THE CHEMISTRY OF THE AMINOCHROMES

PART VI. THE REACTION OF ADRENOCHROME WITH GLUTATHIONE[1,2,3]

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ABSTRACT

The reaction between adrenochrome and glutathione has been studied. The main products result from the 1,4-addition of glutathione to the C_5 unsaturated carbonyl systems in adrenochrome involving the C_6—C_7 and the C_4—C_9 double bonds, and are probably 7-*S*-glutathionyl-5,6-dihydroxy-*N*-methylindole and 9-*S*-glutathionyl-2,3,6,9-tetrahydro-3,5-dihydroxy-6-oxo-*N*-methylindole respectively. 5,6-Dihydroxy-*N*-methylindole is also formed during the interaction of glutathione and adrenochrome. The mechanisms by which these compounds are formed are discussed.

INTRODUCTION

Thiols, such as glutathione, generally react with quinones by 1,4-addition across one of the α,β-unsaturated carbonyl systems. Much of the early work, dating back to 1888 (1), was reviewed by Snell and Weissberger in 1939 (2). The reaction of thiols, including glutathione, with 2-methyl-1,4-naphthoquinone has been described by several workers as a 1,4-addition process (3, 4, 5), although it has recently been suggested that this reaction proceeds by a simple nucleophilic substitution of the quinone ring by the mercaptide anion (6). Most of the previously reported additions of this type involved *p*-quinones; however, there have been a number of examples of the reported addition of thiols, including glutathione, to oxidized catechol derivatives (cf. 7–11) and recently the 1,4-addition of a thiol (1-phenyl-5-mercaptotetrazole) to several quinones, including some *o*-quinones, has been described (12, 13).

Preliminary paper chromatographic studies on the interaction of adrenochrome (I) with glutathione (free acid) in aqueous solution indicated that 5,6-dihydroxy-*N*-methylindole (II) and two other products (non-ether extractable) were formed; the major product (III) had an R_f value* of ca. 0.60 and appeared to have retained the indole nucleus and to contain an α-amino acid grouping (14, 15). Bouchilloux and Kodja subsequently reported that dopachrome solutions were decolorized by glutathione (phosphate buffer, pH = 5.5) with the formation of 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, and compounds described as 4-*S*-glutathionyl-5,6-dihydroxyindole and its 2-carboxy derivative (10, 11). These latter two compounds were also reported to be obtained by the enzymatic oxidation of 5,6-dihydroxyindole or 5,6-dihydroxyindole-2-carboxylic acid respectively in the presence of glutathione (10, 11). This paper reports the results of an extensive spectroscopic and paper chromatographic examination of the products obtained by the interaction of adrenochrome and glutathione.

EXPERIMENTAL

Materials

Adrenochrome (16), *N*-ethylnoradrenochrome (17), *N*-isopropylnoradrenochrome (17), adrenochrome methyl ether (17), adrenochrome ethyl ether (17), and 5,6-dihydroxy-*N*-methylindole (18) were prepared

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[2]*Part V. Can. J. Chem. 41, 139 (1963).*

[3]*A preliminary report of part of this investigation appeared in Arch. Biochem. Biophys. 107, 352 (1964).*

**All the R_f values referred to in this paper were obtained using 2% acetic acid in water as the running solvent (see Experimental section for details).*

by the methods described in the literature. Glutathione, its monosodium salt, and oxidized glutathione were obtained from the Nutritional Biochemicals Corporation.

Spectroscopy

The spectra were recorded on either a Beckman DK-2 or Unicam SP-800 recording spectrophotometer. The reaction mixtures studied were prepared from adrenochrome (5.0 mg) and glutathione (free acid or monosodium salt) (25.0 mg) dissolved in water (1.0 ml). Suitable dilutions were prepared for measurement of the spectra. When required, ether extractions were carried out with peroxide-free ether (4 × 1 ml). In experiments designed to study the effects of added reagents, the reaction mixture was prepared by the method described above; ether extracted (if necessary) and a slight excess of the solid reagent (i.e. silver oxide, sodium hydrosulfite, sodium bisulfite, or sodium acetate) were added directly to the solution. In the experiments designed to find the optimum pH for the formation of a given product the reaction mixture was prepared as described above, except that a suitable acetate or phosphate buffer was used as the reaction medium in place of water.

Paper Chromatography

The aminochrome (10 mg) was dissolved in water (1.0 ml) and the solution was treated with a slight excess of glutathione (free acid or monosodium salt).* When the red color of the aminochrome had been completely discharged the reaction mixture was filtered and a sample (ca. 25–50 μl) of the clear filtrate applied to the chromatographic paper (acid-washed Whatman No. 1 paper). The chromatography was carried out by the descending technique, using 2% acetic acid in water as the running solvent. In all cases the solvent was allowed to descend about 15 to 16 in. (this required approximately 2.5–3 h running time). After drying (in air, at room temperature), the developed chromatograms were examined for fluorescence in ultraviolet light and individual chromatograms were sprayed with one of the following chromogenic reagents:† (*a*) Ehrlich's reagent, (*b*) cinnamaldehyde, (*c*) *p*-dimethylaminocinnamaldehyde, (*d*) Gibb's reagent, (*e*) diazotized *p*-nitroaniline, (*f*) ninhydrin/pyridine, and (*g*) ferric chloride.

In some cases the aqueous solution of the aminochrome reduction products (prepared as described above, 1.0 ml) was extracted, after filtration, with peroxide-free ether (4 × 1.0 ml). Spots consisting of ca. 120–150 μl of the dried (Na_2SO_4) ethereal extract and ca. 40–60 μl of the aqueous mother liquors were applied to the paper separately and the chromatography carried out as described above.

In the experiments designed to obtain relatively pure solutions of the individual products, the reaction mixture was prepared in the manner described above and a sample (ca. 500 μl) was applied as a "streak" across the origin on acid-washed Whatman 3 MM paper (23 cm × 57 cm). After development with 2% acetic acid, the appropriate zones were located by spraying a narrow strip, cut from the edge of the chromatogram. The zones were cut out and eluted with water.

To ascertain if any reaction occurred between 5,6-dihydroxy-*N*-methylindole (II) and glutathione (or oxidized glutathione), an aqueous solution of (II) (10 mg in 1 ml) was treated with an excess of the amino-acid and the reaction mixture examined chromtographically in the manner described above, after it had been allowed to stand at room temperature for a period of time comparable to that required for the adrenochrome–glutathione reaction to go to completion.

RESULTS

Addition of glutathione (monosodium salt) to an aqueous solution of adrenochrome (I) resulted in the discharge of the red color of the solution; the resulting pale yellow solution showed well-defined absorption maxima of approximately equal intensity in the regions of 300 mμ and 350 mμ (see Fig. 1). When the reaction was carried out using glutathione, as the free acid rather than its monosodium salt, the absorbance at 300 mμ was ca. 70% greater than that at 350 mμ (see Fig. 1). In view of the higher absorbances in the 350 mμ region of reaction mixtures obtained using the sodium salt of glutathione. the effect of pH on the relative intensities of the main absorption peaks was investigated, Reaction mixtures containing adrenochrome (I) and glutathione were prepared in a series of acetate and phosphate buffers in the pH range 1–7, and it was observed that formation of the product (λ_{max} = ca. 350 mμ) (i.e. (IV)) occurred optimally at pH = 4.7 (see Fig. 2); the amount of (IV) formed fell off sharply at pH values above and below 4.7 and did not form at all at pH values > 6.

Paper chromatograms of these reaction mixtures on Whatman No. 1 paper with 2%

**An analogous experiment was carried out using oxidized glutathione in place of glutathione, but the red color of the adrenochrome solution was not discharged in a comparable period of time.*

†The chromogenic reagents were prepared by the methods commonly described in the literature (cf. (19)).

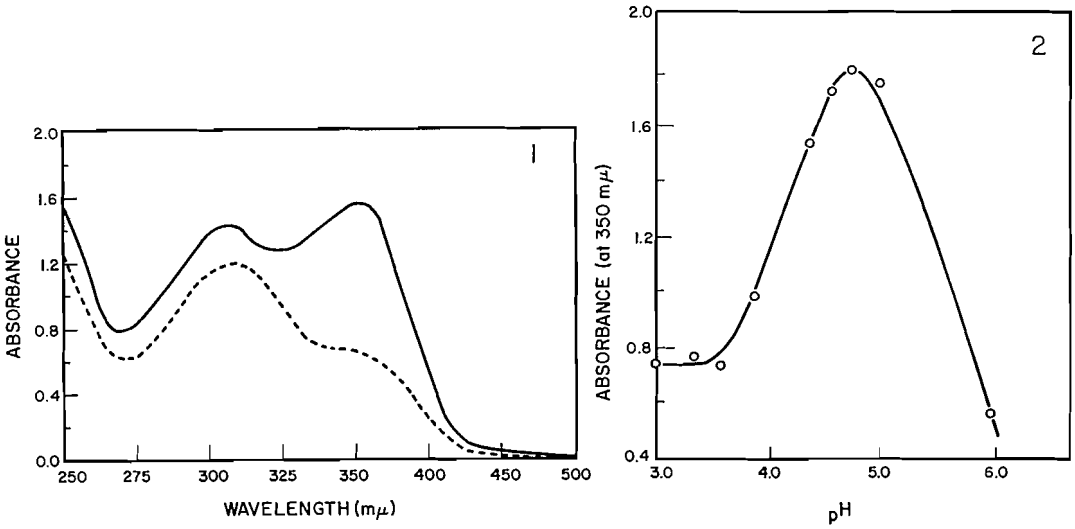


FIG. 1. Absorption spectra of (*a*) adrenochrome/glutathione reaction mixture (broken line) and (*b*) adrenochrome/glutathione (monosodium salt) reaction mixture (solid line).

FIG. 2. The effect of pH on the absorbance at 350 mμ of buffered glutathione/adrenochrome reaction mixtures.

acetic acid as the developing solvent (cf. 14, 15) showed three spots (which gave blue colors after spraying with Ehrlich's reagent) with R_f values of 0.43, 0.64, and 0.85 respectively (see Table I). A yellow spot (R_f = ca. 0.90), which appeared overnight on the sprayed chromatograms, was probably due to unchanged glutathione. The substance with an R_f of 0.85 showed a distinct yellow fluorescence in ultraviolet light; the substance with an R_f of 0.64 (i.e. (III)) did not fluoresce and the product with an R_f of 0.43 showed the weak "blue-mauve" fluorescence in "long wavelength" ultraviolet light characteristic of (II). The absorption maxima for the products were obtained by elution of the appropriate zones from a developed chromatogram of the adrenochrome–glutathione reaction mixture; these were as follows: R_f, 0.64 (i.e. (III)), 303 mμ and R_f, 0.85 (i.e. (IV)), 352 mμ. The unidentified product (III) exhibited color reactions expected for (i) an indole, (ii) a phenol, and (iii) an α-amino acid (see Table I). The color reactions of the product (IV) were a little less certain, due to the contamination of the zone (R_f, 0.80–0.90) on the chromatograms with unchanged glutathione; however, it did appear to react slowly with Ehrlich's reagent, cinnamaldehyde, diazotized *p*-nitroaniline, and ferric chloride (see Table I). The colors obtained from (IV) were similar to those given by the adrenochrome – sodium bisulfite addition product with these reagents (20).

N-Ethylnoradrenochrome and *N*-isopropylnoradrenochrome behaved similarly to adrenochrome on treatment with glutathione in aqueous solution. Paper chromatographic examination of the reaction mixtures showed that three products were formed in each case; the expected *N*-alkyl-5,6-dihydroxyindole and products probably analogous to (III) and (IV). Adrenochrome methyl ether and adrenochrome ethyl ether also behaved in a similar manner to adrenochrome on treatment with glutathione (see Table I). These results suggest that the 1- and 3-positions of the adrenochrome nucleus are not materially involved in any of the reaction sequences. There was no reaction between adrenochrome and oxidized glutathione; consequently, the thiol group of glutathione must have been involved in all these reactions. The possibility of secondary product formation by the

TABLE I

Paper chromatography of the products obtained from the reaction of some aminochromes with glutathione

Aminochrome reduced	Average R_f values (×100) of the major products				
Adrenochrome	43*		64†,‡	85§,‖	90¶
N-Ethylnoradrenochrome		51**	66	84	90¶
N-Isopropylnoradrenochrome		55††	69	85	90¶
Adrenochrome methyl ether	44*		65†	85	90¶
Adrenochrome ethyl ether	45*		66†	85	92¶

*5,6-Dihydroxy-*N*-methylindole (II).
†7-*S*-Glutathionyl-5,6-dihydroxy-*N*-methylindole (III).
‡Color reactions of (III). Ehrlich's reagent, blue–violet; cinnamaldehyde, red–pink → grey–violet; *p*-dimethylaminocinnamaldehyde, blue–green → grey–blue; diazotized *p*-nitroaniline, magenta → violet–brown; Gibb's reagent, brown; ninhydrin/pyridine, blue–grey (slow at room temperature).
§9-*S*-Glutathionyl-2,3,6,9-tetrahydro-3,5-dihydroxy-6-oxo-*N*-methylindole (IV).
‖Color reactions of (IV). Ehrlich's reagent, blue; cinnamaldehyde, violet–brown; *p*-dimethylaminocinnamaldehyde, grey–blue; diazotized *p*-nitroaniline, yellow → brown; Gibb's reagent, brown; ferric chloride, grey–brown; ninhydrin/pyridine, probably blue–grey, but this area is confused by the proximity of excess glutathione.
¶Excess glutathione (and possibly oxidized glutathione).
**N*-Ethyl-5,6-dihydroxyindole.
††5,6-Dihydroxy-*N*-isopropylindole.

interaction of 5,6-dihydroxy-*N*-methylindole (II) with glutathione (or oxidized glutathione) was also ruled out, since no reaction occurred between (II) and either glutathione or oxidized glutathione in aqueous solution, over periods of time comparable with those required for the adrenochrome/glutathione (or monosodium salt) reactions to go to completion.

It was shown by paper chromatography that ether extraction of the adrenochrome–glutathione reaction mixtures completely removed the substance with an R_f of 0.43 (i.e. (II)). The ultraviolet spectrum of the aqueous mother liquors, still showed peaks at ca. 300 mμ and 350 mμ, although the intensity of the 300 mμ peak was much reduced, because of the removal of (II) which also absorbs in this region. The reaction mixture, after removal of (II) by ether extraction, was initially yellow, but on the addition of solid sodium acetate or on the cautious addition of aqueous sodium hydroxide the solution became red. Dilution (ca. 100 times) of the ether-extracted reaction mixture with water, also led to the formation of an orange–red product, after 2 h at room temperature; the color intensified on standing overnight. These color changes did not appear to be affected by bubbling oxygen through the solution. The change in color of the solution was accompanied by a modification of the absorption spectrum; the intensity of the absorption at ca. 350 mμ was markedly reduced, and eventually disappeared; at the same time there was an increase in the absorbance at 300 mμ and a new peak at 485 mμ appeared. The red color of the modified reaction mixture was discharged on addition of excess sodium hydroxide, with the formation of a yellow product which exhibited an intense yellow–green fluorescence, usually associated with adrenolutin (5,6-dihydroxy-*N*-methylindoxyl), in ultraviolet light (cf. 21). The red product was also decolorized by the addition of sodium hydrosulfite or sodium bisulfite. In the former case an ether extractable product was obtained which was shown by paper chromatography and spectroscopy to be identical with 5,6-dihydroxy-*N*-methylindole (II), and in the latter case the red color could be restored by the cautious addition of alkali; addition of excess alklai resulted in the discharge of the red color and the formation of a fluorescent product similar to that described above. These reactions with sodium bisulfite and alkali suggests that the red product is derived from (IV) and that it is probably adrenochrome (I) (λ_{max} 300 mμ and 487 mμ (cf. 22)). The ether extracted adrenochrome–glutathione reaction mixture is capable of further modification by oxidizing and reducing agents. After treatment of the ether

extracted reaction mixture with silver oxide, the solution became deep yellow and the original peaks at ca. 300 mμ and ca. 350 mμ were replaced by peaks at 285 mμ* and 399 mμ; this suggests that both compounds can undergo oxidation. The peak at ca. 350 mμ (due to (IV)) disappeared and the 300 mμ peak increased in intensity on treatment of the original reaction mixture (ether extracted) with sodium hydrosulfite. Paper chromatography showed that reduction with this reagent resulted in the elimination of the faster running substance (i.e. (IV)) and the formation of (II) which could be extracted with ether and identified by chromatography and spectroscopy.

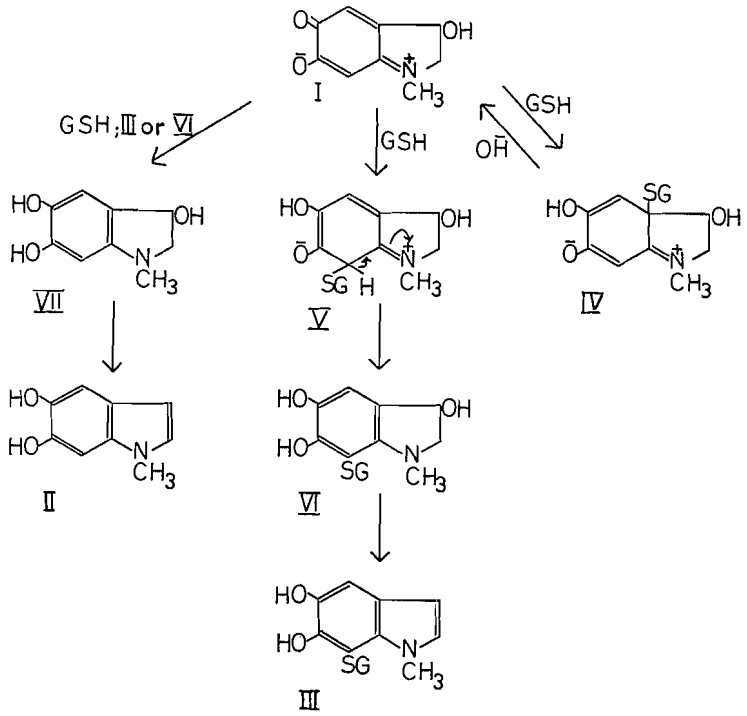
DISCUSSION

On the basis of the observed spectroscopic, paper chromatographic, and chemical data, it is possible to propose likely structures for the products obtained by the action of glutathione on adrenochrome; the formation of all the reaction products can also be explained by reasonable mechanisms.

It is well known that 5,6-dihydroxy-*N*-methylindole (i.e. (II)) is usually produced, to some extent, when adrenochrome reacts with a reducing agent (cf. 22), and it has previously been suggested that the product with an R_f value of 0.43 (i.e. (II)) was, in fact, 5,6-dihydroxy-*N*-methylindole; the results of the present investigation are in agreement with this suggestion. The paper chromatographic and spectroscopic properties of (II) were identical to those of pure 5,6-dihydroxy-*N*-methylindole. The formation of 5,6-dihydroxy-*N*-methylindole (II) could result from the direct reduction of adrenochrome (I) by glutathione. It could also result from the reduction of (I) by (III) (or possibly its immediate precursor (i.e. (VI)); the reduction of a quinone by the corresponding hydroquinone containing an electron-donating group is well known (cf. 23). To test the plausibility of the latter type of reaction occurring, a solution of *N*-isopropylnoradrenochrome was treated with (II); paper chromatography of this reaction mixture showed that interaction had, in fact, occurred, with the formation of 5,6-dihydroxy-*N*-isopropylindole (i.e. the dehydrated reduction product of *N*-isopropylnoradrenochrome). In view of the difficulty of detecting oxidized glutathione, in the presence of glutathione (with the chromatographic system used in this investigation), and the uncertainty that its presence, if found, was not merely due to autoxidation, it is not possible to be sure if the direct reduction mechanism is operating to any extent in the adrenochrome–glutathione system. However, since 5,6-dihydroxy-*N*-methylindole (II) is not the major product of the adrenochrome/glutathione reaction and the *N*-isopropylnoradrenochrome/5,6-dihydroxy-*N*-methylindole interaction resulted in appreciable formation of 5,6-dihydroxy-*N*-isopropylindole, in a time comparable to that required for the completion of the adrenochrome/glutathione reaction, it is most probable that the majority of the (II) present in the glutathione/adrenochrome system resulted from the reduction of adrenochrome by (III) or (VI) which gave "leuco-adrenochrome" (VII) which is known to dehydrate spontaneously and form 5,6-dihydroxy-*N*-methylindole (II) (cf. 24).

There are two possible ways by which glutathione could add, by a 1,4-addition mechanism, to adrenochrome. Addition could occur across the α,β-unsaturated C_5-carbonyl system, which includes the C_6—C_7 double bond, to form the adduct (V); subsequently this product could undergo an "enolization" of the polarized C_6-carbonyl

**The position of the 285 mμ peak could not be precisely determined since end absorption due to oxidized glutathione is significant below 290 mμ; this was partially compensated by the addition of oxidized glutathione to the solution in the reference beam of the spectrophotometer.*



group to give 7-*S*-glutathionyl-3,5,6-trihydroxy-*N*-methylindoline (VI). The 3,5,6-trihydroxyindoline structure is known to rapidly undergo an intramolecular dehydration to give the 5,6-dihydroxyindole structure (cf. 24), consequently, (VI) would be expected to dehydrate to give 7-*S*-glutathionyl-5,6-dihydroxy-*N*-methylindole (III). This structure is quite compatible with the observed properties of (III), i.e. the typical indole absorption maximum at ca. 300 mμ and the color reactions expected for an indole, catechol, and an α-amino acid. It is also water soluble and was not extracted from its aqueous solutions by ether as would have been expected for a molecule containing a large amino acid residue. This structure is similar to that postulated by Bouchilloux and Kodja for one of the products obtained from dopachrome by the action of glutathione, except that the French authors suggested that the glutathione residue was attached to the 4-position of the indole nucleus (11). Alternatively, 1,4-addition of glutathione to the α,β-unsaturated C_5-carbonyl system, with the double bond between the C_9-bridge-head carbon atom and C_4 would result in structure (IV), i.e. 9-*S*-glutathionyl-2,3,6,9-tetrahydro-3,5-dihydroxy-6-oxo-*N*-methylindole. This structure is similar to that proposed by Tse and Oesterling for the adrenochrome – sodium bisulfite addition product (25) and is compatible with the properties of the glutathione/adrenochrome interaction product (IV) with $R_f = 0.85$ and $\lambda_{max} = 352$ mμ. There are a number of chemical similarities between (IV) and the bisulfite addition product (cf. 25, 26) although the glutathione adduct would appear to be less stable. In the first place, there is the apparent regeneration of adrenochrome (λ_{max}, ca. 300 and 487 mμ) on treatment with mild alkali (cf. 14, 26); both products exhibit an absorption maximum in the 350–360 mμ range; both compounds show a weak yellow fluorescence in ultraviolet light and (IV) and the adrenochrome – sodium bisulfite addition product show similar paper chromatographic behavior, e.g. they both have high

R_f's in 2% acetic acid and they both react relatively slowly with indole reagents, such as Ehrlich's reagent and cinnamaldehyde.

Reactions between thiols and aminochromes, such as those described above may be of considerable significance in several biological processes. A possible mode of attachment of melanins to proteins could be by a sulfide linkage formed by an interaction of one of the thiol groups in the latter with one of the indole-5,6-quinone units in the melanin structure (cf. 27, 28); such interactions could also occur with the distinct aminochrome units which are considered by some workers to be present in the melanin structure (cf. 29, 30). It has been suggested that the sulfide linkage occurs at the 4-position in the indole nucleus (11, 31), however, the present investigation would suggest that the possibility of such linkages occurring at the 7-position of the indole nucleus should not be overlooked.

The formation of aminochrome–thiol addition products such as the compound (IV) offers the intriguing possibility that some thiols could conceivably act as "aminochrome-carriers", which could readily regenerate the aminochrome under the appropriate conditions. Hadler *et al.* have recently described the somewhat analogous conjugation of cysteine during its oxidation by 2,6-dichlorophenolindophenol, and suggested that reactions of this type maybe of biological significance (32, 33).

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