THE CATABOLISM AND TRANSPORT OF ARGININE BY *PSEUDOMONAS AERUGINOSA*

by

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B.Sc., University of British Columbia, 1967

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the Department of

of

Microbiology

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February, 1971

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ABSTRACT

Pseudomonas aeruginosa was shown to constitutively degrade arginine via the arginine dihydrolase pathway to ornithine, which was converted both to glutamate and to putrescine. The conversion of ornithine to glutamate appeared to be the major route of arginine degradation in this organism, and was induced to higher activity after growth of the cells with arginine as the sole source of carbon and nitrogen. *P. aeruginosa* did not further degrade putrescine constitutively. However, growth of the cells in arginine resulted in a partial induction of succinic semialdehyde dehydrogenase, an enzyme functioning in putrescine degradation. The anabolic ornithine transcarbamylase of *P. aeruginosa* was repressed after growth of the organism in the presence of arginine.

Pseudomonas putida and *Pseudomonas fluorescens* also possessed the ability to constitutively convert arginine to putrescine via the intermediates, citrulline and ornithine. However, these organisms did not oxidize arginine to the same extent as did *P. aeruginosa*.

P. aeruginosa grew in a mixture of glucose and arginine in the presence of ammonium ions without exhibiting a diauxie effect. Glucose and arginine were oxidized concomitantly when supplied as a mixed substrate, by both growing cells and resting cell suspensions. However, assimilation studies showed that the two substrates were

used to serve somewhat different biosynthetic needs.

Growth of P. aeruginosa in arginine caused an increase in the rates of transport of arginine, lysine, ornithine and citrulline. Kinetic studies of arginine uptake demonstrated the presence of two uptake systems with different affinities for arginine. Inhibition studies indicated that arginine was transported by two uptake systems: a permease specific for arginine, and, with a lower affinity, for ornithine; and a general permease, which transported all the basic amino acids. Polyamines appeared to be transported by an uptake system which was induced to higher levels after growth of the cells with either arginine or putrescine as the sole source of carbon and nitrogen.

P. aeruginosa was found to maintain a stable pool of putrescine when supplied with exogenous ^{14}C-arginine or ^{14}C-putrescine, even when the organism had previously been induced to degrade these substrates. A physical fractionation of the cells indicated that the major portion of this pool was located in the soluble cytoplasm.

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ACKNOWLEDGEMENTS

I would like to express gratitude to my supervisor, Dr. A.F. Gronlund, for her encouragement, advice and criticism throughout the course of this work.

I am also extremely grateful to Dr. H.R. MacMillan and the H.R. MacMillan Family Fund for financial assistance during the first two years of this study.

I would also like to thank very much Mrs. Iris Yu for her help with transport experiments, and my colleagues and my friends for their help throughout my graduate studies program.

INTRODUCTION

The enzymes of arginine degradation have been shown to be inducible and to be subject to catabolite repression in several microorganisms (Ramos et al. 1967; Laishley and Bernlohr, 1968). Jacoby (1964) found that the ability of Pseudomonas fluorescens to oxidize eighteen different amino acids was repressed by glucose. Kay (1968) obtained results which indicated that arginine was degraded constitutively by Pseudomonas aeruginosa at a relatively rapid rate. Kay (1969) found that cells of P. aeruginosa which were supplied with low external concentrations of ^{14}C-arginine in glucose minimal medium accumulated a large pool of putrescine, which was extremely stable, being maintained for periods as long as twenty four hours during starvation for an exogenous carbon source. Putrescine has been shown to bind to deoxyribonucleic acid and ribonucleic acid in vitro and is thought to play a role in translation and, possibly, in transcription (Stevens, 1970). Several studies have indicated that putrescine may be required for cell division (Davis, Lawless and Port, 1970; Hirshfield, et al. 1970; Inouye and Pardee, 1970)

It was the object of this investigation to determine the pathway by which arginine was degraded by P. aeruginosa and the effect of glucose on the catabolism of arginine by this organism.

A further investigation of the basic amino acid uptake systems of P. aeruginosa, which were partially characterized by Kay (1968), was also carried out.

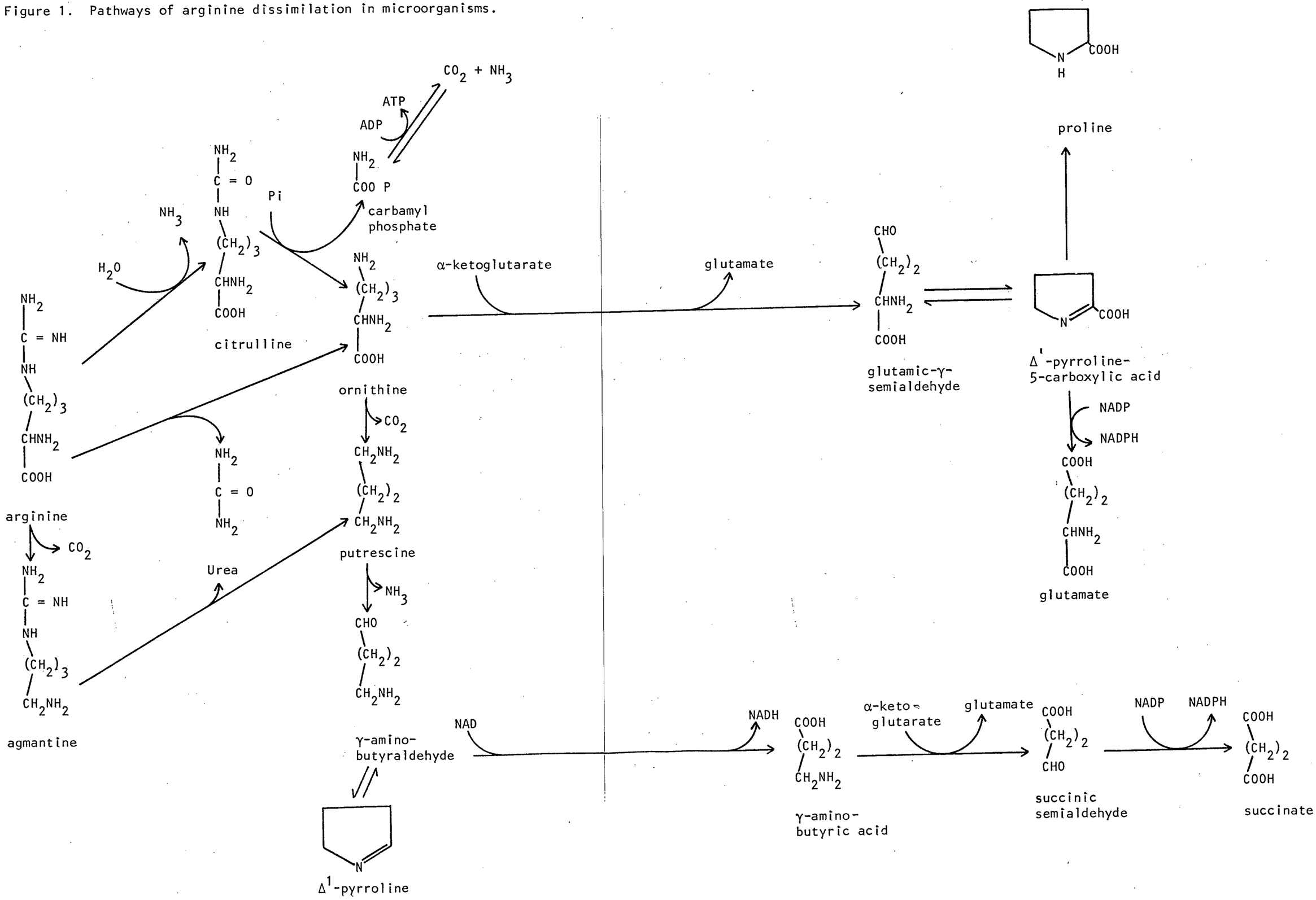
LITERATURE REVIEW

I. Pathways of Arginine Degradation in Microorganisms

The pathways of arginine dissimilation in microorganisms have been found to be varied and complex (Fig. 1). In Neurospora crassa (Castanada, Martuscelli, and Mora, 1967; Meister, 1965), Saccharomyces cerevisiae (Middelhoven, 1964), and two species of Bacillus (de Hauwer, Lavalle and Wiame, 1964; Laishley and Bernlohr, 1968), arginine is first hydrolysed by arginase, resulting in the production of ornithine and urea. Ornithine is then converted by ornithine-γ-transaminase to glutamic-γ-semialdehyde, an unstable intermediate, which spontaneously cyclizes to Δ^1-pyrroline-5-carboxylic acid. The latter compound may be converted by a dehydrogenase to glutamate, or by a reductase to proline. An exception to the ornithine transaminase reaction has been found in Clostridium botulinum, where ornithine is converted to glutamic-γ-semialdehyde by a nicotine adenine dinucleotide (NAD)-linked dehydrogenase (Costilow and Laycock, 1969).

Arginase, ornithine transaminase, and Δ^1-pyrroline-5-carboxylate dehydrogenase are coincidently induced by arginine or by ornithine in Bacillus licheniformis (Laishley and Bernlohr, 1968) and Bacillus subtilis (de Hauwer, Lavalle and Wiame, 1964).

Figure 1. Pathways of arginine dissimilation in microorganisms.



The latter workers found evidence that ornithine was not the actual inducer of these enzymes in B. subtilis. Ornithine was first converted to arginine via the arginine biosynthetic pathway, and arginine then served as the inducer. They also isolated a mutant of B. subtilis which was constitutive for arginine transport, arginase, and ornithine transaminase, indicating that the structural genes for these functions may form an operon. Δ^1-pyrroline-5-carboxylate dehydrogenase remained inducible, and thus must be under separate control.

deHauwer, Lavalle and Wiame (1964) showed that the Δ^1-pyrroline-5-carboxylate dehydrogenase functioning in arginine degradation in B. subtilis was a different enzyme from the one functioning in proline catabolism. In this organism, arginine induced only the arginine degrading enzymes, and proline induced only the proline degrading enzymes. Laishley and Bernlohr (1968), on the other hand, found that arginine caused a partial induction of proline oxidase in B. licheniformis, and proline caused a partial induction of arginase. They therefore hypothesized that Δ^1-pyrroline-5-carboxylic acid, an intermediate common to the pathways of oxidation of both proline and arginine, was the actual inducer of the two pathways.

Organisms lacking an arginase can convert arginine to ornithine by the arginine dihydrolase pathway. Arginine is first converted to citrulline by the enzyme arginine deiminase, which has been

found in Pseudomonads, Streptococci, and Clostridia (Oginsky, 1955). These organisms also contained an enzyme which, in the presence of substrate amounts of phosphate, split citrulline to produce ornithine, NH_3, and CO_2. This reaction required magnesium and either adenosine monophosphate (AMP) or adenosine diphosphate (ADP), and resulted in the production of adenosine triphosphate (ATP). Oginsky called this enzyme citrulline phosphorylase. Its activity, in a cell-free extract of Pseudomonas aeruginosa, was inhibited by ornithine. Subsequent workers discovered that carbamyl phosphate was an intermediate in this reaction, and, therefore, the enzyme was a catabolic ornithine transcarbamylase (Meister, 1965). The carbamyl phosphate was degraded by a carbamate kinase, and this was the ATP producing step.

Stalon et al. (1967a) found that Pseudomonas fluorescens and P. aeruginosa contained two ornithine transcarbamylases separable by ammonium sulphate fractionation. One enzyme, assumed to function in catabolism, was induced by growth in the presence of arginine, whereas the other, assumed to have an anabolic function, was repressed under these conditions. Although the anabolic enzyme was completely irreversible, the catabolic enzyme was capable of catalysing both the synthesis and breakdown of citrulline in vitro but not in vivo (Ramos et al. 1967). Since the pH optima for the activity of the catabolic enzyme in the two directions differed greatly, it was hypothesized that localization in an

acidic compartment might prevent this enzyme from functioning in the anabolic direction *in vivo*. However, Stalon *et al.* (1967b) offered an alternative explanation for the *in vivo* irreversibility of the catabolic ornithine transcarbamylase. They reported that the catabolic enzyme was subject to allosteric inhibition by carbamyl phosphate at concentrations which saturated the anabolic enzyme. The catabolic ornithine transcarbamylase was also inhibited by ATP and was activated by ADP.

Gale (1940) discovered that *Escherichia coli* possessed inducible arginine and ornithine decarboxylases when grown at low pH values. Arginine was decarboxylated to form agmantine, and putrescine was produced from ornithine. The pH optimum of ornithine decarboxylase was 5.0, and that of arginine decarboxylase was 4.0.

Morris and Pardee (1965) found that when *E. coli* was grown in minimal medium, it contained a constitutive ornithine decarboxylase with a pH optimum of 7.5. This enzyme was present at the same level in cells grown in the presence of ornithine at low pH values, but, under these conditions, a high level of ornithine decarboxylase with a pH optimum of 5.3 was also present. The authors hypothesized that the constitutive enzyme served a biosynthetic function, synthesizing the normal cellular concentration of putrescine, whereas the inducible enzyme appeared to serve a catabolic function, degrading ornithine when present in the cell in excess. Morris and Pardee (1966) found a second pathway for the

synthesis of putrescine in E. coli. This consisted of a constitutive arginine decarboxylase converting arginine to agmantine, and a constitutive agmantine ureohydrolase, hydrolysing agmantine to produce putrescine and urea. Although the synthesis of the enzymes of both pathways of putrescine biosynthesis is constitutive in E. coli, Morris and Koffron (1969) discovered that the relative flow through the two pathways varied considerably in vivo. With cells grown in minimal medium, the majority of the putrescine was produced by the decarboxylation of ornithine, whereas, in the presence of exogenous arginine, the direct decarboxylation of arginine was the preferred route. Tabor and Tabor (1969a) have reported that the ornithine and arginine decarboxylases of a strain of E. coli auxotrophic for ornithine were both repressed and feedback inhibited by putrescine and spermidine.

Gale (1942) showed that P. aeruginosa possessed the constitutive ability to oxidize the diamines putrescine, agmantine and cadaverine to completion and could be adapted to partially oxidize histamine and tyramine. Zeller (1963) has reviewed the properties of diamine oxidases from plant, animal, and microbial sources. The enzyme catalysed the oxidative deamination of a number of diamines, resulting in the formation of the corresponding amine aldehydes. When putrescine was the substrate, the product, γ-aminobutyraldehyde, rapidly cyclized to form Δ^1-pyrroline. Evelyn (1967a, b) demonstrated the conversion of putrescine to Δ^1-pyrroline, with the concomitant

uptake of oxygen by cell-free extracts of putrescine grown Mycobacteria. Satake and Fujita (1953) studied the oxidation of putrescine and histamine by a cell-free extract of Achromobacter and concluded that the activity was the result of the action of two substrate-specific enzymes rather than a single enzyme reacting with both diamines. Moreover, the enzymes were dehydrogenases linked to the electron transport system, rather than oxidases reacting directly with molecular oxygen. Kim and Tchen (1962) found that a mutant of E. coli capable of degrading putrescine catalysed the conversion of putrescine to γ-aminobutyraldehyde by a transaminase rather than by an oxidase.

Jakoby and Fredericks (1959) discovered that growth of P. fluorescens in the presence of putrescine induced the synthesis of an enzyme, γ-aminobutyraldehyde dehydrogenase, that oxidized Δ^1-pyrroline to γ-aminobutyric acid, with the concomitant reduction of NAD. γ-aminobutyric-glutamate transaminase catalyzed the conversion of γ-aminobutyric acid to succinic semialdehyde, which was further oxidized to succinate by succinic semialdehyde dehydrogenase. γ-Aminobutyric-glutamic transaminase was induced by growth in either γ-aminobutyrate or putrescine. Succinic semialdehyde dehydrogenase was present constitutively at a fairly high level. Growth in putrescine did not appreciably increase the activity of this enzyme; however, growth in γ-aminobutyrate did increase it somewhat. These three enzymes also function in

putrescine degradation in the E. coli mutant (Kim and Tchen, 1962) and Mycobacteria (Evelyn, 1967) mentioned above. P. aeruginosa grown in the presence of γ-aminobutyrate also oxidized this compound to succinate via succinic semialdehyde (Bachrach, 1960).

Nakamura (1960) discovered that P. aeruginosa possessed two succinic semialdehyde dehydrogenases; one linked to NAD, the other to NADP. Padmanabhan and Tchen (1969) have further studied these enzymes in a Pseudomonad. The NADP-linked enzyme was constitutive, and could not be induced to a higher level. The NAD-linked succinic semialdehyde dehydrogenase activity was resolved into three peaks by chromatography on DEAE-Sephadex, only one of which was specific for succinic semialdehyde. This enzyme was induced to high levels by growth of the cells in γ-aminobutyrate or in putrescine. The other two peaks were aminoaldehyde dehydrogenases acting on 3-aminopropanal and γ-aminobutyraldehyde in addition to succinic semialdehyde. One peak was much more reactive with the amino-aldehydes than with succinic semialdehyde and this enzyme could be induced to a high level by growth in putrescine, but not in γ-aminobutyrate. Thus, it may correspond to the γ-aminobutyraldehyde dehydrogenase studied by Jacoby and Fredericks (1959). Very low activities of the NAD-linked dehydrogenases were detectable under all growth conditions. It was therefore hypothesized that the organism was continuously synthesizing and degrading putrescine.

In summary, arginine may be decarboxylated to agmantine, or it may be converted to ornithine, either by an arginase or by the arginine dihydrolase pathway. Both agmantine and ornithine can be converted to putrescine, which can be further degraded to succinate by some organisms. In addition, ornithine can be directly degraded to glutamate.

An additional pathway of arginine degradation has been described in Streptomyces griseus (van Thoai, 1965). In this organism, arginine undergoes decarboxylating oxygenation resulting in the formation of γ-guanidobutyramide, which is then converted to γ-aminobutyrate via the intermediate γ-guanidobutyrate. S. griseus also contains a transamidinase functioning in streptomycin biosynthesis, and catalysing the transfer of the guanidine group of arginine to glycine, forming ornithine and glycocyamine (Walker, 1965). This enzyme is also present in E. coli (Wilson and Holden, 1969a) and C. botulinum (Mitruka and Costilow, 1967).

More than one pathway of arginine degradation may be operative in a single organism. Wilson and Holden (1969a) have reported that, although the majority of exogenous arginine was converted to putrescine via agmantine in E. coli, 4 - 8% was converted to glutamate, presumably via ornithine and glutamic-γ-semialdehyde. Mitruka and Costilow (1967) demonstrated that C. botulinum possessed an arginine transamidinase in addition to the enzymes of the arginine dihydrolase pathway. Moreover, this organism degraded

ornithine by two pathways, 20% being decarboxylated to putrescine, while 75% was degraded via δ-aminovaleric acid.

II. Catabolite Repression of Amino Acid Degradation

Epps and Gale (1942) were the first to study the repression of enzymes catabolizing amino acids, which was caused by the addition of glucose to the growth medium. In E. coli, ornithine decarboxylase, tryptophanase, aspartase and alanine, serine, and glutamate deaminases were subject to this repression, whereas arginine, lysine, and histidine decarboxylases were unaffected.

The induction of many enzymes responsible for the catabolism of carbohydrates and of amino acids is repressed by glucose. This phenomenon was termed catabolite repression by Magasanik (1961) who considered it to be a type of end-product repression, since the catabolites formed by the action of the glucose-sensitive enzymes could be more readily obtained from glucose. Thus, any situation where limitation of anabolism caused an accumulation of catabolic intermediates (e.g., nitrogen or phosphate starvation) produced a similar repression. Recently, it has been discovered that glucose and other rapidly metabolizable carbon sources cause the repression of enzyme synthesis in E. coli by lowering the intracellular concentration of cyclic adenosine monophosphate (cyclic AMP). The addition of cyclic AMP to

cultures of E. coli overcame the catabolite repression of tryptophanase, D-serine deaminase, thymidine phosphorylase, and permeases and catabolic enzymes specific for several sugars. (see review by Pastan and Perlman, 1970).

Jacoby (1964) found that glucose repressed the ability of P. fluorescens to oxidize 18 different amino acids. He further studied the oxidation of tyrosine and histidine and observed that glucose did not cause a decrease in the rate of uptake of these amino acids. However, the induction of enzymes specific for the catabolism of these amino acids was repressed by glucose.

Lessie and Neidhardt (1967) found that the histidase activity of P. aeruginosa was subject to repression by a number of carbon sources, but that partial derepression occurred when ammonium ions were omitted from the medium. A similar derepression has been demonstrated in Aerobacter aerogenes (Neidhardt and Magasanik, 1957); however ammonium ions did not repress the synthesis of histidase in the absence of glucose. Thus, histidase production was maximal when histidine was required as a source of carbon and energy and was somewhat reduced when histidine was required only as a nitrogen source. It was severely repressed when all of the products of histidine catabolism could be supplied by a more rapidly metabolizable energy source. It is interesting to note that the repression of a carbohydrate degrading enzyme, myo-inositol dehydrogenase, was not affected by ammonium ions. These

workers have proposed that the compound that is physiologically active in the catabolite repression of amino acids is a nitrogenous compound which is readily formed from the catabolites of glucose.

Castanada, Martuscelli and Mora (1967) showed that the presence of ammonium ions in the growth medium of N. crassa decreased the level to which arginase and ornithine transaminase were induced. Wiame (1965) has reported that the presence of ammonium ions, but not glutamate, affected the utilization of arginine by B. subtilis. Middelhoven (1970) has hypothesized that the level of arginase and ornithine transaminase in S. cerevisiae is controlled by a nitrogenous repressor. The induction of these enzymes was not affected by glucose, but was inhibited by ammonium sulphate and a few of the more readily assimilable amino acids. This was not end-product repression due to the formation of glutamate, since glutamate itself was not a strong inhibitor. Moreover, starvation of S. cerevisiae for nitrogen derepressed arginase and ornithine transaminase to a lavel as high as that induced by arginine. This resulted in a depletion of the cellular aginine pool. Derepression could be inhibited by the addition of one of a number of nitrogen containing compounds. It was specific for the arginine degrading enzymes; other enzymes involved in protein and amino acid degradation were not affected.

Lessie and Neidhardt (1967) also noted that succinate, which produced the most severe catabolite repression of histidase synthesis

in P. aeruginosa, also inhibited the histidase activity in vivo. Hug, Roth and Hunter (1968) found that succinate competitively inhibited urocanase, the second enzyme of histidine degradation, in Pseudomonas putida. Urocanate was a competitive inhibitor of histidase. Thus, the presence of succinate caused an in vivo repression of histidase by sequential feedback. However, Jensen and Neidhardt (1969) found that, in A. aerogenes, in vivo inhibition of histidase activity did not require the presence of succinate, but could be caused by restricting growth in histidine in a chemostat by limiting an essential nutrient. This inhibition appeared to be immediately released when the biosynthetic restriction was released, allowing an immediate increase in growth rate before the histidase level had increased significantly. Thus, catabolite inhibition appears to act as a fine control mechanism similar to the feedback inhibition of biosynthetic pathways. Catabolite inhibition of the utilization of a number of sugars has been noted in E. coli (McGinnis and Paigen, 1969).

As far as the enzymes of arginine degradation are concerned, Ramos et al. (1967) found that arginine deiminase, carbamate kinase, and the catabolic ornithine transcarbamylase were subject to catabolite repression in P. fluorescens. These enzymes were derepressed when growth was limited by the carbon source, citrate. Laishley and Bernlohr (1968) showed that the induction of arginase, ornithine transaminase, and Δ^1-pyrroline-5-carboxylate dehydrogenase

was repressed by glucose in B. licheniformis; however, Middelhoven (1970) observed that the arginase and ornithine transaminase of S. cerevisiae were not subject to catabolite repression.

Padmanabhan and Tchen (1969) found that glucose did not repress the induction of succinic semialdehyde dehydrogenase by γ-aminobutyrate. However, when putrescine was the inducer, the levels of both succinic semialdehyde dehydrogenase and aminoaldehyde dehydrogenase were lowered. These workers therefore felt that glucose caused the repression of γ-aminobutyraldehyde dehydrogenase, thereby inhibiting the induction of succinic semialdehyde dehydrogenase by putrescine, by lowering the concentration of the inducer, γ-aminobutyrate.

III. The Biological Importance of Polyamines in Microorganisms

Kay (1969) found that P. aeruginosa, when supplied with exogenous arginine, formed a high intracellular pool of putrescine which was stable over 24 hours of starvation for an exogenous carbon source. Thus, putrescine appeared to be an important product of arginine degradation in this organism, and may be maintained within the cell in a bound state.

Putrescine and the higher polyamines, spermidine and spermine, which are synthesized from putrescine, are present in varying concentrations in microorganisms, plants, and animals. P. aeruginosa

has been reported to contain 45% of its polyamines as putrescine; 32% as spermidine, and 23% as spermine (Weaver and Herbst, 1958). In E. coli, on the other hand, putrescine comprises 90% of the total cell polyamine, with spermidine constituting the remainder (Tabor and Tabor, 1964).

There is indirect evidence that polyamines perform an important function in E. coli. The intracellular putrescine content is the same in cells grown in minimal medium as in cells grown in the presence of arginine or ornithine (Morris and Koffron, 1969). Tabor and Tabor (1969a) found that an ornithine auxotroph continued to synthesize polyamines, even when ornithine limitation caused a three-fold reduction in growth rate. Although the putrescine content was markedly decreased under these conditions, the spermidine level was only slightly reduced.

Studies with mutants of E. coli (Hirshfield et al. 1970) and N. crassa (Davis, Lawless and Port, 1970) have demonstrated that the growth rate of these organisms was greatly reduced when putrescine synthesis was repressed. Many snake forms were observed, indicating that cell division may have been affected. Normal growth was restored by the addition of spermine, spermidine, or putrescine. Inouye and Pardee (1970) have presented evidence that the ratio of putrescine to spermidine may be a critical factor for cell division in E. coli.

The biochemical action of polyamines has been recently reviewed (Stevens, 1970). Polyamines bind strongly to deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and synthetic polynucleotides *in vitro*. They stabilize the double helical structure of DNA, and appear to cause RNA to assume a more compact structure. Polyamines do not appear to be preferentially associated with DNA *in vivo*, but may associate with RNA. In *E. coli*, an increase in the intracellular concentration of spermidine, but not putrescine, resulted in an increased rate of RNA synthesis. The spermidine concentration appears to be related to RNA synthesis in animal tissues, also. Moreover, the addition of polyamines to a cell-free system causes an increase in RNA polymerase activity, appearing to increase the number of available initiation sites.

Polyamines also promote the association of ribosomal subunits and the binding of messenger RNA and amino acyl transfer RNA to ribosomes. They can partially replace magnesium ions in an *in vitro* protein synthesizing system. Since optimal growth of *E. coli* occured at intracellular magnesium concentrations much lower than the concentration required for *in vitro* protein synthesis, Hurwitz and Rosano (1967) hypothesized that polyamines may play an important role in *in vivo* protein synthesis, binding to many of the sites where magnesium binds in the *in vitro* system. Weiss and Morris (1970) found that up to 70% of the magnesium bound to ribosomes could be replaced by spermidine or putrescine

without affecting their activity in cell-free protein synthesis. However, a critical level of magnesium was required for the structural and functional integrity of ribosomes, and could not be replaced by polyamines.

The order of activity of polyamines in all the previously mentioned functions is spermine>spermidine>putrescine. Thus, although E. coli contains much more putrescine than spermidine, the latter may be physiologically more important. It is interesting to note that, in a Pseudomonad unable to synthesize spermidine, the binding of hydroxyputrescine, which possesses the same number of binding sites as spermidine, to ribosomes varies with the magnesium concentration in the same way as does spermidine in E. coli (Rosano and Hurwitz, 1969).

In summary, polyamines bind to and stabilize DNA, RNA, and ribosomes. They stimulate protein synthesis in vitro, and RNA synthesis both in vitro and in vivo. It is possible that polyamines do not have a single specific function in cell metabolism, but may act as polyvalent cations stabilizing nucleotide-nucleotide binding, replacing many of the magnesium ions required for in vitro reactions.

IV. Transport of the Basic Amino Acids by Microorganisms

The transport and accumulation of amino acids by microorganisms has been reviewed extensively (Kepes and Cohen, 1962; Britten and McClure, 1962; Kay, 1968; Kabak, 1970). Bacteria have been found to possess many transport systems, some of which are specific for single amino acids and others of which are specific for "families" of structurally related amino acids. Kay (1968) identified 11 such amino acid transport systems in P. aeruginosa.

The transport of the basic amino acids has been studied in several microorganisms. Schwartz, Maas, and Simon (1959) isolated a mutant of E. coli which was defective in the uptake of canavanine, arginine, ornithine, and lysine, and therefore concluded that these compounds must be transported by a single permease. Citrulline uptake by also affected somewhat by this mutation (Maas, 1965). The studies of Wilson and Holden (1969a) indicated that E. coli possessed at least two systems for the uptake of basic amino acids: one highly specific for arginine, and one with a high affinity for lysine and a lower affinity for arginine. Wilson and Holden (1969b) isolated four proteins, from the osmotic shock fluid of E. coli, which bound arginine, but showed no affinity for lysine. Two of these proteins were studied further, and were capable of restoring the ability of shocked cells to transport arginine.

Kay (1968) obtained evidence for the existence, in P. aeruginosa, of two systems for the transport of basic amino acids: one specific

for arginine and ornithine, and the other having a high affinity for lysine and lower affinities for arginine, ornithine, citrulline, and histidine. Grenson (1966) and Grenson et al. (1966) demonstrated that S. cerevisiae also possessed two systems for the uptake of basic amino acids: one was highly specific for lysine; the other had a very high affinity for arginine, and a lower affinity for lysine, ornithine and canavanine. Pall (1970) showed that N. crassa possessed a basic amino acid transport system with a high affinity for arginine, lysine, canavanine, and ornithine, and a much lower affinity for histidine.

Grenson, Hou and Crabeël (1970) showed that the only mechanism of citrulline uptake by S. cerevisiae was via the general amino acid permease, which was capable of transporting most amino acids. Thwaites and Pendyala (1969) demonstrated that citrulline was also transported solely by the general amino acid transport system of N. crassa.

Ames (1964) and Ames and Roth (1968) have shown that Salmonella typhimurium possessed a highly specific system for the transport of histidine. This organism could also take up histidine via a general aromatic permease, which functioned in the transport of tyrosine, phenylalanine, and tryptophan. On the other hand, Kay (1968) found that all basic amino acids competed strongly with histidine, and therefore concluded that histidine must be transported mainly by the basic amino acid uptake systems in this

organism. Pall (1970) has shown that N. crassa can transport histidine via three transport systems: the basic amino acid transport system, the neutral amino acid transport system, and the general amino acid transport system.

MATERIALS AND METHODS

I. Organisms and Media

Pseudomonas aeruginosa (ATCC 9027) was used throughout this study. *Pseudomonas fluorescens* A.3.12 (W.A. Wood) and *Pseudomonas putida* (ATCC 4359) were also used for one experiment. Stock cultures were maintained at 6 C in glucose ammonium salts minimal medium and were checked periodically for purity by streaking onto Difco plate count agar. Cultures of *P. aeruginosa* were also checked for production of the species characteristic pigment, pyocyanine, by streaking onto Kings medium (King, Ward, and Raney, 1954).

Cells were grown in a medium containing 0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4 and 0.5 ppm $FeSO_4.7H_2O$ at pH 7.2. $MgSO_4.7H_2O$ and the appropriate carbon source were sterilized separately and added aseptically to give a final concentration of 0.05% and 0.2% respectively. When putrescine or arginine were used as sources of both nitrogen and carbon, $NH_4H_2PO_4$ was replaced by 0.35% KH_2PO_4. When arginine was used as the sole carbon source, 1 ml of 1M potassium phosphate buffer (pH 7.0) was added to 20 ml of the minimal medium described above, to prevent an increase in pH during growth.

II. Growth of Cells

For most experiments, Erlenmeyer flasks (250 ml) equipped with 13 mm test tube sidearms and containing 30 ml quantities of medium were inoculated to a final concentration of 2% with a cell suspension previously grown in the same medium, and were then incubated at 30 C in a model G77 water bath (New Brunswick Scientific Co., New Brunswick, N.J.) rotating at 220 rev/min. Growth was followed with a Klett-Summerson colorimeter (red filter) and cells from the logarithmic phase were harvested at 110 - 125 Klett units [equivalent to 0.7 - 0.8 optical density units measured at 660 nm with a model B spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.)].

For some experiments, cells were grown in Roux flasks containing 100 ml of the appropriate medium. A 1% innoculum was used, and the cells from the early stationary phase of growth were harvested after 20 hours at 30 C.

III. Preparation of Cell Suspensions

1. Resting cell suspensions for respirometry

Cells were harvested by centrifugation at 10,000 x g for 7 minutes at 6 C. They were washed three times with cold 0.9%

NaCl (pH 7.4) and resuspended in cold 0.05 M Tris(hydroxymethyl)-aminomethane-HCl (Tris) buffer (pH 7.4). In some cases, cells were resuspended in 0.067 M potassium phosphate buffer (pH 7.4). The final cell concentration was approximately 5 mg of cells (dry weight)/ml, unless otherwise specified.

2. Cell suspensions for transport studies.

Cells in the logarithmic phase of growth were harvested as described above, but at room temperature, and washed twice with minimal salts medium without carbon source. They were resuspended in glucose minimal medium to approximately 1.35 mg of cells (dry weight)/ml and kept at room temperature until required for experimentation.

IV. Preparation of Cell-free Extracts

Cells were harvested from the logarithmic phase of growth by centrifugation at 10,000 x g for 7 minutes at room temperature, and washed twice with 0.9% NaCl (pH 7.4). When necessary, dry cell pellets were stored at -70 C. They were resuspended in cold 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% mercapto-ethanol, to a final concentration of approximately 20 mg of cells (dry weight)/ml. Deoxyribonuclease (Worthington Biochemical Corp.,

Freehold, N.J.) was added to a final concentration of 80 µg/ml. The cells were broken by dropwise expulsion from a precooled French pressure cell under 15,000 psi pressure. Unbroken cells and large cellular debris were removed by centrifugation at 10,000 x g for 7 minutes at 6 C.

V. Manometric Procedures

The oxygen uptake of cells respiring endogenously or in the presence of exogenous substrates was followed in the conventional manner with the Warburg respirometer. A typical reaction mixture contained 1.0 ml cell suspension [approximately 5 mg of cells (dry weight)/ml], 1.9 ml 0.05 M Tris buffer (pH 7.4), 2.5 µmoles substrate in 0.1 ml, and 0.15 ml 20% KOH in the center well. In the endogenous control, 0.1 ml distilled water replaced the substrate. For some experiments the Tris was replaced by 0.067 M phosphate buffer (pH 7.4).

VI. Uptake of Labelled Compounds

The incorporation of ^{14}C-labelled compounds into whole cells, protein, and pools was determined by the Millipore filtration procedure of Britten and McClure (1962). Experiments were carried out at 30 C, in 25 ml or 50 ml Erlenmeyer flasks containing glucose

minimal medium, stirred with a Mag-Jet underwater stirrer (Bronwell Scientific, Rochester, N.Y.) driven by a Lauda K2 circulating pump (Brinkman Instruments, Westbury, N.Y.). Unless otherwise specified, cells were added to give a final concentration of 0.135 mg of cells (dry weight)/ml, and substrates were added to give an external concentration of 2.5×10^{-5}M, and 0.05 µc ^{14}C/ml. Samples of the cell suspension were removed at appropriate time intervals and either filtered immediately or added to an equal volume of cold 10% trichloroacetic acid. Whole cells or trichloroacetic acid-insoluble material were filtered on 0.45 µ pore size filters (Millipore Corp., Bedford, Mass.) in an E8B precipitation apparatus (Tracerlab, Waltham, Mass.) and immediately washed with 2 ml of glucose minimal medium (whole cells) or 2 ml of distilled water (trichloroacetic acid-insoluble material). Filters were dried under an infra-red lamp and placed in vials containing 5 ml of scintillation fluid (Liquifluor, New England Nuclear Corp., Boston, Mass.). The vials were assayed for radioactivity in a model 725 liquid scintillation spectrometer (Nuclear Chicago Corp., Des Plaines, Ill.). Corrections were made for background. In order to reduce statistical deviation, at least 1000 counts were recorded. The counting efficiency of the instrument was 80 per cent under the conditions employed.

VII. Inhibition of Transport by Compounds Structurally Related to the Substrate

To determine inhibition, the unlabelled inhibitor was added to a concentration of 2.5×10^{-3}M immediately prior to the addition of the ^{14}C-labelled substrate to a concentration of 2.5×10^{-5}M. Uptake of radioactivity into the whole cells was followed, and the degree of inhibition was calculated from the reduction in the rate of incorporation of the ^{14}C-labelled substrate relative to the appropriate control.

VIII. Assay of Succinic Semialdehyde Dehydrogenase

Succinic semialdehyde dehydrogenase was measured as described by Jakoby (1962) by coupling the reaction to γ-aminobutyrate-glutamic transaminase. The pH of the reaction mixture was 8.0 and 5 µmoles of both γ-aminobutyrate and α-ketoglutarate were added. The reduction of 0.25 µmoles of NAD or NADP was measured at 340 nm at 34 C in a model 2000 spectrophotometer (Gilford Instrument Laboratories Incorp., Oberlin, Ohio). Appropriate controls were carried out to show the requirement of the reaction for both γ-aminobutyrate and α-ketoglutarate. Specific activities are expressed as mµmoles of substrate utilized per min per mg of protein.

IX. Chemical Fractionation of Whole Cells

Cells were fractionated according to the procedure of Roberts et al. (1955) with the modification of Clifton and Sobek (1961). The hot trichloroacetic fraction was prepared by heating the sample at 90 C for 20 min rather than at 100 C for 30 min. Samples of the cell fractions were plated in duplicate onto stainless steel planchets, dried under an infra-red lamp, and counted at infinite thinness with an automatic low background planchet counting system (Model 4342, Nuclear Chicago Corp., Des Plaines, Ill.). Corrections were made for background. In order to reduce statistical deviation, at least 1000 counts were recorded when possible. The counting efficiency of the instrument was 28%.

X. Physical Fractionation of Whole Cells

Cells were harvested by centrifuging the contents of the Warburg cups at 10,000 x g for 7 minutes at 6 C, and were re-suspended in 0.05 M Tris buffer (pH 7.4) containing 10^{-2}M $MgCl_2$ to approximately 20 mg of cells (dry weight)/ml. The suspension was passed once through a French pressure cell and whole cells and large debris were removed by centrifugation for 7 minutes at 5,000 x g. The resulting cell-free extract was fractionated

according to the procedure of Campbell, Hogg and Strasdine (1962), omitting the washing of the initial 25,000 x g pellet. The magnesium concentration of the 25,000 x g supernatant fluid was adjusted to 10^{-3}M before centrifugation. The pellets were resuspended in 0.05 M Tris (pH 7.4).

XI. Chromatography of Supernatant Fluids from Warburg Cups Containing ^{14}C-labelled Substrates

Protein was precipitated from the supernatant fluids by the addition of trichloroacetic acid to a final concentration of 5%. After 20 min on ice, the precipitated material was removed by centrifugation at 10,000 x g for 10 minutes at 6 C. The supernatant fluids were then extracted 6 times with ethyl ether to remove trichloroacetic acid, and evaporated to dryness using an Evapomix instrument (Buchler Instruments, Fort Lee, N.J.). The dried samples were dissolved in the desired volume of distilled water.

When phosphate was present in the reaction mixture, the samples were applied to a column of Dowex 50 H^+. The column was washed with distilled water until no further radioactivity was eluted, removing the neutral and acidic compounds, including phosphate. The adsorbed compounds were eluted with 4 M ammonium hydroxide, the eluate was evaporated to dryness 4 times to remove ammonia,

and the residue was dissolved in the desired amount of distilled water.

Samples were quantitatively applied to thin layer plates spread with cellulose powder MN300 (Macherey and Nagel, Püren, Germany) and the radioactive compounds were separated two-dimensionally by the method of Jones and Heathcote (1966). The chromatograms were then exposed for one week to medical X-ray film (Eastman Kodak Co., Rochester, N.Y.). The films were developed and the radioactive areas detected by this method were scraped loose from the plates and drawn, by vacuum, into scintillation vials which were subsequently filled with 10 ml of scintillation fluid and assayed for radioactivity in a liquid scintillation spectrometer. The addition of from 5 to 60 mg of cellulose caused no increase in quenching under these conditions.

In some instances, the presence of radioactivity in aqueous samples was assayed directly by liquid scintillation spectrometry. Up to 100 µl of the sample was placed in a vial containing 10 ml of a scintillation fluid consisting of 40 volumes of methanol and 60 volumes of toluene plus luquifluor. Values obtained by this method were multiplied by 1.3 to convert them into values comparable to those obtained with the previously mentioned scintillation fluid.

XII. Assay of Ornithine Transcarbamylase

The assay procedure of Stalon et al. (1967a) was followed. In order to establish a reaction rate, the reaction was carried out in a total volume of 25 ml containing 1 ml of cell-free extract, and 2.5 ml samples were removed at various times, added to an equal volume of 1 N HCl in heavy-walled centrifuge tubes standing in ice, and treated as in the procedure of Stalon et al. (1967a). Sampling times were as follows: 2, 4, 6, 8, 10, 15, 20 and 30 minutes when cell-free extracts of glucose grown cells were used; and 5, 10, 15, 20, 25 and 30 minutes when cell-free extracts of cells grown in the presence of added arginine were used.

Citrulline was assayed by a modification of the method described by Oginsky (1957). To the sample,contained in a 2.0 ml volume, was added 1.0 ml of a mixture of 1 volume of concentrated H_2SO_4 to 3 volumes of concentrated H_3PO_4, and 0.13 ml of a 3% aqueous solution of 2,3-butadionemonoxime. The contents of the tubes were mixed vigorously and placed in boiling water for 10 minutes in the dark, and cooled in ice water in the dark. Each tube was removed immediately prior to being read. Optical density was recorded at 490 nm. This assay measured 15 to 50 µg of citrulline.

XIII. Analytical Methods

Protein was determined by the method of Lowry et al. (1951). The presence of ammonia in Warburg supernatant fluids was determined by a modification of the Conway (1950) microdiffusion technique. Values between 0 and 20 µg of ammonia could be measured under the conditions used.

XIV. Chemicals

All chemicals used in this study were purchased from commercial sources. γ-Aminobutyric acid-4-^{14}C and uniformly labelled L-^{14}C-arginine were obtained from Schwartz Bioresearch Inc., Orangeburg, N.Y., and putrescine-^{14}C (tetramethylenediamine-1,4,-^{14}C dihydrochloride) and DL-ornithine-1-^{14}C from Amersham/Searle Corp., Des Plaines, Ill. L-citrulline-ureido-^{14}C and the ornithine used for transport studies, DL-ornithine-5-^{14}C, were purchased from New England Nuclear Corp., Boston, Mass.

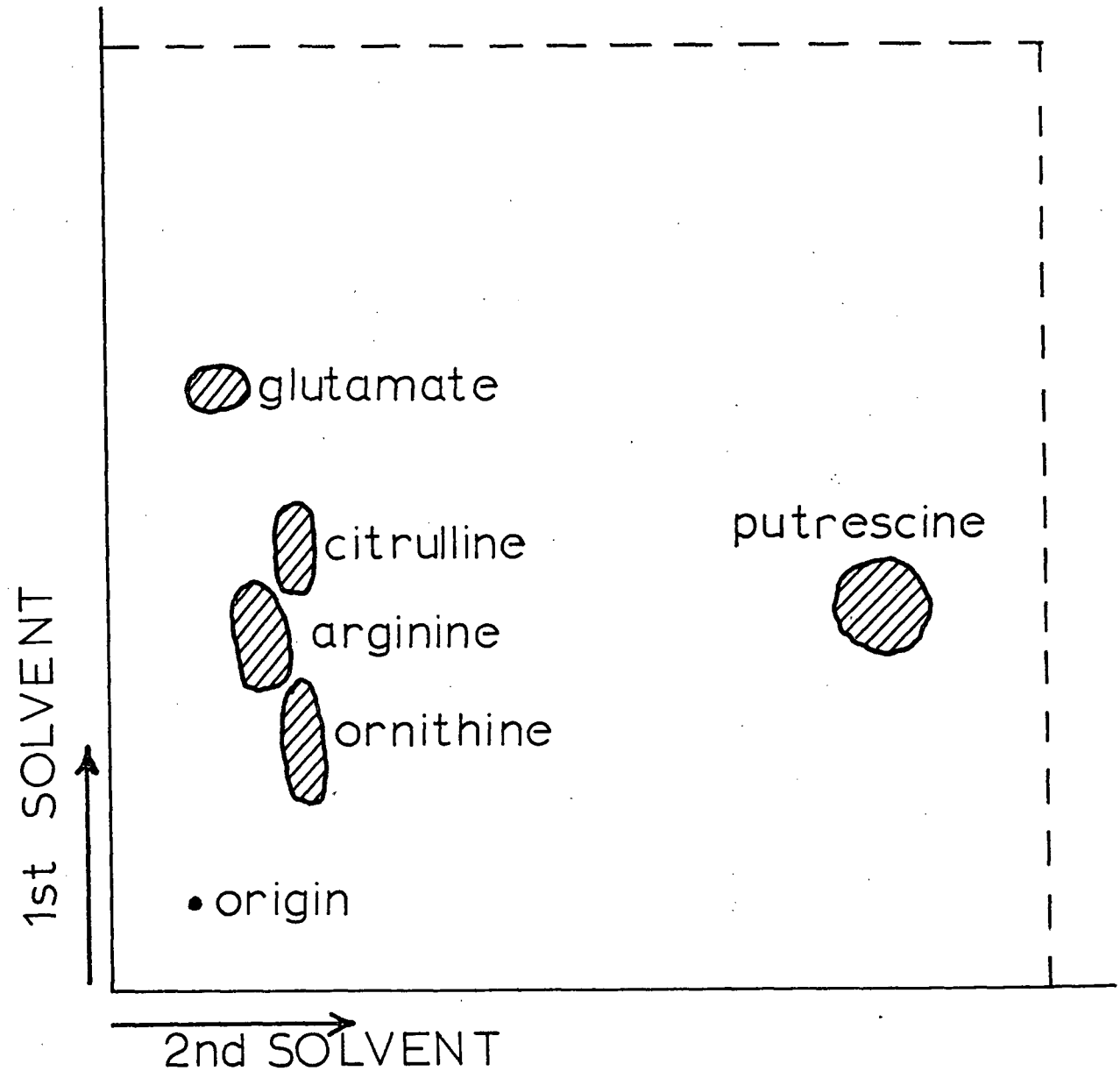
RESULTS AND DISCUSSION

I. Pathways of Arginine Degradation in P. aeruginosa

1. Accumulation of intermediates of arginine degradation

When glucose grown cells of P. aeruginosa were incubated with ^{14}C-arginine in a conventional Warburg apparatus, subsequent thin-layer chromatography and radioautography of the supernatant fluid demonstrated the presence of radioactive citrulline, ornithine, putrescine and glutamate (Figure 2). Small amounts of a number of unidentified radioactive compounds, which were not amino acids or intermediates of the tricarboxylic acid (TCA) cycle, were also present. Both citrulline and ornithine were present after short incubation periods (10 and 30 minutes) in relatively high concentrations, but were virtually absent at later times. These results confirmed the presence, in this strain of P. aeruginosa, of the arginine dihydrolase pathway described by Oginsky (1955). Both putrescine and glutamate appeared to accumulate in the Warburg supernatant fluid as products of arginine degradation, indicating that the organism may possess both an ornithine decarboxylase and the enzymes for the conversion of ornithine to glutamate via glutamic -γ-semialdehyde (Ramaley and Bernlohr, 1966).

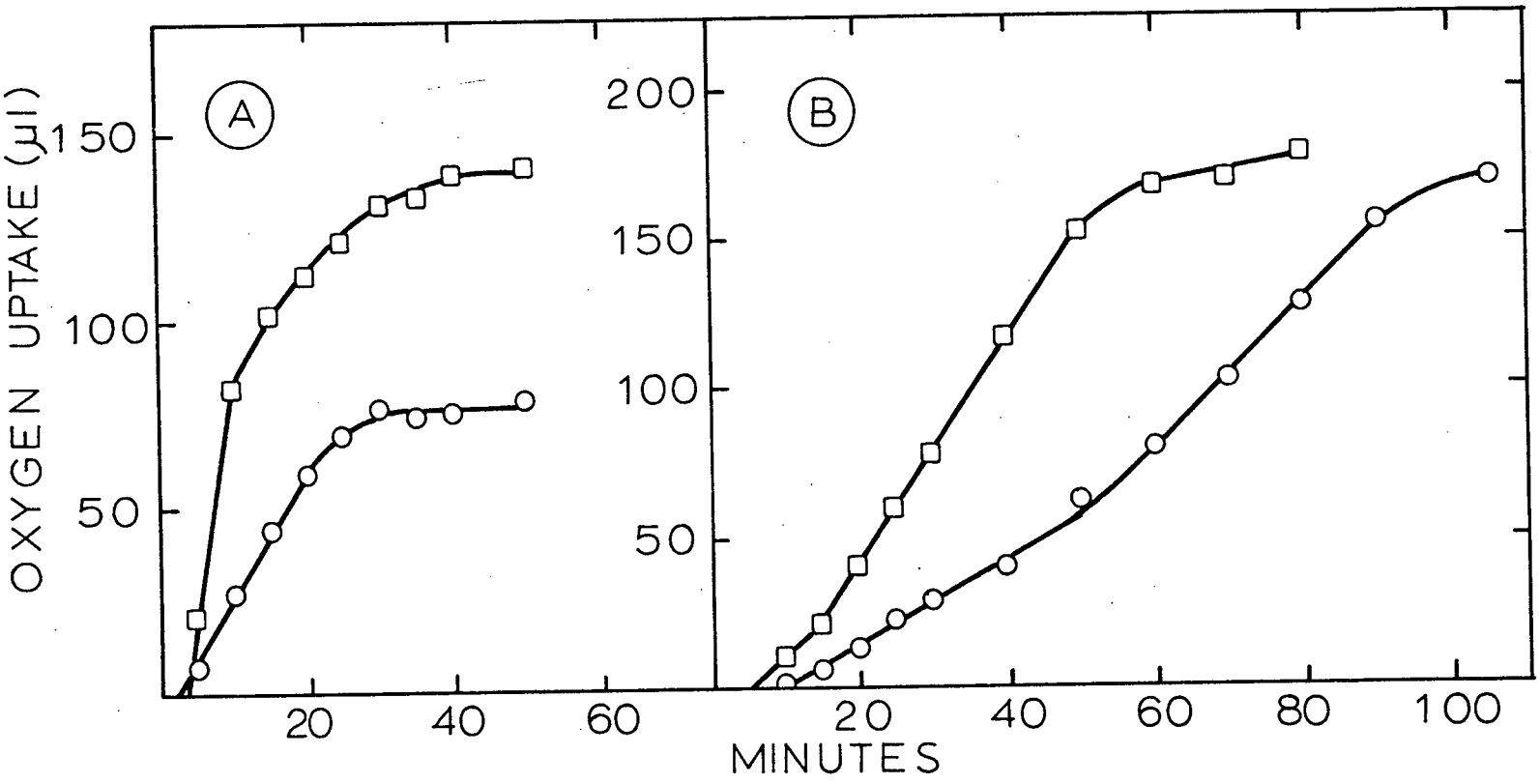
Fig. 2. Radioautogram of a thin-layer chromatogram of the supernatant fluid after the incubation of glucose grown cells with ^{14}C-arginine for 30 minutes under conventional Warburg conditions.



2. Inhibitory effect of Tris buffer on the oxidation of arginine by whole cells

Preliminary manometric experiments, using arginine as substrate, showed that the initial rate of oxygen uptake of arginine grown cells suspended at the usual concentration [5 mg of cells (dry weight)/ml] was so rapid that diffusion of oxygen into the reaction mixture was probably rate-limiting. In addition, the final pH of the reaction mixture was 8.0. Therefore, the cell concentration was decreased by one-third, and the concentration of Tris was increased to 0.067 M. However, under these conditions, oxygen uptake was extremely slow, and stopped at a lower level than would be expected if normal oxidation and assimilation had occured. Thus, oxygen uptake in the presence of 0.067 M Tris buffer (pH 7.4) and in the presence of 0.067 M phosphate buffer (pH 7.4) were compared. With arginine as substrate, both the rate of oxygen uptake and the total oxygen consumption were lower in the presence of Tris buffer than in the presence of phosphate buffer (Figure 3A). Although Tris buffer did not affect the total oxygen uptake when putrescine was the substrate, it did cause a decreased rate of oxygen consumption and a much longer period of adaptation before the most rapid rate was reached (Figure 3B). Repetition of this experiment using ^{14}C-arginine as substrate showed that, upon completion of oxygen uptake, the supernatant fluid from the reaction mixture with

Fig. 3. Oxidation of arginine (A) and putrescine (B) in the presence of Tris and phosphate buffers. Arginine grown cells were used at a concentration of 1.6 mg of cells (dry weight)/cup. Each cup contained 2.5 µmoles of substrate. Symbols: 0, Tris buffer; □, phosphate buffer. Note the change in the ordinate in B.


A
B
OXYGEN UPTAKE (µl)
150
100
50
200
150
100
50
20
40
60
20
40
60
80
100
MINUTES

Tris buffer contained 3 times more radioactivity than that from the reaction with phosphate buffer. Although thin-layer chromatography and radioautography of the supernatant fluids showed that this radioactivity was not present as a single intermediate, glutamate accumulated to much higher concentrations in the presence of Tris buffer than in phosphate buffer. Thus Tris may have had a direct inhibitory effect on the enzymes responsible for degrading glutamate, causing a decrease in the total oxygen consumption when arginine, but not putrescine, was the substrate. The inhibitory effect of Tris on several enzyme reactions, including the oxidation of succinate by mitochondria, has been described by Good et al. (1966).

The decreased rates of oxidation in the presence of Tris may have been caused by an alteration in cell permeability, possibly resulting in some lysis. Other workers have obtained evidence that Tris affects cell permeability. Leive and Kollin (1967) found that exposure of E. coli to cold Tris caused a decrease in the rate of RNA synthesis and a loss of acid precipitable UV-absorbing material. Neu, Ashman and Price (1967) found that exposure of E. coli to Tris for one hour caused a release of the nucleotide pool and degradation of RNA and nucleotides. Eagon and Asbell (1966) showed that the ability of osmotically fragile P. aeruginosa to transport and oxidize certain substrates could be restored in phosphate buffer but not in Tris buffer. Cheng, Ingram, and Costerton (1970) found that washing P. aeruginosa

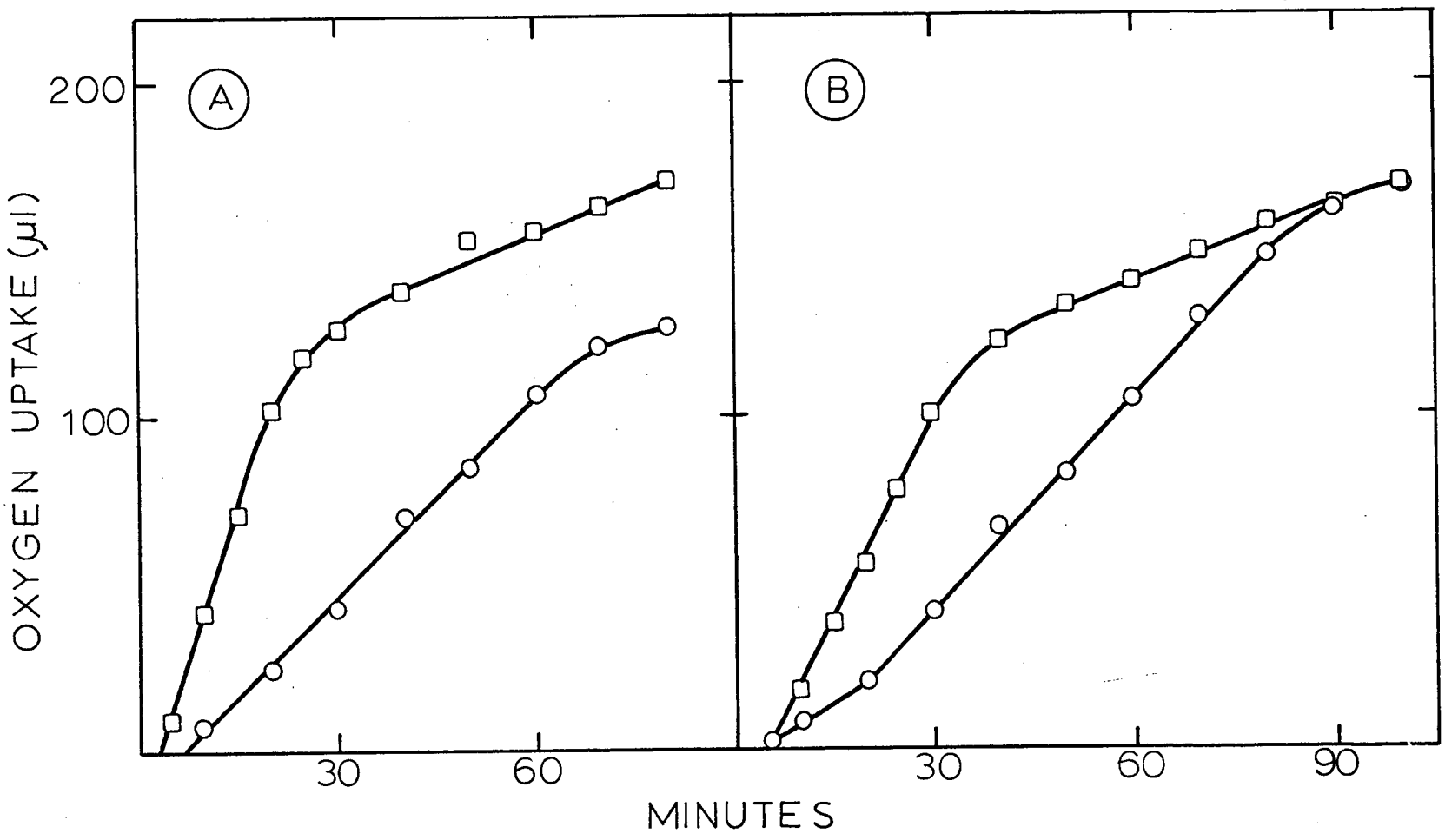
with Tris buffer caused partial release of the periplasmic enzyme, alkaline phosphatase.

The possibility also exists that exogenous phosphate may stimulate the rate of conversion of citrulline to ornithine, which is a phosphate requiring reaction. It is interesting to note that some radioactive citrulline was present in the supernatant fluid from the reaction mixture containing Tris buffer, but not in that containing phosphate buffer.

3. Oxidation of intermediates of arginine degradation

Further evidence that ornithine, citrulline, putrescine, and γ-aminobutyrate were intermediates in the degradation of arginine was obtained by manometric studies. Glucose grown cells oxidized arginine, ornithine, and citrulline constitutively; however, the rate of oxidation of these substrates was increased 5 to 8 fold by growth of the cells in arginine as the sole source of nitrogen and carbon (Fig. 4, Fig. 6A, Table I). Citrulline was oxidized much more slowly than the other substrates, and growth with citrulline as the sole carbon and nitrogen source was also poor. Evidence will be described later suggesting that citrulline uptake may have been rate limiting. Arginine grown cells also oxidized putrescine and γ-aminobutyrate more rapidly than glucose grown cells (Fig. 5, Fig. 6B, Table I). The maximal rate of putrescine

Fig. 4. Oxidation of arginine (A) and ornithine (B).
Symbols: 0, glucose grown cells [3.8 mg of cells (dry weight)/cup]; □, arginine grown cells [1.5 mg of cells (dry weight)/cup]. Phosphate buffer was used.


A
B
200
100
OXYGEN UPTAKE (µl)
30
60
30
60
90
MINUTES

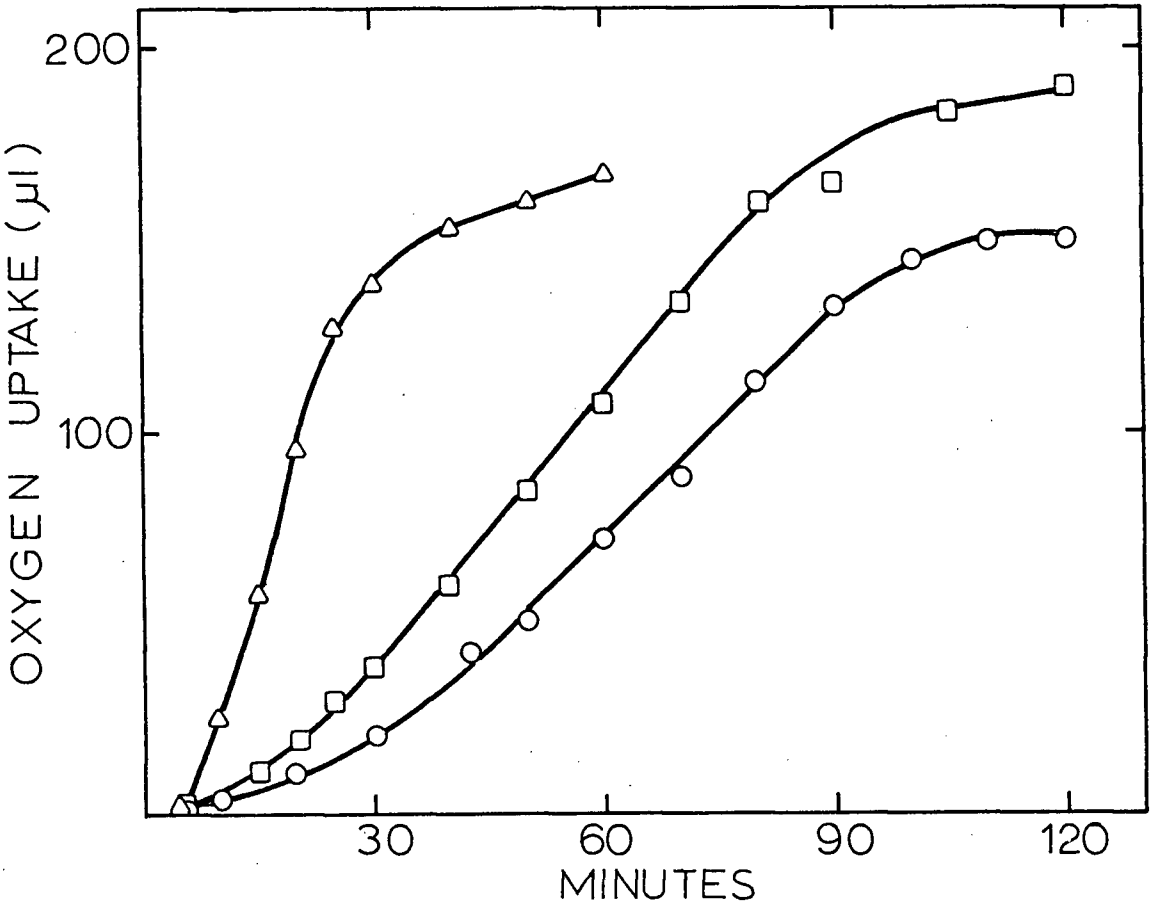


Fig. 5. Oxidation of putrescine. Symbols: O, glucose grown cells [3.8 mg of cells (dry weight)/cup]; □ ,arginine grown cells [1.5 mg of cells (dry weight)/cup]; Δ, arginine grown cells [3.8 mg of cells (dry weight)/cup] . Phosphate buffer was used.

Fig. 6. Oxidation of citrulline (A) and γ-aminobutyrate (B).
Symbols: 0, glucose grown cells; □, arginine grown cells.
Cell concentration was 3.8 mg of cells (dry weight)/cup.
Phosphate buffer was used.

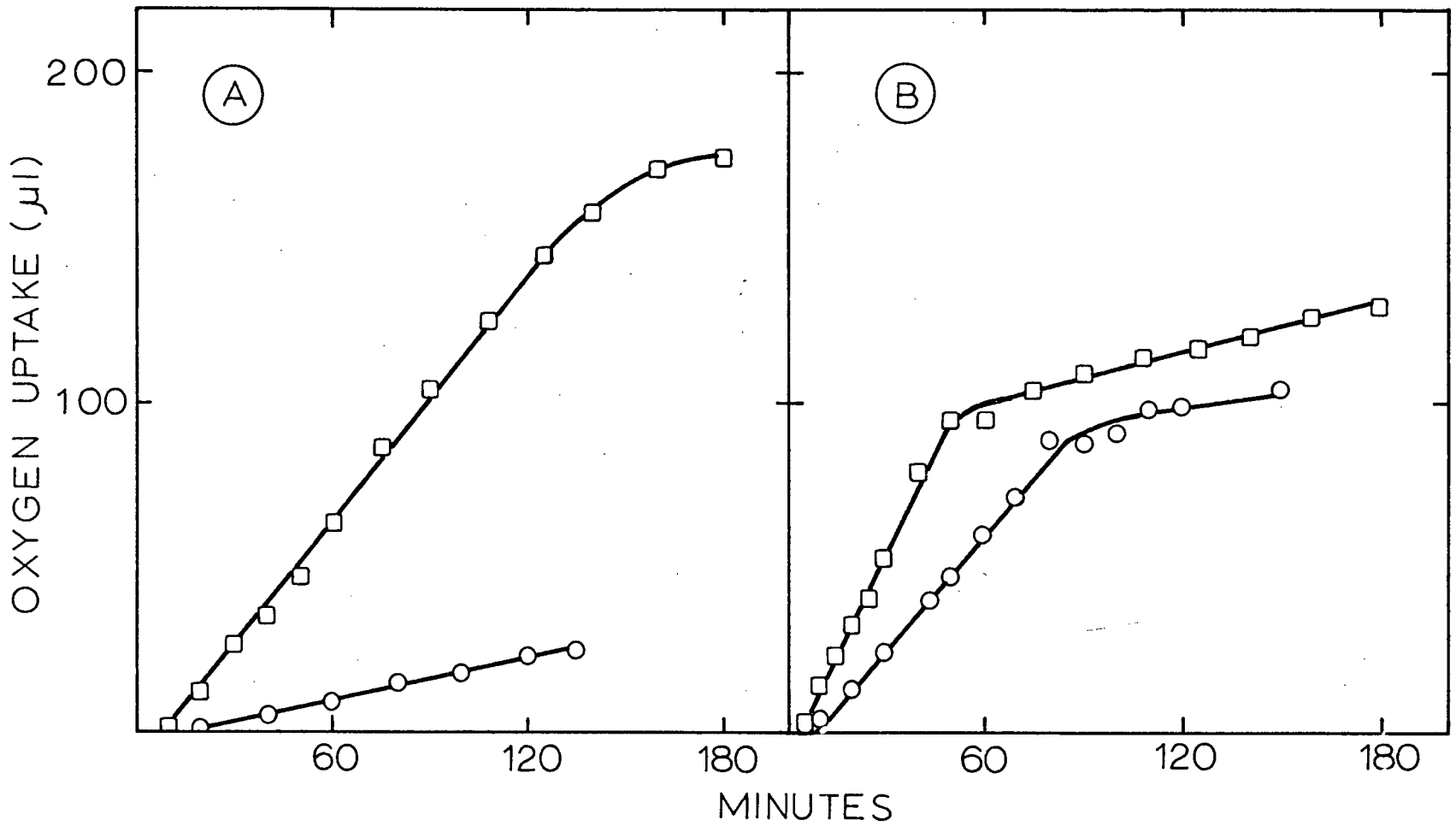

A
B
200
100
OXYGEN UPTAKE (µl)
60
120
180
60
120
180
MINUTES

Table I. Rates of oxygen uptake by P. aeruginosa with arginine and suspected intermediates as substrates.

Substrate[a]	Growth medium	
	arginine-salts	glucose-NH_4^+-salts
	QO_2	
arginine	241	30
Ornithine	164	33
citrulline	19	3.7
putrescine	94	30
γ-aminobutyrate	31	18

[a] Substrate concentration was 2.5 μmoles/Warburg vessel.

oxidation by glucose grown cells was reached only after 35 minutes, whereas arginine grown cells at the same concentration [3.8 mg of cells (dry weight)/cup] demonstrated a lag of only 5 minutes. This lag was not due to the induction of an uptake system for putrescine, because subsequent experiments showed that this compound was transported constitutively at a high rate. Since a similar lag period did not occur before the oxidation of γ-aminobutyrate, the lag may represent a period of induction of the enzymes converting putrescine to γ-aminobutyrate. The fact that γ-aminobutyrate was oxidized at a much slower rate than putrescine indicated that exogenous γ-aminobutyrate was possibly not as available for oxidation as endogenous γ-aminobutyrate, possibly due to a slow rate of uptake of this substrate.

4. Conversion of ornithine to glutamate

Since ^{14}C-glutamate was recovered in relatively high concentrations during the oxidation of ^{14}C-arginine, an attempt was made to determine the presence, in a cell-free extract of arginine grown P. aeruginosa, of the enzymes converting ornithine to glutamate; ie. ornithine transaminase and Δ^1-pyrroline-5-carboxylate dehydrogenase. An attempt was made to couple the two reactions using the conditions of Ramaley and Bernlohr (1966) for the measurement of ornithine transaminase, but by adding NADP or

NAD and measuring the reduction of the latter cofactors at 340 nm. However, no activity was obtained, although the assay was attempted in both phosphate and Tris buffers at several pH values.

It was therefore decided to determine whether P. aeruginosa degraded ornithine-1-^{14}C to ^{14}C-glutamate. Resting cell suspensions of both arginine grown and glucose grown cells from the logarithmic phase of growth were incubated in 50 ml Erlenmeyer flasks containing 6 ml volumes of a typical Warburg reaction mixture buffered with phosphate, and stirred at 30 C as described for uptake experiments. Two µmoles of unlabelled glutamate were added to trap ^{14}C-glutamate, and the reaction was started by the addition of 5 µmoles (15 µCi) of ornithine-1-^{14}C. Two ml samples were removed at appropriate time intervals, added to 2 ml of 10% trichloroacetic acid at 0 C, and treated as described for the thin-layer chromatography of Warburg supernatant fluids. Intermediates of putrescine degradation could not become labelled in this experiment, because the conversion of ornithine-1-^{14}C to putrescine would result in the loss of all the label as $^{14}CO_2$.

The results of this experiment are summarized in Table II. Approximately the same amount of radioactivity was lost from the supernatant fluid after 45 minutes in the presence of glucose grown cells as was lost after 20 minutes in the presence of arginine grown cells. These results indicated that under these conditions, arginine grown cells oxidized ornithine approximately twice as rapidly as

Table II. Degradation of ornithine-1-^{14}C by arginine grown and glucose grown cells of P. aeruginosa.

Radioactivity	Growth substrate			
	Arginine		Glucose	
	Incubation Time (min)			
	20	40	45	90
% of total ^{14}C recovered in supernatant fluid	44%	17%	47%	30%
% of supernatant fluid ^{14}C[a] found as:				
ornithine	49.6%	24.3%	75%	74.4%
acidic and neutral compounds	20%	35.5%	10.8%	1.0%
glutamate	3.4%	6.1%	1.6%	4.6%
proline	7.4%	10.3%	0	0

[a] Supernatant fluid radioactivity unaccounted for in this table was present as several unidentifiable compounds.

glucose grown cells. However, examination of the composition of the supernatant fluids at these times showed that much more of the original ornithine remained in the presence of the glucose grown cells.

From the results of the previous manometric experiments, it was expected that arginine grown cells would have oxidized the majority of the ornithine by 40 minutes (Fig. 4B), and only 4% of the original label was recovered as ornithine at this time. Glucose grown cells should have oxidized the majority of the ornithine by 90 minutes; however, 22% of the original label was recovered as ornithine, a value closely comparable to the amount of label recovered at 20 minutes from induced cells. Thus, it appeared that the oxidation of ornithine by glucose grown cells was slower under these conditions than under conventional Warburg conditions, perhaps due to oxygen limitation. Moreover, ^{14}C-ornithine was supplied as a mixture of the D and L isomers, and it is possible that the D isomer was oxidized more slowly that the L isomer.

Arginine grown cells accumulated much more of the added label as glutamate, proline, and acidic and neutral degradation products than did glucose grown cells. These results indicated that the conversion of ornithine to glutamate was rate limiting in the glucose grown cells and that glutamate was dissimilated as rapidly as it was formed. On the other hand, arginine grown cells appeared

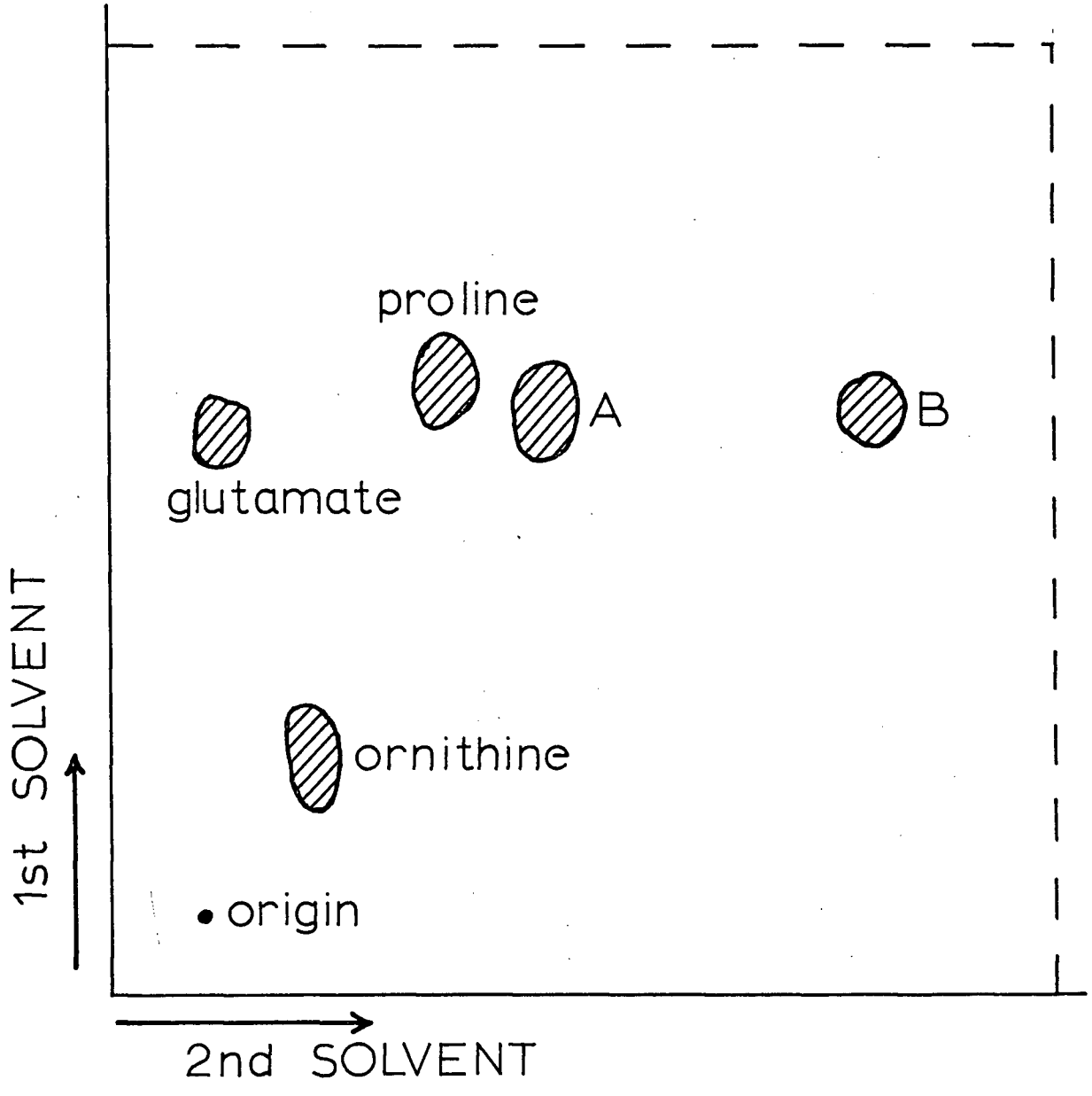
to convert ornithine to glutamate more rapidly than the latter compound could be dissimilated.

Thin-layer chromatography and radioautography of the basic fractions of the supernatant fluids showed two unidentified spots containing considerable radioactivity (spots A and B, Fig. 7). It is possible that these compounds were Δ^1-pyrroline-5-carboxylic acid and/or its breakdown products. Strecker (1960) found that solutions of Δ^1-pyrroline-5-carboxylic acid were unstable, forming at least two products: one was formed both at room temperature and at -15 C, and the other, thought to be a polymerization product, was formed only at -15 C. Since treatment of the supernatant fluids involved storage at -20 C, treatments at room temperature, and several evaporations at 45 - 50 C, it is possible that breakdown products of this compound were formed.

The radioautograms of the thin-layer chromatograms of the basic fractions of the supernatant fluids from glucose grown cells also showed low levels of 8 to 10 unidentified compounds. These compounds were also present in the 20 minute sample from arginine grown cells. These basic compounds were not amino acids and were presumed to be biosynthetic products or intermediates.

From these experiments, it was concluded that the conversion of ornithine to glutamate was an important pathway of arginine degradation in P. aeruginosa. The enzymes of the pathway appeared to be present in glucose grown cells, but were induced to greater activity after growth of the organism in arginine.

Fig. 7. Radioautogram of a thin-layer chromatogram of the basic fraction of the supernatant fluid after incubation of arginine grown cells with ornithine-1-^{14}C for 40 minutes.



5. Succinic semialdehyde dehydrogenase activity

In an attempt to assess the importance of putrescine as an intermediate of arginine degradation in P. aeruginosa, the activity of succinic semialdehyde dehydrogenase, an enzyme participating in putrescine metabolism, was measured in arginine grown cells, putrescine grown cells (induced) and glucose grown cells (uninduced). This enzyme was assayed by linking it to γ-aminobutyrate transaminase, and thus enzyme activity was actually a measurement of the rate of conversion of γ-aminobutyrate to succinate.

It was possible, however, that the induction by arginine of the enzymes degrading γ-aminobutyrate might occur without the degradation of putrescine. Gale (1940) found that growth of E. coli in the presence of glutamate induced an enzyme which decarboxylated glutamate, forming γ-aminobutyrate. If P. aeruginosa possessed the latter enzyme, γ-aminobutyrate might be formed from glutamate rather than from putrescine, and succinic semialdehyde dehydrogenase activity would be indicative of the rate of conversion of ornithine to glutamate and the subsequent degradation of the latter compound, rather than of the oxidation of putrescine. Therefore, succinic semialdehyde dehydrogenase activity was also measured in cells grown with glutamate as the sole source of carbon and nitrogen.

Nakamura (1960) found that a strain of P. aeruginosa contained two succinic semialdehyde dehydrogenases: one linked to NADP,

and the other to NAD. Unlike the enzyme studied by Padmanabhan and Tchen (1969) in an unidentified Pseudomonad, the NADP linked succinic semialdehyde dehydrogenase of P. aeruginosa appeared to be inducible (Table III). Jakoby and Fredericks (1959) found that synthesis of succinic semialdehyde dehydrogenase was constitutive in P. fluorescens, although its activity was increased somewhat after growth in γ-aminobutyrate. However, the synthesis of γ-aminobutyrate transaminase was inducible in this organism, and the specific activity of this enzyme was always lower than that of the dehydrogenase. Since the assay used in the present study depended on γ-aminobutyrate as a source of succinic semialdehyde, it is possible that the NADP linked succinic semialdehyde dehydrogenase was constitutive, but that its activity was limited by an inducible γ-aminobutyrate transaminase. A comparison of the specific activities of the NADP linked and NAD linked enzymes shows that γ-aminobutyrate transaminase was not rate limiting in the assay of the NAD linked succinic semialdehyde dehydrogenase. The ratios of the specific activities of the NADP linked to the NAD linked enzymes were similar under all growth conditions, indicating that the two enzymes may have been coordinately induced.

Both the NAD and the NADP linked enzymes were induced to the highest levels by growth in putrescine. Partial induction of both enzymes was caused by growth with either arginine or glutamate as

Table III. Succinic semialdehyde dehydrogenase activities of cell-free extracts of induced and uninduced cells of P. aeruginosa.

Growth substrate	Specific activity[a]	
	NADP	NAD
putrescine	576	84
arginine	82.3	14.5
glutamate	58.5	7.3
glucose	16.1	<2.5

[a] Specific activity expressed as mμmoles of substrate oxidized x min^{-1} x mg^{-1} of protein.

the sole source of carbon and nitrogen. The fact that growth in glutamate induced only 10% of the activity induced by growth in putrescine indicated that decarboxylation was not the major pathway of glutamate degradation in P. aeruginosa. Arginine grown cells contained significantly higher levels of both enzymes than glutamate grown cells, and it was concluded that this increased activity was due to degradation of putrescine formed during arginine catabolism. Glucose grown cells contained a low constitutive level of the NADP linked enzyme, and extracts from these cells had negligible activity with NAD.

II. Repression of Arginine Biosynthesis in P. aeruginosa by Exogenous Arginine

Radioautograms of thin-layer chromatograms of the supernatant fluids from the experiments examining the degradation of ornithine-1-^{14}C showed that glucose grown cells synthesized ^{14}C-arginine from ^{14}C-ornithine, but that arginine grown cells did not; this indicated that arginine biosynthesis was repressed during growth in the presence of arginine. Repression of the anabolic ornithine transcarbamylase of P. aeruginosa and P. fluorescens by arginine has been reported by Stalon et al. (1967a) and Ramos et al. (1967). Ukada (1966) found that arginine repressed the synthesis of this enzyme in 19 different microorganisms. In order to confirm that repression of

this enzyme occurred in P. aeruginosa, cell-free extracts were assayed for ornithine transcarbamylase activity. Cells were grown in Roux flasks in glucose minimal medium in the presence and absence of 0.05% arginine, and were harvested from the late logarithmic phase of growth at 14 hours. The specific activity of ornithine transcarbamylase was three to four fold greater in cell-free extracts of cells grown without added arginine than in those grown in the presence of arginine.

III. The Effects of Glucose on the Degradation of Arginine by P. aeruginosa.

1. Growth in a mixture of glucose and arginine.

P. aeruginosa grew in a medium containing glucose, ammonium ions, and arginine without showing a diauxie effect, despite the fact that the inoculum had not been adapted to growth in the presence of arginine (Fig. 8 and 9). The doubling time for growth in this mixture was the same as the doubling time in glucose alone; ie. 1.25 hours. The growth rate was not affected by prior adaptation of the inoculum to growth in the presence of arginine. P. aeruginosa grew somewhat more slowly with arginine as the sole source of carbon and nitrogen, having a doubling time of approximately 1.6 hours under these conditions. The lag period was also longer,

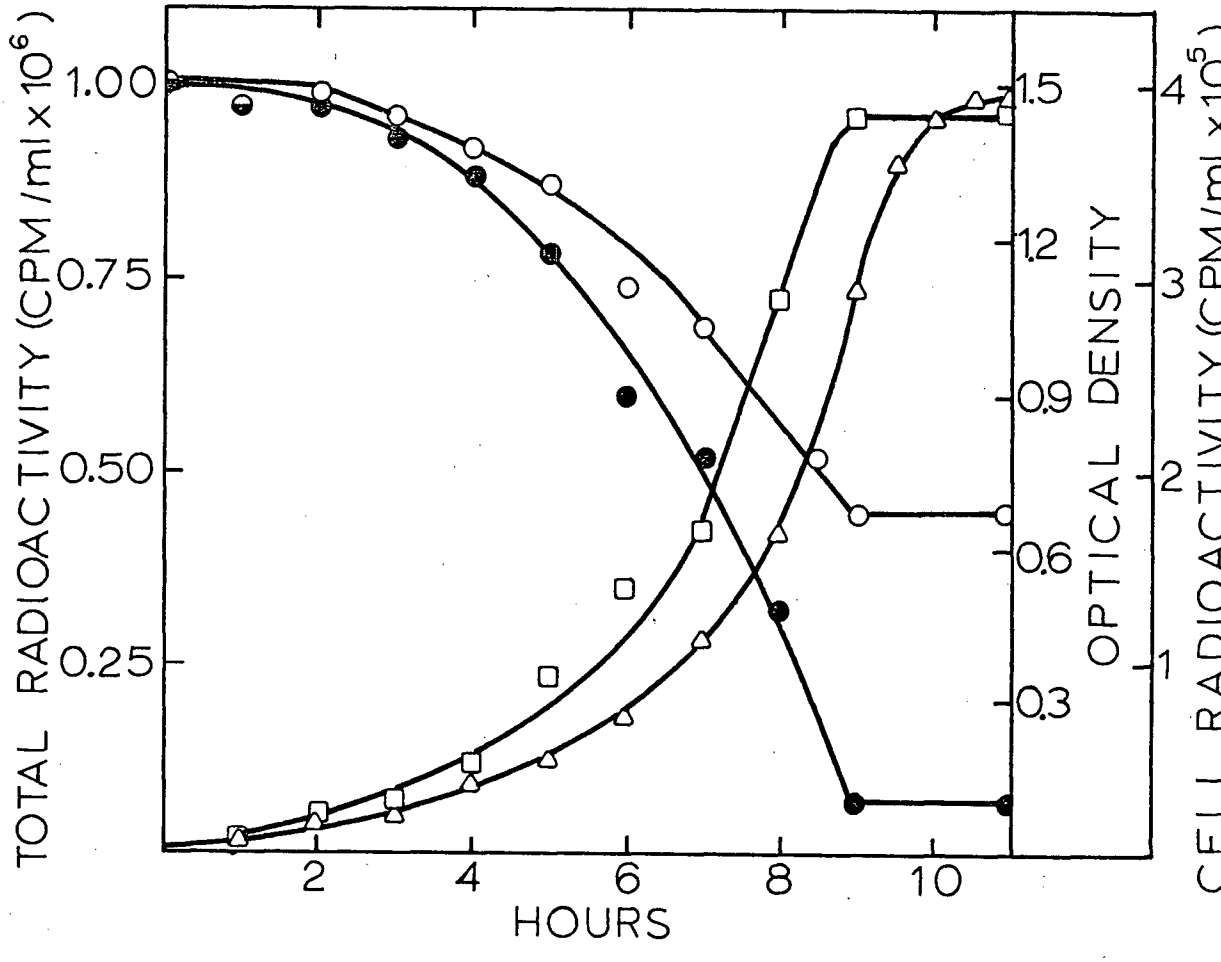


Fig. 8. The utilization of ^{14}C-glucose during growth of P. aeruginosa in a medium containing glucose, arginine and ammonium ions. Symbols: O, total radioactivity in the flask; □ ,radioactivity present in whole cells; Δ, optical density; ●, radioactivity of the supernatant fluid. Cell radioactivity has been plotted on a different scale from total and supernatant fluid radioactivity.

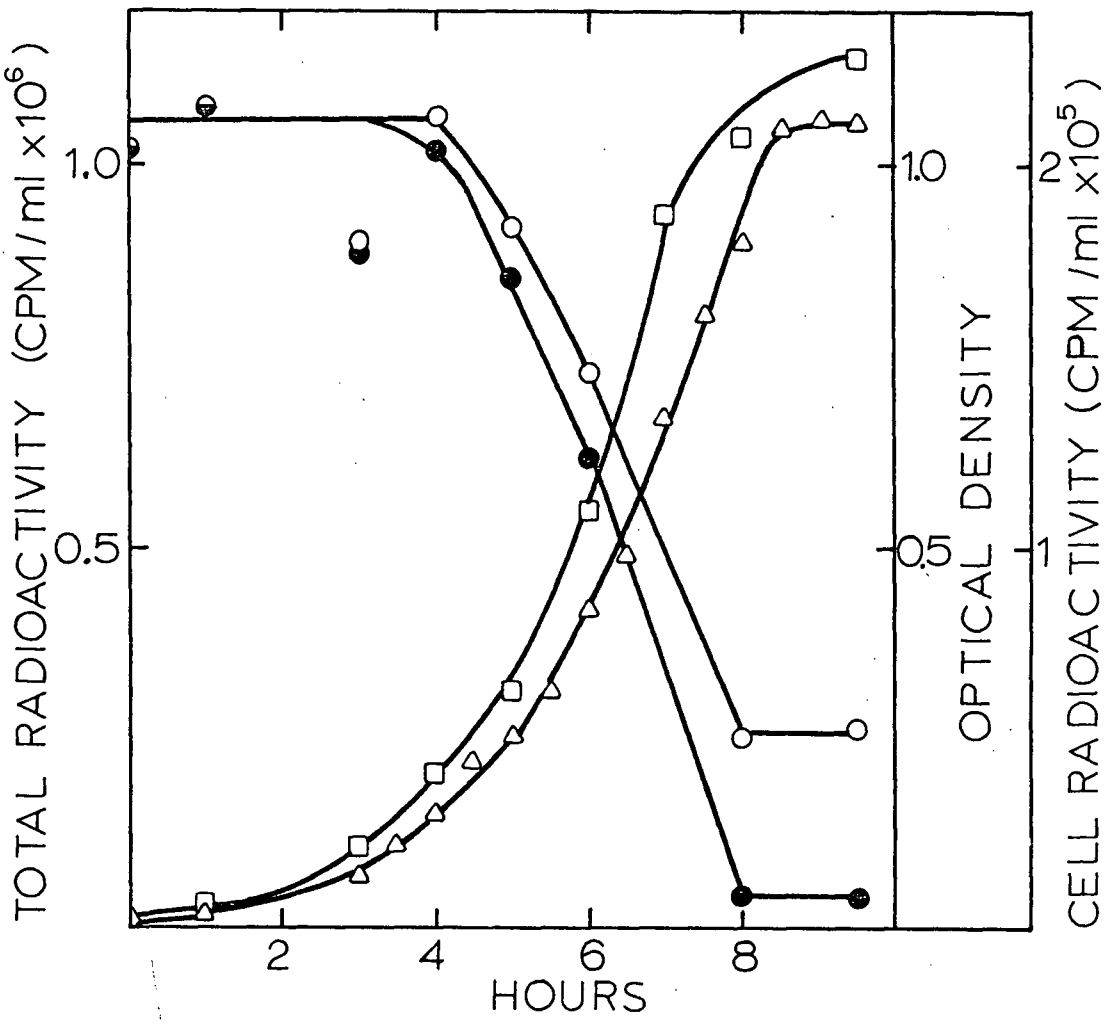


Fig. 9. The utilization of ^{14}C-arginine during the growth of P. aeruginosa in a mixture of glucose and arginine. Symbols: are the same as in Fig. 8. Note that cell radioactivity has been plotted on a different scale than that in Fig. 8.

despite prior adaptation of the cells.

The following experiments were therefore performed to determine whether glucose and arginine were degraded concurrently during growth in the presence of both substrates. Two side-arm flasks of ammonium salts minimal medium, each containing 0.1% glucose and 0.1% arginine, were inoculated with glucose grown P. aeruginosa. ^{14}C-arginine was added to one flask to give a final concentration of 0.5 µCi per ml, and ^{14}C-glucose to the other flask at the same concentration. Optical density was followed using a Klett-Summerson colorimeter equipped with a red filter, and 1 ml samples were taken at one hour intervals and immediately filtered as in transport studies, and washed with 2 ml of medium. The supernatant fluids were collected in a test tube placed in the filtration flask, and were later made up to 5 ml volumes and assayed for radioactivity.

The results of these experiments are shown in Figures 8 and 9. During growth in either labelled substrate, the radioactivity in the whole cells increased concomitantly with the optical density. The degree of assimilation of the radioactive substrates was determined by calculating the ratio of cell radioactivity to optical density. During the first three hours, slightly more glucose than arginine was assimilated per optical density unit of cells, resulting in a cellular glucose to arginine ratio of 1.1. As the period of the most rapid growth was reached, the amount of arginine

per optical density unit of cells decreased slightly, whereas the amount of glucose increased considerably. During this period, the ratio of glucose assimilated per optical density unit of cells to arginine assimilated per optical density unit of cells was 1.9. As the cells entered the stationary phase of growth, they appeared to utilize a portion of their assimilated arginine, and almost half of their assimilated glucose; thus, the ratio of glucose per optical density unit of cells to arginine per optical density unit of cells in the early stationary phase was 1.2, similar to that of cells prior to the initiation of growth.

The decrease in total radioactivity was used as a measurement of the amount of labelled substrate carbon lost as $^{14}CO_2$. Carbon dioxide production from glucose-^{14}C was apparent after two hours, at a time when an increase in optical density became noticeable, and continued at an increasing rate until one hour before stationary phase was reached. Detectable amounts of CO_2 did not appear to be lost from the medium containing ^{14}C-arginine for the first four hours, after which time CO_2 was produced rapidly until half an hour before stationary phase was reached. During the period of maximum increase in optical density, both arginine and glucose were degraded to CO_2.

A greater proportion of the label was lost from ^{14}C-arginine than from ^{14}C-glucose. However, the cells did not grow to as high

an optical density in the flask containing labelled arginine, resulting in the assimilation of only 21% of the ^{14}C-arginine as compared to 38% of the ^{14}C-glucose, although the amount of each substrate assimilated per optical density unit of cells was approximately the same. Since a smaller proportion of the arginine was assimilated, more was available for complete degradation to CO_2.

2. The effect of glucose on the degradation of arginine by resting cell suspensions.

The oxidation of arginine by glucose grown cells harvested from the stationary phase of growth was compared in the presence and absence of glucose (Fig. 10). No break in the oxygen uptake curve was observed when both substrates were present, indicating that glucose and arginine were oxidized concurrently.

The amount of ammonia present in the supernatant fluid was measured at various times during oxygen uptake. The maximum amount of ammonia detected in the supernatant fluid at any time was equivalent to 2.4 µmoles per µmole of arginine originally added. Because arginine contains 4 µatoms of nitrogen per µmole, the ammonia released accounted for only 60% of the arginine nitrogen. Presumably, the remaining 40% was assimilated.

Cells which had been grown in glucose minimal medium supplemented

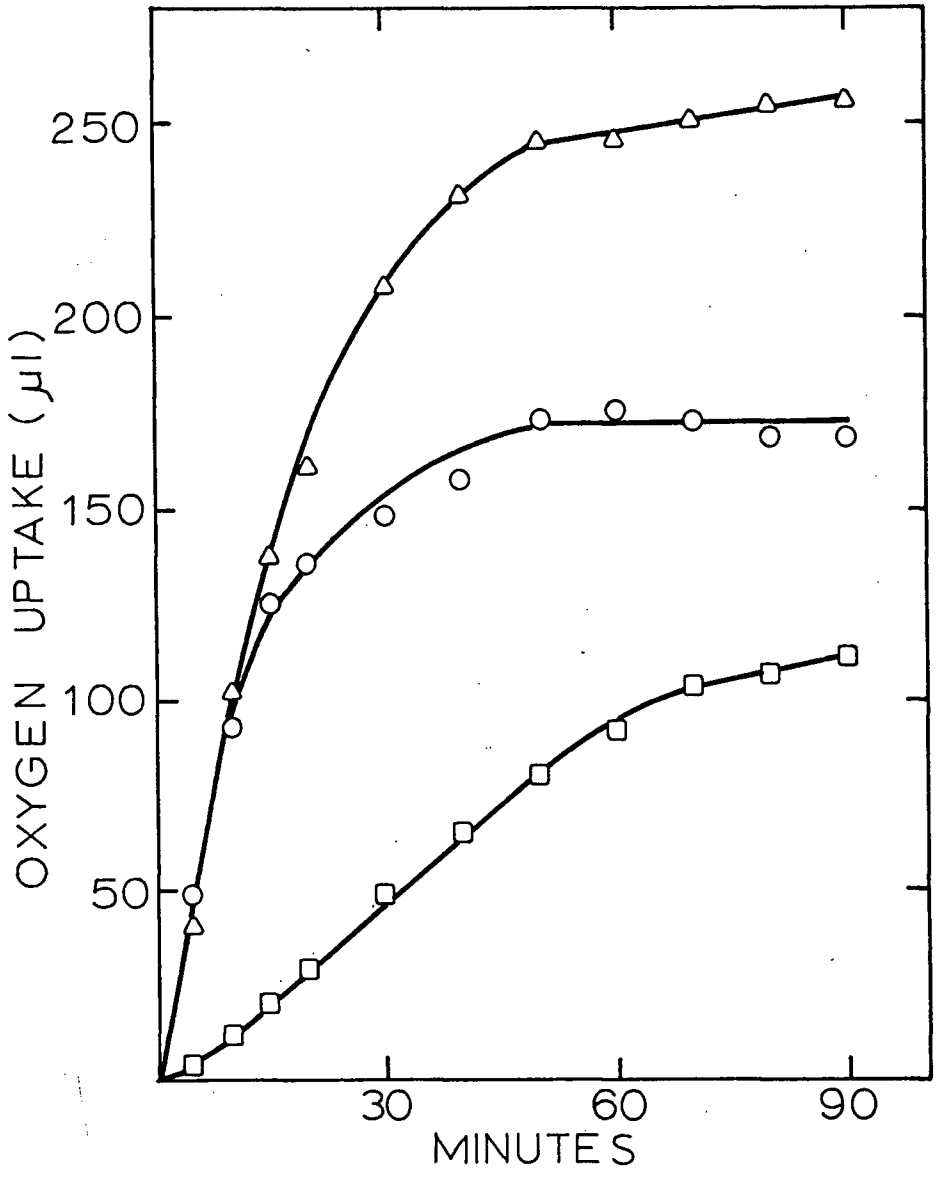


Fig. 10. The oxidation of glucose, 0; arginine, □ ; and a mixture of glucose and arginine, Δ . Glucose grown cells [approximately 5 mg of cells (dry weight)/cup] were used. Each cup contained 2.5 µmoles of each substrate and Tris buffer.

with 0.1% arginine had released this maximum amount of ammonia after 30 minutes of incubation in a Warburg cup with arginine as the only substrate. Since oxygen uptake was not complete by this time, it is likely that, although greater than 50% of the arginine ammonia had been released, ammonia production was not complete, and that further release was masked by assimilation. Cells grown without added arginine released ammonia more slowly. Only 67% of the maximum value was reached by 30 minutes, and 92% by 60 minutes. Thus, the addition of arginine to a growth medium containing glucose induced a more rapid rate of arginine degradation. When both glucose and arginine were present in the Warburg cup, induced cells released 83% of the maximum amount by 30 minutes. The presence of glucose during arginine oxidation may therefore have caused a slight decrease in the rate of degradation of arginine by induced cells. However, it is also possible that the assimilation of ammonia was increased when glucose was being oxidized, resulting in a slightly lower concentration of ammonia in the supernatant fluid at 30 minutes.

The oxidation of 2.5 µmoles of ^{14}C-arginine (1 µCi/µmole) by uninduced cells was examined in the presence and absence of glucose. The changes in the radioactivity of the supernatant fluid, whole cells, and CO_2 in the absence of added glucose are shown in Figure 11. The values obtained when glucose was present were very similar. Radioactivity was lost from the supernatant

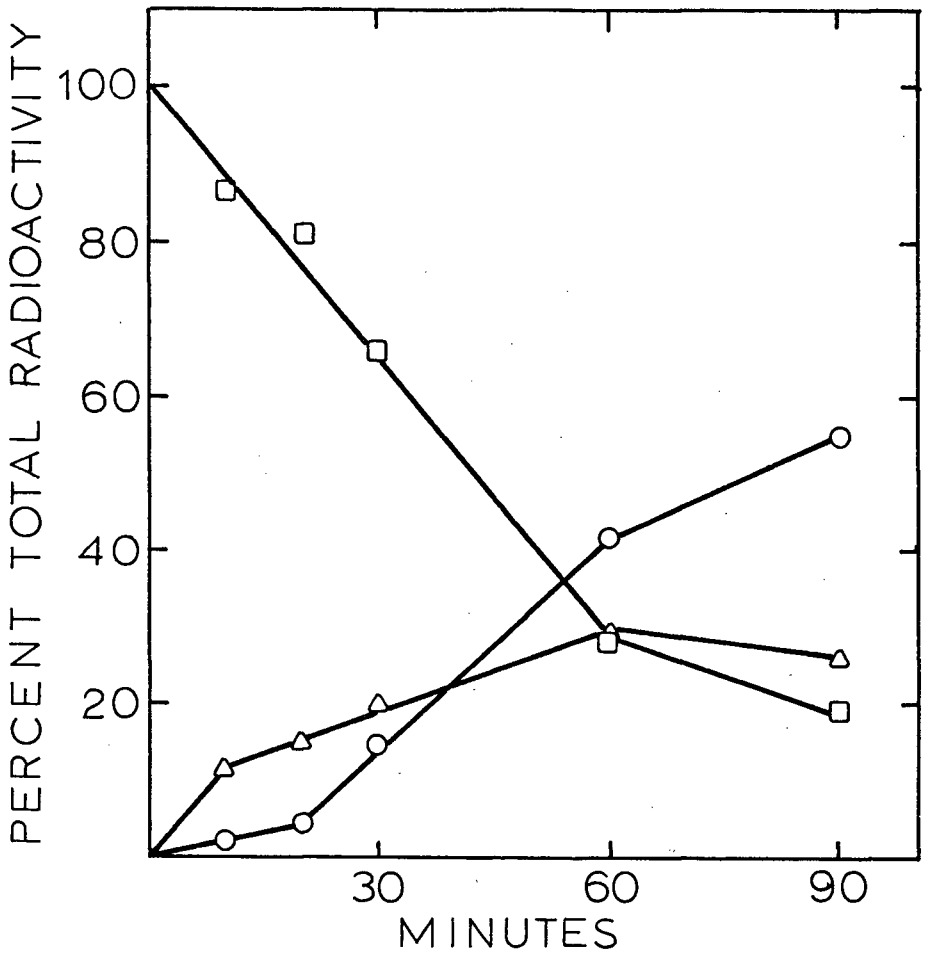


Fig. 11. Distribution of radioactivity during the incubation of P. aeruginosa with ^{14}C-arginine under Warburg conditions. Glucose grown cells [approximately 5 mg of cells (dry weight)/cup] and Tris buffer were used. Each cup contained 2.5 µmoles of ^{14}C-arginine (specific activity = 1 µCi/µmole). Symbols: O, CO_2; □ , supernatant fluid; Δ, whole cells. The radioactivity given off as $^{14}CO_2$ was determined by measuring the radioactivity present in the center well of the Warburg cup.

fluid throughout the period of oxidation, despite the presence of glucose. Moreover, the assimilation of radioactivity into the cells and the evolution of $^{14}CO_2$ were not appreciably altered by the addition of glucose. In both cases, the rate of $^{14}CO_2$ evolution was slow for the first 20 minutes, after which time the amount of $^{14}CO_2$ released increased rapidly. Thirty-five to forty percent of the final cellular radioactivity was assimilated within the first 10 minutes, and assimilation then proceeded at a slower rate, reaching the maximum value by 60 minutes. Thus, arginine was assimilated into cellular material during the initial stages of oxidation and was subsequently degraded to CO_2. These results were similar to those obtained in the growth experiments.

Thin-layer chromatography and radioautography of the supernatant fluids from the 30 minute samples showed that, although the total radioactivity of the two samples was essentially the same, the various intermediates were present in different amounts (Table IV). Much more citrulline and ornithine were present in the cup to which glucose had been added. At all times, the putrescine content of the supernatant fluids was slightly greater in the absence of glucose. By 90 minutes, the majority of the radioactivity was present as glutamate and putrescine, with some also present in unknown spots 1 and 2. Some ornithine remained in the sample in which glucose had been present. Thus, it is possible that the addition of glucose did cause a slight decrease in the rate of ornithine degradation.

Table IV. Composition of the supernatant fluids after oxidation of ^{14}C-arginine by P. aeruginosa in the presence and absence of glucose.[a]

Compound	% of supernatant radioactivity[b]	
	^{14}C-arginine	^{14}C-arginine + ^{12}C-glucose
arginine	39.2	34.0
citrulline	5.9	13.8
ornithine	5.3	15.2
putrescine	3.8	2.5
glutamate	6.7	7.1
unknown 1	1.0	1.2
2	1.4	2.5

[a] 5 mg of cells (dry weight) were incubated for 30 minutes under conventional Warburg conditions with 0.05 M Tris buffer and 2.5 µmoles of substrate. The specific activity of the ^{14}C-arginine was 1 µCi/µmole.

[b] the supernatant radioactivity unaccounted for in this table was present in a number of unidentified compounds.

3. Assimilation of arginine and glucose

The effects of arginine on the assimilation of ^{14}C-glucose, and of glucose on the assimilation of ^{14}C-arginine, were examined. The conditions were the same as in the previously described experiments, and the cells were fractionated at 90 minutes, after the rate of oxygen uptake in the presence of arginine had decreased. The data presented in Table V showed that the addition of arginine caused an increase in the amount of glucose assimilated, and a decrease in the amount released as CO_2. The addition of arginine did not greatly affect the percentage distribution of glucose between the various cell fractions. More glucose was assimilated into the acid ethanol soluble fraction and less into the hot trichloroacetic acid insoluble fraction in the presence of arginine. The addition of 2.5 µmoles of NH_4Cl affected the assimilation of glucose in a similar way; however, the effect exerted by arginine was stronger. This was to be expected, because arginine contains 4 µatoms of nitrogen per µmole and could, therefore, provide four times as much nitrogen.

However, arginine was not acting solely as a nitrogen source in the presence of glucose, because arginine carbon was assimilated under these conditions (Table VI). The addition of glucose caused a slight decrease in the amount of arginine assimilated, with a concomitant increase in the amount released as CO_2. However, the pattern of arginine incorporation into the cell fractions was

Table V. The effect of arginine and ammonium ions on the assimilation of ^{14}C-glucose by a resting cell suspension of P. aeruginosa.[a]

Fraction	% total radioactivity		
	^{14}C-glucose	^{14}C-glucose + arginine	^{14}C-glucose + NH_4^+
CO_2	59.5%	51.9%	54.5%
supernatant fluid	15.7%	14.9%	13.9%
cells	27.7%	33.4%	30.8%
	% cell radioactivity		
cold trichloroacetic acid soluble	9.3%	9.4%	10.3%
acid ethanol soluble	26.3%	30.8%	28.1%
hot trichloroacetic acid soluble	13.6%	14.3%	12.4%
hot trichloroacetic acid precipitate	50.8%	45.5%	49.0%

[a] 5 mg of cells (dry weight) were incubated for 90 minutes under conventional Warburg conditions with 0.05 M Tris buffer. 2.5 µmoles of each substrate were added. The specific activity of the ^{14}C-glucose was 1 µCi/µmole.

Table VI. Assimilation of ^{14}C-arginine by a resting cell suspension of P. aeruginosa in the presence and absence of glucose.[a]

Fraction	% total radioactivity	
	^{14}C-arginine	^{14}C-arginine + ^{12}C-glucose
CO_2	55.1%	60.6%
supernatant fluid	18.8%	17.0%
cells	26.1%	24.2%
	% cell radioactivity	
cold trichloroacetic acid soluble	31.5%	35.4%
acid ethanol soluble	19.1%	13.3%
hot trichloroacetic acid soluble	12.5%	6.8%
hot trichloroacetic acid precipitate	36.9%	44.5%

[a] Experimental conditions were the same as given in Table V. The specific activity of the ^{14}C-arginine was 1 μCi/μmole.

noticeably affected by the presence of glucose. When glucose was present, the proportion of arginine incorporated into the cold trichloroacetic acid soluble pool and into protein was increased, and the proportion incorporated into the acid ethanol and hot trichloroacetic acid soluble fractions was decreased. Greater than 95% of the radioactivity in the acid ethanol soluble fraction was lipid.

Thus, in the presence of both arginine and glucose, P. aeruginosa preferentially incorporated glucose into lipid and nucleic acids, and arginine into protein and the trichloroacetic acid soluble pool. This pattern of utilization would prove economical for the cell, since glucose can be converted into lipid and pentoses more readily than arginine, which is degraded to intermediates of the TCA cycle. On the other hand, many of the amino acids used in protein synthesis, and the amino acids and other basic substances present in the cell pool, may be obtained more readily from arginine than from glucose.

IV. Degradation of Arginine by P. putida and P. fluorescens

Kay (1968) found that P. fluorescens did not catabolize arginine actively, and that the majority of the radioactivity incorporated into the cells during arginine transport studies was incorporated into protein. P. putida, however, catabolized more

of the arginine and formed a larger intracellular pool. The oxidation and assimilation of ^{14}C-arginine by these organisms was therefore studied and compared to that by P. aeruginosa. Manometric studies showed that uninduced cells of P. fluorescens oxidized arginine only slightly, taking up only 1 µmole of oxygen in the presence of 2.5 µmoles of arginine (Fig. 12). P. putida demonstrated a greater constitutive capacity to oxidize arginine, but the initial rate and final extent of oxidation were much lower than with P. aeruginosa (Fig. 12).

The assimilation patterns after incubation of these three organisms in the presence of 2.5 µmoles of ^{14}C-arginine (1 µCi/µmole) for 100 minutes under Warburg conditions were compared (Table VII). More ^{14}C-arginine was assimilated into cellular material and metabolized to CO_2 by P. aeruginosa than by P. putida, and less radioactivity remained in the supernatant fluid. The radioactivity of the trichloroacetic acid soluble extractable pool was similar in these two organisms. The majority of the radioactivity remained in the supernatant fluid after incubation of P. fluorescens with ^{14}C-arginine. Only 8.8% of the radioactivity was recovered as $^{14}CO_2$, and this correlated well with the low oxygen uptake obtained with this organism. The majority of the radioactivity taken into the cells of this organism was not incorporated into cellular material but remained in the intracellular pool. The latter results are different from those obtained by Kay (1968); however, resting

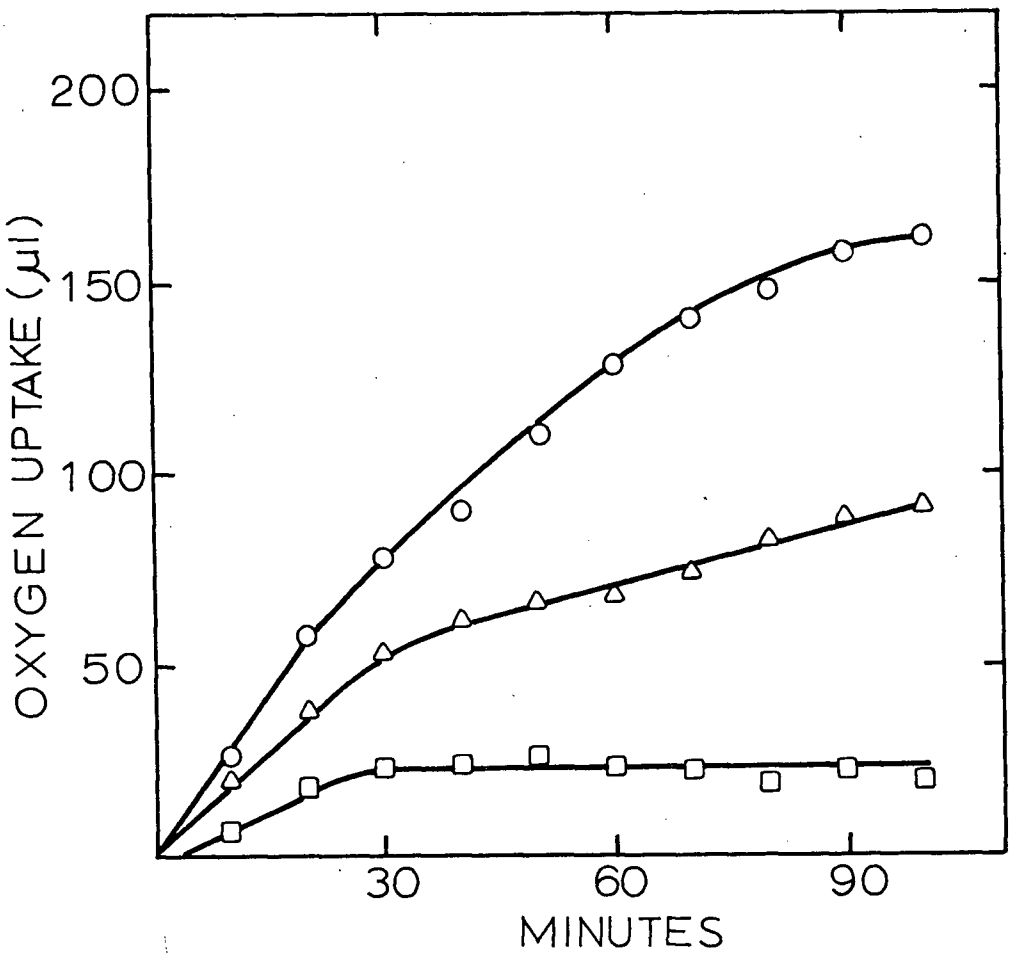


Fig. 12. Oxidation of arginine by glucose grown cells of P. aeruginosa, O; P. fluorescens, □ ; and P. putida, Δ .
Cell concentrations were approximately 5 mg of cells (dry weight)/ml and Tris buffer was used.

Table VII. Comparison of the assimilation of ^{14}C-arginine by *P. aeruginosa*, *P. fluorescens*, and *P. putida*.[a]

Fraction	% total radioactivity		
	P. aeruginosa	*P. putida*	*P. fluorescens*
CO_2	46.7%	27.4%	8.8%
supernatant fluid	37.6%	61.8%	85.5%
cold trichloroacetic acid soluble	8.9%	8.4%	5.5%
cold trichloroacetic acid insoluble	10.3%	2.4%	0.2%

[a] 5 mg of cells (dry weight) were incubated for 100 minutes under conventional Warburg conditions with 0.05M Tris buffer and 2.5 µmoles (2.5 µCi) of arginine.

cells from the stationary phase of growth and high substrate concentrations were used in these experiments, whereas Kay (1968) used cells from the logarithmic phase of growth and low substrate concentrations. The amount of radioactivity in the pool of P. fluorescens was lower than that in P. aeruginosa and P. putida, confirming the results of Kay (1968).

Thin-layer chromatography and radioautography of the supernatant fluids showed that all the radioactivity of the supernatant fluid from P. putida was present as putrescine. Thus, 70% of the total radioactivity was accounted for as putrescine, since the intracellular pool formed by these three organisms in the presence of arginine has been shown to consist of putrescine (Kay, 1968). In addition to putrescine, radioactive arginine and citrulline were found in the supernatant fluid from P. fluorescens. Thus, both P. putida and P. fluorescens had the capacity to constitutively convert arginine to putrescine, presumably via citrulline and ornithine. P. putida was more active in this respect than P. fluorescens. Stanier, Palleroni and Doudoroff (1966) described the presence of a constitutive arginine dihydrolase pathway as characteristic of fluorescent Pseudomonads.

Both the oxygen uptake and assimilation data showed that P. putida could further degrade arginine, whereas P. fluorescens was almost inactive in this respect. P. aeruginosa was the organism most active in the degradation of arginine, and although putrescine

was the major radioactive compound found in the supernatant fluid with this organism, radioactive glutamate was also present. It is therefore possible that the slow rate of oxygen uptake by P. putida was due to the further degradation of putrescine, whereas the faster rate of arginine degradation by P. aeruginosa was due to the additional ability of this organism to degrade ornithine via glutamate.

V. Uptake of Basic Amino Acids and Polyamines by P. aeruginosa

1. Induction of uptake

de Hauwer, Lavalle, and Wiame (1964) found that the rate of transport of arginine by B. subtilis was greatly increased after growth in the presence of arginine. Growth of P. aeruginosa with arginine as the sole source of carbon and nitrogen resulted in a five-fold increase in the rate of arginine transport (Table VIII). In addition, the rates of ornithine and lysine transport were increased seven-fold; the rates of citrulline and putrescine transport, three-fold. Thus, growth in arginine induced an increase not only in the rate of uptake of arginine, but also in that of other basic amino acids and of putrescine.

P. aeruginosa transported citrulline at a much lower rate than the other basic amino acids. It is possible, however, that the rate of citrulline uptake as measured in these experiments was lower than the actual rate, because the label was in the ureido carbon

Table VIII. Induction of transport

Substrate	Rate of transport[a]		
	Growth medium		
	glucose	arginine	putrescine
arginine	10.0	52.1	4.9
ornithine	5.5	41.5	4.9
lysine	5.6	38.2	-[b]
citrulline	1.3	3.4	-[b]
putrescine	11.0	31.5	37.1
γ-aminobutyrate	0	0	2.04

[a] expressed as mμm x min^{-1} x mg^{-1} of cells (dry weight). The reaction mixture contained 0.135 mg of cells (dry weight)/ml and substrates were added to a final concentration of 2.5 x 10^{-5}M. All radioactive substrates had a specific activity of 2 μCi/μmole.

[b] not tested

of the citrulline, and would be lost as $^{14}CO_2$, upon the conversion of citrulline to ornithine. Since arginine grown cells oxidized citrulline more rapidly than glucose grown cells (Table I), it is likely that the discrepancy between the actual rate of citrulline transport and the observed rate would be greater in induced cells. The low rates of citrulline transport may have been the limiting factors responsible for the low rates of citrulline oxidation observed in the respirometry studies.

P. aeruginosa transported putrescine very rapidly, and the rate of transport was induced to a higher level after growth in either arginine or putrescine. The rate of transport of all substrates by putrescine grown cells may be even more rapid than that observed, because the cells were clumped, a phenomenon that was shown to reduce drastically the rate of arginine transport by arginine grown cells. Thus, growth in putrescine probably results in a greater induction of putrescine uptake than does growth in arginine.

Neither glucose grown cells nor arginine grown cells possessed the capacity to transport γ-aminobutyrate. However, this substrate was transported slowly by putrescine grown cells.

2. Kinetics of arginine uptake

A study of the kinetics of arginine uptake by P. aeruginosa was carried out by determining the initial rates of ^{14}C-arginine uptake at varying substrate concentrations (Fig. 13). Glucose grown cells did not demonstrate normal saturation kinetics, but, rather, the rate of arginine uptake continued to increase with increasing substrate concentrations (Fig. 13A). A Michaelis-Menton plot demonstrated the presence of two permeases: one with a Km of 2.2×10^{-7}M which functioned at low arginine concentrations, and one with a Km of 5.4×10^{-6}M which functioned at arginine concentrations greater than 1.3×10^{-5}M (Fig. 14). Kay (1968) found that the uptake of glutamate, proline, and leucine by P. aeruginosa followed similar kinetics. Ames (1964) demonstrated the existence of two kinetic components for the transport of histidine in S. typhimurium: a high affinity permease specific for histidine, and a low affinity permease which also functioned in the transport of the aromatic amino acids. Mutants defective in each of the permeases have been studied (Ames and Roth, 1968). Reid, Utech and Holden (1970) showed that Streptococcus faecalis possessed two kinetic components for the transport of glutamate and aspartate, and have isolated a mutant lacking the high affinity permease for these amino acids (Utech, Reid and Holden, 1970).

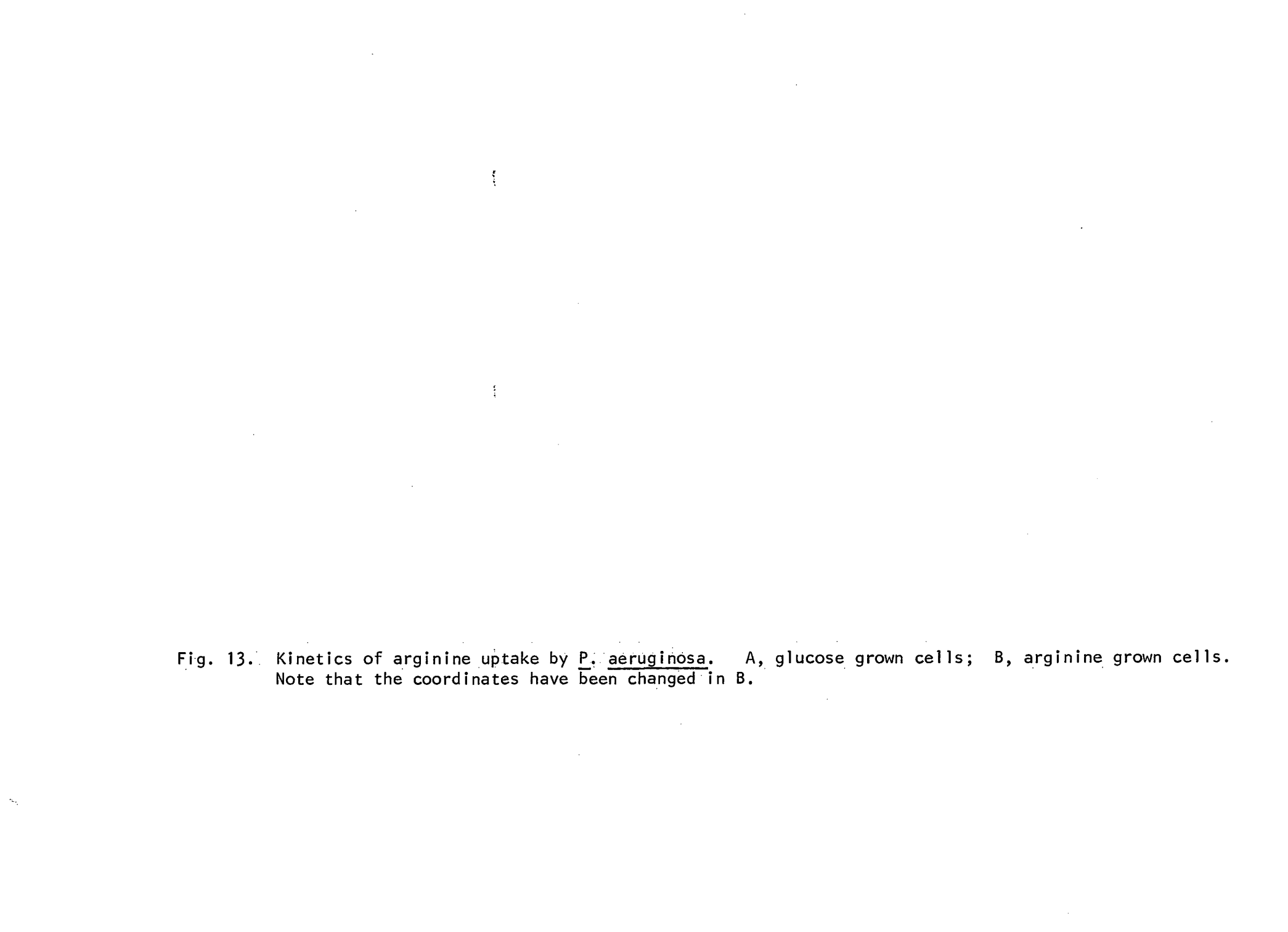
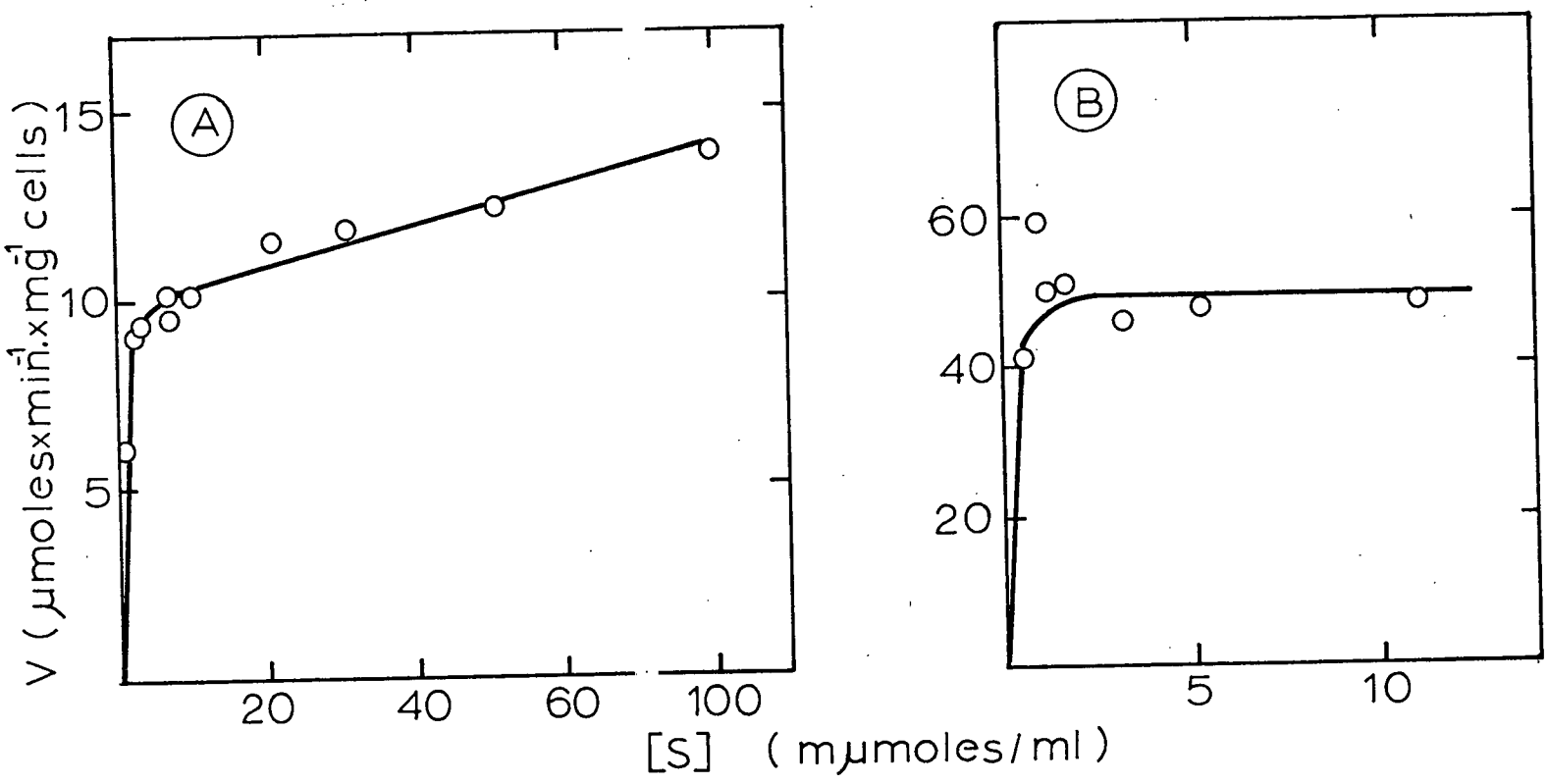


Fig. 13. Kinetics of arginine uptake by P. aeruginosa. A, glucose grown cells; B, arginine grown cells. Note that the coordinates have been changed in B.


A
B
V (µmolesxmin-1.xmg-1 cells)
15
10
5
20
40
60
100
60
40
20
5
10
[S] (mµmoles/ml)

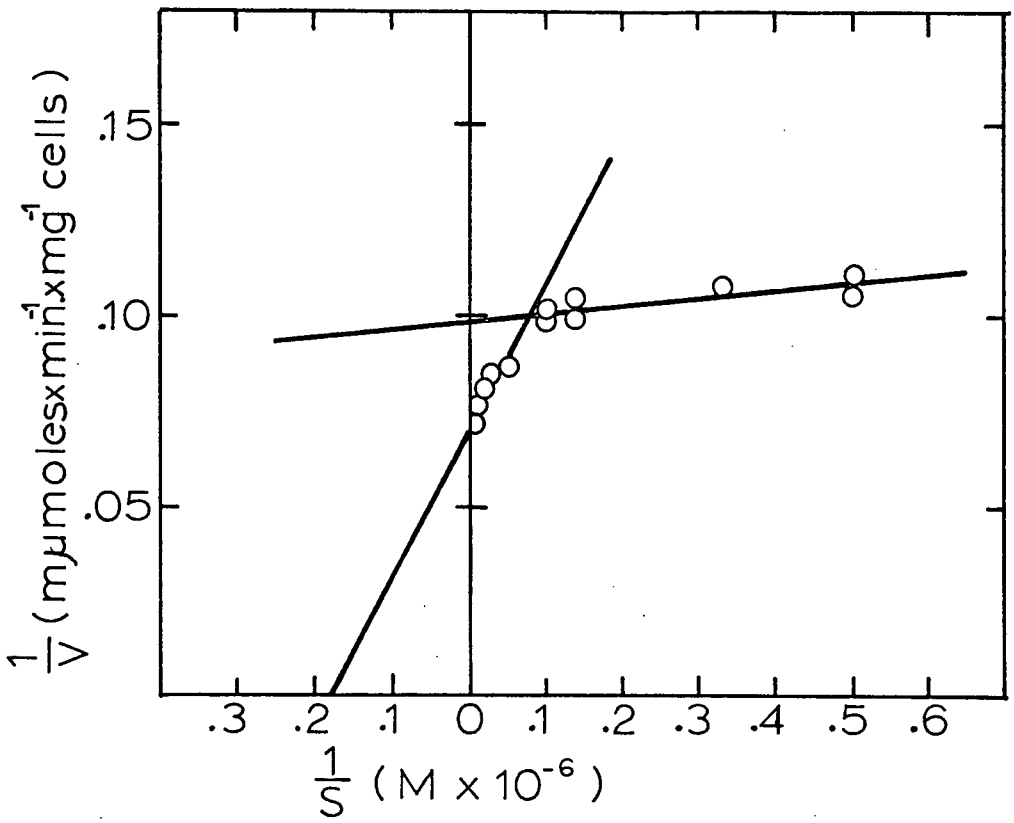


Fig. 14. Kinetics of arginine uptake by glucose grown cells. Lineweaver-Burk plot.

The rates of uptake of low concentrations of arginine by arginine grown cells were too rapid to be measured, and therefore the cell concentration was decreased 15-fold to approximately 9 µg of cells (dry weight)/ml of the final reaction mixture. The uptake of arginine by these cells followed normal saturation kinetics (Fig. 13B), and they did not appear to possess a low affinity permease at high substrate concentrations. However, it is possible that the activity of the latter permease could not be detected at the low cell concentration which was used, or that this activity was obscured due to the rapid degradation of arginine by induced cells. The high affinity permease of arginine grown cells had a Km of approximately 1.7×10^{-7}M, which was similar to the value obtained with glucose grown cells. However, the velocity maximum (V_{max} - mµmoles x min^{-1} x mg^{-1}) of arginine uptake by arginine grown cells was approximately 55, whereas that of glucose grown cells was 10. Thus, growth of the cells in arginine caused increased synthesis of the high affinity permease, but did not induce the synthesis of a new permease.

3. Inhibition of transport

a. Basic amino acids

The differences in the degrees to which the rates of transport of the different substrates were induced indicated that

several uptake systems were involved. Kay (1968) obtained data which indicated that P. aeruginosa possessed two transport systems for the basic amino acids: permease bas 1, which was specific for arginine and had a lower affinity for ornithine, and permease bas 2, which transported lysine, arginine, ornithine, citrulline and histidine, in order of affinity.

Inhibition studies reported here further confirmed the presence of these two basic amino acid uptake systems in this organism. Ornithine was the most effective inhibitor of arginine uptake in both induced and uninduced cells (Table IX). Glucose grown cells appeared to transport arginine mainly by the permease bas 1, since the inhibition exerted by lysine was low, and that exerted by citrulline and histidine was negligible. However, growth of the cells in arginine resulted in a marked increase in the degree of inhibition exerted by these compounds. Because growth of P. aeruginosa in arginine also caused an increase in the rates of transport of lysine and citrulline (Table VIII), the ratio of the activity of the general permease (bas 2) to the specific permease (bas 1) must have increased after induction.

Ornithine uptake was completely inhibited by arginine in both induced and uninduced cells (Table X). Lysine was also a potent inhibitor of ornithine uptake with both types of cells, being almost completely inhibitory with induced cells. Citrulline and histidine were also relatively effective inhibitors of ornithine

Table IX. Inhibition of arginine transport.[a]

Inhibitor	% inhibition	
	Growth substrate	
	glucose	arginine
histidine	10	20
citrulline	11	25
lysine	20	52
ornithine	58	61
arginine	100	100

[a] The reaction mixture contained 0.135 mg of cells (dry weight)/ml and ^{14}C-arginine was added to a final concentration of $2.1 \times 10^{-5}M$ (specific activity = 2.4 µCi/µmole). Unlabelled inhibitors were added immediately prior to the addition of the labelled substrate to a final concentration of $2.5 \times 10^{-4}M$.

Table X. Inhibition of ornithine transport.[a]

Inhibitor	% inhibition	
	Growth substrate	
	glucose	arginine
putrescine	7	12
histidine	48	56
citrulline	69	72
lysine	84	97
arginine	100	99
ornithine	100	100

[a] Conditions were the same as for Table IX with the exception that ^{14}C-ornithine was added to give a final concentration of 2.5×10^{-5}M (specific activity = 2 µCi/µmole).

uptake, with citrulline exerting a somewhat stronger inhibition than histidine. These results indicated that ornithine was transported by both permeases in both types of cells; and they also confirmed the increase in the activity of the general permease (bas 2) after growth of the cells in arginine.

It is important to note that the concentrations of labelled arginine and ornithine used in these studies were greater than 2 x 10^{-5}M, and thus the low affinity permease may have played an important role in transport.

b. Polyamines

Tabor and Tabor (1966) found that E. coli transported putrescine very rapidly, and spermine and spermidine more slowly. They hypothesized the existence of at least two systems of polyamine uptake in this organism, one having a high affinity for putrescine. Putrescine uptake in P. aeruginosa was inhibited only slightly by arginine and ornithine, more strongly by spermine, and very strongly by spermidine (Table XI). Thus, P. aeruginosa probably possesses a general polyamine uptake system with a high affinity for putrescine and spermidine, and a lower affinity for spermine. It is possible that a portion of the inhibition exerted by spermine and spermidine was due to adsorption of the latter compounds to the cell surface, a phenomenon that was observed to occur in E. coli by Tabor and

Table XI. Inhibition of putrescine transport.[a]

Inhibitor	% Inhibition	
	Growth substrate	
	Glucose	Putrescine
arginine	21%	25%
ornithine	20%	21%
γ-aminobutyrate	-[b]	0
spermine	48%	40%
spermidine	83%	60%
putrescine	100%	100%

[a]The reaction mixture contained approximately 27 µg of cells (dry weight)/ml and ^{14}C-putrescine was added to a final concentration of 2.5×10^{-5}M (specific activity = 2 µCi/µmole). Inhibitors were added immediately prior to the addition of the labelled substrate to a final concentration of 2.5×10^{-4}M.

[b]Not tested.

Tabor (1966). The inhibition exerted by arginine and ornithine may have been non-competetive, because putrescine inhibited ornithine uptake only very slightly (Table X). Thus, it is unlikely that significant putrescine uptake occurrs via the basic amino acid transport systems. Growth of the cells in putrescine resulted in a slight decrease in the degree of inhibition exerted by spermidine and no significant alteration in the inhibition exerted by any of the other compounds, indicating that no new transport systems had been induced. The possibility that growth in spermine or spermidine might induce transport systems specific for these polyamines was not investigated.

4. Pool formation

a. Arginine

The effect of induction on pool formation and stability was investigated using ^{14}C-arginine as substrate. The initial patterns of pool formation differed in induced and uninduced cells. In glucose grown cells, the incorporation of ^{14}C-arginine into protein followed a time course similar to that of the total uptake (Fig. 15). Thus, the intracellular pool increased in size during the first 10 minutes of incubation, after which time it decreased slightly due to continued protein synthesis, and then remained

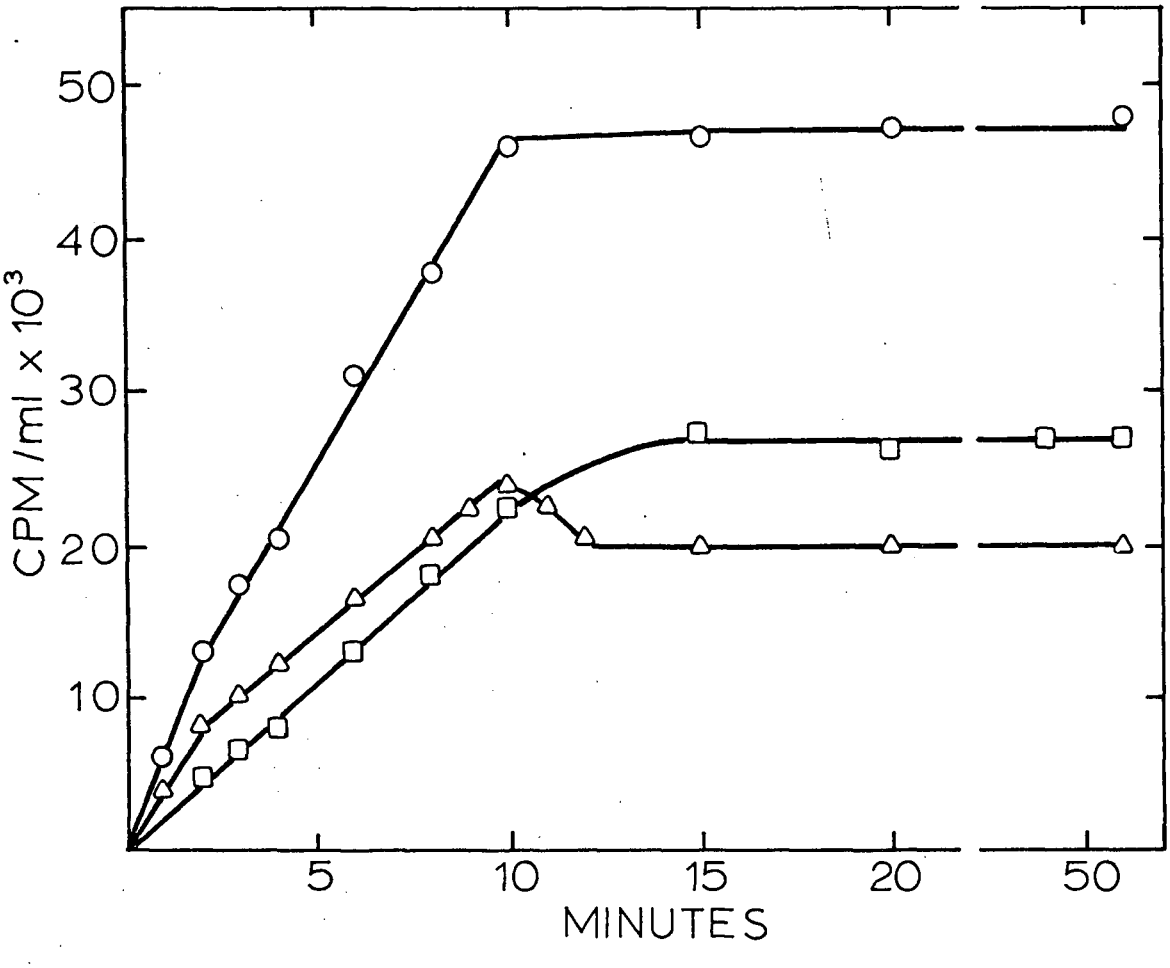


Fig. 15. Formation of an intracellular pool using glucose grown cells supplied with ^{14}C-arginine.
Symbols: 0, whole cells; □ , protein; Δ, trichloroacetic acid soluble pool. The cell concentration was 0.135 mg of cells (dry weight)/ml, and the arginine concentration was 2.1 x 10^{-5}M (specific activity = 2.4 μCi/μmole).

stable for the next 40 minutes. Arginine grown cells, on the other hand, demonstrated a lag of 90 seconds before the maximal rate of incorporation of ^{14}C-arginine into protein was reached (Fig. 16). This lag presumably represented dilution through a pool of unlabelled arginine, which had been established during growth and had not been removed by washing. Thus, after 90 seconds of incubation in ^{14}C-arginine, induced cells had accumulated an extremely high pool, which decreased in size by approximately 50% during the following 6 minutes. This decrease mainly represented incorporation into protein, although a slight decrease in the total cell radioactivity also occurred, probably due to oxidation. The intracellular pool of induced cells remained stable after the first 8 minutes of incubation. The radioactivity remaining in the supernatant fluid after filtration of the whole cells was measured at certain times. Approximately 45% of the original radioactivity was lost, presumably as $^{14}CO_2$, during the first 15 minutes of incubation with induced cells, and a similar amount was lost during the first 30 minutes of incubation with uninduced cells.

Kay (1969) found that the intracellular pool formed by P. aeruginosa from arginine consisted of putrescine, and was stable for periods as long as 24 hours. Arginine grown cells also appeared to maintain a stable pool which was of a similar size to that formed in uninduced cells. Thus, under the conditions used in these experiments, cells which were induced for arginine degradation degraded

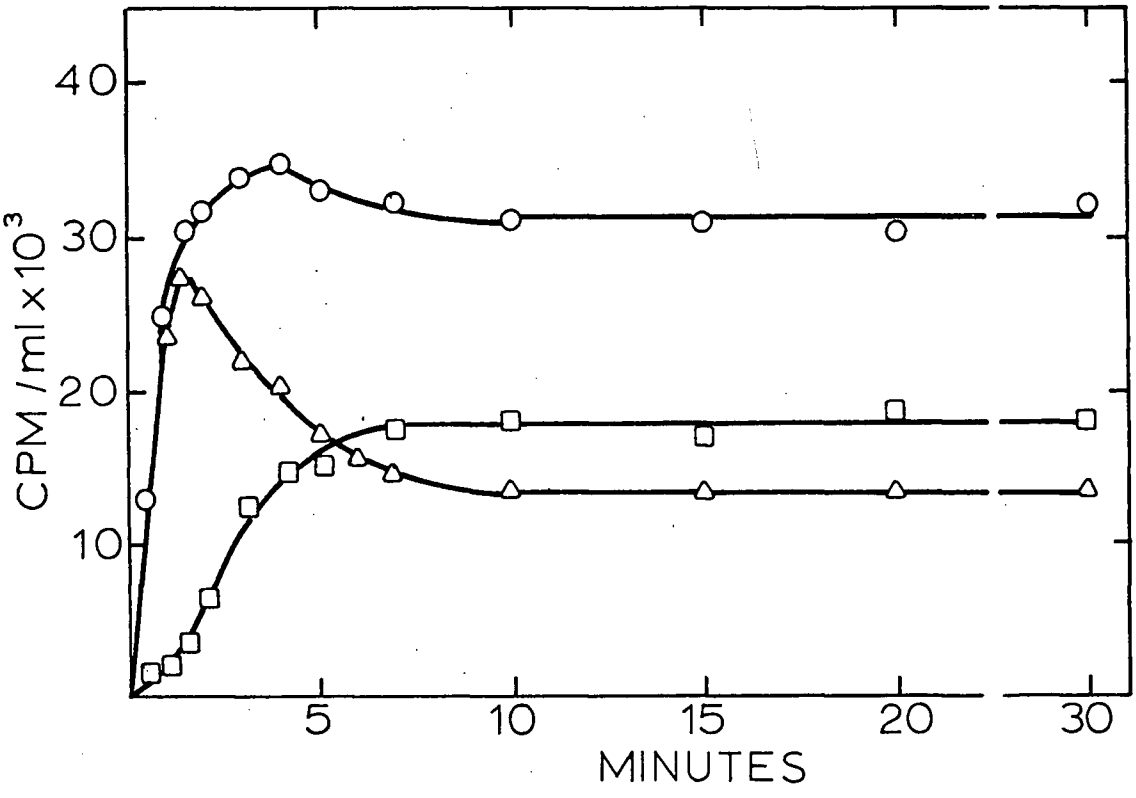


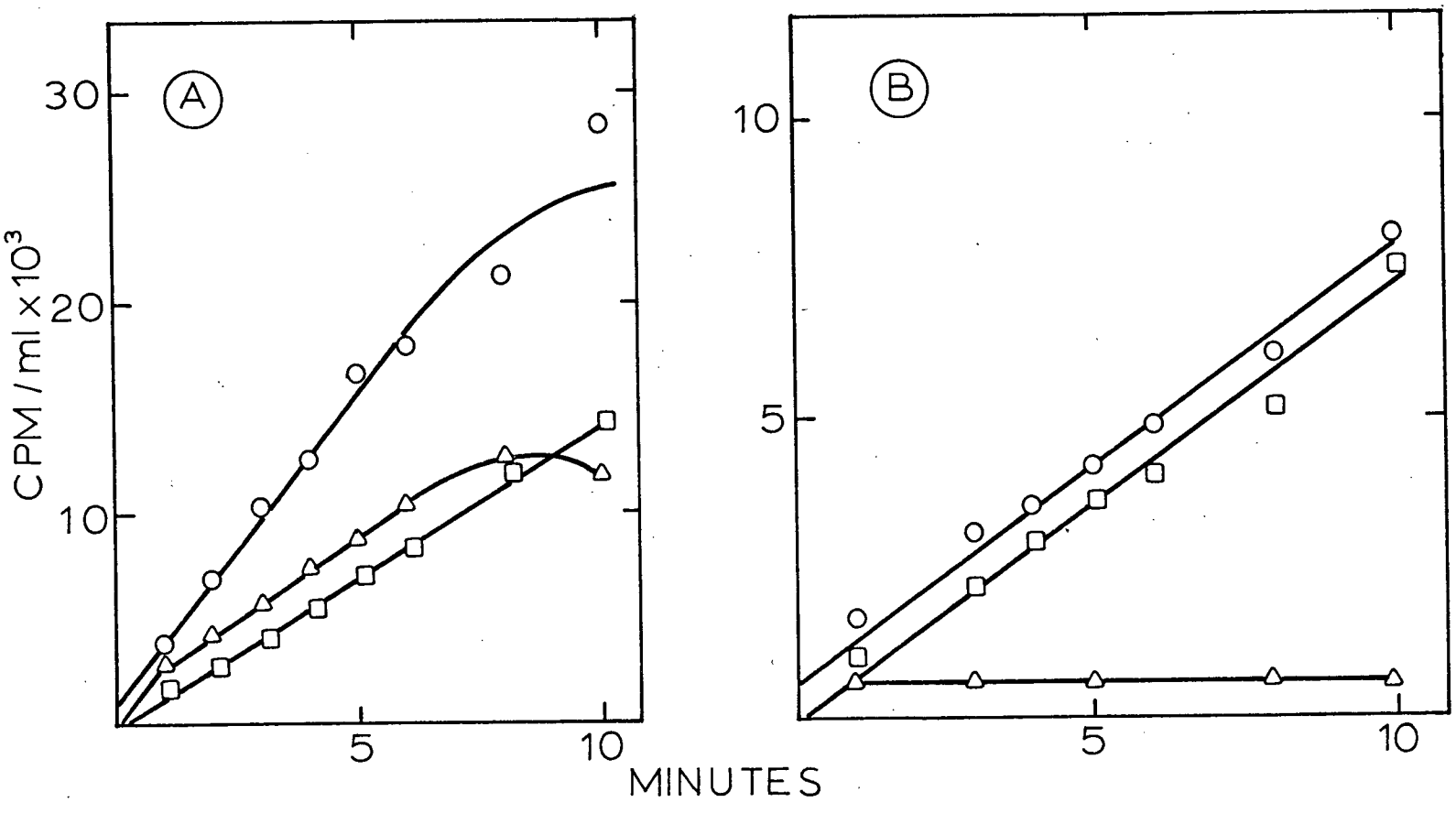
Fig. 16. Formation of an intracellular pool of arginine by cells grown with arginine as the sole source of carbon and nitrogen. Symbols: 0, whole cells; □ , protein; Δ , trichloroacetic acid soluble pool. The concentrations of cells and ^{14}C-arginine were the same as in Fig. 15.

only the same proportion of arginine as uninduced cells, and retained the remainder in the trichloroacetic acid soluble pool. Assuming that putrescine was the only radioactive compound in the pool, it was calculated that approximately 23% of the original arginine label was retained in this pool in induced cells, and 33% in uninduced cells. The difference may not be very significant due to the low concentrations involved.

b. Ornithine and citrulline

The uptake of ornithine by glucose grown cells of P. aeruginosa followed an initial pattern similar to that of arginine (Fig. 17A). After the first minute of incubation, citrulline was incorporated into protein as rapidly as it was taken up (Fig. 17B). On the other hand, neither citrulline nor ornithine were incorporated into protein by arginine grown cells during the first 5 minutes of incubation, and these compounds were accumulated solely in the intracellular pool. These results were due to the fundamental differences in the metabolic fate of these compounds in the two types of cells. Glucose grown cells are induced for arginine biosynthesis, and thus would rapidly convert ornithine and citrulline to arginine, which would then be incorporated into protein. Arginine grown cells have been shown to be induced for the degradation of arginine, and repressed for its biosynthesis. Thus, citrulline

Fig. 17. Formation of intracellular pools of ornithine (A) and citrulline (B) by glucose grown cells.
Symbols: O, whole cells; □, protein; Δ, trichloroacetic acid soluble pool. The cell concentration was 0.135 mg of cells (dry weight)/ml, and the labelled substrates were added to a final concentration of 2.5×10^{-5}M (specific activity = 2 μCi/μmole).


A
B
30
20
10
10
5
5
10
CPM / ml x 10³
MINUTES

and ornithine would not be converted to protein arginine by these cells.

c. Putrescine

The stability of putrescine pools formed by induced and uninduced cells of P. aeruginosa in the presence of external ^{14}C-putrescine was also studied. The total uptake of putrescine into glucose grown cells reached a maximum at 15 minutes, after which time the total cell radioactivity did not change (Fig. 18). However, a gradual incorporation of label into trichloroacetic acid insoluble material commenced after 20 minutes, resulting in a concomitant decrease in the level of the trichloroacetic acid soluble pool.

In the presence of external ^{14}C-putrescine, putrescine grown cells rapidly accumulated a high intracellular pool, which reached a maximum at 6 minutes, and dropped rapidly for the next 4 minutes, due to continued rapid incorporation into protein (Fig. 19). The slight lag in incorporation of radioactivity into protein presumably represented dilution through a pool of unlabelled putrescine which had been established during growth. The intracellular pool remained stable for approximately 10 minutes, and then began to decrease slowly, losing 64% of its radioactivity during the next 60 minutes. During this time the radioactivity present in trichloroacetic acid

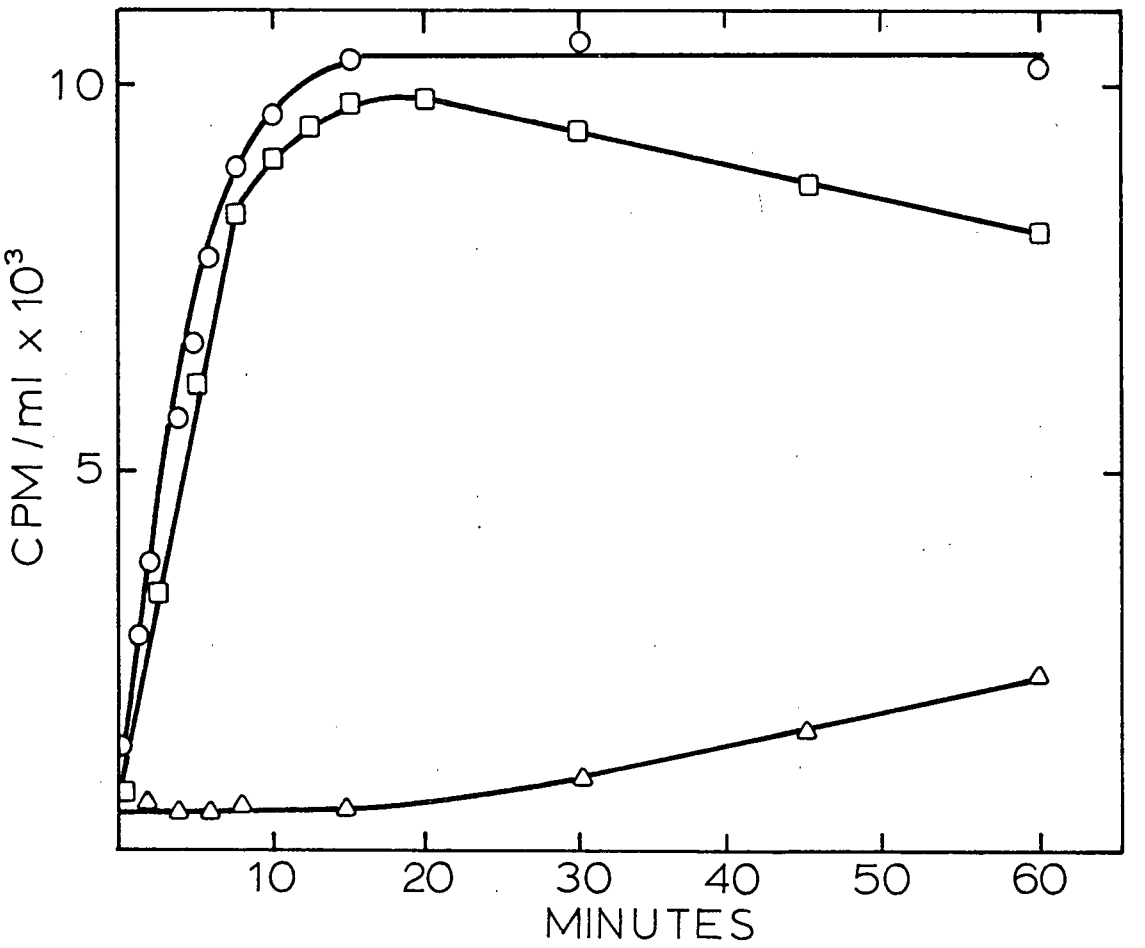


Fig. 18. Formation of an intracellular pool of putrescine by glucose grown cells.
Symbols: O, whole cells; □ , trichloroacetic acid soluble pool; Δ, protein. The cell concentration was 27 µg of cells (dry weight)/ml, and the ^{14}C-putrescine concentration was 2.5×10^{-5}M (specific activity = 2 µCi/µmole).

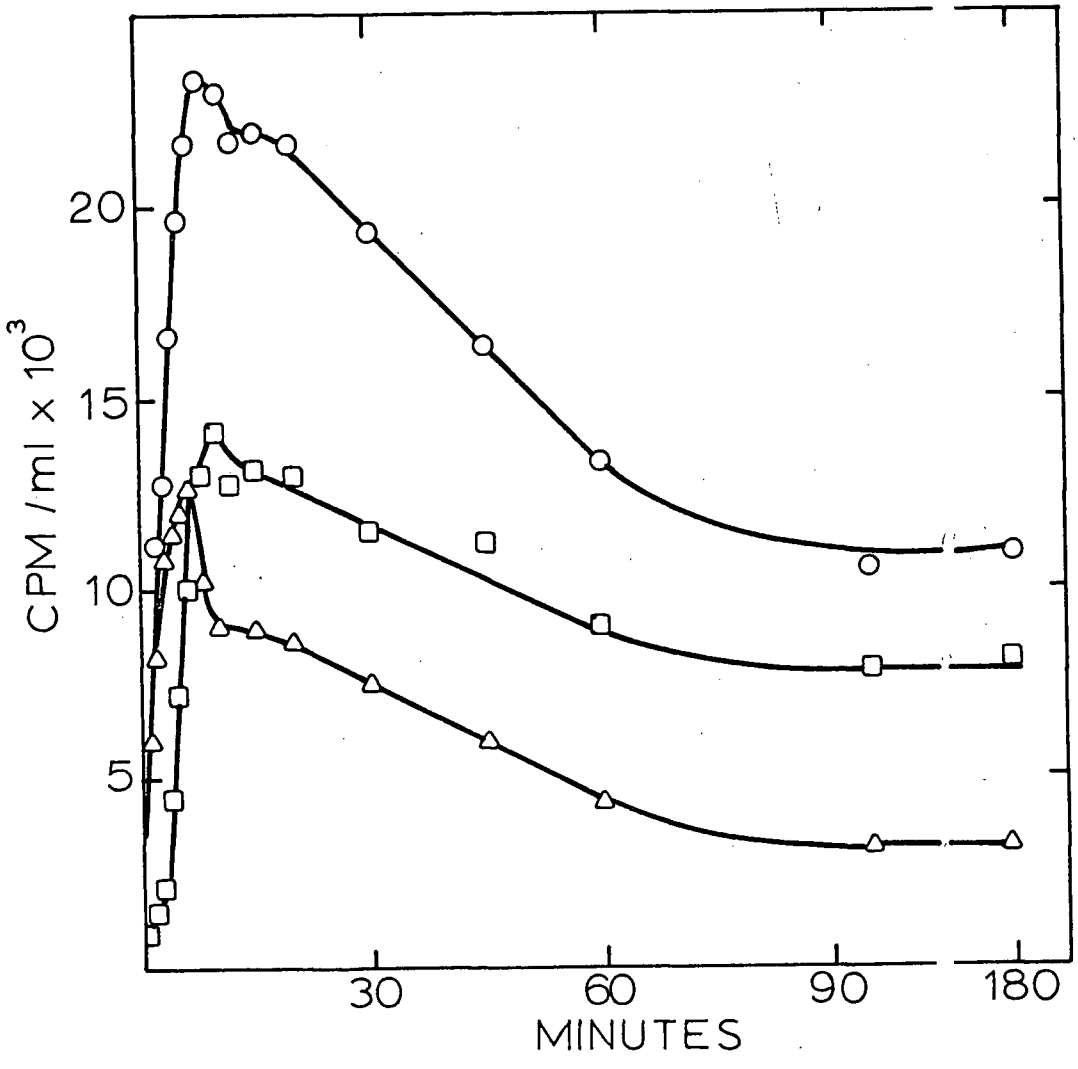


Fig. 19. Formation of an intracellular pool of putrescine by putrescine grown cells.
Symbols: O, whole cells; □ , protein; Δ, trichloroacetic acid soluble pool. The concentrations of cells and of ^{14}C-putrescine were the same as in Fig. 18.

insoluble material decreased by 43%, indicating that lysis had occurred. However, the decrease in pool radioactivity was greater than that due to lysis, indicating that approximately 20% of the pool had been oxidized. Measurements of the radioactivity of the supernatant fluids supported this hypothesis. Forty-eight per cent of the added label had been lost from the reaction mixture after 10 minutes of incubation, and 76% after 60 minutes. A stable pool was maintained from 80 to 180 minutes.

Arginine grown cells also accumulated a high intracellular pool of putrescine, which reached a maximal level at 15 minutes, and then decreased during the next 45 minutes (Fig. 20). The decrease in pool radioactivity which occurred during the first 10 minutes of this period was accounted for by an increase in protein radioactivity. However, the radioactivity of the cell protein remained constant after the first 25 minutes, and thus the further decrease in pool radioactivity presumably represented the oxidation of intracellular putrescine. A stable pool was maintained between 60 and 90 minutes, after which the radioactivity of the trichloroacetic acid insoluble material decreased slowly, indicating that lysis was occurring. During this period, the total cell radioactivity decreased rapidly, and no intracellular pool remained after 3 hours of incubation. Measurements of the radioactivity of the supernatant fluid showed that 39% of the total label had been lost by 15 minutes, and 65% by 60 minutes. After

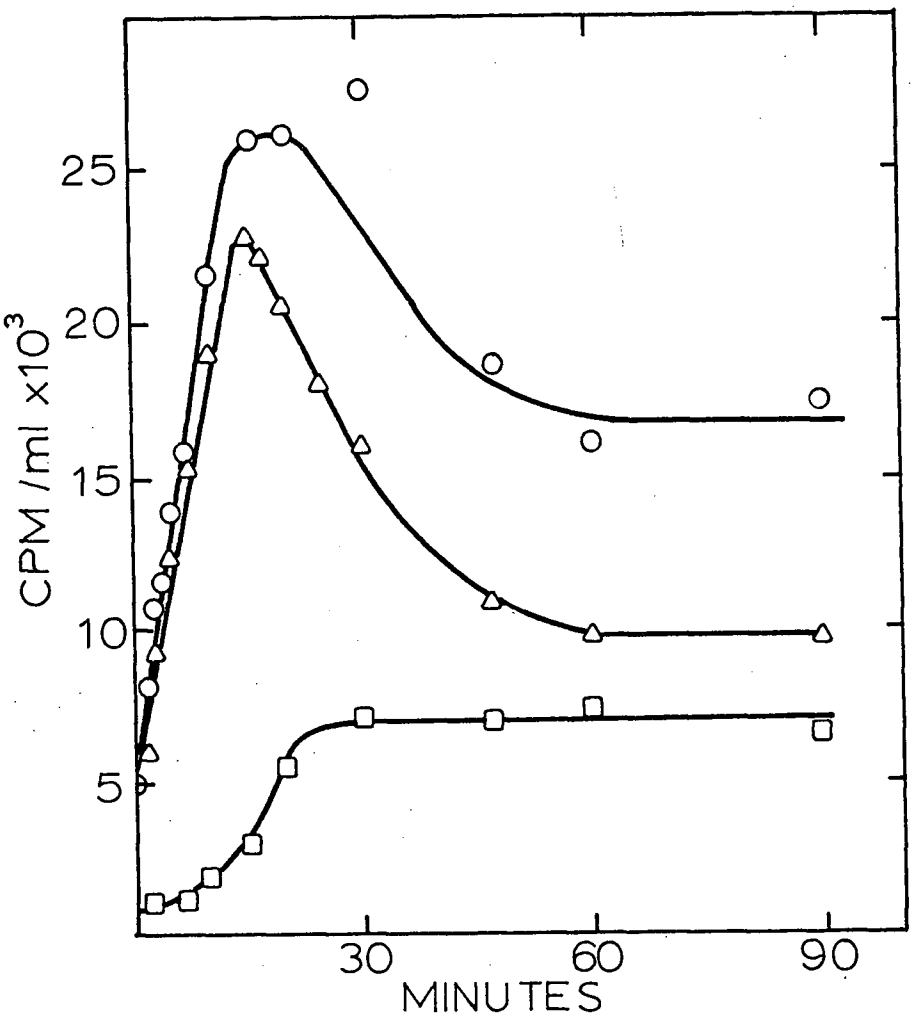


Fig. 20. Formation of an intracellular pool of putrescine by arginine grown cells.
Symbols: O, whole cells; □ , protein; Δ, trichloroacetic acid soluble pool. The concentrations of cells and of ^{14}C-putrescine were the same as in Fig. 18.

3 hours, 3% of the original label was present in the cells, 8.5% in the supernatant fluid, and the remainder had presumably been lost as $^{14}CO_2$.

The size of the stable intracellular putrescine pools was difficult to determine due to the errors arising from the use of low cell concentrations, and the fact that lysis had occurred. However, the concentration of putrescine in the intracellular water was approximately 2 to 3 x 10^{-2}M.

5. Location of intracellular putrescine pool

Kay (1969) showed that the stable pool formed in P. aeruginosa from arginine consisted of putrescine. Since this pool did not require energy for its maintenance, he hypothesized that putrescine may be bound to some component within the cell (Kay, 1968). An attempt was therefore made to determine the location of this intracellular pool by physical fractionation procedures.

Cells were harvested from the stationary phase of growth in glucose minimal medium and resuspended to a concentration of 5 mg of cells (dry weight)/ml. Ten ml of the cell suspension were incubated under conventional Warburg conditions with 5 ml of 0.05 M Tris buffer in a large Warburg cup with a single side-arm to which was added 15 µm arginine (specific activity 0.67 µCi/µmole).

The cells were harvested 90 minutes after the addition of the arginine, and were subjected to physical fractionation. The radioactivity of the fractions was measured and compared with that of the cell-free extract. Samples of each fraction were extracted with cold 10% trichloroacetic acid, and the distribution of the radioactivity between the precipitate and the supernatant fluid was determined.

The results showed that the majority of the trichloroacetic acid extractable pool was present in the soluble cytoplasm (Table XII). Although the membrane and ribosomal fractions also contained considerable radioactivity, 65% to 70% of this was trichloroacetic acid precipitable material, presumably protein, which would have been labelled during the incubation of the cells with ^{14}C-arginine. Thus, 36% of the total label was present in the soluble cytoplasm as trichloroacetic acid extractable material, whereas only 7.5% was present in this form in the ribosomal fraction, and a similar amount in the membrane fraction. Thin-layer chromatography of the trichloroacetic acid extract of the soluble cytoplasm, followed by radioautography, showed that putrescine was the labelled compound.

The soluble cytoplasm has been found to be the location of intracellular putrescine in other organisms. Kim (1966) found that 80% to 90% of the intracellular putrescine of a Pseudomonad was located in the soluble cytoplasm, with the remaining 10% to 20% located in the ribosomal fraction. Tabor and Kellogg (1967) obtained similar results with E. coli, in which 8% to 11% of the intracellular

Table XII. Distribution of radioactivity after physical fractionation of cells incubated in the presence of ^{14}C-arginine.

Fraction	% of total radioactivity	% of the label in the fraction which was extracted by cold trichloroacetic acid
membranes	23	35.4
ribosomes	25	30.7
110,000 x g supernatant fluid	59	59

polyamines were associated with the ribosomes, and the remainder were located in the soluble cytoplasm. However, the latter workers also showed that E. coli ribosomes could take up or lose polyamines upon alteration of the concentration of magnesium or amines in the suspension medium. Thus, although a greater proportion of the trichloroacetic acid soluble putrescine was associated with ribosomes in P. aeruginosa than was observed in a Pseudomonad by Kim (1966) and in E. coli by Tabor and Kellogg (1967), the latter workers used a 10 fold higher magnesium concentration during ribosome isolation. Thus, polyamines are probably redistributed after disruption of the cell, and fractionation results may be invalid.

GENERAL DISCUSSION

Stanier, Palleroni, and Doudoroff (1966) have shown that the possession of a constitutive arginine dihydrolase pathway is a characteristic of fluorescent Pseudomonads, and the three species examined in this study were shown to excrete the intermediates of this pathway during the oxidation of arginine. In addition, all three organisms synthesized putrescine from ornithine, the end-product of the action of the arginine dihydrolase system. Thus, it is likely that the enzymes of the arginine dihydrolase pathway may function in the biosynthesis of putrescine in these organisms.

There is much indirect evidence in the literature that polyamines may play a role in translation, and possibly also in transcription. Several workers have obtained evidence that these compounds are necessary for the growth of E. coli which synthesizes a high pool of putrescine constitutively. It is not known whether P. aeruginosa synthesizes putrescine during growth in glucose minimal medium. However, when supplied with exogenous arginine, this organism retained a large portion as a stable pool of putrescine, even when the enzymes of arginine degradation were fully induced. It is likely that this pool was present in a bound form, since putrescine has been found to bind to DNA, RNA, and ribosomes under in vitro conditions.

Of the three Pseudomonads examined, only P. aeruginosa appeared to have the constitutive ability to completely oxidize arginine, converting ornithine to glutamate and further degrading the latter compound, presumably via conversion to α-ketoglutarate. Cells were unable to constitutively degrade the putrescine formed from arginine, as they exhibited a long lag before synthesis of protein from putrescine commenced in uptake experiments, and a long lag before induction of the ability to oxidize putrescine in manometric experiments.

Growth of P. aeruginosa with arginine as the sole source of carbon and nitrogen resulted in the induction of a greatly increased rate of arginine oxidation, which primarily represented an increase in the rate of degradation of ornithine via the glutamate pathway. Presumably,higher levels of the enzymes of the arginine dihydrolase system were also induced. The results of the succinic semialdehyde dehydrogenase assays showed that growth in arginine resulted in a partial induction of the enzymes of γ-aminobutyrate degradation, to levels higher than those induced by growth in glutamate. These results indicated that some putrescine was being degraded during growth in arginine. However arginine grown cells demonstrated a short lag before oxidizing putrescine under Warburg conditions, and, in uptake experiments, a short lag before putrescine carbon was incorporated into protein. These lag periods were much shorter

than those exhibited by glucose grown cells, and the rate of putrescine oxidation was much higher. These results are similar to those obtained with cells which are induced for the oxidation of a compound but not for its uptake; however, arginine grown cells of P. aeruginosa transported putrescine very rapidly. Thus, arginine grown cells had a greater potential ability to oxidize putrescine than glucose grown cells, but appeared to be blocked in one step. It is possible that a key enzyme of putrescine degradation was not induced during growth on arginine, or that it was synthesized but its activity was inhibited. Such a control mechanism would assist in the maintenance of the large pool of putrescine found to be formed from arginine by P. aeruginosa.

The rate of constitutive arginine oxidation by P. aeruginosa was relatively high. The degradation of endogenously synthesized arginine by the constitutive enzymes of P. aeruginosa would be a disadvantage to the cell, and it is therefore likely that the activity of these enzymes is, in some way, controlled. It is possible that the arginyl-tRNA synthetase has a much higher affinity for arginine than do the degradative enzymes, so that, at low endogenous concentrations, arginine would be preferentially incorporated into protein. On the other hand, the enzymes of arginine biosynthesis and those responsible for its incorporation into protein may be, in some way, separated from the enzymes responsible for arginine degradation, preventing endogenous arginine

from being oxidized. Sercarz and Gorini (1964) obtained evidence for such a compartmentalization in E. coli, where endogenously synthesized arginine was used preferentially for protein synthesis, and exogenous arginine for repressor formation. Tabor and Tabor (1969b) showed that E. coli synthesized putrescine preferentially from exogenous rather than endogenous arginine, whereas protein appeared to be synthesized equally well from both sources. N. crassa has been shown to utilize exogenous arginine and ornithine mainly for catabolism, and endogenous arginine and ornithine for protein synthesis (Castaneda, Martuscelli, and Mora, 1967; Davis, 1968).

Ramos et al. (1967) and Jacoby (1964) found that arginine degradation was subject to catabolite repression in several strains of P. fluorescens. However, the latter worker found that the oxidation of arginine was repressed to a lesser extent than was that of other amino acids. Arginine degradation was not subject to catabolite repression in P. aeruginosa; both glucose and arginine were oxidized concurrently when present as a mixture in the presence of growing cells or resting cell suspensions. This lack of repression was probably due to the high constitutive level of the arginine degrading enzymes. Chemical fractionation results did indicate that, in the presence of a mixture of arginine and glucose, cells utilized the two substrates to serve slightly different biosynthetic needs.

P. aeruginosa possessed at least two systems for the transport of arginine. Kinetic studies demonstrated the presence of a low affinity system and a high affinity system in glucose grown cells. The low affinity system could not be measured in arginine grown cells; however, these cells possessed increased levels of the high affinity system. Inhibition studies also demonstrated the presence of two basic amino acid transport systems; one which was specific for arginine and, with a lower affinity, for ornithine, and a second general system which transported all the basic amino acids. The general uptake system was induced to a greater extent than was the specific system by growth of the organism in arginine as the sole source of carbon and nitrogen. It is important to note that inhibition studies were carried out in the concentration range in which the low affinity permease was operative in glucose grown cells. However, the degree to which this permease contributed to total uptake could not be ascertained, and therefore no definitive correlation could be made between the two permeases identified by kinetic studies and those identified by inhibition studies.

Putrescine appeared to be transported by a general polyamine permease in *P. aeruginosa*. It was interesting to note that growth of *P. aeruginosa* in arginine resulted in almost complete induction of putrescine transport. This may have been a result of induction of the polyamine transport system by the large intracellular pool of putrescine which was formed during growth in arginine.

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