0.5 g. of 2-hydroxy-3-acetylaminofluorene, m.p. 215–216°. This material was soluble in dilute alkali and was reprecipitated upon addition of acid. After four more crystallizations from 50% ethanol the compound had a constant melting point of 225° dec., with some charring at 219°. The spectrum showed maxima at 242 mμ (ε 17,300), 275 (ε 14,600), 320 (ε 8,000), and minima at 233 mμ (ε 15,600), 260 (ε 11,600) and 300 (ε 4,800).

Anal. Calcd. for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48; N, 5.85. Found: C, 75.15; H, 5.59; N, 5.81.

B.—Reduction of 0.5 g. of pure 2-hydroxy-3-nitrofluorene with 5 g. of zinc dust and 0.5 g. of calcium chloride in 100 ml. of 75% ethanol[11] gave 0.2 g. of crude amine. Acetylation in acetate buffer gave 0.183 g. of product which charred at 220°, melted at 221–223°. Crystallization from dilute ethanol yielded 0.11 g. of 2-hydroxy-3-acetylaminofluorene, darkening at 220°, m.p. 225° dec., identical with that prepared by procedure A and with the material isolated from rat urine.

Anal. Calcd. for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48. Found: C, 75.33; H, 5.72.

Isolation Experiments.—Urine was collected from rats kept in metabolism cages while fed a diet containing 0.25 g. of 3-acetylaminofluorene per kg. The rats consumed 1400 g. of diet containing 0.35 g. of 3-AAF during this period while a total of 1540 ml. of urine was collected. The daily collection of urine was filtered and stored at 5° until used. The urine (*p*H 6) was extracted with ether in a continuous liquid–liquid extractor for 12 hours. The ether extract (500 ml.) was washed with two 20-ml. portions of 1% sodium bicarbonate solution, then with 20 ml. of water, 20 ml. of 0.1 *N* hydrochloric acid followed by washing with water (70 ml.) until the ether extract was no longer acidic. The hydrochloric acid wash and the water washes were combined and tested for the presence of 3-aminofluorene by diazotizing, coupling with R-salt and reading the red color.[13] The acid washes contained diazotizable material equivalent to 2.3 mg. of 3-aminofluorene.

The washed ether extract was taken to dryness on the steam-bath, the residue dissolved in 25 ml. of ethanol and refluxed with 15 mg. of Norit. The mixture was filtered, boiled down to approximately 10 ml. and water (about 10 ml.) added to incipient cloudiness.

After standing in a refrigerator overnight shiny brown crystals were obtained which weighed 40 mg., m.p. 210–212°. This material was crystallized once from benzene and three times more from dilute ethanol to yield 6.4 mg. of shiny tan plates, which sintered and charred at 218° and melted to a black paste at 225°. A mixture with synthetic 2-hydroxy-3-acetylaminofluorene charred at 219° and melted at 225°. The ultraviolet absorption spectra of the isolated and synthetic material were practically identical.

Anal. Calcd. for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48. Found: C, 75.14; H, 5.71.

(13) B. B. Westfall and H. P. Morris, *J. Natl. Cancer Inst.*, **8**, 17 (1947).

LABORATORY OF BIOCHEMISTRY
NATIONAL CANCER INSTITUTE
NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE
U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE
BETHESDA 14, MARYLAND

Separation of the Three Isomeric Components of Synthetic α,ε-Diaminopimelic Acid[1]

BY ELIZABETH WORK,[2] SANFORD M. BIRNBAUM, MILTON WINITZ AND JESSE P. GREENSTEIN

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The symmetrical α,ω-diaminodicarboxylic acids of which cystine is the most common representative exist in two racemic modifications, one a mixture of externally compensated isomerides, the other as a non-resolvable internally compensated *meso* form. These modifications may be described in terms of three isomeric components whose optical configurations are represented by L,L and D,D which together form the racemate, and by L,D which is the *meso* form. Among this class of compounds is α,ε-diaminopimelic acid, which is of particular contemporary interest because of its presence in bacterial products,[3,4] and because of its role as a precursor in the biosynthesis of lysine.[5,6]

For further biological studies on this compound, and for purposes of identification of the products isolated from bacterial sources, it was considered desirable to have available all three isomeric forms. To accomplish this purpose, a synthetic mixture of the three forms of diaminopimelic acid was converted into the diamide and treated with a hog kidney amidase-Mn^{++} preparation, a method successfully employed in this Laboratory to resolve the racemic amides of proline,[7] histidine,[8] S-benzylcysteine,[8] and *t*-leucine.[9] In the present instance, the action of this exclusively L-directed enzyme led to a mixture of the free L,L-diaminopimelic acid, the D,D-diamide, and the L-diaminopimelic acid D-monoamide. Paper chromatography (phenol, NH_3) of the protein-free reaction mixture revealed the three components as distinct ninhydrin-reactive spots and was subsequently employed to follow their separation on an XE-64 Amberlite cation-exchange resin. The separated amides were hydrolyzed to the respective free diaminopimelic acid isomers. The optical rotation values (Table I) indicate that resolution was achieved.

Experimental

α,ε-Diaminopimelic Acid.—The general procedure for the synthesis of amino acids developed by Sheehan and Bolhofer[10] was employed here.

To 203 g. of diethyl α,ε-dibromopimelate,[11] dissolved in 920 ml. of dimethylformamide, was added 296 g. of potassium phthalimide. The reaction mixture was heated over the steam-bath for 2 to 3 hours, with occasional shaking. After cooling to room temperature, 1040 ml. of chloroform was added and the mixture then poured into 4 l. of water. The aqueous layer was separated and extracted twice with 800-ml. portions of chloroform. The combined chloroform extract was washed once with 0.1 *N* sodium hydroxide and twice with water. After drying with anhydrous sodium sulfate, concentration of the chloroform layer under reduced pressure yielded 275.5 g. of a clear oil. The oil was dissolved in 2.5 l. of absolute methanol, 34.9 ml. of anhydrous hydrazine added and the solution refluxed over a steam-bath for 3 hours. The resulting suspension was concentrated under a stream of air, 1.3 l. of water added and the remaining methanol driven off under reduced pressure. After the addition of 1.3 l. of concd. hydrochloric acid, the mixture was refluxed for 4 hours. The acid hydrolysate was cooled to 0° and the precipitate (phthalyl hydrazide)

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(2) On leave from University College Hospital Medical School, London. Aid from the Anna Fuller Fund for a travel grant is gratefully acknowledged.

(3) E. Work, *Biochem. J.*, **49**, 17 (1951).

(4) E. Work and D. L. Dewey, *J. Gen. Microbiol.*, **9**, 394 (1953).

(5) D. L. Dewey and E. Work, *Nature*, **169**, 533 (1952).

(6) B. D. Davis, *ibid.*, **169**, 534 (1952).

(7) D. Hamer and J. P. Greenstein, *J. Biol. Chem.*, **193**, 81 (1951).

(8) L. Levintow, V. E. Price and J. P. Greenstein, *ibid.*, **184**, 55 (1950).

(9) N. Izumiya, S-C. J. Fu, S. M. Birnbaum and J. P. Greenstein, *ibid.*, **205**, 221 (1953).

(10) J. C. Sheehan and W. A. Bolhofer, THIS JOURNAL, **72**, 2786 (1950).

(11) R. Willstätter, *Ber.*, **28**, 660 (1895).

filtered off. The filtrate was concentrated to a paste under reduced pressure, 200 ml. of water added, and the concentration repeated. The residue was taken up in the minimal amount of water and the *p*H of the solution adjusted to 6.2 with saturated lithium hydroxide. The solution was decolorized with activated charcoal and filtered (total volume at this point was 1 l.). Five volumes of absolute ethanol was added and the mixture placed at −10° overnight. The precipitated oil was separated by decantation and dissolved in 600 ml. of hot water. Upon the addition of five volumes of alcohol, a white precipitate formed which was filtered off after 12 hours at −10°. Recrystallization was effected once more in a similar manner; yield 95.6 g.

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.4; α-COOH-N, 7.8. Found: C, 43.8; H, 7.4; α-COOH-N, 7.8.

Sörensen and Andersen have reported a preparation of this compound *via* another procedure.[12]

Diaminopimelic Acid Diamide Dihydrochloride.—Twenty-three grams of synthetic diaminopimelic acid was esterified in methanol–HCl mixture, and solvent and excess HCl removed *in vacuo* over NaOH. The sirupy product was treated at 2° with saturated NH_3–methanol and, after the solution had stood at this temperature for 4 days, the solvent was removed by distillation. The residual diamide dihydrochloride, which was a yellow oil, was purified by repeated precipitation from methanol (Norit) with dry ether whereby it was converted to a white amorphous solid soluble only in a relatively large volume (1500 ml.) of methanol. Concentration of this solution under a jet of air to about 50 ml. yielded a deliquescent crystalline precipitate (8 g.) which was recrystallized twice from methanol. A further fraction was obtained from the original mother liquor by treatment with either absolute ethanol or *n*-butanol. The deliquescent precipitate which separated was recrystallized from methanol by addition of *n*-butanol; yield 6.4 g. Both fractions on paper chromatography using S and S 598 paper and phenol–NH_3 as solvent yielded a single ninhydrin spot with R_f = 0.95.

Anal. Calcd. for $C_7H_{18}O_2N_4Cl_2$: C, 32.2; H, 6.9; N, 21.4; Cl, 27.2; amide N, 10.7. Found: C, 32.3; H, 7.0; N, 21.1; Cl, 26.6; amide N, 10.2.

The less soluble fraction was mainly the *meso* form with a relatively small amount of the racemate present. The more soluble fraction consisted chiefly of the racemic form. This distribution was determined by digesting 13 mg. of each fraction with the amidase-Mn^{++} enzyme as described in the next section and by subsequent paper chromatography of the deproteinized reaction products at various times during the hydrolysis. The less soluble form yielded mainly a substance having an R_f of 0.69 (subsequently identified as the monoamide derived from the *meso* form of diamide). This compound increased continuously during the course of the digestion. A small amount of free amino acid (from the L,L-diamide) also appeared, and an equal amount of unhydrolyzed diamide remained (D,D-isomer). When the more soluble diamide was hydrolyzed, a monoamide again appeared in the earlier stages of hydrolysis, but later it decreased in concentration due to conversion to the free amino acid which showed a corresponding rise in concentration with time. The final mixture consisted mainly of equal amounts of free amino acid and unchanged diamide with only a minor amount of monoamide remaining.

Inasmuch as the two diamide components could not be completely separated from each other, it was considered best to recombine them and resolve the mixture enzymically.

Diaminopimelic Acid Diamide Dipicrate.—The most soluble portion of the diamide was converted to the dipicrate for further characterization. The crystalline picrate could not be recrystallized owing to insolubility, and was purified by washing with boiling water; decomp. point 260°.

Anal. Calcd. for $C_{18}H_{22}N_{10}O_{16}$: C, 35.3; H, 3.4; N, 21.7. Found: C, 35.4; H, 3.9; N, 21.7.

Resolution of Diaminopimelic Acid Diamide Dihydrochloride.—Fourteen grams of the mixed diamide hydrochlorides was dissolved in water, the solution adjusted to *p*H 8.0 by addition of LiOH, and $MnCl_2$ solution added to bring the final Mn^{++} concentration to 0.01 *M*. A volume of 80 ml. of dialyzed hog kidney amidase preparation[13] containing 285 mg. N was added together with sufficient water to bring the final volume to 570 ml. Progress of the digestion at 37° was followed by manometric ninhydrin–CO_2 determinations and when, after 7 hours, the α-COOH-N value had reached a constant level, more enzyme solution was added and the mixture incubated further to ensure complete hydrolysis. No change in the α-COOH-N level was evident, and the mixture was dialyzed at 2° against several changes of water to separate the digestion products from protein. The pooled dialysates were concentrated *in vacuo* below 35° to 50 ml.

The concentrate was adjusted to *p*H 9.2 with LiOH and immediately run into a column (3.5 × 90 cm.) of Amberlite XE-64 cation-exchange resin which had been converted to the Li form, buffered with lithium acetate to *p*H 6.5 and washed with water. All effluents were examined on filter paper for ninhydrin reaction, and those giving a positive reaction were subjected to paper chromatography using S and S 598 paper and phenol–NH_3 solvent. Water (1.1 l.) was run through until the effluent was ninhydrin negative. The main band in the aqueous effluent consisted of diaminopimelic acid, R_f = 0.27, which came sharply off the column. It was slightly retarded in relation to a small amount of diamide, R_f = 0.92, which leaked through owing probably to its being left originally as the hydrochloride through insufficient neutralization. There were also other small bands of unidentified materials (R_f = 0.15, 0.55, 0.62) derived probably from proteolysis in the enzyme preparation. These were easily removed during subsequent recrystallizations. On passing to 1% (v./v.) acetic acid as eluant, the first band to come off the column was lysine (R_f = 0.78) whose origin is at present unknown. The lysine was well separated (by 2 l.) from the monoamide which came off as a large extended band (R_f = 0.69) overlapping somewhat with diamide (R_f = 0.92). The overlap was fortunately insufficient to interfere with the resolution, and pure monoamide and diamide fractions were obtained, the overlapping portion being worked up separately.

L,L-Diaminopimelic Acid.—The aqueous effluent containing free diaminopimelic acid (R_f = 0.27) was adjusted to *p*H 6.2 with dilute HCl, concentrated *in vacuo* to a low bulk, and treated with Norit. Excess ethanol was added to the filtrate, producing an intractable gel. The gel was filtered, dehydrated, redissolved in water, and a further ethanol precipitation was attempted with the same production of a gel. Crystallization of the compound was finally effected by slow evaporation of the aqueous solution at 25° in a vacuum desiccator over P_2O_5. The yield was 2.0 g. Recrystallization in the same manner yielded 1.5 g. of the compound. On drying *in vacuo* at 100° it lost 9% in weight, equivalent to one molecule of water of crystallization.

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.42; N, 14.8. Found: C, 43.9; H, 7.33; N, 14.8.

The mother liquor from the recrystallization was treated with a few drops of concentrated HCl, and acetone was added slowly until a slight cloudiness developed. On further standing at 25° the monohydrochloride crystallized.

Anal. Calcd. for $C_7H_{15}O_4N_2Cl$: C, 37.1; H, 6.63; N, 12.3; Cl, 15.7. Found: C, 36.5; H, 6.65; N, 12.6; Cl, 15.5.

The optical rotations of these compounds are described in Table I.

TABLE I

OPTICAL ROTATIONS OF THE ISOMERIC COMPONENTS OF α,ε-DIAMINOPIMELIC ACID

Compound	H_2O (*c* 5)	$[\alpha]^{24}D$ in 5 *N* HCl (*c* 2.6)
L,L-Diaminopimelic acid	+8.14	+45.1°
L,L-Diaminopimelic acid·HCl	....	+38.5° (+45.8° as free compound)
D,D-Diaminopimelic acid	−8.45	−44.6°
D,D-Diaminopimelic acid·HCl	....	−38.0° (−45.2° as free compound)
L,D-Diaminopimelic acid (*meso*)	....	0°

(12) S. P. L. Sörensen and A. C. Andersen, *Z. physiol. Chem.*, **56**, 283 (1908).

(13) *Cf.* S. M. Birnbaum, "Enzyme Preparations," Vol. II, Academic Press, Inc., New York, N. Y., in press.

L-Diaminopimelic Acid D-Monoamide Hydrochloride.—The acetic acid effluents (3,460–6,300 ml.) containing only material with R_f = 0.69 were combined and concentrated *in vacuo* to a sirup. On prolonged standing at 0° a bulky mass of lithium acetate separated. The supernatant solution was decanted and acidified to *p*H 2.0 with HCl. Addition of ethanol produced only a small amount of oil, but subsequent addition of acetone yielded a solid precipitate. Both solid and oil were dissolved in about 1 l. of boiling methanol. On concentration, 4.0 g. of a microcrystalline solid was obtained, which was purified by twice dissolving in water, treating with Norit, and precipitating with ethanol; m.p. 225° dec.

Anal. Calcd. for $C_7H_{16}O_3N_2Cl$: C, 37.1; H, 7.5; N, 18.5. Found: C, 36.8; H, 7.6; N, 18.3.

***meso*-Diaminopimelic Acid.**—The previous compound was dissolved in 3 *N* HCl and the solution refluxed for 5 hours. The solvent was removed *in vacuo*, the residue dissolved in a little water and adjusted to *p*H 6.2 with LiOH. From this solution, the free *meso*-diaminopimelic acid was precipitated as a solid by addition of excess ethanol. It was recrystallized twice as the free amino acid by adding ethanol to incipient turbidity of its aqueous solutions, and once as the hydrochloride from aqueous acetone. The *meso* form differs from the L,L- (and D,D) form in crystallizing readily from ethanol–water instead of forming a gel. It is also less soluble in water.

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.42; N, 14.8. Found: C, 43.6; H, 6.84; N, 14.6.

D,D-Diaminopimelic Acid.—The column eluate (7,000–7,800 ml.) containing only the diamide of D,D-diaminopimelic acid was concentrated *in vacuo* to a sirup, the residue taken up in 200 ml. of 2 *N* HCl, and the solution refluxed for 2 hours. Paper chromatography of the neutralized hydrolysate showed that hydrolysis had been incomplete, and that there was present a mixture of diamide, monoamide and free amino acid in the approximate ratios of 1:4:1. Hydrochloric acid was thereupon added to 3 *N* concentration and the solution refluxed for 5 hours. At the end of this period only free amino acid remained. The solution was evaporated to dryness *in vacuo* to remove excess HCl, the residue was dissolved in a little water, and the solution brought to *p*H 6.2 with LiOH. Addition of ethanol produced a gel as in the case of the L,L-isomer. The compound was therefore crystallized from water by slow evaporation in a vacuum desiccator. The yield of dried amino acid was 0.85 g. Its optical rotation is given in Table I.

Anal. Calcd. for $C_7H_{14}O_4N_2$: C, 44.2; H, 7.42; N, 14.8. Found: C, 43.9; H, 7.41; N, 14.9.

The monohydrochloride was prepared as in the case of the L,L-isomer.

Anal. Calcd. for $C_7H_{15}O_4N_2Cl$: C, 37.1; H, 6.63; N, 12.3; Cl, 15.7. Found: C, 36.5; H, 6.28; N, 12.1; Cl, 15.8.

Carbobenzoxylation of Diamide and Monoamide Mixture.—The fraction of the eluate from the overlapping area (6,300–7,000 ml.) of the bands for the L,D-monoamide and D,D-diamide was treated in molar K_2CO_3 solution at 0° with 10 g. of carbobenzoxy chloride alternately with 5 ml. of 10 *N* K_2CO_3. The mixture was stirred at 0° for 4 hours and allowed to stand at this temperature for 18 hours. A semisolid mass separated which was filtered, washed with cold dilute K_2CO_3 solution and subsequently with water, and finally dried *in vacuo* over P_2O_5. It was extracted with an ether–petroleum mixture to remove excess carbobenzoxy chloride. The residual powder, weighing 3.9 g., was extracted several times with boiling 1:1 chloroform–ethyl acetate mixture, and this residue weighed 3.0 g. It was crystallized from ethanol in a yield of 1.8 g., m.p. 236°. Analysis revealed the compound to be dicarbobenzoxydiaminopimelic acid diamide. From the crystallization mother liquor a less pure fraction weighing 0.6 g., with m.p. 220°, could be isolated.

Anal. Calcd. for $C_{23}H_{28}O_6N_4$: C, 60.5; H, 6.14; N, 12.3. Found: C, 60.0; H, 6.48; N, 12.5.

A part of the pure dicarbobenzoxydiaminopimelic acid diamide was dissolved in an ethanol–acetic acid mixture and hydrogenated in the presence of palladium. Paper chromatography confirmed the presence of the pure diaminodicarboxylic acid diamide. The filtered solution was evaporated *in vacuo* to dryness, the residue dissolved in water, and the aqueous solution extracted with ethyl acetate. The aqueous layer was neutralized and mixed with buffer at *p*H 8.0, $MnCl_2$ solution, and amidase preparation. After incubation at 37° for 7 hours, the solution was deproteinized and examined by paper chromatography as above. Only a single ninhydrin spot characteristic of the diamide of diaminopimelic acid was observed. This resistance to the enzyme would be expected of D,D-diaminopimelic acid diamide.

The K_2CO_3 filtrate from the carbobenzoxylation procedure should presumably have contained the carbobenzoxy derivative of L-diaminopimelic acid D-monoamide. Acidification of this filtrate, however, yielded no evidence of the expected precipitate of this compound. Inasmuch as sufficient amounts of this *meso* form were obtained from the pure chromatographic band, it was considered unprofitable at this time to pursue its whereabouts in the present fraction any further.

Dicarbobenzoxydiaminopimelic Acid.—1.9 grams of the synthetic mixture of diaminopimelic acid dissolved in 27 ml. of NaOH was shaken at 0° with 3.8 g. of carbobenzoxy chloride. After acidification, the mixture was extracted with ethyl acetate, the extract dried over Na_2SO_4, and condensed in volume. Treatment with petroleum ether led to the separation of crystals which were crystallized twice from chloroform; m.p. 137–141°.

Anal. Calcd. for $C_{23}H_{26}O_8N_2$: C, 60.3; H, 5.63; N, 6.11. Found: C, 60.2; H, 5.63; N, 6.12.

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