column. Elution with Skelly B solvent and then with Skelly B–benzene (1:1) gave, respectively, 0.94 g. of II, m.p. 218–220°, and 0.11 g. of II, m.p., 276–278°.

The aqueous phase remaining after extraction with benzene was made alkaline and extracted with ether. There was obtained 0.34 g. of aniline.

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

The Conformation of Lysine on its Site of Biological Utilization[1]

BY ALVIE L. DAVIS,[2] CHARLES G. SKINNER AND WILLIAM SHIVE

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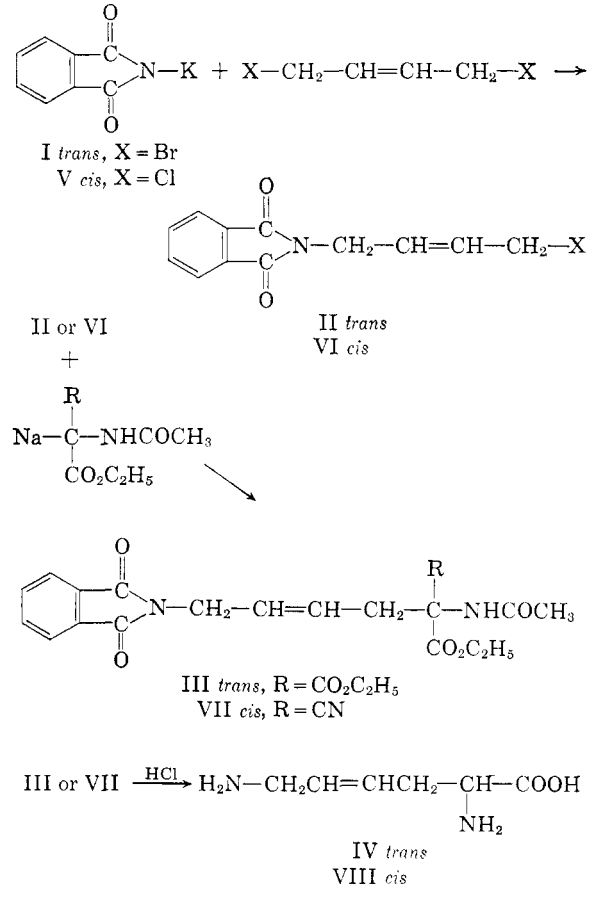
In order to study the conformation of lysine bound to its enzymic site of biological utilization, *cis*- and *trans*-2,6-diamino-4-hexenoic acids (4,5-dehydrolysines) were synthesized. The appropriate *cis*- or *trans*-1,4-dihalo-2-butene was treated with potassium phthalimide, and the resulting N-(4-halo-2-butenyl)-phthalimide was condensed either with ethyl acetamidocyanoacetate in the case of the *cis*- isomer or with ethyl acetamidomalonate in the case of the *trans*- isomer. Acid hydrolysis of the corresponding condensation products gave the isomeric 4,5-dehydrolysines. The biological activities of the two isomers indicate that the conformation of lysine in association with its enzymic site of utilization in several organisms is such that the β- and ϵ-carbons are in a *trans*-like configuration.

3-Aminocyclohexanealanine and 3-aminomethylcyclohexaneglycine both bind at the site of utilization of lysine and competitively inhibit its utilization in certain microörganisms.[3,4] In contrast, 4-aminocyclohexaneglycine apparently does not form such a complex since it is not antagonistic to lysine utilization in these assay systems.[4] The conformation of lysine in forming this enzyme-substrate complex may be such that the β- and ϵ-carbons are in a *trans*-like configuration since both of the active analogs, in contrast to the inactive derivative, can exist in an analogous *trans*-like configuration. Although substituents on either β- or ϵ-carbons of lysine as in the two active cyclohexane analogs did not prevent antagonism of lysine, the inactivity of 4-aminocyclohexaneglycine could possibly result from the effect of substituents on both of these carbons which might result in sufficient steric hindrance to prevent interaction with the enzyme utilizing lysine.

In order to demonstrate that a biologically active lysine analog must be able to conform to the structure in which the β- and ϵ-carbons are in a *trans*-like configuration, lysine analogs with no side chain substituents, but with restrictions on the relative positions of the β- and ϵ-carbons, would be required to eliminate the possibility of steric hindrance accounting for the inactivity of 4-aminocyclohexaneglycine. Accordingly, *trans*- and *cis*-4,5-dehydro-DL-lysine were prepared with the anticipation that the *trans*- but not the *cis*-isomer should prevent utilization of lysine.

trans-4,5-Dehydrolysine was prepared as indicated in the accompanying equations (I) to (VIII). Two equivalents of *trans*-1,4-dibromo-2-butene were treated with one equivalent of potassium phthalimide to yield N-(*trans*-4-bromo-2-butenyl)-phthalimide (II) which subsequently reacted



with the sodium salt of ethyl acetamidomalonate, and the resulting intermediate (III) was converted by acid hydrolysis to *trans*-4,5-dehydrolysine (IV).

As anticipated, the *trans* form of dehydrolysine inhibits utilization of lysine. The analog completely inhibits growth of *Leuconostoc dextranicum* 8086, *Lactobacillus arabinosus* 17–5 and *Streptococcus lactis* 8039 in media devoid of lysine at concentrations of 0.2, 0.6 and 0.6 μg. per ml., respectively. For each organism, the growth inhibition

(1) Presented at the 16th Southwest Regional American Chemical Society Meeting, December, 1960, Oklahoma City.

(2) Taken in part from the Ph.D. dissertation of A. L. Davis, The University of Texas, August, 1960.

(3) A. L. Davis, J. M. Ravel, C. G. Skinner and W. Shive, *Arch. Biochem. Biophys.*, **76**, 139 (1958).

(4) A. L. Davis, C. G. Skinner and W. Shive, *ibid.*, **87**, 88 (1960).

TABLE I

REVERSAL OF INHIBITION OF *trans*-4,5-DEHYDROLYSINE BY LYSINE IN *Leuconostoc dextranicum*

trans-4,5-Dehydrolysine, μg./5 ml.	DL-Lysine, μg./5 ml. Galvanometer readings								
	0	0.5	1	2	5	10	20	50	100
0	60	60	58	61	59	59	60	60	59
0.5	51	58							
1.0	9	53	56						
2.5		8	33	52					
5			2	17	54				
10				9	31	54			
25					9	27	50		
50						9	26	50	
100							8	9	52
250								6	25
500									4

is competitively reversed by lysine over a broad range of concentrations. For example, the toxicity of *trans*-4,5-dehydrolysine is competitively reversed over a two hundred-fold range of increasing concentrations of lysine (Table I) with an inhibition index (ratio of analog to metabolite concentrations) of about 5. For *Escherichia coli* 9723, in contrast to the lactic acid bacteria, the analog was not inhibitory even at concentrations of about 200 μg. per ml.

Since the *trans* form of 4,5-dehydrolysine is a very effective inhibitor for lactic acid bacteria, inactivity of the corresponding *cis* form would indicate that the conformation of lysine on the enzymic site corresponds closely to a *trans*-like configuration. Accordingly, the *cis* isomer was synthesized by a procedure similar to that used for the *trans* derivative. *cis*-2-Butene-1,4-diol was converted to *cis*-1,4-dichloro-2-butene (V) using PCl_3 as the chlorination agent.[5] Two equivalents of V was treated with one equivalent of potassium phthalimide to yield N-(*cis*-4-chloro-2-butenyl)-phthalimide (VI) which subsequently was condensed with the sodio derivative of ethyl acetamidocyanoacetate. The resulting intermediate, ethyl *cis*-2-acetamido-2-cyano-6-phthalimido-4-hexanoate (VII) then was heated in the presence of concentrated hydrochloric acid for about 24 hr. to yield *cis*-4,5-dehydrolysine (VIII). Paper chromatograms of the reaction mixture containing the product (VIII) showed the presence of only one ninhydrin positive component. *cis*-4,5-Dehydrolysine, because of its hygroscopic character, is difficult to purify and it was finally isolated, although in low yield, as a crystalline dihydrochloride salt.

The *cis* isomer did not have biological activity comparable to the *trans*-4,5-dehydrolysine in inhibiting the growth of lactic acid bacteria. However, the *cis* preparation at 100 times the concentration of the *trans* isomer did exert the same growth inhibitory effects in *L. dextranicum*, *L. arabinosus* and *S. lactis*. This identical ratio of activity of the two isomeric products in several different microbial systems indicated that the *cis* preparation was contaminated with about 1% of the *trans* isomer.[6] Since 2-butene-1,4-diol is obtained by the catalytic addition of hydrogen to 2-butyne-1,4-diol, it may be anticipated that even though the major product is the *cis* isomer, a trace of the *trans* isomer is probably present. This conclusion was further substantiated by additional purification of the originally isolated *cis* derivative through fractional crystallization. The inhibitory activity of thrice recrystallized *cis*-4,5-dehydrolysine dihydrochloride was decreased to about one-fourth that of the original material.

Both the *cis* and *trans* 4,5-dehydrolysine on catalytic hydrogenation formed lysine, which was then determined using *Streptococcus faecalis* 8043, an organism which requires lysine for growth. The biological activities of the reduced dehydrolysines were comparable with lysine in promoting growth of this organism. In addition, the R_f values of both of the hydrogenated products and lysine were identical in butanol:acetic acid:water (3:1:1) and 65% pyridine, indicating that the catalytic hydrogenation of *cis*- and *trans*-4,5-dehydrolysine produces lysine.

It is thus demonstrated that the *trans* isomer of 4,5-dehydrolysine is a very effective antagonist of lysine while the *cis* isomer is either ineffective or, at most, only weakly active in comparison to the *trans* isomer. Thus, it appears that the conformation of lysine in forming a complex with the enzyme site is such that the β and ε carbons are in a *trans*-like configuration so that lysine lies stretched out upon the enzyme rather than partially coiled.

Experimental[7]

Biological Assays.—For the lactic acid bacteria a previously described amino acid medium[8] was modified by the addition of calcium pantothenate (1.0 μg./tube), by the omission of lysine from the basal media and with the additional modifications noted for each organism. For *Leuconostoc dextranicum* 8086, the concentrations of DL-aspartic acid and DL-tryptophan were decreased to 15 μg./tube; 0.1 μg./tube pantethine was added; and the phosphate concentration was increased fourfold. For *Lactobacillus arabinosus* 17-5 and *Streptococcus lactis* 8039 the concentration of DL-glutamic acid was increased to 300 μg./tube. The concentration of DL-aspartic was decreased to 20 μg./tube for *L. arabinosus*. For *Streptococcus faecalis* 8043, 300 μg./tube glutamic acid was added and 100 μg./tube glutamine was added aseptically. All of the above assays were incubated at 30° for approximately 20 hr. with the exception of *L. arabinosus* which was grown for approximately 40 hr. For *Escherichia coli* 9723 a previously described medium[9] was employed, and the organism was incubated at 37° for about 16 hr.

***cis*-1,4-Dichloro-2-butene.**—Using the general procedure of Mislow and Hellman,[5] from 52 g. of *cis*-2-butene-1,4-diol there was recovered 33 g. of product, b.p. 148° (uncorr.).[10]

N-(*cis*-4-Chloro-2-butenyl)-phthalimide.—To 33 g. of *cis*-1,4-dichloro-2-butene (two equivalents) was added in small increments 24 g. potassium phthalimide with frequent shaking. The reaction mixture was heated in an oil-bath at 150° throughout the addition period. After the addition of potassium phthalimide was completed, the reaction mixture was heated for an additional hour at 150°, during which time

(5) K. Mislow and H. M. Hellman, *J. Am. Chem. Soc.*, **73**, 244 (1951).

(6) The inhibitory properties of even closely related metabolite antagonists would be expected to have different inhibition indices depending upon the microbial assay system.

(7) The authors are indebted to Messrs. C. Hedgcoth, A. Lane and J. D. Glass for the elemental analyses, and to Dr. J. M. Ravel and Mrs. J. Humphreys for assistance with the microbial assays. All melting points were determined by the capillary technique and are uncorrected. The paper chromatograms were determined by the ascending technique using the solvents indicated, and the spots were developed with ninhydrin reagent.

(8) J. M. Ravel, L. Woods, B. Felsing and W. Shive, *J. Biol. Chem.*, **206**, 391 (1954).

(9) E. H. Anderson, *Proc. Natl. Acad. Sci., U. S.*, **32**, 120 (1946).

(10) Mislow and Hellman[5] reported a boiling point of 152.5° at 758 mm.

it slowly became dark brown in color. The reaction mixture was cooled, extracted with 150 ml. of ether, and the ether phase finally reduced in volume to yield a precipitate which was filtered, recrystallized from absolute alcohol and dried over calcium chloride in a vacuum desiccator to yield 14.7 g. of product, m.p. 75–76°.

Anal. Calcd. for $C_{12}H_{10}NO_2Cl$: C, 61.15; H, 4.28; N, 5.94. Found: C, 61.14; H, 4.50; N, 6.14.

Ethyl *cis*-2-Acetamido-2-cyano-6-phthalimido-4-hexenoate.—A sample of 6.5 g. of ethyl acetamidocyanoacetate was added to a solution of sodium ethoxide prepared from 1.4 g. of sodium treated with 100 ml. of magnesium-dried ethanol. The reaction mixture was warmed during the addition to effect complete solution of the cyanoacetate, and 10 g. of N-(*cis*-4-chloro-2-butenyl)-phthalimide then was added in small increments. After the latter addition was completed, the resulting mixture was heated under reflux for about 2 hr. After cooling, the sodium chloride was removed by filtration and the filtrate was reduced to dryness *in vacuo*. The residue was extracted three times with 50 ml. portions of carbon tetrachloride, and the combined organic extract was finally washed with water. The solvent now was removed *in vacuo* to yield 4.5 g. of residue which was crystallized from ethanol–water to yield 3.5 g. of product, m.p. 97–98°.

Anal. Calcd. for $C_{19}H_{19}N_3O_6$: N, 11.38. Found: N, 11.21.

***cis*-2,6-Diamino-4-hexenoic Acid (*cis*-4,5-Dehydrolysine).**—A mixture of 3 g. of *cis*-2-acetamido-2-cyano-6-phthalimido-4-hexenoate and 50 ml. of concentrated hydrochloric acid was heated under reflux for about 24 hr. The reaction mixture was cooled, the phthalic acid which precipitated was removed by filtration and the filtrate was reduced to dryness *in vacuo*. After the repeated addition and removal of small volumes of ethanol *in vacuo*, the dried residue was crystallized from ethanol–ethyl acetate to yield about 200 mg. of hygroscopic product. The hygroscopic nature of the sample was such that a melting point could not readily be determined. The material isolated gave a positive test with ninhydrin and the R_f values of this product in butanol:acetic acid:water (3:1:1) and 65% pyridine were 0.11 and 0.33, respectively. The sample was carefully weighed under anhydrous conditions using a weighing "pig" for an elemental analysis.

Anal. Calcd. for $C_6H_{12}N_2O_2 \cdot 2HCl$: N, 12.90. Found: N, 12.93.

N-(*trans*-4-Bromo-2-butenyl)-phthalimide.—A 40 g. sample of *trans*-1,4-dibromo-2-butene was heated to its melting point in an oil-bath under anhydrous conditions and, after it had completely melted, 8 g. of potassium phthalimide was added in small increments with frequent shaking. Upon completion of the addition (about 2 hr.), the reaction mixture was heated at about 100–105° for an additional 2 hr. The resulting dark brown reaction mixture was extracted with ether, and the ether extract was reduced to dryness with a water aspirator. The residue was dissolved in absolute ethanol, treated with Darco G-60, filtered and an equal volume of Skelly B then was added to the clear filtrate. While standing in the cold overnight, 10 g. of product separated, m.p. 95–96°.

Anal. Calcd. for $C_{12}H_{10}NO_2Br$: C, 51.45; H, 3.67; N, 5.00. Found: C, 51.77; H, 3.58; N, 5.04.

Ethyl *trans*-2-Acetamido-2-carboethoxy-6-phthalimido-4-hexenoate.—A sample of 8 g. of ethyl acetamidomalonate was added to a solution of sodium ethoxide prepared from 1 g. of sodium treated with 100 ml. of magnesium-dried ethanol, and, after the malonate derivative had dissolved, 10 g. of N-(*trans*-4-bromo-2-butenyl)-phthalimide was added in small increments with frequent shaking. The reaction mixture then was heated under reflux for about 4 hr. The sodium bromide which precipitated during the reaction was removed by filtration, and the filtrate was reduced to dryness *in vacuo*. The residue was dissolved in a small amount of ethanol; after which an equal volume of Skelly B was added, and the resulting solution was placed in the refrigerator for about one week. There was recovered about 7 g. of product, m.p. 126–127°.

Anal. Calcd. for $C_{21}H_{24}N_2O_7$: N, 6.72. Found: N, 6.81.

***trans*-2,6-Diamino-4-hexenoic Acid (*trans*-4,5-Dehydrolysine).**—A mixture of 7 g. of ethyl *trans*-2-acetamido-2-carboethoxy-6-phthalimido-4-hexenoate and 50 ml. of concentrated hydrochloric acid was heated under reflux for about 18 hr. The phthalic acid which formed during the hydrolysis was removed by filtration, and the filtrate was reduced to dryness *in vacuo*. The excess hydrochloric acid was removed by repeated addition and evaporation of small quantities of ethanol *in vacuo*. The resulting residue was crystallized from ethanol–ethyl acetate to yield 700 mg. of product, m.p. 180–185°. The R_f values of this compound in butanol:acetic acid:water (3:1:1) and 65% pyridine were 0.11 and 0.33, respectively. The ninhydrin spray reagent produced a yellow spot which slowly turned purple on standing.

Anal. Calcd. for $C_6H_{12}N_2O_2 \cdot 2HCl$: C, 33.19; H, 6.50; N, 12.90. Found: C, 33.57; H, 6.59; N, 12.66.

Catalytic Hydrogenation of *cis* and *trans*-4,5-Dehydrolysine.—Both of the *cis* and *trans* isomers of 4,5-dehydro-DL-lysine were reduced in the same manner. A solution of 10 mg. of the appropriate isomer in 10 ml. of water was agitated in the presence of 50 mg. of palladium black under about 45 lb. of hydrogen pressure for 1 hr. The catalyst was removed and the appropriate dilutions were made from this 1 mg. per ml. solution for the microbiological assays using *Streptococcus faecalis*. The basal medium[8] was modified by omitting the DL-lysine, and the assays were supplemented with the components indicated and incubated at 30° for about 20 hr. The growth response curves obtained with the two hydrogenated samples were quantitatively identical with that found using an authentic sample of DL-lysine. The R_f values of both of the hydrogenated products were identical with that of lysine in several paper chromatographic systems.

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

A Conformation of Methionine Essential for its Biological Utilization

BY CHARLES G. SKINNER, JEROME EDELSON AND WILLIAM SHIVE

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The synthesis of the *cis* and *trans* forms of 2-amino-4-hexenoic acid (crotylglycine) and of 2-amino-3-methyl-4-pentenoic acid by condensation of the appropriate halide with ethyl acetamidocyanoacetate followed by hydrolysis of the condensation product is accompanied by an allylic rearrangement to give mixtures of crotylglycine and 2-amino-3-methyl-4-pentenoic acid. Biological properties previously ascribed to the *trans* form of 2-amino-4-hexenoic acid result from a small contamination of rearranged product, and purified samples are ineffective as an amino acid antagonist for *Escherichia coli*. In contrast, the *cis* form is a competitive antagonist of methionine for *E. coli*, so that the conformation of methionine on its site of utilization appears to be one in which the terminal group and the β-methylene group are in a *cis*-like configuration structurally resembling *cis*- rather than *trans*-crotylglycine.

Studies of various lysine analogs with restricted rotation have demonstrated that an essential conformation of lysine for binding at its site of utilization is such that the β and ϵ carbons are in a *trans*-