TABLE I

N-(ARYLAMINOMETHYL)-PHTHALIMIDE DERIVATIVES

Amine used	M.p., °C.	Yield, %	Formula	Nitrogen, % Calcd.	Found
Aniline[a]	144.5–145	86	$C_{15}H_{12}N_2O_2$	...	...
o-Bromoaniline	117–118.5	47	$C_{15}H_{11}BrN_2O_2$	8.45	8.53
m-Bromoaniline[b]	167.5–168.5	83	$C_{15}H_{11}BrN_2O_2$	8.45	8.53
p-Bromoaniline[b]	215–216	91	$C_{15}H_{11}BrN_2O_2$	8.45	8.45
2-Bromo-4-methylaniline	117–119	43	$C_{16}H_{13}BrN_2O_2$	8.11	8.14
o-Chloroaniline	141–141.5	32	$C_{15}H_{11}ClN_2O_2$	9.77	9.69
m-Chloroaniline	169	50	$C_{15}H_{11}ClN_2O_2$	9.77	9.56
p-Chloroaniline[b]	207–208	73	$C_{15}H_{11}ClN_2O_2$	9.77	9.59
2,4-Dichloroaniline	139–140		$C_{15}H_{10}Cl_2N_2O_2$	8.72	8.70
p-Iodoaniline[b]	168–169 d.	58	$C_{15}H_{11}IN_2O_2$	7.41	7.30
o-Toluidine	137–138	72	$C_{16}H_{14}N_2O_2$	10.52	10.45
m-Toluidine	138–139	40	$C_{16}H_{14}N_2O_2$	10.52	10.47
p-Toluidine[b]	174.5–175.5	85	$C_{16}H_{14}N_2O_2$	10.52	10.56
2,4-Dimethylaniline	130–132.5	67	$C_{17}H_{16}N_2O_2$	10.00	10.08
2,5-Dimethylaniline	131–133	84	$C_{17}H_{16}N_2O_2$	10.00	10.01
o-Anisidine	112–112.5	90	$C_{16}H_{14}N_2O_3$	9.93	10.15
p-Anisidine	145	87	$C_{16}H_{14}N_2O_3$	9.93	9.72
o-Phenetidine	119–120	85	$C_{17}H_{16}N_2O_3$	9.46	9.66
p-Phenetidine	152–153	63	$C_{17}H_{16}N_2O_3$	9.46	9.55
p-Aminoacetanilide	211	73	$C_{17}H_{15}N_3O_3$	13.59	13.70
p-Aminoacetophenone[c]	165–165.5	52	$C_{17}H_{14}N_2O_3$	9.52	9.66
2-Aminobiphenyl	193–193.5	66	$C_{21}H_{16}N_2O_2$	8.53	8.32
4-Aminobiphenyl[b]	189–189.5	99	$C_{21}H_{16}N_2O_2$	8.53	8.72
Diphenylamine[d]	169.5–170	12	$C_{21}H_{16}N_2O_2$	8.53	8.59
2-Aminopyridine[e]	184	23	$C_{14}H_{11}N_3O_2$	16.60	16.52
Anthranilic acid[b,c]	188–189 d.	56	$C_{16}H_{12}N_2O_4$	9.46	9.71
m-Aminobenzoic acid[b,c]	200–200.5 d.	73	$C_{16}H_{12}N_2O_4$	9.46	9.44
p-Aminobenzoic acid[b,c]	232 d.	50	$C_{16}H_{12}N_2O_4$	9.46	9.54
Ethyl *p*-aminobenzoate[c]	176.5	47	$C_{18}H_{16}N_2O_4$	8.63	8.43
Carbazole[e]	241–242	27	$C_{21}H_{14}N_2O_2$	8.59	8.62

[a] Sachs[1b] reported m.p. 144–145° (Beilstein, "Handbuch der Organischen Chemie," Vol. XXI, fourth edition, p. 477). [b] Recrystallized from dioxane–petroleum ether (65–110°). [c] Reaction medium refluxed one hour. [d] Reaction medium refluxed 2.75 hours. [e] Reaction medium refluxed two hours.

350 ml. of water are refluxed until a clear solution results. The hot solution is filtered and cooled overnight, and the white, crystalline product obtained is filtered and dried. The yield is 112.7 g. (90%), m.p. 138–141° (reported 137–141°).

N-(Anilinomethyl)-phthalimide.—Two and one-half grams (0.0141 mole) of N-hydroxymethylphthalimide is dissolved in 25 ml. of boiling 80% ethanol. One and one-half grams (0.0161 mole) of aniline is added, and the solution immediately turns yellow. The reaction medium is refluxed for 30 minutes, cooled, and the yellow crystalline product is collected; yield 2.5 g. (70%), m.p. 145–145.5°.

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2-Hydroxy-3-acetylaminofluorene, A Metabolite of 3-Acetylaminofluorene in the Rat[1]

BY ELIZABETH K. WEISBURGER

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As part of a study of the comparative carcinogenic effects of positional isomers of 2-acetylaminofluorene, it was desired to test the activity of 3-acetylaminofluorene. The intermediate, 3-aminofluorene, had been synthesized previously by Hayashi and Nakayama[2] and also by Campbell and Stafford[3] but by procedures which could not readily be adapted for the preparation of relatively large quantities of 3-aminofluorene.

In the present investigation the methods of Bradsher and Jackson[4] for the preparation of 5-nitro-2-cyanobiphenyl and of Ray and Barrick[5] for the synthesis of 3-aminofluorenone from the cyanobiphenyl were used. The 3-aminofluorenone was readily reduced to 3-aminofluorene by use of the Huang-Minlon[6] modification of the Wolff–Kishner reaction. The melting points of the 3-aminofluorene and the acetyl derivative agreed with those reported by Hayashi and Nakayama.[2] However, the ultraviolet absorption spectra showed maxima and minima at lower wave lengths which were not reported by the Japanese authors.

If hydroxylation occurred during metabolism of 3-acetylaminofluorene, three possible compounds might result. *o*-Hydroxylation could yield either

(1) Presented before the Biological Chemistry Division at the 126th National Meeting, New York, September, 1954.

(2) M. Hayashi and A. Nakayama, *J. Soc. Chem. Ind. Japan*, Suppl. binding, **36**, 127B (1933).

(3) N. Campbell and W. H. Stafford, *J. Chem. Soc.*, 299 (1952).

(4) C. K. Bradsher and W. J. Jackson, Jr., THIS JOURNAL, **74**, 4880 (1952).

(5) F. E. Ray and J. G. Barrick, *ibid.*, **70**, 1492 (1948).

(6) Huang-Minlon, *ibid.*, **68**, 2487 (1946).

the 2- or 4-hydroxy derivative. If hydroxylation took place in the ring not containing the acetylamino group, 3-acetylamino-7-hydroxyfluorene would probably be produced.

Isolation studies were carried out on the urine of rats fed 3-acetylaminofluorene.[7] The presence of the deacetylation product, 3-aminofluorene, in the ether extract of the urine was demonstrated by colorimetric methods. In addition, a small quantity of material analyzing correctly for a hydroxyacetylaminofluorene was isolated. By comparison of the ultraviolet absorption spectra and by mixture melting points, this material was shown to be 2-hydroxy-3-acetylaminofluorene. This compound had been synthesized previously by Ruiz[8] who reported it as melting at 215°. Ray and Hull[9] claimed later that 2-hydroxy-3-acetylaminofluorene melted at 163° while 2-acetoxy-3-acetylaminofluorene was the compound melting at 215°.

In this investigation 2-hydroxy-3-aminofluorene[8] was acetylated in aqueous acetate buffer, which generally does not attack the phenolic hydroxy group. The product had an apparent constant melting point of 216° but did not analyze correctly. Further repeated crystallizations raised the melting point to 225° whereupon the material analyzed correctly for 2-hydroxy-3-acetylaminofluorene.

The analysis of the compound, m.p. 161–162°, obtained by acetylation of 2-hydroxy-3-aminofluorene according to Ray and Hull, indicated that it was an oxazole, probably 2-methyl-9H-[3,2]-oxazole. The ultraviolet spectrum of the oxazole indicated ring formation while the infrared spectrum[10] lacked hydroxyl and amide bands. On the other hand the infrared spectrum of the 2-hydroxy-3-acetylaminofluorene showed a strong hydroxyl band at 3.15 μ and amide bands at 6.13 and 6.55 μ.

Nitration of 2-hydroxyfluorene[9] followed by chromatography of the product on alumina gave 2-hydroxy-3-nitrofluorene and a red compound thought to be 1,3-dinitro-2-hydroxyfluorenone since its ultraviolet absorption spectrum showed only one major peak.[11]

Experimental[12]

5-Nitro-2-biphenylcarboxylic Acid.—A solution of 24 g. of 5-nitro-2-cyanobiphenyl in 200 ml. of concentrated sulfuric acid, 200 ml. of water and 100 ml. of glacial acetic acid was refluxed for six hours. The precipitate obtained after dilution with water was worked up to yield 19 g. (73%) of 5-nitro-2-biphenylcarboxylic acid instead of 46% as reported by Ray and Barrick.[5]

3-Aminofluorene.—A mixture of 35 g. of crude 3-aminofluorenone, prepared from 5-nitro-2-biphenylcarboxylic acid according to the method of Ray and Barrick,[5] 21 g. of potassium hydroxide, 400 ml. of diethylene glycol and 50 ml. of 95% hydrazine hydrate was refluxed for 2.5 hours. The condenser was removed until the temperature of the boiling mixture reached 205°. The solution was refluxed 1.5 hours more. After cooling the mixture was poured into 1 l. of ice-water, yielding a white precipitate, m.p. 149–150°. Crystallization from ethanol gave 27 g. of product as shiny light tan plates, m.p. 152–153°.

The ultraviolet absorption spectrum in ethanol had maxima at 245 mμ (ϵ 19,900), 262 (ϵ 10,500), 274 (ϵ 10,100), 296 (ϵ 2,820), 320 (ϵ 5,200) and minima at 232 mμ (ϵ 16,500), 261 (ϵ 10,300), 272 (ϵ 9,770), 294 (ϵ 2,620) and 300 (ϵ 2,720).

The **acetyl derivative**, prepared in benzene with acetic anhydride, melted at 189–190° as reported by Hayashi and Nakayama.[2] The spectrum had maxima at 245 mμ (ϵ 31,260), 300 (ϵ 6,300), 312 (ϵ 6,800), and minima at 227 mμ (ϵ 16,100), 287 (ϵ 3,650) and 307 (ϵ 5,100).

3-Benzoylaminofluorene after crystallization from ethanol formed lustrous flat crystals, m.p. 204–205°.

Anal. Calcd. for $C_{20}H_{15}NO$: C, 84.18; H, 5.30. Found: C, 84.07; H, 5.34.

3-*p*-Toluenesulfonamidofluorene, prepared from 3-aminofluorene and *p*-toluenesulfonyl chloride in pyridine, crystallized from dilute ethanol as fine white needles, m.p. 149–150°. A mixture with 3-aminofluorene melted at 115–120°.

Anal. Calcd. for $C_{20}H_{17}NO_2S$: C, 71.61; H, 5.11; N, 4.18. Found: C, 71.49; H, 5.18; N, 4.31.

2-Methyl-9H-fluoreno[3,2]oxazole.—One gram of 2-hydroxy-3-aminofluorene, prepared according to the method of Ruiz from 2-hydroxyfluorene and benzenediazonium chloride, was acetylated according to the method of Ray and Hull.[9] After cooling the crude 2-hydroxy-3-acetylaminofluorene was filtered off and the filtrate poured into ice-water. The precipitate obtained was dissolved in benzene which was percolated through an alumina column. The benzene eluate yielded 0.34 g. of white crystals. After several crystallizations from benzene or benzene–petroleum ether there was obtained 0.152 g. of 2-methyl-9H-fluoreno-[3,2]oxazole, m.p. 161–162°. The spectrum had maxima at 236 mμ (ϵ 27,500), 261 (ϵ 15,300), 284 (ϵ 3,900), 304 (ϵ 13,100), 310 (ϵ 13,100) and 317 (ϵ 16,700) while minima appeared at 234 mμ (ϵ 26,600), 255 (ϵ 13,600), 281 (ϵ 3,200), 286 (ϵ 3,500), 307 (ϵ 11,600) and 313 (ϵ 11,100), with shoulders at 266 (ϵ 13,800) and 299 (ϵ 8,300).

Anal. Calcd. for $C_{15}H_{11}NO$: C, 81.42; H, 5.01; N, 6.33. Found: C, 81.52; H, 4.82; N, 6.38.

2-Hydroxy-3-nitrofluorene.[8,9]—Three grams of 2-hydroxyfluorene in 75 ml. of glacial acetic acid was nitrated with 2.1 ml. of 1:1 nitric acid–water at 17° and stirred one hour longer at room temperature. The crude product weighing 3.7 g. sintered and melted from 110–170°. It was dissolved in benzene and chromatographed on alumina, the lower reddish-brown band being eluted by benzene. The benzene eluate was percolated through a second alumina column and taken to dryness. The orange product, wt. 2.1 g., melted at 132–134°. After further crystallization from benzene, dilute ethanol, or after vacuum sublimation it melted at 133°. The spectrum had maxima at 268 mμ (ϵ 26,500), 295 (ϵ 12,000), and 384 (ϵ 2,900) with minima at 230 mμ (ϵ 6,500), 293 (ϵ 11,200), and 334 (ϵ 1,100).

Anal. Calcd. for $C_{13}H_9NO_3$: C, 68.72; H, 3.99; N, 6.17. Found: C, 68.76; H, 4.48; N, 5.99.

1,3-Dinitro-2-hydroxyfluorenone.—The dark red material (m.p. >250°) obtained from the acetic acid eluates from chromatographing crude 2-hydroxy-3-nitrofluorene was dissolved in ethanol, filtered, diluted with water and acidified with 10 ml. of concentrated hydrochloric acid. The tan precipitate was filtered off and washed, wt. 0.8 g., m.p. sintering at 187°, melting at 245–247°. Sublimation of 0.3 g. at a pressure of 40–60 μ and 250 gave 0.1 g. of orange-red material, m.p. 256°. This was crystallized from dilute acetone to give 84 mg. of red needles, m.p. 255–256°. The ultraviolet spectrum in ethanol had maxima at 269 mμ (ϵ 31,000) and 370 (ϵ 3,800) and minima at 230 mμ (ϵ 14,000) and 354 (ϵ 3,600) with a shoulder at 311 mμ (ϵ 9,100).

Anal. Calcd. for $C_{13}H_6N_2O_5$: C, 54.55; H, 2.11; N, 9.79. Found: C, 54.76; H, 2.29; N, 9.27.

2-Hydroxy-3-acetylaminofluorene. A.—A solution of 1 g. of 2-hydroxy-3-aminofluorene[8] in 350 ml. of water and 3.5 ml. of concentrated hydrochloric acid was filtered, cooled and the *p*H adjusted to 5 by addition of 6.5 g. of crystalline sodium acetate. The mixture was placed in an ice-bath, stirred vigorously and 6 ml. of acetic anhydride added. After being stirred for 6 hours in the ice-bath, the precipitate was filtered off and washed with water. The yield was 0.97 g., m.p. 204–206°. Crystallization from 50% ethanol gave

(7) The details and results of the long term biological experiments will be reported by Dr. Harold P. Morris.

(8) C. Ruiz, *Anales assoc. Quim. Argentina*, **16**, 170 (1928).

(9) F. E. Ray and C. F. Hull, *J. Org. Chem.*, **14**, 394 (1949).

(10) The infrared spectra of these compounds were kindly recorded by Mr. Robert Koegel.

(11) E. K. Weisburger and J. H. Weisburger, *J. Org. Chem.*, **19**, 964 (1954).

(12) Analyses by Dr. William C. Alford and Mr. Robert Koegel. The ultraviolet absorption spectra were determined on a Cary recording spectrophotometer by Miss Rita McCallum.

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).

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

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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).

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

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

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