to twenty minutes the stirring was stopped and the mixture allowed to cool, a light yellow solid usually separating. Approximately 200 ml. of Skellysolve "B" was then added. After shaking, the solid 4-hydroxyquinoline derivative was filtered off, washed several times with Skellysolve and recrystallized from water in the case of 2-methyl-4-hydroxyquinoline and from a mixture of water and ethanol in the other cases.

Acetoacetanilides were prepared from 0.1 mole each of ethyl acetoacetate and aromatic amine either by Method D described above or by refluxing the reactants three to four minutes[15] (Method E). The solid, obtained on cooling the mixture, was recrystallized from acetic acid and water and then from ethanol and water yielding acetoacetanilide (m. p. 82–83°)[3a] in 52% yield and acetoaceto-*o*-toluidide (m. p. 107–108°)[15] in 55% yield.

Benzoylacetanilides were prepared from 0.1 mole each of ethyl benzoylacetate and aromatic amine either by heating the reactants at 150° for five hours (Method F) or by refluxing the mixture for fifteen minutes (Method G). After recrystallization as described for acetoacetanilide, benzoylacetanilide (m. p. 107–108°)[11] was obtained in 50% yield, and benzoylaceto-*o*-toluidide (m. p. 130–131°) in a 65% yield.

Anal. Calcd. for $C_{16}H_{15}NO_2$: C, 75.82; H, 5.96. Found: C, 75.51; H, 5.72.

The anilides were cyclized in concentrated sulfuric acid at 80–90° as described in "Organic Syntheses"[16] or by heating on the steam-bath for fifteen minutes. The 2-hydroxyquinoline derivatives were recrystallized from a mixture of water and ethanol. An attempt to cyclize acetoacetanilide (II) in Dowtherm at 250–260° as described above for the crotonate was unsuccessful.

The yields and the melting points of the quinoline derivatives are given in Table I. Admixture of the various samples of the same derivative showed no depression in melting point, but admixture of isomeric 2- and 4-hydroxyquinolines depressed the melting point.

2-Methyl-4-hydroxyquinoline (0.5 mole scale).—A mixture of 46.5 g. (0.5 mole) of aniline, 65 g. (0.5 mole) of ethyl acetoacetate, 100 ml. of commercial absolute ethanol, 135 g. of Drierite, and 1 ml. of glacial acetic acid was refluxed on the steam-bath for four hours. After removing the Drierite and the solvent, the residue was fractionated through a 30-cm. Vigreux column yielding 58 g. (57%) of ethyl β-anilinocrotonate, b. p. 155° at 10 mm.[17] The crotonate was cyclized in 200 ml. of Dowtherm yielding 38 g. (50%) of 2-methyl-4-hydroxyquinoline (m. p. 229–230°).

4-Phenyl-2-chloroquinoline.—A mixture of 10 g. (0.045 mole) of 4-phenyl-2-hydroxyquinoline (m. p. 259°) and 30 ml. of phosphorus oxychloride was heated in an oil-bath at 120° for two hours, the excess oxychloride distilled under reduced pressure and the light brown viscous oily residue poured onto ice. After standing in the refrigerator for one day the solidified oil was recrystallized from absolute ethanol yielding 10 g. (93%) of white crystals of 4-phenyl-2-chloroquinoline, m. p. 87–88°.

Anal. Calcd. for $C_{15}H_{10}NCl$: C, 75.15; H, 4.21; N, 5.86; Cl, 14.8. Found: C, 74.88; H, 4.70; N, 6.12; Cl, 14.68.

Conversion of Crotonate to Anilide.—To 0.1 mole of ethyl β-anilinocrotonate, b. p. 155° at 10 mm., was added 2 g. of water and five drops of concentrated hydrochloric acid and the mixture stirred and heated in an open flask in an oil-bath at 130–140° for three or four hours. The resulting crude anilide was cyclized[16] to form 4-methyl-2-hydroxyquinoline, m. p. 222–223°, in 35% over-all yield.

Conversion of Anilide to Crotonate.—A mixture of 0.1 mole of acetoacetanilide (m. p. 82–83°), 30 ml. of commercial absolute ethanol and 30 g. of Drierite was refluxed four hours and the Drierite then filtered off. After distilling the solvent, the residue was fractionated giving a 50% yield of ethyl β-anilinocrotonate (b. p. 139–143° at 6 mm.) which was cyclized in Dowtherm to 2-methyl-4-hydroxyquinoline, m. p. 229–230°, in 39% over-all yield from the anilide.

Summary

1. The factors governing the formation of crotonates and anilides from β-keto esters and aromatic amines have been considered.

2. Crotonates and anilides, prepared by various methods, have been cyclized to form 4- and 2-hydroxyquinolines, respectively.

3. In contrast to reports in the literature the anilide from ethyl benzoylacetate and aniline was found to form 4-phenyl-2-hydroxyquinoline on cyclization.

(15) Ewins and King, *J. Chem. Soc.*, **103**, 104 (1913), effected the reaction in one and one-half minutes.

(16) Lauer and Kaslow, "Organic Syntheses," Vol. 24, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 68.

(17) Distillation of the residue at a bath temperature of 120° until the forerun was removed and then at 140–160° gave ethyl β-anilinocrotonate, b. p. 128–130° at 2 mm., in 60–70% yield.

DURHAM, NORTH CAROLINA RECEIVED MARCH 19, 1948

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

The Bacterial Activity of "Racemized Casein," Caseose, and the Four Diastereoisomeric Leucylleucines[1,2]

BY SIDNEY W. FOX, YUTAKA KOBAYASHI,[3] SAMUEL MELVIN[3] AND FREDERICK N. MINARD[4]

Several of the antibiotics, particularly penicillin and gramicidin, are notable for their content of D-amino acid residues. In the case of penicillin, the D-amino acid residue is one of a number of critical structural features, since the L-analog is without activity.[5] D-Amino acids have been shown experimentally to inhibit bacterial growth[6,7] at relatively high concentrations. Recent reports, however, indicate medical utility of some of the simple amino acids, when used in relatively large amounts, in the control of infection.[8] In view of the above observations it is of interest to determine the antibacterial activity of structures re-

(1) Journal Paper No. J-1514 of the Iowa Agricultural Experiment Station, Project 897, in coöperation with the Veterinary Research Institute, and Project 980.

(2) The experiments with "racemized casein" were described before the American Society of Biological Chemists, May, 1947, at Chicago.

(3) This work was supported in part by the Industrial Science Research Institute of Iowa State College.

(4) Upjohn Company Fellow.

(5) du Vigneaud, Carpenter, Holley, Livermore and Rachele, *Science*, **104**, 431 (1946).

(6) Fox, Fling and Bollenback, *J. Biol. Chem.*, **155**, 465 (1944).

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

(8) Mario, *Minerva med.*, **38**, I, 578 (1947).

lated to the D-amino acids and to the antibiotics in which these simple units are incorporate.[9]

Substances which include the D-amino acid residue repeated within a single large molecule are under investigation in this laboratory; interest in such materials and their behavior has also been expressed elsewhere.[10] The present report describes two types of such material, with some reference to their effects on bacteria.

The simpler type is represented by D-leucyl-D-leucine. For comparative experiments in the present study, all four diastereoisomeric leucylleucines were synthesized by the method of Fischer and Koelker.[11] D-Leucyl-D-leucine was of particular interest because of its relationship to D-valyl-D-valine, which had been isolated from partial hydrolyzates of gramicidin as the benzoyl derivative.[12] In the other type of molecule the D-amino acid residues were linked in the main-chain through both the amino and carboxyl groups. Such material was available through the racemization procedure of Dakin.[13,14] The fractions called by Dakin "racemized casein" and caseose were shown to contain inverted residues of valine, leucine, phenylalanine, proline, tyrosine, aspartic acid, glutamic acid, arginine, lysine and histidine. D-Isomers of the first three amino acids represent all of the D-forms which have been found in gramicidin and tyrocidine.[15–19]

TABLE I

GALVANOMETER READINGS OF *Escherichia coli* CULTURES IN NUTRIENT BROTH CONTAINING LEUCINE AND LEUCYLLEUCINE ISOMERS

Figures presented represent per cent. transmission.

Substance	Concentration, mg. per ml. 10	5.0	2.5	1.25	0.6	0.3
L-Leucine	70	74	71	74	72	75
D-Leucine	99	82	76	74	72	70
L-Leucyl-L-leucine	71	70	70	69	72	70
D-Leucyl-D-leucine	72	70	67	67	69	70
L-Leucyl-D-leucine	79	71	70	69	69	70
D-Leucyl-L-leucine	78	71	71	70	68	71

TABLE II

GROWTH OF *Escherichia coli* ON MEDIA CONTAINING CASEIN AND RACEMIZED CASEIN DERIVATIVES

O = no visible growth; S.G. = slight visible growth; G = visible growth

	Time, days 1	2	3
Smaco casein	O	O	O
Racemized casein	O	G	G
Caseose	S. G.	S. G.	S. G.

(9) Fling, Minard and Fox, THIS JOURNAL, **69**, 2466 (1947).
(10) Bergel, *Biochem. J.*, **41**, xxxvi (1947).
(11) Fischer and Koelker, *Ann.*, **354**, 39 (1907).
(12) Christensen, *J. Biol. Chem.*, **154**, 427 (1944).
(13) Dakin, *ibid.*, **13**, 357 (1912–1913).
(14) Dakin and Dudley, *ibid.*, **15**, 263 (1913).
(15) Hotchkiss, *ibid.*, **141**, 171 (1941).
(16) Christensen, Edwards and Piersma, *ibid.*, **141**, 187 (1941).
(17) Hotchkiss, *J. Bact.*, **45**, 64 (1943).
(18) Gordon, Martin and Synge, *Biochem. J.*, **37**, 86 (1943).
(19) Gordon, Martin and Synge, *ibid.*, **37**, 313 (1943).

In none of the tests with the described D-amino acid derivatives was antibacterial activity equal to that of D-leucine noted (Table I). The lack of activity of D-leucyl-D-leucine, as an analog of D-valyl-D-valine, is in agreement with the rapid loss of activity of gramicidin under gentle hydrolytic conditions.[20,21] The activity of gramicidin thus seems not to be even partially represented by contiguous D-amino acid residues. The experiments with racemized casein and with caseose indicate that a polypeptide preparation containing a larger proportion of D-amino acid residues than occurs in tyrocidine is without appreciable antibacterial activity.

The results recorded by Dakin and Dudley[22] for the action of pancreatic microbial cultures on racemized casein was in the main confirmed in this work, with *Escherichia coli*. It should be noted that in the present experiments, the effect of *E. coli* on native casein also was tested; no appreciable growth was observed.

The quantitative nature of the behavior of the leucylleucines as sources of L-leucine for *L. arabinosus 17-5* is striking. Table III represents a typical experimental result. The bacterium was capable of utilizing fully, during the experimental incubation period, the L-leucine residues of L-leucyl-L-leucine and of D-leucyl-L-leucine. As might be expected, it could not utilize D-leucyl-D-leucine. *L. arabinosus* was also unable to use L-leucyl-D-leucine. This behavior is in contrast to the utilizability of D-leucyl-L-leucine.[23]

TABLE III

L-LEUCINE ACTIVITY OF LEUCYLLEUCINE ISOMERS FOR GROWTH OF *Lactobacillus arabinosus 17-5* IN LEUCINE-FREE MEDIUM

Peptide	L-Leucine activity of peptide, %	
L-Leucyl-L-leucine	98	102
L-Leucyl-D-leucine	0	0
D-Leucyl-L-leucine	50	50
D-Leucyl-D-leucine	0	0

Experimental

Leucylleucines.—The D–D, D–L, L–D, and L–L leucylleucines were prepared by the same general procedure employed by Fischer and Koelker[11] and by Fischer.[24] The intermediate bromoisocaproylleucines prepared in the present work had the following m. p.'s (cor.): L-α-bromoisocaproyl-D-leucine, 145–7° (F. & K. 149°), L-α-bromoisocaproyl-L-leucine, 126–7°, (F. & K. 128°), D-α-bromoisocaproyl-D-leucine, 125–7°, (F. & K. 128°), and D-α-bromoisocaproyl-L-leucine, 144–6° (F. 149°). Each of the dipeptides was assayed for L-leucine content. Samples of 20 mg. of each dipeptide were dissolved in 5 ml. of 20% hydrochloric acid and autoclaved for three hours at 15 lb. steam pressure. The solutions were then neutralized with sodium hydroxide and each was diluted to 100 ml. The resultant solutions were then assayed against a stand-

(20) Schales and Mann, *Arch. Biochem.*, **13**, 357 (1947).
(21) Itschner and Fox, unpublished experiments.
(22) Dakin and Dudley, *J. Biol. Chem.*, **15**, 276 (1913).
(23) Taken in conjunction with the work of Ågren: *Acta. Physiol. Scand.*, **13**, 347 (1947), these results emphasize the importance of position of the amino acid residue.
(24) Fischer, *Ber.*, **39**, 351 (1906).

ard solution of DL-leucine turbidimetrically on a Coleman Model 11 Spectrophotometer at 600 mμ. The organism employed was *Lactobacillus arabinosus 17-5*, which responds only to the L-form of leucine. The medium employed was that of Kuiken, *et al.*[25]

Within the probable limits of error of the assay, the identity of the four leucylleucines was established. The L-L assayed 90–100% L-leucine, the L-D and D-L each 50% L-leucine (±5%), and the D-D hydrolyzate gave 0- < 1% L-leucine.

Partially Racemized Casein Fractions.—Smaco vitamin-free casein was partially racemized by the procedure of Dakin.[12] The $[\alpha]^{27}D$ for the original casein, the acid-precipitable racemized casein and the water-soluble caseose (precipitated by ammonium sulfate) were, respectively: $-104.7° \pm 0.8°$, $-52.0° \pm 0.8°$, and $-37.5° \pm 2.0°$ (0.3 g. in 25 ml. 0.5 *N* sodium hydroxide solution).

Inhibition Experiments.—The leucylleucines and leucines were first tested against *E. coli* in a dilution series. Twenty mg. of each compound was weighed into a small test-tube. Two ml. of nutrient broth[26] was added to each tube and solution was effected by warming. Half of this was mixed with medium in another tube, and this process repeated through a series of a total of six tubes. The tubes were plugged with cotton and autoclaved for ten minutes at 15 lb. steam pressure. The tubes were then inoculated from a fresh subculture of *E. coli*.

After twenty-five hours of incubation at 37°, the turbidities of the cultures were assessed in a Coleman Model 11 spectrophotometer at 650 mμ. The results are presented in Table I. The experiment was repeated with a synthetic medium consisting of 0.5% disodium phosphate, 0.5% dipotassium phosphate, 0.5% ammonium chloride, 0.02% magnesium sulfate and 0.5% glucose, brought to *p*H 7 with phosphoric acid. The visual results were similar to those in nutrient broth; only D-leucine at 10 mg./ml. showed total inhibition. No inhibition was found for any of the peptides tested against *L. arabinosus 17-5* in a yeast extract medium[27] with all other conditions the same as in the *E. coli* experiments. Because of the conceivability of racemization of the D-peptides during autoclaving,[28] the experiment of Table I was repeated with the glucose added aseptically after autoclaving, and again with the glucose replaced by glycerol. In both cases the results were identical with those in synthetic medium.

At concentrations of 10 mg./ml. of added racemized casein and of caseose, *E. coli* grew under the experimental conditions given above. *E. coli* was grown for the other inhibition experiments with nutrient broth as above, except for the added casein or racemized preparations. The racemized casein was dissolved in 2 *N* sodium hydroxide solution, neutralized, and Seitz-filtered for the tests. There was no inhibition of *L. arabinosus 17-5* by racemized casein at a concentration of 3.5 mg./ml. nor by caseose at a concentration of 15 mg./ml. in yeast extract medium at 37° for seventy-two hours.

A corresponding set of experiments with racemized gelatin (not isolated) gave similar results.

Experiments on Support of Growth.—For experiments on support of growth, casein, racemized casein, and caseose were made up in concentrations of 100 mg./ml. of 0.8% sodium carbonate solution containing traces of calcium chloride, magnesium sulfate and trisodium phosphate. No other protein was present. *E. coli* was the organism used. The results are presented in Table II.

Tests of replaceability of L-leucine by the leucylleucines were run in leucine-free synthetic medium[25] inoculated with *L. arabinosus*. The results are presented in Table III.

(25) Kuiken, Norman, Lyman, Hale and Blotter, *J. Biol. Chem.*, **151**, 615 (1943).

(26) Difco Laboratories, "Difco Manual," Detroit, Mich., 1943, p. 42.

(27) Difco Laboratories, "Difco Manual," Detroit, Mich., 1943, p. 178.

(28) Fling and Fox, *J. Biol. Chem.*, **160**, 329 (1945).

Summary

Racemized casein and caseose, containing numerous D-amino acid residues per molecule, failed to inhibit the growth of cultures of *Escherichia coli* or *Lactobacillus arabinosus 17-5*. None of the four isomeric leucylleucines showed as much antibacterial activity as D-leucine. The relationship of these experiments to the antibacterial activity of D-amino acids and of the antibiotics which are D-amino acid derivatives has been discussed.

The replaceability of L-leucine by leucylleucine isomers, in the medium for *L. arabinosus 17-5*, has been studied. The L-residues of L-leucyl-L-leucine and D-leucyl-L-leucine were fully utilized. None of the residues of L-leucyl-D-leucine nor of D-leucyl-D-leucine were available to this organism.

AMES, IOWA RECEIVED JANUARY 31, 1948

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Action of Heat on D-Fructose. Isolation of Diheterolevulosan and a New Di-D-fructose Dianhydride

BY M. L. WOLFROM AND MARY GRACE BLAIR[1]

It is probable that the well-established tendency of D-fructose to form bimolecular cyclic anhydrides[2] accounts for some of the molasses formation occurring in cane sugar house processing. Such a view has been expressed by Sattler and Zerban.[3] These authors have investigated the non-fermented products formed on heating a concentrated aqueous solution of D-fructose. They established that the complex mixture obtained closely approximated in composition that required for a mixture of isomeric di-D-fructose dianhydrides. This work has now been repeated in our laboratory and the non-fermented products have been subjected to separation by chromatography on clay, a procedure established by Lew, Wolfrom and Goepp.[4] Two crystalline products were isolated in pure form and characterized. The one was the diheterolevulosan of Pictet and Chavan[5]

(1) Sugar Research Foundation Fellow of The Ohio State University Research Foundation (Project 190).

(2) For a review of the di-D-fructose dianhydrides see Emma J. McDonald, *Advances in Carbohydrate Chem.*, **2**, 253 (1946).

(3) L. Sattler and F. W. Zerban, *Ind. Eng. Chem.*, **37**, 1133 (1945).

(4) B. W. Lew, M. L. Wolfrom and R. M. Goepp, Jr., THIS JOURNAL, **67**, 1865 (1945); **68**, 1449 (1946).

(5) A. Pictet and J. Chavan, *Helv. Chim. Acta*, **9**, 809 (1926).