AMINO ACID TRANSPORT AND POOL FORMATION IN

PSEUDOMONAS AERUGINOSA

by

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M.Sc. (Microbiology),
University of British Columbia, 1966

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Microbiology

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May, 1968

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ABSTRACT

Pseudomonas aeruginosa has been shown to actively transport
and accumulate twenty common amino acids by systems with enzymatic
properties; that is the systems are energy dependent, temperature
sensitive, are saturated at high amino acid concentrations and
are lost by mutation.

During growth on a synthetic, amino acid free medium this
microorganism maintained a low, but significantly concentrated
heterogeneous pool of amino acids for syntheses and this pool
(native pool) was found to be in equilibrium with low levels of
exogenous amino acids with at least one exception.

Amino acid pools established from an exogenous source were
found to behave differently. Whereas some amino acids were
unchanged during the passage through the intracellular pool others
underwent extensive degradation. Some amino acids or their
degradation products were shown to be compartmentalized or made
unavailable for metabolism. Proline did not form large pools under
physiological conditions due to an imbalance between the rate of
transport and the rate of protein synthesis. A multiplicity of
intracellular proline pools was elucidated by inhibitors and
studies at low temperatures.

The amino acid transport systems operative at very low
exogenous amino acid concentrations were shown to be strongly

stereospecific. Several transport systems were elucidated by competitive inhibition studies and were found to recognize amino acids with similar chemical properties. Also very specific amino acid transport systems were demonstrated within the aromatic and basic amino acid families. The multiplicity of amino acid carrier functions was confirmed by pool displacement studies and by the selection of appropriate transport negative (Tr⁻) mutants.

Low affinity amino acid permeases or carriers were shown to operate at high amino acid concentrations for most of the amino acids tested. Low and high affinity permeases could be separately identified by kinetic studies.

Amino acid transport was found to be induced to high levels by growth in the presence of the appropriate amino acid. Some evidence was presented to suggest that the control is coordinately linked to amino acid degradative enzymes.

The constitutive levels of amino acid degradative enzymes were found to be lowered in the presence of glucose. With the exception of arginine, constitutive deaminases were inhibited by inorganic ammonia, whereas for the most part the constitutive transport functions were not changed. Induced transport levels were not markedly influenced by the presence of these nutrients.

A novel mechanism for the transport and accumulation of amino acids was formulated. This mechanism provides for the accumulation of high and low intracellular amino acid pools by an energy dependent mechanism.

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ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude to Dr. A.F. Gronlund for her encouragement, direction, and constructive criticism of both the research and writing of this thesis, and for her exhaustive editing of the manuscript.

I would also like to thank Dr. J.J.R. Campbell for his encouragement and interest and for editing the thesis.

I would like to thank Dr. R.A.J. Warren for his kind interest and his helpful suggestions concerning mutagenesis and selection of bacterial mutants and for other aspects of this work.

Certainly not the least of my gratitude is extended to my wife Judith for her tolerance, forbearance and enthusiasm during the research and especially for her typing and exhaustive editing of this thesis.

Lastly my thanks also go to my fellow-students, especially N. Medveczky, for aiding in my experiments, and to Mrs. Rita Rosbergen for the typing of this final manuscript.

INTRODUCTION

The cell membrane has long been known to be selectively permeable to a great variety of low molecular weight metabolites. The degree of permeability and selectivity differs markedly however, with different life forms.

Bacteria not only exhibit the properties of selective permeability but also are able to concentrate metabolites to a great extent from the external environment. In recent years investigators have tried to elucidate the biochemical mechanisms involved in both the processes of selectivity and accumulation common to most active transport systems.

It was the object of this investigation to study the transport and accumulation of amino acids in Pseudomonas aeruginosa, a microorganism which does not rely on exogenous amino acids for growth but is able to catabolize most amino acids as a source of carbon or nitrogen.

LITERATURE REVIEW

The permeability of cells to low molecular weight metabolites
such as amino acids has long been recognized as an important
cellular function. This preliminary step to metabolism could
profoundly influence cell growth, the rate of substrate utilization,
enzyme induction or repression and the feedback inhibition of
biosynthetic enzymes. Consequently, the transport and accumulation
of amino acids by microorganisms has received much attention in
recent years and several excellent reviews have been written on this
subject (Holden, 1962; Kepes and Cohen, 1962; Britten and McLure, 1962;
Wilbrandt, 1963; Quastel, 1964).

I. Accumulation of Amino Acids

The first observation that selective amino acid accumulating
systems existed in microorganisms was made by E.F. Gale (1947).
Gale showed that amino acids entered Staphylococcal cells, not by
a process of simple diffusion, but rather by a uni-directional
transfer mechanism which, in the case of some amino acids, required
a metabolizable energy source. These amino acids were then
concentrated into what Gale termed "amino acid pools".

The properties of the systems which transport amino acids into
microorganisms received little attention until the problem was
reinvestigated by using labelled amino acids to follow the uptake

of these compounds into cell suspensions (Cohen and Rickenberg, 1956). The investigators at the Pasteur Institute showed that _Escherichia coli_ K12 accumulated amino acids by several distinct stereospecific, energy-dependent systems, which could maintain intracellular amino acid concentrations up to 500 times that of the external medium and which could also function in the absence of protein synthesis. As a result of their work a model - essentially the same as the model described for carbohydrate transport (Rickenberg, Cohen, Buttin, and Monod, 1957) - was postulated to describe the sequence of events mediating amino acid accumulation in _E. coli_. This model was subsequently modified by Kepes (1960), for carbohydrate transport, to include not only a stereospecific enzyme-like permease but also a less specific "transporter" for movement of metabolites across the cell membrane.

At approximately this same time, investigators at the Carnegie Institute (Britten and McLure, 1962) developed a sensitive Millipore filtration technique to follow the rapid accumulation of amino acids by _E. coli_. These workers found several distinct amino acid accumulating systems in _E. coli_ and compiled a most comprehensive description of the properties of these amino acid transport systems. They found that many properties of amino acid pool behaviour were not commensurate with the permease model proposed by the workers at the Pasteur Institute. As a result, an alternate model - the "carrier" model - was proposed to explain their observations of amino acid transport and pool maintenance. These workers also considered in some detail, the osmoregulation of the amino acid pools and found that maintenance and establishment of intracellular amino acid pools were intrinsically affected by the osmolarity of the external medium.

Some amino acid concentrating systems in <u>E. coli</u> have been shown to be subject to loss by mutation. Schwartz, Mass and Simon (1959), isolated mutants of <u>E. coli</u> W resistant to the arginine analogue, canavanine, or to the glycine analogue, D-serine. These mutants were subsequently found to be unable to concentrate the respective natural amino acids.

Although the accumulation process for amino acids in microbial cells has been shown to have rigid structural requirements, to obey saturation kinetics (Holden, 1962), and to be subject to loss by mutation, recent evidence has suggested that these observations may be secondary consequences of the activity of specific amino acid recognition sites or "permeases" residing at the cell membrane surface.

By selecting <u>E. coli</u> W auxotrophs which were unable to grow in the presence of low concentrations of the required amino acids, Lubin, Budreau and Gross (1962), isolated several mutants defined as transport-negative (Tr⁻), which were linked to permease mutants of the β-galactoside system. These workers demonstrated that under conditions in which the primary means of entry of proline was by diffusion (low temperature, high external concentration, or presence of 2,4-dinitrophenol), the rates of proline entry into both the wild type and mutant cells were almost identical. From comparative studies with the Tr⁻ mutant and the parent strain, these workers have argued against the "binding-site theory" of Britten and McLure (1962). Kessel and Lubin (1962), demonstrated that the process of exchange at 0 C was markedly reduced in the Tr⁻ strain, thereby firmly establishing a relationship between uptake and exchange processes. Kaback and Stadtman (1966), corroborated the observations of Kessel and Lubin

by carrying out experiments with isolated whole membrane preparations from both wild type (W6) and a proline transportless mutant (W157). Membranes from the wild type were able to incorporate and concentrate proline, whereas membranes from the mutant were unable to carry out these functions. In addition, membranes from the mutant were unable to exchange accumulated proline with exogenous proline at 0 C. Kessel and Lubin (1962), suggested that the mechanisms for proline uptake and exchange appeared to be closely related.

Tristram and Neale (1968), selected a number of 3,4-dehydroproline and azetidine-2-carboxylic acid resistant strains of E. coli K10, which had defective transport mechanisms. It was found that the rate of exchange at 0 C between labelled intracellular and unlabelled extracellular proline was independent of the initial external proline concentration in the range of 10^{-7} to 7.5×10^{-4} M. At 10 C the maximum intracellular proline concentration was dependent upon the external proline concentration. This suggested that the pool size was a function of permease activity but that exchange at 0 C was not. However, a direct correlation was demonstrated between the ability of proline analogues to inhibit proline uptake and the ability to affect change.

Also, an azetidine-resistant strain, with a diminished proline permease, lacked the ability to exchange intracellular and extracellular proline, 3,4-dehydroproline or azetidine. This suggested that uptake and exchange are in fact related processes in E. coli. As the previous workers have shown, the systems of amino acid accumulation in micro-organisms are exceedingly complex and recently no attempts have been made to further elucidate the proposed models or to formulate new

models. Currently, the attention of most investigators has been focused on a singular aspect of amino acid uptake systems, namely the stereospecificity of the transport process.

II. The Transport of Amino Acids by Microorganisms

FerroLuzzi-Ames (1964), investigated the aromatic transport system of Salmonella typhimurium and from kinetic analyses of their system demonstrated the existence of both specific and non-specific permeases for these amino acids. Specific, high-affinity permeases, as well as a non-specific, low-affinity general permease, were found for phenylalanine, tyrosine, tryptophan and histidine. These results were confirmed by the isolation of Tr^- mutants of S. typhimurium deficient either in the general or in the histidine specific permease (Shifrin, Ames, FerroLuzzi-Ames, 1966).

Although most studies have indicated that bacterial amino acid-incorporating systems are strictly stereospecific, recognizing only structurally related amino acids, recent studies with Agrobacterium tumefaciens have indicated that general non-specific permeases exist in this microorganism. Behki and Hochster (1966, 1967), compared both the valine and proline uptake systems in tumorogenic and non-tumorogenic strains of A. tumefaciens. The general properties of the accumulation process and the rigid structural requirements in the non-tumorogenic strain, with valine, were similar to those observed in E. coli. The tumorogenic strain, however, displayed both an independence from an external energy requirement and a non-specific competition for transport by structurally unrelated amino acids. Both

strains showed a marked lack of structural specificity for the proline incorporating system, as a number of unrelated amino acids inhibited the uptake of this amino acid.

III. Control of Amino Acid Transport

Halpern and Lupo (1965), investigated the glutamate transport of E. coli, strains H and K12, in a novel manner. These workers obtained mutants which, unlike the parent strains, could grow on glutamate as the sole carbon source. The mutation resulted in increased ability of these cells to transport glutamate and its analogues. Extensive kinetic analyses of this phenomenon (Halpern and Even-Shoshan, 1967), led to the demonstration that the glutamate permease of E. coli was allosterically inhibited by glutamate.

It was found that the structurally similar compounds, α-ketoglutarate and aspartate, non-competitively inhibited transport activity. The authors suggested that these compounds also bind to the allosteric site. Subsequently, Marcus and Halpern (1967), mapped the gene determining glutamate transport (glt C) in E. coli K12. The important observations that the transport gene was genetically similar to the glutamate decarboxylase gene (gad), and that the glt C gene was probably a regulator gene for this uptake system, were made. Since both parent and mutant strains had similar affinities for the substrate, the authors suggested that the glutamate permease was under genetic control, being partly repressed in the wild-type and derepressed in glt C^{+} mutants.

There are only a few reports in the literature of inducible

transport systems. Boezi and DeMoss (1961), demonstrated an inducible tryptophan transport system in E. coli T3A, however, the induction inexplicably required the presence of casamino acids, indicating that induction by a singular inducer was not the mechanism. These workers demonstrated that amino acid transport systems, including the tryptophan transport system, were inhibited by pyruvate and stimulated by formate, thereby suggesting that the intermediary metabolism of the organism regulated the activity of the transport process. An inducible glutamate transport system in Mycobacterium tuberculosis and M. smegmatis has been demonstrated (Lyon, Rogers, Hall and Lichstein, 1967). It was found that glutamate-catabolizing enzymes were constitutive in these organisms and that the oxidative lags exhibited by resting cells were due to the induction of a specific glutamate permease.

Inui and Akedo (1965), reported the first concrete evidence for the repressibility of amino acid transport systems in E. coli K10. These workers found that the uptake of cycloleucine and leucine was reduced when cells had previously been grown in a medium containing cycloleucine, L-leucine or L-methionine, while other amino acid transport systems were unaffected. Kinetic studies of repressed cells indicated that both the rate of influx and efflux were altered.

IV. Amino Acid Transport in Microorganisms Other Than Bacteria

The mold Neurospora crassa and the yeast Saccharomyces cerevisiae show extensive similarities to mammalian systems with respect to cell permeability. Many observations have suggested a lack of specificity

of amino acid transport and thus demonstrate the resemblance of the
fungal to the mammalian systems. Recently, however, contrary evidence
has been accumulating which indicates a multiplicity of amino acid
transporting systems both in Neurospora sp. and yeasts.

Zalokar (1961), studied proline uptake in N. crassa mycelial
mats and demonstrated saturation kinetics for the uptake of this amino
acid, indicating that a permease-like function existed. The kinetics
and energy requirements for phenylalanine uptake into conidial
suspensions were subsequently demonstrated by DeBusk and DeBusk (1965).
However, competition studies between phenylalanine and twenty-three
other amino acids showed that there existed a lack of specificity of the
uptake process for phenylalanine.

Lester (1966), found that mutants of N. crassa resistant to
4-methyl-tryptophan were defective not only in the transport of
tryptophan and its methyl-analogues but also in the transport of a
number of other unrelated amino acids. This implied a lack of specificity
for the amino acid uptake systems. Stadtler (1967), qualitatively
corroborated these results but also found that revertants of transport
negative mutants resistant to 4-methyltryptophan consequently recovered
the ability to actively transport aromatic amino acids. These results
were most simply explained by the occurrence of supressor mutations
in another gene controlling a different transport system. It was
suggested that the second mutation had modified a different uptake
system, resulting in the expansion of its substrate range. Subsequently
this second system was shown to govern the transport of basic amino
acids as had been demonstrated previously by Bauerle and Garner (1964).
They found that N. crassa incorporated arginine, lysine, canavanine,

and citrulline with respectively decreasing affinities for a single basic amino acid permease. Wiley and Matchett (1967), showed that N. crassa actively transported tryptophan by a stereospecific transport system which was specific for a family of neutral amino acids that were not necessarily structurally related. These investigators found that an α-amino group next to a carboxyl group and an uncharged side chain were the minimal structural requirements for activity with the transport site.

It is important to realize, as Kappy and Metzenberg (1967) have emphasized, that general transport defects can occur, not only through an alteration of specific or general uptake systems, but also by membrane alterations affecting the activity of membrane-associated proteins such as permeases. These authors isolated a temperature conditional lethal mutant of N. crassa which was resistant to neutral amino acid analogues by virtue of a decreased ability to transport these analogues and their natural cogenera across the cell membrane. They also showed, by several criteria, that this mutant likely possessed a structural membrane defect which could account for the negative amino acid permeability. Holden (1965), showed that a defect in glutamate transport by a vitamin B_6 requiring strain of Lactobacillus plantarum could be restored by the addition of acetate, ammonium ions, and vitamin B_6. It was suggested that the vitamin influences transport indirectly, either by modifying the tertiary structure of membrane components or by participating in their synthesis.

Studies on the amino acid transport systems of the yeast Saccharomyces cerevisiae by Surdin (1965), suggested that the substrate specificity of transport was considerably less than that observed with

bacterial systems. It was shown that although the accumulation system could concentrate amino acids up to 1000 fold, all of the amino acids studied were concentrated by a single permease but at widely varying rates. Surdin also isolated a mutant with a ten fold lower level of permease and found that the specificity properties of this residual permease are essentially the same as those of the parent strain.

Recently, conflicting evidence has accumulated regarding the specificity of yeast amino acid permeation. Maw (1963), studied the incorporation of ^{35}S-labelled amino acids into brewers' yeast and found that the accumulation of the sulfur amino acids was inhibited by certain other amino acids having a close structural relationship to them. Grenson (1966), postulated that in S. cerevisiae both general and specific amino acid permeases were operative. Grenson found that only those amino acids that were structurally related to arginine would compete for that particular transport mechanism and the other naturally occurring amino acids had absolutely no effect on the incorporation of arginine. It was also shown by kinetic analysis, that is, the comparison of K_m and K_l values, that the inhibitory amino acids were being transported by the same mechanism as arginine. These results were confirmed by the selection of a transportless mutant common for the competitive amino acid inhibitors. However, it should be noted that their mutant recovered the ability to transport arginine, without reversion, when ammonium ions were removed from the medium. It was suggested that this transport system was under metabolic control. Another mutant selected in this study was similar to the mutant isolated by Surdin et al. (1965), in that it was affected at the level of general amino acid permeation. Grenson, however, objected to the conclusions

of Surdin et al., primarily because of the ten fold difference between

the K_m for transport of an amino acid and the apparent Michaelis

constant for the same amino acid when acting as an inhibitory agent (K_I)

in the transport of another amino acid. Grenson (1966), has also

demonstrated the existence of a specific lysine permease in yeast

which doesnot recognize other basic amino acids. A specific lysine

transportless mutant was isolated which did not affect the uptake of

other basic amino acids.

V. Localization and Isolation of Transport Functions

Recently some progress has been made in the actual biochemical

identification of the components of the active transport system.

Kaback and Stadtman (1966), reported that carefully isolated membrane

preparations from E. coli were able to actively incorporate and

concentrate proline. In a comparative study with the wild-type (W6)

and transportless strains (W157) isolated by Lubin et al. (1962), these

workers found that the uptake process in the wild type cell membranes

demonstrated saturation kinetics, whereas the amino acid passed through

the mutant membranes only by simple diffusion.

Neu and Heppel (1965), and Nossal and Heppel (1966), showed

that cold osmotic shock treatment of bacteria caused the loss of

certain enzymes and proteins associated with the cell envelope.

Piperno and Oxender (1966), used this procedure to isolate and purify

a protein from E. coli which would bind either leucine, isoleucine,

or valine. The dissociation constants for the leucine and the isoleucine

protein complexes were found to be indistinguishable from their respective

K_m values for transport into whole cells. These results suggest that the binding protein is a component of the transport system responsible for the concentrative uptake of leucine, isoleucine, and valine by E. coli K12. All attempts to restore the transport process to shocked cells by adding back the binding protein were unsuccessful.

MATERIALS AND METHODS

I. Organisms and Media

Pseudomonas aeruginosa ATCC 9027 was used throughout most of this study. Also the following organisms were used: a histidine-requiring strain of P. aeruginosa WK4; P. aeruginosa P5 and P6, proline transport negative strains; P. aeruginosa A5, IB9 and IB10, TA3 and TA10 which were arginine, isoleucine, and tyrosine transport negative strains; and P22, a strain unable to catabolize proline. Stock cultures were maintained at 6 C on ammonium salts minimal agar slants with glucose as the sole carbon source. Histidine (50 µg/ml) was added to maintain WK4, the histidine auxotroph.

Cells required for experimental procedures were grown from a 20 hr inoculum in Roux flasks at 30 C in a medium containing 0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4 and 0.5 ppm iron as $FeSO_4 \cdot 7H_2O$ at pH 7.4, and supplemented with 50 µg/ml histidine for WK4. Glucose and $MgSO_4 \cdot 7H_2O$ were added separately, after sterilization, from 10% stock solutions to give final concentrations of 0.2% and 0.05% respectively (minimal medium).

Cultures were routinely checked for purity and for production of the species characteristic pigment pyocyanin by streaking cells on King's medium (King, Ward and Raney, 1954). WK4 was routinely checked for reversion by streaking cells on minimal medium. Transport negative strains were checked for reversion by streaking cells on agar medium containing the appropriate amino acid as a

sole carbon source.

II. Preparation of Cell Suspensions.

Cells from late logarithmic phase were harvested by centrifugation at room temperature and were quickly resuspended to the desired concentration in minimal medium. The concentration of cells was established by measuring the optical density at 650 mμ using a Model B Beckman spectrophotometer. The cell suspensions were stirred on a Troemner submergeable magnetic stirrer (Henry Troemner Inc., Philadelphia, Pa.) for 10 min prior to the addition of the radioactive amino acid. This procedure was found to give reproducible incorporation data for any one amino acid.

III. Selection of Mutants

1. Mutagenesis

Wild type logarithmic phase cells were harvested from minimal medium by centrifugation at 13,000 x g for 10 min, resuspended in pH 5.5 citrate buffer to a density of approximately 10^9 cells per ml, and shaken at 30 C for 1 hr. N-methyl-N-nitro-N'-nitrosoguanidine was added to give a final concentration of 100 μg/ml and the cells were shaken for 40 min. The culture was quickly chilled to 0 C and centrifuged at 6 C. The cells were washed twice with minimal medium, then resuspended to the original volume in supplemented media to enrich for the desired mutant and

then incubated at 37 C for 2-6 hr.

2. Amino acid auxotrophs

The mutagenized culture was incubated for 6 hr in minimal medium supplemented with 50 µg/ml of the required amino acid. The cells were diluted and plated directly on minimal medium containing 1 µg/ml of the desired amino acid. The plates were incubated for 48 hr and auxotrophs were detected as single minute colonies. These isolates were checked by patching on both minimal agar and minimal agar supplemented with 50 µg/ml histidine.

3. Transport negative strains

Mutagenesis was carried out on cells which had been first adapted to grow on the desired amino acid as the sole carbon source. After washing with medium the treated cells were grown in minimal medium for 2 hr, then plated on solid medium with 0.1% of the amino acid as the sole carbon source. After a 48 hr incubation, minute colonies were picked, washed in drops of saline, and patched onto both amino acid minimal and glucose minimal plates. After further incubation and daily scoring, those colonies which grew slowly on amino acid minimal plates and normally on glucose minimal plates were selected for further studies. These selected colonies were grown in minimal medium and amino acid uptake experiments were carried out to determine the initial (30 sec) rate of ^{14}C-amino acid incorporation at a concentration of 10^{-6} M. Growth curves

on the mutants with defective transport systems were carried out to make sure no other genetic function had been altered.

IV. Uptake of Labelled Amino Acids

The incorporation of ^{14}C-amino acids into cells and cellular constituents was studied by two different procedures, the choice of which depended upon the nature of the experiment.

In the first procedure, the incorporation of ^{14}C-amino acids into whole cells, protein, and pools were determined by the Millipore filtration procedure of Britten and McLure (1962). Cells were filtered on a Tracerlab E8B precipitation apparatus (Tracerlab, Waltham, Mass.) and immediately washed with 2 ml of minimal medium. This procedure did not remove pool amino acids. Dried filters were placed in vials containing 10 ml of scintillation fluid (Liquifluor, New England Nuclear Corporation) and the vials were assayed for radioactivity in a Nuclear Chicago liquid scintillation spectrometer model 725.

In the second procedure, the rate of incorporation of amino acids into whole cells was determined essentially by the method of FerroLuzzi-Ames (1964), which was modified as follows: 1 ml of a vigorously stirred, heavy cell suspension was added to a test tube containing 3 ml of minimal medium and the ^{14}C-amino acid, at the desired specific activity. The reaction mixture was stirred on a Troemner magnetic stirrer using miniature stirring bars. Aliquots were removed for filtration at 15 sec time intervals. Filters were

dried and assayed for radioactivity and rates of amino acid transport were calculated from the earliest linear incorporation data. This method was used for the demonstration of saturation kinetics, and to determine the kinetics of competitive inhibition, since it permitted a series of reactions to be completed within a short period of time and thereby eliminated variation in rates of transport due to cell proliferation.

Experiments, designed to determine the rates of amino acid transport as a function of incubation temperature, were performed in water-jacketed reaction vessels maintained at the desired temperature with a Lauda K-2/R temperature-controlled circulating water pump (Brinkmann Instruments, Inc., Westbury, N.Y.). Cell suspensions were allowed to equilibrate at the desired temperature for 15 min prior to the initiation of the transport experiment by the addition of the ^{14}C-amino acid. The reaction mixtures were continuously agitated with a magnetic stirrer.

Studies on the maintenance of amino acid pools at low temperatures were carried out in the water-jacketed reaction vessels described for transport studies, which had been precooled.

V. Competitive Inhibition of Amino Acid Transport

The rate of uptake of 10^{-6} to 10^{-7} M ^{14}C-amino acids in the presence of 10^{-4} or 10^{-3} M ^{12}C-amino acids was determined as follows: 3 ml of minimal medium, containing the amino acids to be tested for competitive inhibition, and the test ^{14}C-amino acid were added to an 18 mm test tube. These reaction tubes were equilibrated at 30 C and

1 ml of a dense cell suspension (0.4 mg dry cells/ml) was added

with continuous agitation to initiate the reaction. Samples were

collected with a 1 ml tuberculin syringe at 15 sec intervals and

filtered. The degree of competitive inhibition was calculated

from the reduction in the rate of ^{14}C-amino acid incorporation

relative to the appropriate control.

VI. Competitive Pool Displacement

Cell suspensions were preincubated with 200 µg/ml chloram-

phenicol for 30 min at 30 C prior to the addition of ^{14}C-amino

acid to a concentration of 10^{-6} M. Pool formation was followed

and when maximal, the incubation temperature was reduced to 10 C.

The point of maximal pool formation had been determined in preliminary

experiments. All preformed pools were found to be stable at this

temperature and protein synthesis was negligible. To initiate

the exchange reaction, a competitive ^{12}C-amino acid was added to

a final concentration of 10^{-4} M and the rate of exchange determined

from the loss of pool label over a 15 min time interval.

VII. Chromatographic Techniques and Measurement of Amino Acids

in the Cell Pool and in the Culture Medium

A sensitive quantitative measurement of pool amino acids

and amino acids excreted into the growth medium was devised as follows:

cells were grown in 10 ml of minimal medium containing 111 µmoles of

uniformly labelled ^{14}C-glucose with a specific activity of 2 mc̄/mmole

and logarithmic or stationary phase cultures were harvested by
centrifugation at 10,000 x g at room temperature. The supernatant
fluid was carefully decanted and the tube wiped free from adhering
liquid. The resulting packed cells were extracted with 5 ml of 5%
trichloroacetic acid (TCA) and the residual cellular material was
removed by centrifugation at 10,000 x g for 10 min at 6 C. This
procedure was repeated twice and the TCA extracts were combined.
The growth medium was deproteinized with cold 5% TCA in a similar
manner. The TCA was removed from the fractions by extracting 5
times with cold ethyl ether. The resulting aqueous samples were
evaporated to dryness in an air stream. Each dried sample was
dissolved in distilled water and applied to a column (1.2 x 10 cm)
of Dowex 50 (H^+ form). The column was washed with distilled water
until no further radioactivity was eluted and the adsorbed compounds
were then eluted with 100 ml of 4M ammonium hydroxide. The eluate was
evaporated to dryness and the residue dissolved in 0.1 ml of distilled
water. Samples were quantitatively applied to cellulose thin layer
plates (cellulose powder MN 300) and the radioactive compounds were
separated two-dimensionally by the method of Jones and Heathcote (1967).
The resulting chromatograms were exposed for 1 week to medical x-ray
film (Eastman Kodak Co., Rochester, N.Y.). The films were developed
and the radioactive areas detected by this method were scraped loose
from the plates and drawn, by vacuum, into scintillation vials which
were subsequently filled with a toluene scintillation fluid and assayed
for radioactivity in a liquid scintillation spectrometer. The addition
of from 5 to 60 mg of cellulose caused no increase in quenching under
these conditions. It was found by this method that amino acids could

be detected in amounts as low as 1×10^{-8} μmoles.

Confirmatory qualitative amino acid analyses were carried out by two-dimensional chromatography on both thin layer and paper. Thin layer plates were coated with Silica Gel G, spotted with the radioactive sample, and developed in a solvent system of chloroform-methanol-ammonia (2:2:1 v/v) and in the second dimension in phenol-water (75:25 v/v) (Randerath, 1963). Paper chromatograms, spotted with the radioactive samples, were developed in the first dimension in a solvent system of n-butanol-acetic acid-water (120:30:50 v/v) and in the second dimension in phenol-ammonia (200:1 v/v) (Smith, 1960). Chromatograms from both these methods were analyzed by radio-autography and by ninhydrin detection of the appropriate carrier ^{12}C-amino acids.

Culture supernatant fluids, which were collected by filtration, were reacted with 10^{-2} M 2,4-dinitrophenylhydrazine in 2N HCl for 1 hr at 38 C. The keto-acid hydrazones were extracted into ethyl acetate and purified by extraction with 1 M tris-(hydroxymethyl)amino-methane buffer (Tris) pH 11.0. The acid hydrazones were further extracted into ethyl acetate, dried to a small volume, and the derivatives were separated by paper chromatography in a solvent system of n-butanol-ethanol-ammonia (0.5 N) (70:10:20 v/v) as described by Smith (1960).

VIII. Amino Acid Composition of Cellular Protein

Cells from a culture in logarithmic phase were harvested by centrifugation and fractionated according to the procedure of Roberts et al. (1955). The protein residue remaining after extraction with

hot TCA was further extracted twice with ethyl ether to remove residual TCA and the samples were air-dried. The protein was hydrolyzed with 6N HCl for 18 hr at 100 C in sealed glass vials. After hydrolysis, the acid was removed by flash evaporation and the water-washed residue was dried and dissolved in 0.1 M citrate-buffer (pH 5.5). Amino acid analyses were carried out using a Beckman Model 120C amino acid analyzer.

Qualitative ^{14}C-amino acid analyses of the protein of cells which were grown in the presence of low concentrations of radioactive amino acids were carried out by paper chromatography as described above.

IX. Chemical Fractionation of Whole Cells

The chemical fractionation procedure used was essentially that of Roberts et al. (1955), with the modification by Clifton and Sobek (1961). The hot TCA soluble fraction was prepared by heating the samples in 5% TCA for 20 min at 90 C rather than at 100 C for 30 min. Aliquots of the cell fractions were plated in duplicate onto stainless steel planchets, dried under an infra-red lamp, and were counted at infinite thinness with a thin end window Geiger tube attached to a Nuclear Chicago scaler model 181A equipped with an automatic gas-flow counter. Corrections were made for background. In order to reduce statistical deviation, at least 1000 counts were recorded when possible.

X. Chemicals

Amino acids used as carriers for ^{14}C-amino acids were
obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
L-tryptophan (methylene-^{14}C) was obtained from Nuclear-Chicago
Corp., Des Plaines, Ill. The other radioactive amino acids and
^{14}C-glucose were purchased from Schwarz Bioresearch Inc.,
Orangeburg, N.Y., and were obtained as the ^{14}C uniformly labelled
product with the exception of L-hydroxyproline-^{3}H (hydroxyl-^{3}H).
All radioactive amino acids were checked for purity by thin layer
chromatography (Jones and Heathcote, 1967) and radioautography.
N-methyl-N-nitro-N'-nitrosoguanidine was obtained from Aldrich
Chemical Co. Inc., Milwaukee, Wisc.

RESULTS AND DISCUSSION

I. General Properties of Amino Acid Transport

  1. Uptake of amino acids

The initial rate of incorporation of amino acids into whole cells during exponential growth was first order with respect to cell mass and was reproducible under the experimental conditions used (Fig. 1).

The time course of amino acid uptake and distribution within the cells was studied. Figure 2 shows the results of an experiment measuring the uptake of ^{14}C-tyrosine. Tyrosine was taken into the cell immediately and rapidly until the supply in the medium became exhausted. The entry of labelled tyrosine into TCA precipitable material was linear, and after two to three minutes continued at the expense of the amino acid pool. At three minutes, the tyrosine concentration in the pool was 13,350 times greater than the tyrosine concentration of the medium. This pattern of incorporation corresponds to the patterns of amino acid uptake into E. coli (Britten and McLure, 1962; Tristram and Neale, 1968).

  2. Precursor-Pool relationships

The incorporation of tyrosine (Fig. 2) was typical of

many of the amino acids studied. The negligible lag in the
entry of amino acid into the protein fraction suggested
either that passage of the amino acid through the pool was not
obligatory for entry into protein, or possibly, that the amino
acid concentration in the native pool of growing cells was very
low and thus had a negligible influence on the specific activity
of the labelled amino acid which was transported into the cells.

Valine was selected as the amino acid to test these
possibilities. When added to a concentration of 10^{-6} M, the amino
acid was completely removed from the external medium by 90-120
sec, precisely the time at which the pool level was maximal
(Fig. 3, inset). In a similar experiment, a 90 sec preincubation
with ^{12}C-valine at 10^{-6} M was followed, at zero time, by a pulse
of ^{14}C-valine at the same concentration. The prolonged lag of
^{14}C-valine incorporation into TCA insoluble material (Fig. 3)
was due therefore to the equilibration of the radioactive amino
acid with the ^{12}C-valine pool.

These results suggested that exogenously supplied amino acids
entered the cell and accumulated in an intracellular metabolic pool,
and that entry into this pool was necessary for incorporation into
protein. Similar results were obtained for the incorporation of
^{14}C-proline into E. coli (Britten and McLure, 1962). To account for
the negligible lag of ^{14}C-amino acid entry into cellular protein
in the absence of a preloaded pool, concentrations of de novo
synthesized amino acids in the native pool of growing cells must,
therefore, be relatively low.
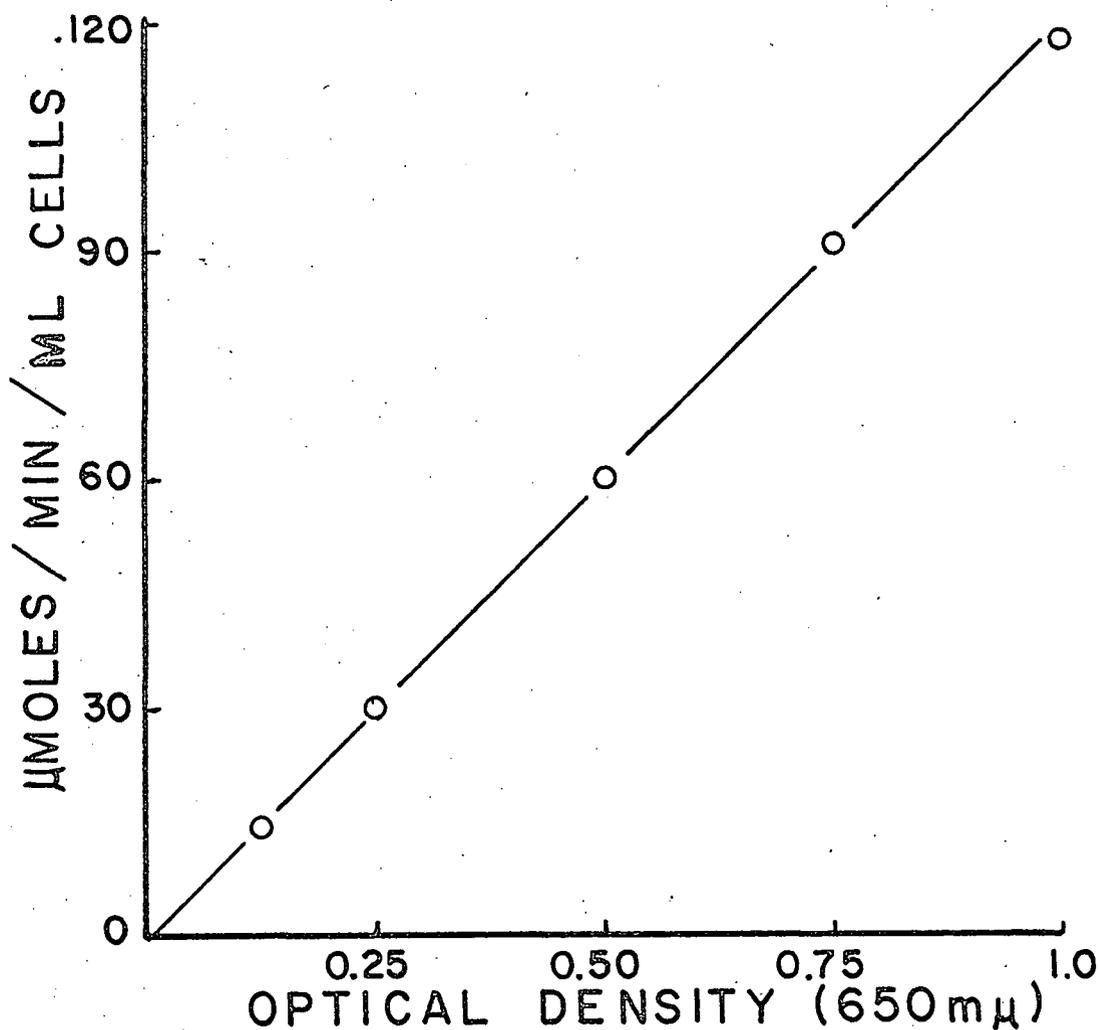
Fig. 1. Increase in the rate of proline uptake with increasing
cell mass. External proline concentration was 10^{-6} M;
specific activity, 25 µc/µmole (O.D.$_{650}$ of 0.50
equals 0.2 mg dry weight/ml).
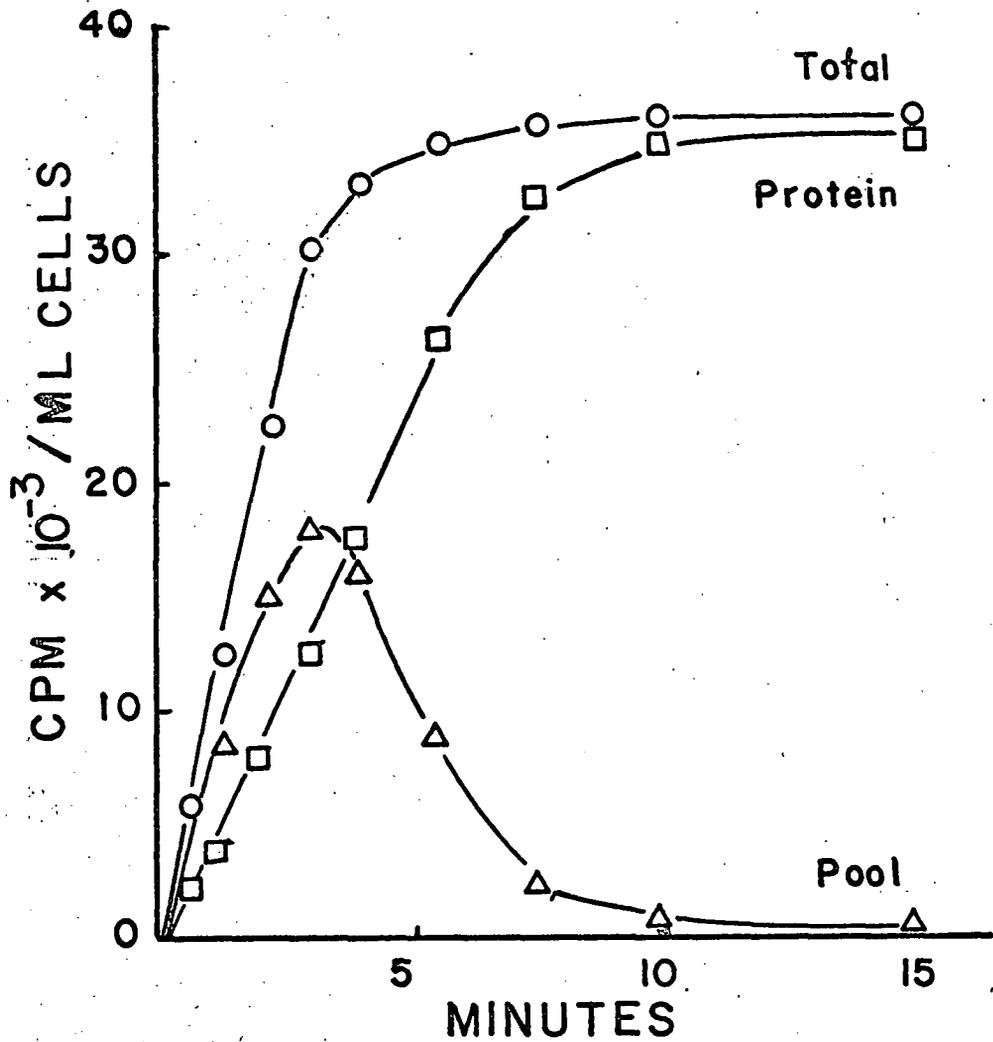
Fig. 2. Time course of tyrosine uptake and incorporation into protein by a growing suspension of P. aeruginosa (0.2 mg dry weight/ml). External tyrosine concentration was 1×10^{-8} M; specific activity, 12.5 µc/µmole.

When the time course of ^{14}C-amino acid uptake was determined for all the commonly occurring amino acids, it was found that only the incorporation of glycine, serine, and glutamate exhibited a lag prior to entry into protein. Figure 4 illustrates the incorporation of ^{14}C-glutamate into whole cells and into the TCA insoluble material of P. aeruginosa. With most amino acids tested, ^{14}C entry into protein was essentially linear from zero time.

These results suggested that most amino acids were present in the pool of growing cells at low levels, but that a few, such as glutamate, were present at relatively high concentrations. Glutamate has also been reported to be present in relatively high concentrations in the pool of E. coli (Britten and McLure, 1962).

3. Comparative rates of transport

From the results of rate studies of amino acid uptake, it became obvious that a large variation in rates of transport for different amino acids existed. Experiments to determine ^{14}C-amino acid incorporation rates were performed for all the common amino acids by rapid sampling in order to obtain accurate, comparative values. From the results of Table I, it can be seen that there is approximately a forty-fold variation in the rates of transport; from a low of 1.92×10^{-4} μmoles/min/mg dry weight of cells for cysteine to 80×10^{-4} μmoles/min/mg dry weight for leucine. The basic and aromatic amino acids were incorporated much more rapidly than the acidic or neutral amino acids.
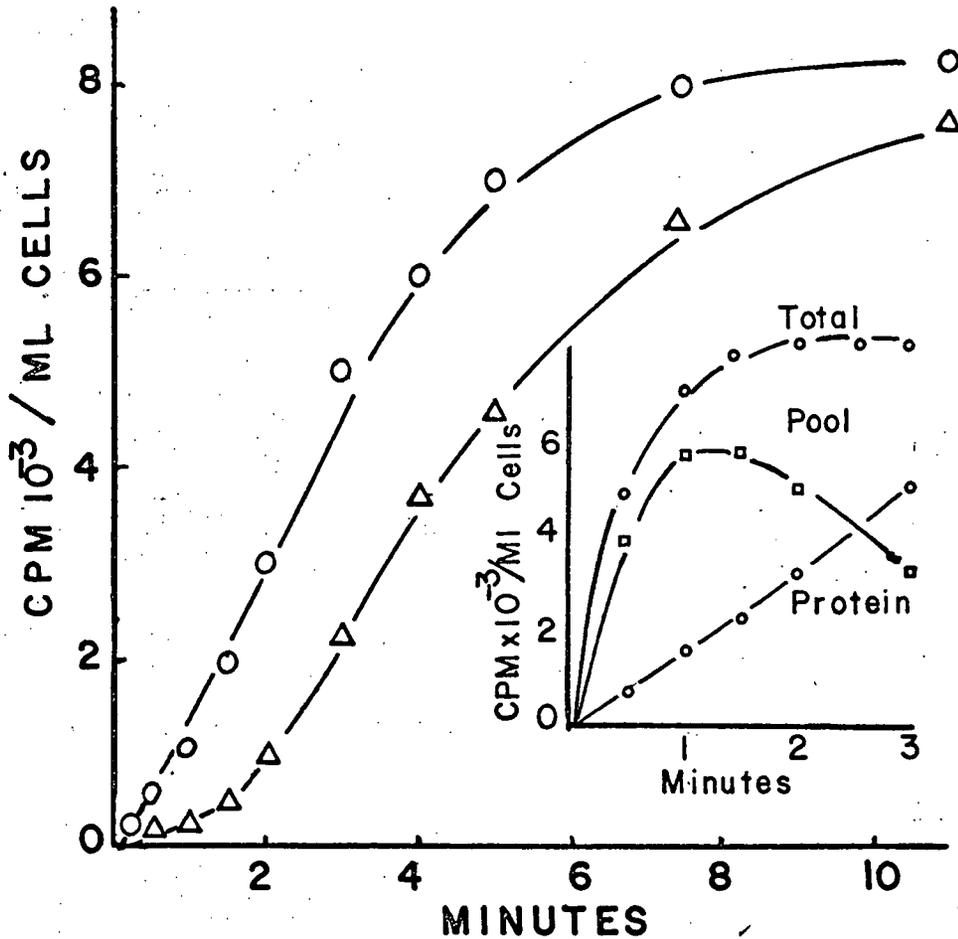
Fig. 3. Incorporation of ^{14}C-valine into the protein of
P. aeruginosa. Symbols: O, 10^{-6} M ^{14}C-valine;
△, $\overline{10^{-6}$ M ^{14}C-valine added 90 sec. after preloading the
cells with 10^{-6} M ^{12}C-valine. Insert: incorporation of
10^{-6} M. ^{14}C-valine into whole cells, protein and pool.
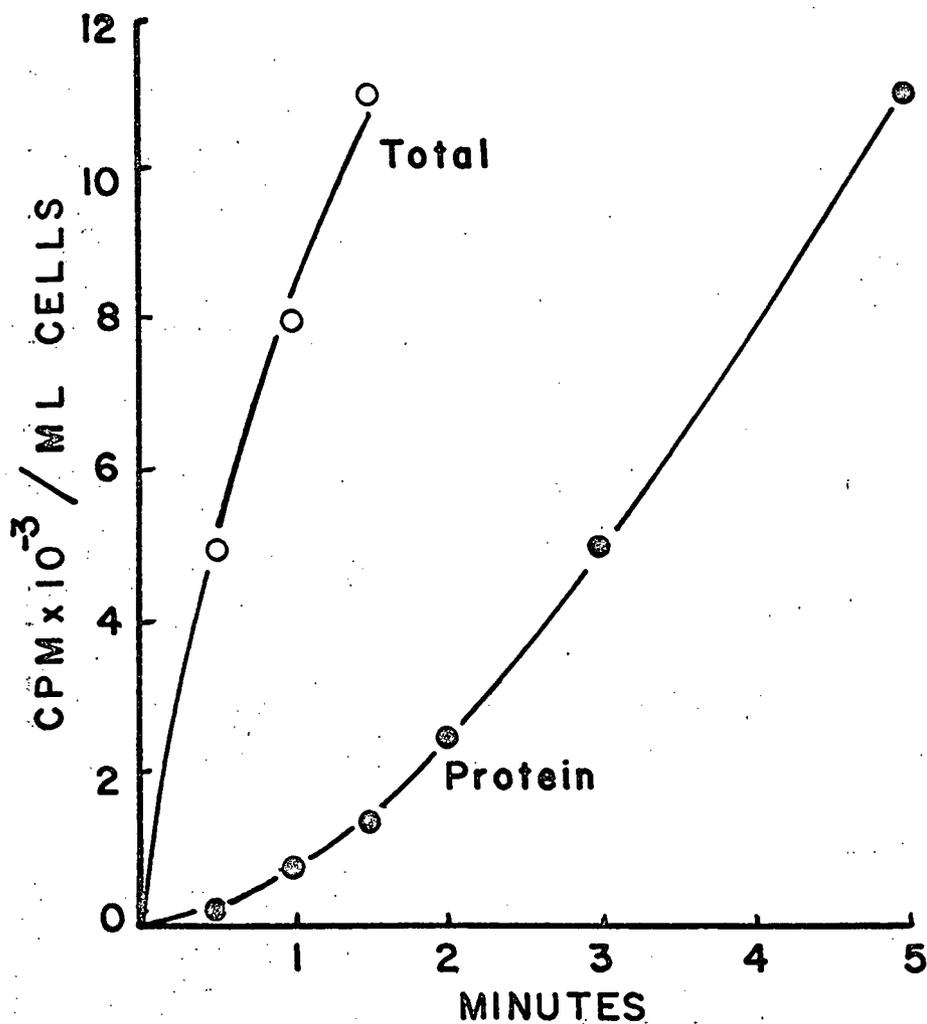
Fig. 4. Total uptake of ^{14}C-glutamate and incorporation into protein of cell suspensions (0.1 mg dry weight of cells/ml). External glutamate concentration was 1×10^{-6} M; specific activity, 12.5 µc/µmole.

The only amino acid tested to which these cells were
impermeable was hydroxy-L-proline. Experiments to detect uptake
of this amino acid showed that no radioactivity was incorporated
either into the amino acid pool or into the protein. Kaback and
Stadtman (1965), reported that hydroxyproline could exchange
with ^{14}C-proline concentrated in membrane preparations of E. coli.
This suggested that the proline concentration mechanism also
functioned with hydroxyproline.

4. Distribution of radioactive amino acids in the cell

To establish whether or not the exogenously supplied
^{14}C-amino acids which were incorporated into the TCA insoluble
material remained unchanged during passage through the intracellular
pool, cultures were allowed to incorporate specific ^{14}C-amino acids
added at low concentrations and the distribution of radioactivity within
the cells was determined by chemical fractionation. For ^{14}C-histidine,
^{14}C-isoleucine, ^{14}C-phenylalanine, and ^{14}C-proline, approximately
90 percent of the radioactivity was incorporated into the protein
fraction, whereas with ^{14}C-arginine, ^{14}C-glycine and ^{14}C-serine only
58.3 percent, 50.7 percent, and 56.7 percent respectively were found
in the hot TCA insoluble fraction (Table II). A significant percentage
of the label found in the cold TCA soluble fraction with the
^{14}C-arginine experiment accounted for this deviation. With ^{14}C-glycine
a large percentage of the added radioactivity was found, understandably,
in the nucleic acid fraction. Also, the nucleic acid fraction

Table I. Rates of transport of amino acids into whole cell
of P. aeruginosa.

AMINO ACID	RATE OF TRANSPORT	RELATIVE RATE"
10^{-6} M	µmoles/min/mg dry weight x 10^{-4} M	
hydroxyproline	0	0
cysteine	1.92	1.00
threonine	3.54	1.85
aspartate	4.11	2.14
serine	4.20	2.19
glutamate	4.67	2.43
proline	5.75	2.99
glycine	5.90	3.08
tyrosine	14.40	7.50
methionine	19.90	9.90
valine	23.60	12.30
histidine	27.40	14.30
alanine	27.60	14.40
phenylalanine	32.30	16.80
lysine	33.60	17.50
isoleucine	39.20	20.42
tryptophan	52.00	27.10
arginine	80.00	41.60
leucine	80.00	41.60

accounted for a large percentage of the label when cells were

grown in the presence of ^{14}C-serine. This was attributed,

presumably, to the presence of the transhydroxymethylase reaction.

The relatively low amounts of label found in the acid-ethanol

soluble fractions were assumed to be present as alcohol soluble

protein, however, the higher proportion of label in this fraction

from cells labelled with ^{14}C-serine was naturally attributed

to the synthesis of lipids or phospholipids.

5. Energy requirement for amino acid incorporation

Most metabolite transport systems in microorganisms are

sensitive to poisoning by metabolic inhibitors such as dinitrophenol,

azide, or other uncouplers of energy metabolism. Cell suspensions

of P. aeruginosa were tested for their ability to transport amino

acids when treated with such inhibitors. When cells were preincubated

for 30 min at 30 C in the presence of 30 mM sodium azide or with

30 mM sodium azide plus 1 mM iodoacetamide, a marked reduction in

amino acid transport ability was observed as measured by ^{14}C-proline

incorporation into whole cells (Fig. 5). Preincubation of the cell

suspensions for 30 min with sodium azide inhibited the rate of

incorporation of this amino acid by 97 percent and by 99 percent

when both sodium azide and iodoacetamide were added to the

preincubating mixture. This second, relatively minor increase in

inhibition of proline uptake with iodoacetamide was presumably

due to the termination of substrate level phosphorylation.

Table II. Distribution of radioactivity in the cell fractions of P. aeruginosa grown in the presence of ^{14}C-amino acids.

^{14}C-AMINO ACID	FRACTION			
	cold TCA soluble	acid ethanol soluble	hot TCA soluble	residual protein
	% of total radioactivity[a]			
arginine	35.5	3.8	2.4	58.3
glycine	4.1	4.8	40.4	50.7
histidine	2.5	3.7	2.7	91.1
isoleucine	0.7	4.3	4.7	90.3
phenylalanine	1.1	4.6	4.2	90.1
proline	2.0	6.5	3.7	87.8
serine	6.3	11.9	25.1	56.7

[a] Percent of total radioactivity incorporated into cellular material.
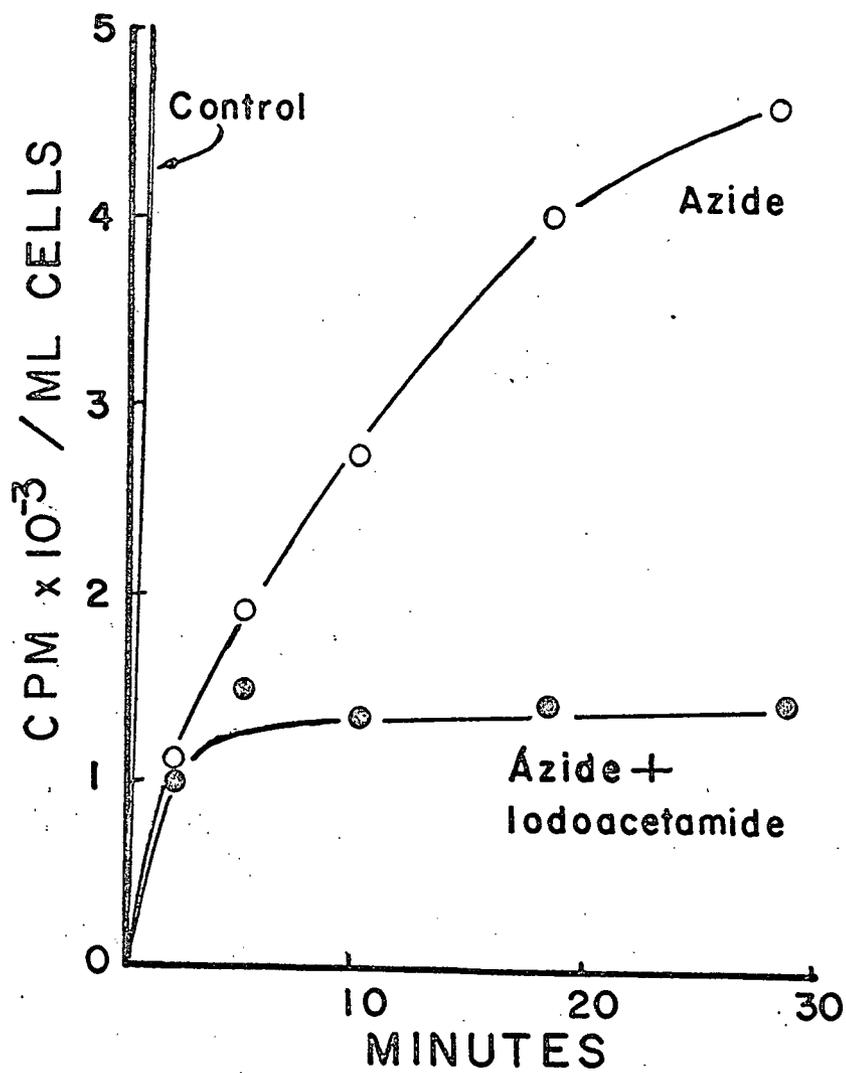
Fig. 5. Effect of sodium azide and sodium azide plus iodoacetamide on the active transport of ^{14}C-proline. Cells were preincubated with the inhibitors for 30 min at 30 C.

The conclusion that the incorporation of amino acids
was energy dependent was substantiated by the observation that
cell suspensions of P. aeruginosa did not incorporate ^{14}C-proline
at 0 C. Cell suspensions which were chilled to 0 C for 10 min
prior to the addition of ^{14}C-proline at a level of 10^{-6} M
showed no significant amount of ^{14}C-proline uptake over a
3 hr time interval.

6. Effect of temperature on the rate of amino acid transport

The uptake of amino acids into whole cells of P. aeruginosa
is temperature dependent. The rate of ^{14}C-valine incorporation
into cell suspensions at various temperatures is shown in Figure
6. Below 10 C the rate of ^{14}C-valine incorporation was negligible,
but increased rapidly with increasing temperature to the maximum
rate, exhibited at 45 C. At temperatures exceeding 45 C the rate
of ^{14}C-valine uptake declined sharply, a characteristic common to
most enzyme systems. Incubation of whole cells for 30 min at 55 C
was found to inactivate permanently the valine transport system.
The fact that transport of valine could be dissociated from growth
was evident from the observation that the rate of valine transport
was high at 45 C, whereas, growth of the organism does not occur
at this temperature. The Q_{10} for valine uptake was found to be
approximately 2.75.

7. Dependence of the rate of transport on substrate concentration

The rate of uptake, for most of the amino acids studied,
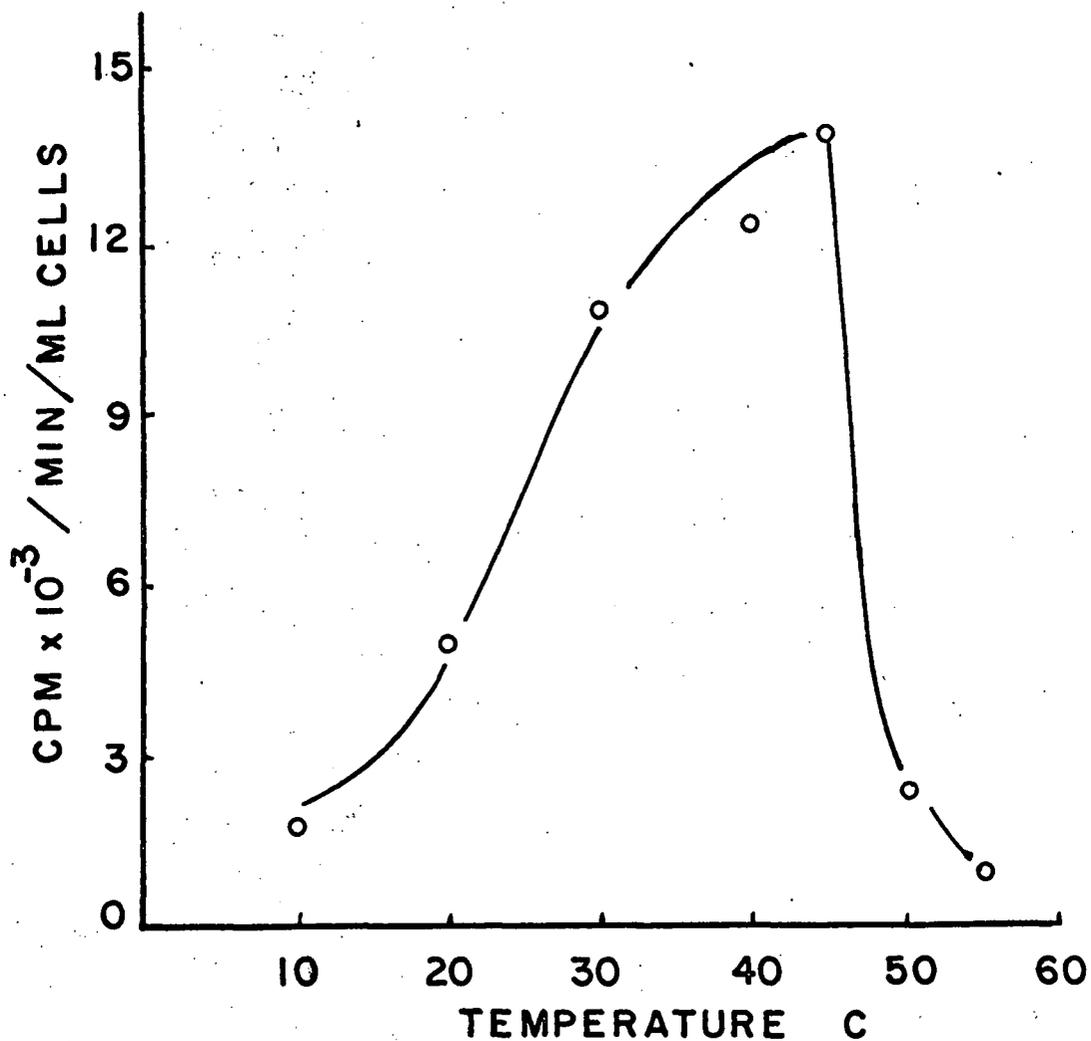
Fig. 6. Rate of 1 x 10^{-6}M ^{14}C-valine incorporation into whole
cells of P. aeruginosa as a function of temperature.
Cells were equilibrated for 10 min at the appropriate
temperature prior to the addition of label. Samples
were removed at 30 sec intervals to obtain initial
uptake velocities.

increased concomitantly with the exogenous amino acid
concentration. The uptake system, however, tended to become
saturated at high external amino acid concentrations. The
kinetics of incorporation of ^{14}C-phenylalanine are shown in
Figure 7. Michaelis' constants were of the order of 10^{-7} M
to 10^{-6} M for most of the amino acids studied. The adherence
of the uptake process to Michaelis-Menton kinetics, the
dependence upon energy metabolism, temperature sensitivity,
and the loss of this uptake process by mutation strongly suggest
that the amino acid incorporation process is mediated by a
protein, presumably a permease.

8. Concentration of amino acids in the intracellular pool

From the kinetics of amino acid incorporation into the protein
fractions, it was assumed that a wide variation in concentrations
of the different amino acids existed within the native amino acid
pool. Direct measurement of these amino acids was carried out to
determine whether or not a correlation existed between native
pool composition and the variation in rates of transport. The
amino acid composition of the growth medium was also determined in
order to establish the concentration gradients maintained between
the intracellular and extracellular amino acids.

The composition of the amino acid pool of cells growing
logarithmically was found to be extremely heterogeneous. Figure
8 represents the distribution of 35 positively charged compounds
present in the pool of P. aeruginosa, only 11 of which were
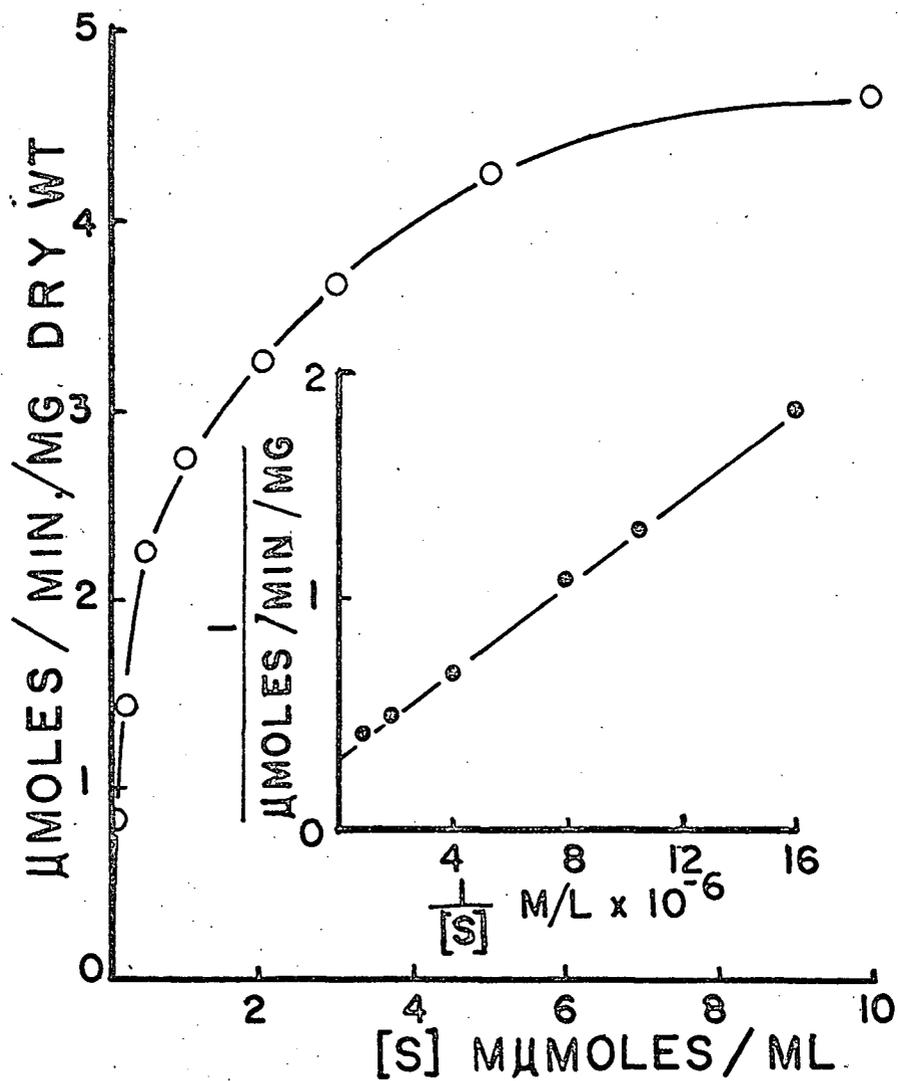
Fig. 7. Saturation kinetics of phenylalanine incorporation by
P. aeruginosa. Cells, in minimal medium, were
incubated for 30 sec at 30 C in the presence of increasing
concentrations of ^{14}C-phenylalanine. Inset: Lineweaver-
Burk plot.

identified as amino acids. The quantitative compositions of the pool, the culture medium, and the concentration ratios are listed in Table III.

The linear amino acid incorporation kinetics into the protein fraction for most of the amino acids tested previously, suggested that the concentration of some amino acids in the native pool was low. Glycine, serine, alanine, and glutamate were present in concentrations approximately 7 to 30 fold higher than the other amino acids found in the native pool, and the incorporation data for glycine, serine, and glutamate (Fig. 4), demonstrate "precursor-pool" kinetics. However, as can be seen from Figure 9, alanine deviated from this trend. This suggests that of the amino acids tested, alanine was the only one which did not first equilibrate with all of the native alanine amino acid pool prior to entry into protein. These results, therefore, suggested that intracellular pools may be compartmentalized in different ways.

From the amino acid concentration ratios (Table III), it can be seen that concentration gradients of approximately 100 to 400 fold are maintained within the cell during logarithmic growth.

An identical experiment, with cells that had been in the stationary growth phase for 3 hr, demonstrated that all intracellular and extracellular amino acids had been utilized with the exception of methionine, which was found in high concentration in the stationary phase culture medium.

From the data of Tables I and III, it can be seen that,
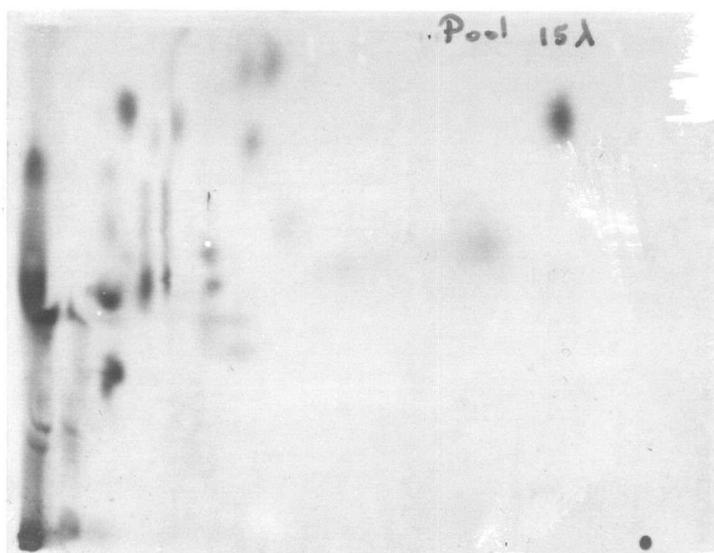
Fig. 8. Radioautograph of the nitrogen pool extracted
         from cells growing logarithmically in the
         presence of U-^{14}C-glucose

Table III. Composition of the amino acid pool and culture
supernatant fluid of P. aeruginosa during
logarithmic growth.

AMINO ACID	POOL	SUPERNATANT FLUID	POOL / SUPERNATANT FLUID
		µmoles/ml	
isoleucine	1.14×10^{-4}	4.67×10^{-7}	244
phenylalanine	1.25×10^{-4}	4.53×10^{-7}	277
leucine	1.35×10^{-4}	4.91×10^{-7}	232
valine	2.39×10^{-4}	1.41×10^{-4}	169
tyrosine	2.55×10^{-4}	0	
threonine	3.72×10^{-4}	2.00×10^{-4}	186
glycine	7.78×10^{-4}	5.60×10^{-4}	139
serine	1.63×10^{-3}	3.80×10^{-6}	427
alanine	1.66×10^{-3}	6.99×10^{-6}	237
glutamate	3.00×10^{-3}	7.73×10^{-6}	388
glutamine	3.93×10^{-3}	0	
glucosamine	0	1.81×10^{-5}	
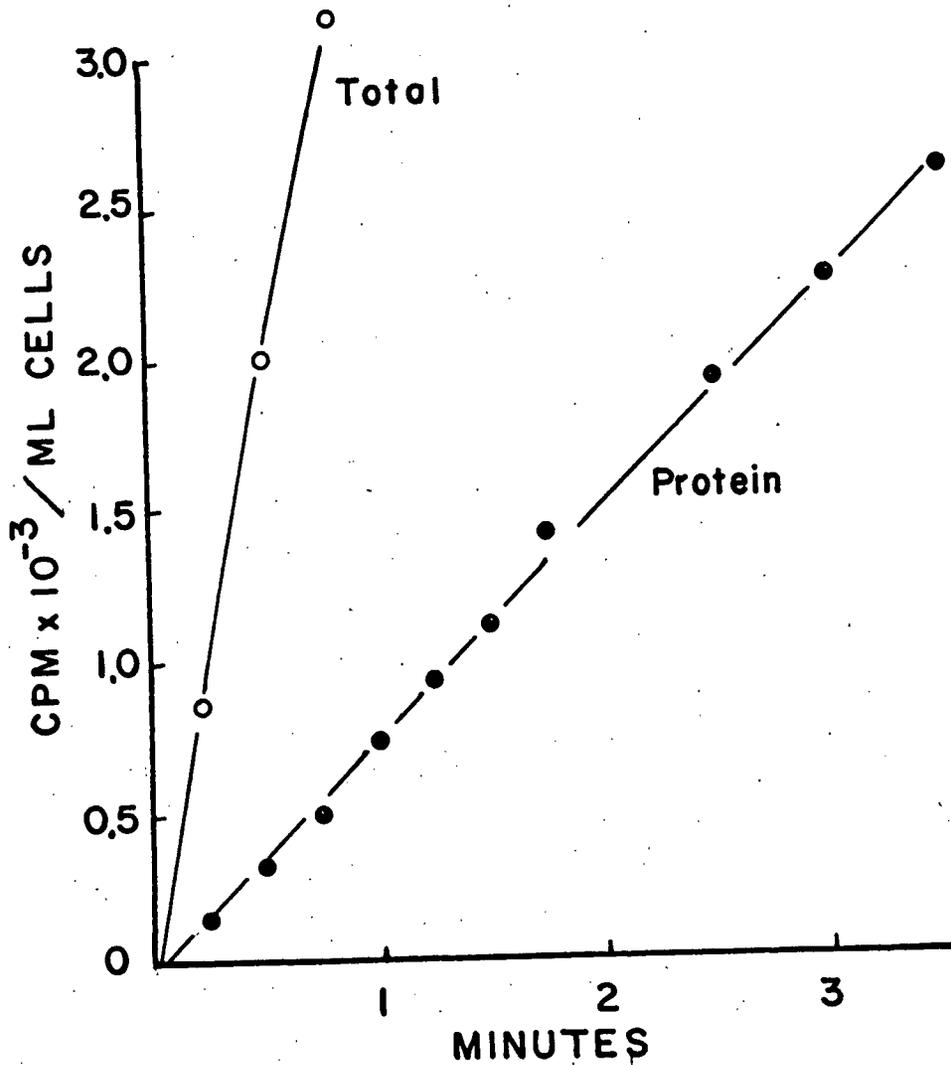
Fig. 9. Early time course of 1 x 10^{-6}M ^{14}C-alanine uptake by
a growing suspension of P. aeruginosa (0.1 mg dry
weight/ml).

with the exception of alanine, amino acids present in the
native intracellular pool at high concentrations are transported
at a relatively low rate into P. aeruginosa.

In this organism the intracellular concentrations of most
amino acids in the native pool were found to be surprisingly
low in relation to reported pool levels of other microorganisms
(Holden, 1962), and some amino acids were not detectable by the
sensitive method employed for their estimation. However, it is
important to emphasize that most investigators have used less
direct methods to determine the levels of amino acids present in
the pool and many analyses must be regarded with some caution
since the pool may contain a great variety of nitrogenous
compounds as has been demonstrated for the pool of P. aeruginosa
(Fig. 8).

The total amino acid pool levels for P. aeruginosa when
grown on minimal medium, are approximately one percent of those
reported for E. coli (Holden, 1962). Few reports are available
on the composition of the amino acid pool of Gram-negative
bacteria and the data reported here represent the lowest native
amino acid pool levels determined for any microorganism to date.
Also, these data are the first reported for an obligate aerobe.

When the individual components of the intracellular pool
were examined, striking differences in the amino acid concentrations
were again observed. For instance, FerroLuzzi-Ames (1964),
reported intracellular concentrations of histidine of 0.06 μmoles/
gm dry weight of S. typhimurium but we have not been able to detect
this amino acid in the native pool of P. aeruginosa. In fact,

the only amino acid which approached this concentration was

glutamate. Glutamate concentrations of 0.5 - 2 μmoles/gm dry

weight of E. coli were reported by Britten and McLure (1962),

and this is 10 fold greater than the glutamate pool level in

P. aeruginosa.

Amino acids such as glutamate, alanine, glycine, and serine,

which have multiple physiological roles, not only as direct

precursors for protein, cell wall and cell membrane synthesis

but also as precursors for other cellular metabolites, would

be expected to be present in the pool in higher concentrations

than most other amino acids. One also would expect, on first

analysis, that these amino acids would be transported more

efficiently than those for which the cell has a lower requirement.

It is reasonable, however, that the low transport rates found

for these amino acids are due to the fact that the cell does not

rely on an exogenous supply of these amino acids, since they may

be readily synthesized from the carbon skeletons available from

the metabolism of glucose.

9. Amino acid composition of the cellular protein

In order to determine if the composition of the cellular

protein reflected the efficacy of amino acid transport, protein

was isolated from logarithmic phase cells and submitted to amino

acid analyses. The results (Table IV) were compared to the

tabulated amino acid transport rates (Table I).

Although the transport rates of some of the 18 amino acids

tested (proline, valine, leucine, and alanine) compared favourably

with their relative abundance in the protein of P. aeruginosa,

the majority of the amino acids showed no rigid correlation.

However, when the data of Table III was compared with that of

Table IV, it was observed that those amino acids that are present

in the native amino acid pool are essentially those present in

highest concentrations in the protein. The amino acids present

in the pool in relatively low concentrations, that is leucine,

isoleucine, valine, and phenylalanine were transported rapidly,

whereas the amino acids present in relatively high concentrations,

were transported slowly.

II. General Properties of Pool Formation and Maintenance

1. Time course of pool formation

When experiments,designed to determine the rate of

incorporation of amino acids, were performed for all of the

amino acids commonly occurring in protein, significant variations

in these incorporation kinetics were found. Figure 10 represents

the four patterns of incorporation found with these amino acids.

Most of the aliphatic, aromatic, and basic amino acids demonstrated

the pattern of uptake illustrated by Figure 10A, and when pools

were chromatographically analyzed the amino acid was found, for

the most part, to maintain its chemical integrity. For example,

chromatography and radioautography of the isoleucine pool is

demonstrated in Figure 11. Most of these amino acids also

Table IV. Composition of the cellular protein of
P. aeruginosa.

AMINO ACID	RELATIVE CONCENTRATION
methionine	1.00
histidine	1.38
lysine	1.85
tyrosine	2.27
phenylalanine	3.09
isoleucine	3.76
proline	3.95
serine	4.28
arginine	4.46
threonine	4.54
glutamic acid	5.48
valine	6.52
glycine	8.40
leucine	8.51
aspartic acid	8.94
alanine	11.95

demonstrated high transport rates (Table I), and the results

agree with the kinetics of amino acid incorporation in E. coli

(Britten and McLure, 1962). However, when incorporation studies

were carried out under identical conditions, several amino acids

were found to deviate from this general pattern. Figure 10B

illustrates the incorporation kinetics common to the amino acids

glycine, serine, and glutamate. It can be seen that pool formation

commences rapidly upon the addition of the labelled amino acid,

but the pool is only partially removed for protein synthesis;

the remaining fraction being lost only very slowly over a long

period of time - if at all.

To elucidate the nature of this phenomenon, pools were

collected at time intervals indicated by arrows in Figure 10, and

were analyzed by radioautography (Table V). Radioautography of

compounds in the early glutamate pool is demonstrated in Figure

11. At 5 min, 66% of the radioactivity in the pool was found to

be glutamate and a "bound" or slowly metabolized compound was

found to account for approximately 90% of the pool at a later time.

This compound did not chromatograph discretely and resisted

identification by several methods.

Glycine and serine also formed heterogeneous pools and,

in fact, these amino acids did not account for the majority of

the radioactivity of the intracellular pool. The major labelled

component was found not to be an amino acid, purine, pyrimidine,

nucleoside, or nucleotide, and did not form a hydrazone. This

material proved to be heterogeneous and from its solubility in

chloroform and chloroform-NaCl was assumed to be lipid and
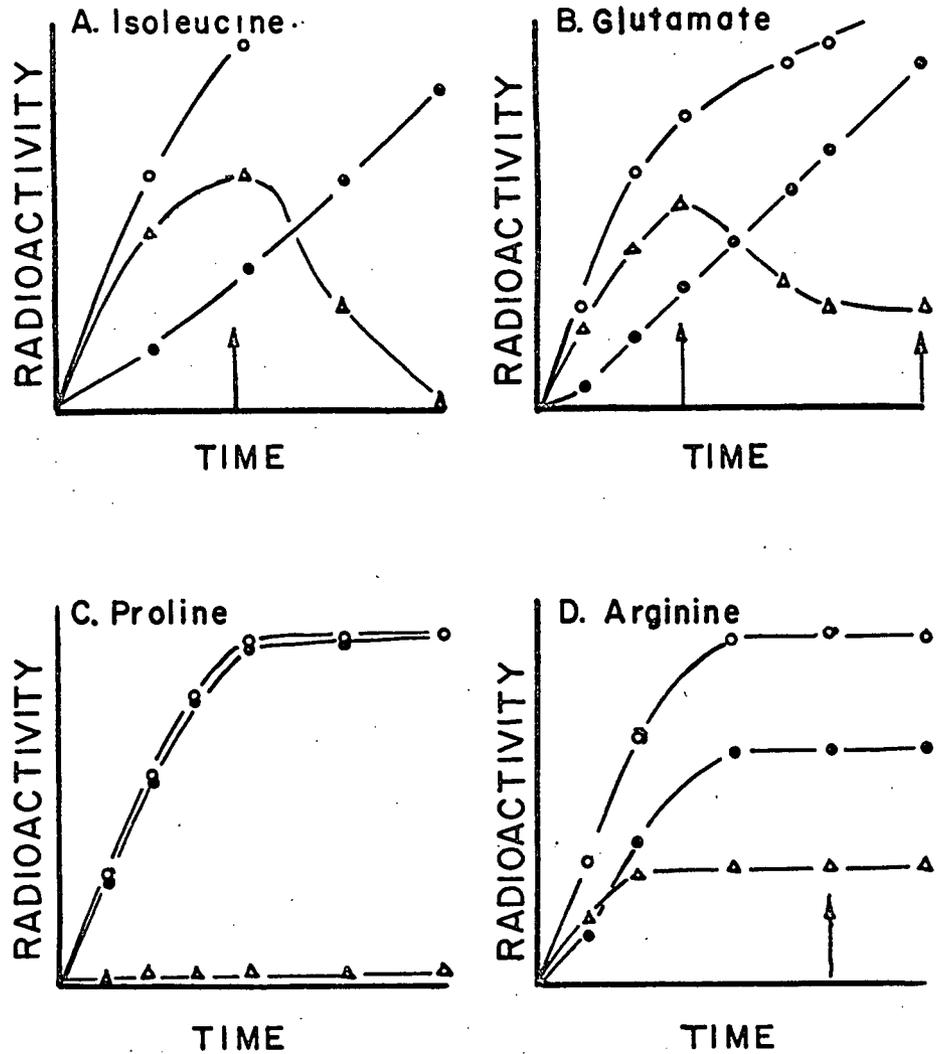
Fig. 10. Patterns of ^{14}C-amino acid uptake into whole cells 0, protein 0, and the calculated pool $\triangle$, of P. aeruginosa.
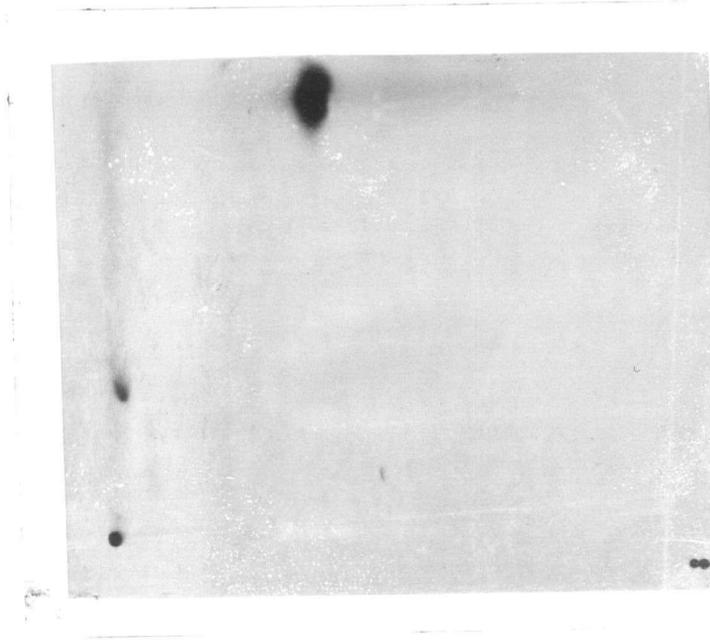
Fig. 11. Radioautograph of the amino acid pool established
with ^{14}C-isoleucine
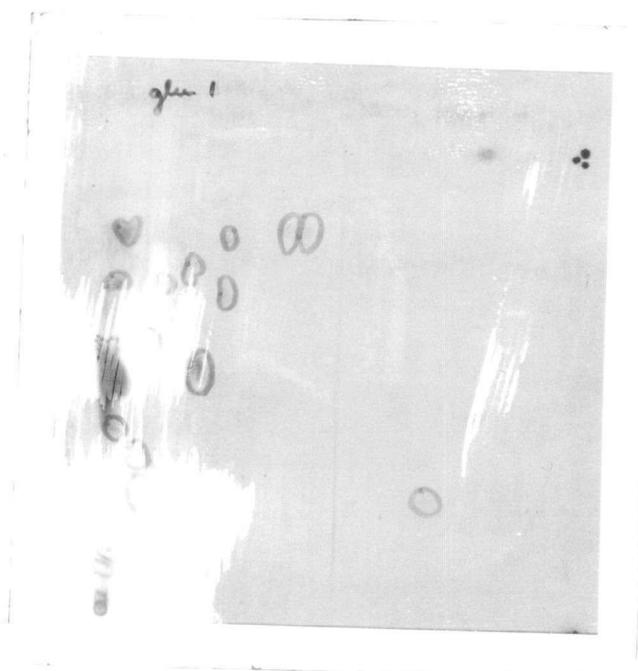
Fig. 12. Radioautograph of the amino acid pool established
with ^{14}C-glutamate.
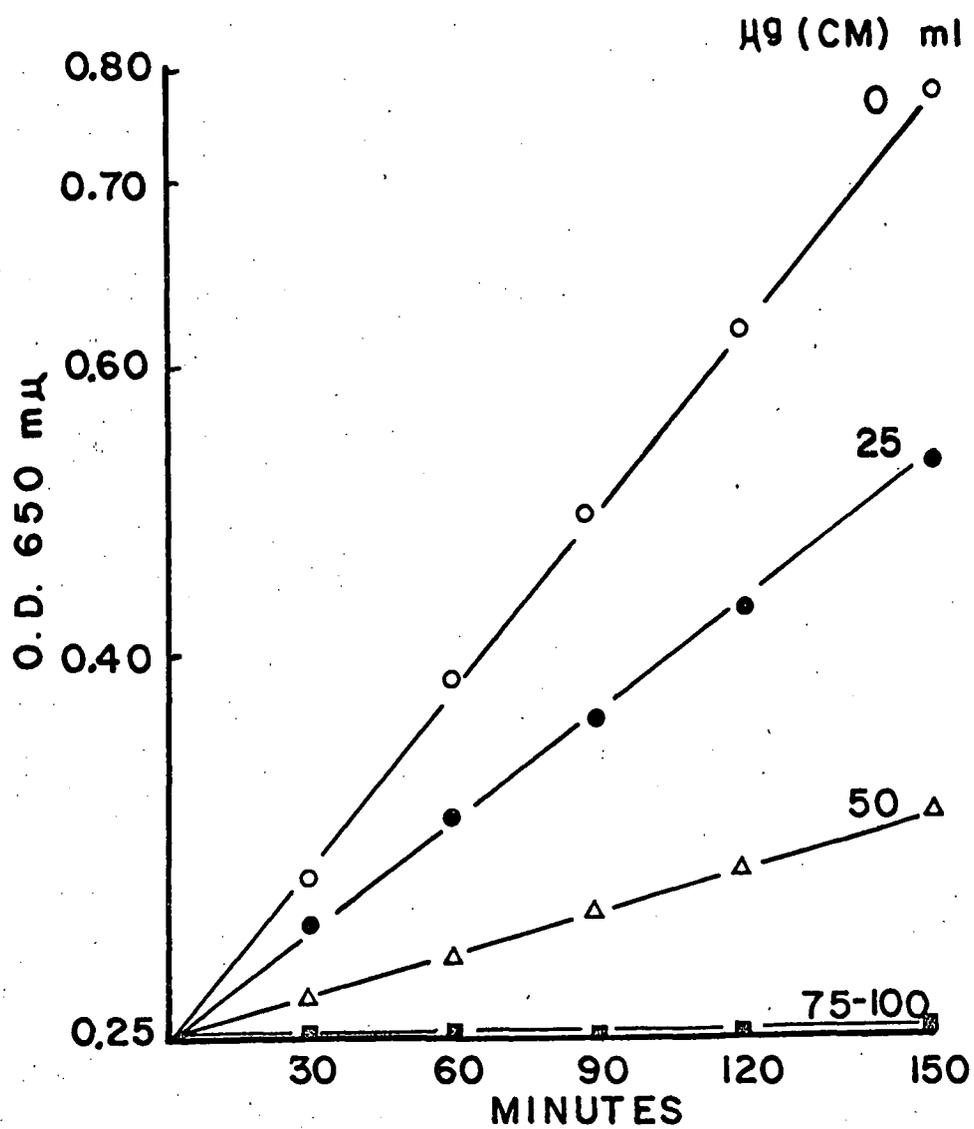
Fig. 13. The inhibition of growth in minimal media
as a function of the chloramphenicol (CM)
concentration.

Table V. Chromatographic analyses of ^{14}C-amino acid pools.

^{14}C-Amino Acid Added	^{14}C Compounds in the pool	Percent of Total Pool Radioactivity	
		early pool	late pool
isoleucine	isoleucine	93.7	-
proline	proline	95.2	-
	glutamate	4.8	-
glutamate	glutamate	65.9	-
	compound X	13.4	89.5
	alanine	0.7	-
	valine	9.4	-
	isoleucine	0.4	-
	leucine	0.3	-
	proline	0.2	-
glycine	compound Y	93.3	92.6
	glycine	1.2	0.9
	glutamate	1.2	1.6
	threonine	0.9	1.2
	serine	0.6	0.3
serine	compound Z	86.8	89.2
	glutamate	1.2	-
	aspartate	1.1	-
	threonine	1.0	0.6
	serine	0.9	1.7
	glycine	0.8	0.3
arginine	glutamate	43.5	6.8
	arginine	21.2	-
	putrescine	15.2	93.2
	γ-aminobutyrate	2.6	-

phospholipid. Since only a minor proportion of the added
^{14}C-amino acid was identified in the intracellular pool, it was
concluded that under the conditions of the transport experiments,
much of the amino acid was converted to lipid and phospholipid
without first entering the free amino acid pool. It was felt
that this conversion may have taken place at the cell membrane.

The third class of uptake kinetics was observed with ^{14}C-
proline and ^{14}C-threonine (Fig. 10C). In this case, essentially
no pool formation occurred during the course of the experiment;
that is, the amino acid entering the cell was immediately
converted into TCA insoluble material. In order to investigate
this deviation from normal pool behaviour, attempts were made
to selectively inhibit protein synthesis without altering the
ability of the cells to transport proline. It was found (Fig.
13) that the degree of protein synthesis could be manipulated by
the controlled addition of chloramphenicol (CM). At concentrations
greater than 100 µg/ml, growth was completely inhibited. The
rates of ^{14}C-proline incorporation into chloramphenicol treated
cell suspensions as a function of the inhibitor concentration
are shown in Figure 14. It can be seen that over the range of
chloramphenicol concentrations employed, the rate of ^{14}C-proline
incorporated into the cell was relatively constant, whereas the rate
of entry of the label into the protein fraction was inhibited in a
manner proportional to the inhibitor concentration. As a result,
accumulation of proline in the intracellular pool increased
significantly.

Preliminary experiments showed that the initial rate of proline incorporation into whole cells did not vary greatly as a function of temperature; however, the rate of growth diminished markedly at low temperatures. These observations were used to confirm the results obtained with chloramphenicol, since chloramphenicol has been shown to adversely affect pool formation in E. coli (Britten and McLure, 1962). When the incorporation of ^{14}C-proline into cell suspensions was followed at different temperatures, results similar to those with chloramphenicol, were obtained (Fig. 15). At 10 C - 20 C, the rate of protein synthesis (as determined by incorporation of ^{14}C-proline into TCA insoluble material) was significantly lowered. Low rates of protein synthesis were coincident with high proline pool levels. At higher temperatures, large pools were not formed due to the rapid incorporation of the labelled amino acid into protein. These results demonstrate that the rate of pool formation and the size of the intracellular pool are functions of both the capacity of the cell to transport an amino acid and to utilize that amino acid for protein synthesis. Therefore, any demonstration of pool formation must be the result of a balance of the above variables, and not necessarily attributable to the pool forming mechanism per se. Thus, if the time course of ^{14}C-proline uptake is followed at 20 C (Fig. 16A) or at 30 C when the rate of protein synthesis has been decreased due to starvation for a required amino acid, then pool formation (Fig. 16B) follows a course similar to that reported for E. coli (Britten and McLure, 1962). Chromatography of the labelled pool material, under these conditions, showed that
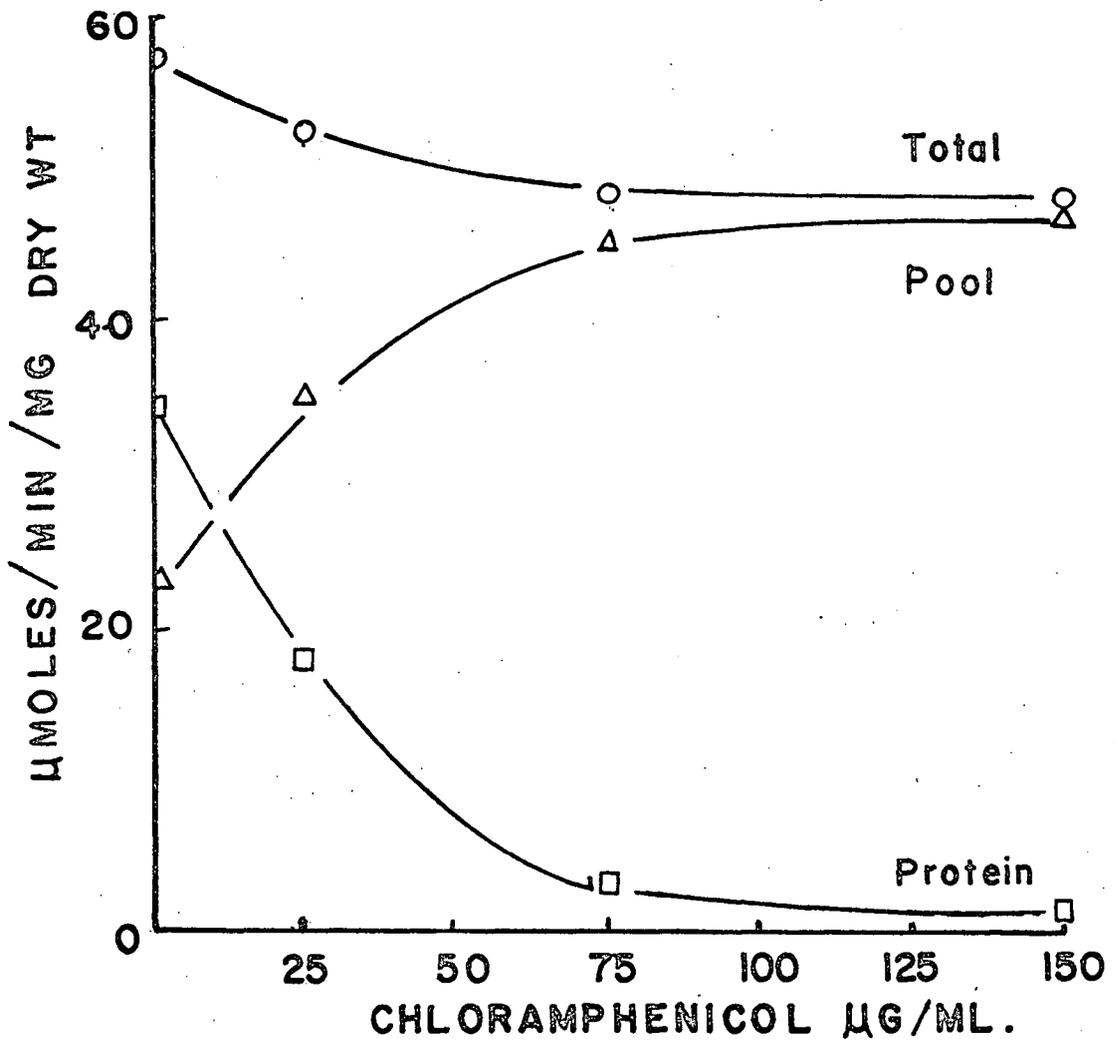
Fig. 14. Effect of chloramphenicol on the rate of ^{14}C-proline incorporation into whole cells and cell fractions of P. aeruginosa. Cells were preincubated for 30 min at 30 C prior to the addition of ^{14}C-proline and samples were removed at 30 sec time intervals to determine initial incorporation velocities.
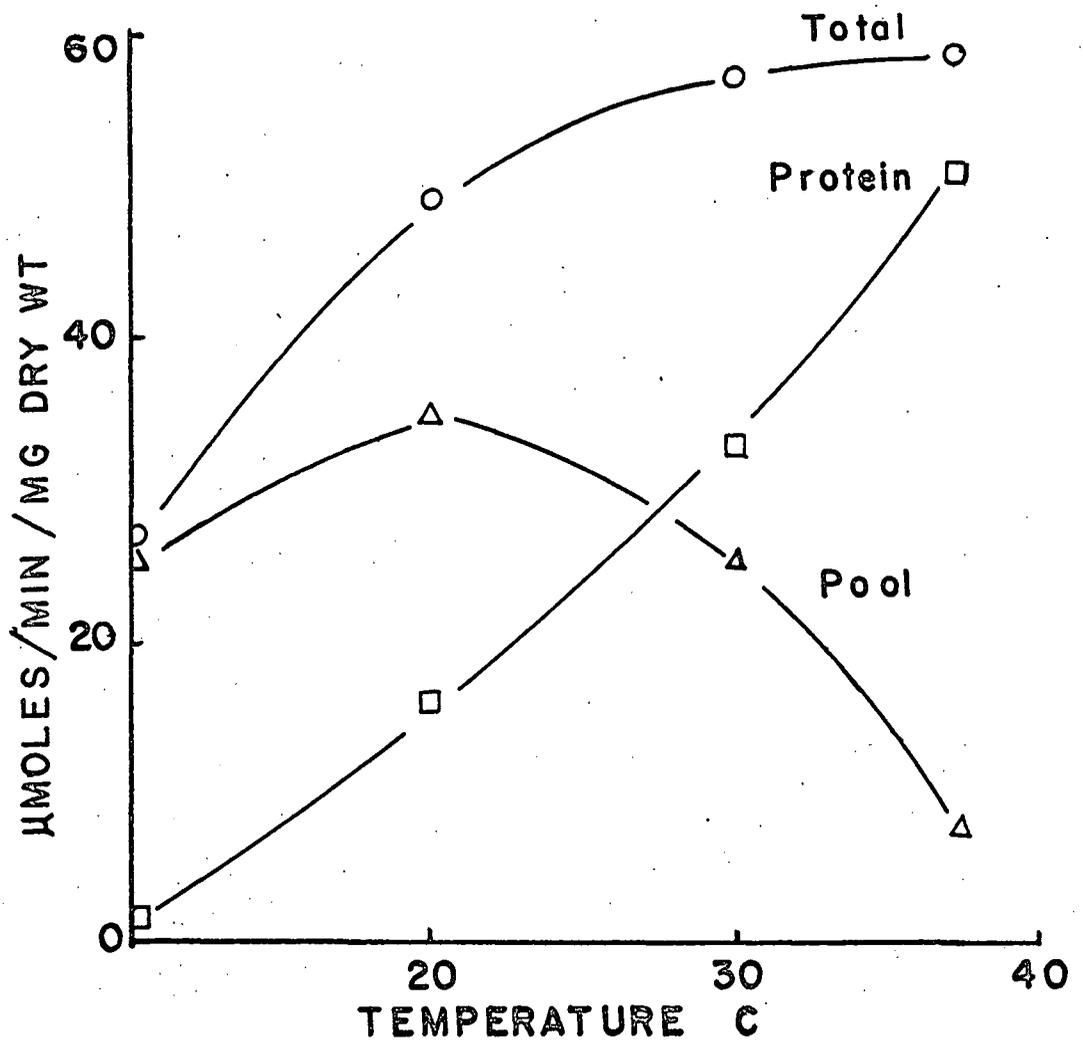
Fig. 15. Effect of temperature on the rate of ^{14}C-proline incorporation into whole cells and cell fractions of P. aeruginosa. Cells were equilibrated at the appropriate temperatures for 10 min prior to the addition of ^{14}C-proline and samples were removed at 30 sec intervals to determine initial incorporation velocities.

the amino acid retained its chemical integrity.

2. The fate of intracellular arginine

The fourth class of uptake kinetics (Fig. 10D), was observed

with the amino acids arginine and cysteine. These amino acids

formed a relatively high pool level and this pool was maintained

for extended periods of time. The arginine pool was studied

further and Figure 17 demonstrates the kinetics and stability

of ^{14}C-arginine pool formation over an extended period. Once

formed, the intracellular pool was stable and remained constant

even during cell growth. During starvation for an exogenous

carbon source, the pool was maintained for periods as long as

twenty-four hours. The pool was shown to exchange with ^{12}C-

arginine when the unlabelled amino acid was added after formation

and stabilization of the ^{14}C-pool. However, the pool exchanged

at a greater rate when the polyamine, putrescine, was added to

the cell suspension (Fig. 18). No exchange occurred with ^{12}C-

spermine. The results of these experiments suggested that the

stable pool formed from arginine was not the original amino acid

but perhaps a degradation product. In addition, ^{14}CO$_2$ recoveries

from these experiments indicated that the oxidation of arginine was

constitutive in P. aeruginosa. It has been demonstrated that E.

coli catabolizes both arginine and ornithine to putrescine (Morris

and Pardee, 1966), and that P. fluorescens catabolizes putrescine

by way of γ-aminobutyrate and succinic semialdehyde to succinate

(Jakoby and Fredericks, 1959). Consequently, the pools were extracted
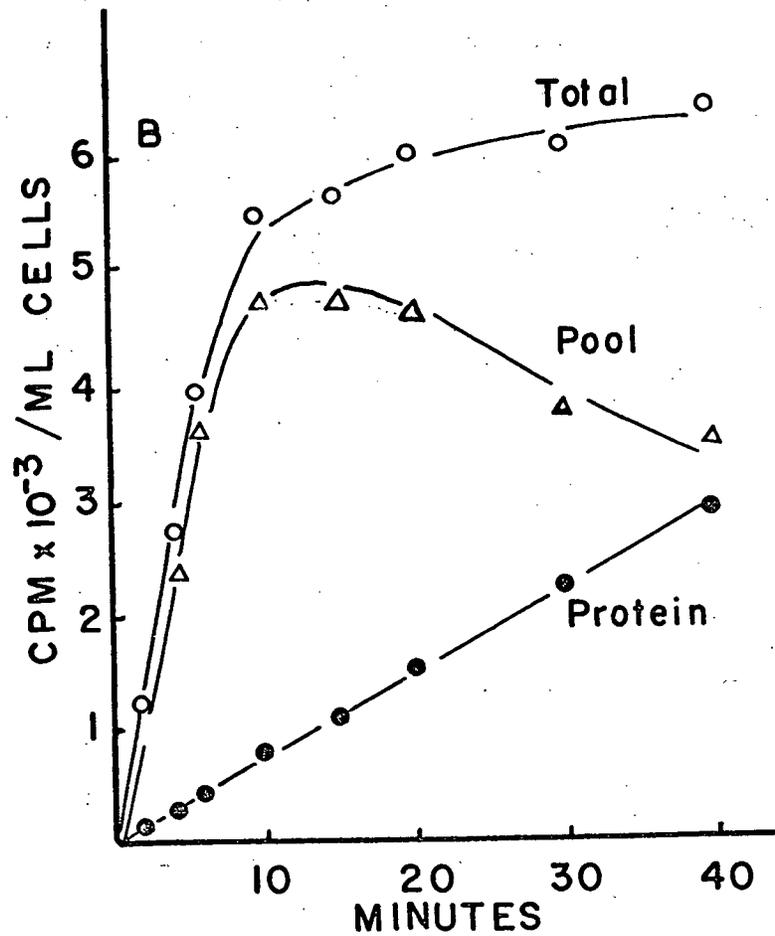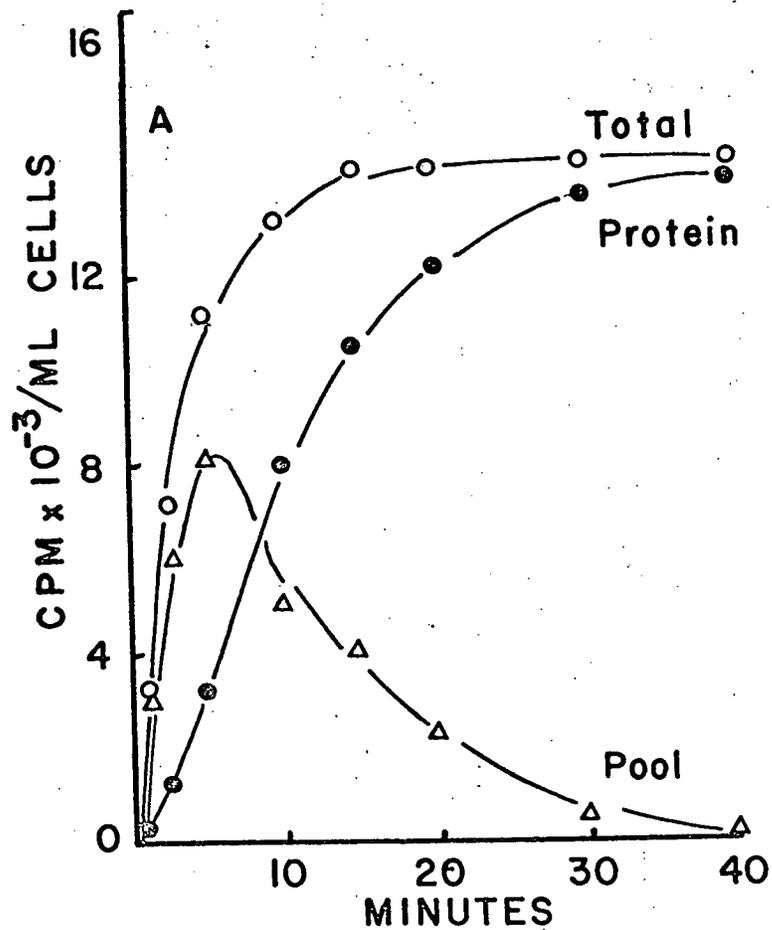
Fig. 16. Time course of ^{14}C-proline uptake into whole cells and cell fractions of P. aeruginosa. A. At 20 C. B. At 30 C, by a histidine auxotroph (WK4) deprived of histidine for 30 min prior to the addition of ^{14}C-proline.
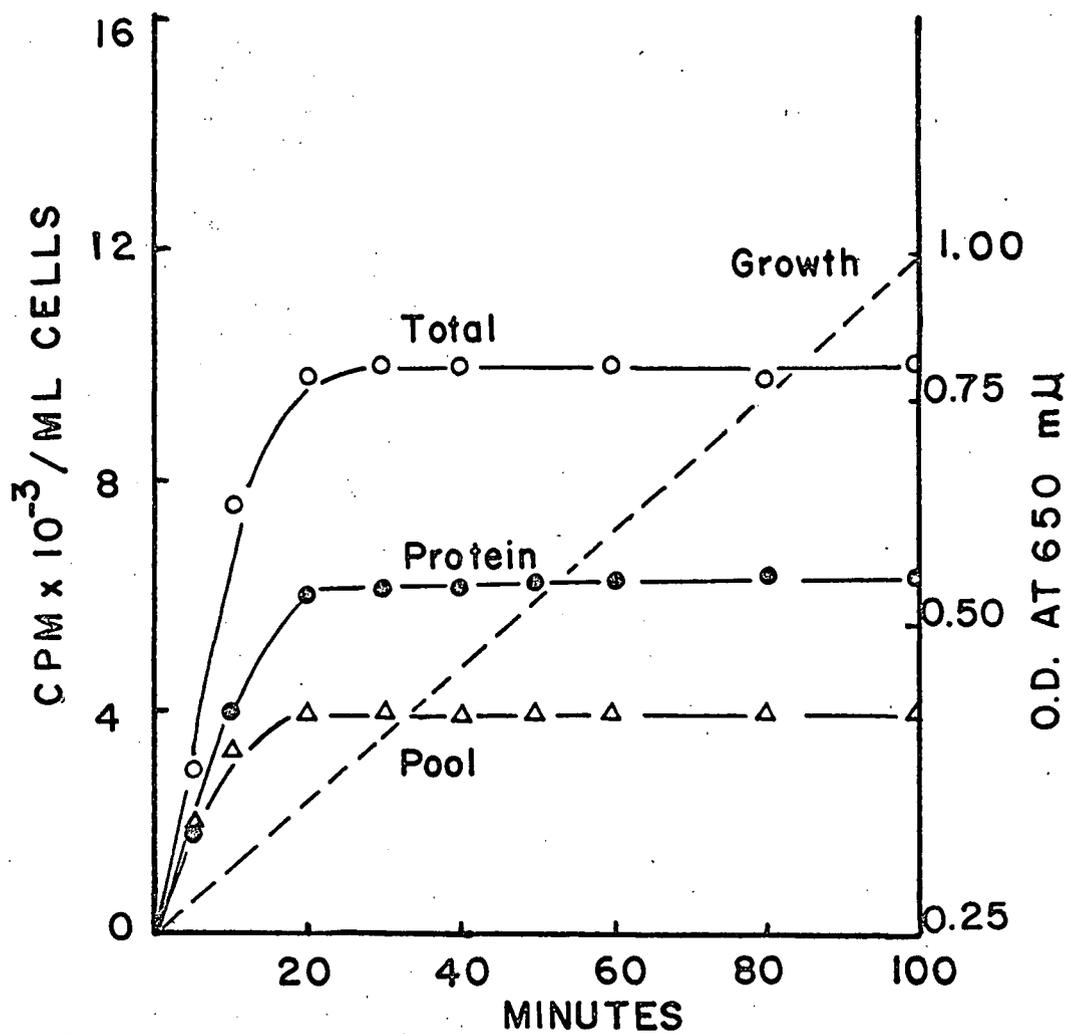
Fig. 17. The formation and maintenance of the pool
derived from ^{14}C-arginine (2 x 10^{-5}M) by a
growing culture of P. aeruginosa.
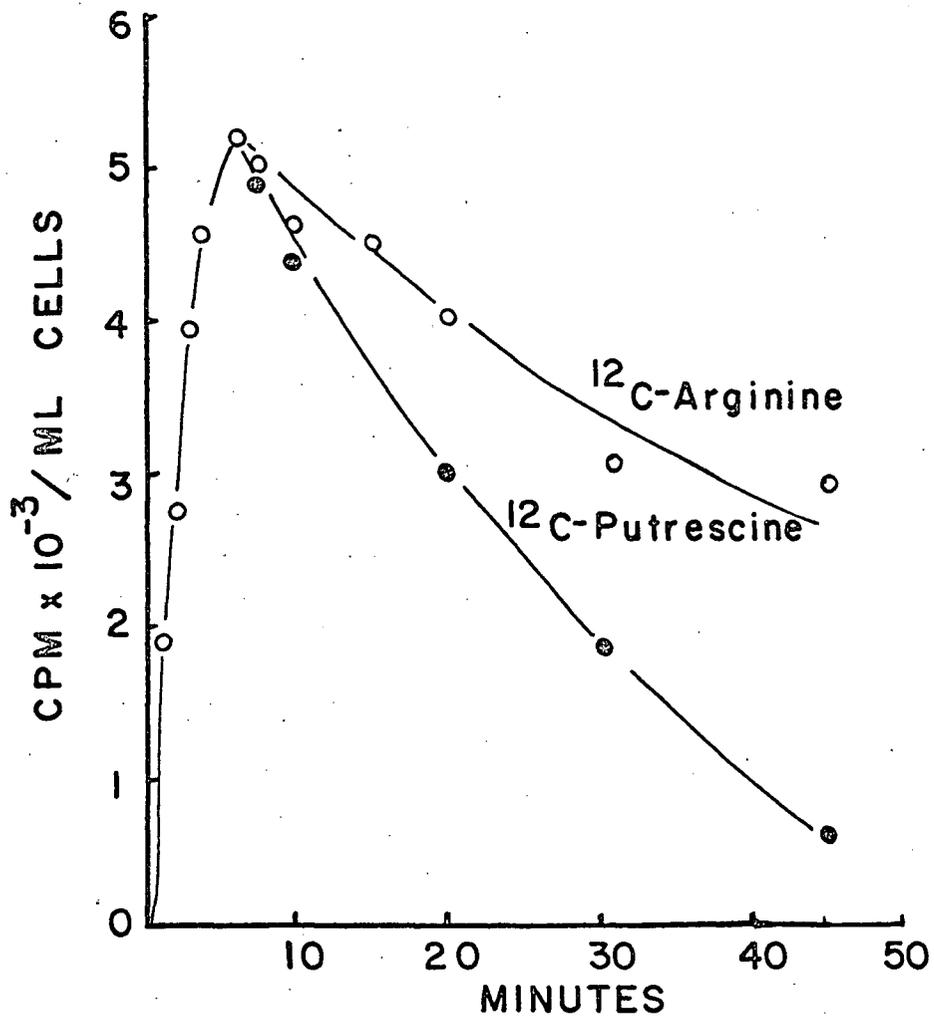
Fig. 18. The displacement of the ^{14}C-pool derived from $2 \times 10^{-5}M$ ^{14}C-arginine by ^{12}C-arginine ($2 \times 10^{-3}M$) or ^{12}C-putrescine ($2 \times 10^{-3}M$) added 6 min after the initial ^{14}C-arginine.

at the times indicated by the arrows in Figure 10D. Subsequent
chromatography indicated that putrescine and γ-amino butyric
acid were formed very quickly and that after the pool had stabilized,
the majority of the radioactivity was found to be present in
putrescine. Putrescine was formed as a product of arginine
catabolism and was not degraded further.

Some investigators have implicated polyamines as structural
components mediating the stabilization of the quaternary structure
of the ribosomes (Silman, Artman and Engelberg, 1965; Choi and
Carr, 1967). This suggested that the intracellular putrescine
was not a "pool" of free putrescine, but rather putrescine bound
to intracellular structures, perhaps ribosomes or even nucleic
acids, and that treatment with TCA may have neutralized the charge
and permitted chemical extraction. However, when cells containing
a ^{14}C-putrescine pool were extracted with a variety of reagents
(Table VI), it appeared that the maintenance of the putrescine
"pool" was also markedly dependent on cellular integrity. The
degree of extraction of the putrescine pool was commensurate
with conditions which were known to cause cell lysis; that is,
low or high pH's or high alcohol concentrations.

Since the putrescine pool was maintained during long periods
of carbon starvation, it was thought that energy may not have been
required for its maintenance. To test this hypothesis, large
pools were allowed to form from ^{14}C-arginine by first preincubating
the cells with high concentrations of chloramphenicol (Fig. 19).
In the complete absence of protein synthesis (evidenced by the
termination of the entry of ^{14}C-amino acid into protein) amino

Table VI.   Lability of the putrescine pool.

REAGENT	PERCENTAGE OF THE POOL[a] LOST
10 ml media	12.0
0.25% TCA	80.6
5.0 % TCA	100.0
10 % ETOH	30.3
20 % ETOH	60.2
40 % ETOH	98.0
pH 10.5 (NaOH)	122.0
pH 8.0 (NaOH)	100.0
pH 5.0 (HCl)	80.6
pH 3.0 (HCl)	85.0
pH 1.0 (HCl)	87.0

[a] Samples of a cell suspension containing putrescine were added
to reagents to give the conditions described in the first
column. After 10 min at 0 C these suspensions were filtered
and the filtrate collected and assayed for radioactivity. The
percentage of the pool lost was calculated from the increase
(if any) in the radioactivity of the filtrate.

acid pools are unstable and "efflux" readily. However, the pool formed from ^{14}C-arginine was stable under these conditions and essentially was unaffected by the addition of 30 mM NaN$_3$ (Fig. 20). This treatment afforded only a minor loss of the pool which was attributed to unmetabolized arginine. This demonstrated that putrescine was not maintained in the intracellular pool by an energy requiring mechanism, since energy deficient cells rapidly lose their amino acid pools (Fig. 27). When ^{12}C-arginine, at high concentration, was added to the culture after this large pool had been formed the resulting exchange rate followed two isotherms (Fig. 21). The rapidly exchanging component was the minor one and was attributed to the presence of residual arginine in the pool, whereas the second more slowly exchanging component was the putrescine pool. Linear exchange rates previously illustrated in Figure 18 occurred when the ^{14}C-pool was homogeneous as was found for pools formed from low levels of exogenous ^{14}C-arginine. Unlike the normal amino acid pools in P. aeruginosa, the putrescine pool was also shown to be stable at 0 C.

From these kinds of data it was concluded that intracellular putrescine must be physically bound or oriented in an unusual manner to some intracellular structural component and is not found free in the pool as in the case of other metabolic intermediates.

Exogenously supplied ^{14}C-arginine has been shown to have a double metabolic role: the conversion to putrescine, and the incorporation into protein. When protein synthesis was inhibited with chloramphenicol, the majority of the incorporated arginine was converted to putrescine (Fig. 20). This demonstrated that the
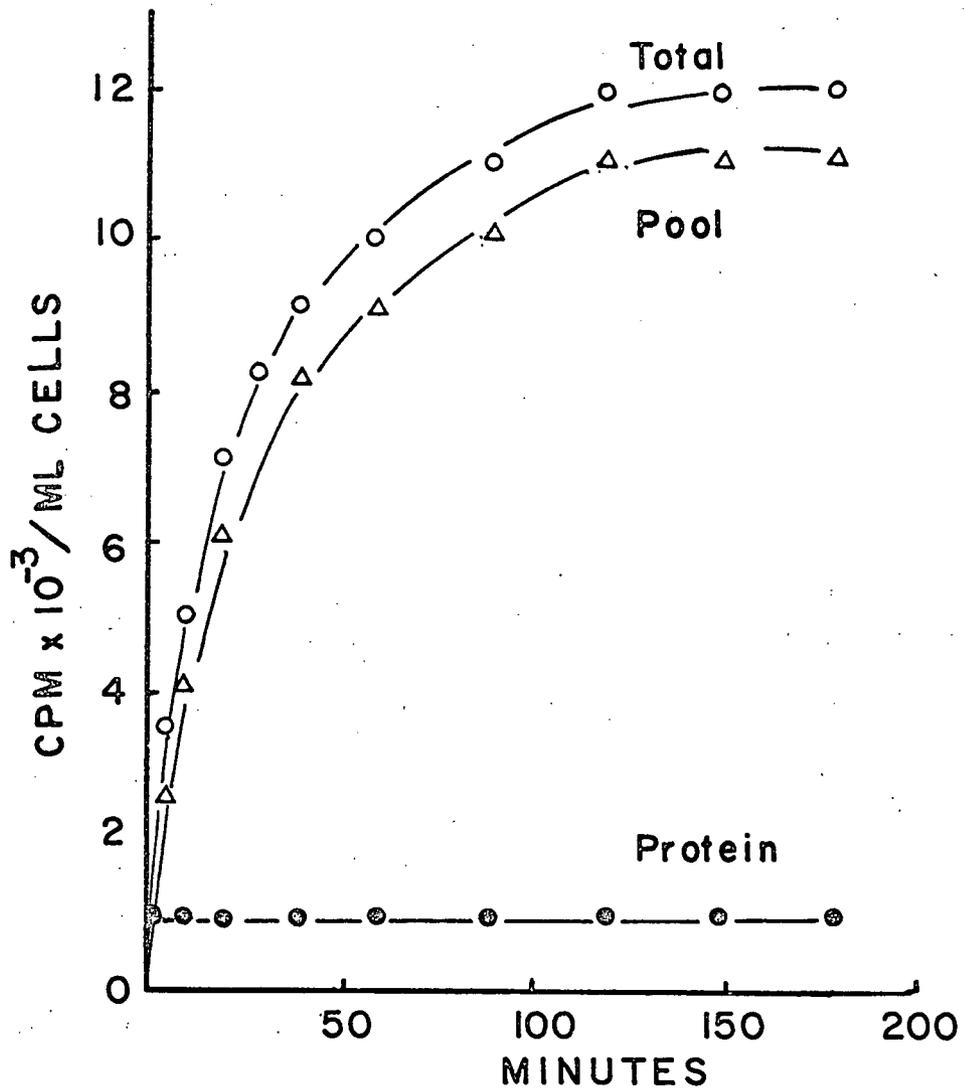
Fig. 19. The Incorporation of ^{14}C-arginine into cell fractions of P. aeruginosa poisoned with 2 mg/ml chloramphenicol.
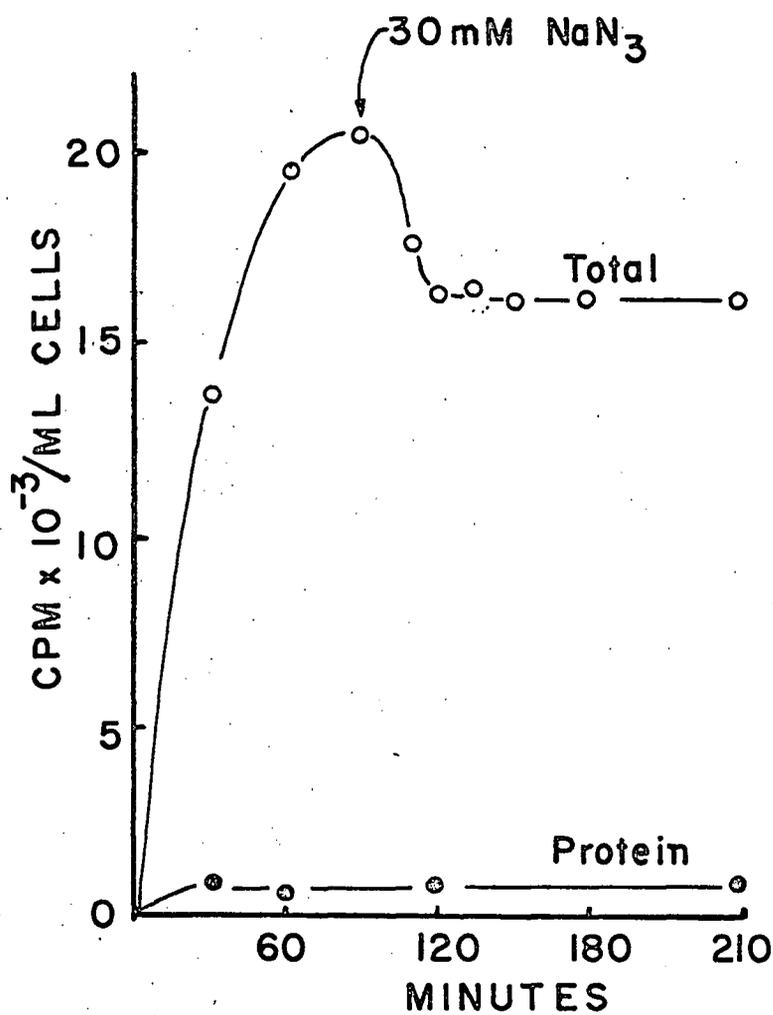
Fig. 20. Partial efflux of the ^{14}C-arginine-^{14}C-putrescine pool in the presence of 30 mM NaN$_3$.
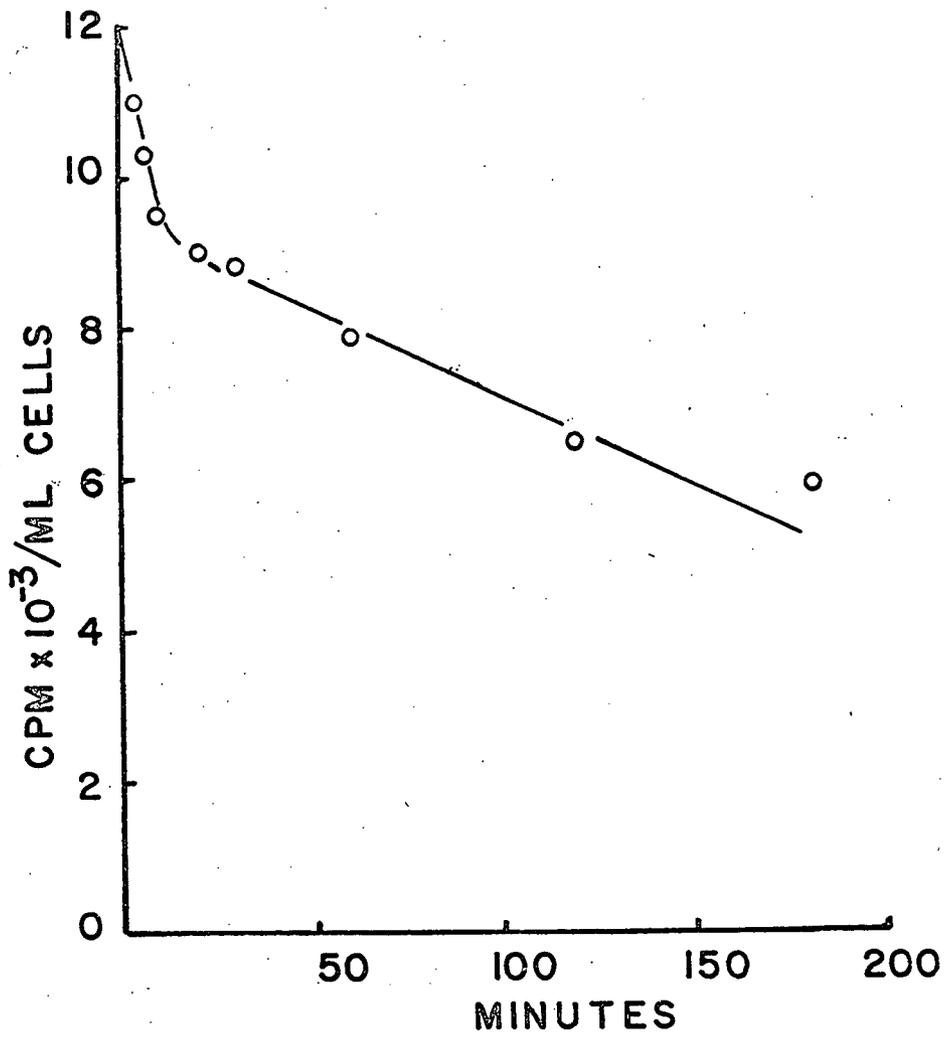
Fig. 21. The exchange of the ^{14}C-arginine-^{14}C-putrescine
pool by 2 x 10^{-3}M ^{12}C-arginine.

fate of the arginine pool was a result of the competition between protein synthesis and catabolism. However, no putrescine was found in the pools extracted from cells grown on high specific activity U-^{14}C-glucose. These results implied that arginine synthesized de novo was not as available for catabolism as for anabolism.

To determine whether or not arginine which was synthesized de novo was unavailable for catabolism, an experiment was designed in which arginine synthesized de novo would be inhibited and at the same time an evaluation of the degree of exogenous arginine catabolism could be determined.

At high exogenous arginine concentrations, arginine synthesized de novo would be terminated by feedback inhibition of ornithine transcarbamylase. When a cell suspension was incubated with 0.4 mM ^{14}C-arginine (Fig. 22), the accumulation of ^{14}C-putrescine in the pool was found to be low relative to the incorporation of ^{14}C-arginine into protein. This was expected due to the increased competition for pool arginine for the purpose of protein synthesis. However, during the time interval when the exogenous arginine was becoming exhausted (80-100 min), a greater proportion of the incorporated exogenous arginine was converted to putrescine. This coincides with the time at which arginine synthesized de novo would again return to normal. Had the newly synthesized ^{12}C-arginine equilibrated with ^{14}C-arginine committed to catabolism, no "apparent" increase in the putrescine pool would have been observed since the specific activity of the putrescine synthesized from an equilibrium mixture of exogenous ^{14}C-arginine and ^{12}C-arginine
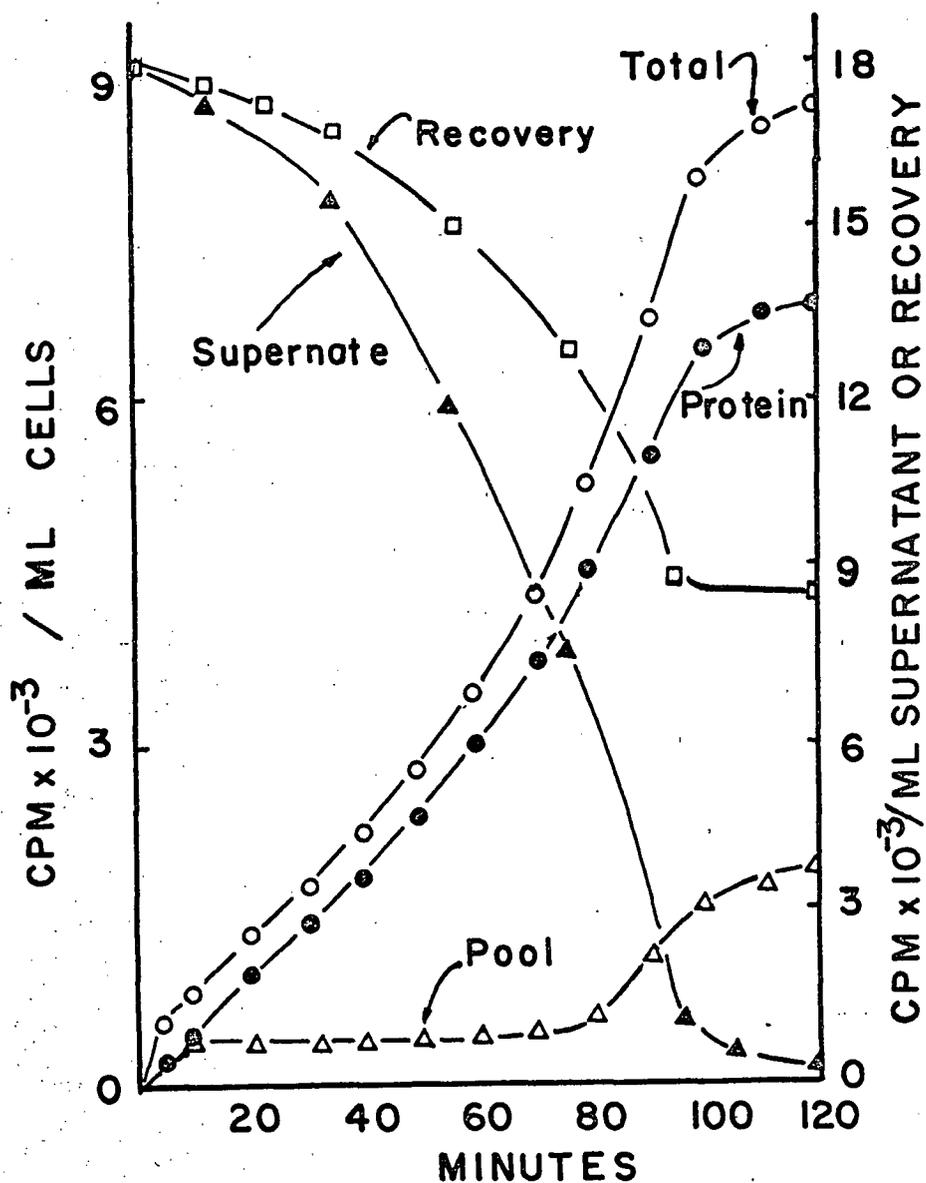
Fig. 22. Time course of ^{14}C-arginine incorporation into cell fractions of P. aeruginosa and the disappearance of exogenous label. ^{14}C-arginine was present at 4×10^{-4} M.

synthesized de novo would have lessened. It was, therefore,
concluded that arginine synthesized for incorporation into
protein was unavailable for catabolism. Possibly this could
be explained by a gross difference in affinity of the arginine
catabolizing enzymes and the amino acid activating enzymes for
the substrate arginine.

To determine whether or not the formation, and presumably
the binding, of intracellular putrescine was peculiar to P.
aeruginosa alone, ^{14}C-arginine incorporation experiments were
carried out with two different species of Pseudomonas; P.
putida and P. fluorescens. From the results of Figures 23A and
23B it can be seen that this same phenomenon occurs in these
species but to different degrees. From these incorporation
experiments it was evident that when the catabolism of arginine
was most active, evidenced by a loss of label from the medium,
more intracellular putrescine was formed in P. putida. This
stresses the significance of the observation made with P.
aeruginosa that the fate of intracellular arginine was dependent
on the competition between catabolism and anabolism.

The formation of putrescine by the catabolism of arginine
in Pseudomonads is undoubtedly ubiquitous; however, the results
of this investigation suggest that putrescine has no essential
physiological role, since it was not formed in detectable amounts
when cells were grown on minimal medium. In the course of this
investigation, several mutants were isolated, which were unable
to catabolize arginine but these mutants grew normally on minimal
medium. This observation emphasizes the non-essential nature of
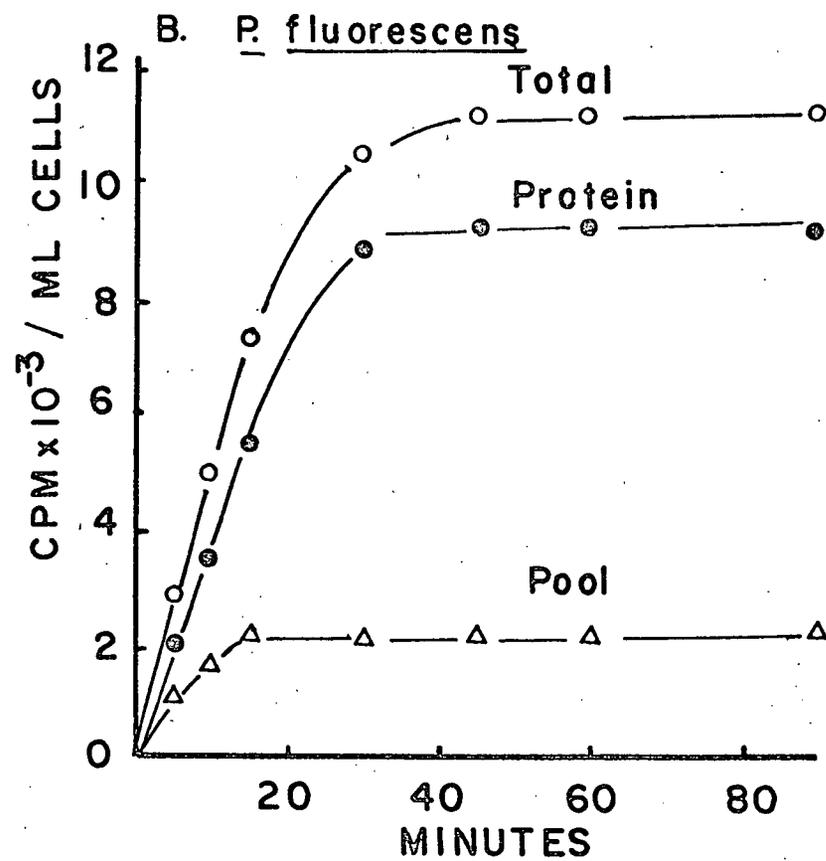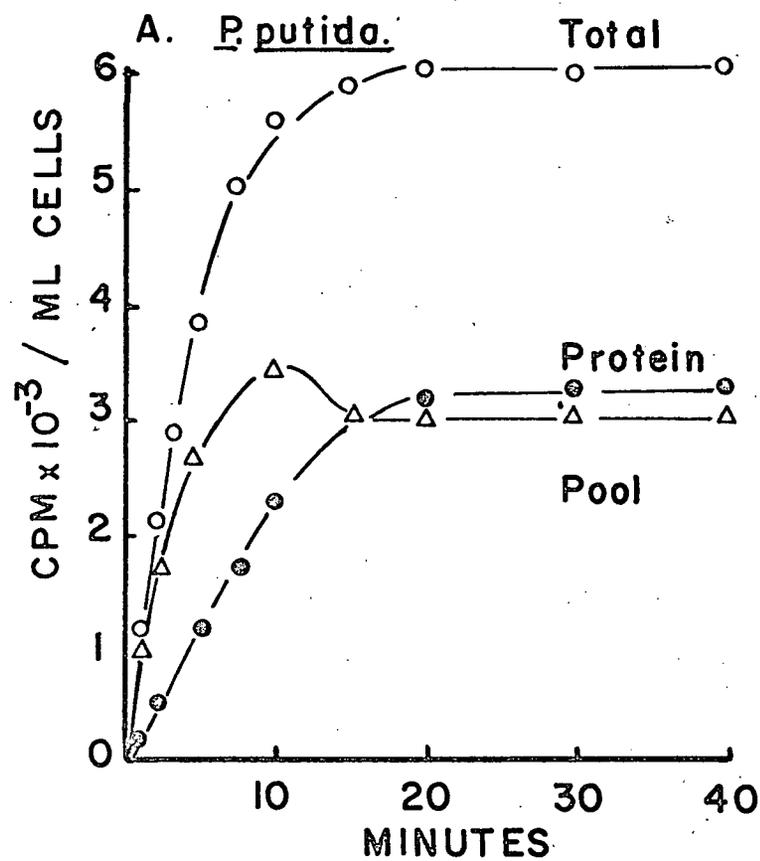
Fig. 23. The time course of ^{14}C-arginine incorporation into whole cells and cell fractions of (A) P. putida and (B) P. fluorescens.

putrescine formation. It is possible, however, that putrescine

biosynthesis may be important to structural integrity in the

absence of magnesium.

Cysteine was also found to accumulate intracellularly in

P. aeruginosa; as much as 50% of the added radioactivity was found

in the TCA soluble fraction of the cell. However, unlike the pool

formed from ^{14}C-arginine, the pool formed from ^{14}C-cysteine was

found, by chromatography and radioautography, to retain its chemical

identity. The nature of this intracellular accumulation has not

been investigated.

3. Pool formation and maintenance

a. Formation

Under optimum conditions for pool formation, that is when

protein synthesis was partially inhibited by low temperature without

affecting the amino acid transport rate, amino acid pools were

saturated at a particular concentration determined by the level

of the exogenous amino acid. Figure 24 demonstrated the maximum

pool level of proline obtained at an exogenous proline concentration

of 10^{-6}M with a proline oxidizeless mutant (P22) of P. aeruginosa.

At this concentration 40% of the exogenous amino acid is incorporated

into the cellular pool. The concentration ratio established between

the internal and external proline was found to be in the range of

1000 fold. At higher external proline concentrations the capacity

of the pool became largely dependent on the external concentrations,
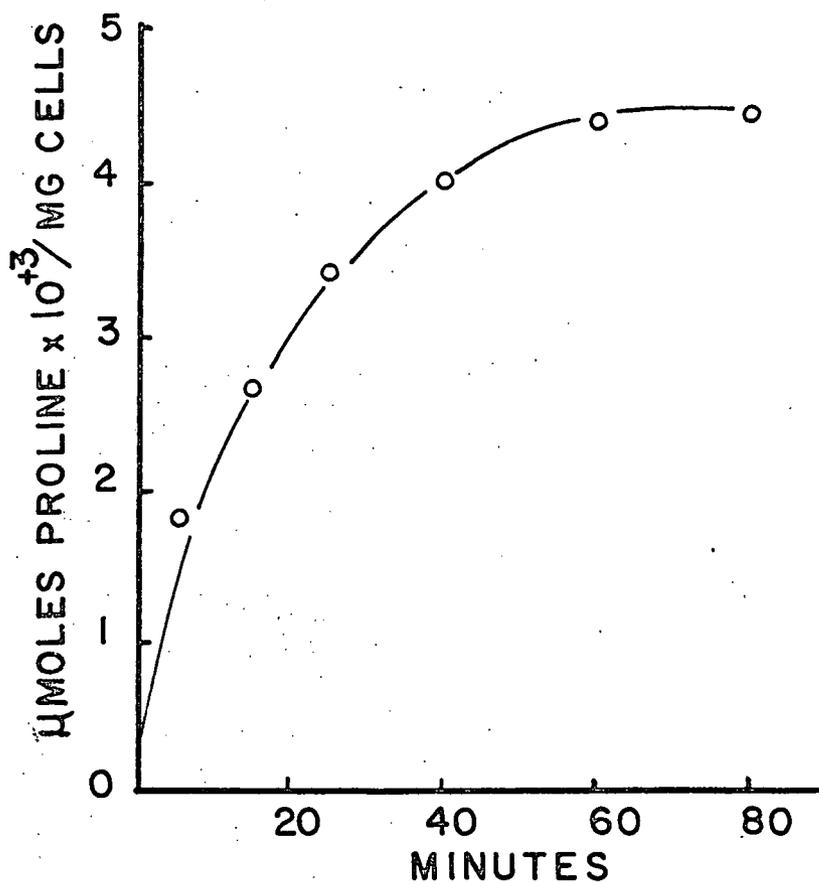
Fig. 24. Maximum proline pool obtained at 10 C with
10^{-6} M ^{14}C-proline by P. aeruginosa P22 growing
in minimal medium. Cells were preincubated
at 30 C for 30 min with 200 µg/ml
chloramphenicol and equilibrated for 10 min at
10 C prior to the addition of ^{14}C-proline.

and the resulting concentration ratios were found to decrease...

When 10^{-6} M proline was allowed to form a maximum pool at 10 C

(Fig. 25A), the addition of all other amino acids, each to a

level of 10^{-4} M, resulted in no significant change in the internal

proline pool. Consequently, the formation of proline pools in

this organism is specific. Also, a pre-established proline pool

did not greatly influence the incorporation of ^{14}C-leucine at

this temperature (Fig. 25B). Therefore, it was concluded that

the maintenance of large pools is structurally specific and that

the maximum accumulation of one amino acid is entirely independent

of the accumulation of another unrelated amino acid. Pool

formation and maintenance were found to be closely related processes.

At high external amino acid concentrations the pool capacity

increased and an equilibrium was established between the intra-

cellular and extracellular proline. The pool did not increase without

exchanging with preloaded intracellular proline. In the experiment

represented by Figure 26, an intracellular proline pool was established

with 10^{-6} M ^{14}C-proline at 10 C, and then ^{12}C-proline was added to a

level of 10^{-4} M at the time indicated. Rapid exchange of the pre-

loaded pool occurred until the specific activity of external and

internal proline were approximately the same.

b. Maintenance

The maintenance of high intracellular amino acid pools was

found to be an energy dependent function. When leucine was allowed

to accumulate at 15 C and NaN$_3$ (30 mM) and iodoacetamide (1 mM) were
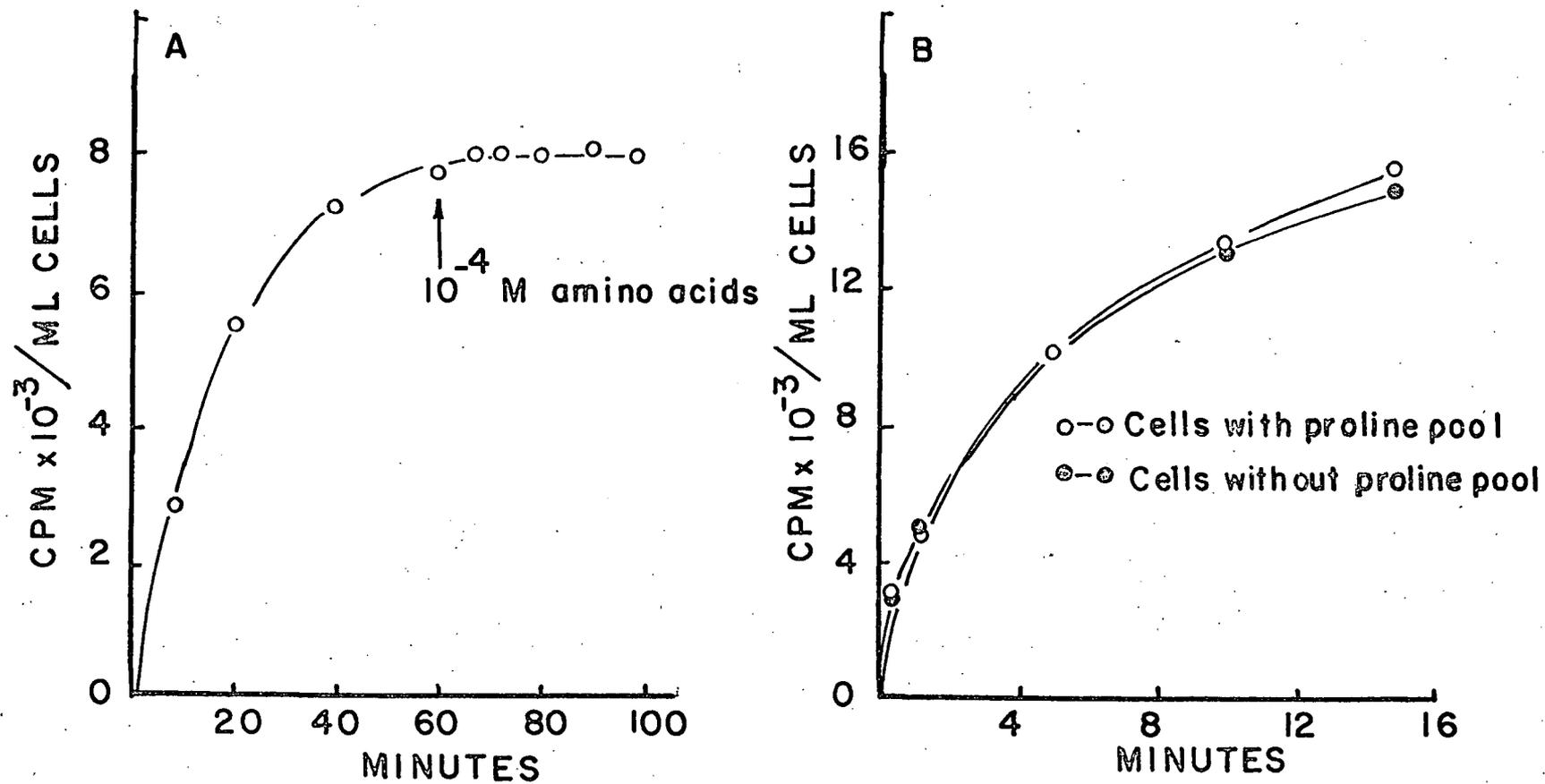
Fig. 25. Specificity of maintenance of the proline pool in P. aeruginosa P22. A. The time course of proline uptake and proline pool maintenance in the presence of a 10^{-4}M concentration of each of 18 amino acids added at 60 min. B. The time course of ^{14}C-leucine uptake into cells which had been preloaded with a maximum level of ^{12}C-proline by previous incubation with 10^{-6}M proline. Control has no preestablished proline pool.

added, an immediate efflux of the accumulated amino acid occurred
(Fig. 27).

4. Pool multiplicity

Both Britten and McLure (1962), and Kessel and Lubin (1962),
postulated from exchange kinetics with "preloaded" E. coli pools,
that a multiplicity of pools existed in the organism. That is,
internal proline exchanged with external proline in a manner
described by two classical isotherms. P. aeruginosa pools are
not stable to 0 C and such exchange reactions are impossible to
perform with the same degree of precision. However, several other
criteria were established to demonstrate that the internal proline
pool of P. aeruginosa was associated with the cell in different
ways. The efflux of proline from the pool in the presence of
30 mM NaN_3 and 1 mM iodoacetamide is shown in Figure 28. Unlike the
leucine pool (Fig. 27), a significant fraction of the proline pool
was lost very slowly in the absence of energy production and this
loss was due to the oxidation of the amino acid as evidenced by the
loss of label from the reaction mixture. The NaN_3 stable pool was
subsequently shown to be exchangeable with ^{12}C-proline when added
at 10^{-4} M. Control experiments demonstrated that the exchange rate
was the same in the absence or presence of NaN_3. Therefore, the
NaN_3 stable component of the pool was exchanged by a non-energy
requiring mechanism. Perhaps this component of the proline pool
is associated with some internal structure of the cell in such a
way as not to be considered a "free" internal pool amino acid.
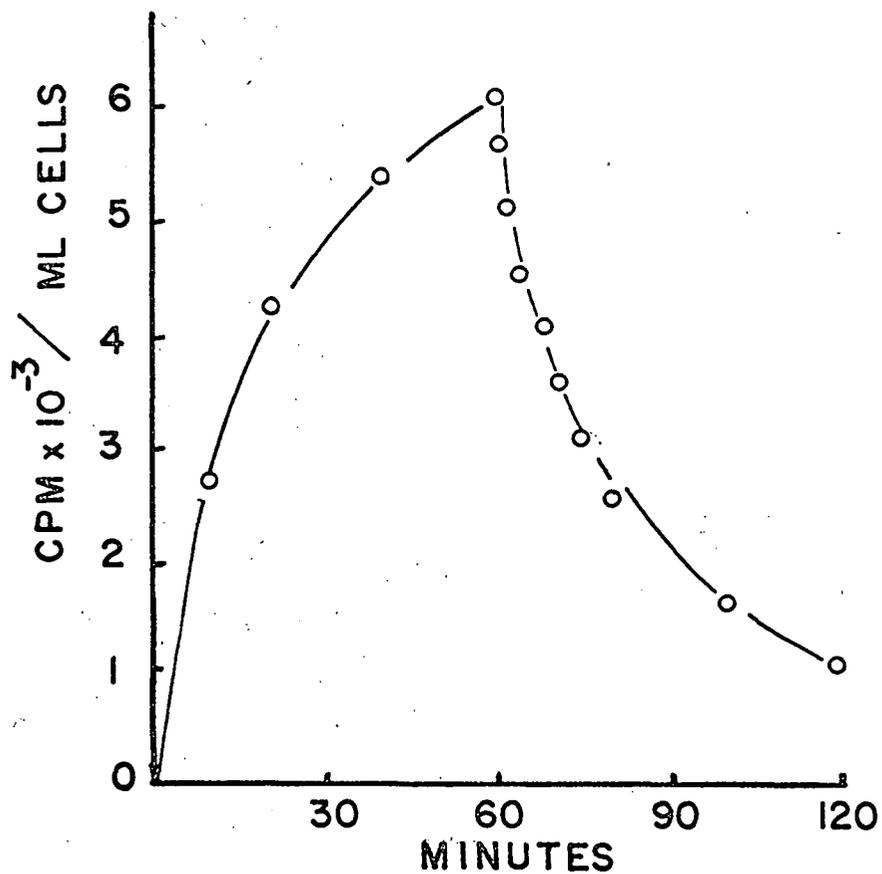
Fig. 26. Exchange of a preloaded ^{14}C-proline pool of
P. aeruginosa P22 with 10^{-4}M ^{12}C-proline
added at 60 minutes. Cells were preincubated
with 200 ug/ml chloramphenicol prior to
initiation of the experiment and the incorporation
study was performed at 10 C.
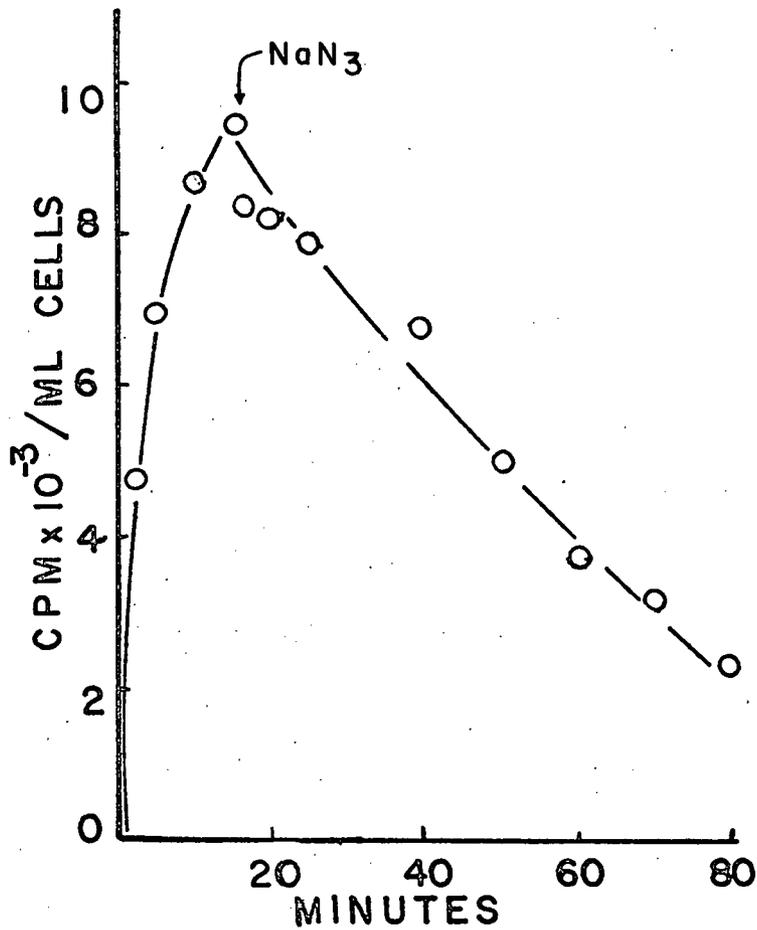
Fig. 27. Efflux of a preformed leucine pool in the
presence of 30 mM NaN₃ and 1 mM iodoacetamide.
Cells were preincubated with 100μg/ml
chloramphenicol for 30 min prior to addition
of 2 x 10⁻⁶M ¹⁴C-leucine at 15 C.
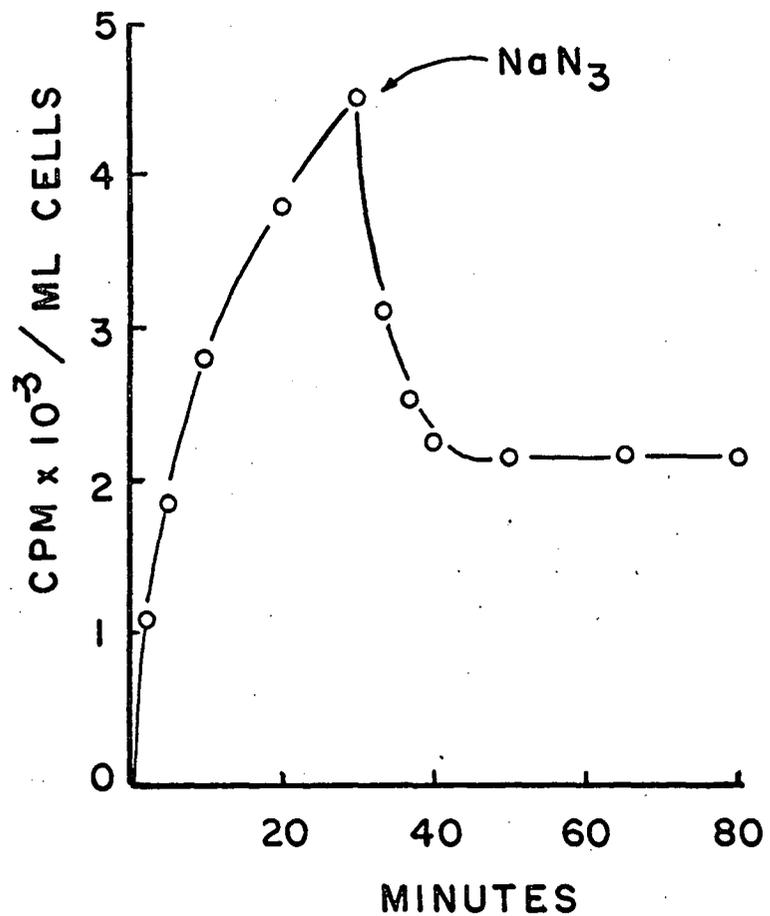
Fig. 28. Partial efflux of the intracellular proline pool of $\underline{P.\ aeruginosa}$ on the addition of 30 mM NaN_3. Cells were preloaded by incubating with $10^{-6}M$ ^{14}C-proline after an initial 30 min preincubation with 200 ug/ml of chloramphenicol. The experiment was conducted at 10 C.

However, even this "stable" proline pool was found to be effluxed at 0 C, although much more slowly than the majority of the pool. When the proline intracellular pool was allowed to accumulate at 10 C and then the cells quickly chilled to 0 C, rapid efflux of most of the pool occurred within ten minutes of chilling and this was followed by a slower efflux of a second component of this pool (Fig. 29).

Since one component of the proline pool was shown to be insensitive to NaN_3 and since the proline pool was effluxed at two distinct rates at 0 C, it was thought that perhaps a critical temperature may exist at which only one component of the pool would efflux. When the proline pool was allowed to accumulate at 10 C and then the cell suspension quickly reduced to 5 C, only one major component of the proline pool was lost by efflux (Fig. 30). The second pool remained stable for 90 min. However, when the temperature was brought back to 10 C the first pool quickly reformed. These data are interpreted as evidence for the existence of multiple proline pools in P. aeruginosa.

Chilling the cell suspensions to 0 C was not found to cause permanent damage to the pool forming mechanisms. When cells were cooled to 0 C, held for 30 min, and then rewarmed to 30 C for 15 min, they were found to incorporate ^{14}C-proline at the normal rate.
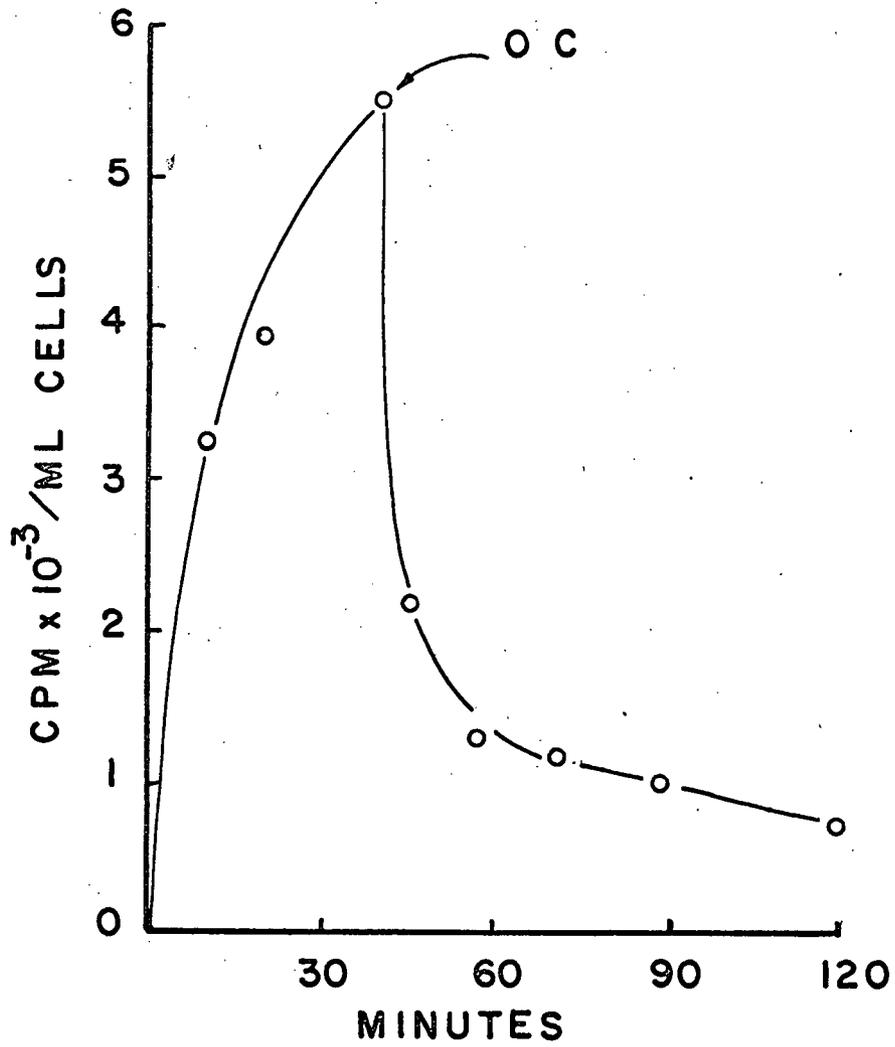
Fig. 29. Efflux of the proline pool of P. aeruginosa
at 0 C. Chloramphenicol treated cells were
preloaded with 1 x 10^{-6}M ^{14}C-proline at 10 C
then placed in an ice bath at 40 min.
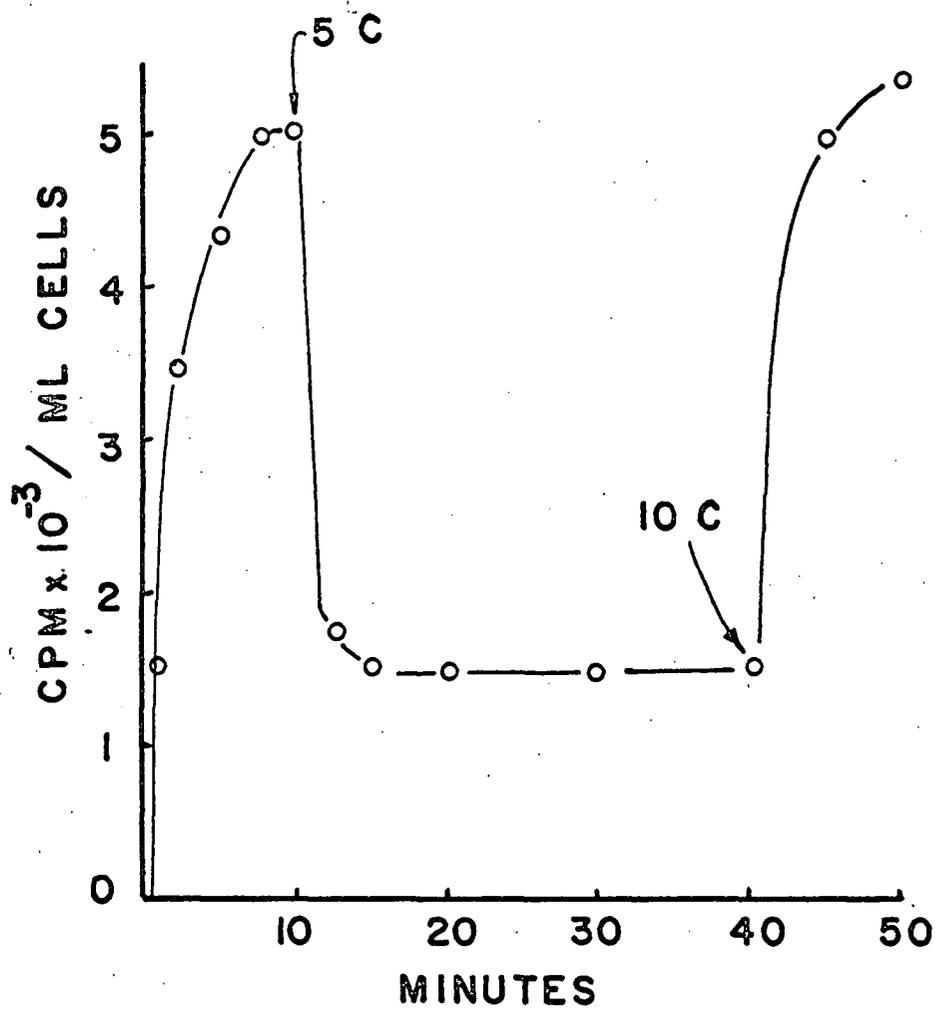
Fig. 30. Efflux of the proline pool of P. aeruginosa
at 5 C and its re-establishment at 10 C.
Chloramphenicol treated cells were preloaded
with 1 x 10^{-6}M ^{14}C-proline at 10 C.

III. Specificity of the Amino Acid Uptake System

1. Competition for amino acid uptake

Cohen and Rickenberg (1956) and Britten and McLure (1962),
observed that amino acid pool formation was a competitive process
for structurally related amino acids. Both groups demonstrated
that strong competitive interactions existed between the various
aliphatic amino acids for the amino acid concentrating process.
Britten and McLure (1962), have also shown that a specific concentrating
mechanism is operative for the accumulation of proline in E. coli.

From the comparative rates of transport of amino acids at low
substrate concentration (Table I), it was observed that structurally
similar amino acids, or those with similar properties, were transported
at approximately the same relative rate. Thus the aliphatic, aromatic,
and basic amino acids were transported more efficiently than the
acidic, neutral, or sulfur containing amino acids. These results
suggested that there may be "families" of transport systems operative
in P. aeruginosa at low external amino acid concentrations.

In order to determine the specificity of the amino acid
incorporating processes, competition experiments were carried out
in a manner that would reveal reductions in the rate at which
particular amino acids were transported into P. aeruginosa in the
presence of high concentrations of other amino acids. When
competitive experiments were performed it became obvious that

several interacting transport systems were present in the organism. A typical experiment is presented in Figure 31A, in which the initial rate of proline transport was rapid at 10^{-6} M ^{14}C-proline. The addition of nineteen naturally occurring amino acids, including hydroxyproline but excluding proline, each present at 10^{-4} M caused no inhibition of uptake of the amino acid. These results demonstrated that proline uptake in _P. aeruginosa_ is a specific process as has been shown previously in _E. coli_ (Britten and McLure, 1962). Kaback and Stadtman (1965), found that hydroxyproline was a competitive inhibitor of proline accumulation in _E. coli_ membranes. This was not the case with _P. aeruginosa_ whole cells since 10^{-4} M hydroxyproline exerted no competitive inhibition of proline transport. This result was expected as no uptake of hydroxyproline could be detected previously (Table I). Specific uptake mechanisms were also found for glutamic acid and aspartic acid, suggesting that these two processes were very specific indeed, in order to discriminate between such structurally similar amino acids.

Unlike the transport of proline, glutamic acid and aspartic acid, the transport of all other amino acids tested in this manner were competitively inhibited by structurally related amino acids. Figure 31B demonstrates the inhibition of the incorporation of ^{14}C-lysine by the basic amino acids. At concentrations of 10^{-4} M, only the basic amino acids inhibited lysine transport. Similarly, amino acids classed as aliphatic (leucine, isoleucine, valine, and alanine) competitively inhibited the uptake of ^{14}C-isoleucine (Fig. 32A), and amino acids classified here as aromatic (tryptophan,
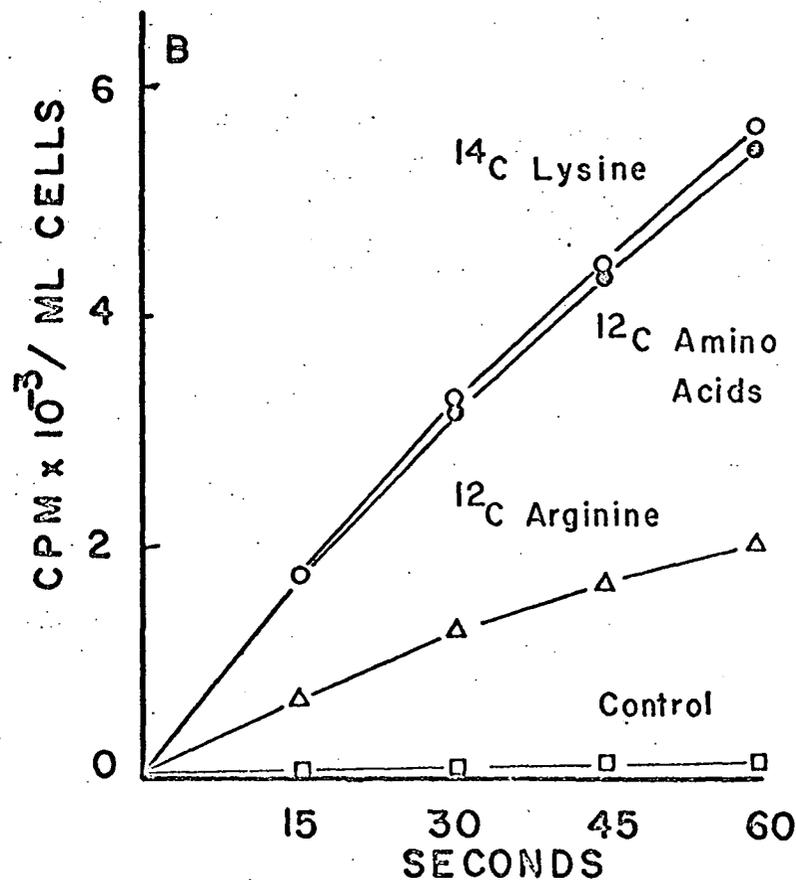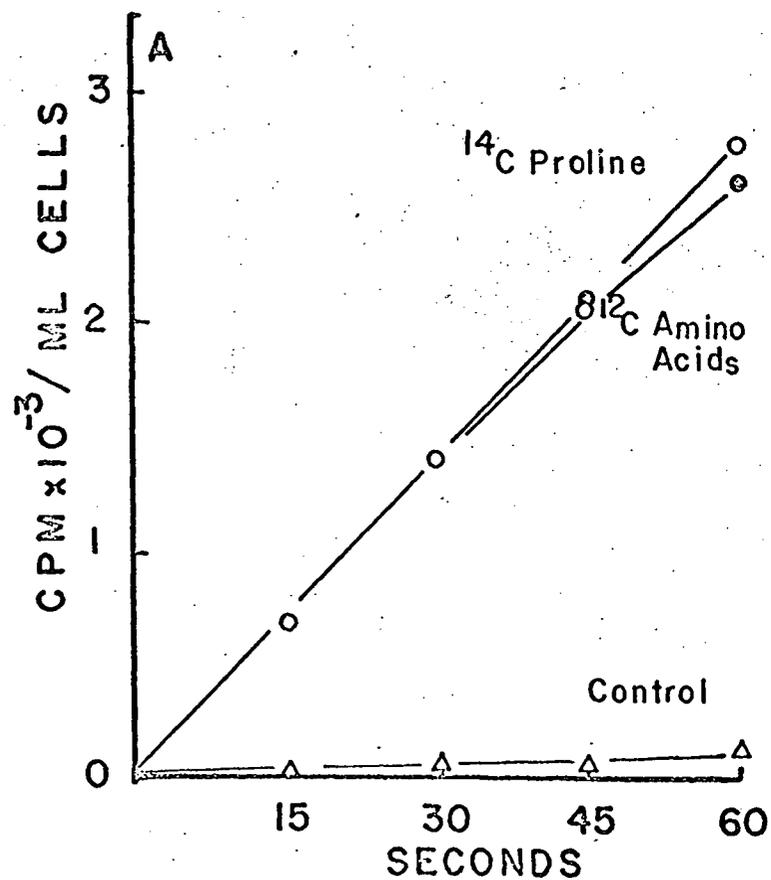
Fig. 31. Competition for amino acid uptake in P. aeruginosa. The rate of incorporation of 10^{-6}M ^{14}C-amino acid was followed in the presence or absence of 10^{-4}M ^{12}C-amino acids. × A. Competition for ^{14}C-proline uptake. ^{12}C-amino acids-10^{-4}M of all amino acids except proline. B. Competition for ^{14}C-lysine uptake. ^{12}C-amino acid-10^{-4}M of all amino acids except basic amino acids. Control - 10^{-4} M ^{12}C-lysine. ×Control - 10^{-4} M ^{12}C-proline.
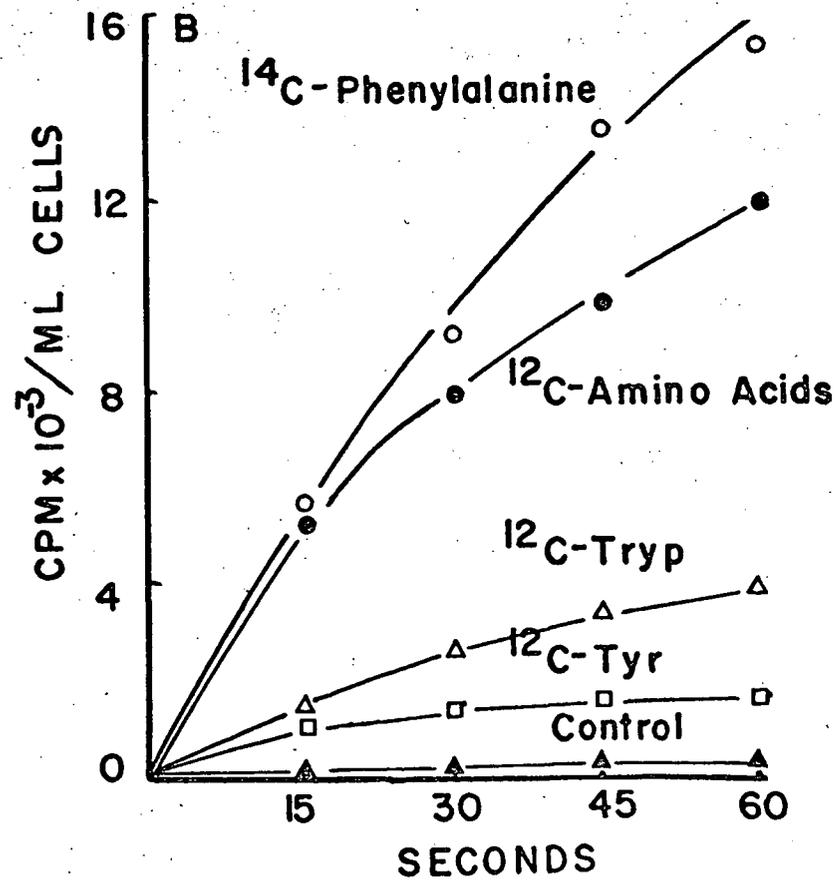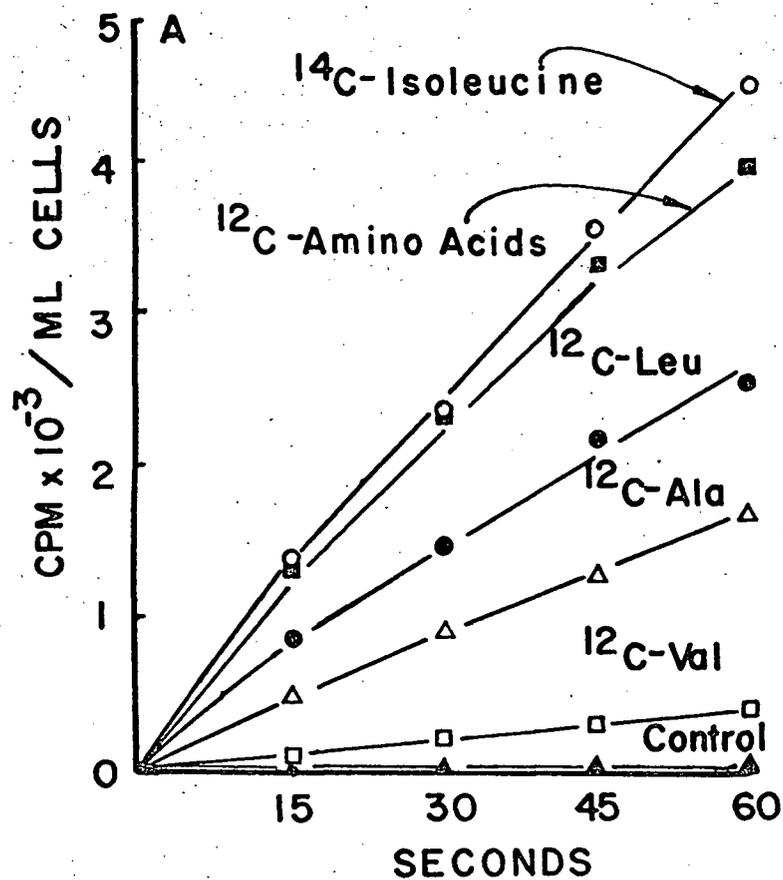
Fig. 32. Competition for aliphatic and aromatic amino acid uptake in P. aeruginosa. A. Isoleucine uptake. ■-■, ^{12}C-amino acids minus aliphatic amino acids. ▲-▲, control contained ^{14}C-isoleucine plus 10^{-4} M ^{12}C-isoleucine. B. Phenylalanine uptake. ●-●, ^{12}C-amino acids minus aromatic amino acids. ▲-▲, control contained ^{14}C-phenylalanine plus 10^{-4} M ^{12}C-phenylalanine.

tyrosine, and phenylalanine), competitively inhibited the uptake of ^{14}C-phenylalanine (Fig. 32B).

In addition to these apparent families of transport systems, other interactions were found. A low molecular weight neutral amino acid transport system was also elucidated. This system was found to recognize alanine, glycine, serine, and threonine. Another more specific uptake system was found to recognize only cystine and cysteine, but not methionine. The incorporation of methionine appears to present a special case, as this amino acid was found to enter on the permease functioning for the aliphatic amino acids, but very weakly, since it caused only minor inhibition of isoleucine uptake when present at 10^{-4} M. However, methionine is incorporated relatively efficiently by P. aeruginosa (Table I), and it was found that the aliphatic amino acids inhibited the uptake of this amino acid only to a moderate degree. It was concluded, therefore, that methionine is recognized by a specific methionine permease with high affinity for this amino acid and also by the aliphatic permease which has a low affinity for this amino acid. The reverse was also true, that is, the aliphatic amino acids entered the cell chiefly by a permease of high affinity, however, these amino acids are recognized also by the methionine permease but with low affinity. These competitions only become significant at external amino acid concentrations exceeding 10^{-3} M.

Complicating the specificity of the permease systems even further, was the observation that the aromatic amino acids weakly inhibited isoleucine uptake at 10^{-4} M but at concentrations

exceeding 10^{-3} M, the degree of competition was considerably more significant. Again, the reverse of this observation was shown to be true, but to a lesser degree. For example, the uptake of ^{14}C-tyrosine was found to be very weakly inhibited by other amino acids, including the aliphatic amino acids. This inhibition was found to be additive and only significant when aliphatic amino acids were each present at 10^{-4} M. Thus, it was demonstrated that amino acid specific and family specific permeases functioned at low amino acid concentrations but that the same degree of specificity was not maintained at high substrate concentrations. Britten and McLure (1962), concluded from a study of the interactions of the aliphatic amino acid accumulating system of E. coli that there were specific mechanisms for the formation and maintenance of small amino acid pools and less specific, or completely nonspecific, mechanisms for the formation of very large pools. However, it should be noted that the "very large pools" referred to with E. coli are not found in P. aeruginosa (Table III), and also that the competitions observed in this study are focused primarily on the transport systems elucidated by rapid sampling. In these experiments, the maximum pool capacity for any one amino acid tested was not reached.

2. Kinetics of competitive inhibition

Since Boezl and DeMoss (1961), and Halpern and Lupo (1965), have shown that certain metabolites can non-competitively inhibit the uptake of some amino acids into E. coli, it was necessary

to demonstrate that the interactions observed between groups of structurally similar amino acids were competitive. When rates of amino acid incorporation into whole cells were determined as a function of the external amino acid concentration, saturation kinetics were observed. For example, the kinetics of phenylalanine incorporation into growing cells of P. aeruginosa in the presence of structurally similar amino acids has been demonstrated (Fig. 7). Double-reciprocal plots of the ^{14}C-amino acid incorporation data showed that the observed inhibitions of amino acid uptake by similar amino acids, were competitive. The competitive inhibition of leucine uptake by valine at two different valine concentrations is shown in Figure 33. Figure 34 demonstrates the competitive inhibition of ^{14}C-lysine transport by ^{12}C-arginine and Figure 35 demonstrates the inhibition of ^{14}C-phenylalanine uptake by ^{12}C-tyrosine. Therefore, the observed interactions between structurally related amino acids for their respective transport systems was shown to be competitive.

3. Specific and general transport systems

FerroLuzzi-Ames (1964), and Grenson (1966), demonstrated that more than one permease may function in the transport of a particular amino acid in microorganisms.

When the relative degrees of competitive inhibition were examined in light of predetermined transport rates, various incongruities became apparent.
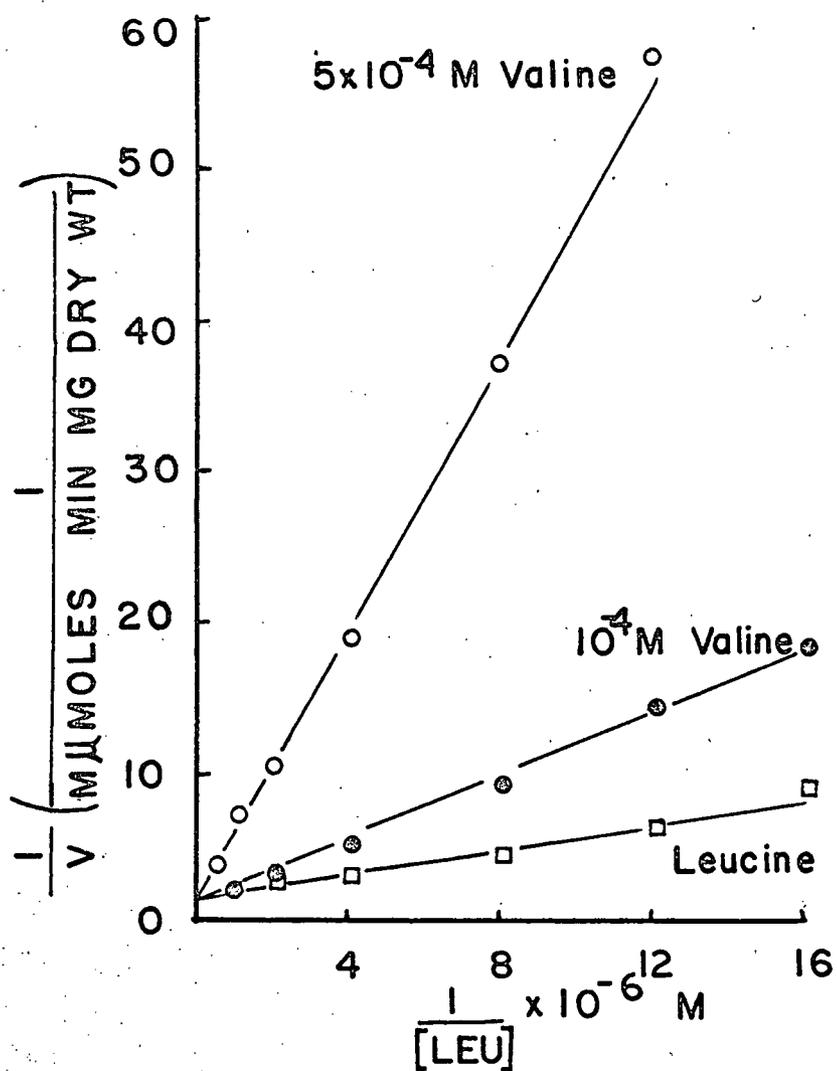
Fig. 33. Competitive inhibition of leucine uptake by ^{12}C-valine. Rates of ^{14}C-leucine incorporation were calculated from samples taken at 15 and 30 sec.
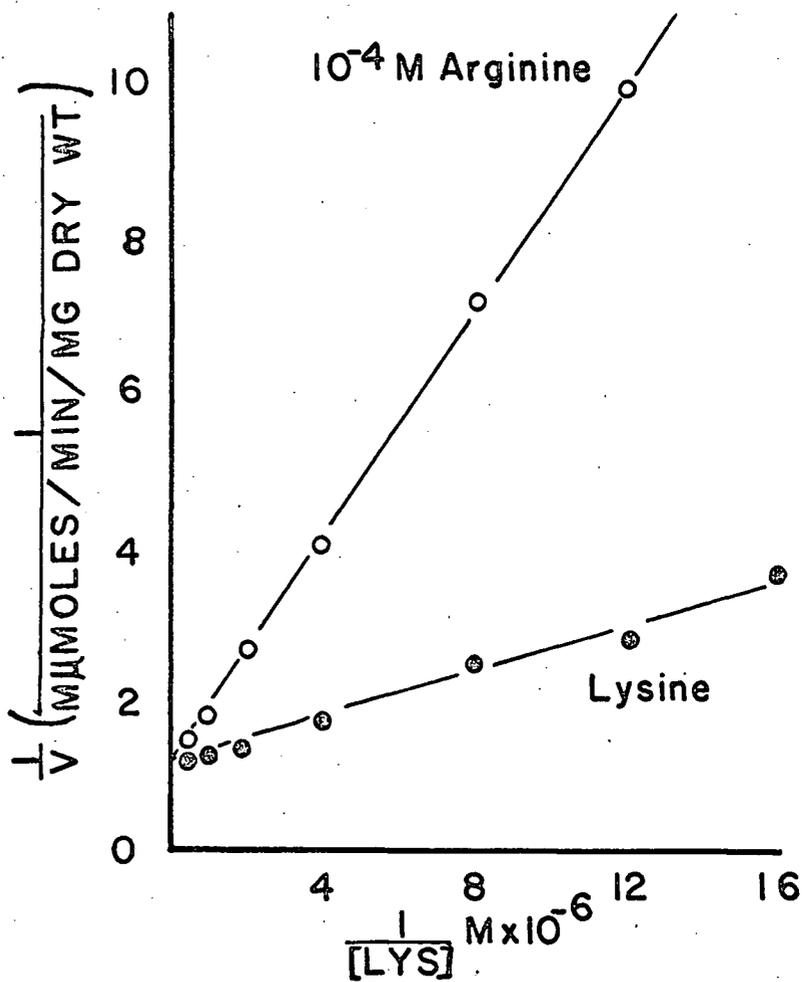
Fig. 34. Competitive inhibition of ^{14}C-lysine uptake by ^{12}C-arginine; ●-● ^{14}C-lysine uptake; O-O ^{14}C-lysine in the presence of 10^{-4} M ^{12}C-arginine.
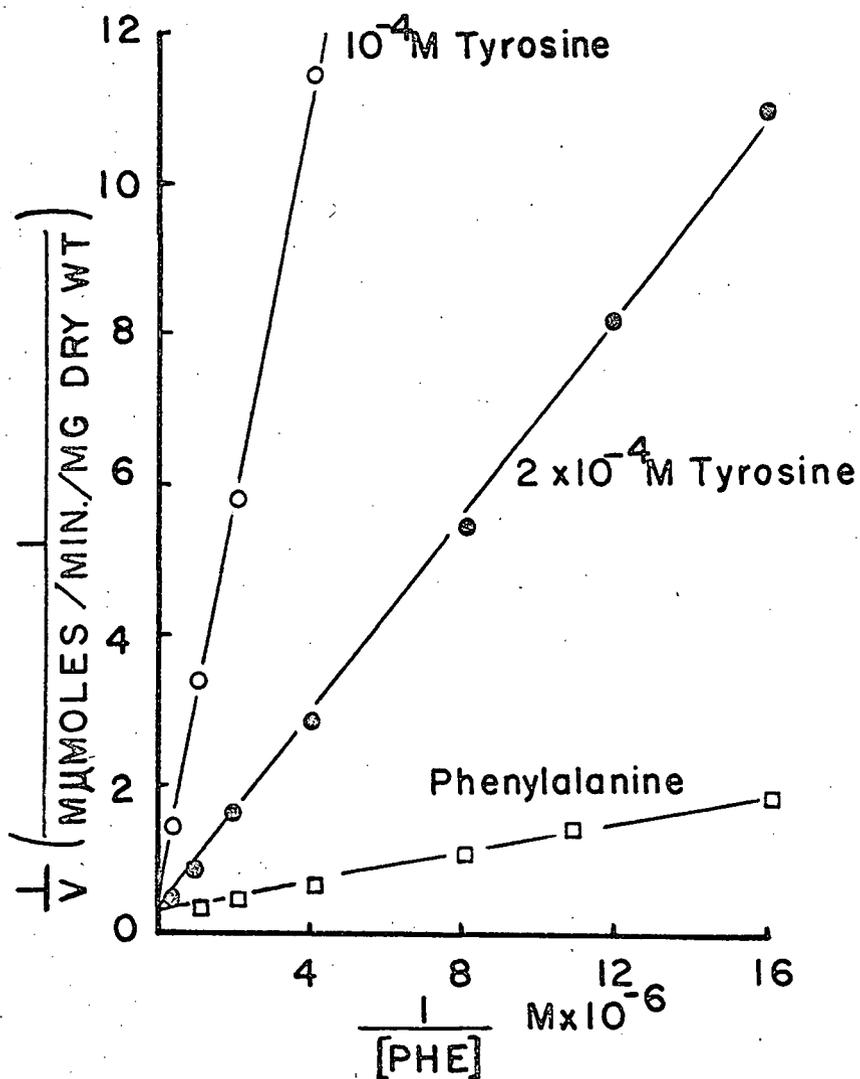
Fig. 35. Competitive inhibition of ^{14}C-phenylalanine uptake by ^{12}C-tyrosine. - , ^{14}C-phenylalanine uptake. 0-0, ^{14}C-phenylalanine uptake in the presence of 10^{-4} M ^{12}C-tyrosine. 0-0, ^{14}C-phenylalanine uptake in the presence of 2 x 10^{-4} M ^{12}C-tyrosine.

a) Basic amino acids

Arginine transport was the most efficient system found with the basic amino acids (Table I), and arginine was incorporated nearly twice as fast as lysine. However, arginine inhibited lysine incorporation only slightly. (Fig. 31, Table VII). These results are construed as being evidence for the multiplicity of high affinity permeases operative with the basic amino acids. Arginine does not compete as effectively as ^{12}C-lysine for the lysine specific permease, but since arginine does show some competition for the permease, then arginine must also be recognized, with a lower affinity, by the lysine transport system. The degree of competition of arginine for the lysine permease was, as expected, a linear function of the log of the arginine concentration (Fih. 36). By extrapolation, it was concluded that 4×10^4 mµmoles/ml of arginine would be necessary to competitively inhibit lysine incorporation completely. These results confirm the existence of a separate basic amino acid permease which recognizes arginine with less affinity than lysine.

When the basic amino acids were used to compete against one another for their respective transport functions, various degrees of competition were observed. From Table VII it can be seen that there exists two high affinity permeases for the basic amino acids. It can also be seen from the list of amino acids recognized by these permeases (Table XI) that for permease, bas 1,

Table VII. Competitive inhibition of basic amino acid uptake.

^{12}C-Competitor	Percent decrease in the incorporation rate of		
	^{14}C-Lysine	^{14}C-Arginine	^{14}C-Histidine
10^{-4} M		10^{-6} M	
Ornithine	33.4	31.6	83.2
Citrulline	19.1	0	73.5
Arginine	66.7	99.5	87.7
Histidine	14.1	0	73.7
Lysine	99.7	0	64.9
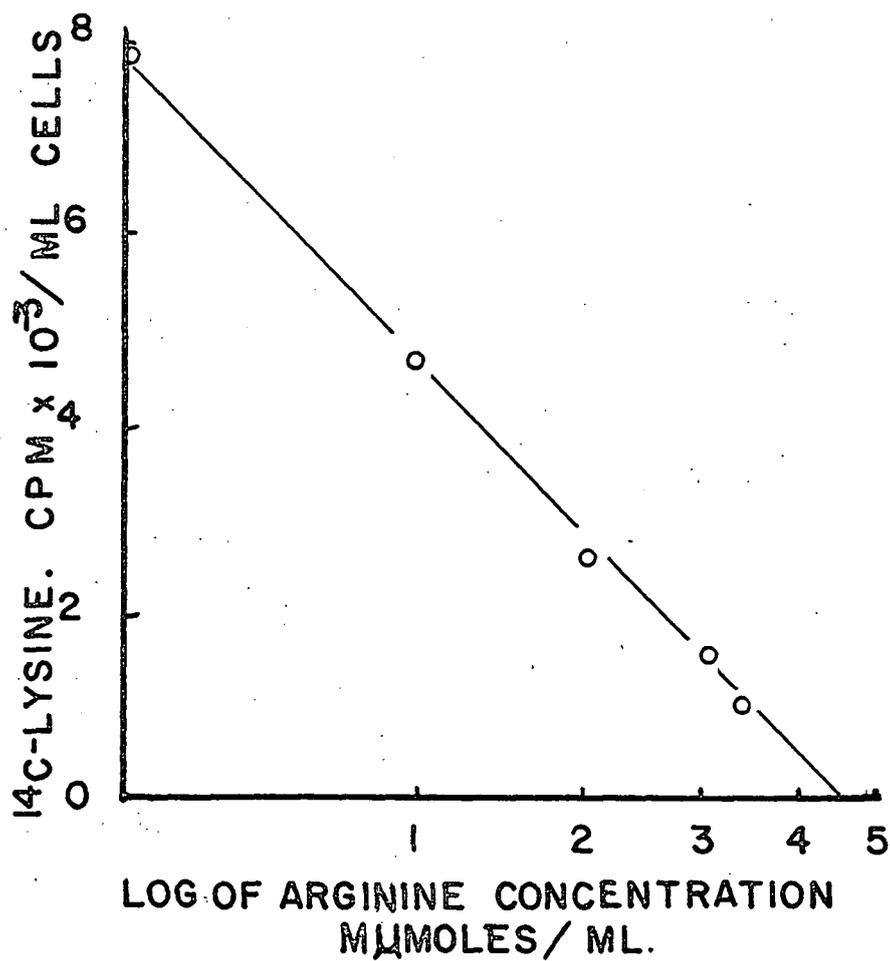
Fig. 36. Inhibition of ^{14}C-lysine uptake by ^{12}C-arginine. ^{14}C-lysine (10^{-6} M) and ^{12}C-arginine at various concentrations were added simultaneously to a growing cell suspension.

which transports arginine, histidine, lysine, ornithine, and

citrulline, the existence of a primary amine other than the

α-amino group would seem to be the factor most important for

recognition by this transport system. Permease, bas 2, appears

to be specific for arginine and ornithine. However, the nature

of the ornithine inhibition has not as yet been investigated and

it may, in fact, be non-competitive. It was concluded that histidine

was transported principally by both permeases since most of the

basic amino acids were more competitive for ^{14}C-histidine transport

than was ^{12}C-histidine itself. The transport of histidine in P.

aeruginosa markedly differs from that reported for S. typhimurium

(FerroLuzzi-Ames, 1964), where histidine transport was shown to

be mediated by a specific high affinity permease and a low affinity,

non-specific aromatic permease.

b) Aliphatic amino acids

Again, incongruity was found between the competition data for

amino acids and the rate of transport (Table I) with the aliphatic

amino acids. Of the aliphatic amino acids tested, leucine was

transported at the fastest rate but it was not as effective a

competitive inhibitor for isoleucine uptake as was valine or

isoleucine. When the various aliphatic amino acids were tested

against one another for relative degrees of competition for the

transport system, it was found that the results were not compatible

with the existence of multiple permeases within this family of

amino acids. The transport competition data (Table VIII), showed

Table VIII. Competitive inhibition of aliphatic amino acid
uptake.

^{12}C- Competitor	Percent decrease in the incorporation rate of			
	^{14}C-Alanine	^{14}C-Isoleucine	^{14}C-Valine	^{14}C-Leucine
	10^{-4} M		10^{-6} M	
Alanine	99.1	59.1	62.1	47.6
Isoleucine	73.6	95.5	99.0	95.2
Valine	79.2	88.2	99.2	88.0
Leucine	41.6	56.9	60.2	23.8
Methionine	-a)	19.4	-	-
Methionine 10^{-3} M	-	40.0	-	-

a) Not tested.

Table IX. Competitive inhibition of aromatic amino acid uptake.

^{12}C-Competitor	Percent decrease in the incorporation rate of		
	^{14}C-Phenylalanine	^{14}C-Tyrosine	^{14}C-Tryptophan
10^{-4} M		10^{-6} M	
Phenylalanine	94.2	98.2	53.9
Tyrosine	88.4	97.0	52.0
Tryptophan	73.8	94.4	88.2

that a single aliphatic permease existed which strongly

recognized isoleucine and valine and, to a lesser degree,

alanine and leucine. The order of the affinity of the aliphatic

amino acids for the transport system is shown in Table IX.

This interpretation, at first, would seem incongruous with

the rapid transport rate for leucine at 10^{-6} M (Table I);

however, these transport rates were determined at a single amino

acid concentration and are of value as approximate comparisons

between amino acids. As such, in no accurate way do these

incorporation rates describe the relative kinetic parameters

for the uptake process. Competition data reflect relative orders

of amino acid affinity for the permease but do not accurately

reflect relative transport rates since the turnover numbers for

amino acids being transported into the cell by a common uptake

system may differ by a large factor whereas the apparent affinity

constants may differ only slightly.

Alanine was also shown to enter by a permease recognizing

the neutral amino acids. From competition studies (Table X), it

was found that this transport mechanism recognized alanine most

efficiently and to a lesser degree glycine, serine, and threonine.

c) Aromatic amino acids

FerroLuzzi-Ames (1964), demonstrated that Salmonella

typhimurium possessed specific high affinity permeases for

histidine, phenylalanine, tyrosine, and tryptophan as well as

a general permease for aromatic amino acids. The results obtained

Table X. Competitive inhibition of neutral amino acid
uptake.

^{12}C-Competitor	Percent decrease in the incorporation rate of		
	^{14}C-Alanine	^{14}C-Glycine	^{14}C-Serine
10^{-4} M		10^{-6} M	
Alanine	92.9	76.1	82.1
Glycine	73.9	77.6	75.1
Serine	50.2	17.7	74.2
Threonine	40.0	10.2	53.4

with P. aeruginosa differ largely with respect to the specificity

of the permeases for aromatic amino acids. Of the aromatic amino

acids, tryptophan exhibited the greatest transport rate in this

organism (Table I); however, this amino acid did not compete

for uptake of ^{14}C-phenylalanine or ^{14}C-tyrosine to the same

degree as ^{12}C-phenylalanine or ^{12}C-tyrosine. Again another permease,

less specific for tryptophan, must be operative which mediates

aromatic amino acid transport in P. aeruginosa. In accordance

with the other permeases described so far, this permease will be

referred to as aro 2 (Table XI). Tryptophan does compete for

the second aromatic permease, although not as efficiently as

tyrosine or phenylalanine. From the competition data of Table

XI, it can be seen that the simplest description of the activity

of permease aro 2, is that it recognizes phenylalanine and tyrosine

with high affinity and tryptophan with a lower affinity. However,

aro 1 was found to be more specific for tryptophan than for

phenylalanine or tyrosine; although these latter two amino acids

competitively inhibited its activity, they did not do so to the

same degree as ^{12}C-tryptophan.

3. The isolation and properties of transportless mutants

The isolation of transport negative (Tr$^-$) mutants for amino

acids was attempted by several methods. First, attempts to isolate

a proline Tr$^-$ mutant by the method of Lubin and Kessel (1962),

proved fruitless since P. aeruginosa was found to be strongly

Table XI. Amino acid permeases of P. aeruginosa.

FAMILY	PERMEASE	AMINO ACIDS[a]
Specific	sp 1	aspartate
	sp 2	glutamate
	sp 3	methionine
	sp 4	proline
Neutral	nt 1	alanine
		glycine
		serine
		threonine
Sulfur	sul 1	cysteine
		cystine
Aliphatic	al 1	isoleucine
		valine
		alanine
		leucine
Aromatic	aro 1	phenylalanine
		tyrosine
		tryptophan
	aro 2	tryptophan
		phenylalanine
		tyrosine
Basic	bas 1	arginine
		ornithine
		histidine
	bas 2	lysine
		ornithine
		arginine
		citrulline
		histidine

[a] Amino acids are listed in decreasing orders of affinity for their respective permeases.

resistant to penicillin. Adaptation of the method by growing
mutagenized cultures of a proline auxotroph of P. aeruginosa
in low concentrations (1 μg/ml) of the required amino acid
with subsequent enrichment by growth recycling in the presence
of 400 μg/ml dihydrostreptomycin, only resulted in the isolation
of strains resistant to the effects of the antibiotic.

Since some resistant strains of microorganisms have been
shown to be transportless for the natural amino acid and the
analogue, attempts were also made to isolate mutants resistant
to the following amino acid analogues: thiazolidine-2-carboxylic
acid (thioproline), 3,4-dehydroproline, canavanine, ethionine,
thiosine, p-fluorophenylalanine, and 5-methyl tryptophan. However,
growth experiments indicated that P. aeruginosa, unlike most
microorganisms, was resistant to low concentrations of these
analogues. Consequently, attempts were made to isolate strains
resistant to very high concentrations of the analogues. Thirty
resistant colonies isolated with each analogue were first tested
for derepression of amino acid synthesis by cross-feeding experiments
with the appropriate auxotroph. None of the mutants tested were
derepressed for the synthesis of the amino acid and, therefore,
were not excretor mutants. However, amino acid uptake experiments
with these mutants did not reveal any significant differences
relative to the wild-type strain. No attempts were made to
isolate strains resistant to 5-methyl-tryptophan because this
organism was found to utilize the amino acid for growth even at
very high concentrations. The resistance to most of these amino
acid analogues was thought to be due to the presence of
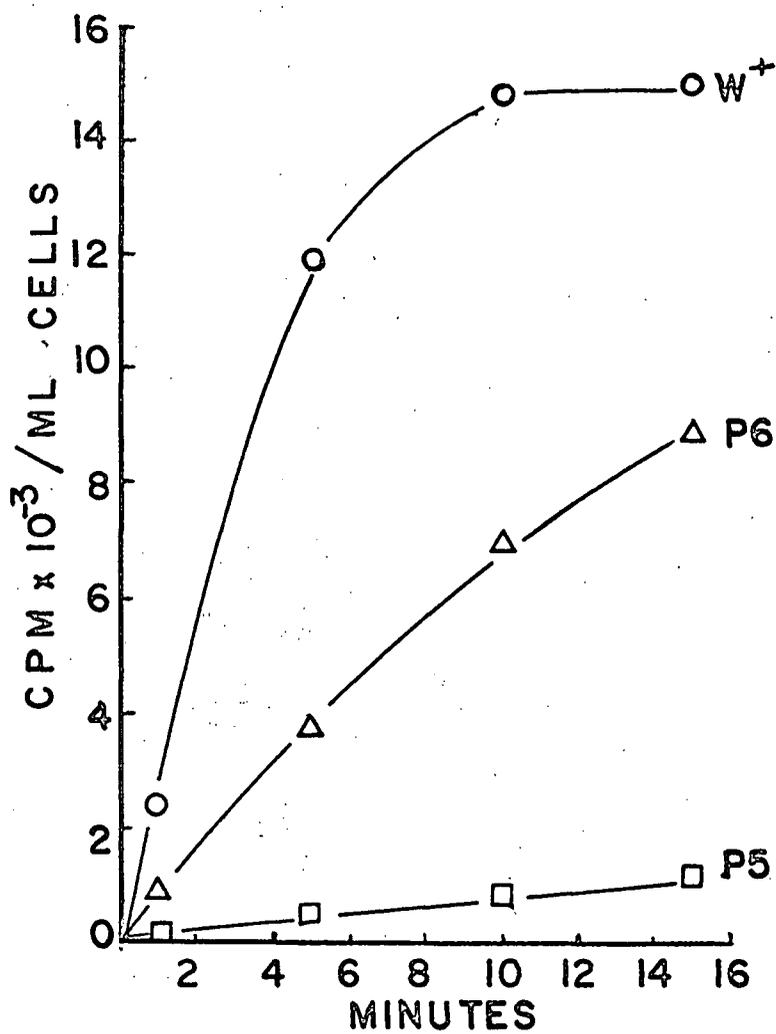
Fig. 37.   Uptake of ^{14}C-proline at 30 C by W$^+$ and
proline Tr$^-$ mutant strains (P5, P6) of
P. aeruginosa.

constitutive oxidizing enzymes active on the analogues as well as the amino acids. Cell suspensions incubated with thioproline grew at a normal rate and catabolized this analogue.

The third method tested was designed to take advantage of the cells ability to catabolize amino acids. It was reasoned that cells defective for the transport of a single amino acid would grow either slowly or not at all when plated on solid media containing that amino acid as the sole carbon source. When colonies isolated in this manner were routinely screened for the incorporation of ^{14}C-amino acid added at low concentrations, it was found that the mutants fell into two general classes. Figure 37 represents the uptake of ^{14}C-proline at 10^{-6} M by the wild type and two strains isolated by this method. One mutant, P5, in which the transport ability for proline was altered approximately 10 fold, was isolated. Most mutants isolated by this method, however, exhibited transport rates approximately the same as mutant P6 (Fig. 37). Mutant P5 incorporated all other amino acids rapidly from a ^{14}C-protein hydrolysate and only failed to incorporate proline at a significant rate. Therefore, this mutation was considered specific for the proline transport system and, as such, agreed with amino acid competition data (Fig. 31B).

Table XII lists the mutants isolated by this method for the various groups of amino acids. Again, most transport defective mutants obtained in this manner were found to be only partially defective when tested at low substrate concentration. Only one transport defective mutant (A5) was isolated for the basic amino

Table XII. Transport negative (Tr⁻) mutants of P.
aeruginosa.

MUTANT	DEFECTIVE PERMEASE
P5	sp 4 (proline)
P6	sp 4
P9	sp 4
P11	sp 4
A5	bas 1
TA3	aro 2
TA10	aro 1
IB9	al 1

acids. When tested for the ability to incorporate arginine,
lysine, and histidine the mutant was found to be more defective
for lysine transport than for arginine transport. Only
partial reduction of uptake with these amino acids was expected,
however, due to the presence of multiple permeases. This mutant
could, therefore, be classified as a lysine transportless mutant
with a defect for the amino acid permease bas 1. Unexpectedly, A5
transported histidine more effectively than did the wild-type
strain. The reason for this has not been determined, however,
Grenson (1966), reported identical results with a canavanine
insensitive, transport negative mutant of $\underline{S}$. $\underline{cerevisiae}$ which was
isolated by resistance to the arginine analogue canavanine. It
was also shown by the competitive inhibition of arginine transport
that, in this yeast, histidine was incorporated on the basic
amino acid permease.

Of the two aromatic transport mutants examined, TA3 behaved
as a complete transport negative mutant analogous to P5, the proline
Tr⁻ mutant. This mutant was most strongly defective for the tryptophan
permease suggesting that the aromatic permease aro 2 was defective
but that the test amino acid was slowly incorporated via permease
aro 1. This interpretation was corroborated by the isolation of
Tr⁻ mutant TC10 which transported tryptophan at nearly the same
rate as the wild type strain and was only moderately defective for
phenylalanine or tyrosine transport. The transport defect could
be most simply described as a mutation at the level of permease
aro 1.

Two mutants for the aliphatic uptake systems were also

isolated. The first, however, was found to be equally defective for all aliphatic amino acids as well as unrelated amino acids. This mutant, IB16, may possess a general membrane defect affecting transport function (Stadtler, 1967), but it was not analyzed in detail. The second mutant isolated, IB9, was found to be defective only for transport of the aliphatic amino acids.

When multiple high affinity permeases function to transport a particular amino acid, then it is obvious that the transport rate observed with growing cultures would represent the cumulative rates of transport between at least two distinct transport systems.

IV. Competition for the Amino Acid Pool

Britten and McLure (1962), and Rickenberg and Cohen (1956), showed that the structurally similar aliphatic amino acids could competitively displace one another from pre-established amino acid pools in E. coli. This exchange process was carried out at 0 C where incorporation of the competitive amino acids was negligible and where preformed pools were stable. Without exception, the amino acid pools of P. aeruginosa rapidly effluxed at 0 C; however, the rate of transport of most amino acids with the exception of proline, was found to be markedly reduced at 10 C (Fig. 6), and exchange reactions were, therefore, performed at this temperature. Figure 38 demonstrates the exchange of a preloaded ^{14}C-valine pool by ^{12}C-valine, ^{12}C-isoleucine, ^{12}C-leucine, ^{12}C-alanine, and ^{12}C-methionine, each added at 10^{-4} M to the stabilized, preloaded culture. When rates of exchange were
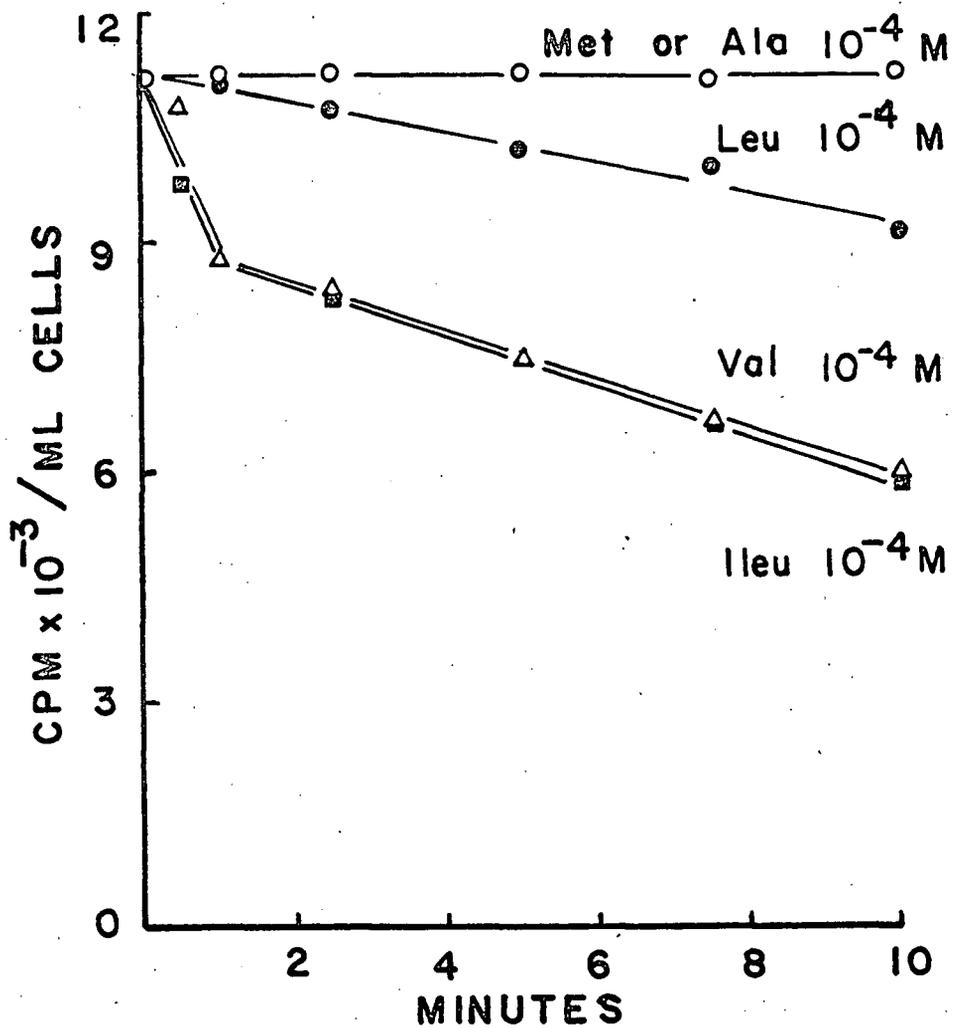
Fig. 38. The displacement of the ^{14}C-valine pool by structurally related amino acids. ^{12}C-amino acids were added at 10^{-4}M. The valine pools were pre-established in the presence of chloramphenicol by extended incubations at 10 C.

Table XIII. Exchange of the aliphatic amino acid pools.

| Pool amino acid | % of Pool exchange/min with | | | |
	Leucine	Isoleucine	Valine	Alanine
			10^{-4} M	
Leucine	0	10.7	4.3	0
Isoleucine	3.1	28.6	20.1	0
Valine	2.2	21.7	20.8	0

Table XIV.   Exchange of the preformed basic amino acid pools.

Pool amino acid	% of Pool exchanged/min with		
	Lysine	Arginine	Histidine
	10^{-4} M		
Lysine	28.2	31.8	21.0
Arginine*	-	-	-
Histidine	14.3	23.3	17.2

*Arginine pools were not formed due to the secondary effects of putrescine accumulation.

calculated for pools preloaded and exchanged with the different

aliphatic amino acids (Table XIII), it was obvious that a very

close correlation existed between the amino acid uptake and

competition data (Table VIII). Both valine and isoleucine

exchanged readily with pools preloaded with these amino acids,

and isoleucine exchanged with the ^{14}C-leucine pool more readily

than valine. Neither alanine, methionine, nor lysine exchanged

with pre-established aliphatic amino acid pools. Clearly from

these observations it can be seen that the exchange process for

the aliphatic amino acids was stereospecific for members of this

family of amino acids, and followed the same competitive relation-

ships as the uptake process. Leucine was somewhat of an exception

as, although it was incorporated at a high rate in growing cultures

(Table I) and was found to exchange isoleucine and valine pools

rather poorly, it would not exchange with its own preformed pool.

Similar exchange data were obtained for the basic amino acids

(Table XIV). The histidine pool was less efficiently exchanged by

histidine than by arginine. The lysine pool was most readily

exchanged by arginine and lysine and to a lesser degree by histidine.

The displacement of amino acid pools with the basic amino acids was

shown to be family specific and closely correlated with the relative

affinities for the uptake system.

Aromatic amino acid pools were also displaced only by members

of this family of amino acids. All three aromatic amino acids

effectively displaced pre-established phenylalanine or tyrosine

pools, but only tryptophan displaced its own pool (Table XV). It

appeared, generally, that the displacement of an amino acid from

Table XV. Exchange of the aromatic amino acids.

Pool amino acid	% of Pool exchanged/10 min with		
	Phenylalanine	Tyrosine	Tryptophan
	10^{-4} M		
Phenylalanine	84.2	51.3	31.1
Tyrosine	78.3	77.9	26.7
Tryptophan	4.6	5.2	72.7

the pool of P. aeruginosa occurred by a process closely related to

the uptake systems and seemed to be strongly influenced by both

the structural relatedness of the exogenous amino acid, the

pre-established amino acid in the pool, and the affinity constants

of the transport system for the amino acids involved. Therefore,

the displacement of amino acids from the pool is a function of the

uptake process and also of a family specific exit process which may

differ from the uptake process by its relative affinities for the

amino acids which constitute a family. Thus, whereas phenylalanine

or tyrosine pools are displaced effectively by tryptophan, the

tryptophan pool is displaced very slowly, if at all, by these

amino acids.

V. Kinetics of Amino Acid Transport at High Substrate Concentrations

From a calculation of the maximum amount of amino acid capable

of entering P. aeruginosa at concentrations saturating the high

affinity permeases (i.e. at V_{max}), it was deduced that the amino

acid would not approximate the necessary carbon requirements for

normal growth. The wild-type strain catabolized nearly all the

commonly occurring amino acids (Table XVI), and it was a natural

assumption that to serve as growth substrates the amino acids would

have to enter the cell perhaps by mechanisms other than those

revealed at low amino acid concentrations. The kinetics of amino

acid uptake determined at high substrate concentrations revealed

the presence of a second permease with a greatly reduced affinity

for the amino acid. Figure 39 illustrates the kinetics of both
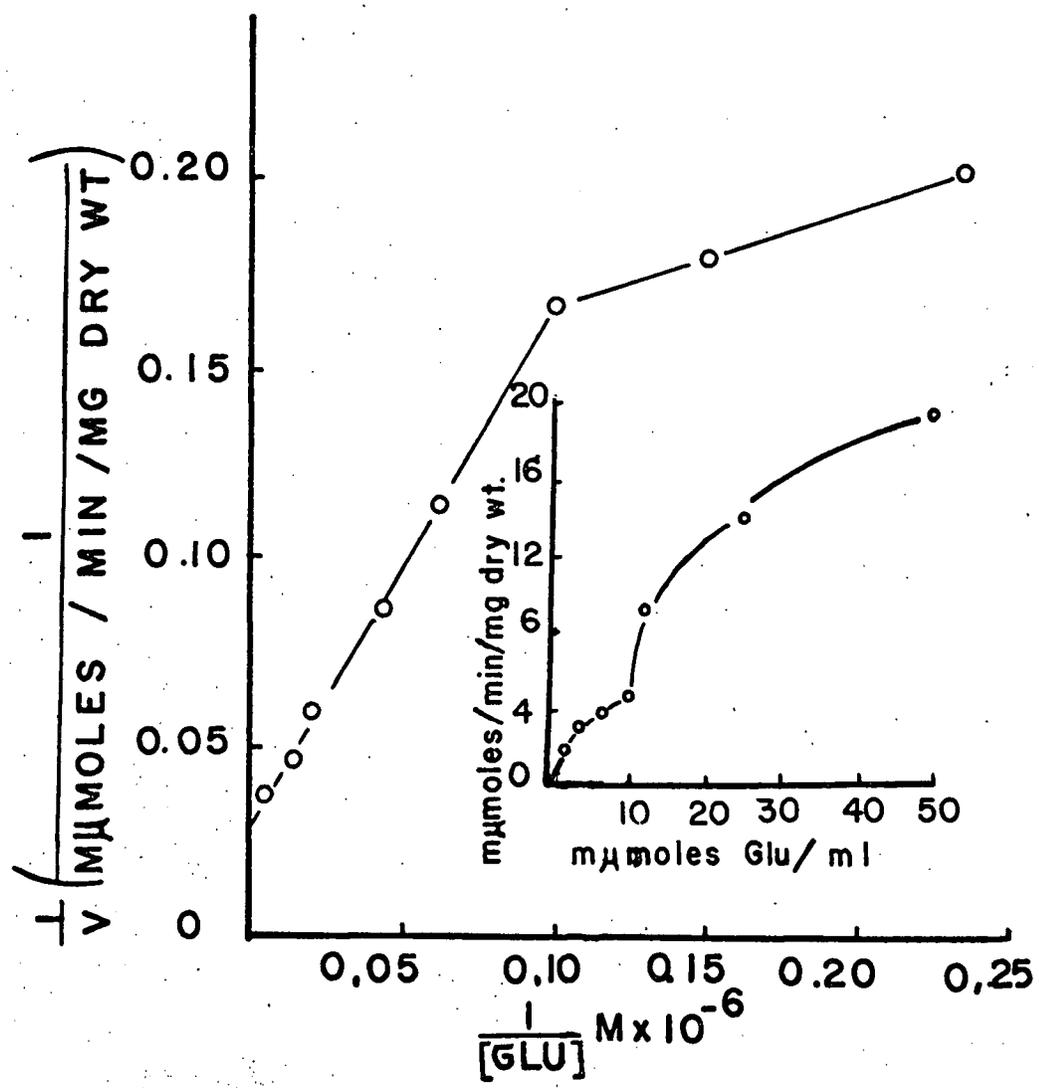
Fig. 39. Kinetics of glutamate uptake at 30 C with varying concentrations of ^{14}C-glutamate. Lineweaver-Burk plot. Inset: saturation kinetics.

the high and low affinity permeases for the transport of glutamic

acid. At concentrations exceeding 10^{-5} M, the second permeability

mechanism becomes the more important means of amino acid entry

into the cell. From the kinetics of amino acid uptake at high

amino acid concentrations (exceeding 2×10^{-5} M), it was found

that low affinity permeases or transport mechanisms were operative

for proline, arginine, and leucine incorporation, but none was

evident for phenylalanine incorporation. It was, then, reasonable

to assume that these probably are the permeability mechanisms which

supplied the cell with high amino acid concentrations for catabolism.

FerroLuzzi-Ames (1964), also demonstrated a non-specific aromatic

permease in S. typhimurium which was operative at high amino acid

concentrations. However, S. typhimurium does not catabolize the

aromatic amino acids and the actual function of this permease

remains a mystery. The nature of the stereospecificity of the low

affinity permeases has not been investigated.

It is postulated that permeability defects such as those

illustrated by mutant P6, a proline Tr$^-$ strain, are in fact mutations

at the level of the low affinity permease; however, at present no

data are available to substantiate this.

VI. Control of Amino Acid Transport

Very few clear demonstrations of the induction of permeability

functions in bacteria have been reported. Lyon et al. (1967), found

that glutamate transport was induced to high levels in

Mycobacterium smegmatis and M. tuberculosis when grown in the presence of glutamic acid, but the nature and properties of the induced and uninduced glutamate transport systems were not thoroughly investigated. De Hauwer, Lavalle, and Wiame (1964), observed that the arginine incorporation process of Bacillus subtilis was induced by growth on arginine and also that a mutant derepressed for the catabolism of arginine was coordinately derepressed for the arginine transport function. This suggested that the control of permeability was linked to catabolism, not anabolism. FerroLuzzi-Ames (1964), found that the histidine transport system was neither induced nor repressed by the presence of histidine, and concluded that the gene for the uptake system was not within the histidine operon.

High rates of proline transport were found to be inducible in P. aeruginosa. Growth in minimal medium containing 0.1% proline increased the rate of the transport of proline when tested at low substrate concentrations. The concomitant addition of high concentrations of chloramphenicol and proline to a logarithmic phase culture prevented the induction of proline transport above the constitutive level. Figure 40 demonstrates the incorporation rate of ^{14}C-proline at 10^{-6} M into the pool of induced and noninduced P. aeruginosa. The control cells were also preincubated with chloramphenicol prior to the initiation of the transport experiment in order to normalize any secondary effects of the inhibitor on the transport process. The kinetics of proline incorporation in induced and noninduced cells are shown in Figure 41. The affinity constant for both induced and noninduced cells was of the order
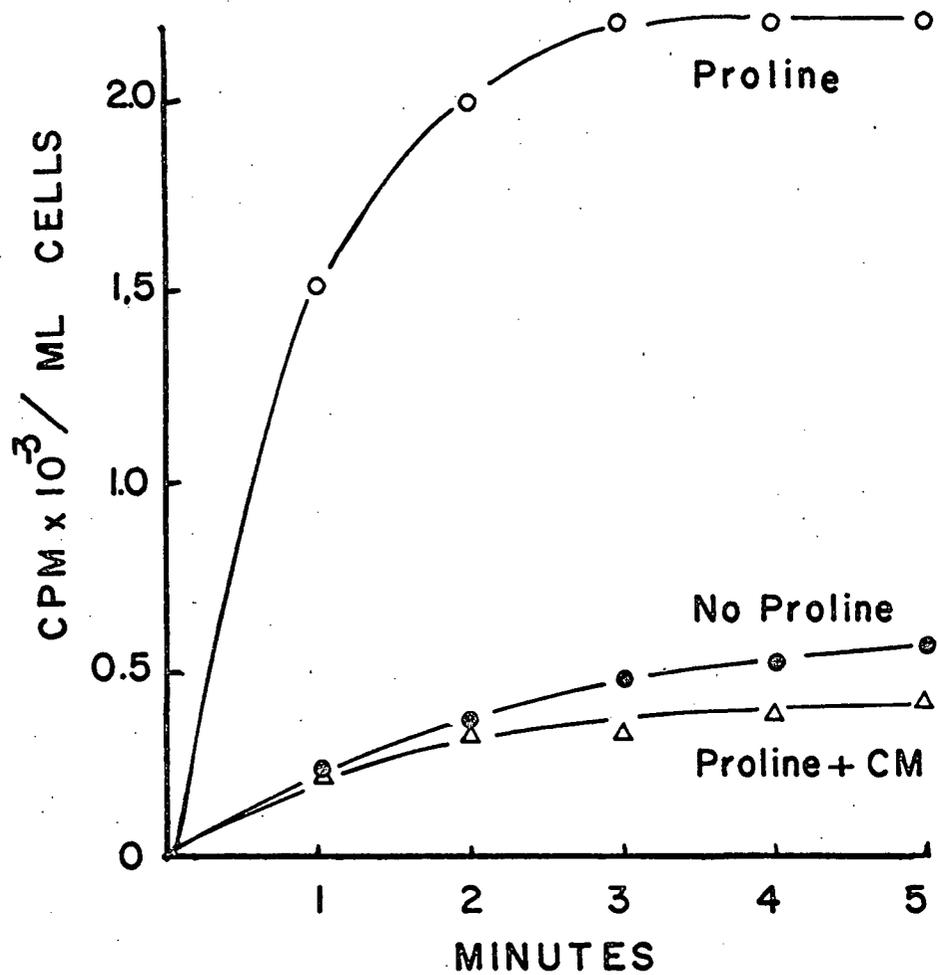
Fig. 40. Time course of pool formation at 30 C
with P. aeruginosa previously grown in
minimal medium in the presence or
absence of 0.1% proline.
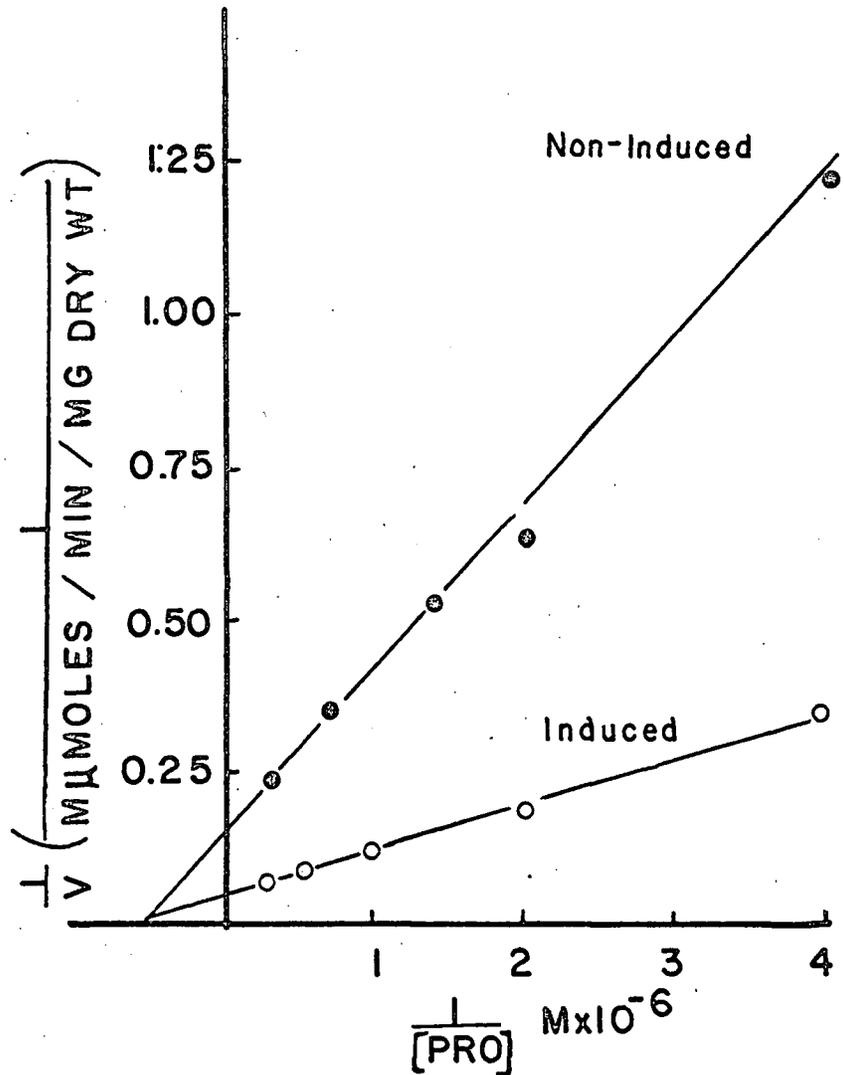
Fig. 41. Kinetics of proline uptake in P. aeruginosa. ●-●, Cells were grown in minimal medium. O-O, Cells were grown in minimal medium plus 0.1% proline.

of 2×10^{-7} M.

Although the control of the incorporation mechanisms for other amino acids has not been investigated, some evidence gained from the study of transport mutants suggested that the control of amino acid transport in P. aeruginosa is quite general. Four mutants of P. aeruginosa unable to catabolize arginine as a carbon source were able to transport arginine at increased rates relative to the wild-type strain. Although the nature of these mutations has not been thoroughly investigated, it is presumed that a lesion in the arginine degradative pathway resulted in the accumulation of an inducer for the transport system.

Similarly, two mutants (TC2 and TC12) were isolated which catabolized tyrosine poorly. These mutants also were able to transport tyrosine at a rate at least twice as fast as the wild-type strain. No investigation of the stereospecificity of these transport alterations has yet been attempted.

VII. Amino Acid Transport and Pool Formation in Starved Cell Suspensions

1. Amino acid transport

When E. coli cells were starved for glucose or maintained at 0 C, no significant pool losses were observed; in fact, highly concentrated amino acid pools were found to be stable for many hours under these conditions (Britten and McLure, 1962). In direct contrast to these observations, the amino acid pools of P. aeruginosa were found to be rapidly depleted during carbon or nitrogen starvation. When cells were prelabelled by growth on high specific

Table XVI. Growth of P. aeruginosa on amino acids as carbon
or nitrogen sources.

Amino Acid	Nitrogen Source	Carbon Source
Alanine	+	+
Arginine	+	+
Asparagine	+	+
Aspartate	+	+
Cysteine	±	-
Glutamate	+	+
Glutamine	+	+
Glycine	+	±
Histidine	±	+
Isoleucine	+	+
Leucine	+	+
Lysine	±	±
Methionine	-	-
Phenylalanine	+	±
Proline	+	+
Serine	+	-
Threonine	±	-
Tryptophan	+	+
Tyrosine	+	+
Valine	+	+

    —   no growth

    ±   slow growth after 48 hr

    +   growth at 24 hr

activity ^{14}C-glucose, and then allowed to exhaust the exogenous

glucose, the native amino acid pool was rapidly depleted.

Radioautography of the extracted pool and culture supernatant

fluid revealed that all amino acids, with the exception of

methionine, had been removed from both the culture supernatant

fluid and the intracellular pool.

Unlike most microorganisms, members of the genus Pseudomonas

have very simple nutritional requirements and catabolize a wide

range of substrates (Stanier et al., 1966). P. aeruginosa can

utilize any of the common amino acids as a sole nitrogen

source and most of them as a sole carbon source (Table XVI).

It was obvious then, that in the absence of glucose or other

non-nitrogenous energy sources, P. aeruginosa undoubtedly

catabolizes the residual amino acids present exogenously and

in its pool.

These preliminary results suggested that the catabolism and

perhaps even the transport of amino acids might be influenced by

the presence of an oxidizable energy source such as glucose.

Transport rates for amino acids representative of various

amino acid families were determined at frequent time intervals

during periods of either carbon or nitrogen starvation. With

most of the amino acids tested, no drastic impairment of transport

ability was evident over the starvation period (Table XVII). The

rates of glycine, alanine, and glutamate transport increased to

levels twice as high as those found with non-starved suspensions

(Fig. 42). The opposite effect was observed with members of the

aliphatic amino acids for the rate of transport of these amino

Table XVII.   Effect of carbon deprivation on amino acid transport.

Amino acid	Rate of amino acid transport percent of control		
	Control Non-starved	Starved 90 min	Re-fed[*]
Alanine	100	172	92
Glutamate	100	222	110
Glycine	100	120	100
Leucine	100	42	101
Valine	100	64	104
Phenylalanine	100	106	100
Proline	100	95	102
Lysine	100	98	95

[*]Glucose was added to the culture 60 sec prior to the determination of transport rate.
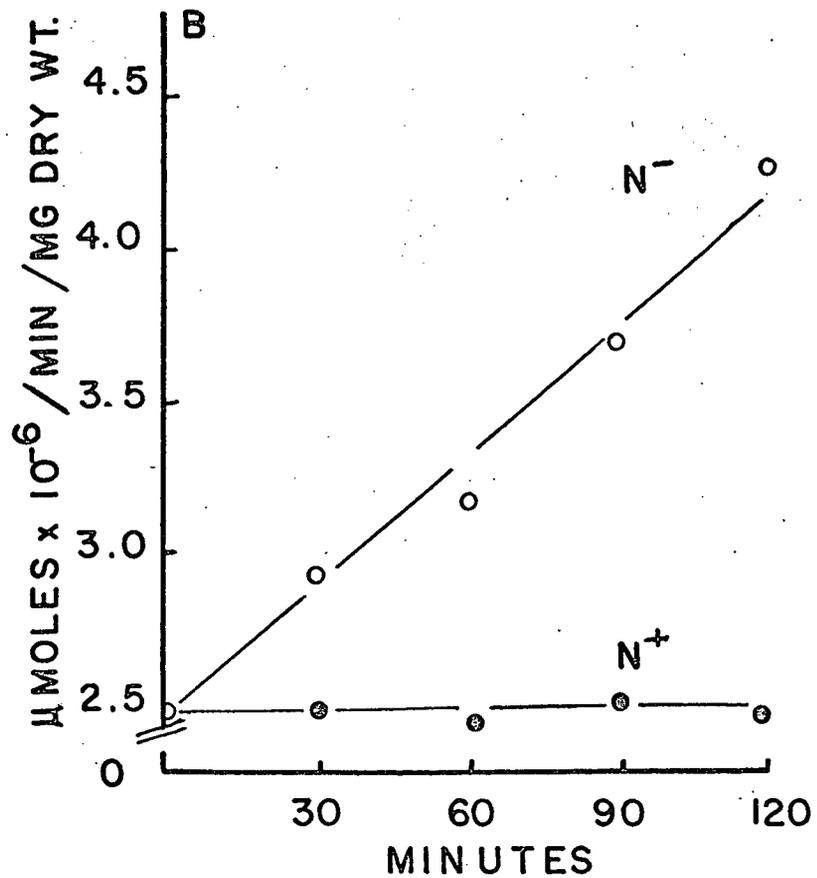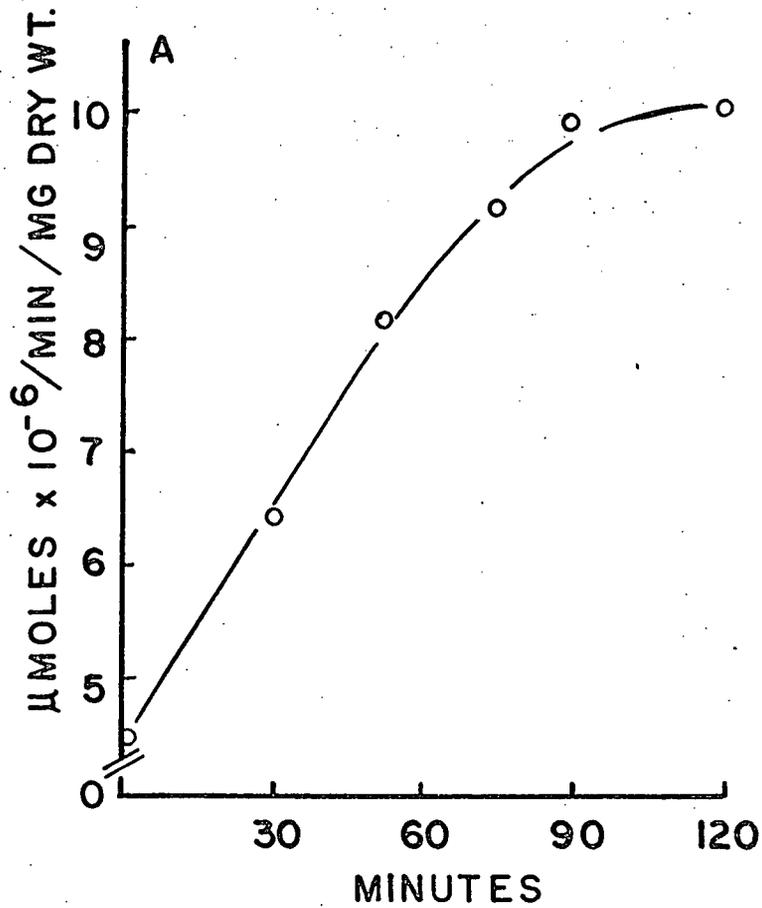
Fig. 42. The change in ^{14}C-glutamate transport in P. aeruginosa during nutrient deprivation. Samples were removed at zero time and at various intervals during: A Carbon starvation; B Nitrogen starvation.
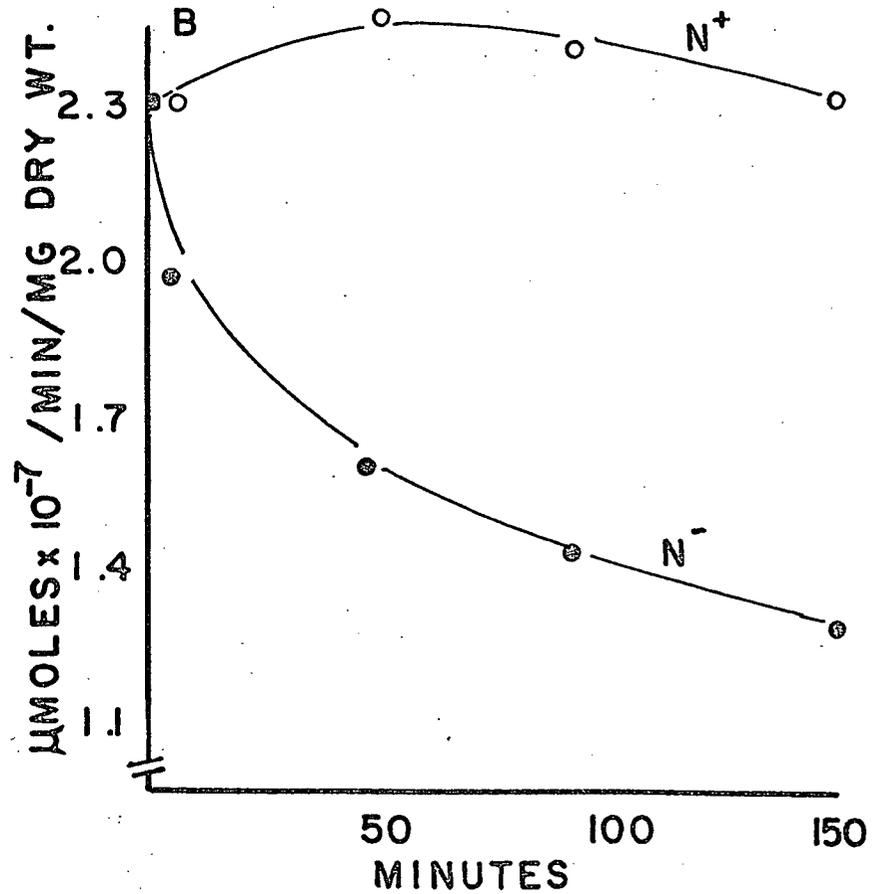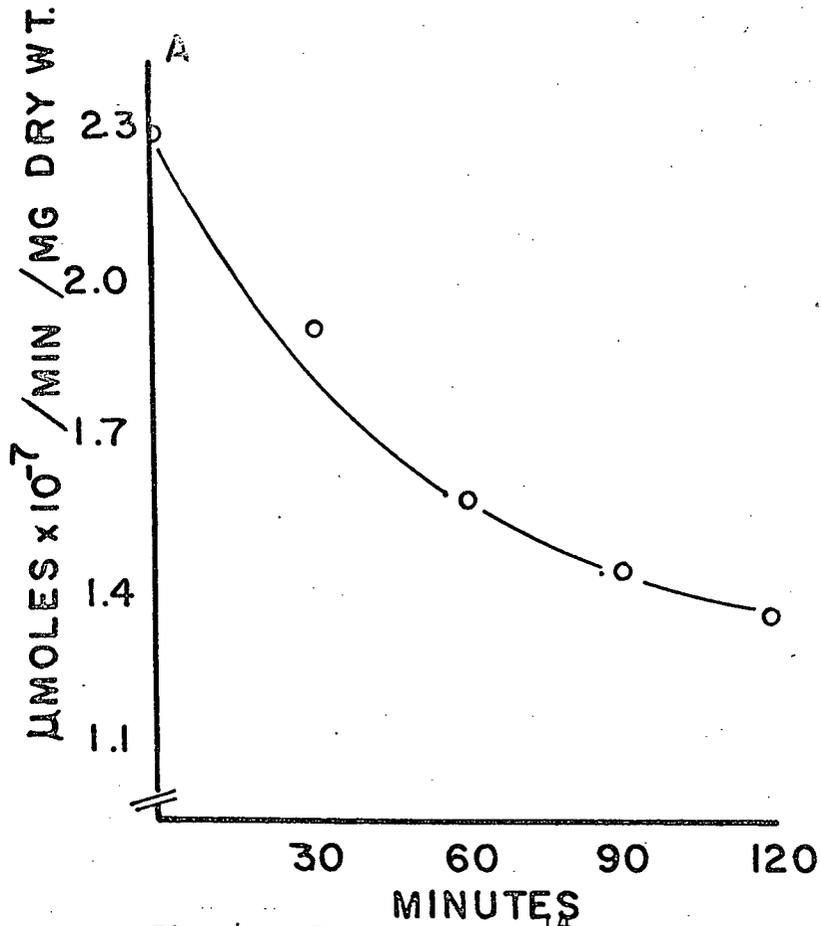
Fig. 43. The change in ^{14}C-valine transport in P. aeruginosa during nutrient deprivation. Samples were removed at zero time and at various externals during: A carbon starvation; B nitrogen starvation.
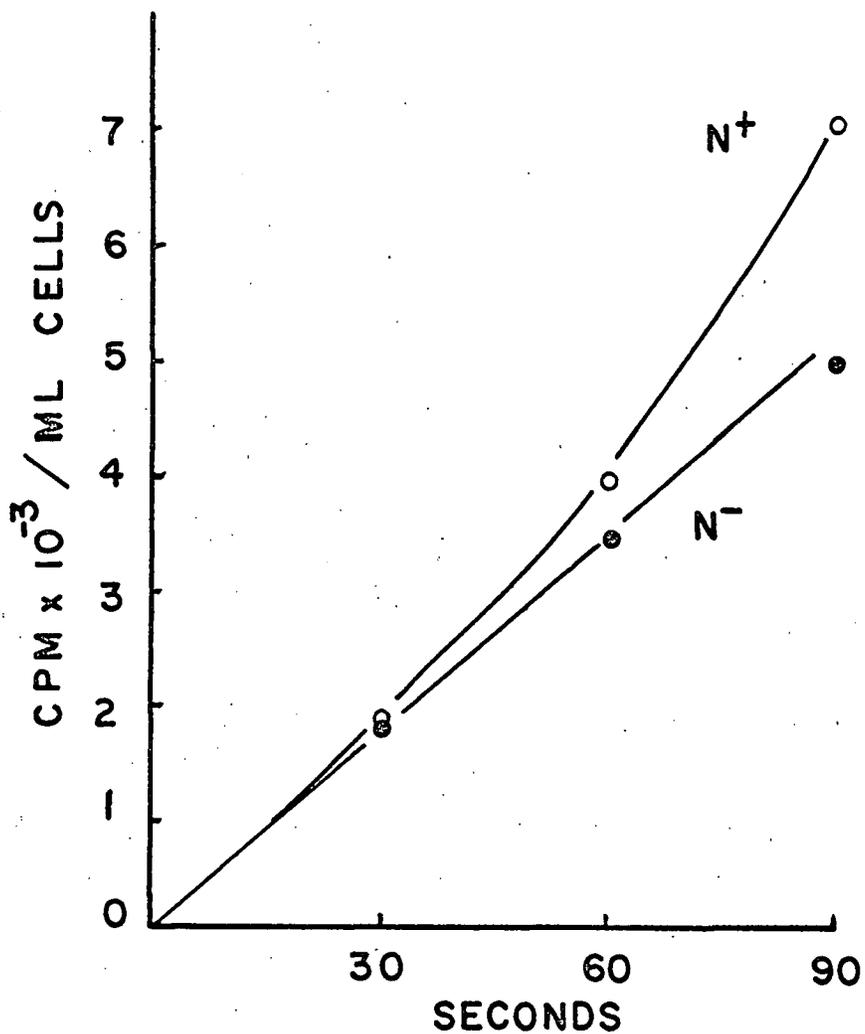
Fig. 44. Rate of transport of ^{14}C-valine into whole cells of P. aeruginosa. Symbols: ●, nitrogen starved cells; ●, nitrogen starved cells with 7.5×10^{-3}H $(NH_4)_2SO_4$ added at zero time.
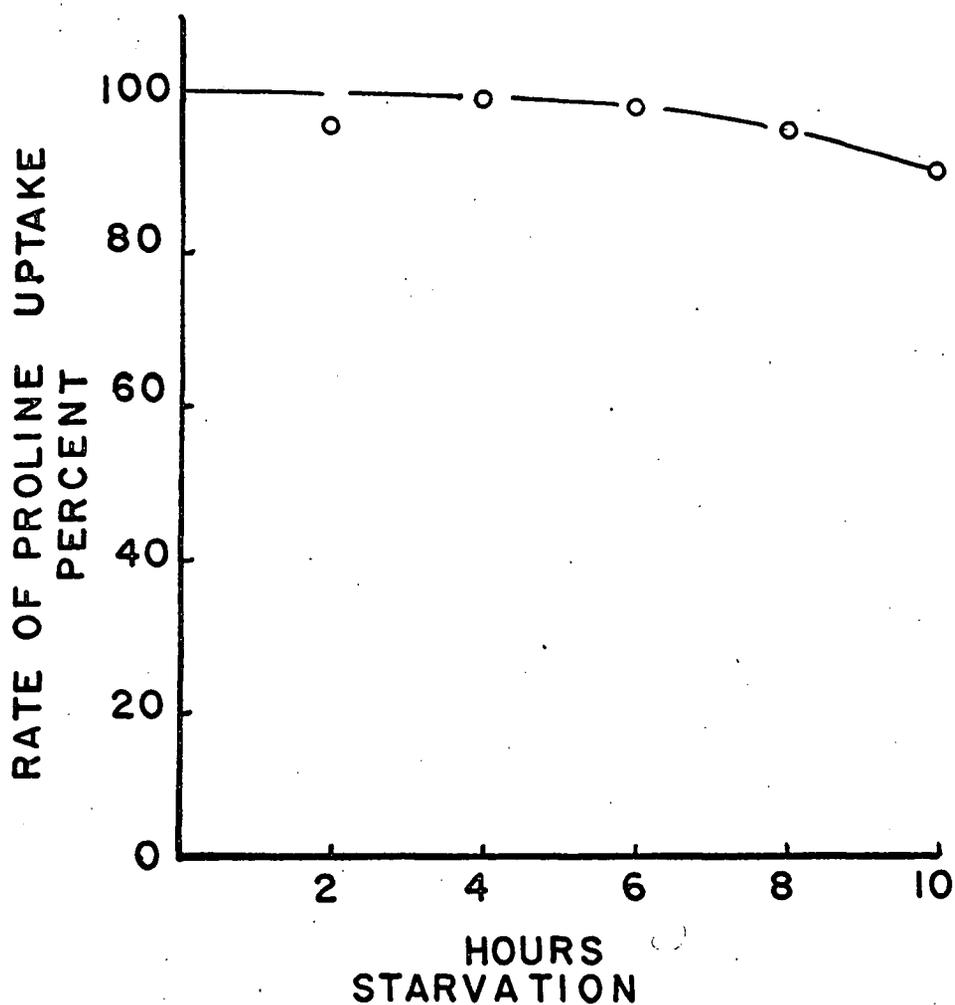
Fig. 45. Proline transport in P. aeruginosa P22 during carbon starvation. Rates of ^{14}C-proline transport were determined at intervals during the starvation period and are expressed as percentage of the non-starved control.

acids decreased significantly during carbon or nitrogen

starvation (Fig. 43). The amino acid transport rates demonstrated

in Figure 42 and 43 were altered by a factor of approximately

two. Extended periods of starvation caused no further change.

From the results in Table XVII it can be seen that short pre-

incubation with the deprived nutrient effectively restored the

transport rate to levels observed prior to starvation. The time

course for the restoration of ^{14}C-valine transport by the

addition of ammonium sulfate to cells previously starved for

nitrogen is shown in Figure 44.

The nature of the maintenance of amino acid transport functions

during extended periods of nutrient deprivation was investigated

further. Cultures were deprived of an energy source and transport

ability for ^{14}C-proline was observed at time intervals up to ten

hours. Only minor changes in the efficacy of proline transport

were observed (Fig. 45), and this was presumably due to cell

death.

It was obvious from a consideration of protein turnover rates

(Mandelstam, 1960), that by the end of the starvation period it

was likely that over 50% of the cellular protein had been subjected

to turnover. Clearly then, the amino acid transport proteins in

P. aeruginosa were exempt from turnover.

It was reasonable to assume that the selective maintenance

of these transport systems during extensive nutrient deprivation

served some physiological function. As a result, the influence

of carbon or nitrogen starvation on pool behaviour was investigated.
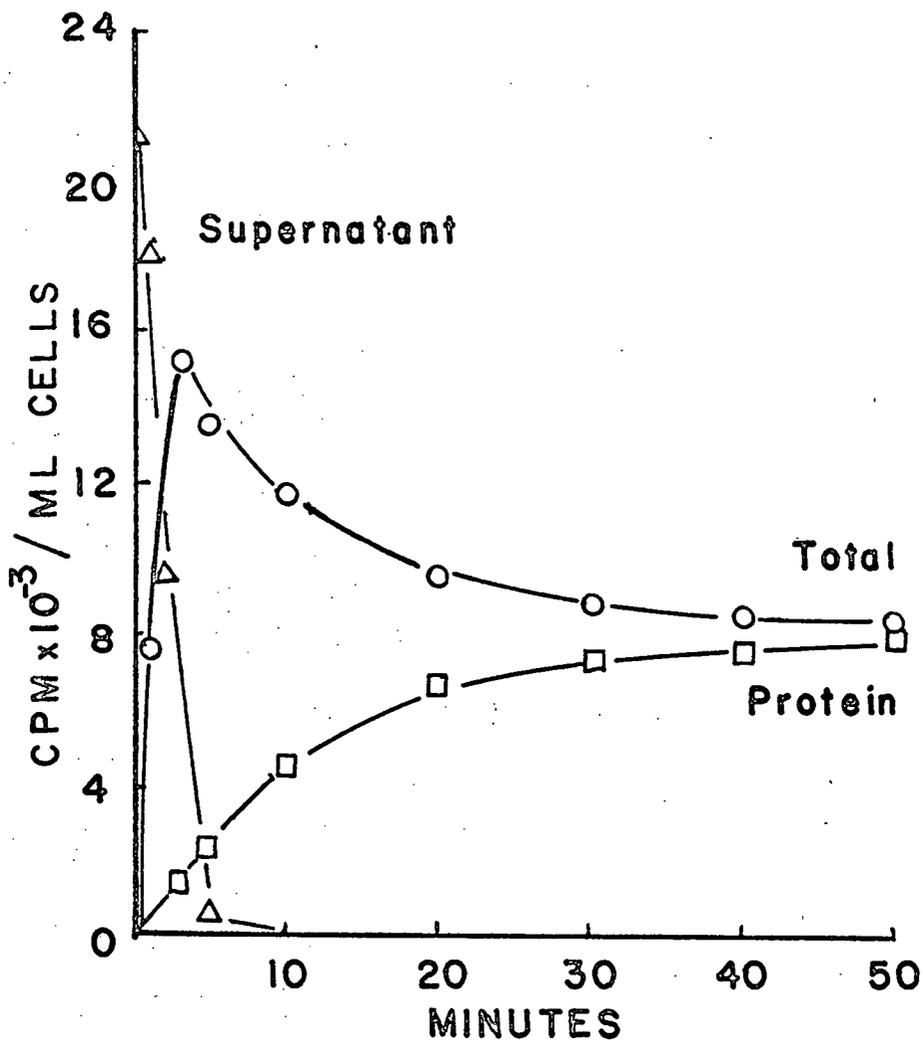
Fig. 46. Uptake of ^{14}C-proline into whole cells and protein of P. aeruginosa during carbon starvation. Cell suspensions were starved for carbon for 45 min prior to the addition of 10^{-6}M ^{14}C-proline.
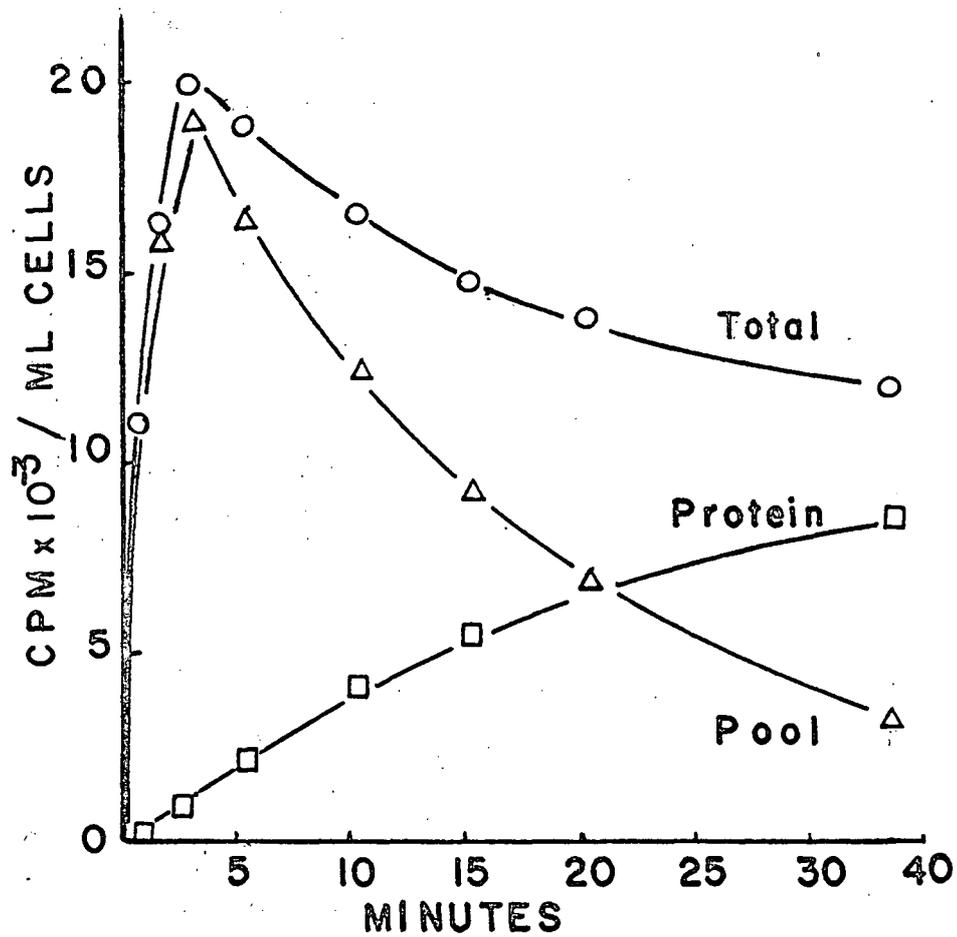
Fig. 47. Uptake of ^{14}C-proline into P. aeruginosa cells and cell fractions. Cells were starved for nitrogen for 45 min prior to the addition of 10^{-6}M ^{14}C-proline.

2. Pool formation and maintenance during carbon or nitrogen
   starvation

a. Time course of amino acid incorporation

When proline uptake was followed in cultures that were
starved for carbon, the pools formed from the exogenous amino
acid were found to be metabolically unstable (Fig. 46). The
initial incorporation of label was rapid and the resulting
high pool level formed within 2.5 min was subsequently lost due
to both catabolism and protein synthesis. The relatively high
loss of radioactivity was unusual and the results suggested
that the catabolism of proline must be regulated in some manner
by the presence of glucose.

When cultures were starved for nitrogen and time course
experiments for ^{14}C-proline incorporation carried out, quite different
results were obtained (Fig. 47). Again the initial uptake of proline
was rapid, and a very large transient pool was formed at 2.5 min,
due primarily to the reduction in the rate of protein synthesis.
However, this large amino acid pool was not depleted by catabolism
but a significant fraction of the pool was excreted back into the
medium. All the radioactivity not incorporated into the cell
could be accounted for in the culture supernatant fluid. It was
obvious then that the fate of the intracellular amino acid pool
differed depending on the nature of the starvation. These results
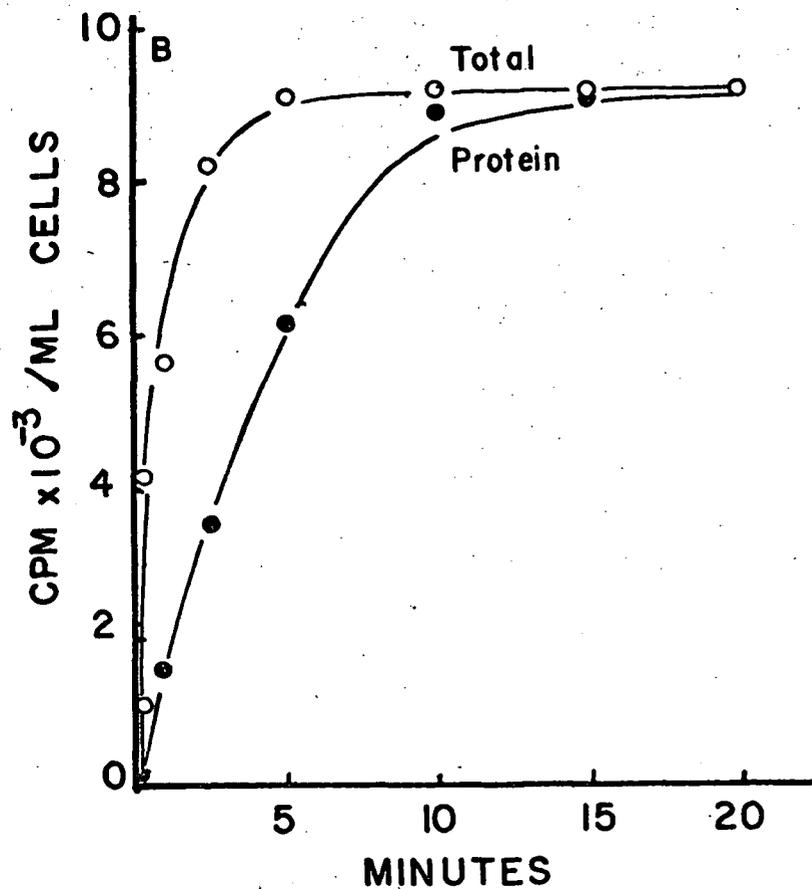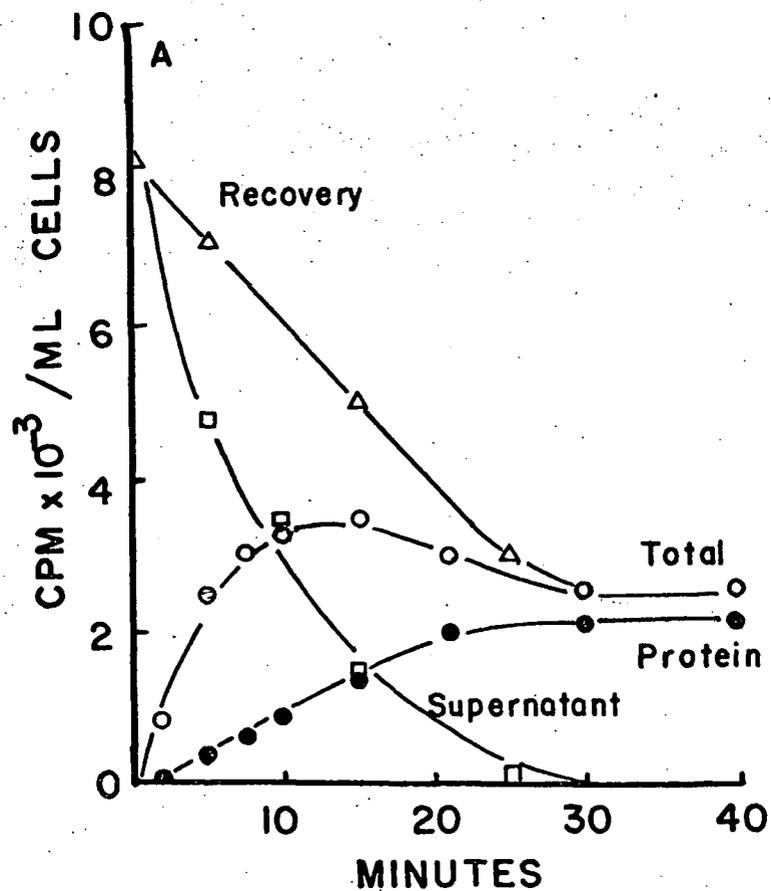
Fig. 48. Time course of ^{14}C-phenylalanine uptake into cells and cell fractions during nutrient deprivation. (A) During carbon starvation (B) During nitrogen starvation.
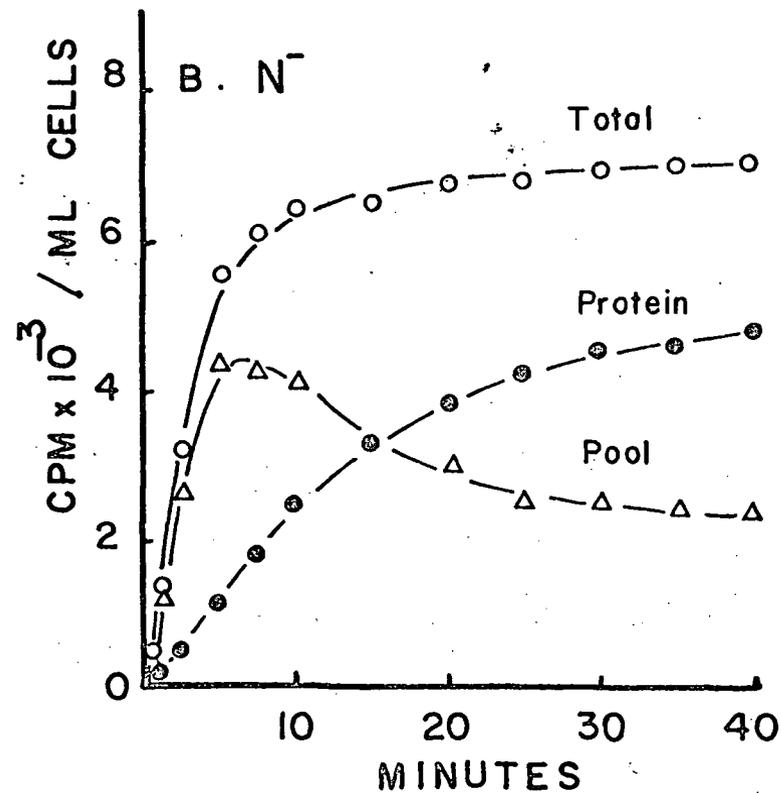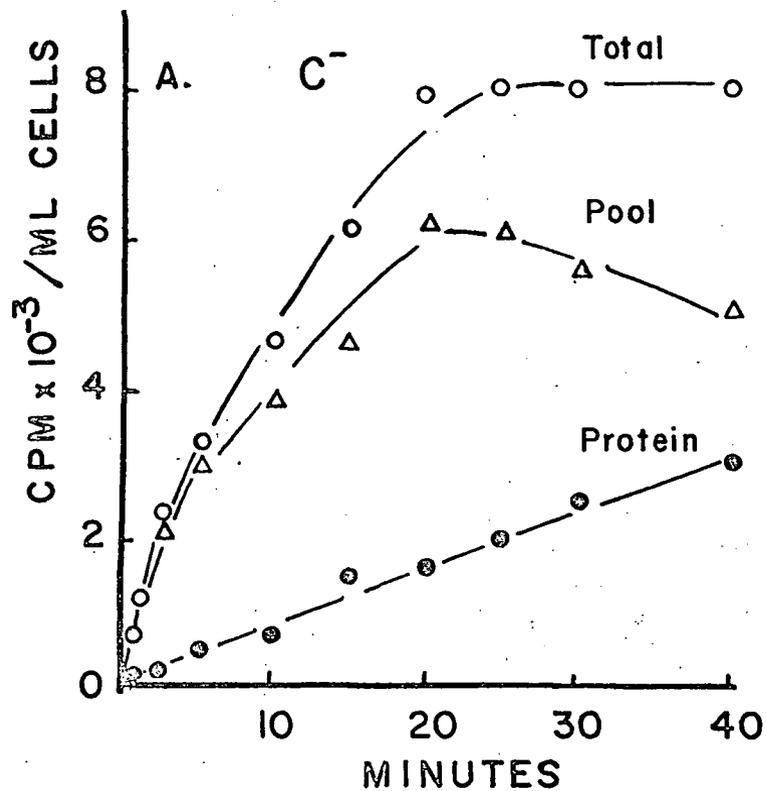
Fig. 49. Time course of ^{14}C-proline uptake in cell suspensions of P. aeruginosa P22 during nutrient deprivation. (A) During carbon starvation (B) During nitrogen starvation.

reinforced the observation that the catabolism of amino acids
was controlled by the presence of glucose.

Phenylalanine transport was also relatively unaffected by
carbon or nitrogen starvation; however, this amino acid is
catabolized relatively slowly by P. aeruginosa, and as a result,
phenylalanine was selected for pool behaviour studies during
nutrient deprivation. As was demonstrated for proline, the ^{14}C-
phenylalanine which entered the pool of carbon starved cells was
rapidly oxidized (Fig. 48A), and only 31% of the added label was
recovered at the end of the experiment. When a similar experiment
was carried out under conditions of nitrogen starvation (Fig. 48B),
the results differed considerably from the experiment with ^{14}C-
proline. Quantitative recovery of radioactivity again indicated
that the oxidation of phenylalanine was repressed by glucose or
a product of glucose degradation. Approximately 10% of the added
label was recovered in the cells and the rest was found in the
culture supernatant fluid. The pool was devoid of any radioactivity.

Time course experiments with a mutant, P22, which was unable
to catabolize proline as a carbon source, demonstrated that carbon
or nitrogen deprivation caused no secondary alterations of the
intracellular pool (Fig. 49). With this organism, all the
incorporated radioactivity as ^{14}C-proline was accounted for by the
label found in the protein fraction at the end of the experiment
either under conditions of carbon or nitrogen starvation. The pool
was found to be stable under these starvation conditions. This
mutant grew well on minimal medium, thereby indicating that the
synthesis and catabolism of proline in P. aeruginosa are not

Table XVIII.   Fate of ^{14}C-amino acids incorporated into
nitrogen starved cells of P. aeruginosa.

Amino Acid	Recovery[a] of ^{14}C	Hydrazones	% ^{14}C in Hydrazones
^{14}C-Glutamate	85.6	Pyruvate	81.4
		α-ketoglutarate	18.6
^{14}C-Aspartate	92.5	Pyruvate	90.5
		α-ketoglutarate	9.5
^{14}C-Alanine	98.5	Pyruvate	89.3
		α-ketoglutarate	10.7
^{14}C-Proline	90.5	Pyruvate	65.5
		α-ketoglutarate	34.5

[a] Percent recovery of ^{14}C from supernatant fluid

Table XIX. Fate of ^{14}C-amino acids incorporated into
nitrogen starved cells.

^{14}C-Amino Acid	% of Recovered ^{14}C		% Total[a] Recovery
	Deaminated	Non-deaminated	
Lysine	83.6	16.4	87.6
Histidine	31.1	68.9	93.2
Phenylalanine	88.0	12.0	83.3
Isoleucine	95.1	4.9	95.0

[a] Percent recovery of total ^{14}C from culture supernatant fluid.

mediated by the same enzymes.

b. Fate of the amino acid pool

It was assumed that the amino acids added to nitrogen starved cells were being deaminated. Two groups of amino acids were examined to determine the nature of the products excreted during nitrogen starvation. The first group of amino acids included proline, glutamate, aspartate, and alanine. These were selected as being amino acids which could be degraded easily due to their close relationship to main catabolic pathways in this microorganism. The second group of amino acids included lysine, histidine, phenyl-alanine, and isoleucine, and these were considered to be more remote from such pathways.

Typical amino acid uptake experiments were carried out with each amino acid under conditions of nitrogen starvation. Culture supernatant fluids were collected after centrifugation and were analyzed either by extraction of reacted 2,4-dinitrophenylhydrazones, or by elution from a Dowex 50 (H^+ form) column. The results of these experiments are listed in Tables XVIII and XIX. All of the radioactivity from the amino acids of the first group (Table XVIII) was extracted as the keto-acid hydrazones. It was, therefore, concluded that these amino acids were rapidly deaminated and the resulting ^{14}C-keto-acids were exchanged with exogenous ^{12}C-keto-acids which had accumulated during glucose catabolism (McKelvie, 1965).

Culture supernatant fluids from the second group of amino acids were passed through Dowex 50 (H^+ form) columns, and in each case, essentially all of the label passed through the column, indicating that the amino acids had undergone deamination (Table XIX). The only exception to this pattern was histidine, however, the ring nitrogen may not have been completely removed and, as a result, this amino acid would have adsorbed more strongly to the column.

Thus, it appeared that under conditions of carbon or nitrogen starvation, the controls which repressed the activity of enzymes concerned with the oxidation or deamination of incorporated amino acids were released. Control cultures which had been starved for nitrogen for forty-five minutes, were reincubated in the presence of 0.1% $(NH_4)_2SO_4$ for the same time interval and then were allowed to incorporate ^{14}C-phenylalanine. It was found that the time course of uptake had been restored to the normal state. The failure to excrete significant quantities of label was taken as evidence that the presence of ammonium ions influences the activity of the enzymes which deaminate amino acids.

Since amino acids synthesized from glucose accumulate in the native pool of P. aeruginosa, albeit at low levels, it would be paradoxical physiologically to simultaneously catabolize these amino acids which are necessary for protein synthesis. Thus the organism undoubtedly evolved control mechanisms which regulate the constitutive levels of amino acid dissimilatory enzymes. However, these same controls were observed to be ineffectual with

respect to the high induced levels of enzymes involved in amino

acid dissimilation, since these enzymes were induced even in the

presence of high glucose concentrations. This too would seem

to be physiologically effective and as a result would allow the

organism to degrade glucose for energy, pentoses, trioses,

et cetera, and at the same time catabolize amino acids to supply

carbon skeletons for the synthesis of other essential metabolites

with a related chemical structure.

In conclusion, P. aeruginosa does not maintain the internal

pool of amino acids during nutrient deprivation as has been shown

for E. coli (Britten and McLure, 1962), but rather controls its

levels of degradative enzymes in such a way as to allow maximum

use of both its pool amino acids and also amino acids present in

the environment in extremely low concentration.

VIII. The Mechanism of Amino Acid Transport and Accumulation

Unlike animal or plant cells, bacteria are able to concentrate

low molecular weight metabolites to a very great extent over the

external environment. For instance, Britten and McLure (1962),

have reported amino acid concentration ratios greater than

28,000 for E. coli. As a result of this unique ability, concerted

efforts have been directed at elucidating the mechanisms involved

in this process.

All the available evidence concerned with active transport

of carbohydrates across biological membranes is consistent with

the hypothesis that this process must consist of at least two

distinct components. The basic components of this process have

been outlined by Winkler and Wilson (1966), for the transport

of β-galactosides into E. coli (Fig. 50). The system involves

a substrate-specific membrane "carrier" which facilitates

movement of the substrate across the permeability barrier, and

also, an unknown mechanism which couples metabolic energy to

the carrier function which ultimately permits a net movement

of substrate from the extracellular environment into the cell

against a concentration gradient. In the absence of energy,

the carrier facilitates the equilibration of internal and external

substrate concentrations. The process is not regarded as simple

diffusion since it still demonstrates substrate specificity and

saturation kinetics. The glucose transport systems of yeast

(Burger, Hajmova, and Kleinzeller, 1959), and erythrocytes (Wil-

brandt, 1963; Le Fevre, 1961), also have been demonstrated to

operate in this manner and have been designated as carrier-mediated

transport or facilitated diffusion.

Various workers have shown, in both animal and bacterial

cells, that when energy metabolism is terminated with specific

inhibitors the membrane carrier remains intact, but the movement

of substrates against a concentration gradient is prevented. Thus

the active transport systems for carbohydrates were converted to

facilitated diffusion (Bibler, Hawkins, and Crane, 1962; Koch,

1964; Winkler and Wilson, 1966).

The transport and accumulation of amino acids in bacteria

has received less attention than carbohydrate transport systems

OUTSIDE | MEMBRANE | INSIDE

$S_{out} \longrightarrow$ $+ C \longleftrightarrow C +$ S_{in}

$\updownarrow Kt\ entry$ $\quad \updownarrow Kt\ exit$

$CS \longleftrightarrow CS$
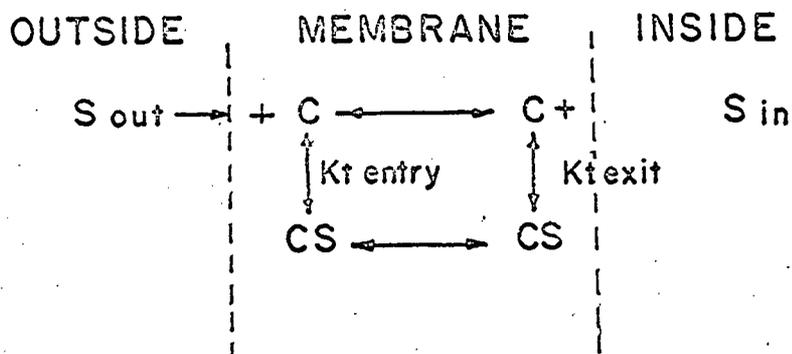
In the steady state:

ENTRY = EXIT

$$Vmax \left(\frac{S_{out}}{S_{out} + Kt\ entry} \right) = Vmax \left(\frac{S_{in}}{S_{in} + Kt\ exit} \right)$$

FOR FACILITATED
DIFFUSION:

FOR ACTIVE
TRANSPORT:

$Kt\ entry = Kt\ exit$ $\qquad\qquad Kt\ entry < Kt\ exit$

Fig. 50. Model for an energy-uncoupled active transport system.
S, substrate (designated as inside or outside the
cell); C, membrane carrier; CS, membrane carrier-
substrate complex through the membrane; Kt, equilibrium
constant for the reaction $S + C \longrightarrow CS$. It is assumed
that (a) the chemical reactions at each interface are
much more rapid than the diffusion of carrier or
substrate-carrier complex (b) a linear concentration
gradient of both C and CS exists in the membrane
(c) the diffusion constants for C and CS are the same.
(Winkler and Wilson, 1966). It is obvious, however,
that in this model Kt should be considered as the
affinity constant for amino acid-carrier formation,
and not the equilibrium constant for this reaction.

as the processes have been found to be fraught with complexities. The amino acid accumulation ability is less amenable to definitive experimentation as more than one process is involved; that is, the accumulation of amino acid pools ultimately is concerned with both the cellular synthesis of amino acids and the incorporation of amino acids from the external environment. The active transport of amino acids across membranes has been shown to exhibit some properties common to carbohydrate transport, however, the process also was shown to possess many unique properties. The active transport system for amino acids as visualized for E. coli by Britten and McLure (1962), is shown in Figure 51. Similar to sugar transport, amino acids presumably are transported across the membrane by a stereospecific carrier to the intracellular milieu, where they are then concentrated by association with specific sites. The site-amino acid association was described as being the energy-dependent process in active transport.

An even more complex system has been encountered with P. aeruginosa. As described previously, the organism constantly maintains a low level of native pool amino acids for protein synthesis, at least during periods of nutrient abundance, but like E. coli, this organism can also concentrate amino acids to a very high level above the exogenous concentration. Unlike E.coli, however, these cells constitutively catabolize the accumulated amino acids especially during carbon or nitrogen deprivation.

OUTSIDE   MEMBRANE   INSIDE

$$A \longrightarrow +C$$

$$K_2 \updownarrow K_1$$

Energy coupled

$$AC \rightleftharpoons +R \underset{K_4}{\overset{K_3}{\rightleftharpoons}} AR + C$$
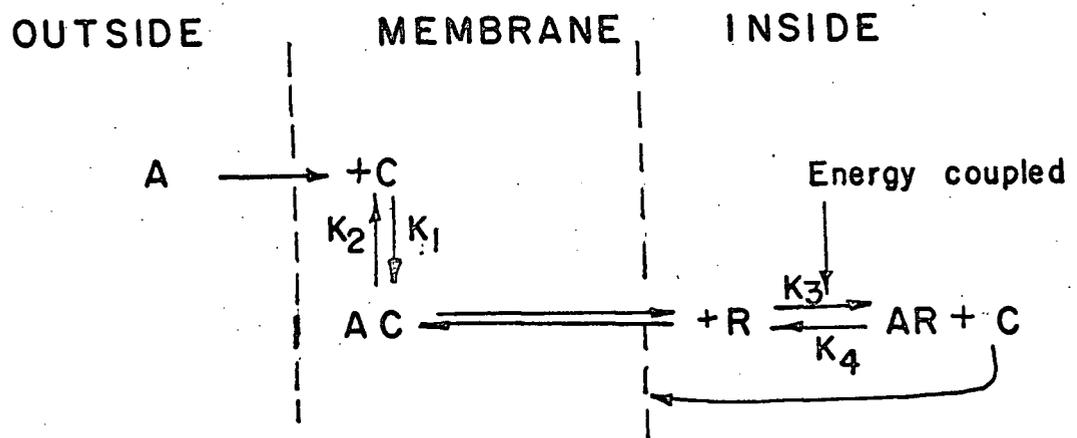
In the steady state:

$$ENTRY(\text{into pool}) = EXIT(\text{into protein})$$

and $\quad AR + C \longrightarrow AC + R$
reduces available C.

FOR ACTIVE
TRANSPORT:

$$K_1 \lll K_2$$

Fig. 51. The carrier model for amino acid transport. A, amino acid; C, membrane carrier; AC, mobile membrane carrier-amino acid complex; R, non-mobile sites; AR, amino acid-site complex (Britten and McLure, 1962).

Preliminary studies on pool maintenance phenomena
demonstrated that internal amino acid pools which resulted from
the transport of amino acids added at low exogenous concentrations
were effluxed in the absence of energy metabolism (Fig. 26-29).
This demonstrated that the ability to maintain significant
concentration gradients had been interfered with. Such data
do not provide insight into the nature of this efflux phenomenon;
however, rapid but limited initial amino acid uptake was observed
when cells were poisoned with NaN_3 (Fig. 5). This suggested that
the actual transport process may have been unaffected by the
inhibitor and that the ability to maintain amino acid pools had
been lost.

In order to pursue this observation further, comparisons
of transport rates were made with cells under normal conditions,
cells poisoned with NaN_3 and iodoacetamide, and with cells
maintained at 0 C. Cell suspensions were exposed to a concentration
of ^{14}C-proline which saturated the high affinity permease (2 x
10^{-5} M) and then filtered at five second intervals during the
incubation period. Transport against a concentration gradient
was completely abolished in the cells treated with NaN_3 (Fig.
52). The amino acid, however, rapidly entered the cell until
the intracellular concentration was approximately the same as
that in the incubation medium. Protein synthesis was completely
inhibited by the preincubation with 30 mM NaN_3 and 1 mM iodoacetamide.
The proline Tr^- mutant, P5, also had a limited transport capacity
at these proline concentrations. Preincubation with NaN_3 did not
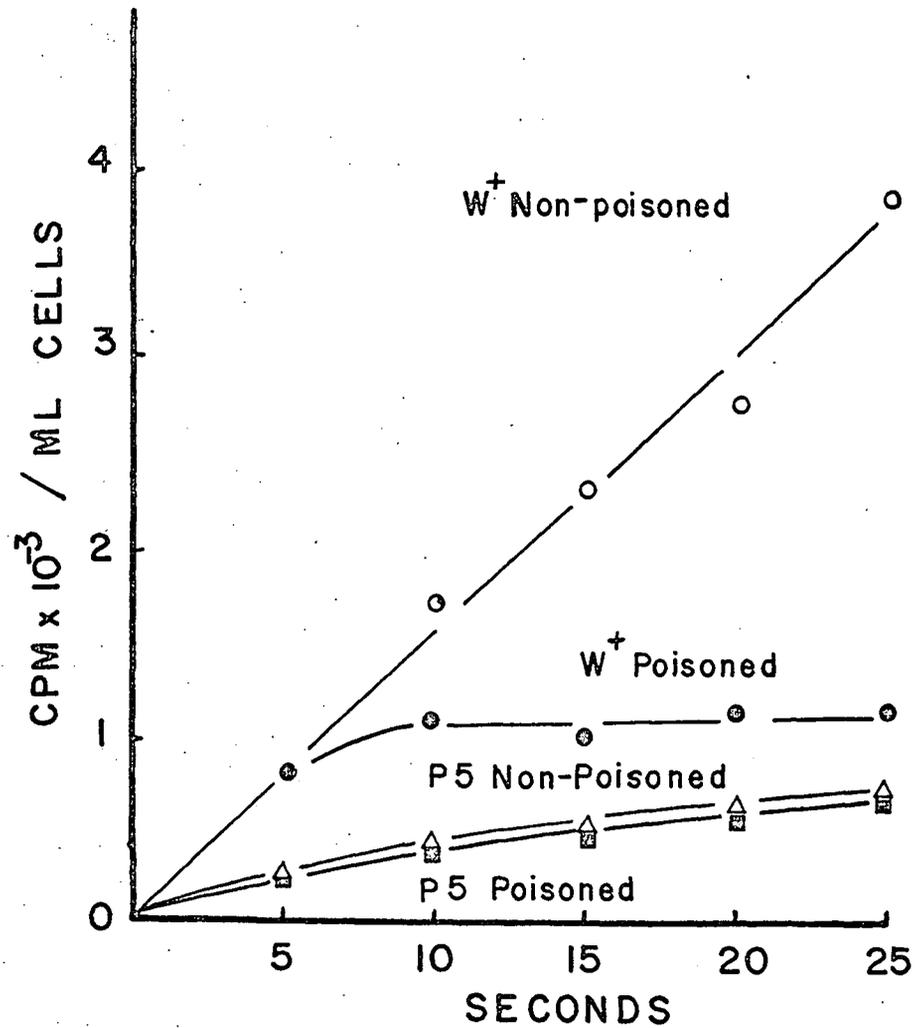
Fig. 52. The uptake of 2×10^{-5}M ^{14}C-proline at 30 C into NaN$_3$ treated and untreated cells of wild-type and proline Tr$^-$ mutant (P5) P. aeruginosa.

significantly alter the transport rate and these inhibited cells only incorporated proline until the internal proline pool had equilibrated with the external medium.

When kinetics of transport into poisoned wild-type cells were determined (Fig. 53), it was observed from incorporation data obtained with five and ten second sampling times, that the affinity constant for uptake at low proline concentrations was significantly reduced, and also that V_{max} was significantly lowered. This suggested that either the high affinity transport function had been altered by treatment of the cells with NaN_3, or that initial rates were not being measured but rather, a composite of the entrance plus an additional efflux component. The K_m of amino acid entrance was reduced approximately 10 fold and the significance of this reduction in the K_m for uptake will be discussed later. These data are then commensurate with the hypothesis that the uptake or transport of proline occurs by an energy-independent mechanism, and that another energy-dependent function was involved, presumably for the accumulation of the amino acid.

The incorporation of certain amino acids was previously shown to be mediated by both high and low affinity permeases (Fig. 38). Proline transport was not an exception, for this amino acid is incorporated into P. aeruginosa by permeases with widely varying K_m values. It was also demonstrated previously that the ability to transport proline was induced by growing cells in the presence of the amino acid (Fig. 40). This presented a working model for studying the effect of increased transport levels on the ability
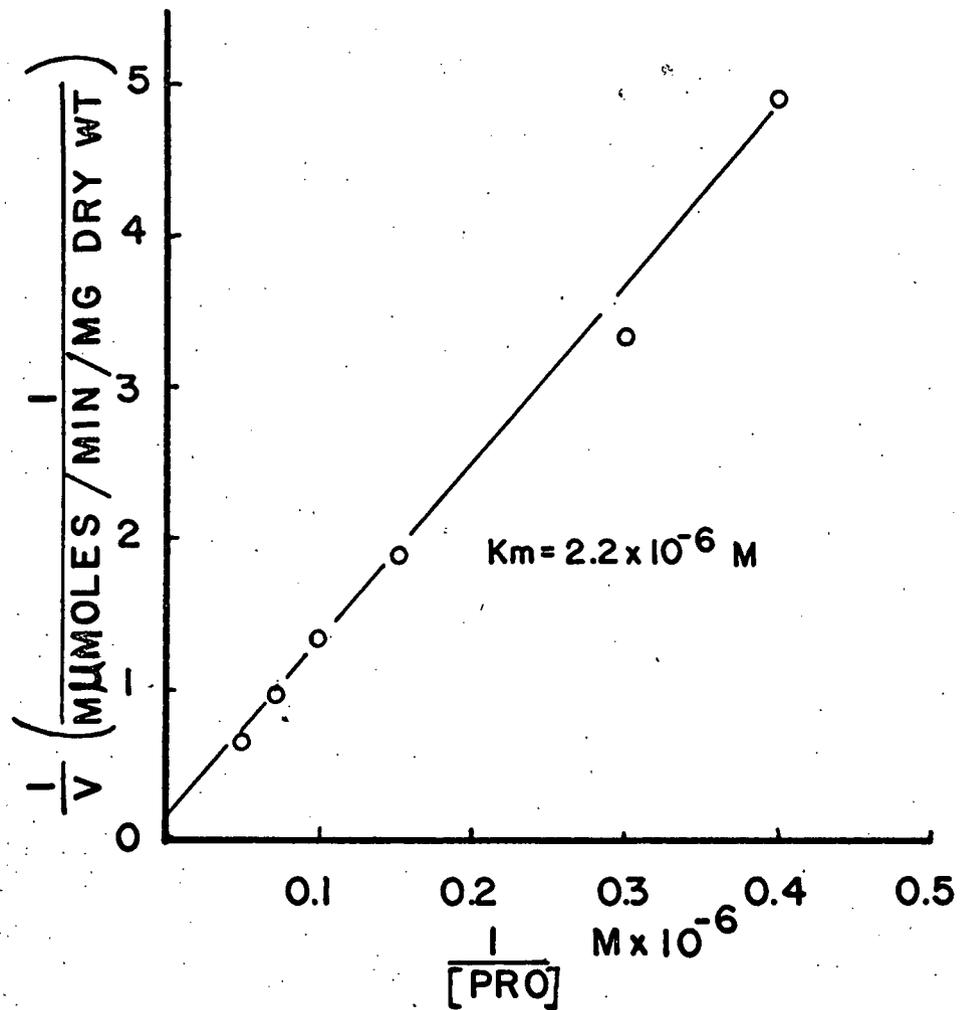
Fig. 53. Lineweaver-Burk plot of proline incorporation into cells of P. aeruginosa. Cell suspensions were preincubated with 30 mM NaN₃ + 1 mM iodoacetamide for 30 min at 30°C prior to the addition of ¹⁴C-proline.
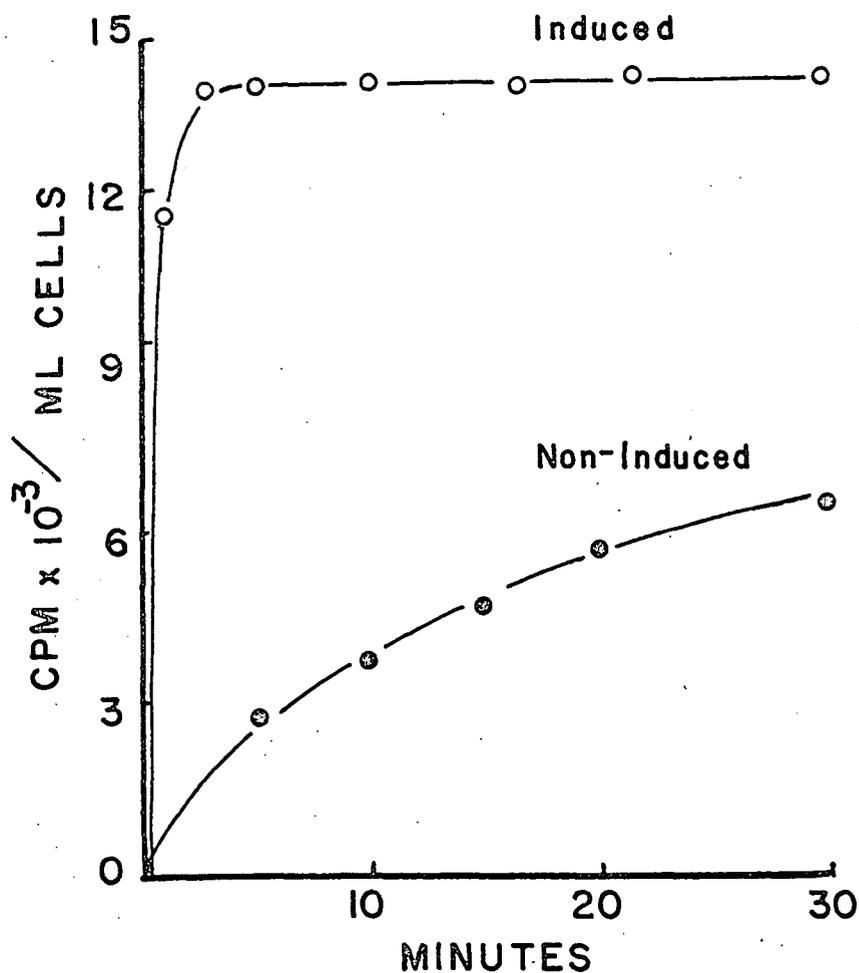
Fig. 54. The incorporation of ^{14}C-proline (10^{-6}M) into the pool of induced and non-induced cells of P. aeruginosa P22 at 10 C. Induced cells were grown in minimal medium with 0.1% proline. The cells were pre-incubated at 30 C for 30 min with 200 ug/ml chloramphenicol prior to the addition of ^{14}C-proline.

of the cell to concentrate amino acids.

Mutant P22, a strain with a lesion in the catabolism of proline, was induced for this transport function, by growth of the organism in the presence of this amino acid. Subsequently, maximum pool levels were established from 10^{-6} M ^{14}C-proline in the absence of protein synthesis (Fig. 53). The induced cells accumulated proline to levels twice those found with the uninduced cells and the resulting concentration gradient increased approximately 10 fold. This indicated that the transport ability and accumulation function were coordinately induced, or that the increased accumulation capacity was a consequence of increased amino acid transport ability.

According to the model visualized by Winkler and Wilson (1966) for β-galactoside transport (Fig. 49), an increased influx component would result in an increased concentration capacity of the cell, but in their investigation and in the studies of Koch (1964), the additional influx component would be ineffectual in establishing high concentration gradients when active transport was effectively converted to facilitated diffusion by poisoning the cells with a metabolic inhibitor such as NaN_3.

Figure 55 demonstrates that this was also found to be the case when induced cells were allowed to transport 2×10^{-5} M ^{14}C-proline after preincubation with NaN_3. The cells did not accumulate intra-cellular proline against a concentration gradient and therefore, the increased transport capacity of P. aeruginosa did not permit
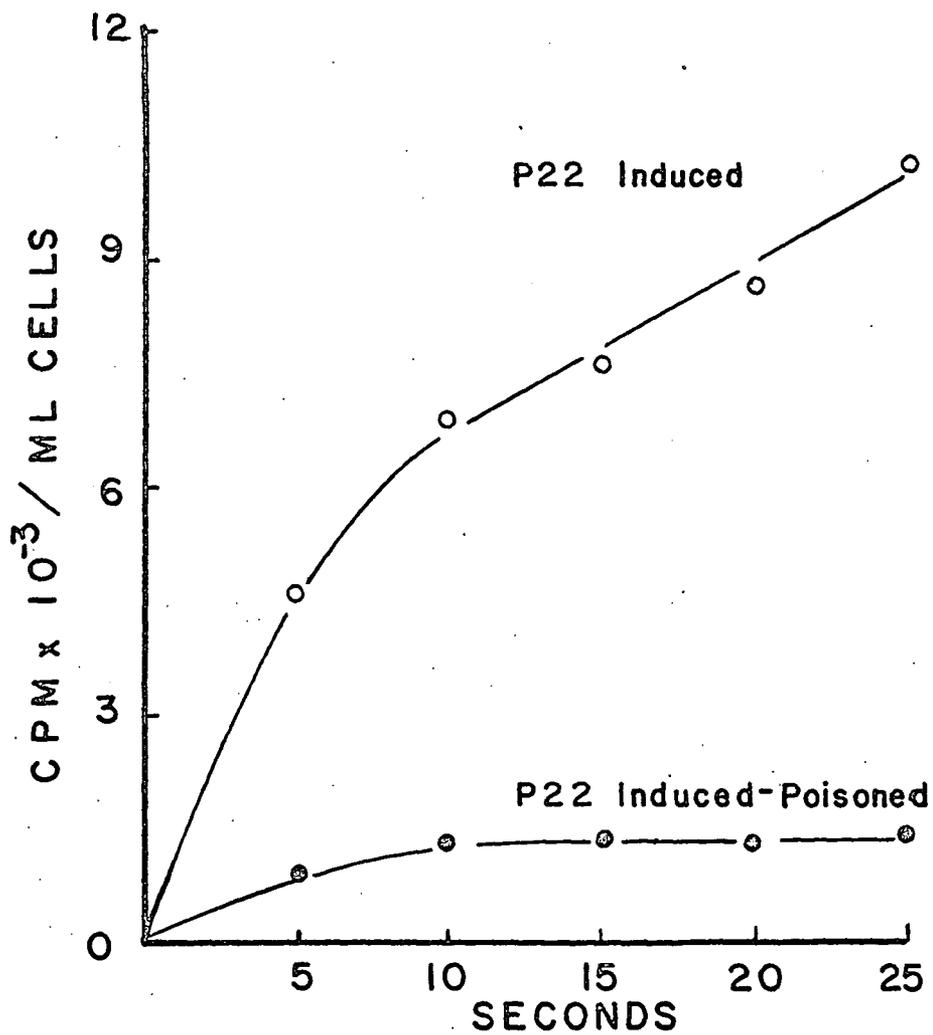
Fig. 55. Uptake of $2 \times 10^{-5}M$ ^{14}C-proline into
NaN$_3$ treated and untreated cells of
previously grown P. aeruginosa. These
cells were grown in minimal medium with
the addition of 0.1% proline.

intracellular amino acid accumulation in inhibited cells.

Further investigation into the nature of the induced uptake system for proline was attempted. The kinetics of the low affinity transport systems for both induced and uninduced cells were determined and are demonstrated in Figure 56. The K_m values for uptake were found to be identical with both kinds of cells and V_{max} was moderately increased with induced cells, thereby indicating that the low affinity permease was also inducible. However, it was felt that the increase in V_{max} could have been due to the influence of the greatly increased levels of the high affinity proline transport function.

The K_m values for amino acid incorporation determined with NaN_3 poisoned cells (Fig. 53), were found to be essentially the same as that of the low affinity permease operative in non-poisoned cells at high substrate concentrations. The effect of NaN_3 on the affinity constant for the high affinity permease could not be determined since the equilibration of external and internal proline concentrations at low exogenous proline levels likely would have occurred at a rate impossible to measure by these methods.

The increased concentrating ability of cells induced for amino acid transport can therefore be interpreted in light of the model described in Figure 50. It is feasible that the rate of exit of an amino acid from the intracellular pool is only moderately affected, if at all, by induction, and that the rate of amino acid entrance into the cell at low exogenous amino acid concentrations was greatly increased due to the induced levels of the high affinity permease (Fig. 54). Accordingly, this interpretation implicatēs
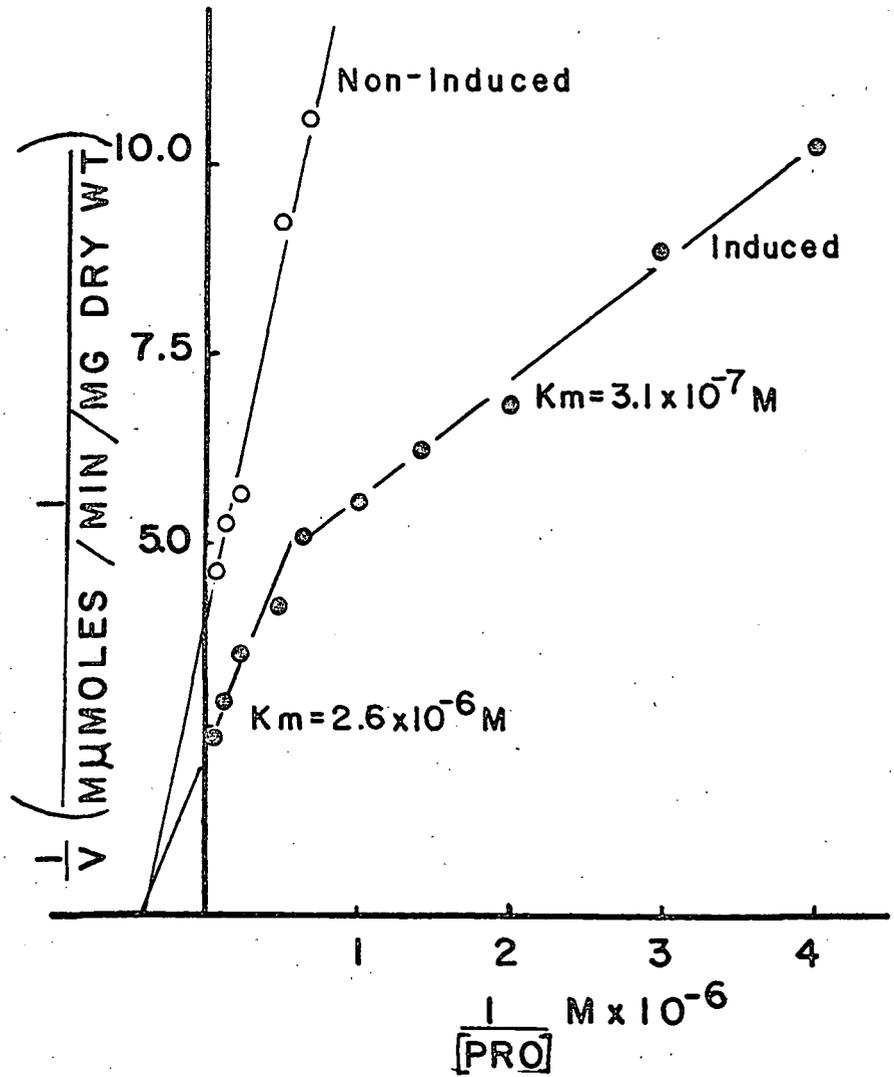
Fig. 56. Lineweaver-Burk plot of proline uptake into induced and non-induced cells of P. aeruginosa.
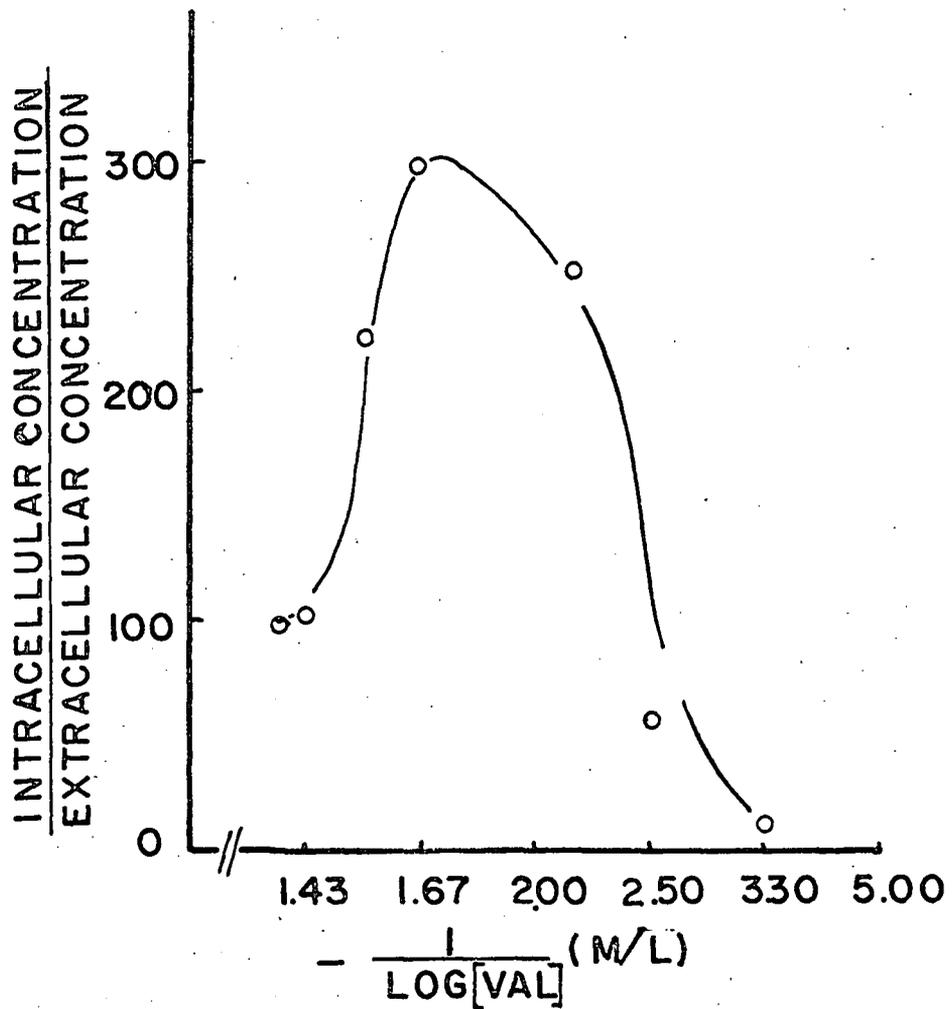
Fig. 57. The ratio of intracellular to extracellular valine concentration as a function of the exogenous valine concentration.

the low affinity permease as the entity mediating efflux observed in Figures 26-29, and it operates in efflux essentially only when high internal amino acid concentrations are present.

   To test the accuracy of this hypothesis, efflux and influx studies were carried out as functions of internal and external amino acid concentrations. If the intracellular amino acid concentration is a function of either permease, then differences in accumulating capacity of the microorganism would be revealed at external amino acid levels which would be conducive to the operation of a particular permease, since these permeases have greatly differing K_m entrance values. These studies were performed with the amino acid valine to obviate secondary effects of efflux due to multiple pool components previously described for proline (Fig. 28-30). The experiment described in Figure 57 substantiates the validity of this hypothesis. Between 10^{-7} and 10^{-6} M external valine concentrations, the accumulating capacity as revealed by the internal to external proline concentration ratios, increased markedly. At valine concentrations exceeding 10^{-6} M the ratios decreased. The maximum concentration ratios observed were approximately 300 fold, under the conditions of the experiment. Thus, at low external amino acid concentrations (where the majority of the supplied ^{14}C-amino acid enters via the high affinity permease) the concentration ratio is high. At higher intracellular amino acid concentrations (where efflux via the low affinity permeases becomes operative) the concentration ratio decreased concomitantly with increased exogenous amino acid, presumably due to an increased component of efflux. The precipitous decline in the internal to

external amino acid ratio when the exogenous valine concentrations exceeded 10^{-5} M, suggested that a large difference exists in the ability to transport the amino acid out of the cell relative to transport into the cell. This difference in influx and efflux capacity could feasibly be attributed to the affinity for the transport protein if the affinity was drastically reduced on the inside of the membrane surface.

Figure 58 shows the increase in pool size as a function of external amino acid concentrations. The pool increased with increasing exogenous amino acid concentration. Thus, at amino acid concentrations exceeding 10^{-6} M, while the intracellular to extracellular concentration ratios decreased, the pool size increased. These data strongly suggested that at high intracellular pool levels a difference in the affinity of the amino acid for the transport function on the inner membrane surface would be overcome and would result in an increasing efflux rate which ultimately would cause the observed decline in the concentration ratio (Fig. 57).

To determine accurately the contribution of each permease to the efflux process, various pool levels were pre-established at different exogenous valine concentrations. The cells were then immobilized on membrane filters and continuously washed for pre-determined time intervals. Rates of efflux from high internal amino acid concentrations to low external concentrations were then calculated and plotted as a function of the pool size.

From the results demonstrated in Figure 59, it was observed that the rate of exit or efflux of internal valine was a function
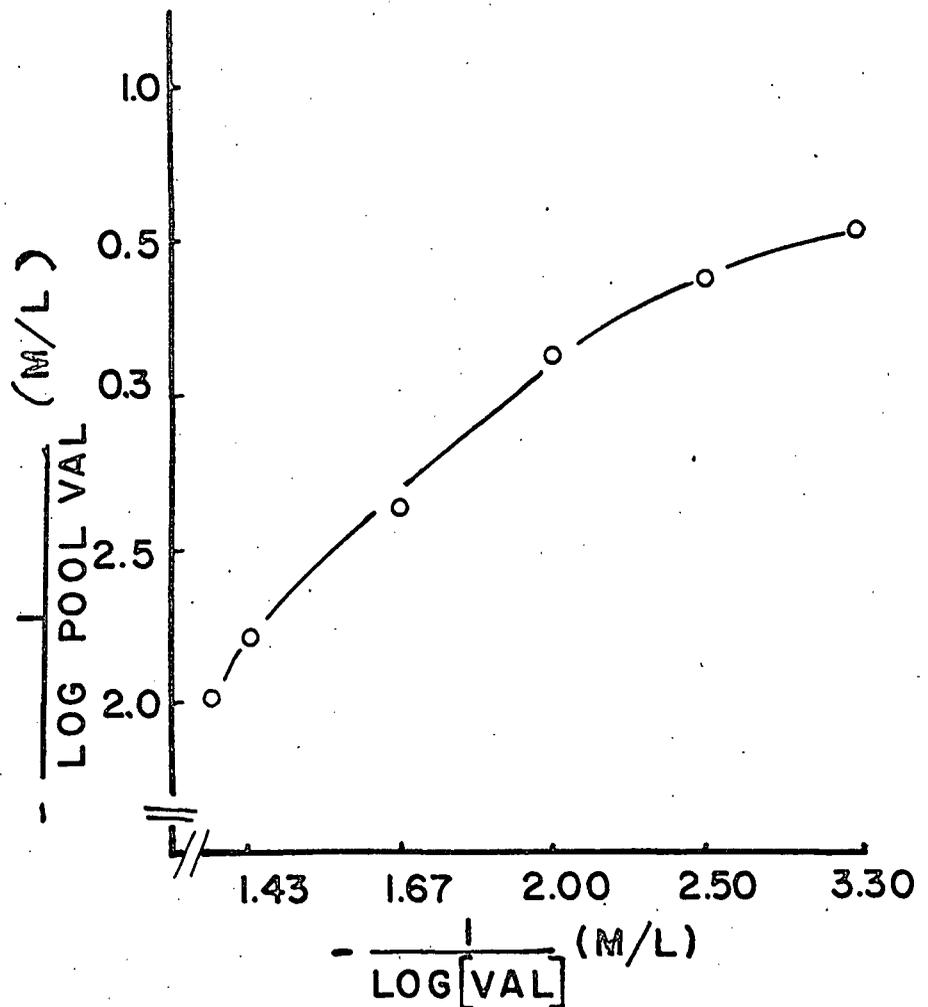
Fig. 58. The effect of the exogenous valine
concentration on the valine pool size
(double negative reciprocal log-log plot).
Cells were preincubated with 100 ug/ml
chloramphenicol at 30 C for 30 min then
pulsed with the appropriate concentration
of ^{14}C-valine at 15 C. Samples were
filtered at regular time intervals until
the maximum pool level had been obtained
in each case.

of the pool valine at levels greater than 10^{-5} M; a concentration

at which the low affinity permease became operative. However,

at lower pool concentrations, where the high affinity permease

was operative, no apparent changes in efflux rates were observed.

When affinity constants for the exit process were calculated,

very striking results were found. Compared to the affinity

constants for valine uptake via the low affinity permease the

affinity of the amino acid for the permease on the inside of the

membrane was found to have been reduced by a factor of approximately

5000. Theoretically, this would permit concentration gradients of

this magnitude to be formed. This has been found to be true for

β-galactoside accumulation in E. coli (Winkler and Wilson, 1966).

These investigators found that the maximum concentration ratio

for β-galactoside was 100 to 200 fold, and that qualitatively

at least, the ratio of K_m of exit to K_m of entrance was substantially

high.

The magnitude of the difference in K_m of entrance and K_m

of exit in this study is considered to be somewhat unique. The

low affinity permease has a K_m of entrance approximately 10 fold

less than the high affinity permease, and as a result the efflux

rate would theoretically saturate at high internal valine concentrations.

However, as already demonstrated (Fig. 52), there is in addition,

an energy dependent function which is responsible for the

accumulation of high levels of amino acid in the pool. This energy

is visualized to act in a manner similar to the β-galactoside

accumulation system studied in E. coli; that is, that energy is
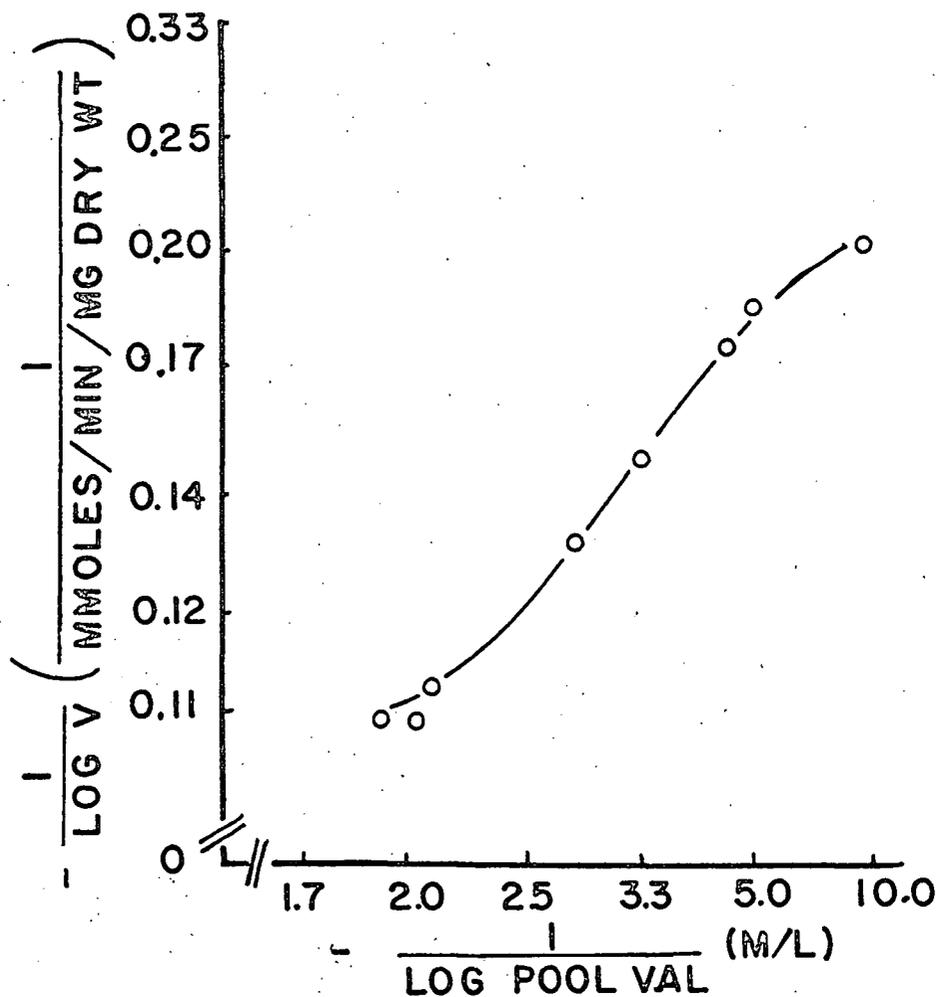
Fig. 59. Rate of efflux of ^{14}C-valine as a function of
the intracellular valine pool size. Cells
were preincubated with 100µg/ml of
chloramphenicol for 30 min at 30 C prior to
the establishment of ^{14}C-valine pools of various
sizes at 15 C. Efflux rates were determined by
continuously washing cells with amino acid free
minimal medium for time intervals up to 3 min.

expended to prevent the amino acid on the inside of the cell

membrane from recombining with the transport "carrier". As a

result, the apparent K_m of efflux would be much greater than

K_m for influx.

It is felt that in P. aeruginosa the combination of low

affinity of the amino acid for the efflux carrier and the energy

function cause a concerted inhibition of efflux. With the

additional component of a high affinity permease accelerating

influx, the net result is a great intracellular accumulation.

Thus the transient high concentration gradients exhibited in

transport experiments at low amino acid concentrations (Fig. 2)

seemingly have a rational explanation.

The data obtained so far explain the behaviour of relatively

large intracellular amino acid pools. However, the maintenance

of relatively low intracellular amino acid pools requires special

consideration, since P. aeruginosa does not normally accumulate amino

acids to very high levels when they are being synthesized de novo.

From Figure 59, it can be observed that at low pool levels valine

is not easily removed from the pool by washing and the rate of

efflux does not greatly depend upon the intracellular amino acid

concentration. Furthermore, the amino acid present in the pool

at low concentrations cannot be considered to be "bound" or

compartmentalized (with the exception of proline), for if they were

immobilized in such a manner, then the amino acid theoretically

would not be removed at all by excessive washing. A measurable

rate of efflux does occur at low amino acid concentrations

(Fig. 59), thereby suggesting that perhaps the high affinity

permease does contribute somewhat to the efflux phenomenon.

The mechanism for the maintenance of low amino acid pool levels at high concentration ratios is visualized as operating in a manner similar to the maintenance of high pool levels; that is, that the affinity of the amino acid for the high affinity carrier must be drastically reduced on the inner surface of the cell membrane. The ability of the cell to establish high concentration ratios at low exogenous amino acid levels (Fig. 57), further implies that the difference in the K_m of influx relative to the K_m for efflux must be very large indeed to account for these data.

This interpretation would seem to be borne out by the concentrations of amino acids in the native pool (Table III), and also by the apparent concentration ratios which are established between the native pool and the exogenous fluid. The average concentration for amino acids found in the native pool was 1.93 x 10^{-6} M, and the average concentration ratio was found to be 255. These values are extremely close to the concentration data found experimentally for the amino acid valine (Fig. 57).

It is possible that some degree of compartmentalization of intracellular amino acids occurs at low concentrations and that such a process would prevent the recombination of the amino acid to the carrier which would inevitably result in a concentration phenomenon. However, it is felt that this concept may unnecessarily complicate the actual pool forming mechanism in P. aeruginosa. In E. coli, the pool maintenance ability was attributed to a combination of

the amino acids with "sites" which resulted in the compartment-alization of the amino acid pool (Britten and McLure, 1962). This model was formulated largely to explain why pools in $\underline{E.}$ $\underline{coli}$ are maintained under adverse conditions where they might be expected to leak out. $\underline{P.}$ $\underline{aeruginosa}$ pools are certainly not maintained either at 0 C, in the absence of energy, during nutrient deprivation, or during excessive washing with amino acid free media. Tristram and Neale (1968) also have suggested that the intracellular pool size in $\underline{E.}$ $\underline{coli}$ was a function of permease activity.

Considerable variations between the affinity constants for exit and entrance of amino acids in $\underline{P.}$ $\underline{aeruginosa}$ present an attractive basis for a model for maintaining intracellular pools.

The intracellular space of microorganisms consists partly of an accumulation of metabolic intermediates, any one of which might allosterically affect the function of a transport carrier in a way that would reduce the affinity for the specific transport substrate. This hypothesis is not merely conjecture, since in $\underline{E.}$ $\underline{coli}$, Halpern and Even-Shoshan (1967), demonstrated that the glutamate permease is non-competitively inhibited by aspartate and α-ketoglutarate. Also Boezi and DeMoss (1961), showed that pyruvate inhibited the transport of tryptophan in $\underline{E.}$ $\underline{coli}$. These observations may explain some facets of the accumulation phenomenon in $\underline{E.}$ $\underline{coli}$.

The NaN_3 sensitivity of pool maintenance in $\underline{P.}$ $\underline{aeruginosa}$ may indicate that a high energy intermediate is involved in the alteration of transport functions intracellularly.

Fig. 60(A). Single carrier model for amino acid transport.

A_{out}, amino acid in the external environment;

C, membrane localized carrier protein;

AC, amino acid-carrier complex;

A_{in}, amino acid in the internal amino acid pool;

$K_1 - K_2$, rate constants for the formation and dissociation

of the carrier-amino acid complex.

Fig. 60(B). Double carrier model for amino acid transport in

P. aeruginosa.

Al, amino acid present at low external concentrations ($<10^{-5}$ M);

Ah, amino acid present at high external concentrations ($>10^{-5}$ M).

Ah can react with both C_1 and C_2;

C_1, high affinity permease or membrane carrier;

C_2, low affinity permease or membrane carrier;

AlC_1, amino acid-carrier complex formed at low amino acid

concentrations;

AhC_1, high affinity carrier-amino acid complex formed at

high amino acid concentrations;

AhC_2, low affinity carrier-amino acid complex formed at

high amino acid concentrations;

$K_t$entry and K'_tentry, equilibrium constants for the

reaction A+C $\leftrightarrow$ AC at the outer-membrane surface;

$K_t$exit and K'_texit, equilibrium constants for the

reaction A+C $\leftrightarrow$ AC at the inner-membrane surface;

Diffusion constants for occupied and unoccupied carriers

are presumed to be equal.

During metabolic energy production, $K_t$exit and K'_texit

are essentially negligible possibly through allosteric deactivations

of C_1 and C_2 carrier functions.

OUTSIDE | MEMBRANE | INSIDE

(A)

$$A_{out} + C \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AC \underset{K_4}{\overset{K_3}{\rightleftharpoons}} C + A_{in}$$
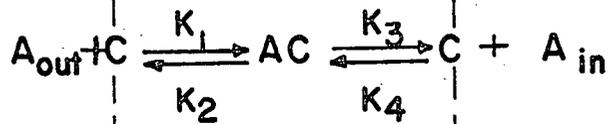
In the steady state: Entry $=$ Exit

For facilitated diffusion: $K_1 = K_4$ assuming $K_2 = K_3$ or

K_2 cannot $> K_3$

For active transport: $AC \underset{K_4}{\overset{K_3}{\rightleftharpoons}} A + C$
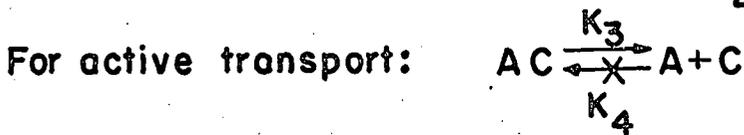
$K_4 < K_1$ due to inactivation of K_4 by a product of energy metabolism.

Similarly for a double carrier system:

(B)
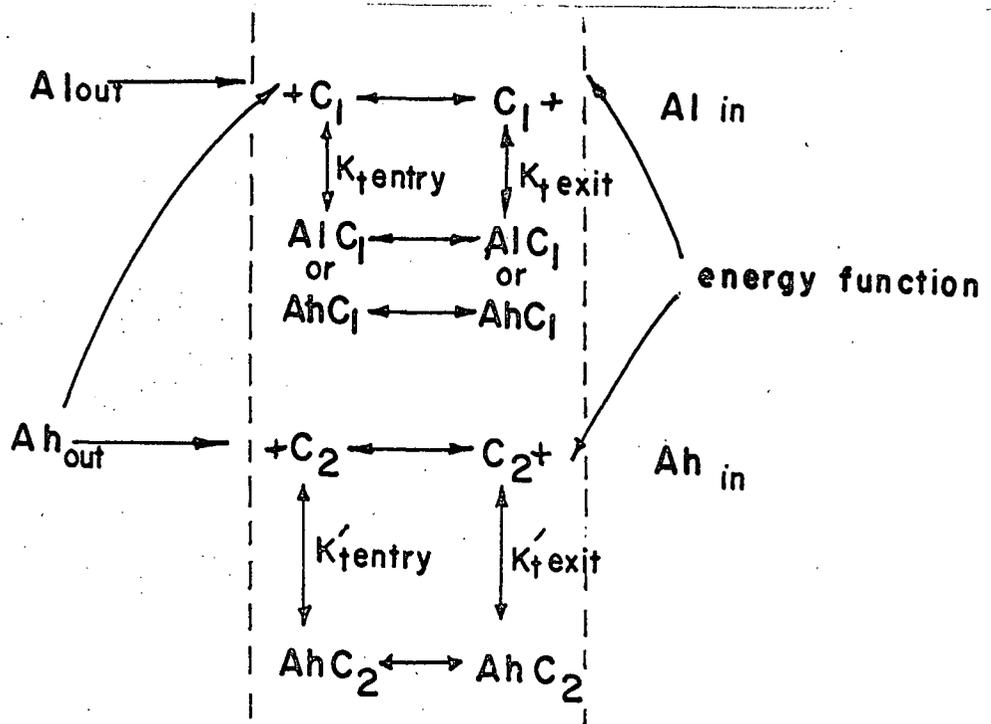
Figure 60 illustrates a hypothetical model devised to explain the amino acid transport and accumulation phenomenon in P. aeruginosa. The amino acid is visualized to collide with stereospecific mobile carriers, to be transported into the cell, and released at the inner membrane surface. The carrier is prevented from recombining with the intracellular amino acid by an energy dependent process and returns to the outer membrane surface unoccupied. This model provides for essentially a unidirectional transport process at low amino acid concentrations with an increasing component of efflux at high amino acid concentrations.

GENERAL DISCUSSION

The enzymatic nature of metabolite incorporation into
cells, first demonstrated with carbohydrates by Rickenberg et
al. (1956), has been extended in recent years to encompass the
amino acids as well as other compounds. The amino acid transport
systems of P. aeruginosa are no exception. These systems have
all the properties ascribed to active transport systems; that
is, transport is seemingly energy dependent, temperature
sensitive, saturated at high substrate concentrations, is lost
by mutation, and results in the accumulation of the incorporated
metabolite at concentrations far exceeding those of the external
environment. The amino acid transport system, as well as accumulation
and catabolism, are under genetic control and these functions may
possibly constitute an operon.

The property of substrate stereospecificity is also
demonstrated by bacterial transport systems. However, this property
varies greatly with the nature of the microorganism. Bacteria have
now been shown to possess a high degree of substrate selectivity,
or stereospecificity, with regards to both sugars and amino acids
(Kepes and Cohen, 1965; Britten and McLure, 1962; FerroLuzzi-Ames,
1964; Behkï and Hochster, 1967). It would seem that this special-
ization diminished with the evolution of the procaryotic to the
eucaryotic cells. Whereas the bacteria maintain highly specialized

permeability functions, Neurospora and Saccharomyces species
tend to lose this property of specificity (Maw, 1963; Grenson,
1966; DeBusk and DeBusk, 1965). This change has been observed
even within one species of bacterium, Agrobacterum tumefaciens
(Behki and Hochster, 1967). The parasitic strain has seemingly
lost the stereospecificity of amino acid transport demonstrated
by the non-parasitic strain.

Apparently animal cells do not possess the high affinity
permeases demonstrated for microorganisms. Although several
distinct, but strongly interacting, amino acid transport systems
have recently been demonstrated with animal cells (Begin and
Scholefield, 1965; Eavenson and Christensen, 1967; Christensen,
Liang, and Archer, 1967), the affinity constants measured are high
(10^{-2} to 10^{-4} M) relative to bacterial affinity constants (10^{-6}
to 10^{-7} M). These animal permeation systems would seem to
correspond more to the low affinity bacterial permeases, which
function at high amino acid concentrations (FerroLuzzi-Ames, 1964),
or to bacterial sugar transport systems (Winkler and Wilson, 1966).

The intracellular metabolic pools of microorganisms are extremely
complex. These pools contain all the soluble intermediates of
anabolism and catabolism and it is not unreasonable that this multitude
of compounds is oriented in different ways within the cell depending
on their metabolic fate. Thus, P. aeruginosa has been shown to
"compartmentalize" putrescine, proline, and probably alanine
intracellularly. It has also been suggested that proline is compart-
mentalized in E. coli (Britten, 1965). Secarz and Gorini (1964),

postulated that endogenous and exogenous arginine contributed

differently toward repressor formation in E. coli. Two distinct

metabolically active pools of tryptophan were demonstrated in

N. crassa (Matchett and DeMoss, 1964). Biosynthetically generated

tryptophan was used preferentially for protein synthesis and

exogenous tryptophan was preferentially oxidized via the

tryptophan cycle.

The maintenance of high intracellular concentrations of amino

acids would seem to be a function of the degradative capacity of the

particular microorganism. Whereas P. aeruginosa was shown to

maintain low internal amino acid concentrations during growth,

E. coli and some fungi maintain high pool levels. However, E.

coli does not deplete its intracellular amino acid pool under

conditions of nutrient deprivation. Hoch and DeMoss (1966),

showed that a constitutive tryptophanase in Bacillus alvei kept

the intracellular pool level of this amino acid to low levels.

P. aeruginosa can concentrate amino acids many thousand

times that of the external environment demonstrating that this

organism can express a high amino acid accumulation capacity

transiently, but it normally does not do so.

The observation that nutrient deprivation (carbon or nitrogen)

does not significantly alter the amino acid transport capacity of

P. aeruginosa may underline the true significance of the existence

of high affinity amino acid, or other metabolite, permeases. McGrew

and Malette (1962), and Marr, Wilson, and Clark (1963), demonstrated

that E. coli utilized small amounts of glucose for energy in

maintaining viability without concomitant growth. Since then
P. aeruginosa catabolizes most amino acids, it would seem logical
that under carbon or nitrogen starvation conditions the high
affinity permeases function to maintain viability, but not to
support growth. In this role, these permeases should indeed be
considered biologically advantageous to the survival of the
species.

The formation and maintenance of intracellular pools of
metabolites is a ubiquitous phenomenon amongst microorganisms.
Mechanisms for concentrating metabolites must have been an early
evolutionary characteristic among life forms, since the ready
availability of precursors for growth would certainly be advantageous
for rapid multiplication. It is feasible that the evolution of
the accumulation process paralleled the decline in available
nutrients which is presumed to have existed in the primordial
soup. However, in the experimental elucidation of transport
mechanisms for amino acids studied in P. aeruginosa, a conscious
attempt was made to formulate a mechanism which was not merely
commensurate with recent models devised for carbohydrate transport,
but which also embodied the facets of simplicity and physiological
feasibility. Thus the unknown "energy function" operating on
the inner membrane surface was postulated to be a high energy
intermediate such as ATP perhaps acting as an allosteric inhibitor
of the amino acid complexing activity of the carrier. Koch (1964),
calculated that one molecule of ATP was required to permit the
active transport of one molecule of β-galactoside in E. coli.

This may be true, but such a mechanism would certainly seem to waste energy and would likely be lost in lieu of a less wasteful mechanism during the course of evolution.

Perhaps one of the most puzzling and as yet unsolved properties of almost all models of carrier-mediated transport is the property of carrier mobility. The distance to be transversed across the membrane exceeds 70 A and it is difficult to visualize how the carrier-amino acid complex moves across the structural barrier of the bacterial membrane.

The multifunctional nature of the bacterial cell membrane underlines not only its importance to the integrity of the cell but also emphasizes its staggering complexity. A large number of specific transport systems for metabolites have now been demonstrated in microorganisms. In addition to the rather large number of permeases described here for amino acids, several have been demonstrated, and in some cases isolated, for carbohydrates (Ganesan and Rotman, 1965), nucleic acids (Peterson and Koch, 1966), polyamines (Tabor and Tabor, 1966), and ions (Pardee, 1966; Peters and Warren, 1968). As more low molecular weight metabolites are tested, increasing numbers of transport functions will undoubtedly be found. Since more than one function has been implicated in the transport of some metabolites, for example the sugars (Winkler and Wilson, 1966), the complexity of the bacterial membrane appears overwhelming.

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