AMINO ACID TRANSPORT IN PROLINE AUXOTROPHS OF *E. COLI*

by

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Presented to the Dean and
Faculty of Graduate Studies
in partial fulfilment of
the requirements for the
Degree of Doctor of Philosophy.

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McGill University, Montreal.

21st March 1966.

ACKNOWLEDGMENTS

The author is greatly indebted to Dr. D. W. Woolley, Rockefeller Institute, New York, for valuable discussions prior to the initiation of this work.

To Dr. Martin Lubin, Harvard Medical School, the author is grateful for the gift of the E. coli auxotrophs used throughout these investigations.

For sustained interest and support during the course of this study, the author is grateful to Dr. J. S. L. Browne, McGill University, to Dr. J. T. Holden, City of Hope Medical Centre, Duarte, California, and to Doctors Alan Ross and Clarke Fraser, Montreal Children's Hospital.

For thoughtful and diligent direction throughout the course of these studies, the author is deeply grateful to Dr. C. R. Scriver.

Thanks are due to Doctors R. M. Johnstone, John Spencer, E. C. Preddie, and P. G. Scholefield for helpful discussions.

For assistance with the determination of Free Fatty Acids and column chromatography of amino acids, the author is grateful to Miss Dorothy Cameron and Miss Eluned Davies and Mr. Jacques Gold.

The author acknowledges the invaluable assistance of Mr. and Mrs. Louis Hebert and Mr. Lapointe during the preparation of this thesis.

To Mrs. Ileana Archdall, the author is greatly indebted for unfailing devotion throughout the typing of this thesis.

The author acknowledges the award of the Edgar M. Wilson Scholarship by McGill University during the session 1964-65.

This work was supported by National Institute of Health Grant No. 05117, and by Medical Research Council Grant No. MT-1085.

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ABBREVIATIONS

ASP	-	Alcohol-soluble protein
ATP	-	Adenosine triphosphate
ATPase	-	Adenosine triphosphatase
CM	-	Chloramphenicol
DNA	-	Deoxyribonucleic acid
DNAse	-	Deoxyribonuclease
DNP	-	2,4-Dinitrophenol
ECF	-	Extracellular fluid
ICF	-	Intracellular fluid
LAC	-	Lipid-amino acid complexes
LUC	-	Lipid-uridine complex
mμC	-	Millimicro-curie
mμM	-	Millimicro-mole
PM	-	Puromycin dihydrochloride
RNA	-	Ribonucleic acid
m-RNA	-	Messenger (informational) ribonucleic acid
s-RNA	-	Soluble (transfer) ribonucleic acid
TCA	-	Trichloroacetic acid
Tr^{-}pro.	-	Inability to transport L-proline actively because of mutation
Tr^{+}pro.	-	Ability to transport L-proline actively
Tris	-	2-Amino-2-(hydroxymethyl)-1,3-propanediol

I. HISTORICAL REVIEW

A. INTRODUCTION

In 1913 Van Slyke and Meyer[1] showed that amino acids are rapidly absorbed from the blood by the tissues of various animals. The authors found that the intracellular concentrations are in excess of those in the blood and concluded that the mode of entry into the cells must be by a mechanism other than simple diffusion. Almost forty years elapsed before these observations were confirmed by Krebs *et al*[2] in 1949. In the meantime, microbes had not been considered as being capable of absorbing (transporting) amino acids from their environment. However, a number of perplexing observations concerning amino acid inter-relationships in the nutritional requirements of micro-organisms had been reported. These observations centred around the ability of structurally related amino acids to permit growth in the presence of inhibitory concentrations of certain amino acids or analogues, or to prevent growth in the presence of an adequate supply of a required amino acid. Thus Gladstone[3] reported that the inhibition of *Bacillus anthracis* by leucine or valine could be overcome by the simultaneous presence of both. A similar relationship was observed between serine and threonine. Aspartate inhibition of *Lactobacillus casei* is

relieved by glutamate[4] and inhibition of Escherichia coli by parahydroxyaspartate is antagonized by aspartate or glutamate[5]. Porter and Meyers[6] observed inhibition of Proteus morganii by threonine, norvaline and norleucine; several amino acids prevent the effects but only methionine consistently prevents inhibition by norleucine. Similar observations concerning the inter-relationship between norleucine and methionine were made by Lampen and Jones[7] with E. coli. Inhibition of Saccharomyces cereviseae by β-2-thienylalanine was found to be antagonized by phenylalanine[8]. In a lysine-requiring mutant of Neurospora crassa, Doermann[9] observed that exogenous arginine inhibits growth although an adequate supply of lysine is available to the cells. An isoleucine/valine-requiring strain of the same organism was found to be inhibited by altering the relative concentrations of these two amino acids[10].

Many of the authors cited drew little or no distinction between utilization and synthesis of the amino acids concerned. In those studies dealing with amino acid auxotrophs, however, it seemed clear that the point of interaction had to do with utilization, rather than with synthesis. In others, speculations as to possible metabolic interconversions were presented but were not substantiated.

In 1947, Gale et al[11-13] and Taylor[14] demonstrated the existence of amino acid transport systems in several gram-positive micro-organisms. The phenomenon makes itself manifest by the appearance of exogenous amino acids in the intracellular water of the cells and provides an acceptable explanation for the amino acid inter-relationships mentioned earlier, and for others to be mentioned later. Taylor[14] reported that gram-negative bacteria, including E. coli, do not accumulate free amino acids from the external medium. This conclusion was based on experiments in which the cells were washed with distilled water prior to extraction of the intracellular pool. Such treatment is now known to remove all internal amino acids from E. coli[15].

B. PROPERTIES OF AMINO ACID TRANSPORT SYSTEMS

Similar to the conclusions drawn by Van Slyke and Meyer[1] for animal tissues, simple diffusion has been ruled out as the mechanism underlying microbial accumulation of amino acids from the external environment. Several unique characteristics, which will be presented shortly, support this view. However, attention should be drawn to a number of terms used in relation to transport phenomena before proceeding to describe these characteristics. Active Transport is used to define the translocation of substrates

across the plasma membrane against a concentration gradient[16] - this is accomplished at the expense of metabolic energy; Facilitated Diffusion defines the transfer of substrates by a mechanism other than simple diffusion, but which still leads to equilibration[17], i.e. transport does not occur against a concentration gradient; Exchange Diffusion defines a mode of access whereby one molecule gains access to the cell's interior by exchanging with another[18]. Although these terms were derived from studies in animal tissues, they are relevant to microbial transport as will be shown later.

1) Dependence of Microbial Amino Acid Transport on Cell Metabolism

Amino acid accumulation is dependent on the presence of a fermentable carbohydrate in the incubation medium. This is one criterion of Active Transport phenomena[16]. The observation was originally made by Gale and Taylor[19] for glutamate accumulation in Staphylococus aureus and was soon shown to be the same for yeast[20]. Halvorson et al[21] made similar observations for glutamate transport in yeast and found that the accumulation of lysine and arginine in this organism has the same requirement. In E. coli, Britten et al[22] found that proline accumulation also requires the presence of glucose, being minimal in its absence or

at 0°C. Cohen and Rickenberg[23] made similar observations for a variety of amino acids in the same organisms. More recently Holden and Holman[24] and Snell et al[25,26] have shown that amino acid accumulation by Lactobacillus arabinosus and by Lactobacillus casei and Strep. faecalis respectively, is dependent on the presence of glucose in the incubation medium. Apparently, other molecules may serve as energy sources. This was pointed out by Gale[27] who found that pyruvate and arginine have some activity in S. aureus.

Further evidence as to the dependence of amino acid transport on cell metabolism has been derived from studies with molecules known to interfere with oxidative metabolism. Thus DNP and azide have been shown to suppress tryptophan accumulation in E. coli[28]; several amino acids in E. coli[23] and various amino acids in Sacch. cereviseae[21,29]. It is possible, however, that these substances affect accumulation other than by uncoupling oxidative phosphorylation. Hence, Gale[30] observed that, at some concentrations, DNP and azide actually increase glutamate accumulation in S. faecalis. This effect was attributed to differential effects of the compounds on the protein synthetic machinery and the metabolic reactions into which glutamate normally

enters. Holden[31] has found the glucose-dependent accumulation of glutamate by L. arabinosus to be markedly resistant to DNP and azide except at high concentrations. Some findings suggest that the exit of amino acids from the cell may be more sensitive to DNP than entry[31].

Generally, sensitivity of accumulation to temperature changes support the view that the entry process involves mechanisms other than simple diffusion. In most cases a drastic reduction in accumulation rate is observed at 0-4°C as compared with 25 or 37°C[22,24]. Temperature coefficients in excess of 1.8 in the range between 18°C-35°C have been observed[11,21].

The pH optimum of accumulation usually corresponds to that at which the fermentation rate is maximal. Hence, in this regard accumulation and metabolism seem to be inseparable. However, lysine uptake in S. faecalis was found to increase steadily as the pH was raised to 9.5, a value very close to the isoelectric point of this amino acid[11], while the uptake of tryptophan in E. coli is optimal at pH 8-8.5[28]. Tryptophan isoelectric point is pH 5.9[32].

Unlike amino acid transport in animal tissues, little is known about the sensitivity of microbial transport to variations in external sodium and potassium

concentrations. Davies et al[33] found that accumulation of glutamate by Staph. aureus, S. faecalis, and Sacch. fragilis is accompanied by uptake of K^+ and that K^+ stimulates glutamate uptake. In the absence of available K^+, accumulation is restricted and is accompanied by Na^+ uptake. Conway and Duggan[34] showed that in yeast the accumulation of basic amino acids is accompanied by a 94-55% increase in Na^+ excretion, while the dicarboxylic and some neutral amino acids cause an increase averaging 11%. The aromatic amino acids, as well as glutamine, asparagine, cysteine, and leucine, give rise to an excretion averaging 41%. These results seem to correlate with the isoelectric points of the amino acids concerned. On the other hand, Avi-Dor and Miller[35] found that in E. coli and Pasteurella tularensis K^+ increases the rate of glutamate accumulation. Mora and Snell[26] observed that amino acid accumulation by protoplasts of S. faecalis respond to variations in Na^+ and K^+ concentrations in a manner similar to mammalian cells, i.e. low K^+ and high Na^+ inhibit, while high K^+ and low Na^+ enhance accumulation. Krebs et al[36] have shown that in Alcaligenes faecalis K^+ loss is accompanied by a loss in respiration.

2) Non-linearity between Rate of Accumulation and External Concentration

The rate of accumulation of extracellular amino acids falls off with increasing external concentration until a maximum is reached. This suggests interaction between the external amino acid and a cellular component or components in limiting quantity and represents another criterion of Active Transport[16]. The observation was made originally by Gale[11] for glutamate accumulation in Streptococcus faecalis. Similar observations have been made by Halvorson et al[21] for glutamate, arginine, and lysine accumulation in S. cereviseae; by Cohen and Rickenberg[37] for valine in E. coli; by Holden and Holman[24] for glutamate uptake in Lactobacillus arabinosus; by Britten and McClure[15] for valine and proline accumulation in E. coli; by Mora and Snell[26] for glycine, L- and D-alanine in S. faecalis; and by Ames[38] for histidine uptake in Salmonella typhimurium. The variation between rate of uptake and external concentration does not indicate whether the rate-limiting component(s) of the process is concerned with the uptake phenomenon per se or with the enzyme systems involved in the energy requirements of the process.

Few exceptions to the generally observed non-linearity between uptake rate and external concentration have been reported. Notable examples are lysine accumulation by S. faecalis[11] and Staph. aureus[19]. However, this exceptional behaviour of lysine does not seem to be true for all strains of S. faecalis, nor for micro-organisms in general, since Holden[39] has observed saturation in another strain of S. faecalis. Halvorson et al[21] has made the same observation for Sacch. cereviseae.

Accumulation in gram-negative bacteria is virtually complete in 1 min. at 37°C[37], a phenomenon which renders accurate measurements difficult to achieve. The process may involve a number of intermediate components but there is no certain knowledge of the rate-limiting process(es). Accumulation rates in gram-negative and gram-positive organisms are comparable, although they are achieved at substantially different external concentrations[31]. Gram-negative organisms accumulate amino acids to 1/5 - 1/20 the level observed in gram-positive ones. This is apparently attributable to differences in cell walls and hence to differences in abilities to withstand high internal osmotic pressures. Thus Britten and McClure[15] have reported that the extent to which proline is accumulated by E. coli is a function of the external osmotic pressure.

This aspect will be dealt with in more detail in a later section.

The accumulated amino acids may enter more than one type of cellular compartment. Cowie and McClure[40] have shown that Candida utilis accumulates amino acids into an expandable, osmotically labile pool from which exchange is possible, and also into an internal (metabolic) pool which is not readily removed from the cells by osmotic shock. This latter pool does not exchange with exogenous amino acids and is composed of endogenously synthesised amino acids in the absence of an exogenous supply. Halvorson and Cohen[29] also found evidence of metabolically distinguishable pools in Sacch. cereviseae, while Zalokar[41] has shown that in Neurospora, exogenous amino acids can bypass a portion of the pool during incorporation into protein. Britten and McClure[15] and Britten et al[22] have observed that proline enters a very specific pool when the extracellular levels are low, whereas at high concentrations, a much larger and relatively non-specific pool is formed. The size of this pool can be increased to very high levels by increasing the osmolarity of the external medium. Ames[38] has found in Salmonella, that each of the amino acids, histidine, phenylalanine, tyrosine, and tryptophan, is accumulated by a highly specific

system, provided the external concentrations are low and that all are transported by a more general non-specific system when the concentrations are high. Information on amino acid pools in gram-positive organisms generally is sparse, but Hancock's[42] findings suggest that in Staph. aureus, there are different proline pools having distinctive exchange properties.

Apart from the complexity of the accumulation process due to its relationships with the energy-yielding systems of the cell, it is further complicated by its apparent ability to discriminate between high and low external concentrations and hence to determine the type of pool that is formed. Thus non-linearity between uptake rate and external concentration may be a reflection, not of interaction between external amino acid and one cellular component present in limited quantity, but rather of interactions between amino acid and two or more cellular components, each present in limited quantity.

3) Competition between Structurally Related Substrates

More recent investigations relating to amino acid interactions during transport have been foreshadowed by earlier reports dealing with inter-relationships in microbial nutrition. Even after the demonstration of

amino acid transport phenomena in micro-organisms, there appeared a number of reports in which interactions between structural analogues were rarely considered in terms of competition during transport. Thus Meinke and Holland[43] reported on the antagonism between serine and threonine in Lactobacillus delbrueckii, Lactobacillus caseii, Leuconostoc mesenteroides and Strep. faecalis. Similar observations were made with regards to D-serine, glycine and alanine in E. coli[44]. Harding and Shive[45] showed that norleucine inhibition of E. coli growth may be antagonised by the addition of methionine, while Brickson et al[46] reported on the antagonism between valine, leucine and isoleucine in Lactobacillus arabinosus and Leuconostoc mesenteroides. They also found that aspartate inhibition of L. arabinosus is relieved by glutamate. E. coli K 12 inhibition by valine is reversed by leucine or isoleucine[47,48]. This observation was confirmed by Cohen et al[49] and by Rawley[50] for several other strains of the same organism. An arginine-requiring mutant of Neurospora is inhibited by exogenous lysine in the presence of an adequate supply of arginine[51,52]. Prescott et al[53] reported that alanine inhibition of Lactobacillus delbrueckii is relieved by serine, while Amos and Cohen[54] showed that a threonine-requiring strain of E. coli is inhibited by the simultaneous presence of

serine. Leucine and lysine auxotrophs of E. coli are inhibited by isoleucine and valine[55] and by various diamines[56] respectively. Haas et al[57] studied a series of histidine-requiring mutants of Neurospora and found their growth to be inhibited by lysine, arginine, and ornithine.

Competitive effects by structurally related amino acids and their analogues provide additional evidence that external amino acids interact with cellular components during transport. Cohen, Umbarger and Brown[58] found that isoleucine interferes with valine uptake in E. coli, and Cohen and Rickenberg[37] showed with the same organism that leucine, valine and isoleucine interact in a common accumulation process which is relatively unreactive with other amino acids. Phenylalanine and methionine accumulation also are reduced only by structurally related substances. In all cases, the process is specific for the L-isomer. A study with valine analogues showed that the amino and carboxyl groups must be unsubstituted. Britten and McClure[15] made essentially the same observations and, in addition, drew attention to the strict structural specificity of a proline transport system. Additional evidence for specificity comes from exchange studies in which related

amino acids and analogues displaced previously accumulated amino acid from the cell. Very good agreement has been observed between the ability of a substance to reduce accumulation and its ability to displace previously accumulated amino acid from the cell[15,37]. A high order of specificity has been reported also for glutamate accumulation in L. arabinosus[24], and for histidine, tryptophan, phenylalanine, and tyrosine in Salmonella[38].

The rather strict structural requirements cited above do not seem to be uniformly encountered. Thus Mandelstam[59] showed that in E. coli and Bacterium cadaveris lysine uptake is inhibited by cadaverine and various diamines, while Mathieson and Catcheside[60] found that histidine accumulation by Neurospora is inhibited by a number of amino acids and various combinations thereof. The D-isomers of methionine in Alcaligenes faecalis[61], of phenylalanine in Sacch. cereviseae[29] and of tryptophan in E. coli[28] reduces the accumulation of the corresponding L-isomer, while D-alanine strongly inhibits the effects of its L-isomer in germinating spores of Bacillus anthracis[62]. The passage of lysine into cells of Strep. faecalis is reduced by acidic amino acids[63]. Broader specificity of the Sacch. cereviseae system is indicated by the ability of valine to reduce phenylalanine accumulation and of

phenylalanine and methionine to reduce valine accumulation[29]. Azaserine has been shown to interfere with tyrosine, phenylalanine, and tryptophan uptake in *E. coli*[64,65].

Accumulation of proline from very dilute solutions is highly specific in *E. coli*, being unaffected by a mixture of fifteen other amino acids present to a concentration 100 times greater than proline[22]. However, when the external concentration of proline is raised, much larger pools are formed and competitive interactions become evident. This suggests that there may be two accumulating systems - one highly specific, the other not so specific. The observation of two apparent Km values (2.5×10^{-6}M and 4×10^{-5}M) lends support to this view. Similar duality in *E. coli* transport of isoleucine, threonine, serine and alanine[66] and in *S. faecalis* glutamate transport[67] have been reported. Transport mutations affecting a specific amino acid also support the suggestion. Lubin *et al*[68] have isolated a number of *E. coli* transport mutants for glycine, proline, histidine, and phenylalanine. Each mutation seems to be independent of the others. Holden and Utech[67] have reported on a mutant of *S. faecalis* in which a high affinity system for glutamate transport is almost completely lacking. On the other hand,

Schwartz *et al*[69] have isolated transport mutants of *E. coli*, in which groups of amino acids (lysine, arginine and ornithine on the one hand, and glycine and alanine on the other) are affected. Maas[70] has recently isolated mutants defective in the uptake of canavanine, arginine, lysine and ornithine. Kepes[66] has suggested that a highly specific transport system may be associated with protein synthesis, while the less specific one may be associated with the establishment of large pools to be used for metabolic purposes. This suggestion gains support from the observation by Ames[38] that in the absence of glucose, the highly specific individual systems do not function, whereas under these conditions, the aromatic amino acids, as a group, enter the cells only by the less specific system. In *E. coli*, however, the competitive interactions between isoleucine, leucine, and valine were observed even at very low extracellular concentrations[15], and in *Strep. faecalis*, glycine, L- and D-alananine share a common system[26].

Further confirmation as to the specificity of amino acid accumulating systems is implied by the induction of specific systems. Boezi and DeMoss[28] have reported on an inducible tryptophan system in E. coli, while

Bibb and Straughn[71] reported on one for citrulline in Strep. faecalis.

Peptides have been shown to be taken up rapidly by bacteria, but by apparently independent systems from those responsible for amino acid uptake. In E. coli, valine peptides were found not to interfere significantly with valine retention by the cells[23], suggesting that they do not interact with the valine accumulating system. These peptides support growth of valine-requiring strains. Similar observations have been made by Kessel and Lubin[72] in that proline peptides support growth of a $Pro^{-}Tr^{-}$(pro.) strain of E. coli. A mutant of E. coli with reduced ability to accumulate glycine was found to grow well on glycine peptides[73]. Leach and Snell[25] showed that glycine peptides are taken up more rapidly than glycine in Lactobacillus casei, while Kessel and Lubin[74] have isolated a mutant of E. coli, which lacks the transport system for glycyl-glycine but which has the ability to concentrate glycine. Thus it appears that separate systems exist for the accumulation of peptides as opposed to amino acids.

4) Retention and Exchange of Accumulated Amino Acids

Gale[27] reported that little or no loss of accumulated glutamate occurs when Strep. faecalis is incu-

bated in water or buffer at 37°C. However, with Staph. aureus, there is some leakage which could be reduced significantly in the presence of glucose. This amino acid is similarly retained by L. arabinosus[31]. This ability to maintain large amino acid pools under adverse osmotic conditions, such as suspension in distilled water, points up a fundamental difference between gram-positive and gram-negative organisms in that under similar conditions E. coli rapidly loses accumulated amino acids[22]. The difference seems to be associated with the relative abilities to withstand osmotic shock and might be related to the differences in cell-wall composition[75]. Although E. coli rapidly accumulates amino acids from buffer at 25°C, little loss occurs under identical conditions[15]. On the other hand, at 0°C the situation is reversed in that the rate of accumulation is much less than the rate of loss, the former being decreased while the latter is increased relative to the 25°C values[15]. The rate of loss is not dependent on the presence of glucose in the medium. These apparently strange characteristics put rather stringent requirements on any scheme that may be presented to account for the mechanism of amino acid accumulation. At 25°C progressively larger fractions of

the proline pool are lost by E. coli as the osmotic strength of the medium is reduced by dilution with water[15].

As indicated earlier, amino acids and analogues which compete with a substrate for uptake are capable of eluting (by exchange) previously accumulated substrate[15,37]. Thus Holden[31] has shown that, although L. arabinosus is capable of maintaining large glutamate pools even in the presence of distilled water, the pool acid is rapidly eluted by external glutamate. Glucose is not required for this exchange. Britten and McClure[15] found that, while the rate of proline uptake by E. coli is reduced by a factor of almost 300 between 25°C-0°C, the exchange rate is only reduced by a factor of about 2. Again, exchange was found to be independent of an exogenous energy supply. It was also found that proline exchange at 0°C does not follow a simple exponential curve, suggesting that there are at least two separate pool components with different exchange rates. This observation was confirmed by Kessel and Lubin[72]. The 0°C exchange displays the same structural specificity as does the uptake process, i.e. small pools of proline are not displaced by a mixture of 15 other amino acids each at a concentration 100 times

that of proline[15,22]. In agreement with their interaction during uptake, excess leucine and isoleucine displace radioactive valine from a pool at 0°C. The rates of exchange are independent of external concentration and are dependent only on the internal pool size[15].

The energy-independent retention of large amino acid pools under physiological conditions is not compatible with the permease concept[76] which predicts a sizeable leak under these conditions. However, it is not a necessary and sufficient condition for the conclusion drawn by Britten and McClure[15] that intracellular absorptive sites exist. This aspect will be discussed further under "Accumulation Mechanisms".

5) Miscellaneous Observations related to Pool Formation

A variety of compounds other than those expected to interfere with cell metabolism or to compete with substrates during transport have been found to interfere with amino acid accumulation. Gale[77] showed that glutamate accumulation in Staph. aureus is reduced by 8-hydroxyquinoline at levels below those which inhibit glycolysis. This effect is reversed by Mg^{2+} and Mn^{2+}. Britten and McClure[15] found that in Mg^{2+}- deficient media the ability of E. coli to accumulate amino acids

is markedly reduced. In this regard Boezi and DeMoss[28] observed an enhancement by Mg^{2+} of tryptophan uptake in E. coli. However, the effects of these manoeuvres on energy supply has not been excluded.

Penicillin has been found to decrease glutamate accumulation by Staph. aureus only after the cells have been allowed to grow in its presence for 30-60 minutes[78]. It is likely that this effect is due to the inhibitory action of the antibiotic on the synthesis of cell-wall components with the result that osmotically fragile cells are produced[75] thereby inducing extensive lysis. However, Maas[79] has suggested that penicillin primarily affects the accumulation mechanism and that the effects on wall synthesis are secondary. The work of Holden[31] on L. arabinosus does not support this contention. The author has found that the antibiotic has little effect on glutamate accumulation by late exponential cultures, while early exponential ones are much more sensitive. Furthermore, the effects of penicillin on the accumulation process can be prevented by high concentrations of sucrose or KCl. It was also pointed out that early exponential cells contain less wall material and that they enhance their ability to accumulate glutamate under conditions permitting further deposition of cell wall in the absence of detectable cell division. Hancock[80]

also found that penicillin interference with amino acid accumulation by Staph. aureus is prevented by the addition of sucrose to the medium.

Gale and Paine[78] reported that chloramphenicol has little or no effect while aureomycin appreciably decreases glutamate accumulation by Staph. aureus at concentrations which do not significantly interfere with glycolysis. Holden[31] has found that chloramphenicol has a consistently slight stimulatory effect on the accumulation of glutamate by L. arabinosus. With E. coli Britten and McClure[15] reported that the antibiotic at 20 μg/ml enhances proline accumulation when the external amino acid concentrations are low and reduces accumulation by a factor of 2 when the concentrations are high. The former effect is explicable in terms of a differential effect of the antibiotic on protein synthesis and on accumulation, but the reasons for the latter effect is not immediately clear.

In L. arabinosus bacitracin, tyrocidin and gramicidin have been found to reduce glutamate uptake[31]. Gale and Taylor[12] showed that tyrocidin causes the release of accumulated glutamate from S. faecalis. It is presumed that this effect is due to interference with the "permeability barrier". Deoxycorticosterone has been reported to interfere with the ability of

germinated conidia of Neurospora to accumulate amino acids[81], however, Holden[31] has not been able to observe any effect by this steroid on glutamate accumulation in L. arabinosus.

Britten and McClure[15] have found that when E. coli cells are subjected to 20,000 p.s.i. pressure for a few minutes, a large portion of the amino acid pool is released to the medium. This is rapidly reaccumulated after restoration of normal pressure but growth is not detected for an hour or more subsequent to restoration.

C. ACCUMULATION MECHANISMS

The information in the preceding sections indicates that, during transit into the cell, external amino acids interact with a cellular constituent present in limiting quantity. It is generally accepted that this constituent resides near the cell surface and is associated with the cell (plasma) membrane. It is also generally assumed that the transported substrates are in the free state within the cell. If this assumption is true, then the plasma membrane may be viewed as a true osmotic barrier in relation to low molecular weight solutes. Any proposal as to the manner in which accumulation takes place must take these aspects into consideration.

1) The Cell (Plasma) Membrane

The plasma membrane was discovered in 1844 by the German botanist, Karl Nageli, who described its physiological importance and suggested its semipermeable properties[82]. Almost a century elapsed before Chambers[83,84] aroused new interest in this structure by virtue of his micro-dissection studies of starfish eggs, amoebae and a variety of other cells. His experiments involved the injection of various dyes into the cells, whereupon immediate colouration of the cells' interior was observed. No such phenomenon occurred when the dyes were placed outside the cell and in this way, the first visual demonstration of the semipermeable character of the plasma membrane was made[82].

Because of the relative ease with which erythrocytes could be obtained, much of the subsequent research dealing with membrane permeability was done with these cells. It soon became apparent from osmotic effects induced by water permeation, that the plasma membrane is not an homogenous structure. The presence of a layer of fatty material which delimits the internal and external environments of the cell was indicated[85]. The existence of such a layer was subsequently demonstrated by Gough[86] who extracted the lipid from erythrocyte

ghosts and concluded that this lipid could best be accounted for if it were to occupy a layer 20-30 Å thick in the membrane.

The rate of permeation of several molecular species into the erythrocyte was found to be strikingly pH-sensitive and metabolically dependent. Thus the Q_{10} values were incompatible with the concept of simple diffusion[87]. Such observations led Davson and Reiner[88] and Danielli[89] to suggest the existence of enzyme-like structures in the membrane and to propose the bimolecular leaflet structure, which assumes a central lipid layer bound on both sides by a layer of protein.

Election microscopy and better staining techniques have aided in the emergence of the concept of the unit membrane[90]. This structure appears as a triple-layered structure of about 75 Å thick. The central layer has been shown to be lipid, while the outer ones are protein. Perhaps the most convincing evidence in support of the Davson-Danielli concept of the plasma membrane is to be found in the work of Stoeckenius[91]. The author has shown that tissue-extracted lipids, when put into an aqueous environment, aggregate to form a 25 Å thick lamellar structure. If protein is then added

in appropriate proportions, this latter associates with the lipid to form a structure identical in appearance to the plasma membrane when viewed by means of the electron microscope.

In all tissues studied to date, from mammalian[90] to bacterial[92], the unit membrane structure has been observed. However, there are marked chemical differences in the percentage composition of the major constituents in membranes of different species. For example, it has been shown that the gross composition of rat liver cell[93] and erythrocyte membranes[94] is about 60% protein, 22% lipid, and 18% phospho-lipoprotein. On the other hand, Weibull and Bergstrom[95] have reported the composition of Bacillus megaterium membranes to be about 68% protein, 18.6% lipid, and 5% carbohydrate, while Gilby et al[96] reported 50% protein, 28% lipid, and 14% carbohydrate for those of Micrococcus lysodeikticus.

Despite these chemical differences, the gross structural arrangement of the constituents is essentially the same. Thus the existence and overall structure of the plasma membrane can be considered as one of the major generalizations in Biology. Its unique structural and compositional characteristics can be expected to

manifest themselves in the form of equally unique functional properties.

2) Evidence indicating the existence of an Osmotic Barrier

Depending on the solubility properties of the migrating molecule, passage across the membrane may be hindered or facilitated by the lipid. Thus the passage of lipophilic molecules is expected to be facilitated while that of hydrophilic molecules is hindered. It has been shown, however, that hydrophilic molecules do gain access to the cell's interior. Such access could be accounted for if one assumes that the membrane has pores[97] large enough to permit the passive diffusion of the permeating molecule. To date such pores have not been demonstrated, although "pore-like" structures have been reported by Goldstein and Solomon[98]. The diameter of these "pores" has been calculated to be about 4.5 Å which is not enough to permit the passage of molecules such as amino acids. It is obvious then that there must be other means by which most molecules of biological importance gain access to the cell's interior.

The existence of "mobile carriers"[18] in the plasma membrane has been proposed to account for transport phenomena. The carriers are assumed to be molecules which render the penetrating solutes more lipophilic

after complexing with them. Several transport models have been constructed on the carrier hypothesis. Facilitated Diffusion[17], assumes stereospecificity and does not lead to the establishment of a concentration gradient. Active Transport[16], which is similar to Facilitated Diffusion, but in addition assumes an irreversible interaction between the carrier and the metabolic processes of the cell, leads to the establishment of a concentration gradient. In both cases it is presumed that the transported solute is in the free state within the cell.

If these models are adequate then, in the absence of an energy source, low-molecular weight solutes should not be concentrated by the cell, i.e. Active Transport should not take place, and Facilitated Diffusion should be the primary process by which the molecules enter the cell. Roberts et al[99], working with E. coli, showed that small molecules, including amino acids, enter the cells giving an internal water space of about 72%. On the other hand, Mitchell and Moyle[100,101] showed that Staph. aureus and protoplasts of M. lysodeikticus are impermeable to small molecules, including lysine and glutamic acid. These conflicting reports led to considerable controversy, which in turn has been responsible

for a great deal of activity in the field. Although the controversy has not been resolved, it is possible that the differences may be related to differences in endogenous respiration since Facilitated Diffusion may be energy dependent[102], differing from Active Transport only in its ability to establish a concentration gradient.

The observations of Mitchell and Moyle[100] are of great importance in demonstrating the existence of an osmotic barrier near the cell surface. These workers showed that the cell wall is not the osmotic barrier since protoplasts of M. lysodeikticus exhibit the same properties of impermeability as intact cells of Staph. aureus[101]. This observation has been confirmed by Weibull[103], who concluded that "the osmotic barrier for small molecular weight solutes is some part of the plasma membrane". These data were obtained from influx experiments and therefore only demonstrate impermeability at the external interface of the membrane. With respect to impermeability relative to the internal interface of the plasma membrane, Mitchell and Moyle[104] and Gilby and Few[105] have shown that protoplasts of B. megaterium and M. lysodeikticus respectively behave as osmometers, implying that molecules within the cell

are osmotically active. Additional evidence comes from the work of Avi-Dor *et al*[106] who reported that the osmotic effects induced by certain solutes in *Pasteurella tularensis* are peculiar to living cells and are dependent on respiration. The ability of intact cells to retain large pools of accumulated substrates when suspended in buffer or distilled water[15,31] may also indicate the presence of an osmotic barrier, but this assumes that the transported material is in the free state.

3) State of Accumulated Amino Acids within the Cell

The fact that accumulated amino acids are freely extractable from bacterial cells with boiling water[11-14] or with cold trichloroacetic acid (TCA) and other solvents, including cold water in the case of *E. coli*[15], does not prove that the substrates are in the free state within the cell. The demonstration of an osmotic barrier near the cell surface does not prove this point either. It is possible that a large portion of the accumulated amino acid is reversibly absorbed to polymers[15] and that treatment with various solvents causes its release to the external medium.

Mitchell and Moyle[101] have shown that the internal osmotic pressure of *Staph. aureus* is about 20-30 atmospheres, a value consistent with the view that the total cold TCA-extractable material is in the free

state within the cell. However, these studies do not indicate to what extent amino acids contribute to the observed osmotic pressure. Mitchell and Moyle[100,104], Gilby and Few[105] and Avi-Dor et al[106,107] have observed optical density changes (indicative of volume changes) when cells and protoplasts of various organisms are exposed to permeating solutes, though generally not with amino acids. Sistrom[108] made similar observations with E. coli spheroplasts when these were exposed to various galactosides. Abrams et al[109] have observed swelling in protoplasts of S. faecalis in the presence of excessive concentrations of amino acids. This observation is not associated with Active Transport phenomena since the amino acids moved down a concentration gradient. On the other hand, Holden[31] has observed swelling is protoplasts of the same organism during active accumulation of glutamate, and further showed that this swelling is enhanced when glucose is added. Wachsman and Storck[110] have described an energy-dependent swelling of B. megaterium protoplasts induced by a variety of amino acids, while Marquis[111] observed this effect in protoplasts of the same organism during α-amino-isobutyric acid accumulation. These data imply that the accumulated substrates are osmotically active but, as Holden[31] points out, it has yet to be proved that

the osmotically active material is in fact the transported amino acid.

Additional evidence suggesting that accumulated amino acids may be in the free state within the cell has been derived from studies with nutritionally defective cells. Holden[112] and Holden and Holman[24] have studied glutamate accumulation in Vitamin B_6-deficient cells of L. arabinosus and have found that the initial rate of accumulation is not modified by the deficiency. There is, however, a marked impairment in their ability to accumulate large pools of this amino acid. This is related to morphological abnormalities[113] which in turn allow the cells to leak unusually large amounts of their internal constituents to the medium[114]. Addition of high concentrations of sucrose[115] or KCl[116] to the incubation medium prevents this leakage.

Electron microscopic examination of B_6-deficient cells showed them to have a thinner than normal wall, while chemical analysis revealed that only about half the normal amount of wall material is associated with these cells. Normal glutamate accumulation could be restored if the cells were allowed to deposit more cell wall when provided with B_6, acetate and NH_4^+[115]. This return to normal is coincidental with the specific deposition of wall material and is not accompanied by any detectable cell division[117,118].

These observations have led the author (Holden) to conclude that the role of Vitamin B_6 in microbial amino acid transport is as a cofactor for the enzyme systems responsible for the deposition of adequate amounts of cell wall, which in turn prevents undue distention of the plasma membrane. This distention is thought to be responsible for the abnormal leakage of internal constituents by B_6-deficient cells. The effect of high sucrose concentrations and adequate amounts of wall material therefore is to counterbalance the internal osmotic pressure of the cells caused by their low-molecular weight solutes. Holden was also able to deduce that there may be various pools of accumulated amino acids which must be osmotically active and that the plasma membrane serves as an osmotic barrier[31]. These conclusions gain support from observations relating to cells having other vitamin deficiencies. Thus biotin- and panthothenate-deficient cells, whose ability to accumulate amino acids is impaired, can be induced to accumulate normally if allowed to deposit adequate amounts of wall material following incubation with acetate plus the required vitamin[119]. It is interesting therefore that Britten and McClure[15] found that <u>E. coli</u> could be induced to accumulate larger than

normal pools of proline if the external osmotic pressure is increased.

4) Schemes proposed to account for Amino Acid Accumulation

At present there are two well-known models which have been presented, each in its own way accomodating a major portion of the experimental data.

a) The Permease Model[76]: This model is based on the following assumptions:

i) The cell is enclosed by an osmotic barrier which is highly impermeable to amino acids;

ii) The impermeability is not absolute and leakage may occur, tending to equilibrate the internal and external concentrations;

iii) Specific transport catalysts (permeases) are associated with the membrane and are capable of complexing with the amino acid;

iv) The complex associates and dissociates reversibly with amino acid on either interface of the membrane and catalytically activates the equilibration of internal and external concentrations;

v) When coupled with cell metabolism, the internal association reaction is inhibited so that the amino acid accumulates inside the cell;

vi) As the internal concentration increases, the rate of leak increases until it equals the rate of influx, thus leading to equilibration;

vii) The amino acid is presumed to be in the free state within the cell.

It has been established that the cell is contained by a semipermeable plasma membrane. However, this semipermeability is related to different molecules and not to the same molecular species under different circumstances. Thus Mitchell and Moyle [100,101] have shown that certain molecules permeate the plasma membrane of resting bacterial cells, while others, lysine and glutamate included, do not. In addition, large pools of amino acids are not lost by both gram-positive and gram-negative cells when they are suspended in buffer at 25°C[15,31]. These observations do not support the permease model since it predicts: 1) that in the absence of an energy source, amino acids should enter the cell; and 2) when the cells have a large internal pool, a sizeable leak should take place.

Observations concerning temperature dependence, non-linearity between rate of accumulation and external concentration, and the specificity of accumulating systems are compatible with this model. However, as Britten and McClure[15] have pointed out, one of the characteristics of the Permease Model is that the circulating flow should be proportional to

the pool size and that this flow in turn should be identical with the initial rate of pool formation at equilibrium. In other words, the pool should inhibit its own formation. The experimental data do not support this prediction.

b) The Carrier Model[15]: This model was conceived in an attempt to account for the observed characteristics of amino acid pools[15]. It assumes the following:

i) The cell contains a small quantity of mobile stereo-specific carriers which freely form complexes (AE) with amino acids, without participation of energy donors;

ii) The cell also contains a relatively large quantity of non-mobile groups (the sites) which form complexes (AR) with the amino acids;

iii) The site complex, AR, can be formed only through a reaction with the carrier complex, AE, and this reaction is coupled to an energy donor;

iv) Exchange may occur between the site-associated and carrier-associated amino acids without coupling to energy donors;

v) Exchange also occurs between free amino acids and carrier-associated amino acids, but not between free amino acids and site-associated amino acids;

vi) There are several classes of sites, some stereospecific and others nonspecific;

vii) There may be an osmotic barrier near the cell surface and free amino acids may diffuse through the protoplasm at the same rate as in water, but the formation of the carrier complex nevertheless occurs at a sufficient rate without participation of an energy donor.

An interesting feature of this model is that, unlike the Permease Model, the plasma membrane plays no active role in the accumulation process. Instead, a number of intracellular carriers have been postulated. The impermeability of the cell to amino acids in the absence of an energy source is not compatible with this concept.

The main feature of this model involves the transfer of carrier-associated amino acid to the binding sites which are supposed to hold the accumulated amino acid within the cell and thus account for the various characteristics of the pool. Thus it implies that intracellular amino acids cannot be osmotically active. Information presented earlier, relative to the state of accumulated substrates within the cell, cannot be accomodated by such a scheme. Furthermore, it has been shown that <u>L. arabinosus</u>

can lose as much as 25% of its internal RNA and substantial amounts of protein without significant alteration in its capacity to accumulate glutamate[31]. Apart from the absorption of lysine to the cell walls of M. lysodeikticus[120,121], there is little direct evidence for the association of sizeable amounts of pool amino acids with subcellular fractions.

5) Possible Nature of Transport Catalysts

Monod[122] and Cohen and Monod[76] have shown that the inducible capacity of E. coli to accumulate β-galactosides is inhibited by inhibitors of protein synthesis. Schwartz et al[69] and Lubin et al[68] have presented evidence showing that mutation can affect the transport of individual amino acids in E. coli. Maas[70] has shown that mutation can also affect the transport of a group of amino acids in this organism. Boezi and DeMoss[28] and Bibb and Straughn[71] reported on the inducibility of a tryptophan accumulating system in E. coli and of a citrulline system in S. faecalis respectively. Lark and Lark[123] showed that the methionine accumulating activity of synchronously dividing Alcaligenes faecalis remains constant throughout the division cycle, indicating that the synthesis of the accumulation machinery is closely synchronized with

the reproductive cycle of the cell. These data imply that the accumulation catalysts may be protein in nature.

The activating enzymes, known to be involved in protein synthesis, have been suggested as amino acid transport catalysts[124]. This view gains support from reports indicating that these enzymes may be associated with the plasma membrane[125,126]. To account for the non-lethal effect of amino acid transport mutations which might result if these enzymes function as transport catalysts, Mitchell[127] has suggested that such mutants may have a change in the location of the specific activating enzyme so that it may be synthesised without being incorporated into the membrane. The extreme specificity of these enzymes is not compatible with a role as transport catalysts, however, since, as has been shown earlier[15], amino acid accumulating systems can be relatively non-specific.

Christensen *et al*[128,129] observed that Vitamin B_6 increases the accumulation of amino acids by Ehrlich ascites tumor cells. The possibility was entertained that this vitamin may serve as an amino acid carrier during transport, but the observations of Holden[112-119] and Mora and Snell[26] do not support this view.

Christensen[130] has subsequently shown that B_6 does not participate in the entry of amino acids into Ehrlich ascites cells.

A number of lipid complexes of amino acids have been postulated as being involved as carriers during transport. Thus Gaby *et al*[131] and Tria and Barnabei[93] have concluded that such complexes are involved in amino acid transport in Ehrlich ascites cells and rat liver cells respectively. Hendler[132] has presented data implicating a "proteo-lipid" in the transport of amino acids in hen oviduct. Hunter and James[133], on the basis of compositional differences between the lipid of a "lipo-arginine" complex and those of neutral amino acid complexes, have suggested that these may function as amino acid carriers during transport in *B. megaterium*. On the other hand, Hunter and Goodsall[134], Macfarlane[135], Brady[136], and Gale and Folkes[137], oppose this view. Britten and McClure[15] have suggested that the "lipid-amino acids" found by Hendler[138] to be formed in *E. coli* may serve as the intracellular carriers proposed by their model.

In other transporting systems, evidence in support of the participation of metabolic enzymes is beginning to accumulate. Thus the movement of sodium and potassium

across nerve[139] and erythrocyte[140-144] membranes has been shown to be associated with a membrane adenosine triphosphatase (ATPase). Lubin and Kessel[145] and Shultz and Solomon[146] have isolated mutants of E. coli which are defective in their ability to accumulate potassium. It has been shown that in these mutants, the Na+-K+-dependent membrane ATPase activity is greatly reduced[147].

The interrelationship between cation transport and that of organic molecules was first demonstrated by Riklis and Quastel[148]. At about the same time, Riggs et al[149] made the observation that amino acid influx into Ehrlich ascites cells is regulated by the intracellular K^+ concentration, while Heinz[150] called attention to the regulatory role of external Na^+ concentration. Similar observations have been made for a variety of other tissues[151-153]. Thus the indirect participation of the membrane ATPase in transport phenomena generally, can be envisioned. However, there is no substantive evidence for this view in bacterial amino acid transport except for the isolated case reported by Mora and Snell[26]. It would be of interest to know how amino acid transport and the transport of other organic molecules, is affected in the K^+ transport mutants to which reference was made earlier[145,146].

Such information may give some idea as to how metabolic energy is utilized during transport.

D. SUMMARY

Amino acid transport in micro-organisms is stereo-specific and metabolically dependent. The phenomenon obeys Michaelis-Menten kinetics and is associated with the plasma membrane. Structurally related amino acids interact competitively during transport, giving rise to interpretative problems as to the specificity of transport systems, since it appears that there are two types of systems for each amino acid or group of amino acids. Accumulated substrates are probably in the free state within the cell because of osmotic effects which are related to the accumulated solutes. The maintenance of large pools of accumulated amino acids is energy independent, being insensitive to the absence of an energy source (fermentable carbohydrate). In gram-positive organisms, these pools are stable even when the cells are subjected to severe osmotic stress; while in gram-negative organisms, the pools are lost under similar conditions. This difference is attributable to differences in the nature of the cell walls of these two types of organisms. The mechanism by which amino acids enter the cells is unknown, although it can be said with certainty that simple diffusion does not contribute significantly to the process. Each model presented

accounts for a considerable portion of the observed characteristics of amino acid transport but has been found to have serious defects. A compromise, in which most of the major features of both models are maintained, is conceivable and would likely be more adequate in defining the mechanism underlying amino acid transport.

II. PURPOSE OF THE STUDY

On the basis of transport in mammalian tissues, amino acids can be grouped into at least four main classes[154,155]:

a) the dibasic amino acids and cystine, affected as a group in the heritable disorder known as cystinuria[156];

b) the "neutral" amino acids, affected as a group by the Hartnup mutation[157,158];

c) the imino acids and glycine, spared as a group (neutral) by the Hartnup mutation[159,160];

d) the dicarboxylic amino acids, shown by Webber[161] to be segregated from the other groups on the basis of infusion studies in dogs.

Because of the considerable evidence favouring the above groupings, little had been done to determine whether the members of a group share a common transport system, or whether each member has a preferential system with some common locus uniting them into a group. Recently, however, Scriver and Wilson[154] have shown that the imino acids and glycine are not transported by a common system in the rat kidney, but instead by three non-identical systems, each bearing some resemblance to the others.

In micro-organisms the situation is more complicated relative to amino acid grouping on the basis of transport. For some amino acids (e.g. proline) highly specific systems

are present while for others the degree of specificity is considerably less; e.g. valine, leucine and isoleucine. Observations to the effect that each amino acid or group of amino acids may have two types of transport systems[15,38] further complicates the picture. Studies with transport mutants have indicated that the accumulation of a specific amino acid[68,69] or a group of amino acids[38,70] may be affected. If it could be shown that a group of amino acids interact during accumulation for the same reasons as in the rat kidney, then the complex situation could be clarified significantly; i.e. inhibition by related amino acids could be due to the existence of individual non-identical systems with overlapping affinities.

An important question raised by such a possibility involves the nature of a transport mutation. If accumulating systems consist of a reactive site and a carrier component as suggested by Christensen[130], then a transport mutant conceivably could have a mutation involving either of these structures. The concept of a reactive site is well supported by experimental data but there is little to substantiate that of separate carrier molecules. Various "lipid-amino acid complexes" have been suggested by some workers[15,131-133] as transport intermediates although this view has been opposed by others[134-137].

The purpose of the studies to be reported is therefore fourfold:

a) to investigate the "commonness" of the valine-leucine-isoleucine and lysine-arginine transport systems in *E. coli*;

b) to investigate the identity or non-identity of the two transport systems which may exist for each amino acid or group of amino acids;

c) to investigate the expression of an amino acid (proline) transport mutation;

d) to investigate the possible role of "lipid-amino acid complexes" as transport intermediates.

III. EXPERIMENTAL

A. SPECIFICITY OF AMINO ACID TRANSPORT SYSTEMS AND THE EFFECT OF MUTATION ON THE L-PROLINE SYSTEM OF E. COLI

1) Materials

a) Compounds:

L-proline-U-C^{14} (200 mC/mM), L-lysine-U-C^{14} (223 mC/mM), L-arginine-U-C^{14} (222 mC/mM), L-valine-U-C^{14} (208.5 mC/mM), L-leucine-U-C^{14} (222.6 mC/mM), and L-isoleucine-U-C^{14} (250.2 mC/mM) were obtained from the New England Nuclear Corp. Hydroxy-L-proline-H^3 (12 mC/mM) was purchased from Volk Radiochemicals. Chloramphenicol and Penicillin G were obtained through the hospital pharmacy. Agar, L-proline, L-arginine, L-lysine, L-valine, L-leucine, L-isoleucine, OH-L-proline, L-ornithine, and L-canavanine were purchased from Nutritional Biochemicals Corp. DL-pipecolic acid was obtained from Mann Research Laboratories. Eastman chromatogram sheets (Type K30 IR - Silica Gel) were obtained from Fisher Scientific Co. All other compounds were of reagent grade.

b) Organisms:

E. coli strains W-6(Tr^+pro.) and W-157(Tr^-pro.) were kindly donated by Dr. Martin Lubin, Harvard Medical School. Both strains are proline auxotrophs

having a block between L-pyrroline carboxylate and proline[162]. W-157 differs from its parent W-6 in its ability to accumulate L-proline actively[72].

c) Media:

The basic medium used throughout these studies was that described by Davis and Mingioli[163] and consisted of the following: K_2HPO_4-0.7%, KH_2PO_4-0.3%, Na_3-citrate-0.05%, $MgSO_4$-0.01%, $(NH_4)_2SO_4$-0.1%, glucose-0.2%. Liquid media were supplemented with 250 or 500 µg/ml L-proline. Solid media were prepared by adding 1.5% agar and supplemented with 0, 10 or 500 µg/ml L-proline.

2) Methods

a) Maintenance of Strain Purity:

Contamination by virtue of back mutation was prevented by the following procedures: W-6 was maintained by suspending the organism in 25 ml sterile medium to a density of 1 mg/ml (O.D. of 0.3 at 650 mµ) and supplemented with 2000 IU/ml Penicillin G. The suspension was shaken at 35-37°C in a water bath for 8-12 hours. At the end of this time the surviving cells were harvested by centrifugation and washing. This was followed by resuspension in 10 ml sterile medium supplemented with 10 µg/ml L-proline and 10% glycerol. The resuspended cells

were stored at -20°C. W-157 was first suspended in 25 ml sterile medium containing 10 µg/ml L-proline and 2000 IU/ml Penicillin G. After shaking for 8-12 hours at 35-37°C, the surviving cells were harvested, washed and resuspended in the same volume of medium containing only Penicillin at 2000 IU/ml. They were then taken through the cycle as with W-6. The surviving cells were suspended in 10 ml sterile medium containing 10 µg/ml L-proline and 10% glycerol and stored at -20°C.

b) Growth Conditions:

Portions of sterile medium, containing 250 or 500 µg/ml L-proline, were inoculated with 1 ml of bacterial suspension and the cells allowed to replicate at 37°C. Aerobic cells were grown with forced aeration while anaerobic cells were grown without aeration. After 16 hours, at which time the cells were in the early stationary phase, they were harvested by centrifugation and washed with two 100 ml portions of saline. The cells were then resuspended in minimal medium without proline supplements.

c) Incubation with labelled amino acids:

Aliquots of cell suspensions of desired density (2.5 - 3 mg/ml dry wt.) were preincubated (starved for proline) for 30 mins. at 37°C with shaking.

Chloramphenicol(CM) was then added to 100 µg/ml and preincubation allowed to continue for an additional 5 mins. At zero time, the C^{14}-amino acid was added (0.02 µC/ml) with varying concentrations of C^{12}-amino acid. H^3-hydroxyproline was added to 0.16 µC/ml. At the desired times aliquots were withdrawn for assessment of radioactivity.

d) Extraction and counting of radioactivity:

Labelled aliquots of cell suspensions were added to 2.5 volumes of ice-cold medium and centrifuged at 0-4°C. The clear supernatant was decanted and the insides of the tubes swabbed dry. The cells were then extracted with 1 ml distilled H_2O at 100°C for 5 mins. Cell debris was sedimented by centrifugation and 0.2 ml aliquots of the clear supernatant assessed for radioactivity. After drying on glass planchettes C^{14} was assessed by means of a gas-flow counter operating at 20% efficiency. H^3-labelled preparations were first taken to dryness in counting vials, redissolved in 0.2 ml anhydrous-methanol and then 10 ml of scintillation mixture* were added. Radioactivity was assessed in a

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* 50 mg 1,4-bis-2 (5-phenyloxazolyl)-benzene and 2 gm 2,5-diphenyloxazole dissolved in 500 ml toluene.

Packard Tricarb Scintillation Counter operating at 20% efficiency.

e) Determination of ICF: ECF ratios:

Anaerobic cell suspensions of 2.5-3 mg/ml dry wt. were incubated in the presence of 10 µg/ml of labelled amino acids and the internal radioactivity assessed. ICF volume was taken as 3 µl/mg dry wt. according to Kessel and Lubin[72]. Counts/min/ml of ICF were obtained by means of the following formula:-

$$\frac{\text{Total cpm/aliquot}}{\mu\text{l/aliquot}} \times \frac{1000}{1}$$

These values were expressed as fractions of the known (added) cpm/ml ECF to give ICF:ECF ratios.

f) Identification of transported material:

Aliquots of pooled cell extracts were concentrated by evaporation at 80-90°C and applied to Silica Gel G plates. These were developed for 3 hours in n-butanol:acetic acid:H_2O(4:1:1 v/v), dried and cut into squares of 1 cm which were serially counted in a gas-flow counter. The positions of the radioactive spots were compared with those of adequate controls. OH-L-proline was not done.

g) Kinetic Analyses:

i) Determination of Km values:

The filter technique of Roberts et al[99] as modified by Kessel and Lubin[72], in conjunction with the reciprocal plots according to Lineweaver and Burk[164], was used throughout these studies. A low and high concentration range was used for each amino acid investigated. One ml of cell suspension (1 mg/ml wet wt.) was added to varying amounts of unlabelled amino acid - [S] + 0.02 μC C^{14} or 0.16 μC H^3 contained in 25 ml erlenmeyer flasks at room temperature. After a 30 sec. exposure, 0.5 ml was withdrawn and poured onto a millipore filter assembly attached to a vacuum motor. The medium was drawn off within 1 sec. The filters were removed, cemented to aluminium planchettes and air dried. Radioactivity was assessed, corrected for a blank and expressed in μM/min/mg (v). Km values were determined by plotting the reciprocals of v versus [S] and taking the intercept on the x-axis as (- 1/Km). The intercept on the y-axis was taken as (+ 1/V max).

ii) Determination of Ki values:

Various amino acids and analogues were used as inhibitors. The technique was as described above, i.e. substrate and inhibitor were present in the flask prior to the addition of the cells. The new intercept on the x-axis was taken as (-1/Kmi) - the apparent Km of the substrate in the presence of the inhibitor. Ki values were derived from the following equation:

$$Ki = \frac{[I]}{(Kmi/Km)-1}$$

where [I] is the inhibitor concentration.

Similar values were not derived when the inhibitor was non-competitive.

iii) Determination of Identity or Non-identity of Transport Systems:

The principle of the "A-B and C-test", as introduced by Ahmed and Scholefield[165] and as utilised by Scriver and Wilson[154], was used. The principle assumes the following:

a) there are two (or more) transport systems A and B which may or may not be identical;

b) two substrates "a" and "b" are transported preferentially by A and B respectively;

c) substrate "a" also demonstrates some affinity for B and substrate "b" some affinity for A - inhibition must be competitive.

If A and B are identical, then the Km value of "a" on A should equal the Ki value of "a" on B. Conversely Km of "b" should equal Ki of "b". On the other hand, if the Km and Ki values of each substrate are not comparable, then the non-identity of A and B is indicated. Confirmation comes from the use of a third (or fourth) substrate "c", the Ki values of which (when used to inhibit "a" and "b") should be comparable.

h) Cell Density and the Accumulation of L-proline:

Suspensions varying in density from 1-8 mg/ml wet wt. were incubated with 10 µg/ml C^{14}-proline. After 1 min. exposure, aliquots containing 1 mg of cells were poured onto millipore filters and the medium removed. The dried filters were assessed for radioactivity and the values expressed as a percentage, taking that of the 1 mg/ml suspension as 100%.

3) Results

a) Purity of Strains:

After 24 hours W-6 (Tr^+pro.) grew well on plates supplemented with 10 µg/ml L-proline but did not on plates without this supplement. W-157 (Tr^-pro.)

grew on 500 µg/ml supplements but not on 10 µg/ml nor on plates without supplemental L-proline.

b) Identity of transported material:

The radioactive materials extracted from the cells after incubation with C^{14}-amino acids for 1 min. at 37°C were found to be comparable with those added to the medium. Table I shows that there is close agreement between the R_f values of the extracted radioactivity and those of controls. Only one radioactive spot was found for each amino acid.

TABLE I

COMPARISON BETWEEN HOT WATER-EXTRACTED C^{14}-MATERIAL AND THAT ADDED TO THE MEDIUM PRIOR TO INCUBATION

Amino acid-C^{14}	R_f-values	
	Control	Extracted
L-leucine	0.47	0.47
L-isoleucine	0.46	0.43
L-valine	0.35	0.33
L-proline	0.19	0.20
L-arginine	0.08	0.10
L-lysine	0.05	0.06

c) Distribution (ICF:ECF) Ratios:

The distribution ratios between intracellular and extracellular environments after 1 min. exposure at 37°C are shown in Table II. Those for W-157

compare favourably with those of W-6 with the notable exception of L-proline. The results also indicate that hydroxy-L-proline is not accumulated actively by these cells.

TABLE II

ICF:ECF RATIOS AFTER 1 MIN. EXPOSURE
(HIGH DENSITY SUSPENSIONS)
TO 10 μg/ml LABELLED AMINO ACIDS AT 35-37°C

Amino Acid	Distribution (ICF:ECF) Ratios W-6(Tr^+pro.)	W-157(Tr^-pro.)
L-lysine-C^{14}	9.0	8.8
L-arginine-C^{14}	7.5	7.6
L-valine-C^{14}	8.5	8.6
L-leucine-C^{14}	9.0	9.2
L-isoleucine-C^{14}	8.0	7.8
OH-L-proline-H^3	1.0	1.0
L-proline-C^{14}	10.0	1.0

d) Km and V max. values for Transport in W-6:

Table III shows the kinetic constants which describe the relationship of the substrates to their transport systems over two widely differing concentration ranges. It is clear that for each amino acid there are two types of systems which saturate at significantly different external concentrations and which have different maximum rates of accumulation. Fig. 1 (curve labelled a-b) shows the type

TABLE III

KINETIC CONSTANTS DESCRIBING RELATIONSHIP OF SUBSTRATES TO THEIR TRANSPORT SYSTEMS IN W-6

Amino Acid	Concentration Range* µM/ml	Km	V max. µM/min/mg
L-lysine-C^{14}	0.34 - 2.1	$2.5 \times 10^{-6}M$	0.67
	6.80 - 27.3	$8.0 \times 10^{-5}M$	12.50
L-arginine-C^{14}	0.29 - 1.15	$2.5 \times 10^{-6}M$	0.57
	5.70 - 22.80	$5.7 \times 10^{-5}M$	10.00
L-valine-C^{14}	0.43 - 1.71	$3.3 \times 10^{-6}M$	0.67
	3.42 - 17.10	$5.0 \times 10^{-5}M$	8.00
L-leucine-C^{14}	0.38 - 1.53	$2.5 \times 10^{-6}M$	0.57
	3.10 - 15.30	$4.0 \times 10^{-5}M$	6.70
L-isoleucine-C^{14}	0.38 - 1.53	$1.4 \times 10^{-6}M$	0.35
	3.10 - 15.30	$1.7 \times 10^{-5}M$	3.30
L-proline-C^{14}	0.43 - 2.61	$2.5 \times 10^{-6}M$	0.77
	8.80 - 34.80	$1.25 \times 10^{-4}M$	25.00
OH-L-proline-H^3	7.2 - 30.50	$4.80 \times 10^{-5}M$	0.43

* These were the concentration ranges used throughout the studies except where otherwise stated.

of curve obtained for each amino acid over these concentration ranges. In all cases the ratio between the high and low Km values is greater than that between the corresponding V max. values. This is in accord with the observations of Britten and McClure[15] regarding the formation of proline pools through transport. Hydroxy-L-proline does not seem to have

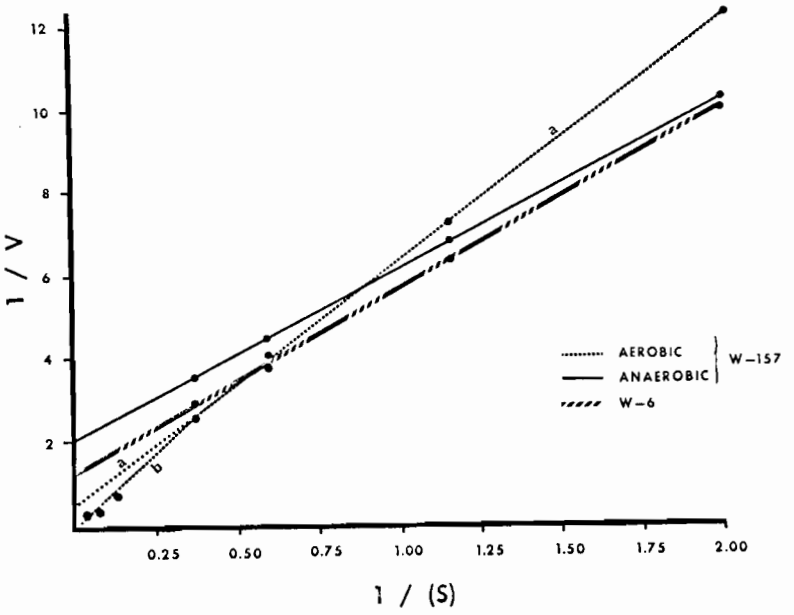

Fig. 1 Reciprocal plots of C^{14}-proline accumulation by low density suspensions of W-6 and W-157 at 25°C.

been accumulated actively since its V max. value is about 60 times less than that of L-proline at a comparable concentration range. This is in accord with its ICF:ECF ratio - Table II.

e) Specificity of Transport Systems in W-6:

i) The Lysine-Arginine Systems:

These systems were found to be independent of the valine-leucine-isoleucine and the proline

TABLE IV

THE LYSINE-ARGININE SYSTEMS

a. Relationship of Competitive Inhibitors to the L-lysine-L-arginine High-affinity (low Km) System. Concentrations were: L-lys. 1.4, L-arg. 0.6, L-ornith. 0.75, and L-canav. 0.60 μM/ml.

		Competitive Inhibitors - Ki values			
Substrate	Km	L-lysine	L-arginine	L-ornithine	L-canavanine
L-lysine	2.5×10^{-6M}	-	1.0×10^{-6M}	8.6×10^{-7M}	0.00
L-arginine	2.5×10^{-6M}	3.6×10^{-6M}	-	2.2×10^{-6M}	2.2×10^{-6M}

b. Relationship of Competitive Inhibitors to the L-lysine-L-arginine Low-affinity (high Km) System. Concentrations were: L-lys. 27.4, L-arg. 11.5, L-ornith. 15.0, and L-canav. 11.5 μM/ml.

		Competitive Inhibitors - Ki values			
Substrate	Km	L-lysine	L-arginine	L-ornithine	L-canavanine
L-lysine	8.0×10^{-5M}	-	2.7×10^{-5M}	1.7×10^{-5M}	6.0×10^{-5M}
L-arginine	5.7×10^{-5M}	4.3×10^{-5M}	-	4.9×10^{-5M}	1.8×10^{-5M}

systems in that members of these groups had no inhibitory effects on either the low Km or high Km systems within the concentration ranges used. However, lysine competitively inhibited arginine accumulation and vice-versa. L-ornithine and L-canavanine also showed competitive effects - Tables IVa and IVb. Note that in no case is there a correspondence between the constants of each amino acid, a fact which is not in accordance with identity of the systems involved.

ii) <u>The Valine-Leucine-Isoleucine Systems</u>:

These systems are also independent of the lysine-arginine and the proline systems. The three amino acids were found to competitively inhibit each other's uptake by both high and low affinity systems. However, with the high affinity (low concentration range) studies, the Km and Ki values of each substrate do not correspond, implying non-identity of the systems - Table Va. On the other hand, studies on the low affinity system showed that the Km and Ki values of each substrate do correspond - Table Vb. Note that on this system isoleucine

TABLE V

THE VALINE-LEUCINE-ISOLEUCINE SYSTEMS

a. Reciprocal relationships between L-valine, L-leucine, and L-isoleucine to the High-affinity Transport System. Concentrations were: L-val. 1.7, L-leu. 1.5, and L-ileu. 1.5 μM/ml.

		Competitive Inhibitors - Ki values		
Substrate	Km	L-valine	L-leucine	L-isoleucine
L-valine	3.3×10^{-6M}	-	1.8×10^{-6M}	9.0×10^{-7M}
L-leucine	2.5×10^{-6M}	9.5×10^{-6M}	-	3.7×10^{-6M}
L-isoleucine	1.4×10^{-6M}	1.2×10^{-5M}	1.1×10^{-5M}	-

b. Reciprocal relationships between L-valine, L-leucine, and L-isoleucine to the Low-affinity Transport System. Concentrations were: L-val. 12.2, L-leu. 11.0, and L-ileu 11.0 μM/ml.

		Competitive Inhibitors - Ki values		
Substrate	Km	L-valine	L-leucine	L-isoleucine
L-valine	5.0×10^{-5M}	-	3.9×10^{-5M}	1.8×10^{-5M}
L-leucine	4.0×10^{-5M}	4.8×10^{-5M}	-	1.8×10^{-5M}
L-isoleucine	1.7×10^{-5M}	4.9×10^{-5M}	3.8×10^{-5M}	-

has the highest affinity (lowest Km value) and lowest V max. value, while the reverse is true for valine - cf. Table III. This suggests that for any system a reciprocal relationship exists between affinity for a substrate and its maximum accumulation rate.

iii) The Proline Systems:

These systems were found to be independent of those previously described. The high affinity system was found to be resistant to 1.6 mM OH-L-proline and 3.3 mM DL-pipecolate, indicating extreme specificity. On the other hand, the low affinity system was susceptible to 15 mM OH-L-proline and 33 mM DL-pipecolate - Table VI. Note that the Km and Ki values of proline and of OH-L-proline are comparable. The implications of this observation is clear. However, the non-competitive effect of DL-pipecolate on OH-L-proline is not immediately clear.

f) Effect of Mutation on the L-proline System:

i) Variations in Km and V max. values with growth conditions:

Early stationary phase aerobically grown cells of W-157 ($Tr^-pro.$) were found to have the

TABLE VI

THE PROLINE SYSTEMS

Relationship of Competitive Inhibitors to the Low-affinity Proline System. Concentrations were: L-pro. 17.4, OH-L-pro. 15.0, and DL-pip. 33.0 μM/ml.

		Competitive Inhibitors - Ki values		
Substrate	Km	L-proline	OH-L-proline	DL-pipecolate
L-proline	1.25×10^{-4M}	-	4.8×10^{-5M}	9.4×10^{-5M}
OH-L-proline	4.8×10^{-5M}	1.2×10^{-4M}	-	N.C.*

* non-competitive effect.

same kinetic constants as W-6 (Tr^{+}pro.) relative to the lysine-arginine and valine-leucine-isoleucine systems. However, these cells showed a difference in Km and V max. values for proline on the high affinity system - Table VII. The Km value of 1×10^{-5M} indicates that in aerobically grown cells, the system has an affinity for proline lower than that of comparable cells of W-6. Anaerobically grown cells showed an interesting difference from control in that the Km and V max. values changed drastically. The Km value changed from 1.0×10^{-5M} to 1.5×10^{-6M}, indicating that the affinity for proline increased by a factor of about 7 - cf. Fig. 1.

TABLE VII

EFFECT OF MUTATION ON THE PROLINE SYSTEM

Comparison between W-6 (Tr^+pro.) and W-157 (Tr^-pro.) relative to proline transport after aerobic and anaerobic growth.

E. coli Strain	Concentration Range μM/ml	Km	V max. μM/min/mg
W-6 (aerobic)	0.43 - 2.61	2.5×10^{-6}M	0.77
(anaerobic)	" "	2.4×10^{-6}M	0.77
W-157 (aerobic)	" "	1.0×10^{-5}M	2.00
(anaerobic)	" "	1.5×10^{-6}M	0.50
W-6 (aerobic)	8.8 - 34.8	1.25×10^{-4}M	25.00
(anaerobic)	" "	1.25×10^{-4}M	25.00
W-157 (aerobic)	" "	1.25×10^{-4}M	25.00
(anaerobic)	" "	1.25×10^{-4}M	25.00

On the other hand, the kinetics of proline uptake by W-6 were not altered by these manoeuvres - Table VII. These observations imply that the mutant system may be relatively flexible with the result that its interaction with the energy producing system can be altered depending on the conditions of growth. Note that the low affinity system under these conditions is not affected by the mutation nor by growth conditions. The high affinity system of W-157, though altered by mutation, like W-6, demonstrated no affinity of OH-L-proline or

DL-pipecolate within the concentration ranges used. The low affinity system, again like W-6, showed affinity for OH-L-proline and DL-pipecolate giving Ki values of 4.9×10^{-5M} and 9.3×10^{-5M} respectively - cf. Table VI.

ii) Variation in Proline Accumulation with Suspension Density:

Fig. 2 shows the decrease in rate of L-proline-C^{14} accumulation (from 10 µg/ml external concentration) with increase in cell suspension density. The decrease is presumed to be due to a decrease in the respiration rate as pointed out by Marquis and Gerhardt(166). Note that the rate of fall is much greater for W-157 than for W-6. The amount of proline accumulated by the two strains at 1 mg/ml was identical under these conditions - cf. Table II.

iii) Relationship between Proline Kinetic Constants and Culture Age of W-6 and W-157:

Stationary phase cells of W-6 and W-157 were suspended in 100 ml of medium + 250 µg/ml L-proline and incubated at 37°C under anaerobic conditions. Growth was followed by O.D. readings at 650 mµ. At 1 hour intervals aliquots were withdrawn, washed and prepared for Km and

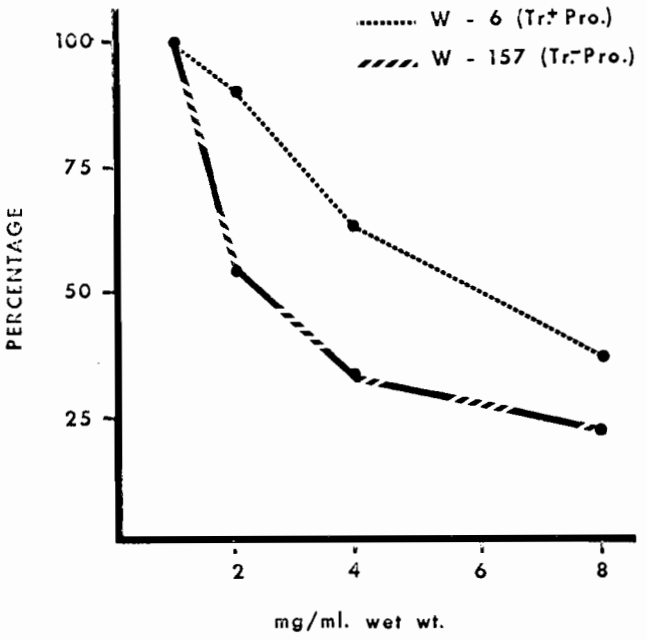

Fig. 2 Comparison between W-6 and W-157 relative to the effects of increasing cell density on C^{14}-proline accumulation.

V max. determinations. The results are shown in Table VIII. It can be seen that the kinetic constants of the high-affinity system of W-6 do not change throughout the growth cycle. In W-157, however, no evidence of the function of this system could be found after the late lag phase; it reappeared in the late exponential - early stationary phase.

TABLE VIII

EFFECT OF CULTURE AGE ON KINETICS
OF PROLINE TRANSPORT IN W-6 AND W-157

Strain		Km		V max.	
		High affinity	Low affinity	High affinity μM/min/mg	Low affinity μM/min/mg
W-6	(Stationary)	2.5×10^{-6}M	1.25×10^{-4}M	0.77	25.00
	(Late Lag)	2.5×10^{-6}M	1.25×10^{-4}M	0.77	25.00
	(Early Expon.)	2.5×10^{-6}M	4.00×10^{-5}M	0.80	10.00
	(Mid Expon.)	2.5×10^{-6}M	4.00×10^{-5}M	0.80	10.00
	(Late Expon.)	2.5×10^{-6}M	-	-	-
W-157	(Stationary)	1.5×10^{-6}M	1.25×10^{-4}M	0.50	25.00
	(Late Lag)	1.5×10^{-6}M	1.25×10^{-4}M	0.50	25.00
	(Early Expon.)	4.0×10^{-5}M*	4.00×10^{-5}M	5.00	5.00
	(Mid Expon.)	4.0×10^{-5}M*	4.00×10^{-5}M	5.00	5.00
	(Late Expon.)	1.5×10^{-6}M	-	-	-

* Concentration range: 0.86 - 2.60 mM. Below 0.86 mM uptake values were very low giving a Km value of ∞ - suggesting entry by a diffusion-like process.

Fig. 3 shows that under these conditions there is no break in the reciprocal plot to suggest the functioning of two systems - cf. Fig. 1.

The kinetic constants of the low-affinity system showed a change in both strains. The affinity increased by a factor of 3 (Km changed from 1.25×10^{-4}M to 4×10^{-5}M) while V max.

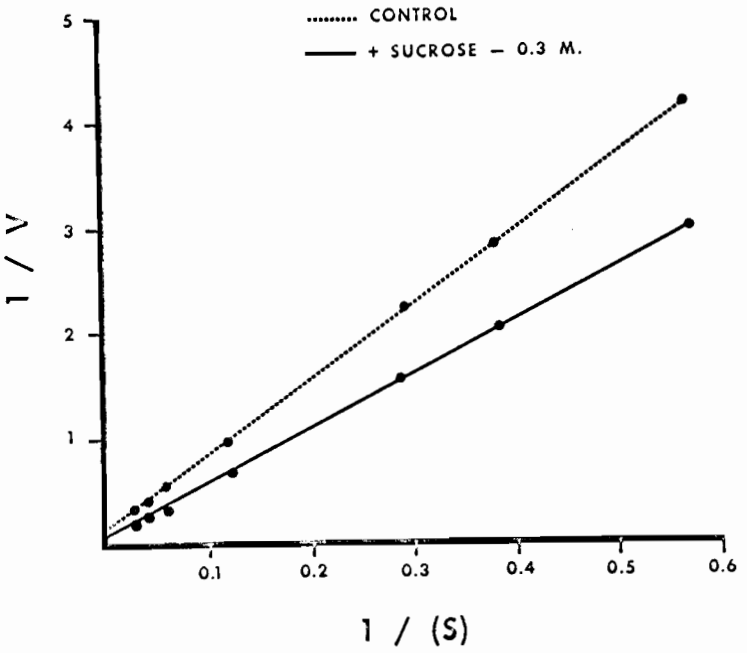

Fig. 3 Reciprocal plots for C^{14}-proline uptake by mid-exponential cells of W-157 and the effect of 0.3 M sucrose on this uptake - cf. Fig. 1

decreased by a factor of 2.5 in the case of W-6 - Table VIII. With W-157, the V max. decreased by a factor of 5, implying that in exponentially growing cells, this system has half the capacity for proline accumulation as compared with W-6. This reduced capacity was restored by the addition of 0.3 M sucrose - Fig. 3. Table IX shows that with valine, under

TABLE IX

EFFECT OF CULTURE AGE ON KINETICS
OF VALINE TRANSPORT IN W-6 AND W-157

Strain		Km		V max.	
		High affinity	Low affinity	High affinity μM/min/mg	Low affinity μM/min/mg
W-6	(Stationary)	3.3×10^{-6}M	5.0×10^{-5}M	0.67	8.0
	(Mid Expon.)	3.3×10^{-6}M	2.0×10^{-5}M	0.67	4.0
W-157	(Stationary)	3.2×10^{-6}M	5.0×10^{-5}M	0.67	8.0
	(Mid Expon.)	3.3×10^{-6}M	2.0×10^{-5}M	0.67	4.0

identical conditions, the kinetic constants of the high-affinity system showed no change as compared with stationary phase cells. However, the low-affinity showed changes in both Km and V max. values, from 5.0 to 2.0×10^{-5}M and from 8.0 to 4.0 μM/min/mg respectively. There was no difference between the two strains as was found with proline - cf. Table VIII.

4) Discussion

The results presented in Table I show that the radioactive materials extracted with boiling water after the cells had been incubated for 1 min. had the same chromatographic properties as the appropriate reference compounds. This is taken to mean that the bulk

of the transported material was the amino acids added to the medium. On the basis of the distribution ratios of the seven amino acids used, the transport mutation of W-157 seems to be specific for proline. Under these conditions, the only difference between the two strains involved proline accumulation. Hydroxy-L-proline was not accumulated actively by either strain - cf. Table II.

The marked difference between the Km and V max. values for accumulation over the two concentration ranges used indicates that, for lysine and arginine, valine, leucine and isoleucine, and proline, there are two types of transport systems - Table III and Fig. 1. This is in accord with observations made by others (15,38,66). The ratio between the high and low Km values for any one amino acid is always greater than the ratio between the corresponding V max. values. This indicates that the half-saturation concentration may vary greatly without a corresponding change in the capacity for accumulation, and confirms an observation originally made by Britten and McClure[15] for proline uptake in E. coli. The Km value of 2.5×10^{-6}M for proline on the high-affinity system is identical to that reported earlier[15], while a Km of 3.3×10^{-6}M

for valine on a similar system is in good agreement with that reported by Cohen and Rickenberg[37] for the same organism. The proline Km on the low-affinity system (stationary phase cells) - Table III - is not in accord with that reported by Britten and McClure[15]. However, the value obtained with exponential phase cells - Table VIII - is identical to that obtained by these authors using cells under similar conditions.

A V max. value of 0.43 μM/min/mg for hydroxy-L-proline is some 60 times less than that of proline at a similar concentration range. This is not compatible with active transport phenomena in these cells and confirms an earlier observation - cf. Table II.

The Km and Ki values of lysine and of arginine, when used as mutually competitive inhibitors on the high affinity system, are not comparable - Table IVa. According to the predictions of the "A-B test", the two amino acids are not transported by identical systems. This view is supported by observations relating to the differences between the Ki values of L-ornithine and of L-canavanine when used as inhibitors of the two amino acids (the "C-test") - Table IVa. Similar conclusions can be drawn for the low-affinity system - cf. Table IVb. Thus it appears that the "lysine-arginine system" actually consists of four non-identical,

yet similar, systems; two high-affinity and two low-affinity systems.

For the valine-leucine-isoleucine high-affinity system, the Km and Ki values for each amino acid do not correspond - Table Va - hence the existence of three similar systems is indicated. On the other hand, there appears to be only one low-affinity system to serve all three amino acids since the Km and Ki values for each amino acid are identical - Table Vb. This is reminiscent of the situation with the aromatic amino acids in Salmonella[38]. It is interesting to note that isoleucine, which has the highest affinity for this system, is the most potent inhibitor of this group and at the same time it has the lowest V max. value. The reverse is true for valine while leucine is intermediate. These observations point up an interesting characteristic in that it appears that there is a reciprocal relationship between affinity for a system and the capacity of this system to concentrate a given substrate.

Whereas the high-affinity systems discussed above do not demonstrate extreme specificity, that for proline does. Thus OH-L-proline and DL-pipecolate show no affinity for this system while they inhibit proline uptake by the low-affinity system at comparable relative

concentrations - Table VI. This observation indicates that the high-affinity system can discriminate between the presence and absence of an hydroxyl group or an extra carbon atom in the ring structure of the amino acid, while the low-affinity system cannot. This represents a strong argument in favour of the existence of two distinct types of systems for each amino acid. Note that OH-L-proline has a higher affinity for the low-affinity proline system than proline itself, and at the same time, it is not concentrated by the cells.

Two conclusions may be drawn from these observations: a) that too high or too low an affinity for a given system can result in the failure of the cells to accumulate a substrate; b) that an amino acid does not have to be concentrated in order to inhibit competitively the uptake of another. Note also that the Km and Ki values of proline and of OH-L-proline are identical - Table VI - indicating the identity of the system being discussed. The significance of the non-competitive effect of DL-pipecolate on OH-L-proline uptake is not clear.

The transport of proline by the high-affinity system of the mutant (W-157) presents an interesting problem. As opposed to the transport of the other amino acids in this strain and all in W-6, the kinetic constants of uptake of this amino acid showed some peculiar

changes when the cells were grown or incubated under different conditions - Table VII and Fig. 2. For aerobic stationary phase cells the Km value was found to be 1 x 10^{-5}M, while for anaerobic cells this value was 1.5 x 10^{-6}M. This represents an increase in affinity by a factor of 6.7, while the V max. value decreased by a factor of 4. No such phenomena were observed for proline accumulation by the low-affinity system in either strain. When the cells were incubated at various suspension densities, the rate of decline in proline accumulation was found to be greater for W-157 than for W-6 - Fig. 2. These data imply that the mutant high-affinity system is more sensitive to changes in the rate of respiration as compared with the wild type.

Another interesting observation concerns the disappearance of the ten-fold difference between W-6 and W-157 - cf. Table II - when aerobic stationary phase cells were incubated under conditions of low suspension density. This observation is not in agreement with that of Kessel and Lubin[72] who used exponentially growing cells in their studies. The possibility exists therefore that this disparity may be related to the fact that exponential cells have less wall material than stationary ones - (cf. ref. 31). The observations

relating to the disappearance of the activity of the W-157 high-affinity proline system after the late lag phase and its reappearance in the late exponential - early stationary phase - Table VIII - supports this suspicion.

A similar explanation, relative to the amount of wall material associated with exponential cells, obtains with the low-affinity proline and valine systems in both strains - Tables VIII and IX. Thus the Km value of proline changed from 1.25×10^{-4M} to 4.0×10^{-5M} between the stationary and exponential phases. Here the affinity for proline increased by a factor of 3, while the V max. decreased by a factor of 2.5 in W-6. In W-157, the decrease in V max. was by a factor of 5 and was rectified (to a factor of 2.5) by the addition of 0.3 M sucrose - Fig. 3. This difference between the V max. values of the two strains is not compatible with earlier observations - Table VII - but is in accord with those of Kessel and Lubin[72]. The inability of the exponential cells of W-157 to take up adequate amounts of proline from concentrations below 0.86 mM suggests that the concentration must be at a certain level before the low-affinity system is activated.

The restoration of proline accumulation capacity in W-157 to normal with 0.3 M sucrose - Fig. 3 - suggested

that a secondary effect of the transport mutation might involve the synthesis of wall material, so that exponential cells of W-157 might have less wall material associated with them than those of W-6. However, the failure to observe a comparable difference between the strains relative to valine accumulation - Table IX - does not support this view. Thus it appears that the mutation primarily affects the high-affinity proline system with secondary effects on the low-affinity one. It is possible that the two systems are physically integrated in such a manner that when the high-affinity system does not function, the capacity of the low-affinity one is reduced

Observations pertaining to the mutant system suggest that it may be more flexible than its wild-type counterpart. It is possible that because of this flexibility its conformation alters appreciably with differences in growth conditions and amounts of wall associated with the cells at any one time. An interesting point is raised by these observations in that a transport mutant may go undetected if stationary phase anaerobic cells are used for kinetic or time-course studies - under these conditions the Km values were 1.5 and 2.5 x 10^{-6M} for W-157 and W-6 respectively.

Data obtained by the "A-B and C-Test" show that there are groups of structurally related high-affinity systems. Each system may preferentially transport one amino acid, but related amino acids may be transported by any one or all of these systems. In the light of this information one can speculate as to how a mutation may affect the accumulation of a group of related substrates. Apparently conflicting observations concerning the ability of mutation to affect the transport of a single amino acid[68,69] or a group of amino acids[38,70] could then be reconciled. One possibility suggested by Scriver and Wilson[154] is that a macromolecular component may be common to each of the similar systems which comprise a "common" group. Thus a mutation affecting this component would affect the transport of the group as a whole. A mutation affecting any other component might then affect only one member of the group. When a group of similar low-affinity systems is involved, this suggestion is equally valid. However when there is a single low-affinity system for a group of amino acids (e.g. valine-leucine-isoleucine), a mutation affecting any component of the transport system could be expected to have comparable effects on the accumulation of any member of the group.

B. "LIPID-AMINO ACID COMPLEXES" AS TRANSPORT INTERMEDIATES

1) Materials

a) Compounds:

L-proline-U-C^{14}, L-leucine-U-C^{14}, L-lysine-U-C^{14}, and L-arginine-U-C^{14} were used throughout these studies. Penicillin G and Chloramphenicol (CM) were also used. Difco yeast extract, peptone and nutrient broth were obtained through Fisher Scientific Co. DNAse I was purchased from Worthington Enzymes. All other compounds were of reagent grade. Silica Gel Sheets (Type K301R) were obtained from Fisher.

b) Organisms:

E. coli W-6 ($Tr^+pro.$) and W-157($Tr^-pro.$) were used throughout these studies.

c) Media:

The minimal medium described in Section A was used in conjunction with a peptone medium described by Peck and Gest[167]. This consisted of the following: Yeast extract - 0.2%; peptone - 0.2%; nutrient broth - 0.8%; KH_2PO_4 - 1.4%; Na_2HPO_4 - 1.4%; and glucose - 1.0%. L-proline was added to 250 µg/ml.

2) Methods

a) Strain Purity:

The purity of the strains was maintained as described in Section A.

b) Growth Conditions:

Aerobic and anaerobic cells were prepared in both types of media as already described. All cells were in the early stationary phase at the time they were used.

c) Preparation of "Wall-membrane Complex":

Cells were suspended to 1 mg/ml wet wt. in minimal medium supplemented with 250 μg/ml L-proline, 2000 IU/ml Penicillin G and 0.3 M sucrose. The suspension was shaken gently for 2.5 hours at 35-37°C at the end of which time the "spheroplasts" were harvested by centrifugation, washed with medium + 0.3 M sucrose and finally lysed by resuspension in minimal medium + 2.0 μg/ml DNAse I. Lysis was followed by optical density changes at 650 mμ and was aided by repeatedly forcing the suspension through a 26G hypodermic needle. The "wall-membrane complexes" were isolated by removing intact cells at 3500 rpm for 5 mins. followed by centrifugation of the supernatant at 5000 rpm for 15 mins. all at 0-4°C in an MSE magnum centrifuge. The final pellet was washed with minimal medium + 2 μg/ml DNAse I and resuspended in minimal medium to the required optical density.

d) Incubation with Radioactive Materials:

Cell or "wall-membrane complex" suspensions of 2.5 - 3 mg/ml dry wt. were incubated at 35-37°C with 0.2 μC/ml C^{14}-amino acid for 10 mins. At the end of this time 5 ml aliquots were withdrawn and added to an equal volume of ice-cold 10% trichloroacetic acid (TCA). The precipitates were collected by centrifugation and washed with two 5 ml portions of ice-cold 5% TCA.

e) Extraction of C^{14}-labelled "Lipid" Fraction:

Washed TCA precipitates were extracted with 2 ml portions of anhydrous methanol at 55°C for 15 mins. In some cases, this was followed by addition of 4 ml of chloroform and the extraction allowed to continue for an additional 30 mins. at room temperature. Following extraction cell debris was removed by centrifugation or by filtration through glass wool. Aliquots were assessed for radioactivity in a gas-flow counter operating at 20% efficiency. Activity was expressed as mμC/gm dry wt. of suspension.

f) Chromatography and Radioautography of "Lipid" Fraction:

Aliquots of the "lipid" extract were concentrated by vacuum evaporation at room temperature and

spotted on to Silica Gel Thin Layer Sheets with the corresponding amino acid as control. These were developed for 120 mins. in Chloroform:Methanol:H_2O (65:25:4 v/v). Developed plates were sprayed with either 10% phosphomolybdic acid in ethanol[168] or 0.2% ninhydrin in n-butanol:10% Acetic Acid (19:1 v/v)[169] and heated for 20 mins. at 110°C. Duplicate plates were exposed to X-ray film for a period of 2-3 weeks. The exposed film was developed and the R_f values of the radioactive spots calculated.

g) Removal of a "Protein" from the "Lipid" Fraction:

Protein was removed from the "lipid" fraction by one of two methods:

i) shaking the $CHCl_3$:MeOH extract with two 3 ml portions of 0.9% NaCl - which caused the protein to aggregate at the interface - and removing the $CHCl_3$ phase with a pasteur pipette after centrifugation;

ii) adding two volumes of peroxide-free diethyl ether to the methanol extract and allowing it to stand overnight at 4°C - the resulting precipitate was removed by centrifugation.

h) Hydrolysis of Deproteinized Lipid Fraction:

Pooled aliquots of this fraction were hydrolyzed according to Hunter and Goodsall[134] - i.e.

by saponification with 0.1N NaOH followed by acid hydrolysis with redistilled 6N HCl for 18 hours at 110°C in sealed ampules. The acid was removed by evaporation under vacuum and the residue taken up in methanol. Aliquots were chromatographed as in Section A, followed by radioautography. The R_f values of the radioactive spots were compared with those of appropriate controls.

i) Preloading Cells with L-proline:

Aerobic cell suspensions of both strains were cooled to 0-4°C and L-proline added to 500 µg/ml. After 30 mins. the cells were recovered by centrifugation in the cold, washed with ice-cold medium and resuspended in cold medium to the original density. C^{14}-proline was then added to 0.2 µC/ml and the suspension kept at 0-4°C for an additional 30 mins., after which the cells were recovered, washed and again resuspended in cold medium to the original density. These preloaded suspensions were then incubated, in the absence of further supplements, at 35-37°C with shaking for 10 mins. The usual extraction procedures were followed.

j) Zero-time Experiments:

These experiments were done by adding 5 ml aliquots of cell suspensions to an equal volume of

ice-cold 10% TCA containing enough radioactivity to bring the final concentration to 0.2 μC/ml. The precipitates were prepared and extracted as described above.

3) Results

a) Preliminary Studies:

Initially experiments were done with anaerobic cells which had been grown in the complex peptone medium. It was observed that extraction with $CHCl_3$: MeOH(2:1 v/v), as opposed to methanol, made no difference to the amount of label extracted; extraction of whole cells as opposed to TCA precipitates also made no significant difference to the activity of the deproteinised lipid fraction. Subsequent experiments were therefore carried out on TCA precipitates extracted with anhydrous methanol to which 2 volumes of $CHCl_3$ were added after the removal of cell debris by centrifugation. Deproteinisation by shaking with 0.9% NaCl was the procedure followed.

b) Composition of Methanol Extracts:

Chromatography of crude methanol extracts showed a total of four spots when sprayed with phosphomolybdate and ninhydrin - Fig. 4. The first(I) remained at the origin and gave a ninhydrin and

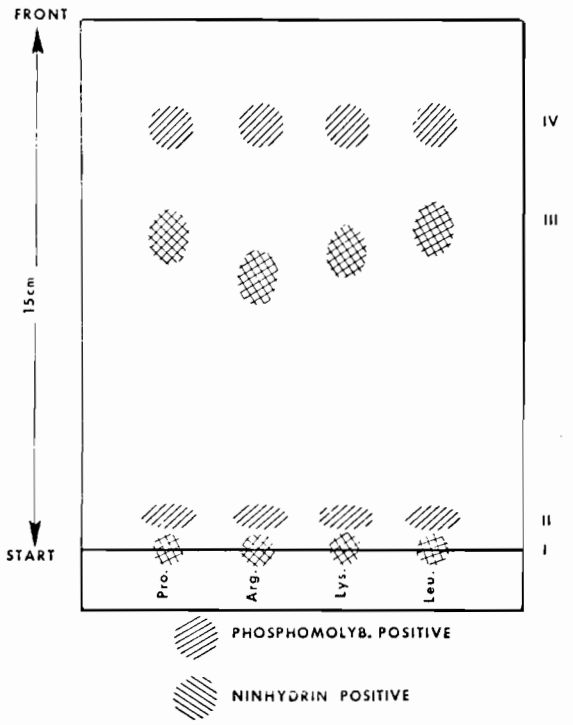

Fig. 4 Tracing of chromatograms of crude Methanol Extracts developed in $CHCl_3$:MeOH:H_2O(65:25:4 v/v).

phosphomolybdate positive reaction. Its chromatographic behavious and reaction with ninhydrin is reminiscent of an alcohol-soluble protein fraction isolated from E. coli under similar conditions by Roberts et al[99]. Further studies on this fraction will be reported in Section C. The second spot(II) was phosphomolybdate positive and had an R_f value of 0.05; the third(III) with an R_f of

0.6-0.7, depending on the amino acid used, was both ninhydrin and phosphomolybdate positive; the fourth (IV) had an R_f of 0.85 and was phosphomolybdate positive. Radioautography showed I and the leading portion of III to be the only radioactive spots; III was therefore taken as the lipid-amino acid complexes (LAC). The R_f values of these complexes as compared with those of the corresponding free amino acids are given in Table X.

TABLE X

COMPARISON BETWEEN R_f VALUES OF "LIPID-AMINO ACID COMPLEXES"(LAC) AND THOSE OF THE CORRESPONDING FREE AMINO ACIDS IN $CHCl_3$:MeOH:H_2O(65:25:4 v/v)

	R_f values	
Amino Acid	Free	Complex
L-proline-C^{14}	0.06	0.70
L-arginine-C^{14}	0.00	0.62
L-lysine-C^{14}	0.00	0.65
L-leucine-C^{14}	0.14	0.70

c) Zero-time Experiments and Recovery of C^{14} amino acids from Hydrolysates of the Deproteinised Lipid Fraction:

Zero-time experiments showed that under none of the growth conditions was any lipid-amino acid formed. The R_f values of the C^{14}-materials recovered from acid hydrolysates of the deproteinised

lipid fraction were found to be comparable with those of the original amino acids - cf. Table I, Section A.

d) Effect of Aerobic versus Anaerobic Growth on LAC Formation in W-6 cells grown in Minimal Medium:

Table XI shows that under the conditions employed, no evidence of lipid-amino acid formation could be found in anaerobically grown cells. On the other hand, some were formed when the cells were grown aerobically. Note that less lipid-proline is formed than for the other amino acids. This is probably due to differences in pool sizes since L-proline was added to the growth medium.

TABLE XI

EFFECT OF AEROBIC VERSUS ANAEROBIC GROWTH ON LAC FORMATION IN W-6 GROWN IN MINIMAL MEDIUM

Amino Acid	LAC Activity - mμC/gm dry wt. Susp. Anaerobic	Aerobic
L-proline-C^{14}	0.00	6.1
L-arginine-C^{14}	0.00	16.0
L-lysine-C^{14}	0.00	16.2
L-leucine-C^{14}	0.00	15.8

e) Effect of Aerobic versus Anaerobic Growth on LAC Formation in W-6 grown in Complex Medium:

Table XII shows that when the cells were grown in a complex medium, considerably more lipid-amino acids were formed than when grown in minimal medium - cf. Table XI. This difference is of the order of twenty-fold for aerobic cells. The effect of growth under anaerobic conditions is also shown and is comparable with that observed for cells grown in minimal medium. Again there is less activity associated with the lipid-proline complex than with the others.

TABLE XII

EFFECT OF ANAEROBIOSIS AND CHLORAMPHENICOL ON LAC FORMATION IN W-6 GROWN IN COMPLEX MEDIUM

	LAC Activity - mμC/gm dry wt. susp.		
Amino Acid	Anaerobic	Aerobic	CM
L-proline-C^{14}	45.0	134.6	7.1
L-arginine-C^{14}	85.6	249.8	12.5
L-lysine-C^{14}	89.5	269.0	13.2
L-leucine-C^{14}	81.8	240.3	12.4

f) Effect of Chloramphenicol on LAC Activity in Aerobic W-6 cells grown in Complex Medium:

Chloramphenicol(CM) at 100 μg/ml added 5 mins. prior to the addition of C^{14}-amino acids reduced

LAC Activity by a factor of about 20 - Table XII.

g) Effect of Mutation on the Formation of Lipid-proline by cells grown in Complex Medium:

The only difference between the activities of the lipid-amino acid complexes formed by aerobic cells of W-6(Tr^+) and W-157(Tr^-) involved that of the lipid-proline complex - Table XIII. The difference between the two strains is about four-fold which is to be expected under these conditions by virtue of the transport mutation - cf. Section A, Table II.

TABLE XIII

COMPARISON BETWEEN ACTIVITIES OF LAC FORMED IN W-6 AND W-157 CELLS GROWN AEROBICALLY IN COMPLEX MEDIUM

	LAC Activity - mμC/gm dry wt. Susp.	
Amino Acid	W-6(Tr^+)	W-157(Tr^-)
L-proline-C^{14}	134.6	30.2
L-arginine-C^{14}	249.8	252.5
L-lysine-C^{14}	269.0	268.6
L-leucine-C^{14}	240.3	239.8

h) Effects of Elimination of Transport Phenomena on the Activity of the Lipid-proline Complex of W-6 and W-157:

Table XIV shows that when transport phenomena were eliminated by preloading the cells with proline the difference between the two strains disappeared

- cf. Table XIII. The same observation was made when "wall-membrane complexes" of the strains were incubated with C^{14}-proline under identical conditions - Table XIV.

TABLE XIV

EFFECT OF ELIMINATION OF TRANSPORT PHENOMENA ON THE LIPID-PROLINE COMPLEX ACTIVITY OF W-6 AND W-157

Material	Lipid-proline Activity - mμC/gm dry wt. Susp.			
	Minimal Medium		Complex Medium	
	W-6	W-157	W-6	W-157
Preloaded Cells	13.4	12.8	90.6	89.5
Wall-membrane Complex	36.4	36.6	258.2	260.1

4) Discussion

Lipid-amino acid complexes have been isolated from a variety of cell types and have had one of two roles ascribed to them. Gaby, Naughten and Logan[170] have isolated such complexes from Penicillium chrysogenum, while Westley, Wren and Mitchell[171] have reported that most of the non-protein amino acids in young larvae of Drosophila melanogaster are found in a lipid-soluble fraction. Similar complexes have been found in hen oviduct[172], rabbit liver[173], ascites tumor cells[131,174], B. megaterium[133,134], E. coli[138],

Clostridium welchii[175], S. aureus[137], Pseudomonas aeruginosa[176], and broad bean leaf[136].

There has been some controversy concerning the question of artefacts. Thus Wren[174], and Gaby and Silberman[177] demonstrated that although artefacts are formed under certain circumstances, the bulk of the complexes are real, their formation being dependent on cell metabolism. The results reported here, relative to zero-time, aerobic and anaerobic experiments show that their formation, in E. coli at any rate, is metabolically dependent - Tables XI and XII.

The roles which have been ascribed to lipid-amino acid complexes are: i) intermediates in transport phenomena[15,131-133]; and ii) intermediates in protein synthesis[134,178,179]. Although the data presented here cannot decide as to whether or not these complexes are involved as intermediates in protein synthesis, it seems clear that they cannot function as transport intermediates. This view is based on the observation that the four-fold difference in the lipid-proline activity between W-6(Tr^+pro.) and W-157(Tr^-pro.) disappeared when transport phenomena were eliminated - Table XIV. This implies that lipid-amino acids are formed after transport and hence cannot regulate accumulation. The complexes seem to be formed at the level

of the "wall-membrane complex" and confirms an earlier finding by Hendler[138].

The "inhibition" of LAC formation by chloramphenicol is in agreement with observations made by Hunter and Goodsall[134] but it is not clear as to whether this "inhibition" is due to an enhanced turnover rate, a suppressed incorporation rate, or both. The possibility that they may be involved in the synthesis of ribosomal protein, as suggested by Wilson and Scriver[180] has not been ruled out by the data. This aspect has not been investigated further for the purposes of the present study and presentation.

It should be noted that it appears that there are more than one type of "lipid-amino acid complex". Macfarlane[135] has shown that in Clostridium welchii these are: O-amino acid esters of phosphatidyl glycerol, while Axelrod et al[181] have observed N-acyl-amino acid formation in animal tissue. A similar type of complex has been isolated from Mycobacteria[182]. The structure of those complexes found in hen oviduct has not been elucidated, but Hendler[179] has shown that they serve as intermediates in de novo synthesis of characteristic proteins in this tissue.

C. AN ALCOHOL-SOLUBLE PROTEIN FRACTION FROM TCA-PRECIPITATES OF E. COLI - ITS RELATIONSHIP TO AMINO ACID TRANSPORT

1) Materials

a) Compounds:

L-proline-U-C^{14}, L-arginine-U-C^{14}, L-lysine-U-C^{14}, and L-leucine-U-C^{14} were used throughout unless otherwise stated. Uracil-2-C^{14} (8.62 mC/mM) and Uridine-2-C^{14} (20 mC/mM) were purchased from New England Nuclear Corp. Puromycin dihydrochloride (PM) was obtained from Nutritional Biochemicals Corp. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was purchased from Fisher Scientific Co. Penicillin G, Chloramphenicol (CM) and DNAse I were also used. All other compounds were of reagent grade.

b) Organisms:

Stationary phase aerobic cells of W-6 and W-157 were used, except when otherwise stated.

c) Media:

The basic minimal medium described in Section A was used throughout. Sulphate-free medium was prepared by substituting NH_4Cl and $MgCl_2$ for the corresponding sulphates in the original basic medium.

2) Methods

a) Maintenance of Strain Purity and Growth Conditions:

These were as described in Section A.

b) Preparation of "Wall-membrane Complexes" and Pre-loading Experiments:

These were as described in Section B.

c) Incubation with Labelled Compounds:

i) Cell suspensions of 2.5 - 3 mg/ml dry wt. were preincubated for 30 mins. and then incubated with C^{14}-materials added to 0.2 μC/ml. When CM or PM was used, these were added 10 mins. after the addition of the label. Aliquots of 5 ml were withdrawn at 10, 20, 40 and 60 mins. and added to an equal volume of ice-cold 10% TCA. The precipitates were collected by centrifugation and washed with two 5 ml portions of cold 5% TCA;

ii) Suspensions of "wall-membrane complex" of both W-6 and W-157 were incubated with C^{14}-proline as in Section B. After 10 mins., aliquots were withdrawn, added to an equal volume of cold 10% TCA and the precipitates collected and washed as above;

iii) Cells of W-6 were resuspended to 1 mg/ml wet wt. in 150 ml minimal medium + 10 μg/ml C^{12}-proline and allowed to resume growth during a 30 min. period at 37°C with shaking. At zero-time, 1 μC of C^{14}-amino acid was added. CM or

PM, when used, was added 10 mins. after the addition of the label - to 100 μg/ml. Aliquots of 30 ml were withdrawn at 10, 20, 40, and 60 mins. thereafter and added to 10 ml cold 20% TCA. The samples were chilled and the precipitates collected and washed as above. Growth was followed by O.D. readings at 650 mμ.

d) Extraction and Assessment of Radioactivity:

Washed TCA precipitates were first extracted with anhydrous methanol as in Section B. The alcohol-soluble protein was separated from the lipid fraction by precipitation in the cold with peroxide-free diethyl ether - cf. Section B. The defatted precipitates were collected by centrifugation and extracted with two 1 ml portions of 5% TCA at 95°C for 15 mins. to give a "nucleic acid" fraction - (cf. ref. 99). The final precipitates were partially hydrolysed with 3N HCl for 18 hours at 110°C to give the "protein" fraction. Aliquots of these fractions were assessed for radioactivity as in Section B. Activity was expressed as mμM/gm dry wt. of Suspension.

e) Prelabelling Experiments:

i) Cells of W-6 (2.5 - 3 mg/ml dry wt.) were preincubated for 30 mins. and then prelabelled for

10 mins. with C^{14}-lysine, C^{14}-arginine, or C^{14}-leucine. The prelabelled cells were then collected and washed with cold medium by centrifugation at 0-4°C. They were then resuspended in cold medium and a zero-time sample taken and added to an equal volume of 10% TCA. The remainder of the suspension was divided into four equal parts and added to flasks containing the following:-

a) C^{12}-amino acid to 2.5 mM;

b) C^{12}-amino acid + CM to 100 µg/ml;

c) C^{12}-amino acid + C^{12}-proline to 100 µg/ml;

d) C^{12}-amino acid + C^{12}-proline + CM.

These were then incubated at 37°C with shaking, aliquots withdrawn at 10, 20, and 30 mins. and added to an equal volume of cold 10% TCA.

With C^{14}-proline labelled cells, these were resuspended in sulphate-free medium, a zero-time sample taken and the remainder divided into 4 equal portions as above. In this case the C^{12}-amino acid was proline and Na_2SO_4 was substituted for C^{12}-proline in flasks (c) and (d). This procedure was repeated with C^{14}-leucine labelled cells. The TCA precipitates were extracted and assessed for radioactivity as above - (d).

ii) Penicillin spheroplasts of W-6 were prepared as in Section B. These were prelabelled for 10 mins.with C^{14}-leucine or C^{14}-lysine in the presence of penicillin. They were then harvested, washed with cold medium + 0.3 M sucrose, and resuspended to a density of 2.5 mg/ml in cold minimal medium + C^{12}-amino acid (2.5 mM), CM and C^{12}-proline both to 100 µg/ml and 0.3 M sucrose. A zero-time sample (half the volume) was taken and the remainder of the suspension incubated for 30 mins. at 37°C with gentle shaking. At the end of the incubation period, fractionation was carried out as schematised in Fig. 5. Lysis was induced as in Section B, except that 0.05 M Tris-HCl (pH 8) + 2 µg/ml DNAse I was used as the lysing medium.

f) Chromatography and Radioautography:

The procedures for amino acids and crude methanol extracts have been described in Section B. For uracil and uridine, chromatography was carried out on Whatman 3 MM paper developed in n-butanol:formic acid (1:1 v/v) according to Markham and Smith[183].

g) Determination of Inorganic Phosphate in Acid Hydrolysates of Alcohol-soluble Protein:

The method of Fiske and SubbaRow[184] was scaled down to one-fifth for these determinations. Ten mg

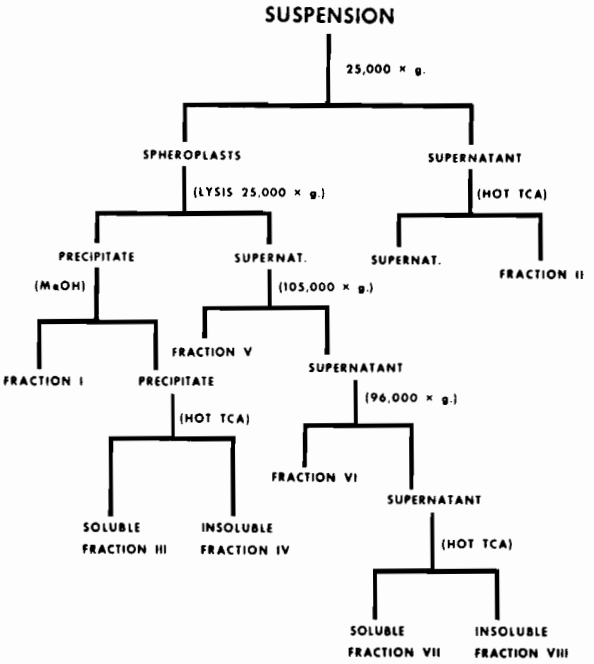

Fig. 5 Scheme for Fractionation* of labelled spheroplasts of W-6.

* Labelled spheroplasts (zero-time and zero + 30 mins.) were separated from the medium by centrifugation in the cold (0-4°C) for 5 mins. at 25,000 x G. The supernatant was treated with TCA (to 5%) and heated to 95°C for 10 mins. The precipitate was taken as Fraction II. The spheroplasts were lysed and the suspension centrifuged at 25,000 x G for 10 mins. (at 0-4°C) to give a sediment and a supernatant. The sediment was extracted with anhydrous methanol and the alcohol-soluble protein (Fraction I) prepared as in Section B. The defatted material was then extracted with 5% TCA at 95°C, as previously described, to give a nucleic acid fraction (Fraction III). The final precipitate was taken as protein representing the "wall-membrane complex" - Fraction IV. The 25,000 x G - 10 min. supernatant was centrifuged at 105,000 x G for 120 mins. (at 5°C) to give a pellet (presumably ribosomes) - Fraction V. The resulting super-

natant was centrifuged at 96,000 x G for 11 hours to give another pellet - Fraction VI. The final supernatant was treated with hot 5% TCA to give a soluble Fraction VII and an insoluble Fraction VIII.

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of dried protein was hydrolysed with 4 ml 6N HCl in a sealed ampule for 18 hours at 110°C. Aliquots (0.1 ml) of the filtered hydrolysate were assessed for inorganic phosphate. The mean of duplicate determinations was taken and expressed as mg per gm protein.

h) Determination of Free Fatty Acids after NaOH Hydrolysis of Alcohol-soluble Protein:

Ten mg of dried protein was suspended in 2 ml 0.1N NaOH and allowed to stand at room temperature for 30 mins. with occasional shaking. The suspension was then extracted with 4 ml n-heptane and the free fatty acid (F.F.A.) titrated with thymol blue as indicator according to Dole[185]. The values were expressed as milli-equivalents per litre per gram of protein.

i) Determination of Amino Acid Molar Ratios of Various Protein Fractions:

Fractions I, II, IV, V, VI and VIII (Fig. 5) were hydrolysed with 6N HCl as above. The acid was removed by evaporation under reduced pressure and the residue dissolved in NaOH-Citric Acid Buffer of pH 2.2. The amino acid content was determined by

chromatography on a Beckman Spinco automatic analyser according to Moore et al[186]. Amino acid contents were expressed as ratios of alanine.

3) Results

a) Composition of Alcohol-soluble Protein:

The alcohol-soluble protein fraction (ASP) was found to contain the entire spectrum of naturally occurring amino acids - cf. Table XXII - cyst(e)ine was not found under these conditions of hydrolysis. In addition, it contained 6.2 mg of inorganic phosphorus per gram and 3.4 m.e.q. per litre per gram of free fatty acid after treatment with NaOH. The latter observation is in accord with the reaction of this fraction with phosphomolybdic acid - cf. Fig. 4. Upon acid hydrolysis, the radioactive material was recovered as the free amino acid, as judged by chromatography - cf. Table I, Section A.

b) Time Course of Amino Acid Incorporation into Alcohol-soluble Protein (ASP) by W-6 and W-157:

Figs. 6 and 7 show that aerobic cells of both strains incorporated C^{14}-lysine, C^{14}-leucine, and C^{14}-arginine into ASP at the same rate. With C^{14}-proline, however, there was a marked difference between the two, illustrating the effects of the transport mutation - Fig. 7.

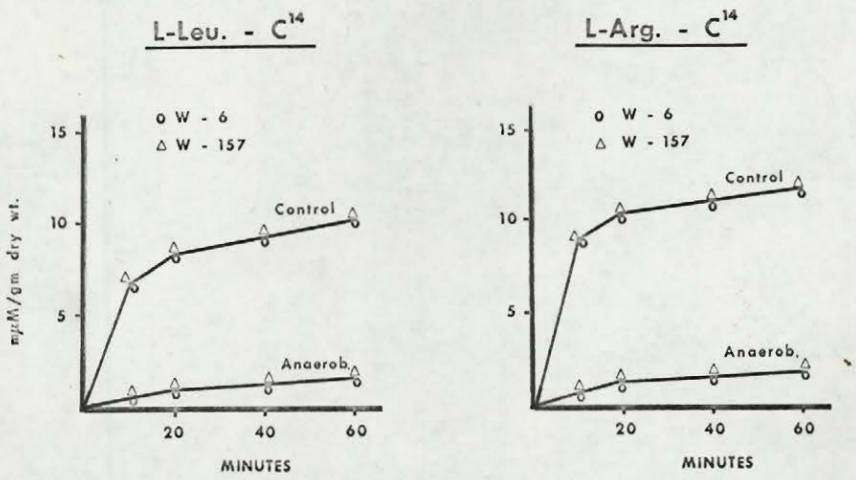

Fig. 6 Time course of C^{14}-leucine and C^{14}-arginine incorporation into the alcohol-soluble protein fraction of W-6 and W-157 grown aerobically and anaerobically.

c) Effect of Anaerobic Growth on Amino Acid Incorporation:

Figs. 6 and 7 also show that anaerobically grown cells incorporate 5-7 times less radioactivity into the alcohol-soluble protein fraction than aerobically grown ones. With C^{14}-proline in W-157, this difference is of the order of two-fold.

d) Effect of Elimination of Transport Phenomena on the Incorporation of C^{14}-proline by W-6 and W-157 into ASP:

Upon elimination of transport phenomena by pre-loading the cells or by incubating "wall-membrane complexes" with C^{14}-proline, the difference between

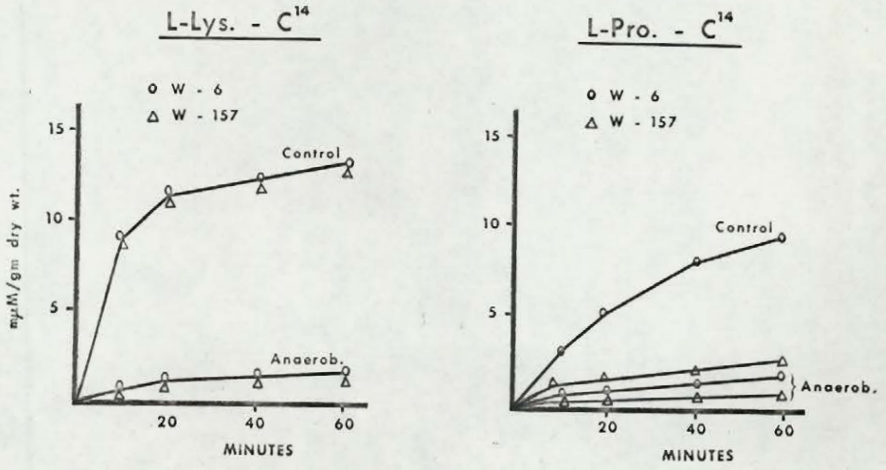

Fig. 7 Time course of C^{14}-lysine and C^{14}-proline incorporation into the alcohol-soluble protein fraction of W-6 and W-157 grown aerobically and anaerobically.

the activities of alcohol-soluble protein of W-6 and W-157 disappeared - Table XV. Note that the activity of the fraction was increased by these manoeuvers.

e) <u>Relationship between Alcohol-soluble Protein Activity and Growth of W-6 and the Effects of CM and PM on this Relationship</u>:

Figs. 8, 9, 10, and 11 show that there was a linear relationship between the activity of this fraction (ASP) and growth. A similar relationship

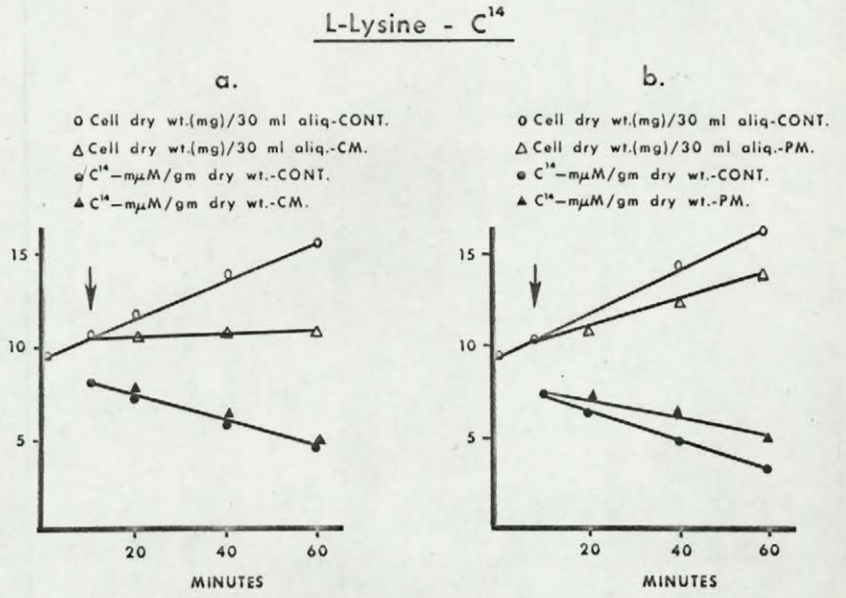

Fig. 8 Relationship between Alcohol-soluble Protein Activity and Growth of W-6 and the Effects of CM and PM on this Relationship - Studies with C^{14}-lysine.

TABLE XV

EFFECT OF TRANSPORT PHENOMENA ELIMINATION ON THE DIFFERENCE BETWEEN W-6 AND W-157 ALCOHOL-SOLUBLE PROTEIN ACTIVITY RELATIVE TO C^{14}-PROLINE LABELLING

	Alcohol-soluble Protein Activity - mμM/gm dry wt. Susp.		
Strain	Control	Preloaded Cells	Wall-membrane Complex
W-6 (Tr^+)	2.60	7.00	17.60
W-157(Tr^-)	0.80	7.00	18.00

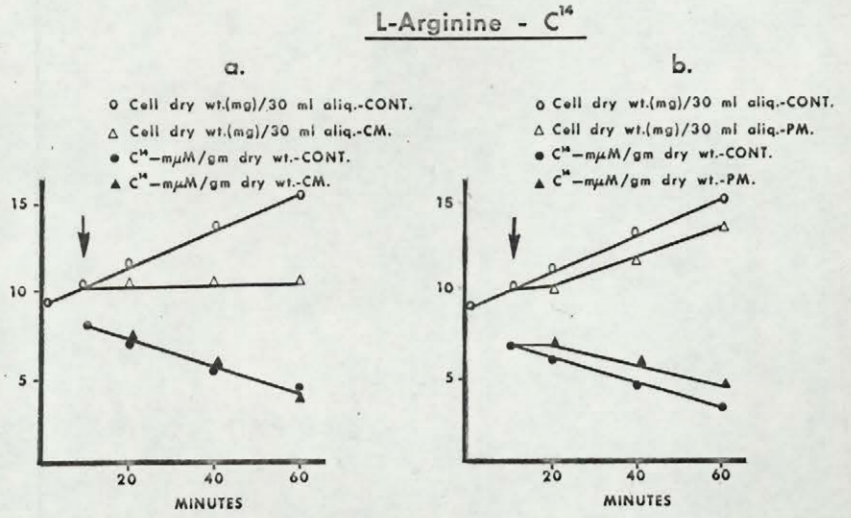

Fig. 9 Relationship between Alcohol-soluble Protein Activity and Growth of W-6 and the Effects of CM and PM on this Relationship - Studies with C^{14}-arginine.

was found to exist when the cells were treated with CM or PM added to 100 μg/ml. It is interesting that CM did not alter the slope of the line when lysine or arginine was used, although there was no appreciable increase in cell mass - Figs. 8a and 9a. However, when leucine or proline was used, the slope was altered - Figs. 10a and 11a. With PM, in all cases, the slope of the line was altered apparently

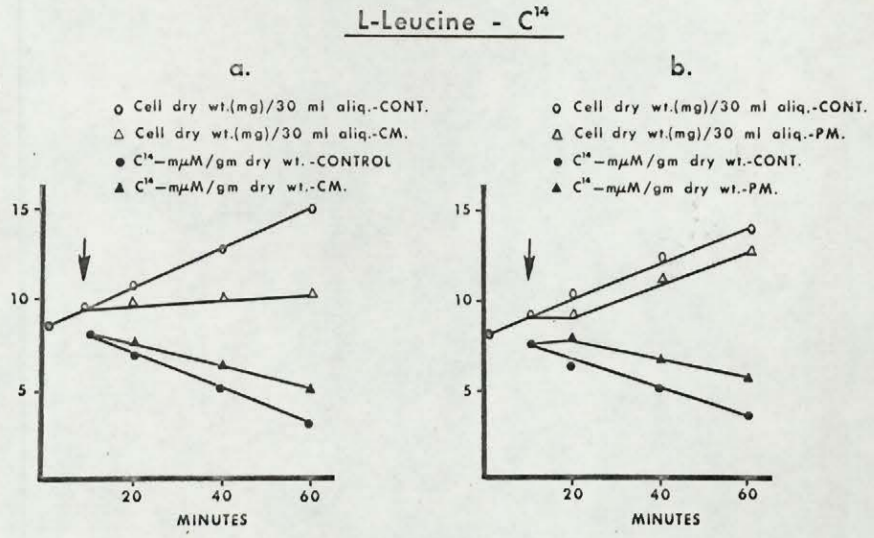

Fig. 10 Relationship between Alcohol-soluble Protein Activity and Growth of W-6 and the Effects of CM and PM on this Relationship - Studies with C^{14}-leucine.

in a manner corresponding to the altered rate of cell mass increase - Figs. 8b, 9b, 10b, and 11b. These observations become more interesting when the change in the number of counts in this fraction is examined as a function of time. Table XVI shows that CM-treated cells actually lost more counts than controls when lysine and arginine were studied; with proline and leucine, there was no difference between these cells and those of controls. PM-treated cells lost less counts for lysine and arginine

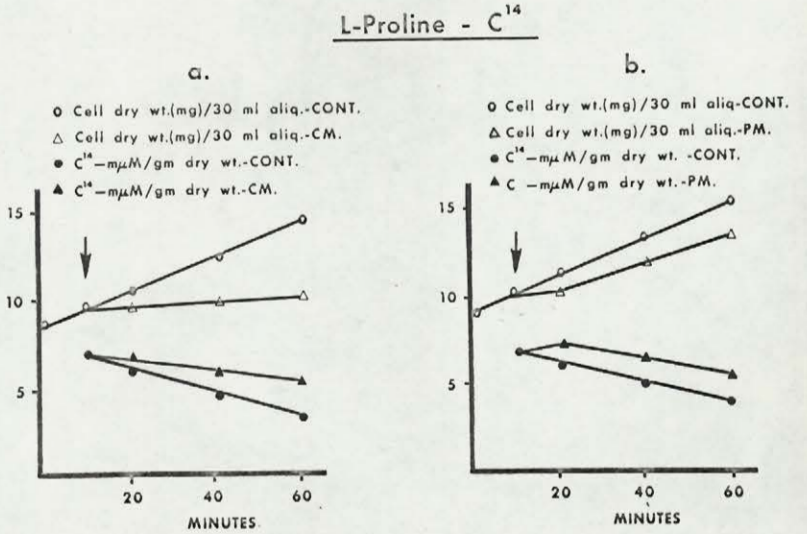

Fig. 11 Relationship between Alcohol-soluble Protein Activity and Growth of W-6 and the Effects of CM and PM on this Relationship - Studies with C^{14}-proline.

TABLE XVI

EFFECT OF CM AND PM ON THE NUMBER OF COUNTS ASSOCIATED WITH THE ALCOHOL-SOLUBLE PROTEIN OF W-6 UNDER GROWING CONDITIONS

Counts per minute per 30 ml Aliquots

Time:	C^{14}-arginine			C^{14}-lysine			C^{14}-leucine			C^{14}-proline		
mins.	Cont.	CM	PM	Cont.	CM	PM	Cont.	CM	PM	Cont.	CM	PM
10	7630	7620	7620	7950	7940	7930	6260	6280	6100	3460	3470	3420
20	7560	6920	7630	7830	7620	7890	5930	5880	6380	3410	3400	3830
40	6870	5800	6980	6810	6610	7160	5870	5820	6610	3200	3210	4000
60	6000	5080	6340	6600	5480	6800	5710	5690	6740	2900	2900	4120

than control cells; with proline and leucine, the number of counts actually increased. After 10 mins. of incubation, no radioactivity was found in the extracellular medium.

f) Effect of CM and C^{12}-Amino Acid on C^{14}-Amino Acid Incorporation into ASP by W-6 under Non-growing Conditions:

Figs. 12 and 13 show that CM (100 µg/ml) interfered with further incorporation of radioactivity into the alcohol-soluble protein fraction. However, this datum does not indicate whether CM inhibited further incorporation of radioactivity, enhanced the removal of radioactivity, or both. C^{12}-amino acid inhibited further incorporation by reducing the specific activity of the pools. PM at 100 µg/ml had no effect under these conditions.

g) Turnover of Radioactivity in Alcohol-soluble Protein of W-6 - Studies with Prelabelled Cells:

Upon addition of prelabelled* cells to medium containing C^{12}-amino acid (2.5 mM), there was a further incorporation of radioactivity into ASP during the first ten minutes; after this time there was no further change - Figs. 14 and 15. C^{12}-amino acid + proline showed the same result except with lysine,

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* using C^{14}-lysine, C^{14}-arginine, and C^{14}-leucine.

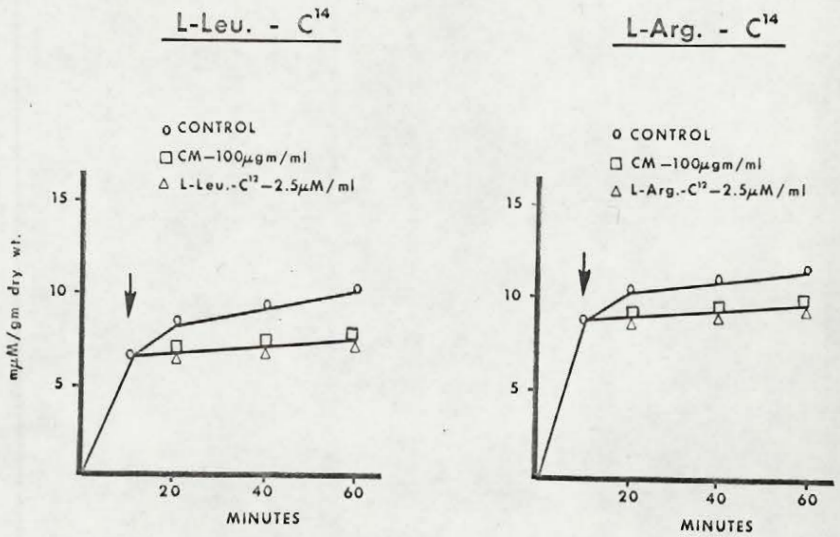

Fig. 12 Effect of CM and C^{12}-Amino Acid on C^{14}-Amino Acid Incorporation into ASP by W-6 under Non-growing Conditions - Studies with C^{14}-leucine and C^{14}-arginine.

in which case there was a slight but consistent stimulatory effect - Fig. 14. C^{12}-amino acid + CM inhibited further incorporation, the level of radio-activity remaining constant throughout the 30 min. incubation period. C^{12}-amino acid + CM + proline resulted in a linear decrease in the activity of this fraction throughout the incubation period. Comparable experiments with C^{14}-proline, using sul-phate-free medium, showed that the activity decreased

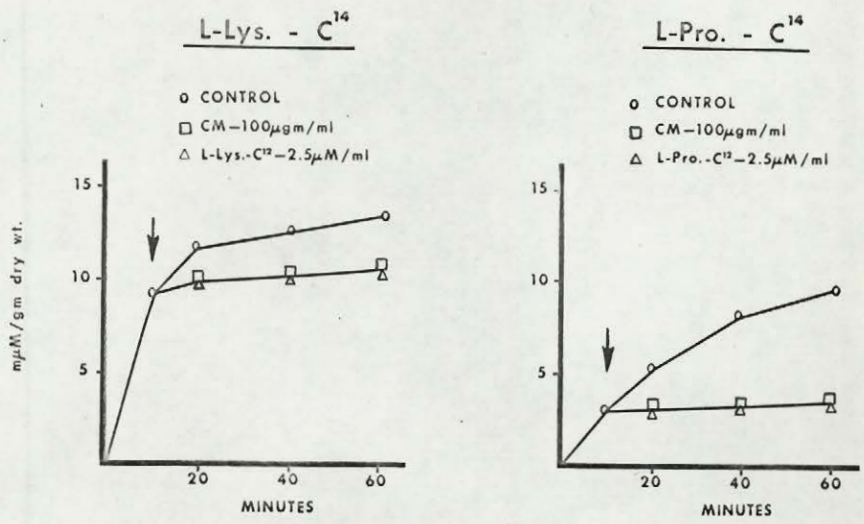

Fig. 13 Effect of CM and C^{12}-Amino Acid on C^{14}-Amino Acid Incorporation into ASP by W-6 under Non-growing Conditions - Studies with C^{14}-lysine and C^{14}-proline.

only when CM and Na_2SO_4 were present together - Fig. 15. The same observation was made with C^{14}-leucine labelled cells suspended in sulphate-free medium - Fig. 16.

h) <u>Effect of CM and PM on the Incorporation of C^{14}-Amino Acid into the Hot TCA-insoluble (Protein) Fraction of W-6</u>:

Table XVII shows that under conditions of crowding (2.5 mg/ml dry wt.), there was little increase in the activity of this fraction (the hot TCA-

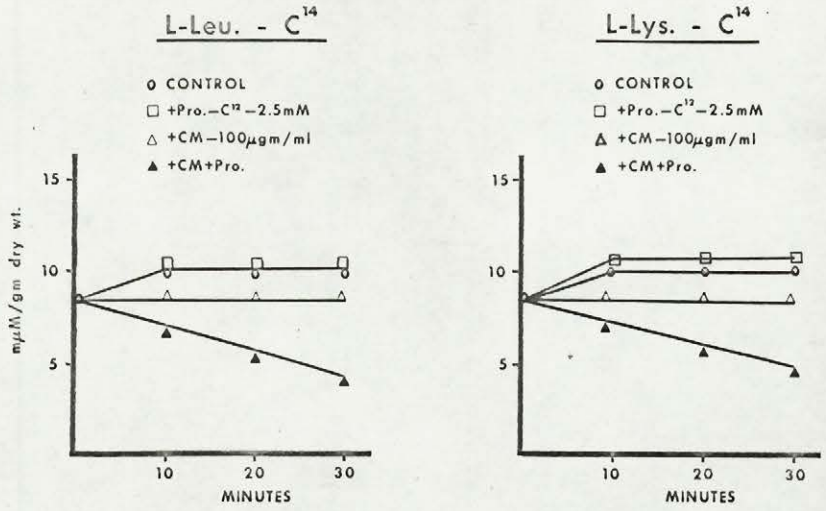

Fig. 14 Turnover of Radioactivity in Alcohol-soluble Protein of W-6 - Studies with C^{14}-leucine and C^{14}-lysine prelabelled cells suspended in Proline-free Medium.

insoluble fraction of the defatted precipitates) throughout the incubation period. With C^{14}-proline there appeared to be considerable protein synthesis but this can be attributed to the specific activity of the proline pool, since these cells are auxotrophic for this amino acid. CM caused a progressive decrease in the activity of this fraction. This is likely due to the fact that protein was being degraded without being resynthesised. In this

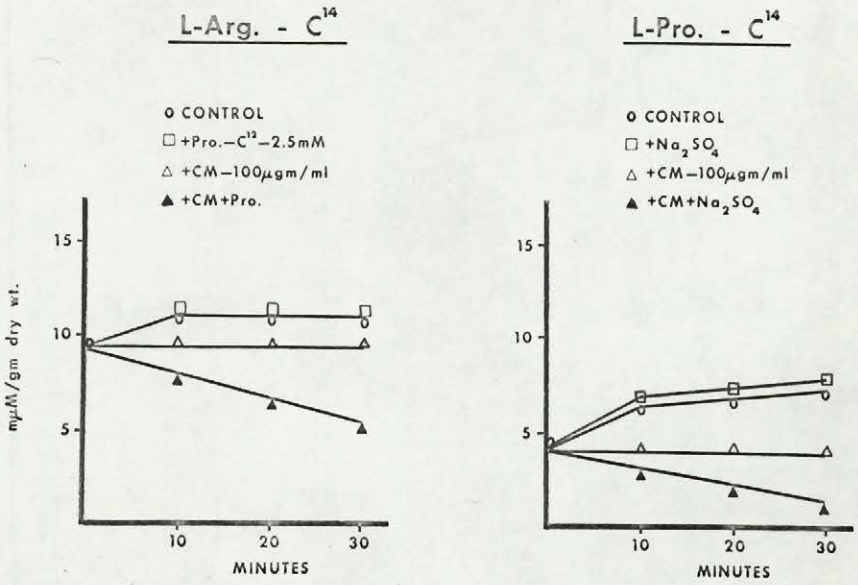

Fig. 15 Turnover of Radioactivity in Alcohol-soluble Protein of W-6 - Studies with C^{14}-arginine Prelabelled Cells suspended in Proline-free Medium and with C^{14}-proline Prelabelled Cells suspended in Sulphate-free Medium.

regard the alcohol-soluble protein is different from the bulk of the cellular proteins since its activity did not change under these conditions - cf. Figs. 12 and 13. PM showed no appreciable effect on the activity when C^{14}-leucine, C^{14}-lysine or C^{14}-arginine was used. With C^{14}-proline, however, incorporation was reduced by about 50% - Table XVII. This is in agreement with the effect of the antibiotic on the growth of these cells - cf. Figs. 8b - 11b.

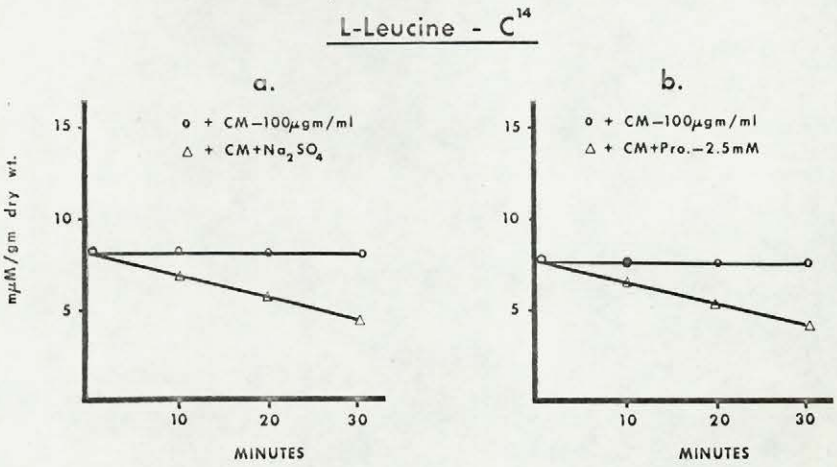

Fig. 16 Turnover of Radioactivity in Alcohol-soluble Protein of W-6 - Comparative studies with C^{14}-leucine Prelabelled Cells suspended in Sulphate-free and Proline-free Media.

TABLE XVII

EFFECT OF CM AND PM ON THE ACTIVITY OF THE BULK OF THE CELLULAR PROTEINS OF W-6

Activity - mµM/gm dry wt. Suspension

Time:	C^{14}-lysine		C^{14}-arginine		C^{14}-leucine		C^{14}-proline		
mins.	Cont.	CM	Cont.	CM	Cont.	CM	Cont.	CM	PM
10	27.5	27.5	29.0	29.0	24.0	24.0	104.2	103.2	103.0
20	29.0	25.0	29.3	26.1	28.6	22.0	248.6	97.3	120.0
40	32.5	23.5	29.7	25.5	33.1	20.5	326.5	79.6	150.0
60	36.0	22.0	30.0	24.3	33.4	19.0	364.4	77.1	175.0

i) Effect of CM on C^{14}-amino acid Incorporation into Nucleic Acid Fraction of W-6:

Table XVIII shows the effect of CM on C^{14}-amino acid incorporation into the nucleic acid fraction (presumably s-RNA) of W-6. It can be seen that the antibiotic had little effect when C^{14}-lysine, C^{14}-arginine or C^{14}-leucine was used. However, with C^{14}-proline, there was a marked increase during the incubation period. This apparent difference can be attributed to the relatively high specific activity of the proline pool and is in accord with the suggestions of Kurland and Maaloe[187] relative to the control of RNA synthesis in this organism.

TABLE XVIII

EFFECT OF CM ON C^{14}-AMINO ACID INCORPORATION INTO THE RNA FRACTION OF W-6

Activity - mμM/gm dry wt. Suspension

Time:	Lysine		Leucine		Arginine		Proline	
mins.	Cont.	CM	Cont.	CM	Cont.	CM	Cont.	CM
10	0.90	0.90	0.80	0.80	0.90	0.90	0.50	0.50
20	0.80	1.00	0.60	0.70	0.60	0.70	1.00	1.00
40	1.00	1.20	0.80	1.00	0.70	0.80	1.10	2.50
60	1.00	1.30	1.00	1.20	0.70	0.80	1.30	3.20

j) Effect of CM on C^{14}-Uridine Incorporation by W-6:

C^{14}-uridine was incorporated into two fractions - the lipid fraction and the nucleic acid (RNA) fraction. Radioautography of the lipid-associated radioactivity showed it to have an R_f of 0.7 as compared with 0.10 for free uridine (chromatography in $CHCl_3$:MeOH:H_2O-65:25:4 v/v). C^{14}-uracil was also incorporated into the lipid fraction which had the same R_f value. Saponification followed by acid hydrolysis showed the radioactive material (from either C^{14}-uridine or C^{14}-uracil labelled lipid) to move with an R_f comparable with that of free uridine when chromatographed according to Markham and Smith[183].

Table XIX shows the relationship between the activity of the lipid-uridine complex and growth, and the effect of CM on this relationship. Note that with time, the CM-treated cells showed a greater loss in activity than the control, although the cell mass did not increase appreciably.

Table XX shows the effect of CM on the incorporation of C^{14}-uridine into the RNA fraction of W-6 under non-growing conditions (suspensions of 2.5 mg/ml dry wt.) - the cells were not supplied with proline. Note that CM enhanced the amount of radioactivity

TABLE XIX

EFFECT OF CM ON THE "LIPID-URIDINE COMPLEX" ACTIVITY IN W-6 UNDER GROWING CONDITIONS

Time: Mins.	Activity - mμM/gm dry wt. Susp. Control	CM-treated	O.D. at 650 mμ Control	CM-treated	cpm/aliquot Control	CM
10	9.5	9.5	0.43	0.42	920	910
20	6.5	4.8	0.47	0.42	700	460
40	4.3	3.6	0.57	0.45	540	380
60	4.4	3.4	0.62	0.46	600	350

incorporated into this fraction. This datum is in accord with the established effects of the antibiotic on RNA synthesis in this organism(187).

TABLE XX

EFFECT OF CM ON C^{14}-URIDINE INCORPORATION INTO THE RNA FRACTION OF W-6 UNDER NON-GROWING CONDITIONS

Time: mins.	Activity - mμM/gm dry wt. Susp. Control	CM-treated
10	15.5	15.5
20	20.9	44.0
40	28.1	160.0
60	30.7	253.1

k) Search for Amino Acid Radioactivity lost from Alcohol-soluble Protein Fraction of W-6 Spheroplasts:

Table XXI shows the changes in radioactivity of the various fractions* obtained from W-6 spheroplasts prelabelled with C^{14}-leucine or C^{14}-lysine. Fractionation was according to Fig. 5. It is clear that the radioactivity lost from the alcohol-soluble protein fraction (I) can be accounted for by that gained in the hot-TCA insoluble fraction (II) obtained from the external medium. Note that this is the only fraction which gained in radioactivity during the incubation period.

* Fraction I - Alcohol-soluble protein.

Fraction II - Hot TCA-insoluble material obtained from external medium.

Fraction III - Hot TCA-soluble material obtained from "wall-membrane complex" after lysis of spheroplasts.

Fraction IV - Defatted, hot TCA-extracted residue of "wall-membrane complex".

Fraction V - Cytoplasmic material sedimented at 105,000 x G for 120 mins - presumably ribosomes.

Fraction VI - Cytoplasmic material sedimented at 96,000 x G for 11 hours.

Fraction VII - Hot TCA-soluble material extracted from 96,000 x G supernatant.

Fraction VIII - Hot TCA-insoluble material from 96,000 x G supernatant - presumably cytoplasmic proteins.

TABLE XXI

CHANGES IN RADIOACTIVITY OF THE VARIOUS FRACTIONS OF W-6 SPHEROPLASTS UNDER CONDITIONS PERMITTING DECREASE IN THE ACTIVITY OF THE ALCOHOL-SOLUBLE PROTEIN

		L-leucine-C^{14}		L-lysine-C^{14}	
Fraction	Time	cpm x 10^3	Δcpm x 10^3	cpm x 10^3	Δcpm x 10^3
I	0.00'	13.00	- 3.00	10.45	- 3.05
	0+30'	10.00		7.40	
II	0.00'	0.55	+ 2.90	0.95	+ 3.10
	0+30'	3.45		4.05	
III	0.00'	0.45	- 0.10	1.95	- 0.35
	0+30'	0.35		1.60	
IV	0.00'	87.70	-16.30	76.20	-17.95
	0+30'	71.40		58.25	
V	0.00'	1.10	+ 0.10	1.05	- 0.05
	0+30'	1.20		1.00	
VI	0.00'	1.60	- 0.25	2.70	- 0.30
	0+30'	1.35		2.40	
VII	0.00'	0.00	0.00	0.00	0.00
	0+30'	0.00		0.00	
VIII	0.00'	3.05	+ 0.05	2.55	0.00
	0+30'	3.10		2.55	

1) Amino Acid Molar Ratios obtained from Various Fractions of Prelabelled W-6 Spheroplasts:

In Table XXII are shown the amino acid molar ratios (relative to alanine) for Fractions I, II, IV, V, VI and VIII (Fig. 5) of W-6 spheroplasts. Attention is drawn to the fact that the ratios for Fractions I and II are different from each other and from those of the other fractions.

TABLE XXII

AMINO ACID MOLAR RATIOS FOR THE VARIOUS FRACTIONS ISOLATED FROM SPHEROPLASTS OF W-6

Amino Acid	Fraction Number					
	I	II	IV	V	VI	VIII
Aspartic	1.14	0.74	0.96	0.94	0.99	1.03
Threonine	0.46	0.37	0.53	0.42	0.46	0.47
Serine	0.44	0.34	0.46	0.29	0.35	0.32
Proline	0.25	0.32	0.44	0.40	0.45	0.44
Glutamic	0.97	0.77	1.00	0.96	1.02	1.10
Glycine	0.66	0.91	0.84	1.42*	0.91	0.86
Alanine	1.00	1.00	1.00	1.00	1.00	1.00
Valine	0.64	0.48	0.60	0.77	0.71	0.72
Cyst./2	-	-	0.06	0.16	0.11	0.07
Methionine	0.03	0.11	0.09	0.02	0.05	0.02
Isoleucine	0.41	0.33	0.57	0.50	0.59	0.63
Leucine	0.76	0.91	0.81	0.72	0.83	0.90
Tyrosine	0.20	0.15	0.25	0.07	0.13	0.14
Phenylalanine	0.28	0.19	0.34	0.29	0.32	0.33
Lysine	0.64	0.53	0.65	0.64	0.61	0.62
Histidine	0.01	0.14	0.05	0.17	0.15	0.19
Arginine	0.50	0.42	0.45	0.45	0.37	0.41
Ammonia	0.83	0.78	0.90	5.76*	1.97	1.57

* These are probably high values since this fraction likely represents ribosomes, the proteins of which were not freed of nucleic acids before hydrolysis. The presence of nucleic acids under these conditions give glycine and ammonia values which are too high since the purines break down under these conditions of acid hydrolysis(188).

4) Discussion

The data presented indicate that the alcohol-soluble protein contains some lipid, phosphorus and a complete spectrum of amino acids (except cyst(e)ine). The presence of phosphorus in this fraction is in agreement with observations of Roberts et al[99] while the presence of lipid is in accord with our earlier observations relative to staining with phosphomolybdate - cf. Fig. 4, Section B. It therefore appears that this fraction is lipo-protein in nature. The observation that the difference in its activity (relative to W-6 and W-157 when labelled with C^{14}-proline) disappeared when transport phenomena were eliminated - Table XV - suggests that, unlike the lipo-protein believed to be involved in the transport of β-galactosides in E. coli[189], it cannot function as an amino acid transport intermediate.

The linear relationship between the activity of this fraction and growth indicates that the removal of label is synchronised with the reproductive cycle of these cells - cf. Figs. 8-11. The differential effects of CM and of PM on the lysine and arginine labelled cells on the one hand, and on the leucine and proline labelled ones on the other, is not immediately clear - cf. Table XVI. It seems, however, that CM permits the removal of label, while PM interferes with this process. On the other hand, CM inhibits the incorporation of

C^{14}-amino acids into this fraction, while PM shows no apparent effect - Figs. 12 and 13.

Under non-growing conditions prelabelled cells lost no label from this fraction except in the simultaneous presence of the required amino acid (proline) and CM - Figs. 14 and 15. In the case of C^{14}-proline labelled cells, no loss occurred in sulphate-free medium, unless CM and SO_4= were supplied simultaneously - Fig. 15. Similar observations were made with C^{14}-leucine labelled preparations suspended in sulphate-free medium - Fig. 16. (It is presumed that in sulphate-free medium these cells were unable to synthesise adequate amounts of the sulphur-containing amino acids.) Thus it appears that the loss of label from this fraction is dependent on the simultaneous presence of all of the amino acids plus CM. These data are not compatible with the views expressed by Pine[190] to the effect that differences in the amounts of alcohol-soluble protein extracted are due to artefacts and that alcohol-soluble proteins represent end-products rather than precursors.

Because the alcohol-soluble protein fraction did not lose radioactivity in the presence of CM under conditions permitting significant reduction of label in the bulk of the cellular proteins, it is suggested that

the metabolism (or rate of C^{14}-amino acid turnover) of this protein is different from the other proteins of the cell - cf. Table XVII. A similar suggestion obtains regarding the lack of effect of PM on amino acid incorporation into this fraction when incorporation into the bulk proteins (relative to C^{14}-proline) was inhibited by about 50%. It is interesting to note that these cells are relatively resistant to PM (cf. Figs. 8b -11b) since Reeve and Bishop[191] have shown that E. coli cells which are resistant to CM are also resistant to PM. These cells (W-6) have been found by Kessel and Lubin[72] to be resistant to 50 μg/ml CM.

Chloramphenicol (CM) was found to increase the activity of the RNA fraction (relative to C^{14}-proline) - Table XVIII - and to enhance the incorporation of C^{14}-uridine into this fraction (Table XX) under conditions when the alcohol-soluble protein does not lose any of its label - cf. Figs. 14 and 15. These data suggest that the alcohol-soluble protein is not associated with the derepression of RNA synthesis in the absence of a required amino acid[187,192].

The observation that the number of counts lost from the alcohol-soluble protein can be accounted for by hot TCA-insoluble material, obtained from the extracellular medium of prelabelled spheroplasts, suggests

that it may serve as a precursor to a special class of proteins - cf. Table XXI. Although the data give no definitive proof that de novo protein synthesis did occur, the dependence on the required amino acid (proline) plus CM suggests that this may be so. This is in accord with the observations of Roberts et al[99], who found that S^{35}-methionine lost from an alcohol-soluble protein fraction of E. coli could be accounted for by that gained by protein during the "secondary" phase of growth.

The amino acid molar ratios of the extra-cellular protein fraction (II) - Table XXII - do not permit a decision as to the type of protein which may have been synthesised under our experimental conditions. However, they do indicate that this fraction is quite different from the alcohol-soluble protein and from the bulk of the cellular proteins - Fraction IV, Table XXII. If this fraction (II) really represents de novo protein synthesis, then an interesting point is raised in that some form of protein synthesis may occur apparently not on ribisomes. CM has been shown to inhibit protein synthesis by interfering with the attachment of messenger RNA (m-RNA) to the ribosome[193].

The bulk of the cellular proteins were found to be associated with the "wall-membrane complex" (Fraction IV, Fig. 5) as judged by the amount of label

associated with it - Table XXI. This observation is in accord with those of Pine[194] who found that under non-growing conditions, the bulk of E. coli proteins is associated with the "wall-membrane complex".

C^{14}-uridine and C^{14}-uracil were observed to be incorporated into a lipid fraction. This observation is similar to those of Hendler[195] who found similar complexes for adenine in the hen oviduct. Strominger[196] has apparently made similar observations (relative to the four ribonucleosides) with bacteria.

The behaviour of the lipid-uridine complex, relative to changes in its activity, when cells were allowed to grow and when treated with CM - Table XIX - suggests that this complex may be associated with some form of RNA synthesis.

IV. GENERAL DISCUSSION

Data presented in Section A (Experimental) showed that stationary phase cells of W-157(Tr⁻pro.) are capable of accumulating L-proline almost as efficiently as W-6(Tr⁺) (when grown anaerobically) and better than W-6 when grown aerobically. However, the cells do not replicate unless provided with high concentrations of this amino acid. The question therefore arises as to why growth of the transport mutant does not take place in 10 μg/ml L-proline. Transport data (Section A) also showed that during growth, after the lag phase, the high-affinity (low capacity) proline system does not operate in W-157. If this system were responsible for the delivery of the amino acid to a special pool which is associated with protein synthesis, (as suggested by Kepes[66]) then one could begin to understand the reasons for the failure of W-157 to grow in media containing low supplements of L-proline.

Although the function of lipid-amino acid complexes is not yet clear, it is fairly well accepted that they represent important intermediates in amino acid metabolism. In Section B (Experimental), it was shown that the transport mutation has secondary effects on the activity of the lipid-proline complex of W-157. The implications of this observation in relation to proline metabolism is therefore apparent.

In Section C (Experimental) data have been presented showing that the transport mutation also affects the extent of C^{14}-proline labelling of an alcohol-soluble protein fraction. This fraction seems to be involved in an unusual form of protein synthesis in that, under non-growing conditions, it is dependent on the presence of chloramphenicol plus the required amino acid (proline). The process of protein synthesis is believed to proceed according to the following scheme: amino acids are first activated[197] by means of an enzyme-mediated condensation with adenosine triphosphate (ATP) to give adenosine monophosphate-amino acid complexes and pyrophosphate; activated amino acids are then transferred by amino acid-specific enzymes to amino acid-specific RNA's (s-RNA)[198,199]; these s-RNA-amino acid complexes find their way to the ribosome where they are assembled into protein[200,201]. The information that dictates the sequence of amino acids in the polypeptide chain has been postulated to be contained in a molecule of DNA-like RNA (m-RNA)[202-205] which is attached to the ribosome to form a complex; the s-RNA's interpret and translate the sequence of the m-RNA nucleotides into a sequence of amino acids[206,207]. Chloramphenicol has been shown to inhibit protein synthesis by interfering with the attachment of m-RNA to the ribosome[193]. Thus the protein synthesis with which the alcohol-soluble protein seems to be

involved may be carried out by processes not involving ribosomes in the same manner as those responsible for other syntheses.

Although there is little doubt that the scheme outlined above is a valid one, it does not answer the question as to the mode of synthesis of the proteins which go toward the formation of ribosomes. Calvin and Calvin[208] have expressed the view that the four cellular organelles essential for successful evolution are:

a) the plasma membrane;

b) the mitochondrion;

c) the plastid;

d) the ribosome.

Recent evidence has shown that the mitochondrion[209] and the plastid[210] are capable of self-replication. It may be that the ribosome and the plasma membrane are also capable of self-replication.

It appears unlikely that the alcohol-soluble protein fraction being discussed is involved in the synthesis of ribosomes because of the differential effects of chloramphenicol and puromycin on its metabolism in otherwise growing cells of W-6. These two antibiotics have been shown to permit the synthesis of immature ribosomes[211]. By a process of elimination, proteins of the plasma membrane may be the likely candidates to which the alcohol-soluble protein acts as

precursor. There are, however, some indications that the lipo-protein component of the E. coli cell wall may also be the component with whose synthesis the alcohol-soluble protein synthesis is involved. Thus Roberts et al[99] have observed a quantitative transfer of S^{35}-methionine from a similar fraction to protein during the "secondary phase" of E. coli growth. Chloramphenicol has also been shown to permit the deposition of wall material in gram-positive organisms[212].

At this stage it is of relative unimportance as to the type of protein to which the alcohol-soluble protein fraction may serve as precursor. What is important is the fact that, because of the transport mutation, W-157 does not adequately label this fraction when supplied with low levels of the required amino acid - proline. It is clear then that the cells would be unable to grow in low supplements of this amino acid if lipid-amino acid complexes and the alcohol-soluble protein fraction represent important intermediates in the metabolism of amino acids.

V. SUMMARY

1. In E. coli W six naturally occurring amino acids were found to be transported by two types of systems - a high-affinity (low Km) and a low-affinity (high Km). Contrary to a widely held belief, leucine, valine and isoleucine were not transported by a single "common" system, but instead by three non-identical high-affinity systems with overlapping affinities, and by a common low-affinity system. Lysine and arginine were accumulated by two non-identical high-affinity systems and by two non-identical low-affinity systems, all with overlapping affinities. The high-affinity proline system was extremely specific being unreactive with OH-L-proline and DL-pipecolate. The low-affinity system was less specific, being reactive with the two proline analogues. OH-L-proline was not transported actively by these cells and had a greater affinity for the low-affinity proline system than proline itself.
2. The only detectable difference between W-6(Tr^+pro.) and W-157(Tr^-pro.) was in their ability to concentrate C^{14}-proline. For proline uptake by the high-affinity system in stationary phase (anaerobically or aerobically grown) cells of W-157, the kinetic constants were different from those of comparable cells of W-6. Aerobic and

anaerobic cells of W-6 had the same kinetic constants; whereas in W-157 there was a seven-fold difference in Km and a four-fold difference in V max. between aerobic and anaerobic cells. In growing cells of W-157 all activity of the high-affinity proline system disappeared after the lag phase and reappeared in the late exponential-early stationary phase. No such phenomenon was observed for valine uptake in this strain or for valine and proline uptakes in W-6.

3. In both strains the kinetic constants for proline and valine accumulation by the low-affinity systems were found to vary with the growth cycle; affinity for substrates increased while maximum rate of accumulation decreased after the lag phase. No difference was found between the two strains relative to valine. With proline, however, after the lag phase W-157 showed only half the maximum rate of accumulation (relative to W-6), although the Km value was the same for both strains. Addition of 0.3M sucrose to mid-exponential phase suspensions of W-157 improved the V max. value for proline accumulation by this system without affecting its Km or the inactivity of the high-affinity system.
4. Lipid-amino acid complexes (LAC) were not detected in high-density suspensions of cells grown anaerobically in minimal medium. Small amounts of LAC were found in

cells grown aerobically in this medium. With cells grown in a complex peptone medium, considerably more LAC were found. The only difference between similarly grown cells of W-6 and W-157 was in the extent of LAC labelling with C^{14}-proline. This difference disappeared when active transport phenomena were eliminated by pre-loading the cells or by incubating "wall-membrane complexes" with C^{14}-proline. Thus it appears that LAC are formed post transport and therefore cannot regulate accumulation.

5. Alcohol-soluble protein (ASP) labelling was greatly reduced when cells were grown anaerobically. The fraction consisted of a complete spectrum of amino acids (except cystine) and contained lipid and phosphorus. The only difference between the two strains involved the extent of labelling by C^{14}-proline. Again the difference disappeared upon elimination of active transport phenomena. Thus ASP cannot regulate amino acid accumulation by these cells.

6. The activity (relative to C^{14}-label) of ASP appeared to be closely synchronised with the growth cycle of these cells. Under non-growing conditions prelabelled cells lost radioactivity from this fraction only when supplied with the required amino acid (proline) plus chloramphenicol. A similar observation was made with C^{14}-proline

labelled cells suspended in sulphate-free medium; label was not lost unless $SO_4^=$ plus chloramphenicol were added to the medium. The label lost from ASP by high-density suspensions of W-6 spheroplasts (in the presence of proline plus chloramphenicol) was accounted for by that which appeared in an extracellular hot TCA-insoluble fraction. This fraction had an amino acid molar ratio different from that of ASP and from that of the other protein fractions of the cell. Thus it may be that ASP is associated with an unusual form of protein synthesis.

7. C^{14}-uridine and C^{14}-uracil were found to be incorporated into a lipid fraction giving a complex (LUC) which, when hydrolysed, gave C^{14}-material with the same chromatographic properties as free uridine. LUC activity (relative to C^{14}-uridine) decreased in control and chloramphenicol-treated cells in a manner suggestive of its involvementin some form of RNA synthesis.

VI. CLAIMS TO ORIGINAL RESEARCH

1. In E. coli W amino acids are transported by two types of systems: a high-affinity (low Km) and a low-affinity (high Km). Valine, leucine and isoleucine are transported by a single low-affinity system and by three non-identical high-affinity systems with overlapping affinities. Lysine and arginine are transported by a total of four systems (two of each) all with overlapping affinities. Proline is transported by an extremely specific high-affinity system and by a less specific low-affinity system.
2. The proline transport mutation of W-157(Tr$^-$pro.) primarily affects the high-affinity system with secondary effects on the low-affinity one when exponential phase cells are studied. In these cells the high-affinity system does not function while the low-affinity one operates at half capacity (relative to similar cells of W-6). Time course studies cannot differentiate between wild and mutant systems if stationary phase cells are used in low-density suspensions. Under these conditions the kinetic constants for the high-affinity system are only slightly different from those of W-6 (when grown anaerobically) and vastly different from those of W-6 when grown aerobically. On the other hand, there are no

differences between the kinetic constants of the low-affinity systems whether the cells are grown aerobically or anaerobically.

3. There appears to be an optimal affinity range (substrate for system) within which adequate accumulation may occur; that is, too high or too low an affinity can result in failure to be accumulated. Thus OH-L-proline, with little or no affinity for the low Km proline system, and with an affinity for the high Km system twice that of proline, is not actively accumulated by these cells. It also appears that within the optimal affinity range there is a reciprocal relationship between maximum rate of accumulation (V max.) and affinity. Thus valine has the lowest affinity for the common valine-leucine-isoleucine high Km system, while isoleucine has the highest affinity; the V max. of valine accumulation by this system is greatest while that of isoleucine is least.
4. Lipid-amino acid complexes (LAC) and alcohol-soluble protein (ASP) do not serve as amino acid transport intermediates. ASP appears to be associated with an unusual form of protein synthesis, apparently not involving ribosomes in the manner now accepted, since radioactivity from this fraction appears in another protein fraction under conditions prohibiting the normal functioning of ribosomes.

5. C^{14}-uridine and C^{14}-uracil are incorporated into a lipid fraction to give a complex (LUC) which seems to be associated with some form of RNA synthesis.

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