11. *The Formation of Melanin from Adrenochrome.*

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The non-oxidative transformation of adrenochrome into melanins in acid solution is controlled in rate by a second-order reaction between adrenochrome and acid. The product of this step cannot be isolated but is probably 1-methylindole-5,6-quinone and gives a derivative with 1,4-naphthaquinone; it polymerises rapidly by way of dimers and oligomers, with absorption spectra characteristic of indolylindolequinone groups. The growing polymer chain contains oxidisable groups which reduce part of the monomer quinone to 5,6-dihydroxy-1-methylindole. The monomer quinone also reacts with indole itself, giving a melanin-like copolymer.

STUDIES of melanin formation *in vitro* have mostly been concerned with oxidative processes, enzymic or otherwise, especially the oxidation of 5,6-dihydroxyindole (a probable intermediate *in vivo*) and analogous compounds. The conversion of these into melanins involves oxidation of the diol system and polymerisation, and the products are black polymeric pigments, apparently without regular structure, containing reversibly oxidisable and reducible groups and susceptible of further oxidation. The most questionable details are of two kinds: first, the site of reactions between the indole nuclei and, secondly, the nature of the reacting species. The first has received considerable attention without reaching finality; the second, in particular the extent to which oxidation proceeds, accompanies, or follows the polymerisation steps, is the subject of this paper. Consider the formation of a hypothetical linear phenolic polymer (I) from 5,6-dihydroxyindole; such a polymer, corresponding to minimal oxidation, is isomeric with indole-5,6-quinone (II), and in terms of the model reaction, indole + quinone ⟶ indolylquinol,[1] could be formed from the quinone (II) by self-combination. However, it could equally well arise by initial attack of the quinone or the corresponding semiquinone free-radical on the parent dihydroxyindole, with most of the oxidation occurring between or after polymerisation steps.

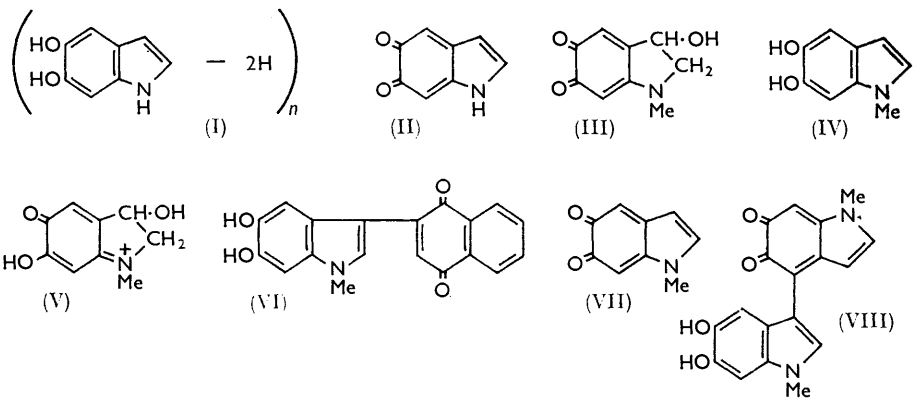
For the study of such a situation a non-oxidative process of melanin formation offers advantages; such a process occurs when solutions of adrenochrome (III) are stored, especially at acid pH, even in the absence of air. Observations on this instability of adrenochrome have been summarised by Heacock.[2] Visually the reaction has three phases. First, addition of acid to adrenochrome causes partial or complete discharge of the red colour, restored only by immediate neutralisation; secondly, various rather dirty

[1] Bu'Lock and Harley-Mason, *J.*, 1951, 703.
[2] Heacock, *Chem. Rev.*, 1959, **59**, 181.

purple shades appear; thirdly, a black precipitate or suspension is formed. With only traces of acid the phases are merged. Bouvet [3] noted that below pH 2 adrenochrome was reversibly transformed into a labile yellow material; Harley-Mason [4] observed that an unbuffered solution of fairly pure adrenochrome, stored in absence of air, afforded *ca.* 75% of black pigment, with 5,6-dihydroxy-1-methylindole (IV) as the main by-product. Melanins formed in this way are similar to those formed oxidatively, differing mainly in a somewhat greater solubility in pyridine or aqueous alkali, which suggests a lower molecular weight or a higher proportion of phenolic groups. The presence of the *N*-methyl group is unlikely to have a significant effect since oxidation of compound (IV) yields a normal melanin.[4]

In the present work the decomposition of adrenochrome in acid solutions, at a range of concentrations and acidities, was followed quantitatively with a recording spectrophotometer. After preliminary experiments in which air was excluded without affecting the reaction, no special precautions were taken, and in general the experiments were very reproducible. The effect of various added substances on the overall process was also studied.

On addition of acid to adrenochrome (III) ($\lambda_{max.}$ 300, 485 mμ) there is formed, in less than one second and reversibly, a yellow salt (conjugate acid), probably (V), since its absorption spectrum, seen briefly in strongly acid solutions or deduced from difference spectra (Fig. 1), shows $\lambda_{max.}$ *ca.* 400 mμ and somewhat resembles that of simple *para*-quinones. For the transformation of the quinone (III) into product (V) there are isosbestic points at 315 and 410 mμ, and from measurements over a range of adrenochrome and acid concentrations the titration curves of Fig. 2 can be calculated, from which the p*K* of adrenochrome is *ca.* 1·2. If the mixture of compounds (III) and (V) is immediately neutralised with alkali, most of the adrenochrome is regenerated but otherwise the solution shows marked instability. When free adrenochrome predominates in the mixture (*i.e.*, at pH > p*K*) the decomposition can be followed by the falling absorption at 485 mμ, as



shown in Fig. 3, and in such sets of curves a regular sequence of events is observed. First, the maximum at 485 mμ steadily diminishes and there are isosbestic points at 410 and *ca.* 540 mμ, but then the maximum, whilst still falling in intensity, begins to move to longer wavelengths, up to but not more than 540 mμ, and successive curves no longer intersect at 540 mμ. Next the maximum at 540 mμ falls away without changing its position. Finally there is a much slower (and possibly not anærobic) phase in which the absorption throughout the visible region may increase slightly. The phase with $\lambda_{max.}$ *ca.* 540 mμ is the " purple " phase observed visually, and in slow reactions (small amounts of acid) it is less well marked as a distinct phase; the following phase is that in which

[3] Bouvet, *Ann. Pharm.*, 1949, **7**, 514.
[4] Harley-Mason, *J.*, 1950, 1276.

black pigment is formed. The observations are summarised in Fig. 4, which shows the change in optical density at 485 and 540 mμ in solutions of various acid concentrations.

Allowance being made for the equilibrium between compound (III) and (V), the results of series of observations like those of Fig. 3 were used to derive the rates of disappearance of adrenochrome from solutions of varying composition, and these rates

FIG. 1. *Absorption spectra*: (1) *adrenochrome*, (2) *conjugate acid of adrenochrome*, (3) *melanin from adrenochrome*. (*All solutions*: 0·05 *mg. per ml. in* 1 *cm. cell.*)

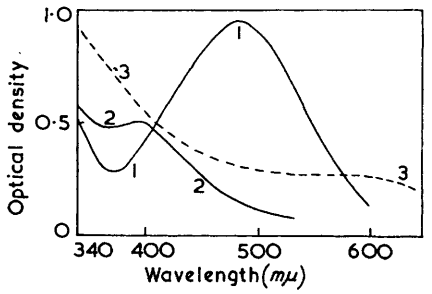


FIG. 2. *Equilibrium between adrenochrome base and conjugate acid.*

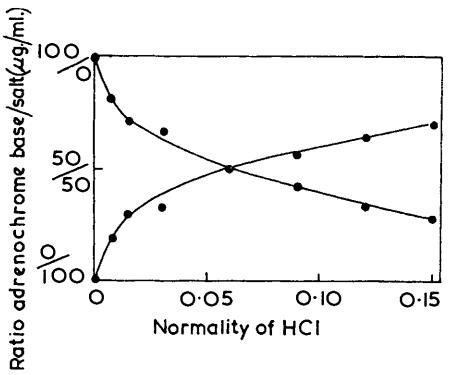


FIG. 3. *Successive absorption spectra* (2-*minute intervals*) *of adrenochrome* (1·0 *mg. per ml.*) *in* 0·03N-HCl. (*Absorption max.* ▲.)

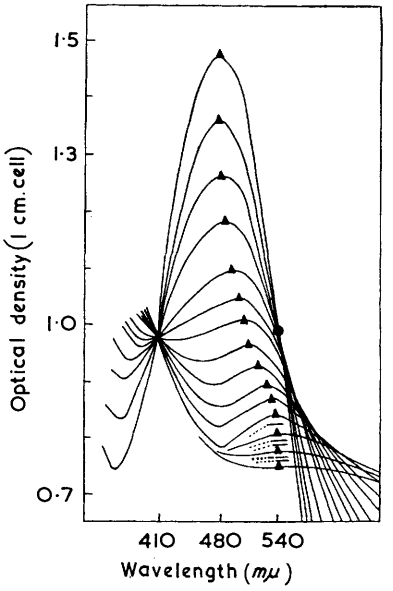
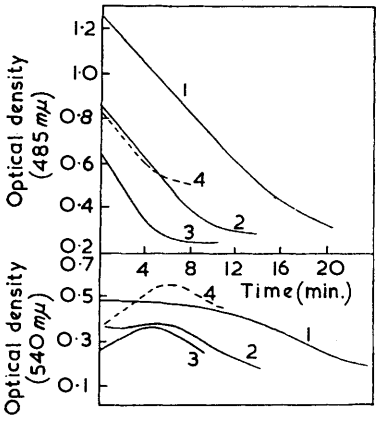


FIG. 4. *Variations of optical density at* 485 mμ (*upper curves*) *and* 540 mμ (*lower curves*): (1) *acid* 0·03N, (2) *acid* 0·07N, (3) *acid* 0·09N, (4) *as* (2) *plus* 1·0 *mg. of indole per ml.* (*Adrenochrome* 0·1 *mg. per ml. in all cases*; 1 *cm. cell.*)



were found to be directly proportional to the concentration of the salt (V) (with $k = 1{\cdot}0$ min.$^{-1}$), or, what is equivalent, to the product of the concentrations of adrenochrome base (III) and mineral acid. This relation holds over the range of adrenochrome concentrations of 0·05 to 0·5 mg. per ml. and with acid solutions of up to nearly 0·1N. In more acid solutions containing initially a preponderance of compound (V), the process is so rapid that its study is more difficult: declining absorption at 400 mμ appears to be accompanied by an increase below 400 mμ and followed by development of a maximum at *ca.* 540 mμ

(shifted towards 600 mμ in solutions more than 1N in acid) which subsequently declines into general absorption.

The overall reaction in the decomposition of adrenochrome in the absence of air is thus limited in rate by an acid-catalysed reaction of the first order in adrenochrome. It is also apparent (cf. especially Fig. 4) that the product of this reaction is not the material with absorption at 540 mμ, but a precursor of that material, itself not detected spectroscopically. Some indications of its nature are available. For instance, if acidification of the quinone (III) is carried out in the presence of 1,4-naphthaquinone no melanin is formed, but from the blue-green product the indolylnaphthaquinone derivative (VI) can be isolated. This is known to be formed from the dihydroxyindole (IV) and 1,4-naphthaquinone,[1] but the quinol (IV) is only formed from the quinone (III) in reductive processes, so that in the present circunstances the formation of product (VI) is best ascribed to a reaction of 1-methylindole-5,6-quinone (VII). The reactions of acidified adrenochrome with indole and cysteine and the formation of quinol (IV) as a by-product of polymerisation, as discussed later, also point to the formation of a quinone (VII) from adrenochrome on acidification. This is also probable on more general grounds: (VII) is the likely product of an acid-catalysed reaction (dehydration), and the 3-hydroxy-group of adrenochrome must be removed at an early stage in the melanin-forming process, since the later stages are closely analogous to those in oxidative melanin formation from 5,6-dihydroxyindole, in which a 3-hydroxy-group is not involved.[4] The formation of quinone (VII) being rate-limiting, and its disappearance as well as its formation being acid-catalysed, no accumulation of it need be expected under the experimental conditions. Other workers have attributed to quinones such as (VII) absorption maxima at *ca.* 360 mμ [5] or at *ca.* 470 mμ,[6] a disagreement which the present evidence does nothing to resolve.

To indole-5,6-quinones such as (VII) has also been attributed [7] the absorption at *ca.* 540 mμ in the " purple " phase of melanin formation, but, as shown by the present work, this material is not the *initial* product of the decomposition of adrenochrome. On the other hand, it is closely related spectroscopically to the final polymeric product. Robertson and his co-workers [5] have particularly considered this phase in oxidative melanin formation and pointed out the resemblance between the absorption spectra obtained and those of indolylbenzoquinones.[1] The comparison is apt, and on general grounds such a chromophore can only be accommodated in a dimer, *viz.*, a dihydroxyindolylindolequinone or a related oligomer. One possible structure for such a dimer is (VIII); its formation from the quinone (VII) would be analogous to the postulated reaction between (VII) and 1,4-naphthaquinone, giving (VI), and to the general reaction [1] between quinones and indoles.

However, the " purple " stage is not attributed uniquely to the formation of an intermediate dimer, and indeed its *gradual* transformation into black polymer indicates a more complex situation. Some insight into the process, as viewed spectroscopically, can be had from *a priori* considerations. If an oxidative transformation of benzene through biphenyl into polyphenyls could be observed spectroscopically, the spectra [8] would show a continuing shift to longer wavelengths for the *para*-polyphenyls, where conjugation is extended without limits, but no comparable shift for the *ortho*- or *meta*-series where increasing conjugation is subject to steric and electronic restrictions. An analogous actual case [9] is the oxidation of 1,8-dihydroxynaphthalene to a 4,5-linked polymer, in which a shift to longer wavelengths occurs when the dimer (a tetrahydroxybinaphthyl) is formed, but is not continued during the further transformation into a sterically hindered polymer. The effective chromophore of this polymer is therefore that of successive dimer units in the chain, but since these are not all quite identical, their combined spectrum

[5] Beer, Broadhurst, and Robertson, *J.*, 1954, 1947.
[6] Cromartie and Harley-Mason, *Biochem. J.*, 1957, **66**, 713.
[7] Mason, *J. Biol. Chem.*, 1948, **172**, 83.
[8] Gillam and Hey, *J.*, 1939, 1170.
[9] Allport and Bu'Lock, *J.*, 1960, 654.

shows a marked broadening of the " fundamental " peaks. The case of a melanin-like polymer is more complex though still analogous; there are present both oxidised (quinonoid and reduced aromatic units, but for long-wavelength absorption the relevant groups are the quinonoid units and their attached neighbours. From considerations of oxidation–reduction equilibria (see below) the attached units will mainly be in reduced forms, and on general (steric) grounds and from data on the spectra [1] of di-indolylquinones and di-quinonylindoles not more than one of them will be greatly effective in extending the quinone chromophore. Though the exact absorption characteristics of each such chromophore will reflect the structure and stereochemistry of a part of the polymer assembly, the basic structure will be that of corresponding dimers. Thus, while transformation of monomer, *e.g.*, (VII), into dimer, *e.g.*, (VIII), modifies the chromophore radically, further transformations into polymer will appear spectroscopically as the gradual imposition of a somewhat random series of distortions until the specific absorption curve is replaced by one of general but analogous absorption. This is precisely what is observed in the final stages of both the oxidative melanisation of 5,6-dihydroxyindole at neutral pH [7] and the acid-catalysed melanisation of adrenochrome as in Fig. 3; the maximum at *ca.* 540 mμ falls whilst the curve as a whole becomes flatter, but there persists a more or less well-defined inflexion at corresponding wavelengths (cf. Fig. 1). The intermediate species with $\lambda_{max.}$ *ca.* 540 mμ are therefore identified as dimers, oligomers, and ultimately " residual chromophores," with 5′,6′-dihydroxyindolylindole-5,6-quinone structures such as (VIII).

The polymerisation process in the acid-catalysed melanisation is therefore envisaged as beginning with self-combinations of the quinone (VII) to give the dimer (VIII) or isomers, such a reaction [1] apparently being acid-catalysed, followed by reaction between dihydroxyindolyl groups in the dimer or oligomers with free or combined indolequinone groups. Since the reacting groups are not necessarily monofunctional a branched or cross-linked polymer will result.[1] If under anærobic conditions this were the only process the final polymer would contain only one quinonoid group per molecule, which is certainly not the case. However, the oxidation potential of an indolequinone such as (VII) should be lowered by a dihydroxyindolyl substituent, as in (VIII); consequently, dihydroxy-indolyl residues in the reduced polymer will be oxidisable by the free quinone (VII), giving quinonoid groups in the polymer and explaining the observed formation [4] of the dihydroxyindole (IV). The extent of such processes will be limited, since the effect of substituting a *quinonoid* group for hydrogen in (VII) would be to *raise* the oxidation potential; consequently, in a linear or random polymer not more than half the units will be readily oxidised by the free quinone. An observed polymer yield of *ca.* 75%, implying about one-third complete oxidation in the polymer, thus seems not unreasonable.

The preceding arguments imply the ready reactivity of quinones such as (VII) both towards indoles with cationoid reactivity and with quinones susceptible to nucleophilic addition. The formation of the naphthaquinone derivative (VI) is evidence for the latter reaction; evidence for the reactivity of (VII) towards indoles was also obtained. As shown in Fig. 4, the addition of indole (or of 5,6-diacetoxyindole) to acidified adrenochrome solutions has no effect on the rate of disappearance of adrenochrome, but markedly accelerates the appearance of " dimer-type " absorption around 540 mμ. Eventually a polymer is precipitated, which is soluble in methanol and thus of rather low molecular weight, and smoke-grey in colour, implying a high proportion of non-quinonoid units. In methanol solution the polymer shows absorption around 300 mμ many times more intense (compared with the visible absorption) than any normal melanin, which is ascribed to the incorporated indole units; on the other hand, the intensity of the visible absorption (compared with that of the original adrenochrome) is greater than in the absence of added indole (cf. Fig. 4), *i.e.*, by supplying a substitute for the reduced groups of the melanin the added indole increases the yield of oxidised chromophoric units from the adrenochrome. Adrenochrome itself does not react at neutral pH with indole or with 1,4-naphthaquinone, and in the acidified system no reaction with a 10-fold excess of tyrosine, phenylalanine,

p-aminobenzoic acid, or tryptophan was detected, though a reaction of indolequinones with free amino-groups might be expected at higher pH. With cysteine a reaction analogous to that observed with enzymically oxidised hydroxyindole [10] apparently competes, in the acidified system, with reduction of the adrenochrome.

The foregoing observations are clearly also relevant to the oxidative route to melanins, in which indolequinones are differently generated. Work now in progress confirms this. The copolymerisation with indoles may also have a bearing on the structure of natural and enzymically produced melanins. Nicolaus *et al.*[11] have recently obtained evidence for the presence of up to 20% of units derived from 5,6-dihydroxyindole-2-carboxylic acid in sepia melanin; this acid could be an alternative product to 5,6-dihydroxyindole in the oxidation of tyrosine and 3,4-dihydroxyphenylalanine, and though not itself oxidisable to a melanin, its incorporation into an indolequinone-derived polymer by a copolymerisation analogous to that established for indole at acid pH now appears very plausible. In the enzymic process a similar direct incorporation of other potentially anionoid substances such as tyrosine or 3,4-dihydroxyphenylalanine might also be expected under conditions where these were present during the generation of the indole-5,6-quinone.

Experimental

Crystalline adrenochrome was prepared by the Sobotka and Austin's method [12] and stored at −25° until required; standard solutions of 1·0 mg. per ml. were then made up in deionised water. From measured volumes of this and of standardised hydrochloric acid, reaction mixtures, with adrenochrome from 0·05 to 1·0 mg. per ml. and acid from 0·007 to 1·5N, were made up directly in 1 cm. optical cells (capacity 4·0 ml.) placed in the beam of a Carey recording spectrophotometer. Spectra were recorded from 650 to 350 mμ at measured intervals of 1·5 to 2·0 min. (cf. Fig. 3). At the scanning speeds used there was an uncertainty of *ca.* 5 mμ in the wavelength scale, and the reported maxima are not necessarily comparable with those measured in static systems.

With an adrenochrome concentration of 0·1 mg./ml. and concentrations of acid C_H from 7×10^{-3} to $1{\cdot}5 \times 10^{-1}$N the values of the optical density at 490 mμ, D_{490}, were measured immediately on mixing. By extrapolation of a plot of D_{490} against $1/C_H$ a value for D_{490} for complete salt formation was obtained and, by using this and the presumption that base and salt absorb independently, values of C_A and C_{HA}, concentrations of adrenochrome base and salt, corresponding to the D_{490} values were computed. The variation of C_A with C_H approximates to the theoretical, and values of C_A and C_{HA} are plotted against C_H in Fig. 2. The spectrum shown for the salt in Fig. 1 was computed from that of a mixture ($C_H = 6 \times 10^{-2}$N) for which C_A and C_{HA} had been thus established. For a rate plot, observed rates of decrease of D_{490} with time were multiplied by the ratio of D_{490} before and immediately after acidification, to correct for the proportions of the total adrenochrome present as two differently-absorbing species.

For copolymerisation with indole, the solution contained 0·1 mg. of adrenochrome per ml., 1·0 mg. of indole per ml., and 0·07N-hydrochloric acid; on completion of the reaction the precipitate was allowed to settle, taken up in aqueous alkali, precipitated with hydrochloric acid, centrifuged down, and dissolved in methanol for measurement of the absorption spectrum. For the preparation of the quinone (VI), 1 drop of concentrated hydrochloric acid was added to 10 ml. of an aqueous-ethanolic solution containing 10 mg. of adrenochrome per ml. and 5 mg. of 1,4-naphthaquinone per ml. The greenish-blue mixture was extracted with chloroform, the extract evaporated, and the residue purified by chromatography on neutral alumina in chloroform–benzene to give 2-(5,6-dihydroxy-1-methyl-3-indolyl)-1,4-naphthaquinone as violet crystals, giving a blue-green colour with alkali and identified with authentic material [1] by absorption spectra and paper chromatography in aqueous butanol.

[10] H. S. Mason and E. W. Peterson, personal communication.
[11] Nicolaus, Piatelli, and Narni, *Tetrahedron Letters*, 1959, No. 21, 14.
[12] Sobotka and Austin, *J. Amer. Chem. Soc.*, 1951, **73**, 3077.

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