[CONTRIBUTION FROM THE CLAYTON FOUNDATION FOR RESEARCH, THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

3-Cyclohexene-1-glycine, an Isoleucine Antagonist

BY JEROME EDELSON, JOHN D. FISSEKIS, CHARLES G. SKINNER AND WILLIAM SHIVE

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3-Cyclohexene-1-glycine was prepared from 3-cyclohexenecarboxaldehyde by conversion of the aldehyde to 5-(3'-cyclohexene)-hydantoin which upon alkaline hydrolysis formed the amino acid. α-Aminocyclohexanevaleric acid was prepared from the corresponding α-bromo acid. Although cyclohexane derivatives of glycine, alanine or α-aminovaleric acid even at high concentrations do not exert any appreciable inhibitory effect on the growth of *Escherichia coli*, 3-cyclohexene-1-glycine is a competitive antagonist of isoleucine for this organism. The biological effects of these analogs have been correlated with their stereochemical structure.

The synthesis and biological activities of several cycloalkyl amino acids have recently been reported, and the structures of such derivatives which act as amino acid antagonists have been correlated with the structure of the inhibited natural amino acid. Cyclopentaneglycine is a specific antagonist of isoleucine in *Escherichia coli*,[1] while the toxicity of 2-cyclopenteneglycine is reversed competitively by a mixture of isoleucine and valine but not either alone.[2] This difference in specificity may be attributed to the more planar structure of the unsaturated analog making it more similar to the planar isopropyl group of valine. 2-Cyclohexene-1-glycine is also an antagonist of isoleucine in *E. coli*, whereas cyclohexaneglycine is not. The cyclohexene ring in the inhibitory analog probably possesses a structure in which carbons 5 and 6 are on opposite sides of the plane containing carbons 1, 2, 3 and 4,[3] since the alternate structure is one which is quite similar to the boat configuration of the inactive cyclohexaneglycine.

In an effort to study further the structural relationships between inhibitory analogs and metabolites, 3-cyclohexene-1-glycine was synthesized, and its biological properties were determined. This analog was observed to inhibit the growth of *Escherichia coli* and to be competitively reversed over a range of inhibitor concentrations by isoleucine.

In the course of preparation of 3-cyclohexeneglycine, several different synthetic approaches were considered. For example, a reduction of *p*-hydroxyphenylglycine and subsequent dehydration of the hydroxycyclohexyl derivative would presumably yield the desired compound. However, the reduction of the phenol derivative, under a variety of conditions, produced primarily hydrogenolysis products, glycine and cyclohexaneglycine, and only a small amount of 4-hydroxycyclohexaneglycine.[4] An alternate, but similar, approach was also attempted involving the hydrogenation of 5-(*p*-methoxyphenyl)-hydantoin. However, the primary product isolated was a cyclohexane derivative, 5-cyclohexylhydantoin.

3-Cyclohexene-1-glycine was prepared in good yield through the corresponding hydantoin derivative which was synthesized from 3-cyclohexenecarboxaldehyde prepared by a Diels–Alder condensation between 1,3-butadiene and acrolein. Alkaline hydrolysis of 5-(3'-cyclohexene)-hydantoin produced the desired amino acid analog.

Cyclohexaneglycine and cyclohexanealanine, previously reported amino acid analogs synthesized by new procedures, and α-aminocyclohexanevaleric acid, prepared from the corresponding α-bromo acid, were studied along with 3-cyclohexeneglycine in the same biological systems. In contrast to 3-cyclohexeneglycine, the cyclohexane derivatives did not inhibit growth of *E. coli* even at concentrations of 1 mg. per ml. Thus, none of the three cyclohexane derivatives possesses a structure sufficiently comparable to that required for attachment to active enzyme sites to compete effectively with the natural amino acids.

Since the introduction of a double bond has been demonstrated to induce inhibitory activity in several cycloalkyl amino acid derivatives,[1-3,5] the biological activity of 3-cyclohexeneglycine is not unexpected. This latter structure can be represented as a modification of isoleucine in which the methyl groups are joined together by an ethylene group, and it is comparable in its biological activity to a previously reported modification in which the methyl groups are joined by a methylene group, cyclopentaneglycine.[1] The cyclohexene ring could theoretically exist in two forms, one a boat-like structure which is not appreciably different from a structure which can be assumed by cyclohexaneglycine, and an alternate structure in which carbons 1 and 6 are on opposite sides of the plane of carbons 2, 3, 4 and 5. The latter structure is the more probable inhibitory form since cyclohexaneglycine, which possesses a boat configuration similar to the initial form of cyclohexeneglycine presented above, is biologically inactive in this assay system.

2-Cyclohexeneglycine similarly is an antagonist of isoleucine in *E. coli*, but it is somewhat less toxic than 3-cyclohexeneglycine. When carbons 5 and 6 of either analog are in such a position that they correspond to the ethyl group of isoleucine, only carbon atoms 3 and 4, which do not have a counterpart in the isoleucine molecule, are different from the natural metabolite. The introduction of the double bond at the carbon 2 position apparently results in this carbon being less similar to the

(1) W. M. Harding and W. Shive, *J. Biol. Chem.*, **206**, 401 (1954).

(2) R. L. Dennis, W. J. Plant, C. G. Skinner, G. L. Sutherland and W. Shive, THIS JOURNAL, **77**, 2362 (1955).

(3) J. Edelson, P. R. Pal, C. G. Skinner and W. Shive, *ibid.*, **79**, 5209 (1957).

(4) These results are similar to those observed by several investigators, including J. H. Billman and J. A. Beuhler, *Proc. Indiana Acad. Sci.*, **63**, 120 (1954), on the hydrogenation of tyrosine and other hydroxyaromatic amino acids.

(5) P. R. Pal, C. G. Skinner, R. L. Dennis and W. Shive, THIS JOURNAL, **78**, 5116 (1956).

methyl group of isoleucine and thereby diminishes its ability to conform to the structure necessary for attachment at the enzyme site.

Inhibition of growth of *E. coli* by 3-cyclohexeneglycine, which is prevented competitively by isoleucine as indicated in Table I, is not affected to any appreciable extent by valine or leucine; however, threonine does prevent the inhibition appreciably. Threonine is known to serve as a precursor of isoleucine in *E. coli* and several other microorganisms, and the reversal by threonine of an isoleucine antagonist has been studied in detail previously.[1,6,7]

Experimental[8,9]

Biological Assays.—A previously described inorganic salts–glucose medium[10] was employed for the assays presented using *Escherichia coli* 9723. This procedure has been recently reported in detail.[11] The inhibitors were dissolved in sterile water and added to the sterile assay tubes without being heated.

5-(3′-Cyclohexene)-hydantoin.—Acrolein (0.15 mole) and butadiene (0.20 mole) were sealed in a macro Carius tube and heated to 160° for 3 hr. After the volatile materials were removed, the residue was fractionally distilled to yield 14.9 g. of 3-cyclohexenecarboxaldehyde, b.p. 116–120° (24 mm.), $n^{19}D$ 1.4729.[12] Ten grams of this aldehyde was dissolved in 200 ml. of 50% ethyl alcohol, in the presence of 13 g. of potassium cyanide and 43 g. of ammonium carbonate, and heated to 60° for 4 hr.[13] After cooling to room temperature, the reaction mixture was acidified under a hood, and the hydantoin crystallized on standing overnight in the refrigerator. After recrystallization from alcohol–water, there was recovered 14.9 g. of material, m.p. 184–186°.

Anal. Calcd. for $C_9H_{12}N_2O_2$: N, 15.55. Found: N, 15.46.

3-Cyclohexene-1-glycine.—The 14.9 g. of 5-(3′-cyclohexene)-hydantoin above was heated to reflux in the presence of 50 g. of barium hydroxide·octahydrate suspended in 50 ml. of water for 5 days. The insoluble material was removed, and the filtrate was neutralized with sulfuric acid. The precipitated barium sulfate was filtered from the hot reaction mixture, and the cooled filtrate yielded a crystalline precipitate. After recrystallization from water, there was recovered 4.1 g. of product, m.p. 312° dec.

Anal. Calcd. for $C_8H_{13}NO_2$: C, 61.91; H, 8.44; N, 9.03. Found: C, 61.80; H, 8.41; N, 9.22.

The N-benzoyl derivative of 3-cyclohexeneglycine was prepared in the usual manner, m.p. 189°.

Anal. Calcd. for $C_{15}H_{17}NO_3$: N, 5.40. Found: N, 5.61.

Catalytic Hydrogenation of 5-(*p*-Methoxyphenyl)-hydantoin.—Using the procedure of Henze and Speer[13] anisaldehyde was converted to its hydantoin derivative, m.p. 199°. A reported m.p. is 195°.[14] Attempts to reduce the phenyl group also resulted in hydrogenolysis of the methoxy group. For example, 13.0 g. of the hydantoin was hydrogenated in the presence of 1.5 g. of Adams catalyst in the presence of 350 ml. of 5% alcoholic hydrochloric acid for 24 hr. After removal of the catalyst, the filtrate was treated with water to faint turbidity, and on cooling, 5.8 g. of product separated. Upon dissolving in alkali and reprecipitation in acid, followed by crystallization from ethanol–water, the product isolated proved to be 5-cyclohexylhydantoin, m.p. 226–227°.

Anal. Calcd. for $C_9H_{14}N_2O_2$: N, 15.38. Found: N, 15.40.

The above product did not depress the melting point of a sample of 5-cyclohexylhydantoin prepared *via* potassium cyanate and cyclohexaneglycine. Further, hydrolysis of the hydantoin isolated above, using barium hydroxide, produced a product which was identical with cyclohexaneglycine in three separate solvent systems when determined by paper chromatography using the ascending technique after developing the chromatogram with ninhydrin reagent.

Catalytic Reduction of *p*-Hydroxyphenylglycine.—The reduction of *p*-hydroxyphenylglycine under varying conditions of temperature (25–150°), pressure (50 lb./sq. in.–2000 lb./sq. in. of hydrogen pressure) and catalyst (platinum oxide, Raney nickel, palladium black), followed by chromatography and development of the chromatogram with ninhydrin, revealed three ninhydrin active materials. Two of these compounds were identified by paper chromatography in several solvents and by chemical analysis as glycine and cyclohexaneglycine. The third compound was subsequently identified as 4-hydroxycyclohexane-1-glycine. Ten grams of *p*-hydroxyphenylglycine was dissolved in 50 ml. of dilute alkali and hydrogenated over Raney nickel W-2 for 9.5 hr. at 2000 lb./sq. in. of hydrogen pressure at 150°. After removal of the catalyst, the filtrate was acidified with hydrochloric acid and reduced to dryness *in vacuo*. The solid was then dissolved in ethanol and precipitated with ether to yield a chromatographically pure product, m.p. 208–209°, which was quite hygroscopic, and proved to be 4-hydroxycyclohexane-1-glycine.

Anal. Calcd. for $C_8H_{16}ClNO_3$: C, 45.82; H, 7.69; N, 6.68. Found: C, 45.71; H, 8.11; N, 6.69.

α-Bromo-α-cyclohexaneacetic Acid.—This compound was prepared by a previously reported procedure; however, in the present instance the product was purified carefully, to obtain analytical data,[15] prior to subsequent ammonolysis to the cyclohexaneglycine derivative. Our product distilled at 154° (2 mm.), upon cooling solidified and, after recrystallization from acetone–water, had a m.p. of 98°.

Anal. Calcd. for $C_8H_{13}O_2Br$: C, 43.45; H, 5.92. Found: C, 43.45; H, 6.13.

Ammonolysis of this compound produced cyclohexaneglycine with R_f values in 95% methyl alcohol, 65% pyridine and butanol:acetic acid:water (4:1:1) of 0.62, 0.76 and 0.67, respectively.

Diethyl α-Acetamido-α-cyclohexylmethylmalonate.—A sample of 15 g. of bromomethylcyclohexane, prepared by the interaction of cyclohexanemethanol and phosphorus tri-

TABLE I

REVERSAL OF 3-CYCLOHEXENE-1-GLYCINE TOXICITY IN *Escherichia coli* 9723[a]

3-Cyclohexene-1-glycine, γ/ml.	Supplement: isoleucine, γ/ml. Galvanometer readings[b]							Threonine, 3 γ/ml.	Leucine, 3 γ/ml.[c]
	0	0.03	0.1	0.3	1.0	3.0	10.0		
0	65	62	63	66	65	68	64	63	64
0.3	65								
1.0	67	67						63	66
3.0	22	67	69					63	66
10.0	5	10	33	68				66	6
30.0		4	4	24	40	65	64	33	
100.0				4	14	47	64	14	
300.0					4	13	57		
1000.0						4	18		

[a] Incubated for 17 hr. at 37°. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100. [c] A supplement of valine at 3 γ/ml. gave a comparable response.

(6) H. E. Umbarger and E. A. Adelberg, *J. Biol. Chem.*, **192**, 883 (1951).

(7) H. E. Umbarger, *J. Bact.*, **65**, 203 (1953).

(8) All melting points were determined on a Fisher–Johns melting point block and are uncorrected. The R_f values were determined by the ascending technique.

(9) The authors are indebted to Dr. J. M. Ravel and her staff at the Biochemical Institute, The University of Texas, Austin, Texas, for the biological testing data, and to Mr. D. L. Ross for the chemical analyses.

(10) E. H. Anderson, *Proc. Natl. Acad. Sci.*, **32**, 120 (1946).

(11) F. W. Dunn, J. M. Ravel and W. Shive, *J. Biol. Chem.*, **219**, 809 (1956).

(12) N. Chayanov, *J. Gen. Chem.* (*USSR*), **8**, 460 (1938), through *C. A.*, **32**, 7905 (1938).

(13) This general procedure of making hydantoins was reported by H. R. Henze and R. J. Speer, THIS JOURNAL, **64**, 522 (1942).

(14) E. K. Harvill and R. M. Herbst, *J. Org. Chem.*, **9**, 21 (1944).

(15) D. Rudman, A. Meister and J. P. Greenstein, THIS JOURNAL, **74**, 551 (1952), reported the general technique of synthesis and a b.p. of 145–150° (3 mm.) but did not characterize this intermediate.

bromide,[16] dissolved in 15 ml. of dry benzene, was added to a solution of 12.0 g. of acetamidomalonic ester dissolved in 100 ml. of magnesium-dried ethanol containing 1.2 g. of sodium. The reaction mixture was heated under reflux for 30 hr., the salt was filtered and the filtrate was reduced to dryness *in vacuo*. The residue was recrystallized from 5 liters of boiling water to yield 5.2 g. of white silky flakes, m.p. 84°.

Anal. Calcd. for $C_{16}H_{27}NO_5$: C, 61.32; H, 8.68; N, 4.47. Found: C, 61.38; H, 8.63; N, 4.73.

Hydrolysis of this intermediate in the presence of 6 *N* hydrochloric acid gave a quantitative yield of cyclohexanealanine, identical in R_f value with a product obtained *via* catalytic reduction of phenylalanine.

α-Aminocyclohexanevaleric Acid.—To a mixture of 18.5 g. of cyclohexanevaleric acid and 0.15 g. of phosphorus trichloride, 19.5 g. of bromine was added dropwise with stirring, while the reaction mixture was cooled in an ice-bath. After addition of bromine was completed, the solution was allowed to stir 1 hr. at room temperature and finally heated to reflux for 48 hr. The reaction mixture was then distilled *in vacuo* to yield 15.5 g. of material, b.p. 155–160° (1.5 mm.). A mixture of 6.5 g. of the crude α-bromocyclohexanevaleric acid and 60 ml. of concentrated ammonium hydroxide was shaken at room temperature for five days. The solvent was removed, the residue was taken up in water and neutralized with concentrated hydrochloric acid. The solid which formed on cooling was filtered and washed with ether. After recrystallization from water there was recovered 0.78 g. of material which starts decomposing at about 200°, melts at 250° dec.

Anal. Calcd. for $C_{11}H_{21}NO_2$: C, 66.29; H, 10.62; N, 7.03. Found: C, 66.56; H, 10.76; N, 7.07.

R_f values of this substance in 95% methanol, 65% pyridine, and butanol:acetic acid:water (4:1:1) were 0.70, 0.83 and 0.82, respectively.

AUSTIN, TEXAS

(16) G. S. Hiers and R. Adams, THIS JOURNAL, **48**, 2385 (1926).

[CONTRIBUTION FROM THE MARY IMOGENE BASSETT HOSPITAL (AFFILIATED WITH COLUMBIA UNIVERSITY)]

The Isolation of Serum Albumin from Specific Precipitates of Serum Albumin and its Rabbit Antibodies[1]

BY THEODORE PETERS, JR.

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When specific precipitates of either bovine serum albumin or chicken serum albumin, each with its respective rabbit antibody, are redissolved at *p*H 2.4, addition of ethanol to 70–80 vol. % at 0° causes precipitation of the antibody but allows most of the serum albumin to remain in solution. At 71 vol. % alcohol and 0.4% protein, recovery of albumin, determined by ultracentrifugation or by the use of I^{131}-labeling, was 88–92%. Purity of the albumin was 82–97%, representing removal of all but 0.4–2.8% of the original antibody. The effects of alcohol concentration and *p*H on the degree of separation were studied. The recovered serum albumin appeared unaltered as determined by ultracentrifugation, electrophoresis, precipitation with specific antiserum and double diffusion in agar. Eight per cent. or less of the antibody could be recovered in a state which would still precipitate with the specific antigen, but the remainder apparently was irreversibly altered.

Introduction

The separation of antigens and antibodies from specific immunological precipitates has been attempted for many years by a variety of procedures.[2] Investigations have been directed mainly at recovery of antibody, and yields have been low. In the present studies, the separation of serum albumin from its rabbit antibodies has been undertaken for a different purpose: (1) to determine the incorporation of C^{14}-labeled amino acids into the serum albumin and (2) to study the nature of apparent albumin precursor compounds which can be precipitated from liver extracts with rabbit antiserum.

That specific precipitates will redissolve in dilute acid or alkali has been known since 1922.[3] More recently, electrophoretic and ultracentrifugal studies have shown that specific precipitates of bovine serum albumin (BSA)[4] and ovalbumin[5] not only redissolve but that the antigen and antibody dissociate completely below *p*H 2.4 or above *p*H 11.7–12.3. This finding suggested that it might be feasible to effect the separation of antigen and antibody by working at high or low *p*H. Little success was achieved in preliminary trials using ammonium sulfate, sodium sulfate, sodium acetate or low concentrations of calcium or in attempted denaturation of antibody by heating. Fair separation was obtained with paper electrophoresis at either *p*H 2.4 or 11.7, but adsorption of albumin limited the recovery. Electrophoresis on starch at *p*H 2.4 effected some separation, but elution of the albumin and antibody from the starch was always incomplete.

The report of Levine[6] that serum albumin is soluble in the presence of high concentrations of alcohol after precipitation with trichloroacetic acid suggested that simple addition of alcohol at *p*H 2.4 might leave the albumin in solution while precipitating the antibodies. Use of *p*H 2.4 was preferred over *p*H 11.7 since the properties of serum albumin have been studied extensively at acid conditions and its antigenic activity, intrinsic viscosity, optical rotation and molecular volume have been shown to be unaffected by exposure of the protein to *p*H 2 and return to neutrality.[7]

At an ethanol concentration of 71 vol. % at *p*H 2.4 and 0.4% protein, it was found that 88–92% of the serum albumin of a specific precipitate is soluble. Purity of the albumin recovered was

(1) Supported by a Grant (H-2751) from the National Heart Institute, U. S. Public Health Service.

(2) *Cf.* (a) W. C. Boyd, "Fundamentals of Immunology," Interscience Publishers, Inc., New York, N. Y., 1956, pp. 70–73; (b) E. A. Kabat and M. M. Mayer, "Experimental Immunochemistry," C. C. Thomas, Springfield, Ill., 1948, pp. 478–488.

(3) V. R. Mason, *Bull. Johns Hopkins Hosp.*, **33**, 116 (1922).

(4) (a) D. H. Campbell, E. Leuscher and L. S. Lerman, *Proc. Natl. Acad. Sci.*, **37**, 575 (1951); (b) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955).

(5) (a) W. J. Kleinschmidt and P. D. Boyer, *J. Immunol.*, **69**, 247 (1952); (b) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 4855 (1955).

(6) S. Levine, *Arch. Biochem. Biophys.*, **50**, 515 (1954).

(7) (a) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954); (b) M. Champagne, *J. Polymer Sci.*, **23**, 863 (1957).