The Identification of 5-Hydroxyl-L-norvaline in Cultures of Pyridoxine Auxotrophs of Escherichia coli B

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THE IDENTIFICATION OF 5-HYDROXY-L-NORVALINE IN CULTURES OF PYRIDOXINE AUXOTROPHS OF *ESCHERICHIA COLI* B[1]

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ABSTRACT.—Gc-ms and hplc have been employed to identify 5-hydroxy-L-norvaline (L-2-amino-5-hydroxypentanoic acid) in the culture fluids of pyridoxine-starved cultures of two pyridoxine auxotrophs of *Escherichia coli* B. The production of 5-hydroxynorvaline is shown to be secondary to pyridoxine starvation of the cultures, and the quantities of 5-hydroxynorvaline are similar to those of the common protein amino acids present in these cultures. This is the first time that 5-hydroxynorvaline has been identified as a product of bacterial metabolism.

5-Hydroxynorvaline (2-amino-5-hydroxypentanoic acid) has been suggested, but not well-established, as a biosynthetic precursor of natural products produced by plants and microorganisms. It was first tested as a precursor in biosynthetic studies of thioglucosides in maturing rape plants, but low incorporations of radioactivity were obtained (1). A later investigation in microorganisms reported the incorporation of [3,4-$^{13}C_2$]-5-hydroxynorvaline into the β-lactamase inhibitor clavulanic acid by *Streptomyces clavuligerus* (2). However, more recent investigations (3,4) have shown that L-ornithine rather than 5-hydroxynorvaline is the precursor of clavulanic acid, and further support for the role of ornithine is provided by the apparent retention of the δ-nitrogen atom of ornithine (5) in the biosynthetic intermediate, clavaminic acid (6), and by the presence of an oxidative deamination reaction as a late step in clavulanic acid biosynthetic pathway (7). In another investigation, a precursor role for 5-hydroxynorvaline is supported by the efficient and specific incorporation of ^{14}C label into carbamoylpolyoxamic acid antibiotics by *Streptomyces cacaoi* var. *asoensis* (8), but the much smaller incorporation of ^{15}N and 2-^{3}H labels observed in these experiments is less consistent with 5-hydroxynorvaline as a direct precursor. Also, an ω-hydroxy-α-amino acid dehydrogenase that catalyzes the interconversion of 5-hydroxynorvaline and glutamic semialdehyde has been partially purified from the mycelia of *Neurospora crassa*, but 5-hydroxynorvaline was not considered as an actual biosynthetic intermediate in this organism (9).

Although 5-hydroxynorvaline is a known constituent in the seeds of higher plants [Jack Bean (10) and *Astragalus* species (11)] and is a component of humilixanthin, a betalin isolated from the fruits of *Rivina humilis* (12), it has not been described as a natural product occurring in microorganisms. We now report, however, that pyridoxine-starved cultures of two pyridoxine auxotrophs of *Escherichia coli* B produce 5-hydroxy-L-norvaline in quantities similar to those of common protein amino acids found in these cultures.

RESULTS AND DISCUSSION

The pyridoxine auxotrophs, *E. coli* WG2 and WG25, were being used in our laboratory to further investigate the biosynthesis of the vitamin. Mutant WG2, which

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is known to lack pyridoxol oxidase activity, synthesizes, accumulates, and excretes pyridoxol and pyridoxol phosphate when it is starved for pyridoxal (13). The protein product of the blocked gene in the PdxA mutant WG25 is not known, but it has been shown in our laboratory (unpublished observations) and in earlier studies by Dempsey and Pachler (14), that culture fluids produced by pyridoxine-starved cultures of the mutant WG25 cross-feed cultures of Group I pyridoxine auxotrophs, such as *E. coli* B WG140. This suggests that, under these culture conditions, mutant WG25 produces an intermediate of the pyridoxine biosynthetic pathway. Furthermore, the cross-feeding activity, and presumably the biosynthetic intermediate, can be detected in the amino acid fraction of the culture fluid. The cross-feeding observation (unpublished) stimulated our investigations of the amino acids produced by these mutant organisms.

The culture fluids of WG2 and WG25 contain complex mixtures of amino acids. A typical amino acid profile, obtained from the lyophilized extract of WG2 (Experiment 12, Table 1), is shown as an ion chromatogram in Figure 1. The mass spectra of the major components were identical to those of the protein amino acids listed in Table 1, except for the mass spectrum (Figure 2, top) corresponding to the major peak at scan #1801 in Figure 1. The presence of a major peak in this mass spectrum at a relatively high *m/z* of 358 implied that, in addition to the α-amino and α-carboxyl groups, a functional group on the side chain of the amino acid had been derivatized. Furthermore, if the even mass number 358 corresponds to the molecular ion, a second nitrogen atom must be present in the amino acid. Under the derivatization conditions employed, amine nitrogens are trifluoroacetylated, and TMSi esters and ethers are formed with carboxyl and alcohol groups, respectively. When the masses of two derivatized amines and a derivatized carboxyl group are combined with that of the α-CH, only 4 mass units are available to account for the remainder of the amino acid side chain. Clearly

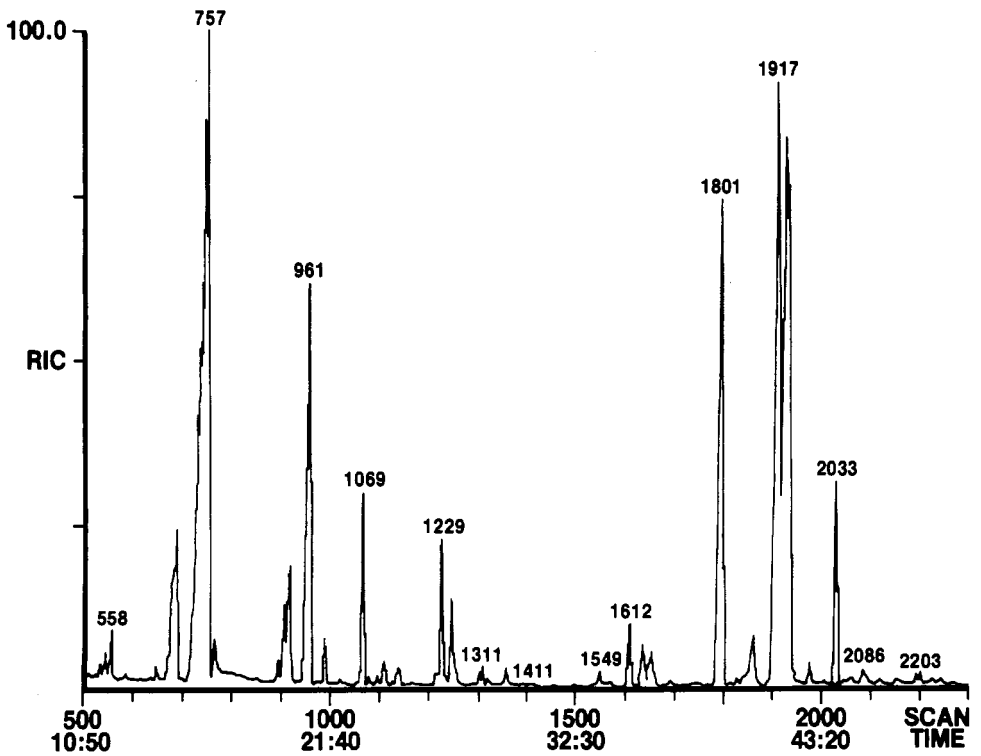

FIGURE 1. Reconstructed total ion chromatogram of the amino acid profile from experiment 12 (mutant WG2). The peak at scan #1801 is 5-hydroxy-L-norvaline.

TABLE 1. Amino Acid Content (μmol/liter) of *E. coli* WG2 and WG25 Culture Fluids.

Amino Acid	Experiment Number[a] and Source of the Sample[b]													
	E. coli WG25						*E. coli* WG2							
	Expt 1 14+P	Expt 2 14+P/ 6−P	Expt 3 14+P/ 23−P	Expt 4 23+P	Expt 5 23+P/ 6−P	Expt 6 23+P/ 23−P	Expt 7 14+P	Expt 8 14+P/ 6−P	Expt 9 14+P/ 23−P	Expt 10 23+P	Expt 11 23+P/ 6−P	Expt 12 23+P/ 23−P	Expt 13 23+P/ 23−P +L-Pro (5 mM)	Expt 14 23+P/ 23−P +L-Thr (5 mM)
Aspartic Acid	833	58	34	159	8	12	554	20	550	23	22	41	218	3
Threonine	20	7	125	11	22	43	39	10	53	—	4	3	5	—
Serine	84	8	11	10	11	20	40	4	12	—	4	3	—	—
Glutamic Acid	4421	150	19	1700	147	29	209	33	635	356	31	184	33	79
Glycine	95	18	35	15	20	28	70	1043	2675	6	182	827	2	378
Alanine	2725	58	47	1531	16	92	219	27	132	180	22	76	18	16
Valine	2020	98	299	896	6	22	79	—	152	290	412	173	48	266
Isoleucine	93	12	28	28	21	30	93	33	141	9	33	50	15	83
Phenylalanine	39	18	582	34	40	245	42	27	490	8	3	129	—	29
5-Hydroxynorvaline	—	33	18	—	17	18	18	83	743	—	23	116	13	19

[a]See Experimental for a full explanation of the culture conditions used in each experiment.

[b]In this table a shorthand notation is used to define the source of the sample, e.g., 14+P is the sample from the 14-h incubation plus pyridoxal, 14+P/6−P is the sample from the 6-h second incubation minus pyridoxal of cells that had been first incubated for 14 h plus pyridoxal. 23+P/23−P is therefore, the sample from the second 23-h incubation without pyridoxal of cells that had been first incubated for 23 h plus pyridoxal.

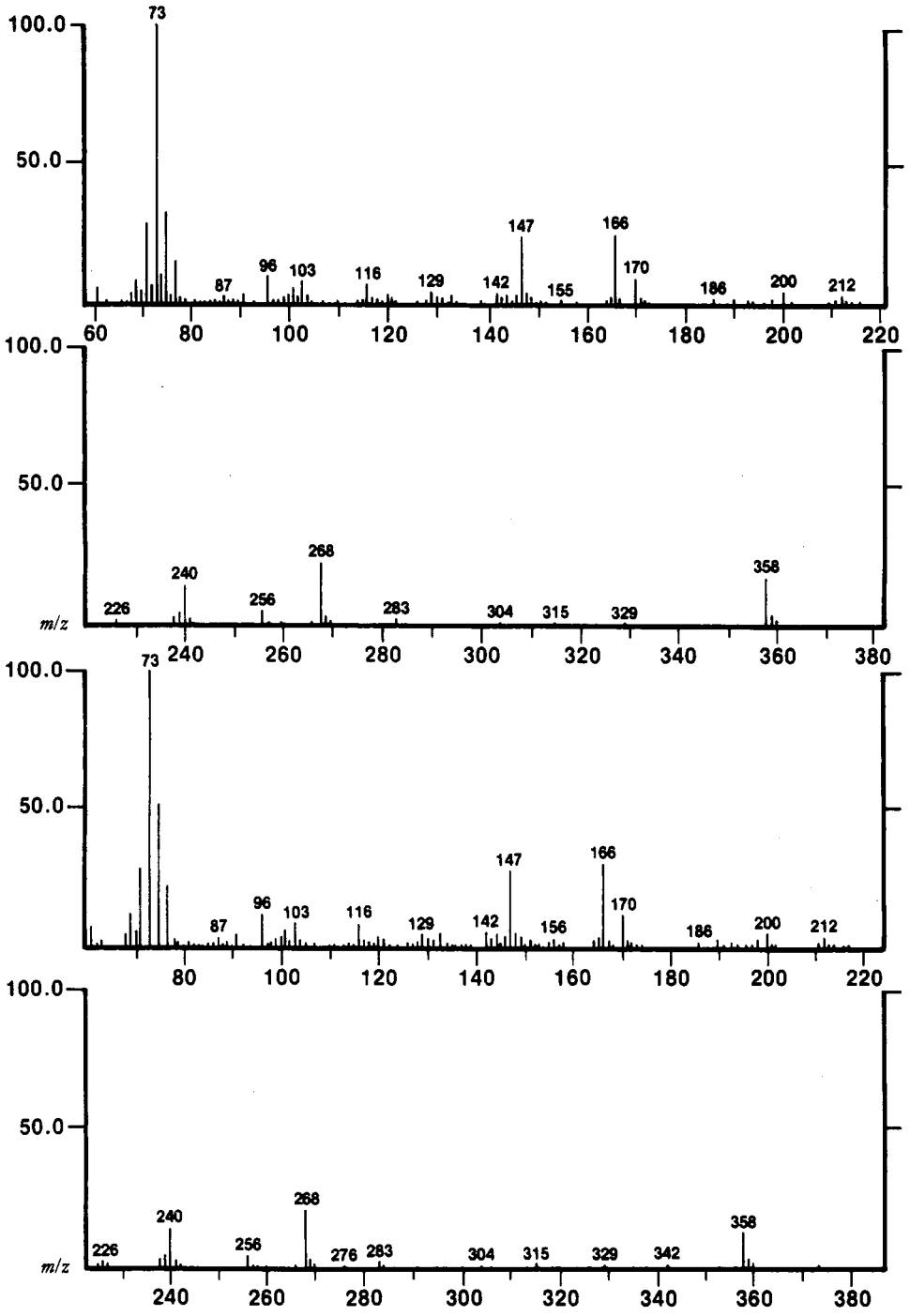

FIGURE 2. Mass spectra (eims) of the peak at scan #1801 in Figure 1 (top) and of authentic 5-hydroxy-L-norvaline (bottom).

this reasoning does not lead to a feasible structure, and it suggests that the peak at *m/z* 358 is not the molecular ion.

However, the loss of Me from TMSi group is a commonly observed fragmentation

in TMSi derivatives (15), and therefore a molecular mass of 373 is plausible. The presence of a second nitrogen atom is excluded by this odd mass number and, assuming that the derivative contains a second TMSi group, the amino acid side chain must correspond to 58 mass units. Two oxygen-containing side chains, $C_2H_2O_2$ and C_3H_6O, are possible, but $C_2H_2O_2$ is identical to the side chain of aspartic acid and can be excluded, since aspartic acid corresponds to another peak in the ion chromatogram. A side chain corresponding to C_3H_6O must contain a hydroxyl group, which would be derivatized with a TMSi group. The presence of this second TMSi group is supported by a peak at *m/z* 166, which is consistent with the loss of Me_3SiCO_2- and Me_3SiO- groups from a molecular ion of mass 373. A peak corresponding to cleavage of the side chain at the α-carbon of the unknown amino acid is not observed in the mass spectrum (Figure 2, top). A survey of fragmentation patterns of TMSi derivatives of amino acids (15) indicated that this observation was associated mainly with amino acids lacking heteroatom substituents on the β and γ carbons. Consequently, the most likely position of the hydroxyl group is the terminal carbon of the side chain, and the unknown amino acid is 5-hydroxynorvaline.

To confirm this interpretation of the mass spectral evidence, an authentic sample of 5-hydroxynorvaline was prepared by borohydride reduction of the γ-ethyl ester of glutamic acid. The mass spectrum of the synthetic sample (Figure 2, bottom) was identical to that obtained for the peak at scan #1801 (Figure 2, top). In addition, the identity of 5-hydroxynorvaline in culture fluids was confirmed by comparison of hplc and amino acid analyzer retention times to those of the synthetic sample.

The stereochemistry of 5-hydroxynorvaline was determined by hplc using modified literature conditions for the separation of amino acid enantiomers as diasteromeric *o*-phthalaldehyde derivatives (16). Figures 3 and 4 show liquid chromatograms of the amino acid fractions obtained from *E. coli* WG25 (Expt. 6) and *E. coli* WG2 (Expt. 9). For each, the 5-hydroxynorvaline co-chromatographs with the authentic L isomer and not the D isomer. Also, no significant peak was observed at the retention time of the D isomer in these chromatograms.

The quantitative data presented in Table 1 show that 5-hydroxynorvaline was present in cultures of both WG2 and WG25 mutants. Small amounts of this amino acid were found in the culture fluid from a 14-h (to late log phase) incubation of *E. coli* WG2 in a pyridoxal-supplemented medium (Expt. 7), whereas no 5-hydroxynorvaline was detected in either the fluid from a 23-h (to stationary phase) incubation of WG2 supplemented with pyridoxal (Expt. 10) or in the 14-h and 23-h culture fluids of mutant WG25 grown in the presence of the vitamin (Expts. 1 and 4). However, transfer of these organisms from the pyridoxal-supplemented medium to a pyridoxal-free medium led to a production of 5-hydroxynorvaline in amounts comparable to those of protein amino acids.

The production of 5-hydroxynorvaline by both *E. coli* mutants demonstrates that the biosynthesis of this amino acid is not a function of the specific PdxA mutation present in WG25. Instead, the results described above suggest that the production of 5-hydroxynorvaline is related to a general depletion of intracellular, biologically active vitamin (pyridoxal and pyridoxamine-5-phosphate) that is secondary to the omission of pyridoxal from the culture medium. Also, neither the D nor the L isomer of 5-hydroxynorvaline supports the growth of mutant WG140 (data not shown). Therefore, the 5-hydroxynorvaline detected in the amino acid fraction of mutant WG25 is not responsible for the cross-feeding ability of this mixture.

The presence of amino acids in the culture fluids is evidence of the fact that amino acid biosynthesis continues even in the absence of pyridoxine (17), and the presence of 5-hydroxynorvaline is evidence that pyridoxine starvation can induce an alternative

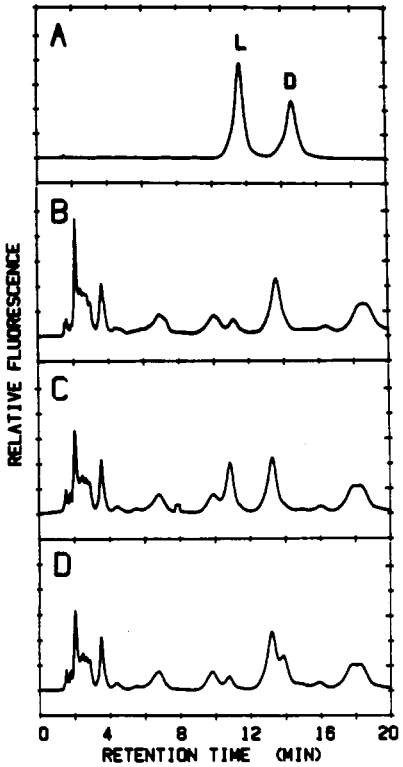

FIGURE 3. Identification of 5-hydroxy-L-norvaline by hplc. (A) Mixture of authentic D- and L-samples of 5-hydroxynorvaline. (B) Extract from experiment 6 (*E. coli* WG25). (C) Extract from experiment 6 supplemented with authentic 5-hydroxy-L-norvaline. (D) Extract from experiment 6 supplemented with authentic 5-hydroxy-D-norvaline.

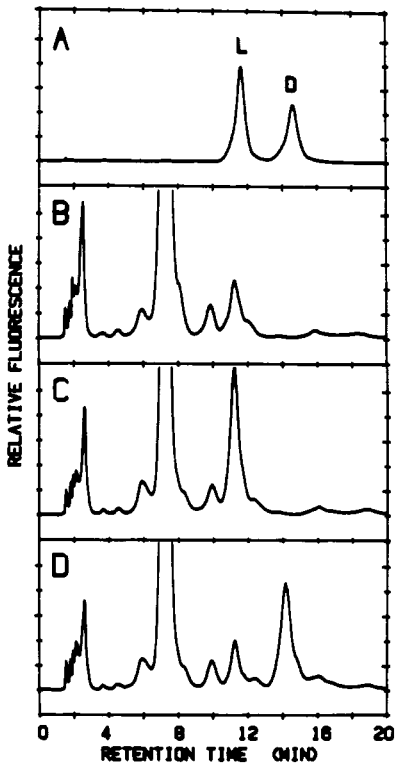

FIGURE 4. Identification of 5-hydroxy-L-norvaline by hplc. (A) Mixture of authentic D- and L-samples of 5-hydroxynorvaline. (B) Extract from experiment 9 (*E. coli* WG2). (C) Extract from experiment 9 supplemented with authentic 5-hydroxy-L-norvaline. (D) Extract from experiment 9 supplemented with authentic 5-hydroxy-D-norvaline.

metabolic pathway. The most obvious biosynthetic route to 5-hydroxynorvaline is by the reduction of glutamic semialdehyde. Although a dehydrogenase that converts the semialdehyde to 5-hydroxynorvaline has been isolated from *N. crassa* (9) and 5-hydroxynorvaline supports the growth of a proline auxotroph of this organism presumably via its conversion to glutamic semialdehyde by the dehydrogenase (18), this reduction has not been reported in bacteria. However, the production of glutamic semialdehyde from glutamate in the course of the biosynthesis of proline by bacteria is well described (19), and the first enzyme of this pathway, glutamate kinase, is subject to feedback inhibition by L-proline (20). Addition of L-proline to the pyridoxine-free resuspension medium resulted in decreased production of 5-hydroxynorvaline (Expt. 13 compared to Expt. 12), but the effect was not specific, as the concentration of other amino acids in the culture fluid showed a similar reduction, and analogous results were obtained when L-threonine, a known inhibitor of aspartate kinase I (21), was included in the pyridoxine-free resuspension medium (Expt. 14).

Alternatively, glutamic semialdehyde may be formed by transamination of L-ornithine by ornithine δ-aminotransferase (22), or 5-hydroxynorvaline may be formed

from *N*-acetylglutamic semialdehyde, an intermediate of arginine biosynthesis, by reduction and deacetylation. The latter route is particularly attractive, since the *N*-acetylation prevents the spontaneous cyclization of glutamic semialdehyde to L-pyrroline-5-carboxylate. Moreover, *N*-acetylglutamic acid might accumulate under conditions of pyridoxine starvation, since the normal fate of *N*-acetylglutamic acid semialdehyde is transamination to *N*-acetylornithine by acetylornithine δ-aminotransferase, an enzyme which requires pyridoxine as a cofactor (23). Further investigations of the biosynthesis of 5-hydroxynorvaline in *E. coli* are required to distinguish among these hypotheses.

EXPERIMENTAL

ORGANISMS.—The pyridoxine auxotrophs of *E. coli* B were induced from the wild type by uv irradiation and isolated by a penicillin enrichment technique (14). *E. coli* WG2 is a mutant of the PdxH gene, which maps at minute 36 on the *E coli* K-12 chromosome (24). It lacks pyridoxol phosphate oxidase activity and requires pyridoxal or pyridoxamine for growth (13). *E. coli* WG25 is a mutant of the PdxA gene, which maps at minute 1 of the chromosome (24). The protein product of this gene is unknown.

CULTURE CONDITIONS.—Stock cultures of WG2 and WG25 were maintained on agar slants of nutrient media ("Nutrient Broth," Oxoid, London, UK) at 4°. Stocks were subcultured onto slants of minimal media containing glycerol (0.5% w/v) as the carbon source (25) and pyridoxal hydrochloride (6×10^{-7} M) and incubated for 24 h (37°). These cultures were used to inoculate the liquid media. All liquid culture incubations were on a rotary shaker at 37° and 400 rpm, and the ratio of flask volume to culture volume was 4:1.

Experiments 1 and 4.—Minimal medium (2×500 ml) containing pyridoxal (6×10^{-7} M) was inoculated with *E coli* WG25. Incubation was for 14 h and 23 h, respectively.

Experiments 2 and 5.—Minimal medium (2×500 ml) containing pyridoxal (6×10^{-7} M) was inoculated with *E. coli* WG25 and incubated for 14 h and 23 h, respectively. The cells were harvested by centrifugation and washed with isotonic saline (3×200 ml). The washed cells were resuspended in the minimal medium (2×1 liter) without pyridoxal and incubated ("second incubation") for 6 h.

Experiments 3 and 6.—Conditions were the same as for experiments 2 and 5, except that the second incubations after the saline washes were for 23 h.

Experiments 7–12.—The conditions for these incubations correspond to experiments 1,2,3,4,5, and 6, respectively, except that *E. coli* WG2 was the organism.

Experiments 13 and 14.—The conditions were the same as for experiment 12, except that the medium for the second incubation (i.e., after the saline wash) contained L-proline (5 mM) in experiment 13, and L-threonine (5 mM) in experiment 14.

ISOLATION AND ANALYSIS OF AMINO ACIDS IN CULTURE FLUIDS.—At the end of the incubations, cells were separated from the culture fluids by centrifugation. Each culture fluid (i.e., the single fluids from experiments 1, 4, 7, and 10, and the fluids from the second incubations of the remaining experiments) was reduced in volume to approximately 75 ml on a rotary evaporator. In each case, this material was acidified with HCl (6M) to approximately pH 2 and applied to a cation-exchange column (Dowex 50×8, 100–200 mesh, H^+ form, 20×2 cm). The column was washed with dilute HCl (0.1 M, 100 ml) followed by H_2O until the pH of the effluent was neutral. Organic bases and amino acids were eluted with dilute NH_4OH (3%). The eluate was taken to dryness, redissolved in 0.1 M HCl (10 ml), and loaded onto a second cation-exchange column (Dowex 50×8, 100–200 mesh, H^+ form, 10×1 cm). The column was washed with H_2O until the effluent was neutral, and the amino acid fraction was eluted with dilute pyridine (0.1 M). The eluate was lyophilized and analyzed for the presence of 5-hydroxynorvaline by conventional amino acid analysis, by gc-ms, and by hplc.

Amino acid analysis.—Portions (ca. 1 mg) of the lyophilized fractions were dissolved in H_2O (100 μl) containing internal standard (*S*-2-aminoethyl-L-cysteine), and the mixture was analyzed by conventional cation-exchange chromatography with ninhydrin detection (Beckman 6300 amino acid analyzer, Beckman, Palo Alto, CA). Each peak in a chromatogram was identified by its absolute retention time and its retention time relative to the internal standard. These retention time data were obtained from the analysis of a calibration mixture of authentic L-amino acids. Peak areas (Model 3390A integrator, Hewlett Packard Corp., Palo Alto, CA) were used to calculate the amino acid concentrations (μmol/liter) presented in Table 1 by reference to the calibration data using the internal standard ratio method.

Gas chromatography-mass spectrometry.—Lyophilized material (ca. 1 mg) was dissolved in about 50 μl

BSTFA (*N-O*-bistrimethylsilyltrifluoroacetamide, Pierce, Rockford, IL) in a sealed vial (Reactivial™) and heated (15 min at 60°C). *N*-Methyl-bistrifluoroacetamide (Pierce, 30 µl) was added, and the mixture was heated for a further 30 min at 60°C. The amino acids were converted to the *N*-trifluoroacyl TMSi esters by this procedure, and the hydroxyl functions of hydroxy-amino acids, such as serine and 5-hydroxynorvaline, were converted to the corresponding TMSi esters.

Gc-ms was carried out on a Finnigan 4500 gc-ms with Incos™ data system (Finnigan-Matt Corp., San Jose, CA). The derivatization mixture (1 µl) was injected by a "cool on column" procedure onto a DB1 fused silica capillary column (30 m×0.32 mm i.d., J and W Scientific, Rancho Verde, CA). The column was interfaced directly onto the ion source of the mass spectrometer. The carrier gas was He (ca. 1 ml/min). Ionization was by electron impact, with electron energy of 70 eV, emission current 0.35 mA, and ion source temperature 150°. The mass spectrometer was tuned to "unit resolution" and the mass spectrum scanned repetitively from 50 to 500 amu at the rate of 1.25 sec/scan. After the injection, the chromatograph was held at 90° for 4 min and then programmed at 2.5°/min up to 250°. The components of the resulting amino acid "profile" were identified by comparing their retention times and mass spectra with those of authentic samples of amino acids.

High performance liquid chromatography.—*o*-Phthalaldehyde reagent [100 µl, prepared from Sigma incomplete reagent (1 ml) and *N*-acetyl-L-cysteine, 5 mg/liter (0.2 ml)] was added to an aqueous solution of lyophilized extract (20 µl). The derivatization reaction proceeded at ambient temperature (15 min), and was stopped by the addition of 100 µl of the chromatographic mobile phase. A portion (20 µl) of this solution was chromatographed on a Nucleosil 5 C_{18} column (250×4.6 mm, Phenomenex, Torrance, CA) in a mobile phase of MeCN (8%), copper (II) acetate (2.5 mM), and L-proline (5 mM) adjusted to pH 6 with ammonium acetate (16,26). The flow rate was 2 ml/min, and fluorescent detection was at 420–650 nm with excitation at 305–395 nm. This system resolves the D and L enantiomers of 5-hydroxynorvaline. The enantiomeric composition of the material in the lyophilized fraction was determined by co-chromatography with authentic samples of D- and L-5-hydroxynorvaline. The enantiomeric composition of the D- and L-glutamic acids used as synthetic starting materials was determined using the above conditions except that a lower concentration of MeCN (2%) was used in the mobile phase.

CHEMICAL SYNTHESIS OF 5-HYDROXYNORVALINE.—The individual D and L isomers of 5-hydroxynorvaline were prepared from D- and L-glutamic acid, respectively, by esterification (27) and reduction of the resulting 5-ethyl esters (10,28,29).

5-Ethyl glutamate.—Glutamic acid (3 g, 0.02 mol, Sigma Chemical Co.) was added to a solution of H_2SO_4 (1.3 ml, 0.025 mol) in absolute EtOH (30 ml) and stirred for 4 h at 45°. A solution of diethylamine (5.1 ml, 0.08 mol) in absolute EtOH (17 ml) was added, and the mixture was kept at 4° for 16 h. The white solid obtained by filtration was recrystallized from aqueous EtOH to yield 1.63 g (46% yield). Tlc [Si gel, *n*-BuOH–HOAc–H_2O (4:1:1)] with ninhydrin detection indicated that 5-ethyl glutamate (R_f 0.25) was free of glutamic acid (R_f 0.06). D-isomer: mp 178–180°; ^{1}H nmr (361.0 MHz, D_2O) δ 4.14 (2H, q, J=7.2 Hz), 3.74 (1H, t, J=6.4 Hz), 2.53 (2H, apparent td, J=7.6 Hz), 2.07–2.18 (2H, m), 1.23 (3H, t, J=7.2 Hz); ^{13}C nmr (90.79 MHz, D_2O) δ 174.3, 173.4, 61.4, 53.5, 29.5, 25.0, 12.8. L-isomer: mp 173–175° [lit. (28) mp 190°, lit. (30) mp 194°]; ^{1}H nmr (361.0 MHz, D_2O) δ 4.16 (2H, q, J=7.1 Hz), 3.76 (1H, t, J=6.4 Hz), 2.54 (2H, apparent td), 2.10–2.21 (2H, m), 1.24 (3H, t, J=7.0 Hz); ^{13}C nmr (90.79 MHz, D_2O) δ 174.3, 173.4, 61.4, 53.5, 29.5, 25.0, 12.8.

5-Hydroxynorvaline.—5-Ethyl glutamate (1.5 g, 8.6 mmol) was added as a solid to a stirred solution of lithium borohydride (0.30 g, 14 mmol) in THF (45 ml, dried over KOH, distilled, and stored over 3Å molecular sieves) at 0°. The solution was warmed to room temperature over 1 h and refluxed for 4 h. Additional $LiBH_4$ (0.30 g, 14 mmol) was added and the mixture refluxed for another 3 h. The reaction mixture was cooled to 0°, H_2O (15 ml) was added, and the mixture was acidfied with 3M HCl (5 ml). The solution was concentrated in vacuo to remove THF and applied to a cation-exchange column (Amberlite IR-120, 20–50 mesh, H^+ form, 44×2.5 cm). The column was washed with H_2O (500 ml) and 0.3 M NH_4OH. Fractions (500 ml) were collected and those containing amino acids (numbers 5 and 6) were combined and evaporated to dryness in vacuo to give a white solid which was recrystallized from aqueous EtOH. Tlc [Si gel, *n*-BuOH–HOAc–H_2O (4:1:1) indicated that 5-hydroxynorvaline (R_f 0.23) was free of glutamic acid (R_f 0.15) and 5-ethyl glutamate (R_f 0.44). D-isomer: mp 221–222; ^{1}H nmr (361.0 MHz, D_2O) δ 3.73 (1H, t, J=6.1 Hz), 3.60 (2H, t, J=6.2 Hz), 1.92–1.85 (2H, m), 1.67–1.53 (2H, m); ^{13}C nmr (90.79 MHz, D_2O) δ 175.2, 61.5, 55.1, 27.72, 27.67. L-isomer: mp 220–221° [lit. (10) mp 216° lit (31) mp 231.5°, lit. (32) mp 220-220.5°]; ^{1}H nmr (361.1 MHz, D_2O) δ 3.74 (1H, t, J=6.1 Hz), 3.62 (2H, t, J=6.3 Hz), 1.94–1.86 (2H, m), 1.67–1.56 (2H, m); ^{13}C nmr (90.80 MHz, D_2O) δ 173.9, 60.3, 53.9, 26.6, 26.5.

The enantiomeric purity of the synthetic products, D- and L-5-hydroxynorvaline (Rt 14.5 and 11.6 min, respectively), and the synthetic starting materials, D- and L-glutamic acid (Rt 23.5 and 15.9 min, respectively), was determined by hplc. Each amino acid (by comparison of relative peak areas) was found to

be >99.5% of the desired enantiomer. Thus no significant racemization occurred during the synthesis of the individual enantiomers.

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