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L-LEUCINE UPTAKE BY SUSPENSION CULTURED CELLS OF *AMMI VISNAGA*

by

© LORITA ROCHELLE BABB

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "L-LEUCINE TRANSPORT BY SUSPENSION-CULTURED CELLS OF *Ammi visnaga*" submitted by LORITA ROCHELLE BABB in partial fulfilment of the requirements for the degree of Master of Science.

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Date June 30, 1975.

ABSTRACT

Uptake of L-leucine by suspension cultured cells of *Ammi visnaga* was investigated. The rate of L-leucine uptake was found to initially increase with the age of the cell culture. The maximum rate of uptake was reached 3-5 days after inoculation, then declined rapidly as cells reached the stationary phase of growth. Nitrogen starvation of cells for up to 24 h resulted in an increase of approximately 10-fold in the L-leucine transport rate.

L-leucine uptake was found to partially fulfill the criteria for an active transport system. Uptake was saturable, indicating carrier mediation. Double reciprocal plots for L-leucine uptake by both nitrogen starved and nitrogen sufficient cells were biphasic. The energy dependence of L-leucine uptake was demonstrated by strong temperature dependence and by sensitivity to dinitrophenol, oligomycin and azide. Uptake against a concentration gradient could not be demonstrated conclusively, since 32% of the transported L-leucine was incorporated into protein and smaller amounts were recovered from other cell fractions. Uptake appeared to be vectorial however, as only a low rate of efflux could be detected.

Uptake of L-leucine was reduced in the presence of a variety of neutral amino acids. The specificity of the L-leucine carrier of *A. visnaga* appeared to be intermediate between the specificities reported for bacterial and fungal transport systems.

The soluble amino acid pool of *A. visnaga* cells decreased during

periods of nitrogen-starvation comparable to those used in determination of transport rates, suggesting a regulatory role for one or more amino acids. Preloading nitrogen starved cells with ammonium sulfate or L-leucine caused a rapid decay of transport activity. Transport of L-leucine by starved and unstarved cells showed different degrees of sensitivity to the inhibitors oligomycin, sodium azide and dinitrophenol, although the effects of the latter two compounds were similar for both groups of cells. These data may reflect the existence of different carriers for L-leucine under different nutritional conditions.

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ABBREVIATIONS

AIB	=	α-amino isobutyric acid
ATP	=	adenosine triphosphate
cpm	=	counts per minute
DNP	=	2,4-dinitrophenol
2,4-D	=	2,4-dichlorophenoxyacetic acid
dpm	=	disintegrations per minute
K_m	=	Michaelis-Menton constant
MES	=	2(N-morpholino)ethane sulfonic acid
PCV	=	packed cell volume
(U)	=	uniformly labelled
V_{max}	=	maximal velocity

I. LITERATURE REVIEW

A. Suspension Culture of Plant Cells

1. General

The first suspension cultures of plant cells that were capable of repeated subculture were isolated about twenty years ago (Muir *et al.*, 1954). Shortly afterwards Nickell (1956) demonstrated the feasibility of growing such cell suspensions as if they were cultures of "microorganisms" and is credited with being the first to utilize suspension cultured cells for biochemical investigations (Nickell and Tulecke, 1959, 1961).

The advances in methodology and application that have occurred in the intervening years are discussed in detail in the comprehensive volume edited by Street (1973a). The present review will deal only with those topics that bear directly on the present work.

2. Growth of plant cells in suspension culture

Most biochemical investigations of suspension cultured cells to date have been conducted with batch suspension cultures. This technique involves the isolation of an inoculum of cells in a finite volume of nutrient medium in a system that is closed except for exchange of gases with the outside air (King and Street, 1973). Cells grown in batch cultures generally remain parenchymatous, with thin walls and large vacuoles (Yeoman and Street, 1973).

The rate of cell growth in such a system depends on the source of tissue, composition of the medium, size of the inoculum and many other factors. Growth may be monitored by changes in dry weight, fresh weight, cell number, packed cell volume, DNA content and nitrogen or carbohydrate accumulation. Doubling times measured by these means for various tissues range from 20 to 48 hours (Dougall, 1972). These values are greater than those of 21-27 hours obtained for cultured, intact tissues (Phillips and Torrey, 1973).

King and Street (1973) have compared the growth pattern revealed by the techniques given above to the classical lag, log, and stationary phases of bacterial growth. The drawbacks to this interpretation are discussed by Rose and Martin (1974). Several lines of evidence suggest that batch cultures possess two discrete growth phases. Henshaw *et al.* (1966) and Fletcher and Beevers (1970), on the basis of cell counts and measurements of cell diameter, distinguished a cell division phase and a cell expansion phase. These phases were characterized primary by changes in ^{14}C-acetate metabolism. Other workers, who have based their conclusions on chemical analyses, have recognized a "cytoplasmic growth phase" and a "maturation phase" (Rose *et al.*, 1972; Rose and Martin, 1974). The cytoplasmic phase is characterized by a high rate of nitrogen metabolism relative to the increase in dry weight. In contrast, the maturation phase exhibits large increments in dry weight relative to increments in cell nitrogen. The two phases may additionally have different pH optima (Nesius and Fletcher, 1973).

3. Nitrogen metabolism of suspension cultured plant cells

Most studies of nitrogen metabolism in suspension cultured cells have dealt with the suitability of various nitrogen-containing compounds as nutrients. In general, a combination of nitrate and ammonium ions is the best nitrogen source for *in vitro* plant cell growth (Gamborg *et al.*, 1968), although some species may utilize nitrate alone (Filner, 1966). Ammonium ions alone will support growth if the culture medium contains an organic acid that is a Krebs Tricarboxylic Acid Cycle intermediate (Gamborg and Shyluk, 1970).

The response of cultured tissue to amino acids added to the medium is variable. Some amino acids may stimulate growth. A number of cell lines, for example, require casein hydrolyzate in the medium for optimal growth. L-glutamine and L-alanine will substitute for ammonium ions to alleviate the requirement of soybean for reduced nitrogenous compounds (Bayley *et al.*, 1972). Cells of *Acer pseudoplatanus* release amino acids into the culture medium shortly after inoculation (Simpkins and Street, 1970). These compounds may be responsible for the stimulation of cell growth by "conditioned medium", that is, medium in which other cells have previously been incubated.

In contrast, the presence of millimolar concentrations of some amino acids, including L-leucine, is inhibitory to growth of soybean (Gamborg, 1970) and Paul's Scarlet rose (Dougall, 1965). L-leucine has been shown to repress nitrate reductase in tobacco cultures (Filner, 1966; Heimer and Filner, 1970), and it has been suggested that such a repression is the mechanism of growth inhibition.

Data from several sources indicate that suspension cultured cells undergo a phase of rapid nitrogen metabolism shortly after inoculation and before large increments in dry weight are achieved (Givan and Collin, 1967; Verma and Marcus, 1974; Rose and Martin, 1974). Total insoluble (Given and Collin, 1967) and soluble nitrogen (Simpkin and Street, 1970) content per cell reach a peak early in the growth cycle, then decline rapidly. Thus, in batch cultures nitrogen metabolism metabolism uncoupled from cell division and the mean composition of cells changes continuously (King and Street, 1973).

The biosynthesis of several amino acids, including L-leucine, by suspension cultured cells has been investigated. Dougall (1965, 1966) and Dougall and Fulton (1967a,b) studied the incorporation of ^{14}C-glucose into protein in the presence of various amino acids. Evidence was obtained that (1) the synthesis of L-leucine proceeds via α-keto-isovaleric acid and (2) that exogenous L-leucine is probably utilized preferentially to endogenous L-leucine as suggested by Oaks (1965a,b).

Fletcher and Beevers (1970), using ^{14}C-acetate, established that protein precursor pools of amino acids are apparently small in rapidly growing cells of Paul's Scarlet rose. This was especially true for L-leucine, which had a very small pool compared to other amino acids. In the same study, the precursor pools of older cells were found to be either expanded or in ready equilibrium with the same constituent elsewhere in the cells.

The two preceeding studies, as well as others (*e.g.* Maretzki, Nickell and Thom, 1969; Maretzki, Thom and Nickell, 1969; Dougall, 1970), have established that the intermediates of amino acid metabolism in cultured plant cells are the same as those of intact plants.

4. Solute transport by suspension cultured plant cells

Solute transport by cultured plant cells has not been extensively studied. Active transport systems have, however, been reported for glucose (Maretzki and Thom, 1972a,b) and L-histidine

(Maretzki and Thom, 1970) in sugarcane, sulfate in tobacco (Hart and Filner, 1969; Smith, 1974, 1975) and L-alanine in soybean (King and Oleniuk, 1973). Energy-dependent proton efflux (or hydroxyl influx) has been described for suspension cultures of bean, rye, and sycamore leaf cells (Fisher and Albersheim, 1974).

B. Leucine Transport Systems in Microorganisms

1. Introduction

The existence of solute transport systems in the plasma membrane of cells has long been recognized. It is generally believed that transport systems provide a mechanism for regulating the internal composition of cells, as well as for capturing scarce but potentially useful compounds from the surroundings.

Amino acid transport has been studied extensively in microorganisms and animal cells and has been found to be a matter of striking complexity. Recent developments in studies of amino acid transport in animal tissues are reviewed by Heinz (1972) and Sanford and Smyth (1972).

The process of amino acid transport is best understood in microorganisms, mainly as a result of their suitability as experimental organisms. In particular, the availability of transport mutants has been an important factor in revealing details of problems such as energetics and mechanisms that cannot be easily resolved by any other method. Reviews of transport in bacteria (Kaback, 1972), bacterial membrane vesicles (Kaback, 1973), and microorganisms in general (Oxender, 1972) have recently become available.

2. Kinetics

The experimental criteria for distinguishing among various types of membrane transport are discussed by Stein (1967). By his criteria, namely saturability, substrate specificity, and competitive inhibition by structurally similar compounds, the transport of natural amino acids appears to be carrier-mediated in most microorganisms.

Plots of the initial rate of a mediated transport process *vs* substrate concentration usually show a hyperbolic curve approaching a maximum similar to the Michaelis-Menton curve observed for enzymatic reactions. Double reciprocal (Lineweaver-Burke) plots of uptake data show a straight line if transport conforms strictly to Michaelis-Menton kinetics. While most transport systems conform to the Michaelis-Menton relation, deviations have been observed. At high concentratons, deviations from linearity may occur, presumably resulting from the existence of a non-saturable diffusion component (Heinz, 1972). In some microorganisms, however, this component exhibits stereospecificity, indicating that a mediated transport system with a high K_m may be operating. A number of Lineweaver-Burke plots for transport systems have been unmistakably non-linear, even at moderate concentrations of amino acids. The transport data obtained for L-valine (Piperno and Oxender, 1968) and L-leucine (Guardiola *et al.*, 1974a) with *Escherichia coli*, L-histidine with *Salmonella typhimurium* (Ames, 1964) and basic amino acids with yeast (Grenson, 1966) have been given biphasic reciprocal plots. These have been generally interpreted as evidence of two distinct transport systems, although alternative interpretations are possible (Nissen, 1974).

Michaelis-Menton constants (K_m) for L-leucine uptake by *E. coli* range from 0.2-4 μ M (Piperno and Oxender, 1968; Claus *et al.*, 1973; Wood, 1974). K_m values for L-leucine uptake by membrane vesicle preparations are generally higher, ranging from 1-18 μ M (Kaback, 1973). Values for fungal systems are still greater, *e.g.*, 110 μ M for *Neurospora* (Pall, 1970), 30-1000 μ M for different strains of yeasts (Bussey and Umbarger, 1970), and 10 μ M for *Penicillium chrysogenum* (Benko *et al.*, 1969). The above values are as given in Table 1.

3. Specificity

Generalization about interactions between the different amino acid substrates of carrier systems is complicated by the large number of different systems proposed for various tissues. The substrate specificities that have been suggested for a number of animal cell and bacterial systems are summarized by Sanford and Smyth (1972).

Carrier specificity for L-leucine has been extensively studied in *E. coli*. L-leucine transport is mediated by at least three distinct systems (Claus *et al.*, 1973; Wood, 1974) and perhaps has as many as five (Guardiola *et al.*, 1974a,b). Penrose *et al.* (1968) first characterized a transport activity that is sensitive to osmotic shock treatment and specific for L-leucine, L-isoleucine, L-valine and L-threonine. Subsequently, a second osmotic shock-sensitive system transporting L-leucine only was detected (Furlong and Weiner, 1970). Both systems are repressed by the presence of L-leucine in the growth medium.

The observation that leucine transport activity was retained in osmotically shocked cells lead to the discovery of a third transport system, which mediates uptake of L-leucine, L-valine and L-isoleucine (Wood, 1974). The existence of this system has been verified by use of

TABLE 1. K_m Values Reported for Transport of Amino Acids by Microorganisms and Plant Cells

Organism or tissue	Amino Acid	K_m value (μM)	Reference
E. coli	L-leucine	0.2 - 4	(Wood, 1974; Claus *et al.*, 1973)
E. coli (membrane vesicles)	L-leucine	1 - 8	(Kaback, 1973)
Saccharomyces sp.	L-leucine	30 - 1000	(Bussey and Umbarger, 1970)
Neurospora crassa	L-leucine	120	(Pall, 1969)
		4 (starved)	
Penecillium chrysogenum	L-leucine	10	(Benko *et al.*, 1969)
Pea leaf fragments	L-leucine	~2.4	(Cheung and Nobel, 1973)
Barley leaf strips	AIB	1000	(Rheinhold *et al.*, 1970)
Susp. cultured sugarcane	L-arginine	100	(Maretzki and Thom, 1970)
	L-lysine	2450	
Susp. cultured soybean	L-alanine	2.5	(King and Oleniuk, 1973)

specific transport mutants (Rahmanian *et al.*, 1973). This shock-resistant system is not repressed by the presence of L-leucine in the medium, and unlike the shock-sensitive systems, is found in membrane vesicle preparations.

Transport systems in fungi appear to be generally broader in specificity for amino acids than those of bacteria, although a number of highly specific transport systems have been described for amino acids other than L-leucine. Under conditions of rapid growth and nitrogen sufficiency yeast (Grenson *et al.*, 1966; Grenson, 1966; Gits and Grenson, 1967; Magaña-Schwenke and Schwenke, 1969), *Penicillium* (Benko *et al.*, 1967) and *Neurospora* (Pall, 1970) possess systems with narrow specificity for certain amino acids. In old, slow-growing cultures, or under starvation conditions, yeast (Gits and Grenson, 1967, 1969), *Penicillium* (Benko, 1967, 1969) and *Neurospora* (Pall, 1970) have additional transport systems that mediate the uptake of a wide variety of amino acids. It has been suggested (Pall, 1970) that the two categories differ in function: The specific systems serve mainly to provide certain amino acids for protein synthesis, while systems that develop under starvation conditions or in old cultures primarily provide a source of reduced nitrogen or carbon.

L-leucine is transported via a general amino acid permease in starved mycelia of *P. chrysogenum* (Benko *et al.*, 1967). L-leucine uptake by nitrogen sufficient mycelia apparently occurs by free diffusion (Hunter and Segel, 1973a). Under starvation conditions, L-leucine influx is followed by efflux of α-ketoisocaproic acid into the incubation medium and is inhibited by preincubation with ammonium chloride or L-leucine (Hunter and Segel, 1973a). Activity of the general amino acid permease (and L-leucine uptake) is strongly but reversibly inhibited by anaerobic conditions and by DNP (Hunter and Segel, 1973b). No constant correlation between transport activity and intracellular levels of ATP was observed.

4. Mechanisms of amino acid transport

The membrane components responsible for transport of amino acids have not been isolated. It has been found, however, that low molecular weight proteins are released from gram-negative bacteria (Furlong and Weiner, 1970; Claus *et al.*, 1970; Wood, 1974) and yeast (Vorisek, 1973) by osmotic shock, with a concomitant loss of L-leucine transport activity. The released proteins do not possess enzymatic activity, but are believed to be the substrate recognition factors of transport systems. The evidence for this role is indirect but of impressive volume. The most convincing observations, as discussed by Rosen and Heppel (1973) are:

1) Osmotic shock causes simultaneous loss of transport activity and appearance of binding activity in the shock fluid.
2) Binding proteins are localized in the cell envelope where transport factors are expected to reside.
3) Parallel repression of transport activity and binding protein synthesis and parallel induction of both have been observed.
4) Transport activity and binding by the released proteins exhibit similar specificity.
5) K_m values for binding and for transport are similar.

Although the molecular basis of amino acid transport has not been elucidated, several general models have been proposed. The term "permease" is prevalent in literature on amino acid transport. The permease concept was originally proposed for β-galactoside uptake by *E. coli* by Cohen and Monod (1956) and later revised by Kepes and Cohen (1961). The revised permease system model invokes two membrane components: (i) an inducible, stereospecific protein permease that combines with external substrates to form a complex and (ii) a transporter that reacts with the permease-substrate complex and an energy source to form a substrate-transporter complex that passively diffuses across the cell membrane. The first step is the rate-limiting reaction. Experimental evidence so far has not supported all features of this model (Oxender, 1972).

An alternative, widely cited model (*e.g.*, Penrose *et al.*, 1970; Hunter and Segel, 1973a) proposes a three-step process consisting of: (i) binding of the solute to a macromolecular mediator in the membrane (ii) translocation of the substrate-mediator complex across the osmotic barrier and (iii) coupling of the process to metabolic energy by conversion of the mediator to a "high energy" form with low affinity for the substrate.

A number of thermodynamic considerations and experimental facts argue against models that invoke mobile carriers. Singer (1974) has, therefore, proposed a generalized transport model invoking a protein-lined pore that spans the cell membrane. A conformational change in the pore proteins translocates a solute binding site across the membrane. An extension of this model provides a possible explanation for the role of the binding proteins with the integral pore proteins discussed above, whereby association of the binding proteins with the integral pore proteins is necessary for the conformational changes that "open" the pore. No direct evidence is yet available to support this model.

5. Energetics

Much experimental effort has been expended to identify the energy donors for amino acid transport and to elucidate the mechanism of the coupling of metabolic energy to transport. Early studies of bacterial transport implicated the high-energy phosphate bond in the coupling process (Schachter, and Minlin, 1969). Subsequent studies with membrane vesicles (see Kaback, 1974) and mutants (Klein and Boyer, 1972; Or *et al.*, 1973) suggested that ATP most likely played an indirect role in transport. Results obtained with uncouplers led to the suggestion that the undefined "high energy membrane state" generated by electron transport is the immediate energy donor for amino acid uptake (Klein and Boyer, 1972; Pavlovsova and Harold, 1969).

Recent work by Berger (1973) and Berger and Heppel (1974) indicates that different modes of energy coupling may exist for different transport systems, depending on whether or not they are sensitive to osmotic shock. Osmotic shock-sensitive transport systems of *E. coli* appear to derive energy directly from ATP, while osmotic shock-resistant systems are driven by the high-energy membrane state. Wood (1974) has partially confirmed this pattern for the shock-sensitive and shock-resistant leucine transport systems of *E. coli*.

Two models for the coupling of metabolic energy to transport are prominent at the present time. Available experimental evidence does not yet permit a clear choice between the two. Kaback and Barnes

(1972), on the basis of membrane vesicle studies, have proposed a model that visualizes amino acid carriers as recyclable electron transport intermediates. Redox changes, occurring as the carriers mediate the flow of reducing equivalents to oxygen, result in translocation of a carrier-substrate complex to the inner membrane surface. Shortcomings of this hypothesis are discussed by Kaback (1974) and Mitchell (1972).

An alternative explanation of energy coupling, which at this time appears to be best supported by experimental evidence, is the chemiosmotic model of Mitchell (1972). According to this model, oxidation of electron donors, or hydrolysis of ATP is accomplished by expulsion of protons into the external medium, resulting in a pH or electrical gradient, or both, across the membrane. A proton-motive force is thus created that tends to pull protons back across the membrane and provides the driving force for solute uptake. Transport of one solute molecule and one or more protons is proposed to occur via a bifunctional carrier. The early evidence for and the implications of this model are discussed by Harold (1972). More direct evidence supporting Mitchell's hypothesis is provided by recent studies (Hirata *et al.*, 1973; Asghar *et al.*, 1973; West, 1973; Hunter and Segel, 1973). Kaback (1974) has discussed those aspects of the model that are not consistent with experimental data.

6. Regulation

Regulation of amino acid transport systems has been studied most extensively in fungi, perhaps because fewer systems are involved than in bacteria. The amino acid transport activity of many fungal systems depends on the availability of suitable nitrogen or carbon sources in the growth medium. Nitrogen starvation frequently results in an increase in transport activity (Benko *et al.*, 1967, 1969; Hackette *et al.*, 1970; Magaña-Schwencke and Schwencke, 1969; Pall, 1969; Grenson *et al.*, 1970) as a result of derepression or deinhibition. Regulatory compounds have not been conclusively identified, but amino acids and ammonium ions are the most likely candidates. Amino acid pools have been observed to decrease with starvation (Bussey and Umbarger, 1970). Transinhibition, which is generally assumed to result from a direct interaction of the intracellular substrate with a component of the transport system, or feedback inhibition by a metabolite of a transport substrate have been suggested as regulatory mechanisms for several systems (Grenson, 1966; Grenson *et al.*, 1966; Gits and Grenson, 1967; Grenson and Hou, 1972; Benko *et al.*, 1967, 1969; Hunter and Segel, 1973a; Pall, 1971; Ring *et al.*, 1970; Bussey and Umbarger, 1970).

Transport of L-leucine by the general amino acid permease of *P. chrysogenum* has been found to be only partially regulated by transinhibition (Hunter and Segel, 1973a). Transport activity was significantly decreased in the presence of substrate, as a result of degradation of a permease component or induction of a regulatory protein such as a transaminase.

Ammonium ions have been suggested as the regulator of the acidic amino acid transport systems of *Aspergillus nidulans* (Robinson *et al.*, 1973). No correlation between the pool size of any amino acid and transport activity was observed. Addition of ammonium ions to conidia resulted in a rapid decrease in transport activity. Studies of mutants suggested that ammonium ions specifically repressed synthesis of the permease.

The mechanism for regulation of amino acid transport in bacteria may differ from that in fungi. Amino acid pools increase in several bacteria under starvation conditions (Ames, 1964; Mandelstam, 1960), although transport rates may also increase. In contrast, L-leucine transport by *Pseudomonas aeruginosa* decreases with starvation, although the L-leucine pool decreases as well (Kay and Gronlund, 1969). At least one transport system for L-leucine in *E. coli* is repressed by the presence of L-leucine in the medium (Wood, 1974). However, detailed studies of L-leucine transport mutants of *E. coli* indicate that the regulation of L-leucine uptake by the multiple systems that exist in this and other bacteria is exceedingly complex (Guardiola *et al.*, 1974a,b).

C. Transport of Leucine and Other Amino Acids by Plant Cells

1. General

Although transport of inorganic nutrients has been extensively studied in plant tissues, transport of amino acids and other organic solutes has received little attention. In fact, few detailed studies of amino acid transport are available. However, in conjunction with studies of other processes uptake has been monitored (*e.g.*, Osmond and Harris, 1971; Leonard and Hanson, 1972a,b).

2. *Chlorella*

Uptake by *Chlorella vulgaris* of L-methionine at external concentrations of 0.1-10.0 μmoles/ml has been found to be energy dependent (Schrift, 1966). Analysis of the accumulated radioactivity showed that more than 90% was in the form of L-methionine suggesting that uptake against a concentration gradient was occurring.

L-proline uptake by *Chlorella pepenvidosa* is most active in growing cultures after depletion of glucose and near-depletion of nitrate from the growth medium (McNamer and Stewart, 1973). L-proline uptake by stationary phase cultures required glucose and occurred only after a long lag phase, suggesting that the accumulation of carbohydrates in the cells regulates transport.

3. Storage tissue

Birt and Hird (1956) were the first to demonstrate the mediated transport of L-leucine and other amino acids in higher plant tissue. The rates of uptake of amino acids by carrot slices were found to increase with prolonged washing of the tissue. Subsequent studies (Birt and Hird, 1959a,b) established that L-leucine was transported against a concentration gradient and that uptake was sensitive to metabolic inhibitors. Competition experiments suggested that carrier specificity was quite broad, though greater affinity for amino acids with lipophilic side chains was observed. The carrier affinity was also greater for L-amino acids than for the corresponding D-amino acids.

4. Hypocotyl tissue

The uptake of L-glutamic acid, L-lysine and glycine by sunflower hypocotyls was investigated by Rheinhold and Powell (1958). L-glutamic acid uptake had a Q_{10} value greater than one and was reduced under anaerobic conditions, leading the authors to conclude that metabolic energy was required for accumulation of this amino acid. Concentration dependency was demonstrated, indicating carrier mediation. Reanalysis of the data by Nissen (1974) suggests that uptake of L-glutamic acid may be represented by a biphasic isotherm.

5. Leaf tissue

The existence of specific carriers for amino acids in leaf cells was first proposed by Mathes and Engelbrecht (1961) to explain the energy-dependent accumulation of glycine by kinetin-treated areas of excised leaves. More recently, Shtarksall *et al.* (1970) studied the uptake of α-aminoisobutyric acid (AIB) by barley leaf strips. AIB was not metabolized by this tissue, and thus difficulties in interpretation resulting from metabolism of the transported compound were eliminated.

Net uptake of AIB was linear with time and proceeded against a concentration gradient. The capacity of leaf strips for accumulation of AIB increased by a factor of four when the tissue was "aged" for several hours in a calcium chloride solution. The stimulation of net uptake was shown to result from increased influx of AIB rather than increased efflux, and was attributed to reversal during the ageing period of wounding effects.

In a continuation of this work, Rheinhold *et al.* (1970) conducted a detailed kinetic analysis of AIB uptake by barley leaf strips. The data indicate that specific uptake mechanisms are present in both fresh and aged strips. Diffusion of AIB into leaf cells represented only a small fraction of net uptake, even at the high AIB concentrations utilized in this work. Concentration dependency curves were found to be biphasic, and were considered evidence for the existence of dual uptake mechanisms.

Uptake of eighteen amino acids by pea leaf fragments has been reported by Cheung and Nobel (1973). The process was strongly temperature-dependent (maximum activity at 29°C) and sensitive to metabolic inhibitors and uncouplers. Metabolic energy derived from either photosynthesis or respiration could support uptake. Competition studies suggested that all naturally occurring amino acids were transported by the same carrier, but that AIB may enter via a separate carrier.

Evidence that plant cells possess shock-sensitive binding proteins analogous to those in bacteria has been obtained (Amar and Rheinhold, 1973). Aged strips of *Phaseolus vulgaris* released 3.5% of their total protein content when subjected to osmotic shock at low temperatures. The loss of protein was accompanied by up to 99% reduction of AIB uptake. This decrease in transport activity could not be attributed to "leakiness" of the cell membranes as a result of nonspecific damage. Although the data suggested a specific role of the released protein in transport, binding of AIB to the released protein could not be demonstrated.

6. Cultured plant cells

Two studies of amino acid transport by suspension-cultured cells are presently available. Uptake of L-arginine and L-lysine by cultured sugarcane cells was found to be a carrier mediated process and was sensitive to uncouplers and inhibitors of the cytochrome system (Maretzki and Thom, 1970). Kinetic data indicate that at least two carrier sites exist for these amino acids. The biphasic concentration dependency curve obtained for arginine uptake was considered to be the result of a specific system operating at low arginine concentrations.

and a non-specific carrier system active at high concentrations. Competition data indicated that the non-specific L-arginine carrier may be the same one responsible for L-lysine uptake.

King and Oleniuk (1973) investigated L-alanine uptake by suspension-cultured soybean cells. Uptake by nitrogen sufficient cells was apparently non-saturable and therefore not a mediated process. Incubation of cells in a nitrogen-free medium resulted in a 100-fold increase in uptake rates. Under nitrogen-starved conditions, uptake was a saturable process with a K_m of 2.55 μ M. DNP and azide rapidly and effectively inhibited transport, indicating that transport was an energy-dependent process. Maximal rates of transport were observed in young, rapidly growing cultures.

It is apparent from a survey of the literature that transport of amino acids by plant cells has, to date, been studied only superficially. While the available data point to the existence of active transport systems for amino acids in plant cells, the key questions of mechanism, energy source and regulation have yet to be investigated.

II. MATERIALS AND METHODS

A. Sources of Materials

1. Chemicals

Routine chemicals were ordered from Fisher Scientific Company and were of reagent grade. Sucrose was obtained from the J.T. Baker Chemical Company. Eastman Chemicals supplied 2,4-diphenoxyacetic acid, and ammonium sulfate was obtained from Mann Research Laboratories. Vitamins, amino acids, oligomycin, Dowex ion exchange resin, and MES (2[N-morpholino]ethane sulfonic acid) buffer were supplied by Sigma Chemical Company. Fine chemicals were of the highest purity available. L-leucine-^{14}C(U), L-leucine (1-^{14}C), L-alanine-^{14}C and Aquasol scintillation fluid were purchased from New England Nuclear Company.

2. Cell cultures

Ammi visnaga was originally obtained as a callus culture from Dr. O. Gamborg of the Prairie Regional Laboratory, Saskatoon, Saskatchewan.

B. Maintenance of Cell Cultures

1. Growth conditions

Cells were grown aseptically in 500 ml erlenmeyer flasks containing 150 ml of suspension. Cells were subcultured every 3.5 days to maintain the cultures in an actively growing state. New flasks were inoculated by transferring a volume of cells sufficient to give an initial density of 1.5 mg dry weight/ml of suspension to a flask containing sterile 1.0 B5 medium (Appendix I). All transfer steps were performed in a lininar flowhood to maintain sterility. Cultures were were incubated at 27°C and under constant light on a New Brunswick Scientific gyrotary shaker operating at 150 rpm.

2. Measurement of growth parameters

a. Dry weight.

Growth was routinely monitored by determination of dry weight. Five ml aliquots of cell suspension were collected on vacuum dried, tared filter paper disks, washed with distilled water to remove debris, and dried overnight at 60°C in a vacuum oven.

b. Packed cell volume.

Packed cell volume was used to estimate and to adjust the density of cell suspensions for experiments. Two to five ml aliquots of cell suspension were pipetted into a 15 ml graduated centrifuge tube and spun for 5 minutes at top speed (approx. 2000x*g*) in a clinical centrifuge. Volumes were read to the nearest 0.1 ml.

c. pH

The pH of the cultured medium was measured by transferring 5-10 ml of suspension to a small beaker, allowing the cells to settle for 5 minutes and measuring the pH with a combination electrode (A.H. Thomas Co.). In older cultures that contained debris, aliquots were first filtered through miracloth, and the pH of the filtrate was determined.

C. Measurement of Amino Acid Uptake by Cultured Cells

The method used for measuring amino acid uptake (Fig. 1) was a modification of the technique used by King and Oleniuk (1973). Cells were harvested when the density had reached approximately 3 mg dry weight/ml of suspension, usually 24-36 h after inoculation. The cells from approximately 150 ml of suspension were collected on miracloth, washed with 250 ml of fresh 0-B5 medium, and quickly transferred to a beaker. The cells were diluted to give a density equivalent to 1.0 ml PCV/2.0 ml of suspension or 15 mg dry weight/ml. Aliquots (2 ml) were pipetted into 10 ml erlenmeyer flasks containing 0.4 ml of 0-B5 medium. 2,4-D was omitted from the B5 medium used for washing and assay since it was found to have an inhibitory effect on L-leucine uptake.

Flasks were preincubated for 20 minutes on a gyrotary shaker at 190 rpm. Assays were usually performed at ambient room temperature. When a water bath shaker was available, assays were performed at 23°C or as otherwise indicated. After preincubation, 100 µl of a solution of unlabelled amino acid containing 10^6 dpm ^{14}C-amino acid (L-leucine or L-alanine) was added by a microliter syringe to each flask. Duplicate flasks were sacrificed at appropriate time intervals by pouring the contents onto tared miracloth disks held under vacuum in Millipore filter holders. The flasks were rapidly rinsed with 10 ml of 0-B5 medium, and this was subsequently passed through the filters to wash the cells. The cells collected on the miracloth disks were used for dry weight determinations.

Uptake was determined by transferring 100 µl aliquots from each filtrate to scintillation vials containing 10 ml of Aquasol universal

MEASUREMENT OF AMINO ACID UPTAKE

Cells grown to 2-3 mg/ml stage

Collect by filtration; wash

Resuspend in fresh growth medium

^{14}C-amino acid of known specific
activity added to cell susp.

Cells collected by filtration wash

Cells

Dry wt determination

filtrate

^{14}C assay

Uptake rate
nmoles/min/mg dry wt.

FIGURE 1. Procedure for assay of amino acid uptake by suspension cultured cells of *Ammi visnaga*.

scintillation fluid. Sufficient isotope had been added to the assay vials to give a zero time reading of 10^4 cpm/100 μl. Vials were counted in a Nuclear Chicago Unilux II scintillation counter. Counting intervals were selected to give ± 1% counting efficiency. Counts were corrected for background and for quenching by the channel ratios method (Wang and Willis, 1965). Total uptake was calculated as dpm in the filtrate at time zero minus dpm in the filtrate after a specified interval, and was expressed on a dry weight basis.

D. Determination of L-leucine Transport at Different Stages of the Growth Period.

A 500 ml flask containing 100 ml of 1.0 B5 medium was inoculated with 3.5 day old cells as described above. Aliquots were aseptically removed at 2 h after transfer and approximately every 24 h thereafter up to 140 h. Cells were washed, concentrated to 1.0 ml PCV/2.0 ml suspension and assayed for uptake of 50 μM L-leucine at 24°C.

E. Nitrogen Starvation of Cells

Cells that had been grown 24 h in 1.0 B5 medium were aseptically collected on miracloth in a Sterifil filtration unit supplied with gentle vacuum. These cells were washed with 250 ml of 1.0 B5 medium lacking inorganic nitrogen (Appendix 1) and transferred aseptically to a 500 ml flask containing 100 ml of the same medium. During the starvation period, flasks were incubated under the conditions described for maintenance of cell cultures.

F. Effect of Duration of Nitrogen Starvation on the Rate of L-leucine and L-alanine Uptake

Cells were starved as described above. Aliquots of 30-40 ml were withdrawn aseptically at the time of transfer to nitrogen-free medium, and at 4 h intervals thereafter up to 24 h. Uptakes of 10 μM L-leucine and L-alanine were assayed separately using triplicate flasks for each time point.

G. Concentration Dependency of L-leucine Uptake by Nitrogen Starved and Nitrogen Sufficient Cells

1. Nitrogen sufficient cells

Cells grown in 1.0 B5 medium for 36 h were assayed for uptake at 8 concentrations of L-leucine (10, 15, 25, 50, 100, 133, 167, and 200 μM).

2. Nitrogen starved cells

Cells were grown for 24 h in 1.0 B5 medium, washed, starved for an additional 12 h, and assayed for L-leucine uptake at 6, 8, 10, 15, 25, 35, 50, 60, 100, and 250 μM.

H. Measurement of L-leucine Uptake at Different pH Values

A nitrogen starved cell suspension (150 ml) was divided into eight equal fractions. Cells from each fraction were collected by filtration and washed twice with 20 ml of pH adjusted nitrogen-free medium containing 0.1 M MES buffer (pH 5.5). The cells were resuspended to give 1.0 ml PCV/2 ml of cell suspension. Uptake of

50 μM L-leucine was assayed at pH values of 5.0, 5.5, 5.7, 5.85, 6.0, 6.2, 6.5 and 7.0.

I. Measurement of L-leucine Uptake at Different Temperatures

One-hundred and fifty ml of nitrogen starved cell suspension were divided into eight equal fractions. Cells from each fraction were collected by filtration, washed and resuspended in 0-B5 medium that had been adjusted to give a pH value of 5.9 at one of the experimental temperatures. The cells were preincubated for 20 minutes at the indicated temperature before addition of labelled amino acid. Uptake of 50 μM L-leucine was assayed at 15, 20, 24, 26, 27, 28, 30, and 35°C. Assays were done in a gyrotary water bath shaker to maintain the desired temperature.

J. Measurement of Efflux Rate

Nitrogen starved (13 h) cells from 250 ml of suspension were collected by filtration and washed with 200 ml of fresh medium. Cells were transferred to a flask containing 40 ml of nitrogen-free medium and incubated 20 minutes on a gyrotary shaker at room temperature. Duplicate aliquots were taken for dry weight determination. At time zero, 1.6 ml L-leucine-^{14}C was added to give an initial extracellular concentration of 50 μM. After 5 minutes, contents of the flask were poured onto miracloth held in a Sterifil unit. The flask was rinsed with 150 ml of medium and this was also filtered through the same miracloth disk. The cells loaded with ^{14}C-leucine were transferred to 40 ml of fresh medium. Aliquots (2 ml) of this suspension were transferred to each of 24 10 ml erlenmeyer flasks. Twelve flasks were preincubated (to minimize any effects as a result of transfer) for 5 minutes before addition of L-leucine-^{3}H. Duplicate flasks were sacrificed at 0, 2, 4, 6, 8 and 10 minutes as previously described. Aliquots (100 µl) of the filtrates were analyzed for ^{3}H and ^{14}C content by the triple channel method of liquid scintillation counting (see Bush, 1964).

Efflux of ^{14}C-leucine into leucine-free medium was followed simultaneously with the remaining 12 flasks. Flasks were sacrificed at the time intervals indicated above. Aliquots (100 µl) of the filtrate were then analyzed for ^{14}C content.

K. Test for Sensitivity of L-leucine Uptake to Inhibition by Other Amino Acids

Nitrogen starved cells were prepared for assay as described above, except that the volume of 0-B5 medium added to the flasks was reduced to 0.35 ml. Potential competing amino acids were added in 50 µl of solution, 15 seconds before the addition of ^{14}C-leucine. The initial extracellular concentration of the amino acids being tested was 1 mM. Control flasks contained 50 µl of distilled water.

L. Recovery of Transported L-leucine-^{14}C

Cells were grown, washed and starved according to the normal procedure. Uptake of ^{14}C-leucine was assayed at room temperature in 50 ml reaction flasks containing 4 ml of cell suspension, 0.8 ml of 0-B5 medium lacking nitrogen and 0.2 ml of isotope solution. Flasks were flushed with air at a rate of 50 ml/min and were shaken continuously throughout the experiment. Flask contents were sacrificed at 2 minutes after addition of ^{14}C-leucine. The cells were removed from the miracloth filters, transferred to 15 ml 80% v/v ethanol and homogenized at 4°C for 5 minutes at 3/4 speed in a Virtis homogenizer. The homogenate was fractionated according to the method of Fletcher and Beevers (1970) (Fig. 2).

M. Determination of Size and Composition of the Soluble Amino Acid Pool

Cells that had been growing in 1.0-B5 medium for 24 h were collected, washed and transferred to nitrogen-free medium. Aliquots of suspension were taken after 0, 12, and 24 h of nitrogen starvation. The cells were collected by filtration, transferred to 15 ml of ethanol (80% v/v) and homogenized as above. The amino acids were separated according to the procedure in Fig. 2, then taken nearly to dryness, and resuspended in 1N sodium citrate buffer (pH 2.2). The amino acids were separated on a Beckman Model 121 Automatic Amino Acid Analyzer with dual columns of Beckman AP-28 resin. The standard assay temperature was 53°C. Basic amino acids were eluted with 0.35 N sodium citrate buffer, pH 5.3. Neutral and acidic amino acids were eluted from the second column with 0.2 N sodium citrate buffer, pH 3.35, followed by the same buffer at pH 4.25. Concentration of the standard amino acids was 100 μM.

N. Effect of L-leucine and $(NH_4)_2SO_4$ on L-leucine Uptake by Nitrogen Starved Cells of *A. visnaga*

Cells that had been incubated 24 h under normal maintenance

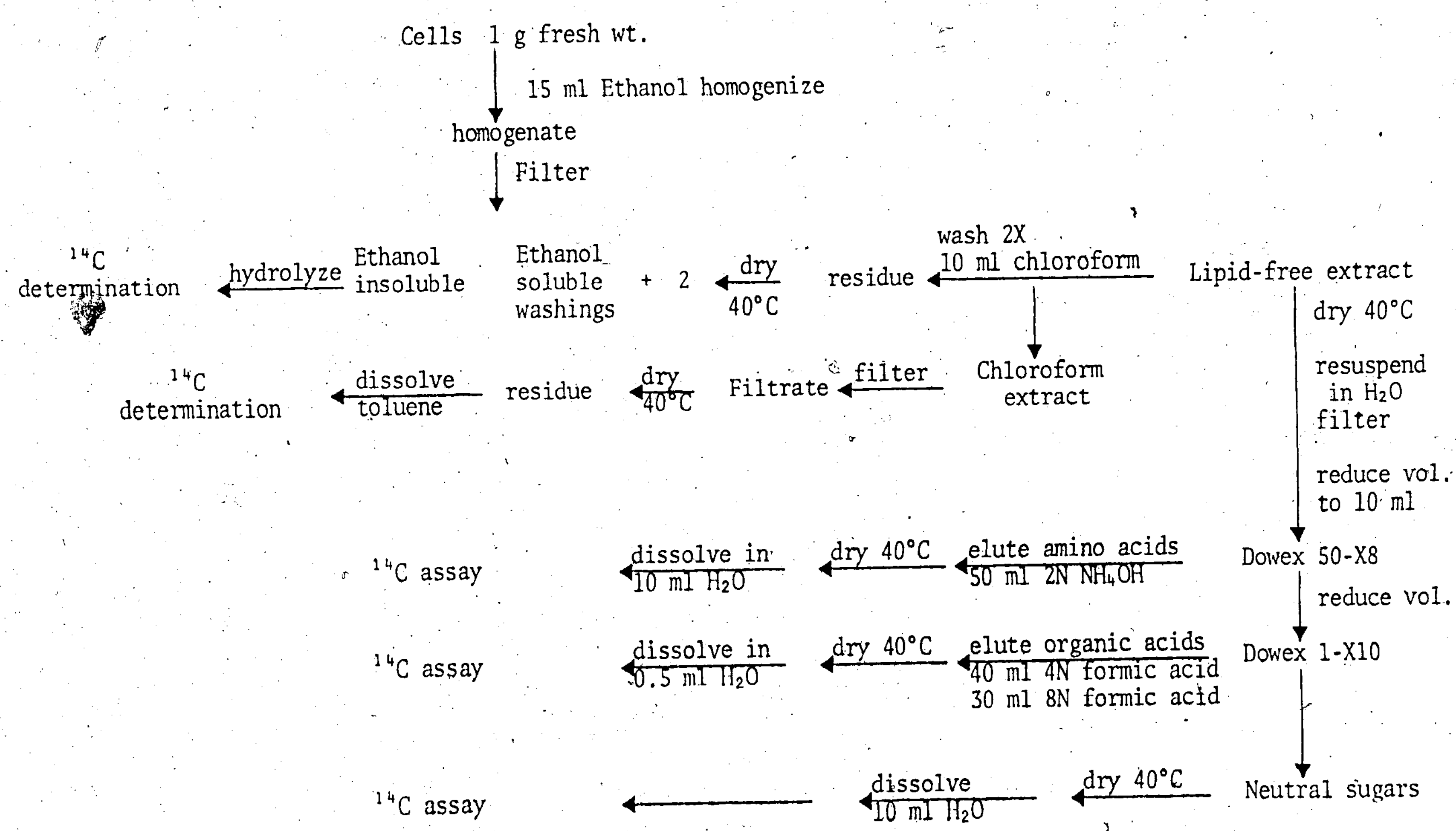

FIGURE 2. Procedure for recovery of transported L-leucine-^{14}C from various cell fractions (after Fletcher and Beevers, 1970).

conditions and subsequently nitrogen starved for 12 h were collected by filtration, washed and resuspended in 0-B5 medium lacking nitrogen to give a PCV of 0.5 ml/2.0 ml of cell suspension. A 10 ml aliquot was withdrawn for assay at time zero and the remaining cells were distributed equally among four 125 ml erlenmeyer flasks. One ml of concentrated stock solution was added to each of three flasks, to give an initial concentration of either 5 mM L-leucine, 1 mM $(NH_4)_2SO_4$ or a combination of 5 mM L-leucine and 1 mM $(NH_4)_2SO_4$. The control flask received 1.0 ml of distilled water. The flasks were incubated at 23°C on a gyrotary shaker operating at 150 rpm. Aliquots of 10 ml were withdrawn from each flask at time intervals of 30, 60, 120 and 180 minutes after addition of the preloading solution. The cells were collected by filtration, washed with 20 ml of 0-B5 medium lacking nitrogen and resuspended in 5 ml of the same medium. Uptake of L-leucine from a 50 μM solution was assayed in duplicate.

O. Long-term Uptake of L-leucine by Nitrogen Starved Cells

Cells that had been grown 24 h under normal maintenance conditions were collected by filtration, washed and starved for 12 h as described previously. Uptake of L-leucine-^{14}C from a 5 mM solution was assayed at 5 minute intervals up to 20 minutes and at 10 minute intervals from 20-100 minutes.

P. Measurement by Warburg Manometry of the Effect of DNP and NaN_3 on Oxygen Uptake by Starved and Unstarved Cells of *A. visnaga*

Aliquots were taken from nitrogen sufficient cell suspensions

24-36 h after inoculation, washed with fresh medium and transferred to calibrated Warburg vessels. Both nitrogen sufficient cells and cells that had been starved for 12 h were treated in the same manner as for assays of L-leucine uptake.

Each Warburg vessel contained 2.0-2.5 ml of cell suspension, 0.2 ml of NaN_3 or DNP in the sidearm and 0.2 ml of 10% potassium hydroxide in the center well. Control vessels contained 0.2 ml of distilled water in the sidearm.

Oxygen uptake was measured at 27°C. Flasks were preincubated with shaking 20 minutes before the first reading was taken. Readings were taken at 5 minute intervals. Oxygen uptake was measured 20 minutes before addition of inhibitors and for an additional 20-30 minutes in the presence of inhibitors.

Q. Determination of the Effects of Respiratory Inhibitors and Uncouplers on L-leucine Uptake

1. Anaerobiosis

Cells that had been nitrogen starved for 12 h were washed and concentrated as described above. Aliquots of 4 ml were transferred to 50 ml reaction flasks containing 0.4 ml fresh 0-B5 medium lacking nitrogen. Flasks were initially flushed with air at 50 ml/min. for 15 minutes and subsequently with either nitrogen or air for an additional 15 or 30 minutes before assay of the uptake of L-leucine from a 50 μM solution of the amino acid.

2. DNP and NaN_3

Uptake of leucine by starved and unstarved cells was assayed as previously described, except that flasks initially contained 2.0 ml of cell suspension and 0.3 ml of fresh 0-B5 medium. Aliquots of 0.1 ml of aqueous solutions of NaN_3 or DNP were added to assay flasks 0.5, 5.0, or 10.0 minutes before the addition of ^{14}C-leucine. Initial extracellular concentration of leucine was 50 μM. Controls consisted of flasks containing solutions of identical composition plus 0.1 ml of distilled water.

3. Oligomycin

Uptake of 50 μM leucine by starved (12 h) and unstarved cells was assayed in the presence of 5, 10 and 20 μg/ml oligomycin. The inhibitor was added as 15 μl aliquots of ethanolic solutions, either 1, 5, or 10 minutes before the ^{14}C-leucine. Control flasks contained 15 μl of ethanol.

III. RESULTS

A. Growth of *A. visnaga* Cells in Suspension Culture

A representative growth curve showing increase in cell mass (dry weight/ml) is illustrated in Fig. 3. When media were inoculated to a concentration of 1.5-2.0 mg/ml of cells from the linear portion of the growth curve, little or no lag phase was observed. Cell mass increased for approximately four days after inoculation, then levelled off as the cells reached stationary phase. Cultures maintained in the stationary phase generally showed a decline in cell mass that was associated with cell death and disintegration.

The external pH of *A. visnaga* cultures fluctuated during the growth period (Fig. 3). The pH dropped to the minimum observed value of 5.6 shortly after inoculation, then recovered to a plateau value of 6.2-6.3 during the logrithmic growth phase. Stationary phase was preceeded by a drop in pH, although the pH was quite variable once the cells actually reached the stationary phase. The same overall pattern was observed in three consecutive growth experiments.

B. Uptake of L-leucine at Different Stages of the Growth Curve

The rate of L-leucine uptake, expressed on a dry weight basis, increased with time after transfer of cells to fresh medium (Fig. 4). The rate reached a plateau lasting from about 64 h to 112 h, and then declined rapidly. The peak rate, observed during late lag phase and early stationary phase, represented a 2.5-fold increase over the rate

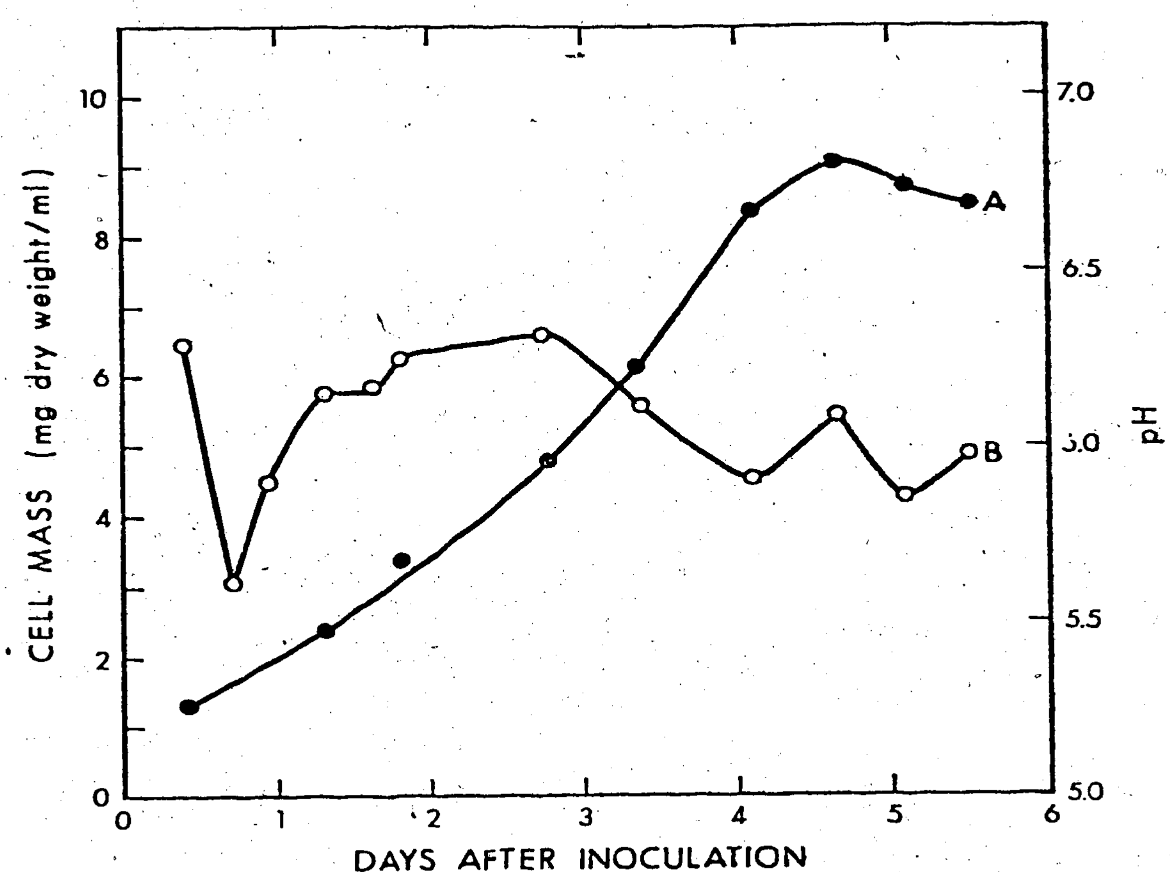

FIGURE 3. Growth (curve A) and pH (curve B) curves for cell suspensions of *A. visnaga* grown at 27°C.

Cells were grown in 500 ml erlenmeyer flasks containing 100 ml of 1.0-BS medium. The flasks were incubated on a gyrotary shaker operating at 150 rpm, under constant light.

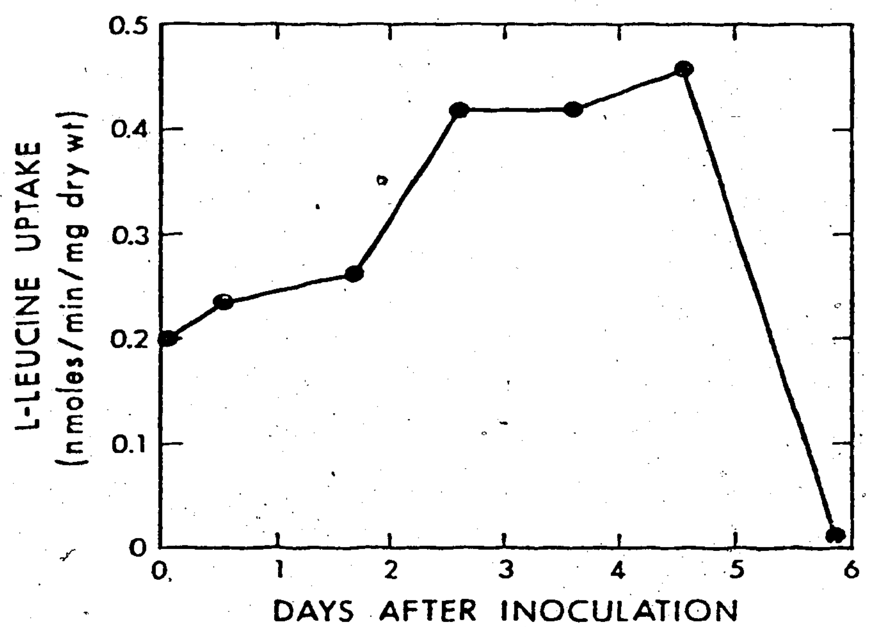

FIGURE 4. Uptake of L-leucine during growth of *A. visnaga* cells in suspension culture.

A 500 ml flask containing 100 ml of 1.0-B5 medium was inoculated and incubated under normal maintenance conditions. Aliquots were removed at intervals of approximately 24 h and uptake of L-leucine from a 50 μM solution was assayed at 24°C. Points are averages of triplicate determinations. Agreement among aliquots was ±5% of the mean.

measured 2 h after inoculation into fresh medium.

C. Effect of Nitrogen Starvation on Growth of *A. visnaga* Cells

Fig. 5 illustrates growth curves for *A. visnaga* cells in 1.0-B5 medium, 1.0-B5 medium lacking nitrogen and 0-B5 medium lacking nitrogen. Growth rates for cells in nitrogen deficient media were comparable to control rates during the first 24 h after inoculation. Beyond 24 h the growth rate of cells in nitrogen-free media declined. Preliminary experiments showed a similar decline in protein and RNA content of the cells after 24 h.

D. Effect of Nitrogen Starvation on L-leucine and L-alanine Uptake

Removal of organic nitrogen from the culture medium resulted in increased uptake rates for both L-leucine and L-alanine (Fig. 6). The greatest increase in uptake rates for both amino acids was observed immediately after transfer to nitrogen-free medium, although the uptake rates continued to increase slowly during subsequent hours of starvation. The rate of L-alanine uptake appeared to plateau after approximately 8 h, while the rate of L-leucine uptake containued to increase slightly throughout the 24 h starvation period. The maximum stimulation of uptake observed during starvation was about 10-fold for both amino acids.

E. Concentration Dependency of L-leucine Uptake

Uptake of L-leucine by both starved and unstarved cells exhibited saturation kinetics, indicating carrier mediation (Lehninger,

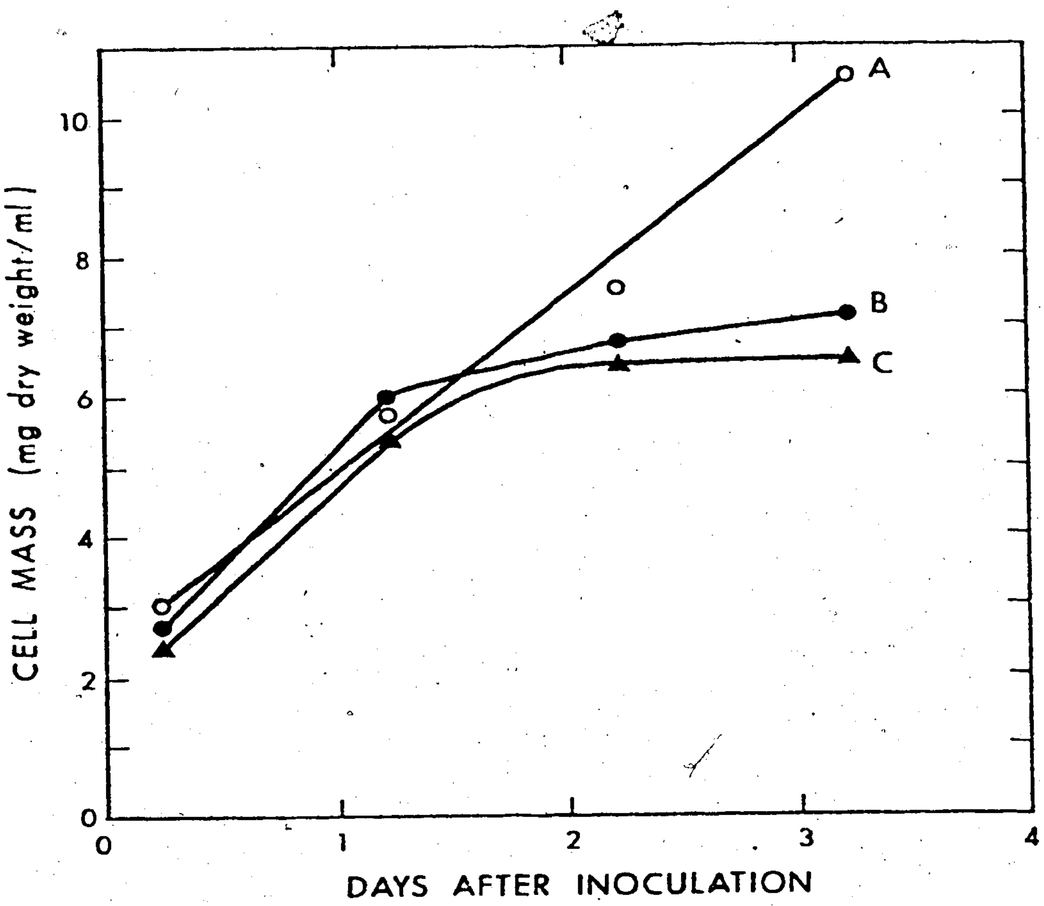

FIGURE 5. Growth of *A. visnaga* cells in 1.0-B5 medium (curve A), 1.0-B5 medium lacking nitrogen (curve B) and 0-B5 medium lacking nitrogen (curve C).

Cells that had been grown 24 h in 1.0-B5 medium were collected by filtration, washed with 250 ml of one of the indicated media and transferred to a 50 ml flask containing 100 ml of the same medium. Flasks were incubated under normal maintenance conditions. Aliquots for dry weight determinations were withdrawn at 24 h intervals.

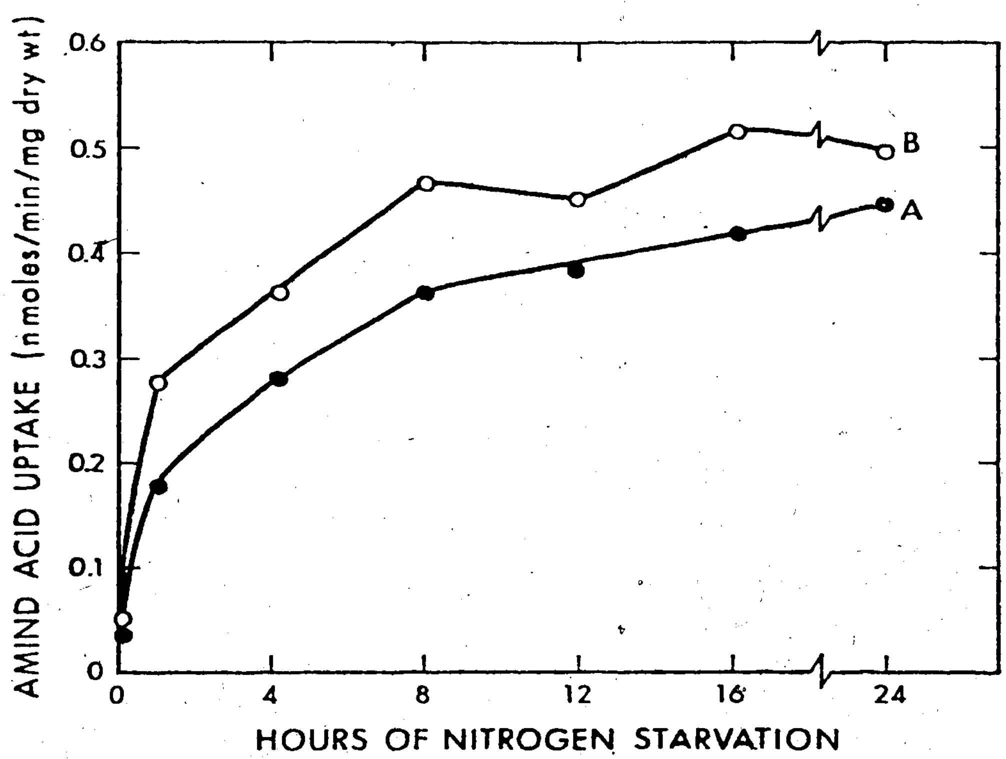

FIGURE 6. Effect of the duration of nitrogen starvation on uptake rates of L-leucine (curve A) and L-alanine (curve B) by *A. visnaga* cells.

Cells that had been incubated 24 h in 1.0-B5 medium were aseptically collected by filtration, washed with 250 ml of 1.0-B5 medium without nitrogen and transferred to a 500 ml flask containing 100 ml of the washing medium. Flasks were incubated under normal maintenance conditions during the starvation period. Aliquots were removed at 4 h intervals and uptake of L-leucine or L-alanine from 10 μM was assayed. Points are averages of duplicate determinations.

1970). When uptake rates were plotted in the double-reciprocal form (Figs. 7 and 8) each of the curves could be resolved into two phases. Apparent Michaelis-Menton constant (K_m) values were calculated for each of the two phases. The values for nitrogen starved cells were 5.6 and 62.5 μM, while values for nitrogen sufficient cells were approximately double, 13.7 and 125 μM.

F. L-leucine Uptake as a Function of pH

Uptake of L-leucine by nitrogen starved cells was maximal near pH 5.8 (Fig. 9). This pH is very close to the pH value of the culture medium when cells were normally harvested for experiments. The transport rates did not vary greatly within the pH range of 5.6-6.3 normally found during growth of the cultures.

G. L-leucine Uptake as a Function of Temperature

L-leucine transport was strongly temperature dependent, as indicated in Fig. 10. The maximum rate of L-leucine transport was reached at approximately 27°C, with a Q_{10} value of about 4 between 15°C and 25°C. The rate of L-leucine uptake decreased rapidly at temperatures exceeding 28°C.

H. L-leucine Efflux from "Preloaded" Cells

The results obtained using the double label method to estimate efflux are summarized in Fig. 11. The initial rate of L-leucine entry into starved cells of *A. visnaga* was 0.543 nmol/min/mg dry weight.

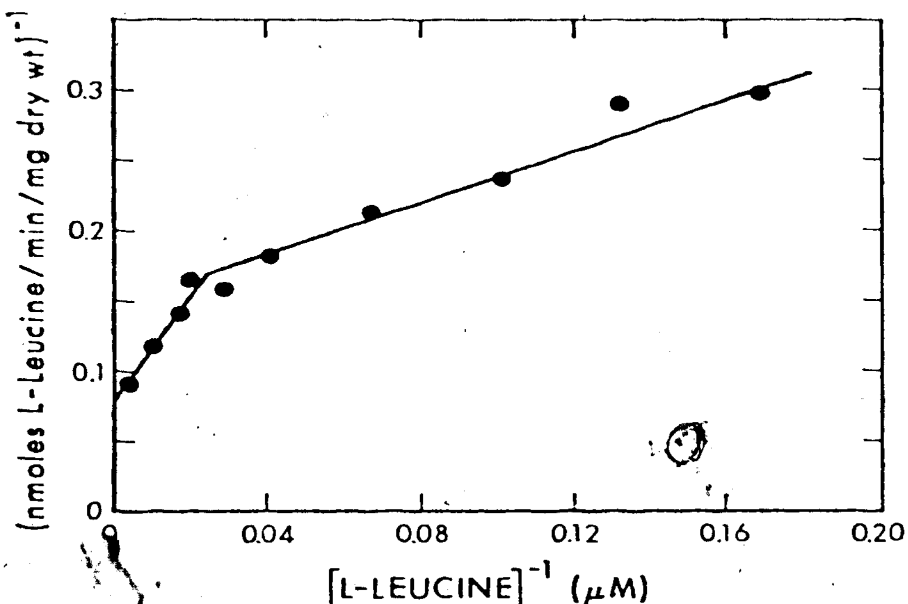

FIGURE 7. Double reciprocal plot for L-leucine uptake by nitrogen starved cells of *A. visnaga*.

Cells were grown 24 h in 1.0-B5 medium, washed, starved for 12 h and assayed at 24°C for uptake of L-leucine at 10 different concentrations. Points are averages of two or more determinations. Agreement among determinations was ±4.5%.

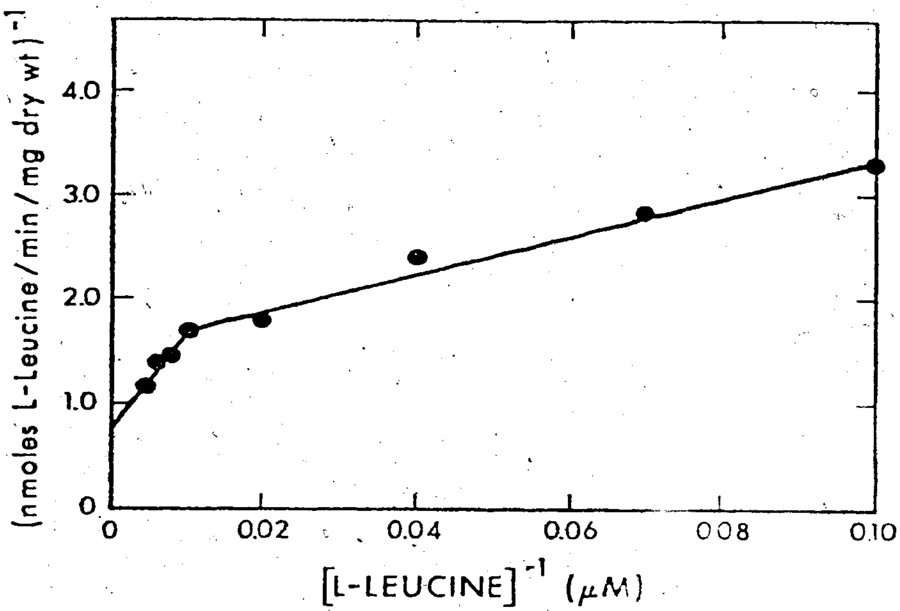

FIGURE 8. Double reciprocal plot for L-leucine uptake by nitrogen sufficient cells of *A. visnaga*.

Cells grown in 1.0-B5 medium for 36 h were collected by filtration, resuspended in fresh medium and assayed at 24°C for uptake of eight concentrations of L-leucine. Points are averages of 2 or more determinations. Agreement among determinations was ±4.5%.

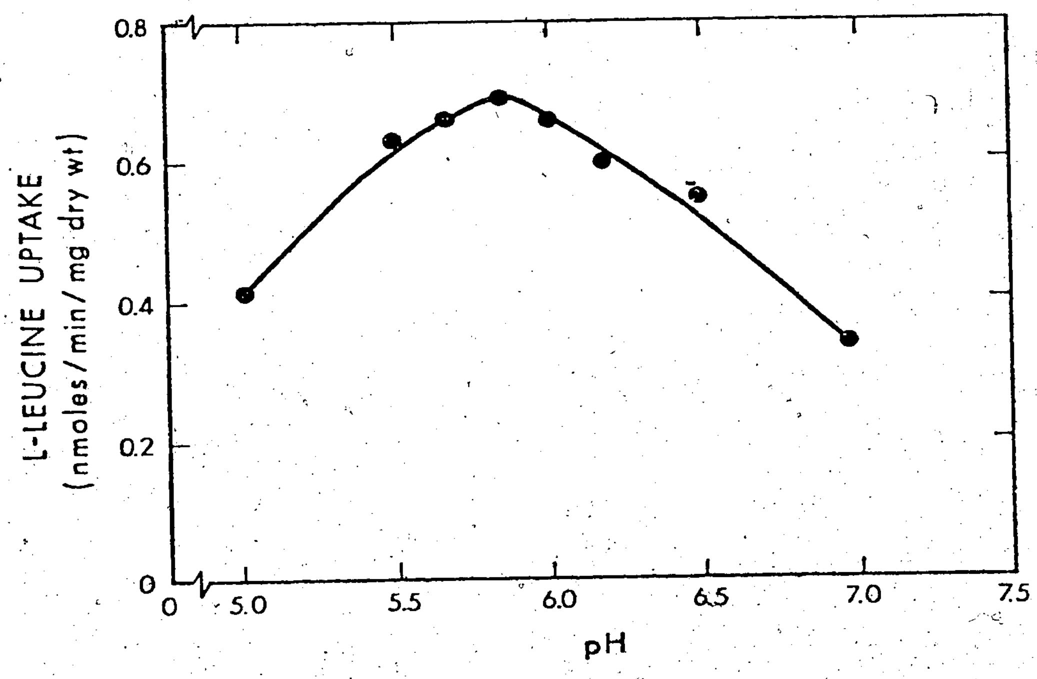

FIGURE 9. Uptake of L-leucine by *A. visnaga* cells as a function of pH.

Nitrogen starved cell suspension (150 ml) was divided into eight fractions. Cells from each fraction were collected by filtration and washed with nitrogen free 0-B5 medium containing 0.1 M MES buffer. The pH values of the washing solutions were the same as those of the assay solutions. Uptake of L-leucine from 50 μM solutions at 8 different pH values was assayed at 24°C. Points are averages of duplicate determinations.

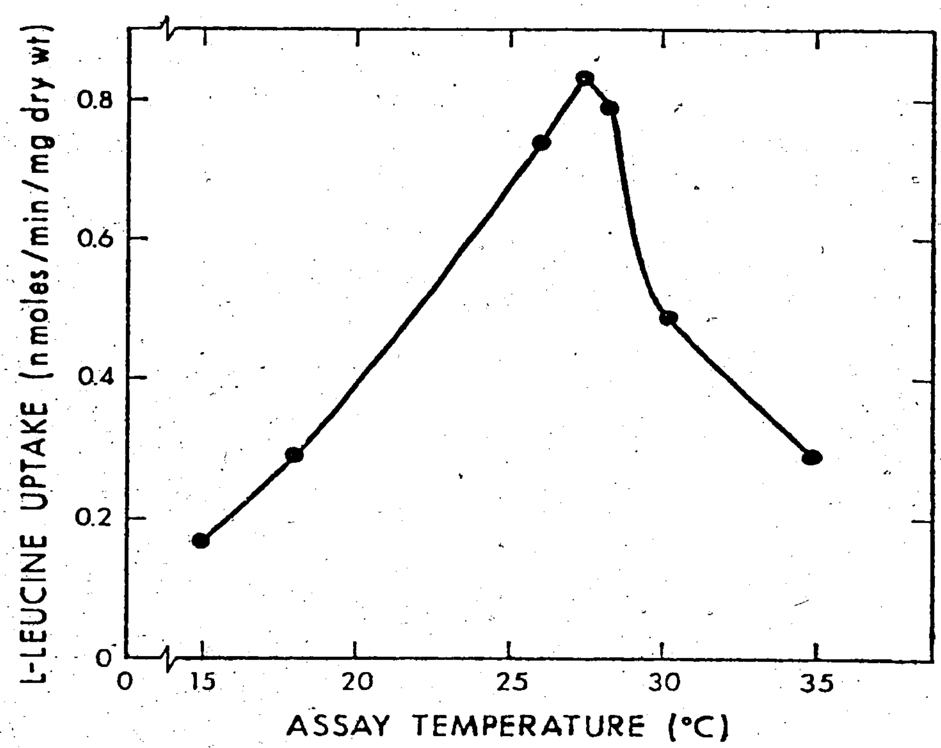

FIGURE 10. Uptake of L-leucine by *A. visnaga* cells as a function of temperature.

Nitrogen starved cell suspension (150 ml) was divided into eight fractions. Each fraction was collected by filtration, washed and resuspended in 0-B5 medium adjusted to a pH value of 5.9 at one of the experimental temperatures. Cells were preincubated 20 min before assay of L-leucine uptake from a 50 µM solution. Points are averages of duplicate determinations.

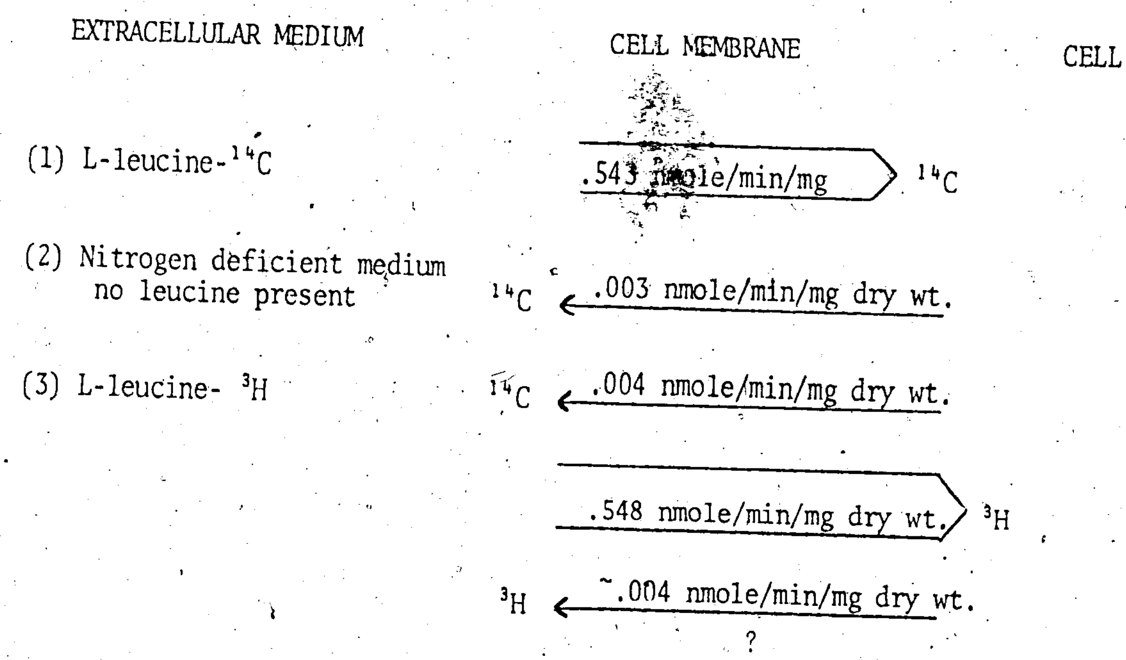

FIGURE 11. Diagramatic representation of influx and efflux components of L-leucine uptake by *A. visnaga* cells.

Nitrogen starved cells (250 ml) were collected by filtration, washed with 200 ml of fresh medium and resuspended in 40 ml of 0-B5 medium lacking nitrogen. At time zero, 1.6 µmole of L-leucine-^{14}C was added to give an initial extra cellular concentration of 50 µM. After 5 min the cells were collected by filtration, washed with 150 ml of 0-B5 medium lacking nitrogen and transferred to 40 ml of fresh medium. Two ml aliquots were transferred to 10 ml erlenmeyer flasks. Twelve flasks were used to assay uptake of L-leucine-^{3}H from a 50 µM solution. Duplicate flasks were sacrificed at 0, 2, 4, 6, 8 and 10 min. Efflux of L-leucine-^{14}C into L-leucine-free medium was followed simultaneously with an additional 12 flasks. Filtrates were analyzed for ^{3}H and ^{14}C.

When preloaded cells were resuspended in 0-B5 medium lacking nitrogen, the rate of L-leucine efflux was calculated to be 0.003 nmol/min/mg dry weight. The rate of ^{3}H-leucine uptake by cells that had been preloaded with ^{14}C-leucine was the same as measured for L-leucine influx into cells that had not been preloaded. This indicated that the degree of preloading utilized did not affect the initial rate of uptake. Thus, conditions employed in the preloading experiments still approximated the conditions normally used for other transport assays. The amount of ^{14}C-leucine present in the external medium was greater when the medium contained L-leucine, presumably because dilution of the isotope by unlabelled L-leucine reduced the amount retransported into the cells. Using this larger quantity, efflux of accumulated L-leucine was estimated to be 0.004 nmol/min/mg dry weight.

I. Recovery of Transported ^{14}C-Leucine and Metabolites

Distribution of ^{14}C in various cell fractions is given in Table 2. The amount of radioactivity recovered was calculated to be 9.7% of that transported. As anticipated, most of the label was recovered in the soluble amino acid and protein amino acid fractions. Approximately 10% of the total counts were recovered in the lipid and organic acid fractions, indicating that some degradation of L-leucine was occurring. Insignificant amounts of radioactivity (less than 0.10%) were recovered in the sugar or CO_2 fractions.

J. Inhibition of L-leucine Transport by Other Amino Acids

The results given in Table 3 indicate that L-leucine uptake by

TABLE 2. Recovery of Transported L-leucine-^{14}C from *A. visnaga* Cells

Fraction	dpm recovered	% of transported dpm
CO_2	834	0.1
Lipids	65500	8.5
Organic acids	7800	1.0
Sugars	1066	0.1
Amino acids	382000	49.7
Protein amino acids	248000	32.3
DPM added as L-leucine-^{14}C	1.079×10^6	
DPM remaining in filtrate	0.309×10^6	
DPM transported	0.770×10^6	
% of transported cpm recovered	91.7	

Uptake of L-leucine-^{14}C from a 50 μM solution was assayed at room temperature in 50 ml reaction flasks. Each flask contained 4 ml of nitrogen starved cell suspension, 0.8 ml 0-B5 medium lacking nitrogen and 0.2 ml isotope solution. Flasks were flushed with air at a rate of 50 ml/min and were shaken continuously. Flask contents were sacrificed 2 min after addition of ^{14}C-leucine and the cells were homogenized in 80% (v/v) ethanol using a Virtis 45 homogenizer. The homogenate was fractionated according to the method of Fletcher and Beevers (1970).

TABLE 3. Effects of Unlabelled Amino Acids on Uptake of L-leucine-^{14}C by Nitrogen Starved *A. visnaga* Cells

Amino Acid	% Inhibition of L-leucine uptake
L-alanine	92
L-isoleucine	78
L-valine	73
L-proline	69
Glycine	62
D-alanine	40
D-leucine	54
L-glutamate	54
L-aspartate	45
L-lysine	30

Uptake of L-leucine-^{14}C from 50 μM solutions was measured in the presence of 1 mM concentrations of unlabelled amino acids. Assay flasks contained 2.0 ml of nitrogen starved cell suspension and 0.35 ml fresh 0-B5 medium lacking nitrogen. Unlabelled amino acids were added in 50 μl of solution 15 sec before the addition of ^{14}C-leucine. Control flasks received 50 μl of distilled water.

nitrogen starved cells is inhibited by a variety of representative neutral, acidic and basic and D-amino acids. Inhibition by other neutral amino acids was most pronounced, suggesting that the carrier possesses some preference for this class. L-alanine, the most effective inhibitor, caused a 92% inhibition of L-leucine uptake, compared to 78% and 73% for L-isoleucine and L-valine respectively, which are structurally more similar to L-leucine. D-alanine and D-leucine were intermediate in their effectiveness as inhibitors, as was L-glutamate. L-aspartate and L-lysine, a basic amino acid, were the least effective inhibitors of the amino acids tested.

K. <u>Qualitative and Quantitative Changes in the Soluble Amino Acid Pool During Nitrogen Starvation</u>

Composition of the cellular soluble amino acid pool of *A. visnaga* is given in Table 4. Relative to other amino acids and consistent with their respective metabolic roles, L-aspartate, L-glutamate, L-serine and L-threonine were present in the greatest concentrations. In contrast, L-leucine was present only in small amounts. The concentrations of all amino acids eventually decreased with starvation, although the concentrations of L-lysine, L-threonine, L-arginine and glycine remained the same or increased slightly after the first 12 h. The depletion was most pronounced for the acid amino acids; in comparison the decrease in size of the L-leucine pool was slight.

TABLE 4. Changes in Size and Composition with Nitrogen Starvation of the Soluble Amino Acid Pool of *A. visnaga* Cells

Amino Acid	0 h	12 h	24 h
Lysine	25.3	41	17
Histidine	48.1	36	14
Arginine	12.4	18	trace
Aspartic acid	730	187	27
Threonine	693	846	201
Serine	984	333	81
Glutamic acid	1566	433	92
Proline	40.2	trace	trace
Glycine	36.1	56	21
Alanine	310	118	27
Valine	272	97	21
Methionine	12	15	8
Isoleucine	68	59	22
Leucine	39	26	11
Tyrosine	58	43	18
Phenylalanine	6	trace	trace

Cells were grown 24 h in 1.0 B5 medium before transfer to nitrogen free medium. Aliquots of cell suspension were sampled after 0, 12 and 24 h of nitrogen starvation. Cells were collected by filtration, homogenized in 15 ml of 80% (v/v) ethanol and fractionated according to the method of Fletcher and Beevers (1970). Amino acids were separated on a Beckman Model 121 Automatic Amino Acid Analyzer.

Suppression of L-leucine Uptake by Ammonium Sulfate and L-leucine

The effect of preloading nitrogen starved cells with high concentrations of L-leucine, $(NH_4)_2SO_4$ or both is illustrated in Fig. 12. All three treatments rapidly and effectively diminished the rate of L-leucine uptake. Preloading with L-leucine reduced uptake to nil, while cells preloaded with $(NH_4)_2SO_4$ retained about 10% of the control activity. Suppression of transport was most rapid when cells were treated with both L-leucine and $(NH_4)_2SO_4$.

M. Long-term L-leucine Uptake by Nitrogen Starved Cells

Results for long term uptake of L-leucine by starved *A. visnaga* cells are given in Fig. 13. The ^{14}C content of the cells increased continuously, although at a decreasing rate, throughout the 100 minute incubation period. No evidence for efflux of substantial amounts of radioactivity was obtained.

N. Inhibition of Respiration by DNP and NaN_3

Dose response curves showing the effects of DNP and NaN_3 on oxygen uptake by starved and unstarved cells of *A. visnaga* are given in Figs. 14 and 15 respectively. DNP administered in final concentrations of 10^{-6} to 10^{-4} M resulted in approximately 30% stimulation of oxygen uptake, consistent with its role as an uncoupler. At concentrations exceeding 10^{-4}M, the rate of oxygen uptake declined, suggesting that secondary effects of the inhibitor were occurring. The response to DNP was quantitatively similar for both starved and unstarved cells.

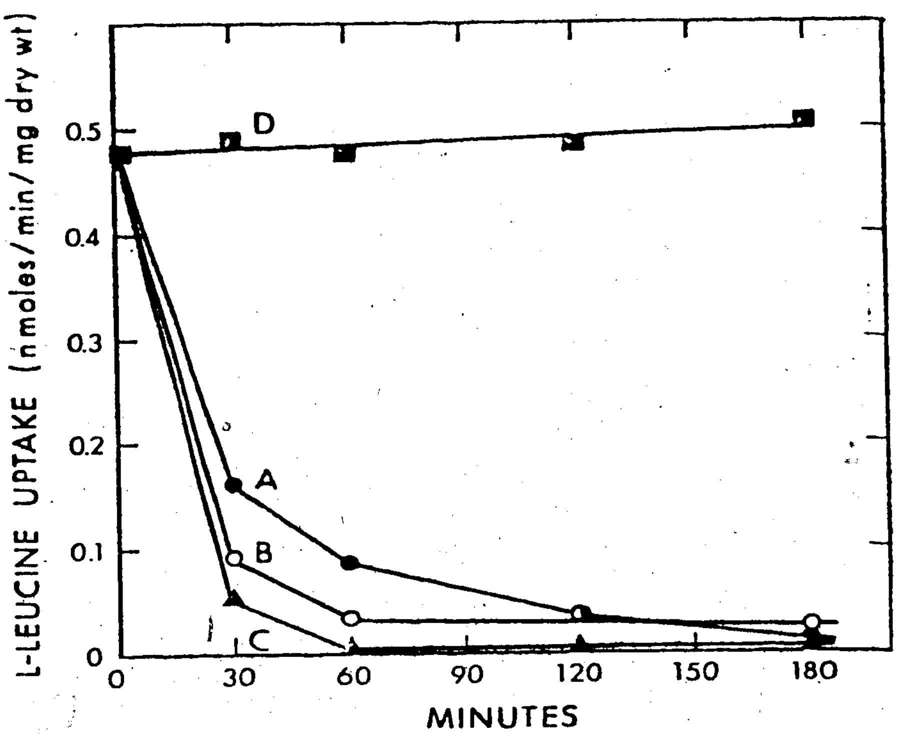

FIGURE 12. Suppression of L-leucine uptake by 5 mM L-leucine (curve A), 1 mM $(NH_4)_2SO_4$ (curve B) or a combination of 5 mM L-leucine and 1 mM $(NH_4)_2SO_4$ (curve C).

Nitrogen starved cells (12 h) were collected by filtration, washed and resuspended in 0-B5 medium lacking nitrogen to give a PCV of 0.5 ml/2 ml of cell suspension. The suspension was then distributed equally among 4 flasks. One ml aliquots of concentrated stock solutions were added to each of 3 flasks to give the indicated concentrations of preloading compounds. The control flask (curve D) received 1.0 ml distilled water. Flasks were incubated at 23°C with constant shaking. Aliquots were removed from each flask at 30, 60, 120 and 180 min after the additions. These cells were washed, resuspended in 0-B5 medium lacking nitrogen and assayed at 23°C for uptake of L-leucine from a 50 μM solution. Points are averages of duplicate determinations.

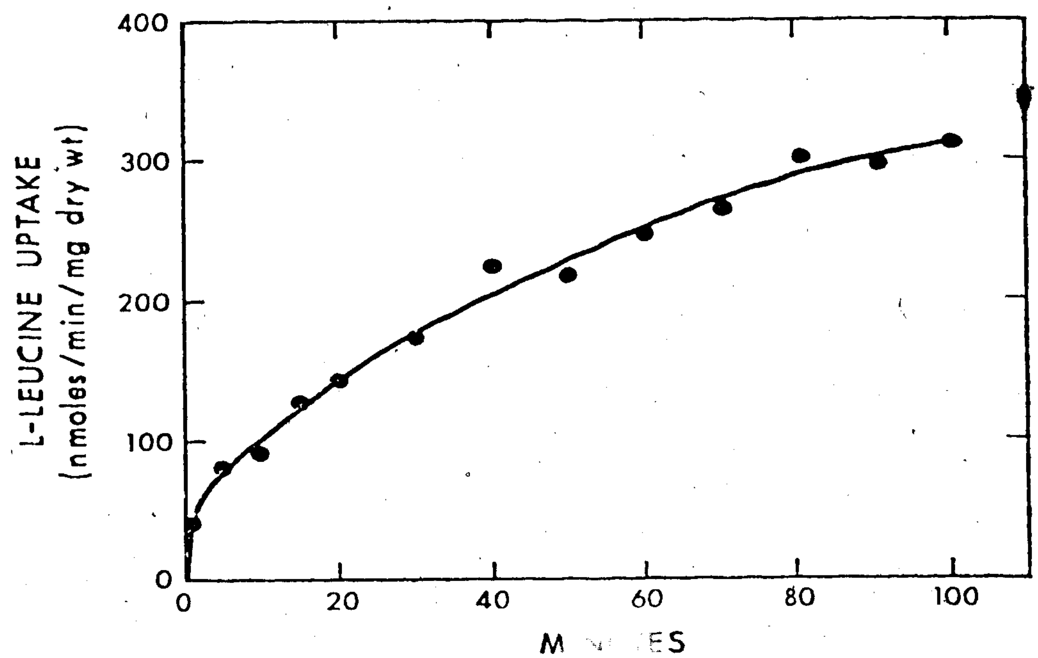

FIGURE 13. Long term uptake of L-leucine by nitrogen starved cells of *A. visnaga*.

Nitrogen starved cells (12 h) were collected by filtration, washed and resuspended in 0-B5 medium lacking nitrogen. Uptake of L-leucine from a 5 mM solution was assayed at 23°C. Points are averages of duplicate determinations.

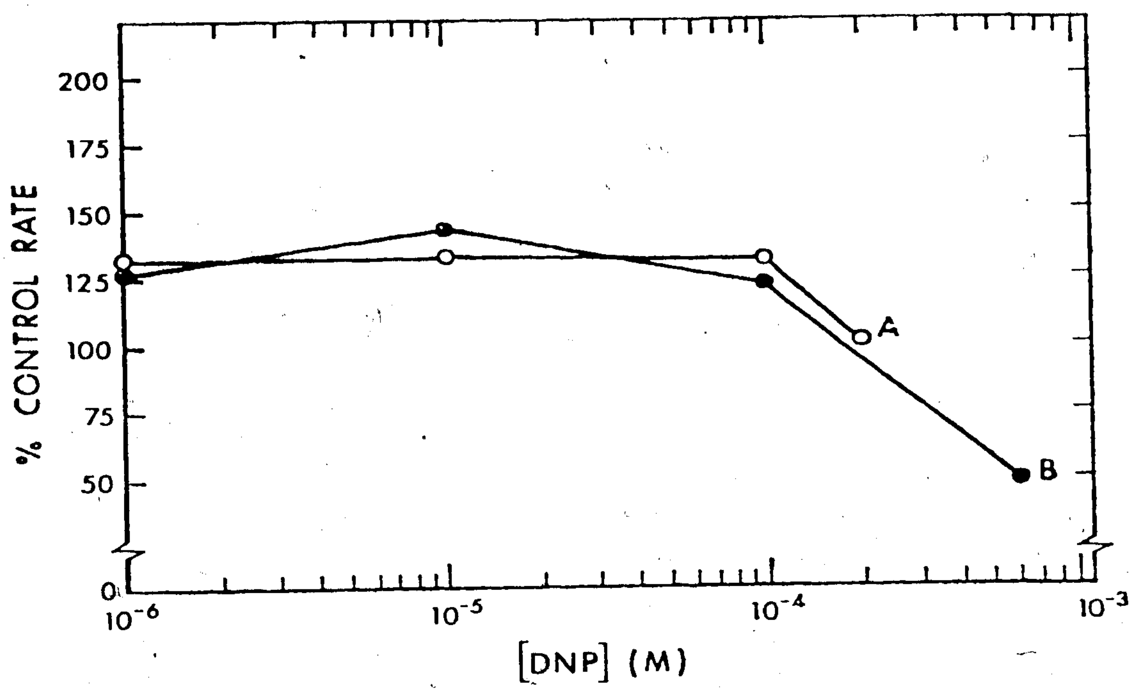

FIGURE 14. Dose response curve for the effect of DNP on oxygen uptake by unstarved (curve A) and nitrogen starved (curve B) cells of *A. visnaga*.

Aliquots were taken from nitrogen sufficient cell suspensions 36 h after inoculation, washed with 0-B5 medium and transferred to calibrated Warburg vessels. Aliquots from nitrogen starved cell suspensions were treated similarly, except that 0-B5 medium lacking nitrogen was used for washing and resuspending. Each Warburg vessel contained 2.0 ml of cell suspension, 0.2 ml of 10% potassium hydroxide in the center well and 0.2 ml of inhibitor solution in the side arm. Control flasks contained 0.2 ml of distilled water in the side arm. Oxygen uptake was measured at 27°C after 20 min of preincubation with shaking.

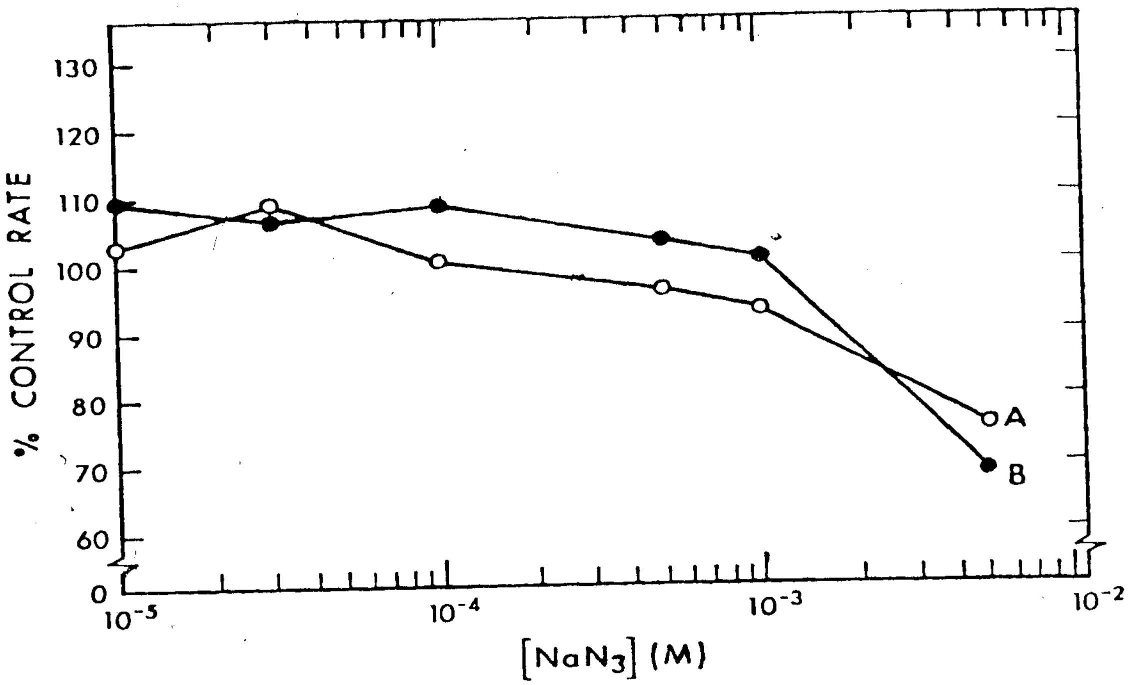

FIGURE 15. Dose response curves for the effect of NaN_3 on oxygen uptake by unstarved (curve A) and nitrogen starved (curve B) cells of *A. visnaga*.

Aliquots were taken from nitrogen sufficient cell suspensions 36 h after inoculation, washed with fresh O-B5 medium and transferred to calibrated Warburg vessels. Aliquots from cell suspensions that had been nitrogen starved for 12 h were treated similarly except that nitrogen-free medium was used for washing and resuspending. Each Warburg vessel contained 2.5 ml of cell suspension, 0.2 ml sodium azide in the side arm, and 0.2 ml 10% potassium hydroxide in the center well. Oxygen uptake was measured at 27°C after 20 min preincubation with shaking.

Nitrogen sufficient cells treated with 10^{-5} to 10^{-3} M NaN_3 showed either a slight stimulation of oxygen uptake or no apparent effect. Nitrogen starved cells responded to the lowest concentrations of NaN with a slight increase in oxygen uptake, but inhibition of oxygen uptake, the classical response to NaN_3, occurred at concentrations greater than 10^{-4} M.

O. Inhibition of L-leucine Uptake by Inhibitors of Respiration and Energy Metabolism

Table 5 summarizes the effects of inhibition of respiration and energy metabolism on L-leucine transport. Inhibitor concentrations, with the exception of oligomycin, were chosen on the basis of their effects on oxygen uptake and were kept as low as possible to minimize nonspecific effects of the inhibitors.

L-leucine transport by nitrogen sufficient and nitrogen starved cells responded differently to the inhibitors employed. L-leucine transport in nitrogen sufficient cells was inhibited 80% by 10^{-4} M DNP, 43% by 10^{-4} M NaN_3 and 43% by 10 μg/ml oligomycin. The corresponding percentages for nitrogen starved cells treated under identical conditions were 35%, 96% and 74% for DNP, NaN_3 and oligomycin respectively (Table 5).

TABLE 5. Inhibition of L-leucine Transport by *A. visnaga* Cells by Inhibitors of Energy Metabolism and Respiration.

Amino Acid	Nitrogen status	Inhibitor	% Inhibition
L-alanine	starved	DNP 10^{-4}M	99
L-leucine	starved	10^{-4}	35
L-leucine	sufficient	10^{-4}	80
L-leucine	starved	NaN_3 10^{-4}	96
L-leucine	sufficient	NaN_3 10^{-4}	43
L-leucine	starved	Oligomycin 10 μg/ml	69
L-leucine	sufficient	Oligomycin 10 μg/ml	43
L-leucine	starved	$-O_2$ (30 min)	51
L-leucine	starved	$-O_2$ (15 min)	51

A. Chemical inhibitors. Nitrogen sufficient cells and cells that had been starved 12-14 h were collected by filtration, washed and resuspended in 0-B5 medium and 0-B5 medium lacking nitrogen respectively. Assay flasks contained 2.0 ml cell suspension and 0.3 ml fresh B5 medium. Aliquots of 0.1 ml aqueous DNP or NaN_3, or .05 ml ethanolic oligomycin were added to assay flasks 5 min before the addition of L-leucine. The initial extracellular concentration of L-leucine was 50 μM. Control flasks contained solutions of identical composition plus 0.10 ml distilled water on 0.05 ml ethanol in place of the inhibitors.

B. Anaerobiosis. Cells that had been nitrogen starved for 12 h were washed and concentrated to a PCV of 1.0 ml 12.0 ml of cell suspension. Aliquots of 4 ml were transferred to 50 ml reaction vessels containing 0.4 ml fresh 0-B5 medium lacking nitrogen. The flasks were initially flushed with air at 50 ml/min for 15 min and subsequently flushed with air or nitrogen for an additional 30 min before assay of L-leucine uptake from a 50 μM solution.

IV. DISCUSSION

A. Growth of *A. visnaga* Cells in Suspension Culture

The growth of *A. visnaga* cells in suspension culture conformed to the pattern observed for growth of cells of other plant species (*e.g.*, Street, 1973). Plant cells in suspension culture typically exhibit a three-stage growth curve, consisting of an apparent lag phase, an exponential phase and a stationary phase. With *A. visnaga*, the lag phase could be reduced in duration or eliminated if new cultures were inoculated to a minimum of 1.5-2.0 mg dry weight/ml of exponentially growing cells.

The doubling time for exponentially growing *A. visnaga* cultures was 30-36 h. This compared favorably with doubling times of 20-48 h observed for suspension cultures of a variety of different plant species (Dougall, 1972).

The factors that influence the pH of the growth medium are numerous (Raven and Smith, 1974), the most important being uptake of ammonium ions, release of carbon dioxide and the preferential uptake of hydroxyl or hydrogen ions. It is not possible at this time to precisely correlate the pH of the culture and the metabolic state of the culture. However, in the context of the present study it is important to note that Velicky and Rose (1973) have suggested that pH changes during the growth of the culture might be an important factor influencing nitrogen metabolism.

B. Uptake of L-leucine at Different Stages of the Growth Curve

Assays of transport activity performed at different points on the growth curve indicated that the rate of L-leucine transport did not remain constant in actively growing cultures (Fig. 4). The peak rate represented a 2.5-fold increase over the rate measured shortly after inoculation into new medium. It is possible that this increase in uptake rate was associated with decreasing levels of inorganic nitrogen in the medium, particularly ammonium ions. Hunter and Segel (1973), for example, found that the presence of ammonium ions in the culture medium inhibited amino acid transport by *P. chrysogenum*. Although inorganic nitrogen levels were not monitored in the present work, other studies (Bayley *et al.*, 1972; Rose and Martin, 1974) have shown that ammonium ions are rapidly depleted from the medium by newly inoculated cultures.

Alternatively, transport rates over the growth curve may be influenced by intracellular levels of soluble nitrogen. Suspension cultured plant cells appear to undergo a rapid phase of nitrogen metabolism shortly after inoculation, causing total insoluble (Givan and Collin, 1967) and soluble nitrogen (Simpkin and Street, 1970) content per cell to peak early in the growth curve, then decline rapidly. It is possible that the transient increase in L-leucine transport rate observed in growing *A. visnaga* cells is inversely correlated with the soluble nitrogen pool or some component of that pool.

From a functional point of view, activation of an amino acid transport system would be advantageous under conditions of nitrogen scarcity, since it would permit recovery of amino acids being translocated from roots or senescing tissues (see Nelson and Gorham, 1959; Muhammad and Kumazawa, 1974), as well as recovery of leaked amino acids from within intracellular spaces.

It should be noted that King and Oleniuk (1973) have reported results for L-alanine uptake by suspension cultured soybean cells that contradict the results obtained for L-leucine uptake by *A. visnaga*. In soybean cells, L-alanine uptake was observed to decrease with the age (cell mass) of the culture. The data presented, however, were not extensive and represent uptake over a limited region (2.4 mg/ml to 3.9 mg/ml dry weight) of the growth curve.

C. Effect of Nitrogen Starvation on L-leucine and L-alanine Uptake

In order to further explore the possibility that uptake of L-leucine was correlated with the nitrogen status of the tissue, uptake was assayed in cells that had been incubated in nitrogen free medium. It is evident from Fig. 6 that nitrogen starvation resulted in an approximately 10-fold increase in L-leucine uptake over the rate measured in nitrogen sufficient cells. While increases of similar magnitude have been observed for the uptake of -aminoisobutyric acid (4x) after "ageing" of barley leaf strips in $CaSO_4$ (Shtarkshall *et al.*, 1970), and for L-leucine (3.5x) and glycine (2x) uptake by corn roots washed in $CaCl_2$ (Leonard and Hanson, 1972a,b), considerably greater increases in transport activity have also been recorded. Increases under nitrogen starved conditions of 80 to 100-fold have been reported for uptake of various amino acids by *P. chrysogenum* (Benko *et al.*, 1967), yeast (Schwencke and Magaña-Schwencke, 1969) and cultured soybean cells (King and Oleniuk, 1973).

The difference in magnitude of the response to starvation of soybean and *A. visnaga* cells was not anticipated. Fig. 6 indicates that the pattern of L-alanine uptake was similar to that of L-leucine in starved *A. visnaga* cells; thus, the difference appears to reflect differences in cell lines rather than being a function of the amino acid used in each study.

D. Concentration Dependency of L-leucine Uptake

Uptake of L-leucine by starved and unstarved cells was apparently carrier mediated. In addition, when transport rates were plotted *vs* concentration of L-leucine in the double reciprocal form (Fig. 7 and 8) each of the curves could be resolved into two phases.

Bi- or multiphasic kinetics have been observed in a variety of other transport systems, but their interpretation remains a subject for debate. In animal cells, such kinetics have been attributed to either increased rates of diffusion at high permeant concentrations or to the existence of multiple carriers for a given permeant (Heinz, 1972). Similar curves for L-leucine uptake by *E. coli* have been interpreted as representing multiple carriers (Guardiola *et al.*, 1974a). This conclusion appears to be verified by studies of transport mutants (Guardiola *et al.*, 1974b).

The interpretation of biphasic uptake kinetics in plant cells has been further complicated by the existence of two cell membranes, the plasmalemma and the tonoplast, with which mechanisms for solute transport could be associated. It has thus been argued that muliphasic isotherms for ion uptake represent either two sites operating in parallel, both in the plasmalemma (Epstein, 1972), or a pair operating in series, one in the tonoplast and the other in the plasmalemma (Laties, 1969). Recently, Nissen (1973) and Linask and Laties (1973) have proposed that uptake of organic and inorganic solutes is mediated by a single structure that is located in the plasmalemma. This structure is postulated to undergo "all-or-none" changes of state at certain distinct permeant concentrations, and these changes can be described by a series of phases, each following Michaelis-Menten kinetics. This model is supported by the kinetic characteristics of plasma membrane-associated ATPase, identified in oat roots, which appears to have a role in ion transport (Leonard and Hodges, 1973).

Analysis or reanalysis by Nissen (1974) of published kinetic data indicates that transport of a wide variety of inorganic ions can be described by multiphasic uptake mechanisms. More relevant to the present work is the fact that amino acid transport by barley leaf strips (Shtarkshall *et al.*, 1970), wheat roots (ref. 37 *in* Nissen, 1974), possibly sunflower hypocotyl segments (Rheinhold and Powell, 1958), and cultured sugarcane cells (Maretzki and Thom, 1970) can be reinterpreted according to the model of Nissen and are in agreement with the results obtained for *A. visnaga*.

The saturability of L-leucine uptake in nitrogen sufficient cells suggests that these cells possess a constitutive transport system for this and perhaps other amino acids. This is in contrast to soybean (King and Oleniuk, 1973) and *P. chrysogenum* (Hunter and Segel, 1973), where L-alanine and L-leucine uptake respectively are apparently non-saturable under nitrogen sufficient conditions and presumably result from diffusion. This may account, at least in part, for the difference in the magnitude of the increase in transport activity brought about by nitrogen starvation.

K_m values reported for L-leucine transport systems of plants and microorganisms are summarized in Table 1. Although direct comparisons of K_m values are difficult to make because of variations in the concentration range employed, nutritional status, etc., the apparent K_m values of 5.6 μM and 62.5 μM obtained for L-leucine transport by nitrogen starved *A. visnaga* cells compare favourably with values obtained for amino acid uptake in other suspension cultured cells, yeast and fungi. The values were much lower than those reported for L-leucine and AIB uptake by leaf tissues (Table 1). However, L-leucine uptake by plant tissues was examined only at high external concentrations; it is possible that uptake at lower concentrations would have shown multiphasic kinetics and additional K_m values. Furthermore, recent evidence has suggested that uptake of AIB, which is used in investigations of amino acid transport primarily because it is not metabolized by most tissues, occurs via a different carrier than the one responsible for transport of naturally occurring amino acids (Cheung and Nobel, 1973).

Pall (1969) has described two systems in *Neurospora crassa* that are responsible for uptake of L-leucine and other neutral amino acids. System I is active in young, rapidly growing cultures and has an apparent K_m for L-leucine uptake of 120 μM; in comparison, system II, active in old, carbon starved cultures, accounts for approximately 75% of uptake and has a K_m of 4 μM.

Once again, from a functional point of view, the lower K_m

(greater affinity for the carrier) would be advantageous to the organism in facilitation of more efficient retrieval of amino acids present at low concentrations in the external (extracellular) medium. The fact that 1) L-leucine transport rates increase when there is insufficient nitrogen in the culture medium and 2) the K_m for L-leucine transport decreases under the same conditions suggests that such a system may be operating in cultured *A. visnaga* cells.

E. L-leucine Uptake as a Function of Temperature

L-leucine transport by nitrogen starved cells was found to be strongly temperature-dependent, a phenomenon that is usually considered characteristic of active, energy dependent systems (Lehninger, 1972). Maximal uptake was observed at 27°C, which was coincidentally the temperature at which the cells were grown. Although reduced energy production is thought to be the main factor in diminishing L-leucine transport at low temperatures, it should be noted that some transport proteins can be extracted by cold osmotic shock (Farrell and Rose, 1971). A second point for consideration, one that is frequently overlooked, is that changes in temperature can initiate phase changes in membrane lipids and hence conformational changes in membrane-associated proteins (see Fox and Tsukagoshi, 1972). It is plausible that such a conformational change could alter the affinity of the carrier for its substrate. In either situation, extraction of proteins or change of conformation, changes in transport activity would be observed independently of energy metabolism.

F. L-leucine Efflux from "Preloaded" Cells

Uptake rates that have been presented thus far represent only net rates, *i.e.*, the difference between influx and efflux. It was considered important to obtain an estimate of the efflux rate alone so that total uptake could be determined under experimental conditions. Efflux is generally measured by preloading cells with labelled amino acids, then resuspending the cells in isotope-free medium. Radioactivity that subsequently appears in the medium is taken as a measure of efflux. Two difficulties are encountered with the "preloading" method: (1) the permeant can undergo metabolic conversion once it enters the cell, thus reducing its concentraton, and (2) solutes lost through efflux may be retransported. Influx and efflux, therefore, would best be measured simultaneously. In the present study, this was attempted by using ^{14}C-leucine as the "preloaded" form of leucine and ^{3}H-leucine to estimate influx. As nearly as can be estimated, efflux of accumulated L-leucine under experimental conditions was less than 1% of the net rate (Fig. 11). Although for the reasons given above, this estimate may be low, it is unlikely that the real values are substantial.

G. Recovery of Transported ^{14}C-leucine and its Metabolites

Extraction and fractionation of the cellular contents of *A. visnaga* cells previously incubated with ^{14}C-leucine indicated that most of the label would be recovered in the soluble and protein amino acid fractions (Table 2). Although L-leucine was not specifically isolated, the work of others (Birt and Hird, 1959; Holleman, 1966, cited in Holleman and Key, 1967; Oaks, 1965; Cheung and Nobel, 1973) suggests that exogenously supplied L-leucine generally does not undergo extensive conversion to other amino acids. However, on the basis of the conversion to protein and other compounds it is difficult to draw firm conclusions about intracellular concentrations of free L-leucine during uptake, or to establish whether uptake occurred against a concentration gradient.

H. Inhibition of L-leucine Uptake by Other Amino Acids

Transport of L-leucine and other amino acids has been found to occur via highly specific carriers in bacteria (Piperno and Oxender, 1968), filamentous fungi (Benko *et al.*, 1967, 1969; Pall, 1969, 1970), yeast (Grenson *et al.*, 1966; Grenson, 1966; Gits and Grenson, 1967) and mammalian cells (Heinz, 1972). Demonstration of carrier specificity ideally involves a detailed kinetic analysis, preferably by the "ABC test" (Scriver and Wilson, 1964), but even without this detailed analysis an indication of carrier specificity can be derived by simple inhibition tests.

L-Leucine uptake by nitrogen starved *A. visnaga* cells was inhibited by a variety of neutral, acidic, basic and D-amino acids, with the neutral amino acids being the most effective inhibitors. Relative to most bacterial and some fungal systems (see pages the transport system responsible for L-leucine uptake in *A. visnaga* can be considered quite broad in its specificity. The published data for transport systems of other plant cells suggest that broad structural specificity may be a common property. Cheung and Nobel

(1973) have found, for example, that a transport system present in fragments cut from pea leaves is responsible for the uptake of a variety of neutral and positively charged amino acids. Similarly, Birt and Hird (1959) found that a number of amino acids were apparently transported by the same carrier, while amino acids with aliphatic side chains were preferentially taken up. However, evidence of a system specific for basic amino acids has been found in suspension-cultivated sugarcane cells (Maretzki and Thom, 1969) and chloroplasts have been found to possess a carrier specific for glycine, L-alanine, L-isoleucine, L-leucine and L-valine (Nobel and Cheung, 1972).

In retrospect, it would have been informative to investigate the inhibition of L-leucine transport by other amino acids in nitrogen sufficient cells as well as in starved cells. Several fungi have been found under nitrogen and carbon sufficient conditions to possess relatively specific transport systems, while under starvation conditions, additional general (non-specific) amino acid transport systems become active (Pall, 1969, 1970; Benko *et al.*, 1967). The data obtained for nitrogen starved *A. visnaga* cells could represent such a general system while masking a more specific system for L-leucine and structurally related amino acids.

I. Qualitative and Quantitative Changes in the Soluble Amino Acid Pool During Nitrogen Starvation

It has been repeatedly sugg ed that amino acid transport systems are regulated by intracellular amino acids (Benko *et al.*, 1969; Ring *et al.*, 1970; Pall, 1971). It has not always been possible, however, to demonstrate any correlation between pool size and transport activity (Robinson et al., 1973; Magaña-Schwencke, 1969). The observation that the pool sizes of nearly all amino acids decrease under conditions of nitrogen starvation (Table 4), while the rate of leucine transport is enhanced, is compatible with a regulatory role for some component(s) of the soluble pool. It is not possible, however, to draw any conclusions about the identity of this component.

The data presented for amino acid pool sizes of *A. visnaga* represent total soluble amino acids. It should be noted that isotope studies conducted with maize root tips (Oaks, 1965) and soybean hypocotyls (Holleman and Key, 1967) have demonstrated the existence of two distinct soluble pools of L-leucine: a "protein precursor" or "metabolic" pool and a "storage" pool. In both studies the protein precursor pool was depleted most rapidly when a source of translocated or exogenous L-leucine was removed. Thus, it is relevant to ask (1) whether multiple soluble pools also exist in *A. visnaga* cells and (2) if so, which pool is responsible for any observed regulatory effects.

J. Suppression of L-leucine Uptake by Ammonium Sulfate and L-leucine

The enhanced uptake of L-leucine by *A. visnaga* cells under starvation conditions was rapidly diminished by preloading cells with L-leucine, $(NH_4)_2SO_4$ or both (Fig. 12). Suppression of transport was most effective with the combination treatment. The degree of suppression (or reinhibition) of transport was greater than that reported for other transport systems (Hunter and Segel, 1973; Pall, 1971).

Several explanations for the observed effects of L-leucine are possible:

1) Intracellular L leucine may transinhibit the carrier. Although (Gross *et al.*, 1970) have postulated that trans-inhibition involves disruption of energy coupling to transport, transinhibition is generally assumed to result from a direct interaction of an intracellular substrate of the transport system with some component of the carrier, thus preventing its translocation.
2) Allosteric inhibition of the transport system by a metabolite of L-leucine that is not a substrate may occur.
3) The transported L-leucine or a metabolite may induce the formation of a regulatory protein that binds to the carrier.

Ammonia (or ammonium ions) has been cited as regulatory for amino acid transport in *Aspergillus nidulans* (Robinson *et al.*, 1973) and *P. chrysogenum* (Benko *et al.*, 1969). The fact that $(NH_4)_2SO_4$ exhibited a similar re-inhibitory effect to that of L-leucine lends credence to those explanations that invoke a role for a metabolite of L-leucine. Unfortunately, this does not narrow the possibilities greatly since point #1 would still apply if the metabolite were a substrate of the transporter. L-glutamate and L-glutamine would be good candidates in this respect, and in fact, these compounds have been shown to increase rapidly when suspension cultured cells are incubated in the presence of ammonium ions (see King and Oleniuk, 1973).

The argument above would not apply if ammonium ions could prevent the actual entry of L-leucine into the cells either by competition for a common transporter or by competition for an energy source utilized by both systems. No information is yet available on the mechanism by which ammonium ions enter plant cells, but *P. chrysogenum* has been found to possess a highly specific transport system (which excludes amino acids) for ammonium ions (Hackette *et al.*, 1970). Furthermore, Hunter and Segel (1973b) have found that ammonium ions exert their inhibitory effect on L-leucine uptake by that organism only under conditions when the ammonium ion transporter is active. They have concluded that their results can best be explained by competition between the two transporters for a common energy coupling system. The relevance of this observation to L-leucine uptake in *A. visnaga* awaits elucidation.

K: Long-term L-leucine Uptake by Nitrogen Starved Cells

Uptake rates for L-leucine were usually determined using short time intervals to insure that measurements reflected initial rates of uptake. It has been observed, however, that events may occur during long-term uptake of amino acids that give an indication of the manner in which the transport process is regulated. Hunter and Segel (1973a) have observed that the ^{14}C-leucine content of nitrogen starved mycelia of *P. chrysogenum* reaches a peak and then declines during prolonged incubation with the labelled amino acid. The disappearance of label was found to result from efflux of α-ketoisocaproic acid back into the suspending medium.

The results, given in Fig. 13, indicate that the ^{14}C content of *A. visnaga* cells increased continuously throughout the long-term incubation period. No evidence for efflux of substantial amounts of label was obtained, suggesting that L-leucine uptake by *A. visnaga* cells does not fit the pattern observed for *P. chrysogenum*.

L. Inhibition of Respiration by DNP and Azide

Inhibitors of respiration and energy metabolism are frequently employed in studies of transport systems to demonstrate the "active", energy dependent nature of the process (*e.g.*, King and Oleniuk, 1973). Before utilizing this technique with *A. visnaga* cells, it was considered essential to demonstrate that the concentrations of inhibitors employed in transport experiments elicited the expected effect on oxygen consumption. As indicated in Fig. 14, DNP administered in final concentrations of 10^{-6} to 10^{-4} M resulted in approximately 30% stimulation of O_2 uptake, consistent with its role as an uncoupler. However, at concentrations exceeding 10^{-4}M the rate of oxygen uptake decreased, suggesting that the inhibitor was exerting secondary effects on the cells. The nature of the secondary effects was not investigated in the present work, but nonspecific effects of DNP on cell membranes have been noted elsewhere (Mircevova, 1973). The implications for studies of membrane transport systems are clear.

Similarly, the interpretation of oxygen uptake data obtained in the presence of different concentrations of NaN_3 (Fig. 14) must be interpreted cautiously. The classical effect of NaN_3 is inhibition of cytochrome oxidase. However, at high concentrations NaN_3 may also affect proton conductance across membranes and inhibit ATPase activity (Harold, 1972). Interpretation is further complicated by the existence in some plant tissues of an additional cyanide-insensitive pathway for oxygen uptake (*e.g.*, Wedding *et al.*, 1973a,b). This system permits oxygen uptake to precede at an undiminished or even stimulated rate, although the conventional pathway via cytochrome oxidase is blocked. The rate of oxygen uptake via the cyanide insensitive pathway appears to depend in part on the tightness, before inhibition, of the coupling of oxidative phosphorylation to electron transport.

The existence of the cyanide-resistant pathway in *A. visnaga* has not been demonstrated, but it is suggested by the fact that uptake of L-alanine and L-leucine was strongly inhibited by NaN_3 concentrations that did not inhibit oxygen consumption. A similar pattern was observed for NaN_3 inhibition of phosphate uptake by beech mycorrhizae (Harley *et al.*, 1953). The slight difference in response of starved *vs* unstarved cells may reflect a difference either in energy coupling or in the degree of operation of the two pathways for each nutritional state.

M. Inhibition of L-leucine Transport by Inhibitors of Respiration and Energy Metabolism

The L-leucine transport rates of nitrogen sufficient and nitrogen starved cells responded differently to the inhibitors DNP, NaN_3 and oligomycin (Table 5). These differences could not be predicted on the basis of the effects of the inhibitors on oxygen consumption. Thus, these data may reflect underlying differences in the coupling of metabolic energy to transport in cells with varying nutritional status. Berger (1973) and Berger and Heppel (1974) have described two general classes of amino acid transport systems in *E. coli*. One class includes systems sensitive to osmotic shock that are associated with periplasmic membrane proteins and are absent in membrane vesicle preparations. The second group includes transport systems whose binding proteins are not released from the cell membrane by osmotic shock. Evidence obtained by the use of a variety of energy donors and inhibitors strongly suggested that shock-resistant systems are activated by the so-called "energized membrane state", while ATP, or an intermediate derived from ATP, is directly involved in driving shock-sensitive systems. It has recently been found (Wood, 1974) that L-leucine uptake by different transport systems in *E. coli* probably fits the same pattern.

To a limited extent it is possible to employ the same line of reasoning to analysis of the inhibitor data for L-leucine uptake by *A. visnaga*. The sensitivity of nitrogen sufficient transport to DNP, which as an uncoupler dissipates the energized membrane state generated by electron transport, suggests that possibly this state rather than ATP directly is the form in which energy is coupled to uptake. This appears [illegible] confirmed by the relatively low sensitivity of nitrogen sufficient transport to oligomycin. Oligomycin is known to deplete ATP pools in plants (Jacoby and Plessner, 1970) apparently by inhibition of mitochondrial ATPase, as no effect on the plasma membrane ATPase of oat roots has been found (Leonard and Hodges, 1973). The fact that oligomycin has any effect at all on L-leucine transport by *A. visnaga* cells can be explained if the energized membrane state is also generated by ATP hydrolysis.

In comparison, the greater sensitivity of L-leucine transport by nitrogen starved cells to oligomycin suggests a direct involvement of ATP, as does the decreased sensitivity to the uncoupler DNP. That

ATP may be supplied by oxidative phosphorylation or at the substrate level is indicated by the 49% of uptake that persists under anaerobic conditions (Table 5). The results obtained with NaN_3 do not support this hypothesis if indeed NaN_3 acts as an inhibitor of cytochrome oxidase or as an uncoupler. However, if NaN_3 acts as an inhibitor of ATPase under the present experimental conditions, the data are compatible.

The major drawback to this manner of interpretation is that it presumes that the results arise only from the initial effects of the inhibitors. In reality, the inhibitors of specific steps of energy metabolism eventually have far-reaching secondary effects as substrates accumulate. While this problem can be circumvented in work on micro-organisms by use of mutants, this alternative is not yet available for the study of plant cell transport systems. With these reservations, it seems plausible that the data reflect different modes of energy coupling, particularly if two discrete transport systems are visualized as being involved.

The mechanism of energy coupling to organic solute transport has not been clearly elucidated for any system. The topic remains highly controversial, even in those microbial systems that have been extensively studied. Evidence is accumulating, however, that suggests that the coupling of energy to solute transport involves a proton gradient as proposed by Mitchell (1972).

Electrogenic pumps have recently been demonstrated in plant cells (Higinbotham *et al.*, 1970; Higinbotham, 1974; Raven and Smith, 1974; Poole, 1974, Lin and Hanson, 1974) and it has been suggested that such pumps play a role in solute transport (Higinbotham *et al.*, 1970;

Higinbotham, 1974; Raven and Smith, 1974; Lin and Hanson, 1974). ATP hydrolysis has been proposed as the driving force for such pumps, and this suggestion seems to be supported by the observation that the activity of the plasma membrane ATPase of oat roots increases under the same conditions that enhance solute uptake (Leonard and Hanson, 1972a,b; Leonard and Hodges, 1973).

Evidence for the involvement of a proton gradient in amino acid uptake has been obtained by Etherton and Nuovo (1974) who measured the transmembrane potentials of oat coleoptile cells in the presence and absence of nine amino acids. Introduction of an amino acid resulted in rapid depolarization of the membrane, followed by partial repolarization. The data obtained were consistent with a model in which amino acids are transported by hydrogen ion co-transport systems as suggested by Mitchell (1972).

V. GENERAL DISCUSSION

Amino acids may cross cell membranes by three mechanisms:

1) Free diffusion along a concentration gradient, characterized by independence from metabolic energy sources, low temperature dependence, lack of competition for uptake among structurally related compounds, and absence of saturability as the amino acid concentration increases.
2) Facilitated diffusion, which involves carrier mediated transport driven by an electrochemical or concentration gradient of unmetabolized substrate.
3) Active transport via a carrier system that relies on a primary (ATP) or secondary (proton or ion gradient) input of metabolic energy and results in net accumulation of unchanged substrate against a concentration gradient.

Evidence obtained for leucine transport by *A. visnaga* indicates that it is carrier mediated, strongly temperature dependent, and energy dependent. Concentrative uptake could not be demonstrated conclusively, but other evidence obtained suggests that L-leucine transport in this tissue is an active process, rather than one of facilitated diffusion as found for this amino acid in many animal tissues.

Although plant cells are not generally thought of as acquiring solutes from their environment in the same manner as heterotrophic cells, functional roles for amino acid transport systems in higher plants can be visualized. Recovery from intracellular spaces of leaked amino acids and capture of amino acids being translocated from other portions of the plant, for example, would likely involve uptake against a concentration gradient, necessitating an active transport system as opposed to simple or facilitated diffusion. In this connection it is interesting to note that specialized "transfer cells" believed to be involved in the active transport of various compounds have been found associated with the vascular tissues of plants. Certain of these transfer cells are thought to specifically retrieve organic nitrogen compounds (Zu and O'Brien, 1971).

The use of cell suspension cultures as experimental systems for the study of biochemical and physiological problems has several advantages compared to intact plant tissues: (1) cells may be propagated rapidly under uniform growth conditions; (2) cultured cells are easily manipulated, and (3) cells may be obtained without excision and subsequent wounding responses.

With respect to the present work, the major disadvantage of batch suspension cultures is the tendency for the cell populations to be heterogeneous in age, genetic complement, and degree of aggregation. Transport data, therefore, represent composite values and do not necessarily reflect the events occurring in an individual cell.

Of the three properties mentioned above, variability in the degree of cell aggregation is probably the most serious problem. In contrast to studies with single cells, L-leucine uptake by cell aggregates undoubtedly reflects both homocellular transport (from medium to cell) and transcellular transport (from cell to cell). Symplastic transport (transport from cell to cell via a cytoplasmic continuum) of amino acid analogues has been observed in plant tissues (Müller and Bräutigam, 1973), and probably occurs for L-leucine in aggregates of *A. visnaga* as well.

In addition, the sizes of cell aggregates, and in some cases, the positions of individual cells in aggregates have been shown to influence the activity of oxidoreductive and hydrolytic enzymes (de Jong *et al.*, 1967), peroxidase isoenzymes (Verma and Van Huystee, 1970b) and rates of protein synthesis (Verma and Van Huystee, 1970a). Extremely relevant to the study of L-leucine transport is the observation that the concentration of free amino acids varies with aggregate size (Verma and Van Huystee, 1970a).

The heterogeneity of plant suspension cultures with respect to age or stage of the growth cycle can similarly be expected to obscure details of regulation of amino acid transport. Studies with synchronous cultures of yeast, for example, have revealed stepwise changes in transport during the cell cycle (Carter and Halvorson, 1973). The stage at which the increase occurs is characteristic for each transport system, and is a potential tool for determining which amino acids are transported by a common carrier.

Methodology for synchronization of suspension cultured cells (King *et al.*, 1973) and maintenance of highly dispersed steady state cultures (Kurz, 1973) have been very recently developed. Use of these techniques in future studies of transport phenomena would appear promising.

A second feature of suspension cultures that could be considered a disadvantage is the problem of relating results obtained to the situation that exists in intact plants. Cells in fine suspension culture do not correspond morphologically to any of the types of tissue cells found in intact plants, are generally unstable, and cannot be induced to follow normal pathways of cyto-differentiation (Street, 1973b). It is, therefore, legitimate to ask how representative the data obtained with these cultures are. Although studies of enzyme activity and metabolic regulation in suspension cultured cells are relatively few, there are many reports on metabolism of various compounds (summarized by Dougall, 1972). The results of these studies indicate that at least the metabolic intermediates are identical with those in intact plants.

Data obtained for L-leucine transport were qualitatively similar to those reported for other plant cells. Quantitative differences in rates and K_m values between cultured tissues and excised tissues can be attributed in part to differences in experimental conditions; it is, therefore, difficult to assess real differences among tissue sources.

VI. SUMMARY AND CONCLUSIONS

Uptake of L-leucine by suspension-cultured plant cells of *Ammi visnaga* has been investigated. This process was found to be carrier mediated and strongly temperature dependent. Transport activity reached a peak several days after inoculation, suggesting that the nitrogen status of the cultures was an important factor. This was verified by studies of nitrogen starved cells, which showed a 10-fold increase in transport rates. Nitrogen starvation concomitantly caused a rapid depletion in the soluble amino acid pool, indicating a possible role in regulation of transport activity for amino acids. Preloading starved cells with L-leucine or ammonium sulfate resulted in a rapid decrease in transport rates, perhaps as a result of feedback inhibition.

Efflux measurements indicate that net uptake rates mainly represent influx. Transported L-leucine was metabolized by nitrogen-starved cells; concentrative uptake was not demonstrated.

Transport of L-leucine b[illegible]gen starved and unstarved cells showed different sensitivity to the [illegible]bitors oligomycin, azide and DNP, although the effects of the latter two compounds on respiration were similar for both groups of cells.

On the basis of these data, it is concluded that (1) L-leucine uptake probably occurs by active transport in starved cells of *Ammi visnaga*; (2) separate systems may be responsible for uptake under different nutritional conditions, analogous to those described in fungi, and (3) suspension cultures are a useful system for elucidation of transport processes in plant cells, although future studies should take advantage of recent improvement in tissue culture technology to provide highly dispersed, steady-state or synchronized cell populations.

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COMPOSITIONS OF TISSUE CULTURE MEDIA

(a) 1.0-B5 Medium

Macronutrients	mg/l
NaH_2PO_4	150
KNO_3	2500
$(NH_4)_2SO_4$	134
$MgSO_4 \cdot 7H_2O$	250
Iron compound (330 Fe)	28
$CaCl_2 \cdot 2H_2O$ (stock solution 15g/100 ml)	150

Micronutrients	Stock solution mg/100 ml	mg/l
$MnSO_4 \cdot H_2O$	1000	
H_3BO_3	300	
$ZnSO_4 \cdot 7H_2O$	200	
$Na_2MoO_4 \cdot 2H_2O$	.25	1.0
$CuSO_4$	2.5	
$CoCl_2 \cdot 6H_2O$	2.5	
KI	75	1.0

Vitamins	Stock solution mg/100 ml	mg/l
Nicotinic acid	10	
Thiamine HCl	100	
Pyridoxine HCl	16	10
m-Inositol	1000	

Sucrose		20g/l
Hormones	1 ppm 2,4-D (50 mg/100ml)	2 ml

Final pH of 1.0-B5 medium adjusted to 5.5 with 0.2 N NaOH

(b) Modifications to 1.0-B5 medium

1. 0-B5 medium

   contains no 2,4-D

2. 1.0-B5 Medium, nitrogen free

   KNO_3 and $(NH_4)_2SO_4$ eliminated. 1.875 g/l KCl added.