procedure, the corresponding peptide was obtained in only 21% yield. However, the following amino acids were tried in the above procedure, but with complete lack of success: *dl*-tryptophan, *dl*-phenylalanine, *l*(+)-glutamic acid, sarcosine and β-alanine.

Although the ethyl ester of glycine gave a peptide ester using method L, glycine amide led to no isolable product.

α-Substituted Carbobenzyloxyglycylaminomalonic Esters. Method N. By Alkylation of I. Preparation of the α-Methyl Derivative.—To a solution of 1.20 g. (0.052 mole) of sodium in 50 ml. of dry ethanol was added with stirring 18.3 g. (0.05 mole) of diethyl carbobenzyloxyglycylaminomalonate. To the viscous yellow solution obtained after 15 minutes of stirring was added in one portion 7.1 g. of methyl iodide. The reaction mixture was then refluxed and stirred for 2 hours, another 4.0 g. of methyl iodide was added, refluxing was continued 8 more hours and then still another 3.1 g. of methyl iodide was added after which refluxing was continued for a final 5 hours. The mixture was cooled and poured into ice with stirring. The separated oil was taken up in ether, washed with water and saturated brine, and dried over anhydrous magnesium sulfate. After filtering, the solvent was evaporated, and the residual oil was dried to constant weight in a vacuum at 50°; yield 14.96 g. (79%). This was hydrogenated directly.

In extending this procedure to the preparation of higher homologs it was found that addition of the alkyl iodide (100% excess) in one portion did not affect the yield.

Method O. By a Michael Reaction of I. Preparation of Diethyl α-Cyanoethylcarbobenzyloxyglycylaminomalonate.—A freshly cut cube of sodium about one-eighth inch on a side was dissolved in 15 ml. of dry ethanol and 12.2 g. (0.0333 mole) of diethyl carbobenzyloxyglycylaminomalonate was added to give a thick slurry. To this was added with stirring 2.00 g. (0.0377 mole) of acrylonitrile dropwise over a period of ten minutes during which time some heat was evolved and the entire mass liquefied to a light yellow solution. After stirring one hour at room temperature, the solution was filtered and allowed to stand overnight at room temperature. Since the product gave no evidence of crystallizing, the solution was poured into water, the precipitated oil was taken up in ether and the ether solution was dried over anhydrous magnesium sulfate. The solvent was removed and the residual oil was dried in a vacuum at 50° to give 12.0 g. (86%) of crude product. This was hydrogenated directly.

Method P. Michael Reactions of II. Preparation of 3-Carbethoxy-3-β-cyanoethyl-2,5-piperazinedione.—A cube of sodium about one-eighth inch on a side was dissolved in 15 ml. of dry ethanol and 3.72 g. (0.02 mole) of 3-carbethoxy-2,5-piperazinedione was added. To this suspension was added 1.10 g. (0.0208 mole) of acrylonitrile dropwise with shaking. Heat was evolved and the reaction mixture set to a solid mass, which was broken up and kept at room temperature for 2 hours with occasional shaking. After removal by filtration, the colorless solid was washed three times with dry ethanol and dried to give 4.10 g. (85%) of crude product, m.p. 199–202°. Recrystallization from 125 ml. of water gave 2.90 g. of colorless plates, m.p. 207–208°. When mixed with a sample, m.p. 205–206°, of the cyano compound prepared by the reductive cyclization (method C) of diethyl β-cyanoethylcarbobenzyloxyglycylaminomalonate (method O), there was no depression of melting point.

Although substitution of ethyl acrylate for acrylonitrile in the above procedure led to the expected product in isolable form (Table IV), acrolein gave only a product in the form of a brittle, hygroscopic glass not successfully converted to a purified form.

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[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGY OF YALE UNIVERSITY]

An Ornithine–Proline Interrelation in *Escherichia coli*[1]

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Ornithine-C^{14}, supplied at low concentration (0.1 μg. per ml.) to growing *Escherichia coli* cells, is incorporated into the proline (and glutamic acid) as well as into the arginine of the bacterial protein. The conversion of ornithine to proline constitutes a quantitatively minor metabolic link and appears to involve glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate as intermediates. When ornithine-C^{14} is supplied at higher concentrations, patterns of labeling result that tend to deëmphasize this ornithine-proline link.

In *E. coli*, the major biosynthetic routes leading to proline and to ornithine have been established.[2] Proline was shown to be formed from glutamate *via* glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate[3]; and ornithine is synthesized from glutamate *via* N-acetylglutamate, N-acetylglutamic γ-semialdehyde and N^{α}-acetylornithine.[4] Studies with N-acetylglutamate-C^{14} have suggested a quantitatively minor pathway (not *via* glutamic acid) contributing to proline synthesis.[5] In the present investigation, tracer incorporation experiments with the same microbial species have demonstrated a limited conversion of *exogenous* ornithine into proline; and isotopic competition experiments have indicated that this conversion proceeds *via* glutamic γ-semialdehyde. In view of these findings, a similar conversion of *endogenous* ornithine to proline may well account for the minor pathway previously reported.

The appreciable *relative* contributions of labeled ornithine to the proline and glutamic acid of the bacterial protein that have been observed in the present incorporation experiments appear to depend on the low concentration (0.1 μg. per ml.) of the ornithine used. At higher ornithine concentrations (*e.g.*, 10 μg. per ml.) the specific activities of protein proline and glutamic acid are decreased relative to that of protein arginine. The latter amino acid is known to be derived biosynthetically from ornithine.[6] In the isotopic competition experiments of Abelson,[6] ornithine used at a still higher concentration (about 100 μg. per ml.) gave rise to almost all the protein arginine, but did not contribute detectably, within the sensitivity of the method employed,[7] to protein proline and glutamic acid.

The present incorporation experiments with uniformly C^{14}-labeled ornithine were carried out by

(1) These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Yale University.

(2) H. J. Vogel, in W. D. McElroy and B. Glass, "Amino Acid Metabolism," The Johns Hopkins Press, Baltimore, Md., 1955, p. 335.

(3) H. J. Vogel and B. D. Davis, THIS JOURNAL, **74**, 109 (1952).

(4) H. J. Vogel, *Proc. Natl. Acad. Sci.*, **39**, 578 (1953).

(5) H. J. Vogel, P. H. Abelson and E. T. Bolton, *Biochim. Biophys. Acta*, **11**, 584 (1953).

(6) P. H. Abelson, *J. Biol. Chem.*, **206**, 335 (1954).

(7) It is estimated that a contribution by exogenous ornithine to proline, amounting to 10 moles per 100 moles proline formed, could have been readily detected.

methods previously described.[5,8] For the isotopic competition experiments[6,5,8] an analogous procedure was followed, except that, in addition to the labeled ornithine, unlabeled competitors were included in the culture medium used. The results obtained are summarized in Table I.

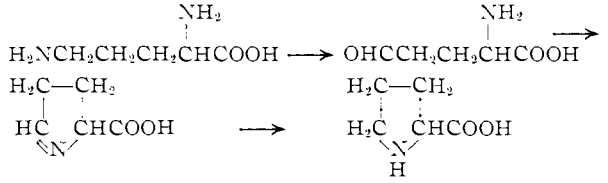
TABLE I

EFFECT OF C^{12}-COMPETITORS ON THE INCORPORATION OF ORNITHINE-C^{14} INTO PROTEIN AMINO ACIDS OF *Escherichia coli* (AS RELATIVE SPECIFIC ACTIVITY)[a]

Competitor added	Arg	Pro	Glu
None	100	22	15
Glutamic γ-semialdehyde	100	1	12
Proline	100	1	12
Arginine hydrochloride	100	28	4

[a] The specific activity figures shown are relative to the respective protein arginine values. Arg, arginine; Pro, proline; Glu, glutamic acid.

It is seen from the results obtained without added competitor that exogenous ornithine contributes not only to arginine, but also to proline and glutamic acid. The competition results show that added glutamic γ-semialdehyde (which is in equilibrium with Δ^1-pyrroline-5-carboxylate) as well as proline, but not arginine, largely suppress the incorporation of the labeled ornithine into protein proline relative to protein arginine. It is therefore concluded that exogenous ornithine forms proline *via* glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate as intermediates, as shown below.



In the experiment without added competitor and in those with the semialdehyde or proline as competitors, the total uptake of radioactivity by the cells was approximately the same. However, with arginine as competitor, this uptake was only about 12% of that obtained without added competitor. In the experiment with added arginine, the low relative specific activity obtained for glutamic acid points to a competition phenomenon, which may involve the formation of unlabeled ornithine from the added arginine. If so, the results suggest that the ornithine thus formed does not equilibrate completely with the labeled ornithine taken up by the cells.

The quantity of proline produced from labeled ornithine is small: the proline so formed, in the absence of competitors, was calculated to be less than 0.1% of the total amount of proline formed by the cells.

The intermediate formation of glutamic γ-semialdehyde in the conversion of exogenous ornithine to proline suggests that the semialdehyde is produced by δ-transamination of the ornithine. Although no appreciable ornithine δ-transaminase activity could be detected in extracts of the B strain of *E. coli*,[9,10] the cells may nevertheless have enough of this activity to make possible the relatively small flow of metabolites involved. However, the possibility of a less direct mechanism for the formation of the semialdehyde has not been excluded.

The minor extent to which exogenous ornithine contributes to proline in *E. coli* contrasts with the ready and substantial conversion of exogenous ornithine to proline in mammals[11] and certain fungi.[8,12] These observations are paralleled by the findings that the latter organisms,[9,13,14] in contradistinction to *E. coli*,[9,10] show appreciable ornithine δ-transaminase activity. *E. coli* has also been found to differ from mammals and fungi in other features of the glutamate–proline–ornithine interrelation.[2]

Experimental

Materials.—Uniformly labeled L-ornithine-C^{14}, prepared from L-arginine-C^{14} isolated from algae grown with $C^{14}O_2$ as carbon source, was obtained through the generosity of Dr. E. T. Bolton; the specific activity of the ornithine-C^{14} was about 30 millicuries per millimole. DL-Glutamic γ-semialdehyde, which is in equilibrium with its spontaneous cyclization product, Δ^1-pyrroline-5-carboxylate, was prepared as previously described.[8] The L-proline and the L-arginine hydrochloride used were commercial products.

Incorporation Experiments.—Ornithine-C^{14} (about 1.5 μg.) was added to unlabeled glucose-salt medium (15 ml.) in which cells of *E. coli*, strain B, were growing exponentially at 37° with aeration. The bacteria were permitted to grow from an optical density (at 620 mμ) of about 0.1 to a density of about 0.2. The organisms were then harvested and extracted successively with cold trichloroacetic acid and with ethanol, ethanol–ethyl ether, and hot trichloroacetic acid. The resulting protein residue was hydrolyzed with hot 6 *N* hydrochloric acid; excess hydrochloric acid was removed by evaporation. The amino acids in the hydrolysate were separated by paper chromatography, and the chromatograms obtained were radioautographed, as previously described.[5,6,8] The relative molar specific activities of the arginine, proline and glutamic acid separated were determined[8] with the aid of a gas flow counter.

Competition Experiments.—The isotopic competition experiments[6,5,8] were carried out analogously, except that C^{12}-competitors (about 100 μg. per ml.) were included in the culture medium used. The resulting relative molar specific activities of the relevant protein amino acids were then determined as before.

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