MOLECULAR PHARMACOLOGY, 15, 428–438

Active Oxygen in Liver Microsomes: Mechanism of Epinephrine Oxidation

JOHN B. SCHENKMAN,[1] INGELA JANSSON,[1] GARTH POWIS[2] AND HERMANN KAPPUS[3]

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

(Received June 7, 1978)
(Accepted October 24, 1978)

SUMMARY

SCHENKMAN, J. B., JANSSON, I., POWIS, G. & KAPPUS, H. (1979) Active oxygen in liver microsomes: Mechanism of epinephrine oxidation. *Mol. Pharmacol.* **15**, 428–438.

Under normal conditions, during the oxidation of NADPH, liver microsomes are shown to generate hydrogen peroxide but not appreciable amounts of superoxide anion. This latter species can be detected, however, if iron pyrophosphate is present. The NADPH-supported microsomal oxidation of epinephrine is non-self-propagating, *i.e.*, requires an external catalyst. It requires NADPH in a stoichiometry of 1 per adrenochrome formed, while utilizing 3 oxygens and producing 3 peroxides. This reaction is inhibited by superoxide dismutase but not by catalase and does not appear to diminish the background rate of NADPH mediated hydrogen peroxide formation, a reaction utilizing equal amounts of oxygen and NADPH for a 1:1:1 stoichiometry with hydrogen peroxide formation. Adrenochrome formation always exhibits a lag period. This is shown here to be due to accumulation of an intermediate of epinephrine oxidation. From the data presented it is concluded that if free superoxide anion is released to the aqueous medium, it is only in small amounts and rapidly dismutes. Epinephrine does not reduce cytochrome P-450. NADH does not support the oxidation of epinephrine to adrenochrome to any appreciable extent, but does allow peroxide formation to occur, presumably by other routes. A schematic mechanism of epinephrine oxidation is shown demonstrating the observed 1:3 stoichiometry of adrenochrome:peroxide formation.

INTRODUCTION

The conversion of epinephrine to adrenochrome has been studied since the early part of this century (see 1 for review). In alkaline solution the reaction was fairly rapid and corresponded to the utilization of one molecule of oxygen per molecule of epinephrine consumed. At neutral or near neutral pH this oxidation was shown to be very slow unless catalyzed by metal ions (1). Hydrogen peroxide formation was reported to accompany the oxidation of catechol and catechol amines, since the consumption of oxygen was diminished by the presence of catalase (2).

The catalysis of epinephrine oxidation by ferritin iron plus hydrogen peroxide (3) was suggested as being mediated by hydroxyl radicals (in analogy with Fenton's reagent). Later studies suggested epinephrine oxidation to involve organic radicals and species

Supported by USPHS grants GM17021 and GM24530 from the NIH. Preliminary reports of this work have appeared: *Pharmacologist* **18**, 119 (1976); *Fed. Proc.* **37**, 464 (1978).

[1] To whom reprint requests should be addressed. Present address: Dept. of Pharmacology, Univ. of Connecticut, Health Center, Farmington, CT 06032.

[2] Current address: Dept. of Oncology, Mayo Clinic, Rochester, MN 55901.

[3] Dept. of Toxicology, Univ. of Tübingen, Tübingen, Germany.

0026-895X/79/020428-11$02.00/0

of active oxygen other than hydroxyl radical (4). In the oxidation of epinephrine catalyzed by xanthine oxidase, the reaction was shown to require the co-oxidation of hypoxanthine (4). An oxygen radical similar to that involved in aerobic reduction of cytochrome c by xanthine oxidase (5), superoxide anion, was implicated in that reaction. That superoxide was indeed involved in the reaction catalyzed by xanthine oxidase was demonstrated (6, 7) by complete inhibition of adrenochrome formation at pH 7.8 and 10.2 by superoxide dismutase.

Two distinct mechanisms have been suggested for the *autoxidation* of epinephrine to adrenochrome (6). One involves a superoxide mediated free radical chain, and the other a superoxide dismutase insensitive pathway driven by an organic radical mediated chain reaction. The latter appeared to predominate at lower pH, adrenochrome formation being completely insensitive to superoxide dismutase below pH 8.5 (6); superoxide dismutase is not inoperable in this range, as it completely inhibits the xanthine oxidase catalyzed reaction even at pH 7.8 (6).

Although attempts to detect superoxide anion generation by microsomes and NADPH were unsuccessful when lactoperoxidase was used as a trap (8), superoxide dismutase inhibition of epinephrine oxidation to adrenochrome by microsomes at pH 8.5 was strongly suggestive of involvement of superoxide anion (9). In this report we have examined epinephrine oxidation to adrenochrome at pH 7.5 and show that two reactions can occur, one of which is superoxide dismutase inhibitable and requires NADPH and the other, which is not inhibitable by superoxide dismutase and does not require NADPH. The former reaction is catalyzed by microsomes and the latter is unaffected by microsomes. The stoichiometries of these reactions are described and their mechanisms are discussed.

MATERIALS AND METHODS

Preparation of microsomes. The microsomal fraction of pooled livers of 200–350 g male Sprague-Dawley rats (Charles River Breeders) was prepared. The animals had been allowed free access to lab chow and water until decapitation. The livers were removed and flushed retrogradely with chilled 0.15 M NaCl to remove blood. The microsomes were prepared by the rapid calcium aggregation technique (10) and were resuspended to 10 mg protein/ml in 0.15 M KCl–10 mM Tris-HCl buffer, pH 7.5. Protein was determined by a biuret method (11).

Assays. Epinephrine oxidation to adrenochrome (3) was measured in a medium (3.0 ml) containing 0.5 mg microsomal protein/ml (unless otherwise indicated) in 0.1 M Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM NaN_3 and 0.4 mM epinephrine. In the presence of catalase NaN_3 was omitted. The medium was equilibrated in two cuvets at 37° in an Aminco DW2 spectrophotometer and the reaction was initiated by rapid addition of 0.5 mM NADPH in the sample cuvet with a spring loaded plunger. Adrenochrome formation was measured as the increase in absorption at 480 nm using an extinction coefficient of 4.02 $mM^{-1}cm^{-1}$ (12). When KO_2 was used to catalyze adrenochrome formation, it was added in anhydrous dimethylsulfoxide, the latter kept stored over anhydrous Na_2SO_4.

NADPH consumption was assayed in the above medium at 37°, and measured by determining fluorometrically the amount of enzymatically active NADPH remaining at various times, using glutamic dehydrogenase (13).

Oxygen consumption was determined in the same assay medium at 37° polarigraphically. A 1 ml waterjacketed chamber supplied with a Yellow Springs Instruments oxygen electrode was used. It was standardized with air saturated medium at 37° (210 nmoles O_2 per ml) and further checked using standarized H_2O_2 solutions (ϵ_{240} nm = 43.6 $M^{-1}cm^{-1}$, [14]) and catalase.

Hydrogen peroxide formation was measured by the ferrox forming reaction (15, 16) with modifications suggested by Hildebrandt and Roots (14). Sodium azide (0.2 mM) was present to inhibit endogenous catalase activity. Assays were at 37° for up to 5 min with microsomes and was initiated by NADPH. The assay makes use of H_2O_2 oxidation of ferrous ions and complexation of the ferric ions with thiocyanate. The red color was measured at 480 nm and quanti-

tated using standard curves prepared *with microsomes* for each assay medium with standardized H_2O_2.

Spectra with lactoperoxidase were performed with an Aminco DW2 spectrophotometer, scanning from 480 nm to 630 nm, and using a similar medium to that of the other assays. For standardization of spectra, KO_2 (Apache Chemicals, Inc.) dissolved in dry dimethylsulfoxide containing 0.3 M recrystallized dicyclohexyl-18-crown under dry argon (gift of J. Valentine), was used to from the superoxide complex and H_2O_2 (Baker Chemicals), diluted in distilled water was used to form the peroxide complex. In all studies 10 μM lactoperoxidase was used and spectra were recorded at 22°; at 37° the spectra are less stable.

RESULTS AND DISCUSSION

Spectra of lactoperoxidase-active oxygen complexes. In order to determine the species of active oxygen formed by liver microsomes 10 μM lactoperoxidase was used to trap and visualize two of these forms, superoxide (O_2^-) and peroxide (H_2O_2). Figure 1 shows the difference spectra of the complexes obtained on addition of peroxide or superoxide to lactoperoxidase. The two complexes exhibit distinct differences, the spectra in agreement with those assigned for these complexes by Yamazaki *et al.* (17). The addition of H_2O_2 to 10 μM lactoperoxidase causes the appearance of two peaks (solid trace), one at 590 nm and one at 552 nm, with isosbestic points at 528 nm and 602 nm. The addition of potassium superoxide to 10 μM lactoperoxidase causes the appearance of two peaks (dashed trace), one at 538 nm and one at 568 nm. Normally, the addition of KO_2 to an aqueous medium causes an immediate effervescence of oxygen due to disproportionation of the superoxide to oxygen and peroxide. A rate constant of about $10^7 M^{-1} sec^{-1}$ has been estimated at neutral pH for this reaction (7, 18), from which it would be expected that the superoxide would be dissipated within a small fraction of a second. However, when lactoperoxidase is present in excess it traps the superoxide in a stable complex and disproportionation does not occur, as seen by the characteristic spectral complex and lack of effervescence. From this result, if superoxide anion is formed and released by the microsomes on the addition of NADPH it would be expected to be trapped by the lactoperoxidase, provided sufficient levels can accumulate.

The addition of 10 μM lactoperoxidase to

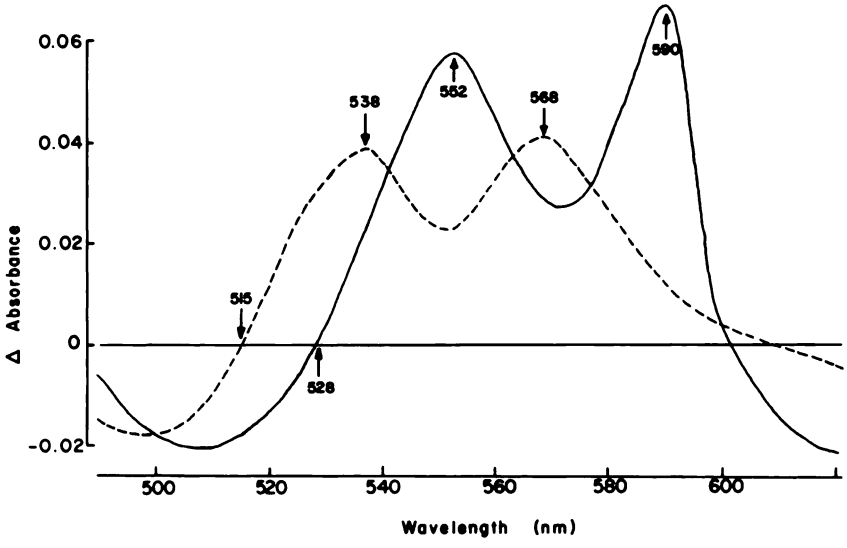


FIG. 1. *Difference spectra of lactoperoxidase complexes with 10 μM KO_2 (----) or 100 μM H_2O_2 (——)* Conditions for spectral measurement are in METHODS section.

the microsomal suspension, followed by NADPH reveals immediate formation and release of H_2O_2 to the medium (Fig. 2). The appearance of isobestic points at 528 nm and 602 nm plus lack of other overlapping spectral changes clearly indicates that hydrogen peroxide, but not detectable levels of superoxide anion, is released to the aqueous suspension, an observation in agreement with that of Debey *et al.* (8). The lack of appearance of a complex indicative of superoxide anion release is not due to inability of the added lactoperoxidase to trap this active oxygen species, but is due to too slow accumulation and too rapid degradation. This was seen on addition of 50 μM iron pyrophosphate, a requirement for lipid peroxidase activity. The addition of this chelate caused rapid appearance of a combined superoxide and peroxide complex (Fig. 3). Thus, if O_2^- is normally released by microsomes, it is at a level below that detectable by lactoperoxidase in the absence of iron chelate. One can provide calculations of the maximal levels of superoxide one can generate (steady-state levels) based upon the rate constant for superoxide dismutation of 10^7 $M^{-1}sec^{-1}$ (7, 18) and some rate of superoxide formation. Choosing a rate similar to that of adrenochrome production in table I, one would expect concentrations of superoxide to remain below 0.3 μM, a level that was not detected using 10 μM lactoperoxidase as a trap.

Epinephrine oxidation catalyzed by the microsomal fraction. Because the autoxidation of epinephrine is not mediated by superoxide anion at pH 7.5 (6), it appeared appropriate to examine the reaction catalyzed by microsomes. Addition of limiting levels of NADPH causes rapid bursts of adrenochrome formation (Fig. 4), at rates more than 50 times the autoxidation rate, followed by cessation of the reaction after depletion of the NADPH. Further addition of NADPH (at arrows) reinstitutes the formation of adrenochrome. NADH was not able to support adrenochrome formation by microsomes. From these results it is clear that the reaction catalyzed by microsomes requires the co-oxidation of NADPH. In this reaction, at limiting NADPH levels (Fig. 4), 6 μM adrenochrome was produced during oxidation of 20 μM NADPH.

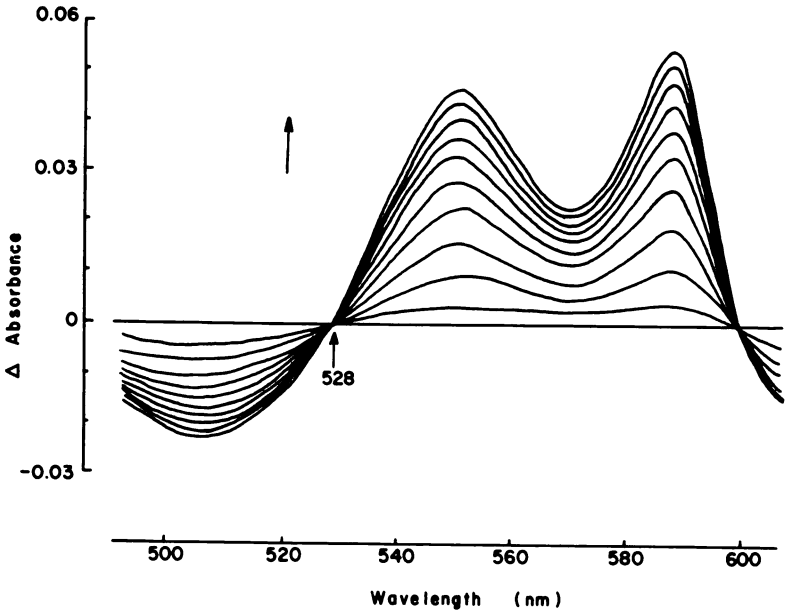


FIG. 2. *Formation of lactoperoxidase complex with H_2O_2 generated by NADPH oxidations in liver microsomes*

Conditions are provided in the METHODS section. Repetitive scans were recorded at a rate of 5 nm/sec after addition of 1 mM NADPH to the medium containing 1.5 mg microsomes and 10 nmoles lactoperoxidase/ml.

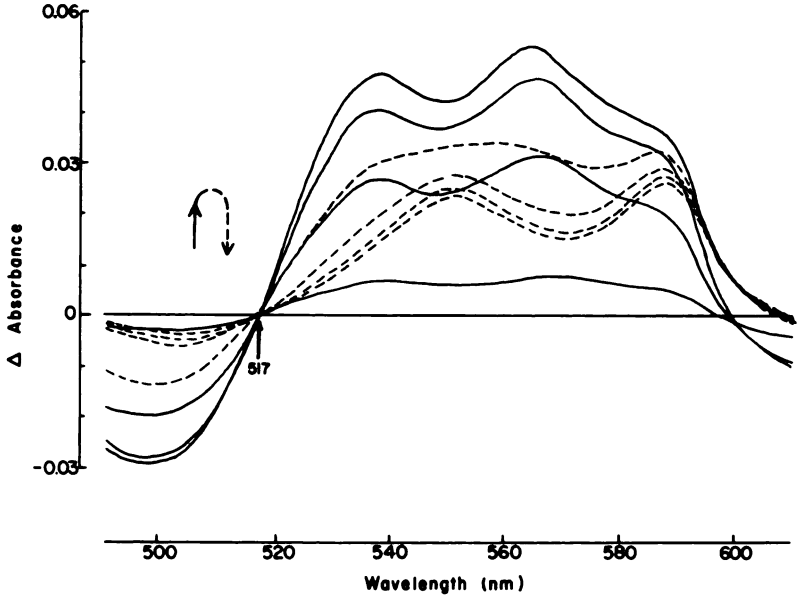


FIG. 3. *Formation of composite lactoperoxidase complex I and complex III spectrum in the presence of microsomes, NADPH and iron pyrophosphate (50 μM)*

Scans of the spectra were at a rate of 5 nm/sec. Conditions were as described in METHODS. Solid lines increase upward with time, followed by decline in absorbance (dashed line). Selected spectra deleted to show changes with time more clearly.

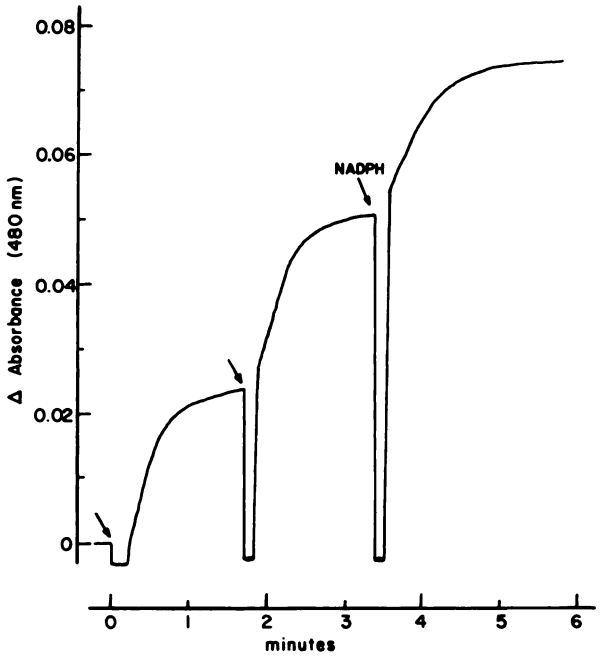


FIG. 4. *Microsomal adrenochrome formation at limiting levels of NADPH*

20 μM NADPH was added as indicated by arrows. Microsomal protein was 1.5 mg/ml.

The stoichiometry of the microsome-catalyzed co-oxidation of epinephrine and NADPH was studied in order to determine the mechanisms and species of active oxygen in the reaction (Fig. 5). The NADPH supported reaction exhibits a lag period before adrenochrome formation occurs. Similar lag periods are observed in NADPH consumption, oxygen consumption and peroxide production by the microsomes. Adrenochrome production is stopped immediately by superoxide dismutase, regardless of when it is added to the medium, indicating the involvement of superoxide in the reaction leading to adrenochrome formation.

The initial rates of utilization of NADPH and oxygen are 28 nmoles/min and 32 nmoles/min per mg microsomes respectively and are accompanied by rates of adrenochrome and peroxide formation of 2 nmoles/min and 32 nmoles/min respectively (Fig. 5). The initial rates vary with different preparations, but by one and a half min the rates of adrenochrome formation are generally maximal and continue linearly for a period. An observed stoichiometry of this latter period is shown in Table 1, along with simultaneous equations drawn to describe the observed reactions (A and B). Because the enhanced rates of oxygen and NADPH consumption and H_2O_2 formation are triggered by epinephrine addition, the rate of adrenochrome formation is taken as the unit value in equation A. The reaction is not self-propagating, but requires a constant supply of NADPH, hence NADPH is added to act as an electron source for activating oxygen to O_2^-. Oxygen reduction by liver microsomes

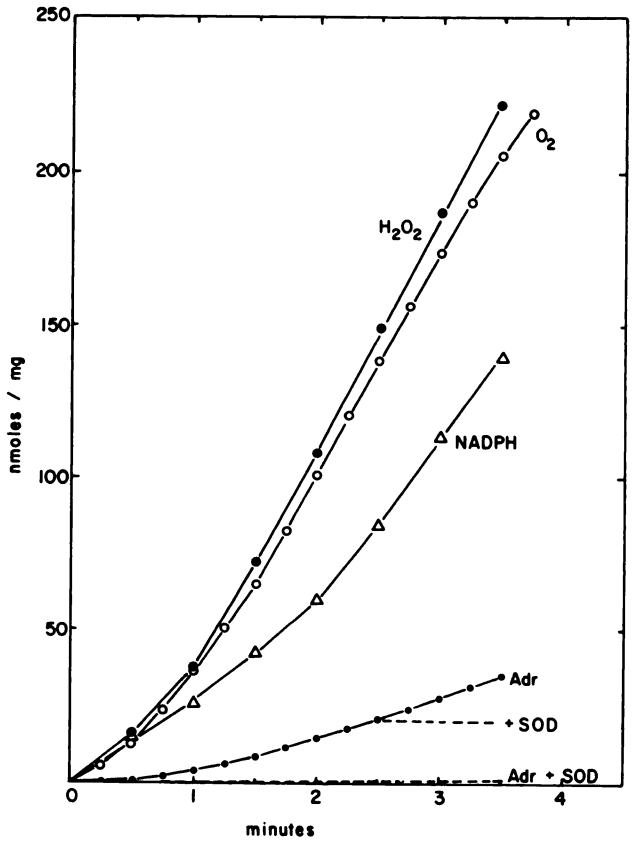


FIG. 5. *Stoichiometry of simultaneous microsomal adrenochrome and H_2O_2 production and NADPH and O_2 consumption*

Conditions are described in METHODS section. This represents results of a typical experiment.

TABLE 1

Stoichiometry of Epinephrine Oxidation[a]

Observed[b]:	13 EPI +	70 $NADPH_2$ +	80 O_2 →	80 H_2O_2 +	13 ADR.
A:	1 EPI +	1 $NADPH_2$ +	3 O_2 →	3 H_2O_2 +	1 ADR.
	(13)	(13)	(39)	(39)	(13)
B:		1 $NADPH_2$ +	1 O_2 →	1 H_2O_2	
		(41)	(41)	(41)	
Σ:	13 EPI +	54 $NADPH_2$ +	80 O_2 →	80 H_2O_2 +	13 ADR.
Δ:		16 $NADPH_2$ → ?			
		−5 $NADPH_2$ → 5 $NMNH_2$[c]			

[a] nmoles/min/mg microsomal protein

[b] mean of 3 experiments

[c] A fluorescence assumed to be reduced nicotinamide ribotide formed in the medium and was not quenched by glutamic dehydrogenase.

stops at the level of H_2O_2 when azide is present to block subsequent reaction by contaminating catalase, and three molecules of oxygen are required to satisfy the stoichiometry of the cooxidation of one molecule each of NADPH and epinephrine.

Taking the rate of adrenochrome formation and relating it to the stoichiometric rates predicted by equation A provides the values shown in brackets (Table 1). The values calculated did not account for the total rates observed. Equation B is provided to account for the excess rates of NADPH and oxygen consumption and peroxide formation and reflects a 1:1:1 stoichiometry. The remaining rate of peroxide produced equals the remaining rate of oxygen consumption (values in brackets), and an equal rate of NADPH consumption is added. Note these observed rates are greater than the initial rates. Equation A and B values in brackets are summed and indicated all components are accounted for except for an excess rate of NADPH disappearance, equal to 16 nmole/min/mg. This value is much lower in younger rats. This excess rate of NADPH consumption (Δ), since it is not accompanied by oxygen uptake, must be supporting nonxoxidative reaction(s). One such reaction is the hydrolytic cleavage of NADPH to reduced nicotinamide ribotide (19), a compound which fluoresces and has UV absorption like NADPH, but will not support reductive reactions; accompanying NADPH disappearance is an increase in fluorescent material in the medium which is not quenched by glutamic dehydrogenase, at a rate of about 5 nmoles/min/mg.

Mechanism of microsomal epinephrine oxidation. The stoichiometry shown in Table 1 equation A would utilize a mechanism like that depicted in Scheme 1, in which superoxide formed by NADPH-supported oxygen reduction, is shown to initiate epinephrine oxidation as well as leuco-adrenochrome oxidation (steps "a" and "d"). The semiquinone free radicals formed are shown to be oxidized by oxygen (steps "b" and "e"); whereas steps "a" and "d" produce hydrogen peroxide with one equivalent each of NADPH and epinephrine, hydrogen peroxide is formed by dismutation of the superoxide molecules from steps "b" and "e" and contains both reducing equivalents derived from epinephrine.

The non-epinephrine associated oxidation of NADPH shown in equation B of Table 1 is faster than the initial rates of the reaction (Fig. 5), and an average rate of H_2O_2 formation of 41 nmoles/min/mg microsomes in 1:1:1 stoichiometry with O_2 consumption and NADPH consumption. This would suggest epinephrine initiates electron flow from NADPH in a pathway not using NADPH in the absence of epinephrine. It would also indicate that pathways not influenced by epinephrine operate simultaneously. As will be indicated below, the enhanced rate in equation B is probably due to reduction of epinephrine radical back to epinephrine.

Based upon the observed stoichiometry (Table 1) during microsomal epinephrine oxidation to adrenochrome other composite stoichiometries and mechanisms may be proposed. For example, one might suggest a second mechanism, in which epinephrine

oxidation begins with NADPH reduction of oxygen to superoxide anion, followed by superoxide reduction by an electron from epinephrine, at point "a," yielding peroxide (Scheme 1). The resulting radical may be further attacked by another superoxide, at point "b," forming another peroxide and epinephrine quinone. This may be followed by ring closure, at point "c," which forms the adrenochrome catechol, leucoadrenochrome. Leucoadrenochrome may be further oxidized by superoxide at point "d," forming a semiquinone radical and more peroxide, and again at point "e," by superoxide yielding a fourth peroxide and adrenochrome. In this mechanism, equation A would have a stoichiometry of 1 adrenochrome: 2 NADPH: 4 O_2: 4 H_2O_2. Equation B would still be in 1:1:1 stoichiometry, but comprising about 2/3 the rate shown in Table 1, *i.e.*, (28):(28):(28), or the initial rate.

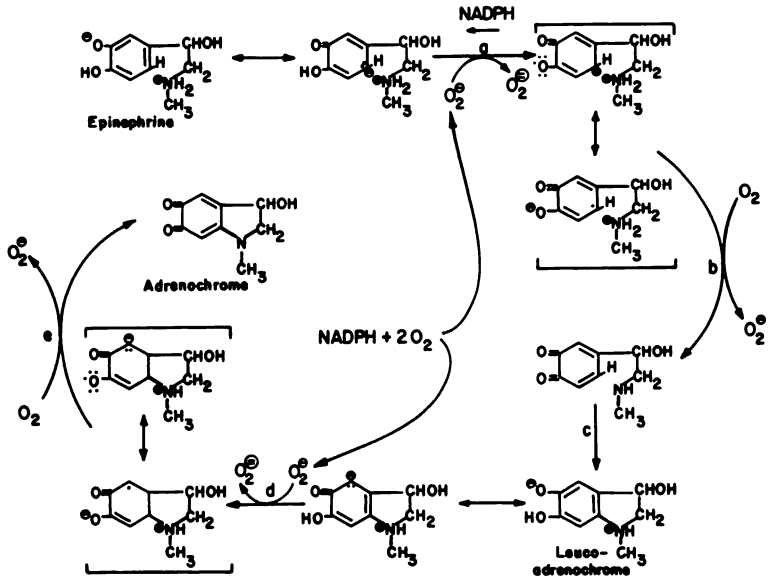
A third possible mechanism can also be drawn, in which the same stoichiometry as mechanism #2 would be obtained. This would be a radical propagated disproportionation, in which only the initiation is mediated by superoxide anion. This can be depicted, using the abbreviation of Misra and Fridovich (6) as follows:

$$2\ NADPH_2 + 4\ O_2 \rightarrow 4\ O_2^- + 2\ NADP^+ \quad (a)$$
$$4\ O_2^- + 4\ RH_3^- \rightarrow 4\ H_2O_2 + 4\ RH_3\cdot \quad (b)$$
$$2\ RH_3\cdot \rightarrow RH_2 + RH_3^- \quad (c)$$
$$RH_2 + RH_3\cdot \rightarrow RH\cdot + RH_3^- \quad (d)$$
$$RH\cdot + RH_3\cdot \rightarrow R + RH_3^- \quad (e)$$

$\Sigma = RH_3^- + 2\ NADPH_2 + 4\ O_2 \rightarrow 4\ H_2O_2 + R$

RH_3^- indicates epinephrine, RH_2 is leucoadrenochrome and R is adrenochrome.

A number of other mechanisms in which stoichiometries of 2 H_2O_2:1 adrenochrome are possible may be omitted here as not fitting the observed stoichiometry or the sensitivity to superoxide dismutase. Mechanisms #2 and #3 could also be eliminated as inconsistent with results obtained with superoxide dismutase: Superoxide dismutase causes immediate cessation of adrenochrome formation, regardless of when it is added to the reaction medium, as indicated above and in Figure 5. However, superoxide dismutase does not have such immediate effect on O_2 consumption of H_2O_2 production (Fig. 6). These are slowed immediately but are well above the background rate observed in the absence of epinephrine. This would suggest that during the period of increasing rate (Fig. 5) there is an accumulation of some metabolite ($RH_3\cdot$?) in a superoxide dependent reac-



SCHEME 1. *Proposed mechanism of epinephrine oxidation by superoxide to adrenochrome*

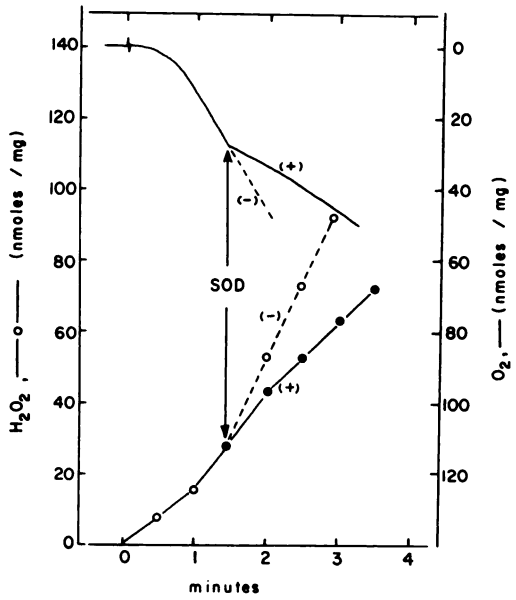


FIG. 6. *The influence of superoxide dismutase (SOD) on microsomal oxygen consumption and hydrogen peroxide formation*

SOD (120 U/ml) was added (+) at 1.5 min and its influence can be compared with the reactions in the absence (−) of SOD. Results are of a typical experiment. Conditions are as described in METHODS section.

tion. This component continues to be oxidized, consuming O_2 and forming H_2O_2, but adrenochrome formation is immediately stopped, when superoxide dismutase is added. The ability to continue consuming O_2 and to form H_2O_2 in the presence of superoxide dismutase would obviate mechanism #2, since this requires O_2^- at all four oxidative steps. Reaction mechanism #3 can also be eliminated based upon the response to superoxide dismutase: accumulation of organic radical intermediates would cause continued adrenochrome production and not immediate cessation, and also contrary to that observed, would cause immediate cessation of enhanced O_2 consumption and H_2O_2 production.

Further indication that Scheme 1 and Table 1 (A) depict the mechanism of adrenochrome formation is seen in Figure 7. The addition of superoxide dismutase to microsomes after NADPH in the absence of epinephrine does not affect the rates of oxygen consumption, as expected, nor H_2O_2 production, since in both instances H_2O_2 contains both reducing equivalents derived from NADPH (either by dismutation of superoxide or by sequential electron transfer from an oxygen activating enzyme). The addition of epinephrine after superoxide dismutase is without effect on oxygen consumption (Fig. 7), indicating superoxide is needed for initiation of the reaction; similarly, the addition does not cause adrenochrome formation nor enhance hydrogen peroxide production.

In the absence of superoxide dismutase epinephrine addition enhances the NADPH supported oxygen consumption (Fig. 7); superoxide dismutase addition immediately slows oxygen consumption. This strengthens the suggestion above that the continued enhanced rates of O_2 uptake and H_2O_2 formation after superoxide dismutase are due to the presence of semiquinone intermediates which can be autoxidized by molecular oxygen. This would also explain the lag in the rate of adrenochrome reaction (Fig. 5) as being due to the accumulation of the semiquinone metabolite to steady state levels.

In further agreement is the observation that NADPH consumption also exhibits a lag period (Fig. 5). This lag in part can be explained (Table 1) as being due to epinephrine oxidation (eq. A). However, nonadrenochrome related NADPH consumption also appears to rise (eq. B) to levels above background. This can be explained in relation to the following observations: the addition of epinephrine to the microsomal suspension after superoxide dismutase does not cause an increase in the rate of NADPH consumption, in agreement with the lack of stimulation of oxygen uptake with these conditions shown in Figure 7. However, unlike the results in Figures 6 and 7, the addition of superoxide dismutase after NADPH and epinephrine, when the reaction has become linear, does *not* cause an appreciable slowing of the enhanced rate of NADPH consumption for at least three minutes. This would indicate that some intermediate which has accumulated is reducible and that the reaction consumes NADPH. The data presented are consistent with epinephrine semiquinone accumulation. This intermediate can be re-

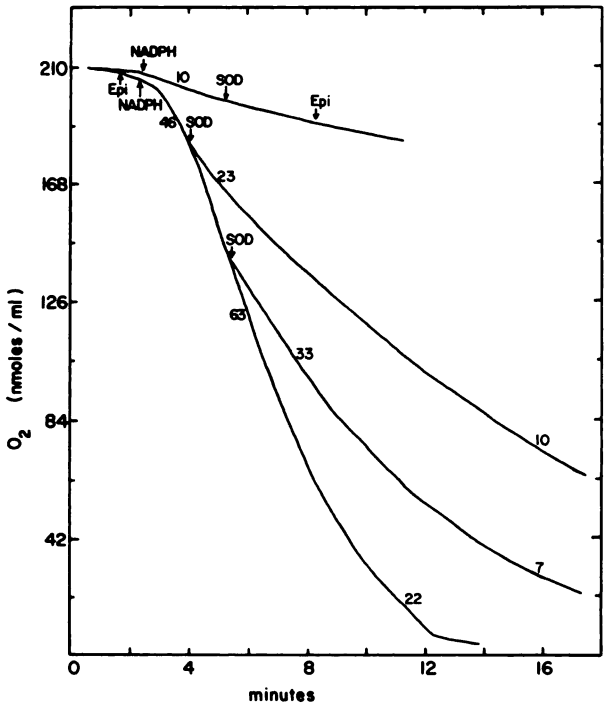


FIG. 7. *Oxygen consumption in the presence of epinephrine (Epi) and NADPH, and the influence of superoxide dismutase (SOD) on the oxygen uptake*

Numbers refer to rates (nmoles O_2/min/mg protein). The reaction was run in the medium containing 0.5 mg microsomes per ml.

duced, consuming NADPH, and oxidized, consuming O_2 and forming O_2^- plus epinephrine quinone. Both reactions are insensitive to superoxide dismutase (see Scheme 1).

Leucoadrenochrome semiquinone radical (the intermediate after step "d" in Scheme 1) would not be expected to accumulate since its oxidation would yield adrenochrome. Similarly, leucoadrenochrome accumulation can be obviated since its oxidation would be prevented by superoxide dismutase. Further, oxidation of leucoadrenochrome is very rapid; this intermediate accumulates when the incubation becomes anaerobic. Adrenochrome also becomes reduced to leucoadrenochrome (colorless) under these conditions (see also 21). However, subsequent aerobic mixing causes almost immediate color reformation.

In conclusion, the NADPH-supported microsomal catalysis is inhibited by superoxide dismutase, indicating an involvement of superoxide anion. The epinephrine must act to pull an NADPH-supported electron flow, since NADPH consumption is also enhanced. However, because the enhanced NADPH consumption is insensitive to superoxide dismutase, a reduction of some accumulated intermediate must have caused the effect. This would agree with the lag observed in adrenochrome production and oxygen consumption, and would indicate that the accumulating intermediate can reduce molecular oxygen and is an epinephrine radical. Thus, the rate-limiting step in epinephrine oxidaton would be the oxidation of this radical (after "a" in Scheme I).

From the results and above discussion it would appear that the microsomal oxidation of epinephrine is accomplished by the ability of this compound to reduce superoxide anion, released by the microsomes, at a rate faster than the superoxide can dismute. This consumption of superoxide does not cause an increase in the rate of NADPH-mediated electron flow itself

(since superoxide dismutase would also be expected to do so), but results in accumulation of a reducible intermediate which does enhance NADPH consumption. Because this enhancement of NADPH consumption is on the same order of magnitude as the rate of adrenochrome production, we can provide a rough calculation to determine as approximate rate of superoxide formation. At steady state conditions, *i.e.*, when adrenochrome production is linear, at 1 mg microsomes per ml the rate of superoxide production would be on the order of 2.5×10^{-7} M sec^{-1}. Calculations using this rate of formation and the second order rate constant for superoxide dismutation of 10^7 M^{-1} sec^{-1} (7, 18) would suggest that levels of superoxide above 0.3 μM would not be reached.

REFERENCES

1. Heacock, R. A. The chemistry of adrenochrome and related compounds. *Chem. Rev.* **59**, 181–237, 1959.
2. Gillette, J. R., D. Watland, & G. Kalnitsky. The catalysis of the oxidation of some dihydroxybenzene derivatives by various metallic ions. *Biochim. Biophys. Acta* **15**, 526–532, 1954.
3. Mazur, A., S. Green & E. Shorr. The oxidation of adrenaline by ferritin iron and hydrogen peroxide. *J. Biol. Chem.* **220**, 227–235, 1956.
4. Valerino, D. M. & J. J. McCormack. Xanthine oxidase-mediated oxidation of epinephrine. *Biochem. Pharmacol.* **20**, 47–55, 1971.
5. McCord, J. M. & I. Fridovich. The reduction of cytochrome c by milk xanthine oxidase. *J. Biol. Chem.* **243**, 5753–5760, 1968.
6. Misra, H. P. & I. Fridovich. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**, 3170–3175, 1972.
7. McCord, J. M. & I. Fridovich. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**, 6049–6055, 1969.
8. Debey, P. & C. Balny. Production of superoxide ions in rat liver microsomes. *Biochimie* **55**, 329–332, 1973.
9. Aust, S. D., D. L. Roerig, & T. C. Pederson. Evidence for superoxide generation by NADPH-cytochrome c reductase of rat liver microsomes. *Biochem. Biophys. Res. Commun.* **47**, 1133–1137, 1972.
10. Cinti, D. L., P. Moldeus, & J. B. Schenkman. Kinetic parameters of drug-metabolizing enzymes in Ca^{2+}-sedimented microsomes from rat liver. *Biochem. Pharmacol.* **21**, 3249–3256, 1972.
11. Gornall, A. G., C. J. Bardawill, & M. M. David. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751–766, 1949.
12. Green, S., A. Mazur, & E. Shorr. Mechanism of the catalytic oxidation of adrenaline by ferritin. *J. Biol. Chem.* **220**, 237–255, 1956.
13. Estabrook, R. W., J. R. Williamson, R. Frenkel, & P. K. Maitra. The fluorometric determination of mitochondrial adenine and pyridine nucleotides. *Methods in Enzymology* **10**, 474–482, 1967.
14. Hildebrandt, A. G. & I. Roots. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. *Arch. Biochem. Biophys.* **171**, 385–397, 1975.
15. Thurman, R. G., H. G. Ley, & R. Scholz. Hepatic microsomal ethanol oxidation: hydrogen peroxide formation and the role of catalase. *Eur. J. Biochem.* **25**, 420–430, 1972.
16. Cheronis, N. D., & J. B. Entrikin. In *Semimicro Qualitative Analysis.* 2nd Ed., Interscience Publ. Inc., N.Y., 1958, 231–232.
17. Yamazaki, I., H. Yamazaki, M. Tamura, T. Ohnishi, S. Nakamura, & T. Iyanagi. Analysis of the O_2 reduction process by the peroxidase system. Oxidation of organic compunds III, *Adv. in Chemistry Series* **77**, 290–306, 1968.
18. Koppenol, W. H. & J. Butler. Mechanism of reactions involving singlet oxygen and the superoxide anion. *FEBS Letters* **83**, 1–6, 1977.
19. Sasame, H. A. & J. R. Gillette. The inhibitory effects of endogenous NADPH-pyrophosphatase on the reduction of cytochrome c and the oxidation of drugs by liver microsomes. *Arch. Biochem. Biophys.* **140**, 113–121, 1970.
20. Sasame, H. A., J. R. Mitchell, & J. R. Gillette. The formation of superoxide as a mechanism of uncoupling between the oxidation of pyridine dinucleotides and drug oxidation by rat liver microsomes. *Fed. Proc.* **34**, 729A, 1975.
21. Uemura, T., E. Chiesara, & D. Cova. Interaction of epinephrine metabolites with liver microsomal electron transport system. *Mol. Pharmacol.* **13**, 196–215, 1977.