.

depletion was usually more notable than any effect on transport (data not shown). Inclusion of glutathione in a preincubation along with leucine chloromethylketone partially reversed the effects of leucine chloromethylketone upon subsequent amino acid transport. However, DL-penicillamine or dithiothreitol at comparable concentration had similar effects. When glutathione and leucine chloromethylketone, each at 5 mM concentration, were incubated together in Krebs-Ringer phosphate buffer but lacking S37 cells for 30 min at 20°C, only 17% of the glutathione remained at the end of the incubation. Incubation of glutathione alone under the above conditions permitted recovery of more than 95% of the glutathione. The product of the reaction between leucine chloromethylketone and glutathione has not been structurally characterized, but it is presumably the alkylation derivative at the thiol group since the assay used depends upon a free thiol group to measure glutathione.

Leucine chloromethylketone inhibition of [^{3}H]glucose uptake

S37 cells were first incubated in the presence or absence of 5 mM leucine chloromethylketone for 1 h at 20°C. Following this, 10 mM [^{3}H]glucose uptake was measured for 1 min at 20°C. The leucine chloromethylketone-preincubated cells showed 74% inhibition of glucose uptake relative to control.

In a comparative study, S37 cells were incubated 5 min with or without 5 mM leucine chloromethylketone at 20°C. The cell preparations were then divided; system L was tested as uptake of 0.1 mM [^{3}H]histidine for 1 min at 20°C; system A was tested as uptake of 10 mM [^{3}H]histidine, and 5 mM glucose uptake was also measured. System L was inhibited 69% relative to control, system A was inhibited 57%, and glucose uptake was inhibited 76%. This demonstrated that glucose uptake was inhibited to a degree comparable to amino acid transport, and also illustrated the variability in degree of inhibition of the transport systems which has been experienced (cf. Fig. 3).

When S37 cells were first incubated with 5 mM leucine chloromethylketone for 5 min at 0°C, inhibitions were all much more modest. Using the same test situations as in the preceding paragraph, system L was inhibited 12%, system A 16%, and glucose uptake 17%.

Effect of leucine chloromethylketone upon sulfate space

S37 cell preparations were divided and incubated at 20°C for 30 min in the absence or presence of 5 mM leucine chloromethylketone. Following this incubation, the exclusion of $Na_2{}^{35}SO_4$ from cell pellets was determined in triplicate, the experiment being carried out on two separate occasions. Sulfate was usually excluded from the cells, so that sulfate space usually represented extracellular space in the pellet. The average sulfate space for controls in these experiments was 19.6%, whereas that for cells exposed to the analog was 35.5% of the mass of the pellet.

Effect of leucine chloromethylketone on vital staining of S37 cells

S37 cells were incubated with 1 or 5 mM leucine chloromethylketone, or with no leucine chloromethylketone present for 1 h at 20°C. Vital staining with erythrosin B was performed, accompanied by counting cells before and after the incubations, and the procedure was carried out on two occasions. In the

first experiment, 9% of the cells were non-viable at zero time and this increased by an additional 1% in control medium in 1 h. 1 mM leucine chloromethylketone caused an additional 6% to absorb vital stain, whereas 5 mM leucine chloromethylketone caused an additional 11% to absorb vital stain. In the second experiment, 4% of the cells absorbed vital stain at zero time and this increased an additional 5% in 1 h. An additional 4% of the cells became non-viable due to 1 mM leucine chloromethylketone, and an additional 8% due to 5 mM leucine chloromethylketone.

Discussion

The implication of the crossover result given by alanine chloromethylketone (Fig. 1) is that two kinds of effect occurred. The slight stimulation seen in the low-concentration region in which system L dominates [5] suggests that alanine chloromethylketone interacts with system L, promoting exchange uptake of [^{3}H]histidine. This could be due to a faster reorientation of a carrier in the membrane when a substrate is bound to it. The preincubation inhibition seen in the high-concentration region in which system A dominates is a somewhat more unusual result, although it has been observed with certain substrates in the case of system A [4]. This could be taken as an indication that system A is less available due to a continued interaction of the substrate with the transport system, but it would not necessarily indicate that the nature of the continuing interaction is covalent bond formation because *trans* inhibition of system A has been observed with substrates unlikely to form covalent linkages with the transport system.

The more dramatic effects of leucine chloromethylketone as a preincubation inhibitor of both systems A and L (Fig. 2) more clearly suggest the possibility of covalent linkages of the inhibitor to the transport system. *Trans* inhibition would be an unlikely effect to find in the case of system L in that this transport system functions very readily in exchange [3]. The probability that amino acid transport system components have been alkylated is further supported by the isolation of a protein fraction labeled with [^{14}C]leucine chloromethylketone from the plasma membrane, and by the unreversed nature of the preincubation inhibition.

It is also of importance to suggest that the analog leucine chloromethylketone interacts with amino acid transport systems in a manner similar to the interaction of the systems with normal substrates. This suggestion is supported by the concentrative uptake of 0.1 mM [^{14}C]leucine chloromethylketone, by the partial protection against preincubation inhibition afforded by leucine, and by the competitive type of inhibition of [^{3}H]leucine transport shown by leucine chloromethylketone. Concentrative uptake is suggestive of entry by active transport. An additional implication of this experiment is that leucine chloromethylketone need not always react with the transport site in an alkylating fashion. The reversal of leucine chloromethylketone inhibition shown by leucine could be interpreted in two ways in the case of system L: first, leucine could be interacting with the transport system preventing access to the system by leucine chloromethylketone; second, the leucine chloromethylketone inhibition could be just as extensive, but leucine could be accelerating the operation

of the remaining portion of the system since system L is a good exchange system. The second explanation is not so satisfactory for the effect on system A, since system A is not as effective an exchange system. A kinetic plot of the form of Fig. 5, competitive inhibition, also implies that substrate and inhibitor are competing for a common binding site. Our suggestion of leucine chloromethylketone binding to transport systems specific for amino acids would be consistent with findings that phenylalanine chloromethylketone alkylates a bacterial amino acid transport system [15,16], that glycine chloromethylketone alkylates a second bacterial transport system [17], and that alanine chloromethylketone and leucine chloromethylketone alkylate bacterial enzymes [8].

There is, however, evidence in the results that, while the modification of the carboxyl group to the chloroketone still permits interaction with the amino acid transport systems to occur, the affinity of the analogs for the binding sites is appreciably weakened. Our previous studies had shown histidine methylketone to be a moderately effective *cis* inhibitor of both transport systems L and A [5]. The weak effect of alanine chloromethylketone upon system A could be interpreted as a poor acceptance of the chloromethylketone substitution for the carboxyl group by system A. The chloromethylketone moiety is somewhat bulkier than the methylketone or the carboxyl group. The advantage of leucine chloromethylketone over alanine chloromethylketone could be that it possesses a side chain which would afford an optimal hydrophobic interaction with the binding site in system L [6] so that an orientation effect could cause some greater acceptance by system L of the substitution of the chloromethylketone moiety for the carboxyl group. Still, comparing the K_m value of 0.11 mM seen for leucine transport with the K_i value of 3 mM for leucine chloromethylketone competitive inhibition of leucine transport indicates that the degree of acceptance of the change is much poorer than it was for the substitution of the methylketone for the carboxyl group. The dramatic preincubation inhibition seen in Fig. 2 must be attributed more to the permanent nature of the inhibitory interaction than to a high degree of affinity of the analog for the initial binding site. It is somewhat surprising that leucine chloromethylketone is as effective in inhibition of system A as system L, since alanine chloromethylketone was such a weak inhibitor. Leucine interacts with both systems A and L, but its preferred route of entry is system L [2,4]. An orientation effect at the binding site would seem an unlikely possibility for system A in that hydrophobic interaction has not been shown to be of significance for system A [4,6]. An alternate possibility which would be consistent with the data is that there is a common component which is a portion of, or supports the operation of, both transport systems A and L, and that the analog forms a covalent bond with this common supporting component.

Garcia-Sancho et al. [18] concluded that free carboxylate groups were required for transport of amino acids by the Ehrlich cell. Their conclusion was based on three lines of evidence. First, they found that transport by system L was markedly decreased at low pH values resembling pK_i for the substrates. They suggested that it could be due to substrate protonation being unfavorable to transport, but noted other explanations were possible. One such explanation would be titration of a group in the binding site. Their second line of evidence

was that the amides of glycine, leucine, and phenylalanine were entirely incapable of inhibiting systems A and L. This does help to define the limits beyond which the carboxyl group cannot be modified. Their third item of evidence was that 2 mM phenylalanine chloromethylketone caused only a slight decrease in uptake through system L, which they judged to be an insignificant change. It could be that phenylalanine chloromethylketone is a slightly less effective analog than leucine chloromethylketone, and that in a *cis* inhibition experiment the degree of inhibition to be observed would be slight. Since modification of the carboxyl group to the chloromethylketone group does appear to decrease the affinity for interaction with the transport systems to occur in the S37 cell, it is also entirely possible that in the Ehrlich cell there is a difference in the active sites forbidding any interaction with the chloromethylketone group.

Depletion of glutathione levels and the labeling of a protein fraction associated with the γ-glutamyltranspeptidase activity are both effects which might be expected to be coincident with transport inhibition if the γ-glutamyl cycle were to support transport [19]. Interaction with the protein fraction is more likely to be the significant effect of leucine chloromethylketone in that alanine chloromethylketone depressed glutathione levels while having minimal effect on transport system activity. Interaction of leucine chloromethylketone with γ-glutamyltranspeptidase could explain the effects seen on amino acid transport, but the relationship of the γ-glutamyl cycle to individual functionally characterized transport systems requires further clarification.

The effects on the aspects of membrane function other than amino acid transport present interesting contrasts and provide difficult questions of interpretation. General destruction of the membrane due to nonspecific interactions has appeared to be limited, since the increase in non-viable cells as judged by vital staining and the increase in sulfate space is not sufficient to account for the inhibition of amino acid transport observed. It does seem unlikely that the marked inhibition of glucose uptake by leucine chloromethylketone could be attributable to any resemblance in structure to glucose. It would seem more reasonable to assume that a secondary effect is occurring. One possibility is that the analog is becoming localized in the plasma membrane on the basis of hydrophobicity and then it is combining with a particularly reactive group which is available there. In this vein, leucine chloromethylketone has been a potent inhibitor of cell-free asparagine synthetase in solution, but has failed to inhibit the enzyme in intact cells (Cooney, D., personal communication). Another possibility to consider is the suggestion of Kimmich [20] that a common intermediate in the plasma membrane energizes various kinds of transport system. It would be premature to state that this is actually the case, or that γ-glutamyltranspeptidase is the common intermediate in question. Metabolism may be the major determinant in glucose uptake so that inhibition of glucose uptake could reflect inhibition of one of the glycolytic enzymes [21]. It has also been suggested that glucose utilization is controlled by the availability of ADP and P_i, and that the $(Na^+ + K^+)$-ATPase is a major factor in determining this availability [22]. If the sodium pump were a locus for the action of leucine chloromethylketone, then the actions on glucose uptake and on amino acid transport through system A might be explained, although not the action on

system L. If the balance of intracellular Na^+ and K^+ were disturbed, uptake through the A system would be slower and its efflux faster [23]. Although leucine chloromethylketone may react with some plasma membrane proteins associated with processes other than amino acid transport, it may nonetheless prove useful in restricting the number of proteins which must be purified and examined as possible components of amino acid transport systems in mammalian cells.

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