ployed, 1.5 ml of supernatant was utilized. Concentrations of all cofactors remained the same and the final volume was similarly made up to 4.0 ml with 0.1 *M* phosphate buffer. After 1 hr of incubation in air with slow shaking at 37° the reaction was stopped by adding 3.0 ml of 15% KOH in 50% EtOH followed by heating at 65–70° in a water bath for 10 min. The partially saponified reaction mixture was transferred to a 20-ml glass-stoppered centrifuge tube and the flask was rinsed with 3.0 ml of the alcoholic KOH solution which was added to the contents of the centrifuge tube. Saponification was completed by heating the tightly stoppered tube in a water bath (75–80°) for 1 hr.

The saponified mixtures were extracted three times with 5.0-ml portions of petroleum ether (bp 60–80°). The combined extracts were diluted to 25.0 ml and dried (Na_2SO_4). Five milliliters of the dried petroleum ether extract was diluted with 10.0 ml of PPO (2,5-diphenyloxazole) solution (0.4% PPO in PhMe–95% EtOH, 70:30 v/v) in standard counting vials. The radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. A sufficient number of counts was taken to reduce the statistical error of counting to less than 5%. In each run, as a further check on the extraction procedure (and as a measure of possible contamination), the contents of one incubation flask was treated with KOH solution prior to incubation; the radioactivity in the petroleum ether extract derived from these alkali pretreated incubations was always found to be negligible.

Gas–liquid partition chromatography[10] of the nonsaponifiable material was accomplished as follows. The petroleum ether extract of nonsaponifiable material (15 ml) was concentrated to dryness (water bath, 50°) under N_2. The residue was dissolved in 0.5 ml of petroleum ether and gas chromatographed on 10% silicone gum rubber (SE-30) on Chromosorb W (80–100 mesh), 4 ft × 0.25 in. glass column with the column temperature 240°, detector temperature 285°, injection port temperature 355°, inlet pressure of 2.8 kg/cm², and carrier gas (He) flow rate 50 ml/min; this gave a retention time for squalene of 13.0 min and for cholesterol, 24.4 min.

Separation of the nonsaponifiable extract into squalene and cholesterol was accomplished by the method of Langdon and Bloch.[11] The petroleum ether extract (15 ml) was concentrated to dryness in a water bath (50°) under N_2. The residue was chromatographed on acid-washed alumina[12] using petroleum ether (60–70°). The squalene fraction was checked for purity by glpc. Sterols were then eluted with Me_2CO–Et_2O (1:1 v/v). The cholesterol fraction was similarly checked for purity using glpc. Squalene- and cholesterol-containing eluates were concentrated to dryness in a water bath (50°) under N_2 in standard counting vials. PPO solution (10 ml) was added and the radioactivity was determined with a Packard Tri-Carb liquid scintillation counter.

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(10) The F and M Model 402 biomedical gas chromatograph equipped with flame ionization detector was employed in these studies.

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(12) Alupharm Chemicals, New Orleans, La.

2-Benzyl-5-aminolevulinic Acid, an Analog of Glycyl-DL-phenylalanine[1a]

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Numerous studies have indicated that peptides have biological activities which differ from the activities displayed by the free constituent amino acids.[2] Interpretation of the results of such studies is complicated by the hydrolysis of the peptides to form free amino acids as often occurs in biological systems. In an attempt to produce a peptide analog whose study would not be complicated by hydrolysis, 2-benzyl-5-aminolevulinic acid was synthesized. 2-Benzyl-5-aminolevulinic acid (I) can be viewed as an analog of glycylphenylalanine wherein the labile CONH group of the peptide has been replaced by the nonlabile $COCH_2$ group.

All compounds used in biological testing were of the racemic form. 2-Benzyl-5-aminolevulinic acid was tested for its effect upon the growth of *Escherichia coli* 9723 and *Leuconostoc mesenteroides* P-60. Growth of *E. coli* was not inhibited by I when tested in the basal salts–glucose medium. However, as shown in Table I,

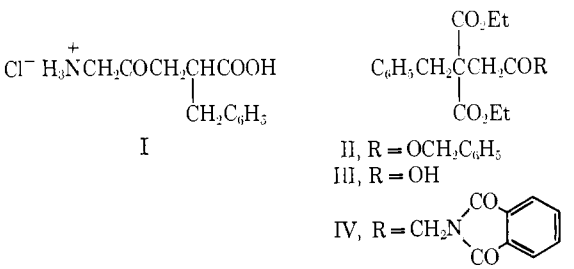
TABLE I

EFFECT UPON GROWTH OF *E. coli* 9723[a] BY 2-BENZYL-5-AMINOLEVULINIC ACID (BALA) IN MEDIA CONTAINING β-2-THIENYL-DL-ALANINE (TA), GLYCYL-β-2-THIENYL-DL-ALANINE (GTA), GLYCYLGLYCYL-β-2-THIENYL-DL-ALANINE (G_2TA), AND GYLCYL-DL-PHENYLALANINE (GPA)

	mg of dry wt of cells/ml of culture — Supplements			
BALA, mμmoles/ml	TA[b]	GTA[c]	G_2TA[d]	TA, GTA, GPA[e]
0	0	0	0	0.13
20				0.12
60		0.01		0.10
200	0	0.08	0	0.06
600	0	0.20	0	0.01

[a] Incubated 15 hr at 37°. [b] Medium contained 2 mμmoles of TA/ml. [c] Medium contained 6 mμmoles of GTA/ml. [d] Medium contained 6 mμmoles of G_2TA/ml. [e] Medium contained 200 mμmoles of TA, 200 mμmoles of GTA, and 20 mμmoles of GPA/ml.

I may either stimulate or inhibit the growth of *E. coli* depending upon the way the medium is supplemented with peptides of β-2-thienylalanine or phenylalanine.



I

II, R = $OCH_2C_6H_5$

III, R = OH

IV, R = CH_2N (phthalimido)

If growth was inhibited by glycyl-β-2-thienylalanine, reversal of inhibition occurred when I was added to the medium. When growth was inhibited by the tripeptide glycylglycyl-β-2-thienylalanine, I was ineffective as a growth stimulant. When the *E. coli* medium was sup-

(1) (a) This investigation was supported by U. S. Public Health Service Grant No. AI03710 from the National Institutes of Health and Biochemistry Training Grant GM-203. (b) Taken in part from the M. S. Thesis of Sherrel C. Smith, University of Tennessee, June 1965. (c) To whom requests for reprints should be directed: Department of Chemistry, Abilene Christian College, Abilene, Texas 79601.

(2) (a) F. W. Dunn, *J. Biol. Chem.*, **234**, 802 (1959); (b) S. Shankman, V. Gold, S. High, and R. Squires, *Biochem. Biophys. Res. Commun.*, **9**, 25 (1962); (c) W. Shive and C. G. Skinner in "Metabolic Inhibitors," Vol. I, R. M. Hochster and J. H. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, Chapter 1, pp 37–39.

plemented by a mixture of β-2-thienylalanine, glycyl-β-2-thienylalanine, and glycylphenylalanine at such levels as to permit growth, increasing concentrations of I caused growth inhibition.

It is possible that I exerts its effects on the growth of *E. coli* by interfering with the uptake of dipeptides by *E. coli*. The uptake of amino acids and peptides by *E. coli* has been reported to occur by separate routes.[3] Other sites of interference may exist. The failure of I to reverse the inhibition of growth produced by glycyl-glycyl-β-2-thienylalanine, while reversing the inhibition caused by glycyl-β-2-thienylalanine, could indicate separate routes of uptake and utilization for the dipeptide and the tripeptide in *E. coli*.

Leuconostoc mesenteroides responded differently. Growth of *L. mesenteroides* P-60 was inhibited by I when phenylalanine was supplied either as the free amino acid or as the dipeptide glycylphenylalanine. In either case, approximately 50% inhibition of growth occurred at a concentration of 2000 mμ-moles of I/ml in the presence of 20 mμmoles of either phenylalanine or glycylphenylalanine/ml.

Substitution of a methylene unit for the NH group of glycylphenylalanine has produced an analog which shows some properties of a dipeptide, *i.e.*, it is similar to glycylphenylalanine in stimulating growth of *E. coli* in the presence of inhibitory levels of glycyl-β-2-thienylalanine. These results demonstrate the availability of a unique type of peptide analog which is not capable of hydrolysis to form free amino acids.

Experimental Section[4]

Benzyl 4-Phenyl-3-dicarbethoxybutyrate (II).—To a solution of 61 g (0.244 mole) of diethyl benzylmalonate[5] in 150 ml of DMF was added (in ten portions) 5.88 g (0.245 mole) of NaH. After the evolution of H_2 ceased, the solution was filtered through Celite, and the Celite was washed with an additional 50 ml of DMF. Benzyl chloroacetate (45 g, 0.244 mole) was added to the combined DMF solutions, and an immediate exothermic reaction took place. The solution was stirred at 90° for 18 hr, and the DMF was removed under vacuum. $CHCl_3$ (150 ml) was added to the residue, the solution was extracted twice with 50 ml of H_2O, and the $CHCl_3$ was removed under reduced pressure. The resulting ester mixture was distilled; the fraction distilling at 190–198° (0.5 mm) weighed 62.35 g (64.1%). A portion was redistilled for analysis; bp 194–196° (0.5 mm). *Anal.* ($C_{23}H_{26}O_6$) H; C: calcd, 69.33; found, 68.65.

4-Phenyl-3-dicarbethoxybutyric Acid (III).—Benzyl 4-phenyl-3-dicarbethoxybutyrate (17.77 g) in 200 ml of 95% EtOH was hydrogenated at 2.8 kg/cm² in the presence of 0.5 g of $PdO_2 \cdot xH_2O$ for 18 hr. The Pd was removed by filtration through Celite. The EtOH was removed *in vacuo*, and the last traces of H_2O were removed azeotropically with C_6H_6. The remaining oil solidified. After drying *in vacuo*, the material had a neutralization equivalent of 311 (theoretical 308) and mp 73–81°; it was used without further purification.

An analytical sample was prepared by dissolving 1.0 g in a saturated $KHCO_3$ solution, extracting twice with Et_2O, acidifying the $KHCO_3$ solution, and extracting the oil which separated into Et_2O. The ethereal solution was concentrated to an oil and seeded to initiate crystallization. The crystalline material was dried *in vacuo*; mp 76–79°. *Anal.* ($C_{16}H_{20}O_6$) C, H.

Ethyl 2-Benzyl-5-phthalimido-2-carbethoxylevulinate (IV). A 13.5-g (0.044 mole) portion of 4-phenyl-3-dicarbethoxybutyric acid was heated at 70° for 2 hr with 6.28 g (0.0528 mole) of $SOCl_2$. After standing overnight at room temperature, the excess $SOCl_2$ was removed *in vacuo*. Anhydrous C_6H_6 (30 ml) was added and removed *in vacuo*. This crude material had the expected ir spectrum for 4-phenyl-3-dicarbethoxybutyryl chloride, and was used without further purification.

Approximately 0.022 mole of 4-phenyl-3-dicarbethoxybutyryl chloride, dissolved in 125 ml of anhydrous Et_2O, was added to approximately 3 g of CH_2N_2 in 200 ml of EtOH-free Et_2O at 0°. The solution was allowed to stand 1 hr at 0° and 4 hr at room temperature. The excess CH_2N_2 was removed under reduced pressure. The oil which remained was dissolved in 150 ml of anhydrous Et_2O, and dry HCl was bubbled into the Et_2O solution until N_2 evolution ceased. A yellow oil remained after the Et_2O and HCl were removed *in vacuo*. Attempts to redistil this material resulted in decomposition; therefore, the crude ethyl 2-benzyl-5-phthalimido-2-carbethoxylevulinate was used without purification.

Approximately 0.02 mole of ethyl 2-benzyl-5-chloro-2-carbethoxylevulinate was added to 2.96 g (0.016 mole) of potassium phthalimide in 30 ml of DMF, and a mild exothermic reaction occurred. After the mixture was stirred for 2 hr at 85°, 70 ml of $CHCl_3$ was added and the $CHCl_3$ solution was extracted twice with 40 ml of H_2O. The $CHCl_3$ was removed under reduced pressure, and a dark brown viscous oil remained. The oil was dissolved in a small volume of Et_2O, and petroleum ether was added to precipitate the oil. The oil crystallized after about 10 hr. The crude material weighed 7.1 g (98.7%, based on 0.016 mole of potassium phthalimide). Five recrystallizations from Et_2O–petroleum ether (bp 37–60°) gave white microcrystals, mp 100–101°. *Anal.* ($C_{25}H_{25}NO_7$) H, N; C: calcd, 66.51; found, 65.96.

2-Benzyl-5-aminolevulinic Acid Hydrochloride (I).—Ethyl 2-benzyl-5-phthalimido-2-carbethoxylevulinate (2.4 g) was dissolved in 30 ml of glacial HOAc. Concentrated HCl was added to the HOAc until a faint permanent precipitate was obtained. The solution was allowed to reflux for 12 hr and was then concentrated to approximately 20 ml under reduced pressure. H_2O (20 ml) was added and the volume was reduced again. The addition of H_2O and subsequent reduction of volume was repeated five times, and then the phthalic acid which separated was removed. The filtrate was concentrated to an oil under reduced pressure. To this oil 10 ml of dioxane was added followed by a large volume of anhydrous Et_2O. After 1 hr, a fluffy mass of crystals had formed in the dioxane and Et_2O solution and was separated from the oil which remained. After drying, the white platelets weighed 0.86 g, mp 57–65°. The remaining oil was covered with anhydrous Et_2O and crystallization occurred after several hours. The slightly yellow crystals weighed 0.16 g, mp 155–164° dec. The total yield was 1.02 g (75.5%). The first crop that was collected, after trituration with hot dioxane, gave the analytical sample, mp 163–169° dec. The ir spectrum was consistent with the assigned structure. With ninhydrin, 2-benzyl-5-aminolevulinic acid hydrochloride gave a yellow color which slowly changed to purple. R_f values were 0.24 in *n*-BuOH saturated with H_2O and 0.75 in pyridine–H_2O (63:35). *Anal.* ($C_{12}H_{16}ClNO_3$) C, H, N.

Microbiological Assays.—For *E. coli*, a previously described[6] inorganic salts–glucose medium was used, and the organism was incubated at 37° for 15 hr. *L. mesenteroides* was grown in a medium which has been reported elsewhere.[7]

In all assays growth was measured with a turbidimeter in which distilled water read 0 and an opaque object read 100. For the data in Table I dry weight of cells was determined by reference to a standard curve in which the weight of dry cells per milliliter was plotted against turbidimeter readings.

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(4) All melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Reduced-pressure evaporations were performed at water aspirator pressures using a rotary evaporator. The paper chromatograms were prepared by the ascending technique using the solvents indicated, and the spots were developed with ninhydrin reagent. The elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Where analyses are indicated only by the symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

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