OXIDATIVE ASSIMILATION OF GLUCOSE BY AEROBIC BACTERIA

by

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Department of Agricultural Microbiology

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ABSTRACT

Oxidative assimilation of glucose-U-C^{14} by several aerobic bacteria was found to involve the assimilation of radioactivity into nitrogenous cell components, principally proteinaceous, in conjunction with the re-incorporation of endogenously produced ammonia. In one of these bacteria, Pseudomonas aeruginosa, if the cells were starved or treated with chloramphenicol prior to glucose-C^{14} the amount of assimilation, especially into protein, was decreased. The incorporation into nucleic acids and lipids was increased by the antibiotic, but was only slightly affected by starvation. A determination of the cytological sites of the assimilated material showed that, in control cell extracts, the soluble proteins of the cytoplasm contained most of the C^{14}. Starved or antibiotic treated cell fractions had substantially less of the label in these proteins, whereas the radioactivity incorporated into the ribosomal ribonucleic acid and the "membrane" lipids was greater.

A study of the aminoacyl-soluble ribonucleic acid synthetases in P. aeruginosa revealed that these enzymes were present only in the cytoplasm. Starving the cells resulted in decreased activity of the synthetases, but they were rapidly reactivated during oxidative assimilation. The large amount of heterologous reactions between bacterial soluble ribonucleic acids and synthetases indicated that little species specificity existed. However, cross reactions between the systems in bakers' yeast and the bacteria were poor, showing that some degree of species specificity was present in these instances.

Preliminary experiments on the route of assimilation of ammonia in P. aeruginosa and in P. fluorescens gave no evidence for the direct amination of pyruvate by alanine dehydrogenase, but did demonstrate a requirement for concurrent substrate oxidation while ammonia was being incorporated. In contrast, several lines of evidence indicated that ammonia was assimilated via α-ketoglutarate in P. aeruginosa.

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PUBLICATIONS

Rothstein, M., and G.A. Tomlinson. 1961. Biosynthesis of amino acids by the nematode Caenorhabditis briggsae. Biochim. Biophys. Acta 49: 625-627.

Tomlinson, G.A., and M. Rothstein. 1962. Nematode Biochemistry. I. Culture methods. Biochim. Biophys. Acta 63: 465-470.

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Tomlinson, G.A., and J.J.R. Campbell. 1963. Patterns of oxidative assimilation in strains of Acetobacter and Azotobacter. J. Bacteriol. 86: 1165-1172.

Abstract

Oxidative assimilation of glucose-U-C^{14} by Pseudomonas aeruginosa, P. fluorescens, Achromobacter B81, A. viscosus, Azotobacter agilis, A. vinelandii, and Acetobacter xylinum was found to involve the assimilation of radioactivity into nitrogenous cell components, principally proteinaceous in nature in conjunction with the reincorporation of endogenously produced ammonia. Acetobacter aceti did not exhibit oxidative assimilation under these circumstances.

Further investigation of oxidative assimilation in P. aeruginosa revealed that if the cells were starved or treated with chloramphenicol prior to glucose-C^{14} oxidation, the amount of assimilation was decreased. The incorporation of radioactivity into protein was severely restricted by both treatments. The amount of labelling of both lipids and nucleic acids was increased in the presence of the antibiotic, but was only slightly affected by starvation. A determination of the cytological sites of the assimilated material showed that, in control cell extracts, the soluble proteins of the cytoplasm contained most of the C^{14}. Starved or antibiotic treated cell fractions exhibited a profound decrease in the label of these proteins, whereas the amount of incorporation into the ribosomal ribonucleic acid and the "membrane" lipids was higher.

A study was made of the aminoacyl-s-RNA synthetases in P. aeruginosa, and these enzymes were shown to be present only in the cytoplasm, even when the cell extracts were prepared and fractionated in a medium of high ionic strength. Starving the cells resulted in a decrease in the activity of the aminoacyl-s-RNA synthetases, but they were rapidly reactivated during oxidative assimilation of glucose. There was found to be little

species specificity between the s-RNA's and synthetases of P. aeruginosa, P. fluorescens, Achromobacter B81, and E. coli, since good cross reactions were obtained, but the heterologous reactions between bakers' yeast and the bacteria were poor, except in the case of the yeast s-RNA and the E. coli enzyme.

Preliminary experiments on the route of ammonia assimilation in P. aeruginosa and in P. fluorescens gave no evidence for the direct amination of pyruvate by alanine dehydrogenase, but did demonstrate a requirement for concurrent substrate oxidation while ammonia was being assimilated. In contrast, several lines of evidence indicated that ammonia was assimilated via α-ketoglutarate in P. aeruginosa.

J.J.R. Campbell

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INTRODUCTION

"Oxidative assimilation" is the term used to describe the incorporation of carbon into cellular components during the oxidation of a substrate by washed cells of microorganisms (100). Energy for this process, which takes place in the absence of added nitrogen, is provided by oxidation of part of the substrate, while the remainder is assimilated. Since the assimilation occurs without added nitrogen, it might be expected that the incorporated carbon would be found exclusively in non nitrogenous cell constituents, designated "primary products", and indeed, this has been found to be true for a number of microorganisms. Recently, however, it has become evident that, in some microorganisms, assimilation takes place into a variety of cell components, including nitrogenous ones; the nitrogen for this process being derived from the endogenous respiration of the cells. Studies in this laboratory have shown that this is the situation found in resting cells of Pseudomonas aeruginosa, and moreover, that a primary product is not formed during oxidative assimilation of glucose (55).

This thesis is concerned with several aspects of oxidative assimilation in aerobic bacteria. Firstly, the experimental approach used by Duncan and Campbell (55) with P. aeruginosa was applied to a number of aerobic bacteria, to determine if, under these conditions, they would form a special reserve product such as carbohydrate or lipid, or whether all cell components would be synthesized. Secondly, the effect of starvation of P. aeruginosa cells on the extent and patterns of oxidative assimilation was determined, and an attempt was made to elucidate the mechanism of ammonia assimilation in P. aeruginosa and P. fluorescens. Thirdly, the cytological sites of oxidative assimilation were investigated in P. aeruginosa, and the

influence of starvation or inhibition of protein synthesis on this process was studied. Finally, since protein was one of the main products of oxidative assimilation in P. aeruginosa (as well as in the other aerobes studied), an investigation was made of the formation of aminoacyl soluble ribonucleic acid (aminoacyl-s-RNA) in P. aeruginosa, and the system present in this microorganism was compared to those in other bacteria, and in yeast.

LITERATURE REVIEW

I. Oxidative Assimilation

H. A. Barker (11) first used the term "oxidative assimilation" to describe the conversion, by the colorless alga Prototheca zopfii, of part of an organic molecule into cellular material, while the remainder was oxidized. This process was manifested by an oxygen uptake which was less than expected, although the substrate had completely disappeared. In addition, the dry weights of the microorganisms had risen somewhat. A similar phenomenon had been noted previously in Escherichia coli by Cook and Stephenson (41), but no reason for its occurrence had been postulated. From the molecular quantities of the reactants, and the fact that one of the products was carbon dioxide, Barker was able to write balanced equations for the oxidation of several substrates. Thus, the equation for the oxidation of acetate was written as follows:

$$CH_3COOH + O_2 \longrightarrow CO_2 + H_2O + (CH_2O)$$

The formula for the product of assimilation of several organic compounds could also be written as CH_2O. Since P. zopfii was known to synthesize and store glycogen, Barker (11) concluded that this was the primary product of oxidative assimilation in the alga, and that it might be used as the source of raw material for cellular synthesis during prolonged incubation or growth.

The Warburg manometric technique was used in the subsequent years by a succession of authors to study oxidative assimilation, and their results supported Barker's hypothesis. Thus, Clifton with Pseudomonas calcoaceti and E. coli (32), Giesberger using Spirillum (70), and Doudoroff with Pseudomonas saccharophila (52), all postulated primary products of

assimilation similar to that suggested by Barker. These results were based on the amount of oxygen consumed, and the carbon dioxide liberated, at the time of the first sharp break in the oxygen curve for the substrate in question. That substrate assimilation was not confined to respiring systems was suggested by the experiments of Winzler and Baumberger (174) on heat production during alcoholic fermentation. From the heats of formation, and the heat produced during exogenous respiration, they concluded that 70.5% of the glucose was fermented to ethyl alcohol and carbon dioxide, and 29.5% stored. Corresponding figures for aerobic dissimilation were 24.5% and 75.5%. Fermentative assimilation was also shown in Candida albicans by van Niel and Anderson (155), but could not be detected in Streptococcus faecalis.

Siegel and Clifton (134,135) have studied the relationship between the amount of assimilation of several carbohydrates and organic acids and the available free energies of these compounds. The extent of assimilation of arabinose was investigated in both growing and resting cells of E. coli (134). From manometry with resting cells, the percent of oxidative assimilation with each sugar was determined, and found to be 40% for arabinose, and about 50% for glucose and lactose. In addition, the free energy changes for the oxidative reactions were calculated from these data, based on the actual amount of each sugar oxidized. The value so calculated for arabinose was found to be twice as high as that for glucose and arabinose, yet from carbon balances done on growing cells, arabinose was assimilated to the greatest extent, glucose less, and lactose the least. Previous studies on the extent of assimilation in resting and growing cells of E. coli had indicated that this occurred to a similar measure in both cases (37). In a second paper, Siegel and Clifton (135) reported on a similar

investigation of the energetics involved in the assimilation of succinate, fumarate, lactate, pyruvate and glycerol by short term cultures of E. coli. The same situation was found with these compounds as with the sugars: a similar amount of carbon was assimilated from pyruvate as from lactate, and from fumarate as from succinate, although the free energy of oxidative assimilation was found to be less for the first compound of each pair. In these experiments, both the amount of oxygen taken up and the carbon assimilated were determined with growing cells, and therefore should be comparable, in contrast to the data on sugars, where resting cells were used in assessing one value, and growing cells for the other. When the amounts of assimilation in resting cells of the acids and the sugars were calculated solely on the basis of oxygen uptake, there appeared to be somewhat less assimilation than in growing cells. The efficiency of utilization of the acids was found to be lower than that of the sugars. This was considered to be due in large measure to the circumstance that some of the molecules of the compounds with fewer carbons had to be oxidized completely to provide energy for the assimilation of others, or for the assimilation of the intermediates derived from them. With substrates having more carbons, the energy evolving processes generated the intermediates needed for synthesis. That the same situation existed in Bacillus subtilis was suggested by the studies of Wilner and Clifton (172) also with organic acids and glucose as substrates for assimilation. Again, fumarate and pyruvate were assimilated to a greater degree than would have been predicted by the free energy changes from oxidation of the molecule. Clifton and his associates have concluded from these investigations that the chemical structure of a compound, which determines how it is metabolized, appears to be more important in determining the

extent of its assimilation than the amount of free energy inherent in the molecule.

Photosynthetic assimilation was first studied by Gaffron (65) using resting cells of purple bacteria. As a result of these investigations, he postulated the presence of a primary assimilatory product with the empirical formula $(C_4H_6O_2)_n$. Small quantities of a polymeric substance with this elementary composition were isolated from cultures grown photosynthetically on butyrate. However, further investigations of this unique polymeric product was discouraged by van Niel's report (154) that the gross composition of purple bacteria corresponded closely to the composition of Gaffron's proposed product of assimilation.

The use of manometric techniques introduced several limitations which, despite the considerable volume of work which was undertaken, prevented the identification of the nature of the assimilated material. One difficulty was the uncertainty of the actual oxygen uptake, since it was not known whether endogenous respiration continued unabated, was stimulated, or was inhibited by, the addition of oxidizable substrate. Qualitative tests were usually carried out on supernatant fluids for predetermined typical products or intermediates in the oxidation of the substrate, and if these were negative, then no other tests were done. However, in experiments where carbon balances were performed, discrepancies were sometimes found, which were due to the presence of unidentified products. Still another question was whether the substrates were assimilated intact, or whether they were cleaved to smaller fragments prior to the assimilatory process. Some of these questions were answered by later experiments with radioisotopes, which showed that the amount of oxidative assimilation was often much less than that indicated by manometric measurements. This

technique also made possible the separation and characterization of the products of assimilation.

Since some bacteria appeared to assimilate carbon as efficiently in the absence of nitrogen as in its presence, it seemed evident that assimilation was not necessarily coupled with general cell synthesis. One could, therefore, conclude that the assimilated material would be found in a limited number of cell constituents, or primary products. In many microorganisms this was found to be the case. The assimilation of carbon by P. zopfii into a product having the empirical formula of glycogen has been discussed. This material, or one with the same empirical formula, was also suggested by the findings of other workers with a number of bacteria.

Polysaccaride is the primary product of assimilation by many bacteria, especially the enteric group, which have been found to contain levels of glycogen as high as 48% of the dry weight of the cells (103), present as a reserve product within the cell, or as structural components, especially capsules (170). Holme and Palmstierna (85) have made detailed studies of an alkali stable polyglucose, apparently identical to glycogen in its properties, formed from glucose by growing or resting cells of E. coli during nitrogen starvation. If the polysaccharide containing cells were transferred to a medium containing inorganic nitrogen, but no carbon, the newly formed polyglucose was broken down, and used for protein synthesis.

The oxidative assimilation of glucose, and other substrates into Sarcina lutea was investigated by Binnie, Dawes and Holms (21). When the cells were grown on peptone, carbohydrate made up 10% of their dry weight, and after oxidation of glucose by freshly harvested or lyophilized endogenous diminished cells, this rose to 28%. The assimilated

material was utilized during endogenous respiration, about 50% disappearing in 3.5 hours, the rest more slowly. When cells which had been allowed to assimilate uniformly labelled glucose were fractionated chemically, the bulk of the radioactivity was found in the alcohol soluble material, and yielded glucose on hydrolysis. From the specific activity of the polyglucose, the authors concluded that glucose had been assimilated directly. The chromatographic behaviour of the polymer suggested that it was of relatively low molecular weight. The use of acetate or pyruvate as substrates did not result in an increase in the carbohydrate of the cells, the radioactivity from these compounds being found in the cold trichloroacetic acid soluble fractions, with other metabolic intermediates.

With the aid of C^{14} labelled substrates, it has been shown that bacteria are able to assimilate carbon into both lipid and carbohydrate. Nocardia corallina, when oxidizing propionate-3-C^{14}, incorporated 37% of the assimilated radioactivity into the lipid fraction, which accounted for 30% of the dry weight of the cells, whereas 52% was incorporated into carbohydrate, which made up less than 8% of the cell material (115). The higher specific activity of the carbohydrate suggested that it was the initial site of assimilation. With some bacteria, the substrate determines the product of assimilation. Thus, with E. coli, the presence of acetate increased the formation of lipid, and decreased glycogen formation, whereas glucose had the opposite effect (171).

Yeasts have been the subject of a number of studies on assimilation, and there seems to be some disagreement as to whether the incorporated material takes the form of lipid or carbohydrate. Winzler (173) found an increase in the reducing sugar content of yeast which had oxidized acetate. This increase was enough to account for 80% of the material

theoretically assimilated. On the other hand, McLeod and Smedly-Mclean (111) found that lipid was synthesized from acetate, without the intermediate formation of carbohydrate. These experiments were generally of a longer duration, and were carried out in the presence of higher concentrations of acetate than employed by Winzler. Perhaps an analysis made earlier in the course of the oxidation would have revealed the primary synthesis of carbohydrate. Pickett and Clifton (125) established that carbohydrate was the primary product of assimilation by Saccharomyces cerevisiae during glucose oxidation, since the increase in readily hydrolysable carbohydrate was equal to the increase in dry weight of the cells. When a second yeast, Torulopsis utilis, was allowed to oxidize sucrose plus acetate-C^{14} in a nitrogen free medium, the radioactivity was incorporated into both carbohydrate and lipid (92). If acetate-C^{14} were used alone, however, nearly 50% of the assimilated material was calculated, by difference, to be "protein," although there was no net increase of protein in the cells during the experiment. This finding, by Jackson and Johnson, of assimilation into protein, was one of the first reports of a nitrogenous product of assimilation.

Although the lipid formed by assimilation into yeast cells is thought to consist of conventional triglycerides (171), the importance of the lipid material poly-β-hydroxybutyrate as a product of assimilation by bacteria has recently been recognized. Although it had been known for years that this lipid was found as a major component of Bacillus species (101,102), it remained for McRae and Wilkinson in 1958 (108) to show that it functioned as an intracellular reserve of carbon and energy in B. megaterium and B. cereus. Glucose, pyruvate or β-hydroxybutyrate served as substrates for synthesis of the polymer by washed cell suspensions, but

acetate could not, although it greatly enhanced the formation of poly-β-hydroxybutyrate in the presence of one of the other substrates. The widespread occurrence of this polymer was soon demonstrated by Forsyth, Hayward and Roberts (60), who found it to be present in many Gram negative bacteria, including Azotobacter species and nonpigmented pseudomonads. The presence of poly-β-hydroxybutyrate in cocci was demonstrated by Sierra and Gibbons (136), who studied the biosynthesis and oxidation of the polymer in Micrococcus halodenitrificans. Doudoroff and Stanier (53) found that poly-β-hydroxybutyrate was the product of photosynthetic assimilation in Rhodospirillum rubrum, thus confirming the much earlier conclusion of Gaffron (65) with purple bacteria. The role of the polymer as an endogenous carbon and energy source in R. rubrum was also demonstrated.

Previous work by Wiame and Doudoroff (167) on the oxidative assimilation of C^{14} labelled substrates by P. saccharophila had shown that, in this microorganism, two carbon fragments were the fundamental building blocks of assimilation. When lactate was used as the substrate, the carboxyl carbon was oxidized almost completely, while the remaining two carbons were used for synthetic reactions. A similar situation occurred with succinate, i.e., the carboxyl carbons were oxidized, and the methyl carbons were assimilated, and whereas both carbons of acetate were assimilated, the methyl carbon was favoured. However, the nature of the assimilated material was not established, although it was suggested, without any supporting data being given, that "the carbon flowed into many different materials in the cell, including protein." The finding that poly-β-hydroxybutyric acid occurred in some pseudomonads, and that two carbon fragments were assimilated by P. saccharophila, led Doudoroff and Stanier (53) to reexamine the products of assimilation in this organism. They

found that freshly harvested cells, when incubated with glucose-C^{14}, assimilated only 21% of the added radioactivity, and of this, two-thirds appeared as poly-β-hydroxybutyrate. However, if the cells were starved before the experiment, more than 50% of the glucose was assimilated, again as the polymer. This was also the main product formed during the oxidation of acetate or butyrate. Doudoroff and Stanier reported that poly-β-hydroxybutyrate could serve as the substrate for endogenous respiration for P. saccharophila in the absence of an oxidizable substrate, but net transfer of polymer carbon to other cell constituents could not be demonstrated.

Up to this time, reports on assimilation by bacteria had concerned the formation of a primary carbonaceous reserve. However, indications that this might not be a universal situation were provided by the experiments of Warren, Ells and Campbell (163), who showed that P. aeruginosa consistently produced considerable quantities of ammonia during endogenous respiration, and that it reincorporated this ammonia when glucose was added. Gronlund and Campbell (74) extended this finding to a number of bacteria, as well as to S. cerevisiae. Bacillus cereus was shown by Clifton and Sobek (38) to produce ammonia endogenously, and later experiments with glucose-C^{14} revealed that 50% of the radioactivity was assimilated by resting cell suspensions of this organism (35). Initially, most of the label was found in the cold trichloroacetic acid soluble pool components, but soon passed into the compounds soluble and insoluble in hot trichloroacetic acid, which would be primarily nucleic acids and proteins. Formation of a polymer such as poly-β-hydroxybutyrate did not appear to occur. Clifton (36) has since reported similar results for a number of bacteria, including Bacillus megaterium, Azotobacter agilis,

B. subtilis, and Hydrogenomonas facilis (45).

Almost simultaneously with Clifton's report on B. cereus, Duncan and Campbell (55), using P. aeruginosa, demonstrated that this microorganism also formed no primary product during oxidative assimilation of glucose, but followed the same route of incorporation of carbon as did B. cereus, i.e., the label appeared first in the cold trichloroacetic acid soluble pool, then passed into compounds soluble and insoluble in hot trichloroacetic acid and alcohol. However, in contrast to the bacillus, and to the conclusions suggested by oxygen uptake data, the pseudomonad assimilated only 16% of the substrate. Duncan and Campbell were able to relate assimilation in P. aeruginosa to the formation of α-ketoglutarate, which was excreted into the medium during the early stages of glucose oxidation, and reincorporated concurrently with ammonia provided by endogenous respiration. Since this microorganism has an active glutamic acid dehydrogenase, Duncan and Campbell believe that "the partial block of glucose oxidation at α-ketoglutarate represents a control mechanism, ensuring that the method of entry for nitrogen will be present as soon as any becomes available by diffusion, leaching, or from endogenous storage products." Assimilation by P. aeruginosa appeared to be limited by the amount of nitrogen available, since the addition of ammonia greatly increased the amount of C^{14} incorporated, and prevented the accumulation of α-ketoglutarate in the suspending fluid. When chloramphenicol was added to resting cell suspensions oxidizing glucose, the amount of incorporated radioactivity was decreased, and assimilation into the lipid, cold trichloroacetic acid soluble pool, and hot trichloroacetic acid soluble fractions was increased, at the expense of the residual fraction, which is mainly protein. The reincorporation of endogenously produced ammonia during

oxidative assimilation has led to the hypothesis, advanced by Duncan and Campbell (55), and by Clifton (36), that oxidative assimilation may serve, at least in part, to replenish the nitrogenous endogenous reserves of some microorganisms.

II. Inorganic Nitrogen Assimilation

In the previous section, the literature on oxidative assimilation was reviewed, and it was shown that this process, although by definition occurring in the absence of added nitrogen, often involves protein and nucleic acid biosynthesis. For this reason, the assimilation of inorganic nitrogen becomes important in the overall phenomenon of oxidative assimilation.

The major pathway by which organisms incorporate inorganic nitrogen into organic compounds involves the ammonium ion. As early as 1926, Quastel and Woolf (127) reported the formation of aspartate from fumarate and ammonia by cells of *E. coli*, and later this was extended to several anaerobes (42). The equilibrium of this reaction was found to favour aspartate formation, with a K_{eq} of approximately 20. Virtanen and Tarnenen (157) extracted aspartase from cells of *Pseudomonas fluorescens*, and demonstrated that the reaction occurred in cell free extracts. The enzyme proved to be fumarate specific, and later work by Ichihara *et al.* (91) indicated that folic acid, reduced glutathione, and cobalt ions were required by the purified enzyme. This report, however, remains to be confirmed or extended, and the mechanism of the action of aspartase is still unknown.

The enzyme alanine dehydrogenase was first described by Wiame and Pierard (168) in a mutant of *B. subtilis* which lacked glutamic acid

dehydrogenase, but oxidized glutamate. It was assumed that transamination of glutamic acid with another keto acid was occurring, and that the amino acid so formed was then oxidized by an enzyme as yet unknown. Examination of cell extracts revealed an alanine dehydrogenase specific for L-alanine and nicotinamide adenine dinucleotide (NAD). Pyruvate was the oxidation product, and alanine was synthesized in the presence of reduced nicotinamide adenine dinucleotide (NADH), pyruvate and ammonium ions. This enzyme has been reported in other bacilli, many of which lack glutamic dehydrogenase, but some of which have both enzymes (58,86,87). Although the enzyme was formerly thought to be specific for L-alanine, it has recently been reported that several other aliphatic, monocarboxylic amino acids are also oxidized (123). The equilibrium constant of the deamination reaction at pH 10 has been reported to be 1.4×10^{-15}, and the free energy change to be +18.6 kcal. Thus, the synthesis of alanine is favoured, unless pyruvate and NADH are removed by oxidation, as they are in bacterial spores, in which the deamination has been shown to occur (122). The reaction mechanism has not yet been elucidated, but an enzyme-bound intermediate has been postulated (71).

Glutamic acid dehydrogenase, which has been exhaustively studied in yeast, bacterial, and animal systems, has always been thought to be the primary route for the incorporation of ammonia into amino acids. In yeast (3), and in *E. coli* (2) cell free preparations, the reaction has been shown to be nicotinamide adenine dinucleotide phosphate (NADP) specific, and to occur in two stages.

$$\text{L-glutamic acid} + \text{NADP} \rightleftharpoons \text{iminoglutaric acid} + \text{NADPH}$$

$$\text{iminoglutaric acid} + H_2O \rightleftharpoons \alpha\text{-ketoglutaric acid} + NH_3$$

At pH 6.5, the free energy change is +17.6 kcal in the direction of α-keto-

glutaric acid formation, and therefore, like alanine dehydrogenase, favours the synthesis of the amino acid (124). NADP specific glutamate dehydrogenases have also been found in Neurospora crassa (59), and in many bacteria (2). The animal enzymes can use either NADP or NAD as hydrogen acceptors. Like alanine dehydrogenase, glutamic acid dehydrogenase was thought to be specific for one substrate only. However, Struck and Sizer (145) found that, under appropriate conditions, certain other aliphatic, monocarboxylic acids were also oxidized by the chicken liver enzyme. These other amino acids were oxidized at a much higher pH than is optimal for glutamate, similar to the conditions found to give the maximum rate for alanine dehydrogenase and alanine. Leucine was the best substrate among these compounds, and alanine was also oxidized slowly. Aspartic acid was not oxidized.

Both glutamic acid (124) and alanine dehydrogenases (71,122,168) are inactivated by iodoacetate and p-chloromercuribenzoate, but not by arsenite, suggesting that thiol, but not dithiol groups are involved in the enzymatic reaction. The inhibition of alanine dehydrogenase activity by the sulfhydral binding reagents can be reversed by L-cysteine (122). Possibly related to this finding are the reports of the inhibition, by shaking, of alanine biosynthesis, from pyruvate and ammonia, by resting cells of B. subtilis (58) and Brucella abortus (7). However, a recent report by Freeze and Oosterwyck (63) indicated that alanine dehydrogenase in B. subtilis is an inducible enzyme, and that induction is repressed by aeration, probably because of the oxidation of NADH to NAD. Thus, no alanine would be available to act as an inducer. Since the levels of the enzyme were low in aerated B. subtilis cells grown in the presence of pyruvate, there must be some mechanism for assimilation of ammonia in these

cells other than the amination of this keto acid to give alanine; however, no glutamic acid dehydrogenase was found in the presence or absence of aeration, nor did L-glutamate act as an inducer for alanine dehydrogenase.

There are several other less important pathways of ammonia incorporation in microorganisms. A single report exists on the formation of glycine from glyoxylate, ammonia, and NADH by an enzyme from Mycobacterium tuberculosis var. hominis (39). The standard free energy of glycine formation was calculated to be -11 kcal per mole. Amides are known to be products of nitrogen fixation (6), and the synthesis of amides from ammonia and either aspartate or glutamate is a major ammonia incorporation reaction of plant tissue (143). Cell free preparations of Staphylococcus aureus (57) and Proteus vulgaris (77), have been shown to synthesize glutamine from glutamic acid, ammonia, adenosine triphosphate (ATP), and magnesium. Nothing is known of the mechanism of asparagine synthesis in bacteria; however, in plants, the cofactor and metal requirements appear to be the same as for glutamine synthesis (165). Mortenson suggests that this is because there is a transamidation reaction from glutamine to aspartic acid, yielding asparagine and glutamic acid (116).

In the opinion of O'Connor and Halvorson (123), alanine and glutamic acid dehydrogenases are the primary routes for assimilation of ammonia in microorganisms. Support for the major role of glutamic acid dehydrogenase is found in the work on nitrogen fixation by Azotobacter vinelandii (27), Clostridium pasteurianum (180), photosynthetic bacteria (161), algae (169), and soybean nodules (181). Exposures of growing cultures of these agents to N_2^{15}, or to $N^{15}H_4^+$, for short periods of time resulted in the highest specific activity of the isotope being found in glutamate. The concentration of N^{15} in the glutamic acid was usually two or more times

that of the nearest compound. C. pasteurianum cell free extracts were found to follow the same route of nitrogen fixation, and the addition of α-ketoglutarate to the reaction mixture reduced the amount of isotope in the ammonia, probably by formation of glutamic acid via glutamic acid dehydrogenase (116). Virtanen et al. (156) also found that yeast cells, exposed to ammonium or nitrate salts after a period of nitrogen starvation, synthesized glutamic acid the earliest, and the most actively of all the amino acids.

The synthesis of alanine from pyruvate and ammonia has been demonstrated to occur in several species of bacteria and actinomycetes (7,20,58,71), in yeast (95), in plants (96), and in animals (15,126), in situations where transamination reactions between pyruvate and glutamate have been eliminated. Altenbern and Housewright (7) demonstrated that the transamination of pyruvate with glutamate played the largest role in the formation of alanine by resting cells of B. abortus, but that reductive amination of pyruvate occurred to about one-third the amount of transamination. Washed cell suspensions of B. subtilis were able to synthesize alanine from pyruvate and ammonia much more rapidly than glutamate from α-ketoglutarate and ammonia, according to Fairhurst et al. (58). In Streptomyces species, the primary reaction for assimilation of ammonia was found to be the amination of pyruvate by ammonia, with that of fumarate being of secondary importance (20). No amination of α-ketoglutarate or oxalacetate could be demonstrated. Burk and Pateman (26) showed that mutants of N. crassa lacked glutamic acid dehydrogenase, but possessed an alanine dehydrogenase specific for NADPH, and were able to synthesize alanine when incubated with pyruvate, ammonia, and NADPH. There have been several reports by Halvorson and his associates on the presence and func-

tion of alanine dehydrogenase in B. cereus spores (109,122,123). The enzyme was found to be able to act in the direction of synthesis in vegetative cells, but it is probable that the function of the enzyme in spores is to deaminate alanine, to yield the pyruvate which has been reported to be necessary for spore germination. Although the enzyme has been found in Rhizobium species, a recent report by Brouvers (25) indicates that alanine dehydrogenase levels in these microorganisms are not related to their nitrogen fixing efficiency. To date, however, despite the proliferation of reports on the presence of alanine dehydrogenase in a number of bacteria, yeasts, and in actinomycetes, there does not appear to have been a case in which the enzyme has been found in a Pseudomonas species.

The necessity for energy utilization by microbial cells during the assimilation of nitrogenous compounds has been suggested by a number of studies. For instance, Winzler et al. (175) reported that the assimilation of ammonia by yeast was dependent both on the biotin content of the cells, and on glycolysis; requiring the presence of glucose in the environment, and being inhibited by azide. Possibly an expenditure of energy is required for active transport across cell barriers, or for endergonic utilization of the compound, or both. McLean and Fisher (110) calculated that, with Serratia marcescens, approximately 2.2 moles of oxygen were consumed per mole of ammonia assimilated. S. faecalis was found to be able to utilize glutamate only in the presence of glucose, or a similar energy source (66,67). Endogenous uptake of added ammonia occurred in M. tuberculosis, the oxygen consumption in the presence of added ammonia being greater than in its absence (19). Heating prevented the assimilation of the ammonia, although oxygen uptake at the level characteristic of the absence of ammonia continued. Following a recovery period after heating,

assimilation of ammonia started once more, and was accompanied by an increase in oxygen uptake. Hence, an oxidative process appeared to be coupled to the endogenous assimilation of ammonia in M. tuberculosis. Bernheim (18) has reported that both an oxidizable substrate and potassium were necessary for the utilization of ammonia by P. aeruginosa under normal conditions, but in the absence of the metal, assimilation could be restored to a significant degree by surface active agents, such as polymyxin B, or benzalkonium chloride.

There has so far been no resolution of the path of ammonia uptake in microorganisms possessing both dehydrogenases. However, in those lacking glutamic acid dehydrogenase, for example, some bacilli (168), it would seem that alanine dehydrogenase must play the major role. The primary function of this enzyme in spores of Bacillus species has been found to be that of providing a supply of pyruvate, which is a requirement for germination. McCormick and Halvorson (109) have recently reported that the alanine dehydrogenase of mature spores of B. cereus was heat resistant. The preferential and very active participation of glutamate in transamination reactions led Braunstein, in 1957 (23), to attribute to glutamic acid dehydrogenase and α-ketoglutarate, the key positions in ammonia assimilation. All indications still are that he was probably correct, with pyruvate amination by alanine dehydrogenase, and the formation of amides, playing supporting roles in some situations.

III. Species Specificity of s-RNA's and Aminoacyl-s-RNA Synthetases

Recently, it has become evident that the process of oxidative assimilation, rather than yielding only a carbonaceous product within the cell, often results in the synthesis of a number of cell constituents, in-

cluding proteins. The problem of how a protein is biosynthesized is one of the most exciting in biochemistry, and one in which rapid advances have been made in the last five years. Within this field, the elucidation of the role played by s-RNA, and how it is played, is receiving much attention. The participation of s-RNA in protein biosynthesis was first indicated, in 1958, by its amino acid accepting activity, and its ability to transfer the attached amino acid to a ribosomal fraction (83). This amino acid acceptor RNA seemed to fulfill the requirements for an "adaptor" between template RNA and protein postulated by Crick (44). At the present time, it is generally believed that the biosynthesis of protein proceeds through the following steps:

$$AA_i + Enz_i + ATP \rightleftharpoons Enz_i(AA_i \sim AMP) + PP \qquad (1)$$

$$Enz_i(AMP \sim AA) + sRNA_i \rightleftharpoons AMP + Enz_i + AA_i \sim sRNA \qquad (2)$$

$$AA_i \sim sRNA_i + AA_j \sim sRNA_j \longrightarrow AA_i\text{-}AA_j + sRNA_i + sRNA_j \qquad (3)$$

where AA_i is a particular natural amino acid, Enz_i is an enzyme specific for the activation of AA_i, s-RNA is a molecule of soluble RNA specific for AA_i, and AA_i-AA_j represents the growing peptide chain. Reaction (3) is catalysed by guanosine triphosphate and ribosomes. It is now agreed that Reactions (1) and (2) are catalysed by the same enzyme, and specific enzymes have been found which activate particular amino acids to form aminoacyl-s-RNA's (16). Data have also accumulated which indicate that there are different s-RNA's for different amino acids (132).

In the last three years, interest has been aroused in the interspecific reactions between s-RNA's and transfer enzymes. Most of the reports concern comparisons of the heterologous reactions between yeast, mammalian and E. coli systems. It appears that there is often a cross

reaction between mammalian enzymes, and yeast s-RNA's, or vice versa, but that yeast and E. coli systems generally react poorly, if at all. The reaction between the bacterial and mammalian systems is variable. Thus, yeast and hog enzymes yielded either yeast or hog tyrosyl-s-RNA, whereas the E. coli enzymes reacted only in homologous systems (31). Mammalian enzymes catalysed the synthesis of yeast valyl-s-RNA, but not E. coli leucyl-s-RNA, according to Zillig et al. (182), and this was confirmed by Doctor and Mudd (51). Benzer and Weisblum (14) reported that there was little or no cross reaction between yeast and E. coli in the formation of tyrosyl- or argininyl-s-RNA, but that there was a cross reaction in the formation of lysyl-s-RNA.

Interbacterial crosses for the leucine system, as well as cross reactions between yeast, rat liver, and E. coli, were studied by Rendi and Ochoa (129), who found that enzymes from Lactobacillus arabinosus, Propionibacterium shermanii, S. faecalis, and A. vinelandii reacted with the s-RNA of E. coli with efficiencies ranging from 10 to 50% of the homologous system, but did not incorporate the amino acid into the s-RNA's of yeast or rat liver. There was no cross reaction between the E. coli enzyme system and either the yeast or rat liver s-RNA's or vice versa. According to Doctor and Mudd (51), however, yeast enzyme reacts as well with E. coli and rat liver leucine s-RNA as it does with its own, suggesting to these authors that the E. coli s-RNA used by Rendi and Ochoa might have lost some of its activity during isolation. This is supported by the findings of Keller and Anthony (94), who demonstrated that rat liver enzyme incorporated leucine into E. coli s-RNA quite readily. One must, therefore, interpret results, especially those obtained with one amino acid only, with caution, since either the enzyme or the s-RNA may have

been damaged during isolation.

Doctor and Mudd (51) have attempted to overcome this problem by a comprehensive study on the interspecific reactions between the s-RNA's and enzymes from yeast, E. coli and rat liver, for 14 amino acids. In most of the cases studied, there was some reaction, indicating that there seems to be no absolute specificity between the enzymes and s-RNA's of a system. In addition, in some of the cross reactions, the heterologous system was more active than the homologous one, especially with the rat liver enzymes. The authors have several suggestions to explain this anomalous finding: damage to s-RNA's or enzymes of the homologous system during isolation; incorporation of an amino acid into a different acceptor RNA (i.e., the specificity between amino acid and s-RNA is not complete); different amounts of s-RNA's in different species; or, finally, recognition of more than one component of a particular amino acid specific s-RNA by the heterologous system, whereas only one is recognized by the homologous system.

That there is more than one component in several s-RNA's corresponding to a single amino acid is well established. Apgar, Holley and Merrill (8) have used counter current distribution to achieve some separation of some acceptor s-RNA's, while Sueoka and Yamane (147) fractionated the aminoacyl-s-RNA's on methylated albumin columns. By these methods, leucine, isoleucine, serine, threonine, glutamic acid and valine s-RNA's from E. coli have been shown to consist of more than one component. In addition, Berg et al. (17) have demonstrated by enzymatic means that E. coli methionine s-RNA has at least two components, since yeast enzyme reacted to only one-third the extent with E. coli s-RNA as did the homologous enzymes. Sueoka and Yamane (148) further reported that, again in E.

coli, this heterogeneity of s-RNA's for certain amino acids was reproducible, and that the relative amount of each component was rather constant under different growth conditions. Moreover, in different microorganisms (E. coli, Micrococcus lysodeikticus, B. subtilis, and yeast), the elution profiles of aminoacyl-s-RNA's were different, although the elution profile of each aminoacyl-s-RNA in an organism tended to resemble that of the same aminoacyl-s-RNA in the others.

An interesting experiment also reported by Sueoka (148) has added a complication to our knowledge of the heterologous reaction. The leucine s-RNA's resulting from the reaction of the yeast homologous system, and from that of the yeast s-RNA and the E. coli enzyme, were subjected to column chromatography. The elution profiles of the two leucyl-s-RNA's proved to be entirely different, although there was a small amount of overlap. However, the significance of this anomalous reaction is not clear, since, in a later paper, Yamane and Sueoka (176) investigated heterologous systems for other amino acids, and found that leucine was the only compound to react in this way. Moreover, the leucyl-s-RNA obtained constituted only one percent of the normal yeast leucyl-s-RNA.

It is not yet known whether there is a corresponding enzyme for each component s-RNA, but this is undoubtedly being investigated. The experiments of Berg et al. (17) reported above, would tend to support multiple enzyme systems, since one would consider that E. coli contains two enzymes, one for each methionine specific s-RNA, while the yeast contains only one enzyme, and therefore can react with only one of the component s-RNA's. Further supporting evidence is found in the experiments of Doctor and Mudd (51), and of Benzer and Weisblum (14), where, although a cross reaction may work one way, the reverse reaction is often inactive.

The existence of several s-RNA's for one amino acid becomes more interesting when this is considered together with the coding problem. Speyer et al. (141), and Nirenberg and coworkers (114,117), have independent evidence for degeneracy in the amino acid code for asparagine, leucine, threonine, and serine. Sueoka and Yamane (148) have reported multiple components for all these amino acids except asparagine, which has not yet been investigated by their methods. Using the counter current distribution technique, Weisblum, Benzer and Holley (166) have found that of two leucine acceptor components in E. coli, one responds to poly UC, and the other to poly UG, in the incorporation of leucine into acid insoluble polypeptides. It may be that there are alternate codes for amino acids, and the one used depends on the activating enzyme which is favoured in a given situation.

Loftfield and Eigner (105), in a recently published paper, take the opposite view of the specificity problem. Their idea of the situation is not a multicomponent system with species differences governing the components present, but a system depending on kinetics for specificity. In kinetic experiments with s-RNA's and enzymes from yeast and E. coli, using valine incorporation as the test, Loftfield and Eigner reported that, although the rate of the heterologous reaction between the E. coli enzyme, and the yeast s-RNA was much slower than that of the homologous system, the Michaelis constants were very similar. The lower incorporation at a given time, then, was due to the slower rate of reaction of the heterologous enzyme-substrate complex to yield valyl-s-RNA. These authors admit that this is a consequence of structural differences, but take the stand that these are not necessarily, or even likely to be in the enzyme recognition area.

Another approach to studies of species differences in various s-RNA's is through hybrid formation with heterologous, heat denatured deoxyribonucleic acid (DNA). Giacomoni and Spiegelman (68) and Goodman and Rich (72) have reported that there was a small, but reproducible amount of hybrid formation when E. coli s-RNA and DNA were combined and heated. The resultant hybrid was ribonuclease (RNase) resistant, whereas the free s-RNA was sensitive to this enzyme. A similar result was obtained with the s-RNA and DNA of Bacillus megaterium (68). With the E. coli system, 0.024% of the DNA was found to consist of sequences complementary to the s-RNA. However, the E. coli s-RNA hybridized to different, lesser extents with the DNA's of other microorganisms. Thus, Goodman and Rich (72) found that there was over 50% as much reaction with the DNA's of the other Enterobacteriaceae as with E. coli, but less than 15% as much with the DNA's of unrelated organisms such as B. megaterium, B. cereus, B. abortus, P. fluorescens, and M. lysodeikticus. McCarthy and Bolton (107) have reported an analogous situation for the hybridization of heterologous DNA and messenger RNA with the DNA of E. coli. The s-RNA-DNA hybridization experiments were extended to other microorganisms by Giacomoni and Spiegelman (68), who found that when the DNA's of P. aeruginosa and B. megaterium were heated with B. megaterium s-RNA, hybridization occurred only in the homologous B. megaterium system. Therefore, despite the findings that the E. coli s-RNA can transmit the genetic message of a rabbit into hemoglobin (159), and react with the enzyme systems of a number of microorganisms, it can still be identified with the genome of its origin.

MATERIALS AND METHODS

I. Oxidative Assimilation into Whole Cells of Aerobic Bacteria

A. Bacteriological methods

P. aeruginosa 120 Na and *P. fluorescens* A 3.12 were grown in a glucose-ammonium phosphate-salts medium as described by Warren *et al.* (163). The cells were harvested by centrifugation in the cold after growth for 20 hr at 30 C, washed twice in cold 0.05 M tris (hydroxymethyl) aminomethane (tris) buffer (pH 7.2), and resuspended to ten times the growth concentration. Unless otherwise noted, this buffer was used throughout the thesis. This procedure was found to yield a cell suspension containing approximately 5 mg (dry weight) of cells per ml. When diluted with 19 volumes of buffer, the suspension gave a reading of 20% transmission at 660 mμ in a Beckman model B spectrophotometer. Accordingly, the other microorganisms used were resuspended so that a 1:20 dilution gave the same reading. This was generally found to correspond to 5 mg (dry weight) per ml; 1 ml of the suspension of the pseudomonads was used per Warburg vessel. Dry weights were established by drying 5 ml of the cell suspensions to constant weight at 100 C.

Achromobacter B81 was grown and harvested as above. The cells were resuspended to 40 times the growth concentration, and 1 ml was used per vessel.

Achromobacter viscosus ATCC 12448 failed to grow in the glucose-mineral salts medium used for the other species, but this situation was remedied by the addition of 0.2% yeast extract (Difco). The cells were harvested after 17 hr of growth, washed as previously described, and resuspended to 25 times the growth concentration; 2 ml of this suspension

were used per vessel.

Azotobacter agilis and Azotobacter vinelandii were obtained through the courtesy of Dr. J. Basaraba, Dept. of Soil Science, The University of British Columbia, and originated from the University of Wisconsin stock culture collection. They were grown in the medium of Warren et al. (163), except that 0.5% glucose was used as a carbon source. The cells were harvested and washed in the same manner as has already been described. Incubation time for A. agilis was 20 hr at 30 C, and for A. vinelandii 40 hr. A. agilis cells were resuspended at 67 times growth concentration, whereas A. vinelandii required resuspension at 100 times growth.

Acetobacter aceti ATCC 8303 was cultured in a medium consisting of 2% glucose, 0.2% yeast extract (Difco), with salts added as above, final pH 6.0. The medium was dispensed in 100 ml portions in 500 ml Florence flasks, and the cultures were incubated for 17 hr on a rotary shaker. The cells were harvested and washed as before, except that the buffer used was 0.05 M tris, pH 6.5. The cells were resuspended to 60 times their growth concentration, and 2 ml were used per Warburg vessel.

Acetobacter xylinum ATCC 10245 was grown in the same manner as A. aceti, but the harvesting procedure required modification because of the formation of cellulose. The culture was freed of cellulose by filtration through several layers of cheesecloth, followed by washing with pH 6.5 tris. The cells were then harvested and washed as for A. aceti, and resuspended to 100 times growth concentration in fresh buffer. One ml of the suspension was used per Warburg vessel.

B. Assimilation studies

Manometric experiments were carried out in a Warburg apparatus at 30 C, using conventional techniques for measuring oxygen consumption.

Each vessel contained 5 µmoles of substrate (approximately 3 to 3.5 µc of glucose-U-C^{14} in radioactive experiments), the appropriate volume of cell suspension, and 0.05 M tris buffer (pH 7.2), to a final volume of 3 ml. In the case of the two Acetobacter species, 0.05 M tris, pH 6.5 was used in place of the pH 7.2 buffer. The volume of cell suspension was chosen so that oxygen uptake was complete in 120 min. At appropriate intervals, to obtain samples for chemical analysis, 2 ml of the cup contents were pipetted into 1 ml of tris buffer in cold centrifuge tubes and centrifuged immediately. From here on, the cells were fractionated according to Duncan and Campbell (55), except that the extraction with hot trichloroacetic acid was carried out at 90 C for 10 min. This procedure yielded five fractions: cold and hot 5% trichloroacetic acid soluble, lipid, alcohol soluble, and residual fractions.

C. Starved cell experiments

1. Starvation procedure

Twenty hour old cells were harvested under sterile conditions, washed twice with sterile tris, and resuspended to ten times their growth concentration in fresh buffer. Twenty-five ml of this suspension were placed in a sterile 250 ml Erlenmeyer flask containing 2 ml of 20% KOH in the centre well, and shaken for 3 hr at 30 C in a Labline shaker water bath having a speed of 110-120 strokes per min. At the end of this period, the cell suspension was removed by pipette, centrifuged at 6000xg for 15 min in the cold, washed once with tris buffer, and resuspended to 25 ml. This cell suspension was then used for assimilation studies. Cells were also starved by incubation without shaking, being harvested, washed, and resuspended as described at the end of the 3 hr period.

2. Assimilation experiments

These were performed in the same type of flask and incubated in the waterbath as outlined above. A total of 25 ml were placed in each of two flasks, consisting of 8.3 ml of cell suspension, 0.83 ml of glucose-U-C^{14} (50 µmoles per ml), approximately 29 µcuries, and tris buffer to volume. Two ml of 20% KOH were added to the centre well. The substrate was added by pipette after 10 min equilibration. Parallel experiments, with nonradioactive glucose or other substrates, were run by the usual Warburg technique, using 1 ml of cell suspension, and 5 µmoles of substrate in a total of 3 ml. When cofactors were used, preincubation time was 20 min. At intervals during the experiments with radioactive glucose, 2 ml of the flask contents were removed, added to 1 ml of cold tris in a chilled centrifuge tube, and centrifuged immediately for 15 min at 6000xg at 4 C. From here on, the procedure was that described in Section I B.

D. Analytical methods

1. Analysis of residual fractions

A portion (2 ml) of each of the 120-min residual fractions was hydrolysed with 1 N HCl for 4 hr at 108 C in a sealed ampoule. The hydrolysate was taken to dryness in a vacuum desiccator over NaOH and $CaSO_4$; then, 1 ml of water was added, an aliquot taken for the determination of radioactivity, and the remainder applied to a Dowex-50 (H) column (50 to 100 mesh; 0.8x10cm). The column was washed with 50 ml of water, followed by 75 ml of 1 N NH_4OH. Both eluates were concentrated to dryness with a flash evaporator; 1 ml of water was added to each, and a sample was taken for determination of radioactivity. Close to 100% of the applied counts were recovered in the two fractions. Both fractions of each residue were

subjected to analysis by paper chromatography and electrophoresis, and radioactive areas on the paper were determined as described in the section on paper chromatography. Control experiments carried out with glucose and β-hydroxybutyric acid showed that these substances were not destroyed by the hydrolysis, and that both compounds appeared in the water eluate from the Dowex-50 column.

2. Analysis of cold trichloroacetic acid fractions

Three ml portions of the cold trichloroacetic acid fractions of cells which had metabolized glucose for 15 min were extracted with four successive 3 ml portions of ether to remove the trichloroacetic acid. The extracts, which were still acidic, were subjected to paper chromatography and electrophoresis. Radioactive areas on the paper were located by scanning.

In an effort to isolate the polymer from the cold trichloroacetic acid soluble fraction of Achromobacter B81, the contents from nine large Warburg vessels, containing a total of 162 ml, were removed after 15 min on the Warburg apparatus at 30 C, pipetted into cold buffer as for the assimilation experiments, and the procedure carried through the point of the trichloroacetic acid extraction. The supernatant fraction (330 ml) from this extraction was extracted with ether in a liquid-liquid extractor overnight, concentrated tenfold by evaporation at 40 C, and the ether extraction repeated. The residual solution (30 to 35 ml) which was still acidic (pH 5.4), was concentrated to 8 ml, and a sample tested for the presence of carbohydrate by the anthrone method (153). Ethyl alcohol was added to the remainder of the solution, to a concentration of 70% (v/v). The flocculent precipitate which formed on standing was collected by centrifugation, washed, and redissolved in water. This opalescent solu-

tion was used for the following tests: reaction with iodine, periodate (by paper chromatography) stability to alkali after hydrolysis at 100 C, composition as revealed by acid hydrolysis, and suitability as a substrate for phosphorylase.

3. Chemical methods

Glucose in the supernatant fluid was determined by the "glucostat" method of Worthington Biochemical Corp., Freehold, N.J.; gluconic acid by the method of Hestrin (82); and keto acids by the technique of Friedemann (64) as modified by Duncan and Campbell (55). Derivatives were prepared from keto acids by use of 2,4-dinitrophenylhydrazine (56). For α-ketoglutarate, pyruvate, oxalacetate and glyoxylate, the reaction mixtures were allowed to stand for 4 hr at room temperature; for 2-ketogluconate, the incubation time was 16 hr. Ammonia was determined by the Conway microdiffusion technique (40).

4. Paper chromatography and electrophoresis

Paper chromatography of the fractions obtained from the supernatant fluids was carried out on Whatman no. 1 paper, by use of secondary butanol-formic acid-water (BFW; 70:10:20, v/v; 130) or ethanol-methanol-water (EMW; 45:45:10, v/v; 120). EMW separates glucose and gluconic acid, whereas BFW distinguishes between Krebs cycle acids. Gluconic and 2-ketogluconic acids were differentiated by chromatography in ethyl acetate-pyridine-sat. aq. boric acid (EPB; 60:25:20, v/v; 73). For chromatography of the 2,4-dinitrophenylhydrazone derivatives of keto acids, the solvent system used was n-butanol-ethanol-ammonia (70:10:20, v/v; 56). Paper electrophoretograms were run for 2 hr in 0.05 M NH_4HCO_3 (pH 7.7) at 13 v per cm. For location of compounds, the following reagents were used (138): amino acids: 0.2% ninhydrin in acetone; sugars and sugar acids: silver

nitrate-sodium hydroxide-thiosulfate dip; organic acids: aniline xylose dip; reducing substances: aniline phosphoric acid dip; and carbohydrates: periodate-benzidine spray.

Radioactive areas on paper chromatograms or electrophoretograms were determined by running strips through a Nuclear-Chicago Model C 100 B Actigraph II, with a gas-flow counter, a Model 1620 B Analytical Count Ratemeter, and a Chart Recorder. In later experiments, the apparatus was modified by replacing the gas-flow counter with a Nuclear-Chicago Model 1032 B 4-pi Counter assembly.

E. Isotopic methods

These were as described by Duncan and Campbell (55). Uniformly labelled glucose-C^{14} was diluted so that it had a specific activity of 0.6 to 0.7 µc per µmole, and 5 µmoles were added per reaction vessel.

II. Inorganic Nitrogen Assimilation by *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*

Nitrogen assimilation studies were performed in the conventional Warburg apparatus, using 1 ml of cell suspension (prepared as described in Section I A from 20 hr cells) per Warburg flask. Ammonium sulphate was used as the source of ammonia. The keto acids, inhibitors, and ammonium sulphate solutions were neutralized to pH 7.2 before use. Ammonia was determined by the Conway microdiffusion technique (40), on supernatant fluids from the Warburg flasks.

III. Oxidative Assimilation into the Cytological Fractions of Normal, Starved, or Chloramphenicol Treated Cells of *P. aeruginosa* ATCC 9027

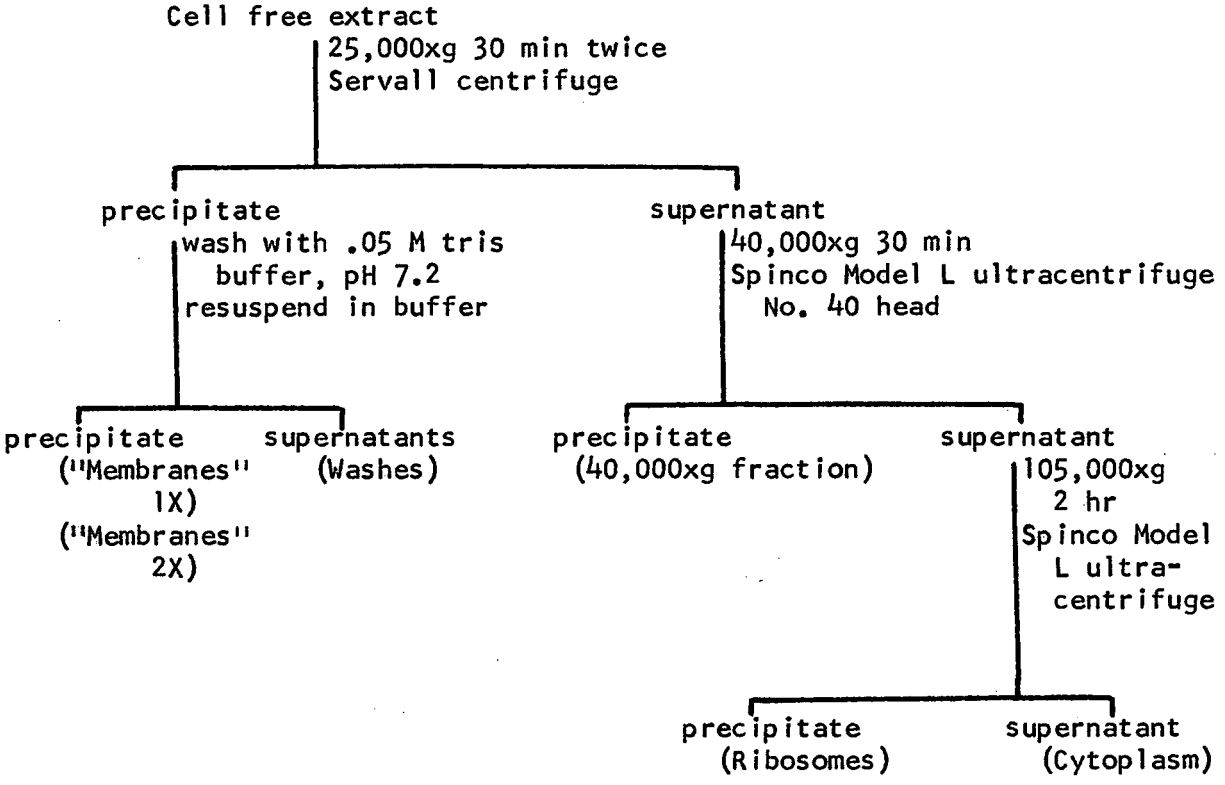
A. Assimilation studies

Cells were grown for 20 hr at 30 C, harvested, and resuspended as before (Section I A). The starved cells were prepared as previously described (Section I C 1), by shaking a 10 times growth suspension under sterile conditions for 3 hr at 30 C, washing, and resuspending the cells to the same volume in fresh buffer. For the assimilation studies, the procedure outlined in Section I C 2 was followed. Chloramphenicol (200 µg per ml) was in contact with the cells for 30 min prior to the addition of the substrate. Oxygen uptake was followed with a Warburg respirometer, using an appropriate aliquot of cells.

At predetermined intervals throughout the experiment (5, 15, 30 and 120 min), 5 ml were removed from each Erlenmeyer flask, added to 2.5 ml of cold tris buffer in a chilled centrifuge tube, and then centrifuged immediately at 6000xg in the cold. The cells from each pair of centrifuge tubes were pooled, resuspended to 6 ml in tris containing 3.7×10^{-2}M Mg^{++}, an aliquot removed for counting, and the remainder held in an ice bath until the end of the experimental period.

B. Preparation of cell fractions

The cells removed at each time interval were broken separately in a chilled French pressure cell at 15,000 to 17,000 lbs pressure, the extracts collected in cold centrifuge tubes, and 0.1 ml of deoxyribonuclease (DNase) (1 mg per ml) added to reduce their viscosity. The extracts were stirred intermittently for five min at room temperature, and then centrifuged twice at 6000xg for 15 min to remove whole cells. An aliquot of each extract was removed for counting, and the remainder fractionated by the following scheme:



C. Analysis of cytological fractions

Large scale preliminary experiments were done, in which the fractions were analysed for RNA (orcinol method) (131), DNA (diphenylamine method) (131), glucose oxidizing activity (manometrically), and for protein (Lowry method) (106), as a means of establishing the purity of various fractions (30).

D. Chemical fractionation of the cytological fractions

The "membranes," ribosomes, and cytoplasm were chemically fractionated as outlined in Section I B.

E. Analysis of chemical fractions

1. "Membrane" residual fraction

The 30 and 120 min residual fractions from the "membranes" were hydrolysed and subjected to column chromatography as previously described (Section I D 1), except that the dimensions of the Dowex-50 columns were 1.2 x 30 cm. However, on paper chromatography of the water eluates of these columns from the 120 min fractions of control and chloramphenicol treated cells, it was found that there was so little radioactivity present that it was impossible to determine whether these materials were acidic or neutral. The water eluates from the 30 min fractions of control and antibiotic treated cells, and those from both the 30 min and 120 min starved cells, were therefore rechromatographed on Dowex-1 (acetate) columns (1.2 x 30 cm), which were washed with 80 ml of water (eluted neutral compounds), followed by 80 ml of 1N HCl (eluted acidic compounds). Each of these eluates was taken to dryness in a flash evaporator, then 1 ml of water was added, and an aliquot counted for its C^{14} content. Paper chromatography and electrophoresis were carried out as previously described (Section I D 4).

2. Cytoplasmic cold trichloroacetic acid soluble fractions

The cold trichloroacetic acid soluble extracts from the cytoplasmic fractions were ether extracted, and subjected to paper chromatography and electrophoresis as given in Section I D 2.

F. Preparation of *P. aeruginosa* s-RNA

P. aeruginosa was cultured in a 20 l bottle containing 17 l of medium, which was aerated by a stream of air from a pump, the air being passed through a sterile cotton filter. In addition, the culture was agitated by means of an overhead stirrer. The medium used consisted of: enzymatic casein hydrolysate (Difco) 3%, glucose 0.3%, KH_2PO_4 0.2%,

$K_2HPO_4.3H_2O$ 0.3%, $FeSO_4$ 0.0005%, pH 7.2. After autoclaving, 10 ml of sterile $MgSO_4.7H_2O$ per 1 of medium was added aseptically. Sterile "G.E. Antifoam" was added at intervals as needed. The medium was inoculated with 500 ml of 24 hr cells, and the culture was incubated for 20 hr at approximately 30 C. The purity of both the inoculum and the bulk culture was checked by Gram staining and plating. Harvesting was done with a Sharples centrifuge at top speed at room temperature. The cells were collected from the cylinder, washed twice with cold tris buffer in a refrigerated Servall centrifuge, the wet paste weighed, and stored frozen.

After being thawed, the bacterial cells (about 150 g) were mixed with enough alumina to give a doughy consistency, and were then broken by grinding in a chilled mortar until liquefaction occurred. Generally, this process required about 10 to 15 min. The mixture was extracted with sufficient tris buffer, containing 10^{-2} M Mg^{++} to give 400 ml of suspension. The viscous suspension was poured into a beaker, 3 mg DNase added, and the mixture was stirred at room temperature for 10 min. By this time the viscosity was greatly reduced. Alumina and unbroken cells were removed by centrifugation twice at 3000xg for 15 min in a refrigerated Servall centrifuge. The supernatant from this centrifugation, amounting to between 170 and 200 ml, was then centrifuged for 2 hr. batchwise, at 105,000xg in a Spinco Model L preparative ultracentrifuge. The upper two-thirds of each tube was removed, and stored frozen overnight. After thawing, this fraction was used for the preparation of s-RNA by the phenol method of Tissieres (151). The yield was approximately 1 mg of s-RNA (dry weight) per 1 g (wet weight) of cells.

G. Assay procedure for incorporation of C^{14} amino acids into s-RNA

A reaction mixture which contained the following components was prepared: tris acetate buffer 0.5M pH 7.4, 1.5 ml; ATP 0.1M, 0.3 ml; $MgCl_2$ 1M, 0.075 ml; KCl 2M, 0.075 ml; ethyl mercaptan 5%, 0.15 ml; Chlorella C^{14} amino acid hydrolysate (Merck, Sharpe and Dohme Co. Ltd., Montreal), 0.03 ml (3 μc). This mixture was stored frozen. The incubation mix consisted of - reaction mixture, 0.15 ml; cytosine triphosphate 0.3M, 0.02 ml; s-RNA 10 mg per ml, 0.05 ml; enzyme, as required. Incubation was for 30 min at 35 C. The reaction was stopped by the addition of 0.03 ml of cold 95% ethanol, followed by 1 ml of alcohol-salt solution (2 parts 95% ethanol to 1 part 1.5M NaCl) with vigorous stirring after each addition. After 10 min in an ice bath, the samples were centrifuged, the supernatants decanted, 1 ml of alcohol-salt solution added, the mixture stirred vigorously, allowed to stand 10 min in an ice bath, and then centrifuged. This washing procedure was repeated twice, the third wash consisting of cold, 95% ethanol. The final precipitate was dissolved in 0.2 ml of 1N NH_4OH, and two 0.02 ml portions were plated at infinite thinness for determination of the C^{14} incorporation into the s-RNA.

H. Isotopic methods

These were as previously described (Section I E).

IV. Species Specificity of s-RNA's and Amino Acyl-s-RNA Synthetases

The microorganisms used in this study were P. aeruginosa ATCC 9027, P. aeruginosa 120 Na, P. fluorescens A 3.12, E. coli B, Achromobacter B81, and Saccharomyces cerevisiae (bakers' yeast).

A. Preparation of s-RNA's

The bacteria were grown, harvested, and cell extracts prepared

as outlined in Section IV A. For E. coli and Achromobacter B81, the concentration of glucose in the medium was raised to 1%. Soluble RNA's were prepared from the 105,000xg supernatant fractions of the cell extracts by the phenol extraction method of Tissieres (151). Yeast s-RNA was a gift of Drs. G.M. Tener and R.V. Tomlinson (13).

B. Preparation of enzymes

The bacteria were grown for 20 hr at 30 C in a glucose-ammonium salts medium (163). For E. coli, 1% glucose was used, instead of the 0.2% normally employed. The cells were harvested, washed, resuspended in buffer, and disrupted by passage through a French pressure cell, and the cell extracts fractionated as before (Section III B). The 105,000xg supernatant fractions, which were stored at 0 C, were used as the synthetase systems.

The yeast enzyme was obtained from Dr. R.V. Tomlinson, and was prepared in essentially the same way as described for the bacterial enzymes, the starting material being Fleischmann yeast cakes.

All the enzymes were tested for activity on the same day that they were prepared. As long as they were not frozen, the enzymes retained their activity for several days in the cold.

C. Preparation of aminoacyl-s-RNA's

C^{14} aminoacyl-s-RNA's were prepared using the assay procedure in Section III G. For the preliminary experiments, it was found that 0.5-0.075 mg of S-RNA gave enough incorporation of C^{14} to determine the reactivity of the systems being tested, but in order to obtain sufficient incorporation so that individual amino acids could be detected, the amount of C^{14} added was increased to 12 μc per sample, and an excess of both enzyme

(0.05 ml), and s-RNA (1.5 mg) were used.

D. Paper chromatography

The aminoacyl-s-RNA's were hydrolysed by allowing the NH_4OH solutions from incorporation experiments to stand at room temperature for 1 hr. Two volumes of cold ethanol were then added to each sample, and after 10 min in an ice bath, the tubes were centrifuged, decanted, stored at -15 C, and later used for paper chromatography. A determination was made of the radioactivity in each of these alcoholic solutions, and it was found that there was complete recovery of the C^{14} present prior to the reprecipitation. Chromatograms were run, in the descending manner, on Whatman no. 1 filter paper, for 30 hr, with n-butanol: glacial acetic acid: water (120:20:50, v/v) as the solvent. About one-third of each sample of C^{14} amino acid was applied to the paper, dried, and then co-chromatogrammed with a set of amino acid standards. After development of the chromatograms, they were cut into strips, and analysed by an Actigraph Model 1032B 4-pi scanner. The strips were dipped into ninhydrin (0.2% in acetone), heated at 60 C for 2 min, and the radioactive peaks were identified by comparison with the standards. The percentage incorporation of each amino acid, or group of amino acids, into the s-RNA was determined by the weight of each peak in the chart, compared to the total weight of all the amino acid peaks.

RESULTS AND DISCUSSION

I. Oxidative Assimilation into Whole Cells of Aerobic Bacteria

A. Patterns of oxidative assimilation into strains of Pseudomonas and Achromobacter

1. Manometric observations, ammonia production and uptake, and excretion of radioactive products into the supernatant fluids during glucose-U-C^{14} oxidation

P. aeruginosa 120 Na oxidized glucose rapidly for about 45 min, at which time 58% of the amount of oxygen required for complete oxidation had been consumed (Figure 1A). Beyond this point, a slow secondary rate obtained until 67% of the theoretical value was reached. Gluconate was oxidized in similar fashion, whereas α-ketoglutarate was oxidized slowly and after a period of induction. The relative Q_{O_2} values for the oxidation of glucose, gluconate, and α-ketoglutarate were 131, 110, and 7.2, respectively. Glucose had disappeared from the supernatant fluid by 15 min, and it would appear that oxygen uptake from this point on was due to oxidation either of intermediates which had been secreted into the supernatant fluid, or of assimilated material. The former explanation is at least partly correct, since gluconate, pyruvate, and α-ketoglutarate were present in the supernatant fluid at 15 min and gradually decreased in concentration after this time, until all had almost completely disappeared by 45 min (Table 1). The break in the oxygen uptake curve at 45 min came, therefore, at a point where exogenous substrate and intermediates were virtually exhausted. No 2-ketogluconate was detected by treatment of the supernatant fluid with 2,4-dinitrophenylhydrazine,

Table 1.

Radioactive compounds in the supernatant fluid during glucose oxidation

Microorganism	Compounds present at		
	15-45 min	60 min*	120 min*
Pseudomonas aeruginosa 120 Na	Gluconate +++ Pyruvate ++ α-Ketoglutarate+		
P. fluorescens A 3.12	Glucose++ Gluconate+++ Neutral compound A+++		
Achromobacter B81	Glucose++ α-Ketoglutarate++ Succinate+ Fumarate+	Succinate++ Fumarate++	Succinate++ Fumarate++
A. viscosus	Glucose+++ Neutral compounds B++	Glucose+ Dicarboxylic acids (trace) Neutral compound B+++	Fumarate (trace) Neutral compound B+

* Radioactive UV-absorbing material was present in all cases at 60 and 120 min.

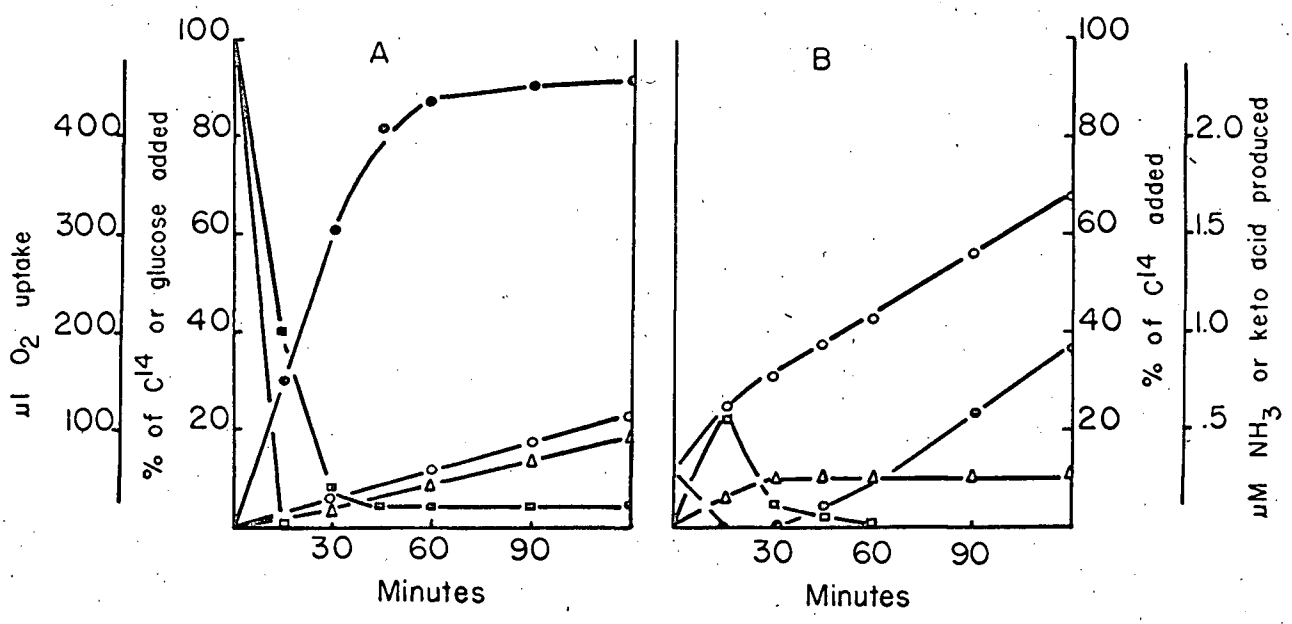


FIG. 1 A. Oxygen uptake with 5 μmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during manometric experiments with washed-cell suspensions of Pseudomonas aeruginosa. Oxygen uptake with glucose, ● ; with α-ketoglutarate, △ ; endogenously, ○ . Disappearance from supernatant fluids of glucose, □ , and C^{14}, ■ . Endogenous oxygen uptake values have been subtracted from the values reported for substrate oxidation.

FIG. 1 B. Time course of NH_3 production, keto acid formation, and C^{14} incorporation into cells of Pseudomonas aeruginosa during oxidation of 5 μmoles of glucose-U-C^{14} by washed-cell suspensions. Production of NH_3 endogenously, ○ ; and in presence of glucose, ● . Keto acid production, □ , and C^{14} incorporation into cells, △ .

followed by extraction, chromatography, and scanning. Endogenously produced ammonia was incorporated into the cells on the addition of glucose, and none could be detected in the supernatant fluid until glucose and ∝-ketoglutarate had disappeared (Figure 1B).

P. fluorescens oxidized glucose in a different manner, there being no sharp break in the curve (Figure 2A). The initial rate was slower than that of P. aeruginosa, and decreased at 25 min, at which time 28% of the amount of oxygen required for complete oxidation had been consumed. Oxygen uptake ceased at 68% of the theoretical total. ∝-Ketoglutarate was oxidized at an initial rate identical to that of glucose, whereas gluconate was attacked more slowly. The Q_{O_2} values for glucose, gluconate, and ∝-ketoglutarate were 124, 65, and 124, respectively. As one might conclude from the manometric data, analyses of the supernatant fluids showed that gluconate was present and keto acids were absent. From radioactive scanning of chromatograms and electrophoretograms, it was found that gluconate concentration was highest at 15 min and then gradually decreased, whereas a second product increased in concentration until 45 min and then disappeared fairly rapidly. This second product, designated as neutral compound A in Table 1, had an R_f of 0.14-0.16 in BFW (R_f of glucose = 0.22), gave a positive silver nitrate reaction, and did not migrate electrophoretically. Similar results were obtained with disaccharides, but attempts to isolate and characterize the unknown compound failed. Oxygen uptake after 30 to 45 min, at which time glucose was exhausted, can be correlated, therefore, with the disappearance of these two products, which apparently are oxidized at a slower rate than is glucose. Endogenously produced ammonia was incorporated into cells on the addition of glucose; however, some remained in

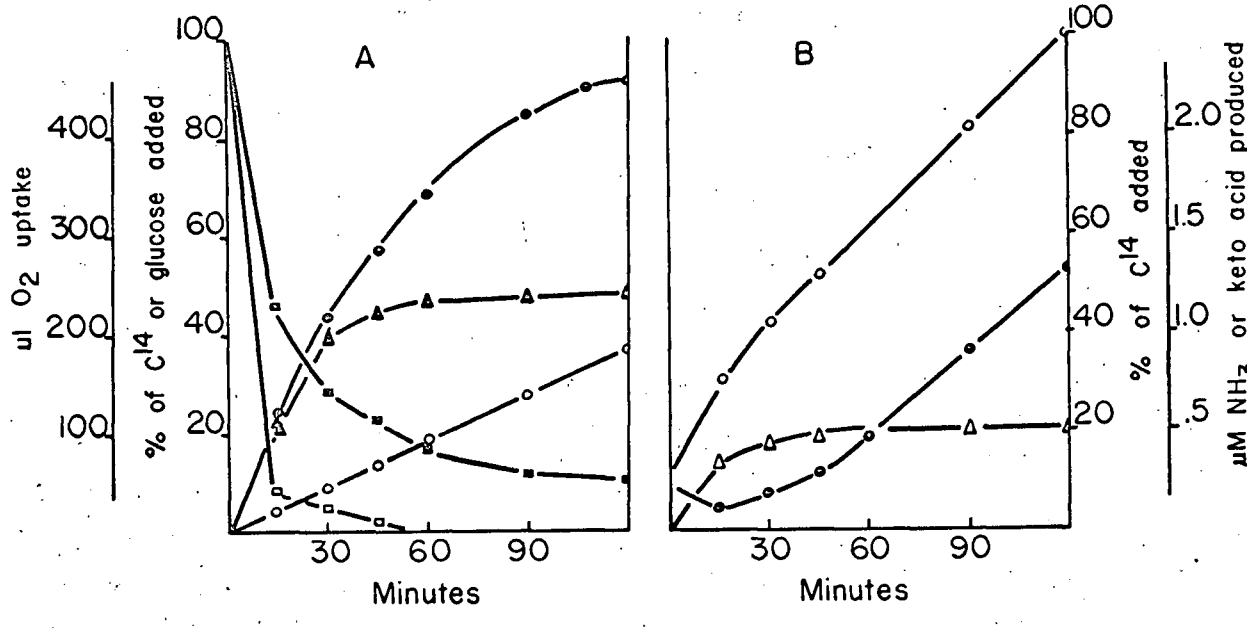


FIG. 2 A. Oxygen uptake with 5 µmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during manometric experiments with washed-cell suspensions of Pseudomonas fluorescens. Oxygen uptake with glucose, ● ; with α-ketoglutarate, ▲ ; endogenously, 0 . Disappearance from supernatant fluids of glucose, □ , and C^{14}, ■ . Endogenous oxygen uptake values have been subtracted from the values reported for substrate oxidation.

FIG. 2 B. Time course of NH_3 production and C^{14} incorporation into cells of Pseudomonas fluorescens during oxidation of 5 µmoles of glucose-U-C^{14} by washed-cell suspensions. Production of NH_3 endogenously, 0 ; and in presence of glucose, ● . C^{14} incorporation into cells, Δ .

the supernatant fluid at all times (Figure 2B).

Achromobacter B81 oxidized glucose at a rapid initial rate with a break in the curve at 30 min, at which time 28% of the amount of oxygen required for complete oxidation had been taken up (Figure 3A). This was followed by a slower secondary oxidation which ceased at 59% of the theoretical total. After a 5 to 7 min period of induction, gluconate was oxidized rapidly, whereas α-ketoglutarate oxidation was characterized by a very slow rate of induction and subsequent oxidation. The Q_{O_2} values for glucose, gluconate, and α-ketoglutarate were 101, 86, and 6.7 respectively.

The main product which accumulated during glucose oxidation was found to be α-ketoglutarate, which disappeared slowly from the supernatant fluid during the interval between 30 and 120 min (Table 1). Pyruvate could not be detected in the supernatant fluids obtained at 15 and 30 min. Succinate, fumarate, and other doubly charged acids identified by cochromatography and scanning, were present but did not disappear from the supernatant fluids. Support for the accumulation of the acids was given by the ultraviolet (UV) spectra, which showed a high end absorption characteristic of dicarboxylic acids. Endogenously produced ammonia was incorporated into the cells on the addition of glucose, and none could be detected in the supernatant fluid until glucose and α-ketoglutarate had disappeared (Figure 3B).

A. viscosus, which required yeast extract for growth, proved to have a delayed oxidation of glucose (Figure 4A). There was no apparent secondary oxidation, and leveling of the curve occurred at 54% of theoretical oxygen uptake. Gluconate and α-ketoglutarate were oxidized after an induction period, the latter being oxidized very slowly. The Q_{O_2}

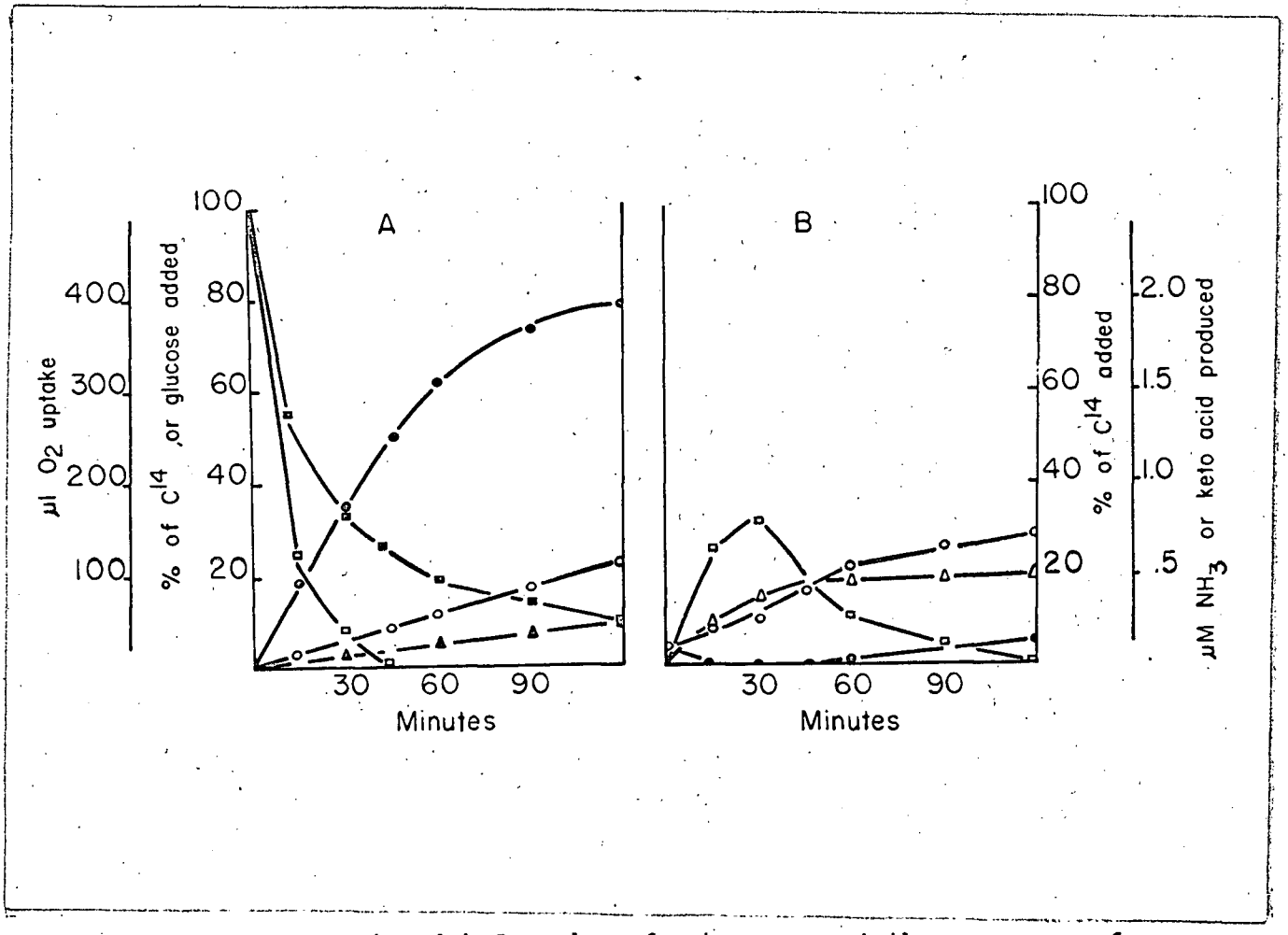


FIG. 3 A. Oxygen uptake with 5 μmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during manometric experiments with washed-cell suspensions of Achromobacter B81. Oxygen uptake with glucose, ● , with α-ketoglutarate, ▲ ; endogenously, O . Disappearance from supernatant fluids of glucose, □ , and C^{14}, ▣ . Endogenous oxygen uptake values have been subtracted from the values reported for substrate oxidation.

FIG. 3 B. Time course of NH_3 production, keto acid formation, and C^{14} incorporation into cells of Achromobacter B81 during oxidation of 5 μmoles of glucose-U-C^{14} by washed-cell suspensions. Production of NH_3 endogenously, O ; and in presence of glucose, ● . Keto acid production, □ ; and C^{14} incorporation into cells, △ .

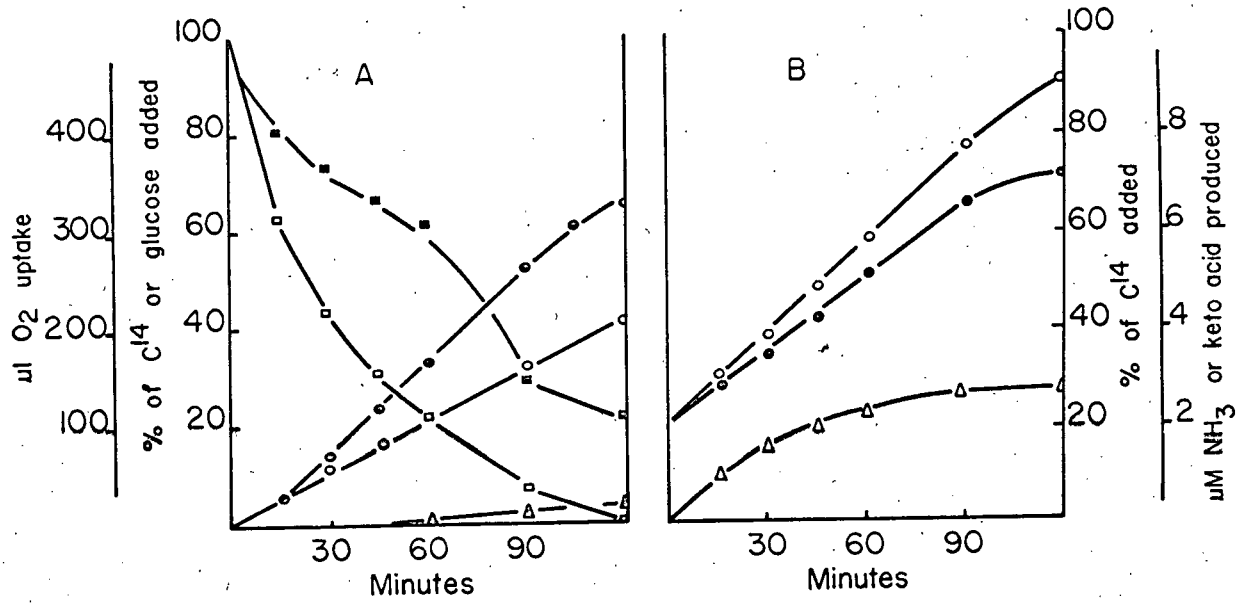


FIG. 4 A. Oxygen uptake with 5 µmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during manometric experiments with washed-cell suspensions of Achromobacter viscosus. Oxygen uptake with glucose, 0 ; with α-ketoglutarate, ▲ ; endogenously, 0 . Disappearance from supernatant fluids of glucose, □ , and C^{14}, ▣ . Endogenous oxygen uptake values have been subtracted from the values reported for substrate oxidation.

FIG. 4 B. Time course of NH_3 production and C^{14} incorporation into cells of Achromobacter viscosus during oxidation of 5 µmoles of glucose-U-C^{14} by washed-cell suspensions. Production of NH_3 endogenously, 0 ; and in the presence of glucose, ● . C^{14} incorporation into cells, Δ .

values for the oxidation of glucose, gluconate, and ∝-ketoglutarate were 17, 13, and 2.1, respectively. Glucose persisted in the supernatant fluid for 60 min, and there was no keto acid or gluconate present at any time. Comparison of the rates of disappearance of radioactivity and glucose from the supernatant fluid revealed that the level of glucose fell more rapidly, indicating that some intermediate compound must be accumulating in the supernatant fluid. Chromatography and electrophoresis of the supernatant fractions revealed the presence of neutral compound B, which increased in amount until 60 min, and then decreased rapidly (Table 1). This compound had properties similar to those of compound A of P. fluorescens, and attempts at its isolation also failed. After 60 min, traces of fumarate and other acids, in addition to compound B, were detected.

The rate of glucose oxidation by this organism was so slow and the endogenous production of ammonia so rapid, that the addition of glucose did not have the pronounced effect on the ammonia content of the supernatant fluid that was evident with the other organisms. However, A. viscosus appeared to take up the largest amount of ammonia of any of the organisms under study (Figure 4B).

In every instance, some radioactivity remained in the supernatant fluid at the termination of the experiment. Most of this could be accounted for by the increase, with time, of UV-absorbing material. Viable-cell counts were not carried out, and so the possibility of some cell lysis cannot be eliminated. In the case of A. viscosus, a comparatively large amount of UV-absorbing material was present at the end of the experiment. Incubation of the supernatant fluid with DNase or RNase, with concurrent determination of changes in OD at 260 mμ, showed that the UV-absorbing

material was a substrate for RNase only. This would suggest that, at least in this instance, the increase in UV-absorbing material was due to the secretion of RNA and not to cell lysis.

A comparison of the results of the oxidation of glucose-U-C^{14} by resting cells of P. aeruginosa, P. fluorescens, Achromobacter B81, and A. viscosus with those obtained by Duncan and Campbell (55), using P. aeruginosa ATCC 9027, shows that there are basic similarities between the bacteria. With all the strains, the two stage glucose oxidation corresponded to the accumulation of intermediate compounds, whose rates of oxidation were limiting for the conversion of glucose to CO_2 and water. With P. aeruginosa these were pyruvate, gluconate, and α-ketoglutarate, with Achromobacter B81 it was α-ketoglutarate, whereas with P. fluorescens it was gluconate. Both P. fluorescens and A. viscosus also accumulated, and later oxidized, unknown neutral compounds. Assuming that all four bacteria studied continued to oxidize their endogenous reserves in the presence of exogenous substrate, (as does P. aeruginosa ATCC 9027 with glucose (121)), with the resultant production of ammonia, then it would appear that each organism reincorporated some ammonia by way of intermediates of glucose oxidation. Since P. aeruginosa and Achromobacter B81 formed α-ketoglutarate, as shown by its excretion into the supernatant fluid, ammonia incorporation could have occurred through the action of glutamic dehydrogenase. P. fluorescens did not accumulate α-ketoglutarate; however, the rate of oxidation of this compound was similar to that of glucose, and one could not, therefore, expect it to be in excess. A. viscosus also failed to accumulate α-ketoglutarate, and because the rate of oxidation of the keto acid was very slow, if it had been produced in excess within the cell, and then excreted, it should have

accumulated in the supernatant fluid during the experiment. However, the high utilization of endogenously produced ammonia indicated that the rate of removal of α-ketoglutarate by amination would be sufficiently rapid to prevent the extracellular accumulation of this keto acid. Despite the finding that keto acids were not excreted into the medium in all cases, each of the bacteria possessed a mechanism for the uptake of ammonia. The excretion of keto acids into the supernatant fluids occurred in the instances where the supply of ammonia from endogenous respiration was low, thus permitting a more efficient utilization of ammonia and glucose for synthetic purposes.

Although there were similarities among the organisms under study, there were also some differences that are perhaps pertinent to this discussion. P. fluorescens and A. viscosus synthesized neutral, low molecular weight polymeric compounds which were excreted into the supernatant fluids, and, in each case, the compounds were utilized when the parent substrate had disappeared, but unlike P. aeruginosa and Achromobacter B81 they did not excrete keto acids. Both strains of Achromobacter formed dicarboxylic acids from glucose, although these compounds did not disappear with time as did the other products detected in the supernatant fluid. The production of these acids was also found in several Achromobacter species by Sguros and Hartsell (133), when the ratio of carbon to nitrogen was high, as it was in the Warburg cup during glucose oxidation.

2. Incorporation of C^{14} into cells during oxidative assimilation

The amount and patterns of incorporation of radioactivity into cells of the bacteria are shown in Tables 2a, 2b, 3a and 3b. In each case, the amount of assimilated material was much less than would be expected from the oxygen uptake, a result similar to that found in P. aeruginosa

Table 2a.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of *P. aeruginosa*

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	2.0	2.0	0.48	0.78	2.6	7.9	7.2
30	2.8	2.7	0.78	1.40	4.5	12.2	11.4
120	2.7	2.9	0.70	1.70	5.6	13.6	12.6
			Per cent of total C^{14} incorporated into cell fractions				
15	25	25	6.1	10	34	100	
30	23	22	6.4	12	37	100	
120	20	20	5.6	13.4	41	100	

* Cold and hot trichloroacetic acid soluble fractions.

Table 2b.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of P. fluorescens

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	5.4	1.9	1.1	1.2	4.8	14.4	14.4
30	5.9	2.5	1.2	1.5	6.4	17.3	17.7
120	5.3	3.0	0.79	1.7	8.5	19.3	19.2
			Per cent of total C^{14} incorporated into cell fractions				
15	38	13	7.4	8.4	33	100	
30	34	14	6.9	8.5	37	100	
100	27	16	4.2	8.8	44	100	

* Cold and hot trichloroacetic acid soluble fractions.

Table 3a.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of Achromobacter B81

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	4.3	0.51	0.31	2.6	3.6	11.3	11.0
30	5.4	0.55	0.39	3.6	5.9	15.8	15.5
120	5.3	0.65	0.56	3.3	9.6	19.4	19.4
			Per cent of total C^{14} incorporated into cell fractions				
15	38	4.5	2.7	23	32	100	
30	34	3.5	2.5	23	37	100	
120	27	3.4	2.8	17	50	100	

* Cold and hot trichloroacetic acid soluble fractions.

Table 3b.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of A. viscosus

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	6.8	0.29	0.25	0.80	2.0	10.1	9.5
30	9.5	0.49	0.50	2.0	3.5	16	16
120	7.0	1.1	0.60	5.0	12.5	26.2	26
			Per cent of total C^{14} incorporated into cell fractions				
15	67	2.9	2.5	7.9	20	100	
30	59	3.1	3.2	12.5	22	100	
120	27	4.2	2.3	19	48	100	

* Cold and hot trichloroacetic acid soluble fractions.

ATCC 9027 (55). The products of glucose oxidation which were excreted into the supernatant fluids appeared to be important in determining the extent of assimilation, not only α-ketoglutarate for its involvement in ammonia uptake, but also compounds such as pyruvate, gluconate, and the unidentified neutral substances formed by P. fluorescens and A. viscosus. There was a correlation between the time required for the disappearance of these oxidizable substrates from the supernatant fluid, and the amount of material assimilated. In the case of A. viscosus, an unusually high degree of oxidative assimilation was achieved by a slow rate of glucose oxidation, and the presence of large amounts of endogenously produced ammonia. The other three organisms oxidized glucose rapidly, but the accumulation of the "pacemaker compounds" ensured that substrate was available over a prolonged period, thus resulting in the reincorporation of endogenously produced ammonia and a fairly high level of oxidative assimilation.

3. Assimilation of C^{14} into the cell fraction soluble in cold trichloroacetic acid

The compounds removed from the cells by treatment with trichloroacetic acid in the cold, and therefore considered to be "pool" constituents, made up a rather large proportion of the total radioactivity present in the cells. As might be expected, if oxidative assimilation involved protein synthesis, labelled glutamate was a usual pool constituent. However, P. fluorescens did not contain free glutamate, perhaps because it possessed a strong ability to oxidize α-ketoglutarate, and therefore did not accumulate this keto acid during glucose oxidation. P. fluorescens did, however, contain a permease which brought about the passage of a high concentration of glucose into the trichloroacetic acid soluble pool.

Other pool components were neutral amino acids and a polymer in P. aeruginosa and P. fluorescens; a polymer in Achromobacter B81; and glucose, amino acids, a neutral compound, and a polymer in A. viscosus. The identity of the polymeric substance present in the cold trichloroacetic acid soluble fractions of each bacterium could not be established, owing to lack of material. However, from a large-scale, non-radioactive experiment with Achromobacter B81, evidence was found that, in this case, the polymer was carbohydrate in nature, but not glycogen. Treatment of whole cells with the anthrone reagent before and after assimilation of glucose, showed that 5% of the dry weight of the cells was made up of carbohydrate material which did not increase during the experiment. A similar analysis on the cold trichloroacetic acid extract revealed that 20% of the carbohydrate was present in this fraction. The remaining carbohydrate was distributed between the hot trichloroacetic acid extract and residue. The alcohol precipitable material from the cold trichloroacetic acid extract reacted with periodate, did not react with iodine, was stable to alkaline hydrolysis, and was not hydrolysed by phosphorylase. The unit compound of the polymer behaved like glucose when analysed by paper chromatography. However, other hexoses, notably galactose, would react similarly. In addition to the polymeric carbohydrate, there was present, before acid hydrolysis, as well as after, a periodate-oxidizable compound, having an R_f in BFW similar to that of glycerol.

4. Assimilation of C^{14} into the cell fractions insoluble in cold trichloroacetic acid

The assumption that all four organisms reincorporated some ammonia during glucose oxidation would seem to be valid, for analysis of the cells by the techniques of Roberts et al. (130), as modified by

Duncan and Campbell (55), revealed that C^{14} appeared in all fractions containing nitrogenous components, as well as the lipid containing ones. (Tables 2 and 3). Moreover, the residual fraction containing the protein was the one which continued to increase in radioactivity with time, and which, in all cases, accounted for almost half the radioactivity of the cell at 120 min. When these residual protein-containing fractions from cells which had respired for 2 hr were hydrolysed and applied to Dowex-50 (H) columns, some of the radioactivity was removed by elution with water (Table 4). The small amount of radioactive material in the

Table 4.

Acid hydrolysis and separation by ion exchange of residual* fraction

Microorganism	"Glucose" content**	Eluted by water (neutral compounds)	Eluted by NH_4OH (ninhydrin + compounds)
		%	%
Pseudomonas aeruginosa	0.6	10	90
P. fluorescens	0.3	7	93
Achromobacter B81	1.0	30	70
A. viscosus	0.5	7.5	92.5

* Insoluble in hot 5% trichloroacetic acid.

** Glucose content was measured by the anthrone test. Results are expressed as per cent of dry weight of cells.

water eluates of three of the organisms could have been due to cell wall constituents; however, in the case of Achromobacter B81, 30% of the residual material was eluted. This high value agrees with the fact that this organism synthesized a carbohydrate polymer which was soluble in cold trichloroacetic acid, as is free glycogen. The compounds in the water eluates should be either neutral or acidic in nature; however, paper chromatography and electrophoresis of these eluates never revealed any acidic components. The sole radioactive peak in each of the water eluates reacted similarly to glucose upon chromatography or paper electrophoresis of a mixture of the eluate with standard glucose. However, the systems used would not distinguish between glucose and galactose. β-Hydroxybutyrate, which, if present, should be in this eluate, was never detected.

Elution of the columns with ammonia removed 70-93% of the total C^{14} of the fractions. Paper chromatography and scanning of the ammonia eluates showed that all radioactive areas were ninhydrin positive. The individual amino acids were not identified.

To a lesser extent than the protein fraction, the fraction soluble in hot trichloroacetic acid, which contained primarily nucleic acids, also increased with time. A. viscosus assimilated an unusually high percentage of the C^{14} of glucose, a considerable amount of which was due to its containing a surprisingly large amount of radioactive material soluble in cold or hot trichloroacetic acid. The relative amount of radioactivity in the hot trichloroacetic acid soluble fractions doubled between 15 and 120 min, and accounted for 19% of the total C^{14} of the cells at the completion of the experiment. Achromobacter B81 also assimilated a large percentage of the added radioactivity into the nucleic

acid fraction, but in this instance the fraction appeared to be most important in the early stages of oxidative assimilation.

In confirmation of the observations of Duncan and Campbell (55), the lipid of P. aeruginosa appeared to be of significance in the early stages of oxidative assimilation. This suggestion is also true for Achromobacter B81, but not for the other two organisms.

The pattern of assimilation of glucose found in these experiments was similar to that reported recently by Duncan and Campbell (55) and Clifton (36), i.e., the radioactivity appeared first in the cold trichloroacetic acid soluble components, from where it was distributed into the other fractions. All four bacteria incorporated a large proportion of the assimilated carbon into nitrogenous cell components, of which protein contained most of the C^{14} in each case. The concept (55) that oxidative assimilation occurred by way of reincorporation of endogenously produced ammonia was found to be tenable. The two Achromobacter species assimilated a high proportion of the radioactivity into the nucleic acid fractions, at the expense of the lipid. This is particularly interesting in the case of A. viscosus, since this organism will not grow in an inorganic salts medium with glucose as the sole source of carbon and without organic nitrogen. Growth experiments with A. viscosus showed that both amino acids and vitamins were required to replace the yeast extract used in the growth medium for assimilation experiments. Despite the fact that this microorganism cannot grow in the inorganic medium, resting cells with no added vitamins or amino acids exhibited the highest amount of oxidative assimilation of any organism studied.

The only bacterium investigated which appeared to have a primary storage product was Achromobacter B81. This conclusion is supported by

the ratio (10.6) of endogenous oxygen uptake to ammonia production, which is a figure twice that normally obtained with obligate aerobes, and similar to that found by Gronlund and Campbell (75) with E. coli. In addition, the apparent incorporation of ammonia by Achromobacter B81 was very low in relation to the amount of material assimilated. Analysis of the cold trichloroacetic acid-soluble pool in the early stages of glucose oxidation, and the fraction insoluble in hot trichloroacetic acid at the end of the experimental period, revealed that the storage product was a polymeric carbohydrate that was not identical to glycogen.

B. Patterns of oxidative assimilation into strains of Acetobacter and Azotobacter

1. Manometric observations, ammonia production, and excretion of radioactive products into the supernatant fluids during glucose-C^{14} oxidation

Acetobacter aceti oxidized glucose slowly, and for the most part only to the gluconic acid stage. A total of 68 µl of oxygen were taken up, while a value of 56 µl would indicate complete conversion of gluconate (Figure 5A). Gluconate was not oxidized by whole cells of this organism, and 91% of the added C^{14} remained in the supernatant fluid at the end of the experiment. Moreover, more than 4 µmoles of gluconic acid were present in the supernatant fluid at 120 min (Table 5). The production of almost stoichiometric amounts of gluconate from glucose by A. aceti was also found by De Ley and Schell (48). The oxygen uptake value, the amount of radioactive material which disappeared from the supernatant fluid, and the specific quantitative analysis for gluconate, indicated that approximately 10% of the glucose was oxidized beyond the gluconic acid stage. This

Table 5.

Radioactive compounds in the supernatant fluid during glucose oxidation

Microorganism	Compounds present at		
	15-45 min	60 min*	120 min*
Acetobacter aceti	Glucose+++ Gluconate+++	Glucose+ Gluconate++++	Gluconate++++
Acetobacter xylinum	Glucose++ Gluconate+++	Glucose+ Gluconate+ Cellulose+	Gluconate+ Cellulose++
Azotobacter agilis	Glucose+		
Azotobacter vinelandii	Glucose+		

* Radioactive UV-absorbing material was present in all cases at 60 and 120 min.

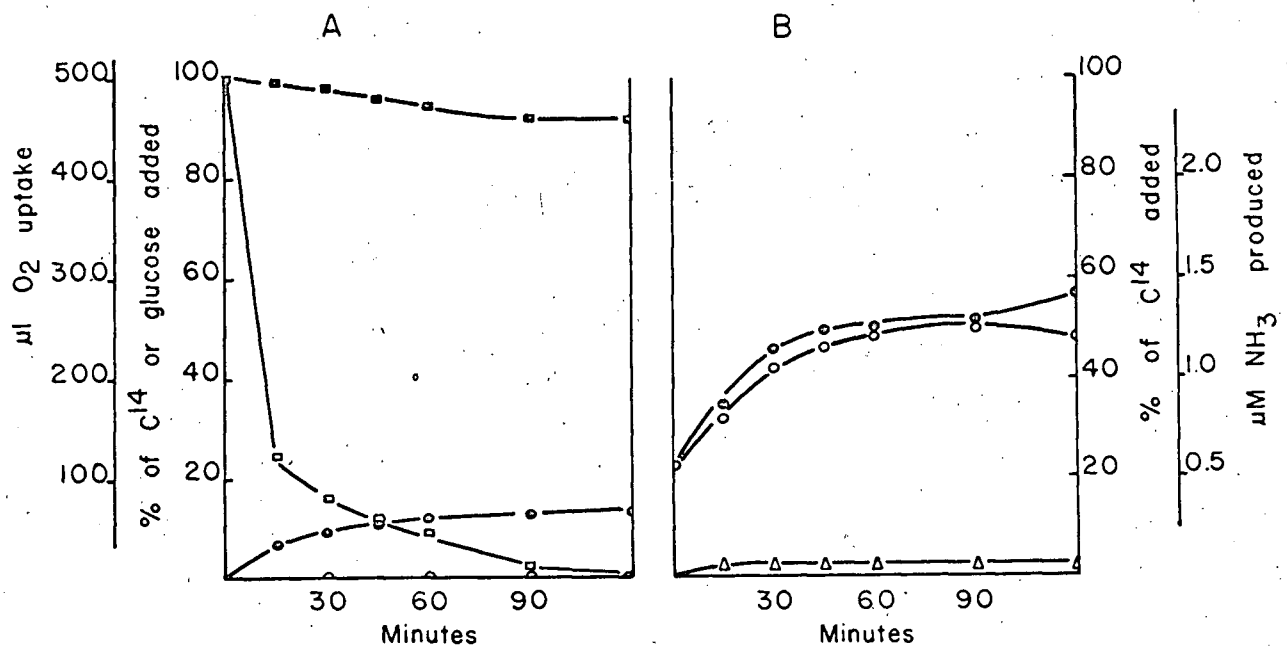


FIG. 5 A. Oxygen uptake with 5 µmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during experiments with washed-cell suspensions of Acetobacter aceti. Oxygen uptake with glucose, 0 ; endogenously, O . Disappearance of glucose, □, and C^{14}, ▣ from supernatant fluids. Endogenous oxygen uptake values were subtracted from the values reported for glucose oxidation.

FIG. 5 B. Time course of NH_3 production and C^{14} incorporation into cells of Acetobacter aceti during oxidation of 5 µmoles of glucose-U-C^{14} by washed-cell suspensions. NH_3 production endogenously, O ; NH_3 production in presence of glucose, 0 ; C^{14} incorporation into cells, Δ .

could have been by pathways not involving gluconate, for whole cells and cell extracts, prepared with a 10 Kc Raytheon sonic oscillator, failed to oxidize this compound. α-Ketoglutarate was oxidized at almost the same rate as glucose, the Q_{O_2}'s for glucose, gluconate and α-ketoglutarate being 19, 0, and 15, respectively. There were traces of glucose present in the reaction mixture at 90 min, and it would appear that the two stage oxidation with A. aceti was due to a decrease in the rate of glucose utilization. There was no obvious reason for the change in rate of glucose disappearance, since gluconolactone, which would be in enzymatic equilibrium with the glucose, and thus might slow the rate of its oxidation, was not detected. There were no keto acids excreted into the medium. Despite the very low endogenous oxygen uptake, ammonia production was relatively high (Figure 5B). However, the presence of glucose did not result in ammonia reincorporation; in fact, ammonia production appeared to be slightly stimulated by glucose. Not surprisingly, only 2% of the radioactivity was incorporated into the cells.

The observation that ammonia was not reincorporated by A. aceti was to be expected, since glucose was oxidized only as far as gluconate, and therefore no α-ketoglutarate would have been available for reductive amination to glutamate. Moreover, experiments on ammonia uptake during the oxidation of α-ketoglutarate and pyruvate by resting cells of A. aceti showed that, although both keto acids were oxidized, the amount of ammonia was the same in the presence and absence of these substrates. Therefore, one can conclude that ammonia cannot be reincorporated into the cell even when the substrate is being oxidized by way of the tricarboxylic acid cycle. This finding is in agreement with the fact that A. aceti required an organic nitrogen source for growth, apparently because it was unable to

utilize inorganic nitrogen for the synthesis of organic compounds.

In contrast to A. aceti, Acetobacter xylinum oxidized glucose at a rapid, constant rate, until the break in the curve at 90 min, when 80% of the theoretical amount of oxygen required for complete oxidation of glucose was achieved (Figure 6A). Gluconate and α-ketoglutarate were oxidized by induced enzymes, the Q_{O_2}'s for glucose, gluconate and α-ketoglutarate oxidation being 78, 74, and 39, respectively. The products detected in the supernatant fluid during glucose oxidation were gluconic acid, which increased until 30 min, and then decreased slowly, and cellulose, which was produced in quantity after 60 min (Table 5). It appeared that glucose and gluconate were being oxidized simultaneously, for some glucose was present until 60 min. No keto acids were detected in the medium. The endogenous oxygen uptake followed a most unusual pattern, there was a rapid initial rate, but all oxidation soon stopped. The same result was obtained repeatedly. Despite the fact that A. xylinum required a complex nitrogen source for growth, endogenously produced ammonia appeared to be reincorporated during the oxidation of glucose, without, however, the excretion of keto acids into the medium (Figure 6B). This is a similar situation to that found with Achromobacter viscosus.

The oxidation of glucose by Azotobacter vinelandii proceeded at a very rapid rate without the accumulation of any intermediate product in the supernatant fluid (Table 5, Figure 7A). However, there was a secondary rate of oxidation which began after 67% of the oxygen for complete oxidation of glucose had been consumed, and continued linearly until the experiment was terminated at 120 min, at which time 89% of the theoretical oxygen uptake had been reached. Part of this oxygen uptake could have been due to the oxidation of glucose which was found in the metabolic pool,

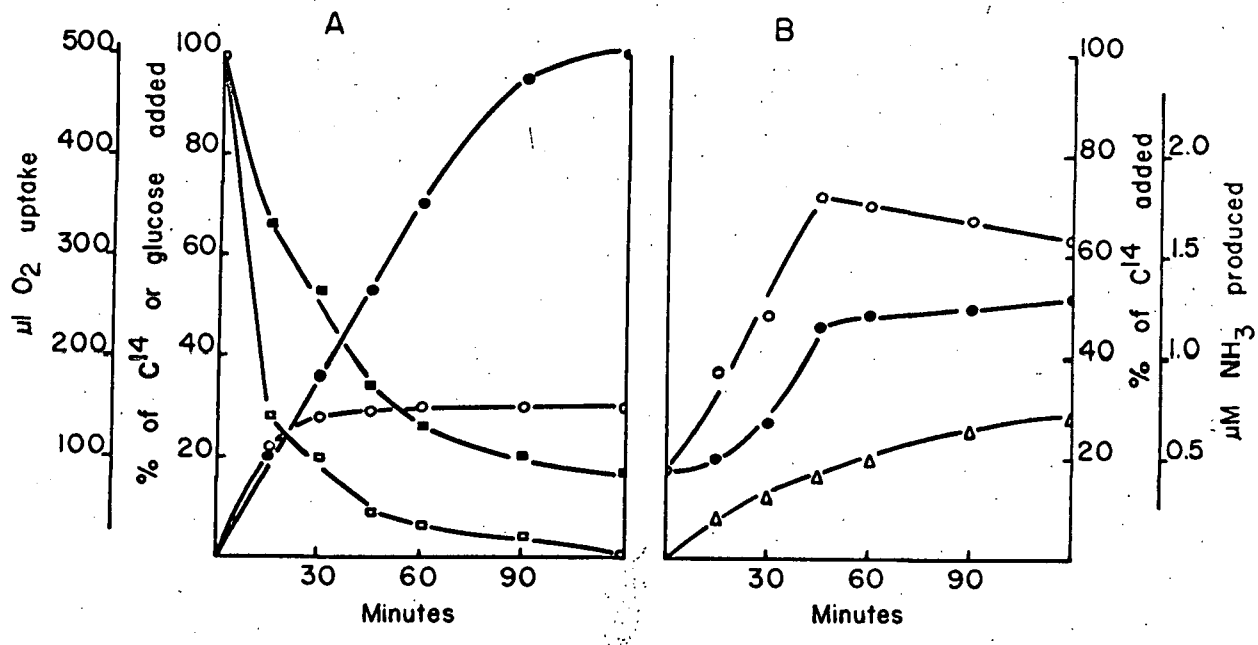


FIG. 6 A. Oxygen uptake with 5 μmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during experiments with washed-cell suspensions of Acetobacter xylinum. Oxygen uptake with glucose, ● ; endogenously, O . Disappearance of glucose, □ , and C^{14}, ■ from supernatant fluids. Endogenous oxygen uptake values were subtracted from the values reported for glucose oxidation.

FIG. 6 B. Time course of NH_3 production and C^{14} incorporation into cells of Acetobacter xylinum during oxidation of 5 μmoles of glucose-U-C^{14} by washed-cell suspensions. NH_3 production endogenously, O ; NH_3 production in presence of glucose, ● ; C^{14} incorporation into cells, Δ .

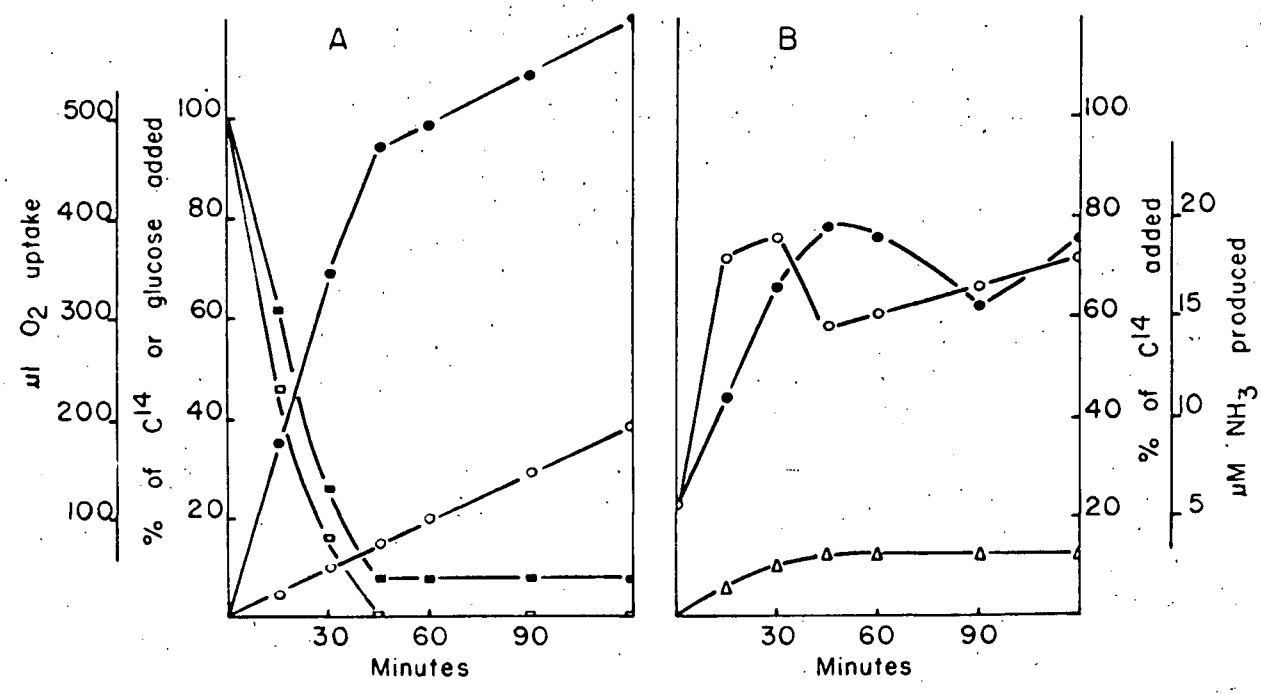


FIG. 7 A. Oxygen uptake with 5 µmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during experiments with washed-cell suspensions of Azotobacter vinelandii. Oxygen uptake with glucose, ● ; endogenously, O . Disappearance of glucose, □ , and C^{14}, ▩ from supernatant fluids. Endogenous oxygen uptake values have been subtracted from the values reported for glucose oxidation.

FIG. 7 B. Time course of NH_3 production and C^{14} incorporation into cells of Azotobacter vinelandii during oxidation of 5 µmoles of glucose-U-C^{14} by washed-cell suspensions. NH_3 production endogenously, O ; NH_3 production in presence of glucose, ● ; C^{14} incorporation into cells, Δ .

but for the rest, one can only conclude that the presence of glucose allowed the organism to draw on some previously acquired reserve material, for this secondary rate of oxidation was considerably in excess of the normal rate of endogenous respiration. Yet, there was no detectable loss of radioactivity from either the cells or from the supernatant fluid after 45 min, although enough oxygen was consumed to oxidize completely 1 µmole of glucose during this period. While this secondary oxidation was going on, A. vinelandii did not release any ammonia, thus indicating that the reserve material being oxidized was non nitrogenous (Figure 7B).

Gluconate was oxidized by induced enzymes, whereas ∝-ketoglutarate oxidation proceeded at a slow, steady rate. The Q_{O_2}'s for glucose, gluconate, and ∝-ketoglutarate oxidation were 180, 20, and 23, respectively. There appeared to be ammonia taken up, because there was a decrease in the amount of ammonia in the early stages when glucose was present. Since the presence of an available energy source such as glucose will increase the fixation of nitrogen by this organism, the ammonia incorporated during glucose oxidation may be much greater than is apparent from Figure 7B.

Azotobacter agilis also oxidized glucose rapidly, but without a secondary rate of oxidation (Figure 8A). Again there was very little assimilation of the glucose supplied, for at 120 min 83% of the theoretical amount of oxygen for complete oxidation had been consumed. Both gluconate and ∝-ketoglutarate were oxidized slowly and by induced enzymes. The Q_{O_2}'s for glucose, gluconate, and ∝-ketoglutarate were 220, 43, and 8.6, respectively. There was a decrease of ammonia in the medium during the early stages of glucose oxidation, suggesting that ammonia was being incorporated into cellular material (Figure 8B). However, A. agilis assim-

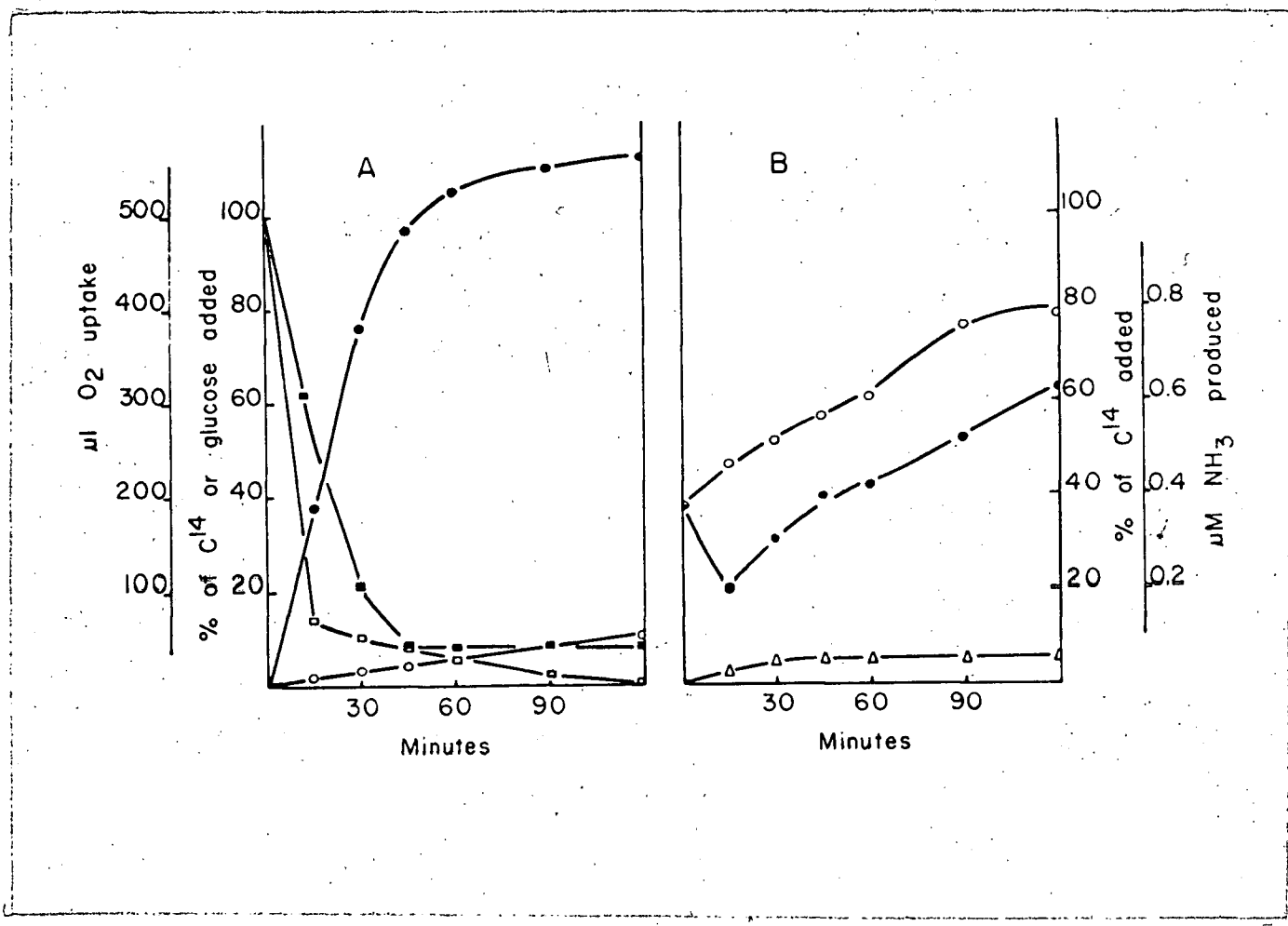


FIG. 8 A. Oxygen uptake with 5 µmoles of substrate and disappearance of glucose and C^{14} from supernatant fluids during experiments with washed-cell suspensions of Azotobacter agilis. Oxygen uptake with glucose, ● ; endogenously, O . Disappearance of glucose, □ , and C^{14}, ▩ from supernatant fluids. Endogenous oxygen uptake values have been subtracted from the values reported for glucose oxidation.

FIG. 8 B. Time course of NH_3 production and C^{14} incorporation into cells of Azotobacter agilis during oxidation of 5 µmoles of glucose-U-C^{14} by washed-cell suspensions. NH_3 production endogenously, O ; NH_3 production in presence of glucose, ● ; C^{14} incorporation into cells, △.

ilated only 5% of the C^{14} into cellular material, in spite of the excess of ammonia available to it.

2. Incorporation of C^{14} into cells during oxidative assimilation

The amount and patterns of the incorporation of C^{14} from glucose are shown in Tables 6a, 6b, 7a and 7b. The amount of assimilated material, unlike that in most cases in the previous section, corresponded fairly well to that which would be predicted by the oxygen uptakes. A. vinelandii, for example, incorporated 11% of the added radioactivity, while oxidizing glucose to 89% of the theoretical amount. None of these bacteria, however, excreted compounds into the medium which could be considered to act as "pacemakers" for oxidative assimilation, since the gluconate produced by A. aceti, and the cellulose by A. xylinum were not metabolized further. Although ammonia was found to be the limiting factor in the experiments of Duncan and Campbell (55) with P. aeruginosa, this could not have been the case with Azotobacter species, which are able to fix nitrogen, and yet they exhibited a very low rate of glucose assimilation. The reason for this apparent anomaly may be that the energy requirements for nitrogen fixation are great enough to limit oxidative assimilation severely. With these bacteria, therefore, the degree of assimilation may be a function of their efficiency of fixing nitrogen, and it is true that A. vinelandii which is known to fix nitrogen very efficiently, and produced large amounts of ammonia during glucose oxidation, assimilated much more radioactivity than did A. agilis.

3. Incorporation of C^{14} into the cell fractions soluble in cold trichloroacetic acid

The compounds extracted from the cells by treatment with cold

Table 6a.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of *A. aceti*

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	1.9	0	0	0	0	1.90	1.9
30	1.9	0.02	0	0.02	0	1.94	2.0
120	2.2	0.02	0.01	0.02	0.02	2.29	2.5
			Per cent of total C^{14} incorporated into cell fractions				
15	100	0	0	0	0	100	
30	98	1	1	0	0	100	
120	96	1	1	0.5	0.5	100	

* Cold and hot trichloroacetic acid soluble fractions.

Table 6b.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of *A. xylinum*

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	1.5	0.58	0.20	0.90	5.1	8.28	8.10
30	2	0.87	0.30	1.29	8.3	12.7	12.5
120	2.3	1.7	0.64	1.88	22	28.5	29
			Per cent of total C^{14} incorporated into cell fractions				
15	18.1	7.1	2.4	11.9	60.5	100	
30	15.5	6.8	2.4	10.1	65.2	100	
120	8.1	6.0	2.2	6.5	77.2	100	

* Cold and hot trichloroacetic acid soluble fractions.

Table 7a.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of A. vinelandii

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	3.6	0.50	0.24	0.21	0.8	5.35	5.2
30	5.0	1.48	0.56	0.59	2.1	9.83	10.2
120	3.1	2.06	0.60	1.69	4.2	11.65	11.7
			Per cent of total C^{14} incorporated into cell fractions				
15	67	9.5	4.5	3.9	15	100	
30	52	15.3	5.7	6.0	21	100	
120	26.5	17.7	5.3	14.5	36	100	

*Cold and hot trichloroacetic acid soluble fractions.

Table 7b.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed-cell suspensions of A. agilis

Time (min)	Cold-soluble*	Lipid	Alcohol-soluble protein	Hot soluble*	Residue	Total in fractions	Unfractionated cells
			Per cent of total C^{14} added to vessel				
15	3.0	0.20	0.10	0.15	0.20	3.65	3.5
30	3.1	0.46	0.34	0.50	0.80	5.20	5.2
120	2.2	0.55	0.55	0.65	1.25	5.20	5.3
			Per cent of total C^{14} incorporated into cell fractions				
15	82	5.6	2.7	4.1	5.5	100	
30	59.5	8.9	6.6	9.6	15.4	100	
120	42	10.7	10.7	12.6	24	100	

* Cold and hot trichloroacetic acid soluble fractions.

5% trichloroacetic acid accounted for essentially all of the label of the cells of A. aceti (Table 6a), and a major amount of the radioactivity in A. agilis and A. vinelandii (Tables 7a and 7b). Radioactive neutral amino acids were present in all four organisms, and glutamate was demonstrated in both Azotobacter species. With the exception of A. agilis, all contained some free glucose at the end of the 2 hr experiments. In addition, pools from the Azotobacter species and A. xylinum contained polymeric compounds, and those from the Acetobacter species contained gluconic acid.

4. Incorporation of C^{14} into the cell fractions insoluble in cold trichloroacetic acid

In each of the bacteria except A. aceti, radioactivity was distributed among all the cell fractions. The two Azotobacter strains exhibited patterns of assimilation very similar to those observed with Pseudomonas species (54,55,152), B. cereus (35), and A. agilis (140). Hydrolysis and column chromatography of the residual fractions of cells which had been respiring for 120 min, revealed a correlation between the amount of "glucose" as shown by the anthrone reagent, and the percentage of counts eluted with water (Table 8). The compounds eluted in this manner would be either neutral or acidic in nature, but paper chromatography in two solvent systems, as well as electrophoresis, showed no acidic compounds; the radioactive peaks cochromatogrammed with glucose only. The lack of acidic components in the residual fractions would indicate that poly-β-hydroxybutyrate was not synthesized during the assimilation experiments, although Sobek and Clifton (140) found low levels of this polymer in A. agilis. Radioactive amino acids were the constituents of the ammonia eluates from the columns (Table 8). Both of the Azotobacter,

Table 8.

Acid hydrolysis and separation by ion exchange of residual* fraction

Microorganism	Per cent C^{14} incorporated	"Glucose" (per cent dry wt)**	Per cent total counts eluted	
			H_2O	NH_4OH
Azotobacter agilis	23	0.4	2.5	97.5
A. vinelandii	34	1.0	20	80
Acetobacter aceti	0.1	0.3	-	-
A. xylinum	80	3.8	80	20

* Insoluble in hot 5% trichloroacetic acid.
** As measured by the anthrone test.

therefore, incorporated the major portion of the assimilated material into nitrogenous compounds, including proteins. The suggestion of Duncan and Campbell (55) that oxidative assimilation of glucose in P. aeruginosa was largely the reincorporation of endogenously produced ammonia can be extended to these Azotobacter species, as well as to the two pseudomonads and the Achromobacter strains previously discussed (152). Since the incorporation of ammonia by Azotobacter species into organic material is known to occur through glutamate (6), and in these experiments glutamic acid-C^{14} was found in the metabolic pool, ammonia was probably assimilated via the α-ketoglutarate formed from glucose.

The small amount of cellular material insoluble in cold trichloroacetic acid confirmed the conclusion that A. aceti did not incorporate measurable amounts of ammonia into the cells (Table 7a). One must

conclude, therefore, that A. aceti did not exhibit oxidative assimilation when glucose was the substrate, since the C^{14} incorporated was almost entirely in the pool components, in the form of free glucose and gluconate.

The ability of A. xylinum resting cells to synthesize cellulose which accumulates extracellularly, complicated the study of oxidative assimilation in this microorganism. Column chromatography of the hydrolysed residual fraction revealed that 80% of the radioactivity was associated with neutral compounds, which on paper chromatography and electrophoresis proved to be glucose and cellobiose. Moreover, a large proportion of the unhydrolysed material was insoluble in water, acid, or base, being soluble only in Schweitzer's reagent. These observations lead to the conclusion that cellulose was the major component of the residual fraction. This does not mean that the cellulose was formed intracellularly, because extracellular fibrils would also be in this fraction. The release of radioactive amino acids by the hydrolysis of the residual fraction indicates that protein was synthesized by A. xylinum. There was also radioactivity incorporated into other nitrogen containing fractions, such as the alcohol soluble protein, and nucleic acids. It can be calculated that about 11% of the added C^{14} was assimilated into cell material, and of this about half is nitrogenous, the result of the reincorporation of endogenously produced ammonia.

C. Oxidative assimilation by starved cells of P. aeruginosa ATCC 9027

1. Manometric observations

Starvation of resting cells of P. aeruginosa for 3 hr was found to decrease their rate of glucose dissimilation, perhaps because it slowed

down the rate of pyruvate oxidation (Figure 9). Oxidation of ∝-keto-glutarate was unaffected. This decrease in the rate of pyruvate oxidation occurred with cells which had been starved with or without shaking, but shaking gave a more pronounced effect. With the shaken cells, the extent of decrease in pyruvate oxidation was found to vary from experiment to experiment, and probably reflected the state of the cells on harvesting, although conditions were standardized as much as possible. There was no stimulation of the rate of oxidation of pyruvate when thiamine pyrophosphate (0.5 µmole per Warburg flask) was added. Since glucose oxidation was more affected when cells were starved with shaking than without, shaken, starved cells were used in the experiments with glucose-U-C^{14}. In the experiment quoted below, the oxygen uptake at 120 min with starved cells was 80% of the theoretical value for the complete oxidation of glucose, as compared to 67% for freshly harvested (control) cells. This higher oxygen uptake would indicate a lower assimilation of glucose by starved cells.

2. Ammonia excretion and uptake

When the amount of ammonia excreted into the medium was determined, it was found that shaking the cells had greatly increased endogenous ammonia production (Table 9, Figure 10). Moreover, although both shaken and non shaken cells reincorporated ammonia during glucose oxidation, the amount and the rate of uptake differed greatly. The curve of ammonia uptake for the non shaken cells paralleled that for control cells, except that less ammonia was assimilated, not all of that available being utilized (Table 9, Figure 10). The cells which had been shaken for 3 hr reincorporated more ammonia in the first 5 min of glucose oxidation than did the others, but the calculated uptake then declined, apparently because of a decrease in the rate of endogenously evolved ammonia. This decrease resulted

Table 9.

Ammonia production and uptake by previously starved cells of *Pseudomonas aeruginosa* during glucose oxidation

Time	Non shaken cells NH_3 present per vessel		NH_3 uptake (calculated)	Shaken cells NH_3 present per vessel		NH_3 uptake (calculated)
	Endogenous	Glucose		Endogenous	Glucose	
Min	µmoles	µmoles		µmoles	µmoles	µmoles
Starvation period						
0	0.010			0.010		
180	0.080			0.550		
Assimilation period						
0	0.200	0.200	0	0.200	0.200	0
5	0.225	0.110	0.115	0.475	0.120	0.355
15	0.310	0.120	0.190	0.490	0.140	0.350
30	0.450	0.180	0.270	0.500	0.155	0.345
120	1.100	0.320	0.780	0.680	0.470	0.210
Total endog NH_3 prod'n	1.180			1.230		

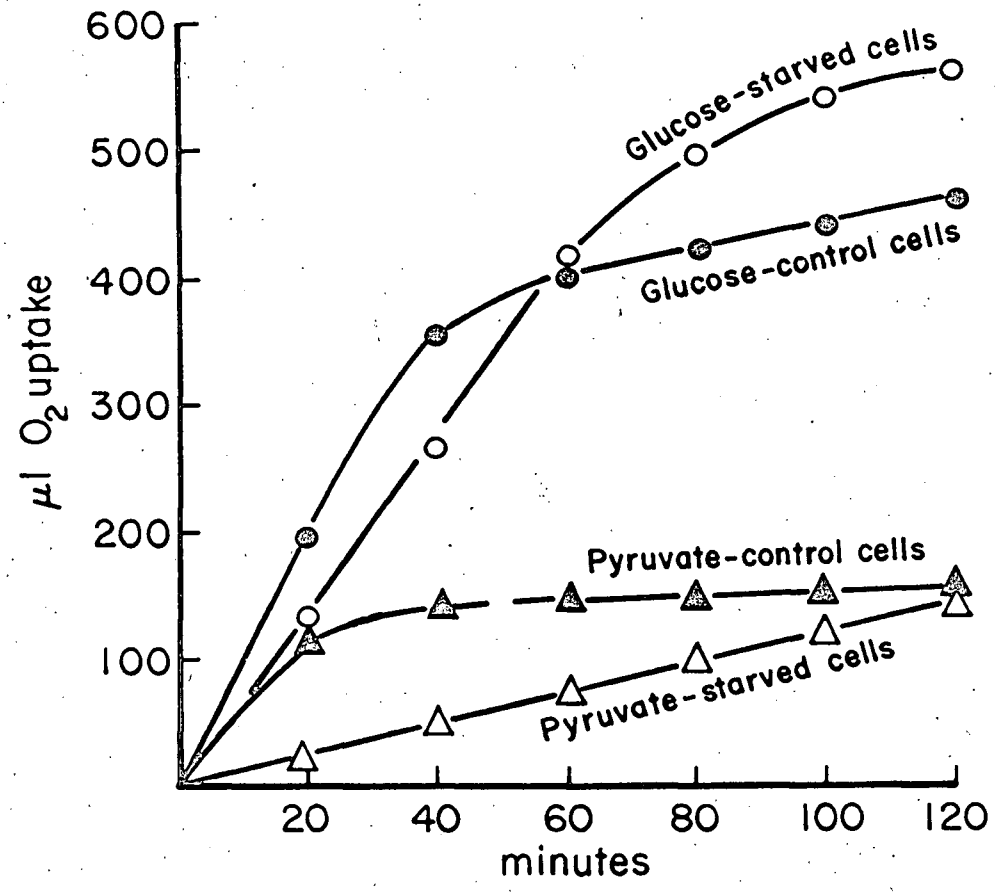


FIG. 9. Oxygen uptake during oxidation of 5 μmoles of pyruvate or glucose by control and starved washed cell suspensions of Pseudomonas aeruginosa.

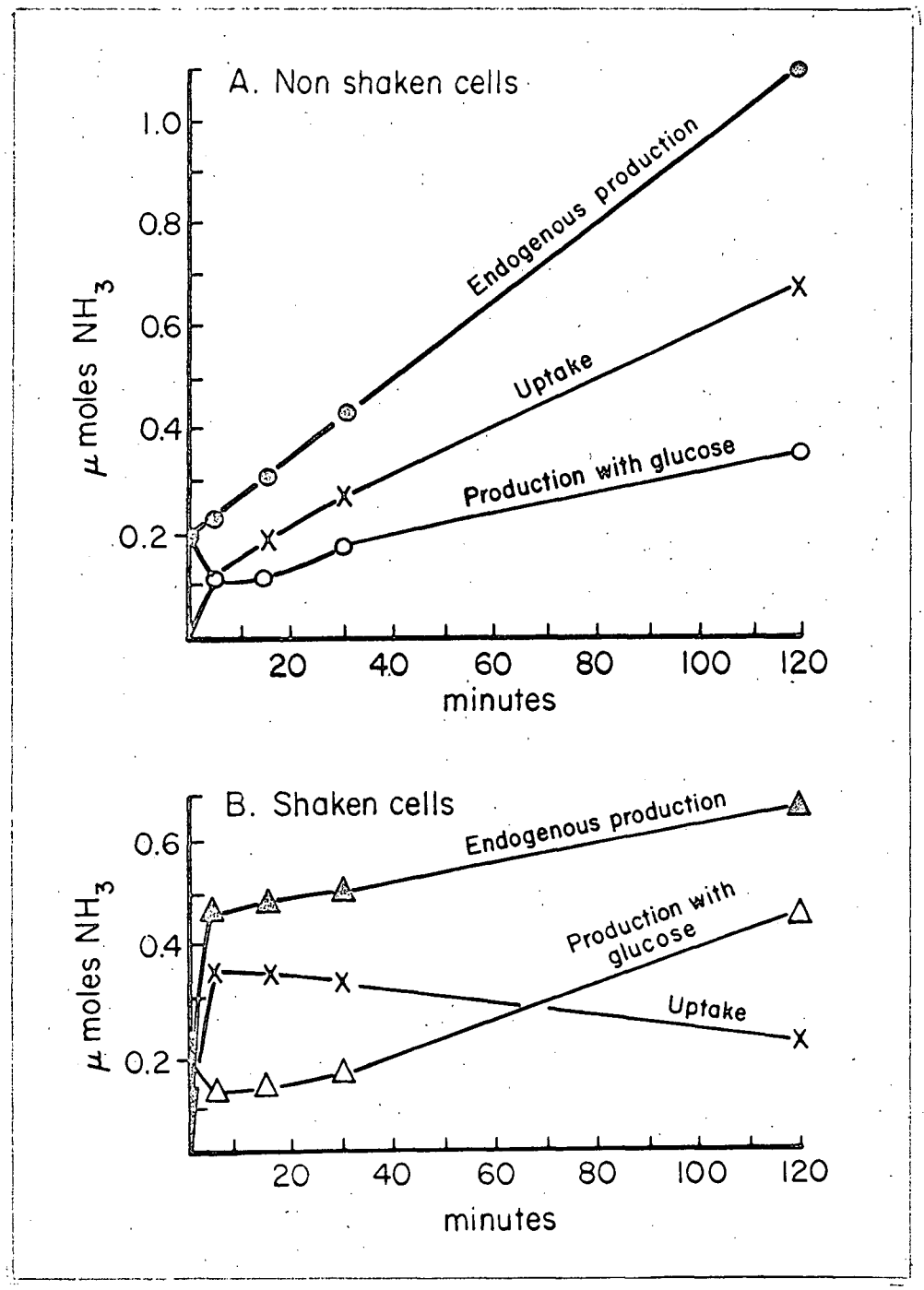


FIG. 10. Production and uptake of NH_3 by washed, starved cells of Pseudomonas aeruginosa.

in the total amount of endogenously produced ammonia being very nearly equal for the two types of cells.

3. Excretion of radioactive products into the supernatant fluids

Since pyruvate oxidation was impaired by starving the cells, it is not surprising that large amounts of the keto acid accumulated in the supernatant fluids during glucose oxidation (Figure 11). In the experiment where shaken, starved cells oxidized glucose-C^{14}, 4 µmoles of pyruvate were present at 30 min, as compared to 2.6 µmoles when freshly harvested cells were used. In addition, chromatography and electrophoresis of the supernatant fluids, followed by scanning, revealed the presence of ∝-ketoglutarate, gluconate, and 2-ketogluconate. The sugar acids were most highly labelled at 5 min, whereas pyruvate and ∝-ketoglutarate contained the highest amount of C^{14} at 30 min. This is a pattern similar to that found with control cells, although in this instance, the pyruvate concentration was highest at 15 min. There was very little ∝-ketoglutarate present in the Warburg supernatants; the ratio of optical density readings at 435mµ and 390mµ was 2.15 in the keto acid assay throughout the experiment which is characteristic of pyruvate. The ratio for ∝-ketoglutarate was 1.05. However, the specific activity of the ∝-ketoglutarate was high, since it could be detected on paper chromatograms by its radioactivity. When non shaken cells were used, pyruvate also accumulated in greater quantity than with freshly harvested cells, reaching a value of 4 µmoles at 15 min.

4. Distribution of C^{14} in the cells

Doudoroff and Stanier (53) reported that when starved cells of P. saccharophila were allowed to oxidize glucose, the oxidative assimilation was more than doubled (to 50% of the added C^{14}), over that of freshly

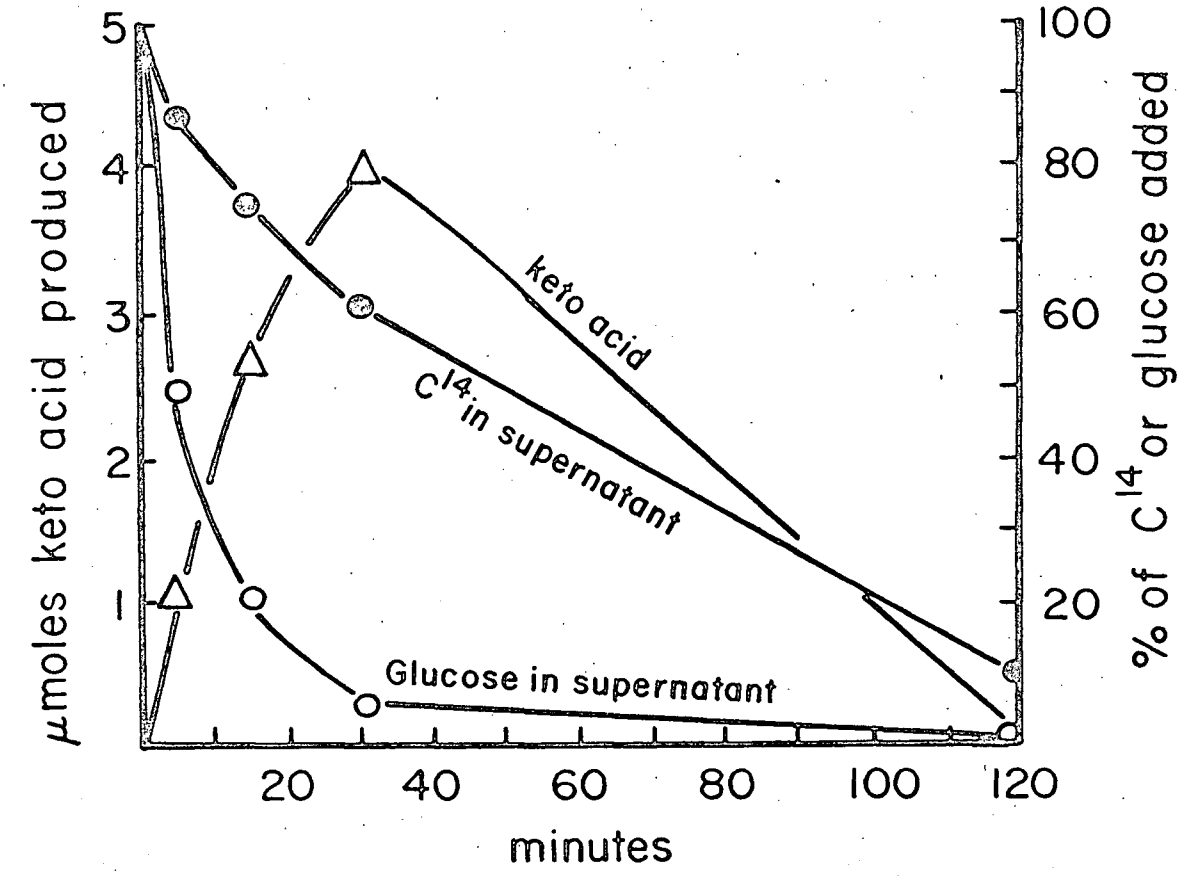


FIG. 11. Disappearance of C^{14} and glucose from, and excretion of keto acid into supernatant fluids during oxidation of glucose-U-C^{14} by washed, starved cells of Pseudomonas aeruginosa.

harvested cells, and that the assimilated material was largely poly-β-hydroxybutyrate. A similar experiment was performed in our laboratory with P. aeruginosa, but with different results. Starved cells of this pseudomonad assimilated only 9.3% of the added glucose, as compared to 16.3% with freshly harvested cells, and 12.5% in the presence of 200 µg of chloramphenicol per ml (Figure 12).

In the early stages of glucose oxidation, the chemical distribution of the assimilated C^{14} in starved cells was more similar to that in chloramphenicol treated, freshly harvested cells (55), than it was to the control cells (Table 10), in that the cold trichloroacetic acid soluble pool components were highly labelled, whereas label in the protein was low. However, although the radioactivity in the cells in the presence of the antibiotic remained mainly in the metabolic pool throughout the experiment, in starved cells it was transferred slowly to protein and nucleic acids.

5. Analysis of the cold trichloroacetic acid soluble fractions

When the cold trichloroacetic acid soluble pools from starved cells were investigated by paper chromatography and electrophoresis, and the results compared to those obtained in experiments with freshly harvested and chloramphenicol treated cells, it was found that all three types of cells incorporated the C^{14} mainly into the free amino acid pools. The amino acids were predominantly those which are neutral at pH 7.6 (alanine, serine, leucine, glycine, etc.), as well as glutamic acid. The glutamate from the control and antibiotic treated cells accounted for most of the radioactivity of the pools at 30 min, decreasing in label thereafter. There was no free glucose present. In the cold trichloroacetic acid soluble fractions from starved cells, however, the main radioactive component during the entire experiment was glutamate, although it decreased in radioactivity

Table 10.

Incorporation of C^{14} from 5 μmoles of glucose-U-C^{14} into washed, starved cell suspensions of *Pseudomonas aeruginosa*

Time (min)	Cold TCA** soluble	Lipid	Alcohol soluble protein	Hot TCA** soluble	Residual fraction	Total in fractions
			Per cent of total C^{14} added to vessel			
5	2.3	0.8	0.3	0.2	0.3	3.9
15	3.0	1.4	0.4	0.3	0.8	5.9
30	4.0	1.5	0.5	0.4	1.1	7.5
120	3.7	1.5	0.6	0.8	2.7	9.1
			Per cent of total C^{14} incorporated into cell fractions*			
5	62 (36)	20 (26)	7 (3)	4 (11)	7 (26)	100
15	51 (25)	23 (25)	7 (4)	5 (11)	14 (35)	100
30	52 (20)	20 (25)	7 (4)	6 (11)	15 (43)	100
120	40 (15)	16 (21)	6 (4)	9 (11)	29 (49)	100

* Figures in parentheses are values for control cells (55).
** Trichloroacetic acid.

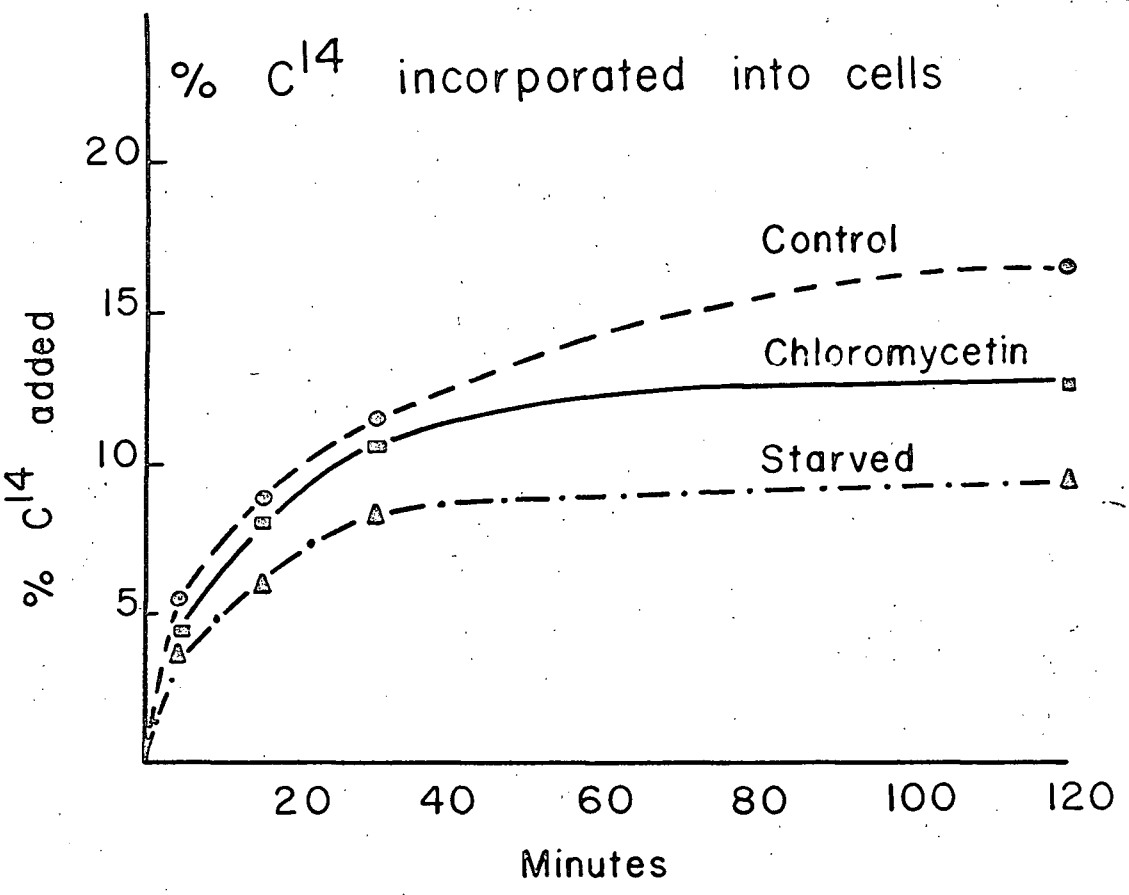


FIG. 12. Incorporation of C^{14} during oxidation of glucose-U-C^{14} by washed, control, chloramphenicol (chloromycetin) treated, or starved cells of Pseudomonas aeruginosa.

considerably during this time. There were also small amounts of radioactive α-ketoglutarate, glucose, gluconic acid, and 2-ketogluconic acid present in the early stages. At 15 min, these four compounds had been replaced by a doubly charged acid whose R_f's in BFW and EPB, and whose $R_{picrate}$ in HCO_3^- buffer corresponded to those of 6-phosphogluconate or 2-keto-6-phosphogluconate. Unlike gluconate and 2-ketogluconate, these phosphorylated derivatives were not separated by EPB. Pyruvate was not present at any time in any of the pools.

6. The influence of vitamin B_6 on oxidative assimilation

It was thought possible that the accumulation of glutamate in the pools of the starved cells might be due to a depletion of the cofactors or enzymes required for transamination. To test the cofactor theory, starved cells were allowed to assimilate glucose-U-C^{14} in the presence of pyridoxal phosphate and pyridoxamine phosphate (8 µg per flask of each). A parallel experiment was run in the absence of the cofactors. However, there was no increase in the amount of C^{14} incorporated into the cells, nor any significant change in the pool when glucose was oxidized in the presence of these cofactors.

Unlike P. saccharophila, which incorporates assimilated carbon into poly-β-hydroxybutyrate, P. aeruginosa does not form any primary product during the oxidative assimilation of glucose (55). This situation was emphasized by the results of these experiments with starved cells. Starvation of P. saccharophila cells resulted in a doubling of assimilated material during glucose oxidation (53), but starvation of P. aeruginosa cells led to a 40% decrease. The reason for this difference seems to be that oxidative assimilation by P. aeruginosa involves protein synthesis,

which is a very complex process. One might postulate two reasons for the lack of protein synthesis in starved cells. First, pyruvate was oxidized completely, but more slowly by these cells, than by freshly harvested ones, which meant that not as much α-ketoglutarate was available for the incorporation of ammonia in the form of glutamate. In addition, highly labelled glutamic acid accumulated in the metabolic pool, and so some ammonia was being reincorporated; however, the glutamate thus formed was not used for synthesis. Therefore, a second reason for the low assimilation could be a defect in transamination of the glutamate to give other amino acids required for protein synthesis. Since the addition of vitamin B_6 to starved cells did not increase assimilation, a lack of transaminases could be responsible. The cells apparently have a control mechanism which prevented unlimited assimilation of carbon unless it could be incorporated into cellular material. During glucose oxidation by P. aeruginosa, carbon was not assimilated directly, but only after conversion to α-ketoglutarate, which was, in turn, aminated to yield glutamate (55). When this glutamate began to accumulate in the pools of starved cells, perhaps as a result of a low transaminase activity, the incorporation of ammonia and carbon was slowed down, and glucose was oxidized more completely than in freshly harvested cells. If P. aeruginosa possessed an alternative assimilatory product such as poly-β-hydroxybutyrate, this sequence of events would not be expected to occur.

II. Inorganic Nitrogen Assimilation

Although the assimilation of ammonia into organic compounds is known to occur in microorganisms primarily by way of α-ketoglutarate and glutamic acid dehydrogenase, recently there has been interest in a pos-

sible additional route via pyruvate and alanine dehydrogenase (58,63,122,168). Both pyruvate and α-ketoglutarate are present in the supernatant fluids during the early stages of oxidative assimilation of glucose by P. aeruginosa, and disappear as the oxidation progresses, with concurrent uptake of ammonia. Since the tricarboxylic acid cycle is functional in this microorganism, pyruvate can be converted to α-ketoglutarate (139). It has been shown that an NADPH dependent glutamic acid dehydrogenase is present in cell extracts of P. aeruginosa, but direct assays for alanine dehydrogenase are difficult, because of the presence of a NADH oxidase. However, there is some evidence that, at least under the conditions of the assay used, alanine dehydrogenase levels are much lower than those of glutamic acid dehydrogenase (160). The aspartase activity of extracts of this strain of P. aeruginosa is also low (160).

Resting cell experiments were done with P. aeruginosa and P. fluorescens to try to differentiate between ammonia uptake directly through pyruvate, and that through the α-ketoglutarate derived from pyruvate. The aspartase route was not investigated. Two approaches were taken. Firstly, assimilation of ammonia by P. aeruginosa was determined during the oxidation of pyruvate or α-ketoglutarate separately, and in various combinations. Secondly, the effects of inhibitors of keto acid metabolism in P. fluorescens and P. aeruginosa were determined.

A. Experiments with P. aeruginosa ATCC 9027

1. Assimilation of ammonia during the oxidation of keto acids

Ammonia, as $(NH_4)_2SO_4$, was added at the concentrations of 5, 7.5, and 15 μmoles per Warburg flask, to resting cell suspensions oxidizing 5 μmoles of glucose, pyruvate, or α-ketoglutarate, and the uptake of both

oxygen and ammonia was followed. In the cases where glucose or pyruvate were the substrates, the addition of ammonia decreased oxygen consumption, although the decrease was much greater with glucose than with the keto acid. The largest reduction was found with 15 µmoles of ammonia. The addition of 5 µmoles of ammonia to cells oxidizing ∝-ketoglutarate resulted in an apparent increase in oxygen consumption at the time the experiment was terminated (80 min) perhaps because the lag period was shorter (Table 11). However, the final oxygen uptake, at the time that the oxidation of the keto acid was complete was not increased. When 5 µmoles of ammonia

Table 11.

Oxygen and ammonia uptake during the oxidation of 5 µmoles of glucose, pyruvate, or ∝-ketoglutarate in the presence and absence of 5 µmoles of ammonia

Substrate	Oxygen uptake at 80 min		NH_3 uptake at 80 min (5 µmoles NH_3 added)
	Per cent theoretical	$+NH_3$	µmoles
Glucose	67	50	2.95
∝-Ketoglutarate	22*	28*	1.80
Pyruvate	80	59	1.00

* Oxygen uptake had not ceased.

were added, a relatively small amount of ammonia was consumed during the oxidation of any of the substrates, and increasing the amount of ammonia did not increase its uptake. More ammonia was assimilated with glucose (on a molar basis) than with the keto acids (Table 11). This may indicate

an energy or cofactor requirement (e.g., reduced pyridine nucleotides) for ammonia uptake not satisfied by the oxidation of the keto acids.

In the experiments where the two keto acids were oxidized simultaneously, the second substrate was not added to the flask until oxidation of the first had proceeded for 40 min. This was done because when α-ketoglutarate is oxidized by glucose grown cells of P. aeruginosa, there is a lag period before oxygen uptake begins; however, pyruvate is oxidized immediately. It is evident that the addition of pyruvate to cells oxidizing α-ketoglutarate neither stimulated nor inhibited ammonia uptake (Table 12).

Table 12.

Oxygen and ammonia uptake during the oxidation of pyruvate and α-ketoglutarate

Initial Substrate	Substrate added at 40 min	Oxygen uptake % theoretical at 100 min	NH_3 uptake Found 60 min	Found 100 min	Expected 100 min
		%	µmoles		µmoles
α-Keto-glutarate	-	28	1.5	1.8	
α-Keto-glutarate	pyruvate	34	-	3.0	2.9 (1.8+1.1)
α-Keto-glutarate	α-ketoglut.	37	-	3.0	3.3 (1.5+1.8)
Pyruvate	-	60	1.1	0.8	
Pyruvate	α-ketoglut.	32	-	3.0	2.3 (1.5+0.8)
Pyruvate	pyruvate	65	-	1.9	1.9 (1.1+0.8)

In fact, all combinations resulted in approximately the amount of am-

monia uptake predicted, except in the situation where α-ketoglutarate was added to cells already oxidizing pyruvate, in which instance 30% more ammonia was incorporated than calculated. It is possible that pyruvate oxidation, like that of glucose, provided cofactors (such as reduced pyridine nucleotides) or energy for the assimilation of ammonia by α-ketoglutarate.

At first glance, these experiments appear to show that there was no competition between the two keto acids for available routes of ammonia assimilation. On closer examination, however, one can detect a flaw in this reasoning, since it was not known whether one system was saturated with substrate when the second was added. Ammonia was in excess, because increasing its amount did not increase its uptake. There did not appear to be a way of ensuring that reduced cofactors and substrate were in excess without radically altering in vivo conditions. One must conclude, therefore, that these experiments did not prove that ammonia was assimilated in the presence of the two keto acids by non competitive routes, even though the data fit this interpretation.

If one assumes that the 60% of theoretical oxygen uptake during pyruvate oxidation was consumed in oxidizing the keto acid to completion, then 40%, or 2 µmoles was available for assimilation. Free pyruvate was not found in the metabolic pool of P. aeruginosa, and therefore, pyruvate did not appear to be assimilated without prior amination to alanine, or oxidation to another compound. If assimilation of the keto acid as alanine had occurred, 2 µmoles of ammonia should have been taken up. However, the actual incorporation of ammonia was only 1.1 µmoles, indicating that probably little or no direct assimilation of pyruvate occurred. A calculation of the amount of ammonia which should be assimilated if this were done only

through the α-ketoglutarate derived from pyruvate is complicated by the fact that we do not know how much of the pyruvate was oxidized to completion, how much only to α-ketoglutarate, and how much of the α-ketoglutarate was, in turn, oxidized.

2. Assimilation of ammonia in the presence of inhibitors

Arsenite, at the concentration of 10^{-3}M, was found to inhibit, by 80-90%, the uptake of both ammonia and oxygen by resting cells of P. aeruginosa in the presence of α-ketoglutarate or pyruvate (Table 13). Endogenous oxidation was also inhibited, although to a lesser extent. Experiments with cell extracts showed that the glutamic acid dehydrogenase of P. aeruginosa, and the alanine dehydrogenase of B. cereus (122) are unaffected

Table 13.

Inhibition of oxygen and ammonia uptake by 10^{-3}M arsenite during the oxidation of pyruvate and α-ketoglutarate by washed cell suspensions of P. aeruginosa

Initial Substrate	Substrate added at 40 min	Inhibition by arsenite Oxygen uptake	NH_3 uptake
		%	%
α-Ketoglutarate	-	93	85
α-Ketoglutarate	pyruvate	90	92
Pyruvate	-	80	80
Pyruvate	α-ketoglutarate	88	76

by this concentration of arsenite, but it is not known whether the inhibitor also prevents oxidative assimilation as a result of the lack of oxidation. In this connection, Fairhurst et al. (58) reported that the

formation of alanine by B. cereus resting cells from pyruvate and ammonia was inhibited 88% in the presence of 2×10^{-3}M sodium arsenite. Since the glutamic dehydrogenase of P. aeruginosa was not inhibited by arsenite, and since there was almost no assimilation of ammonia even in the presence of α-ketoglutarate, it is probable that, in the absence of substrate oxidation which provides both the reduced cofactors, and the energy necessary, ammonia assimilation does not occur.

The second inhibitor used was fluoroacetate, at the concentrations of 10 and 25 μmoles per flask, in hopes of blocking glucose oxidation at citrate. However, glucose dissimilation was affected very little by the inhibitor, and the uptake of ammonia proceeded as usual. It was then learned that neither acetate nor fluoroacetate are converted to citrate by acetate grown cells of P. aeruginosa, although pyruvate is (139). This observation would explain these findings with glucose grown P. aeruginosa cells. It is interesting, therefore, that ammonia was assimilated during acetate oxidation by P. aeruginosa, and that the endogenous oxygen uptake was inhibited 35%. The use of fluoroacetate was abandoned with this strain of P. aeruginosa, and experiments were done with a second strain, P. aeruginosa 120 Na, but this too appeared to be unaffected by the inhibitor, since oxygen uptake with glucose was increased 25%, and endogenous oxygen consumption was decreased 36%.

B. Experiments with P. fluorescens A 3.12

Resting cells of P. fluorescens were found to have an unchanged rate of glucose oxidation in the presence of 10 and 25 μmoles of fluoroacetate, but the total oxygen uptake was reduced 25%. There was a slight initial lag in oxygen uptake, and the curve broke at a different point.

In addition, the endogenous oxygen consumption was found to be decreased 54%. When 50 µmoles of the inhibitor were used per flask, the rates of pyruvate and acetate oxidation were reduced by 82% and 71%, respectively, whereas those for glucose and α-ketoglutarate oxidation were unaffected. The final oxygen uptake with pyruvate was decreased from 67% of theoretical to 45%, but there was no such decrease with acetate. Oxidation of the keto acid appeared to have ceased at the end of the experiment.

A manometric experiment was then done, in which ammonia assimilation was followed during pyruvate oxidation in the presence of 50 µmoles of fluoroacetate per flask. The results are shown in Figure 13 and Table 14. In the control experiment, ammonia uptake followed oxygen uptake, and when oxidation ceased, ammonia was evolved. In the presence of fluoroacetate, both ammonia and oxygen consumption remained linear until the experiment was terminated at 80 min; however, levels were much lower than in the control. At 20 min, the inhibition of both the uptake of ammonia and oxygen was 90%, this decreased to 76% and 52%, respectively at 80 min (Table 14).

Since the incorporation of ammonia and the uptake of oxygen were inhibited to the same extent by fluoroacetate, it is not unlikely that, as with the experiments in the presence of arsenite, ammonia assimilation does not occur in the absence of substrate oxidation.

This series of experiments, therefore, represented a preliminary study on the route of ammonia incorporation by P. aeruginosa and P. fluorescens. It was established that the oxidation of pyruvate by both Pseudomonas species was accompanied by ammonia uptake, but no evidence was obtained for the direct amination of this keto acid. In contrast, ammonia was certainly

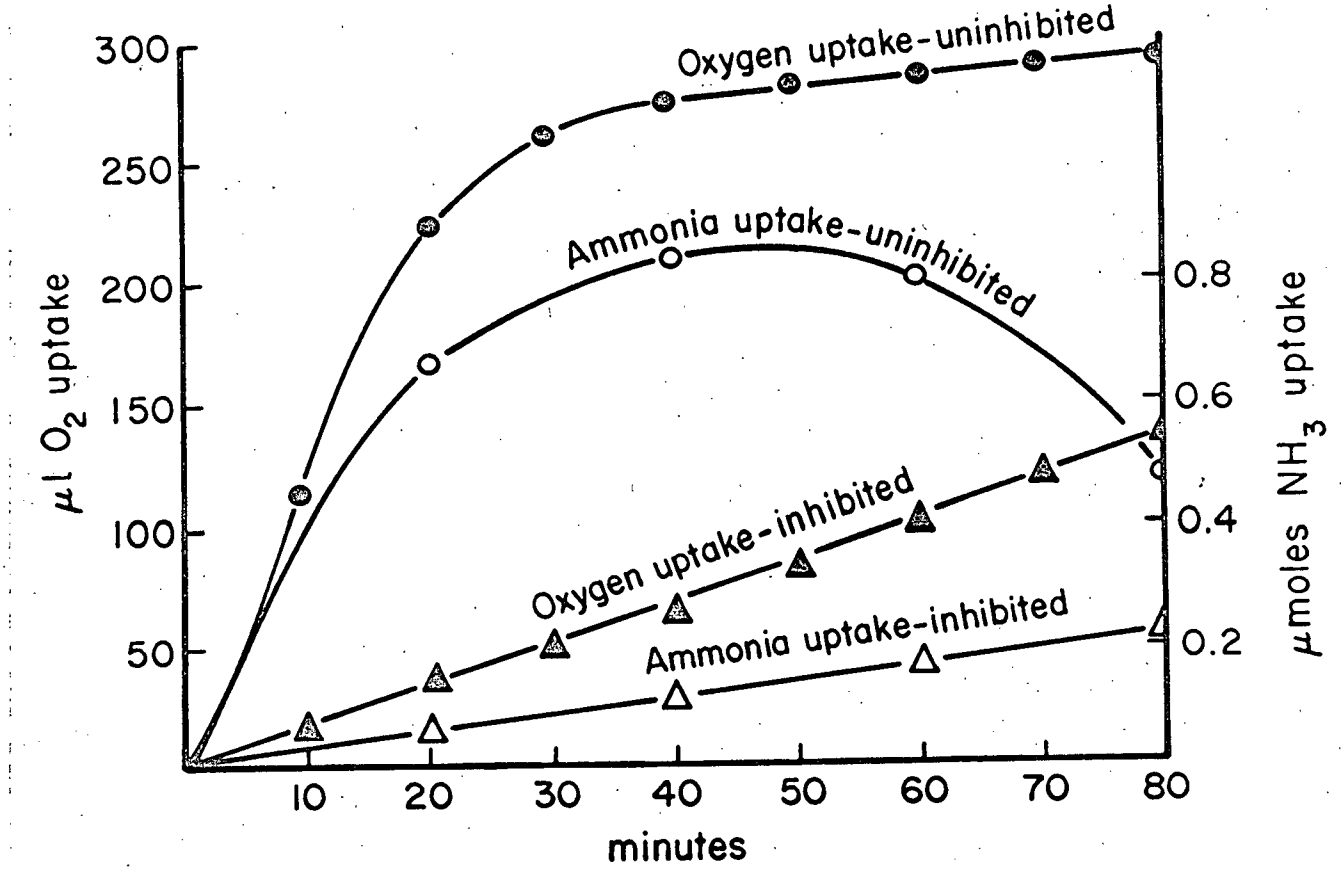


FIG. 13. Uptake of oxygen and NH_3 by washed cells of Pseudomonas fluorescens during the oxidation of 10 μmoles of pyruvate with and without 50 μmoles of fluoroacetate.

Table 14.

Inhibition of uptake of ammonia and oxygen during the oxidation of 10 µmoles of pyruvate in the presence of 50 µmoles fluoroacetate by washed cells of Pseudomonas fluorescens

Time	Control		Plus Fluoroacetate		Inhibition	
	NH_3 uptake	O_2 uptake	NH_3 uptake	O_2 uptake	NH_3	O_2
min	µmoles	µliters	µmoles	µliters	%	%
0	0.000	000	0.000	000	-	-
20	0.675	225	0.067	33	90	87
40	0.837	260	0.120	66	86	81
60	0.812	275	0.176	100	88	64
80	0.470	285	0.210	137	76*	52

* Calculated on basis of 40 min ammonia uptake in control, since oxygen uptake had not stopped at this time.

assimilated via ∝-ketoglutarate in P. aeruginosa, since it was taken up during the oxidation of ∝-ketoglutarate, and there is known to be an active glutamic dehydrogenase present, with a K_{eq} far in favour of amino acid synthesis. Supporting data for the primary role of ∝-ketoglutarate in ammonia assimilation by P. aeruginosa came from the starved cell experiments, where a large amount of labelled glutamate accumulated in the metabolic pool under circumstances where protein synthesis was not occurring. However, there was little radioactive alanine in the pool. One would expect, since pyruvate was present in large quantities in the medium, that, if ammonia were incorporated to any extent via alanine dehydrogenase, then alanine would contain the highest amount of C^{14}, and that glutamic

acid would be of lower specific activity, because of the lack of α-ketoglutarate for its synthesis. In addition, investigation of the metabolic pools of freshly harvested cells of P. aeruginosa after glucose assimilation, showed glutamic acid to be the most highly labelled component. Therefore, the data from these experiments were consistent with the oxidation of pyruvate to α-ketoglutarate, amination to form glutamate, followed by transamination to yield alanine and other amino acids.

Although the experiments with P. aeruginosa and P. fluorescens in the presence of inhibitors gave negative results, they did serve to emphasize the interrelationships between cell processes. Thus, whenever the oxidation of a substrate was prevented, no assimilation of ammonia, and by inference, of carbon, occurred. The energy requirement for uptake of both ammonia and of organic nitrogen by microbial cells has been indicated in a number of experiments (19,34,66,100,110,175), and the cofactor requirements of the dehydrogenases are well known.

It would seem that any further work on alternate pathways for ammonia assimilation by either of these microorganisms will have to take another direction. Alternative approaches would be either the study of cell extracts, or short term experiments, similar to those performed with algae by Calvin's group, to determine whether alanine is formed directly through pyruvate, or only by transamination with glutamate.

III. Oxidative Assimilation into the Cytological Fractions of Normal, Chloramphenicol Treated, or Starved Cells of Pseudomonas aeruginosa ATCC 9027

A. Chemical composition of cytological fractions

Recent work in this laboratory on cytological fractions of P. aeruginosa cells has been done mainly with lysozyme-versene disrupted cells (29,30,75). However, it was discovered that some commercial lysozyme preparations contained a ribonuclease which resulted in degradation of most of the RNA, so that it appeared in the cytoplasm as small fragments (75). Accordingly, an alternate method of breaking cells was sought. The French pressure cell, used at 15,000 to 17,000 lbs pressure, was found to give an efficient breakage of small volumes of P. aeruginosa cell suspensions. Since it was desired to obtain representative "membrane", ribosomal, and cytoplasmic fractions from these cells in as pure a state as possible, several criteria were set up to determine the purity of the fractions, as follows (30): "membranes"- contain most of the glucose oxidizing activity, approximately 5% of the RNA, and little DNA; ribosomes- contain little or no glucose oxidizing activity, most of the RNA, have an RNA:protein ratio approaching unity, and contain little DNA; cytoplasm- contains no glucose oxidizing activity, little RNA, and almost all of the DNA. When the fractionation procedure described in Section II B was used, it was found that these criteria were met, and that the results were reproducible. The data from one of these experiments are shown in Table 15. The properties of these fractions are similar to those described by Campbell et al. (30) for P. aeruginosa, and also resemble those of E. coli fractions (149). If magnesium was omitted from the buffer in which the cells were broken, most of the RNA appeared in the cytoplasm. There is probably contamination of at least the "membrane" fraction with cell wall material, and some particles of "membrane" appear with the ribosomes.

B. Incorporation of glucose-U-C^{14} into cytological fractions

Three experiments were performed:

Table 15.

Distribution of glucose oxidizing activity, protein and nucleic acids in various cytological fractions

Fraction	Glucose oxidizing activity		Protein		RNA		DNA		RNA/ Prot.
	units*	%total	mg	%total	mg	%total	mg	%total	
Cell free extract	675	100	64	100	18	100	3	100	0.30
"Membranes" 1X	381	62	9.8	15.7	1.2	6.7	0.03	1	0.12
"Membranes" 2X	80	20	2.7	4.3	0.3	1.7	0	0	0.11
"Membranes" 1X wash	33	6	4.4	7.0	1.1	6.2	0.10	3.4	0.25
"Membranes" 2X wash	0	0	1.3	2.1	0.7	3.9	0	0	0.54
40T xg pellet	86	14	3.8	6.1	2.1	1.2	0	0	0.55
Ribosomes	20	5	12.5	20	11.7	66	0	0	0.94
Cytoplasm	0	0	28	45	0.7	3.9	2.8	96	0.03
Total	600		62.5		17.8		2.93		
Recovery (%)	89		97.5		99		97.5		

*Units- µl oxygen taken up per mg protein X total protein in fraction.

(a) Cells from a typical assimilation experiment were disrupted, the constituents physically fractionated, and then each of these fractions chemically fractionated. This will be referred to as the "control" experiment, and the cells used will be referred to as "freshly harvested" or "normal".

(b) Cells which had oxidized glucose-C^{14} in the presence of 200 µg of chloramphenicol per ml were disrupted, the constituents physically fractionated, and then each of these fractions chemically fractionated.

(c) Cells which had been starved for 3 hr under aseptic conditions, and then allowed to oxidize glucose-C^{14} were disrupted, the constituents physically fractionated, and each of these fractions chemically fractionated.

When the relative incorporation of radioactivity into these physical fractions was determined, it was found that the "membranes" 1X, ribosomes, and cytoplasm accounted for most of the labelling of the cell extracts. Table 16 gives the incorporation of C^{14} into each of the fractions from the three types of experiments, expressed as a percentage of the radioactivity in the cell extracts.

1. <u>"Membrane" fractions</u>

The percentage of radioactivity which was incorporated into the "membrane" fractions was similar in the three types of cells, and remained quite constant during the experiments, but the actual number of counts increased with time in each case. The control "membranes" contained a slightly lower percentage of the C^{14} of the extracts than did those from chloramphenicol treated or starved cells. On chemical fractionation of the "membranes", the distribution of C^{14} within each fraction was found to vary greatly (Table 17). By far the largest part of the label from control "membranes"

Table 16.

Incorporation of C^{14} into cytological fractions during oxidation of 5 µmoles of glucose-U-C^{14} by resting cells of Pseudomonas aeruginosa (Results expressed as per cent of the C^{14} of the cell extract)

Time (min)	"Membranes" A*	B*	C*	Ribosomes A	B	C	Cytoplasm A	B	C
5	14	16	17	3.4	5.8	3.1	77	72	68
15	14	18	20	2.7	3.9	3.4	78	70	66
30	14	16	18	2.6	4.4	3.7	79	71	73
120	13	16	18	2.2	4.9	5.3	79	75	72

* A- Freshly harvested cells (3.53×10^6 cpm added).
B- Chloramphenicol treated cells (3.70×10^6 cpm added).
C- Starved cells (3.94×10^6 cpm added).

was in the residual fraction, whereas that in chloramphenicol treated or starved cell "membranes" during the early stages of glucose assimilation was found in the lipid (Figure 14). However, as the oxidation of glucose progressed, there appeared to be a marked transfer of radioactivity from the lipid to the protein residue in the starved cell "membranes", and a slight shift from the lipid label to the residue label in the antibiotic treated cells. The lipid from the control cell "membranes" contained little radioactivity, although lipid was found experimentally to make up 21% of the dry weight of this fraction.

There seemed to be less inhibition by chloramphenicol of C^{14} incorporation into the residual fractions, presumably protein, of the "membranes" than into the cytoplasm. At 120 min, based on the percentage of C^{14}

Table 17.

Incorporation of C^{14} into chemical fractions of "membranes" during oxidation of glucose-U-C^{14} by control, chloramphenicol treated, or starved resting cells of Pseudomonas aeruginosa

Time (min)	Alcohol-soluble protein	Lipid	Hot soluble*	Residue
		Control		
	Per cent of C^{14} in cell extract			
5	1.4	1.5	1.2	9.8
30	1.9	1.0	2.2	9.0
120	1.3	0.5	1.5	9.9
	Per cent of C^{14} in "membranes"			
5	11.3	10.6	8.4	70
30	13.6	7.1	15.4	64
120	10.0	4.1	10.7	76
		Chloramphenicol treated		
	Per cent of C^{14} in cell extract			
5	1.3	6.4	1.7	6.3
30	1.1	7.7	2.2	5.0
120	0.9	6.3	2.3	6.5
	Per cent of C^{14} in "membranes"			
5	8.5	41	11.0	41
30	7.0	48	13.5	31
120	5.4	39	14.5	41
		Starved		
	Per cent of C^{14} in cell extract			
5	0.2	12.5	1.0	3.4
30	0.2	11.5	1.6	4.7
120	0.2	8.6	1.6	7.8
	Per cent of C^{14} in "membranes"			
5	1.0	73	6.0	20
30	1.0	64	9.0	26
120	1.0	48	9.0	43

* Hot trichloroacetic acid soluble fraction.

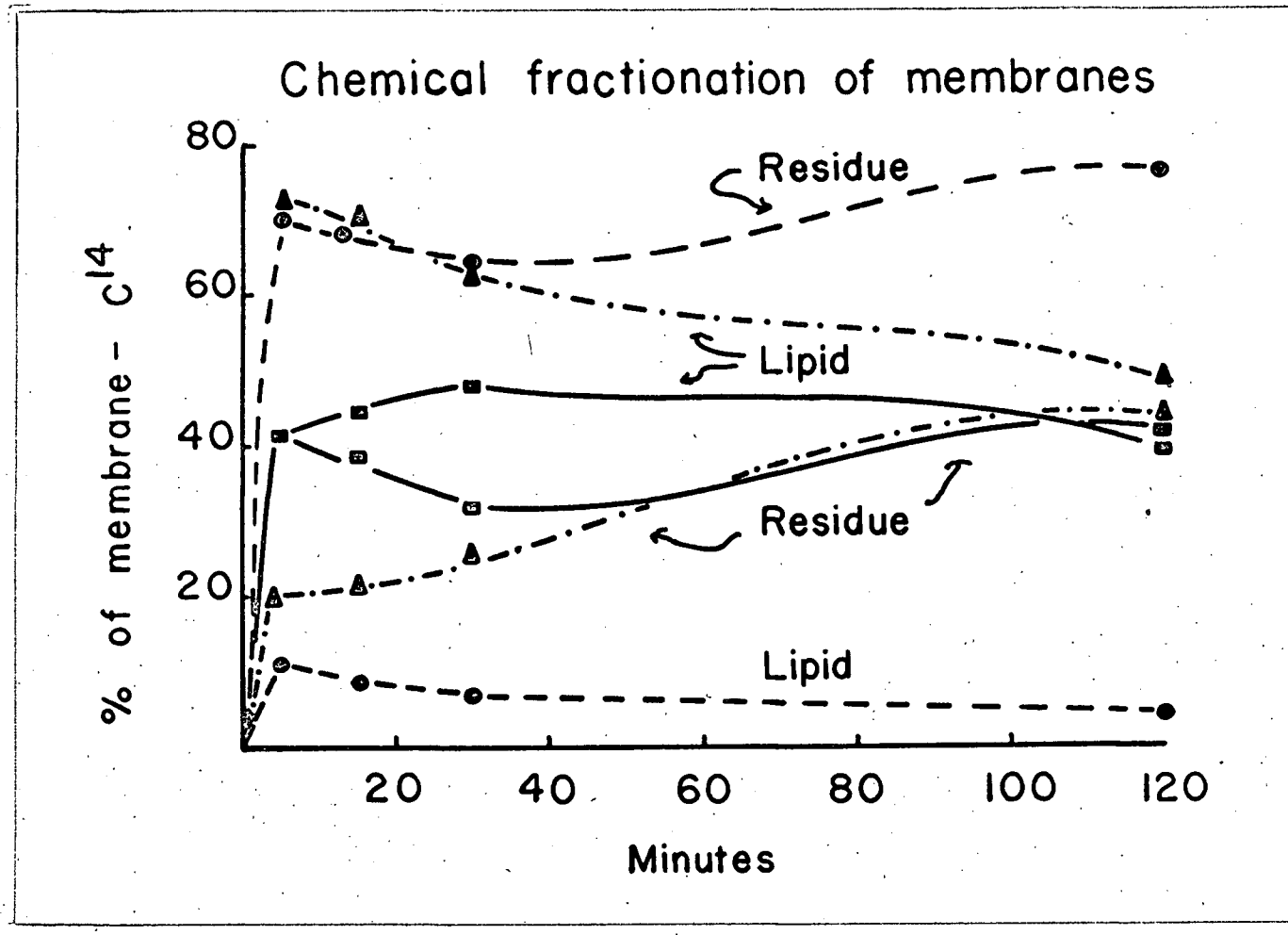


FIG. 14. Incorporation of C^{14} into the protein residue and lipid of the "membrane" fractions during oxidation of glucose-U-C^{14} by washed cells of Pseudomonas aeruginosa. Control cells ●-----●, chloramphenicol treated cells ■ —— ■, starved cells ▲ •—•—• ▲.

in each fraction, the inhibition was 48% and 86%, for "membranes" and cytoplasm, respectively. Accordingly, the 30 and 120 min "membrane" residue samples were hydrolysed, and analysed by column chromatography. As Table 18 shows, there was a high percentage of non amino acid C^{14} in the water eluates of the Dowex-50 columns after chloramphenicol treatment of the cells, but only about 10-15% in the other two.

Table 18.

Column chromatography of the residual fractions from "membranes" of control, chloramphenicol treated, or starved cells

Fraction	Dowex-50 H_2O (neutral and acidic)		Dowex-1 H_2O (neutral)		Dowex-1 HCl (acidic)		Dowex-50 NH_4OH (amino acids)	
	cpm*	%	cpm	%	cpm	%	cpm	%
Control-30	0.99	16	0.48	8	0.51	8	4.68	84
Chloram.-30	1.29	55	0.79	33	0.51	22	1.12	45
Starved-30	0.88	25	0.46	13	0.42	12	2.64	75
Control-120	1.46	11	-	-	-	-	11.30	89
Chloram.-120	1.08	31	-	-	-	-	2.46	69
Starved-120	1.35	15	0.63	7	0.72	8	7.58	85

* cpm x 10^{-3}

The non amino acid radioactivity was found, on further fractionation by column chromatography on Dowex-1 resin, to consist of about equal quantities of neutral and acidic compounds, but there was so little radioactivity in these materials that their identities could not be established.

The neutral compounds may represent cell wall carbohydrates, or sugars from the "membranes" themselves. Studies have shown that, in some bacteria, hexose (glucose or mannose) makes up to 20% of the dry weight of the membranes, probably in the form of a glycolipid (70). The acidic compound found in these "membranes" could come from this glycolipid also, or from the rhamnolipid which has been found to be formed by P. aeruginosa cells (80,81,93). Chloramphenicol treatment of the cells caused little or no inhibition of labelling of these non amino acid components, but did result in a drastic reduction (75%) of incorporation of C^{14} into the "membrane" protein, after correction for non proteinaceous radioactivity. In freshly harvested cell "membrane" fractions, the activity of the protein was high, while in the starved cell "membranes" during the early stages of assimilation, protein was of low specific activity, although this increased rapidly as the experiment progressed.

Radioactive peaks in the NH_4OH eluates from the columns were found, by paper chromatography and scanning, to correspond only to ninhydrin positive compounds. No diaminopimelic acid could be demonstrated.

Table 19 gives the specific activity of the nucleic acids at 5 min and 120 min in each of the "membrane", ribosomal, and cytoplasmic fractions. Although the RNA of the "membranes" made up only 6-7% of the total RNA of the cell extract, it was of high specific activity in each of the three types of cells, but especially so in the 5 min chloramphenicol sample.

2. Ribosomal fractions

The incorporation of radioactivity into the ribosomes, which contained 20% of the protein, and 66% of the total RNA of the cell extracts, was low throughout each experiment, indicating a slow turnover of the

Table 19.

Specific activity of nucleic acid from cytological fractions of control, chloramphenicol treated, or starved cells of *Pseudomonas aeruginosa*

Fraction	mg RNA in fraction		cpm in RNA		Specific activity cpm/mg RNA	
	5 min	120 min	5 min	120 min	5 min	120 min
"Membranes"						
Control	0.058	0.072	760	3,600	13,100	50,000
Chloramphenicol	0.060	0.076	1,120	3,060	18,700	40,300
Starved	0.062	0.062	710	3,080	11,400	49,900
Ribosomes						
Control	0.552	0.552	500	600	905	1,090
Chloramphenicol	0.517	0.545	1,650	3,400	3,190	6,250
Starved	0.560	0.515	700	4,000	1,250	7,750
Cytoplasm						
Control	0.080	0.085	507	3,120	6,340	36,700
Chloramphenicol	0.088	0.088	1,060	2,930	12,100	33,300
Starved	0.075	0.085	213	3,090	2,840	36,400

protein and nucleic acid (Table 16). In the control cells, the percentage of added C^{14} incorporated into the ribosomes decreased with time, although the actual amount of radioactivity remained fairly constant (Figure 15). This decrease in apparent labelling was shown, by a chemical fractionation,

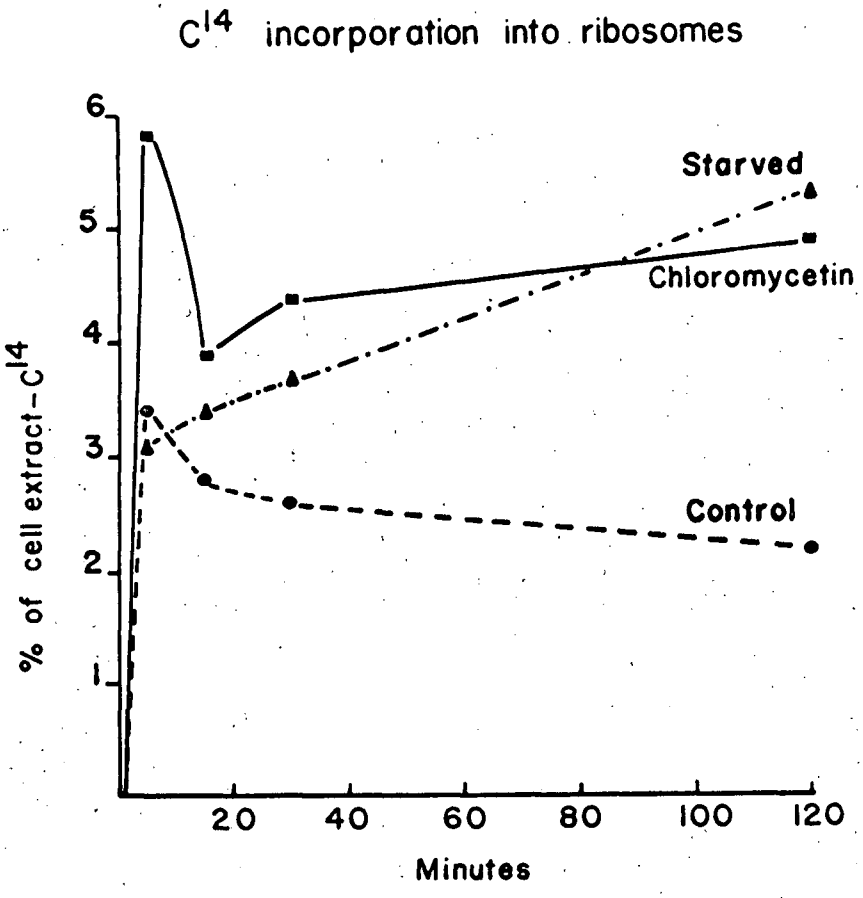


FIG. 15. Incorporation of C^{14} into the ribosomal fractions during oxidation of glucose-U-C^{14} by washed, control, chloramphenicol (chloromycetin) treated, or starved cells of Pseudomonas aeruginosa.

to be the result of a low incorporation of C^{14} into the nucleic acid of the hot trichloroacetic acid extract, while the amount of C^{14} in the protein residue increased during the experiment, and made up most of the radioactivity of the fraction (Figure 16, and Table 20). On the other hand, with chloramphenicol treated or starved cells, there was an increase both in absolute and in relative incorporation of label into the ribosomes over that of the control. The ribosomal RNA of the former cells was much more highly labelled than that of the control cells, whereas protein contained little C^{14}. A calculation of the specific activity of the ribosomal RNA showed that it was low in all three cases (Table 19). However, in antibiotic treated cells early in the experiment, and in starved cell ribosomes in the later stages, the specific activities were much higher than that of the control.

3. Cytoplasmic fractions

In the cells from each type of experiment, the cytoplasm, which contained 45% of the protein of the extract, also contained the bulk of the C^{14} (Table 16). The relative amount of radioactivity incorporated into this fraction did not change significantly during the course of each experiment, but as with the "membranes" and ribosomes, the absolute number of counts increased. Changing the conditions of the experiments proved to cause only a slight variation in the relative amount of cytoplasmic C^{14} (Table 16). The significant change occurred in the distribution of radioactivity among the chemical constituents of the cytoplasm (Figure 17 and Table 21). At 120 min, most of the label in the cytoplasm of the control experiment was found in the protein residue, the C^{14} being incorporated into it at a faster rate than into either the cold trichloroacetic acid pool components, or the lipid and alcohol soluble protein. This cellular compon-

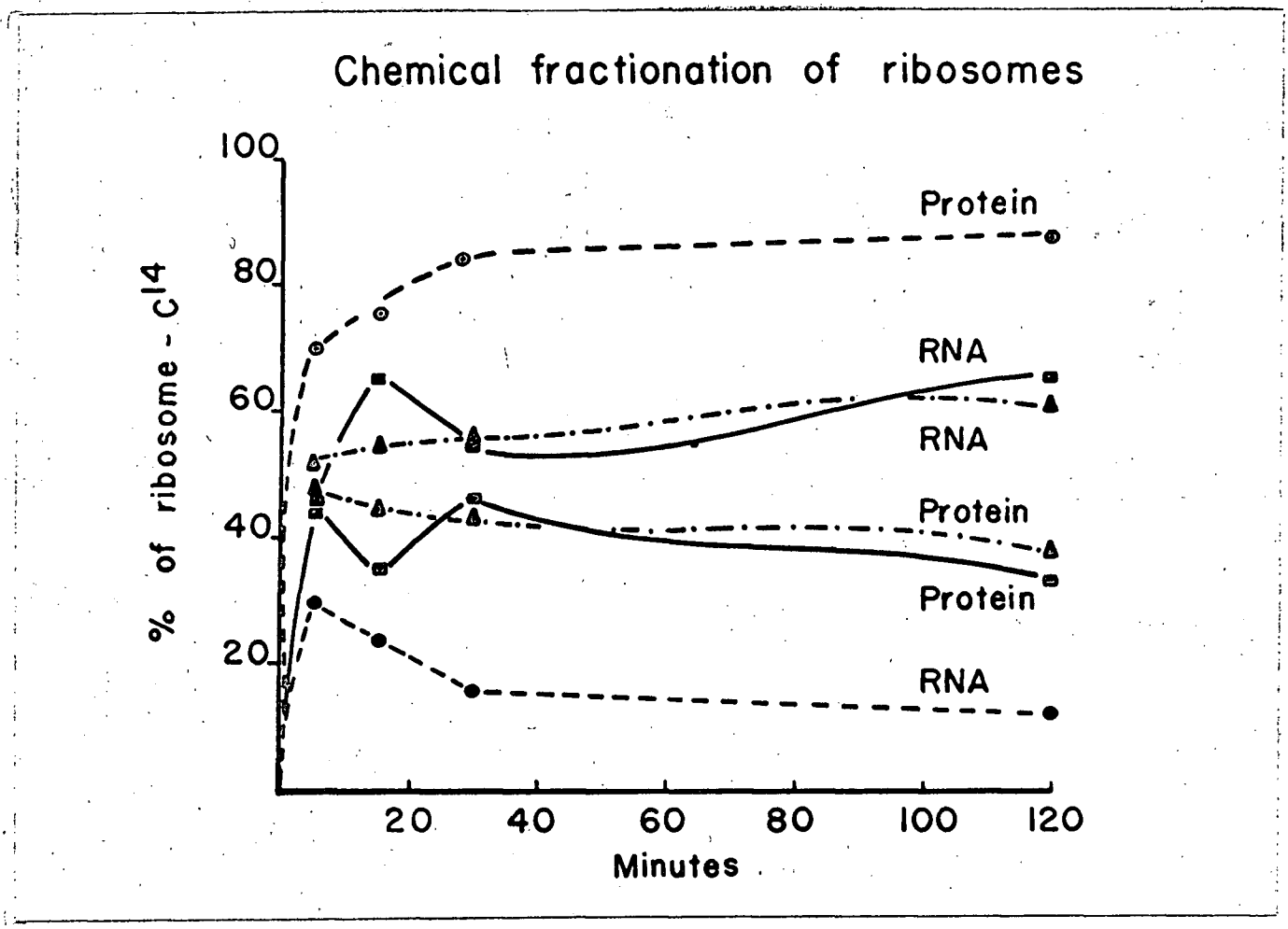


FIG. 16. Incorporation of C^{14} into the RNA and protein residue of the ribosomes during oxidation of glucose-U-C^{14} by washed cells of Pseudomonas aeruginosa. Control cells O-----O, chloramphenicol treated cells □ —— □, starved cells Δ·—·—·Δ.

Table 20.

Incorporation of C^{14} into the protein residue and the RNA of the ribosomal fractions during oxidation of glucose-U-C^{14} by control, chloramphenicol treated, or starved resting cells of Pseudomonas aeruginosa

Time (min)	Hot soluble*	Residue	Hot soluble*	Residue
	Control			
	Per cent of C^{14} in cell extract		Per cent of C^{14} in ribosomes	
5	1.0	2.4	30	70
30	0.41	2.2	16	84
120	0.29	1.9	13	87
	Chloramphenicol treated			
	Per cent of C^{14} in cell extract		Per cent of C^{14} in ribosomes	
5	2.5	3.3	43	57
30	2.4	2.0	54	46
120	3.2	1.7	65	35
	Starved			
	Per cent of C^{14} in cell extract		Per cent of C^{14} in ribosomes	
5	1.6	1.5	52	48
30	2.0	1.7	55	45
120	3.2	2.0	61	39

* Hot trichloroacetic acid soluble fraction.

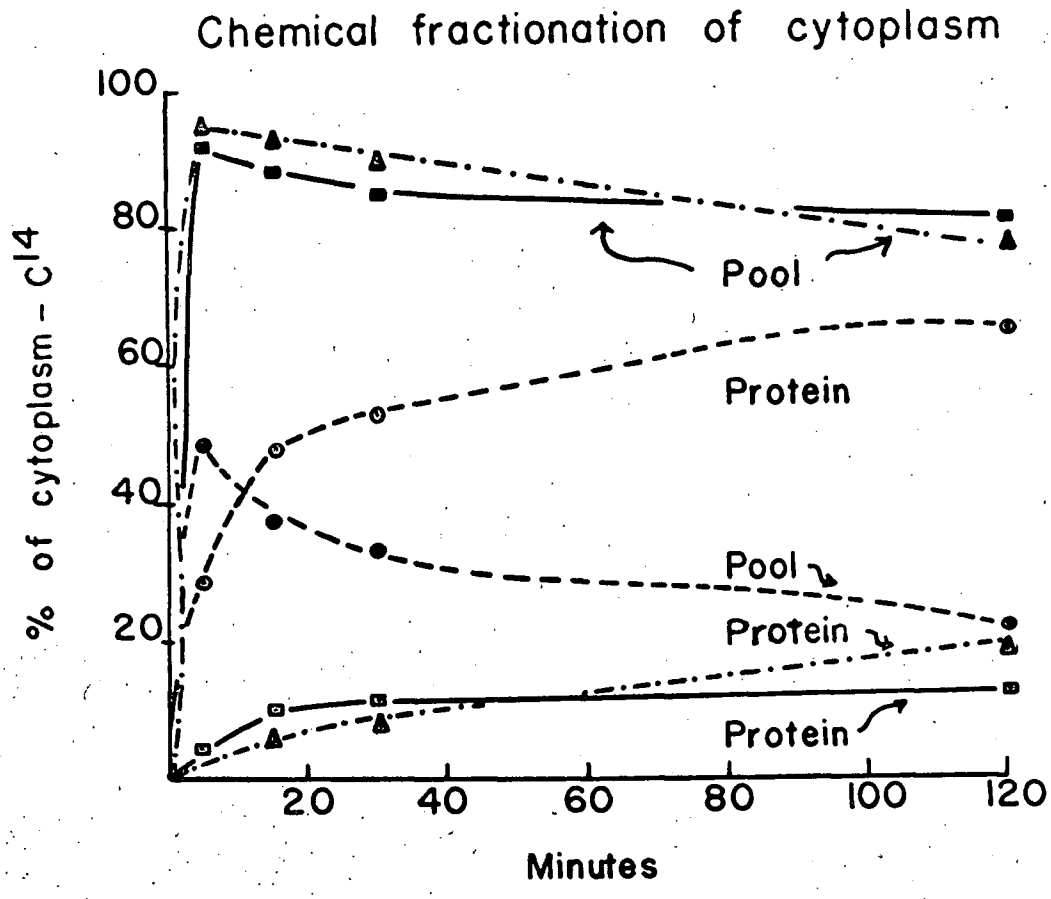


FIG. 17. Incorporation of C^{14} into the cold trichloroacetic acid soluble pools and residual fractions of the cytoplasm during oxidation of glucose-U-C^{14} by washed cells of Pseudomonas aeruginosa. Control cells ●-----●, chloramphenicol treated cells ■ —— ■, starved cells ▲·—·—·▲.

Table 21.

Incorporation of C^{14} into chemical fractions of the cytoplasm during glucose-U-C^{14} oxidation by control, chloramphenicol treated, or starved resting cells of Pseudomonas aeruginosa

Time (min)	Cold soluble*	Alcohol-soluble protein	Lipid	Hot soluble*	Residue
			Control		
		Per cent of C^{14} in cell extract			
5	38	2.6	1.3	0.8	22
30	28	2.9	0.6	1.3	35
120	17	2.9	0.4	1.3	51
		Per cent of C^{14} in cytoplasm			
5	49	4.1	2.1	1.1	29
30	36	3.8	0.7	1.6	44
120	22	2.8	0.5	1.6	65
			Chloramphenicol treated		
		Per cent of C^{14} in cell extract			
5	66	0.9	1.4	1.6	2.6
30	61	1.5	1.2	1.7	8.0
120	60	2.0	1.1	2.2	9.3
		Per cent of C^{14} in cytoplasm			
5	92	1.2	2.0	2.2	3.6
30	85	2.0	1.6	2.2	10.4
120	81	2.6	1.4	3.0	12.4
			Starved		
		Per cent of C^{14} in cell extract			
5	64.5	0.20		0.3	3.0
30	68.0	0.15		0.5	4.5
120	51.5	0.20		1.6	12.9
		Per cent of C^{14} in cytoplasm			
5	95	0.30		0.4	4.4
30	93	0.20		0.65	6.2
120	78	0.35		2.4	19.5

* Cold and hot trichloroacetic acid soluble fractions.

ent was very highly labelled, indicating that a rapid turnover of the soluble proteins was occurring during glucose oxidation. As with the "membranes" and ribosomes, synthesis of the cytoplasmic proteins was greatly decreased in the presence of chloramphenicol, and also by prior starvation of the cells. However, in starved cells, the rate of labelling of the protein continued at a rapid rate until the end of the experiment.

In both the chloramphenicol treated and starved cells, the trichloroacetic acid soluble pool made up a very much larger proportion of the radioactivity of the cytoplasm than it did in the control cells (Figure 17 and Table 21). The pool from starved cells at 5 min contained 95% of the cytoplasmic label, and this decreased only 15% during the course of the experiment, as the label in the protein residue rose. The incorporation C^{14} into lipid and alcohol soluble proteins was extremely low. With chloramphenicol, 92% of the radioactivity was in the metabolic pool at 5 min, decreasing to 81% at 2 hr. The residual protein fraction increased slightly in radioactivity during this period, although there was 86% less C^{14} incorporated than in the control. Ether extraction of the cold trichloroacetic acid soluble pools, followed by chromatography and electrophoresis of the aqueous solutions and scanning to detect the radioactive components, showed that, as with the metabolic pools from whole cells, the C^{14} was mainly in the form of amino acids in all three experiments. The distribution of radioactivity was similar to that found in pools of the different types of whole cells, in that glutamate was highly labelled, and accumulated in the starved cell fractions.

Although the actual amount of C^{14} incorporated into the hot trichloroacetic acid soluble fraction of the cytoplasm was low, a calculation of the specific activity of the nucleic acid in this fraction showed that

it was highly labelled (Table 19). The nucleic acid extracted with hot trichloroacetic acid proved to be almost all RNA, presumably soluble RNA, which has a higher rate of turnover than does ribosomal RNA. The cytoplasmic DNA, which was degraded by the DNase treatment of the extracts to reduce their viscosity, was a part of the cold trichloroacetic acid pool. The initial specific activity of the RNA formed in the presence of chloramphenicol was higher than that in the other two experiments.

C. Experiments with the cytoplasmic proteins

1. Amount of radioactivity contained in the "pH 5 enzyme"

To obtain some estimate of the C^{14} incorporated into the synthetic enzymes of the cytoplasm, the following experiment was performed. Each of the cytoplasmic fractions from the three types of cells was adjusted to pH 5.4 with acetic acid, allowed to stand in the cold 20 min, and centrifuged. Counts were made on aliquots of each fraction before this treatment, and on the supernatant solution after acidification and centrifugation. Since the enzymes responsible for the activation of amino acids, and their transfer to soluble RNA and ribosomes, are precipitated by acidification to pH 5.4, the amount of C^{14} removed at this pH should be a measure of the synthesis of these enzymes during glucose oxidation. The results are given in Table 22.

These data indicate that, under conditions of prior starvation of P. aeruginosa cells, there was a definite decrease in the amount of C^{14} incorporated during glucose oxidation into the fractions containing the enzymes responsible for protein synthesis, as compared to freshly harvested cells. This would help to explain why, in the starved cells, protein synthesis was greatly reduced. Chloramphenicol caused a complete inhibition

Table 22.

Percentage of C^{14} removed by acidification of the cytoplasmic fractions to pH 5.4

Time	Control	Chloramphenicol	Starved
(min)	%	%	%
5	3	0	1
15	15	0	2
30	18	0	3
120	10	3	10

of the incorporation of radioactivity into these fractions, except at 2 hr, when a slight labelling was found, and shows that protein synthesis was occurring.

2. Effect of starvation on the activity of the aminoacyl-s-RNA synthetases

Additional information on the state of the amino acid activating enzymes during starvation of P. aeruginosa cells, and during glucose assimilation by these cells, was gained by assaying the formation of aminoacyl-s-RNA by these synthetases. Preliminary experiments indicated that only the cytoplasmic enzymes were capable of activating the amino acids. An experiment was therefore carried out in which the cells were starved, allowed to oxidize glucose, then disrupted and physically fractionated. Samples were taken at intervals during the starvation and assimilation periods. The amount of enzyme which was limiting for the formation of aminoacyl-s-RNA in the zero time sample was determined, and then the relative activities of the enzymes of the other time intervals were assayed in the same manner.

Protein determinations were done on each fraction, and the specific activity (cpm per µg protein) was calculated. The results are shown in Figure 18 and Table 23.

Table 23.

Incorporation of C^{14} amino acids into s-RNA by cytoplasmic enzymes of starved cells of Pseudomonas aeruginosa

Fraction	Protein	C^{14} incorporated	Specific Activity
	µg/0.003 ml	cpm/0.003 ml	cpm/µg protein
Period of starvation			
zero	7.0	3710	530
1.5 hr	8.2	3780	460
3.0 hr	7.4	3240	440
Period of assimilation			
15 min	6.5	3320	510
30 min	8.0	4130	515
120 min	8.4	4480	535

Therefore, it appeared that, during starvation of P. aeruginosa, the enzymes responsible for amino acid incorporation were degraded, and that they were resynthesized while glucose was assimilated. This would explain the low incorporation of radioactivity from glucose into protein by starved cells during the early stages of assimilation. As glucose oxidation progressed, the protein synthesizing enzymes were quickly regenerated, and incorporation into protein increased. Despite the indication that these

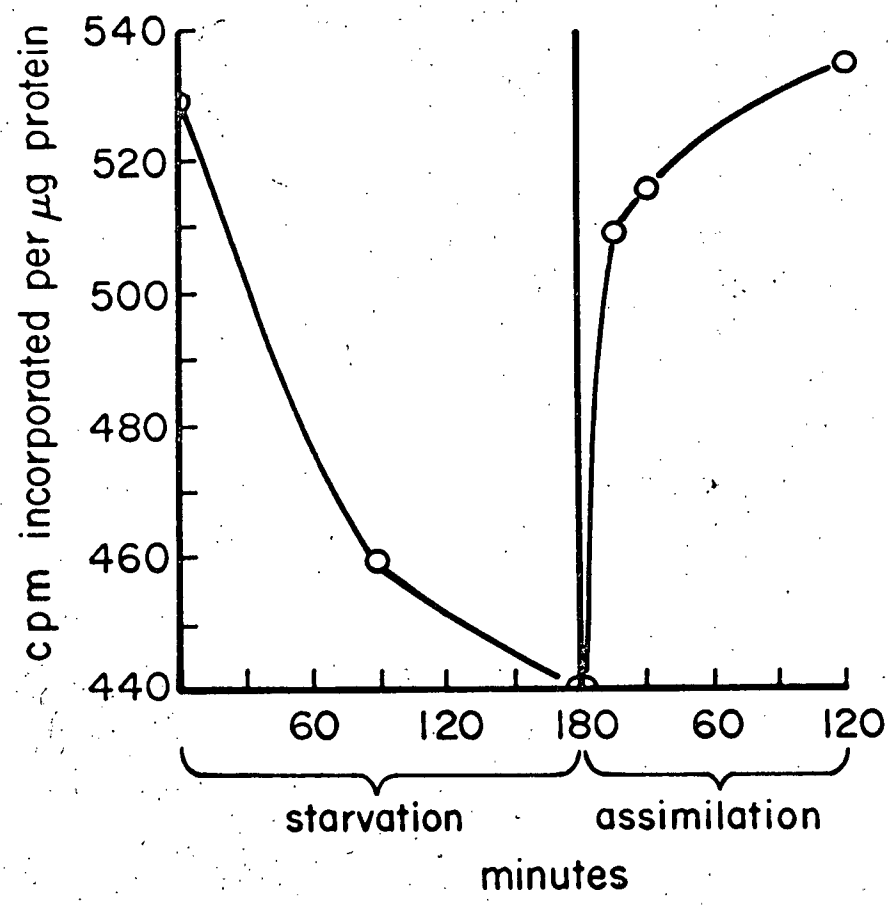


FIG. 18. Incorporation of C^{14} amino acids into s-RNA by cytoplasmic fractions of Pseudomonas aeruginosa.

enzymes were resynthesized while glucose was being oxidized, they accounted for only a small proportion of the cytoplasm of the starved cells, at least during the early stages of the experiment, compared to that for the control cells (Table 22). One would think from the reactivation curve in Figure 18, that the proteins of the pH 5 enzyme would be preferentially synthesized during assimilation, but this did not appear to be the case.

The cytological distribution of the carbon assimilated from glucose was perhaps not surprising, since it was known from a previous chemical fractionation of whole cells (55) that the incorporation of label occurred mainly into proteins, and from preliminary experiments that cytoplasmic proteins constituted by far the largest chemical fraction of the cell. Therefore, the finding that 60-65% of the C^{14} of the cell was in the soluble proteins of the cytoplasm in the control experiment was consistent with these observations (Table 21). However, the high specific activity of the cytoplasmic proteins, which are mainly enzymes, was somewhat unexpected, since the cells were grown on glucose, and one would have thought that most of the necessary enzymes for its oxidation would have already been present. That most of these newly synthesized enzymes were not degradative was shown by the experiment with chloramphenicol, where, although the synthesis of soluble protein was inhibited 86%, glucose oxidation proceeded at a rapid rate. Since nearly 20% of the total radioactivity of the control cytoplasm was found in the pH 5 enzyme fraction, some of the newly synthesized soluble protein was concerned in protein synthesis. The lower incorporation of C^{14} into this fraction in starved cells corresponded to the decrease of protein synthesis in these cells. During endogenous respiration, the activity of the aminoacyl-s-RNA synthetases also declined,

but was restored by the assimilation of glucose. The reduction in these enzymes may be a result of their endogenous breakdown (75).

An effect of chloramphenicol on the metabolic pool components similar to that found in these experiments with P. aeruginosa, has been reported in Vibrio cholera (78). In this case also, the amount of amino acids in the pool was higher, but its qualitative composition was unchanged.

It has been known for some time, that, whereas chloramphenicol inhibits protein synthesis in bacteria, it allows RNA synthesis to continue at an increased rate (67); however, the nature of this "chloramphenicol RNA" is still under discussion. Most reports agree that the RNA is of high molecular weight, having a sedimentation coefficient of 14 to 16 S (119), which rises to 30 S if the centrifugation is done in the presence of 10^{-2} M magnesium ions (47). Aronson and Spiegelman (9) have found that after high speed centrifugation of cell extracts, the chloramphenicol RNA appeared in the particulate fraction with the ribosomes. There are two main schools of thought as to just what this high molecular weight chloramphenicol RNA represents. Gros et al. (76) showed that in E. coli, chloramphenicol RNA possessed properties similar to those of a rapidly labelled RNA, termed "messenger RNA", and that in common with messenger RNA, it stimulated amino acid incorporation into protein. There is normally a rapid breakdown of messenger RNA, but Gros and his group believe that this does not occur unless protein elongation is completed, and that chloramphenicol stabilizes this RNA by stopping protein synthesis. The antibiotic does not inhibit the continued synthesis of the RNA, however. The effects of actinomycin and chloramphenicol on RNA synthesis were investigated by Reich and his associates (128). Actinomycin, which prevents DNA-dependent RNA synthesis, inhibits the synthesis both of the rapidly labelled RNA produced

normally, and of chloramphenicol RNA. Reich et al. (128), and Aronson and Spiegelman (10) take the stand that messenger and chloramphenicol RNA's are merely precursors of ribosomal RNA, since the pulse labelled fraction of RNA enters the ribosomes intact, as does chloramphenicol RNA, providing that the organisms, after removal from the antibiotic, are placed in a medium which permits protein synthesis. The high molecular weight RNA, then, is unstable until it is combined with protein. Thus, there are two proposed templates for protein synthesis: the ribosomal RNA, or a transient, DNA-like messenger RNA.

Despite the proliferation of data indicating that chloramphenicol RNA is of high molecular weight, Yee and Gezon (178), and Yee et al. (177) have reported that the RNA formed by starved cells of Shigella flexneri in the presence of chloramphenicol is a functional s-RNA, with the expected base ratios. The s-RNA of this organism was found to be increased by 160% after 4 hr of incubation in a complete medium with the antibiotic. Extracts of S. flexneri were prepared by grinding, however, which Aronson and Spiegelman (10) found resulted in the solubilizing of ribosomal RNA, as a result of degradation of the ribosomes. This would give an apparent increase of s-RNA in these experiments, but it should not give an increase in functional s-RNA.

The results of the experiments with P. aeruginosa, in which the oxidation of glucose-C^{14} by resting cells in the presence of chloramphenicol gave rise to an increase in the radioactivity of the ribosomal RNA over that of the control (Table 20), are in accordance with previous reports of an increase in synthesis of high molecular weight RNA when protein synthesis is prevented. This chloramphenicol RNA would appear mainly in the ribosomal fraction from high speed centrifugation, and result in a high initial incor-

poration of C^{14} into the ribosomal RNA. Normal ribosomal RNA has a slow turnover (47), as shown by the very low specific activity of this fraction in the control cells, whereas chloramphenicol RNA is constantly turning over (88). Cytoplasmic RNA, although only a minor part of the total RNA, was also of high specific activity in the chloramphenicol treated cells, during the first stages of glucose oxidation. This could be the result of degradation of high molecular weight RNA, or increased synthesis of s-RNA as suggested by Yee and Gezon (178).

The increase in label of the ribosomal RNA in starved cells, on the other hand, occurred late in the experiments (Table 20), and can be more readily explained by its resynthesis after depletion during endogenous respiration, than as a manifestation of the decrease in protein synthesis (29,75). If the latter situation were the case, one would expect that the initial label, like that in the antibiotic treated cells, should be high. Clifton (36), and Duncan and Campbell (55) have suggested that assimilation may serve to replenish the endogenous reserves of microorganisms, and Campbell, Gronlund and Duncan (29) have shown that RNA is a major endogenous substrate for P. aeruginosa.

Hunter et al. (89), Butler, Godson and Hunter (28), and Hunter and Godson (90) have reported that the membrane lipoprotein, which contains most of the cell lipid, was the initial site of incorporation of lysine-C^{14} in B. megaterium. Chloramphenicol or phospholipase inhibited the uptake of amino acid by the lipid. Only the phospholipid component was active, and after extraction of the lipoprotein with hot alcohol, the amino acid was found to be associated with the lipid portion. In this connection, Silberman and Gaby (137) have shown that the lipid of P. aeruginosa contains bound amino acids, but the effect of chloramphenicol on the uptake

of amino acids by this microorganism was not investigated. In contrast to the results of Hunter et al. (28,89,90), in which chloramphenicol was shown to inhibit the incorporation of C^{14} amino acids into B. megaterium membrane lipoprotein, there was a marked increase in the incorporation of C^{14} from glucose into the alcohol-ether soluble fraction of the "membranes" of P. aeruginosa in the presence of the antibiotic (Table 17). Duncan and Campbell (55) have also reported an increased labelling of the total lipid of the cell when protein synthesis was inhibited in this microorganism. This increase may be the result of more substrate being available for lipid synthesis when protein synthesis is not occurring, or it may be due to the formation, by a chloramphenicol insensitive route, of lipo-amino acid complexes in P. aeruginosa, but not in B. megaterium. These lipid-amino acid complexes would then accumulate during the inhibition of protein synthesis. Since glucose C^{14} was used in the experiments with P. aeruginosa, it is not known whether the radioactivity was in the lipid per se, or whether the C^{14} was in the form of amino acids attached to the lipid. Chloramphenicol is known to inhibit the processes involved in transfer of the amino acid residues from s-RNA to the ribosomes (99), and therefore, if the postulated lipid-amino acid complexes were formed prior to the production of aminoacyl s-RNA, one could visualize how the antibiotic would have no effect on their formation. However, Hunter and Godson (90) have proposed that the amino acids are transferred from the s-RNA to phospholipids, and then to the ribosomes.

An even greater initial increase in label of the lipid component of the "membranes" was found in starved cells (Table 17). As the experiment progressed, the rate of C^{14} incorporation into lipid decreased, as protein synthesis increased (Figure 14). This phenomenon may be explained

by either of the two explanations previously considered. Unlike chloramphenicol treated cells, however, the relative label in the total lipids of the starved cells was not higher than that of the control (see Section I, C 4).

Many reports have appeared concerning the presence of small amounts of RNA in the membrane fraction of microorganisms (1,24,30,43,89, 142), and the high specific activity of this RNA after exposure of E. coli cells to P^{32} or C^{14} has also been demonstrated (43,142,158). It has been suggested that this RNA is not merely an artifact arising as a result of the fractionation procedure, but that it may be an integral part of ribosomes attached to the membranes, and represent an RNA active in protein synthesis (43). Campbell et al. (29) were able to remove the RNA from lysozyme prepared "membranes" of P. aeruginosa by treatment with versene, and alternate freezing and thawing. After such treatment, the "membrane" fraction could be separated into two components by centrifugation at 25,000xg, which yielded a pellet ("membranes") and the supernatant from which ribosomes could be sedimented at 100,000xg. McQuillen et al. (112) have shown that some ribosomes of E. coli are associated with the membrane. Suit (149), in P^{32} experiments with E. coli "membranes", was able to find no function for "membrane" RNA in cell wall synthesis, but instead later obtained evidence that it might consist of messenger RNA, since, after infection of the organism with T_2 bacteriophage, "membrane" RNA had base ratios similar to that of the phage DNA (150).

The small amount of RNA associated with washed "membranes" of P. aeruginosa was also found to have a high specific activity, and therefore appears to be different than the ribosomal RNA, whose label is low (Tables 17, 19 and 20). The high incorporation of C^{14} into the "membrane" RNA of

control cells could be explained by its possible function as a messenger suggested by Suit (150), which would result in a rapid turnover, and an increased label. The same interpretation of results could be used for the starved cells, with the additional argument that this RNA may also be utilized as an endogenous reserve, and thus be resynthesized during glucose oxidation. The initial high specific activity of the membrane RNA in antibiotic treated cells would be a manifestation of the formation of chloramphenicol RNA, some of which would be attached to the "membrane" ribosomes.

IV. Species Specificity of s-RNA's and Aminoacyl-s-RNA Synthetases

A. Cytological location of aminoacyl-s-RNA synthetases in P. aeruginosa

When the cytological fractions ("membranes", ribosomes, and cytoplasm) from P. aeruginosa were tested for their aminoacyl-s-RNA synthetase activity with the homologous s-RNA, it was found that the cytoplasm was the only fraction which was active (Table 24). Addition of the "membranes" or ribosomes to the cytoplasm did not stimulate incorporation of C^{14} amino acids into the P. aeruginosa s-RNA, nor did either of these fractions replace the s-RNA. Chloramphenicol (200 µg per ml) produced no inhibition of the formation of aminoacyl-s-RNA by the cytoplasmic enzymes. The low blank values when the s-RNA was omitted showed that none of the cell fractions contained enough s-RNA to support the incorporation of measurable amounts of amino acids. If the amount of cytoplasm was reduced by one-half, there was an increase of 15% in the number of counts incorporated; this could not be duplicated by raising the amount of ATP in the assay, and may have been due to the presence of unlabelled amino acids in the cytoplasmic fractions which

Table 24.

Incorporation of C^{14} amino acids into s-RNA by *Pseudomonas aeruginosa* cell fractions

Fraction	s-RNA	Incorporation of C^{14} into s-RNA
		cpm
Cytoplasm (0.025 ml)	-	700
Cytoplasm (0.050 ml)	-	910
Cytoplasm (0.025 ml)	+	24,210
Cytoplasm (0.050 ml)	+	21,220
"Membranes" (0.050 ml)	-	770
"Membranes" (0.050 ml)	+	1,150
Cytoplasm (0.025 ml) "Membranes" (0.025 ml)	-	800
Cytoplasm (0.025 ml) "Membranes" (0.025 ml)	+	26,130
Cytoplasm (0.050 ml) Ribosomes (0.050 ml)	-	900
Cytoplasm (0.050 ml) Ribosomes (0.025 ml)	+	21,440
Cytoplasm (0.050 ml) Chloramphenicol (80 µg)	+	19,740

would cause a dilution of the C^{14} amino acids added.

Hunter et al. (89) have reported that, when B. megaterium extracts were prepared by lysozyme treatment in a medium of high ionic strength, the enzymes responsible for activation and incorporation of the amino acids were associated with the membrane fraction, whereas, if the ionic strength were low, these enzymes appeared in the cytoplasm. In an effort to determine whether the use of a high ionic strength suspending fluid would cause the amino acid activating enzymes of P. aeruginosa to be associated with the membranes, extracts were prepared in 0.33 M phosphate buffer as was used for the preparation of membranes of B. megaterium. Perhaps because of the deficiency of magnesium ions in the phosphate buffer, it was difficult to obtain an extract of low viscosity by the use of DNase treatment. However, satisfactory extracts were finally prepared, and separation of the fractions by centrifugation proceeded normally. These fractions were tested for their ability to incorporate amino acids into P. aeruginosa s-RNA. The amount of s-RNA used was only one-half of that of the experiments reported in Table 24, and the incorporation of C^{14} was correspondingly lower (Table 25). It is evident that, unlike those of B. megaterium, the aminoacyl-s-RNA synthetases of P. aeruginosa were still found in the cytoplasm, even at a high ionic strength. However, the B. megaterium extracts were prepared by digestion with lysozyme, which is a less severe treatment than the high pressure used in these experiments for breaking P. aeruginosa cells.

Fraser (61) has recently shown with rat liver that heated cytoplasm could be used as a source of s-RNA. Since this would simplify the experiments considerably, this approach was investigated with P. aeruginosa. The heated cytoplasm was prepared as described by Fraser (61), from the

Table 25.

Incorporation of C^{14} amino acids into s-RNA by cytological fractions of Pseudomonas aeruginosa prepared at a high ionic strength

Fraction	s-RNA	Incorporation of C^{14} into s-RNA
		cpm
"Membranes"	+	620
Cytoplasm	+	8940
Cytoplasm "Membranes"	+	9060

fraction used in the experiments reported in Table 24. The control experiment in this case contained 0.5 mg s-RNA (Table 26). The data in Table 26 show that the heated cytoplasm did not cause a stimulation of counts incorporated into the alcohol precipitate. There was an increase of C^{14} incorporated in the presence of the heated cytoplasm, but this was due to the occlusion of C^{14} by the large amount of precipitate, as indicated by the controls in which no enzyme was added. The heated cytoplasmic fraction did not appear to inhibit the incorporation of C^{14} into the system where both enzyme and s-RNA were present.

B. Interspecific reactions between s-RNA's and aminoacyl-s-RNA synthetases

In preliminary experiments with P. aeruginosa ATCC 9027, it was found that yeast s-RNA reacted very poorly with the bacterial enzyme system. A similar situation with yeast s-RNA and E. coli activating enzymes has been reported for a number of amino acids (14,31,51,182), but there has

Table 26.

Incorporation of C^{14} amino acids into the s-RNA present in the heated cytoplasm of Pseudomonas aeruginosa

Enzyme	s-RNA	Heated pH 5 fraction of cytoplasm	Incorporation of C^{14}
		ml	cpm
Cytoplasm	-	-	400
Cytoplasm	+	0.25	7250
Cytoplasm	+	-	5270
Cytoplasm	-	0.10	1460
None	-	0.10	1380
Cytoplasm	-	0.25	2660
None	-	0.25	2530

been little work done on interbacterial systems. Three pseudomonads (P. aeruginosa ATCC 9027, P. aeruginosa 120 Na, and P. fluorescens A 3.12), as well as Achromobacter B81, E. coli B, and bakers' yeast were selected for study. It was thought that, if any species specificity existed between s-RNA's and aminoacyl-s-RNA synthetases, this should be shown by greater heterologous reactions among the Pseudomonas species and Achromobacter B81, than between these bacteria and E. coli or yeast.

The cross reactions were done in all combinations possible, since sometimes an enzyme will show a greater reaction with a heterologous s-RNA than would be indicated by the reverse cross (51,182). Blank tubes containing enzymes, but no s-RNA, were run to rule out a contribution from the s-RNA in the enzyme fraction. The incorporation of C^{14} in these controls

was found to be of the order of 1200 cpm. Table 27 gives the number of counts per minute incorporated into each of the s-RNA's by the homologous and heterologous enzymes. Table 28 shows the percentage of incorporation of C^{14} into each s-RNA, based on the amount of incorporation by the homologous enzyme system, and Table 29 gives the per cent of radioactivity incorporated as compared to the homologous s-RNA system.

These data show that the conditions of the assay were at least adequate for each homologous system, since, with the exception of Achromobacter B81, there was an almost constant amount of C^{14} incorporated into each s-RNA (40,000 cpm). The poor reaction in the Achromobacter system, into which only about half as much C^{14} was incorporated, appeared to be due to the s-RNA, because the synthetases from this organism were able to acylate the s-RNA's from the other bacteria (though not the yeast) with a higher efficiency than the homologous s-RNA. None of the bacterial s-RNA's reacted well with the yeast enzyme, and only the enzyme from E. coli incorporated much C^{14} into the yeast s-RNA. The E. coli synthetases incorporated 68% of the counts of the homologous bacterial, or 74% of the homologous yeast, system into the yeast s-RNA.

A higher amount of incorporation of amino acids into a heterologous s-RNA than into the homologous one was found with two other enzyme systems besides that of the Achromobacter already mentioned. Thus, the P. aeruginosa ATCC 9027 and the E. coli enzymes gave better reactions with the s-RNA's of P. aeruginosa 120 Na and P. fluorescens than they did with their own. Doctor and Mudd (51), studying interspecific reactions between yeast, E. coli, and rat liver systems, reported a similar phenomenon, especially with the rat liver enzymes. They suggested several reasons for these anomalous findings, but none was established as being responsible

Table 27.

Incorporation of C^{14} amino acids into s-RNA's of various microorganisms by homologous and heterologous enzymes

Enzyme / s-RNA	P. aerug. 9027	P. aerug. 120 Na	P. fluor. A 3.12	Achr. B81	E. coli	Yeast
	cpm	cpm	cpm	cpm	cpm	cpm
P. aerug. 9027	41,060	26,080	30,730	23,200	43,830	8,670
P. aerug. 120 Na	54,480	43,230	36,730	37,550	57,480	13,650
P. fluor. A 3.12	51,230	35,980	38,250	48,250	55,130	17,030
Achr. B81	17,370	13,830	13,750	20,980	24,330	6,320
E. coli	32,930	28,180	26,030	33,400	39,930	10,850
Yeast	15,130	8,000	13,380	13,700	26,980	36,150

Table 28.

Percentage of C^{14} amino acids incorporated into s-RNA's based on the amount found in the system homologous for the enzyme

Enzyme / s-RNA	P. aerug. 9027	P. aerug. 120 Na	P. fluor. A 3.12	Achr. B81	E. coli	Yeast
P. aerug. 9027	100	60	82	110	110	24
P. aerug. 120 Na	132	100	96	179	144	38
P. fluor. A 3.12	125	83	100	230	138	47
Achr. B81	42	31	37	100	61	18
E. coli	79	65	70	160	100	30
Yeast	37	19	36	66	68	100

Table 29.

Percentage of C^{14} amino acids incorporated into s-RNA's based on the amount found in the system homologous for the s-RNA

Enzyme / s-RNA	P. aerug. 9027	P. aerug. 120 Na	P. fluor. A 3.12	Achr. B81	E. coli	Yeast
P. aerug. 9027	100	63	75	54	106	21
P. aerug. 120 Na	125	100	87	87	132	33
P. fluor. A 3.12	134	94	100	125	144	44
Achr. B81	83	64	66	100	116	30
E. coli	83	71	65	81	100	27
Yeast	43	22	37	38	74	100

for their observations. In the case of Achromobacter B81 in these experiments, it is probable that the s-RNA was damaged, because neither the enzymes from this microorganism, nor those from any of the others reacted well with it (Tables 27 and 28). As for the increase in some of the heterologous reactions with the E. coli and Pseudomonas species, one of the other explanations advanced by Doctor and Mudd (51) may be invoked: there may be different amounts of s-RNA's in different species, or, where multicomponent s-RNA's exist, the heterologous enzyme may recognize more than one s-RNA.

C. Patterns of amino acid incorporation into homologous systems

Table 30 gives the percentage of each amino acid, or group of amino acids, incorporated into the s-RNA's of the six microorganisms by the homologous enzyme systems. The incorporation of some of the amino acids was found to be very low, especially that of proline, glutamic acid and alanine.

A comparison of the average amino acid composition of several bacteria (species of Enterobacteriaceae, Pseudomonas, and Bacillus) (144, 146), and that of the Chlorella hydrolysate (Merck, Sharpe and Dohme Ltd.) is given in Table 31.

The pattern of incorporation of the amino acids by the bacteria does reflect, to some degree, their availability, and also the composition of the cells. The leucine group was incorporated to the largest extent in all of the homologous reactions, accounting for from 31% (E. coli) to 52% (Achromobacter) of the total radioactivity in the s-RNA's. The basic amino acids were the next highest, making up nearly one-quarter of the counts in P. aeruginosa ATCC 9027, P. fluorescens, and E. coli. However, large amounts of the valine group (valine and methionine) were incorporated into the s-RNA's

Table 30.

The percentage of incorporation of C^{14} amino acids into s-RNA's by homologous enzymes

Organism / Amino acid	P. aerug. 9027	P. aerug. 120 Na	P. fluor. A 3.12	Achromo. B81	E. coli B	Yeast
	%	%	%	%	%	%
Basic group*	19.4	10.6	18.5	14.0	22.3	25.2
Asp. group*	9.5	9.9	9.0	18.4	11.9	6.9
Glu. group*	9.3	2.0	3.2	10.6	3.0	3.5
Alanine	5.8	0.0	0.0	1.6	1.2	1.1
Proline	1.1	0.0	5.1	0.0	3.6	6.4
Tyrosine	5.6	14.4	9.9	0.0	10.1	12.7
Val. group*	12.1	21.1	16.5	19.2	16.2	20.3
Leu. group*	37.2	42.1	37.8	52.4	31.6	24.2

* Basic group - lys, his, arg.
Aspartic acid group - asp, gly, ser.
Glutamic acid group - glu, thr.
Valine group - val, met.
Leucine group - leu, ileu, phe.

Table 31.

A comparison of the amino acid composition of *Chlorella* hydrolysate* and of that of several bacteria (144,146)

Amino acid	Bacteria		*Chlorella* hydrolysate		
	% amino acid		% amino acid		% C^{14}
Lysine	6.4		7.4		
Histidine	2.0	13.7	2.5	18.8	18.5
Arginine	5.9		8.9		
Aspartic acid	10.2		8.8		
Serine	4.6	24.0	2.9	16.1	12.9
Glycine	9.2		4.4		
Glutamic acid	11.2	16.7	10.4	13.3	13.0
Threonine	5.5		2.9		
Alanine	10.4		7.4		5.8
Proline	4.2		7.4		5.8
Tyrosine	2.7		5.9		6.1
Valine	7.2	10.2	5.9		14.2
Methionine	3.0		*		
Leucine	8.7		13.3		
Isoleucine	5.4	17.6	5.9	25.1	21.8
Phenylalanine	3.5		5.9		

* From the data supplied by Merck Sharpe and Dohme, methionine is not present in this hydrolysate. However, if this is so, then the valine is of higher specific activity than the other amino acids.

of most of the bacteria. This was somewhat surprising, for the amounts of these amino acids in the bacteria as shown by analysis (Table 31) (144,146), and their specific activities in the Chlorella extract were about the same as the corresponding values for the aspartate and glutamate groups, and yet incorporation of these amino acids into the s-RNA's was much lower than that of valine and methionine. The incorporation of tyrosine was also higher than one would have expected from the data in Table 31, whereas that of alanine was lower. The only enzyme systems which catalysed the formation of prolyl-s-RNA were those of E. coli, P. fluorescens, and yeast.

The lack of incorporation of glutamate, glycine, and proline has been reported previously. Glutamyl-s-RNA synthetase was found by Alford et al. (4) to be easily inactivated by mild procedures during its isolation, and Zubay (183) was unable to detect any incorporation of glutamate into s-RNA by crude preparations. Extracts of various tissues and organisms which have been examined for glycine activation with ATP have shown little or no ATP-PP exchange (104,118). Although many aminoacyl-s-RNA synthetases are protected by 2-mercaptoethanol (5), it has been reported that alanyl s-RNA synthetase is inhibited by this substance (164). Since 2-mercaptoethanol was added routinely both to enzyme preparations and to assay mixes, the low incorporation of alanine by most of the enzymes might have been due to inactivation of the alanyl synthetase by the sulfhydryl compound. Moreover, it has also been found that alanyl-s-RNA synthetase from pig liver can be preserved in an active form only at the temperature of liquid nitrogen (164), a method of storage not available in this laboratory.

On the other hand, the rather high incorporation of the hydro-

phobic group of amino acids (leucine, isoleucine, phenylalanine, valine, methionine, and tyrosine) is common, and could be the result either of the presence of multicomponent s-RNA's for these amino acids, or the inadvertent selection of conditions favouring their reaction. It is known that in E. coli, there is more than one s-RNA for leucine, isoleucine, valine and methionine, but this is also true for serine, threonine and glutamic acid (8,17,147).

The pattern of amino acid incorporation into yeast s-RNA was somewhat different than that of the bacteria. Instead of the leucine group making up one-third to one-half of the radioactivity of the s-RNA, it accounted, as did the basic amino acids, and valine and methionine, for about 25% of the C^{14}. The remaining 25% was divided between tyrosine (13%) and the aspartic acid group and proline (6% each).

D. Patterns of amino acid incorporation into heterologous systems

The amino acids incorporated by the heterologous s-RNA - enzyme systems followed the same general scheme as that of the homologous combinations (Tables 32 - 37). Inspection of these tables revealed that each enzyme system, and each s-RNA mixture, contained all of the amino acid activities which were tested for, although in some cases, the incorporation was very low. Even with the two Pseudomonas strains, there was no "perfect fit" between heterologous s-RNA's and enzymes. Moreover, the E. coli enzymes appeared to react as well with P. aeruginosa s-RNA as did the enzymes of Achromobacter or P. fluorescens. There were, nevertheless, some interesting findings especially in the cases where the heterologous reaction was greater than the homologous one. In some of these instances, the increase in C^{14} incorporation was due to a corresponding increase in uptake

Table 32a.

Incorporation of C^{14} amino acids by P. aeruginosa ATCC 9027 enzyme into heterologous s-RNA's, and by heterologous enzymes into P. aeruginosa ATCC 9027 s-RNA

Micro-organism	P. aeruginosa ATCC 9027	P. aeruginosa 120 Na		P. fluorescens A 3.12		Achromobacter B81		E. coli B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic*	8.0	4.0	3.3	4.3	6.9	1.7	5.3	3.6	6.0	0.8	2.0
Asp.*	3.9	3.8	3.4	6.5	1.9	3.0	2.4	4.9	4.7	1.7	1.3
Glu.*	3.8	2.0	1.1	2.3	1.2	1.7	1.3	1.6	2.5	0.9	0.4
Ala.	2.4	1.2	0	1.4	0	0	1.9	0.3	0	0	0
Pro.	0.5	0.5	0	1.1	0	0	0	1.0	3.8	0	0.5
Tyr.	2.3	5.5	3.2	3.5	3.9	1.5	1.0	2.9	3.1	0	0.9
Val.*	5.0	10.3	5.0	4.6	5.9	3.3	3.2	5.1	7.0	7.7	1.2
Leu.*	15.2	27.2	10.1	27.4	10.7	6.3	8.1	13.3	16.6	4.0	2.4
Total:	41.0	54.5	26.0	51.2	30.7	17.4	23.2	32.9	43.8	15.1	8.7

* Basic group - his, arg, lys.
Aspartic group - asp, gly, ser.
Glutamic group - glu, thr.
Leucine group - leu, ileu, phi.

*Valine group - val, met.

Table 32b.

Percent of each amino acid incorporated by P. aeruginosa ATCC 9027 enzymes into heterologous s-RNA's, and by heterologous enzymes into P. aeruginosa ATCC 9027 s-RNA

Micro-organism:	P. aeruginosa ATCC 9027	P. aeruginosa 120 Na		P. fluorescens A 3.12		Achromobacter B81		E. coli B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	19.4	7.4	12.8	8.5	22.5	9.6	22.9	10.9	13.8	5.1	23.6
Asp.	9.5	7.0	12.9	12.7	6.3	17.3	10.3	14.7	10.8	11.4	15.2
Glu.	9.3	3.6	4.3	4.5	3.9	9.8	5.6	4.9	5.8	6.1	4.3
Ala.	5.8	2.2	0	2.7	0	0	8.1	0.8	0	0	0
Pro.	1.1	1.0	0	2.2	0	0	0	3.0	8.7	0	5.4
Tyr.	5.6	10.1	12.2	6.9	12.9	8.4	4.5	9.1	7.2	0	10.4
Val.	12.1	18.9	19	8.9	19.3	19.1	13.8	15.5	16	51	14
Leu.	37.2	49.7	38.8	53.5	35	35.8	35	41.3	37.9	26.4	27.2

Table 33a.

Incorporation of C^{14} amino acids by *P. aeruginosa* 120 Na enzyme into heterologous s-RNA's, and by heterologous enzymes into *P. aeruginosa* 120 Na s-RNA

Micro-organism:	*P. aeruginosa* 120 Na	*P. aeruginosa* ATCC 9027		*P. fluorescens* A 3.12		Achromobacter B81		*E. coli* B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic	4.5	3.3	4	3.1	4.2	1.2	9.7	4.6	8.5	0.4	2.9
Asp.	4.3	3.4	3.8	5.8	2.4	2.3	2.8	3.8	6.4	1.6	1.7
Glu.	0.9	1.1	2.0	0	0.6	0.8	1.3	0	1.5	0	0.8
Ala.	0	0	1.2	0	0.7	0.6	2.3	0	0	0	0.2
Pro.	0	0	0.5	0	0	0	1.8	1.2	1.5	0	0.6
Tyr.	6.3	3.2	5.5	4.7	3.9	2.5	3.4	3.2	6.2	0	0.8
Val.	9.1	5.0	10.3	5.4	7.8	2.6	5.0	5.8	8.0	5.9	1.4
Leu.	18.1	10.1	27.2	17.2	17.1	3.8	11.2	9.6	25.5	0	5.4
Total:	43.2	26.1	54.5	36.0	36.7	13.8	37.5	28.2	57.5	8.0	13.6

Table 33b.

Percent of each amino acid incorporated by P. aeruginosa 120 Na enzymes into heterologous s-RNA's, and by heterologous enzymes into P. aeruginosa 120 Na s-RNA

Micro-organism:	P. aeruginosa 120 Na	P. aeruginosa ATCC 9027		P. fluorescens A 3.12		Achromobacter B81		E. coli B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	10.4	12.8	7.4	8.5	11.5	8.4	25.7	16.4	14.7	5.5	21.4
Asp.	9.9	12.9	7.0	16	6.4	16.6	7.5	13.6	11.1	20.4	12.7
Glu.	2.0	4.3	3.6	0	1.6	5.5	3.4	0	2.6	0	6.1
Ala.	0	0	2.2	0	1.9	4.5	6.2	0	0	0	1.6
Pro.	0	0	1.0	0	0	0	4.7	4.1	2.6	0	4.7
Tyr.	14.4	12.2	10.1	13	10.7	18.3	9.1	11.3	10.8	0	6.0
Val.	21.1	19	17.9	14.9	21.1	18.7	13.3	20.4	13.9	74	9.9
Leu.	42.1	38.8	48.7	47.6	46.7	27.5	29.9	34.2	44.5	0	39.6

Table 34a.

Incorporation of C^{14} amino acids by P. fluorescens enzymes into heterologous s-RNA's, and by heterologous enzymes into P. fluorescens s-RNA

Micro-organism:	P. fluorescens A 3.12	P. aeruginosa ATCC 9027		P. aeruginosa 120 Na		Achromobacter B81		E. coli B.		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic	7.1	6.9	4.3	4.2	3.1	1.3	7.9	4.0	7.5	0.9	3.4
Asp.	3.5	1.9	6.5	2.4	5.8	1.6	5.6	2.5	8.8	1.2	2.3
Glu.	1.3	1.2	2.3	0.6	0	0.6	2.0	0	3.1	0	0
Ala.	0	0	1.4	0.7	0	0	1.9	0	0.3	0.4	0
Pro.	2.0	0	1.1	0	0	0	1.0	1.1	6.1	0	1.6
Tyr.	3.8	4.0	3.5	3.9	4.7	0.7	2.7	2.5	5.5	0.5	0.9
Val.	6.3	5.9	4.5	7.8	5.4	2.4	5.8	5.6	7.3	7.1	2.4
Leu.	14.5	10.8	27.3	17.1	17.2	7.1	21.5	10.4	17.7	3.3	6.5
Total:	38.2	30.7	51.2	36.7	36.0	13.7	48.2	26.0	55.1	13.4	17.0

Table 34b.

Percent of each amino acid incorporated by *P. fluorescens* enzymes into heterologous s-RNA's, and by heterologous enzymes into *P. fluorescens* s-RNA

Micro-organism:	*P. fluorescens* A 3.12	*P. aeruginosa* ATCC 9027		*P. aeruginosa* 120 Na		*Achromobacter* B81		*E. coli* B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s.RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	18.5	22.5	8.5	11.5	8.5	9.7	16.4	15.3	13.6	6.6	20.3
Asp.	9.0	6.3	12.6	6.4	16	11.6	11.6	9.8	16	8.6	13.2
Glu.	3.2	3.9	4.5	1.6	0	4.2	4.2	0	5.6	0	0
Ala.	0	0	2.7	1.9	0	0	4.0	0	0.6	2.5	0
Pro.	5.1	0	2.2	0	0	0	2.0	4.3	11.1	0	9.1
Tyr.	9.9	12.9	6.9	10.7	13	5.4	5.5	9.4	9.9	4.0	5.0
Val.	16.5	19.3	8.8	21.1	14.9	18.3	11.9	21.4	13.2	52.9	13.8
Leu.	37.8	35	53.2	46.7	47.6	51.6	44.5	39.9	32.0	25.4	39

Table 35a.

Incorporation of C^{14} amino acids by Achromobacter B81 enzymes into heterologous s-RNA's, and by heterologous enzymes into Achromobacter B81 s-RNA

Micro-organism:	Achromobacter B81	P. aeruginosa ATCC 9027		P. aeruginosa 120 Na		P. fluorescens A 3.12		E. coli B		Yeast	
Amino Acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic	2.9	5.3	1.7	9.7	1.2	7.9	1.4	6.4	3.6	3.0	1.0
Asp.	3.8	2.4	3.0	2.8	2.4	5.6	1.6	3.4	3.1	0.8	1.2
Glu.	2.2	1.3	1.7	1.3	0.8	2.0	0.6	1.1	1.3	0.6	0.3
Ala.	0.3	1.9	0	2.3	0.6	1.9	0	1.2	0.3	0.2	0
Pro.	0	0	0	1.8	0	1.0	0	1.9	1.1	1.4	0.2
Tyr.	0	1.0	1.5	3.4	2.6	2.7	0.8	3.0	1.0	0.4	0.6
Val.	4	3.2	3.3	5.0	2.7	5.8	2.5	6.0	3.5	5.4	1.1
Leu.	11	8.1	6.3	11.2	4.1	21.5	7.3	10.4	11.4	2.1	2.0
Total:	21.0	23.2	17.4	37.5	13.8	48.5	13.7	33.4	24.3	13.7	6.3

Table 35b.

Percent of each amino acid incorporated by Achromobacter B81 enzymes into heterologous s-RNA's, and by heterologous enzymes into Achromobacter B81 s-RNA

Micro-organism:	Achromobacter B81	P. aeruginosa ATCC 9027		P. aeruginosa 120 Na		P. fluorescens A 3.12		E. coli B		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	14	22.9	9.6	25.7	8.4	16.4	9.9	19.1	14.7	22.2	16.2
Asp.	18.4	10.3	17.3	7.5	16.6	11.6	11.9	10.3	12.9	5.7	18.1
Glu.	10.6	5.6	9.8	3.4	5.5	4.2	4.3	3.2	5.2	4.5	4.9
Ala.	1.6	8.1	0	6.2	4.5	4.0	0	3.7	1.1	1.1	0
Pro.	0	0	0	4.7	0	2.0	0	5.8	4.6	10.3	3.3
Tyr.	0	4.5	8.4	9.1	18.3	5.5	5.5	8.9	4.1	2.7	9.1
Val.	19.2	13.8	19.1	13.3	18.7	11.9	18.7	17.8	14.2	39.2	17.6
Leu.	52.4	35	35.8	29.9	27.5	44.5	57.9	31.2	46.7	15.1	30.8

Table 36a.

Incorporation of C^{14} amino acids by *E. coli* enzymes into heterologous s-RNA's, and by heterologous enzymes into *E. coli* s-RNA

Micro-organism:	*E. coli* B	*P. aeruginosa* ATC 9027		*P. aeruginosa* 120 Na		*P. fluorescens* A 3.12		*Achromobacter* B81		Yeast	
Amino Acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic	8.9	6.0	3.6	8.5	4.6	7.5	4	3.6	6.4	6.6	1.7
Asp.	4.8	4.7	4.9	6.4	3.8	8.8	2.5	3.1	3.4	1.2	1.7
Glu.	1.2	2.5	1.6	1.5	0	3.1	0	1.3	1.1	1.5	0.4
Ala.	0.5	0	0.3	0	0	0.3	0	0.3	1.2	1.7	0
Pro.	1.4	3.8	1.0	1.5	1.2	6.1	1.1	1.1	1.9	2.2	0.2
Tyr.	4.0	3.1	2.9	6.2	3.2	5.5	2.5	1.0	3.0	0	1.2
Val.	6.5	7.0	5.1	8.0	5.8	7.3	5.6	3.5	6.0	9.3	1.8
Leu.	12.5	16.6	13.3	25.5	9.6	17.7	10.4	11.4	10.4	4.6	3.8
Total:	39.9	43.8	32.9	57.5	28.2	55.1	26.0	24.3	33.4	27.0	10.8

Table 36b.

Percent of each amino acid incorporated by *E. coli* enzymes into heterologous s-RNA's, and by heterologous enzymes into *E. coli* s-RNA

Micro-organism:	*E. coli* B	*P. aeruginosa* ATCC 9027		*P. aeruginosa* 120 Na		*P. fluorescens* A 3.12		Achromobacter B81		Yeast	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	22.3	13.8	10.9	14.7	16.4	13.6	15.3	14.7	19.1	24.2	15.4
Asp.	11.9	10.8	14.7	11.1	13.6	16	9.8	12.9	10.3	4.5	15.4
Glu.	3.0	5.8	4.9	2.6	0	5.6	0	5.2	3.2	5.6	3.5
Ala.	1.2	0	0.8	0	0	0.6	0	1.1	3.7	6.2	0
Pro.	3.6	8.7	3.0	2.6	4.1	11.1	4.3	4.6	5.8	8.0	2.0
Tyr.	10.1	7.2	9.1	10.8	11.3	9.9	9.4	4.1	8.9	0	10.6
Val.	16.2	16	15.5	13.9	20.4	13.2	21.4	14.2	17.8	34.6	16.2
Leu.	31.6	37.9	41.3	44.5	34.2	32	39.9	46.7	31.2	16.9	36.5

Table 37a.

Incorporation of C^{14} amino acids by yeast enzymes into heterologous s-RNA's, and by heterologous enzymes into yeast s-RNA

Micro-organism:	Yeast	*P. aeruginosa* ATCC 9027		*P. aeruginosa* 120 Na		*P. fluorescens* A 3.12		*Achromobacter* B81		*E. coli* B	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
	cpm x 10^{-3}	cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}		cpm x 10^{-3}	
Basic	9.1	2.0	0.8	2.9	0.4	3.5	0.9	1.0	3.0	1.7	6.0
Asp.	2.5	1.3	1.7	1.7	1.6	2.3	1.2	1.2	0.8	1.7	1.2
Glu.	1.3	0.4	0.9	0.8	0	0	0	0.3	0.6	0.4	1.5
Ala.	0.4	0	0	0.2	0	0	0.4	0	0.2	0	1.7
Pro.	2.3	0.5	0	0.6	0	1.6	0	0.2	1.4	0.2	2.2
Tyr.	4.6	0.9	0	0.8	0	0.9	0.6	0.6	0.4	1.2	0
Val.	7.3	1.2	7.7	1.4	5.9	2.5	7.4	1.1	5.4	1.8	9.3
Leu.	8.8	2.4	4	5.4	0	6.7	3.4	2.0	2.1	3.8	4.6
Total:	36.1	8.7	15.1	13.6	8.0	17.0	13.4	6.3	13.7	10.8	27.0

Table 37b.

Percent of each amino acid incorporated by yeast enzymes into heterologous s-RNA's, and by heterologous enzymes into yeast S-RNA

Micro-organism:	Yeast	*P. aeruginosa* ATCC 9027		*P. aeruginosa* 120 Na		*P. fluorescens* A 3.12		Achromobacter B81		*E. coli* B	
Amino acid:	s-RNA	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.	s-RNA	Enz.
Basic	25.2	23.6	5.1	21.4	5.5	20.3	6.9	16.2	22.2	17.4	24.2
Asp.	6.9	15.2	11.4	12.7	20.4	13.2	8.9	18.1	5.7	18.4	4.5
Glu.	3.5	11.3	6.1	6.1	0	0	0	4.9	4.5	4.1	5.6
Ala.	1.1	0	0	1.6	0	0	2.6	0	1.1	0	6.2
Pro.	6.4	5.4	0	4.7	0	9.1	0	3.3	10.4	2.3	8.0
Tyr.	12.7	10.4	0	6.0	0	5	4.2	9.1	2.7	12.3	0
Val.	20.3	14	51	9.9	74	13.8	55	17.6	39.2	18.7	34.6
Leu.	24.7	27.2	26.4	39.6	0	39	26.4	30.8	15.1	42.5	16.9

of the leucine group. For example, the P. aeruginosa ATCC 9027 enzyme formed nearly double the amount of the P. aeruginosa 120 Na and P. fluorescens leucyl-, isoleucyl-, and phenylalanyl- s-RNA's as that of its own. In the case of the second P. aeruginosa strain, valine - methionine incorporation was also doubled, but neither of the other two pseudomonads reacted as well with the basic amino acids as did P. aeruginosa ATCC 9027. The increase in heterologous reaction between E. coli enzyme, and P. aeruginosa 120 Na s-RNA, could also be attributed mainly to the leucine group, and partly to these amino acids in the s-RNA's of the other strains of P. aeruginosa and of P. fluorescens. The incorporation of proline, for which E. coli has an active enzyme, was nearly tripled into P. aeruginosa 120 Na s-RNA, and quadrupled into the s-RNA of P. fluorescens.

None of the amino acids (except for valine and methionine) was well incorporated into the yeast s-RNA by the bacterial enzymes. Compared to the amount incorporated into the s-RNA's in the interbacterial heterologous systems, the uptake of the leucine group was especially poor in most cases. From the pattern of amino acid incorporation into yeast s-RNA by the homologous enzymes however, it would appear that there may have been less of the s-RNA's for these amino acids in the yeast preparation than in s-RNA's from the bacteria. The lack of incorporation of tyrosine into yeast s-RNA by E. coli enzyme has also been reported by Clark and Ezyaguirre (31), and Benzer and Weisblum (14). However, the results with valine in this heterologous system are in contrast to those of Loftfield and Eigner (105), who, using an assay procedure similar to that employed here, and valine as the sole amino acid present, found that there was only 18% as much incorporation into yeast s-RNA by the E. coli enzyme as into the homologous E. coli s-RNA. The yeast s-RNA and the E. coli s-RNA were shown to be in com-

petition for the valine. The presence in the Chlorella hydrolysate of methionine, which is not separated paper chromatographically from valine, would explain the differences between these results and those of Loftfield and Eigner (105).

The yeast enzymes proved to be much more versatile than the yeast s-RNA, not from the standpoint of the amount of C^{14} incorporated, but from that of the number of amino acids transferred to the bacterial s-RNA's. The yeast enzymes for the leucine and basic amino acid groups reacted fairly well with all of the bacterial s-RNA's, and there was generally a full complement of amino acids incorporated, although the incorporation was low in each case. Despite the high cross reaction exhibited between the E. coli enzyme, and the yeast s-RNA, the reverse cross was no better than that with the other bacteria. The poor incorporation of radioactivity by the yeast enzyme into bacterial s-RNA's may be a problem of slow reaction rates, as previously demonstrated with E. coli enzyme and yeast s-RNA in the case of valine (105).

This study was undertaken in the hope that differences in the species specificity of microbial s-RNA's and amino acyl-s-RNA synthetases could be demonstrated. However, there proved to be a rather large amount of heterologous reaction even between those microorganisms which were not closely related. In the months since this investigation was initiated, there have been several extensive experiments reported in which similar results were obtained to those presented here. The latest demonstration of the lack of specificity between s-RNA's and aminoacyl-s-RNA synthetases has been in the work of Yamane and Sueoka (176), who showed that the enzymes of many microorganism will interact to a greater or lesser extent

with E. coli s-RNA. The one case in which there does appear to be specificity for some amino acids is that of yeast, since many reports have indicated that bacterial enzymes incorporate only poorly a number of amino acids into yeast s-RNA, but that animal enzymes react fairly well. This situation has been verified in these experiments, although the yeast enzyme appeared to show a greater interaction with the bacterial s-RNA than vice versa (Table 37). Since the experiments done in this study were all performed under the same conditions, the results obtained represent a minimum amount of interaction, which very possibly could be increased if optimum conditions were employed for each system.

The experiments of McCarthy and Bolton (107), Giacomoni and Spiegelman (69), and Goodman and Rich (72) on hybrid formation between messenger RNA's and s-RNA's with DNA, are in contrast to these amino acid incorporation studies, since hybrid formation appears to occur only between closely related bacteria, there being, for example, very low hybridization between E. coli and Pseudomonas. The lack of specificity in the amino acid incorporation reactions may be due, then, to the enzymes, rather than to the s-RNA's.

GENERAL DISCUSSION

The underlying theme of the data from the experiments described in this thesis proved to be the importance of protein synthesis in oxidative assimilation by the aerobic bacteria studied. Thus, in both strains of Pseudomonas aeruginosa, and in Pseudomonas fluorescens, Achromobacter B81, Achromobacter viscosus, Azotobacter agilis, Azotobacter vinelandii and Acetobacter xylinum, the major part of the radioactivity incorporated into the cells during glucose-U-C^{14} oxidation was found in the proteinaceous fractions. When more detailed studies were done with P. aeruginosa, under circumstances in which protein synthesis was greatly reduced, oxidative assimilation was also markedly decreased. The addition of chloramphenicol to cells oxidizing glucose-C^{14} resulted in a reduction of about 25% in the total amount of oxidative assimilation, and a large part of the assimilated material was found in the metabolic pool as free amino acids. Starving the cells prior to glucose oxidation, i.e., aerating the cells in a non nutrient medium, proved to decrease oxidative assimilation to a greater extent (40%) than did the presence of the antibiotic. There are several possible explanations for this phenomenon which are, in each instance, based on the use of protein and nucleic acid as major endogenous reserves by P. aeruginosa (29,75). One result of the starvation period was a greatly diminished activity of the aminoacyl s-RNA synthetases, which are necessary for protein synthesis. A second was the accumulation of radioactive glutamate in the metabolic pool, perhaps due to a lack of transaminases. A third result was the decrease in the rate of pyruvate oxidation. This led to a slower formation of α-ketoglutarate, which Duncan and Campbell (55) showed to be a key compound in oxidative assimilation by P. aeruginosa. Therefore, the over-

all effect of the period of starvation was the sacrifice of vital cellular constituents, which were resynthesized as soon as a substrate became available.

The studies of oxidative assimilation in P. aeruginosa were extended to an investigation of the cytological sites of the assimilated material. It was found that the soluble proteins of the cytoplasm, which are mainly enzymes, contained the major portion of the C^{14} of the cell extracts. This indicated that these enzymes were turning over rapidly during glucose oxidation. Experiments with chloramphenicol revealed, that although this turnover was inhibited by 87%, glucose oxidation proceeded at almost the usual rate. Therefore, it was tentatively concluded that, under normal circumstances, most of the enzymes synthesized were synthetic, rather than degradative in function. Support for this conclusion was found in the incorporation of C^{14} during glucose dissimilation, either by freshly harvested or by starved cells, into the fraction of the cytoplasmic protein which includes aminoacyl s-RNA synthetases. Further experiments with starved cells showed that the activity of these enzymes was decreased during the starvation period, but was rapidly restored during oxidative assimilation.

Since protein synthesis seemed to be of such great importance in oxidative assimilation of glucose by P. aeruginosa, some investigations were made on the aminoacyl s-RNA synthetases in this microorganism. As mentioned above, these enzymes were found in the cytoplasm, and further experiments with the ribosomes and "membranes" showed that these fractions were inactive in the formation of aminoacyl s-RNA. Because preliminary experiments had revealed that yeast s-RNA was not charged with amino acids by P. aeruginosa synthetases, the studies were extended to a determination of

species specificity between the enzymes and s-RNA's of other microorganisms. Three of the bacteria which had been found to have a pattern of oxidative assimilation similar to that of P. aeruginosa were selected - a second strain of P. aeruginosa, as well as P. fluorescens, and Achromobacter B81. For comparative purposes, a more distantly related organism, E. coli, which is known to assimilate glucose into a carbonaceous reserve product, was chosen. Yeast was also included because of its poor cross reaction with P. aeruginosa. It was found that there was little specificity exhibited between bacterial enzymes and s-RNA's since good heterologous reactions were obtained, but that the yeast gave a poor cross reaction with each of the bacteria except the E. coli enzyme system. In the past few months, the same general conclusion as to the lack of species specificity has been reached by a number of authors (45,52,176).

Oxidative assimilation also occurred into the nucleic acids, primarily into the RNA. Under normal conditions, the ribosomal RNA showed a very slow turnover, but if starved cells were used, the C^{14} incorporated into the RNA was greatly increased, supporting the contention of Duncan and Campbell (55) and of Clifton (36) that oxidative assimilation takes place to replace endogenous reserves. Chloramphenicol also increased the incorporation of C^{14} into the ribosomal RNA, in agreement with previous reports on its action (9,66,67).

The nitrogen for synthesis of these components in P. aeruginosa was found by Duncan and Campbell (55) to be derived from the ammonia produced during endogenous respiration by this microorganism. A similar conclusion was reached in the cases of the other aerobic bacteria studied here, where the uptake of C^{14} was paralleled by the uptake of endogenously produced ammonia. Like the strain of P. aeruginosa used by Duncan and

Campbell (55), oxidative assimilation by P. aeruginosa, P. fluorescens, Achromobacter B81, A. viscosus and Acetobacter xylinum involved the excretion of pacemaker compounds, whose presence in the surrounding medium during glucose dissimilation increased the extent of oxidative assimilation. Not all of these compounds were keto acids. They served, under the conditions of these experiments, to slow glucose oxidation so that carbon would still be present to permit assimilation of ammonia, as it became available from the oxidation of endogenous substrates. The bacteria which formed these pacemaker compounds assimilated more C^{14} than those, such as the two Azotobacter species, which did not excrete such intermediates into the surrounding medium. These organisms, by virtue of their ability to fix atmospheric nitrogen, should not have lacked nitrogen for the synthesis of cellular material, yet they assimilated the least C^{14} of any of the bacteria studied. From these results, it would seem that an investigation of oxidative assimilation should include a determination of the extracellular products appearing during the course of substrate oxidation.

Preliminary experiments carried out on ammonia assimilation in P. aeruginosa and in P. fluorescens showed that there was a requirement for concurrent substrate oxidation during ammonia assimilation. This oxidation would provide cofactors, such as reduced pyrimidine nucleotides, as well as energy for transport of the aminated compound across cell barriers. No evidence was found for the direct amination of pyruvate through alanine dehydrogenase. In contrast, the presence of labelled glutamate in the metabolic pool, the parallel uptake of ammonia and disappearance of α-ketoglutarate from the surrounding medium (55), and the presence of an active glutamic acid dehydrogenase in P. aeruginosa (55), all supported the assimilation of ammonia through the conversion of α-ketoglutarate to glutamate.

Despite the emphasis on nitrogenous cell components as products of oxidative assimilation in P. aeruginosa and the other aerobes studied, C^{14} from glucose was also incorporated into non nitrogenous constituents. In Achromobacter B81 a polymeric carbohydrate was formed, and lipids were generally highly labelled in most of the bacteria. From a cytological investigation of P. aeruginosa, most of the lipid was found to be in the "membrane" fraction, its label being increased by treatment of the cells with chloramphenicol, or by prior starvation. The reason for this increase was not established, but it may be the result of the channeling of more substrate into lipid when protein synthesis is decreased, the latter process usually taking precedence. This increase, although substantial, especially in the case of the antibiotic treated cells, did not compensate for the loss of protein synthesis, indicating that P. aeruginosa does not form a carbonaceous reserve, even in the absence of protein synthesis.

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