[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

Studies on Antipodes. VII.[1] Observations on D-Amino Acids and on Casein Derivatives Containing D-Amino Acid Residues

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Much of the interest in the inhibitory properties of D-amino acids[3-5] has centered about their intrinsic effects upon growth, their role in providing supporting evidence that proteolytic enzymes also catalyze protein synthesis,[4] and about the evolutionary implications of the occurrence of D-amino acids in a planetary biochemistry based upon a predominantly L-configuration.[6] The D-amino acid is especially a structural anomaly which merits investigation in attempts to prepare artificial antibiotics.

D-Leucine was probably the first pure D-amino acid shown definitely to possess growth-retarding activity,[3,7] as observed with *Lactobacillus arabinosus*.[8] Other reports of inhibition of microbial growth include the effects of D-valine[4] and D-tryptophan[9] upon *L. arabinosus*, the action of atypical[10] forms of alanine,[5] valine,[5] leucine[5] and serine[11] on *Escherichia coli*, the effects of D-lysine and of D-glutamic acid upon *Staphylococcus aureus*,[12] and the inhibition of *Tetrahymena geleii* by the D-forms of leucine, valine and phenylalanine.[13] D-Methionine inhibits the uptake of L-methionine by liver slices.[14] Retardation of mammalian growth by various D- or DL-amino acids has also been reported.[15-17]

The common basis for some retardations of growth is probably an inhibition of proteases.[4] This hypothesis is supported by the facts that D-leucine inhibits the hydrolytic behavior of peptidases from a number of sources[18] and that the D-isomers of phenylalanine, tyrosine, histidine, alanine and isoleucine retard the action of carboxypeptidase.[19]

D-Amino acids other than those mentioned above are also being investigated. In the present work the effects of D-glutamic acid and of D-α,γ-diaminobutyric acid (found in polymyxin[20]) upon the growth of *L. arabinosus*, *E. coli* and *S. aureus* have been assessed. Jeney[12] found D-glutamic acid to be inhibitory to *S. aureus* at 1.0 μM./ml.; in the work described here, the amino acid was not found to be inhibitory at concentrations below 68 μM./ml. The discrepancy may be explainable as due to variation in susceptibility of different strains, or more likely to Jeney's use of a non-proteinaceous medium.

The finding of an antipodal effect with α,γ-diaminobutyric acid (Table I) suggested comparison with other diamino acids. DL-Lysine and DL-ornithine were tested to determine if they had activity at high dilution. The effect of ornithine is of interest since, although it has not been reported as a constituent of protein, the L-isomer has been found in Gramicidin S[21] and in bacitracin.[22] The activities observed did not however stimulate further investigation.

TABLE I

ANTIBACTERIAL ACTIVITY OF SOME DIAMINO ACIDS IN MM./ML.

Compound	*E. coli*	*L. arabinosus*	*S. aureus*[a]
L-α,γ-Diaminobutyric acid·1.5 HCl	>0.11[b]	>0.11	>0.11
DL-α,γ-Diaminobutyric acid·2HCl	.11–0.084	> .11	> .11
D-Diaminobutyric acid·1.5HCl	.058–0.069	> .069	> .069
L-Lysine·HCl[c]	> .17	> .17	> .17
DL-Lysine·HCl[d]	.17–0.14	> .17	> .17
DL-Ornithine·HCl[e]	> .15	.15–0.09	.15–0.09

[a] Also known as *Micrococcus pyogenes* var. *aureus*. [b] Where the symbol ">" appears, the figure given represents the highest concentration tested; this concentration was noninhibitory. [c] Bios Laboratories. [d] Eastman Kodak Co. product recrystallized from aqueous ethanol. [e] H. M. Chemical Co.

The accumulated information on the D-amino acids emphasizes that even though some of them are inhibitory for some organisms the concentrations at which antibiotics containing such residues

(1) Paper VI, Minard and Fox, THIS JOURNAL, **71**, 1160 (1949).

(2) Work supported by the Industrial Science Research Institute of Iowa State College. Taken in part from the M.S. thesis of Yutaka Kobayashi, 1950.

(3) Fox, Fling and G. N. Bollenback, *J. Biol. Chem.*, **155**, 465 (1944).

(4) Fling and Fox, *ibid.*, **160**, 329 (1945).

(5) Kobayashi, Fling and Fox, *ibid.*, **174**, 391 (1948).

(6) Fox, *Can. Med. Assoc. J.*, **56**, 76 (1947).

(7) No earlier report has been found in extensive literature searches in this project. In 1940, Nielsen, (*Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, **23**, 115 (1940)) claimed for the action of leucine on *Bacterium radicicola* in a textual statement unsupported by experimental data, that "—die *d*(−)-Form sich als nicht assimilierbar; ausserdem schien sie auch hemmend zu wirken. Diese Frage wird den Gegenstand einer spateren Untersuchung bilden." The present authors have been unable to find any later experimental report. All Nielsen's experiments in the cited publication were assimilation experiments; D-leucine was of interest as a nitrogen source rather than as an inhibitor.

(8) Failure to inhibit growth of *L. arabinosus* with D-leucine has been claimed by Baumgarten, Mather and Stone in *Cereal Chem.*, **23**, 135 (1946). These authors tested D-leucine at a concentration of 2.2 mg./ml., instead of at 20 mg./ml., which was the concentration in the experiments with which they compared their results. Baumgarten, *et al.*, thus lacked a factual basis for their stated conclusion.

(9) Prescott, Schweigert, Lyman and Kuiken, *J. Biol. Chem.*, **178**, 727 (1949).

(10) The D-configuration of amino acids is often referred to as "unnatural." This form has been found in so many antibiotics, however (*cf.* ref. 1), that the term "atypical" is employed here instead.

(11) Davis and Maas, THIS JOURNAL, **71**, 1865 (1949).

(12) Jeney, *Hung. Acta Physiol.*, **1**, 142 (1948).

(13) Kidder and Dewey, *Proc. Natl. Acad. Sci.*, **33**, 347 (1947).

(14) Simpson and Tarver, *Arch. Biochem.*, **25**, 384 (1950).

(15) Artom, Fishman and Morehead, *Proc. Soc. Exptl. Biol. Med.*, **60**, 284 (1945).

(16) Hankes, Henderson, Brickson and Elvehjem, *J. Biol. Chem.*, **174**, 873 (1948).

(17) Graham, Hier, Waitkoff, Saper, Bibler, and Pentz, *ibid.*, **185**, 97 (1950).

(18) Abderhalden and Abderhalden, *Fermentforschung*, **16**, 445 (1942).

(19) Elkins-Kaufman and Neurath, *J. Biol. Chem.*, **175**, 893 (1948).

(20) Gore and Petersen, *Ann. N. Y. Acad. Sci.*, **51**, 924 (1949).

(21) Synge, *Biochem. J.*, **39**, 363 (1945).

(22) Barry, Gregory and Craig, *J. Biol. Chem.*, **175**, 485 (1948).

exhibit their effects are typically far smaller. Appropriate derivatives of D-amino acids which structurally bridge the gap between the simple unit and the polypeptide antibiotics are therefore of interest. Evidence that gramicidin[23] is cyclic (Gramicidin S[24] and polymyxin[25] are also believed to be cyclic) has led to attempts to prepare active cyclopeptides containing D-amino acid residues[26,27] and to attempts to cyclize racemized casein, which is of interest because of its intramolecularly numerous D-amino acid residues.[28]

Treatment with thionyl chloride was carried out on the basis of an hypothetical acyl chloride formation from free carboxyl groups in the glutamic acid residues known to exist in racemized casein[29] followed by coupling of a type akin to that observed in cyclization studies of penicillin intermediates.[30] Other agents such as hot ethylene glycol,[31] hot β-naphthol,[32] hot glycerol[33] and phosgene,[34] each of which promotes a type of cyclization, gave inactive products. It is of incidental interest that the material recovered from β-naphthol was soluble in 80% ethanol, while that from the phosgene treatment set to an insoluble, vitreous mass which could not be tested.

The thionyl chloride treatment, however, led to products which repeatedly exhibited antibacterial activity against *S. aureus* in ranges down to several hundred gamma per ml., and which occasionally inhibited *L. arabinosus* in the same way. The solids, in suspension, were found to possess activity against all three microörganisms employed in the tests (Table II).

TABLE II

ANTIBACTERIAL ACTIVITY OF SOLID RACEMIZED CASEIN AND DERIVATIVES IN MG./ML.

Compound	*E. coli*, mg.	*L. arabinosus*, mg.	*S. aureus*, mg.
Casein	>5[a]	>5	>5
Casein-T	>5	>5	<5 >2
Casein-R	>5	>5	<5 >3
Casein-RT[b]	2–1	0.8–0.5	1.0–0.25

[a] Where the symbol ">" appears, the figure given represents the highest proportion tested; this proportion was non-inhibitory. [b] The figures for Casein-RT indicate the range within which inhibition was observed. The higher value was always inhibitory while the lower value was variable. 5 mg./ml. was the highest proportion used.

The inadequate knowledge of the constitution of partially racemized casein and its almost certain heterogeneity precluded comparing the activity of the above material (Casein-RT) with that of unavailable optical isomers. Comparisons were however possible for casein treated for racemization alone,[28] or with thionyl chloride alone (Table III). Similarly treated caseose[29] samples exhibited behavior analogous to that of Casein-R. The results indicate that the activity is found only in those casein derivatives obtained through both treatments. Although its use was based on a conceivable ability to produce cycles, no evidence is at hand that such structures have resulted from the treatments. Clarification of the nature of the reaction with thionyl chloride, and an appraisal of the proportion of the active material in Caseose-T and Casein-RT must await the results of further studies.

TABLE III

ANTIBACTERIAL ACTIVITY OF THE WATER-SOLUBLE FRACTION OF SOME CASEIN DERIVATIVES IN MG./ML.

Compound	*E. coli*, mg.	*L. arabinosus*, mg.	*S. aureus*, mg.
Casein-T	>1.5[a]	>1.5	>1.5
Casein-RT	>3.0	>3.0	1.2–0.94[b]
Casein-R-β-naphthol	>1.5	>1.5	>1.5
Caseose	>2.0	>2.0	>2.0
Caseose-T	>2.0	>2.0	0.15–0.13[b]

[a] Where the symbol ">" appears, the figure given represents the highest concentration tested; this concentration was non-inhibitory. [b] Inhibitory in all cases at the higher value while variable at the lower value.

Experimental

D-Glutamic Acid.—D-Glutamic acid was prepared by enzymatic resolution of DL-glutamic acid[35] with papain, and use of a modification of the procedure of Fruton *et al.*,[36,37] employing higher citrate buffer concentration, and hydrolytic cleavage; $[\alpha]^{23}D$ +30.1 ± 0.5° (5 *N* HCl, *c*, 4.09, *l* 1).

Diaminobutyric Acids.—The L-, DL- and D-forms were prepared by the procedure of Adamson.[38] Optically active salts obtained by recrystallization from water and acetone possessed a ratio of 3 molecules of HCl of crystallization to 2 molecules of amino acid. Carter, *et al.*,[39] reported a DL-dihydrochloride which on repeated recrystallization lost HCl. The 3/2 HCl salt was stable in air and was obtainable, by the procedure employed, only for the L- and the D-isomers. A sample of each melted at 212–213°. For the L-isomer, the rotation was $[\alpha]^{26}D$ +12.7 ± 0.3° (water, *c*, 6.9, *l* 1) and for the D-isomer, $[\alpha]^{27}D$ −12.1° ± 0.7° (water, *c*, 5.7, *l* 1).

Anal. Calcd. for $C_8H_{23}O_4Cl_3N_4$: Cl, 30.82; N, 16.21. Found (L-isomer): Cl, 30.4; N, 16.3.

The racemic diaminobutyric acid 2HCl had a m. p. of 199–202°. All m. p.'s were taken in capillary tubes and were corrected.

Partially Racemized Casein Fractions.—Smaco vitamin-free casein was racemized according to the procedure of Dakin and Dudley.[40] The $[\alpha]^{26}D$ in *N*/2 NaOH for the original casein, the acid-precipitated casein designated as casein-R, and the water-soluble caseose (precipitated with ammonium sulfate and dialyzed against distilled water for six days and lyophilized) were, respectively, −105.8 ± 0.8°, −62.5 ± 0.8° and −36.1 ± 0.7°.

Reaction of Thionyl Chloride with Racemized Casein.—Five grams of racemized casein[28] and 20 ml. of thionyl

(23) Gordon, Martin and Synge, *Biochem. J.*, **37**, 86 (1943).
(24) Consden, Gordon, Martin and Synge, *ibid.*, **41**, 596 (1947).
(25) Bell, Bone, English, Fellows, Howard, Rogers, Shepherd and Winterbottom, *Ann. N. Y. Acad. Sci.*, **51**, 897 (1949).
(26) Fling, Ph.D. thesis, Iowa State College, 1946.
(27) Fruton, THIS JOURNAL, **70**, 1280 (1948).
(28) Fox, Kobayashi, Melvin and Minard, *ibid.*, **70**, 2404 (1948).
(29) Dakin and Dudley, *J. Biol. Chem.*, **15**, 263 (1913).
(30) Hunter, Hinman and Carter in Clarke, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., 1949, p. 909.
(31) Sannie, *Bull. soc. chim.*, **9**, 487 (1942).
(32) Lichtenstein, Hestrin, Dimant and Brzoza, THIS JOURNAL, **60**, 560 (1938).
(33) Fodor, *Enzymologia*, **12**, 101 (1947).
(34) Harris, Wolf, Mozingo, Arth, Anderson, Easton and Folkers, THIS JOURNAL, **67**, 2096 (1945).
(35) Arnow and Opsahl, *J. Biol. Chem.*, **134**, 649 (1940).
(36) Fruton, Irving and Bergmann, *ibid.*, **133**, 703 (1940).
(37) Fox, Melvin, Radke and Wilkerson, unpublished experiments.
(38) Adamson, *J. Chem. Soc.*, 1564 (1939).
(39) Carter, Van Abeele and Rothrock, *J. Biol. Chem.*, **178**, 325 (1949).
(40) Dakin and Dudley, *ibid.*, **15**, 267 (1913).

chloride (Eastman Kodak Co.) were refluxed for 30 minutes at 75 ± 5°. The mixture darkened considerably during the process. The excess thionyl chloride was removed at the water-pump, the residue covered with hexane and taken to dryness. This was repeated four times. The solid was removed from the flask and ground in a mortar. The yield was 4.8 g. $[\alpha]^{26}D$ −53.3 ± 10.0° (*N*/2 NaOH, *c*, 0.3, *l* 1). The product was designated Casein-RT.

Reaction of Thionyl Chloride with Casein.—Casein was treated with thionyl chloride in the same manner as was racemized casein: $[\alpha]^{26}D$ −79.1 ± 1.7° (*N*/2 NaOH, c, 1.2, *l* 1). The product was designated Casein-T.

Reaction of Casein-R with β-Naphthol.—Casein-R was treated with β-naphthol at 155 ± 10° according to the procedure of Lichtenstein.[41] Two grams of product was obtained after ether extraction from 2.7 g. of casein-R; $[\alpha]^{26}D$ −34 ± 5.5° (*N*/2 NaOH, *c*, 0.88, *l* 1). This product was designated β-naphthol Casein-R.

Bacterial Tests.—The growth of the microörganisms was observed visually. The majority of the tests were carried out with a final volume of 1.0 ml. consisting of 0.5 ml. of 2X medium and 0.5 ml. of the test solution. The medium was buffered to maintain an initial *p*H of 6.9 ± 0.2 with 0.5 g. each of KH_2PO_4 and K_2HPO_4 per 100 ml. of final medium for all tests with casein and its derivatives. This added salt concentration did not prevent the growth of the organisms as evidenced by the growth of the controls. The *L. arabinosus* 17-5 and *S. aureus* were grown on yeast extract medium[42] and the *E. coli* was grown on nutrient broth.[43] The inocula were prepared from 24-hour cultures incubated at 37°, centrifuged, washed once with saline solution, and resuspended in 5 ml. of saline. The suspended organisms were diluted approximately one to eight and a drop of this suspension was used in each tube.

The solid casein derivatives were tested as follows: a 1.0 ± 0.2 mg. sample was transferred into a test-tube, the sample covered with about 3 ml. of ether, and the ether allowed to evaporate through the cotton plug in a stream of air and under a slight vacuum maintained by the water-pump. This required typically 6–18 hr. for evaporation. Too rapid an evaporation of the ether resulted in the particles acquiring a charge and sticking to the walls of the tube and to the cotton plug. The tube was now sterile, as determined in controls. A measured amount of sterile IX medium, 1.0, 2.0 or 4.0 ml., was pipetted into the sterile tube and inoculated with the proper organisms. The tubes were shaken before incubation at 37°. The tubes were set up in duplicate.

The water-soluble fractions of Casein, Casein-T, Casein-R and Casein-RT were prepared as follows: 100 mg. of material was suspended in 10 ml. of distilled water for 24 hours. The solution was then filtered through an all-glass Seitz filter. For Caseose-T, a 40-mg. sample was treated as above. The solid concentration of the solutions was estimated by taking three 1.0-ml. aliquots and evaporating the solvent from tared beakers in a vacuum desiccator over sulfuric acid. Three levels of test solution were generally used: 0.5, 0.3 and 0.1 ml. per 1.0 ml. of final volume. The volume was adjusted to 1.0 ml. with sterile distilled water where needed. The tubes were inoculated, shaken and incubated at 37°. The tubes were set up in duplicate. The caseose solutions of known concentration could be prepared readily because caseose is water-soluble and was sterilized by Seitz filtration.

The amino acids were tested by weighing into test-tubes, dissolving in water, adjusting the *p*H to 6.9 ± 0.2, bringing to volume with water and sterilizing in the autoclave at 15 lb./sq. in. for 15 minutes. The amino acid solution was then aseptically pipetted into tubes containing sterile medium. Three levels of the amino acid were used: 0.5, 0.3 and 0.1 mg. per ml. of final volume. The volume was adjusted to 1.0 ml. with sterile distilled water where needed. The tubes were inoculated, shaken and incubated at 37°.

For all of the bacterial tests, the growth of the organisms was checked at 18, 24, 36 and 48 hours. Inoculated and uninoculated controls were run simultaneously in all tests. The results recorded are for a minimum of four tests per inhibitor.

Acknowledgment.—Thanks are due to Mrs. Janet Thompson, who repeated the critical tests with Casein-RT and with Caseose-T with the aid of written directions only. The results were in agreement with the others obtained.

Summary

Bacterial growth inhibitions, previously observed with D-leucine, D-valine and D-alanine, have been extended to D-α,γ-diaminobutyric acid, which was active against *Escherichia coli* at a concentration in which the L-isomer was inactive.

Thionyl chloride-treated racemized casein was inhibitory to *E. coli*, *Lactobacillus arabinosus* 17-5, and to *Staphylococcus aureus* in suspension in ranges of 2-1 mg./ml., 0.8–0.5 mg./ml., and 1.0–0.25 mg./ml., respectively. Larger amounts of thionyl chloride-treated casein, or of racemized casein were not inhibitory to the same organisms, in control experiments. In solution, thionyl chloride-treated caseose was inhibitory to a strain of *S. aureus* at 0.15–0.13 mg./ml.

The optically active "sesquihydrochlorides" of α,γ-diaminobutyric acid have been described.

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(41) Lichtenstein, THIS JOURNAL, **66**, 1103 (1944).

(42) McMahan and Snell, *J. Biol. Chem.*, **152**, 83 (1944).

(43) Difco Laboratories, "Manual," 1948, p. 24.