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Preparation of amino acids and derivatives and their effect on the growth of Lactobacillus arabinosus

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NOTE TO USERS

PREPARATION OF AMINO ACIDS AND DERIVATIVES AND THEIR EFFECT ON THE GROWTH OF *LACTOBACILLUS ARABINOSUS*

by

Marguerite Fling

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INTRODUCTION

One of the outstanding characteristics of many compounds isolated from natural sources is the optical activity of such substances. In the case of proteins, not only are the compounds optically active, but the amino acids obtained on hydrolysis all have the same configuration on the alpha carbon atom. This fact has led to the use of the term "natural" to designate the l-configuration of amino acids.

In the past decade, however, derivatives of "unnatural" or d-amino acids occurring in natural products have been reported. Jacobs and Craig in 1935 (1) obtained d-proline from ergot alkaloids. Ivanovics and Bruckner (2) obtained d-glutamic acid from the capsule of Bacillus anthracis. In 1941 Lipmann, Hotchkiss, and Dubos (3) reported that 45% of the amino acids obtained from gramicidin and 20% of those from tyrocidin possessed the d-configuration. The first official report on the war-time work on the structure of the penicillins (4) showed them to be complex d-amino acid derivatives.

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(1) Jacobs and Craig, J. Biol. Chem. 110, 521 (1935).
(2) Ivanovics and Bruckner, Z. Immunitatsf. 91, 175 (1937).
(3) Lipmann, Hotchkiss, and Dubos, J. Biol. Chem. 141, 163 (1941).
(4) Com. on Med. Res., OSRD, Washington, and Med. Res. Council of London, Sci. 102, 627 (1945).

The presence of d-amino acids in antibiotics raises the question of possible correlation between amino acid configuration and the physiological activity of these compounds. The present study was undertaken to determine if inhibitory activity and configuration are related. In addition, simple derivatives of both d- and l-amino acids were tested to see what type of derivative offers most promise in the development of simple effective chemotherapeutic agents.

DEFINITIONS OF TERMS USED

Amino acid refers to the d-, l-, or dl- forms of any of the 22 accepted amino acids as listed in Schmidt (5). Norvaline and allothreonine are also included in the discussion of the amino acids, since both compounds have been tested in conjunction with the accepted amino acids.

Amino acid derivative refers to chemical modifications which still retain the nitrogen of the α-amino group and the carbonyl of the carboxyl group. The closely related sulfonic acids are also considered as derivatives.

Inhibition is the term used to designate a decrease in one or more of the physiological activities of organisms growing in the presence of a medium which allows normal development. In particular, it is desired to differentiate between the use of the word as defined above and the use found frequently in the literature, namely, as a synonym for non-assimiliability.

Reversal of inhibition refers to the action of compounds which are capable of preventing the action of an inhibitory compound.

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(5) Schmidt, "The Chemistry of Amino Acids and Proteins." 2nd Ed. p. 21. Baltimore, Charles C. Thomas. 1944.

HISTORICAL

Effect of Amino Acids on Bacterial Growth

In 1911 Bainbridge (6) showed that some aerobic, non-sporulating bacilli and cocci were unable to digest purified proteins. These same bacteria, however, could grow on protein hydrolyzates. It soon became evident that not all hydrolyzates were of equal value in promoting bacterial growth. Long (7) pointed out that tryptic digests were inferior to peptic digests for promoting the growth of the tubercule bacillus. He suggested that the inferiority of the tryptic digests might be due to their higher content of free amino acids. As a result of this type of work, attention became focussed on the possible deleterious effect of the amino acids.

Wyon and McLeod in 1923 (8) published a preliminary report on the inhibition of bacterial growth by amino acids. They claimed that 11-130 mM per liter of nine different amino acids proved inhibitory to seven different strains of bacteria, including Staphylococcus aureus and Bacillus subtilis.

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(6) Bainbridge, J. Hyg. 11, 341 (1911).
(7) Long, Amer. Rev. of Tuber. 3, 86 (1919-20).
(8) Wyon and McLeod, J. Hyg. 21, 376 (1923).

They found the cyclic amino acids histidine, tyrosine, and tryptophane to be inhibitory at the lower concentrations. They also found a direct relationship between the molecular weight and the potency of the monoaminomonocarboxylic acids glycine, alanine, and leucine. The other acids found inhibitory were cystine, glutamic acid, and phenylalanine. McLeod and associates (9, 10), using lower concentrations of amino acids, ran similar tests. The authors emphasized that the inhibitory effect was manifested only toward the more delicate organisms, such as Gonococcus, and that no marked effect was shown on hardier organisms, such as Staphylococcus and Escherichia coli.

More recently, there have been other reports on the inhibitory effect of amino acids toward various organisms. Sahyun, et al. (11) showed that cysteine and tyrosine at concentrations from 0.6-1.0 mg. per ml. retarded the rate of growth of Escherichia coli. Gladstone (12) demonstrated an interrelationship between dl-valine[a], dl-leucine, and dl-

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[a] Amino acid prefixes refer solely to configurations. The prefixes are omitted when the literature cited contained no indication of the configuration.

(9) Gordon and McLeod, J. Path. Bact. 29, 13 (1926).
(10) McLeod, Wheatley, and Phelon, Brit. J. Exptl. Path. 8, 25 (1927).
(11) Sahyun, Beard, Schultz, Snow, and Cross, J. Infectious Diseases 58, 37 (1936).
(12) Gladstone, Brit. J. Exptl. Path. 20, 189 (1939).

isoleucine and the growth of Bacillus anthracis. If all three amino acids were added to the medium, growth was accelerated and improved. The addition of any one of the three at concentrations as low as mM/5000 inhibited growth for 24 hours.

Pelczar and Porter (13) reported a similar case for Proteus morganii in which dl-norvaline, dl-norleucine, and dl-allothreonine at M/1500 inhibited growth unless added in the presence of a mixture of 18 amino acids. Further work (14) showed that methionine was specific for the dl-norvaline reversal. Four different amino acids could reverse the inhibition caused by dl-norleucine, and any one of 20 amino acids could reverse dl-allothreonine activity.

Greene (15), in an attempt to obtain a synthetic medium for Leptospira canicola, found that a concentration of .025% of dl-alanine, l-arginine, l-glutamic acid, glycine, l-lysine, dl-methionine, dl-phenylalanine, l-tryptophane, dl-tyrosine, or dl-valine in Schuffner's medium caused inhibition. l-Asparagine, dl-aspartic acid, l-histidine, dl-leucine, and l-proline were without effect. A similar study of Neisseria intracellularis by Grossowicz (16) showed asparagine and l-cystine, and, to a smaller degree, glycine and tyrosine to be inhibitory. The activity of asparagine and cystine was re-

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(13) Pelczar and Porter, Arch. Biochem. 2, 323 (1943).
(14) Porter and Meyers, Arch. Biochem. 8, 169 (1945).
(15) Greene, J. Bact. 50, 39 (1945).
(16) Grossowicz, J. Bact. 50, 109 (1945).

versed by the addition of serum. Lanen, Baldwin, and Reher (17), working with crown gall bacteria, found a direct relation between inhibition of bacterial growth and attenuation as caused by dl-aminobutyric acid, dl-threonine, dl-methionine, dl-norvaline, dl-valine, dl-norleucine, dl-isoleucine, glycine, dl-serine, dl-leucine, l-leucine, dl-alanine, and dl-lysine. They found no correlation between molecular weight and activity. dl-Leucine and l-leucine had equal activity. Schwartzman (18), in a report which dealt in part with the effect of amino acids on the activity of penicillin, found that 0.625-3.75 mg./ml. of l-tyrosine, l-proline, dl-methionine, dl-valine, and dl-alanine caused 10-30% growth inhibition of E. coli.

In the above cases, either natural or racemic amino acids were used. Nielsen (19) tested 39 different amino acids to determine if they could be assimilated by Rhizobium leguminosarum. He found that concentrations of .004M of dl-valine, dl-leucine, and dl-isoleucine were less effective than lower concentrations (.0008M). To determine if the organisms could utilize d-amino acids, he tested both forms of leucine. He claimed that the results (which were not published) showed that d-leucine was not assimilated, but appeared in fact to be

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(17) Lanen, Baldwin, and Reher, Sci. 92, 512 (1940).
(18) Schwartzman, J. Exptl. Med. 83, 65 (1946).
(19) Nielsen, Compt. rend. trav. lab. Carlsberg, Ser. physiol. 23, 115 (1940). /C. A. 35, 2549 (1941)./

inhibitory. Since, however, he was using an incomplete medium which did not support growth in the absence of a source of available nitrogen, it is difficult to see how d-leucine could have been considered inhibitory, as defined in this thesis.

Effect of Amino Acid Derivatives on Bacterial Growth

The isolation of a bactericidal fraction from B. brevis was announced in 1939 (20). Since then much work has been done on the structure of the two compounds gramicidin and tyrocidin, isolated from this fraction. As has been pointed out in the introduction, amino acids of the d-configuration were obtained on hydrolysis of both compounds. Gramicidin yielded d-leucine and dl-valine. Christensen (21) isolated d-valyl-d-valine and l-valyl-l-valine from a gramicidin hydrolysis. d-Phenylalanine has been obtained from a tyrocidin hydrolyzate. Gramicidin is active against gram-negative organisms in concentrations of 0.01-1.0 γg./ml. Tyrocidin is active against both gram-negative and gram-positive organisms at concentrations 10 to 50 times higher than gramicidin.

In connection with the occurrence of d-amino acid residues in gramicidin and tyrosine, Hotchkiss (22) referred to unpublished work in which he tested palmityl-l-tryptophane

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(20) Dubos and Cattaneo, J. Exptl. Med. 70, 249 (1939).
(21) Christensen, J. Biol. Chem. 154, 427 (1944).
(22) Hotchkiss, Advances in Enzymology 4, 153 (1944).

and found that it had only slight bacteriostatic and detergent activity. Palmityl-dl-tryptophane showed no increase in activity.

The work of Woods (23) on the mode of action of sulfanilamide, i.e., its interference with the utilization of p-aminobenzoic acid, has led to the synthesis of analogues of many essential metabolites in an attempt to duplicate the activity of the sulfa drugs. Most of these attempts have dealt with vitamin analogues, but some amino acid analogues have been tried.

McIlwain (24) prepared the aminosulfonic acid analogues of glycine, alanine, valine, leucine, phenylalanine, and aspartic acid. The analogues were tested in concentrations of 1.6×10^{-5} to 1.6×10^{-2} M against a group of organisms, including E. coli, Staphylococcus aureus, and Streptococcus hemolyticus. Inhibition occurred with those organisms, such as S. aureus, which have fairly complex nutritional requirements. Strains of S. aureus which were trained to grow without specific amino acids were no longer inhibited by the corresponding aminosulfonic acid analogues. The inhibition could be reversed by the addition of aminocarboxylic acids. This reversal was, in most cases, not specific, since more

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(23) Woods, Brit. J. Exptl. Path. 21, 74 (1940).
(24) McIlwain, Brit. J. Exptl. Path. 22, 148 (1941).

than one aminocarboxylic acid could be used to effect the reversal.

Roblin and co-workers (25) prepared the oxygen analogue of methionine, which they called methoxinine. It proved inhibitory against E. coli and S. aureus in concentrations of 12.5 mg./100 ml. The activity was reversed by l-methionine but not by the d-isomer.

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(25) Roblin, Lampen, English, Cole, and Vaughan, J. Am. Chem. Soc. 67, 290 (1945).

EXPERIMENTAL

Preparation of Compounds

d-Alanine

Benzoyl-dl-alanine was resolved by the procedure of Fischer (26). Thirty-five and eight-tenths g. (0.185 mole) of benzoyl-dl-alanine (laboratory preparation) and 86.4 g. (0.219 mole) of anhydrous brucine (Eastman Kodak) were dissolved in 132 ml. of hot water. The clear solution was placed in the refrigerator for 15 hours. The precipitate which formed was filtered off and recrystallized three times from 55 ml. portions of hot water. Twelve hours were allowed for each recrystallization. The yield of brucinium benzoyl-d-alaninate was 27 g. (51%).

Thirty-two and five-tenths g. (0.057 mole) of the brucine salt was dissolved in 97.5 ml. of hot water. The brucine was precipitated with 57 ml. (0.057 mole) of N potassium hydroxide. After being cooled 10 minutes in an ice bath, the brucine was filtered off. The mother liquor was neutralized and concentrated to dryness under reduced pressure (bath temperature 40-50°). The residue was dissolved in 40 ml. of

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(26) Fischer, Ber. 32, 2451 (1899).

warm water. Upon chilling, benzoyl-d-alanine crystallized out. The yield, after three recrystallizations from water, was 4.2 g. (38% based on the brucine salt). A sample melted at 147°[a].

Four g. (0.021 mole) of benzoyl-d-alanine and 20 ml. of 20% hydrochloric acid were refluxed for five hours. The benzoic acid which crystallized out was filtered off, and the filtrate was extracted with six portions of ether. The aqueous layer was evaporated to dryness on a steam bath. The residue was dissolved in a small amount of warm ethanol which contained a few drops of hydrochloric acid. Ether was then added until precipitation appeared complete. The yield of d-alanine hydrochloride was 1.8 g. (69% based on benzoyl-d-alanine).

$[\alpha]_D = -9.2° \pm 0.2°$ (10.7% aqueous solution).

The free amino acid was obtained by dissolving the hydrochloride in absolute ethanol, chilling, and neutralizing with concentrated ammonium hydroxide. The d-alanine which separated was washed free of chloride with absolute ethanol.

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a All melting points are uncorrected.

Purification of recovered brucine

Recovered brucine was air dried and was purified by the following procedure. To a suspension of 5 g. (0.012 mole) of brucine in 50 ml. of hot water was added 1.5 ml. (0.015 mole) of 10 N sulfuric acid. The small amount of insoluble material was filtered off and the clear filtrate was placed in the refrigerator. The brucine sulfate which crystallized out was filtered off. The yield of brucine sulfate was 5.6 g. (90%). Five and six-tenths g. of brucine sulfate was dissolved in 50 ml. of hot water. Sodium hydroxide was added, with stirring, until the solution was basic. The brucine which precipitated was filtered off and dried in a vacuum oven at 60° C. The yield of purified brucine was 4.2 g. (84% recovery), of which a sample melted from 173-174? Another run, starting with 70 g. of crude brucine, yielded 83% of purified product.

l-Alanine

l-Alanine, kindly furnished by Dr. O. K. Behrens of Eli Lilly and Co., was dissolved in hydrochloric acid solution and cleared with Norit. The crystals of l-alanine hydrochloride obtained by evaporation were dissolved in ethanol and precipitated with ether.

$[\alpha]_D = +9.4° \pm 0.2°$ (8.4% aqueous solution).

The free amino acid was obtained in the same manner as the d- form.

dl-Leucine

The procedure of Marvel (27) was followed with slight variations. One hundred forty g. (1.21 moles) of freshly distilled isocaproic acid (laboratory preparation), boiling point 185-196°, and 293 g. (1.76 moles) of bromine (dried with 198 ml. of concentrated sulfuric acid) were placed in a one-liter flask fitted with a reflux condenser, a safety trap, and a water trap for absorbing hydrogen bromide. Two and seven-tenths g. (0.022 mole) of dry red phosphorus was added cautiously through the top of the reflux condenser. The contents of the flask were then heated for six hours in a water bath at 70-80°. Two hundred ten g. (89%) of α-bromoisocaproic acid, distilling from 128-133°/12 mm., was obtained.

Two hundred ten g. (1.08 moles) of α-bromoisocaproic acid was added to 1230 ml. (18.5 moles) of 15 N ammonium hydroxide. The mixture was allowed to stand at room temperature for four days. The precipitate which formed was filtered off, yielding 58 g. of dl-leucine. The mother liquor was then concentrated to dryness. The residue was dissolved in hot water, an equal volume of ethanol was added, and the mixture was placed in the refrigerator. The precipitate which formed yielded an additional 34 g. of dl-leucine. The total yield of dl-leucine was 92 g. (65%).

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(27) Marvel, Org. Syntheses 21, 74 (1941).

Formyl-dl-leucine

The formylation procedure of du Vigneaud, Dorfman, and Loring (28) for dl-cystine was adapted to leucine. Fifteen g. (0.114 mole) of dl-leucine (Merck) was dissolved in 225 ml. (5.16 moles) of 88% formic acid (Merck). The solution was heated to 60° and kept between 59-61° by the dropwise addition of 75 ml. (0.795 mole) of acetic anhydride (tech.). When the reaction mixture was cool, 37.5 ml. of water was added. The clear solution was then concentrated under reduced pressure to a mushy consistency. This mass was thoroughly mixed with 25 ml. (0.025 mole) of cold N hydrochloric acid, filtered, and washed with a small amount of cold water. The yield of formyl-dl-leucine was 13.7 g. (75%). A sample melted from 111-113°. Hydrolysis of the mother liquor yielded 2.9 g. of dl-leucine, giving a yield of formyl-dl-leucine of 93% (calculated on the basis of reacted leucine). Other runs, in which no attempt was made to recover unreacted dl-leucine, gave yields of 83% and 87%.

Formyl-d-leucine

The resolution procedure of Fischer and Warburg (29) was used. To a solution of 47.2 g. (0.297 mole) of formyl-dl-

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(28) du Vigneaud, Dorfman, and Loring, J. Biol. Chem. 98, 577 (1932).
(29) Fischer and Warburg, Ber. 38, 3997 (1905).

leucine in 3780 ml. of hot absolute ethanol was added 117 g. (0.297 mole) of brucine. The clear solution was placed in the refrigerator only long enough to be chilled thoroughly, or about nine hours. For a smaller run (15 g. of formyl-dl-leucine), this required about five hours. The precipitated brucinium formyl-d-leucinate weighed about 87 g. The clear filtrate containing the brucine salt of formyl-l-leucine was returned to the refrigerator. No further crystallization occurred.

Eighty-seven g. (0.162 mole) of the brucine salt was dissolved in 425 ml. of water. The solution was cooled in an ice bath, and the brucine precipitated with 165 ml. (0.165 mole) of N sodium hydroxide. After the brucine was filtered off, the filtrate was extracted once with chloroform and five times with ether to remove residual brucine. At this point, when one drop of concentrated nitric acid was added to one drop of the filtrate, no color was formed, indicating the absence of brucine. The filtrate was acidified with 21 ml. (0.105 mole) of 5 N hydrochloric acid and was then concentrated under reduced pressure to 200 ml. The concentrated filtrate was placed in an ice bath and 30 ml. (0.15 mole) of 5 N hydrochloric acid was added. After 15 minutes, the formyl-d-leucine was filtered, washed with cold water, and recrystallized from 50 ml. of hot water. The yield was 17.1 g. (72%). A sample melted from 137-140°. In another run, the yield was 77%.

Formyl-l-leucine

The mother liquor containing brucinium formyl-l-leucinate was concentrated to dryness under reduced pressure. The residue was treated in the same fashion as was brucinium formyl-d-leucinate. From 55.8 g. of formyl-dl-leucine, there was obtained 19 g. (68%) of formyl-l-leucine.

d-Leucine

Seventeen g. (0.107 mole) of formyl-d-leucine was refluxed with 170 ml. (0.488 mole) of 10% hydrochloric acid for one and one-half hours. The solution was then concentrated under reduced pressure to dryness. The residue was dissolved in water and the solution evaporated to dryness on a steam bath. This residue was dissolved in a small amount of water and was neutralized with 2 N lithium hydroxide. The precipitate was filtered and washed with absolute ethanol until free from chloride. The yield of d-leucine was 11 g. (78%).

l-Leucine

Formyl-l-leucine was treated in the same manner as was formyl-d-leucine. The yield of l-leucine was 64%.

Phthalyl-l-leucine

This compound was prepared according to the procedure of Reese (30). One g. (0.0077 mole) of l-leucine and 1.1 g. (0.0077 mole) of phthalic anhydride (Eastman Kodak) were heated in an oil bath at 150° until reaction, as evidenced by frothing, had ceased. The light brown mass, which hardened on cooling, was extracted with boiling ether. Hexane was added to the filtered ether extracts until turbidity persisted. The precipitate was filtered after five hours and recrystallized from about 75 parts of cyclohexane. The yield of recrystallized phthalyl-l-leucine was 1.2 g. (62%). A sample melted from 118-119°. Rotation on a product of a subsequent recrystallization from cyclohexane was as follows:

$[\alpha]_D^{27} = -22.1° \pm 1.0°$ (1.00% in absolute ethanol).

Reese reported a melting point of 115-116° and a specific rotation of -21.87° (5% in absolute ethanol).

Phthalyl-d-leucine

The preparation of this compound was identical with that of the l- form. One g. of d-leucine yielded 1.6 g. (83%) of phthalyl-d-leucine. A sample melted from 118-119°. Rotations on products of subsequent recrystallizations from cyclohexane were as follows:

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(30) Reese, Ann. 242, 9 (1887).

$[\alpha]_D$ = +23.3° ± 1.4° (1.99% in absolute ethanol).

$[\alpha]_D^{22}$ = +23.9° ± 1.4° (1.02% in absolute ethanol).

$[\alpha]_D^{27}$ = +22.8° ± 1.0° (0.73% in absolute ethanol).

Anal. Calc'd for $C_{14}H_{15}O_4N$: N, 5.36; Neut. equiv., 261

Found: N, 5.52; Neut. equiv., 261

Glycyl-dl-leucine

The procedure of Fischer and Warburg (31) was used. Ten g. (0.0765 mole) of dl-leucine was dissolved in 76.5 ml. (0.0765 mole) of N sodium hydroxide and cooled in an ice bath. Seventeen g. (0.15 mole) of chloroacetyl chloride (Eastman Kodak) and 270 ml. (0.270 mole) of N sodium hydroxide were added alternately, in small portions, with stirring and cooling. The solution was then acidified with 30 ml. (0.150 mole) of 5 N hydrochloric acid. After cooling, the chloroacetyl-dl-leucine was filtered, washed with cold water, and dried. The yield was 10.5 g. (66%). The 10.5 g. (0.150 mole) was added to a mixture of 52 ml. (0.780 mole) of 15 N ammonium hydroxide and 6.5 ml. of water, and was refluxed for 20 minutes. The solution was then evaporated to dryness under reduced pressure. The residue was dissolved in water and precipitated with ethanol. The yield of glycyl-dl-leucine was 4.7 g. (31% based on dl-leucine).

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a The melting points were run on a melting point block.

(31) Fischer and Warburg, Ann. 340, 157 (1905).

A sample decomposed at 245° [a]. (Fischer and Warburg reported a decomposition point of 242-245°.)

Glycyl-l-leucine

The procedure of Fischer and Steingrover (32) was used. The preparation of chloroacetyl-l-leucine was identical with that of the dl- compound. Five g. of l-leucine yielded 4.3 g. (54%) of chloroacetyl-l-leucine. This amount (0.027 mole) was suspended in 30 ml. (0.390 mole) of 13 N ammonium hydroxide and was placed in an incubator at 37° for three days. A small amount of insoluble material was filtered off and the filtrate was concentrated to dryness under reduced pressure. The residue was recrystallized by dissolving in hot water and adding ethanol until turbid. The yield of glycyl-l-leucine was 3.4 g. (32% based on l-leucine). A sample turned yellow at 232°. and gradually darkened until 245° [a], when decomposition was complete.

$$[\alpha]_D^{29} = -34.9° \pm 0.8° \quad (3.64\% \text{ in water}).$$

Fischer and Steingrover reported that a sample of their product turned yellow at 234° and was completely decomposed at 242°. The specific rotation on their product was as follows:

$$[\alpha]_D^{20} = -35.1° \pm 0.5° \quad (4.16\% \text{ in water}).$$

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[a] The melting points were run on a melting point block.

(32) Fischer and Steingrover, Ann. 365, 167 (1909).

Glycyl-d-leucine

This compound has been prepared by Abderhalden and Geddert (33), from glycyl-dl-leucine by using yeast. In this case, however, the compound was prepared in the same manner as the l- peptide. From 6 g. of d-leucine there was obtained 2.3 g. (29%) of glycyl-d-leucine. A sample started to darken at 230° and was completely decomposed at 244° [a].

$[\alpha]_D^{28} = +34.7° \pm 2.1°$ (1.38% in water).

Abderhalden and Geddert reported:

$[\alpha]_D^{20} = +37.62°$ (4% in water).

$[\alpha]_D^{20} = +37.16°$ (4% in water).

l-Leucine methyl ester hydrochloride

This compound was made by Abderhalden and Spur (34) as an intermediate in the preparation of l-leucine methyl ester. No constants, however, were given for the hydrochloride. Five g. of l-leucine was suspended in 30 ml. of absolute methanol and saturated with dry hydrogen chloride. The clear solution was refluxed 30 minutes. The alcohol was removed under reduced pressure and the residue recrystallized by dissolving in hot absolute ethanol and adding ether until turbid. The yield was 2.3 g. (33%) of the hydrochloride. A sample melted from 145-

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[a] The melting points were run on a melting point block.

(33) Abderhalden and Geddert, Z. physiol. Chem. 74, 407 (1911).
(34) Abderhalden and Spur, Z. physiol. Chem. 107, 5 (1919).

146°.

Anal. Calc'd for $C_7H_{14}O_2NCl$: Cl, 19.6

Found: Cl, 19.6[a]

d-Leucine methyl ester hydrochloride

This compound was made by the method of Smith and Brown (34a). The preparation was similar to that of the l- form, except that the hydrogen chloride was bubbled through the methanolic solution for an additional 30 minutes after saturation, the refluxing being omitted. Two g. of d-leucine yielded 1.6 g. (57%) of the hydrochloride. A sample melted from 148-149°.

dl-Leucine anhydride

The procedure of Sannie (35) was used. Two g. of dl-leucine was refluxed with 12 ml. of ethylene glycol (laboratory stock) for 40 minutes. After the mixture was cooled, the anhydride crystallized out. The precipitate was filtered and recrystallized from absolute ethanol. The yield was 0.4 g. (21.5%). A sample melted at 260°. Salaskin (35a) reported a melting point of 269-270°.

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a Determination run by Mr. Y. Kobayashi.

(34a) Smith and Brown, J. Am. Chem. Soc. 63, 2605 (1941).
(35) Sannie, Bull. soc. chim. 9, 487 (1942).
(35a) Salaskin, Z. physiol. Chem. 32, 595 (1901).

dl-Valine

The procedure of Marvel (36) was followed with slight modifications. Three hundred g. (2.94 moles) of isovaleric acid (Eastman Kodak, practical grade) was dried by adding 150 ml. of benzene and distilling the mixture until the temperature of the distillate reached 100°. The dried acid was placed in a flask which was fitted with a reflux condenser, a safety trap, and a water trap for absorbing hydrogen bromide. To the flask was added 495 g. (6.2 moles) of bromine, (dried with concentrated sulfuric acid). Five g. (0.040 mole) of dry red phosphorus was added cautiously through the top of the reflux condenser. The mixture was refluxed for 15 hours at 70-80°. Three hundred ninety-four g. (74%) of α-bromoisovaleric acid, distilling from 110-125°/ 15 mm., was obtained. In another run made by Miss Lucille Grow, 150 g. of isovaleric acid was brominated for six hours at 120°. The treatment with benzene was omitted. The yield was 200 g., or 72%.

To 2 l. (30 moles) of 15 N ammonium hydroxide was added 287 g. of α-bromoisovaleric acid. The flask was stoppered and allowed to stand at room temperature for seven days. The contents of the flask were then concentrated on a water bath

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(36) Marvel, Org. Syntheses 20, 106 (1940).

to 400-500 ml. Filtration at this point yielded 29 g. of dl-valine. By concentrating the mother liquor to dryness, dissolving the residue in hot water, and adding two volumes of ethanol, an additional 35.3 g. of dl-valine was obtained. The total yield was 65.3 g. (26%). Another run yielded 35% of dl-valine, from 50 g. of the bromo acid. An attempt was made to convert α-bromoisovaleric acid to valine by using ammonia and ammonium carbonate. This method was used by Cheronis and Spitzmueller (37) to prepare α-aminobutyric acid. The yield of valine, however, was only 18%.

Formyl-dl-valine

The acetic anhydride-formic acid procedure (28) was used. Forty g. (0.341 mole) of dl-valine was dissolved in 620 ml. (14.2 moles) of 88% formic acid. The solution was heated to 60°, and 206 ml. (2.19 moles) of acetic anhydride was added at such a rate that the temperature within the flask was maintained between 59-62°. After the mixture had cooled, 104 ml. of water was added. The clear solution was then concentrated under reduced pressure until crystals separated from the mother liquor. Upon filtration, 36.5 g. of formyl-dl-valine was obtained. A sample melted from 141-143°. The mother liquor was then concentrated to dryness under reduced pressure.

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(37) Cheronis and Spitzmueller, J. Org. Chem. 6, 349 (1941).

The residue was ground with cold N hydrochloric acid, washed with water, and dried. This yielded an additional 4.8 g. of formyl-dl-valine, of which a sample melted from 139-142°. Total yield was 84%. Another 40 g. run gave 38 g. (77%) of formyl-dl-valine.

Formyl-d-valine

Formyl-d-valine was resolved according to the procedure of Fischer (38). To a solution of 40 g. (0.276 mole) of formyl-dl-valine in 1200 ml. of hot absolute methanol (Merck) was added 109 g. (0.276 mole) of brucine. The clear solution was placed in the refrigerator for four hours. The precipitated brucine salt of formyl-d-valine weighed 72 g. (100%). Seventy-two g. (0.138 mole) of the brucine salt was dissolved in 432 ml. of water. The solution was cooled in an ice bath, and the brucine was precipitated with 144 ml. (0.144 mole) of N sodium hydroxide. After 15 minutes, the brucine was filtered off. The filtrate was washed once with chloroform and then with ether until the brucine test (red color with nitric acid) was negative. The filtrate was acidified with 60 ml. (0.060 mole) of N hydrochloric acid, and concentrated under reduced pressure (water bath at 40°) until crystals appeared. The concentrated filtrate was

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(38) Fischer, Ber. 39, 2320 (1906).

placed in an ice bath, and 40 ml. (0.040 mole) of N hydrochloric acid was added. After 15 minutes the formyl-d-valine was filtered and washed free of chloride with cold water. The yield was 7 g. (35%). A sample melted from 150-152°. In another run 10 g. of formyl-dl-valine yielded 2.2 g. (44%) of formyl-d-valine.

Formyl-l-valine

The mother liquor containing brucinium formyl-l-valinate was concentrated to dryness under reduced pressure. The residue was treated in the same manner as was brucinium formyl-d-valinate. From 40 g. of formyl-dl-valine, there was obtained 10.3 g. (52%) of formyl-l-valine.

d-Valine

The mother liquor from the 7 g. batch of formyl-d-valine was concentrated to dryness under reduced pressure. The residue was extracted with 100 ml. of warm ethanol. The ethanol was removed under reduced pressure, and the residue hydrolyzed by refluxing with 10% hydrochloric acid for one hour. The solution was evaporated to a small volume, and neutralized with 2 N lithium hydroxide. The precipitate was filtered, and washed free of chloride with absolute ethanol. The yield of d-valine was 5.5 g.

The crystalline formyl-d-valine (7 g.) was hydrolyzed

with 70 ml. of 10% hydrobromic acid, as carried out by Fischer (38). The hydrolyzate was treated in the same manner as above. The yield of d-valine was 4.5 g.

Rotation of d-valine from the HCl hydrolyzate:

$[\alpha]_D = -24.2°$ (1.14% in 20% HCl).

Rotation of d-valine from the HBr hydrolyzate:

$[\alpha]_D = -25.0°$ (1.11% in 20% HCl).

The combined yield of d-valine was dissolved in hot water, and recovered by adding an equal volume of ethanol.

Rotation on recrystallized d-valine:

$[\alpha]_D = -26.6°$ (1.37% in 20% HCl).

The recrystallization was repeated in the same manner:

$[\alpha]_D = -26.2°$ (1.19% in 20% HCl).

l-Valine

The hydrolysis of 10.3 g. of formyl-l-valine with 10% hydrobromic acid yielded 6.3 g. (76%) of l-valine. No attempt was made to obtain an additional amount from the mother liquor of the formyl-l-valine.

Phthalyl-d-valine

The procedure of Reese (30) for phthalyl-l-leucine was adapted to valine. A mixture of 0.9 g. (0.0077 mole) of d-valine and 1.1 g. (0.0077 mole) of phthalic anhydride was heated in an oil bath at 175° until reaction, as evidenced

by frothing, had ceased. The brown mass which hardened on cooling was extracted with boiling ether. Hexane was added to the filtered ether extracts until turbid. The precipitate which formed was recrystallized from about 50 parts of cyclohexane. The yield was 0.9 g. (47%). A sample melted from 113-114°. Rotations on the products of subsequent recrystallizations from about 50 parts of cyclohexane were as follows:

$[\alpha]_D$ = +67.3° ± 0.3° (1.48% in absolute ethanol).

$[\alpha]_D^{27}$ = +69.0° ± 0.9° (1.07% in absolute ethanol).

$[\alpha]_D^{22}$ = +67.4° ± 0.6° (0.78% in absolute ethanol).

Anal. Calc'd for $C_{13}H_{13}O_4N$: N, 5.67; Neut. equiv., 247

Found: N, 5.76; Neut. equiv., 248

Phthalyl-l-valine

A mixture of 0.9 g. (0.0077 mole) of l-valine and 1.1 g. (0.0077 mole) of phthalic anhydride, treated in the same manner as the dl- derivative, yielded 1.3 g. (69%) of phthalyl-l-valine. A sample melted from 114-115°. Rotations on the products of subsequent recrystallizations from about 50 parts were as follows:

$[\alpha]_D$ = -65.6° ± 1.1° (1.45% in absolute ethanol).

$[\alpha]_D^{27}$ = -63.1° ± 1.4° (1.07% in absolute ethanol).

$[\alpha]_D^{22}$ = -62.4° ± 1.0° (0.76% in absolute ethanol).

Anal. Calc'd for C H O N : N, 5.67; Neut. equiv., 247

Found: N, 5.78; Neut. equiv., 247

l-Valine methyl ester hydrochloride

This compound was prepared by using Fischer's general method (39). Dry hydrogen chloride was passed into a suspension of 2 g. of l-valine in 30 ml. of absolute methanol for 45 minutes. The alcohol was removed under reduced pressure. The residue was dissolved in a minimum amount of boiling absolute ethanol. About 40 volumes of ether were added, and the mixture was placed in the refrigerator. The yield of the ester hydrochloride was 1.1 g. (38%). A sample melted at 168°.

Anal. Calc'd. for $C_6H_{14}O_2NCl$: Cl, 21.2

Found: Cl, 21.2 [a]

d-Valine methyl ester hydrochloride

One g. of d-valine was suspended in 15 ml. of absolute methanol. The suspension was saturated with hydrogen chloride, and the resulting clear solution was refluxed for 30 minutes. The alcohol was removed under reduced pressure. The residue was dissolved in about 5 ml. of boiling absolute ethanol, and 200 ml. of ether was added. The solution was allowed to stand for several hours in the refrigerator, after

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a Determination run by Mr. Y. Kobayashi.

(39) Fischer, Ber. 34, 433 (1901).

which the precipitate was filtered off. The yield of the ester hydrochloride was 1.1 g. (77%). A sample melted at 168°.

Anal: Calc'd for $C_6H_{14}O_2NCl$: Cl, 21.2

Found: Cl, 21.1[a]

Sodium δ-hydroxyvalerate

The procedure of Westerfeld (40) for the oxidation of cyclopentanone was modified as follows: Ninety-eight g. of freshly distilled cyclopentanone (b. pt. 126-128°) was oxidized with hydrogen peroxide by adding portions of cyclopentanone and of 300 ml. of 30% hydrogen peroxide alternately with stirring to 600 ml. of 2 N sodium hydroxide. The oxidation was carried out at 35-45° by keeping the flask containing the reagents in an ice bath. The stirring was continued after the reagents had been added until all frothing ceased. The clear, homogenous liquid was then concentrated under reduced pressure to a white, slightly gelatinous mass. Without further treatment, this was converted to the δ-bromo acid.

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a Determination run by Mr. Y. Kobayashi.

(40) Westerfeld, J. Biol. Chem. 143, 177 (1942).

δ-Bromovaleric acid (41)

The gelatinous sodium δ-hydroxyvalerate was dissolved in 100 ml. of 48% hydrobromic acid. To this was added a mixture of 30 ml. of 48% hydrobromic acid and 100 ml. of concentrated sulfuric acid. The combined mass was refluxed for four hours. The mixture was then poured into one liter of water and extracted twice with ether. It was saturated with ammonium sulfate, which caused a separation into two layers. The upper layer was added to the ether extracts and the lower layer was extracted six more times with ether. The ether extracts were combined, washed with a saturated solution of ammonium sulfate, and dried over calcium sulfate. The ether was removed and the residue was distilled under reduced pressure, yielding 32.1 g./3mm. Upon redistillation, 22.6 g. (10.6% based on cyclopentanone), boiling from 116-121°/3mm., was obtained. Another run, starting with 98 g. cyclopentanone, gave 37.8 g. (18%), distilling from 114-119°/2-3mm.

α,δ-Dibromovaleric acid

The procedure of Merchant, Wickert, and Marvel (42) was followed with modifications. Fifty-four g. of δ-bromovaleric acid was reacted with 19.5 ml. of dry bromine and 0.55 g. of

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(41) Cloves, Ann. 319, 367 (1901).
(42) Merchant, Wickert, and Marvel, J. Am. Chem. Soc. 49, 1829 (1927).

red phosphorus for four hours at 120°. This gave 62.5 g. of acid distilling from 125-145°/2-3mm. Redistillation gave 42.5 g. (55%), boiling from 135-142°/2-3mm.

α,δ-Dibromovaleryl chloride

The procedure of Fischer and Suzuki (43) was followed. Phosphorus pentachloride was added to 42.5 g. of α,δ-dibromovaleric acid in a Claisen flask until no more reaction occurred. The mixture was distilled from the Claisen, using a water pump. Forty g. of distillate was collected from 125-135°.

Prolyl-l-leucine

This compound was prepared according to the procedure of Abderhalden and Sickel (44). Six and five-tenths g. of α,δ-dibromovaleryl chloride and 3.3 g. of l-leucine gave 8.4 g. of crude α,δ-dibromovaleryl-l-leucine in the form of a brown oil. In another run, 4.6 g. of l-leucine gave 10.5 g. of oil.

Treating 4.2 g. of , -dibromovaleryl-l-leucine with 20 ml. of concentrated ammonia for eight days at 40° resulted in 0.4 g. (14% based on l-leucine) of prolyl-l-leucine, of which a sample melted at 225°. The dipeptide was isolated by evaporating the ammoniacal solution under reduced pressure and

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(43) Fischer and Suzuki, Ber. 37, 2843 (1904).
(44) Abderhalden and Sickel, Z. physiol. Chem. 159, 166 (1926).

washing the residue with absolute ethanol until free of bromide. Another 4.0 g. portion of α,δ-dibromovaleryl-l-leucine was treated for eight days at 40° with 20 ml. of absolute methanol containing 4 g. of ammonia. The peptide was isolated in the same manner and amounted to 0.3 g. (11% based on l-leucine), of which a sample melted at 218°. Upon recrystallization from ethanol and water, a sample melted at 228°. In a third run, 10.5 g. of , -dibromovaleryl-l-leucine, treated with aqueous ammonia, yielded 0.9 g. (11% based on l-leucine) of the peptide. A sample, after recrystallization, melted at 223°.

$$[\alpha]_D^{28} = -54° \pm 6° \quad (0.33\% \text{ in water}).$$

Abderhalden and Sickel reported a specific rotation of -47.64° ± 2.0°.

Prolyl-d-leucine

Ten and three-tenths g. of α,δ-dibromovaleryl chloride was added alternately in small portions with small portions of 47 ml. of N sodium hydroxide to a solution of 5.2 g. of d-leucine in 66 ml. of N sodium hydroxide. The mixture was acidified with 78 ml. of N hydrochloric acid and extracted with ether. The ether extract was washed with dilute hydrochloric acid and dried with calcium sulfate. Removal of the ether left 12.4 g. of a light brown oily mass. No attempt was made to crystallize it.

Twelve and four-tenths g. of α,δ-dibromovaleryl-d-leucine was mixed with 60 ml. of concentrated ammonia and kept at 40° for six days. The ammonia was then evaporated under reduced pressure and the residue washed with absolute ethanol until free of bromide. The yield was 1.3 g. (14% based on d-leucine). It was dissolved in about 250 ml. of boiling water. An equal amount of ethanol was added and the mixture placed in the refrigerator. The precipitate which formed on cooling weighed 1.2 g. A sample melted at 225°.

$[\alpha]_D^{28} = +57° \pm 6°$ (0.35% in water).

Anal: Calc'd for $C_{11}H_{20}O_3N_2$: N, 12.2

Found: N, 12.3

Prolyl-l-valine

Ten and three-tenths g. of α,δ-dibromovaleryl chloride in small portions was added alternately with portions of 47 ml. of N sodium hydroxide to a solution of 4.6 g. of l-valine in 66 ml. of N sodium hydroxide. The mixture was acidified with 78 ml. of N hydrochloric acid and extracted with ether. The ether extract was washed with dilute hydrochloric acid and dried with calcium sulfate. Removal of the ether left 11 g. of a thick yellow oil. On standing several days in the vacuum desiccator, the oil began to crystallize. On standing a week, the mass was still only partially crystalline. Ten g. of this partially crystalline mass was mixed with 60 ml. of con-

centrated ammonium hydroxide and kept at 40° for seven days. Solution was complete after the first day. The solvent was evaporated under reduced pressure and the residue washed free of bromide with ethanol. A sample melted from 228-230°. The yield was 0.8 g. (10% based on l-valine). It was dissolved in 20 ml. of boiling water. Eighty ml. of ethanol was added and the mixture was placed in the refrigerator. The precipitate which formed on cooling weighed 0.6 g. A sample melted at 221°.

$[\alpha]_D^{28} = -58.1° \pm 5.1°$ (0.58% in water).

Anal. Calc'd for $C_{10}H_{18}O_3N_2$: N, 13.1

Found: N, 12.9

Prolyl-d-valine

This compound was prepared in the same manner as the l-isomer. Four and six-tenths g. of d-valine yielded 10.9 g. of oily α,δ-dibromovaleryl-d-valine. Crystallization was slower than in the case of the l- isomer. Nine and nine-tenths g. of α,δ-dibromovaleryl-d-valine yielded 0.8 g. (10% based on d-valine) of recrystallized prolyl-d-valine, a sample of which melted at 220°.

$[\alpha]_D^{28} = +57.6° \pm 5.9°$ (0.50% in water).

Anal: Calc'd for $C_{10}H_{18}O_3N_2$: N, 13.1

Found, N, 13.0

dl-Tyrosine

Eighteen g. of l-tyrosine, 60 ml. of acetic acid (tech.), and 15 ml. of acetic anhydride (tech.) were placed in a flask fitted with a condenser. The flask was heated in a boiling water bath for two hours. During the heating, the tyrosine went into solution. The mixture was concentrated under reduced pressure to a gummy consistency. This residue was refluxed for one hour with 100 ml. of 5 N hydrochloric acid. The acid solution, after treatment with Norit, was straw colored. The solution was neutralized with 2 N lithium hydroxide. The yield of dl-tyrosine was 9.6 g. (53%).

$\alpha = -.02° \pm .05°$ (0.9 g. in 10 ml. 3 N HCl).

In another run, in which the tyrosine was precipitated from the acid solution with sodium acetate, 600 g. of l-tyrosine yielded 394 g. (65%) of dl-tyrosine.

d-Tyrosine

d-Tyrosine was obtained by using Abderhalden and Sickel's resolution (45) of formyl-dl-tyrosine. The formylation of dl-tyrosine was carried out by using a mixture of formic acid and acetic anhydride. Two hundred forty g. (2.03 moles) of dl-tyrosine was dissolved in 2480 ml. (56.8 moles) of 88%

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(45) Abderhalden and Sickel, Z. physiol. Chem. 131, 277 (1923).

formic acid. The solution was heated to 60° and kept between 60-62° by the dropwise addition of 824 ml. (8.75 moles) of acetic anhydride. When the reaction mixture was cool, 416 ml. of water was added. The clear solution was concentrated under reduced pressure until crystals started to separate. The crystals were filtered off and recrystallized from 400 ml. of water. The yield was 81.5 g., of which a sample melted at 183°. The mother liquor was concentrated to dryness under reduced pressure. The residue was dissolved in boiling water and norited. This treatment yielded 103.5 g., of which a sample melted at 181°. The total yield of formyl-dl-tyrosine was 185 g. (67%).

Eighty g. (0.442 mole) of formyl-dl-tyrosine and 120 g. (0.305 mole) of brucine were dissolved in 2800 ml. of water at 75°. The mixture was allowed to stand at room temperature overnight. The precipitate was filtered. It weighed 109 g. It was recrystallized twice from 1000 ml. batches of hot (75°) water. The yield after the second recrystallization was 70.3 g. A sample melted at 145°, if heated slowly. If heated rapidly, it appeared to melt at 123°.

Seventy g. of the brucine salt was dissolved in 690 ml. of hot water. The solution was rapidly cooled to 40°, and the brucine was precipitated with 101 ml. of 1 N sodium hydroxide. After the mixture was cooled for 15 minutes in an ice bath, the brucine was filtered off. The filtrate was

washed, once with chloroform, twice with ether, and was then acidified with 101 ml. of N hydrochloric acid. The filtrate was next concentrated under reduced pressure to 250 ml. The concentrated filtrate was cooled, and the precipitated formyl-d-tyrosine was filtered. The yield was 12 g. The 12 g. (0.0576 mole) of formyl-d-tyrosine was refluxed three hours with 120 ml. (0.345 mole) of 10% hydrochloric acid. The d-tyrosine was precipitated by neutralizing with sodium acetate. The yield was 8.9 g. Another 7.7 g. was obtained from the mother liquor by hydrolyzing it with 100 ml. of 10% hydrochloric acid. The total yield of d-tyrosine was 16.6 g. (48% based on formyl-dl-tyrosine).

$[\alpha]_D = +8.93° \pm 1.4°$ (6.6% in 3 N HCl).

The sample used for the rotation was recovered by precipitation with sodium acetate, and another rotation was run.

$[\alpha]_D^{26} = +9.20° \pm 1.0°$ (9.4% in 3 N HCl).

The sample was again recovered by precipitation with sodium acetate and this time it was recrystallized from water.

$[\alpha]_D^{26} = +9.39° \pm 0.8°$ (9.5% in 3 N HCl).

3-Nitro-d-tyrosine

The procedure of Waser and Lewandowski (46) for 3-nitro-l-tyrosine was followed. One g. (0.0055 mole) of d-tyrosine

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(46) Waser and Lewandowski, Helv. Chim. Acta 4, 659 (1921).

was made into a paste with 3 ml. of water, and chilled in an ice bath. To this was added 2.6 g. (0.0312 mole) of 12 N nitric acid dropwise with stirring. The mixture was placed in the refrigerator for several hours. The 3-nitro-d-tyrosine nitrate which precipitated was filtered and dissolved in 4 ml. of hot water. The solution was brought to a pH of 4 with concentrated ammonium hydroxide, and the mixture was placed in the refrigerator for several hours. The 3-nitro-d-tyrosine was then filtered and dried. The yield was 0.9 g. (67%). A sample in a sealed capillary tube melted at 216°, when heated rapidly.

$[\alpha]_D^{25} = -3.0° \pm .2°$ (4.0% in 4% HCl).

Anal. Calc'd for $C_9H_{10}O_5N_2$: N, 12.4

Found: N, 12.2

3-Amino-d-tyrosine dihydrochloride

The procedure of Waser and Lewandowski (46) for 3-amino-l-tyrosine was followed. Five g. (0.028 mole) of d-tyrosine was converted to 3-nitro-d-tyrosine nitrate as outlined above. The nitrate was dissolved in 18 ml. of water and brought to a pH of 4 with concentrated ammonium hydroxide. The 3-nitro-d-tyrosine was not filtered off. Twenty ml. of water and 35 ml. of 12 N hydrochloric acid were added, and the mixture was heated to boiling in a flask equipped with a reflux condenser. Eleven g. of tin (30-mesh) was added slowly through the top

of the condenser, and the mixture was refluxed for one hour. The excess tin was precipitated with hydrogen sulfide. The stannous sulfide was filtered off and boiled out with water. The aqueous extract was added to the filtrate containing the 3-amino-d-tyrosine, and hydrogen sulfide was passed into the solution for several days. This resulted in the precipitation of a small amount of stannous sulfide, which was filtered off. When the filtrate was treated a third time with hydrogen sulfide, only colloidal sulfur formed. The sulfur was filtered off and the filtrate concentrated to dryness under reduced pressure. The residue was thoroughly washed with concentrated hydrochloric acid until colorless. The yield of 3-amino-d-tyrosine dihydrochloride was 1.8 g. (28%). A sample decomposed with frothing from 156-158°. The rotation was run on the dihydrochloride, but was calculated for 3-amino-d-tyrosine.

Anal. Calc'd for $C_9H_{16}O_4N_2Cl_2$: N, 9.79; Cl, 24.7

Found: N, 9.62; Cl, 24.6

$[\alpha]_D^{25} = +3.0° \pm .4°$ (1.7% in 4% HCl).

d-Tyrosine ethyl ester hydrochloride

This compound was prepared according to the procedure of Rohmann (47). Ten g. (0.0555 mole) of d-tyrosine was suspended in 120 ml. of absolute ethanol. Dry hydrogen chloride was passed through the suspension until the tyrosine went

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(47) Rohmann, Ber. 30, 1979 (1897).

into solution. One hundred fifty ml. of absolute ethanol was added, and the mixture was refluxed in a water bath for four hours. The ethanol was removed under reduced pressure. The residue was dissolved in 50 ml. of boiling ethanol. To this was added 300 ml. of ether, and the mixture was placed in the refrigerator. The yield of d-tyrosine ethyl ester hydrochloride was 9.8 g. (72%). A sample melted from 164-165°.

l-Tyrosine ethyl ester hydrochloride

The same procedure (47) yielded 11.7 g. (86%) of l-tyrosine ethyl ester hydrochloride from 10 g. of l-tyrosine (Merck). A sample melted at 164°.

N-Benzoyl-d-tyrosine ethyl ester

The procedure of Fox (48) for N-benzoyl-l-diiodotyrosine ethyl ester was followed. Seven and three-tenths g. (0.030 mole) of d-tyrosine ethyl ester hydrochloride was dissolved in 30 ml. of water. Sixty ml. (0.120 mole) of 2 N sodium carbonate was added. The free ester was extracted with two 30 ml. portions of ethyl acetate. The ethyl acetate extracts were filtered, and transferred to a separatory funnel. Three and six-tenths g. (0.026 mole) of benzoyl chloride was added, with shaking. Thirty ml. (0.060 mole) of 2 N sodium carbonate

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(48) Fox, J. Am. Chem. Soc. 68, 194 (1946).

was added. The mixture was shaken until no more carbon dioxide was evolved. The ethyl acetate layer was washed twice with water, dried with calcium sulfate, and evaporated under reduced pressure. The solid residue was recrystallized from 150 ml. of boiling ethyl acetate-benzene mixture (1:9). The yield was 5.5 g. (69%) of N-benzoyl-d-tyrosine ethyl ester. A sample melted from 115-117°.

Anal. Calc'd for $C_{18}H_{19}O_4N$: N, 4.47

Found: N, 4.46

$[\alpha]_D^{25} = -22.7° \pm .6°$ (3.0% in ethanol).

Levene and Mardashew (49) reported a specific rotation of 23.2° for the N-benzoyl-l-tyrosine ethyl ester.

N-Benzoyl-l-tyrosine ethyl ester (50)

The same procedure (48) yielded 9 g. (87%) of N-benzoyl-l-tyrosine ethyl ester, starting from 9.8 g. of l-tyrosine ethyl ester hydrochloride. A sample melted from 118-119°.

N-Benzoyl-d-tyrosine

This compound was prepared by Fischer (51) by the resolution of N-benzoyl-dl-tyrosine. In this case, however,

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(49) Levene and Mardashew, J. Biol. Chem. 117, 179 (1937).
(50) Bergmann, Ulptz, and Camacho, Ber. 55, 2796 (1922).
(51) Fischer, Ber. 32, 3638 (1900).

the procedure of Fox (48) for N-benzoyl-l-diiodotyrosine was followed. Two and five-tenths g. (0.0080 mole) of N-benzoyl-d-tyrosine ethyl ester was heated with 8 ml. (0.040 mole) of 5 N sodium hydroxide for 30 minutes in a boiling water bath. Forty-six ml. of water was added. The solution was then neutralized with 18.5 ml. (0.0555 mole) of 3 N hydrochloric acid. The precipitate was recrystallized from 10 ml. of 30% ethanol. The yield of benzoyl-d-tyrosine was 1.8 g. (79%). A sample did not have a sharp melting point. It pre-melted from 143-144° and was completely melted at 165°.

$[\alpha]_D^{25} = -18.0° \pm .7°$ (6.0% in water containing an equivalent amount of KOH).

N-Benzoyl-l-tyrosine

The same procedure (48) yielded 1.7 g. (65%) of benzoyl-l-tyrosine from 3 g. of benzoyl-l-tyrosine ethyl ester. A sample showed the same melting point characteristics as benzoyl-d-tyrosine.

$[\alpha]_D^{25} = +17.6° \pm .7°$ (6.0% in water containing an equivalent amount of KOH).

Fischer reported a specific rotation of +18.29°.

N-Benzoyl-d-tyrosylamide

The procedure of Bergmann and Fruton (52) was followed. Ammonia was passed into 50 ml. of absolute methanol until the solution had gained 6 g. To this solution was added 3.2 g. of benzoyl-d-tyrosine ethyl ester. The solution was placed in a stoppered flask and was placed in the refrigerator for 17 days. The solution was evaporated under reduced pressure, and the residue recrystallized from boiling ethanol. The yield was 1.2 g. (60%) of benzoyl-d-tyrosylamide. A sample melted at 203°. Bergmann and Fruton reported a melting point of 198-200°.

N-Benzoyl-l-tyrosylamide

The procedure used for the d- isomer was followed (52). Two and two-tenths g. of benzoyl-l-tyrosine ethyl ester was left in a solution of 6 g. of ammonia in 50 ml. of absolute methanol for two days. The solution was evaporated under reduced pressure, leaving a residue which melted below 160°. The residue was dissolved in 50 ml. of absolute methanol saturated with ammonia, and left for four days in the refrigerator. The solution was removed under reduced pressure and recrystallized from boiling ethanol. The yield was 0.7 g.

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(52) Bergmann and Fruton, J. Biol. Chem. 124, 325 (1933).

(35%) of benzoyl-l-tyrosylamide. A sample melted at 204°. Bergmann and Fruton reported a melting point of 198°.

Bacteriological Procedure

The organism used for testing was Lactobacillus arabinosus 17-5, American Type Culture Collection, No. 8014. The stock culture was carried on a 2% yeast extract agar deep (53) by monthly transfers. Loop transfers were made from the stock cultures to tubes containing 10 ml. of yeast extract broth (53), which were then incubated for 24 hours at 30°. These 24-hour cultures were used to inoculate tubes containing the compounds being tested. In the preliminary work on amino acids, the tubes containing the 24-hour cultures were centrifuged, the broth was decanted, and the organisms were suspended in 10 ml. of physiological saline. One drop of this saline suspension was used to inoculate each tube. It was found that the degree of inhibition varied with the number of organisms in the inoculum (Table XVI), and in later work the 24-hour cultures were diluted with saline, 10^{-4} or 10^{-6} dilutions being used.

The basal medium was essentially that of Kuiken, et al. (54). The composition of the complete medium is given in

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(53) McMahan and Snell, J. Biol. Chem. 152, 83 (1944).
(54) Kuiken, Norman, Lyman, Hale, and Blotter, J. Biol. Chem. 151, 615 (1943).

Table I. For convenience, the medium was made up in the form of several stock solutions which were combined as needed. One stock solution contained a tenfold concentration of all the vitamins. Another contained a tenfold concentration of the minerals. For the work requiring complete medium, the amino acids were combined in one stock solution of twofold concentration. For the work on nutritional requirements, the amino acids were made up in separate stock solutions, each containing 10 mg. of the amino acid per ml. Tyrosine was dissolved in 0.1 N sodium hydroxide; cystine was dissolved in 0.1 N hydrochloric acid; the other amino acids were dissolved in water, by heating if necessary. The stock solutions were layered with toluene and stored in the refrigerator. The solutions usually kept well for periods of one month. In some cases, mold growth appeared in some of the amino acid stock solutions. When that happened, the solutions were discarded.

In order to make up medium for any particular run, the proper stock solutions were combined, an aliquot amount of glucose and sodium acetate was added, the pH was adjusted to 6.5-6.8, and the mixture diluted to volume with distilled water.

The final volume of medium in each tube was 2.5 ml., consisting of 1.25 ml. of the basal medium and 1.25 ml. of either distilled water or a solution of the compound being tested.

Table I.

Complete Medium for Lactobacillus arabinosus[a]

Glucose	40 g.	$FeSO_4.7H_2O$	20 mg.
Sodium acetate (anhydrous	14.4 g.	$MnSO_4.4H_2O$	20 mg.
		l-Arginine HCl	400 mg.
Adenine sulfate	10 mg.	dl-Alanine	400 mg.
Guanine HCl	10 mg.	dl-Aspartic acid	800 mg.
Uracil	10 mg.	dl-Glutamic acid.H_2O	800 mg.
Thiamine chloride	200 γ	l-Histidine HCl	400 mg.
Pyridoxine HCl	200 γ	dl-Lysine	800 mg.
Calcium pantothenate	200 γ	dl-Phenylalanine	400 mg.
		l-Proline[b]	400 mg.
Biotin	0.8 γ	dl-Serine	400 mg.
Riboflavin	400 γ	l-Tryptophane[c]	400 mg.
Nicotinic acid	800 γ	dl-Methionine	400 mg.
p-Aminobenzoic acid	1.0 γ	dl-Threonine	400 mg.
		l-Tyrosine	400 mg.
K_2HPO_4	1.0 g.	dl-Leucine	400 mg.
KH_2PO_4	1.0 g.	dl-Valine	400 mg.
$MgSO_4.7H_2O$	400 mg.	dl-Isoleucine	400 mg.
NaCl	20 mg.	l-Cystine	400 mg.

Adjust to pH 6.5 to 6.8 and dilute with water to 1 liter.

[a] A slight modification of the medium given in Kuiken, et al., J. Biol. Chem. 152, 83 (1944).

[b] l-Proline was found to be non-essential for growth and was omitted when none was available.

[c] In some cases 800 mg. of dl-tryptophane was used.

The procedure for the determination of the nutritional requirements of the organism was carried out as reported by Kuiken, et al. (54). The amino acid being tested was omitted from the medium, and graded amounts of a solution containing the proper concentration of the amino acid being tested were added in duplicate to a series of tubes. Cultures for inoculation were prepared by centrifuging the 24-hour cultures, decanting the broth, and suspending the cells in 10 ml. of physiological saline. One drop of this suspension was used for each tube.

Autoclaving was carried out at 15 pounds pressure for 20 minutes. It later developed that 10 minutes was sufficient time to sterilize the small quantities of solutions used.

After autoclaving and inoculation, the tubes were incubated for 72 hours at 30°. Growth was determined by titration with 0.1 N sodium hydroxide or by reading turbidities with a Klett-Summerson photoelectric colorimeter.

Two different procedures were used to test for inhibitory activity. In the first procedure, used on amino acids, the amount tested (usually 50 mg.) was weighed out and added directly to the tubes. Growth was determined after 72 hours by titration with 0.1 N sodium hydroxide.

The second procedure was used by Carol Houck Bollenback in testing the amino acid derivatives. A natural rather than a synthetic basal medium was used. It was identical with the

yeast extract broth used to prepare the 24-hour cultures. The tests were run in serial dilution in twofold steps, with a final volume of 1 ml.

The 24-hour culture was diluted in yeast extract broth and one drop of a 10^{-4} or 10^{-6} dilution was used. Plate counts were run to determine the number of organisms present. The tubes, after autoclaving and inoculation, were incubated at 37° for 24 hours. Growth was determined by visual inspection of the tubes.

RESULTS

Nutritional Requirements of *Lactobacillus arabinosus*

Since the relationship between essential metabolites and antibacterial substances is known to be of importance in the case of vitamin analogues (55), it was desirable to ascertain the nutritional requirements of *L. arabinosus* with respect to alanine, valine, leucine, and tyrosine—the four amino acids dealt with in this thesis.

Table II

The Effect of dl-Alanine on the Growth of *L. arabinosus*

dl-Alanine added	0.100 N acid produced		Average 0.100 N acid produced
γ	ml.		ml.
0	2.71	2.68	2.70
20	3.04	3.00	3.02
40	3.25	3.30	3.28
60	3.31	3.42	3.36
80	3.48	3.51	3.50
200	3.67	3.48	3.58

(55) Roblin, *Chem. Rev.* 38, 270 (1946).

Alanine

dl-Alanine was tested in concentrations of from 0-200 γ in 2.5 ml. of alanine-free medium. The results for a typical test, set up in duplicate, are given in Table II.

The results showed that alanine was not an essential amino acid for the growth of L. arabinosus. The addition of alanine did, however, result in improved growth, and therefore alanine can be classed as an accessory amino acid.

Valine

dl-Valine was tested in concentrations of from 0-25 γ in 2.5 ml. of valine-free medium. In this case the growth after 72 hours was determined turbidimetrically.

The results showed that little growth occurred in the absence of valine. Good growth resulted when small amounts of valine were added, as shown in Table III.

Leucine

dl-Leucine was tested in concentrations of from 0-25 γ in 2.5 ml. of leucine-free medium. Growth after 72 hours was determined turbidimetrically and by titrating. The results are given in Table IV.

The growth response of the organism to leucine was similar to its response to valine. Both may be classified as essential amino acids.

Table III

The Effect of dl-Valine on the Growth of L. arabinosus

dl-Valine Added	Galvanometer Reading[a]		Average Galvanometer Reading
γ			
0	24	26	25
3	33	—	33
6	41	41	41
9	48	49	49
12	60	57	59
15	63	65	64
20	71	72	72
25	75	73	74

Table IV

The Effect of dl-Leucine on the Growth of L. arabinosus

dl-Leucine Added	0.050 N Acid Produced		Average 0.050 N Acid Produced	Average Galvanometer Reading[a]
γ	ml.		ml.	
0	0.85	0.82	0.84	32
3	1.17	1.21	1.19	38
6	1.54	1.49	1.51	48
9	1.99	2.01	2.00	56
12	2.31	2.16	2.24	66
15	2.58	2.58	2.58	75
20	2.94	2.95	2.95	81
25	3.28	3.22	3.25	88

[a] Distilled water reads zero; a reading of 100 corresponds to complete opacity.

Tyrosine

l-Tyrosine was tested in concentrations of from 0-15γ in 2.5 ml. of tyrosine-free medium. After 48 hours growth appeared to be the same in all tubes. Results after 72 hours for the 0 and 15γ tubes are given in Table V.

Table V

The Effect of l-Tyrosine on the Growth of L. arabinosus

l-Tyrosine Added	0.050 N Acid Produced	Galvanometer Reading[a]
γ	ml.	
0	5.55	132
15	6.12	145

[a] As in Table III.

The results showed that the organism grew well in the absence of tyrosine. The response to added tyrosine was similar to the response of added alanine.

Typical curves for the growth response of L. arabinosus to dl-alanine, dl-valine, dl-leucine, and l-tyrosine are depicted in Fig. 1 (p. 54).

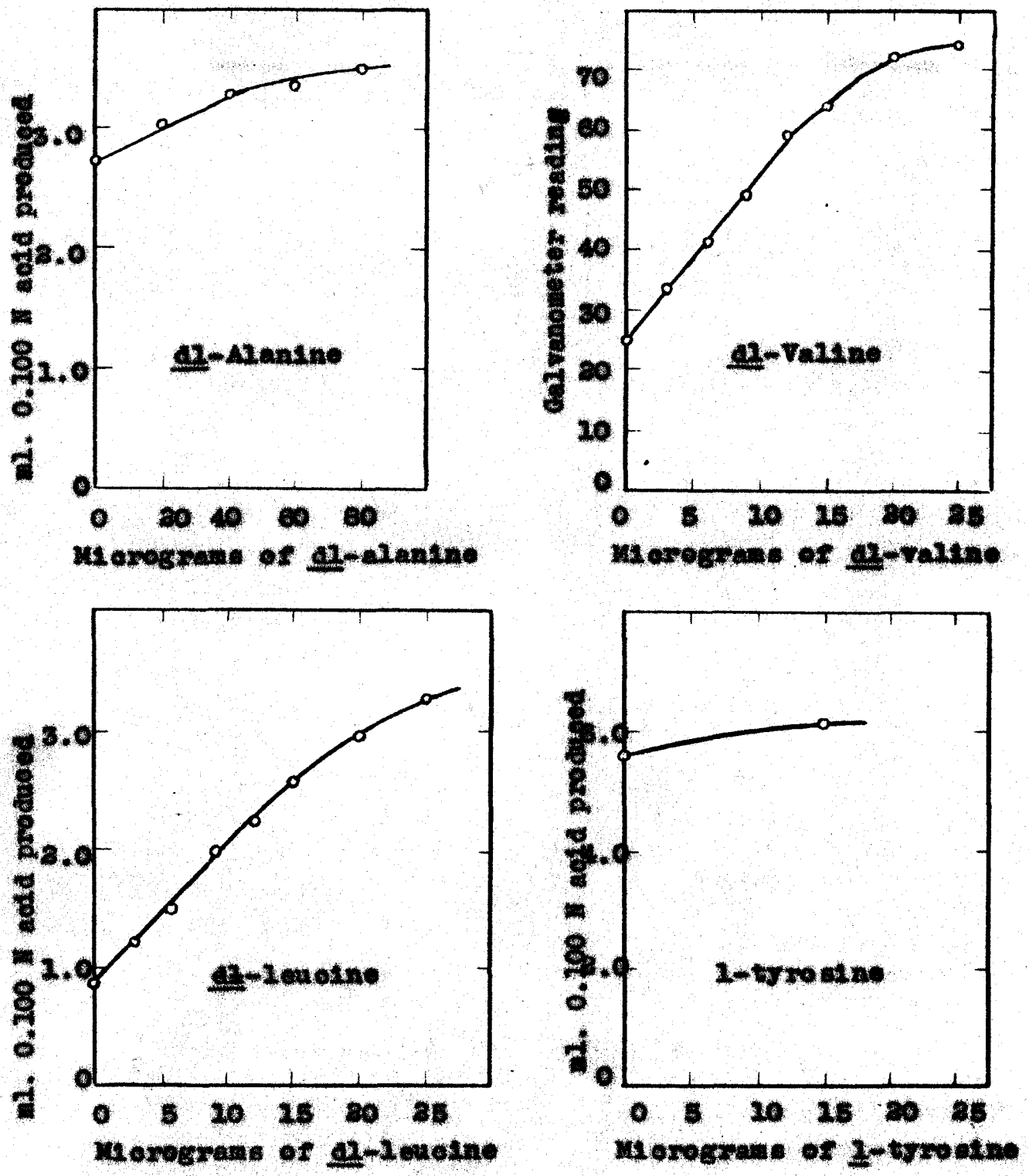

Fig. 1

Growth response of L. arabinosus to dl-alanine, dl-valine, dl-leucine, and l-tyrosine.

Effect of Amino Acid Isomers on the Growth of L. arabinosus

Tests were set up in which 50 mg. samples of amino acid isomers and racemates were added to 2.5 ml. of basal medium.

It was found that this concentration resulted in an increase in titratable acidity quite apart from that produced by the growth of the organisms. This increase can be accounted

Table VI

Titratable Acidity after Autoclaving and Incubation[a]

Addition to basal medium in tube	0.072 N acid produced[b]	
	ml.	ml.
None	0.21	0.29
50 mg. d-leucine	0.50	----
50 mg. l-leucine	0.55	----
50 mg. dl-leucine	0.48	0.54

[a] Fox, Fling, and Bollenback, J. Biol. Chem. 155, 466 (1944).
[b] The tubes were not inoculated.

for by the reaction of the amino acid and the dextrose contained in the medium (56). The increase in titratable acidity due to this effect is indicated in Table VI.

The titers of tubes for which this value has been taken into consideration are designated as "corrected" titers.

---

(56) Frankel and Katchalsky, Biochem. J. 35, 1034 (1941).

The first amino acid isomers to be tested for their possible inhibitory effect were those of leucine. A summary of the results obtained with l-, d-, and dl-leucine is found in Table VII. All the experiments recorded in Table VII were

Table VII

Comparative Effect of l-, d-, and dl-Leucine on L. arabinosus[a]

Test No.	0.100 N acid produced in control	Ratio of growth to growth in control: 50 mg. l-leucine	50 mg. dl-leucine	50 mg. d-leucine
	ml.	%	%	%
1	2.00	106	51	16
2	2.54	92	57	39
3	3.06	86	42	33
4	2.42	62	37	27
5	1.74	98	76	58
6	2.42	87	68	44
7	2.08	107	81	64
8	1.93	104	100	70
9	2.30	80	56	22
10	2.24	105	88	56
Average:	2.27	93	66	43

[a] Fox, Fling, and Bollenback, J. Biol. Chem. 155, 466 (1944).

done at different times with different subcultures (undiluted) of the bacterium. All figures are the averages of duplicate determinations.

Since brucine has a known toxicity (57) and since it was used in the resolution of the amino acids, it seemed necessary to check on the possibility of the presence in the purified d-amino acids of traces of brucine which might have carried through the process of resolution. Such traces might conceivably be physiologically significant although absent by

Table VIII

Comparison of Effect of Leucine Isomers and Brucine Sulfate on L. arabinosus[a]

Addition to basal medium in tube	0.072 N acid produced		Average 0.072 N acid produced (corrected)
	ml.		ml.
None	3.76	3.88	3.53
50 mg. l-leucine	3.84	3.74	3.24
50 mg. d-leucine	1.91	1.72	1.27
50 γ brucine sulfate	3.97	4.00	3.70
500 γ brucine sulfate	3.83	3.77	3.51

[a] Fox, Fling, and Bollenback, J. Biol. Chem. 155, 466 (1944).

chemical criteria. It was found that when 500 γ of brucine sulfate was added to samples of complete basal medium, growth was normal (Table VIII). Solutions containing this amount of brucine gave definitely positive nitric acid color tests. Additional evidence of lack of inhibition due to brucine can

(57) Henry, "Plant Alkaloids." 3rd Ed. p. 530. Philadelphia, Blakiston's Son & Co. 1939.

be derived from the fact that l-amino acids obtained by resolution were as devoid of inhibitory or stimulatory activity as isolated amino acids.

Since autoclaving 50 mg. of amino acid with the medium resulted in an increase in titratable acidity, it was deemed advisable to check if autoclaving had any effect on the growth inhibition caused by d-leucine. For this purpose, tubes were

Table IX

Effect of Autoclaving on Inhibition by d-Leucine

Addition to basal medium in tube	0.072 N acid produced	
	ml.	ml.
None	3.00	2.96
50 mg. d-leucine (autoclaved with medium)	2.25	2.15
50 mg. d-leucine (autoclaved separately and then added aseptically to medium.)	2.28	2.16

set up in which 50 mg. portions of d-leucine, sterilized by autoclaving dry, were added aseptically to sterile 2.5 ml. portions of medium. Results with these tubes were compared to controls in which the leucine was autoclaved together with the medium. The results are given in Table IX. The degree of inhibition is approximately the same in both cases.

The original amount of amino acid used in testing — 50 mg. per 2.5 ml. or 20 mg. per ml.—was chosen as a maximum level at which inhibition would have significance for this study. It was of interest to know if smaller amounts of d-leucine would also show inhibition. Tubes were set up, containing from 0-50 mg. d-leucine. The results of a typical run are given in Table X.

Table X

Effect of Varying Amounts of d-Leucine in L. arabinosus

d-Leucine added	0.100 N acid produced		Average 0.100 N acid produced (corrected)
mg.	ml.	ml.	ml.
0	3.05	3.02	2.88
5	2.48	2.45	2.24
10	2.05	1.98	1.73
20	1.64	1.71	1.43
30	1.55	1.52	1.28
40	1.32	1.33	1.03
50	1.17	1.23	.88

These results are also presented in the form of a graph in Fig. 2, (p. 60), together with the results of a similar experiment run on d-valine. The results showed that amounts less than 50 mg. were effective in inhibiting the growth of L. arabinosus.

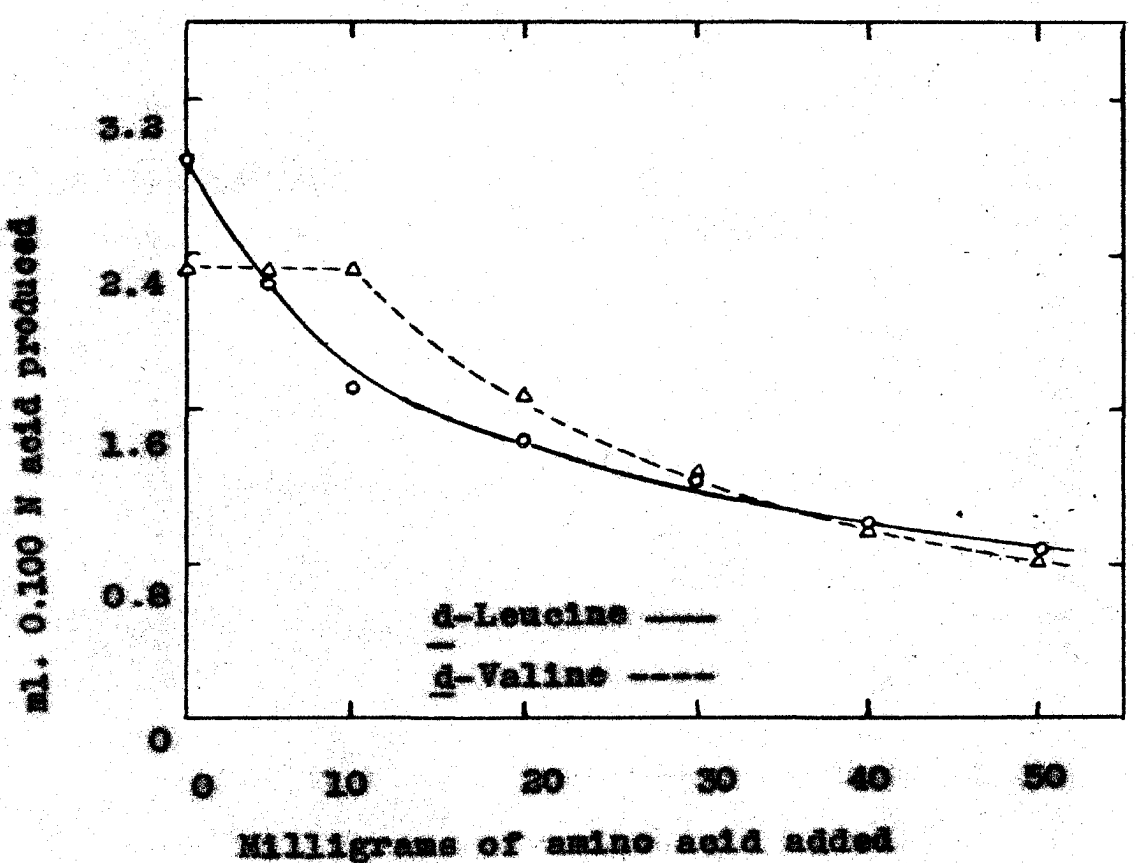

Fig. 2

Growth response of L. arabinosus to varying amounts of d-leucine and d-valine. These curves were constructed from separate experiments.

If sub-inhibitory amounts of d-leucine were autoclaved together with leucine-free medium, it was found that the resultant medium would support more growth than that of a medium in which the leucine was autoclaved separately and added aseptically. This effect was noticed in concentrations of 1-10 mg. of d-leucine per 2.5 ml. of medium. When this type of experiment was tried on less than 1 mg. of d-leucine, no difference

Table XI

Effect of Autoclaving d-Leucine Together With and Separately From a Leucine-Free Medium

Addition to leucine-free medium	Average 0.100 N acid produced			
	Autoclaved Separately		Autoclaved together	
	ml.	ml.	ml.	ml.
None	.27	.29	.23	.28
100 γ d-leucine	.31	.32	.28	.28
250 γ d-leucine	.29	.31	.29	.31
500 γ d-leucine	.30	.30	.34	.33
1000 γ d-leucine	.34	.32	.35	.39
5000 γ d-leucine	.37	.32	.53	.55
None	.39	.30	.32	.35
1 mg. d-leucine	.33	.40	.47	.47
2 mg. d-leucine	.36	.35	.53	.51
5 mg. d-leucine	.40	.42	.72	.75
10 mg. d-leucine	.50	.42	.95	.96

was noticed in the ability of the final media to support growth. The results of two separate experiments, one using 0-5000γ of d-leucine/2.5 ml. medium, and the other 1-10 mg. /2.5 ml., are given in Table XI. In the first experiment, the d-leucine was added in an aqueous solution. In the second, the

amount needed was weighed out and added in solid form. The final volume in each tube was 2.5 ml. and the composition of the original leucine-free medium was the same for both tests.

The results of a series of experiments run on l-, d-, and dl-valine are given in Table XII. A comparison of these results with those for the leucines as given in Table VII shows that the activity of equal amounts of d-valine and dl-valine is much closer than is the case with d- and dl-leucine.

Table XII

Comparative Effect of l-, d-, and dl-Valine on L. arabinosus[a]

Test No.	0.100 N acid produced in control	Ratio of growth to growth in control		
		50 mg. l-valine	50 mg. dl-valine	50 mg. d-valine
	ml.	%	%	%
1	2.79	95	59	62
2	2.08	100	71	65
3	1.93	98	80	68
4	2.24	97	59	60
Average:	2.26	98	67	64

[a] Fox, Fling, and Bollenback, J. Biol. Chem. 155, 466 (1944).

dl-Leucine shows an activity which is approximately equal to the d-leucine it contains. In the case of valine, the activity of the racemic form seems to be greater than can be accounted for by its d-valine content. A comparison of the

effect of varying amounts of d-valine as shown in Fig. 2 (p. 60) also shows a difference in the inhibition caused by these two amino acids. The data for the valine curve in Fig. 2 is given in Table XIII.

Table XIII

Effect of Varying Amounts of d-Valine on L. arabinosus

d-Valine added	0.100 N acid produced		Average 0.100 N acid produced (corrected)
mg.	ml.	ml.	ml.
0	2.47	2.47	2.36
5	2.48	2.48	2.33
10	2.55	2.49	2.33
20	1.91	1.90	1.68
30	1.56	1.53	1.26
40	1.35	1.29	.99
50	1.18	1.24	.81

The isomers and racemates of alanine and tyrosine were also tested. Because of the variation in the degree of inhibition in experiments run at different times, several composite experiments on alanine, leucine, and valine were run at the same time. Tyrosine was tested later and, since no activity was found, no experiments were run which included all four amino acids. In the results given in Tables XIV and XV, alanine, leucine, and valine were run at one time, and tyrosine at another. Because of the low solubility of tyrosine,

Table XIV

Comparison of Amino Acid Isomers and Racemates in Inhibition of Bacterial Growth[a]

Addition to basal medium in tube	0.100 N acid produced		Average 0.100 N acid produced (corrected)
	ml.	ml.	ml.
None	2.39	2.39	2.23
50 mg. l-leucine	2.51	2.71	2.35
50 mg. dl-leucine	2.24	2.21	1.97
50 mg. d-leucine	1.54	1.48	1.25
50 mg. l-valine	2.56	2.53	2.17
50 mg. dl-valine	1.63	1.76	1.32
50 mg. d-valine	1.62	1.77	1.33
50 mg. l-alanine	2.84	3.31	2.84
50 mg. dl-alanine	3.05	3.04	2.81
50 mg. d-alanine	3.03	2.95	2.75

[a] Fling and Fox, J. Biol. Chem. 160, 329 (1945).

50 mg. portions could not be used. Though most of the 15 mg. of tyrosine went into solution on autoclaving, an undetermined portion precipitated out again on cooling.

In an attempt to control the variation in the degree of inhibition, tests were set up to determine the effect of the number of organisms in the inoculum on the amount of inhibi-

Table XV

Effect of d-, l-, and dl-Tyrosine on L. arabinosus

Addition to basal medium in tube	0.100 N acid produced		Average 0.100 N acid produced (corrected)
	ml.	ml.	ml.
None	2.65	2.42	2.37
15 mg. l-tyrosine[a]	2.67	2.69	2.43
15 mg. dl-tyrosine[a]	2.52	2.88	2.47
15 mg. d-tyrosine[a]	2.55	2.76	2.43

[a] Most of the tyrosine went into solution on autoclaving. An undetermined amount precipitated out on cooling. Merck l-tyrosine was used.

tion produced. Two runs were made, one using 50 mg. portions of d-leucine/2.5 ml., and the other with 30 mg. portions. Dilutions of the 24-hour culture were made in 0.9% saline solution up to 10^{-8}. In each case, one drop of the dilution was used for inoculation. The tubes were set up in duplicate and the results in Table XVI show the average titers obtained.

The results showed a relative increase in inhibition with a decrease in the number of organisms in the inoculum. Cell counts were not run when these experiments were set up, and the actual number of cells involved was not known. In most of the tests on the amino acid derivatives, dilutions of 10^{-4} or 10^{-6} were used and plate counts were run.

Table XVI

Effect of Density of Inoculum on Degree of Inhibition

Dilution of 24-hour culture	0.100 N acid in control		0.100 N acid in d-leucine tubes		Ratio of growth to growth in control
	ml.	ml.	ml.	ml.	%
		50 mg. d-Leucine			
Undiluted	2.82	2.78	1.85	1.81	65
10^{-2}	2.26	—	.71	1.15	41
10^{-4}	2.36	—	.71	.86	34
10^{-6}	2.12	—	.57	.75	31
10^{-8}	1.88	—	.59	.49	29
		30 mg. d-Leucine			
Undiluted	2.48	2.47	1.59	1.66	66
10^{-2}	2.19	2.24	1.07	1.09	49
10^{-4}	2.06	2.03	.88	.95	45
10^{-6}	1.79	1.78	.69	.76	41
10^{-8}	1.51	—	.63	.59	40

Since one of the differences in the structures of alanine, leucine, and valine is the length of the side chain attached to the α-carbon, it was of interest to know if this length was critical. The series used consisted of glycine,

dl-alanine, dl-∝-aminobutyric acid, dl-norvaline, and dl-norleucine. This series had the advantage of containing only unbranched side chains, thus eliminating possible differences due to branching. Equimolar amounts were used. The results of two runs are shown in Table XVII.

Table XVII

Effect of Length of Side Chain on Degree of Inhibition

Addition to basal medium	0.100 N acid produced 1st run		2nd run	
	ml.	ml.	ml.	ml.
None	2.18	2.21	1.95	1.92
28.5 mg. glycine	1.36	1.43	1.31	1.32
34.0 mg. dl-alanine	2.34	2.42	2.05	1.83
39.5 mg. dl-aminobutyric acid	.85	.89	1.17	.84
44.5 mg. dl-norvaline	.65	.65	.73	.75
50.0 mg. dl-norleucine	.83	.74	.71	.77

With the exception of glycine, the compounds through norvaline showed an increase in the degree of inhibition with an increase in the length of the side chain.

Snell and Guirard (58) reported glycine to be inhibitory for Streptococcus lactis, the inhibition being reversed by

(58) Snell and Guirard, Proc. Nat. Acad. Sci. 29, 66 (1943).

the addition of pyridoxine or alanine. It was of interest to know if the effect of glycine on L. arabinosus could likewise be reversed by pyridoxine and alanine. The results of an experiment to test this are given in Table XVIII. In the same experiment, alanine and pyridoxine were added to tubes containing d-valine and d-leucine to see if any reversal would take place.

Table XVIII

Effect of Pyridoxine and Alanine on Inhibition Caused by Glycine, d-Leucine, and d-Valine

Addition to basal medium	0.100 N acid produced	
	ml.	ml.
None	2.36	2.41
15 mg. glycine	2.18	2.17
30 mg. glycine	1.42	1.54
30 mg. glycine + 30 mg. l-alanine	2.61	2.51
30 mg. glycine + 30 mg. d-alanine	2.73	2.65
30 mg. glycine + 0.5 mg. pyridoxine	2.32	2.38
30 mg. d-leucine	1.10	1.23
30 mg. d-leucine + 30 mg. l-alanine	1.27	1.23
30 mg. d-leucine + 0.5 mg. pyridoxine	1.41	1.39
30 mg. d-valine + 30 mg. l-alanine	1.40	1.20

The results showed that the inhibition caused by glycine was reversed by alanine or pyridoxine. It is of interest that d- and l-alanine were equally effective. (Snell and Guirard used dl-alanine.) Alanine and pyridoxine seemed to have little

or no effect on the inhibitory action of d-valine and d-leucine.

Effect of Amino Acid Derivatives on the Growth of L. arabinosus

The isomers and racemates of glycylleucine were tested in concentrations of 10 and 50 mg. At the lower concentration, none of the forms had much noticeable effect. At the higher level, they all appeared to be stimulatory. The results (Table XIX) raised the question of the ability of the organism to utilize glycylleucine in a medium deficient in leucine. An aqueous solution of glycyl-l-leucine was tested in concentrations of from 0-30 γ/2.5 ml. medium. The growth response, as shown in Table XX, indicated that the organism

Table XIX

Effect of Glycylleucine Peptides on L. arabinosus

Addition to basal medium	0.100 N acid produced	
	ml.	ml.
None	3.10	3.09
10 mg. glycyl-d-leucine	3.25	3.18
10 mg. glycyl-l-leucine	3.23	3.10
10 mg. glycyl-dl-leucine	3.24	3.20
None	1.92	1.94
50 mg. glycyl-d-leucine	3.17	2.87
50 mg. glycyl-l-leucine	2.85	2.89
50 mg. glycyl-dl-leucine	3.35	3.21

could use glycyl-l-leucine in place of l-leucine. This is in agreement with the results of Kuiken, et al. (54). The addition of 1 mg. of glycyl-d-leucine to a leucine-free medium resulted in no growth. d-Leucine and d-valine retained their inhibitory activity when added to a medium containing glycyl-l-leucine as a source of l-leucine. These results are all shown in Table XX.

Table XX.

Growth Effects with a Leucine-free Medium

Addition to Leucine-free medium	0.100 N acid produced	
	ml.	ml.
None	.45	.46
5 glycyl-l-leucine	.68	.71
10 glycyl-l-leucine	1.02	.94
15 glycyl-l-leucine	1.19	1.18
20 glycyl-l-leucine	1.25	1.28
1 mg. glycyl-d-leucine	.47	.51
20 γ. glycyl-l-leucine + 50 mg. d-leucine	.52	.58[a]
20 γ glycyl-l-leucine + 50 mg. d-valine	.72	.80[a]

[a] Corrected, using correction value from another run.

Leucine anhydride, or 2.5-diisobutyl-diketopiperazine, was tested because of its cyclic structure. The racemic form was used on the assumption that, if the d- form were active, the dl- form would show some of this activity--as is true with dl-valine and dl-leucine. Because of the high insolu-

bility of the compound, 50 mg. levels could not be used. Even the 10 mg. portions used did not go into solution on autoclaving. The portion that did dissolve showed no activity, as is shown in Table XXI.

Table XXI

Effect of Leucine Anhydride on L. arabinosus

Addition to basal medium	0.100 N acid produced	
	ml.	ml.
None	2.44	2.49
10 mg. leucine anhydride	2.43	2.45

Preliminary tests on the rest of the amino acid derivatives were run by Carol Houck Bollenback, using the serial dilution method outlined in the methods of procedure. The tests were run on yeast extract medium in twofold dilutions. The compounds were made up in aqueous solution, with the exception of the N-benzoyltyrosylamides and the N-benzoyltyrosine ethyl esters which were dissolved in 40% ethanol. The pH of each solution was adjusted to 6.5-6.8, with the exception of the leucine and valine methyl esters which were adjusted to 7.5-7.8. The concentration given with every compound in Table XXII was the concentration of the stock solution. The first tube in the series, therefore, contained half that concentration. The varying concentrations of the

Table XXII

Effect of Amino Acid Derivatives on L. arabinosus

- no growth, -? questionable growth, ⊕ slight growth, +- moderate growth, + good growth, ++ heavy growth

	½	¼	⅛	1/16	1/32	1/64	1/128
3×10^{10} cells/ml. One drop of 10^{-4} dilution used.							
d-Leucine 33.3 mg./ml.	-	-	+				
l-Leucine 33.3 mg./ml.	+						
Formyl-d-leucine 100 mg./ml.	+						
Formyl-l-leucine 100 mg./ml.	-	+					
d-Valine 50 mg./ml.	-	+-	+				
l-Valine 50 mg./ml.	+						
Formyl-d-valine 100 mg./ml.	-	+					
Formyl-l-valine 100 mg./ml.	-	+					
2×10^{9} cells/ml. One drop of 10^{-6} dilution used.							
Phthalyl-d-leucine 100 mg./ml.	-	-	-	-	-	+-	+
Phthalyl-l-leucine 100 mg./ml.	-	-	-	-	-	+	
Phthalyl-d-valine 100 mg./ml.	-	-	-	-	-	+	
Phthalyl-l-valine 100 mg./ml.	-	-	-	-	-	+	
1.8×10^{9} cells/ml. One drop of 10^{-6} dilution used.							
3-Amino-d-tyrosine HCl 10 mg./ml.	-	-	+-	+			
3-Amino-l-tyrosine[a] HCl 10 mg./ml.	-	-	-?	+-	+		
3-Nitro-d-tyrosine 5 mg./ml.	⊕	+					
3-Nitro-l-tyrosine[a] 10 mg./ml.	⊕	+					

[a] Obtained from Dr. S. W. Fox.

Table XXII (cont.)

	½	¼	⅛	1/16	1/32
2 x 10^9 cells/ml. One drop of 10^{-6} dilution used.					
d-Tyrosine ethyl ester HCl 20 mg./ml.	-	-	+		
l-Tyrosine ethyl ester HCl 40 mg./ml.	-	-	+		
N-Benzoyl-d-tyrosine 60.7 mg./ml.	+				
N-Benzoyl-l-tyrosine 60.7 mg./ml.	-	+			
N-Benzoyl-d-tyrosylamide 13 mg./ml.	+				
N-Benzoyl-l-tyrosylamide 13 mg./ml.	+				
N-Benzoyl-d-tyrosine ethyl ester 15 mg./ml.	+				
N-Benzoyl-l-tyrosine ethyl ester 15 mg./ml.	+				
1.3 x 10^9 cells/ml. One drop of 10^{-6} dilution used.					
Prolyl-d-valine 20 mg./ml.	+				
Prolyl-l-valine 20 mg./ml.	+				
Prolyl-d-leucine 5 mg./ml.	+				
Prolyl-l-leucine 5 mg./ml.	+				
d-Valine methyl ester HCl 50 mg./ml.	-	+			
l-Valine methyl ester HCl 50 mg./ml.	-	+			
1.3 x 10^9 cells/ml. One drop of 10^{-6} dilution used.					
d-Leucine methyl ester HCl 100 mg./ml.	-	-	-	-?	+
l-Leucine methyl ester HCl 100 mg./ml.	-	-	-	⊕	+

stock solutions were due to the relative insolubility of many of the compounds. In the case of the N-benzoyl-tyrosylamides and the N-benzoyltyrosine ethyl esters, the compounds precipitated out after autoclaving, probably due to loss of alcohol during autoclaving. Growth was determined by streaking agar plates.

The results showed that inhibition was caused by the phthalyl derivatives of leucine and valine, the amino- and nitro-tyrosines, the tyrosine ethyl esters, and the leucine and valine methyl esters. In none of these cases was there a sharp difference in activity between the d- and the l-forms.

DISCUSSION AND CONCLUSIONS

Nutritional Requirements

The results given in Tables II, III, IV, and V show that valine and leucine are essential for the growth of L. arabinosus, while alanine and tyrosine are merely accessory growth factors. These results agree with those reported by Kuiken, et al. (54). The data in Table XX, indicating that glycyl-l-leucine is capable of supporting growth in a medium free of leucine, also check with their findings. In the same paper, Kuiken, et al. reported that dl-leucine had just half the activity of an equal amount of l-leucine and they therefore concluded that only the l-form was utilized. The experiment (Table XI), in which small amounts of d-leucine were added to a leucine-free medium, supports this conclusion.

A conceivable explanation for the availability of d-leucine after it is autoclaved with the medium is the possibility of some racemization occurring in the presence of glucose at the autoclaving temperature. Such an effect would be difficult to check polarimetrically, because of the small changes in rotation involved. The results indicate that the effect is not of importance at the levels normally used in bioassay.

Inhibitory Effect of Amino Acids

Glycine

It is of interest to compare the results obtained by Snell and Guirard (58) with Streptococcus lactis and those reported in this thesis with L. arabinosus. Snell and Guirard found that high levels of glycine inhibited the growth of S. lactis. This inhibitory effect was reversed by alanine or pyridoxine. Tests on the nutritional requirements of S. lactis showed that alanine and glycine were essential for the growth of the organism. Pyridoxine was also required, although large amounts of alanine could replace pyridoxine. The authors concluded that the organism could not synthesize alanine, but that it could use alanine, if present at high enough levels, to synthesize pyridoxine. They interpreted the glycine-alanine effect as the mass action of an antagonist (glycine) interfering with a structurally similar essential metabolite (alanine). To account for the glycine-pyridoxine effect, they offered the alternative suggestion that glycine might block a mechanism involving both alanine and pyridoxine.

Although glycine and alanine appear to have the same interrelated effect on the growth of L. arabinosus, the nutritional requirements of this organism with respect to glycine, alanine, and pyridoxine are different.

Neither glycine nor alanine is an essential amino acid. Alanine has an accessory growth effect, while glycine is reported as being without any effect (54). Pyridoxine is likewise non-essential (59).

The question arises as to whether it is the synthesis or the utilization of alanine and pyridoxine that is blocked by glycine. According to Roblin (55), it is possible to differentiate between the antagonistic effect of a compound which interferes with the synthesis of a metabolite and the antagonistic effect of one which interferes with the utilization or function of a metabolite. In the first case, the minimum amount of the metabolite which is effective against the antagonistic compound is effective against any amount of the antagonistic compound. In the second case, a more or less constant ratio exists between the amount of antagonistic compound and the amount of metabolite necessary to overcome the antagonism.

If these concepts are applied to the glycine antagonism, the possibility of interference with the synthesis of either alanine or pyridoxine is ruled out. Snell and Guirard have demonstrated that when the amount of glycine is increased the amount of alanine or pyridoxine must likewise be increased if growth is to occur. This would indicate that a

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(59) Bohonos, Hutchings, and Peterson, J. Bact. 44, 479 (1942).

utilization of both alanine and pyridoxine was being blocked. The suggestion of Snell and Guirard that the glycine antagonism was tied up with the essential character of alanine and pyridoxine would seem to be weakened by the results obtained with L. arabinosus. It is more likely that a step in a process involving alanine and pyridoxine, carried out by both organisms, is blocked by glycine.

d-Valine and d-leucine

Several interesting correlations can be made from the results of experiments establishing the inhibitory effect of d-valine and d-leucine. One is the possibility of d-valine and d-leucine interfering specifically with the utilization of l-leucine and l-valine. The fact that d-alanine--the isomer of a non-essential acid--is not inhibitory would seem to lend support to this view. (Since d-tyrosine was not tested at the 20 mg./ml. level because of its insolubility, it is not included in this discussion.)

Evidence against this specificity, however, is the activity of dl-valine and dl-leucine. If d-leucine were interfering with the utilization of l-leucine, the addition of l-leucine would be expected to result in overcoming the d-leucine effect. In the case of the racemic compounds, the ratio of d-leucine/l-leucine is 1 and the inhibition is still manifest. The anti-metabolite/metabolite ratio for

most cases of inhibition is greater than 1 (55). For the sulfa drugs, it runs from 50/1 to 5000/1. It therefore seems more likely that the d-amino acids interfere with the utilization of all l-amino acids rather than with just the specific antipodes. This would be in line with McIlwain's work (24) on aminosulfonic acids. He found that any one of a group of aminocarboxylic acids was capable of reversing the action of the inhibitory aminosulfonic acids.

It should perhaps be pointed out that there are two differences between d-amino acids and the type of compounds discussed in Roblin's review. The first is that the d-amino acids differ from the essential metabolites configurationally instead of structurally. The second difference is that the compounds were tested at much higher levels. Since, however, the same high level of l-amino acids was without effect, the activity of the d-amino acids cannot be attributed to the higher concentrations.

A second correlation which may be drawn from the d-amino acid activity relates the configuration of the amino acids found inhibitory toward L. arabinosus to the configuration of amino acids isolated from gramicidin. The amino acids obtained from gramicidin by hydrolysis include d-leucine, dl-valine, and l-valine. When these three amino acids were tested against L. arabinosus, it was d-leucine and dl-valine which proved to be inhibitory. Lipmann, Hotchkiss,

and Dubos (3) originally suggested that the presence of d-amino acid residues might account for some of the antibacterial properties of gramicidin. It would be interesting to test the d- and l- isomers of the other amino acids isolated from gramicidin to see if further correlation is found.

A third correlation that can be drawn between the activity of d-valine and d-leucine on one side and the lack of activity of d-alanine on the other side is that of the length of the side chain to the degree of inhibition. d-Alanine, with its small methyl side chain, has no inhibitory activity. d-Valine and d-leucine, with isopropyl and isobutyl side chains, show marked inhibition. The steric effect of voluminous side chains was pointed out by Bergmann and coworkers in their studies of the antipodal specificity of proteolytic enzymes (60). Bergmann pictured an attraction between the polar groups of the enzyme on one hand, and the polar groups of the substrate on the other. In order for interaction to take place, the active groups attached to the α-carbon had to be in specific spatial arrangement. With α-carbons of the l-configuration, this arrangement of polar groups was such that the side chain did not interfere when the enzyme interacted with the substrate. If the same polar groups were arranged in the d-configuration, the side chain would come between the enzyme and the substrate, and prevent

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(60) Bergmann, Zeras, Fruton, Schneider and Schleich, J. Biol. Chem. 109, 325 (1935).

or slow down the reaction.

In support of this concept, Bergmann and coworkers have synthesized many d- and l- peptides and peptide derivatives. When the side chain was larger than CH_3 or H, the d- compound was hydrolyzed slowly or not at all. Although the authors referred to the "inhibitory" effect of the compounds, they did not actually test the effect of an excess of the d- compounds on the enzymic hydrolysis of the l- compounds.

Edlbacher and Bauer (61), however, tested the effect of d-leucylglycine on the hydrolysis of l-leucylglycine by swine peptidase. They found that not only was d-leucylglycine not hydrolyzed by the enzyme but also that 20-24 moles of d-leucylglycine would prevent the hydrolysis of one of l-leucylglycine. A similar ratio of d-leucylglycine to glycylglycine inhibited the hydrolysis of glycylglycine.

It is entirely conceivable that the enzymes in the intact bacterial cell can likewise be blocked by d-amino acids with voluminous side chains. The results given in Table XVII with racemic amino acids containing varying side chains would tend to support this concept. Results with dl- compounds, however, should be accepted with reservations. Bergman and Fruton, in testing N-benzoyl-dl-tyrosylglycylamide

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(61) Edlbacher and Bauer, Z. physiol. Chem. 270, 176 (1941).

(62), found that the compound was not hydrolyzed at all, even though the l- compound was split both in the presence and absence of the dl- form. They suggested that the explanation might lie in the high stability of the racemate. Such results emphasize the importance of using pure optical forms in all work of this type, as opposed to the practice of using dl- forms and assuming that the dl- compound is equal in activity to the sum of the activities of the d- and l- components.

Effect of Amino Acid Derivatives

The amino acid derivatives were made in the hope of enhancing the activity of the d-amino acids. Some derivatives with greater activity were obtained, but on the whole the derivatives so far obtained do not seem to show much antipodal specificity. The activity of the amino-tyrosines seems to lie with the amino phenol structure (63). The activity of the phthalyl compounds would seem to deserve further study. Is it the trisubstituted amine or the ring structure that is of importance?

More peptide syntheses are indicated. Since the combination of glycine and leucine as glycyl-d-leucine com-

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(63) Barber and Haslewood, Biochem. J. 39, 285 (1945).

pletely removes the inhibitory action of both glycine and d-leucine, it would be of interest to know if the combination of the two as d-leucylglycine would have the same effect.

Lack of antipodal specificity in the derivatives may be due to a change in the polar groups of the amino acids, so that there no longer is the same attraction between the enzyme and the amino acid. It may be necessary to synthesize derivatives containing the peptide linkage intact, with various groups substituted on the ends of the peptide chain.

The preparation of more amino acid derivatives and a study of their effect on bacterial growth should throw more light on both antibacterial action and protein synthesis.

SUMMARY

1. The d- and l- isomers of the following derivatives of valine, leucine, and tyrosine were prepared: the formyl, phthalyl, and prolyl derivatives of valine and leucine; the methyl ester hydrochlorides of valine and leucine; the glycyl derivatives of leucine; benzoyl, 3-amino, and 3-nitro tyrosine; the ethyl esters of tyrosine and benzoyl tyrosine; and benzoyl tyrosylamide.

Of these, the prolyl and phthalyl derivatives of d-leucine, d-valine, and l-valine; the methyl ester hydrochlorides of d- and l-valine; the 3-amino and 3-nitro derivatives of d-tyrosine; and benzoyl-d-tyrosine ethyl ester were new compounds and constants for them were reported.

2. High levels of glycine were found to inhibit the growth of L. arabinosus. The relationship between this inhibition and similar results reported in the literature for Streptococcus lactis was discussed.

3. High levels of d-leucine and d-valine were also found to inhibit the growth of L. arabinosus. The same levels of l-leucine, l-valine, l-alanine, and d-alanine were without appreciable effect. Possible correlations between these results and the nutritional requirements of the organism, the structure of the amino acids, and the amino

acids obtained from gramicidin were discussed.

4. Of the valine, leucine, and tyrosine derivatives made, none seemed to show antipodal specificity. 3-Amino-l-tyrosine, 3-amino-d-tyrosine, d- and l-leucine methyl esters, and the phthalyl derivatives of both d- and l-leucine and valine showed more activity than d-leucine, although none of the activities was very high.

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