THE UNIVERSITY OF MANITOBA

CHARACTERIZATION OF THE AMINO ACID TRANSPORT SYSTEMS OF ACHLYA: Regulatory Aspects Of Cytokinins And Calcium.

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

October, 1974

CHARACTERIZATION OF THE AMINO ACID TRANSPORT SYSTEMS OF *ACHLYA*: Regulatory Aspects Of Cytokinins And Calcium.

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ACKNOWLEDGEMENT

The author wishes to express sincere gratitude to Dr. H.B. LéJohn for his invaluable assistance and encouragement in the course of this investigation.

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ABSTRACT

Specific transport systems mediated the accumulation of amino acids in the coenocytic fungus Achlya. Detailed kinetic analyses coupled with pH and temperature studies demonstrated the existence of nine groups of transport systems responsible for the intake of the twenty L-amino acids commonly found in proteins. According to their interaction with the transport systems of this water-mould, the amino acids have been grouped into the following categories: (i) alanine-glycine; (ii) serine-threonine; (iii) leucine-isoleucine-valine linked with phenylalanine-tyrosine-tryptophan; (iv) aspartic-glutamic acids; (v) lysine-arginine-histidine; (vi) asparagine-glutamine (vii) proline; (viii) methionine; (ix) cysteine.

The transport of each amino acid is believed to proceed by an active process since the uptake of each was inhibited by a variety of agents that affect electron transport and oxidative phosphorylation. The bivalent cation, Ca^{++}, is required for the growth

of this organism. No other bivalent cations can substitute. Amino acid transport was shown to depend upon the availability of Ca^{++}. Calcium was observed to act at two levels in the transport systems for amino acids. The first level is at the membrane and the second level is a close association with a cell wall glycopeptide from which it can be released by plant growth hormones such as cytokinins and auxins. These hormones, like citrate, inhibited the transport of amino acids indicating that calcium may be a common link between metabolite uptake and agents that regulate such intake. In essence, calcium may be the key regulatory element for active transport of amino acids in this water-mould.

LIST OF ABBREVIATIONS

2,4-dinitrophenol, DNP

m-chlorophenyl carbonyl cyanidehydrazone, CCCP

p-hydroxymercuribenzoic acid, pHMB

(0-carboxyphenyl)thioethylmercuri-sodium salt, thimerosal

tris (hydroxymethyl)aminomethane, Tris

Tris adjusted to required pH with acetic acid, Tris-acetate

indole-3-acetic acid, IAA

N^6-hexylaminopurine, 6hAde

N^6-benzylaminopurine, BAP

ethylenediaminetetraacetic acid, EDTA

transfer RNA, tRNA

AMINO ACIDS

alanine, Ala

glycine, Gly

serine, Ser

threonine, Thr

leucine, Leu

isoleucine, Ileu

lysine, Lys

arginine, Arg

histidine, His

phenylalanine, Phe

valine, Val

tyrosine, Tyr

cysteine, Cys

methionine, Met

glutamic acid, Glu

aspartic acid, Asp

tryptophan, Trp

proline, Pro

asparagine, Arn

glutamine, Gln

I N T R O D U C T I O N

INTRODUCTION

The plasma membrane of the living cell is a dynamic structure which mediates the passage of solutes between the extracellular and the intracellular environments. Within this membrane there are molecules which transport metabolites into the cytoplasmic matrix. These "carrier" molecules, often called permeases, can transport substances against concentration gradients. This overall concentrative process is energy dependent and is termed active transport.

Amino acids perhaps constitute the largest group of substances commonly concentrated by active transport in various cells. Numerous workers have attempted to delineate the stratagem employed by organisms to accumulate this important class of nutriments. A combination of kinetic, biochemical, and genetic studies have demonstrated the presence of specific amino acid permeases in a variety of cells.

Recent years have witnessed the isolation of membrane proteins which might be involved in amino acid transport. The specificity of substrate binding by these proteins coincides with that observed in cellular uptake studies. (1, 2)

Genetic studies have centered on the isolation of mutants deficient in the uptake of one or more amino acids. Such mutants, termed permeaseless, are generally lacking in the transport of a small group of structurally related amino acids. (3) Thus they can be useful in delimiting the range of substrate specificity of the missing transport systems. However, some restrictions are encountered in the employment of mutants for delimitating transport systems. Often it is difficult to distinguish permeaseless mutants from those unable to transport substances due to general membrane lesions. This problem can be resolved, in part, by kinetic studies.

It has been observed, in kinetic studies, that analogues of a transport substrate often inhibit competitively the uptake of that substrate. Such competition effects may follow Michaelis-Menten kinetics. Thus by examining the competitive inhibition of the transport of one amino acid by others, the range of specificity of a particular transport system can be determined. The applicability of the kinetic approach is deterred by the existence of more than one transport system with overlapping specificities in a given organism. Further complications may arise due to noncompetitive inhibition of transport by various substances.

This work is concerned with the kinetics of amino acid transport systems in the aquatic mold Achlya. This organism possesses distinct amino acid permeases.

H I S T O R I C A L

HISTORICAL

Active uptake of amino acids has been demonstrated in mammalian cells, bacteria, and fungi. As early as 1913 Van Slyke and Meyer (4) observed that the dog tissue cells actively accumulated amino acids from the plasma. Their work was pursued by Christensen and colleagues (5) who described a similar phenomenon in various mammalian tissue cells.

Elaborate studies on membrane transport have been conducted in bacterial cells. In 1953 Gale and his associates (6) discovered that Staphylococcus aureus and Streptococcus faecalis were able to concentrate lysine and glutamate from a growth medium containing casein hydrolyzate. In successive years a considerable body of evidence emerged, demonstrating active amino acid transport in other bacteria such as Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium and Staphylococcus aureus.

In contrast, the investigations of the transport properties of fungi have been very restricted. Information on amino acid transport in yeasts originates from studies of Saccharomyces cerevisiae. Other fungi which have been examined in this field of study are Neurospora

crassa, Penicillium chrysogenum, Aspergillus nidulans and Achlya sp. (1969) (7).

The amino-acid permeases reside in a protein and lipid matrix, the exact disposition of which is an enigma. The exact energy-coupling mechanism which enables these permeases to function remain unknown. However, transport studies have clearly demonstrated one common characteristic of amino acid permeases - the exhibition of specificity in binding to their substrates.

The specificity of amino acid transport systems is manifested in their preference for the L-stereoisomeric form of their substrates. Chemical modification of selected solutes has shown that both an α-amino and a carboxylic acid group are generally required for substrate interaction with an amino acid permease.(8). An even more pronounced characteristic is the competition by structurally related amino acids for the same permease (9).

Considerable progress has been made in characterizing the amino acid transport systems of various mammalian tissue cells. The work of Nathans (10), Finch and Hird (11) and Munck (92) have elucidated three amino acid transport systems in the rat intestinal mucosa. Each system is responsible for the uptake of one of the following groups of amino acids.-

(a) neutral amino acids (b) basic amino acids, L-cysteine (c) L-proline.

It is interesting to note that the specificities of two systems overlap so that the proline permease is shared by some of the neutral amino acids-leucine glycine, and alanine.

Amino acid reabsorption in the human kidney tubular lumen is facilitated by distinct transport systems, each of which is shared by more or less structurally related amino acids. Rosenberg (13) has gathered evidence for the presence of two lysine transport systems in the human kidney. Scriver and Wilson (14) have described a transport system specific for the imino acids and glycine in the human renal tubular lumen, while Webber (15) has reported the presence of an acidic amino acid permease in the human kidney tissue cells. Schwartzman et al, (16) observed competitive interactions between the basic amino acids: lysine, ornithine, and arginine during their reabsorption in rat kidney slices.

The inference that the high activity of amino acid uptake, in Ehrlich ascites carcinoma cells, might be related to their malignancy, prompted Christensen and his group (17) to investigate the transport characteristics of these cells. The Ehrlich cells possess at least two

distinct carriers for the neutral amino acids. One carrier (L-site) shows a higher affinity for leucine, isoleucine, valine, phenylalanine and methionine, the other carrier (A-site) has a preference for alanine, glycine, serine, threonine, proline and methionine. In addition there is a distinct glutamate transport system (18) as well as a basic amino acid permease (19) in Ehrlich cells.

In membrane studies as in many other areas of biological research Escherichia coli has been intensively exploited. This organism has been quite amenable to the biochemical and genetic manipulations of membrane researchers. Several workers have contributed information on the specificity of at least eight amino acid permeases in E. coli.

Using membrane vesicles prepared from E. coli (W), Kaback and Stadtman (20) studied the effects of various amino acids on the uptake of glycine. Kinetic studies showed that serine, alanine and threonine competed with glycine transport in these membrane vesicles. Mutants of E. coli (W), resistant to D-serine, were unable to transport glycine, serine, threonine and alanine. Proline efflux in E. coli (W6), a proline auxotroph, was inhibited by extracellular proline and hydroxyproline. No other amino acid was able to inhibit the counter-transport of intracellular proline. From this observation, Kaback

and Stadtman (21) concluded that proline was transported by a distinct permease in E. coli (W6).

Kinetic analysis of amino acid uptake in E. coli (K12) has implied the existence of four separate transport systems for the following groups of amino acids:-

(i) leucine, isoleucine, valine (ii) phenylalanine, tyrosine, tryptophan (iii) methionine, (iv) alanine, glycine, serine (22).

Schwartz et al (23) examined the uptake of several amino acids in E. coli (W) mutants which were resistant to L-canavanine. These mutants were unable to concentrate lysine and arginine but were capable of accumulating other acids. Lubin et al (24) succeeded in isolating histidine transport mutants of E. coli (W). These mutants lacked the ability to concentrate histidine but readily accumulated glycine, proline, phenylalanine, and lysine.

Glutamate transport in wild type cells and mutants of E. coli (W) has been surveyed by Halpern and Evenshoshan (25). They found that L-glutamate uptake was competitively inhibited by D-glutamate and derivatives of D-glutamate with both unsubstituted α-amino and α-carboxylic acid groups. Lupo and Halpern (26) observed that mutants* of E. coli (W) were able to concentrate larger amounts of glutamate than that taken up by the

*Cells able to grow on glutamate as their source of carbon.

wild type cells. The kinetics of glutamate uptake in both the wild-type and mutant cells suggested that either two transport systems were operative or there was one permease with allosteric regulatory properties.

Enzymological and physiochemical techniques have clarified the presence of twelve distinct and specific amino acid transport systems in Staphylococcus aureus (27). In this organism, the specificities of the permeases are quite rigid and thus are in contrast to those of the rat intestinal mucosa (Munck (12)). Accordingly, there is one transport system for each of the following groups of structurally similar amino acids:- (i) alanine and glycine (ii) leucine, isoleucine, valine (iii) serine, threonine (iv) aspartate glutamate, (v) asparagine, glutamine (vi) lysine (vii) histidine (viii) arginine (ix) phenylalanine, tyrosine, tryptophan (x) cysteine (xi) methionine and (xii) proline.

The amino acid permeases of fungi generally tend to have wider specificities than those of bacteria. In 1966 Grenson and colleagues (28) described an arginine transport system in Saccharomyces cerevisiae. Kinetic studies on wild-type cells revealed that arginine transport was competitively inhibited by lysine, canavanine, and ornithine. Histidine exerted a mixed-type inhibition

on the uptake of arginine in these cells. In mutants of S. cerevisiae, resistant to canavanine (a structural analogue of arginine), the uptake of arginine, lysine and ornithine was impaired. Subsequently, Grenson (29) examined lysine uptake in S. cerevisiae. Michaelis - Menten kinetic analysis demonstrated that lysine was taken up by two systems in wild type cells. One system had an affinity for arginine but was lacking in canavanine-resistant mutants. The other system was quite specific for lysine as inferred from kinetic competition studies. This specific lysine transport system was absent in the mutant (Lys P_4) which accumulated arginine, ornithine and canavanine. From these results Grenson concluded that a very specific lysine permease exists in S. cerevisiae and that this system was independent of the arginine permease. Lysine could enter the cell through the arginine transport system but arginine could not utilize the specific lysine permease. In 1967 Gits and Grenson (30) deduced from Lineweaver-Burk kinetic studies that methionine uptake in S. cerevisiae proceeded via two permeases for this sulfur-containing amino acid. One permease, specific for methionine, was absent in mutants that were resistant to ethionine (a structural analogue of methionine). The other permease with a low affinity for methionine was

capable of transporting a small group of neutral amino acids.

The effects of nitrogen-starvation on amino acid uptake in Penicillium chrysogenum have been studied by Benko and Segel (31). According to their observation the vegetative mycelial form of this fungus, in a nitrogen-sufficient medium, displayed specific transport systems for L-phenylalanine, L-methionine and L-leucine. However, under nitrogen-starvation conditions, there was a general, non-specific permease for the neutral amino acids. The biosynthesis of this nitrogen-regulated permease was inhibited by cycloheximide, anaerobiosis, and azide. Hunter and Segel (32) have studied the specificities of the acidic and basic amino acid permeases in the mycelial mat of P. chrysogenum. In the presence of excess leucine, which saturated a general amino acid permease, arginine competitively inhibited lysine uptake while glutamate competed with aspartate for a common permease.

A more extensive survey of amino acid transport in the fungi has been achieved by Pall. Through kinetic and genetic studies Pall has demonstrated that five distinct amino acid transport systems exist in Neurospora crassa. From kinetic studies, he deduced that there was a specific transport system (AAi) for the

uptake of the aromatic and neutral amino acids (33). This system was functional in rapidly growing mycelia. A mutant, deficient in tryptophan uptake had been isolated and found unable to concentrate aromatic and neutral amino acids. Another system (AAii) had a broad range of specificity transporting neutral and basic amino acids (33). However, it was active only in mature mycelial pads and due to its low Km values it was assigned a scavenger function. A third system (AAiii) was found to be specific for the basic amino acids, lysine, and arginine (34). Its activity, like that of (AAi), was maximal in germinated conidia and in mycelia at the exponential growth phase. A mutant, designated bat, was found unable to concentrate lysine and arginine. However, it displayed normal transport characteristics for neutral, aromatic and acidic amino acids. The acidic amino acids aspartate and glutamate competed for a common transport system which he assigned the term (AAiv). Like system (AAii), the acidic amino acid permease was most active in carbon and nitrogen-starved mycelia. However, the systems were not identical since (AAii) had a very low affinity for the acidic amino acids (35). A fifth transport system (AAv), specific for methionine, was recognized in sulfur-starved mycelia of N. crassa. The function of this permease was deduced to be that of a scavenger for sulfur amino acids

from the growth medium. A mutant (cys-3) was isolated and found to be deficient in methionine uptake as well as in several sulfur-pathway proteins (36).

Distinct amino acid transport systems have been reported in Aspergillus nidulans. In 1969 Sinha (37) used p-fluorophenylalanine-resistant mutants to study the specificity of an aromatic acid permease in a hyphal suspension of this organism. Kinetic observations revealed that (p-FPA)-resistant mutants were unable to accumulate phenylalanine and tyrosine. Recently Robinson et al (38) described an acidic amino acid permease of A. nidulans. In the germinated conidia of this organism there was a general amino acid permease similar to that observed in P. chrysogenum by Hunter and Segel (32). Robinson et al studied the kinetics of glutamate uptake by germinated conidia of A. nidulans in the presence of excess lysine. This basic amino acid saturated the general amino acid permease. Under those conditions it was observed that aspartate competed with glutamate for the acidic amino acid permease.

The active transport of phenylalanine has been studied kinetically in the fungus Achlya (39). It was reported that calcium ion regulated the uptake of this and other amino acids in this organism. This study is an extension of that work in an attempt to resolve the

amino acid transport systems in Achlya. Elucidation of the transport systems was achieved by competition kinetics and some physical characterization of the different transport systems. Comparative studies of the transport characteristics of various fungi "may not only serve to help discern general patterns of regulation but may also help in phylogenetic studies of fungi" (35).

MATERIALS AND METHODS

MATERIALS AND METHODS

Media, Buffers and Chemicals

Media

GY: 0.5 g yeast extract (Difco), 5.0 g glucose per litre of deionized H_2O.

PYG: 1.0 g bacteriological peptone, 1.0 g yeast extract (Difco), 3.0 g glucose per litre of deionized H_2O.

Buffers

A, (2 x TKGNa): 10 mM Tris-acetate, 2 mM KCl, 2 mM NaCl, 10 mM glucose.

B, (TKGNa) : Buffer A with the concentration of each constituent halved.

C : Buffer A with the concentration of Tris-acetate reduced to 1 mM.

Chemicals

Biochemicals were purchased from Sigma Chemical Co.

Benzyl (8-^{14}C)adenine (24 mCi/mmol) was purchased from Amersham/Searle β-Indoleacetic-2-^{14}C acid (60 mCi/mmol)was purchased from New England Nuclear. $^{45}Ca^{++}$ as $CaCl_2$ in aqueous solution was purchased from Amersham/Searle.

The labelled amino acids were the L-optical isomers and were purchased from Amersham/Searle. The amino acids used were:

[alanine]-2,3-^{3}H (34 Ci/mmole)

[glycine]-2-^{3}H (2 Ci/mmole)

[serine]-3-^{3}H (500 mCi/mmole)

[threonine]-4,5-^{3}H (228 mCi/mmole)

[leucine]-4,5-^{3}H (1 Ci/mmole)

[isoleucine]-4,5-^{3}H(n) (17.7 Ci/mmole)

[valine]-2,3-^{3}H(n) (29 Ci/mmole)

[methionine] methyl-^{14}C (53.7 mCi/mmole)

[cysteine]-^{14}C(U)-HCℓ (38.6 mCi/mmole)

[glutamic acid]-^{14}C(U) (10 mCi/mmole)

[aspartic acid]-^{3}H(G) (178 mCi/mmole)

[lysine]-4,5-^{3}H(n)-HCℓ (250 mCi/mmole)

[arginine]-5-^{3}H-HCℓ (22 Ci/mmole)

[histidine]-2,5-^{3}H (58 Ci/mmole)

[3-phenylalanine] ring-4-^{3}H (58 Ci/mmole)

[tyrosine]-3,5-^{3}H (40 Ci/mmole)

[tryptophan]-^{3}H(G) (47 Ci/mmole)

[proline]-^{3}H(G) (178 mCi/mmole)

[asparagine]-^{3}H(G) (100 mCi/mmole)

[glutamine]-^{14}C(U) (48 mCi/mmole)

Growth of Organism

In this study, the strain of Achlya used was the same as that described previously (7). Stock cultures of this organism were maintained on slants of Cantino's PYG agar (40). Pieces of mycelial threads were transferred from slants to Petri dishes containing 20 mls of GY medium. The plate cultures were then incubated for 48 hrs at 22°C to facilitate growth and sporulation. After this incubation period the mycelial mats from three plates were transferred aseptically to an Erlenmeyer flask containing 125 ml distilled H_2O. The flask was agitated by hand in order to separate the spores from the mycelial mat. The mat was then removed from the flask with a sterile inoculating needle. Two millilitres of this spore suspension were then used to inoculate fresh GY medium in Petri plates or Roux flasks.

Large Scale Spore Production

Achlya was grown as 100 ml GY stationary cultures in Roux flasks. The size of the spore inoculum was 10 ml. After incubation at 22°C for 48 hrs the flasks were shaken vigorously by hand for about 15 seconds and the contents filtered through eight layers of sterile cotton gauze.

Germination of Spores

The spore suspension (approximately 400 mls) obtained from four Roux bottles was then diluted by the addition of 200 mls sterile deionized H_2O. The diluted suspension was incubated at 11°C for 15 hrs allowing the spores to germinate in the spent GY medium. The germinated spores were then collected by filtration through a nylon cloth (61 µm pore size) obtained from Henry Simon Ltd., Stockport, England. The germlings, collected on the nylon cloth, were washed with 100 mls sterile distilled H_2O and resuspended in the pertinent experimental medium.

Cell Viability

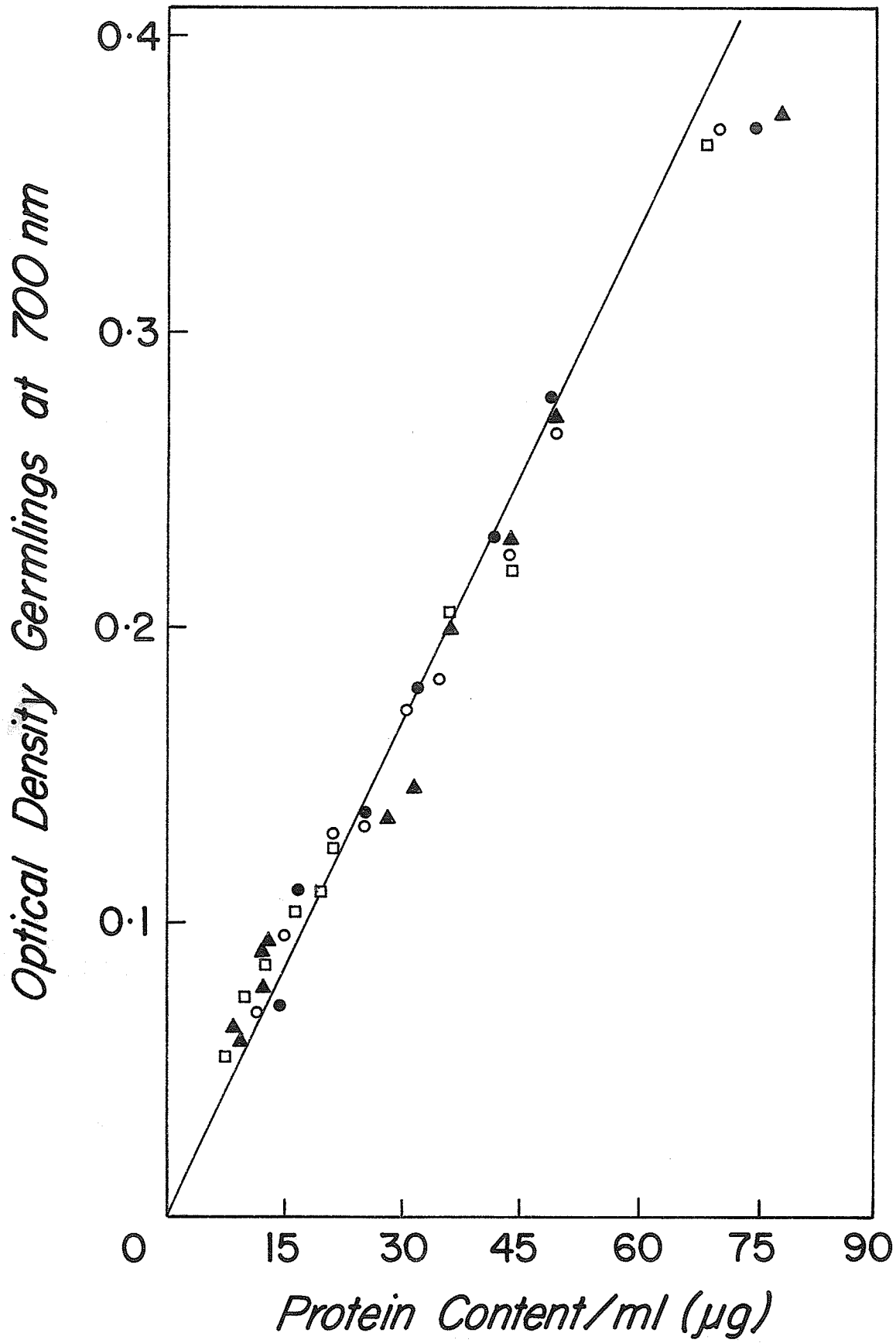
Viability of the cells was evaluated by plating suitable dilutions of cells on GY agar and incubating at 15° for 24 to 36 hours before counting. The appearance of a small mycelial tuft was taken to represent cell viability. All cell and colony counts were made microscopically.

Cell Density

Growth and germinating conditions were standardized

so that an accurate estimate of the cell density was determined from either protein content or optical density. The optical density of germling suspensions was determined at 700 nm, and simultaneously, a suitable aliquot of the cell suspension was filtered through Millipore HAWP filters from which the cells were collected and frozen in liquid N_2 to aid disruption. The cells were subsequently thawed, then triturated with 1 ml of 10 mM Tris-acetate buffer, pH 7, and finally disintegrated by ultrasonic treatment using a model 1000 Insonator (Ultrasonic System Inc., N. Y.). Cell debris was removed by centrifugation and the soluble protein determined by the colorimetric method of Lowry et al (41). Over a wide range of cell densities, a linear relationship existed between protein content and cell number. However, since the viability was only 80 to 85% of the cell number, the correlation used in all of these studies was optical density against protein content (Fig. 1). The optical density at 700 nm of intact cells used in these experiments was maintained at 0.19 ± .01.

Fig. 1. Relationship between protein content of germinated spores of Achlya and density at A_{700}. Each experimental symbol represents an average of three determinations and the different symbols represent analyses performed on independent cell populations prepared over a 2-week period.


Optical Density Germlings at 700 nm
0·4
0·3
0·2
0·1
0
15
30
45
60
75
90
Protein Content/ml (µg)

METHODS

Transport Assays

Germlings were suspended in either buffer A or buffer C and pre-equilibrated for 15 min before the start of an experiment. To ensure that transport activities were minimally affected by macromolecular synthesis, all experiments were carried out in the presence of 28 μM cycloheximide - a concentration that is 10 times greater than the optimum inhibitory concentration of the drug. Reaction was started by the addition of 1 ml of cells (approximately 10^3 germlings) to the reaction solution which was then mixed rapidly. The cells were allowed to concentrate the particular amino acid mixture for 3 min before the reaction was stopped by filtering the cell suspension through pre-soaked Millipore HAWP filters in a 30-chamber Millipore manifold attached to vacuum pumps. Each filter was washed with 15 ml of buffer B at room temperature within 10 seconds. Net accumulation of amino acids was estimated from the total radioactivity on filter minus nonspecific radioisotope adherence to dead cells killed by 0.4% formaldehyde. Washed filters were transferred to 10 ml Bray's solution (42) in a scintillation vial, dissolved, and radioactivity determined in a Beckman LS-230 liquid scintillation spectrometer.

pH Studies

Germlings were preincubated in buffer C. One millilitre of this cell suspension was then transferred to 1 ml of the reaction solution containing 25 mM Tris-acetate of the specified pH, 56 μM cycloheximide, and the amino acid to be transported.

Temperature Studies

Pre-equilibration of the germlings and reaction solution was carried out at the specified temperature for 10 min before they were mixed and the initial reaction rate studied.

Evaluation of Initial Reaction Rate

The quantity of radioactive material taken up as a function of time was determined as described under "Transport Assays". The period during which there was a linear relationship between uptake and time was taken to represent a measurement of initial reaction rate.

Exit Experiments

Cells were preloaded with 0.1 mM labelled amino acid for 30 min at 24° under standard uptake conditions where the incubation medium was buffer A containing 28 μM cycloheximide. The exogenous amino acid was removed by filtering the cells throughnnylon mesh, washed with unlabelled buffer and resuspended in the same volume of buffer A with cycloheximide containing 10 μM CCCP as a metabolic energy-generation inhibitor. The cycloheximide served as an inhibitor of protein synthesis and permitted the determination of exit rates in the absence of endogenous amino acid metabolism into proteins. No attempt was made to account for or exclude amino acid activation to tRNA. Samples were taken at frequent intervals, filtered, washed, and the residual radioactivity in the cells determined.

R E S U L T S

RESULTS

Active Transport of Amino Acids

The transport of each amino acid used in this study exhibited certain characteristics which were indicative of an active uptake process. For example, the phenylalanine transport system showed a saturable initial rate of uptake as a function of external amino acid concentration (Fig. 2a). This amino acid transport system functioned optimally at pH 7 and at 30°C (Fig. 2b, 2c).

Moreover the transport of this aromatic amino acid was hampered by metabolic energy uncouplers and electron inhibitors. The agents azide, 2,4-DNP, cyanide and CCCP were particularly potent inhibitors of phenylalanine transport. The non-competitive inhibitory effects exerted by azide and DNP (Fig. 3) implied that the inhibitors were interacting with a component other than the phenylalanine permeases. Transport of all amino acids was blocked by sulphydryl reagents such as Hg^{++}, Ag^{++}, thimerosal and pHMB. These compounds abolished amino acid transport completely at less than 10 μM under this assay condition. The inhibition was completely abolished by cysteine and 2-mercaptoethanol.

Fig. 2(a) Michaelis-Menten analysis of the initial reaction rate of phenylalanine transport. In this transport study, uptake rate of amino acid in the presence of 1 mM 2,4-DNP carried out simultaneously, was substracted from the non-poisoned cell rate and it is this result that is presented here. Transport study was conducted with cells in medium A (see methods). Cell density, A_{700} was maintained at 0.20.

(b) Influence of pH on the initial reaction rate of uptake of phenylalanine. The buffer used was Tris-acetate 12.5 mM. The amino acid was used at a concentration of 50 μM and cell density A_{700} kept at 0.19.

(c) Influence of temperature on the initial reaction rate of uptake of phenylalanine. Transport studies were conducted in medium A. The amino acid was supplied at a concentration of 50 μM and the density of cells, A_{700}, maintained at 0.19.

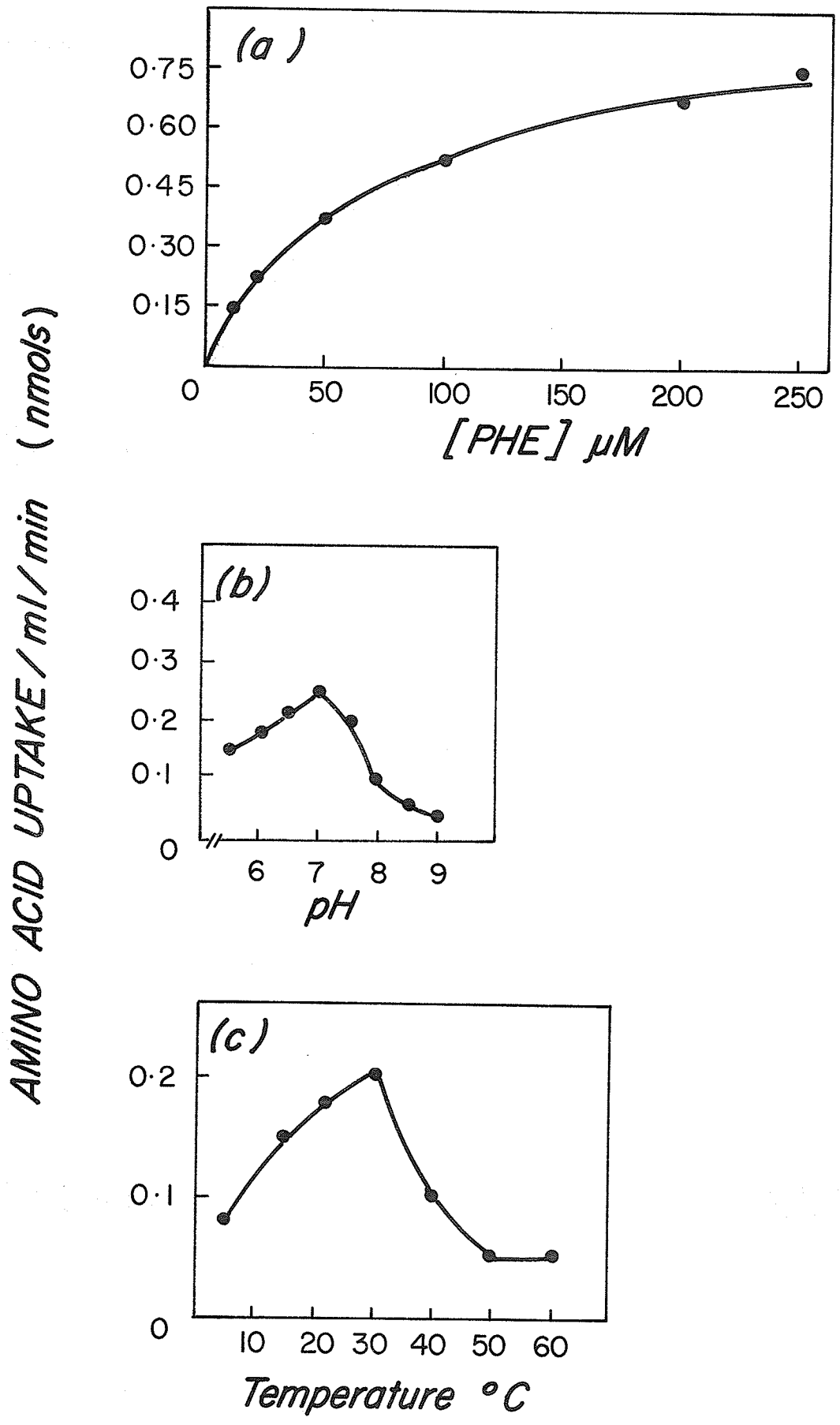

(a)
0·75
0·60
0·45
0·30
0·15
0
50
100
150
200
250
[PHE] μM
AMINO ACID UPTAKE / ml / min (nmols)
(b)
0·4
0·3
0·2
0·1
0
6
7
8
9
pH
(c)
0·2
0·1
0
10
20
30
40
50
60
Temperature °C

Fig. 3. Lineweaver-Burk analysis of the inhibition of the initial reaction rate of phenylalanine transport by (A) azide (▼) 1 mM; (□) 0.25 mM; (△) 0.1 mM; (●) nil; (B) 2,4-DNP (▼) 1 mM; (□) 0.25 mM; (△) 0.1 mM; (●) nil. The cell density, A_{700}, was maintained at 0.20. Reaction was run at 24°C at pH 7.

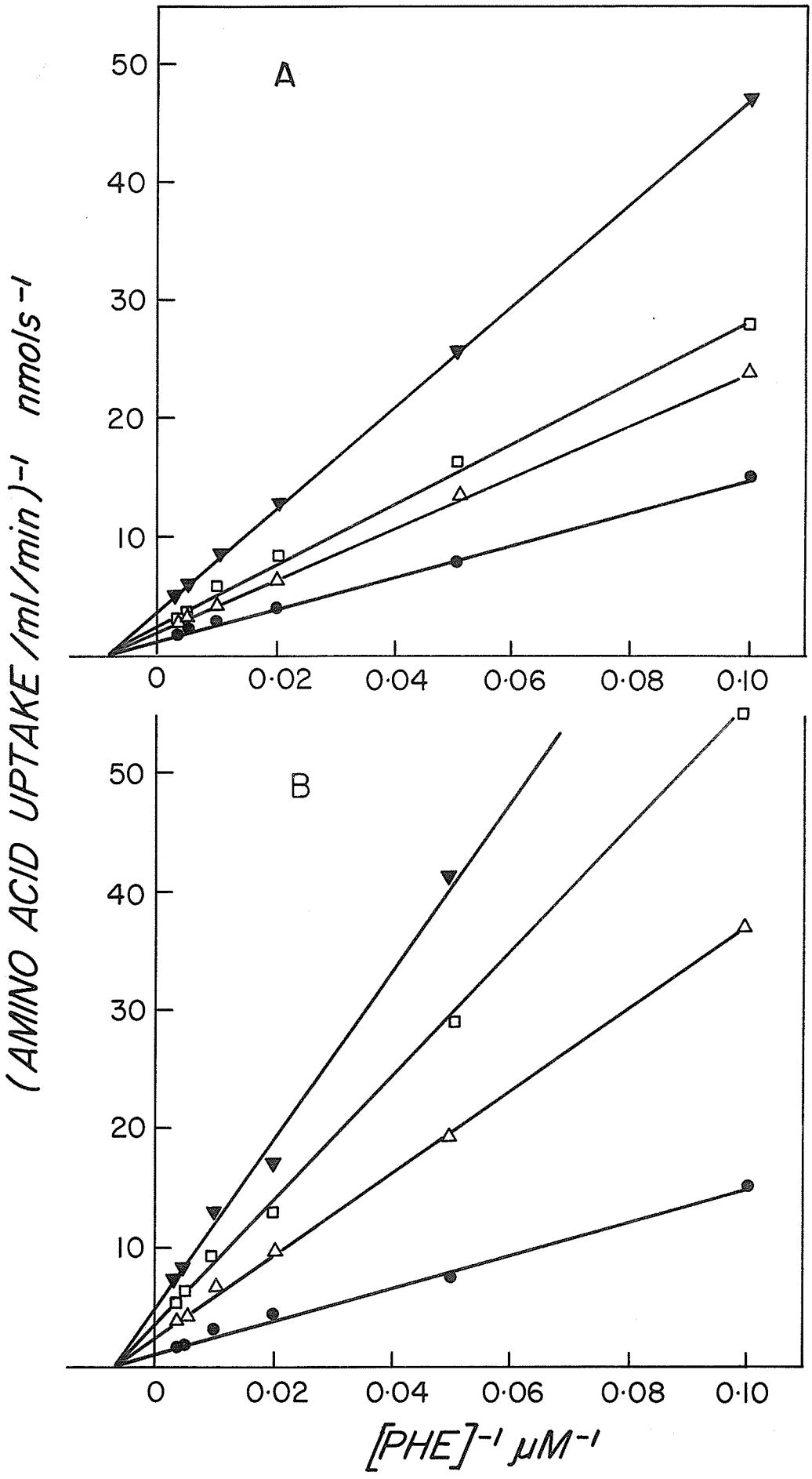

A
B
50
40
30
20
10
0
0·02
0·04
0·06
0·08
0·10
(AMINO ACID UPTAKE /ml/min)-1 nmols-1
[PHE]-1 μM-1

TABLE I

A summary of the $S_{(0.5)}$ values for the transport of amino acids by germinated spores of Achlya.

Amino Acid	$S_{(0.5)}$ (M)
Threonine	2.5×10^{-5} and 2.00×10^{-4}
Arginine	3.33×10^{-5}
Glycine	1.50×10^{-4}
Serine	1.25×10^{-4}
Histidine	1.67×10^{-4}
Methionine	5.13×10^{-6} and 2.0×10^{-4}
Valine	8.3×10^{-5}
Phenylalanine	5.0×10^{-5}
Asparagine	7.5×10^{-5}
Proline	1.50×10^{-4}
Cysteine	7.5×10^{-5}
Tyrosine	3.33×10^{-5}
Isoleucine	6.67×10^{-5}
Glutamate	2.5×10^{-5}
Glutamine	1.5×10^{-4}
Tryptophan	1.67×10^{-4}
Lysine	8.3×10^{-6} and 1.0×10^{-4}
Alanine	1.67×10^{-4}
Aspartate	4.0×10^{-6}
Leucine	1.11×10^{-4}

Determination of Half Saturation Values* $S_{(0.5)}$

Two well-recognized properties of an active transport system are a defined concentration gradient and an apparent saturation of uptake rate. These two characteristics were manifested by all 20 amino acid transport systems. The saturation curves, also shown in Lineweaver-Burk (43) format are presented in (Fig. 4 to 8). The half saturation values calculated from the Lineweaver-Burk plots are summarized in Table I. The constants varied from 10^{-4} to 10^{-6}M. The transport systems for three amino acids, threonine, methionine, and lysine displayed kinetic patterns that deviated from simple linear double reciprocal plots. They were biphasic implying that there might be two saturable components for each of these amino acids.

Influence of pH

The half saturation values for transport of several of these amino acids are very similar making it difficult to determine whether the amino acids utilized the same or

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*The term half-saturation value $S_{(0.5)}$ is used because the constants determined are not true Michaelis-Menten constants that can only be adequately calculated under equilibrium conditions.

Fig. 4. Michaelis-Menten and Lineweaver-Burk analysis of the initial reaction rates of transport of (top left) threonine, (top right) arginine; (bottom right) glycine; and (bottom left) serine. The symbols $\underline{v}$ and $\underline{s}$ refer to transport rate and substrate concentration respectively. See Fig. (2a) legend for other details.

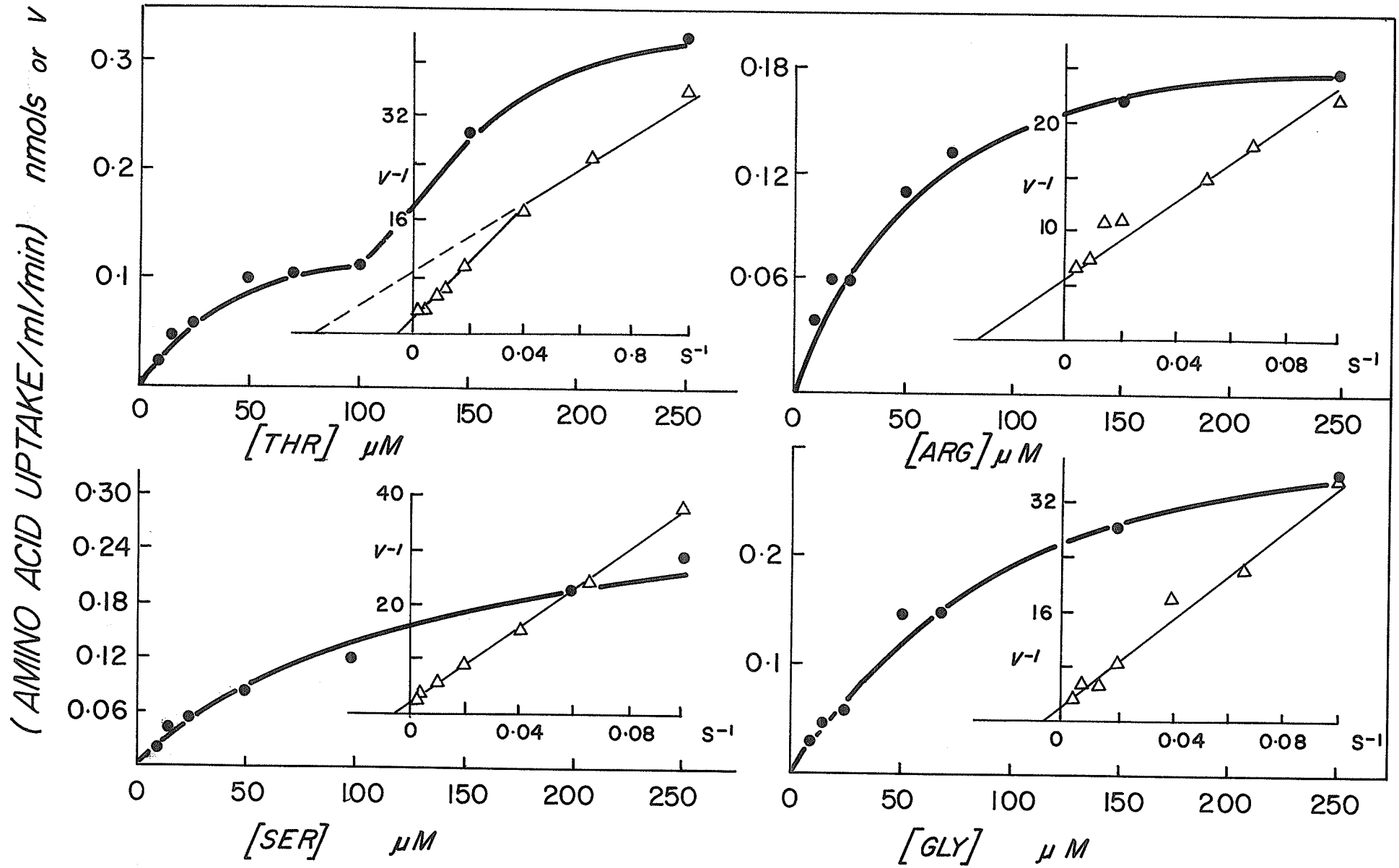

(AMINO ACID UPTAKE/ml/min) nmols or v
0·3
0·2
0·1
0
50
100
150
200
250
[THR] µM
32
v-1
16
0
0·04
0·8
s-1
0·18
0·12
0·06
[ARG] µ M
20
10
0·08
0·30
0·24
0·18
0·12
0·06
[SER] µM
40
0·2
0·1
[GLY] µ M

Fig. 5. Michaelis-Menten and Lineweaver-Burk analysis of the initial reaction rates of transport of (top left) histidine; (top right) methionine; (bottom right) valine; and (bottom left) phenylalanine. See Fig. 4 legend for other details.

The intracellular concentrations of histidine methionine, phenylalanine and valine were respectively three, thirty, fourteen and fifteen times greater than their external concentrations.

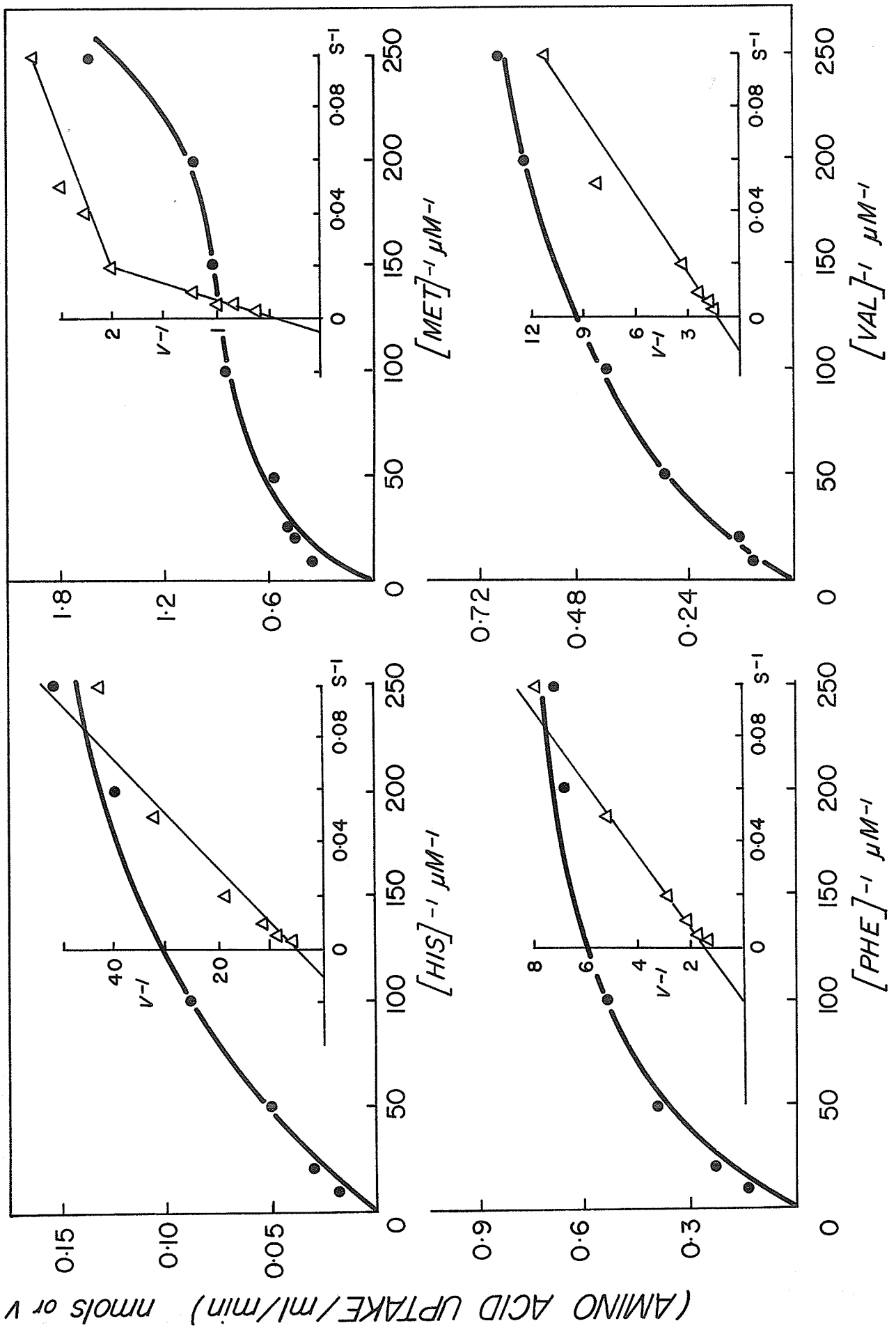

(AMINO ACID UPTAKE/ml/min) nmols or v
[PHE]⁻¹ μM⁻¹
[HIS]⁻¹ μM⁻¹
[MET]⁻¹ μM⁻¹
[VAL]⁻¹ μM⁻¹
v⁻¹
s⁻¹

Fig. 6. Michaelis-Menten and Lineweaver-Burk analysis of the initial reaction rates of transport of (top left) asparagine; (top right) proline; (bottom right) cysteine and (bottom left) tyrosine. See Fig. 4 legend for other details.

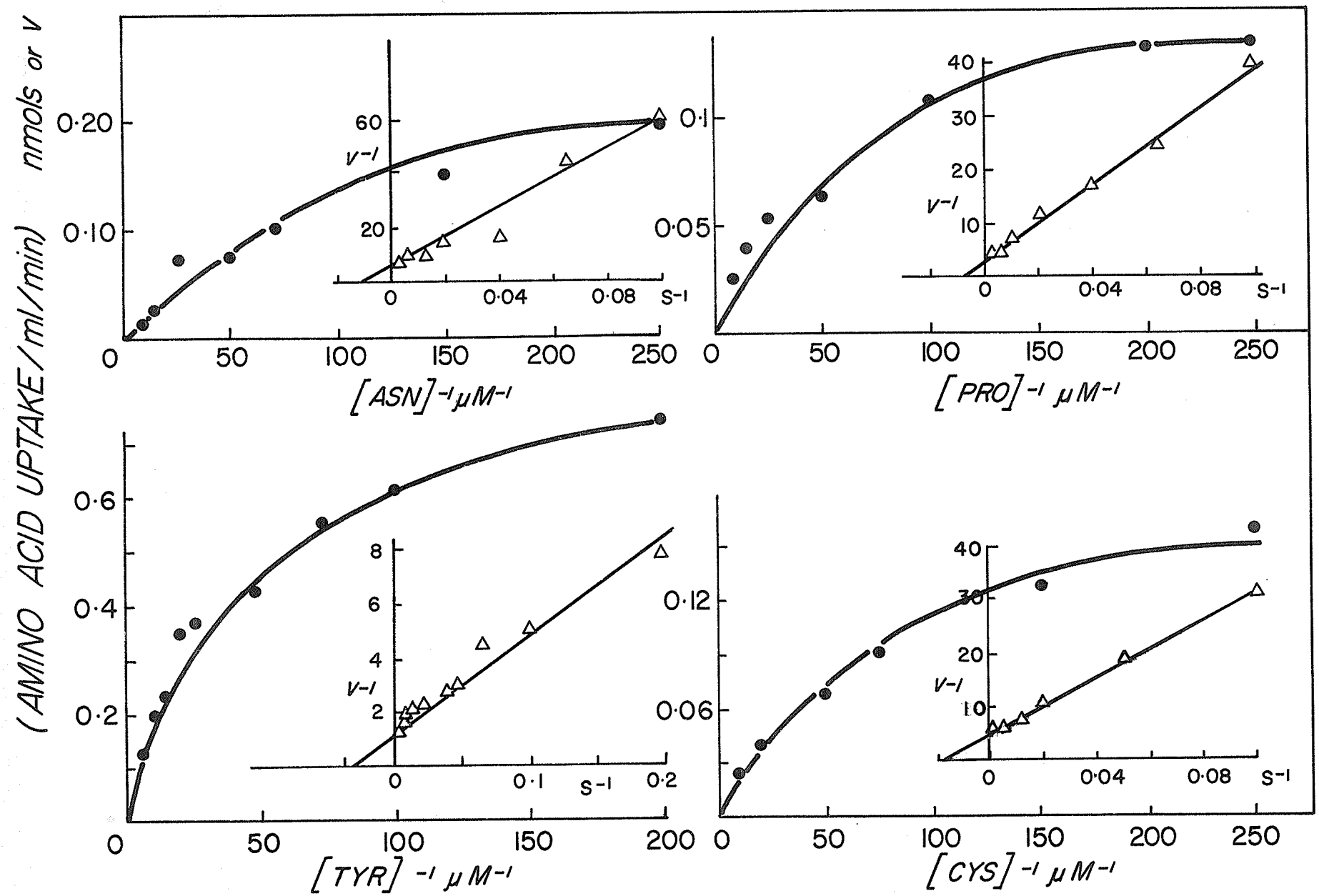

(AMINO ACID UPTAKE/ml/min) nmols or V
0·20
0·10
0
50
100
150
200
250
[ASN]⁻¹ μM⁻¹
60
V⁻¹
20
0
0·04
0·08
S⁻¹
0·1
0·05
40
30
20
10
[PRO]⁻¹ μM⁻¹
0·6
0·4
0·2
200
[TYR]⁻¹ μM⁻¹
8
6
4
2
0·1
0·2
0·12
0·06
[CYS]⁻¹ μM⁻¹

Fig. 7. Michaelis-Menten and Lineweaver-Burk analysis of the initial reaction rates of transport of (top left) isoleucine; (top right) glutamate; (bottom right) glutamine; and (bottom left) tryptophan. See Fig. 4 legend for other details.

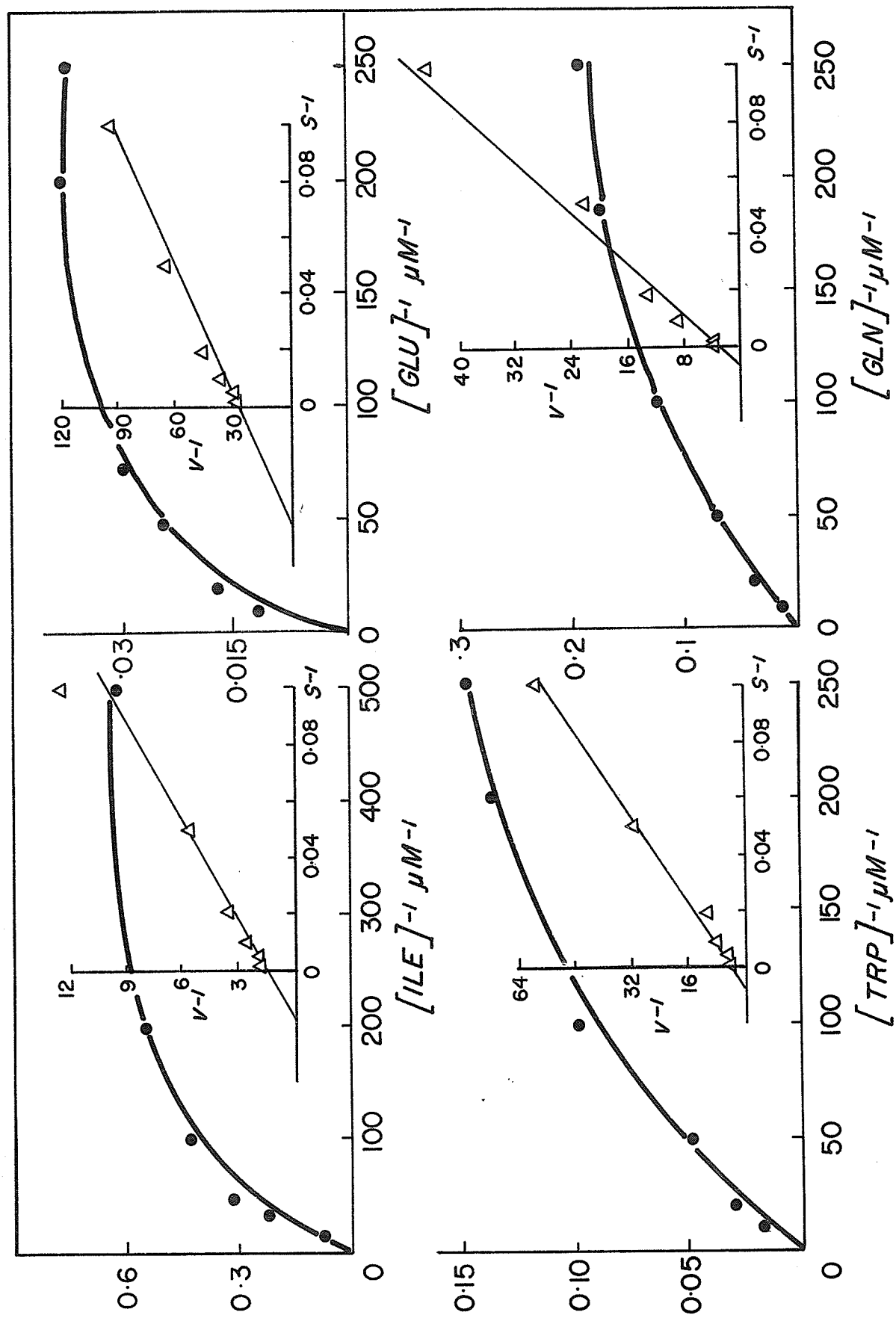
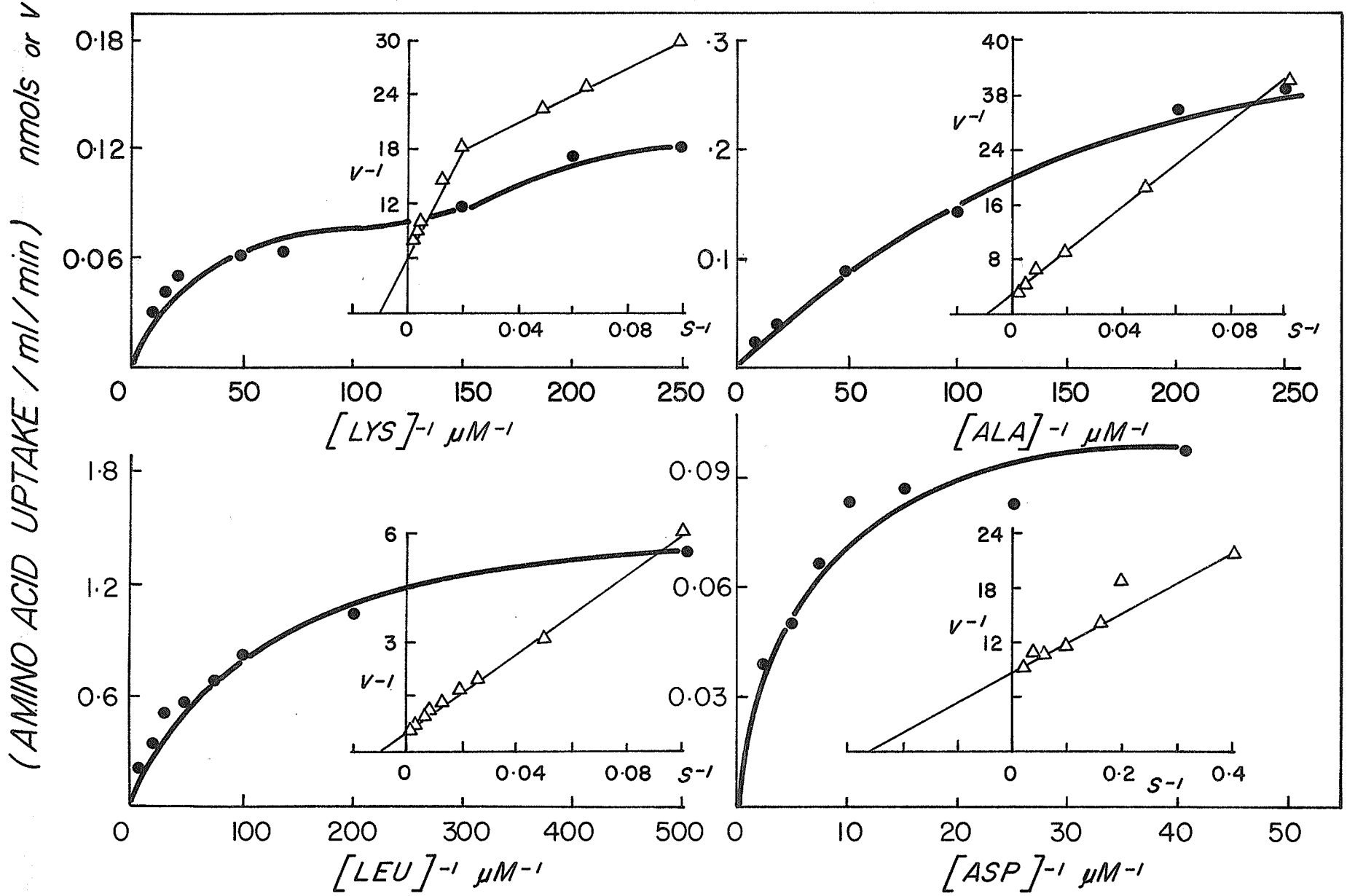

(AMINO ACID UPTAKE/ml/min) nmols or v
[ILE]-1 μM-1
[GLU]-1 μM-1
[TRP]-1 μM-1
[GLN]-1 μM-1
v-1
s-1

Fig. 8. Michaelis-Menten and Lineweaver-Burk analysis of the initial reaction rates of transport of (top left) lysine; (top right) alanine; (bottom right) aspartate; and (bottom left) leucine. See Fig. 4 legend for other details.


(AMINO ACID UPTAKE / ml / min) nmols or v
0·18
0·12
0·06
0
50
100
150
200
250
[LYS]-1 µM-1
30
24
18
12
v-1
0
0·04
0·08
s-1
·3
·2
0·1
40
38
24
16
8
[ALA]-1 µM-1
1·8
1·2
0·6
0
100
200
300
400
500
[LEU]-1 µM-1
6
3
0·09
0·06
0·03
10
20
30
40
50
[ASP]-1 µM-1
0·2
0·4

different transport systems. Further characterization was possible based on pH specificity as shown in (Fig. 9). The optimum pH of transport of the amino acids varied between pH 6.0 and pH 7.5. All of the amino acids displayed conservative pH optima of either 6.5 or 7.0. Histidine was transported maximally at pH 6.0 while arginine, glutamate and cysteine were taken up rapidly at pH 7.5. Interestingly, aspartate and glutamate demonstrated pH optima of 6.5 and 7.5 respectively. As will be discussed later, kinetic studies show that these two acidic amino acids share a common permease.

Effect of Temperature

The effects of temperature on the initial rate of accumulation of amino acids were illuminating. In all cases examined, except that of tryptophan, the transport activity of the cells declined sharply above 30°C. In two cases, that of leucine and alanine, the temperature-dependent transport curves indicated that 25° may be the optimum temperature for their transportation (Fig. 10).

Close scrutiny of the temperature plots revealed that valine, lysine, tryptophan, glutamic acid, and leucine may represent distinct transport systems because

Fig. 9. Influence of pH on the initial reaction rate of uptake of all 20 amino acids. The buffer used was Tris-acetate 12.5 mM. Each amino acid was used at a concentration of 50 μM and cell density, A_{700}, kept at 0.19. (a) Ala (●); Gly (○) (b) Ileu (c) Val (●); Leu (○) (d) Thr (●); Ser (○) (e) Asp (○); Glu (●) (f) Met (○); Cys (●) (g) His (●); Arg (○) (h) Lys (i) Tyr (○); Phe (●) (j) Trp (○); Pro (●) (k) Asn (l) Gln

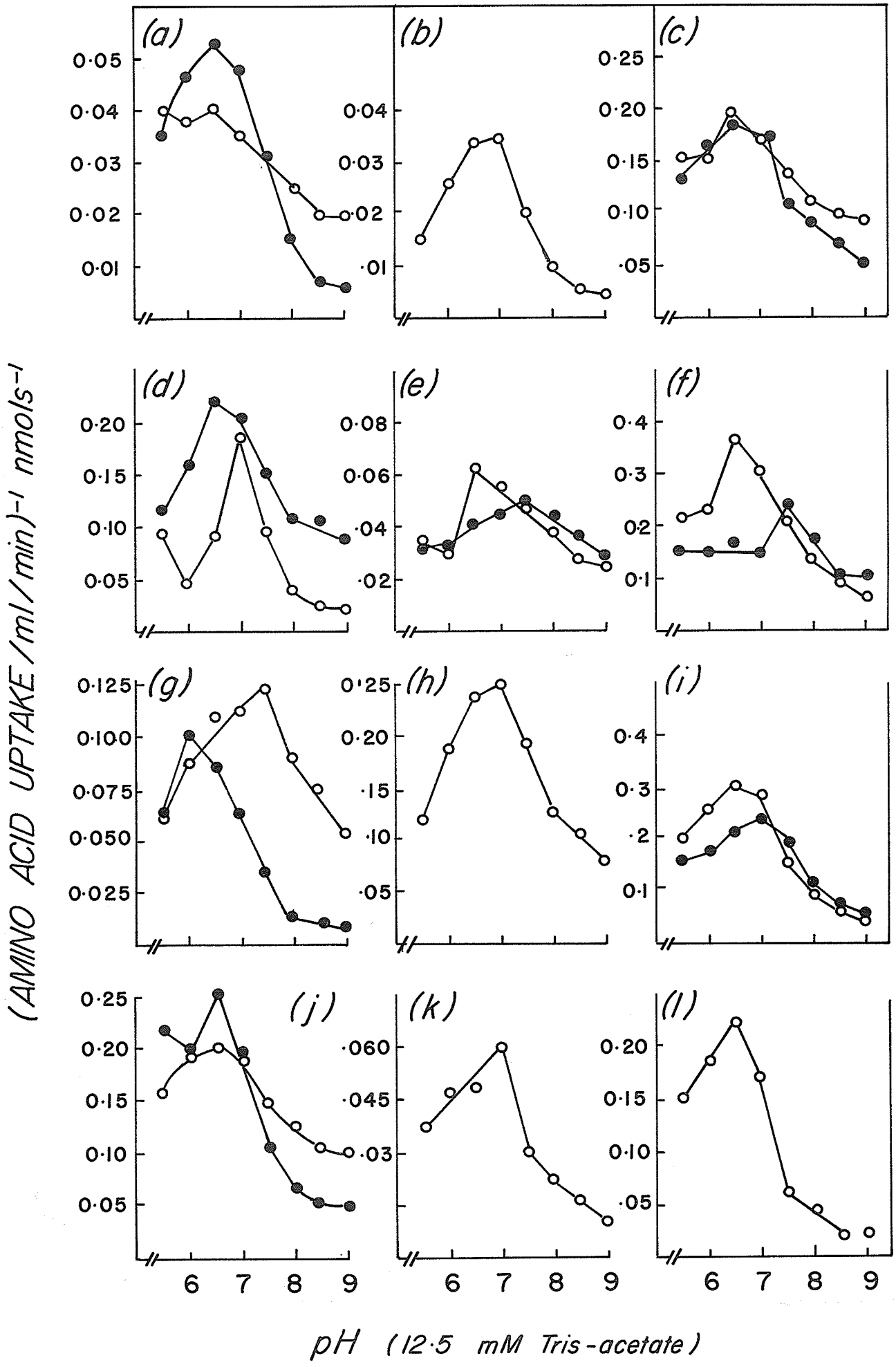
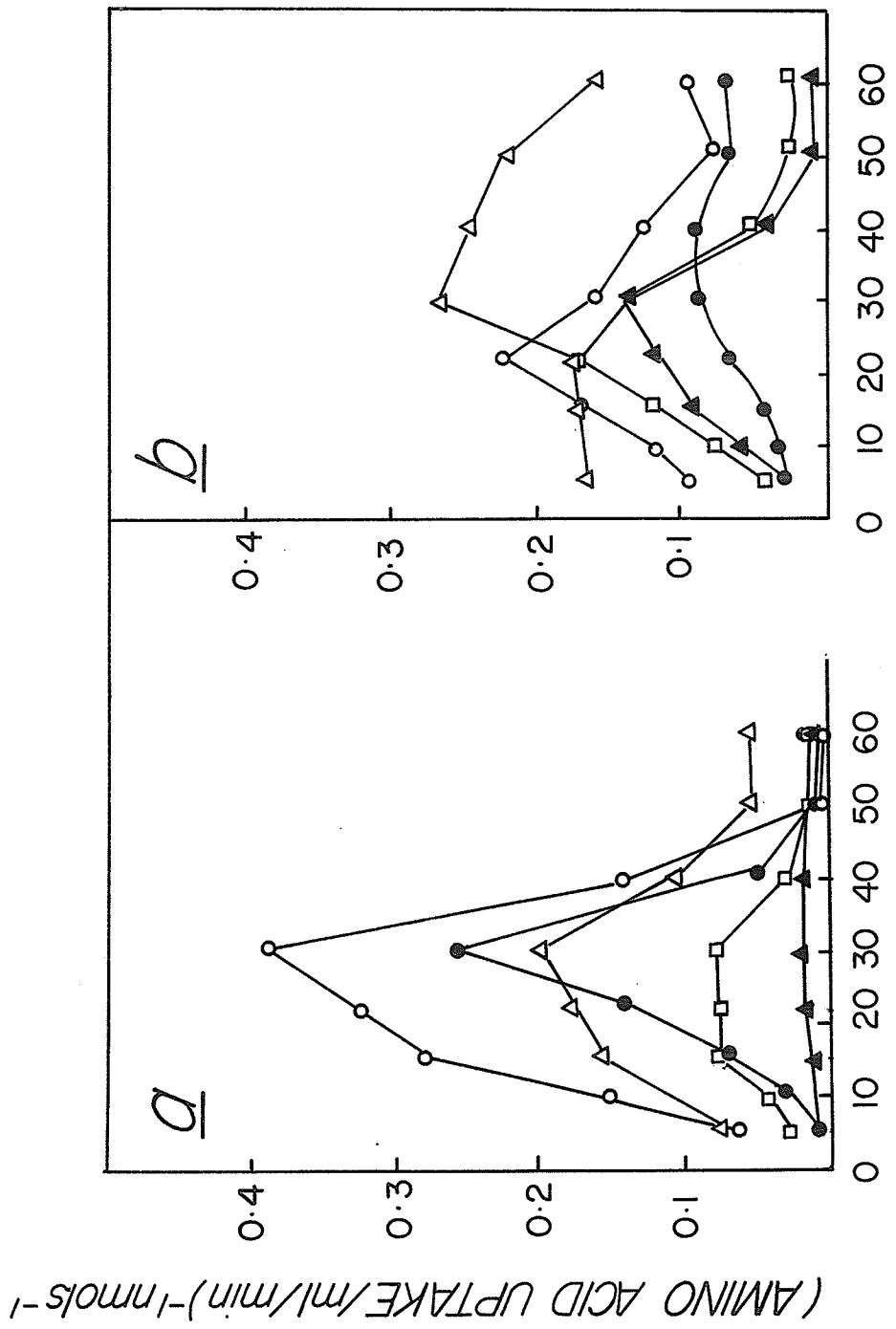

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
0·05
0·04
0·03
0·02
0·01
0·25
0·20
0·15
0·10
·05
0·08
0·06
·04
·02
0·4
0·3
0·2
0·1
0·125
0·100
0·075
0·050
0·025
0'25
·15
·10
·060
·045
·03
6 7 8 9
(AMINO ACID UPTAKE /ml/min)⁻¹ nmols⁻¹
pH (12·5 mM Tris-acetate)

Fig. 10. Influence of temperature on the initial reaction rate of uptake of several amino acids. Transport studies were conducted in medium A and each amino acid was supplied at a concentration of 50 μM. The cell density, A_{700}, was kept at 0.19. (A) Met (○); His (●); Phe (△); Val (□); Lys (▲). (B) Glu (△); Ala (○); Leu (□); Ileu (▲); Trp (●).


a
b
0·1
0·2
0·3
0·4
0
10
20
30
40
50
60
(AMINO ACID UPTAKE/ml/min)-1nmols-1
TEMPERATURE (°C)

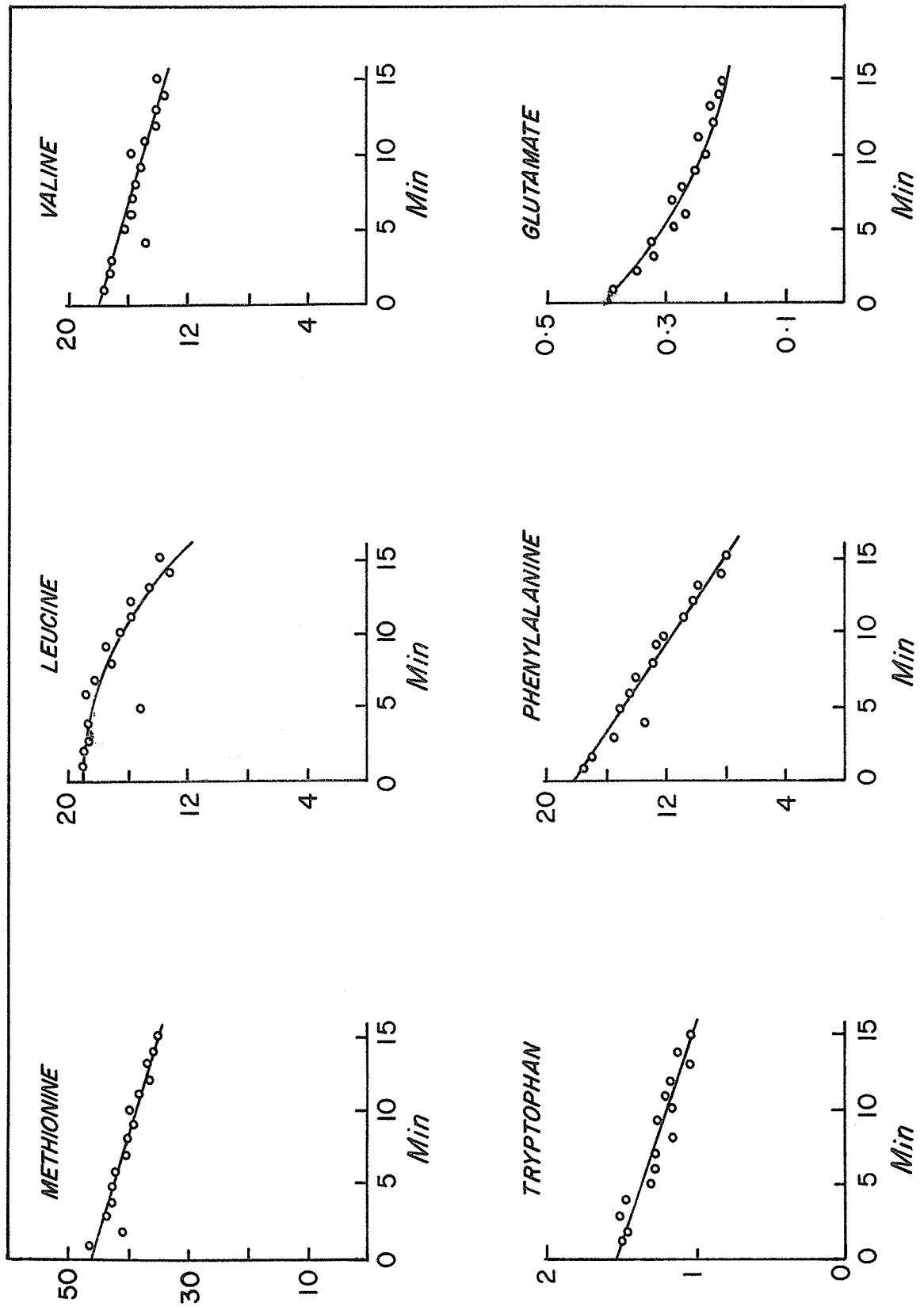
of widely different optima and temperature-rate relations. The transport systemsof the other amino acids were less clearly resolved. Lysine, glutamic acid, and tryptophan permeases appeared to be more stable than the others at temperatures higher than 30°C.

The transport activities for the amino acids were strongly dependent on temperature with Q_{10}'s between 20° and 30° greater than 2, and this indicated that a metabolic transport system requiring high activation energy was involved for each amino acid. The rapid decrease in transport activity above 30° demonstrated that proteins may be involved in the transport process.

Exit Experiments

The results obtained for six amino acids exiting in the presence of CCCP are shown in Fig. 11. Apparently, all of the amino acids do not exit at the same rate or even by the same mechanism. The exit kinetics for glutamate and leucine are uniquely different from those of methionine, valine, tryptophan and phenylalanine. The difference between the valine and leucine exit kinetics is particularly interesting since competition experiments indicate that a common permease may serve for the uptake of these two amino acids. It is possible that different acceptors are used for the entry and exit of the same amino acid.

Fig. 11. Arithmetic plots of the efflux of various amino acids pre-loaded into germinated spores of Achlya. The various cell populations at a density of 0.25 (A_{700}) were independently exposed to the different labelled amino acids for 30 min in medium A in the presence of 28 μM cycloheximide. Experimental conditions are discussed in the "Methods" section.


nmols Amino Acid Remaining in Cells
METHIONINE
LEUCINE
VALINE
TRYPTOPHAN
PHENYLALANINE
GLUTAMATE
Min
Min
Min
Min
Min
Min

Competition Studies

Further characterization of the amino acid permeases was possible through competition studies. A preliminary step involved the measuring of the initial rate of transport of a given labelled amino acid in the presence of unlabelled carrier or other amino acids. The results of such experiments for all twenty labelled amino acids are summarized in Fig. 12.

Quite noticeable in Fig. 12 is the inhibition of the uptake of all amino acids by methionine. However, methionine transport was blocked only by the unlabelled carrier itself. To further understand the nature of the inhibitory effect of methionine, more detailed kinetic studies were necessary. The transport rates of any given labelled amino acid at different concentrations were determined in the presence and absence of a fixed concentration of methionine. The results of such experiments are expressed in Lineweaver-Burk format from Fig. 13 to 16. The transport rates of glycine, leucine, isoleucine, valine, proline, serine, threonine, glutamine, and tryptophan were inhibited non-competitively by methionine.

Fig. 12

Competition between amino acids during transport by Achlya germlings.

Competition for transport of a radioactive amino acid was determined using 20 unlabelled amino acids. The unlabelled amino acid was supplied at 500 µM and the labelled amino acid at 50 µM. Transport assay conditions are discussed in the "Methods" Section. Shaded areas represent inhibition and non-shaded areas, no inhibition.

A value of fifty percent was considered to be significant in the inhibition of the labelled amino acid uptake by the unlabelled amino acid.

AMINO ACIDS

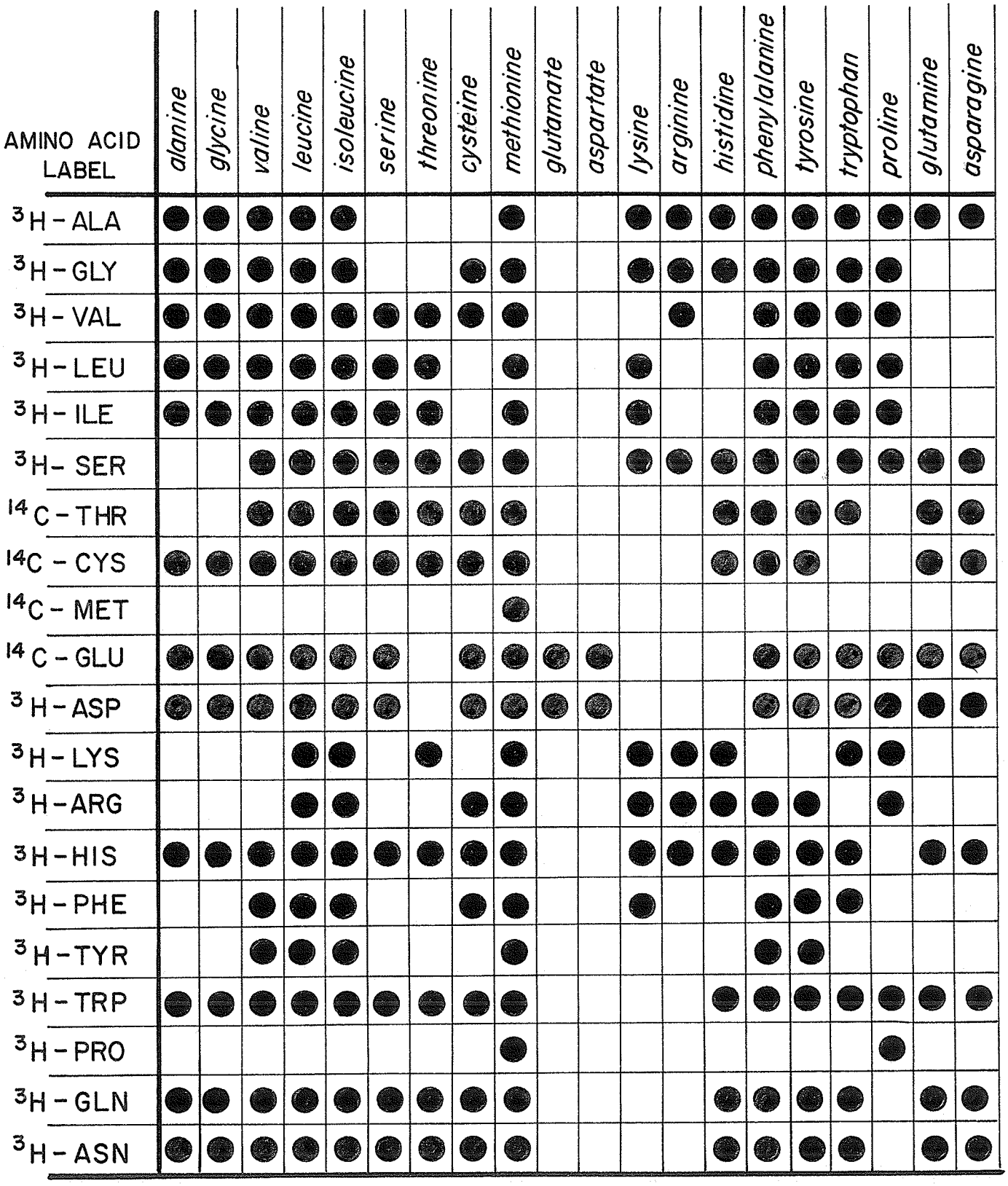

AMINO ACID LABEL
alanine
glycine
valine
leucine
isoleucine
serine
threonine
cysteine
methionine
glutamate
aspartate
lysine
arginine
histidine
phenylalanine
tyrosine
tryptophan
proline
glutamine
asparagine
3H - ALA
3H - GLY
3H - VAL
3H - LEU
3H - ILE
3H - SER
14C - THR
14C - CYS
14C - MET
14C - GLU
3H - ASP
3H - LYS
3H - ARG
3H - HIS
3H - PHE
3H - TYR
3H - TRP
3H - PRO
3H - GLN
3H - ASN

Fig. 13. Lineweaver-Burk analyses of the inhibition of the initial reaction rates of (a) glycine (b) leucine transport by methionine. The concentrations of the inhibitor were 10 μM (△); 100 μM (□); nil (○). The cell density, A_{700}, was kept at 0.20. Reaction medium A was used at pH 7.

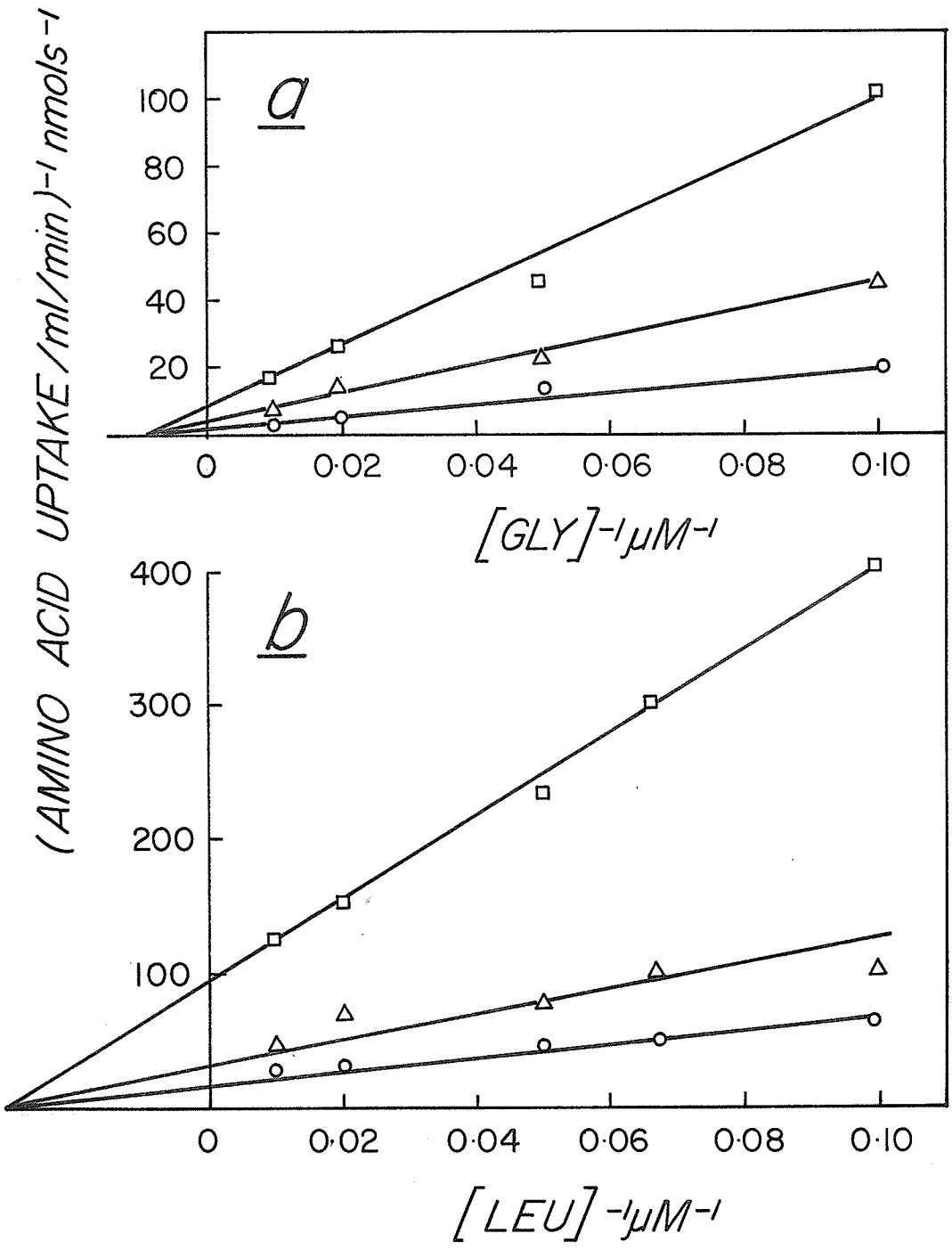

(AMINO ACID UPTAKE/ml/min)$^{-1}$ nmols^{-1}
a
100
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[GLY]$^{-1}$μM^{-1}
b
400
300
200
100
0
0·02
0·04
0·06
0·08
0·10
[LEU]$^{-1}$μM^{-1}

Fig. 14. Lineweaver-Burk analyses of the inhibition of the initial rates of (a) isoleucine (b) valine and (c) proline transport by methionine. Concentrations of the inhibitor were 10 μM (△); 100 μM (□); nil (○). Experimental conditions as in Fig. 13 legend.

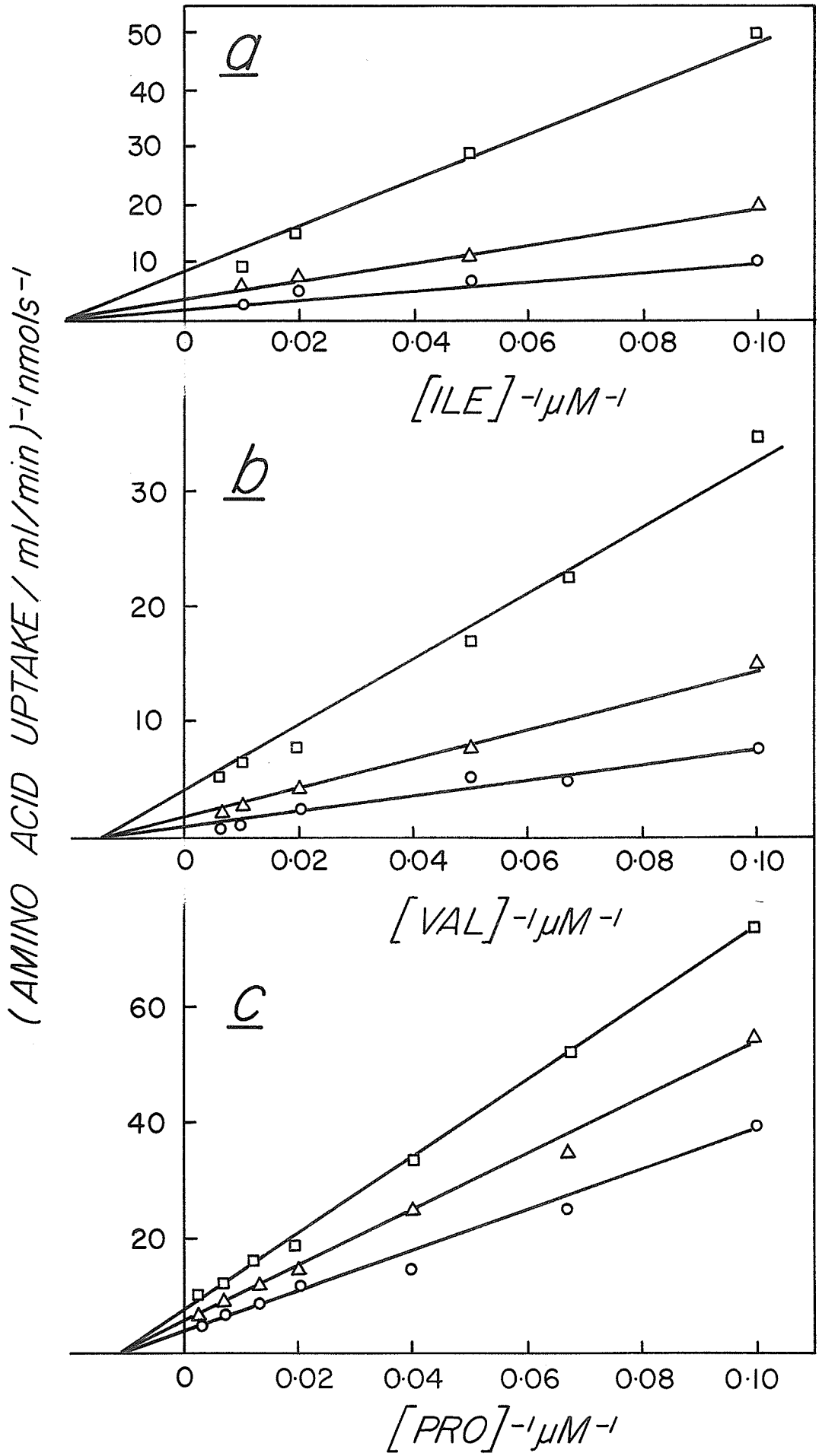

a
b
c
50
40
30
20
10
0
0·02
0·04
0·06
0·08
0·10
[ILE]⁻¹μM⁻¹
[VAL]⁻¹μM⁻¹
[PRO]⁻¹μM⁻¹
(AMINO ACID UPTAKE / ml/min)⁻¹nmols⁻¹
60

Fig. 15. Lineweaver-Burk analyses of the inhibition of the initial reaction rates of (a) serine (b) threonine transport by methionine.

The concentrations of the inhibitor were 10 μM (□); 100 μM (△); nil (○). Experimental conditions as in Fig. 13 legend.

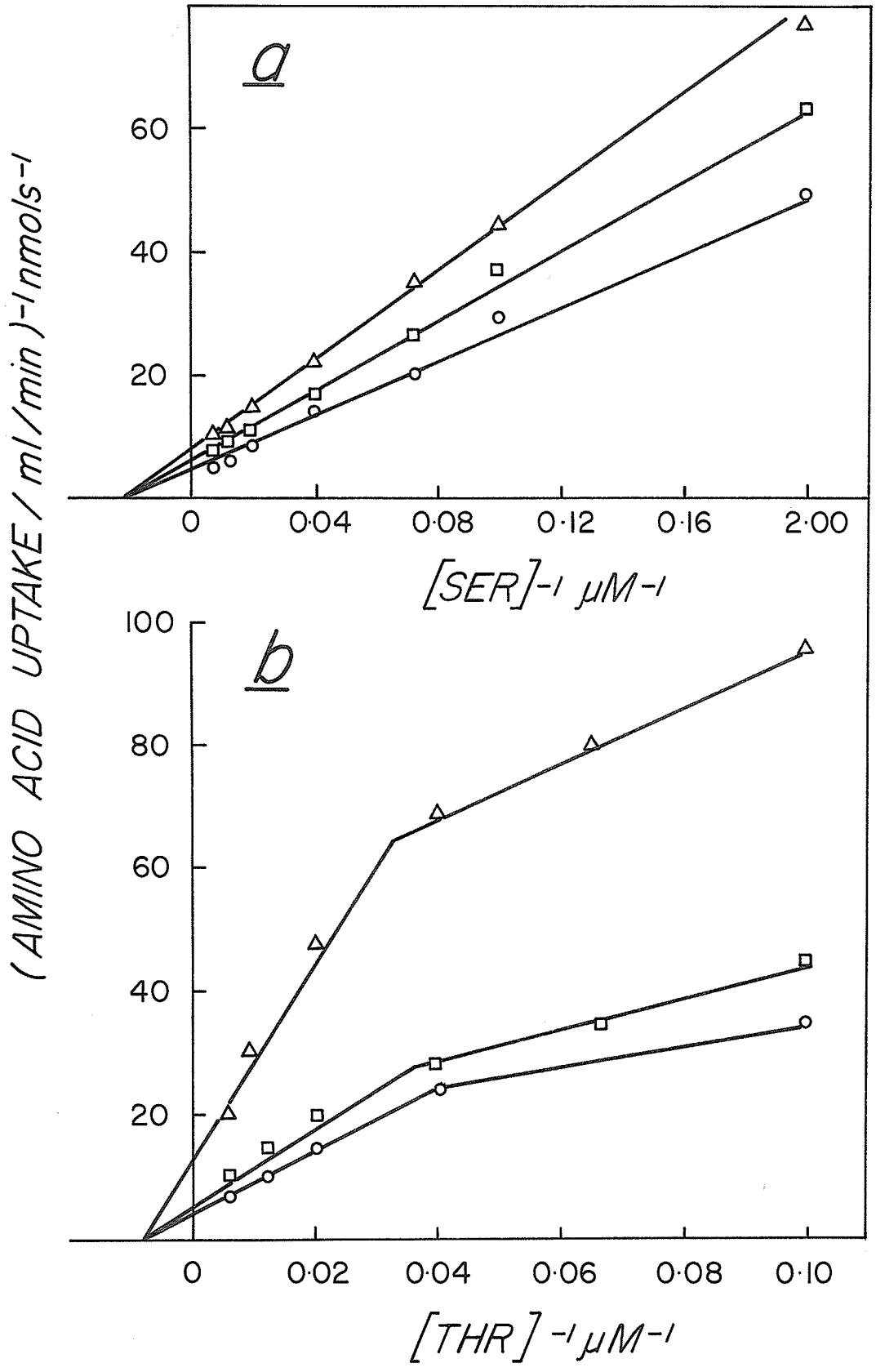
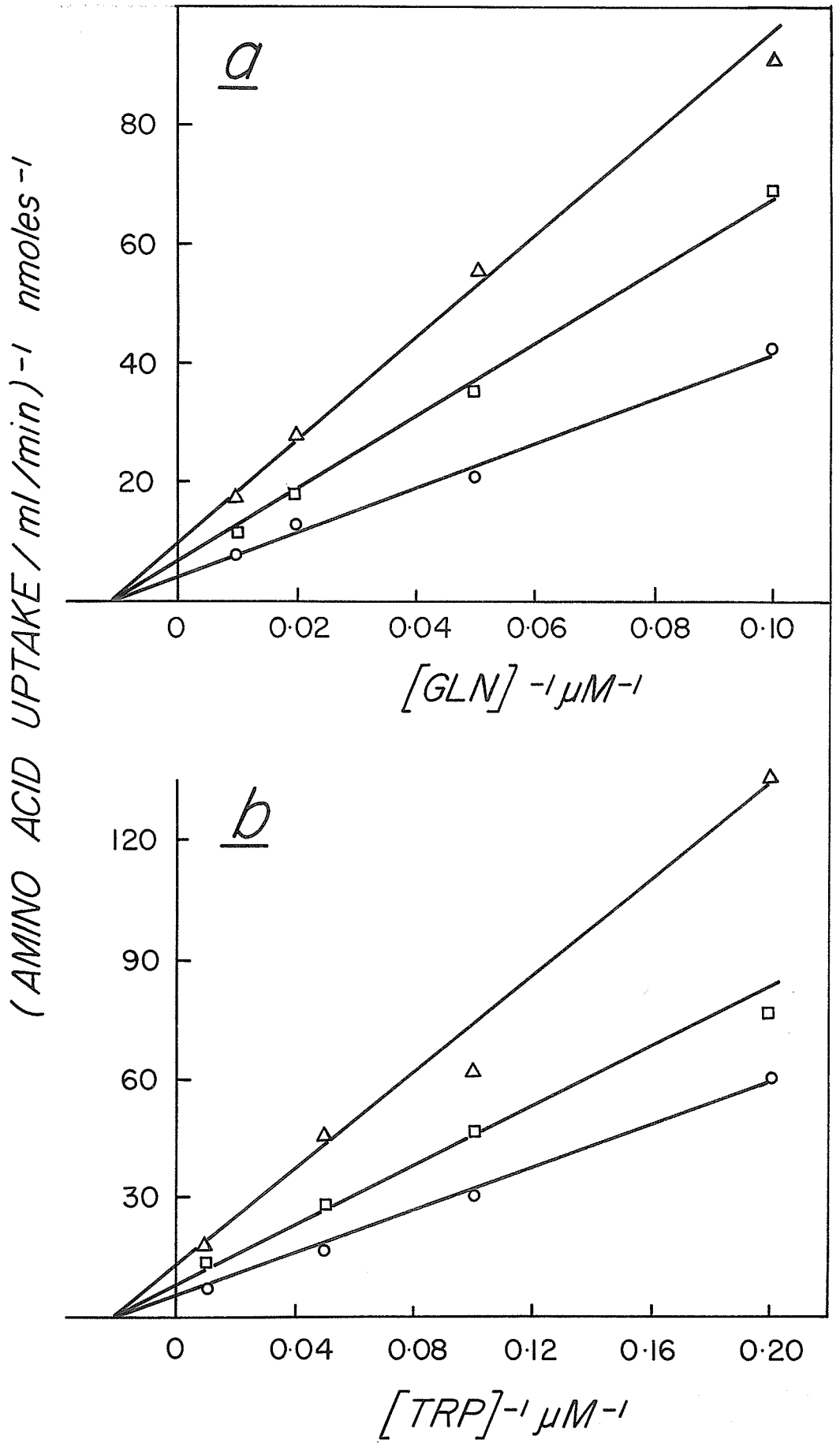

a
60
40
20
0
0·04
0·08
0·12
0·16
2·00
[SER]-1 μM-1
b
100
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[THR]-1 μM-1
(AMINO ACID UPTAKE / ml/min)-1 nmols-1

Fig. 16. Lineweaver-Burk analyses of the inhibition of the initial reaction rates of (a) glutamine (b) tryptophan transport by methionine.

The concentrations of the inhibitor were 10 μM (□); 100 μM (△) nil (○). Experimental conditions as in Fig. 13 legend.


a
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[GLN] -1 μM-1
b
120
90
60
30
0
0·04
0·08
0·12
0·16
0·20
[TRP]-1 μM-1
(AMINO ACID UPTAKE / ml/min)-1 nmoles-1

The preliminary competition studies of Fig. 12 had shown that a given labelled amino acid may be inhibited by both structurally related and unrelated amino acids. To investigate the amino acid specificity of a given permease, it was necessary to determine the initial rates of uptake of that amino acid over a wide concentration range in the presence and absence of the inhibitor. On the basis of such kinetic studies specificity of transport systems were discerned. If the unlabelled amino acid did not compete with the labelled substrate, no inhibition would be observed and it must be assumed that the two amino acids were transported by different systems. One, of course, must take into consideration the affinity differences that may exist.

The inhibition of glycine transport by alanine and phenylalanine is shown in Fig. 17 (a). Glycine uptake was competitively inhibited by alanine but phenylalanine exerted a non-competitive inhibition effect on the transport of this neutral amino acid. Similarly, the transport of threonine was competitively inhibited by the structurally similar amino acid, serine (Fig. 17 b).

The branched-chain amino acids valine, leucine, and isoleucine seemed to share a common transport system for they were all competitive with one another (Fig. 18 a).

Fig. 17. (a) Inhibition of glycine transport by alanine (□); serine (●); and phenylalanine (△). Control (○). The concentration of the inhibitor was 300 μM. See Fig. 13 legend for other details.

(b) Inhibition of threonine transport by alanine (●); serine (■); and phenylalanine (△). Control (○). The concentration of the inhibition was maintained at 300 μM. See Fig. 13 legend for other details.

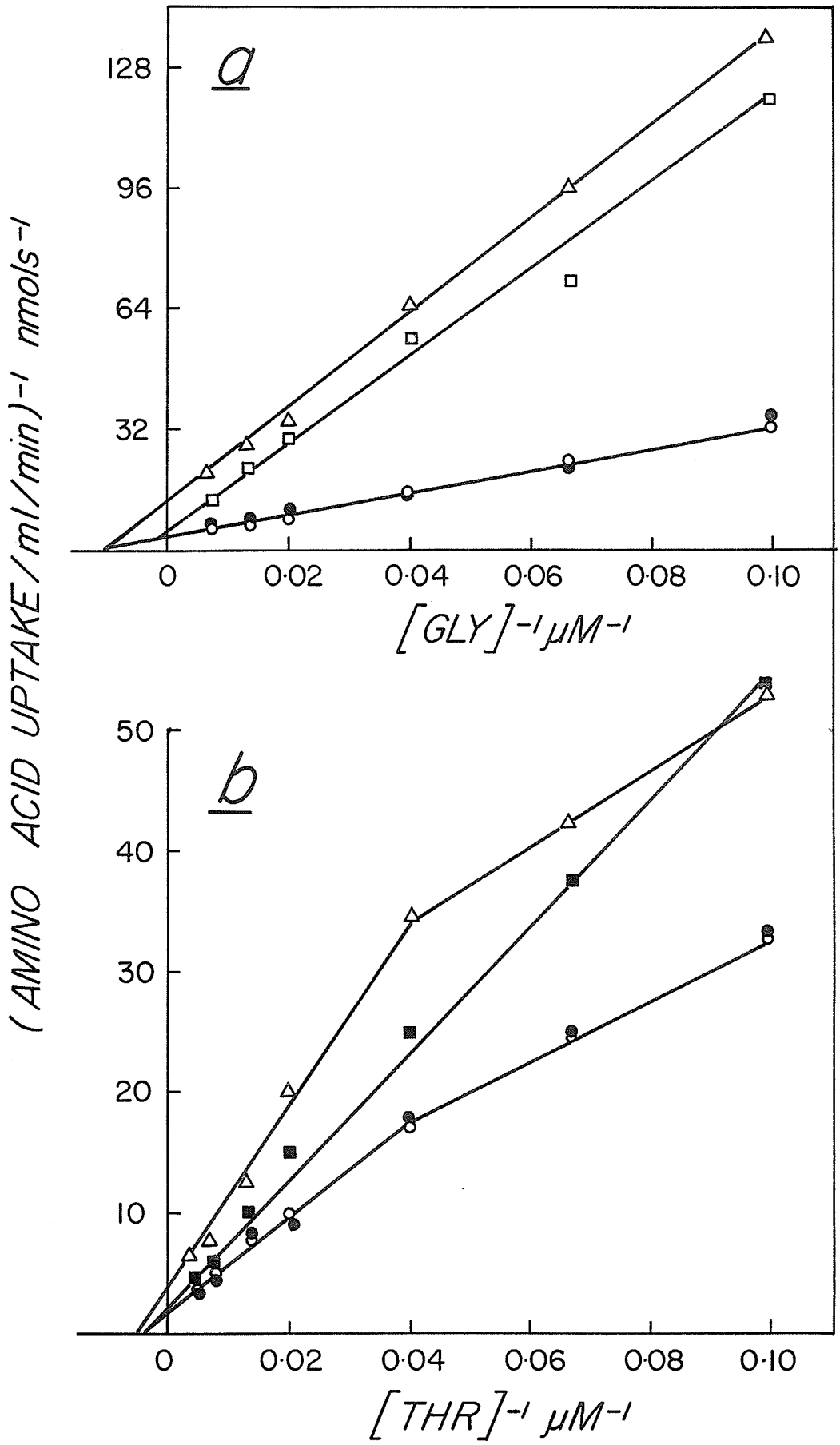

a
128
96
64
32
0
0·02
0·04
0·06
0·08
0·10
[GLY]⁻¹ μM⁻¹
b
50
40
30
20
10
0
0·02
0·04
0·06
0·08
0·10
[THR]⁻¹ μM⁻¹
(AMINO ACID UPTAKE/ml/min)⁻¹ nmols⁻¹

Fig. 18. (a) Competitive inhibition of valine transport by phenylalanine (△); leucine (□); isoleucine (○) and tyrosine (▽). Control (●); control and 500 μM aspartate (▲). The concentration of the inhibitor was maintained at 500 μM. See Fig. 13 legend for other details.

(b) Competitive inhibition of phenylalanine transport by valine (□); tyrosine (△) and leucine (▽). Control (○); control and 500 μM aspartate (●). The concentration of inhibitor was maintained at 500 μM. See Fig. 13 legend for other details.

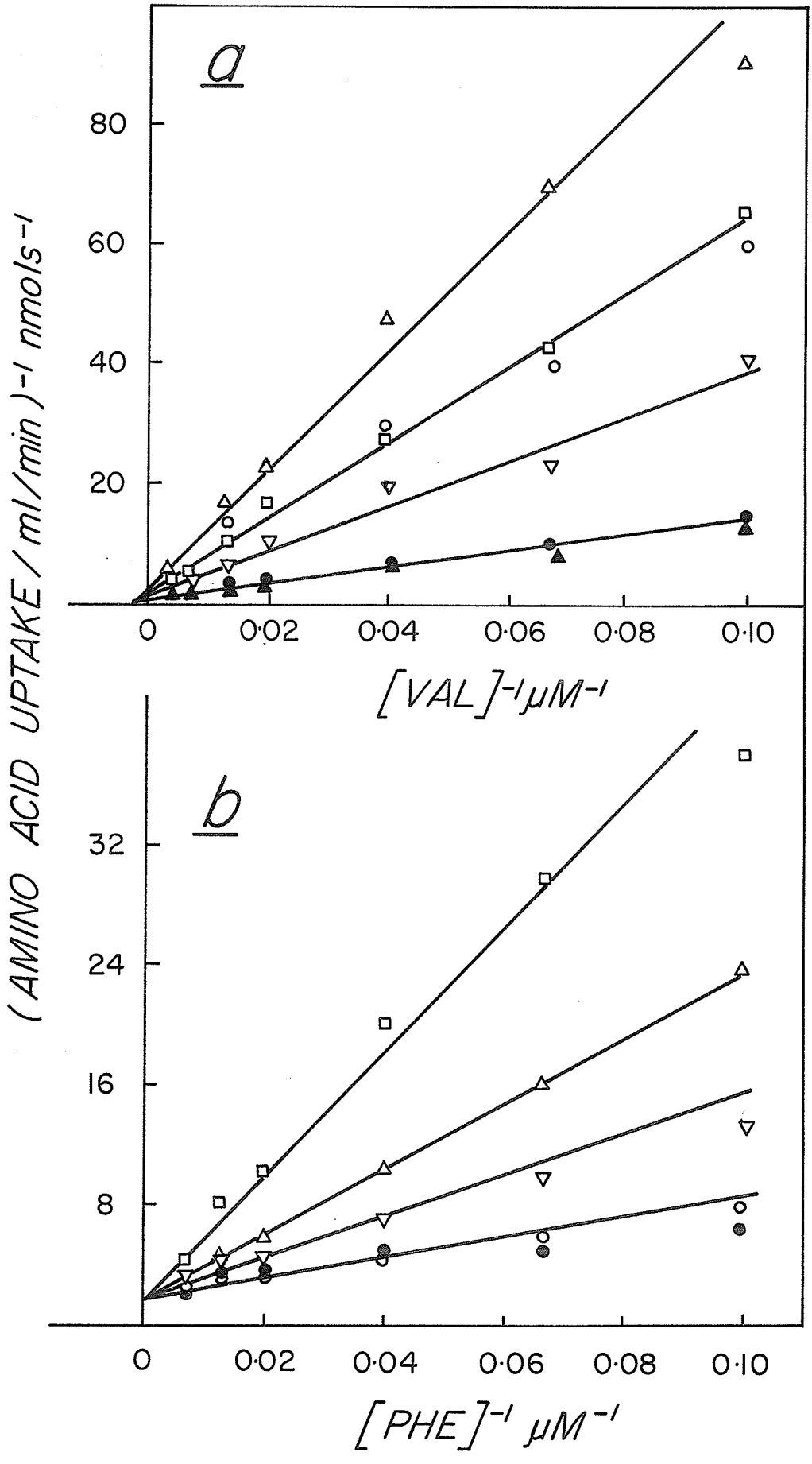

a
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[VAL]⁻¹ μM⁻¹
b
32
24
16
8
0
0·02
0·04
0·06
0·08
0·10
[PHE]⁻¹ μM⁻¹
(AMINO ACID UPTAKE / ml / min)⁻¹ nmols⁻¹

Fig. 19. (a) Competitive inhibition of phenylalanine transport by tyrosine (○) and tryptophan (△). Control (●). Concentration of the inhibitor was maintained at 300 μM. See Fig. 13 legend for other details.

(b) Competitive inhibition of tryptophan transport by valine (●) and phenylalanine (△). Control (○); control and 500 μM proline (□). Concentration of the inhibitor was maintained at 150 μM. See Fig. 13 legend for other details.

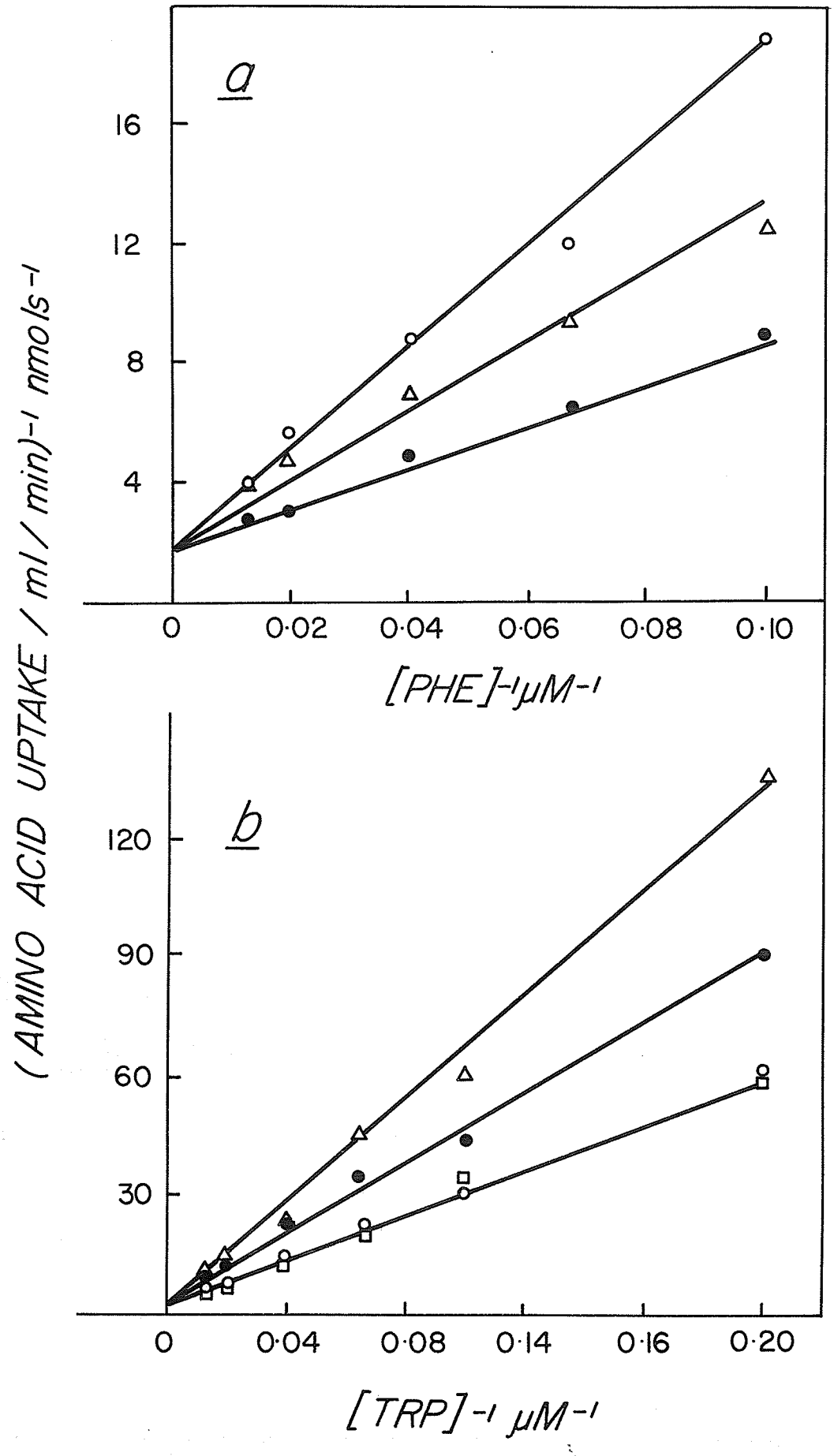
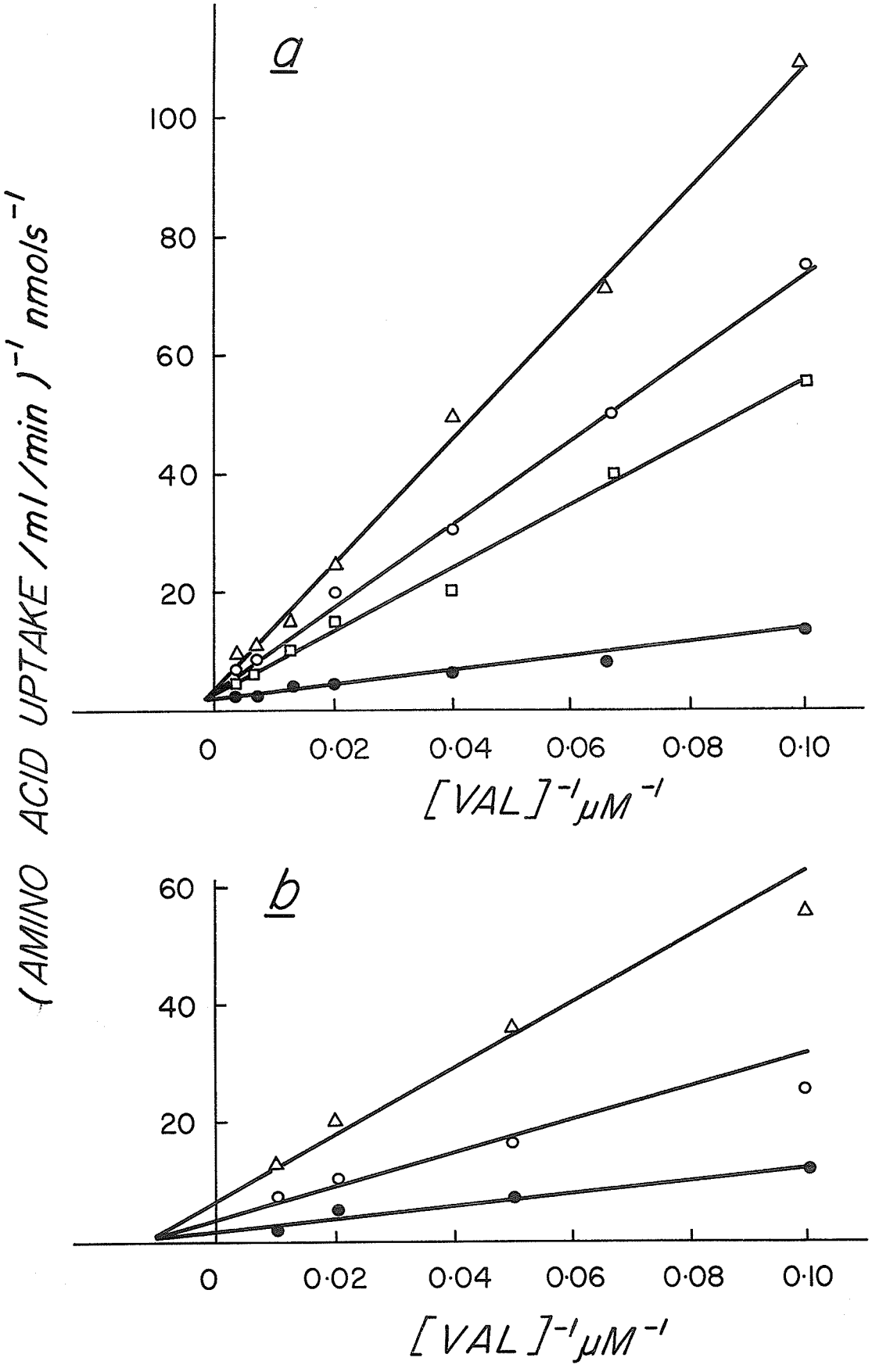

a
16
12
8
4
0
0·02
0·04
0·06
0·08
0·10
[PHE]-1 μM-1
b
120
90
60
30
0
0·04
0·08
0·14
0·16
0·20
[TRP]-1 μM-1
(AMINO ACID UPTAKE / ml / min)-1 nmols-1

Fig. 20. (a) Competitive inhibition of valine uptake by phenylalanine. Concentrations of the inhibitor were 125 µM (□); 250 µM (○); 500 µM (△); nil (●). See Fig. 13 le legend for other details.

(b) Non-competitive inhibition of valine transport by threonine. Concentrations of the inhibitor were 125 µM (○); 250 µM (△); nil (●). See Fig. 13 legend for other details.


a
100
80
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[VAL]⁻¹ μM⁻¹
(AMINO ACID UPTAKE /ml/min)⁻¹ nmols⁻¹
b
60
40
20
0
0·02
0·04
0·06
0·08
0·10
[VAL]⁻¹ μM⁻¹

The aromatic amino acids, phenylalanine and tyrosine, and the heterocyclic tryptophan interacted with a common carrier (Fig. 19 a). Interestingly, there was some degree of overlap between the leucine-isoleucine-valine (LIV) permease and the phenylalanine-tyrosine-tryptophan (PTT) permease (Fig. 18 b, 19 b). The inhibitory effects of phenylalanine on valine uptake are shown in Fig. 20 a. The K_i for phenylalanine (Fig. 21) was 50 μM and this is identical to the $S_{(0.5)}$ value for the uptake of this amino acid.

Kinetic studies showed that the basic amino acids lysine, histidine and arginine interacted with a common carrier. Arginine had a higher affinity for the lysine permease than did histidine (Fig. 22 a). However, lysine significantly inhibited the uptake of both histidine and arginine (Fig. 22 b, 23 a).

The glutamate permease was observed to be shared by the other acidic amino acid, aspartate. Phenylalanine inhibited the transport of glutamate in a non-competitive manner, whereas threonine had no significant effect on the uptake of this acidic amino acid (Fig. 23 b).

Methionine appeared to be transported by a very specific permease. The only amino acid that interacted with the methionine transport system was ethionine, a structural analogue. The biphasic nature of the double reciprocal plot for methionine uptake was altered to a

Fig. 21. Initial rates of valine transport in the presence of increasing phenylalanine concentrations. Competition of phenylalanine for the valine transport system was assayed as described in Fig. 20 (a) at valine concentrations of 12.5 μM (△); 25.0 μM (○) and 50.0 μM (□). The phenylalanine concentration was varied as indicated. Data was plotted by the method of Dixon (44).

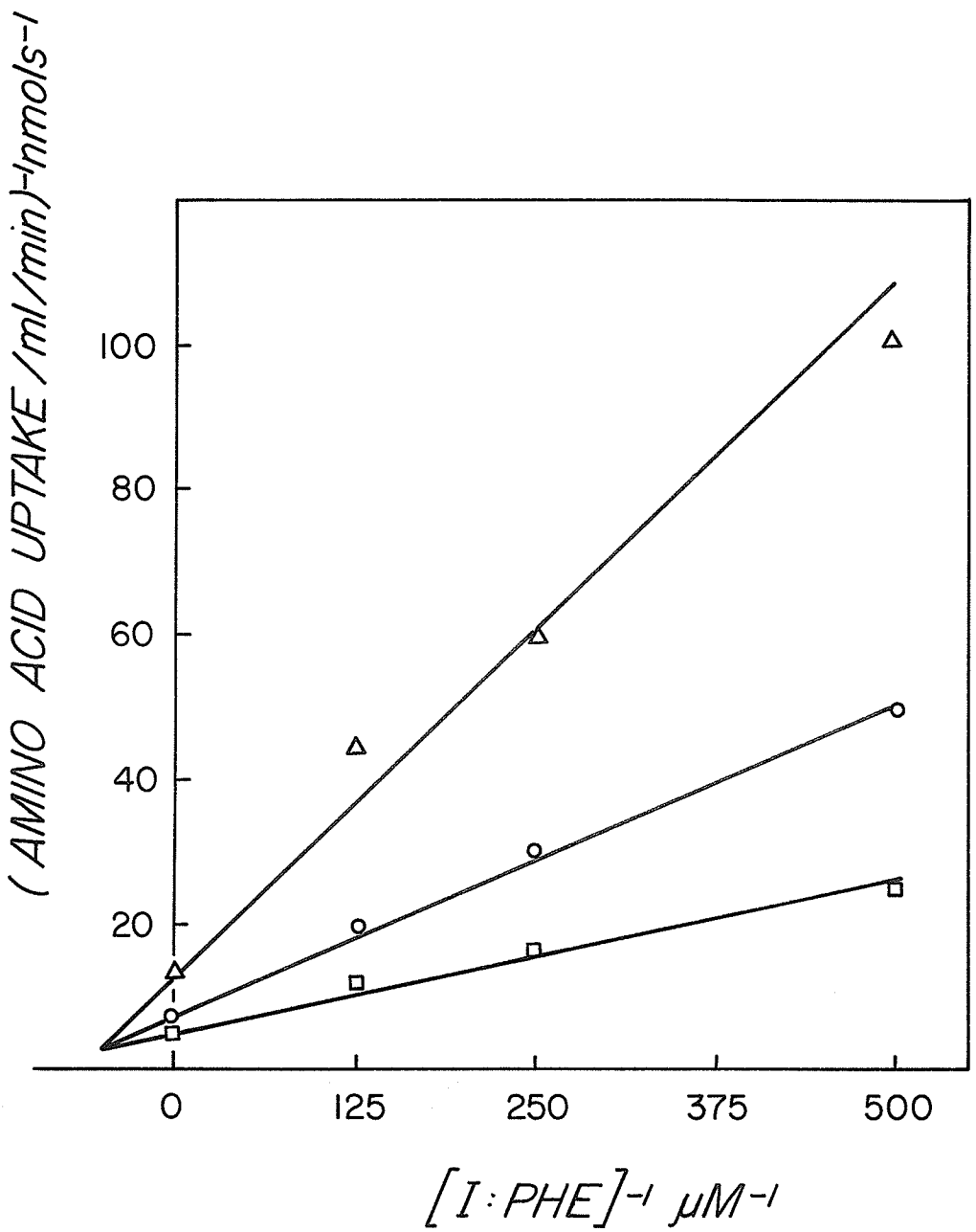

(AMINO ACID UPTAKE/ml/min)-1nmols-1
100
80
60
40
20
0
125
250
375
500
[I:PHE]-1 μM-1

Fig. 22. (a) Competitive inhibition of arginine (△) and histidine (□) for the lysine transport system. Control (●). Concentration of the inhibitor was maintained at 500 μM. See Fig. 13 legend for other details.

(b) Competitive inhibition of the histidine transport by arginine (△) and lysine (□). Control (●). Concentration of the inhibitor was 500 μM. See Fig. 13 legend for other details.

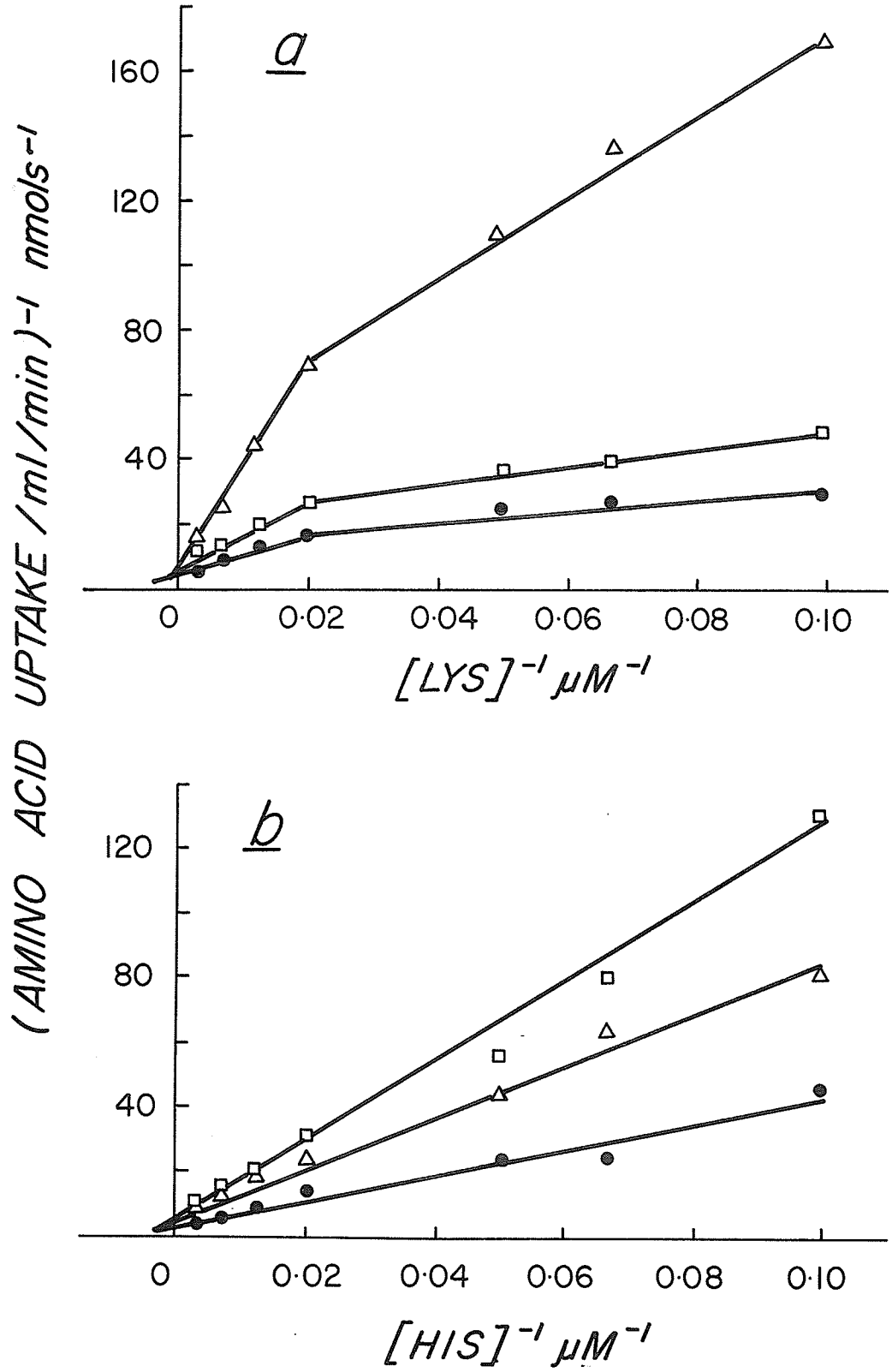

a
160
120
80
40
0
0·02
0·04
0·06
0·08
0·10
[LYS]⁻¹ μM⁻¹
b
120
80
40
0
0·02
0·04
0·06
0·08
0·10
[HIS]⁻¹ μM⁻¹
(AMINO ACID UPTAKE / ml / min)⁻¹ nmols⁻¹

Fig. 23 (a) Competitive inhibition of arginine uptake by histidine (□) and lysine (△). Control plot (○) and with 0.25 mM glutamic acid present (●). The concentration of inhibitor was maintained at 0.25 mM. See Figure 13 legend for other details.

(b) Inhibition of glutamate transport by threonine (●), phenylalanine (○) and aspartate (△). Control plot (□). The concentration of threonine was 1 mM whereas the other inhibitors were added at 0.15 mM. See Fig. 13 legend for other details.

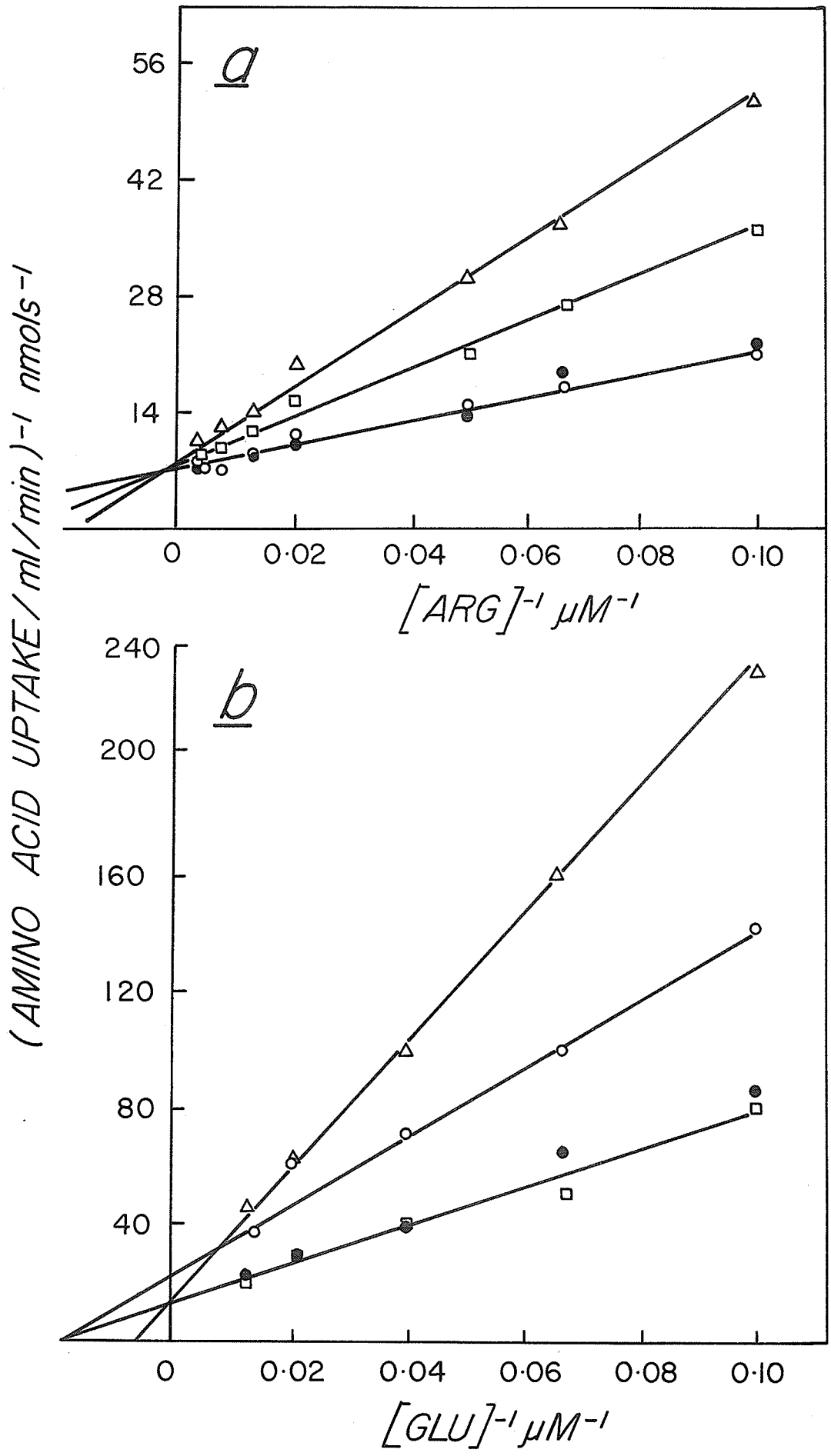
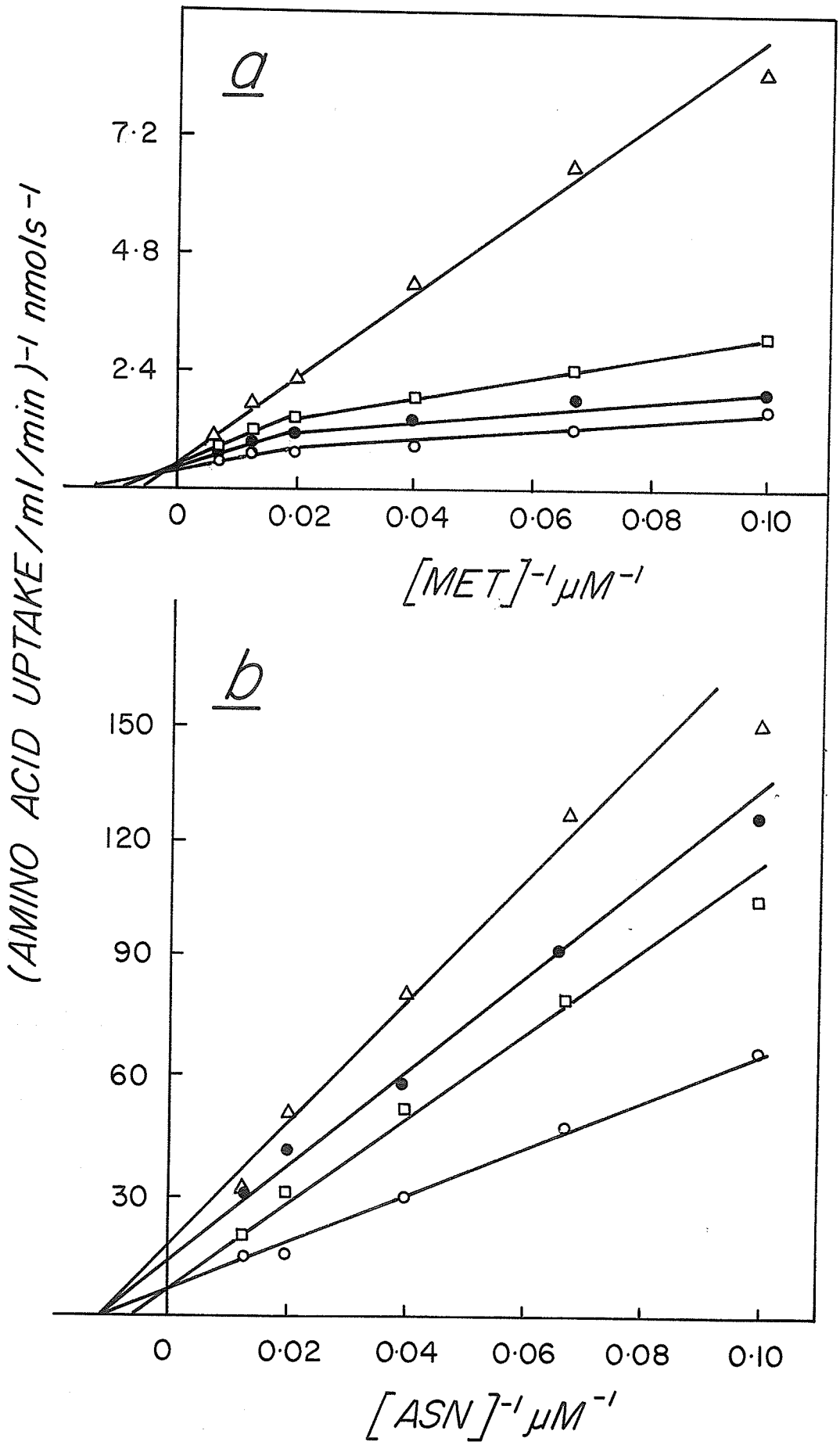

a
56
42
28
14
0
0·02
0·04
0·06
0·08
0·10
[ARG]⁻¹ μM⁻¹
b
240
200
160
120
80
40
0
0·02
0·04
0·06
0·08
0·10
[GLU]⁻¹ μM⁻¹
(AMINO ACID UPTAKE / ml / min)⁻¹ nmols⁻¹

Fig. 24. (a) The effects of ethionine (△); proline (●) and cysteine (○) on methionine transport. Control (□). The concentrations of each effector was maintained at 300 μM. See Fig. 13 legend for other details.

(b) The effects of tyrosine (△); alanine (●) and glutamine (□) on asparagine uptake. Control (○). The concentration of the inhibitor was 150 μM. See Fig. 13 legend for other details.


a
7·2
4·8
2·4
0
0·02
0·04
0·06
0·08
0·10
[MET]⁻¹ μM⁻¹
b
150
120
90
60
30
0
0·02
0·04
0·06
0·08
0·10
[ASN]⁻¹ μM⁻¹
(AMINO ACID UPTAKE/ml/min)⁻¹ nmols⁻¹

straight line in the presence of ethionine. Interestingly, cysteine, also a sulfur-containing amino acid, as did proline enhanced methionine transport (Fig. 24 a).

Glutamine was competitive with asparagine for transport (Fig. 24 b). Tyrosine and alanine significantly inhibited asparagine uptake but the nature of the inhibition was non-competitive.

Like methionine, proline appeared to be transported by a specific permease and methionine was the only amino acid which inhibited its uptake. As shown in Fig. 14 c, the transport of this heterocyclic amino acid was inhibited in a non-competitive way by methionine.

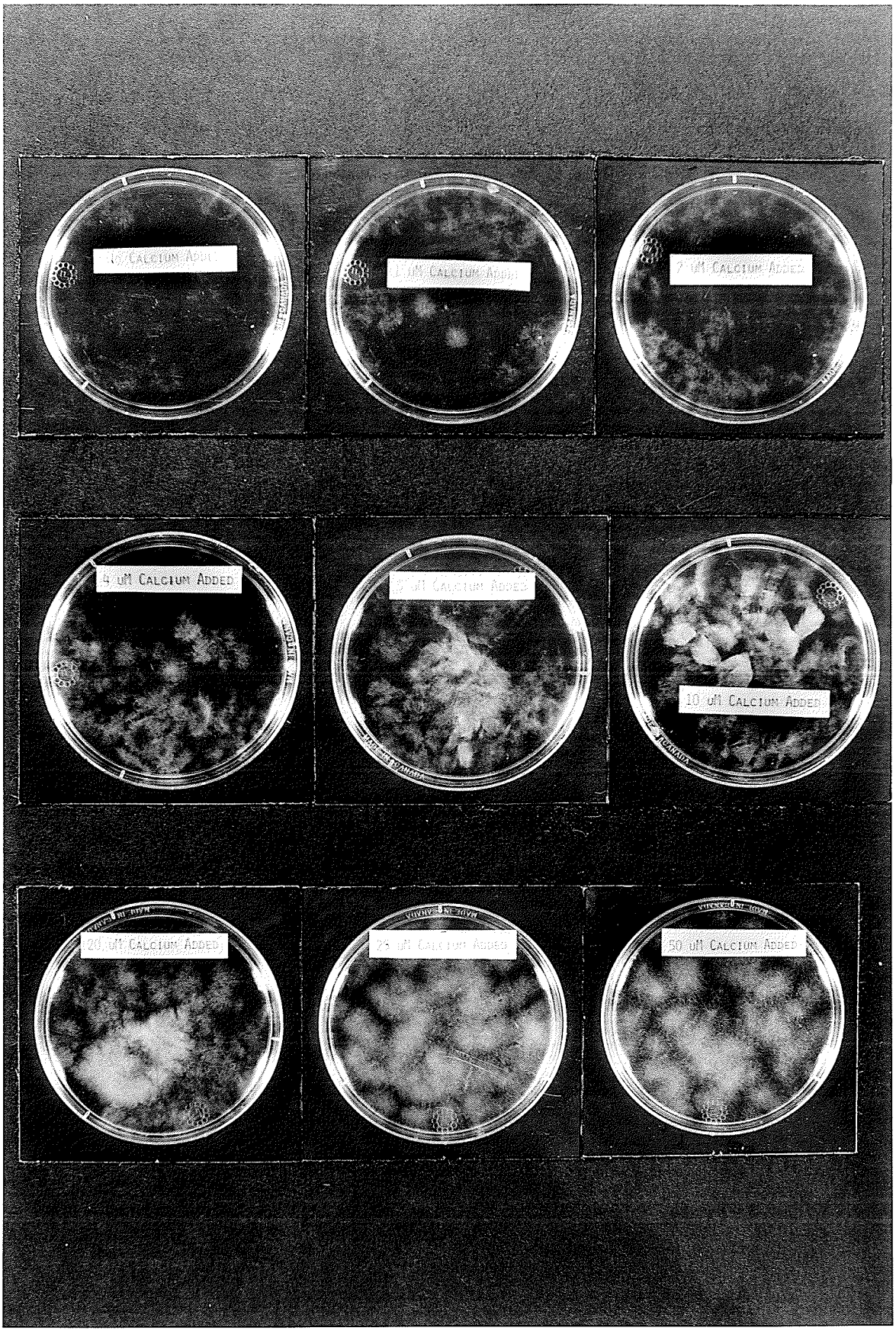
Cysteine transport was enigmatic because it was inhibited by most of the neutral amino acids, but itself did not inhibit the transport of those amino acids that blocked its uptake.

Regulatory Aspects of Amino Acid Transport

Dependence on Ca^{++}

The bivalent cation, Ca^{++}, is essential for all phases of the ontogenic sequence of development of Achlya. Starvation for Ca^{++} promptly terminates development of the organism. The result of the effects of Ca^{++} on

Fig. 25. Influence of Ca ions on the mycelial growth yield and germination of spores of Achlya. The concentration of Ca^{++} provided for each culture is specified in the figure.


0 uM Calcium Added
1 uM Calcium Added
2 uM Calcium Added
4 uM Calcium Added
7 uM Calcium Added
10 uM Calcium Added
20 uM Calcium Added
25 uM Calcium Added
50 uM Calcium Added

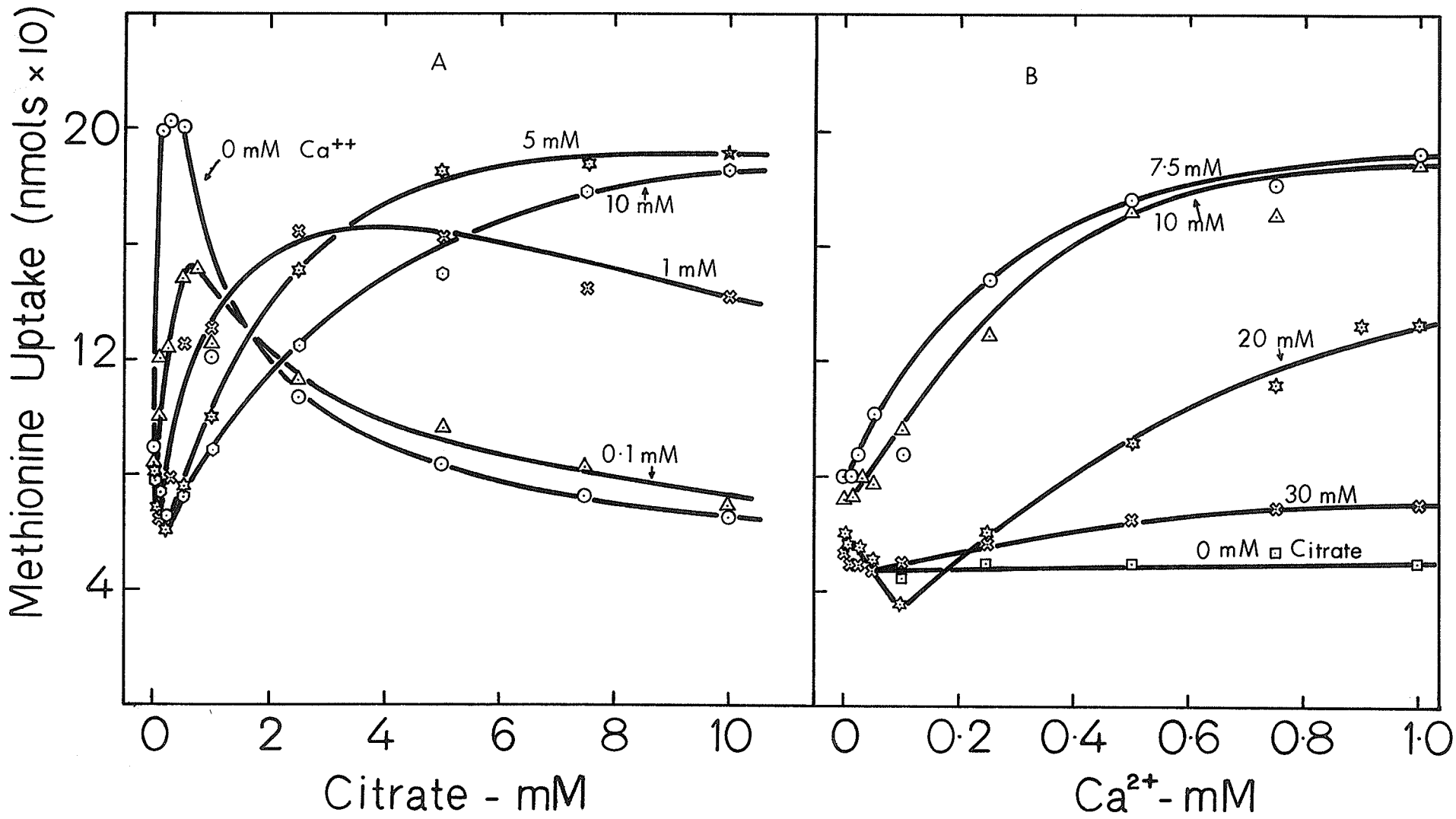
mycelial growth and on sporulation is shown in Fig. 25. Using a modified defined growth medium of Barksdale (58) that lacks both Ca^{++} and EDTA, germination growth and sporulation of the organism were completely retarded at the spore stage. With the addition of trace amounts of Ca^{++}, growth was resumed, but to a limited extent. As the Ca concentration was increased from 1 μM to 10 μM an increasing number of spores germinated and the growth cycle was completed provided the concentration of Ca^{++} added was in excess of 25 μM at a spore density of 10^3 cells per ml. Neither Mg^{++}, Mn^{++}, Sr^{++}, Ba^{++}, Co^{++} and several other bivalent cations used could replace Ca^{++}. In fact, some of them proved to be inhibitory even when used in association with Ca^{++}. Other studies have shown that Sr^{++} and Ba^{++} exclude Ca^{++} uptake by direct competition.

Effect of Calcium and Citrate on Amino Acid Transport

The stimulatory effect of Ca^{++} on amino acid transport is made more apparent by washing the cells with bivalent chelating agents such as citrate or EDTA before studying their transport activity (59). Since the chelating agents cannot penetrate the cell membrane (39) it was concluded that one of the cellular sites

Fig. 26. (a) The effect of citrate on methionine transport when Ca^{++} concentration is varied from 1 μM to 1 mM. The amino acid concentration was 50 μM. The reaction was carried out in buffer A and the cell density, A_{700}, was kept at 0.2.

(b) The effect of Ca^{++} on methionine transport when citrate concentration is is varied from 0.1 mM to 10 mM. Experimental conditions as in Fig. 26 (a) legend.


A
B
Methionine Uptake (nmols x 10)
20
12
4
0 mM Ca++
5 mM
10 mM
1 mM
0·1 mM
7·5 mM
10 mM
20 mM
30 mM
0 mM Citrate
0
2
4
6
8
10
Citrate - mM
0
0·2
0·4
0·6
0·8
1·0
Ca2+ - mM

of Ca^{++} binding must be superficial. The nature of the interaction between Ca^{++}, citrate, and the cell during transport of methionine is described in Fig. 26. In the absence of citrate, Ca^{++} did not affect methionine uptake (Fig. 26 a). However, when citrate was also present, not only was the inhibitory effect of the chelator reversed by Ca^{++}, but transport was significantly stimulated. The stimulatory effect of Ca^{++} was more explicit in the results of Fig. 26 b. In the absence of exogenously added Ca^{++}, methionine transport was maximally stimulated by very low concentrations of citrate, and then transport was inhibited at citrate concentrations greater than 1 mM. Addition of Ca^{++} at concentrations varying from 1 mM to 10 mM led to a reversal of the citrate inhibition but in a novel way. The higher Ca^{++} concentration was less effective at low citrate concentration. This result suggests that citrate may be removing some inhibitory component from the cell surface when used at low concentrations. Therefore, in the absence of exogenously added Ca^{++}, this was observed as stimulation of transport activity in Fig. 26 b. But it is difficult to reconcile that concept with the lack of cellular penetration by citrate and its inhibitory influence on the transport and macromolecular metabolism of protein and nucleic acid precursors (39); and the enhancement shown by Ca^{++} when citrate is present. In

fact, at low concentrations where citrate stimulated transport (Fig. 26 b), Ca^{++} reduced this stimulatory effect considerably. The only logical conclusion that can be drawn, aside from a direct role of Ca^{++} in the transport process, is that Ca^{++} was competing for citrate with the latent inhibitor.

Effect of Cytokinins

Cytokinins, recognized plant growth hormones, can regulate the flow of metabolites and Ca^{++} through the cell membrane of Achlya (60). The chemical, 6hAde, inhibited the transport of all amino acids used in this study except that of tryptophan. The effects of 6hAde (a cytokinin analogue) on the transport of histidine, phenylalanine, methionine and valine are shown in Fig. 27. For each amino acid the K_m value was unaltered and only the V_{max} was affected. The inhibition of valine transport by citrate is shown in Fig. 28. Similar to 6hAde, citrate imposed a noncompetitive inhibitory effect on valine transport.

Binding Properties of Glycopeptide

Removal of a low molecular weight glycopeptide from the cell wall matrix of Achlya germlings by cold osmotic shock treatment resulted in the loss of the

Fig. 27. Lineweaver-Burk plots of the inhibitory effect of 6hAde on (a) histidine, (b) phenylalanine, (c) methionine and (d) valine transport. The concentrations of 6hAde were 0 μM (⊠), 100 μM (△) and 200 μM (○). Experimental conditions as in Fig. 13 legend.

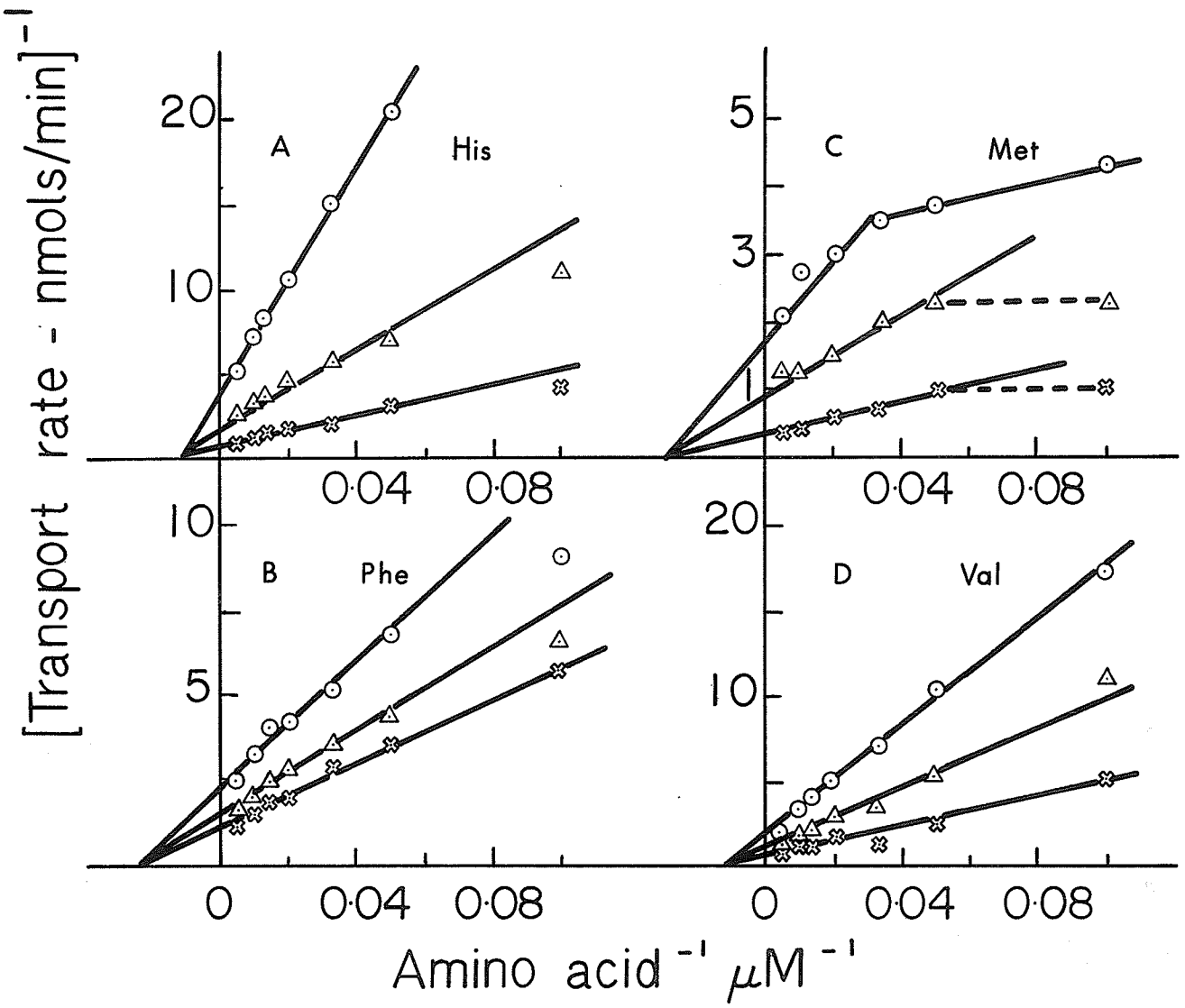
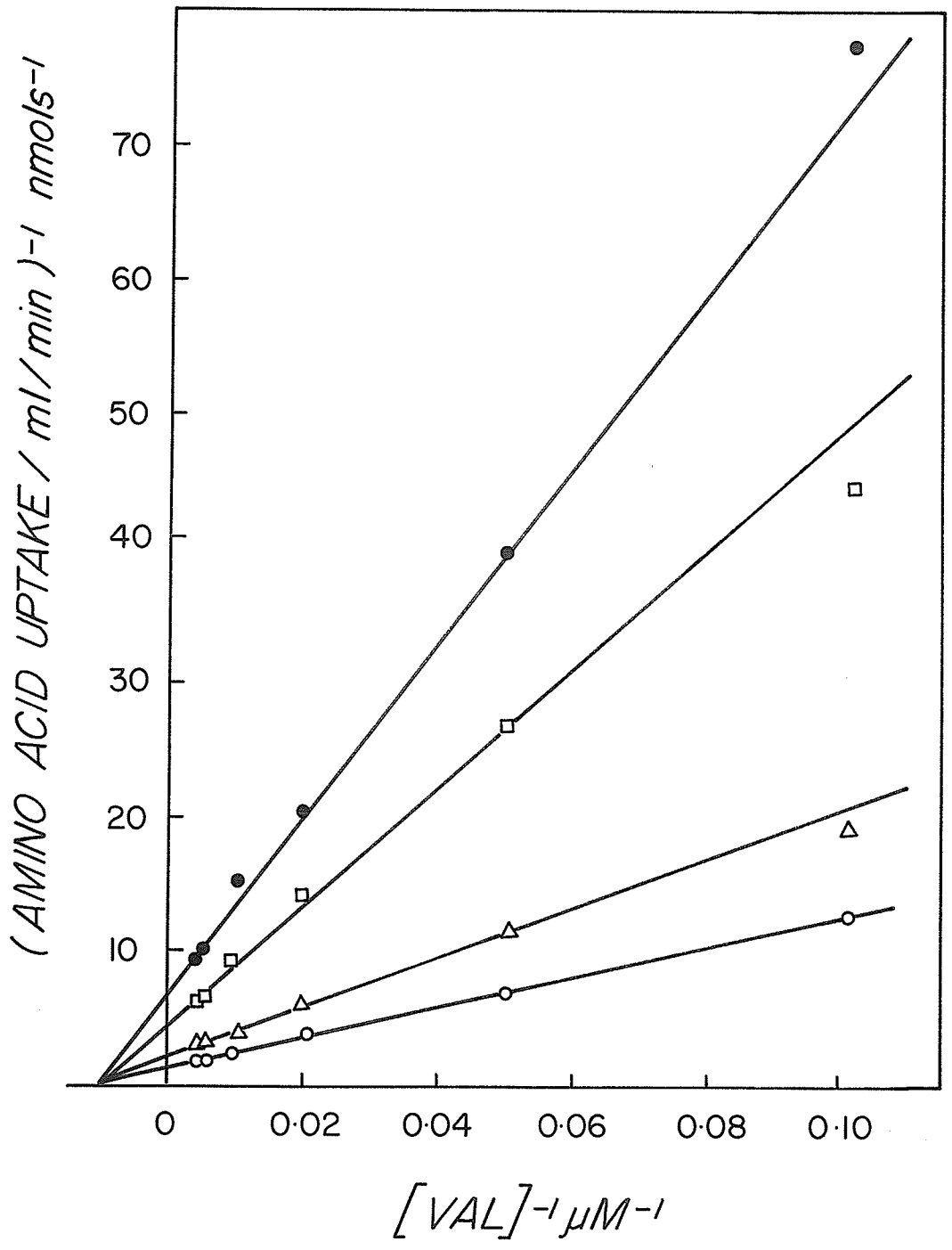

[Transport rate - nmols/min]$^{-1}$
A
His
B
Phe
C
Met
D
Val
20
10
5
3
1
0
0·04
0·08
Amino acid^{-1} μM^{-1}

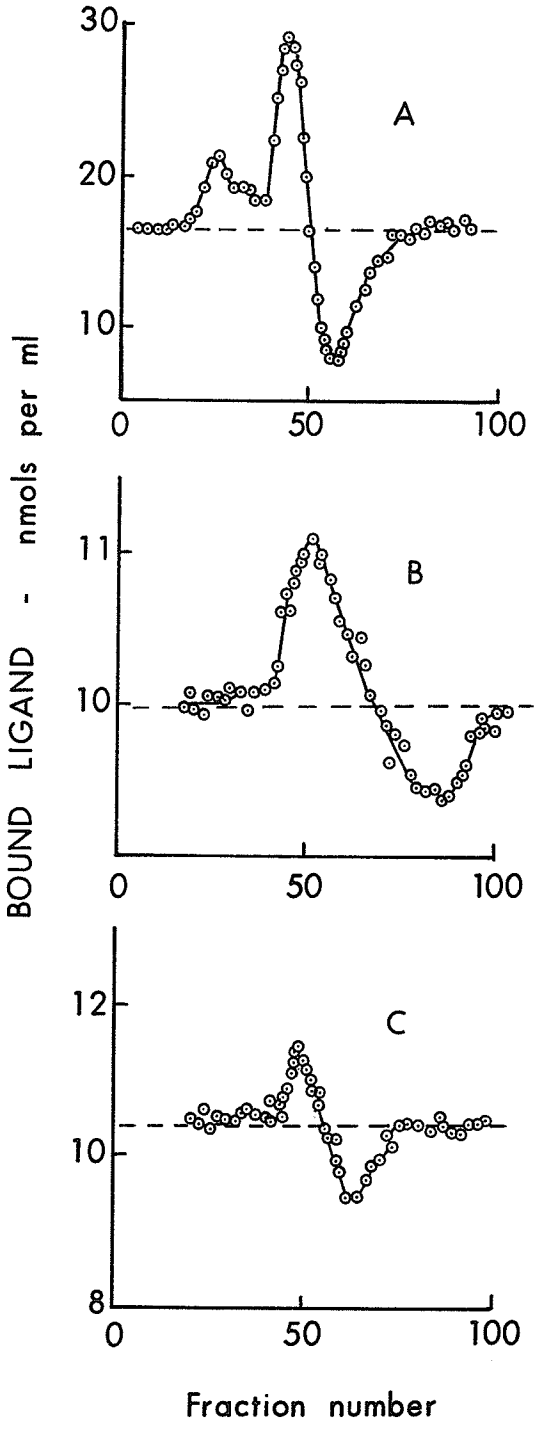
Fig. 28. Lineweaver-Burk analysis of the inhibition of valine transport by citrate. Transport studies were conducted in buffer A and the cell density, A_{700}, was maintained at 0.19. The concentration of citrate were nil (○); 2 mM (△); 5 mM (□) and 10 mM (●).


(AMINO ACID UPTAKE / ml/min)-1 nmols-1
70
60
50
40
30
20
10
0
0·02
0·04
0·06
0·08
0·10
[VAL]-1 μM-1

ability of the cells to actively transport amino acids, to display a cytokinin-dependent binding of tryptophan and indoleacetic acid, and to concentrate Ca^{++} that is easily removed by chelating agents such as EDTA and citrate. The glycopeptide purified as described by LéJohn and Cameron (61, Cameron, L. E. and LéJohn, H.B. manuscript submitted for publication) was examined for its ability to bind a variety of amino acids, auxin BAP, and Ca^{++}. Typical binding curves for Ca^{++}, 6BAP, and indoleacetic acid are shown in Fig. 29. Among the several amino acids examined, tryptophan showed the highest affinity for the glycopeptide.

Fig. 29. Equilibrium binding of (a) $^{45}Ca^{++}$ (b) BAP-^{14}C and (c) IAA-^{3}H to purified Achlya calcium binding glycopeptide using a column of Sephadex-G50 beads. The column (30 cm x 2.5 cm) was equilibrated with the particular radioisotope in 1 mM potassium phosphate buffer, pH 6.5, before applying a constant amount of glycopeptide (130 µg based on protein content) to the column. Equal volumes (0.94 ml) of fraction were collected and 100 µl samples counted for radioactivity. Results are expressed as n mols bound per ml of fraction. The dotted lines represent equilibrium position for binding of ligands to the glycopeptide.

(Data obtained from Cameron, L. E. Ph.D. Thesis Univ. of Man. 1975)


30
20
10
A
0
50
100
11
10
B
0
50
100
12
10
C
8
0
50
100
BOUND LIGAND - nmols per ml
Fraction number

D I S C U S S I O N

DISCUSSION

The results of this study demonstrated that amino acids were actively transported by several different transport systems of Achlya. The transport of most amino acids showed saturation-type kinetics, indicating that they were being mediated by "carriers". The protein nature of the "carriers" was inferred from pH and temperature studies and their susceptibility to inhibition by sulphydryl agents. Moreover the adverse effects of energy uncouplers and electron inhibitors on the transport process revealed that transport of these amino acids was an energy-dependent phenomenon. The exit patterns of six amino acids confirmed the involvement of "carriers" as well as metabolic energy expenditure in the transport of amino acids.

The entry of amino acids in Achlya was achieved by specific transport systems. On the basis of competition kinetic studies there were at least nine categories of amino acid permeases in this coenocytic fungus. The transport systems evinced were as follows:

Alanine - Glycine Permease.

This system is believed to be slightly different from that seen in Escherichia coli W membrane vesicles

(20), where a single common carrier for alanine, glycine, and serine is present. The transport of glycine in Achlya was not affected by serine (Fig. 17 a). Phenylalanine which shared the neutral amino acid permease of Neurospora crassa (33) inhibited glycine transport in Achlya, in a non-competitive manner. Similar to Achlya, Streptococcus faecalis (45) is known to display an alanine-glycine permease system.

Serine - Threonine Permease

Unlike Pseudomonas aeruginosa (46), Achlya possesses a threonine transport system that is also shared by serine. Alanine had no effect on threonine uptake but phenylalanine imposed a non-competitive inhibitory effect on the transport of this neutral amino acid. Like Achlya, Staphylococcus aureus (27) is known to transport serine via the threonine permease.

Leucine - Isoleucine - Valine (LIV) Permease

Both leucine and isoleucine inhibited valine transport in Achlya germlings. A common permease for these three branched-chain amino acids has been recog-

nized in Escherichia coli K12 (22), as well as in yeast (47). The neutral amino acid, threonine, does inhibit valine transport in a non-competitive fashion (Fig. 20).

Phenylalanine - Tyrosine - Tryptophan (PTT) Permease

Kinetic studies showed that the aromatic amino acids phenylalanine and tyrosine may share a common uptake system with tryptophan. A similar observation has been made for Salmonella typhimurium (48) and Comamonas sp. (49). In Achlya the PTT system appears to overlap with the LIV transport system because valine uptake was competitively inhibited by phenylalanine, tyrosine and tryptophan. Also leucine, isoleucine, and valine competed with phenylalanine uptake. The inhibitory constant (K_i) of phenylalanine for valine transport was identical to its K_m value, suggesting that the same permease may be mediating the transport of these two amino acids. This sharing of a transport system by an aromatic and an aliphatic amino acid has been reported for Neurospora crassa (50). Germinated conidia of this fungus were shown to contain a tryptophan transport system which was also used by leucine and phenylalanine. Furthermore, in mammalian tissues leucine and phenylalanine have been found to be transported by a common permease (17).

Aspartic - Glutamic Acid Permease

This system was observed in Neurospora crassa (35), Mycobacterium avium (51), and Aspergillus nidulans (38). In Achlya several neutral amino acids inhibited glutamate transport. For example, phenylalanine was a competitor for this acidic amino acid transport system, but the inhibition pattern was non-competitive. This can be explained if one assumed that a competition for the energy supply was the cause (38).

Lysine - Arginine - Histidine (HLA) Permease

Unlike arginine, histidine was a weak competitor for the lysine permease which exhibited biphasic properties. Grenson (29) observed similar biphasic features for the lysine transport system in yeast. A basic amino acid permease for lysine, arginine and histidine has been seen in Penicillium chrysogenum (32).

Asparagine - Glutamine Permease

Unlike the glutamine transport system of E. coli (52), Achlya contains a permease for both asparagine and glutamine. Glutamine inhibited asparagine competitively but the degree of inhibition was not very pronounced

(Fig. 24 b). This may be due perhaps, to the higher affinity shown by asparagine for the transport system (Table 1). Tyrosine and alanine exerted a non-competitive inhibitory effect on asparagine transport and the reason for this is not clear. Interference with energy supply may be invoked as in previous cases.

Proline Permease

In Agrobacterium tumifaciens (53) proline is known to share a transport system that is common to many amino acids. In contrast, Achlya accumulated proline by a distinct permease. Methionine was the only other amino acid which inhibited proline uptake but the nature of this inhibition was non-competitive (Fig. 12 c). Kaback and Stadtman (21) have shown that the membrane vesicles of Escherichia coli W6 transported proline by a specific permease. Saccharomyces chevaliere (54) possesses a transport system which manifested a high degree of specificity for proline.

Methionine Permease

From kinetic studies it was implied that there were possibly two transport systems for methionine in

Achlya. The biphasic Lineweaver-Burk plot for methionine transport resembles the pattern seen in yeast (30) which has a low affinity (K_m of 7.7×10^{-4}M) and a high affinity (K_m of 1.2×10^{-5}M) system. A similar low affinity saturation value was obtained for Achlya ($S_{(0.5)}$ of 2.0×10^{-4}M) and a high affinity system ($S_{(0.5)}$ of 5.13×10^{-6}M). Benko, Wood and Segel (55) have studied the methionine transport system for Penicillium chrysogenum that also displays biphasic properties. The Achlya methionine permease is uniquely specific, showing absolutely no overlap with the other permeases although this amino acid itself inhibited the transport activities of all other amino acid transport systems. This high degree of specificity reflects perhaps a physiological feature which is that Achlya has an absolute requirement for organic sulphur in growth.

Cysteine permease

This transport system was not as clearly resolved in Achlya as it has been in Staphylococcus aureus (27). Assuming that there is a dire need for sulphur in Achlya then cysteine ought to share the methionine permease, or at least, show similar transport characteristics to

methionine. Kinetic analysis revealed that both proline and cysteine enhanced methionine uptake (Fig. 24 a). Stimulation of one amino acid transport by the extracellular presence of another has been termed "Cis Stimulation" (56). It should be noted that under these assay conditions cysteine was being rapidly oxidized to cystine. This could affect transport systems that may require their proteins in the reduced state.

Under these growth conditions Achlya germlings have specific permeases which participate in amino acid transport. Under varying nutrient conditions it is likely that the specificities of various transport systems may alter as seen in Penicillium chrysogenum (57). It is also possible that at various stages of the growth cycle, Achlya may alter the characteristics of some of its amino acid transport systems and these conclusions about categories of transport permeases may then need revision. (See "Problems relating to Cell Physiology" below).

Ca^{++} Influence on Amino Acid Transport

These results show that the active transport of amino acids depend upon the availability of Ca^{++}. The sequestering of this essential bivalent cation by citrate resulted in a non-competitive inhibition of amino acid

transport. This pattern of citrate inhibition implies that the amino acids and the chelating agent were not interacting directly with the amino acid permeases themselves, but through some intermediary that regulates all amino acid permease activities. Citrate inhibition can be related to Ca^{++} deprivation and arrest of all metabolic energy-linked functions that depend upon Ca^{++} for activity. Such a metabolic energy-utilizing system may be a membrane (Na^{+}, K^{+}) - linked ATPase that is activated by Ca^{++}.

Influence of cytokinin on amino acid transport

Both the cytokinin, BAP, and the auxin, IAA, were capable of binding to a cell wall glycopeptide which sequestered large amounts of Ca^{++}. The pattern of inhibition of amino acid transport by 6hAde indicated that cytokinin did not interact with the amino acid permease itself. The plant growth hormone may affect some basic membrane process upon which amino acid uptake depends. The antagonistic effect of cytokinin on Ca^{++} bound to the cell wall glycopeptide (61) can explain the inhibition of amino acid transport by 6hAde and other cytokinins and analogues but this would require that the glycopeptide regulates amino acid transport which it does not seem to do. The only reasonable

conjecture is that calcium may represent the common factor through which citrate and cytokinins inhibit the transport of amino acids in Achlya. Whereas citrate can chelate Ca^{++}, the cytokinins can release Ca^{++} from the fungal cell wall glycopeptide but not by chelation. Cytokinins can also directly affect an energy-supply system on the cell membrane and consequently reduce the cell's ability to concentrate metabolites. It would appear that this energy-generating system is where Ca^{++} plays a vital role and citrate inhibits metabolite uptake by removal of the essential Ca^{++} from the membrane and the glycopeptide. Cytokinins do the same and thereby cause an under-expenditure of energy for transport activities.

Problems relating to Cell Physiology

To account for V_{max} differences between some amino acids which share a common permease one ought to consider some of the following factors:

(i) The transport systems may not have been uniformly dispersed per experimental population (day to day) e.g. specific versus general permeases;

(ii) Cell physiology changes resulting from

(a) poor synchrony

(b) altered sporulation conditions through nutrient depletion, pH and temperature fluctuations

(c) possibly light effects;

(iii) Cell number and viability were not coincident and may not be standard for all populations; and (iv) Defined medium was not used to grow the cells prior to germination of spores used in the transport studies.

The importance of all or any of the above cannot be underemphasized.

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