INFLUENCE OF PHENYLALANINE ANALOGUES UPON BACTERIAL ACCUMULATION AND INCORPORATION OF PHENYLALANINE

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ABSTRACT

CONWAY, T. W. (The University of Texas, Austin), E. M. LANSFORD, JR., AND W. SHIVE. Influence of phenylalanine analogues upon bacterial accumulation and incorporation of phenylalanine. J. Bacteriol. **85**:141–149. 1963.—Phenylalanine accumulation and its relation to phenylalanine incorporation into trichloroacetic acid-insoluble material in *Escherichia coli* 9723 were studied with a variety of structural analogues of phenylalanine. Inhibition of phenylalanine-C^{14} incorporation was exerted by analogues only when their concentration was sufficient to prevent the formation of 85 to 90% of the accumulated intracellular phenylalanine "pool," indicating that less than about 15% of the "free amino acid pool" of phenylalanine is essential for maintaining a normal rate of incorporation of phenylalanine into protein. Although certain analogues having high activity in inhibiting accumulation of phenylalanine showed low activity in preventing incorporation, no analogue was found that solely prevented accumulation.

Upon the discovery that cells of *Escherichia coli* are capable of concentrating radioactive amino acids several fold from a medium containing low concentrations of the tracer, with the formation of "pools" of intracellular amino acids (Britten, Roberts, and French, 1955; Cohen and Rickenberg, 1956), it was assumed that these pools may be essential intermediates for the utilization of exogenous amino acids. However, more recent work with yeasts (Halvorson and Cohen, 1958; Cowie and McClure, 1959) has indicated that the large amino acid pools are not obligate intermediates for protein synthesis.

The present investigation indicates that the phenylalanine pool can be greatly reduced without affecting the normal rate of incorporation of radioactive phenylalanine into *E. coli* cells, and a comprehensive study using analogues and derived compounds of amino acids reveals some distinctions among several classes of analogues, in terms of their relative effects on accumulation and on incorporation of the amino acid.

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MATERIALS AND METHODS

Amino acids and analogues. Nonradioactive natural amino acids and derivatives (Nutritional Biochemicals Corp., Cleveland, Ohio) and uniformly labeled L-phenylalanine-C^{14} (specific activity 0.57 μc/mg, approximately 3×10^5 counts per min per mg; Schwarz Laboratories, Inc., Mt. Vernon, N.Y.) were commercial products. The amino acid analogues were obtained as follows: β-phenyl-DL-serine (Nutritional Biochemicals Corp.) and DL-*p*-fluorophenylalanine (California Corp. for Biochemical Research) were commercial products; β-2-thienyl-L-alanine, glycyl-β-2-thienyl-DL-alanine, and *p*-tolyl-DL-alanine were gifts from F. W. Dunn; β-1-naphthalene-DL-alanine and 3-thianaphthene-DL-alanine were furnished by Eli Lilly & Co., Indianapolis, Ind.; and all other analogues were generously supplied by C. G. Skinner.

Preparation of bacterial cells. E. coli (ATCC 9723) cells maintained on nutrient agar were used to inoculate 5-ml broth cultures in salts-glucose medium (Anderson, 1946). The 5-ml culture was used to inoculate 200 ml of the same medium, which was then incubated at 37 C with aeration for 10 to 12 hr. In all media, separately sterilized glucose was used. The cells were collected by centrifugation (5,000 $\times$ *g*), and were then resuspended in appropriate cold (4 C) medium. A curve relating culture turbidity to dry weight of cells was used to determine the amount of medium for resuspension.

Filtration method of measurement of phenyl-

alanine-C^{14} uptake. The membrane-filter technique of Britten et al. (1955) was used for measurement of radioactive phenylalanine accumulation and incorporation, as well as for measurement of inhibition of these processes exerted by analogues. The total amount of radioactive amino acid absorbed in the cells recovered on the membrane filter is designated as uptake. The amount of radioactivity precipitable by 5% trichloroacetic acid and counted after filtration on a separate membrane is designated as incorporation; this is assumed to be an approximate measure of the amount of protein-bound radioactive amino acid. Subtraction of incorporation radioactivity from total uptake-radioactivity yields a remainder designated as accumulation, which is taken as a measure of the free amino acid "pool" (soluble in 5% trichloroacetic acid).

In uptake experiments, chilled (4 C) cell suspensions (0.5 to 1.0 mg dry wt/ml) were equilibrated to 30 C, and then at time zero were added to tubes (also at 30 C) containing in growth medium (Anderson, 1946) at pH 7.0 sufficient L-phenylalanine-C^{14} to produce a final concentration of 2 μg/ml, with or without appropriate analogues or other supplements. At carefully timed intervals, 4.0 ml of each cell suspension were quickly withdrawn with a pipette; of this sample, 2.0 ml were placed on a membrane filter, and 2.0 ml were transferred to a tube containing 2.0 ml of 10% trichloroacetic acid. The 50-mm membrane filters (type A Bac-T-Flex; average porosity, 0.5 μ; Schleicher and Schuell Co., Keene, N.H.) were cut into 1-in. circles for use in a stainless steel filtering funnel (Tracerlab Inc., Waltham, Mass.). Although only a few seconds were required to remove the radioactive medium, each membrane was left on the funnel under suction for exactly 1 min to insure uniform removal of liquid. Appropriate controls were used to determine the amount of phenylalanine-C^{14} adsorbed to the membrane from the solution. The samples of cells which had been suspended in trichloroacetic acid (5% final), after standing about 15 min, were filtered through identical membranes and washed on the filter with 2 ml of water. Each dried membrane was fastened with a small amount of rubber cement to the bottom of a 1-in. stainless-steel planchet. The radioactivity of the samples was determined with a thin-window gas-flow detector tube and a model 192A scaler (Nuclear-Chicago Corp., Des Plaines, Ill.). At least 1,600 total counts, and usually 2,000 to 4,000 counts, were accumulated from each sample before counting was terminated.

For all experiments, chilled washed cells were used to prevent cell growth during the time required for manipulations, before commencement of the timed incubation at 30 C. In some experiments, chilling of the cells was also used as a means of terminating absorption or incorporation processes, prior to permitting resumption of these processes in an altered medium. Under these conditions, it is recognized that synchronous synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) is possible (DeLamater, 1956). In the present experiments, chilling lowered somewhat the rate of incorporation subsequently observed; but, the longer the cells remained at 0 C, the closer their subsequent rate of incorporation of phenylalanine-C^{14} returned to that obtained with unchilled cells. All incubations using previously chilled cells were continued for no longer than 15 min (less than the generation time of *E. coli*); in experiments for comparison of the relative effectiveness of analogues, controls containing radioactive phenylalanine (without analogue) were run at regularly spaced intervals, e.g., every fifth sample within a group of incubation mixtures.

Measurement of effectiveness of analogues in displacement of accumulated phenylalanine. A suspension of cells (1 mg dry wt/ml), containing 4 μg of radioactive phenylalanine per ml, was allowed to accumulate the amino acid at 30 C for exactly 5 min; the vessel was then submerged in isopropanol-solid CO_2 and twirled until the cell suspension had started to freeze, and the slushy suspension was pipetted into cold centrifuge tubes. After centrifugation ($8{,}000 \times g$) at 0 C for 10 min, the radioactive supernatant liquid was discarded. The cells were resuspended in medium at 0 C and recentrifuged; the medium was then discarded. The pellet in each tube was resuspended in warm medium (30 C), containing the appropriate analogue and other components, by vigorously expelling the medium from a syringe onto the cell pellet; at timed intervals, samples were then taken for radioactivity measurement.

Results and Discussion

Effects of natural amino acids. Certain natural amino acids influenced the uptake of phenylalanine (Table 1). The natural amino acids most

TABLE 1. *Inhibition by natural amino acids of accumulation and incorporation of phenylalanine-C^{14}*

I/S ratio†	Inhibition of "pool" (*a*) and of incorporation (*b*)*									
	L-Phenylalanine-C^{12}		Tyrosine		L-Tryptophan		L-Leucine		Histidine	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	56	59	45	9	50	−3				
3			61	7						
10			82	21	83	8				
30			91	35	86	44				
50							12	−77		
100			94	53	90	55				
300					98	64				
500							44	−66		
1,000							69	−17	48	−2
5,000							77	−1		
6,500							88	66		

* Both inhibition of "pool" and inhibition of incorporation are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. The variation between the controls was within ±8% for accumulation and ±12% for incorporation. The controls contained an average of 389 counts per min per mg of dry cells in the "pool," and an average of 100 counts per min per mg of dry cells was incorporated, during the 5-min incubation period.

† I/S = ratio of molar concentrations (supplementary amino acid/phenylalanine-C^{14}) calculated for the L isomers. Except where indicated, all amino acids were DL forms. Serine, glutamic acid, aspartic acid, proline, arginine, lysine, and glycine, at an I/S ratio of 1,000, produced no significant inhibition of either accumulation or incorporation. Inhibition of accumulation by valine, isoleucine, methionine, alanine, and threonine was less than 25% (I/S = 1,000).

closely related to phenylalanine (e.g., tryptophan and tyrosine) were highly effective in preventing the accumulation of phenylalanine, exerting half-maximal inhibition at concentrations equimolar with phenylalanine-C^{14}. This suggests that if a single process of enzymatic nature is involved in concentrating phenylalanine, then tryptophan and tyrosine each have affinity for the site approximately equal to that of phenylalanine. It is of interest that for inhibition of incorporation, 100 times as much tryptophan (or tyrosine) was required as for comparable inhibition of accumulation. Histidine, which also resembles phenylalanine structurally, was capable at high concentrations of inhibiting accumulation of phenylalanine (Table 1).

Leucine at moderately low concentrations stimulated the incorporation of phenylalanine, but at higher concentrations was capable of inhibiting both pool formation and incorporation of phenylalanine. It appears possible that leucine limits protein synthesis in *E. coli* under these conditions; thus, leucine supplements increase the rate of protein synthesis. Alternatively, leucine may prevent the enzymatic loss or destruction of phenylalanine.

Other amino acids (threonine and cysteine), at high concentrations, appear to inhibit protein synthesis without comparable inhibition of accumulation of phenylalanine. It is likely that this is the result of amino acid antagonisms unrelated to phenylalanine metabolism. Still other amino acids give slight effects, which cannot be attributed to specific effects upon phenylalanine utilization.

Effects of phenylalanine peptides and derivatives. Inhibition of accumulation and incorporation of phenylalanine by its peptides (Table 2) appears to involve equilibration of the phenylalanine moiety of the peptide with phenylalanine

TABLE 2. *Effects of peptides on accumulation and incorporation of phenylalanine-C^{14}*

I/S†	Inhibition of "pool" (*a*) and of incorporation (*b*)*					
	Supplementary peptide:					
	Glycylphenylalanine		Phenylalanylphenylalanine		Glycylthienylalanine	
	a	*b*	*a*	*b*	*a*	*b*
10	51	45	46	53	44	0
100	66	55			53	8
1,000	74	61				4
2,000					64	

* Both inhibition of "pool" and inhibition of incorporation are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. The variation between controls was ±8.5% for accumulation and ±8% for incorporation. The controls contained an average of 343 counts per min per mg of dry cells in the "pool", and an average of 143 counts per min per mg of dry cells was incorporated, during the 5-min incubation.

† See footnote to Table 1.

absorbed from the medium, since the extent of inhibition of accumulation was the same as the extent of inhibition of incorporation. This equality of effects would be expected to result from dilution of the isotopic tracer with nonradioactive phenylalanine released from the peptide. Inhibitions to an equal extent of "pool" formation and of incorporation were exerted similarly by phenylalanine ethyl ester, phenylpyruvate, and phenyllactate. In the case of a peptide containing a phenylalanine analogue (glycylthienylalanine; Table 2), the accumulation process was inhibited in preference to incorporation into protein, and, even at very high levels, glycylthienylalanine inhibited accumulation to only a limited extent. This suggests that the catalytic sites that convert glycylthienyalanine to an active form of thienylalanine are saturated at low levels of the peptide.

Effects of growth-inhibitory phenylalanine analogues. The four compounds included in Fig. 1, which are typical growth-inhibitory phenylalanine analogues for *E. coli*, were excellent inhibitors of phenylalanine accumulation; at high levels, they also inhibited phenylalanine incorporation. *p*-Fluorophenylalanine, a potent growth inhibitor, showed effects on accumulation and incorporation closely resembling those of thienylalanine; its effect on accumulation was previously studied by Cohen and Rickenberg (1956). Of the analogues in Fig. 1, β-phenylserine was relatively ineffective for inhibiting incorporation; however, the inhibition index (ratio of inhibitor to metabolite for a stated degree of growth inhibition) for β-phenylserine was relatively high, and the effectiveness of an analogue as an inhibitor of incorporation would be expected to be inversely proportional to its inhibition index.

Although there were some minor variations from one inhibitor to another in this group, in general about 85 to 90% of the amino acid was displaced from the pool before the incorporation was reduced by 5 to 15%. At this degree of inhibition, the molar ratio of inhibitor to phenylalanine-C^{14} was approximately the same as the inhibition index (for complete growth inhibition).

p-Aminophenylalanine inhibits growth as a result of inhibition of the biosynthesis of shikimic acid, rather than of inhibition of utilization of endogenous phenylalanine (Fradejas, Ravel, and Shive, *unpublished data*); thus, for this analogue pool, inhibition cannot be related to an inhibition index for growth.

Effects of alicyclic analogues. Some structural

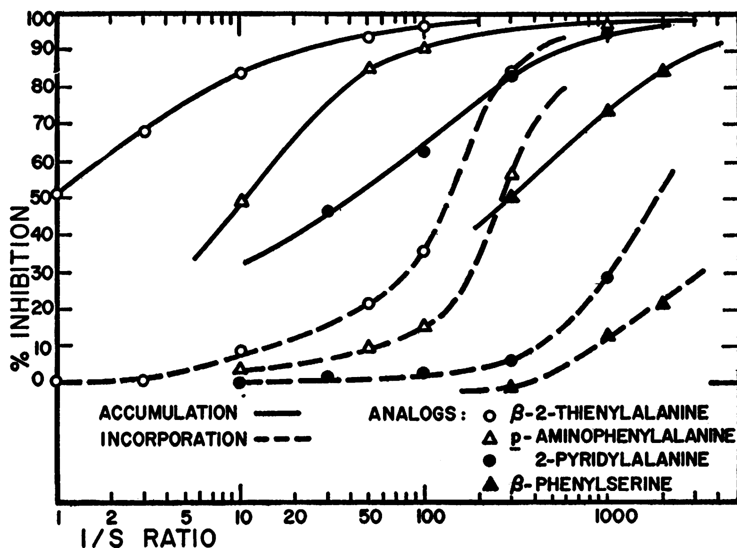

FIG. 1. *Effects of growth-inhibitory phenylalanine analogues. I/S, see footnote to Table 1. Inhibitions are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. Variation between controls was within ±8.5% for accumulation and ±10% for incorporation. The controls contained an average of 389 counts per min per mg of dry cells in the "pool," and an average of 103 counts per min per mg of dry cells was incorporated, during the 5-min incubation.*

requirements for inhibition of accumulation of phenylalanine are reflected by the data of Table 3, which indicate that 1-cyclohexenealanine and 1-cyclopentenealanine, known to be growth-inhibitory antagonists of phenylalanine for *Leuconostoc dextranicum* 8086 (Shive and Skinner, 1958) and also inhibitory for growth of a phenylalanine-requiring strain of *E. coli* 9723, are good inhibitors of accumulation of phenylalanine. In contrast, the saturated compounds cyclohexanealanine, inactive as a growth inhibitor, and cyclopentanealanine, a leucine antagonist (Pal et al., 1960), were ineffective as inhibitors of phenylalanine uptake. 3-Cyclohexenealanine, an antagonist of leucine for *L. dextranicum* (Edelson et al., 1959*a*), was as effective in inhibiting phenylalanine uptake as the other two unsaturated analogues; but it did not inhibit growth of *E. coli* 9723, wild type. Superficially, it appears that 3-cyclohexenealanine deviates considerably from the structural requirements for binding at the enzymatic sites normally associated with phenylalanine. However, inspection of the structure of one of the conformations of 3-cyclohexenealanine reveals that the β carbon and the 2, 3, 4, and 5 carbons of the ring (in a "half-chair" conformation) are very close to the same plane, whereas carbon atoms 1 and 6 are slightly out of the plane on opposite sides. Such a structure appears to be sufficiently close to that of the benzyl group for the compound to serve as an antagonist of exogenous phenylalanine utilization. This was supported by the observation that the analogue was inhibitory to the growth of a phenylalanine-requiring mutant of *E. coli* 9723, and the inhibitory effect was reversed by phenylalanine.

TABLE 3. *Effects of alicyclic analogues*

I/S†	Inhibition of "pool" (*a*) and of incorporation (*b*)*					
	Supplementary amino acid:					
	1-Cyclohexene-alanine		3-Cyclohexene-alanine		1-Cyclopentene-alanine	
	a	*b*	*a*	*b*	*a*	*b*
30					54	5
100					98	15
300					99	36
500	91	47	89	45		

* Both inhibition of "pool" and inhibition of incorporation are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. The variation between controls was within ±7%. The controls contained an average of 393 counts per min per mg of dry cells in the "pool", and an average of 120 counts per min per mg of dry cells was incorporated, during the 5-min incubation.

† See footnote to Table 1.

TABLE 4. *Effects of acyclic amino acid analogues*

Analogue	Approx I/S* ratio required for indicated per cent inhibition†		
	Accumulation		Incorporation
	50%	90%	50%
2-Amino-4-methyl-4-hexenoic acid	7	75	180
2-Amino-4-ethyl-4-hexenoic acid	170	750	1,500
2-Amino-4-heptenoic acid	10	5,000	2,000
Allylglycine	160	2,000	450
Methallylglycine	18	400	520
Ethallylglycine	25	220	500
trans-Crotylglycine	15	300	300

* I/S, see footnote to Table 1.

† Inhibitions are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. Variation between controls was within ±6% for accumulation and ±12% for incorporation. The controls contained an average of 393 counts per min per mg of dry cells in the "pool", and an average of 120 counts per min per mg of dry cells was incorporated, during the 5-min incubation.

Effects of acyclic amino acids. 2-Amino-4-methyl-4-hexenoic acid is a growth-inhibitory analogue of phenylalanine, and also weakly inhibits leucine utilization, for *L. dextranicum* (Edelson et al., 1959*b*). This compound (Table 4) was also an effective inhibitor of phenylalanine accumulation and incorporation in *E. coli* 9723. Structural comparison of this analogue with the saturated analogue (2-amino-4-methylhexanoic acid), a leucine antagonist for *E. coli* 9723 (Edelson et al., 1959*b*) but virtually inactive with respect to phenylalanine accumulation, suggests that planarity in the substituent attached to the α carbon of the alanine chain is a requirement for prevention of phenylalanine accumulation. However, this is not the only requirement, since

2-amino-4-ethyl-4-hexenoic acid, which has the proper planar configuration, only moderately inhibited phenylalanine accumulation and incorporation (Table 4) and was not a growth inhibitor for *E. coli* 9723 (Smith, Skinner, and Shive, 1961). Possibly the effect of the unsaturated linkage on the adjacent ethyl group is to force the terminal methyl into a conformation which is sterically hindered in binding at the active site. The corresponding saturated compound, 2-amino-4-ethylhexanoic acid, a leucine antagonist for *L. dextranicum* (Smith et al., 1961), did not inhibit phenylalanine uptake any more effectively than leucine itself.

Table 4 also shows that ethallylglycine is a slightly more active inhibitor of phenylalanine accumulation than methallylglycine, which in turn is more active than allylglycine. Ethallylglycine, visualized in one of its conformations, is structurally the most similar of the three to phenylalanine, and it is an isomer of the more potent analogue 2-amino-4-methyl-4-hexenoic acid. This relationship suggests that the size of the planar group on the α carbon was important in determining the effectiveness of the analogue in preventing phenylalanine accumulation. *Trans*-crotylglycine, a growth inhibitor for *L. dextranicum* that is competitively reversed by phenylalanine (Norton et al., 1962), inhibited the utilization of exogenous phenylalanine in *E. coli* (Table 4); neither this analogue nor ethallylglycine inhibited growth of *E. coli* 9723, wild type.

The stimulatory effect of leucine upon phenylalanine incorporation (Table 1), and of certain structurally related compounds having a similar effect (e.g., methallylglycine), may possibly be due to action by these compounds in preventing the destruction of phenylalanine, or in serving as nitrogen donors to form limiting amino acids essential for protein synthesis.

Effects of aromatic amino acid analogues not inhibitory to growth of E. coli 9723. None of the compounds listed in Fig. 2 inhibited growth of *E. coli* 9723 (wild type), although they prevented reversal by exogenous phenylalanine of glycylthienylalanine inhibition (Dunn, 1958) and utilization of phenylalanine by a phenylalanine-requiring mutant of *E. coli* 9723. It is noteworthy that all were excellent inhibitors of phenylalanine accumulation, as good as or better than the growth inhibitory analogues. *m*-Tolylalanine was

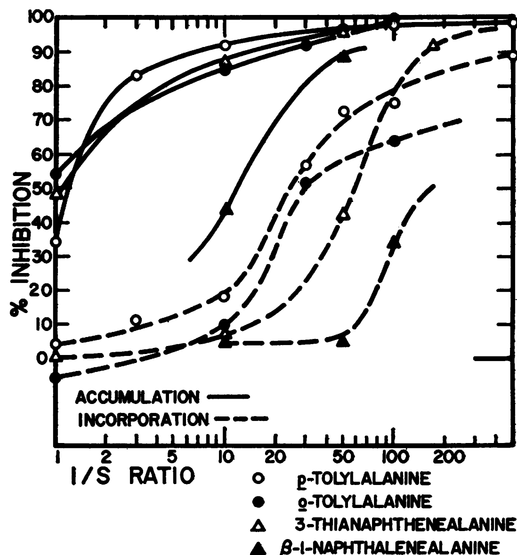

FIG. 2. *Effects of aromatic amino acid analogues not inhibitory to growth of Escherichia coli 9723. I/S, see footnote to Table 1. Inhibitions are expressed as per cent reduction of the number of counts measured in the unsupplemented controls. Variation between controls was within ±9% for accumulation and ±10% for incorporation. The controls contained an average of 387 counts per min per mg of dry cells in the "pool," and an average of 144 counts per min per mg of dry cells was incorporated, during the 5-min incubation.*

relatively ineffective, although the *o* and *p* isomers were excellent inhibitors, and another *m*-substituted phenylalanine, *m*-nitrophenylalanine, was a moderately active inhibitor of accumulation. Certain alanine-substituted double-ring compounds (3-thianaphthenealanine and 1-naphthalenealanine) were effective inhibitors of phenylalanine accumulation, yet 2-naphthalenealanine, at a ratio of analogue to phenylalanine of 50 (maximal solubility), inhibited accumulation less than 30% and had no effect on incorporation of phenylalanine.

Inhibitions by miscellaneous compounds. Structural analogues of tryptophan and tyrosine, like the parent amino acids (Table 1), were effective inhibitors of phenylalanine pool formation; examples are *m*-fluorotyrosine, 5-hydroxy-2-pyridinealanine, and 5-methyltryptophan. The ineffectiveness of phenylglycine, *p*-aminophenylglycine, and 4-piperidylglycine, as inhibitors, indicated that the alanine side chain is essen-

tial for preventing accumulation of phenylalanine, but the presence of the alanine chain attached to an aromatic ring is not a sufficient condition for activity since compounds such as *m*-tolylalanine were not highly inhibitory. Certain aromatic compounds (anthranilic acid, benzimidazole, adenine, *p*-aminobenzoic acid, phenylmercaptoacetic acid, shikimic acid) were effective inhibitors of phenylalanine accumulation only at very high concentrations. β-Phenylethylamine, *trans*-cinnamic acid, and β-phenylethanol were inhibitory in vitro to a phenylalanine-activating enzyme derived from *E. coli* 9723 (Conway, Lansford, and Shive, 1962), but were not effective inhibitors of phenylalanine accumulation.

Comparison of effectiveness of analogues as accumulation inhibitors with their effectiveness as incorporation inhibitors. Considerably higher concentrations of phenylalanine antagonists were required for inhibition of incorporation than for inhibition of accumulation. However, the ratio of analogue concentration required to inhibit incorporation (for a given degree of inhibition) to analogue concentration required to inhibit accumulation was unity when unlabeled phenylalanine (Table 1), or any compound metabolically converted to phenylalanine (Table 2), was used as the inhibitor. A ratio of about 20 to 30 was obtained with many of the most effective inhibitors of pool formation (e.g., *p*-fluorophenylalanine, *p*-aminophenylalanine, *o*- and *p*-tolylalanine, ethallylglycine; see Fig. 1 and 2, and Table 3). The highest ratios (about 100) were shown by tryptophan and tyrosine (Table 1); thus, these compounds represented the more specific inhibitors of pool formation. Neither tryptophan nor tyrosine inhibited growth of *E. coli* 9723, nor did they inhibit growth of a phenylalanine-requiring mutant strain of this organism, except at very high concentrations. Ratios less then unity were shown by compounds, structurally unrelated to phenylalanine, which are general inhibitors of protein synthesis.

Displacement of accumulated phenylalanine pool by analogues. The effect of an analogue on the utilization of a previously accumulated natural amino acid may occur in two ways: the analogue may displace the amino acid so that the isotopic tracer is released to the medium, or the analogue may inhibit the utilization of the "pool" amino acid for protein synthesis or other essential functions. Both processes may occur, and the former should greatly influence the extent of inhibition of incorporation of the accumulated amino acid. Since both the accumulation and displacement processes are known to be rapid (Cohen and Rickenberg, 1956), we expected that any displacement action of the analogue might occur quickly enough to permit distinction between pool displacement and inhibition of pool utilization. As seen from Fig. 3, thienylalanine quickly displaced (in about 0.5 min) most of the accumulated phenylalanine-C^{14}, without reducing the rate of incorporation, and phenylalanine-C^{14} which was very early displaced was evidently reutilized for incorporation into protein. A low concentration of thienylalanine was sufficient for fast displacement of most of the accumulated amino acid. It appears that, during even such rapid displacement, the rate of incorporation into protein depends on the maintenance of a very small pool of phenylalanine, i.e., 10 to 15% of the initial total. This was approximately the magnitude of the minimal pool necessary for an optimal rate of incorporation, in the presence of sufficient amounts of structural analogue to prevent the accumulation of the major portion of the pool.

Possible functions of amino acid pools. The existence of permeases or transporting systems in bacteria can at present be demonstrated only in terms of the formation of amino acid (or other metabolite) pools, by whole cells or protoplasts. The present work has clearly established that amino acid analogues can abolish the major portion of the phenylalanine pool without decreasing the rate of incorporation of the amino acid into protein. The minimal pool of accumulated phenylalanine necessary for optimal incorporation is about 0.5 mμmole/mg of dry cells. Labeling of soluble ribonucleic acid (sRNA) with phenylalanine-C^{14} catalyzed by a phenylalanine-activating crude enzyme preparation (Conway et al., 1962) achieved at most the transfer of about 0.5 mμmole/mg of sRNA. Thus, simple calculations, based on the approximate abundance of phenylalanine-specific sRNA, indicate that probably only a very small part of the residual (10 to 15%) amino acid pool required for optimal incorporation rate could consist of phenylalanyl-sRNA. Assuming that amino acyl-sRNA is an essential, although small, part of this minimal pool, present evidence warrants no more than speculation as to whether the major

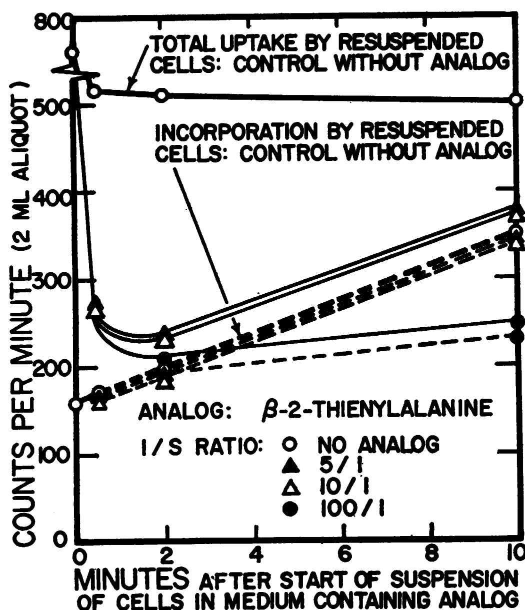

FIG. 3. *Inhibition by β-2-thienylalanine of the utilization of previously accumulated phenylalanine-C^{14}. Preincubation (see Materials and Methods) of Escherichia coli cells (0.5 mg of dry cell wt/ml, final concentration) with 4 μg of L-phenylalanine-C^{14} per ml (0.57 μc/mg) produced a total uptake of 757 counts per min per mg of dry cell wt during the 5-min incubation period. The preincubation was terminated as described in Materials and Methods. The media for resuspension of cells included a control of unsupplemented medium, and β-2-thienylalanine at the following concentrations: 20.7 μg/ml (I/S ratio, 5/1; see footnote to Table 1), 41.4 μg/ml (10/1), and 0.44 mg/ml (100/1). The solid lines correspond to the total uptake of radioactivity measured at the indicated times; the dashed lines indicate the portion of the radioactivity which remained trichloroacetic acid-insoluble (i.e., incorporation). Similar rapid and extensive displacement of the phenylalanine-C^{14} "pool," without substantial inhibition of incorporation, was exerted by 3-thianaphthenealanine (I/S ratio 10/1), p-aminophenylalanine (I/S ratio, 10/1), and p-fluorophenylalanine (I/S ratio, 1/1).*

portion of the pool (in the absence of analogues) involves phenylalanine bound in a labile conjugated form, or present as the free amino acid retained by some impermeable subcellular membrane barrier.

Since 85 to 90% of the amino acid pool could be displaced without affecting the rate of utilization of amino acid for protein synthesis or other incorporation, the function of this major portion of the accumulated pool is of interest. Observations of phenylalanine uptake into nitrogen-deficient cells provided a clue for the postulation of one function. Accumulation into nitrogen-starved cells and into normal cells is very similar, except that glucose is an effective inhibitor of the process in nitrogen-starved cells. In this respect, the system resembles the inducible tryptophan pool (Boezi and DeMoss, 1961), which also forms under conditions of unbalanced growth. From kinetic data (Table 5), it can be seen that the amino acid pool forms first, and glucose then exerts its inhibition, probably by accelerating biosynthesis of the amino acid or the breakdown of intracellular protein (as has been shown with yeast; Halvorson, 1958) with the release of amino acids, which in turn cause the displacement of the accumulated phenylalanine-C^{14}. It is possible, thus, that the major part of the amino acid pool represents a storage form, both of exogenous nutrients and of substances resulting from intracellular breakdown. However, it also appears possible that the large concentration of amino acids, comprising the 85 to 90% of the pool not essential for maintaining the normal rate of protein synthesis, may have functions related to metabolic control mechanisms, such as enzyme repression or inhibition of biosynthetic reactions.

TABLE 5. *Effect of simultaneous addition of glucose and tracer on rate of uptake of phenylalanine-C^{14} in nitrogen-deficient Escherichia coli**

Incubation time	L-Phenylalanine (mμmoles/mg of dry cells)		
	Total uptake	Incorporation	Accumulation
min			
2	4.68	0.64	4.04
4	1.81	1.30	0.51
8	2.40	1.50	0.90

* Nitrogen-deficient *E. coli* 9723 cells were prepared by resuspending normal cells in growth medium (Anderson, 1946) lacking ammonium salts, and incubating for 1 hr at 37 C. These cells (0.45 mg dry wt/ml) in 35 ml of medium containing no nitrogen compound or carbohydrate were poured at time zero into a tube containing 70 μg of L-phenylalanine-C^{14} and 0.20 ml of 20% glucose solution. Incubation was at 30 C.

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LITERATURE CITED

ANDERSON, E. H. 1946. Growth requirements of virus-resistant mutants of *Escherichia coli* strain "B". Proc. Natl. Acad. Sci. U.S. **32**:120–128.

BOEZI, J. A., AND R. D. DEMOSS. 1961. Properties of a tryptophan transport system in *Escherichia coli*. Biochim. Biophys. Acta **49**:471–484.

BRITTEN, R. J., R. B. ROBERTS, AND E. F. FRENCH. 1955. Amino acid adsorption and protein synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **41**:863–870.

COHEN, G. N., AND H. V. RICKENBERG. 1956. Concentration specifique reversible des amino acides chez *Escherichia coli*. Ann. Inst. Pasteur **91**:693–720.

CONWAY, T. W., E. M. LANSFORD, JR., AND W. SHIVE. 1962. Purification and substrate specificity of a phenylalanine-activating enzyme from *Escherichia coli* 9723. J. Biol. Chem. **237**:2850–2854.

COWIE, D. B., AND F. T. MCCLURE. 1959. Metabolic pools and the synthesis of macromolecules. Biochim. Biophys. Acta **31**:236–245.

DELAMATER, E. D. 1956. Bacterial chromosomes and their mechanism of division. Sixth Symp. Soc. Gen. Microbiol. p. 215–260.

DUNN, F. W. 1958. Inhibition studies with β-2-thienylalanine, β-1-naphthylalanine, *p*-tolylalanine and their peptides. J. Biol. Chem. **233**:411–414.

EDELSON, J., C. G. SKINNER, J. M. RAVEL, AND W. SHIVE. 1959*a*. 3-Cyclohexene-1-DL-alanine, an analog of leucine. Arch. Biochem. Biophys. **80**:416–420.

EDELSON, J., C. G. SKINNER, J. M. RAVEL, AND W. SHIVE. 1959*b*. Structural specificity of some amino acid antagonists. J. Am. Chem. Soc. **81**:5150–5153.

HALVORSON, H. O. 1958. Intracellular protein and nucleic acid turnover in resting yeast cells. Biochim. Biophys. Acta **27**:255–266.

HALVORSON, H. O., AND G. N. COHEN. 1958. Incorporation of the endogenous and exogenous amino acids in yeast proteins. Ann. Inst. Pasteur **95**:73–87.

NORTON, S. J., M. C. MAUNG, C. G. SKINNER, AND W. SHIVE. 1962. Structural requirements for phenylalanine antagonism. III. Some unsaturated straight-chain amino acids. Arch. Biochem. Biophys. **96**:287–292.

PAL, P. R., C. G. SKINNER, R. L. DENNIS, AND W. SHIVE. 1956. Cyclopentane-alanine and 1-cyclopentene-1-alanine, inhibitory analogs of leucine and phenylalanine. J. Am. Chem. Soc. **78**:5116–5118.

SHIVE, W., AND C. G. SKINNER. 1958. Metabolic antagonists. Ann. Rev. Biochem. **27**:643–678.

SMITH, L. C., C. G. SKINNER, AND W. SHIVE. 1961. Structural requirements for phenylalanine antagonism. Arch. Biochem. Biophys. **94**:443–448.