mate of 0.50 μ^3 for the volume of the cellular unit of exponentially growing *S. faecalis*. Using this value and the estimates (above) of 1.00, 1.26, and 0.64 for the relative cellular volumes of the three cell types studied, we obtain the spherical diameters shown in Table IV. These diameters and the percentage values for wall and membrane (Table II) permit the calculation of the thickness of wall and membrane as the two outer layers of ideal spheres. The results, shown in Table IV, suggest as a first approximation (no attempt has been made to evaluate relative densities of the components) that wall thickness has doubled in both the valine- and threonine-deprived cells, while membrane thickness has doubled only in the valine-deprived cells. Obviously, other more complex explanations involving "polynuclear" cells or folded membrane structures (Edwards, 1962) cannot be disregarded at this stage. Verification of the conclusions regarding cell size and cell number is complicated by chain formation (Toennies *et al.*, 1961), and new techniques must be devised. The electron-microscopic study of cellular anatomy is also being explored.

TABLE IV
ESTIMATED CELL DIMENSIONS (mμ)

	Cell Type		
	Exponential Phase	Valine-Deprived	Threonine-Deprived
Diameter of cell	984	1072	846
Thickness of outer layer (wall)	43	76	78
Thickness of second layer (membrane)	35	73	27

Quantitative determination of bacterial membrane substance has not so far been possible. The present work presents two procedures, applicable to the organism studied, for this purpose, and indicates that withdrawals of single bacterial nutrients may produce major and diverse shifts in cell morphology. Additional observations seem desirable before concepts of enzyme repression and feedback inhibition (Davis, 1961) or other theoretical tools are applied.

REFERENCES

Burton, K. (1956), *Biochem. J. 62*, 315.
Chargaff, E. (1959), *in* The Nucleic Acids, vol. 1, Chargaff, E., and Davidson, J. N., editors, New York, Academic Press, Inc., p. 359.
Davis, B. D. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 1.
Edwards, M. R. (1962), *8th Int. Congr. Microbiol., Abstracts*, p. 31.
Knaysi, G. (1941), *J. Bacteriol. 42*, 575.
Kolb, J. J., Weidner, M. A., and Toennies, G. (1963), *Anal. Biochem. 5*, 78.
Marshak, A., and Vogel, H. J. (1951), *J. Biol. Chem. 25*, 262.
Miller, H. K. (1958), *in* Methods of Biochemical Analysis, vol. 6, Glick, D., editor, New York, Interscience Publishers, Inc., p. 47.
Ogur, M., and Rosen, G. (1950), *Arch. Biochem. 25*, 262.
Shockman, G. D. (1959), *J. Biol. Chem. 234*, 2340.
Shockman, G. D., Conover, M. J., Kolb, J. J., Riley, L. S., and Toennies, G. (1961), *J. Bacteriol. 81*, 44.
Shockman, G. D., Kolb, J. J., Bakay, B., Conover, M. J., and Toennies, G. (1963), *J. Bacteriol. 85* (Jan.).
Shockman, G. D., Kolb, J. J., and Toennies, G. (1957), *Biochim. Biophys. Acta 24*, 203.
Shockman, G. D., Kolb, J. J., and Toennies, G. (1958), *J. Biol. Chem. 230*, 961.
Toennies, G., Bakay, B., and Shockman, G. D. (1959), *J. Biol. Chem. 234*, 3269.
Toennies, G., Iszard, L., Rogers, N. B., and Shockman, G. D. (1961), *J. Bacteriol. 82*, 857.
Toennies, G., and Shockman, G. D. (1959), *Proc. IVth Int. Congr. Biochem.*, Vol. 13, London, Pergamon Press, p. 365.
Vendrely, R. (1959), *in* The Nucleic Acids, vol. II, Chargaff, E., and Davidson, J. N., editors, New York, Academic Press, Inc., p. 155.
Wilkinson, J. F., and Duguid, J. P. (1960), *Intern. Rev. Cytol. 9*, 1.

Accumulation of L-Homolanthionine by an *Escherichia coli* Mutant*

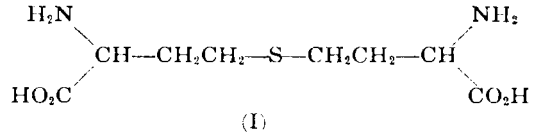
H. T. HUANG

From the Fermentation Research Department, Chas. Pfizer and Co., Inc., Groton, Connecticut

Received September 24, 1962

A methionine-requiring mutant of *E. coli* has been found to accumulate in submerged culture a sulfur-containing amino acid, which was isolated and identified as L-homolanthionine. This is the first record of the occurrence of homolanthionine in a living system.

Weiss and Stekol (1951) have reported the synthesis of the three diastereoisomers of *S*-bis(γ-amino-γ-carboxypropyl) sulfide or homolanthionine (I). They had established previously (Stekol and Weiss, 1948, 1949) that (I), without specifying which particular diastereoisomer, can be converted to cystine in the rat. So far, the occurrence of (I) in a living system has not been observed.



(I)

In the course of experiments on amino acid accumulation by mutants of *E. coli* (Huang, 1961), a methionine-requiring auxotroph 31–12 was developed and found to accumulate significant amounts of a sulfur-containing amino acid which resembled cystathionine in its behavior upon paper chromatography. The material was concentrated from a batch of fermentation broth by adsorption on IR-120 (H+) and elution with ammonium hydroxide. A pure product was obtained by chromatography on Permutit Q (H+) and recrystallizations from water-methanol. Elementary analysis gave an empirical formula of $C_8H_{16}O_4N_2S$. A Kuhn-Roth determination indicated that no C-methyl

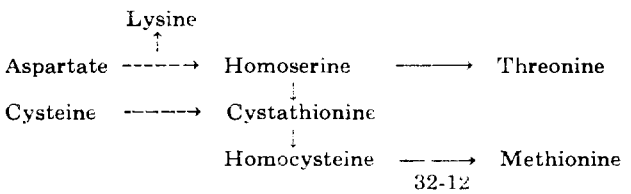
* Presented at the 142nd National Meeting, American Chemical Society, Atlantic City, N. J., Sept. 9–14, 1962.

was present. Desulfurization with Raney nickel yielded only one amino acid, identified as α-aminobutyric acid by paper chromatography. From these data, it is clear that the structure of the compound isolated is given by (I).

The pure product is optically active, and its specific rotation agrees reasonably well with that reported for L-homolanthionine (Weiss and Stekol, 1951). The rotation in neutral solution is more negative than that in aqueous acid. The compound is completely oxidized by the L-amino acid oxidase of snake venom, and it is inert to the D-amino acid oxidase of hog kidney. Thus, there is little doubt that the compound isolated is L-homolanthionine.

The α-aminobutyric acid obtained by nickel desulfurization is largely oxidized by snake venom oxidase, and a small part of it is oxidized by hog kidney D-amino acid oxidase. Partial racemization evidently occurs during the nickel treatment.

No L-homolanthionine could be detected in the culture broth or in the cells of the parent W strain of *E. coli*, grown under the same conditions as those described for the mutant. It is presumed, by analogy with the biosynthetic formation of L-cystathionine, that L-homolanthionine is synthesized in the mutant from L-homocysteine and L-homoserine. Examination of the currently accepted pathway suggests that L-homolanthionine is a byproduct rather than an intermediate in methionine biosynthesis.



The mutant producing L-homolanthionine can utilize L-methionine, D-methionine, and DL-methionine sulfoxide for growth. It does not respond to homocysteine, homocystine, cystathionine, or homoserine. The genetic block is, therefore, between methionine and homocysteine. It is reasoned that the block has resulted in the accumulation of homocysteine and homoserine within the cell. These are metabolized to form homolanthionine which is subsequently excreted as a byproduct.

Experimental

Mutants.—Mutants of W strain of *Escherichia coli* were isolated *via* ultraviolet treatment and penicillin selection (Davis, 1949), and screened as previously described for accumulation of amino acids (Huang, 1961). The culture medium of an L-methionine-requiring mutant 31–12 was found to contain a polar amino acid which in its paper chromatographic behavior did not correspond to any of the naturally occurring amino acids reported in the literature. The mutant could also utilize as a growth factor D-methionine and DL-methionine sulfoxide but not cystathionine, *allo*-cystationine, homocysteine, homocystine, or homocysteine thiolactone and homoserine.

Fermentation.—Inoculum for the fermentor stage was grown in a Fernbach flask containing 1 liter of medium with the following composition: minimal salts (Davis and Mingioli, 1950), 200 mg DL-methionine, and 10 g glycerol in 1 liter of water, sterilized at 120° for 20 minutes. After inoculation from slants the flask was incubated on a rotary shaker at 28° for 18 hours.

Large-scale growth of culture was carried out in 4-liter glass fermentors (Shull and Kita, 1961), with 2 liters of medium containing, in grams/liter, glycerol (9.0), crude beet molasses (26.0), corn steep liquor (26.0), ammonium hydrogen phosphate (10.0), $MgSO_4 \cdot 7H_2O$ (0.1); the *p*H was adjusted to 7.5 with 50% KOH before sterilization at 120° for 1 hour. Soy bean oil (5 ml/fermentor) was used as antifoam. After inoculation with 100 ml of inoculum, each fermentor was stirred at 1750 rpm, aerated at a rate of one volume of air per volume of liquid per minute, and incubated at 28° for 26 hours. The broth was estimated by paper chromatography to contain approximately 0.5 g/liter of the unknown amino acid.

Isolation of the Unknown Amino Acid.—The combined broth from nine fermentors was brought to *p*H 2.0 with sulfuric acid, filtered, and passed through a column containing 7 liters of IR 120 (H+). The column was washed with 7 liters of water and eluted with 1.15 N ammonium hydroxide. One-liter fractions were taken and analyzed by paper chromatography for the presence of amino acids. Eight fractions containing most of the desired material were evaporated *in vacuo*. The concentrate (*p*H 8.1) was filtered and passed through a column containing 250 ml of IRC 50 (H+). The effluent and the water wash, totaling 2500 ml at *p*H 4.5, were stirred with 20 g of Darco G-60 at 80°, filtered, and evaporated *in vacuo* to 200 ml. Methanol (200 ml) was added and the clear solution refrigerated overnight. The precipitate was collected and dried at 100°. The crude product, 11 g, contained mostly the unknown amino acid, with valine, glutamic acid, and cystine as the major impurities.

This product was dissolved in 150 ml of water, treated with 2 g of Darco G-60, and filtered. The combined filtrate and wash was passed through a column containing 125 ml of Permutit Q (H+) and eluted with 0.1 N ammonium hydroxide. Fractions of 40-ml volume were collected and analyzed for amino acid content. Fractions 54–84 were evaporated to 100 ml and mixed with 50 ml of methanol. After overnight refrigeration, the precipitate was collected and dried; the yield was 6.4 g. Two reprecipitations from methanol-water afforded 3.3 g of microcrystalline powder, which was essentially pure with just a perceptible trace of valine and cystine.

The analytical sample was recrystallized from water. $[\alpha]_D^{25} = +39.4°$ (*c*, 2 in 1 N HCl); $-10.7°$ (*c*, 2 in H_2O); reported by Weiss and Stekol (1951), $[\alpha]_D^{25} = +37.3°$ (*c*, 1 in 1 N HCl). *Anal.* Calcd. for $C_8H_{16}O_4N_2S$: C, 40.66; H, 6.83; N, 11.86; S, 13.57%. Found: C, 40.80; H, 7.05; N, 11.75; S, 13.77%.

Raney Nickel Desulfurization.—Fifty mg of the product in 25 ml of water was refluxed for 1 hour with

Table I

Action of L and D Specific Oxidases on $C_8H_{16}O_4N_2S$ Acid and Desulfurized Product

Amino Acid Substrate	Amino Acid Oxidase	Found After Incubation[a,b] (mg/ml substrate)
$C_8H_{16}O_4N_2S$[a]	L-Oxidase	None
	D-Oxidase	1.08
	None	1.09
Desulfurized product[b]	L-Oxidase	0.17
	D-Oxidase	0.45
	None	0.64

[a] $C_8H_{16}O_4N_2S$ 2.5 mg, L-oxidase 2.0 mg (or D-oxidase 8.0 mg) in 2.5 ml 0.08 M tris(hydroxymethyl)aminomethane-HCl buffer at *p*H 7.2, shaken in air for 3 hours. [b] Desulfurized product 2.0 mg, enzymes as in *a* in 2.5 ml Tris buffer, shaken in air for 5 hours.

1 g of Raney nickel. On cooling the mixture was filtered, and the catalyst was washed twice with 25 ml of water. The combined filtrate and wash was lyophilized to give 20 mg of a white powder. Paper chromatography indicated the presence of only one amino acid, indistinguishable from α-aminobutyric acid.

Action of L- *and* D-*Amino Acid Oxidases.*—L-Amino acid oxidase was a lyophilized snake venom preparation (*Crotalus adamanteus*) from the Ross Allen Reptile Institute, Silver Springs, Fla. D-Amino acid oxidase was a hog kidney preparation from Worthington Biochemical Corporation, Freehold, N. J. Quantitative amino acid analyses were carried out by paper chromatography.

Results on the oxidation of the isolated amino acid and of the α-aminobutyric acid from the nickel desulfurization by these enzymes are summarized in Table I. It is clear that the $C_8H_{16}O_4N_2S$ acid is completely oxidized by the L-oxidase and inert to the D-oxidase. The α-aminobutyric acid is about 70% L-isomer and 30% D-isomer.

References

Davis, B. D. (1949), *Proc. Nat. Acad. Sci. U. S. 35*, 1.
Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60*, 17.
Huang, H. T. (1961), *Appl. Microbiol. 9*, 419.
Shull, G. M., and Kita, D. A. (1955), *J. Am. Chem. Soc. 77*, 763.
Stekol, J. A., and Weiss, K. (1948), *J. Biol. Chem. 175*, 405.
Stekol, J. A., and Weiss, K. (1949), *J. Biol. Chem. 179*, 67.
Weiss, S., and Stekol, J. A. (1951), *J. Am. Chem. Soc. 73*, 2497.

The Isolation and Characterization of L-Homoarginine from Seeds of *Lathyrus sativus**

S. L. N. RAO, L. K. RAMACHANDRAN,† AND P. R. ADIGA

From the Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

Received August 8, 1962

A method of isolation of the new plant amino acid, L-homoarginine, from seeds of *Lathyrus sativus*, in a yield of 0.96 g per kg, is presented. The amino acid has been characterized on the basis of the properties of the free base, the monoflavianate, and the monopicrate. The properties of L-homoarginine monopicrate and the infrared spectrum of the monoflavianate are reported. It is suggested that homoarginine may act as a precursor to lathyrine in *L. tingitanus*.

The study of the chemical constituents of seeds of *Lathyrus sativus* is of considerable interest for two reasons. The seed meal is widely used by people in certain parts of India as a principal dietary constituent. Such people, in course of time, develop a nervous disorder termed "lathyrism" and are afflicted by varying degrees of physical disability, particularly of the lower limbs (Ganapathy and Dwivedi, 1961). During an investigation of the toxic constituents of the above seed, the presence of L-homoarginine in high concentration was discovered, and we report below the method of isolation of this constituent and its characterization. On completion of this work a report appeared in the literature (Bell, 1962a,b) on the recognition of homoarginine in three species of *Lathyrus* (*sativus*, *cicera*, and *cymenum*) and on the isolation of the amino acid from the species *L. cicera*. The method of isolation and identification used by Bell (1962b) differs substantially from techniques used by us. Our independent work confirms the presence of the new naturally occurring amino acid, L-homoarginine, in the plant kingdom.

Experimental and Results

L. sativus seed meal (3 kg) was extracted for 90 minutes, under refluxing conditions, with 4.5 liters of 75% EtOH. The extraction was repeated thrice, with fresh portions of solvent. The pooled extracts were filtered and concentrated to an eighth of the volume *in vacuo* (40–45°). Paper chromatographic examination [solvent–*n*-butanol (4), pyridine (1), H_2O (1), HOAc (2)] of the concentrate revealed the presence of two ninhydrin-reacting constituents with R_F values 0.09 and 0.24, which according to tests described later turned out to be different from other known constituents of plants. The concentrate was extracted with 1.5 liters of $CHCl_3$, and the $CHCl_3$ layer containing most of the color and lipids was rejected. The aqueous layer (1.5 liters, *p*H 5–6) was applied to a 180-ml column of Dowex 50-X8 (200–400 mesh) in the H form. After the passage of the sample the column was washed with 2 liters of water, securing the complete elution of the constituent with R_F 0.09 whose nature has been dealt with in a separate communication (Adiga *et al.*, 1962). Then 2.5 liters of 1.0 N HCl was passed through the column and the eluate rejected. After this stage, 1.5 N HCl was used as eluant and fractions of 10-ml volume were collected. All the arginine in the extract emerged in fractions 11–28, and the compound with R_F 0.24 emerged in fractions 30–216. The contents of tubes 30–216 were pooled and diluted four-fold with water and applied to a 99-ml column of Dowex 50-X8 in the H form. The column was washed with water till the effluents were Cl^- free, and the adsorbed amino acid was eluted with 1 N NH_4OH, completion of elution being checked by paper chromatography. The eluate was lyophilized. The yield was 2.8 g, and the material, which was slightly hygroscopic, was stored dry in a desiccator.

The substance in solution in water gave an alkaline reaction and in the test for elements was found to contain nitrogen but neither sulfur nor phosphorus. The ninhydrin reaction for amino groups (Rosen, 1957) and the Sakaguchi test for guanidino groups (MacPherson, 1946) were answered by the compound, while

* Aided by grants from the Rockefeller Foundation and the Council of Scientific and Industrial Research, India.

† Present address: Department of Biochemistry and Biophysics, University of Hawaii, Honolulu 14, Hawaii.