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ADRENALIN OXIDATION REVISITED. NEW PRODUCTS BEYOND THE ADRENOCHROME STAGE.

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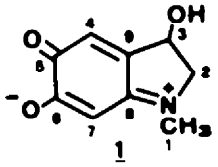
Abstract. In neutral aqueous buffer adrenochrome (1), the first isolable intermediate in the oxidation of adrenalin, undergoes rearrangement to give, besides adrenolutin (2), a yellow compound which was assigned the dimeric structure 6. Under anaerobic conditions, compound 6 is the major reaction product (about 60 % yield). In the presence of air a more complex pattern of products is formed including, besides 6, the hitherto unknown 5,6-dihydroxy-1-methyl-2,3-indoledione (10) and a related compound identified as the 4,4'-dimer 11.

Since 1937 it has been known that the enzymic or chemical oxidation of adrenalin leads in the early stages to a red unstable compound generally referred to as adrenochrome (1).[1,2,3] Interest in the chemistry of 1 was promptly roused by the wide range of physiological and psychological activity ascribed to this aminochrome, which has continued unabated over the years.[4] As a result of these studies it was apparent that the most characteristic feature of 1 is its tendency to undergo rearrangement in the presence of alkali[5] or metal cations (e.g. Zn^{2+})[6,7] to give a strongly fluorescent substance identified by Lund[8] as 5,6-dihydroxy-1-methylindoxyl (adrenolutin, 2). The structure elucidation of 2 appeared to have definitively settled the question of the fate of 1. Accordingly, only sporadic attention was devoted to the formation of other oxidation products of adrenalin following the adrenochrome stage. In 1945 Cohen[9] suggested that the oxidation of 1 proceeds in the early stages to give a relatively simple product, i.e. the 5,6-o-quinone of 1-methylindoxyl (3), designated "oxoadrenochrome". Subsequent attempts by Harley-Mason[10] to obtain oxoadrenochrome by oxidation of 2 were, however, unsuccessful, and the only recognisable product formed was a greenish-black pigment loosely described as the indigo 4. Moreover, Harley-Mason[10] found that, when left in aqueous solution under nitrogen, 1 slowly changes into an amorphous dark precipitate referred to as "adrenalin black".



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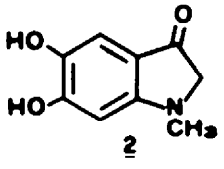

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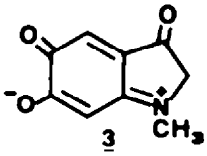

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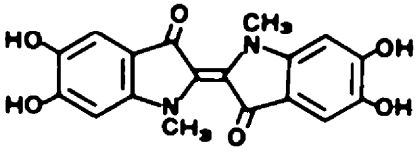

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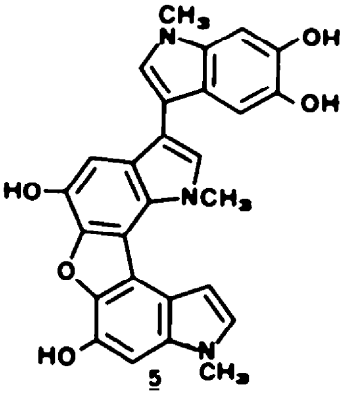


5

This was regarded as a polymer arising by dehydration and repeated condensation of 1 through the 3- and 4-positions of the indole ring[11], but new evidence indicates that adrenalin black is in fact a mixture of indole oligomers of the type 5.[12]

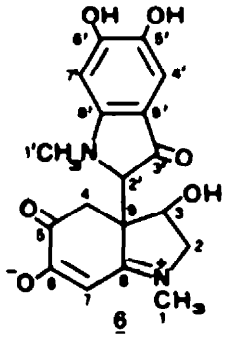
In connection with our interest in the oxidation chemistry of catecholamines[13,14] we have now examined the rearrangement of 1 anew and have found that at neutral pH the major reaction product is not 2, as commonly believed, but a non-fluorescent, $FeCl_3$-positive compound which tends to separate from the reaction medium. HPLC analysis showed that this product is formed in higher yields (about 60%) in the absence of oxygen and is relatively stable to autoxidation in neutral or slightly acidic media, while at pHs higher than 7 it readily decomposes to chromatographically ill defined polymeric species. Hence, the isolation of the compound was best achieved by careful ion exchange chromatography on Dowex 50 W X2 (H+ form) of a rearrangement mixture of 1 in Hepes buffer[15] at pH 6.8. Elution with water afforded the desired compound as a yellow amorphous powder, $C_{18}H_{18}N_2O_6$[16], homogeneous by TLC and HPLC, with a characteristic absorption maximum at 339 nm; on treatment with $NaBH_4$ it underwent reduction to give a spectrum (λ max 311 and 397 nm) closely similar to that of 2 under the same conditions (λmax 317 and 393 nm).

The 1H-NMR spectrum comprised the entire set of resonances of 1[17] with the sole exception of the H-4 proton signal, which was replaced by a pair of doublets at δ 2.01 and 2.76 (J = 16 Hz) typical of diastereotopic geminal protons adjacent to a carbonyl group. In the 13C-NMR spectrum features characteristic of both 1 and 2 were clearly recognisable[18], with the additional presence in the sp^3 region of a doublet at δ 70.33, in place of the C-2 methylene group of 2, and a singlet at δ 60.08, attributable to an angular quaternary carbon on the ring skeleton of 1.

Taken together, these data can only be accommodated by the gross structure 6, in which an adrenolutin moiety is linked through the 2 position to the angular 9-position of 1. Structure 6 has three asymmetric centres at positions 3, 9 and 2', of which the first derives from adrenalin and is therefore configurationally established. Thus, four diastereoisomers can in principle be described for 6. By HPLC analysis and careful inspection of the 1H- and 13C-NMR spectra, however, no indication was obtained of a mixture of compounds, which suggests that one diastereoisomer is preponderant. Nuclear Overhauser enhancement difference (NOED) experiments failed to provide conclusive information on the stereochemistry of 6, and attempts to grow suitable crystals for X-ray analysis were defeated under a variety of conditions.

As far as the origin of 6 is concerned, a most obvious explanation would be a direct nucleophilic addition of 2 to the electrophilic 9 position of 1. However, this possibility is not supported by separate experiments showing that under the usual reaction conditions 1 does not react with 2. Thus, the possibility remains that in the course of the rearrangement of 1 to 2 a highly reactive intermediate is transiently generated, which is efficiently trapped by the unchanged aminochrome present in the reaction mixture. A most likely candidate is 3,5,6-trihydroxy-1-methylindole (7), the enolic tautomer of 2, which apparently meets the requirements for high nucleophilicity at the 2-position owing to the concurrent electron releasing effects of the hydroxyl groups at the 3- and 6-positions.

The observed reactivity of the angular 9-position of 1 is not unprecedented. Adducts at C-9 such as 8 and 9 have long been known to form by reaction of 1 with sodium bisulphite or thiols at neutral pH values, and have been adequately characterised by 1H-NMR spectroscopy.[18,20]



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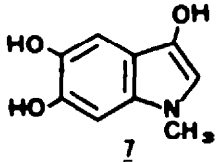

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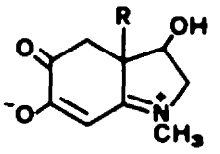


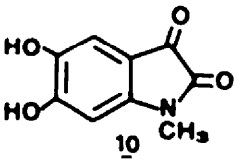
8 R=SO_3^-

9 R=$SCH_2CH_2CO_2H$

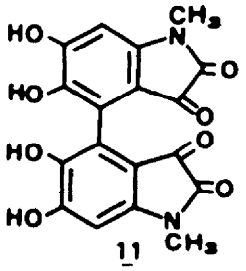
When a solution of 1 in Hepes buffer at pH 6.8 was left in air for a prolonged period of time, a slow oxidation reaction took place. Hplc and tlc (cellulose) monitoring provided evidence that under these conditions 2 is transiently formed and progressively changes into a red compound with λ_{max} 363, 509 nm which is subsequently oxidised to a closely related compound with absorption maxima at 352, 512 nm. These two products were then isolated by gel filtration on Sephadex G-10 or LH-20 of an autoxidation mixture of 2 in Hepes buffer at pH 6.8, followed by freeze drying of the appropriate fractions.

The first formed compound was readily identified as 5,6-dihydroxy-1-methyl-2,3-indoledione (10) mainly on the basis of the mass spectrum, exhibiting the molecular ion peak at m/z 193 and fragment peaks at m/z 165 (M^+ - CO) and 136 (base peak, M^+ - 2CO - H). The 1H- and 13C-NMR spectra displayed features consistent with the above structural assignment.

The mass spectrum (EI) of the second compound showed a weak molecular ion peak at m/z 384, suggesting a dimer of 10. The 1H-NMR spectrum consisted only of two resonances for a shielded aromatic proton and the N-methyl group, suggesting that the two monomer isatin units are linked symmetrically through the 4-position. Consistent with this interpretation, the 13C-NMR spectrum displayed only 9 signals, of which that at δ 96.42 (C-7) appeared as a doublet. The compound was therefore assigned structure 11.



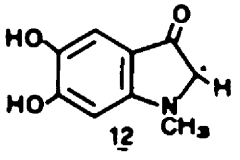
10



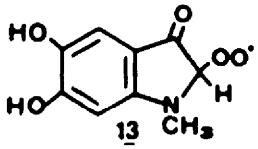
11

In subsequent experiments it was found that the yields of 10 and 11 formed by autoxidation of 2 vary significantly with the nature of the buffer system used, being higher in Hepes and lower in phosphate and Tris. We have at present no explanation of this effect, although we surmise that trace metal impurities may play a role.

Experiments designed to investigate the mechanism of formation of 10 and 11 showed that these compounds can be obtained from 2 only by autoxidation, since other oxidising systems such as Fe^{2+}-EDTA-O_2, ammonium persulphate, peroxidase-H_2O_2 are totally ineffective in producing 10 and 11. Moreover, no significant inhibition of the autoxidation reaction of 2 was observed in the presence of superoxide dismutase and catalase, which are efficient scavengers of superoxide ions and hydrogen peroxide. All these observations would point to the occurrence of an oxidative pathway of 2 involving the initial generation of a radical at C-2 (12). This readily interacts with oxygen to give an intermediate peroxy radical (13) which is eventually converted to 10. Such a mechanism closely parallels that commonly thought to be operative in the autoxidative conversion of activated methylene groups to carbonyl groups (as for anthrones,[21] benzoxazines,[22] etc.).



12



13

It is noteworthy that the autoxidation of 2 is characterised by the lack of formation of indigoid species, which markedly contrasts with the usual behaviour of indoxyls. This divergence can be ascribed to the presence on the benzene ring of the electron releasing hydroxyl groups, which apparently exert a destabilising effect on the initially formed radical 12, thereby favouring its rapid interaction with oxygen over dimerisation.

The fate of the isatin 10 under oxidative conditions is clearly dictated by the reactivity of the catechol hydroxyls. Whether the dimer 11 arises from coupling of a phenoxyl radical from 10 or from an ionic process requiring the intermediacy of the hypothetical 5,6-quinone is difficult to test owing to the discouraging features of the chemistry involved.

In conclusion, the structure characterisation of products 6, 10 and 11 provides for the first time some insight into the later stages of the oxidative pathway of adrenalin, throwing light on some basic aspects of the chemistry of 1 that have escaped the attention of previous studies.

EXPERIMENTAL

UV spectra were recorded on a Perkin Elmer Mod. 550S spectrophotometer. 1H-NMR spectra (270 MHz) and 13C-NMR spectra (67.9 MHz) were recorded on a Bruker AC 270 spectrometer (δ values are referred to TMS as an internal standard). Electron impact mass spectra were determined with a Kratos MS-80 mass spectrometer. Elemental analyses were performed by Analytische Laboratorien, Gummersbach 1 Elbach, F.R.G. All solvents were reagent grade from Carlo Erba. Inorganic salts and all other chemicals were of the highest purity available. R-(-)-adrenalin and Hepes were from Fluka. All enzymes were from Sigma. Dowex 50 W X2 resin (200-400 mesh) was from Bio Rad.
HPLC analyses were carried out with a Beckmann model 330 instrument using a 4 mm x 25 cm RP-18 Lichrosorb column (Merck). The mobile phase was 0.25 M $Na_2B_4O_7$-MeOH 96.5:3.5 v/v, pH 6 and the flow rate was maintained at 3 ml/min. Detection was carried out with a Knauer UV absorbance detector (λ =300 nm). Sephadex LH-20 and G-10 used for column cromatography were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Analytical thin layer chromatography was carried out on cellulose plates (0.25 mm, Merck) using water as the eluent. The chromatograms were visualised by UV irradiation at 254 and 366 nm and by spraying with an ethanolic solution of $FeCl_3$. Adrenochrome has an Rf of 0.8 and adrenolutin of ca. 0.45. Glass distilled water and buffers were carefully freed from heavy metal impurities by passage through a column of Chelex 100 resin (Na+ form). Glassware was washed with 1M HCl, 0.01 M EDTA and 10-15 rinses of deionised water.
Adrenochrome was prepared by silver oxide oxidation of adrenalin according to Sobotka and Austin[23] and was stored dry at -80 °C. Adrenolutin was prepared by a literature procedure.[10]

Isolation of the dimer 6.

Adrenochrome (500 mg) was dissolved in 0.05 M Hepes buffer at pH 6.8 (200 ml) and left at 27 °C in a water bath under a nitrogen atmosphere. After about 4 h, when the dark red colouration had turned to brown, the mixture was rapidly filtered, acidified to pH about 2 and filtered again. The yellowish solution thus obtained was chromatographed on a Dowex 50 W X2 (H+ form) column (3.5 x 15 cm) using water as the eluent, and fractions of 10 ml were collected. After some brownish products, the yellow dimer 6 was eluted in fractions 80-125, which were combined and carefully concentrated at 30 °C under vacuum. When the volume was reduced to about 15 ml, 6 separated from the cooled mixture as an amorphous precipitate which was filtered, washed with cold methanol and dried over phosphorus pentoxide. The yield was about 80 mg, provided that the work up was sufficiently rapid. In some preparations the compound was obtained in the form of yellow microcrystals darkening without melting at about 170 °C, Rf = 0.9, λ max (H_2O) 339(log ε 4.08), (H_2O, H+) 293, 351 nm (log ε 4.14 and 4.13), $C_{18}H_{18}N_2O_8$ (found: C, 60.16; H, 5.07; N, 7.70. Calc.: C, 60.3; H, 5.0; N, 7.8); 1H-NMR (DMSO-d_6): δ 2.01 (1H,,J=16 Hz,H-4b), 2.52 (3H,s, CH_3), 2.76 (1H,d,J=16 Hz, H-4a), 2.99 (3H,s,CH_3), 3.32 (1H,s,H-2'), 3.39 (1H,d,J=11 Hz, H-2b), 4.22 (1H,dd,J=11,2 Hz,H-2a), 5.12 (1H,d,J=2 Hz,H-3), 5.46 (1H,s,H-7), 6.12 (1H,s,H-7'), 6.68 (1H,s,H-4'); 13C-NMR (DMSO-d_6):δ 33.51 (q,CH_3), 38.37 (q,CH_3), 60.08 (s,C-9), 62.45 (t,C-2), 66.62 (d,C-3), 70.33 (d,C-2'), 96.74 (d,C-7'), 99.13 (d,C-7), 106.89 (d,C-4'), 115.12 (s,C-9'), 141.66 (s,C-8'), 156.61 (s,C-5'), 160.79 (s,C-6'), 168.91 (s,C-8), 176.01 (s,C-6), 192.86 (s,C-5), 197.29 (s,C-3'). It should be noted that the signal corresponding to the C-4 methylene group was not detected, probably owing to masking of the pertinent chemical shift range by the intense DMSO-d_6 signal.

Isolation of 5,6-dihydroxy-1-methyl-2,3-indoledione (10).

To a solution of 2 (100 mg) in methanol (20 ml), 0.1 M Hepes buffer at pH 7.0 (30 ml) was added and the mixture was stirred at room temperature. The reaction course was monitored by TLC on cellulose. When most of the adrenolutin had disappeared (about 4 h), the red solution was carefully concentrated at 30°C under reduced pressure and chromatographed on a column (2.2 x 100 cm) of Sephadex G-10, eluent water. The fractions containing the isatin were pooled and freeze dried. About 60 mg (55%) of 10 were thus obtained as a dark red powder, Rf=0.73, λ max (H_2O) 363, 509 nm (log ε 3.91 and 3.42); EIMS m/z 193 (M+, found: 193.0382, $C_9H_7N\,O_4$ requires 193.0375), 165, 136, 122; 1H-NMR (CD_3OD): δ 3.14 (3H, s, N-CH_3), 6.47 (1H, s, H-7), 6.96 (1H, s, H-4); 13C-NMR (DMSO-d_6): δ 25.40 (q, N-CH_3), 98.12 (d, C-7), 104.24 (s, C-9), 107.40 (d, C-4), 142.73 (s, C-8), 149.60 (s, C-5), 161.22 (s, C-6), 163.57 (s, C-2), 177.21 (s, C-3).

Isolation of 5,5',6,6'-tetrahydroxy-1,1'-dimethyl-4,4'-biindolyl- 2,2',3,3'-tetrone (11).

A solution of 2 (100 mg) in Hepes buffer-methanol at pH 7.0 (50 ml) was left to autoxidise as above. After about 30 h , when all the initially formed isatin 10 had disappeared, the dark red solution was carefully concentrated at 30°C under reduced pressure and chromatographed on a

column (2.5 x 80 cm) of Sephadex LH-20 , eluent 95% ethanol-water 80 : 20 v/v. The fractions containing the red dimer were pooled and freeze dried. About 50 mg (45%) of 11 were thus obtained as a dark red powder sparingly soluble in water, Rf=0.95, λ max (H_2O) 352, 512 nm (log ε 4.07 and 3.58) ; EIMS m/z 384 (M+, found: 384.0581 , $C_{18}H_{12}N_2O_8$ requires 384.0594), 369, 356, 340, 328, 314;1H-NMR (CD_3OD): δ 3.13 (3H x 2, s, N-CH_3, N'-CH_3), 6.38 (1H x 2, s, H-7,H-7'); 13C-NMR (DMSO-d_6): δ 25.11 (q, N-CH_3, N'-CH_3), 96.42 (d, C-7, C-7'), 100.21 (s, C-9, C-9'), 114.88 (s, C-4, C-4'), 141.54 (s, C-8, C-8'), 150.80 (s, C-5, C-5'), 163.24 (s, C-6, C-6'), 169.44 (s, C-2, C-2'), 173.24 (s, C-3, C-3').

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15. Hepes: 4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid.
16. Compound 6 did not give a satisfactory mass spectrum under a variety of conditions.
17. 1H-NMR data of 1 (CD_3OD): δ 3.18 (3H,s,CH_3), 3.64 (1H,dd,J=14,2 Hz, H-2b), 4.11 (1H,dd, J=14,6 Hz, H-2a), 5.05 (1H,ddd, J=6,2,2 Hz,H-3), 5.53 (1H,s,H-7), 6.51 (1H,d, J=2 Hz, H-4). 1H-NMR data of 2 (DMSO-d_6): δ 2.84 (3H,s,CH_3), 3.58 (2H,s,CH_2), 6.33 (1H,s,H-7), 6.70 (1H,s,H-4).
18. 13C-NMR data of 1 (CD_3OD): δ 35.54 (q,CH_3), 64.87 (t,C-2), 66.58 (d,C-3), 93.08 (d,C-7), 127.83 (d,C-4), 156.35 (s,C-9), 161.96 (s,C-8), 173.83 (s,C-6), 186.30 (s,C-5). 13C-NMR data of 2 (DMSO-d_6) δ 34.04 (q,CH_3), 60.96 (t,C-2), 95.89 (d,C-7), 106.96 (d,C-4), 112.74 (s,C-9), 139.16 (s,C-8), 156.05 (s,C-5), 160.05 (s,C-6), 195.61 (s,C-3).
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