Stability of α-Hydrogen of Amino Acids during Active Transport*

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ABSTRACT: In *Escherichia coli* W, glycine and DL-alanine, both tritiated in the α- position, were used to measure the possible loss of an α-hydrogen atom during active transport.

Radioactive glycine that had been transported by the cells was trapped in glutathione. No significant loss of α-tritium from either glycine or DL-alanine was found.

Although a variety of simple chemical substances are actively transported by bacterial cells, little information exists on the biochemical reactions that occur during the process of transport. This paper reports the results of experiments designed to detect one possible chemical change in substrate molecules that are actively accumulated by cells of *Escherichia coli* W.

Schwartz *et al.* (1959) showed that glycine, D-serine, and probably L-alanine are actively transported by the same system in *E. coli*. Each of these amino acids has a free α-hydrogen atom. We found that aminoisobutyric acid, a glycine analog that lacks an α-hydrogen, is not concentrated by cells of *E. coli*. These observations suggested that the presence of an α-hydrogen might be essential for active transport of glycine and its analogs in *E. coli*.[1] Therefore the loss of tritium counts during active transport of α-tritiated glycine and DL-alanine was measured, both in wild-type strains and in mutants that are defective in transport of these amino acids.

Experimental Procedures

Amino Acids. Nonradioactive amino acids, D-cycloserine, D-[1-^{14}C]alanine, L-[1-^{14}C]alanine, and [1-^{14}C]-aminoisobutyric acid were obtained from California Corp. for Biochemical Research and Mann Research Laboratories. Uniformly labeled [^{14}C]glycine, tritiated glycine, and tritiated water were purchased from New England Nuclear Corp. Chloramphenicol was a gift from Parke-Davis Co.

Preparation of α-Tritiated Alanine. α-Tritiated alanine was prepared by a modification of a method previously used to prepare deuterated lysine (Clark and Rittenberg, 1951). DL-Alanine (500 mg), glacial acetic acid (4.7 ml), and anhydrous sodium acetate (450 mg) were heated under reflux for 1 hour, and acetic anhydride (1.65 ml) was added. After 2 hours at 100°, tritiated water (1 ml, 20 mc) was added and heating was continued for 1 hour. The solvents were removed by lyophilization, and the residue was taken up in 2 N HCl (30 ml) and heated under reflux for 6 hours. After removal of HCl under reduced pressure, the product was dissolved in water and poured through a 2 × 12-cm column of Dowex 50 (acid form). The column was thoroughly washed and then eluted with 2 M ammonia. Tritiated DL-alanine was obtained by evaporation under reduced pressure and was recrystallized from ethanol-water. The product was chromatographically homogeneous in two solvent systems (1-propanol-water, 4:1, and 80% phenol) and had a specific activity of 80,000 cpm/mg.

Location of the tritium in the alanine molecule was confirmed by enzymatic transamination with glutamic-pyruvic transaminase (Rowsell, 1962). Upon isolation and purification, the pyruvate prepared from the tritiated alanine contained less than 400 cpm/mg.

Mutants and Cultures. Two strains of *E. coli* W were used: (a) Tr_{gly}^+, a glycine auxotroph, was obtained from Dr. B. D. Davis; (b) Tr_{gly}^-, a mutant that requires high levels of glycine (500–1000 μg/ml) for rapid growth, was derived from Tr_{gly}^+. (The symbols Tr_{gly}^+ and Tr_{gly}^- denote properties of glycine transport, and are sometimes referred to in the text that follows as the wild-type and mutant strains, respectively.) Related studies on these strains and the methods for isolation are described elsewhere (Lubin *et al.*, 1960; Lubin, 1962; Kessel and Lubin, 1963).

All strains were stored at −20° in growth medium supplemented with 5–10% glycerol. Thawed cells were inoculated into glucose-salts medium (medium A; Davis and Mingioli, 1950) supplemented with glycine at 1 mg/ml, and well-aerated cultures were grown at 37° to a density of about 150 Klett units (420-mμ filter).

Measurement of Transport of Glycine and Glycine Analogs. Chloramphenicol (200 μg/ml) was added to exponentially growing cultures and, after an addi-

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[1] Mammalian cells, however, have been shown to concentrate aminoisobutyric acid (Christensen, 1960; Maio and Rickenberg, 1962; Paine and Heinz, 1960). Christensen *et al.* (1958) found that ^{18}O was not lost from aminoisobutyric acid as a result of active transport.

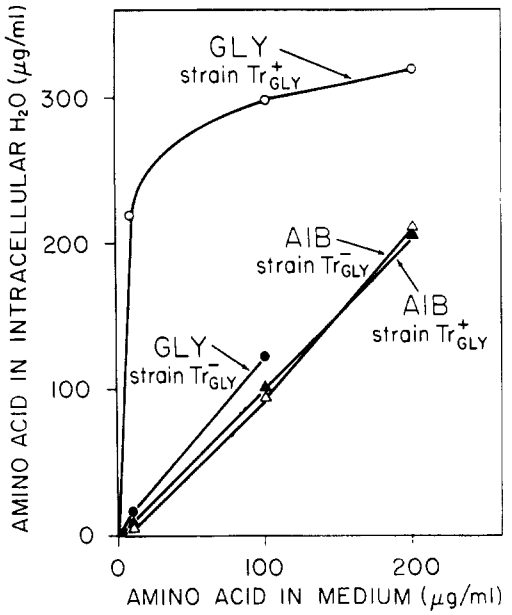

FIGURE 1: Uptake of [^{14}C]glycine and aminoisobutyrate by cells of strain Tr_{gly}^{+} and Tr_{gly}^{-}. Cultures were incubated for 2 minutes at 24° in medium containing radioactive substrate, as described under Methods. Samples were then collected on Millipore filters and radioactivity was determined. The data of Figure 1 represent the average values from five experiments, none of which deviated by more than ±5% from the reported values. Glycine (GLY) (○ or ●); aminoisobutyrate (AIB) (△ or ▲).

tional 30 minutes of incubation at 37°, the cells were collected by centrifugation. They were then washed twice at 0° in CM-glucose buffer (medium A supplemented with 200 μg chloramphenicol). Uptake of substrates was unimpaired by storage of cells at 0° for as long as 2 hours. The final suspension contained 3.3 mg of cells (wet wt) per ml.

Suspensions of cells in 0.5-ml portions were shaken in a water bath at 24° for 2 minutes. Radioactive amino acids were then added, usually in a volume of 5 μl. In some experiments, nonradioactive compounds to be tested for inhibitory activity were also added at this point. Incubation was then continued for another 2 minutes.

Samples of 0.2 ml were collected in duplicate on HA-Millipore filters (0.45-μ pore size) and washed briefly in CM-glucose buffer at 24°, and the radioactivity retained on the filter was measured with a Nuclear-Chicago thin-window gas-flow counter (background 2 cpm).

Measurement of Radioactive Glycine Trapped in Glutathione. To measure incorporation of radioactive glycine into intracellular glutathione, 100 mg of cells (wet wt) was suspended in 35 ml of CM-glucose buffer containing [^{12}C]glycine (at a concentration of either 0.1 or 10 mM), [^{14}C]glycine, and tritiated glycine. After incubation for 5 minutes at 24° with shaking, the cells were collected by centrifugation at 6000 × *g* for 5 minutes at 0° and blotted dry with filter paper. Cells were extracted for 5 minutes with 6 ml of 5% trichloroacetic acid containing 30 mg of carrier glutathione, and isolation of the copper-glutathione complex was carried out by the method of Bloch and Snoke (1960).

For each determination the dried copper-glutathione complex was suspended in 15 ml of toluene-Liquifluor (Pilot Chemicals Inc., Watertown, Mass.) in 20-ml vials initially two-thirds filled with powdered Thixotropic Gel (Packard Instrument Corp.). Radioactivity from ^{14}C and tritium was determined in a Packard scintillation counter.

To measure biosynthesis of glutathione in the cell-free system, extracts of Tr_{gly}^{+} and Tr_{gly}^{-} strains were prepared by the method of Samuels (1953). Glutathione was isolated and radioactivity was determined as before.

Measurement of Radioactive Alanine in Intracellular Pool. Uptake of a mixture of DL-[1-^{14}C]alanine and α-tritiated DL-alanine was determined by incubation of 100 ml of a suspension containing 1 g of cells (wet wt) in CM-glucose buffer containing radioactive substrate. After incubation at 24° for 5 minutes with shaking, the cells were collected by centrifugation at 0° (6000 × *g* for 5 minutes) and blotted dry with filter paper, and the pool of small molecules was extracted into 5 ml of 5% trichloroacetic acid containing 1 mg of DL-[^{12}C]-alanine.

Cell debris was removed by centrifugation at 12,000 × *g* for 10 minutes, and the supernatant fluid was poured through a 0.8 × 15-cm column of Dowex 50 (hydrogen form). The resin was then washed with 20 ml of deionized water, and absorbed amino acids were eluted with 2 M ammonia. After removal of the ammonia by lyophilization, the residue was dissolved in 0.1 ml of water and an aliquot was streaked on Whatman 3MM paper. The chromatogram was developed for 36 hours in 80% propanol, the alanine spot was cut out and eluted with 3 ml of water into a 20 ml vial, 14 ml of Bray's solution (Bray, 1960) was added, and the radioactivity from ^{14}C and tritium was determined. About 80% of the total radioactivity in the pool was recovered as alanine.

Results and Discussion

Glycine and DL-*Alanine Uptake by Wild-Type and Mutant Strains.* When Tr_{gly}^{+} cells of *E. coli* were suspended in CM-glucose buffer with 7.5 μg/ml of [^{14}C]glycine, the counts that accumulated within a few minutes corresponded to an intracellular concentration of 220 μg/ml (Figure 1). However, chromatographic analysis showed that most of the radioactivity was in metabolites of glycine, including glutathione, purines, and carboxylic acids. This finding prompted us to determine if glycine was actively transported before conversion to other products occurred.

Therefore, a suspension of Tr_{glv}^{+} cells was mixed

with [^{14}C]glycine for 1 minute. The cells were rapidly collected on large Millipore filters (0.45-μ pore size, 47-mm diameter) and washed briefly with CM-glucose buffer. The pool of small molecules was extracted with ethanol and was examined for glycine by chromatography. The intracellular concentration was found to be about 50 μg/ml, or 7-fold greater than the concentration in the medium.

For mutant Tr_{gly}^-, in contrast, the ratio of intracellular to extracellular radioactivity after a 2-minute pulse of glycine was close to 1. Aminoisobutyric acid was not actively transported either by mutant or by wild-type strains; the concentration in the intracellular pool was close to that of the medium (Figure 1).

In the wild-type strain Tr_{gly}^+, the kinetics of uptake of radioactive glycine were nonlinear: the rapid phase of uptake of counts lasted only 1–2 minutes, and over the next 20 minutes the counts further increased by 100%. For L-alanine, the kinetics of uptake were similar to those of glycine. For both glycine and L-alanine, the slow phase of uptake of radioactivity presumably was due to conversion of these substrates to other products. For D-alanine, uptake was nearly complete at the end of 2 minutes; during the next 20 minutes the counts increased by only about 5%. The initial rates of entry of D-alanine and L-alanine were similar.

In mutant strain Tr_{gly}^-, uptake of [^{14}C]glycine and D-[^{14}C]alanine was depressed to about one-twentieth of the normal rate; uptake of L-[^{14}C]alanine was depressed to only one-half of the normal rate.

Studies of Competition of Substrates for the Glycine Transport System. In studies of competition for uptake, both D- and L-alanine at levels of 75 μg/ml were found to decrease uptake of [^{14}C]glycine (7.5 μg/ml) to 20% of the control level. In contrast, addition of aminoisobutyric acid, or a variety of other amino acids (sarcosine, L-valine, L-serine, L-proline, D-isovaline), resulted in only a slight decrease of uptake of radioactive counts, invariably by less than 10%. A few D-alanine analogs (D-amino-*n*-butyrate, D-serine, and D-cycloserine) inhibited uptake of [^{14}C]glycine, D-[^{14}C]alanine, and L-[^{14}C]alanine by 30–40%.

Growth of wild-type cells (Tr_{gly}^+) on solid media was clearly inhibited by D-cycloserine[2] (3 μg/ml) or by D-serine (25 μg/ml) (Davis and Maas, 1949; Maas and Davis, 1950). The mutant strain (Tr_{gly}^-) grew well in the presence of these levels of D-cycloserine and D-serine. Six mutants, derived from separate clones of wild-type strain, were selected for resistance to D-serine or D-cycloserine; these mutants also showed decreased uptake of [^{14}C]glycine, D-[^{14}C]alanine, and L-[^{14}C]alanine comparable to results with mutant Tr_{gly}^-.

Thus, by several criteria, glycine, D-alanine, and D-cycloserine appear to be on the same transport system. L-Alanine and D-serine also seem to be transported by the glycine transport system, but may have additional means of rapid access into the cell. Similar results were reported by Mora and Snell (1963) for *Streptococcus faecalis:* they found that glycine, D- and L-alanine, and D-cycloserine were on a common transport system.

Fate of α-Tritium Atom in Glycine during Active Transport. The failure of *E. coli* W to concentrate aminoisobutyrate suggested that active transport of glycine analogs might require the presence of an unsubstituted α- position.[3] According to this hypothesis, D- or L-alanine could be transported on the glycine system, because one α-hydrogen is unsubstituted. The uptake of tritiated glycine and DL-alanine was therefore investigated. If the tritium attached to the α-carbon atom were exchanged during transport, the amino acid derived from the internal pool after active transport

TABLE 1: Uptake of a Mixture of α-[^{3}H]Glycine and [2-^{14}C]Glycine by *E. coli* W.[a]

Strain	External Concn. of Glycine (mM)	Counts Present in Glutathione as ^{14}C	as ^{3}H	^{3}H/^{14}C
A Tr_{gly}^+	0.1	1,000	10,000	10.0
		1,050	12,000	11.4
		1,050	11,000	10.5
B Tr_{gly}^+	10.0	700	8,000	11.4
C Tr_{gly}^-	0.1	80	1,000	12.5
		100	1,100	11.0
D Tr_{gly}^-	10.0	200	2,300	11.5
		200	2,140	10.7
E Tr_{gly}^+ (cell-free)	32.0	11,700	138,000	11.8

[a] System: 100 mg of cells, wet wt, was suspended in 35 ml of CM-glucose buffer and was incubated for 5 minutes with radioactive glycine at 24°. In both A and C, the specific activities were 7000 cpm of ^{14}C and 84,000 cpm of ^{3}H per μg (^{3}H/^{14}C ratio of 12.0); in both B and D the specific activities were only 70 cpm of ^{14}C and 840 cpm of ^{3}H. After incubation, cells were extracted as described under Methods. The cell-free experiment (E) was done under conditions specified in Figure 2; 1 μc of [^{14}C]glycine and 10 μc of [^{3}H]glycine were present. The data above are from a single experiment. Two repetitions yielded ^{3}H/^{14}C ratios of 11.5 ± 1.2 for all samples.

[2] D-Cycloserine inhibits synthesis of D-alanyl-D-alanine in *E. coli* (Strominger, 1962).

[3] In *E. coli*, α-methylamino acids did not inhibit uptake of analogs containing free α-hydrogen. Thus, isovaline failed to inhibit uptake of [^{14}C]valine or leucine, and cycloleucine failed to inhibit uptake of [^{14}C]leucine. Dr. Sondra Schlessinger has found (personal communication) that α-methylhistidine did not interfere with histidine uptake in *Salmonella typhimurium;* we obtained the same results with *E. coli.*

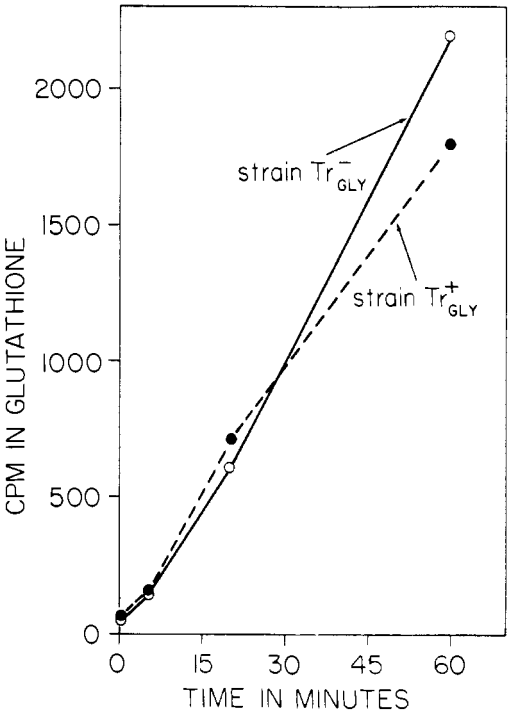

FIGURE 2: Incorporation of [^{14}C]glycine into glutathione in cell-free extracts of *E. coli* W. System: 1.0 ml of an extract of alumina-ground cells (200 mg of cells, wet wt, per ml); 250 μmoles of potassium phosphate buffer (*p*H 7.7); 32 μmoles each of glycine, cysteine, and glutamate; 6 μmoles of ATP; 0.6 μmole of DPN; 66 μmoles of Mg hexose diphosphate; 1 μc of uniformly labeled [^{14}C]glycine, all in a total volume of 3.0 ml. The reaction was stopped by addition of 3 ml of 10% trichloroacetic acid. After high-speed centrifugation, supernatant fluid was used for isolation of glutathione, as described under Methods. Strain Tr_{gly}^- (○—○); strain Tr_{gly}^+ (●—●).

had occurred should show markedly decreased radioactivity.

One possible misinterpretation, however, might result from an isotope effect, which is pronounced for tritium in most chemical systems, and which could cause an increased or decreased selection of tritium atoms, compared with hydrogen, in attachment of labeled glycine to the transport system. With α-tritiated alanine, the results would be expected to be less ambiguous. Any labeled molecule must lose its tritium atom during active transport if exchange were a necessary part of this biochemical process.

The rapid conversion of glycine to other products in the intracellular pool complicated the experimental design. Although our data, as stated above, indicate that counts of [^{14}C]glycine were rapidly accumulated by *E. coli*, the bulk of radioactivity was not found in glycine, but in glutathione and other glycine metabolites. We initially found that the biosynthesis of glutathione did not involve loss of α-hydrogen from glycine

TABLE II: Uptake of DL-[^{14}C]Alanine + DL-[α-^{3}H]Alanine by *E. coli* W.[a]

Strain	External Concn of DL-Alanine (mM)	Intracellular DL-Alanine cpm as ^{14}C	Intracellular DL-Alanine cpm as ^{3}H	^{3}H/^{14}C
Tr_{gly}^+	0.1	225	1700	7.6
	2.0	350	2700	7.7
Tr_{gly}^-	0.1	40	350	8.8
	2.0	188	1560	8.3

[a] System: Suspensions of 1 g of cells, wet wt, were incubated in 100 ml of CM-glucose buffer containing doubly labeled DL-alanine. Specific activity throughout was 80 cpm/μg for DL-[^{3}H]alanine and 9.0 cpm/μg for DL-[^{14}C]alanine; ^{3}H/^{14}C ratio = 8.9. After 5 minutes at 24°, cells were collected by centrifugation, and the alanine pool was isolated as described under Methods. Figures reported are for a typical experiment. Two repetitions yielded results which did not differ from these by more than 10%. At least 80% of the radioactive material accumulated by the cells was recovered as alanine.

(Table I, E), and that the kinetics of synthesis of glutathione from [^{14}C]glycine proceeded at the same rate in extracts of mutant and wild type (Figure 2). Therefore the glycine taken up could be determined by measuring the pool of intracellular glutathione.

To measure the fate of α-tritiated glycine during active transport, cells of strain Tr_{gly}^+ and Tr_{gly}^- were suspended in CM-glucose buffer containing [^{3}H]glycine and [^{14}C]glycine, with a ratio of ^{3}H/^{14}C counts in the medium of 12:1. After 5 minutes of incubation at 37°, the cells were harvested and the pool of small molecules was extracted as described under Methods. The ratio of ^{3}H/^{14}C counts was determined for glycine both in the medium and in the glutathione pool.

Little or no change in the ratio of ^{3}H/^{14}C glycine occurred as a result of transport. The ratio of counts in glutathione was at least 90% of the input ratio of counts in the medium (Table I, A, B).

A control experiment was carried out to test the possibility that a change in the ^{3}H/^{14}C ratio might result from metabolic processes unrelated to transport. In mutant Tr_{gly}^-, any such loss would presumably have occurred to the same extent as in wild-type cells. The results in Table I (C and D) show that no greater loss occurred in mutant Tr_{gly}^-, compared with wild-type Tr_{gly}^+, in the process of entry of glycine and conversion to glutathione. (This control would have been crucial if a large loss of tritium counts had been observed during transport of glycine by wild-type cells.)

As stated previously, D-alanine and, probably, L-alanine are on the same transport system as glycine.

This conclusion is based on studies of competition for entry and on measurements of uptake of alanine by mutants that have lost the transport system for glycine (Schwartz *et al.*, 1959; and present study). In experiments similar to those with α-tritiated glycine, cells of wild-type (Tr_{gly}^+) and mutant (Tr_{gly}^-) strains were incubated for a brief interval with α-tritiated DL-alanine and DL-[^{14}C]alanine. The pool was isolated and the ratio of $^3H/^{14}C$ counts was measured. More than 80% of the counts in the intracellular pool, as noted before, were recovered as alanine, as shown by chromatographic methods. With DL-alanine in the medium at a concentration of 0.1 mM, the radioactivity recovered from the intracellular pool of small molecules corresponded to an apparent 20-fold concentration of DL-alanine.

The ratio of $^3H/^{14}C$ in the medium was 8.9, and in the extracted pool the ratio ranged from 7.6 to 8.8 (Table II). The small difference between the results obtained with Tr_{gly}^+ and Tr_{gly}^- strains was probably not significant. Therefore active transport of DL-alanine does not appear to result in loss of tritium from the α- position.

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