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Amino Acid Pool Formation in *Pseudomonas aeruginosa*

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The accumulation and behavior of various amino acids in the pool of *Pseudomonas aeruginosa* (ATCC 9027) were investigated. Patterns of pool formation and maintenance varied with different amino acids tested and were dependent, to a considerable extent, upon the ability of the organism to catabolize the particular amino acid. The establishment of steady-state amino acid pool levels depended upon the activity of the amino acid permease involved and upon the rate of protein synthesis. The presence of a relatively large specific amino acid pool did not affect the formation of a pool of a structurally different amino acid, and a preformed steady-state pool was not displaced by structurally unrelated amino acids. Steady-state amino acid pools decreased rapidly in the presence of inhibitors of energy metabolism and at 0 C. Steady-state internal amino acid pools were found to be in equilibrium with the corresponding external amino acid, present at low levels. A multiplicity of proline pools was demonstrated.

The composition and behavior of the amino acid pools of microorganisms have been well documented in recent years (7). The pools of *Escherichia coli*, in particular, have been carefully investigated (2, 3), and this has resulted in the formulation of specific models to describe both amino acid transport and pool behavior.

However, investigations of amino acid pool characteristics had not been attempted for the pseudomonads, organisms which are able to catabolize and utilize most amino acids both as carbon and nitrogen sources. In a previous publication (10), the properties of high-affinity amino acid transport systems in *Pseudomonas aeruginosa* were described. During the investigation, it became obvious that, although unrelated amino acids entered the cell by similar mechanisms, the metabolic fates and the maintenance of amino acid pools varied with the different amino acids examined.

The purpose of the present investigation was to establish the general properties of amino acid pools in *P. aeruginosa*, to relate pool formation and maintenance to rates of protein synthesis, and to describe observations on the compartmentalization of amino acid pools in this microorganism.

MATERIALS AND METHODS

The organisms used throughout this study were *P. aeruginosa* ATCC 9027; *P. aeruginosa* P22, a strain unable to use proline as a sole carbon source; and *P. aeruginosa* WK4, a histidine auxotroph. The growth media, conditions of culture, and maintenance of stock cultures have been described previously (10).

The procedure of Britten and McClure (2) was used to establish general patterns of amino acid pool formation; however, the modified procedure (10) of FerroLuzzi-Ames (5) was employed in rate studies.

Chromatography. The intracellular amino acid pools were extracted with cold trichloroacetic acid. The trichloroacetic acid was removed by extraction with cold ether, and the amino acids were separated by the combined thin-layer chromatography–radioautographic technique (10). Lipids were extracted and chromatographed by one- and two-dimensional chromatography on Silica Gel G thin-layer plates (9).

Inhibition experiments. Cell suspensions were preincubated for 30 min in minimal medium (10) with various metabolic inhibitors, prior to the initiation of incorporation studies by the addition of the ^{14}C-labeled amino acid. Subsequently, the cells were collected by filtration and washed with minimal medium containing the appropriate concentration of inhibitors.

Low-temperature experiments. Uptake experiments were conducted in water-jacketed reaction vessels maintained at the desired temperature with a Lauda K-2R circulating water pump (Brinkmann Instruments, Inc., Westbury, N.Y.). The cultures were stirred with a magnetic stirrer during the ^{14}C-amino acid uptake experiments, and the cells were filtered with a 0.45-μm pore size filter (Millipore Corp., Bedford, Mass.) in a Tracerlab E8B filtration apparatus (Tracerlab, Waltham, Mass.).

Selection of mutants. Wild-type exponential-phase cells were harvested from minimal medium by centrifugation at 13,000 $\times$ *g* for 10 min, suspended in cit-

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rate buffer (*p*H 5.5) to a concentration of approximately 10^9 cells per ml, and shaken at 30 C for 1 hr. *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine was added to give a final concentration of 100 μg/ml; the cells were shaken for 40 min, then the culture was quickly chilled to 0 C and centrifuged at 6 C. The cells were washed twice with minimal medium, suspended to the original volume in supplemented media to enrich for the desired mutant, and incubated at 37 C for 2 to 6 hr.

The mutagenized culture was then incubated for 6 hr in minimal medium supplemented with 50 μg of the required amino acid per ml. The cells were diluted and plated directly onto solid minimal medium containing 1 μg/ml of the desired amino acid. The plates were incubated for 48 hr and auxotrophs were isolated as minute colonies.

Mutagenesis was carried out on cells which first had been adapted to grow on proline as the sole carbon source. After washing with medium, the treated cells were grown in minimal medium for 2 hr, then plated onto solid medium with 0.2% proline and 0.01% asparagine as the sole sources of carbon. After 48 hr of incubation, minute colonies were picked, washed in drops of saline, and patched on both proline and minimal agar plates. After further incubation, cells which grew on glucose but not on proline were isolated. The isolation of these mutants was necessary because of the constitutive ability of *P. aeruginosa* to catabolize amino acids.

Chemicals. All radioactive amino acids were purchased and utilized as previously described (10). Chloramphenicol was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Kinetics of pool formation and composition of the pools. Significant variations were found in the kinetics of incorporation of the amino acids commonly occurring in protein. Figure 1 illustrates the four distinct patterns of incorporation and pool formation observed with the amino acids when added to growing cells at a low concentration. Most of the aliphatic, aromatic, and basic amino acids demonstrated the pattern of uptake shown in Fig. 1A. When these pools were chromatographically analyzed, the test amino acid was found, for the most part, to have maintained its chemical integrity. For example, chromatography and radioautography of the isoleucine pool, when the amount of ^{14}C in the pool was maximal, demonstrated that 94% of the total radioactivity was present as ^{14}C-isoleucine. These results agree with those of Britten and McClure (2) for the kinetics of amino acid incorporation into *Escherichia coli*. However, under identical conditions, several amino acids deviated from this general pattern. Figure 1B illustrates the incorporation kinetics common to the amino acids, glycine, serine, and glutamate. Pool formation commenced rapidly after the addition of the labeled amino acid, but the pool was only partially removed for protein synthesis after the exogenous supply of the amino acid had been exhausted. The remaining fraction of the pool was lost very slowly or not at all.

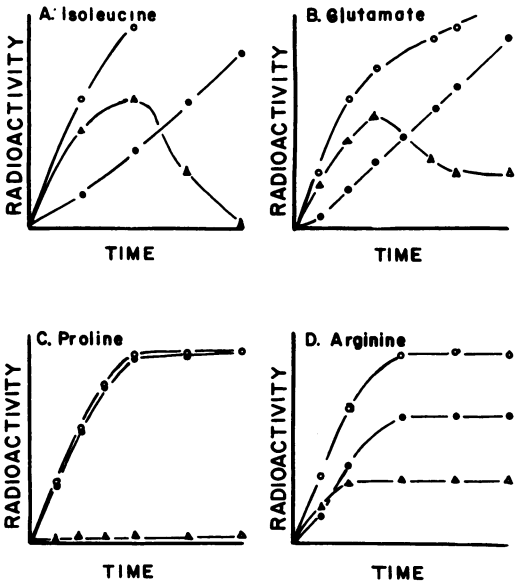

FIG. 1. *Patterns of ^{14}C-amino acid uptake into whole cells (●), protein (○), and the calculated pool (△) of P. aeruginosa. Procedure: Britten and McClure (2)*

To elucidate the nature of this phenomenon, pools were collected at various time intervals and were analyzed by radioautography (Table 1). With glutamic acid, 66% of the radioactivity in the pool, collected 5 min after the addition of label, was found to contain glutamate. A "bound" or slowly metabolized compound (compound X) accounted for approximately 90% of the ^{14}C in the pool at the later time. This compound did not chromatograph discretely and resisted identification by several methods.

Glycine and serine also yielded heterogeneous pools; in fact, these amino acids did not account for the majority of the radioactivity in the intracellular pool. The major labeled components (Y and Z) were found not to be amino acids, bases, nucleosides, or nucleotides, and did not form hydrazones. The material was eluted with water from a Dowex 50 (H^+) column. From their solubility in chloroform and chloroform-sodium chloride and movement during thin-layer chromatography, they were assumed to be lipid and phospholipid. Since only a minor proportion of the added ^{14}C-glycine or serine was identified in the intracellular pool, it is suggested that, under the conditions of the transport experiments, the amino acids were possibly converted to lipid or phospholipid without first entering the free pool. It was felt that such a conversion could perhaps

have taken place at the cell membrane. However, when isolated membrane preparations, soluble cytoplasm, or combinations of these physical fractions were incubated at 37 C in a nitrogen-free medium with 10 μg of ^{14}C-serine or ^{14}C-glycine (specific activity, 67 μc/μmole) and chromatographed by thin-layer chromatography, no radioactivity could be detected in lipid or phospholipid by radioautography.

The third class of uptake kinetics was observed with ^{14}C-proline and ^{14}C-threonine (Fig. 1C). With these amino acids, essentially no pool formation occurred during the course of the experiment; that is, as the amino acids entered the cell, they were immediately converted into trichloroacetic acid-insoluble material. To investigate this deviation from apparently normal pool behavior, attempts were made to selectively inhibit protein synthesis without altering the ability of the cells to transport proline. The rate of protein synthesis was manipulated by the controlled addition of chloramphenicol. At concentrations greater than 100 μg/ml, growth was completely inhibited. The rates of ^{14}C-proline incorporation into chloramphenicol-treated cell suspensions as a function of the inhibitor concentration are shown in Fig. 2A. Over the range of chloramphenicol concentrations employed, the rate at which ^{14}C-proline was transported into the cell remained relatively constant, whereas the rate of entry of the label into the protein fraction was inversely proportional to the inhibitor concentration. As a result, accumulation of proline in the intracellular pool increased significantly. The quantitative difference between the proline pool in Fig. 1C and that in Fig. 2A, in the absence of chloramphenicol, is a result of the fact that two different procedures were employed in these experiments.

Preliminary experiments showed that, in contrast to the valine transport system (10), the initial rate of ^{14}C-proline transport into whole cells did not vary greatly as a function of temperature; however, the rate of growth diminished markedly at low temperatures. These observations were used to confirm the results obtained with chloramphenicol, since chloramphenicol was shown by Britten and McClure (2) to affect pool formation adversely in *E. coli*. When the incorporation of ^{14}C-proline into cell suspensions was followed at different temperatures, results similar to those with chloramphenicol were obtained (Fig. 2B). At 10 to 20 C, the rate of protein synthesis (as determined by incorporation of ^{14}C-proline into trichloroacetic acid-insoluble material) was significantly lowered. Low rates of protein synthesis were coincident with high proline pool levels. At higher temperatures, large pools were not formed because of the rapid incorporation of the labeled amino acid into protein. These results demonstrate that the rate of pool formation and the size of the intracellular pool are functions of both the capacity of the cell to transport the amino acid and the ability of the cell to utilize that amino acid for protein synthesis. When the time course of ^{14}C-proline uptake was followed with the wild type at 20 C (Fig. 3A) or with the histidine-requiring auxotroph WK4 at 30 C (Fig. 3B), in which the rate of protein synthesis was decreased by starvation for a required amino acid, then pool formation followed a course similar to that reported for *E. coli* (2). Chromatography of the labeled pool material

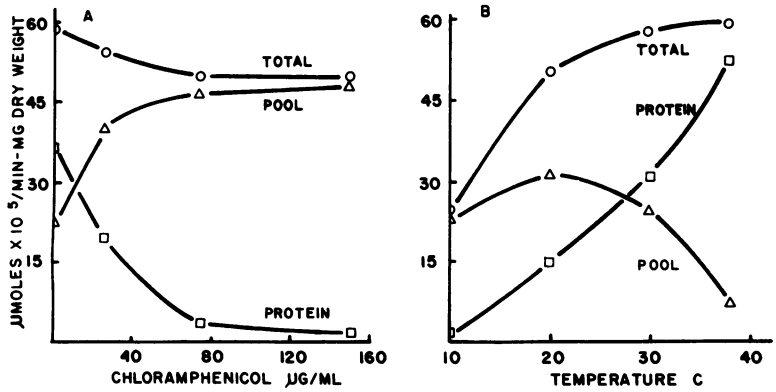

FIG. 2. *Effect of chloramphenicol and temperature on the rate of ^{14}C-proline incorporation into whole cells and cell fractions of P. aeruginosa. (A) Cells were preincubated in the presence of chloramphenicol for 30 min at 30 C prior to the addition of ^{14}C-proline. Samples were removed at 30-sec intervals to determine initial incorporation velocities. (B) Cells were equilibrated at the appropriate temperatures for 10 min prior to the addition of ^{14}C-proline. Samples were removed at 30-sec intervals. Procedure: FerroLuzzi-Ames (5)*

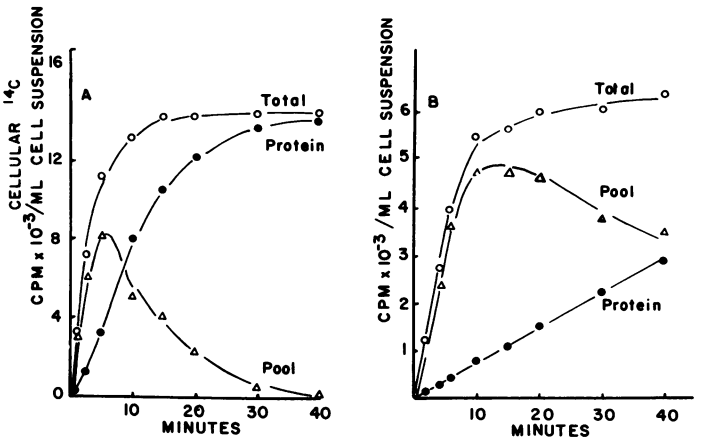

FIG. 3. *Time course of ^{14}C-proline uptake into whole cells and cell fractions of P. aeruginosa. (A) Wild type, at 20 C; (B) at 30 C, by a histidine auxotroph (WK4) deprived of histidine for 30 min prior to the addition of ^{14}C-proline. Procedure: Britten and McClure (2).*

under these conditions showed that the amino acid had retained its chemical integrity.

Pool formation occurred readily at relatively high external proline concentrations (Fig. 4). At proline concentrations less than 8×10^{-6} M, pool formation was limited by the rate of protein synthesis. This exogenous proline level has previously been termed the "limit concentration" (5), and represents the minimal external concentration at which organisms stop the biosynthesis of amino acids and use only the exogenous supply.

When cells were incubated with high concentrations of chloramphenicol, preformed amino acid pools decayed concomitantly with the cessation of protein synthesis. When ^{14}C-proline was added to cells which had been preincubated with 2 mg of chloramphenicol per ml for 30 min, rapid pool formation occurred, but the pool effluxed into the medium at 15 min (Fig. 5), the time at which protein synthesis had stopped.

The fourth class of uptake kinetics (Fig. 1D) was observed with the amino acids arginine and cysteine. These amino acids formed relatively high intracellular pool levels, and the pools were maintained for extended periods of time. Figure 6 demonstrates the kinetics and stability of the ^{14}C-arginine pool over a long period of time. Once formed, the intracellular pool was found to be stable and remained at a constant size even during cell growth. During starvation for an exogenous carbon source, the pool was maintained for periods as long as 24 hr. However, chromatography of the labeled pooled material (Table 1) showed that this stable pool was, in fact, putrescine, a degradation product of arginine metabolism.

Cysteine accumulated intracellularly in *P. aeruginosa*, and as much as 50% of the added radioactivity was found in the trichloroacetic acid-soluble fraction of the cell. Unlike the pool formed from ^{14}C-arginine, the ^{14}C-cysteine pool was found by chromatography and radioautography to retain its chemical identity. The nature of this intracellular accumulation has not been investigated as yet.

Pool formation and maintenance. Under optimal conditions for pool formation, that is, when protein synthesis was partially inhibited without affecting the amino acid transport rate, steady-state amino acid pools were formed at a particular concentration determined by the concentration

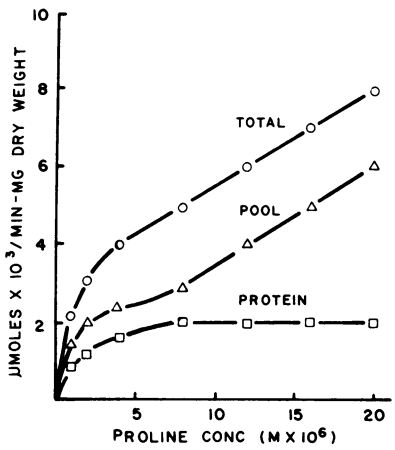

FIG. 4. *Rate of ^{14}C-proline uptake by whole cells and cell fractions as a function of the exogenous proline concentration. Procedure: FerroLuzzi-Ames (5).*

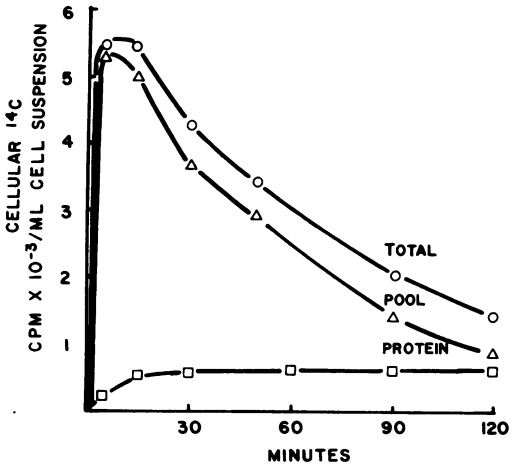

FIG. 5. *Influence of protein synthesis on the maintenance of the proline pool in P. aeruginosa. Cells were preincubated with chloramphenicol (2 mg/ml) for 30 min prior to the addition of ¹⁴C-proline. Procedure: Britten and McClure (2).*

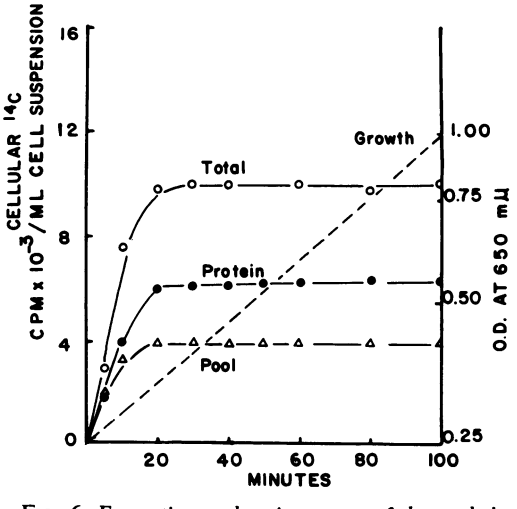

FIG. 6. *Formation and maintenance of the pool derived from ¹⁴C-arginine (2 × 10⁻⁵ M) by a growing culture of P. aeruginosa. Proceduce: Britten and McClure (2).*

of the added exogenous amino acid. The proline pool obtained at steady-state at 10 C with an exogenous proline concentration of 10^{-6} M is shown in Fig. 7. At this external concentration, 40% of the exogenous amino acid was incorporated into the cellular pool. The concentration ratio established between the internal and external proline was in the range of 1,000-fold. The pool size increased with increasing external proline concentrations; however, the resulting concentration ratios decreased (*unpublished data*). The addition of 10^{-4} M of each of 18 other amino acids, including hydroxyproline, did not result in a significant change in the internal proline pool (Fig. 8A). Consequently, the maintenance of proline pools in this organism is specific. Also, a preestablished proline pool did not greatly influence the incorporation of ¹⁴C-leucine at 30 C (Fig. 8B). It was concluded, therefore, that the maintenance of amino acid pools in *P. aeruginosa* is structurally specific and that the maximal accumulation of one amino acid is independent of the accumulation of another unrelated amino acid.

Pool exchange. Pool formation and maintenance were found to be closely related processes. At high external amino acid concentrations, the pool size increased and an equilibrium was established between the intracellular and extra-

TABLE 1. *Chromatographic analyses of ¹⁴C-amino acid pools*

¹⁴C-amino acid added	¹⁴C compounds in the pool	Total pool radioactivity (%)	
		Early pool[a]	Late pool[b]
Isoleucine	Isoleucine	93.7	—[c]
Proline	Proline	95.2	—
	Glutamate	4.8	—
Glutamate	Glutamate	65.9	—
	Compound X	13.4	89.5
	Alanine	0.7	—
	Valine	9.4	—
	Isoleucine	0.4	—
	Leucine	0.3	—
	Proline	0.2	—
Glycine	Compound Y	93.3	92.6
	Glycine	1.2	0.9
	Glutamate	1.2	1.6
	Threonine	0.9	1.2
	Serine	0.6	0.3
Serine	Compound Z	86.8	89.2
	Glutamate	1.2	—
	Aspartate	1.1	—
	Threonine	1.0	0.6
	Serine	0.9	1.7
	Glycine	0.8	0.3
Arginine	Glutamate	43.5	6.8
	Arginine	21.2	—
	Putrescine	15.2	93.2
	γ-Aminobutyrate	2.6	—

[a] Sample taken at maximal pool level.
[b] Sample taken when a steady-state pool had been reached.
[c] No detectable radioactivity.

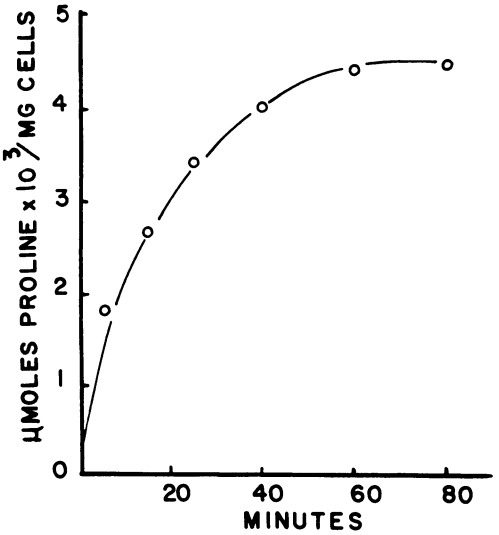

FIG. 7. *Maximal proline pool obtained at 10 C with 10^{-6} M ^{14}C-proline by P. aeruginosa P22 in minimal medium. Cells were preincubated at 30 C for 30 min with 200 μg of chloramphenico pler ml and equilibrated for 10 min at 10 C prior to the addition of ^{14}C-proline. Procedure: Britten and McClure (2)*

cellular proline. However, the pool did not increase without exchanging with preloaded intracellular proline. When ^{12}C-proline was added at 10^{-4} M to a cell suspension with a preestablished ^{14}C-proline pool, rapid exchange of the preloaded pool occurred until the specific activity of external and internal proline were approximately the same (Fig. 9). No evidence for two exchange rates, as has been found with *E. coli* (2), was observed.

Energy requirement for pool maintenance. The maintenance of intracellular amino acid pools was found to be energy-dependent. When valine was allowed to accumulate intracellularly at 15 C and then NaN_3 (30 mM) and iodoacetamide (1 mM) were added, an immediate efflux of the accumulated amino acid occurred at a constant rate (Fig. 10), but the rate was much lower than that of pool formation.

Pool multiplicity. Britten and McClure (2) and Kessel and Lubin (11) postulated, from exchange kinetics with "preloaded" *E. coli* proline pools, that a multiplicity of pools existed in the organism. That is, internal proline exchanged with external proline in a manner described by two classical isotherms. Pools in *P. aeruginosa* are not stable at 0 C, and such exchange reactions are impossible to measure with the same degree of precision. At higher temperatures, uptake cannot be completely dissociated from exchange. However, other criteria were established to demonstrate that the internal proline pool of *P. aeruginosa* was maintained within the cell in different ways. The efflux of proline from a steady-state intracellular pool in the presence of NaN_3 (30 mM) and iodoacetamide (1 mM) is shown in Fig. 11. Unlike the valine pool (Fig. 10), a significant fraction of the proline pool was either not lost (P22) or lost very slowly (wild type) in the absence of energy production. The proline concentration

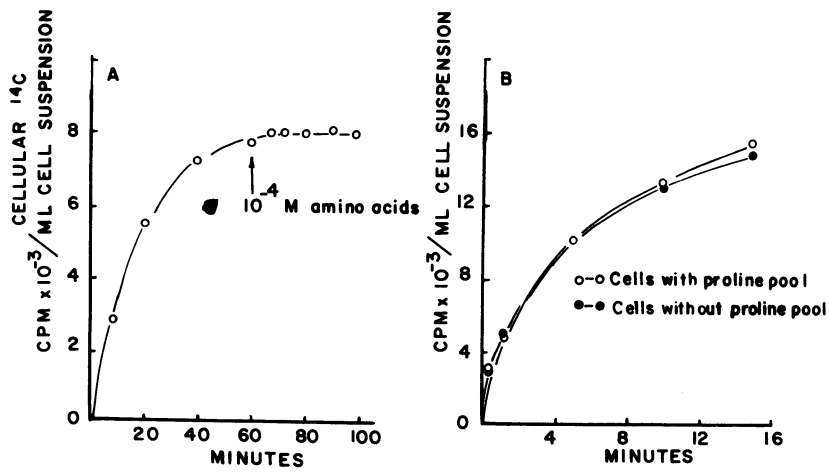

FIG. 8. *Specificity of maintenance of the proline pool in P. aeruginosa P22. (A) The time course of proline uptake and proline pool maintenance in the presence of a 10^{-4} M concentration of each of the 18 amino acids added at 60 min. (B) The time course of ^{14}C-leucine uptake into cells which had been preloaded with a maximal level of ^{12}C-proline by previous incubation with 10^{-6} M proline. Control has no preestablished proline pool. Procedure: Britten and McClure (2)*

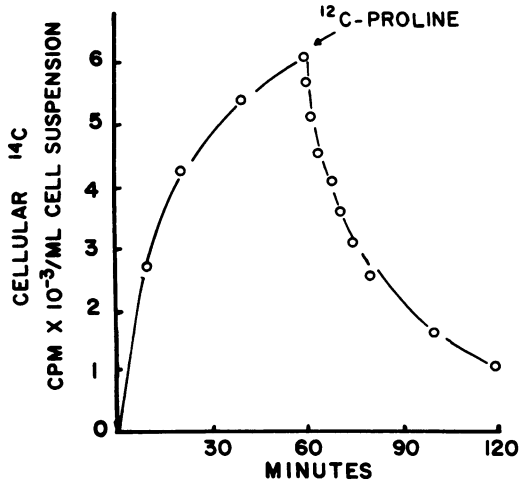

FIG. 9. *Exchange of a preloaded ^{14}C-proline pool of P. aeruginosa P22 with 10^{-4} M ^{12}C-proline added at 60 min. Cells were preincubated with 200 μg of chloramphenicol per ml prior to initiation of the experiment, and the incorporation study was performed at 10 C. Procedure: Britten and McClure (2)*

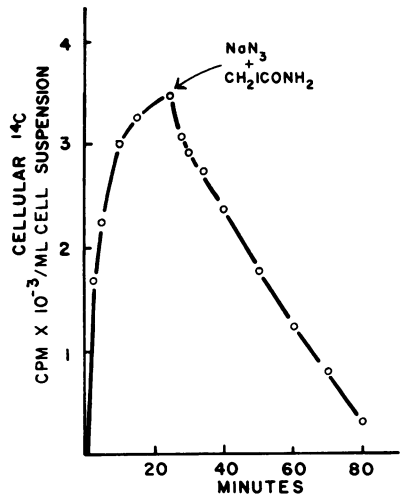

FIG. 10. *Efflux of a preformed valine pool in the presence of NaN_3 (30 mM) and iodoacetamide (1 mM). Cells were preincubated with 100 μg of chloramphenicol per ml for 30 min prior to addition of 2 × 10^{-6} M ^{14}C-valine at 15 C. Procedure: Britten and McClure (2)*

gradient, expressed as the amount of proline per milliliter of cell water versus the amount of proline per milliliter of external medium, was 800 at the time of addition of the inhibitor and 300 at the time of establishment of the stable pool. The slow pool loss was due to a low constitutive rate of oxidation of the amino acid by the wild-type strain. The NaN_3-stable pool was subsequently shown to be exchangeable when ^{12}C-proline was added at 10^{-4} M. Control experiments also demonstrated that this exchange rate was the same in the absence or in the presence of NaN_3. Therefore, at least the NaN_3-stable component of the pool was exchanged by a reaction which did not require metabolic energy.

Effect of temperature on pool maintenance. When proline was allowed to accumulate intracellularly at 10 C and the cells were quickly chilled to 0 C, rapid efflux of most of the pool occurred within 10 min of chilling. This event was followed by a slower efflux of a second component of this pool (Fig. 12). No amino acid pools were stable at this temperature, with the exception of the putrescine pool established with ^{14}C-arginine. One component of the proline pool was shown to be insensitive to NaN_3 (Fig. 11); however, even this "stable" pool effluxed slowly at 0 C. This suggested that the loss of amino acid pools in *P. aeruginosa* at low temperatures may possibly be due to an inactivation of the pool-forming mechanism or to the "cold shock" phenomenon (13).

Since the proline pool effluxed at two distinct rates at 0 C, it was thought that perhaps a critical temperature existed at which only one component of the pool would efflux. When the proline pool was allowed to accumulate at 10 C and then

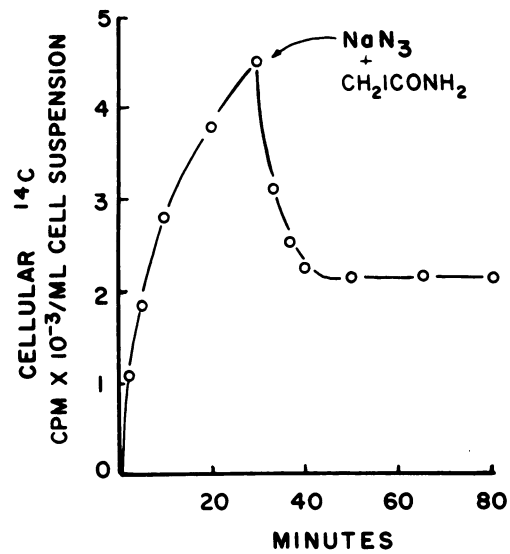

FIG. 11. *Partial efflux of the intracellular proline pool of P. aeruginosa on the addition of NaN_3 (30 mM) and iodoacetamide (1 mM). Cells were preloaded by incubating with 10^{-6} M ^{14}C-proline after an initial 30-min preincubation with 200 μg of chloramphenicol per ml. The experiment was conducted at 10 C. Procedure: Britten and McClure (2)*

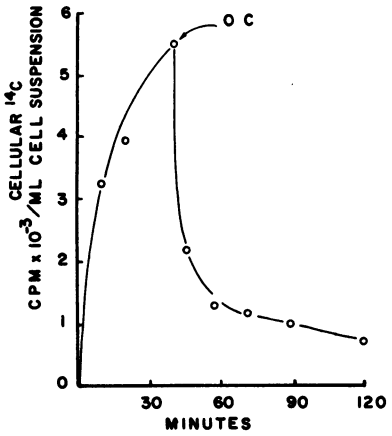

FIG. 12. *Efflux of the proline pool of P. aeruginosa at 0 C. Chloramphenicol-treated cells were preloaded with 10^{-6} M ^{14}C-proline at 10 C, then placed in an ice bath at 40 min. Procedure: Britten and McClure (2)*

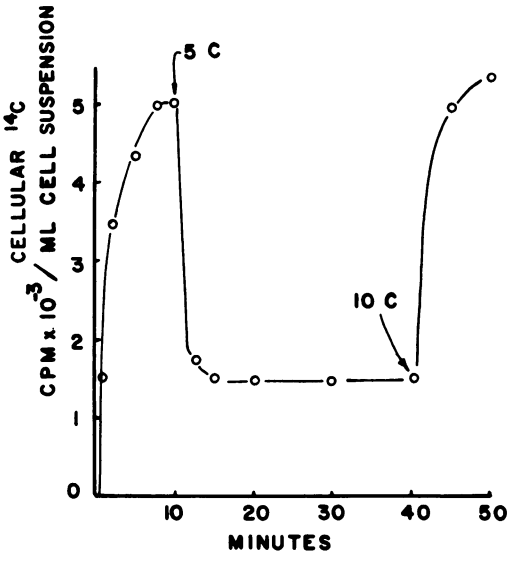

FIG. 13. *Efflux of the proline pool of P. aeruginosa at 5 C and its reestablishment at 10 C. Chloramphenicol-treated cells were preloaded with 10^{-6} M ^{14}C-proline at 10 C. Procedure: Britten and McClure (2)*

the temperature of the cell suspension was quickly reduced to 5 C, only part of the proline pool was lost by efflux. When the temperature was raised to 10 C, the first pool quickly reformed (Fig. 13). The proline concentration gradient was 890 at 10 C and 220 at 5 C.

Chilling the cell suspensions to 0 C did not cause permanent damage to the pool-forming mechanisms. When cells were cooled to 0 C, held for 30 min, and then rewarmed to 30 C for 15 min, they were found to incorporate ^{14}C-proline at the normal rate.

DISCUSSION

The patterns of amino acid pool formation in *P. aeruginosa* varied considerably with the amino acid studied. The amino acids, such as glutamate, serine, and glycine, which have multiple roles in cellular metabolism, are degraded during pool formation, but are mostly incorporated into cellular protein. Some degradative products of these amino acids accumulate in the trichloroacetic acid-soluble fraction of cells even after the exogenous amino acid supply has been exhausted. Kaback and Stadtman (9) have shown that *E. coli* membranes are responsible for the uptake and conversion of glycine or serine to phospholipids. This function could not be demonstrated with preparations from *P. aeruginosa*. The most striking example of the accumulation of a degradation product from amino acid catabolism during pool formation was that of the putrescine pool arising from arginine catabolism by this microorganism. This accumulated product must be "compartmentalized" within the cell in such a manner as to make it unavailable for cellular metabolism, since it was maintained even in starved cultures. The organism is able to utilize putrescine as a sole source of carbon and nitrogen for growth (*unpublished data*).

The majority of amino acids tested remained unchanged during the short interval that they resided in the amino acid pool during these experiments. This observation was suggested previously by chromatographic analyses of the amino acids found in the protein of cells grown in the presence of low levels of ^{14}C-amino acids (10).

The steady-state amino acid intracellular pools established in *P. aeruginosa* at low exogenous amino acid concentrations are the result of a balance between the rate of uptake into the cell and the rate of protein synthesis. Thus, high pool levels may be established with high exogenous amino acid concentrations which effectively saturate the transport system, or under conditions which selectively reduce the rate of protein synthesis, such as inhibition by chloramphenicol, lowered temperatures, or starvation for a required metabolite.

It was demonstrated previously that the uptake of ^{14}C-proline was prevented in the presence of an inhibitor of energy metabolism, such as sodium azide (10). At least part of this inhibition, however, may occur at the level of pool maintenance against a concentration gradient in addition to actual amino acid transport, since preformed amino acid pools decayed rapidly in the presence of sodium azide. This observation is consistent with those reported for the maintenance of intracellular galactoside pools in *E. coli* (12, 15) and

for the amino acid pools of other microorganisms (1).

In general, the maintenance of amino acid pools in *P. aeruginosa* differs in many respects from the pools described for *E. coli*. Amino acid pools in *E. coli* have been found to be maintained under conditions of nutrient or energy deprivation, or at 0 C (2). Preformed pools in *P. aeruginosa* decay rapidly in the absence of energy metabolism or at low temperatures, and amino acid pools are not maintained during carbon or nitrogen starvation but rather are rapidly catabolized (*unpublished data*). These data strongly suggest that the mechanism by which intracellular amino acid pools in *P. aeruginosa* are maintained may differ significantly from that proposed for *E. coli*.

The maintenance of the intracellular proline pool of *P. aeruginosa* represents a special case. The retention of a significant fraction of the intracellular pool in the presence of azide is unusual when compared with the pools of other amino acids. These results suggest that a multiplicity of intracellular proline pools may exist in this microorganism. Proline pool multiplicity has been demonstrated in *E. coli* (2, 11). However, the two proline pools found for *P. aeruginosa* must be maintained by different mechanisms, only one of which is energy-dependent.

The proline pool which is stable to the effects of azide was maintained under conditions that would not permit proline pool formation and this, therefore, dissociates the maintenance of this pool from the transport process. Furthermore, the exchange data indicate that this pool was still in some state of equilibrium with exogenously added proline. The partial efflux of the proline pool at 5 C and the reestablishment at 10 C support the hypothesis of a double pool which was indicated by the effect of azide poisoning. The most obvious explanation would be a change in the rate constant of the pool-forming mechanism at 5 C; however, control experiments indicated that uptake of proline did not occur with *P. aeruginosa* at 5 C.

The behavior of this stable pool is in accord with the model proposed by Britten and McClure (2) for *E. coli*, but with *P. aeruginosa* this proline pool would seem to be the exception rather than the rule.

The effect of high chloramphenicol concentrations on the maintenance of preformed amino acid pools was unexpected. Since these pools decayed rapidly at the complete cessation of protein synthesis, it first seemed logical that protein synthesis, even at very low levels, would be required during the active formation of intracellular pools. However, protein synthesis at 10 C may also be terminated with as little as 200 μg of chloramphenicol per ml. Under these conditions, rapid pool formation still occurred. This suggested that the maintenance of intracellular pools did not depend upon protein synthesis and that chloramphenicol had other effects upon pool maintenance. In high concentrations (600 to 2,000 μg/ml), chloramphenicol has been implicated in energy-uncoupling processes. Hanson and Hodges (6) found that chloramphenicol greatly depressed oxidative phosphorylation in maize mitochondria. Stoner, Hodges, and Hanson (14) postulated that chloramphenicol interferes at the level of high-energy intermediates. If chloramphenicol interrupts oxidative phosphorylation, one proline pool would have been expected to be retained, as was shown to be the case for azide poisoning. The majority of the pool was lost in the presence of 2 mg of chloramphenicol per ml, suggesting that perhaps a less specific alteration in permeability properties occurred in this organism. Britten and McClure (2) have shown that chloramphenicol concentrations exceeding 20 μg/ml interfered in some way with the formation of large pools in *E. coli*, but that at lower chloramphenicol concentrations an increase in the pool size occurred, as would be expected from the removal of the drain from the pool into protein.

The loss of amino acid pools at 0 C in *P. aeruginosa* may have been due to a "cold shock" phenomenon described for this organism (4, 13). It is technically difficult to separate the effects of cold shock from permease inactivation at low temperatures. Farrel and Rose (4) have shown that cold shock in *P. aeruginosa* could be prevented by growth of a psychrophilic strain at 10 C. Although our strain will grow for a short time at 10 C, continued growth at this temperature does not occur. At 0 C, cells grown at 17 C (minimal growth temperature for this strain) still lost the amino acid pools at a rapid rate.

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