more relaxed. There was no effect on temperature, pulse, respiration, urine or blood; thus, administration is safe for trial in a number of diseases.

RAHWAY, N. J. RECEIVED MAY 23, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

The Synthesis, Resolution and Proof of Configuration of the Isomers of *m*-Tyrosine[1]

BY ROBERT R. SEALOCK, MERRILL E. SPEETER[2] AND RICHARD S. SCHWEET

m-Tyrosine has been obtained by hydriodic acid decomposition of 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone in 60–70% yields. The oxazolone, in turn, was prepared by an Erlenmeyer condensation between acetylglycine and *m*-hydroxybenzaldehyde in yields of 67–75%. Resolution was accomplished by means of the brucine salt of the formyl derivative. Of the resulting amino acids, the levorotatory isomer was shown to possess the L-configuration by means of the D-amino acid oxidase and the Lutz-Jirgenson methods. Results with the D-isomer were in complete accord. Catalytic reduction of acetamido-*m*-hydroxycinnamic acid yielded acetylcyclohexylamine.

In a study of the role of ascorbic acid in phenylalanine and tyrosine metabolism, the individual optical isomers of *m*-tyrosine were required. The amino acid was therefore synthesized and resolved and the configuration of the respective isomers established.

By means of the classical Erlenmeyer-oxazolone procedure, the racemic compound has been previously prepared by Blum[3] and Abderhalden and Schairer.[4] Likewise, the synthesis has been achieved by the diketopiperazine method.[5,6] The methods, however, were unsatisfactory in view of the large amounts required for subsequent resolution and the extensive feeding and metabolic experiments to be conducted. As an alternative, Dakin's acetylglycine modification[7] of the Erlenmeyer scheme proved more useful. Condensation of acetylglycine and acetic anhydride with *m*-hydroxybenzaldehyde yielded 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone which was readily converted to DL-*m*-tyrosine in one step by the hydriodic acid procedure of Lamb and Robson.[8]

Resolution was accomplished with the formyl derivative, prepared by the method of Clarke as described by du Vigneaud and Meyer.[9] The resolving agent was *l*-brucine with which an alcohol insoluble levorotatory and a water insoluble dextrorotatory salt were obtained. The resulting formyl derivatives proved to be levorotatory and dextrorotatory, respectively. Each in turn yielded a free amino acid exhibiting a rotation of opposite direction from that observed with the formyl compound. The one corresponding to the insoluble brucine salt proved to be dextrorotatory and the unnatural isomer. The other proved to be levorotatory and to be the amino acid possessing the natural configuration. Thus *m*-tyrosine affords a further illustration of amino acids exhibiting opposite direction of rotation when in the form of an acyl derivative.

The configuration of the two amino acid isomers was determined by the application of the specific D-amino acid oxidase method originally suggested by Krebs.[10] The levorotatory isomer was not oxidized by the enzyme, whereas the dextrorotatory isomer was readily oxidized to the alpha-keto acid. Confirmation of the configuration was obtained by the physical-chemical method of Lutz and Jirgenson[11,12] which depends upon the natural isomer exhibiting a positive shift in rotation with increasing concentrations of acid.

In the process of arriving at the final synthesis, catalytic reduction of acetamido-*m*-hydroxycinnamic acid was attempted. The reduced product proved to be acetyl-β-cyclohexylalanine instead of the desired acylamino acid. The proof of the structure of the compound was accomplished by means of appropriate derivatives particularly those described by Herbst and Shemin.[13] The latter authors obtained the same compound from the catalytic reduction of acetamidocinnamic acid.

Experimental

m-Hydroxybenzaldehyde was prepared from commercial *m*-nitrobenzaldehyde[14] and acetylglycine was likewise prepared by a method already in the literature.[15]

2-Methyl-4-(3'-acetoxybenzal)-5-oxazolone.—In a liter flask were thoroughly mixed 61 g. (0.5 mole) of *m*-hydroxybenzaldehyde, 58.6 g. (0.5 mole) of acetylglycine, 41 g. (0.5 mole) of anhydrous sodium acetate and 143.5 ml. (1.5 moles) of acetic anhydride. The mixture was placed in a boiling water-bath for six hours with a reflux condenser attached. It was allowed to cool to room temperature with the resultant formation of a solid mass of crystalline material, at which point 300 ml. of cold water were gradually worked into the mixture. After storage in the cold overnight, the product was separated by filtration and thoroughly washed with several 100-ml. portions of ice-cold water. The air-dried material, which was canary yellow in color, melted at 116–118°. In different preparations 82–92 g. corresponding to 67–75% yields were obtained. The crude material could be recrystallized from hot ethyl acetate through careful addition of Skellysolve B, in which case the melting point was 118–120°. Ordinarily the crude material was not recrystallized but was used directly in the next reaction.

Anal. Calcd. for $C_{13}H_{11}O_4N$: N, 5.71. Found: N, 5.73, 5.68, 5.68.

(1) Presented before the Division of Biological Chemistry at the 110th Meeting of the American Chemical Society in Chicago, Illinois, September, 1946.

(2) The Upjohn Company, Kalamazoo, Michigan.

(3) L. Blum, *Arch. Exp. Path. Pharmakol.*, **59**, 269 (1908).

(4) E. Abderhalden and W. Schairer, *Fermentforschung*, **12**, 295 (1931).

(5) H. Ueda, *J. Biochem. (Japan)*, **8**, 397 (1928).

(6) H. Ueda, *Ber.*, **61**, 146 (1928).

(7) H. D. Dakin, *J. Biol. Chem.*, **82**, 439 (1929).

(8) J. Lamb and W. Robson, *Biochem. J.*, **25**, 1231 (1931).

(9) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

(10) H. A. Krebs, *Biochem. J.*, **29**, 1620 (1935).

(11) O. Lutz and B. Jirgenson, *Ber.*, **64**, 1221 (1931).

(12) O. Lutz and B. Jirgenson, *ibid.*, **63**, 448 (1930).

(13) (a) R. M. Herbst and D. Shemin, "Org. Syntheses," 2nd edition, Coll. Vol. 2, 491 (1946); (b) D. Shemin and R. M. Herbst, THIS JOURNAL, **61**, 2471 (1939).

(14) R. B. Woodward, *Org. Syntheses*, **25**, 55 (1944).

(15) R. M. Herbst and D. Shemin, "Org. Syntheses," 2nd edition, Coll. Vol. 2, 11 (1946).

DL-*m*-Tyrosine.—The 2-methyl-4-(3′-acetoxybenzal)-5-oxazolone was converted into the free acid by treating 49 g. (0.2 mole) with 250 ml. of glacial acetic acid, 250 ml. of hydriodic acid (sp. gr. 1.70) and 20 g. of red phosphorus at reflux for 2.5 hours. The hot mixture filtered through asbestos was concentrated to dryness *in vacuo* and reconcentrated with three 50-ml. portions of water. The semi-crystalline amino acid hydroiodide was dissolved in 25 ml. of hot water, treated with Norite and neutralized with concentrated ammonium hydroxide until basic to congo red and acid to litmus. One hundred milliliters of absolute alcohol was added and the mixture allowed to stand twenty-four hours in the cold. The product was filtered, washed with several small portions of ice water and finally dried with alcohol. Further purification was achieved by dissolving the crude preparation in 25 ml. of water with the aid of a few drops of hydriodic acid. After treatment with Norite the acid was neutralized with sodium hydroxide and the solution made slightly acid with acetic acid. After crystallization was well underway at room temperature, 6 volumes of alcohol were added. After twenty-four hours in the cold, 21–24 g. (60–70%) of compound was obtained. It melted at 283° with decomposition when the sample was introduced into the bath preheated to 250°. Blum[3] reported a value of 280–281°. As in the preceding reaction 0.5 or 1.0 mole quantities gave essentially the same percentage yield.

Anal. Calcd. for $C_9H_{11}O_3N$: N, 7.73. Found: N, 7.59, 7.59.

Formyl-DL-*m*-tyrosine.—Clarke's formylation procedure as applied by du Vigneaud and Meyer[9] yielded the desired compound. From 35.5 g. a total of 36.5 g. (89%) of plate crystals melting at 136–138° were obtained, recrystallization being accomplished with the minimum of hot water. Prior to use in the resolution each preparation was thoroughly dried in a vacuum desiccator over solid potassium hydroxide.

Anal. Calcd. for $C_{10}H_{11}O_4N$: N, 6.69. Found: N, 6.67, 6.66.

Resolution of Formyl-DL-*m*-tyrosine.—A mixture of 37 g. (0.176 mole) of the acylamino acid and an equimolar amount, 69.3 g., of anhydrous brucine was dissolved in 1900 ml. of hot 95% ethyl alcohol. The solution was filtered and allowed to cool slowly to room temperature during which time crystallization began. With frequent stirring the process was allowed to continue for four days in the cold. The 81.7 g. which was obtained exhibited a rotation of $[\alpha]^{26}D$ −22.4° (1% solution in water). Three crystallizations from 20 volumes of alcohol in each case yielded 43.5 g. (82%) of salt with a rotation of $[\alpha]^{25}D$ −37.3°. Additional recrystallizations did not increase the rotation observed, although with later resolutions on a larger scale an additional treatment was sometimes needed in order to achieve maximum rotation. All rotations were determined after drying over phosphorus pentoxide at 100°. The alcohol insoluble salt when air-dried contained one molecule of water of hydration.

In order to obtain the other diastereoisomer, all the alcoholic mother liquors were combined and concentrated to dryness *in vacuo*. The residue was dissolved in 3 volumes of water and treated with Norite. After a somewhat slow crystallization in the cold (it may be hastened by seeding) 37 g. of anhydrous salt was obtained. Two recrystallizations gave a maximum rotation of +0.73° (1% of anhydrous salt in water). The total yield calculated on the anhydrous basis was 27 g. corresponding to a 46% yield. The salt as obtained proved to be the tetrahydrate. In subsequent resolutions concentration of the aqueous mother liquors gave variable but significant increases in the total yield of this salt. Likewise, resolutions carried out with 0.5 mole quantities proved equally successful.

Formyl-D-*m*-tyrosine.—The alcohol insoluble salt obtained above was dissolved in 20 volumes of hot water and quickly cooled to 40°. The brucine was precipitated by making the solution alkaline to phenolphthalein with 2 *N* sodium hydroxide. After cooling overnight, the brucine was removed and the last traces extracted with six 50-ml. portions of chloroform. To the alkaline solution 6 *N* sulfuric acid was added in an amount exactly equivalent to the sodium hydroxide. After concentration to a thick sirup *in vacuo*, the compound was extracted with acetone. The acetone was removed *in vacuo* and the residue crystallized from 1 volume of water. A 40% yield of plate crystals melting at 146–148° was obtained. The mother liquor was reserved for isolation of the free amino acid after hydrolysis. This procedure is of particular advantage if the free amino acid is the primary requirement, for the formyl derivative is relatively soluble even in the cold. If desired, the isolation of the formyl derivative may be omitted entirely.

Anal. $[\alpha]^{27}D$ −44.7°, 1% in water. Calcd. for $C_{10}H_{11}O_4N$: N, 6.69. Found: N, 6.51, 6.56.

Formyl-L-*m*-tyrosine.—The same procedure applied to the water insoluble brucine salt gave a 36.5% yield of compound melting at 148–149° and possessing a rotation of +45.7°. The mother liquor was again reserved for hydrolysis.

D-*m*-Tyrosine.—The formyl-D-*m*-tyrosine was refluxed for four hours with 10 volumes of 10% hydrochloric acid. The excess acid was removed *in vacuo* and the residue dissolved in 1 volume of water. A slight excess of concentrated ammonium hydroxide was added and immediately afterward sufficient glacial acetic acid to make the reaction slightly acid to litmus. The immediate crystallization was complete in twenty-four hours in the cold. The crystals were carefully washed with ice-cold water in small portions and finally with alcohol and ether. A 56% yield of plate crystals was obtained, which melted with decomposition at 275–276°. Recrystallization could be accomplished from either hydrochloric acid or hot water.

Anal. Calcd. for $C_9H_{11}O_3N$: N, 7.73. Found: N, 7.87, 7.68.

L-*m*-Tyrosine.—The identical procedure described above was used with the formyl-L-*m*-tyrosine. A quantitative yield of amino acid identical in all respects except with regard to direction of rotation was obtained.

Configuration of *m*-Tyrosine Isomers.—The amino acid isomers were incubated with a preparation of the D-amino acid oxidase according to the procedure previously described.[16] From 0.1 to 1.0 mg. was used in 5 ml. of pyrophosphate buffer. Since the keto acid corresponding to this amino acid was not available, calculations were made with the calibration factor for *p*-hydroxyphenylpyruvic acid.

The isomer possessing a positive rotation and which had been obtained from the alcohol insoluble brucine salt yielded keto acid values corresponding to 96.4–108% of the theoretical as may be seen in Table I and was designated as the D- or unnatural isomer. Conversely, the other isomer was designated as the L- or natural isomer.

TABLE I

m-TYROSINE ISOMERS AND D-AMINO ACID OXIDASE

Isomer	Amount incubated, micromoles	Keto acid obtained[a] Micromoles	%
Levorotatory	0.552	0	0
	1.10	0	0
	5.52	0	0
Dextrorotatory	0.552	0.533	96.6
	1.10	1.19	108.0
	5.52	5.86	106.0

[a] The keto acid values were calculated using the calibration factor for *p*-hydroxyphenylpyruvic acid.

The method of Lutz and Jirgenson[11,12] was also used. Rotations in the presence of different ratios of acid and alkali gave the results recorded in Fig. 1, and completely confirmed the enzymatic results.

α-Acetamido-*m*-hydroxycinnamic Acid.—In a solution of 12 g. (0.33 mole) of sodium hydroxide in 200 ml. of water, 24.5 g. (0.1 mole) of 2-methyl-4-(3′-acetoxybenzal)-5-oxazolone was suspended and heated to 60° with stirring. The resulting solution was treated with 10 g. of Norite and the warm filtrate was neutralized; yellow crystals were obtained after cooling. Upon recrystallization with further treatment with Norite, 18 g. (80%) of white crystals melting at 140–142° were obtained. The compound proved to be a monohydrate.

Anal. Calcd. for $C_{11}H_{11}O_4N.H_2O$: N, 5.86; H_2O, 7.53. Found: N, 5.97, 5.97; H_2O, 7.83.

(16) R. R. Sealock, *Science*, **94**, 73 (1941).

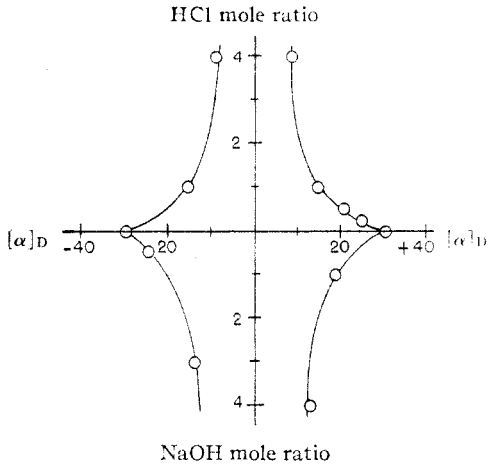

Fig. 1.—The optical rotation of the two isomers of *m*-tyrosine with different ratios of acid or base. The left hand curve corresponds to the L- or natural isomer and the right curve to the D- or unnatural configuration. In each instance the amino acid was present in 0.05 *M* solution.

N-Acetylcyclohexylalanine.—A solution of 11.1 g. (0.05 mole) of acetamido-*m*-hydroxycinnamic acid in 150 ml. of glacial acetic acid was reduced at 40 pounds pressure for two hours in the presence of 0.25 g. of platinum oxide catalyst. The catalyst was removed by filtration and the acetic acid by vacuum distillation. Crystallization of the thick oil from boiling water yielded plate crystals melting at 174–175°. A Millon's test was negative. Herbst and Shemin[13] record a melting point of 178° (cor.).

Anal. Calcd. for $C_{11}H_{19}O_3N$: N, 6.57. Found: N, 6.45, 6.43.

β-DL-Cyclohexylalanine.—Five grams of the above reduction product was refluxed twelve hours in 100 ml. of *N* hydrochloric acid. The amino acid was obtained by isoelectric precipitation. The 4.3 g. obtained melted at 229–230° with decomposition and gave a negative Millon test and a positive ninhydrin reaction.

Anal. Calcd. for $C_9H_{17}NO_2$: N, 8.18. Found: N, 8.09, 8.18.

N-Formyl-DL-cyclohexylalanine.—The formyl derivative was prepared from 4 g. of the amino acid according to the procedure of Clarke.[9] The residue upon recrystallization in 10 ml. of water, gave 3.2 g. (80%) of crystals melting at 135–136°.

Anal. Calcd. for $C_{10}H_{17}O_3N$: N, 7.03. Found: N, 7.13, 7.07.

N-Benzoyl-DL-cyclohexylalanine.—The above amino acid was benzoylated in the usual fashion and the resulting derivative exhibited a melting point of 184–185°. Herbst and Shemin[13] reported a value of 182–183.5°.

AMES, IOWA RECEIVED DECEMBER 22, 1949

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Streaming Orientation Studies on Denatured Proteins. III. Denaturation of Ovalbumin in the Presence of Urea[1]

BY JOSEPH F. FOSTER AND EDWARD G. SAMSA[2]

Studies have been made on the streaming birefringence of ovalbumin denatured in the presence of concentrated aqueous solutions of urea. The data indicate both unfolding and aggregation of the molecules. At *p*H values near 2.5 or above 9.0 aggregation is least serious and the measurements indicate that the preparations are nearly homogeneous with respect to molecular length. Such values of length lie invariably in the range 500–700 Å., a value taken as characteristic of the unfolded molecule in the absence of aggregation. At *p*H 2.5 denaturation for one hour at 37° in 7.5 *M* urea yields lengths which are substantially independent of protein concentration. This provides further suggestive evidence that the reaction under these conditions is essentially an intramolecular unfolding.

In the preceding papers of this series[3] an investigation was made of the heat denaturation of ovalbumin using the technique of streaming birefringence. It was shown that interpretation of the results on a molecular basis is not in general possible because of aggregation, even at *p*H values rather far removed from the isoelectric point. Evidence was given, however, that unfolding to lengths of 300 to 600 Å. does occur, depending on the conditions of denaturation.

One of the chemical agents often used for denaturing globular proteins is concentrated aqueous urea.[4] In this medium the denatured proteins usually remain soluble, a result which is of particular interest since it suggests that aggregation might be less serious in such media. The present paper summarizes some of the principal results of a study of the streaming birefringence of ovalbumin denatured in aqueous urea.

Experimental

Preparation of Solutions.—The weighed samples of ovalbumin[5] were dissolved in a small volume (x) of buffer or water, usually about 2.5 ml. The urea, usually 10.5 g., was dissolved in ($15 - x$) ml. of buffer or dilute HCl and brought to 37°. The solutions were mixed, held for the desired period of time at the desired temperature and cooled rapidly to 20–25°. Glycerol (42.0 g.) was then added, the solution filtered through sintered glass to remove any traces of floating debris, centrifuged at 20,000 × *g* to remove any smaller suspended impurities, and degassed by evacuating under a water aspirator. Such a solution is approximately 7.5 *M* in urea at the denaturation stage, and the final solution is 58.9% in glycerol (viscosity 18.6 centipoise at 25° as determined with an Ostwald pipet).

Streaming Birefringence Measurements.—The instrument used has been described previously.[3,6] In some of the measurements a new outer cylinder providing an annular gap of 0.50 mm. replaced the previously used cylinder (gap 1.0 mm.). This modification was desirable because of the lower birefringence obtained in the urea-containing media

(1) Journal Paper No. J-1947 of the Iowa Agricultural Experiment Station, Project 978. Supported in part by a grant from Swift and Company. Presented in part before the Division of Biological Chemistry at the Chicago Meeting of the A. C. S., Sept. 1950.

(2) Postdoctoral Fellow, Dept. of Chemistry, Iowa State College, 1950–1951. Present address: A. E. Staley Mfg. Co., Decatur, Ill.

(3) (a) J. F. Foster, and E. G. Samsa, THIS JOURNAL, **73**, 3187 (1951); (b) E. G. Samsa and J. F. Foster, *ibid.*, **73**, 3190 (1951).

(4) For a review of the literature in this field see H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(5) The ovalbumin was recrystallized at least three times with $(NH_4)_2SO_4$ by the method of Sørensen and Høyrup (*Compt. rend. trav. lab. Carlsberg Sér. chim.*, **12**, 12 (1917)), dialyzed free of salt and lyophilized.

(6) J. F. Foster and I. H. Lepow, THIS JOURNAL, **70**, 4169 (1948).