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# HEPATIC MICROSOMAL METABOLISM OF EPINEPHRINE AND ADRENOCHROME BY SUPEROXIDE-DEPENDENT AND -INDEPENDENT PATHWAYS

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**Abstract**—Factors affecting the formation of adrenochrome from epinephrine by the hepatic microsomal fraction in the presence of NADPH have been studied. High oxygen concentrations produced up to a 2-fold increase in the rate of adrenochrome formation, but had no effect upon the rate of superoxide formation by the microsomal fraction, while $10^{-5}$ M KCN produced a 4-fold increase in the rate of adrenochrome formation from epinephrine and a 50 per cent decrease in the rate of aerobic adrenochrome metabolism. The metabolism of adrenochrome by the microsomal fraction was biphasic. The initial, slower phase of metabolism was oxygen dependent and was inhibited by superoxide dismutase. The second, faster phase occurred only when the dissolved oxygen had been used up and represented the one electron reduction of adrenochrome by NADPH-cytochrome *c* reductase to form adrenochrome semiquinone, which then disproportionated to a zwitterionic tautomer of adrenochrome and leucoadrenochrome. The leucoadrenochrome was relatively stable and could be converted back to adrenochrome by reaeration of the medium. 5,6-Dihydroxy-*N*-methylindole, which has been suggested to be formed from leucoadrenochrome, could not be detected by the techniques employed. In the presence of oxygen, the adrenochrome semiquinone formed by the reduction of adrenochrome was oxidized back to adrenochrome with the formation of superoxide. Superoxide catalyzed the conversion of adrenochrome to adrenolutin.

The chemistry of the oxidation of epinephrine and related compounds has been studied extensively [1–4], and the oxidation of epinphrine to adrenochrome is now used frequently as an assay for the formation of superoxide or for the presence of superoxide dismutase [5–8]. The physiological and pharmacological activity of adrenochrome has been studied in some detail [2, 9]. Although the presence of adrenochrome in mammalian tissues has not been demonstrated unequivocally, as has been pointed out by Heacock [2], this does not rule out the possibility that it may have a transient and even important role to play in the metabolism of epinephrine. Detailed schemes for the chemical oxidation of epinephrine have been proposed recently [3, 10] and experimental evidence for the existence of some of the intermediates has been presented [3]. More recently, a pathway for the oxidation of epinephrine to adrenochrome by the hepatic microsomal fraction has been proposed [11]. During the course of these studies, it was noted that adrenochrome formation reached a peak followed by the conversion of the pink adrenochrome to a colorless product and that part of this metabolism could be blocked by superoxide dismutase, despite reports that superoxide would neither oxidize nor reduce adrenochrome [3], and that adrenochrome, unlike epinephrine, did not use superoxide formed by the microsomal fraction [12]. The purpose of the present investigation was to determine the relative contribution of superoxide-dependent and -independent pathways in the hepatic microsomal metabolism of epinephrine and adrenochrome.

## MATERIALS AND METHODS

Male albino rats of the Wistar strain, weighing between 200 and 250 g, were killed by a blow on the head and exsanguinated; the livers were removed and flushed retrogradely through the hepatic vein with 50 ml of 0.9% NaCl at 4°. Hepatic microsomes were prepared by the method of Ernster *et al.* [13] and suspended in 0.15 M KCl at a protein concentration of 6 mg/ml. Protein was determined by the method of Lowry *et al.* [14] with crystalline bovine serum albumin as a standard. NADPH-cytochrome *c* reductase was prepared from hepatic microsomes by the method of Yasukochi and Masters [15]. Microsomal metabolism was determined with an incubation medium containing Tris–HCl buffer, pH 7.4 (300 μmoles), $MgCl_2$ (15 μmoles), KCl (75 μmoles), microsomal protein (3 mg) and epinephrine or adrenochrome (0.6 μmoles) or cytochrome *c* (0.18 μmole), all in a final volume of 3 ml at 37°. The reaction was initiated by the addition of NADPH (3 μmoles) dissolved in a volume of 10 μl. Alternatively, the incubation medium contained Tris–HCl buffer, pH 7.4 300 μmoles), EDTA (0.3 μmole), either xanthine oxidase (50–400 μg) or NADPH-cytochrome *c* reductase (18–140 μg) and the substrates above, in a final volume of 3 ml at 37°. The reaction was initiated with either xanthine sodium (0.5 μmole in 20 μl) or NADPH (3 μmoles in 10 μl) for xanthine oxidase and NADPH-cytochrome *c* reductase respectively. Where necessary the incubation medium was bubbled with a gas mixture in a sealed cuvette for 5 min before the assay but, unless stated, the medium was not gassed during

the assay. The formation and disappearance of adrenochrome were followed at 480 nm, using an extinction coefficient of 4.02 $mM^{-1} cm^{-1}$ [16]; the reduction of cytochrome *c* was followed at 550 nm, using an extinction coefficient of 19.6 $mM^{-1} cm^{-1}$ [17]. Fluorescence measurements were made on an Aminco Bowman fluorometer most commonly at an excitation wavelength of 400 nm and an emission wavelength of 500 nm. Hepatic microsomal oxygen utilization was measured with a Clark oxygen electrode assembly (Rank Brothers, Bottisham, Cambridge, U.K.). Cyclic voltammetry was performed essentially as described by Adams [18] with a 10 $\mu$l flow-through electrode chamber and carbon paste electrodes (graphite/Nujol) scanning from −0.3 V to +1.2 V and back every minute at 25°. The concentration of KCl in the incubation medium was increased to 0.1 M and, because the products of metabolism were destroyed during each scan, fresh medium was pulsed into the chamber just prior to the commencement of each scan. Standards were prepared in deaerated buffer just prior to use.

Adrenochrome and 5,6-dihydroxy-*N*-methyl indole were prepared by the method of Harley-Mason [1] and adrenolutin (3,5,6-trihydroxy-*N*-methyl indole) by the method of Bu'Lock and Harley-Mason [19]. These standards were washed extensively and recrystallized and their purity and identity confirmed, following the formation of their trimethylsilyl derivatives, by gas chromatography mass spectrometry. Epinephrine bitartrate and xanthine were obtained from the Sigma Chemical Co, St. Louis, MO. NADPH, NADH, xanthine oxidase, catalase and cytochrome *c* from the Boehringer Co, Indianapolis, IN, and superoxide dismutase from Miles Laboratories, Elkhart, IN.

## RESULTS

A typical recording of the oxidation of epinephrines to adrenochrome by the microsomal fraction under aerobic conditions (Fig. 1a) shows the characteristic lag period followed by a short linear phase of adrenochrome formation reaching a plateau and then a rapid bleaching of the pink-colored solution. Reaeration of the medium at this point led to the almost instantaneous reappearance of adrenochrome at about 75 per cent of the original plateau level. Adrenochrome formation was dependent upon the oxygen concentration, and continuous gassing with 100% $O_2$ produced a 2-fold increase in the rate of adrenochrome formation and prevented the secondary bleaching of the medium. The metabolism of adrenochrome under aerobic conditions (Fig. 1b) was clearly biphasic and reaeration following the second phase of metabolism resulted in the partial reappearance of the pink color to the medium. Gassing with 100% oxygen completely abolished the second phase of metabolism and also produced some reduction in the first phase of metabolism. Lowering the amount of oxygen available shortened the initial phase of metabolism (Fig. 1b), and it was absent under completely anaerobic conditions (Fig. 2). Measurement of oxygen utilization revealed that the onset of both the second phase of adrenochrome metabolism and the bleaching of the adrenochrome formed during the oxidation of epinephrine corresponded to the disappearance of all the oxygen from the incubation medium. Epinephrine and adrenochrome (0.2 mM) increased the rate of NADPH-dependent oxygen utilization by the microsomal fraction under aerobic conditions at 37° from 3.6 to 35.4 and 27.6 nmoles/min/mg, respectively, con-

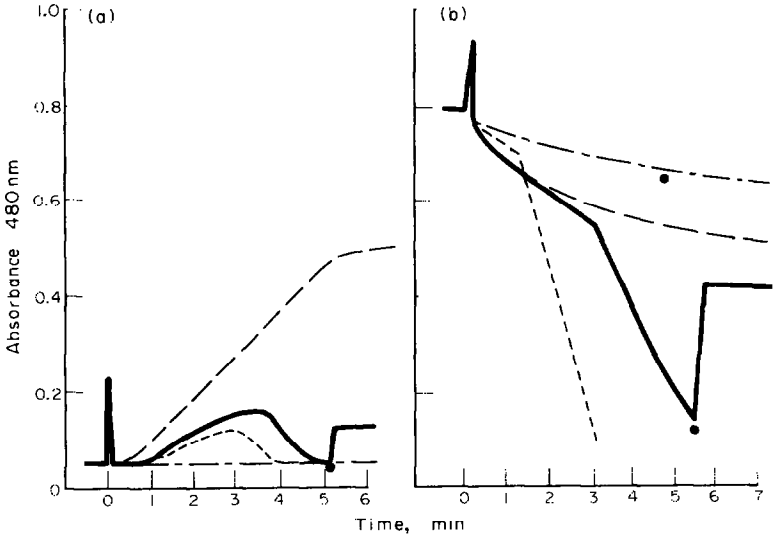


Fig. 1. Epinephrine and adrenochrome metabolism by the hepatic microsomal fraction. The metabolism of epinephrine (a) and adrenochrome (b) by the hepatic microsomal fraction was measured as the appearance and disappearance of adrenochrome at 480 nm, as described in the text, at 37°, with medium pregassed with 10% oxygen in nitrogen (.....), with air (— — —), or continually gassed with 100% oxygen (— · —). Blanks in the absence of NADPH are shown (· · · · ·). Air was bubbled through the cuvettes at the point shown by a closed circle (●).

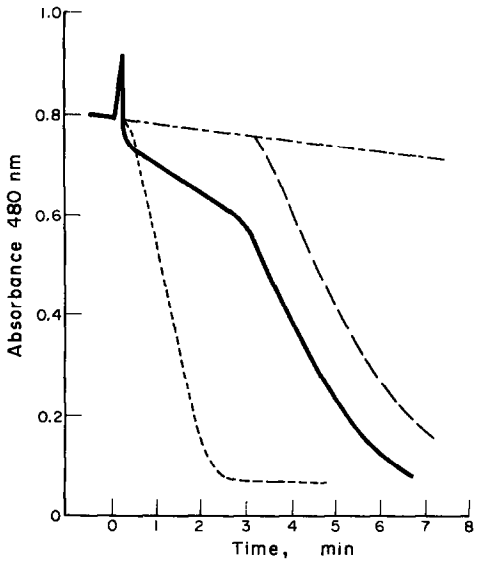


Fig. 2. Factors affecting hepatic microsomal adrenochrome metabolism. The metabolism of adrenochrome by the hepatic microsomal fraction was measured as the change in optical density at 480 nm, as described in the text, at 37° under aerobic conditions, in the absence (———), and in the presence (– – –) of superoxide dismutase, 33 μg/ml, or after gassing with nitrogen (.....). The aerobic blank in the absence of NADPH is shown (–·–·–·–·–).

siderably in excess of that reported for normal substrates of the microsomal mixed function oxidase [20], with the result that the medium rapidly became anaerobic. The increased rate of adrenochrome formation at high oxygen concentrations was not due to an increase in the rate of microsomal superoxide

Table 1. Factors affecting the hepatic microsomal metabolism of epinephrine and adrenochrome*

| | Epinephrine | Adrenochrome | |
|---|---|---|---|
| | Aerobic | Aerobic | Anaerobic |
| Control | 100 (13.6) | 100 (18.7) | 100 (91.2) |
| NADH† | 1 | 67 | 12 |
| CO | 0 | NA‡ | 114 |
| CO–$O_2$ (8:2) | 97 | 96 | NA |
| SKF-525-A (0.5 mM) | 90 | 81 | 95 |
| Superoxide dismutase (33 μg/ml) | 0 | 16 | 106 |
| Catalase (67 μg /ml) | 88 | 98 | 121 |
| Dicoumarin (50 μM) | 96 | 115 | 106 |
| KCN (40 μM) | 416 | 56 | 91 |

*Metabolism was determined at 37°, as described in the text. Epinephrine oxidation was measured during the linear phase of adrenochrome formation following the initial lag period, and adrenochrome metabolism over the first 2 min under aerobic and anaerobic conditions. Shown are typical results from three sets of observations. Figures in parentheses represent the control rates of metabolism in nmoles/mg of microsomal protein/min.

†One mM NADH replaced 1 mM NADPH.

‡Not applicable.

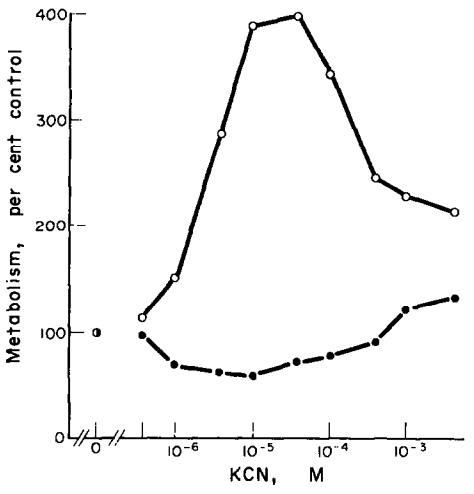


Fig. 3. Effect of cyanide upon the aerobic metabolism of epinephrine and adrenochrome. The metabolism of epinphrine (○) and the first phase of adrenochrome metabolism (●) under aerobic conditions was measured at 37°, as described in the text.

formation. This was measured by the reduction of acetylated ferricytochrome *c* [21], with and without superoxide dismutase (these results not shown). The aerobic phase of adrenochrome metabolism was inhibited by superoxide dismutase, but there was no effect upon the anaerobic phase of metabolism (Fig. 2 and Table 1). Other factors affecting microsomal epinephrine and adrenochrome metabolism are shown in Table 1. NADH was less effective than NADPH in supporting epinephrine oxidation and anaerobic adrenochrome metabolism although surprisingly effective at supporting aerobic adrenochrome metabolism. Neither carbon monoxide nor SKF-525-A, both inhibitors of cytochrome P-450 mediated reactions [22], had any marked inhibitory effect upon metabolism. The failure of catalase to inhibit metabolism rules out hydrogen peroxide as a factor in metabolism and probably also hydroxyl radicals, which may be formed by the interaction of superoxide with hydrogen peroxide [23]. Dicoumarin, an inhibitor of microsomal diaphorase [24] which catalyses the reduction of a variety of quinones [25], similarly had no effect upon metabolism. The effect of KCN in stimulating adrenochrome formation from epinephrine and in decreasing aerobic adrenochrome metabolism is shown in more detail in Fig. 3. In both cases, the maximum effect was noted at $10^{-5}$ M KCN which is much too low to be accounted for by an inhibition of microsomal fatty acid desaturase [26] or cytochrome P-450 [27]. The effect of KCN in stimulating adrenochrome formation was found to be additive with that of 100% oxygen. KCN is a potent inhibitor of superoxide dismutase [28], but this could not account for the effect upon microsomal metabolism since the microsomes contained no superoxide dismutase, as shown by the absence of an effect upon the oxidation of epinephrine by xanthine oxidase and xanthine. KCN had no effect upon epinephrine-stimulated oxygen utilization and has been

Table 2. Metabolism by NADPH-cytochrome *c* reductase and xanthine oxidase*

| Substrate | NADPH-cytochrome *c* reductase | | Xanthine oxidase | |
|---|---|---|---|---|
| | Without SOD (nmoles/min/mg) | With SOD (nmoles/min/mg) | Without SOD (nmoles/min/mg) | With SOD (nmoles/min/mg) |
| Cytochrome *c* | 2367 | 2294 | 87 | 0 |
| Epinephrine | 293 | 0 | 44 | 0 |
| Adrenochrome (air) | 35 | 2 | 27 | 0 |
| Adrenochrome ($N_2$) | 251 | 216 | ND† | ND |

* Metabolism was determined at 37°, as described in the text, with and without 33 μg/ml of superoxide dismutase (SOD). Epinephrine oxidation was measured during the linear phase of adrenochrome formation following the initial lag period, and adrenochrome metabolism over the first 2 min under aerobic and anaerobic conditions.

† Not determined.

reported to have no effect upon epinephrine-stimulated microsomal NADPH oxidation [29]. It is probable, therefore, that KCN produced its apparent effect upon epinephrine oxidation by decreasing the rate at which adrenochrome was removed.

Hepatic microsomal NADPH-cytochrome *c* reductase will catalyze the reduction of a variety of quinones [30, 31], and it has been suggested that it might be responsible for the reduction of adrenochrome under anaerobic conditions [12]. This has now been confirmed using purified NADPH-cytochrome *c* reductase (Table 2). NADPH-cytochrome *c* reductase will also generate superoxide in the presence of NADPH and oxygen [7], which accounts for the aerobic metabolism of epinephrine and adrenochrome shown in Table 2. The superoxide-dependent metabolism of adrenochrome is also seen with the simpler superoxide-generating system of xanthine oxidase and xanthine (Table 2). A comparison of the results of Table 2 showing cytochrome *c* reduction (2367 nmoles/min/mg) and the superoxide-dependent oxidation of epinephrine (293 nmoles/min/mg) by NADPH-cytochrome *c* reductase with the rates of microsomal cytochrome *c* reduction (171 nmoles/min/mg) (Table 3) and epinephrine oxidation (14 nmoles/min/mg)

Table 3. Stimulation of hepatic microsomal NADPH-cytochrome *c* reductase by adrenochrome*

| | Without SOD (nmoles/min/mg) | With SOD (nmoles/min/mg) |
|---|---|---|
| Control | 171 | 169 |
| Adrenochrome (60 μM) | 218 | 174 |
| (200 μM) | 391 | 198 |

* Metabolism was determined, as described in the text, at 37° as the initial rate of cytochrome *c* reduction, with and without 33 μg/ml of superoxide dismutase (SOD). Shown are typical results from three sets of observations.

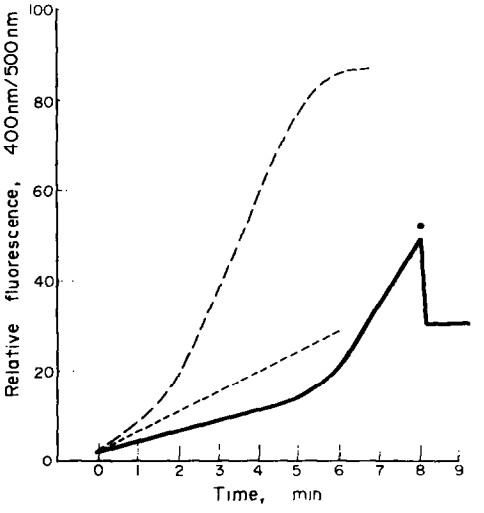


Fig. 4. Formation of fluorescent products from adrenochrome. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 500 nm. Products resulting from the hepatic microsomal metabolism of adrenochrome under aerobic conditions ( ) and under anaerobic conditions (– – –), and from metabolism by 30 mg/ml of xanthine oxidase and 0.17 mM xanthine (.....), with the incubation conditions described in the text are shown. The initially aerobic cuvette was bubbled with air at the point shown by a closed circle (●).

(Table 1) reveals tht NADPH-cytochrome *c* reductase activity is sufficient to account for all of the superoxide produced by the microsomal fraction.

The products of both the aerobic and anaerobic metabolism of adrenochrome exhibited a fluorescence at an excitation wavelength of 400 nm and an emission wavelength of 500 nm (Fig. 4). The product formed during the aerobic, superoxide-dependent, phase of metabolism exhibited a maximum fluorescence at 397/520 nm which corresponded to that of an adrenolutin standard. The product formed under anaerobic conditions exhibited a maximum fluorescence at 408/516 nm and disappeared upon reaeration of the medium. This probably represented leucoadrenochrome, although the compound has proved too unstable to be prepared as a reference compound [1, 3]. Leucoadrenochrome has been reported to undergo a proton catalyzed dehydration to 5,6-dihydroxy-*N*-methyl indole [3]. This compound had a characteristic maximum fluorescence at an excitation/emission wavelength of 323/410 nm, but no such fluorescence could be detected in the incubation medium.

Cyclic voltammetry was used in an attempt to provide a positive identification of the unstable leucoadrenochrome which was thought to be the product of the microsomal metabolism of adrenochrome under anaerobic conditions. Hawley *et al.* [32] have reported that under suitable conditions adrenochrome gives rise to characteristic cathodic and anodic peaks corresponding to the reduction of adrenochrome to leucoadrenochrome and the re-oxidation of leucoadrenochrome to adrenochrome

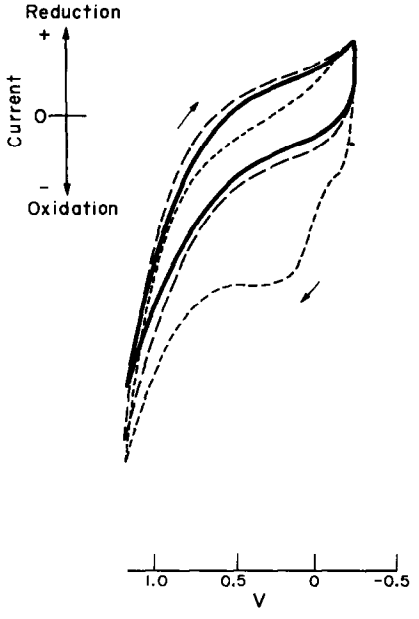


Fig. 5. Cyclic polarogram of the products resulting from the hepatic microsomal metabolism of adrenochrome. Cyclic scans were performed from −0.3 V to +1.2 V and back every minute (3.0 V/min); the arrows indicate the direction of the scan. The incubation medium was as described in the text and contained 0.2 mM adrenochrome. It was equilibrated initially with air, but was held in a closed container during the incubation at 25°, and fresh medium was pulsed into the electrode chamber just before commencing each scan at −0.3 V. The figure shows scans commencing at 0 min (———), 3 min (– – – –) and 6 min (.....), by which time all of the adrenochrome had been reduced to the colorless product.

respectively (peaks 3 and 4 in Fig. 2, a cyclic polarogram of epinephrine, in Ref. 32). In the study by Hawley *et al.* [32], the anodic peak corresponding to the oxidation of leucoadrenochrome was apparent only on the second cyclical scan due to the fact that adrenochrome had first to be formed electrochemically from epinephrine and then reduced to give leucoadrenochrome. In the present study, the microsomal metabolism of adrenochrome resulted in the appearance of two anodic peaks, corresponding to two readily oxidizable species, and a decrease in size and a shift of the cathodic peak, at a time corresponding to the anaerobic phase of metabolism (Fig. 5). The peaks at −0.1 V corresponded in position to the adrenochrome–leucoadrenochrome couple which could be seen with the adrenochrome standard at pH 3.0. The anodic leucoadrenochrome peak was present on the first cyclical scan after introduction of fresh medium into the electrode chamber at 6 min, indicating that the leucoadrenochrome was a product of metabolism and not formed by the electrochemical reduction of adrenochrome. The second anodic peak at +0.2 V could not be assigned to either adrenolutin or 5,6-dihydroxy-*N*-methyl indole, since both reference compounds gave anodic peaks in the same region.

It might be expected that the reduction of adrenochrome by NADPH-cytochrome *c* reductase, which can catalyze the one electron reduction of a variety of quinones [30], would proceed through a semiquinone intermediate which under aerobic conditions would be expected to oxidize readily to adrenochrome with the generation of superoxide from molecular oxygen. Uemura *et al.* [12] have presented evidence for the formation of superoxide in the presence of adrenochrome based upon the adrenochrome enhancement of microsomal cytochrome *c* reduction. This is not conclusive evidence, however, since the semiquinone might react directly to reduce cytochrome *c* [30]. Table 3 shows that the adrenochrome enhancement of cytochrome *c* reduction is due almost exclusively to superoxide in that it can be inhibited by superoxide dismutase. The ratio of the formation of superoxide in the presence of 0.2 mM adrenochrome and oxygen to the rate of adrenochrome reduction under anearobic conditions (Table 1) is 2:1, which may be fortuitously close to the theoretical ratio, but it does suggest that the adrenochrome semiquinone must be released from the enzyme and react efficiently with dissolved oxygen to form superoxide. The lag phase in the microsomal oxidation of epinephrine (Fig. 1) might thus be explained by the time taken to form a limiting concentration of adrenochrome by the action of superoxide produced by the microsomal fraction, to act as a catalyst for the further production of superoxide.

## DISCUSSION

A suggested pathway for the metabolism of epinphrine by the hepatic microsomal fraction is shown in Fig. 6. The oxidation of epinephrine (I) to adrenochrome (III) is known to be accompanied by the formation of peroxide with superoxide acting as an initiator and a self-propagating catalyst [11], thus explaining the ability of superoxide dismutase to inhibit the reaction. A more detailed pathway for the conversion of epinephrine to adrenochrome through the postulated epinephrine quinone intermediate (II), based upon that suggested by Misra and Fridovich [10], has been presented elsewhere [11]. The involvement of molecular oxygen (steps 1 and 2) might explain why an increased oxygen concentration produced an increase in the rate of adrenochrome formation with no change in the rate of microsomal superoxide formation. The ability of the carbon monoxide–oxygen mixture (9:1) to inhibit the microsomal oxidation of epinephrine has been interpreted previously as evidence for the involvement of cytochrome P-450 in microsomal superoxide formation [11, 34]. It is more likely to be due, however, to the low oxygen concentration compared to air (21% oxygen), and in the present study an 8:2 carbon monoxide–oxygen mixture was found to have no effect upon epinephrine oxidation. This would suggest that cytochrome P-450 is not involved in microsomal superoxide formation, and NADPH-cytochrome *c* reductase is capable of accounting for all of the microsomal superoxide formation. NADPH-cytochrome *c* reductase also catalyzes the one electron reduction of adrenochrome to adrenochrome semiquinone (V, step 4), which under aerobic conditions reacts readily with molecular

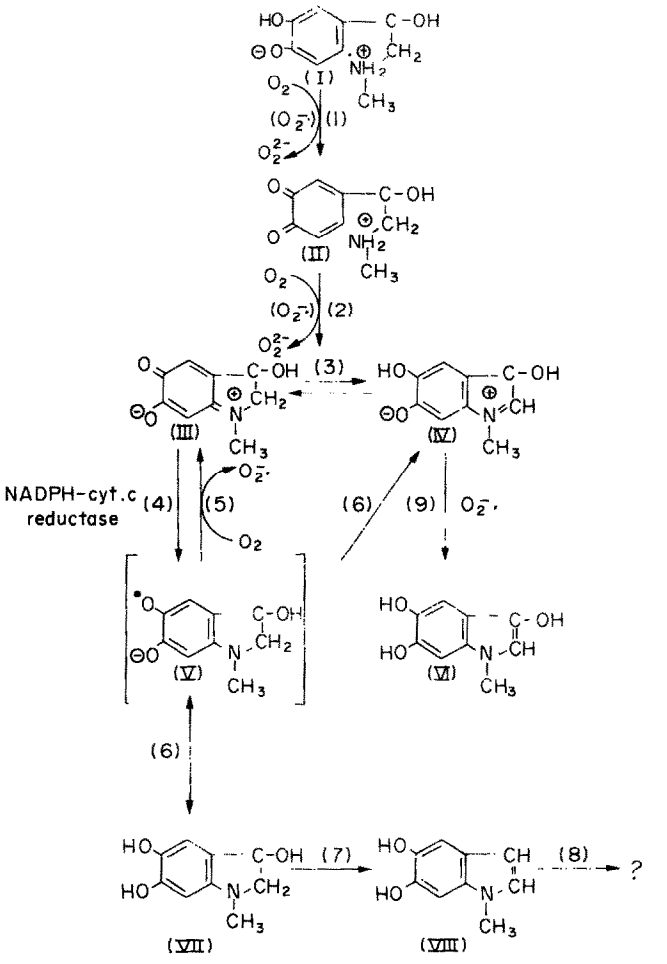


Fig. 6. Suggested pathway for the metabolism of epinephrine and adrenochrome by the hepatic microsomal fraction. I, epinephrine; II, epinephrine quinone; III, adrenochrome as the zwitterionic *p*-quinoneimine suggested by Harley-Mason [33]; IV, tautomeric form of adrenochrome; V, adrenochrome semiquinone; VI, adrenolutin; VII, leuco-adrenochrome; and VIII, 5,6-dihydroxy-*N*-methyl indole.

oxygen to form superoxide and regenerate adrenochrome (step 5). In the absence of oxygen it is probable that the adrenochrome semiquinone undergoes bimolecular disproportionation (step 6), as suggested by Harley-Mason [1], to form leucoadrenochrome (VII) and a zwitterionic tautomeric form of adrenochrome (IV). This appears to be more likely than two sequential one electron reductions of adrenochrome, as proposed by Uemura *et al.* [12], since the adrenochrome semiquinone is clearly released from the enzyme for it to be able to react with oxygen. Similarly, Iyanagi and Yamazaki [30] have proposed the one electron reduction of benzoquinone by NADPH-cytochrome *c* reductase with the release of the semiquinone followed by dismutation to the quinone and the hydroquinone. The leucoadrenochrome (VII) formed under anearobic conditions appears to accumulate and be stable enough to be reoxidized back to adrenochrome upon aeration of the medium, despite reports that it is rapidly converted to 5,6-dihydroxy-*N*-methyl indole [1, 3]. In this study, no evidence could be found for the formation of 5,6-dihydroxy-*N*-methyl indole (that is for step 7), although it is possible that it was formed and then further oxidized (step 8). Standard solutions of 5,6-dihydroxy-*N*-methyl indole were found to lose their characteristic fluorescence when mixed with equal amounts of adrenochrome indicating, perhaps, further oxidation. Adrenochrome administered to whole animals has been shown to be metabolized and excreted in the urine as conjugates of 5,6-dihydroxy-*N*-methyl indole and adrenolutin [35]. Superoxide anions clearly catalyzed the conversion of adrenochrome to adrenolution (VI), probably through the zwitterionic tautometer of adrenochrome (IV, step 9). This reaction is known to occur readily under alkaline conditions [1] and superoxide could be acting as a Brønsted base, although this is by no means certain. A lower steady-state concentration of the adrenochrome tautomer (IV) in the presence of oxygen, due to cycling between intermediates III and V, might explain why a high oxygen concentration produces a decrease in the rate of superoxide-dependent adrenochrome metabolism. The superoxide-dependent conversion of adrenochrome shown in step 9 would not produce any oxidized or reduced

adrenochrome intermediates, thus explaining the findings by Bors *et al.* [3], nor would adrenochrome decrease the levels of superoxide in the medium, as reported by Uemura *et al.* [12], particularly in view of the great excess of superoxide produced through steps 4 and 5.

Recent work has suggested that a more cautious approach should be adopted toward the simplistic superoxide initiated and superoxide dismutase inhibited view of epinephrine oxidation because of the ease of formation of the semiquinone of epinephrine by hydroxyl radicals [3]. To this should now be added the caution that, in biological systems where the adrenochrome can be further metabolized, various factors may appear to affect the rate of adrenochrome formation from epinephrine without actually having an effect upon the rate of superoxide formation.

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