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2-ACETYLAMINO-5-HYDROXYFLUORENE, A METABOLITE OF THE CARCINOGEN 2-ACETYLAMINOFLUORENE[1]

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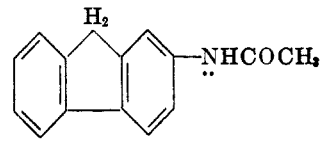
Although hydroxylation is an important reaction in the metabolism of aromatic compounds by animals, the intimate mechanism of this reaction is not clear (1). It was found in those cases studied adequately that where the aromatic ring bore an electron-releasing grouping hydroxylation occurred *orthopara*. Where the first substituent acted as an electron sink further attack was on the *meta* carbon, although some *para* substitution also took place. Thus, from such known cases predictions can be advanced on the points of attack in a molecule of more complex structure.

Electrophilic substitution, for example, nitration, of fluorene involves the 2 position. Neish (2) has also shown that biological hydroxylation of fluorene yields 2-hydroxyfluorene. Disubstitution of this hydrocarbon occurs mostly at the 2, 7 and to a lesser extent at the 2, 5 positions (3), reflecting a concentration of negative charges under the influence of the attacking positive ion at the 2, 5, and 7 positions.



When fluorene is substituted at the 2 position with an electron-releasing grouping such as acetylamino it might be expected that the free electron pair of the nitrogen would contribute increased electron density at certain parts of the ring system. As demonstrated by the formulas the higher charges will be expected to concentrate on carbons 1, 3, 5, and 7. It is to be noted that the 5 and 7 carbons are thus favored not only by the increased density owing to the nitrogen electron resonance but also by mesomeric effects in the fluorene ring system itself. Similar reasoning can be applied to the case of biphenyl and the corresponding 4-acetylaminobiphenyl.

The supplementary effects of the 5-membered ring and particularly of the aliphatic type 9-carbon atom should therefore be considered. The 5-membered ring contributes in maintaining the planarity of the molecule (4–6) which facilitates free charge transfer in the molecule as a whole and between the two six-membered rings in particular.

The 9-carbon atom can be expected to participate in the electronic configura-

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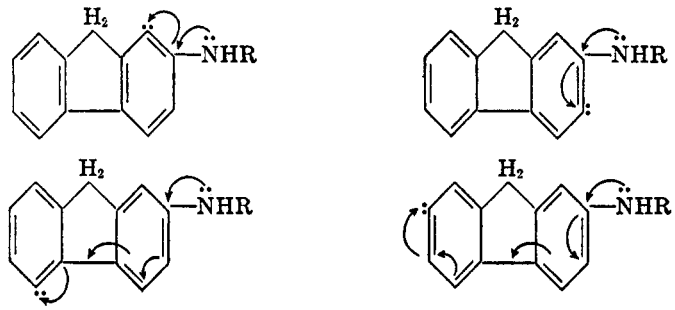
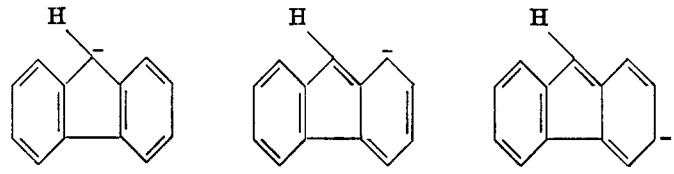
tion of the molecule by way of a hyperconjugative process. Experimental evidence for this is derived from the readiness with which fluorene forms the corresponding anion in the presence of alkali metals or hydroxides under anhydrous



conditions (7, 8). The potentially available partial negative charge in fluorene by resonance will add partial charges on carbons 1 and 3, as shown.



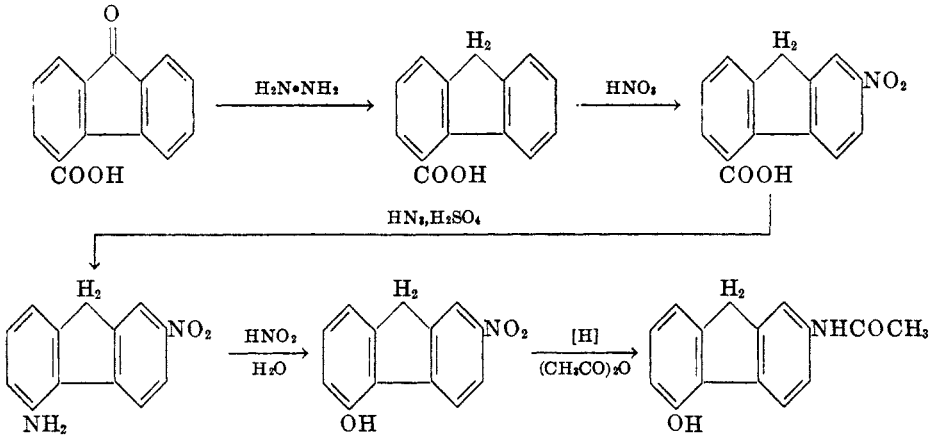
In 2-acetylaminofluorene, however, this contribution must be minimal (9) in the face of the available free electron pair from the nitrogen atom. In any case, the effect of the 9-carbon would be additive on the 1 and 3 positions, so that no competition exists. Experimental evidence for the activation of some of these positions is adduced from the isolation of 3-nitro- and 7-nitro-2-acetylamino fluorene in the nitration of 2-acetylaminofluorene (10).

These considerations, therefore, support the hypothesis that biological hydroxylation of 2-acetylaminofluorene by an electrophilic or a related mechanism (11) should attack the 1, 3, 5, and 7 positions to produce the corresponding phenols. 2-Acetylamino-7-hydroxyfluorene has been isolated from the urine of rats (12) and dogs (13) fed 2-acetylaminofluorene. Furthermore, we have already demonstrated the presence of 2-acetylamino-1- and 3-hydroxyfluorene in the urine of rats fed 2-acetylaminofluorene (14).

If 2-acetylamino-5-hydroxyfluorene could be shown to be a metabolite of 2-acetylaminofluorene, it would be the first demonstration of hydroxylation in a polynuclear aromatic system at the theoretically predictable positions. It would mean concordance of the positions attacked by ordinary chemical substitution and by biological hydroxylation. Development of a method of synthesis of this hitherto unknown compound was therefore important for reference purposes in our metabolism studies.

Polyphosphoric acid cyclization of commercial diphenic acid afforded fluorenone-4-carboxylic acid (15). Reduction of this material by the Huang-Minlon

modification of the Wolff-Kishner reaction yielded 71 % of fluorene-4-carboxylic acid.



In the nitration of this acid the nitro group can be expected to enter the ring not bearing the carboxy substituent, attacking the 5 and preferably the 7 position in conformity with the known behavior of fluorene bearing a *meta*-directing substituent. The proof of structure demonstrated that the nitro group indeed entered the 7 position. However, the appreciable loss of product during the recrystallization of the crude material suggests that some nitration also took place on the 5 position. The nitration had to be carried out with efficient stirring and control of the temperature to prevent the formation of nitrous fumes. Without this precaution some oxidation at the 9 position with production of 2-nitrofluorenone-5-carboxylic acid (16), m.p. 262°, occurred.

A Schmidt reaction on 2-nitrofluorene-5-carboxylic acid smoothly replaced the carboxy by an amino group. The resulting 2-nitro-5-aminofluorene was reduced to the known 2,5-diaminofluorene and deaminated with production of 2-nitrofluorene thus definitely assigning the location of the nitro group.

Conversion of the nitro-amino derivative to the corresponding nitrophenol followed by reduction of the nitro group led to 2-amino-5-hydroxyfluorene. Acetylation in buffered aqueous medium which does not affect the phenolic hydroxy group yielded the desired 2-acetylamino-5-hydroxyfluorene.

This compound, both free and combined as a glucuronide, was found to be a metabolite of 2-acetylaminofluorene by means of carrier experiments (to be reported in detail elsewhere) on the urine of rats fed 2-acetylaminofluorene-9-C^{14}. Thus, hydroxylation takes place at the positions expected by theoretical considerations.

EXPERIMENTAL[2]

Fluorene-4-carboxylic acid. Diphenic acid (90 g.) was dissolved in 900 g. of polyphosphoric acid. The mixture was heated with stirring to 160°, cooled and added to 2 volumes of ice to

[2] Microanalyses by Dr. W. C. Alford and his staff. The ultraviolet absorption spectra of the compounds were determined on a Cary instrument as 3.9×10^{-5} molar solutions in ethanol by Miss Rita McCallum.

precipitate the fluorenone-4-carboxylic acid. The crude product was dissolved in 500 ml. of 0.75 *N* sodium hydroxide, treated with Norit, and precipitated with acid, wt. 84 g., m.p. 222°. This material was dissolved in a mixture of 600 ml. of diethylene glycol, 52 g. of sodium hydroxide, and 49 ml. of 85% hydrazine hydrate and refluxed for 2.5 hours. The temperature was raised to 205° and refluxing was continued 3 hours longer. The solution was poured on ice and neutralized with hydrochloric acid. The crude fluorene-4-carboxylic acid was dissolved in sodium hydroxide solution, treated with Norit, and reprecipitated with hydrochloric acid. The light tan product, wt. 73.5 g., melted at 179°. Recrystallization from ethanol gave 56 g., m.p. 191–192° (17).

2-Nitrofluorene-5-carboxylic acid. A solution of 10 g. of fluorene-4-carboxylic acid in 150 ml. of glacial acetic acid was cooled to 30°. After addition of 40 ml. of fuming nitric acid (*sp. gr.* 1.5) the solution was warmed gradually to 65–70° whereupon a reaction took place. The temperature was held between 65 and 70° with vigorous mechanical stirring for 20 minutes and the mixture then was cooled. The yellow precipitate, m.p. 253–255°, wt. 9 g., was filtered off and washed with cold 50% acetic acid and water. Recrystallization from 250 ml. of acetic acid gave 8 g. of material, which when crystallized further from 1100 ml. of ethanol yielded 7.6 g. of fluffy needles, m.p. 273°. The UV spectrum had a maximum at 332 mμ (ϵ = 18,120) and a minimum at 270 mμ (ϵ = 3,445).

Anal. Calc'd for $C_{14}H_9NO_4$: C, 65.88; H, 3.55; N, 5.49.

Found: C, 65.62; H, 3.61; N, 5.27.

2-Nitro-5-aminofluorene. 2-Nitrofluorene-5-carboxylic acid (12.5 g.) was dissolved in a mixture of 85 ml. of concentrated sulfuric acid and 10 ml. of oleum (30% sulfur trioxide). After addition of 95 ml. of chloroform the mixture was stirred at 40–42° and 4.5 g. of sodium azide was added in small portions. The mixture was stirred 2 hours longer, the chloroform layer was separated, and the sulfuric acid layer was poured onto ice. Neutralization gave 9.8 g. of a reddish-brown precipitate, m.p. 153–155°, which was crystallized from benzene to give 7.8 g. of orange needles, m.p. 161–163°. After further recrystallization a sample melted at 162.5–163°. The spectrum of 2-nitro-5-aminofluorene showed maxima at 275 (ϵ = 7,400), 329 (ϵ = 10,550) and 377 mμ (ϵ = 10,720) while minima appeared at 260 (ϵ =6,380), 295 (ϵ = 6,130) and 349 mμ (ϵ = 9,520).

Anal. Calc'd for $C_{13}H_{10}N_2O_2$: C, 69.01; H, 4.46; N, 12.39.

Found: C, 68.93; H, 4.55; N, 12.39.

Reduction of 2-nitro-5-aminofluorene. A mixture of 150 mg. of the compound, 0.1 g. of calcium chloride, and 0.95 g. of zinc dust in 25 ml. of 80% ethanol was refluxed for 1.5 hours and filtered while hot. Partial evaporation of the filtrate yielded 97 mg. of purple crystals, m.p. 171°, which gave almost colorless needles, m.p. 173°, from 50% ethanol. Acetylation in benzene afforded 2,5-di(acetylamino)fluorene, m.p. 296°, undepressed by the authentic compound prepared from 2,5-dinitrofluorene. Morgan and Thomason (18) reported 2,5-diaminofluorene with a m.p. of 175°, and the diacetyl derivative, m.p. 289°.

Deamination of 2-nitro-5-aminofluorene. A solution of 75 mg. of 2-nitro-5-aminofluorene in 10 ml. of glacial acetic acid, 1 ml. of sulfuric acid, and 2 ml. of water was cooled in an ice-bath. Upon addition of 30 mg. of sodium nitrite in 1 ml. of water the precipitated amine sulfate gradually dissolved. After ½ hour urea was added followed 15 minutes later by 4.8 ml. of 50% hypophosphorus acid. The mixture was kept in the cold overnight, water was added and the yellow precipitate was filtered off. This material was chromatographed in benzene on an alumina column and was crystallized from dilute acetic acid to yield 40 mg. of 2-nitrofluorene, m.p. 153–154°. It did not depress the m.p. of authentic 2-nitrofluorene and had an ultraviolet absorption spectrum corresponding to that of authentic material.

2-Nitro-5-acetylaminofluorene. Acetylation of 0.1 g. of 2-nitro-5-aminofluorene in 10 ml. of benzene with 1 ml. of acetic anhydride gave 0.101 g. of yellow needles, m.p. 283°, after recrystallization from acetic acid. The UV absorption spectrum had a maximum at 327 mμ (ϵ = 16,850) and a minimum at 270 mμ (ϵ = 2,300).

Anal. Calc'd for $C_{15}H_{12}N_2O_3$: C, 67.15; H, 4.51; N, 10.44.

Found: C, 67.00; H, 4.63; N, 10.25.

2-Nitro-5-hydroxyfluorene. To a hot solution of 5 g. of 2-nitro-5-aminofluorene in 300 ml. of glacial acetic acid, there was added 50 ml. of 18 *N* sulfuric acid. On cooling to 15° a gray slush crystallized. After the addition of 1.8 g. of sodium nitrite in 150 ml. of water and stirring for 1 hour most of the material dissolved. Excess nitrite then was removed in 0.5 hour with 2 g. of urea. The mixture was added over a period of 15–20 minutes to 575 ml. of refluxing 5 *N* sulfuric acid. The crude material, m.p. 240°, isolated upon cooling weighed 5 g. Upon crystallization from xylene 3.8 g. of tan-yellow needles, m.p. 240–242°, were obtained. Further crystallization from ethanol (1 g./40 ml.) using Norit gave orange-yellow needles, m.p. 241–242°. The UV spectrum had maxima at 256 (ϵ = 7,020) and 353 mμ (ϵ = 14,810) with minima at 247 (ϵ = 5,230) and 278.5 mμ (ϵ = 2,300).

Anal. Calc'd for $C_{13}H_9NO_3$: C, 68.72; H, 3.99; N, 6.17.
Found: C, 68.82; H, 4.27; N, 6.14.

2-Amino-5-hydroxyfluorene. A mixture of 0.87 g. of 2-nitro-5-hydroxyfluorene, 50 ml. of ethanol, 6 ml. of water, 0.3 g. of calcium chloride, and 3 g. of zinc dust was refluxed for 2 hours. The solution was filtered off into 5 ml. of concentrated hydrochloric acid, the ethanol removed under a current of nitrogen, and the amine, m.p. 255°, obtained by treatment with sodium carbonate or ammonium hydroxide. The amine crystallized from 50% ethanol to give 0.59 g. of pink needles, m.p. 255–256°. The melting point was raised to 257.5° by another recrystallization. The UV absorption spectrum showed maxima at 279 (ϵ = 19,900) and 302 mμ (ϵ = 18,200). Minima appeared at 250 (ϵ = 4,340) and 291 mμ (ϵ = 15,100).

Anal. Calc'd for $C_{13}H_{11}NO$: C, 79.16; H, 5.62; N, 7.10.
Found: C, 79.07; H, 5.68; N, 7.31.

This compound, diazotized and coupled with R salt according to the test for diazotizable amine developed by Westfall and Morris (19), gave a dye with a maximum absorption at 543 mμ. Under the standard method for the test, an equimolar amount of 2-amino-5-hydroxyfluorene gave 70% of the absorption of 2-aminofluorene at 525 mμ.

This compound also reacted in the test developed for the isomeric 2-amino-7-hydroxyfluorene (20) but at 450 mμ gave only 35% of the absorption given by the 7-isomer.

2-Acetylamino-5-hydroxyfluorene. The crude amine prepared from 3 g. of 2-nitro-5-hydroxyfluorene was dissolved in 300 ml. of warm 0.2 *N* hydrochloric acid. The solution was filtered and 10 g. of sodium acetate was added to bring the solution to *p*H 4. The suspension was cooled in an ice-bath and stirred for 6 hours after the addition of three 5-ml. portions of acetic anhydride at hourly intervals. There was obtained 2.5 g. of crude acetyl derivative, m.p. 205–207° (decompn.). Two crystallizations from dilute ethanol gave 1.7 g. of fine needles, m.p. 209–210°. The spectrum had maxima at 283.5 (ϵ = 25,500), 300 (ϵ = 22,000) and 311.5 mμ (ϵ = 20,000) with minima at 248 (ϵ = 3,320), 291.5 (ϵ = 18,800) and 308 mμ (ϵ = 19,400).

Anal. Calc'd for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48; N, 5.85.
Found: C, 75.01; H, 5.77; N, 6.09.

This compound couples with diazotized *p*-nitroaniline under the conditions described in a previous publication (21) to give a red-brown dye at *p*H 5. The dye turns violet at *p*H 11.

SUMMARY

Theoretical and experimental considerations suggested that one of the metabolites of the carcinogen 2-acetylaminofluorene could be 2-acetylamino-5-hydroxyfluorene.

The latter compound was synthesized by a series of reactions starting from fluorenone-4-carboxylic acid.

Carrier isotope dilution experiments showed this hydroxy derivative to be a metabolite of 2-acetylaminofluorene. Hydroxylation thus occurred at all the theoretically predicted positions in this polynuclear ring system.

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