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Oxidation of α-Methyldopa and Other Catechols by Cytochrome P-450-Generated Superoxide Anion: Possible Mechanism of Methyldopa Hepatitis

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SUMMARY

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Renewed interest in the hepatic injury produced by α-methyldopa has been stimulated by recent reports that the antihypertensive drug may initiate chronic active liver disease, occasionally with a fatal outcome. To determine whether the toxicity might be due to a reactive metabolite, [^{3}H]α-methyldopa was incubated with rat liver microsomes in the presence of an NADPH-generating system. A large amount of covalent binding occurred, but only in the presence of NADPH and oxygen (V_{max} = 0.5 nmol/mg/min; K_m = 50 μM). The binding was inhibited by a CO-O_2 atmosphere (9:1), indicating the involvement of cytochrome P-450. However, α-methyldopa did not show P-450 binding spectra (type I, II, or III), and its covalent binding was inhibited by superoxide dismutase, ascorbic acid (1 mM), ethylenediamine (20 mM), and glutathione (1 mM). This indicated that α-methyldopa was being oxidized by cytochrome P-450-generated superoxide anion to a reactive semiquinone and/or quinone. The covalent binding was inhibited by analogues such as *l*-dopa, dopamine, epinephrine, norepinephrine, and catechol, but not by 3-*O*-methyldopa, indicating a requirement for the unsubstituted catechol nucleus. Additional studies demonstrated that the rat microsomal system could be replaced by human hepatic microsomes or by a xanthine oxidase system and the binding was again inhibited by superoxide dismutase. Metabolic activation by superoxide anion may play a role in the hepatotoxicity of this and other catechols, including hydroxylated estrogens.

INTRODUCTION

α-Methyldopa is a widely used antihypertensive drug with a catechol nucleus similar to that of epinephrine. Many reports have shown that it produces mild, clinically covert, hepatic injury in up to 36% of recipients (1) when liver function

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tests are routinely monitored during drug administration. More recent evidence indicates that the drug initiates chronic active hepatitis in a smaller percentage of patients, occasionally with a fatal outcome (1–4).

In preliminary experiments (5, 6) we have found that α-methyldopa produces scattered single cell necrosis with acidophilic bodies in Fischer rats given single large doses (500 mg/kg intraperitoneally). The lesion was not increased by prior treatment with inducers of drug metabolism such as phenobarbital, 3-methylcholanthrene, or 5,6-benzoflavone. In previous studies we have found that many hepatotoxic drugs, such as acetaminophen (paracetamol), furosemide, isoniazid, and iproniazid (7), are metabolically activated to chemically reactive, toxic metabolites by cytochrome P-450 oxidases, and that the toxicities from some of these reactions are not always increased by prior treatment with phenobarbital (8, 9).

Recently Sasame *et al.* (10) have shown that superoxide is formed by cytochrome P-450 in hepatic microsomes, and that the amount is not increased by prior treatment of rats with phenobarbital or 3-methylcholanthrene. Since superoxide is known to oxidize epinephrine to adrenochrome by way of reactive intermediates (11), we have examined the possibility that α-methyldopa might also be metabolically activated by superoxide to a chemically reactive and potentially hepatotoxic metabolite.

MATERIALS AND METHODS

Chemicals. [^{3}H]α-Methyldopa [L-[^{3}H]3-(3,4-dihydroxyphenyl)-2-methylalanine; specific activity, 1.25 Ci/mmole] was purchased from Amersham/Searle. Its radiochemical purity was determined by thin-layer chromatography on Avicel plates, 250 μm (Analtech), in two different developing systems: 2-propanol–2 N hydrochloric acid (65:35) and methyl ethyl ketone–acetic acid–water (75:25:30). Bands (0.5 cm) from each plate were removed, mixed with 0.2 ml of water in 15 ml of 2,5-bis[2′-(5′-*tert*-butylbenzoxazolyl)]thiophene scintillation fluid (Yorktown), and counted. The tritiated α-methyldopa was shown to be at least 99% radiochemically pure in these two systems. L-[*G*-^{3}H]3,4-Dihydroxyphenylalanine ([^{3}H]dopa; specific activity, 5.92 Ci/mmole), L-4-hydroxy-3-methoxy[*U*-^{14}C]phenylalanine ([^{14}C]3-*O*-methyldopa; specific activity, 337.5 mCi/mmole), DL-[7-*N*-^{3}H]epinephrine L-bitartrate (specific activity, 8.4 Ci/mmole), and DL-[7-*N*-^{3}H]norepinephrine L-bitartrate (specific activity, 9.8 Ci/mmole) were purchased from New England Nuclear. [^{3}H]Dopamine hydrochloride (specific activity, 3.2 Ci/mmole) was obtained from Amersham/Searle. Unlabeled α-methyldopa was obtained from Calbiochem; unlabeled dopa, dopamine, 3-*O*-methyldopa, epinephrine, and norepinephrine were purchased from Sigma. Superoxide dismutase from bovine erythrocytes, catalase from bovine liver, xanthine oxidase (grade IV) from buttermilk, and mushroom tyrosinase were also purchased from Sigma. Other chemicals were of the best available commercial grades.

Liver microsomes. Male Fischer rats (Charles River Laboratories, 150 g) and male Swiss-Webster mice (Taconic Farms, 20 g) were killed by decapitation. Livers were removed and homogenized with a motor-driven glass-Teflon homogenizer in 2 volumes of 1.15% KCl containing 20 mM Tris buffer, pH 7.4. The homogenate was centrifuged at 9000 × *g* for 20 min in a Sorvall RC-2 centrifuge, and the supernatant was decanted and recentrifuged for 1 hr at 105,000 × *g* in a Beckman model L5-65 preparative ultracentrifuge. The microsomal pellet was resuspended and washed in the Tris-KCl buffer and recentrifuged for 60 min at 105,000 × *g*. The washed microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4, before incubation.

Microsomes were prepared in an identical fashion from Fischer rats and Swiss-Webster mice that had been treated as follows: (a) phenobarbital (75 mg/kg intraperitoneally daily for 3 days); (b) 5,6-benzoflavone in sesame oil (80 mg/kg intraperitoneally 48 hr prior to death); (c) cobaltous chloride (20 mg/kg subcutaneously twice daily for 2 days in rats; 30 mg/kg

intraperitoneally twice daily for 2 days in mice); or (d) piperonyl butoxide (1360 mg/kg intraperitoneally 30 min prior to death). Control rats and mice were injected with 0.9% NaCl or sesame oil at similar intervals. Microsomal cytochrome P-450 and P-448 were measured according to Omura and Sato (12).

Incubation mixtures. Ice-cold reaction vessels contained liver microsomal protein (usually 2 mg/ml); phosphate buffer, pH 7.4, 83 mM; [^{3}H]α-methyldopa or other catechol analogues (1500–2500 dpm/nmole) at 0.5 mM unless otherwise stated; and an NADPH-generating system (NADP, 0.64 mM; glucose 6-phosphate, 15.5 mM; glucose 6-phosphate dehydrogenase, 2 units/ml; $MgCl_2$, 10 mM). In experiments with antibody prepared against NADPH–cytochrome *c* reductase or cytochrome b_5 (13), 1.5 ml of incubation mixture contained goat preimmune or immune γ-globulin at 10 mg/ml, microsomal protein at 1.0 mg/ml, and the NADPH-generating system described above. In other experiments the NADPH-generating system was replaced by 0.22 mM xanthine and xanthine oxidase (5 μg/ml) or by 100 units/ml of mushroom tyrosinase. In some experiments ethylmorphine (5 mM) was substituted for [^{3}H]α-methyldopa, and formation of formaldehyde was estimated according to Nash (14). All reactions were started by the addition of microsomes and carried out at 37° in a shaking water bath incubator under air. They were stopped at the desired time (usually 10 min) by adding 1 ml of 30% trichloroacetic acid.

Extraction of microsomal protein. The protein pellet was washed repeatedly with trichloroacetic acid, methanol, and ethanol–ether until no more radioactivity could be extracted; the pellet was extracted at least four times with 5% trichloroacetic acid, four times with 80% methanol–water, and twice with ethanol–ether (1:1). The extracted protein was dissolved in 1.0 ml of 1 M NaOH, and an aliquot was transferred to 15 ml of 2,5-bis [2′-(5′-*tert*-butylbenzoxazolyl)] thiophene scintillation fluid and counted in a Packard liquid scintillation spectrometer. Radioactivity was corrected for background quenching (external standardization) and expressed as nanomoles irreversibly bound per minute per milligram of microsomal protein. The protein concentration was determined according to Lowry *et al.* (15), using crystalline bovine serum albumin as standard. Kinetic data were plotted according to Lineweaver and Burk (16); points were fitted to the line by the method of least squares.

Characterization of glutathione conjugates. α-Methyldopa (0.5 mM) was incubated with glutathione (1 mM), microsomes, and cofactors as above. Five milliliters of ice-cold methanol were added to terminate the reaction. Protein was centrifuged and carried through the washing procedures described above to determine the irreversible binding of radiolabel. The supernatant from each incubation was evaporated under a stream of nitrogen, and remaining traces of water were removed by lyophilization. Each residue was then taken up in 200 μl of distilled water, and 50 μl were spotted on Avicel-F plates (Analtech, 500 μm) and developed in methyl ethyl ketone–acetic acid–water (75:25:30). The chromatograms of incubations without added glutathione and those with glutathione but lacking the NADPH-generating system showed only the presence of [^{3}H]α-methyldopa (R_F 0.55–0.62). Chromatograms of incubations containing both glutathione and the NADPH-generating system showed the presence of an additional radioactive peak (R_F 0.13–0.20), as determined by scanning with a Packard model 385 recording rate meter. This was also the major peak observed when [^{3}H]α-methyldopa was incubated with tyrosinase and glutathione. These peaks, containing suspected α-methyldopa–glutathione conjugates, gave positive ninhydrin (light pink) and positive potassium dichromate–silver nitrate reactions (yellow against a red-brown background).

The microsomal and tyrosinase incubations showing the presence of a conjugate were chromatographed separately on Sephadex G-10 columns (1.5 × 90 cm), using water as the eluent at a flow rate of 18 ml/hr. Two-milliliter fractions were collected; radioactive peaks were found in

fractions 22-24 (unknown), 30-36 (suspected [^{3}H]α-methyldopa-glutathione conjugate), and 58-66 ([^{3}H]α-methyldopa). The fractions containing the suspected glutathione conjugate peak were combined and lyophilized. Thin-layer chromatography of the residue on Avicel-F (500 μm) showed an R_F for the conjugate of 0.21 in the methyl ethyl ketone system described above and an R_F of 0.90 in water-ethanol (9:1). An ultraviolet spectrum of this conjugate showed maxima at 256.5 and 295 nm, similar to those observed by Bouchilloux and Kodja (17) for a dopa-glutathione conjugate.

Indirect assays for superoxide formation from microsomes. The rate of formation of adrenochrome and α-methyldopachrome was determined in a cuvette containing 0.5 mg of microsomal protein per milliliter, 1.0 mM epinephrine or α-methyldopa, and the cofactors used in the incubation mixtures described above. Microsomes were obtained from control animals or from animals treated with phenobarbital, cobaltous chloride, or piperonyl butoxide as described above. In some experiments SKF 525-A was added to microsomes in a concentration of 0.2 mM. The difference in absorbance between 485 and 620 nm was adjusted to zero in an Aminco DW-2 spectrophotometer, and the rate of increase in absorbance was determined after the addition of 20 μl of 50 mM NADPH.

RESULTS

Involvement of superoxide anion. Incubation of [^{3}H]α-methyldopa with rat or mouse hepatic microsomes in the presence of an NADPH-generating system led to its irreversible binding to microsomal proteins (Table 1A). The binding reaction had a maximal velocity of approximately 0.5 nmole/mg of protein per minute in rats and 0.4 nmole/mg of protein per minute in mice (Fig. 1), proceeded at a constant rate for at least 10 min, and was directly proportional to enzyme concentration up to 2 mg of microsomal protein per milliliter. Superoxide dismutase was found to inhibit the covalent binding almost completely (Table 1B). Addition of ascorbic acid (1 mM), which can reduce superoxide anions (18), semiquinones, and quinones (19), totally blocked the binding of [^{3}H]α-methyldopa (Table 1B); this concentration of ascorbic acid did not affect the *N*-demethylation of ethylmorphine.

Further indirect evidence for the in-

TABLE 1

Conditions for irreversible binding of [^{3}H]α-methyldopa to rat and mouse liver microsomal protein in vitro

For each incubation, rat or mouse microsomes were prepared as described in MATERIALS AND METHODS and incubated under air with [^{3}H]α-methyldopa (0.5 mM) and an NADPH-generating system, except as noted. Values are representative results from at least six experimental or 20 control determinations. Values obtained on several experimental days from different microsomal preparations never varied more than ±11% from the values shown.

Reaction mixture	α-Methyldopa bound	
	Rat	Mouse
	nmole/mg protein /min	
A. Complete	0.469	0.381
B. +Superoxide dismutase (15 μg/ml)[a]	0.008	0.007
+Ascorbic acid (1 mM)[a]	0.003	0.000
C. +Catalase (0.2 mg/ml)	0.468	—[b]
+Sodium benzoate (10 mM)	0.514	—[b]
D. −NADPH; + xanthine oxidase (5 μg/ml) + xanthine (0.22 mM)	0.288	—[b]
−NADPH; + xanthine oxidase (5 μg/ml) + xanthine (0.22 mM) + superoxide dismutase (15 μg/ml)	0.016	—[c]
E. −NADPH; + NADH	0.028	0.060
$-O_2$ (100% N_2 atmosphere)	0.000	0.006
$+N_2$-O_2 (9:1 atmosphere)	0.410	0.349
$+CO$-O_2 (9:1 atmosphere)	0.183	0.157
+Preimmune γ-globulin[c]	0.470	—[b]
+Immune γ-globulin[c] (NADPH-cytochrome *c* reductase antibody)	0.240	—[b]
+Immune γ-globulin (cytochrome b_5 antibody)	0.555	—[b]

[a] Ethylmorphine *N*-demethylase activity was unchanged by these additions.

[b] Not determined.

[c] Each incubation contained 10 mg of partially purified preimmune or immune γ-globulin per milligram of microsomal protein, as previously described (13). Data are expressed as nanomoles of α-methyldopa bound per milligram of microsomal protein.

volvement of superoxide in the oxidative activation of [^{3}H]α-methyldopa was obtained by studies with catalase and sodium benzoate (Table 1C). Catalase had no effect on the binding, which showed that hydrogen peroxide was not the oxidizing agent. Benzoate also did not inhibit the binding of [^{3}H]α-methyldopa to microsomal protein, which indicated that the hydroxyl radical was not involved. Hydrogen peroxide is a product of the dismutation of superoxide, and a hydroxyl radical could have arisen from the Haber-Weiss reaction (20).

Irreversible binding of [^{3}H]α-methyldopa to microsomal proteins also occurred when the NADPH-generating system was replaced by xanthine and xanthine oxidase (Table 1D), an enzyme that is known to generate superoxide anions (21). This binding was also markedly reduced in the presence of superoxide dismutase (Table 1D).

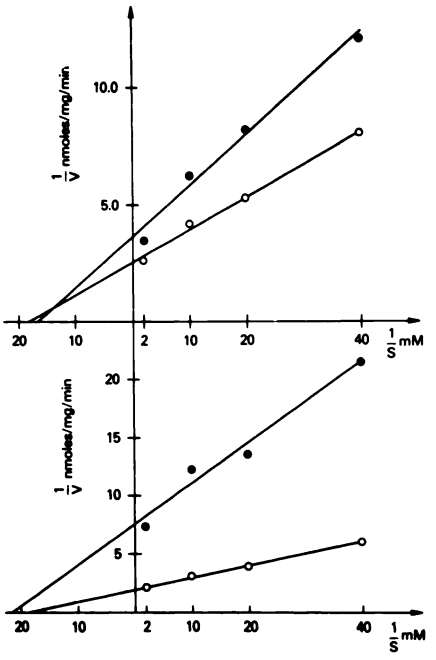

FIG. 1. *Lineweaver-Burk plots of irreversible binding of [^{3}H]α-methyldopa to rat and mouse liver microsomal protein in vitro*

Upper: Plot for [^{3}H]α-methyldopa incubated with microsomes from control mice (○; K_m = 5.5 × 10^{-5} M; V_{max} = 0.39 nmole/mg/min) and from mice treated with piperonyl butoxide (●; K_m = 6.0 × 10^{-5} M; V_{max} = 0.28 nmole/mg/min). Lower: Plot for [^{3}H]α-methyldopa incubated with microsomes from control rats (○; K_m = 5.4 × 10^{-5} M; V_{max} = 0.53 nmole/mg/min) and from rats treated with cobaltous chloride (●; K_m = 4.6 × 10^{-5} M; V_{max} = 0.13 nmole/mg/min). Values are the averages of two incubations.

Involvement of cytochrome P-450. Although α-methyldopa did not yield difference spectra with microsomes (type I, II, or III), as usually found for substrates of cytochrome P-450 oxidases (22), the irreversible binding of [^{3}H]α-methyldopa to microsomal proteins did require microsomes, oxygen, and an NADPH-generating system (Table 1E). The binding was inhibited by a carbon monoxide–oxygen atmosphere (9:1) and by an antibody against NADPH–cytochrome *c* reductase (Table 1E). In contrast, the irreversible binding may have been slightly increased by an antibody against cytochrome b_5 (Table 1E), although this difference was not statistically different in the present experiments ($p > 0.5 < 0.10$).

Prior treatment of animals with cobaltous chloride, which blocked the synthesis and thereby reduced the concentration of cytochrome P-450, lowered the apparent V_{max} without changing the apparent K_m (Fig. 1 and Table 2). However, neither induction of the cytochrome P-450 system with phenobarbital nor induction of cytochrome P-448 with 5,6-benzoflavone increased the binding reaction to any great extent. Prior treatment of animals with piperonyl butoxide significantly reduced binding of [^{3}H]α-methyldopa (Fig. 1 and Table 2) without changing the apparent K_m, whereas addition of SKF 525-A to incubations did not affect binding although the rate of ethylmorphine *N*-demethylation was decreased (Table 2). Paralleling these findings, the rate of oxidation of epinephrine to adrenochrome by liver microsomes was inhibited approximately 50% by prior treatment of animals with piperonyl butoxide, whereas the addition of SKF 525-A had no effect (data not shown).

Nature of reactive species. Addition of glutathione to incubation mixtures almst completely abolished the binding reaction (Table 3A). A glutathione conjugate of α-methyldopa was isolated from these reactions (as described in MATERIALS AND

TABLE 2

Effects of prior treatments on irreversible binding of [^{3}H]α-methyldopa to rat and mouse liver microsomal protein in vitro

Microsomes were prepared as described in MATERIALS AND METHODS from control and treated animals and incubated with [^{3}H]α-methyldopa (0.5 mM) and an NADPH-generating system. SKF 525-A (0.2 mM) was added directly to incubations containing microsomes from control but not from treated animals. This concentration inhibited ethylmorphine *N*-demethylase activity by 58%. Values represent means ± standard errors from four incubations from individual rats or from four incubations from pooled livers of 10 mice.

Prior treatment	[^{3}H]α-Methyldopa binding	Cytochromes P-450 and P-448
	nmole/mg/min	*nmoles/mg*
Rat liver microsomes		
Controls	0.455 ± 0.019	0.76
Phenobarbital	0.371 ± 0.018	2.00
Benzoflavone	0.545 ± 0.045	1.46
Piperonyl butoxide	0.169 ± 0.045	0.81
Cobaltous chloride	0.169 ± 0.007	0.45
SKF 525-A (0.2 mM)	0.436 ± 0.017	
Mouse liver microsomes		
Controls	0.323 ± 0.011	0.95
Phenobarbital	0.326 ± 0.010	0.95
Benzoflavone	0.323 ± 0.024	1.15
Piperonyl butoxide	0.175 ± 0.017	0.81
Cobaltous chloride	0.288 ± 0.010	0.86
SKF 525-A (0.2 mM)	0.346 ± 0.015	

METHODS) which had chromatographic properties (gel filtration on Sephadex and thin-layer chromatography on Avicel) almost identical with those of a glutathione-dopa conjugate described by others (17, 23). This α-methyldopa conjugate was not found in control incubations lacking NADPH cofactor or in incubations with NADPH but lacking glutathione. The conjugate showed a positive ninhydrin reaction and a positive reaction for divalent sulfur when sprayed with potassium dichromate–silver nitrate (24).

Ethylenediamine, a known *o*-quinone-trapping agent (25), blocked the binding reaction of α-methyldopa to microsomes by approximately 40% (Table 3A), indicating that a substantial portion of the binding may involve oxidation of α-methyldopa to an electrophilic *o*-quinone, which then reacts with nucleophilic tissue components.

To test this hypothesis, the action of tyrosinase on the binding reaction was investigated. Tyrosinase apparently does not form discrete semiquinone intermediates, since the first detectable product is the quinone (26). Tyrosinase caused marked irreversible binding of [^{3}H]α-methyldopa to microsomal proteins; the binding was not inhibited by superoxide dismutase but was decreased about 40% by ascorbate (Ta-

TABLE 3

Effects of radical and quinone scavengers on irreversible binding of [^{3}H]α-methyldopa to tissue protein

Reaction mixture	[^{3}H]α-Methyldopa bound	
	Rat	Mouse
	nmoles/mg/min	
A. NADPH-generating system[a]	0.444 ± 0.016	0.377 ± 0.012
+Glutathione (1 mM)	0.15 ± 0.003	0.016 ± 0.002
+Ethylenediamine (20 mM)	0.308 ± 0.010	0.220 ± 0.008
B. Tyrosinase[b]	1.49 ± 0.075	
+superoxide dismutase (15 μg/ml)	1.55 ± 0.090	
+Glutathione (1 mM)	0.08 ± 0.003	
+Ascorbic acid (1 mM)	0.87 ± 0.005	
+Ethylenediamine (20 mM)	1.29 ± 0.088	

[a] For each incubation, rat or mouse microsomes were prepared as described in MATERIALS AND METHODS and incubated under air with [^{3}H]α-methyldopa (0.5 mM) and an NADPH-generating system. Values are means ± standard errors for four determinations. The addition of glutathione or ethylenediamine at the concentrations shown did not decrease ethylmorphine *N*-demethylase activity.

[b] Microsomes were incubated, without an NADPH-generating system, with [^{3}H]α-methyldopa (0.5 mM) and mushroom tyrosinase (100 units/ml) alone or with the additions shown. Values are the means ± standard errors for three incubations.

ble 3B). Glutathione again abolished the binding reaction, concomitantly with the formation of a glutathione conjugate indistinguishable from that formed with hepatic microsomes. Ethylenediamine also decreased the binding reaction, although to a lesser extent than in the microsomal system.

Other catechols. Catechols other than α-methyldopa were tested for their ability both to inhibit binding of [^{3}H]α-methyldopa (Table 4) and to become bound irreversibly in the microsomal system (Table 5). Structural analogues such as dopa, dopamine, epinephrine, and norepinephrine, as well as catechol, inhibited the irreversible binding of [^{3}H]α-methyldopa by 42–79%. Kinetic plots of the binding reaction of [^{3}H]α-methyldopa in the presence of dopa or dopamine showed apparent competitive inhibition kinetics (Fig. 2); that is, the apparent V_{max} of α-methyldopa binding was unchanged and the K_m was increased 1–2-fold in the presence of dopa and dopamine. Similar plots were obtained with [^{3}H]-α-methyldopa when epinephrine was added to the incubations. Like α-methyldopa, two catechols, dopa and dopamine, as well as other catechols, were bound irreversibly to microsomal proteins after metabolic activation (Table 5). This binding was blocked by superoxide dismutase (Table 6). Superoxide generated from xanthine oxidase also led to irreversible binding of these radiolabeled catechols, and this reaction was again blocked by superoxide dismutase (Table 6). In contrast, an *O*-methylated catechol, 3-*O*-methyldopa, did not inhibit [^{3}H]α-methyldopa binding at any concentration of α-methyldopa (Table 4), and radiolabeled 3-*O*-methyldopa was not significantly bound to microsomal proteins (Table 5).

TABLE 4

Effects of various catechol analogues on irreversible binding of [^{3}H]α-methyldopa to rat microsomal protein in vitro

Microsomes were incubated with [^{3}H]α-methyldopa (0.5 mM) and an NADPH-generating system alone or in the presence of analogues (0.5 mM). Values are the means ± standard errors of three incubations.

Addition	[^{3}H]α-Methyldopa bound
	nmole/mg/min
Control	0.380 ± 0.011
Dopa	0.190 ± 0.007
Dopamine	0.079 ± 0.006
Epinephrine	0.221 ± 0.009
Norepinephrine	0.214 ± 0.008
Catechol	0.112 ± 0.005
3-*O*-Methyldopa	0.394 ± 0.012

TABLE 5

Irreversible binding of catechol analogues to rat microsomal protein in vitro

Microsomes were incubated with radiolabeled analogues (0.5 mM) and an NADPH-generating system. Values are the means ± standard errors of three incubations.

Analogue tested	Label bound
	nmole/mg/min
[^{3}H]α-Methyldopa	0.558 ± 0.041
[^{3}H]Dopa	0.496 ± 0.058
[^{3}H]Dopamine	0.357 ± 0.030
[^{3}H]Epinephrine	0.778 ± 0.065
[^{3}H]Norepinephrine	0.772 ± 0.062
[^{14}C]3-*O*-Methyldopa	0.015 ± 0.011

DISCUSSION

The results presented here show that superoxide anion radicals can be formed by hepatic microsomal cytochrome P-450, and this in turn leads to the formation of reactive electrophiles of α-methyldopa and other catechols. An oxygenated cytochrome P-450 complex is thought to dissociate to form oxidized cytochrome P-450 and superoxide anion. The radical anion then escapes into the surrounding medium and can react with easily oxidizable substrates such as catechols.

Support for this hypothesis comes from studies on the formation of the highly colored adrenochromes from epinephrine and α-methyldopa when incubated with microsomes and NADPH. The color formation from both catechols is completely blocked by superoxide dismutase or ascorbic acid. An antibody to cytochrome b_5 (13), which increases the rate of superoxide anion production from microsomes in the presence of ethylmorphine (10), increases the rate of colored complex formation both from epinephrine (10) and from α-methyldopa.[3]

[3] Unpublished observations.

Moreover, α-methyldopa inhibits the rate of adrenochrome formation from epinephrine.[3]

Just as similar colored complexes are formed from both epinephrine and α-methyldopa, each forms some oxidized product which irreversibly binds to microsomal protein in a reaction that is inhibited by superoxide dismutase and the other inhibitors described in this paper. These inhibitors have no effect on another microsomal P-450-mediated reaction, the rate of *N*-demethylation of ethylmorphine (Table 1B, footnote *a*). This *N*-demethylation requires ternary complex formation of substrate, oxygen, and P-450, whereas no typical P-450 enzyme-substrate spectral change is observed with either α-methyldopa or epinephrine. Suspected inhibitors of the formation of a P-450-oxygenated complex, such as carbon monoxide, an antibody to NADPH-cytochrome *c* reductase, and piperonyl butoxide, markedly reduce both the irreversible binding reaction of radiolabeled catechols and adrenochrome formation from epinephrine. However, SKF 525-A, which competitively inhibits many P-450-mediated reactions, inhibited neither the binding reaction nor adrenochrome formation from epinephrine. These results indicate that univalent reduction of oxygen by the P-450 system is necessary for irreversible binding of α-methyldopa and other catechols to microsomal protein, but that interaction of the catechols with the P-450 system in a manner typical of most substrates is not necessary.

Nonetheless, apparent kinetic constants (K_m and V_{max}) can be derived for the reaction, and competitive inhibition by analogues is observed. In such studies these parameters more accurately reflect some rate-limiting step in the over-all reaction

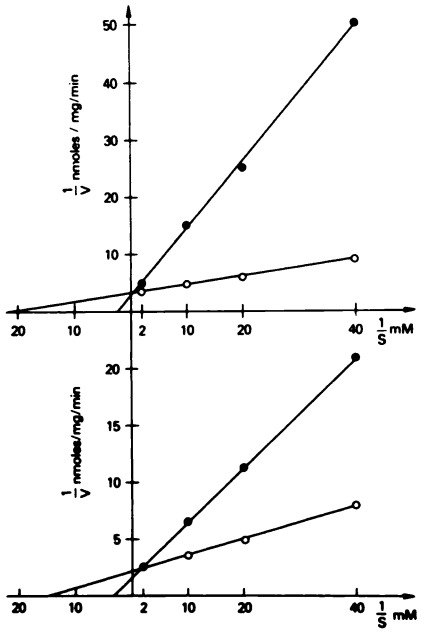

FIG. 2. *Lineweaver-Burk plots of irreversible binding of [³H]α-methyldopa to rat and mouse liver microsomal protein in vitro*

Upper: Plot for [³H]α-methyldopa incubated with mouse microsomes without (○; K_m = 4.6 × 10^{-5} M; V_{max} = 0.31 nmole/mg/min) and with (●; K_m = 4.4 × 10^{-4} M; V_{max} = 0.44 nmole/mg/min) the addition of dopamine, 0.2 mM. Lower: Plot for [³H]α-methyldopa incubated with rat microsomes without (○; K_m = 6.7 × 10^{-5} M; V_{max} = 0.47 nmole/mg/min) and with (●; K_m = 3.0 × 10^{-4} M; V_{max} = 0.61 nmole/mg/min) the addition of dopa, 0.2 mM. Values are the means of two incubations.

TABLE 6

Effect of superoxide dismutase on irreversible binding of catechols to mouse microsomal protein in vitro mediated by NADPH-dependent cytochrome P-450 or xanthine oxidase

Microsomes were incubated with radiolabeled analogues (0.5 mM) and either an NADPH-generating system or a xanthine oxidase system as described in MATERIALS AND METHODS, with or without the presence of superoxide dismutase (15 μg/ml). Values are the means ± standard errors of three incubations.

Catecholamine	NADPH	NADPH + superoxide dismutase	Xanthine oxidase	Xanthine oxidase + superoxide dismutase
	nmole/mg/min	*nmole/mg/min*	*nmole/mg/min*	*nmole/mg/min*
Dopa	0.254 ± 0.022	0.000 ± 0.009	0.243 ± 0.015	0.000 ± 0.010
Dopamine	0.394 ± 0.035	0.020 ± 0.004	0.299 ± 0.024	0.009 ± 0.004
Epinephrine	0.648 ± 0.049	0.026 ± 0.010	0.287 ± 0.020	0.001 ± 0.008

rather than represent true kinetic parameters of a discrete enzymatic step. At present only limited information is available on this rate-limiting step. Some form of cytochrome P-450 appears to be involved, since decreased levels led to a reduced rate of binding, as shown by the studies with cobaltous chloride (Table 2). However, other factors must also be important, since the increased levels of cytochrome P-450 obtained after phenobarbital treatment did not increase the rate of [^{3}H]α-methyldopa binding. This may result because phenobarbital does not change the steady-state concentration of the monoreduced form of cytochrome P-450 or its dissociation to superoxide.

Currently only suggestive information on the nature of the reactive catechol intermediate(s) is available. Catechols bind irreversibly to microsomal proteins, whereas a 3-*O*-methylated derivative fails to react. Nucleophilic *o*-quinone- and semiquinone-trapping agents block the binding reaction. These results suggest that the catechol nucleus is oxidized to its semiquinone free radical by superoxide (Fig. 3) and that the radical then either reacts with tissue macromolecules directly or is further oxidized to an electrophilic quinone, which then reacts.

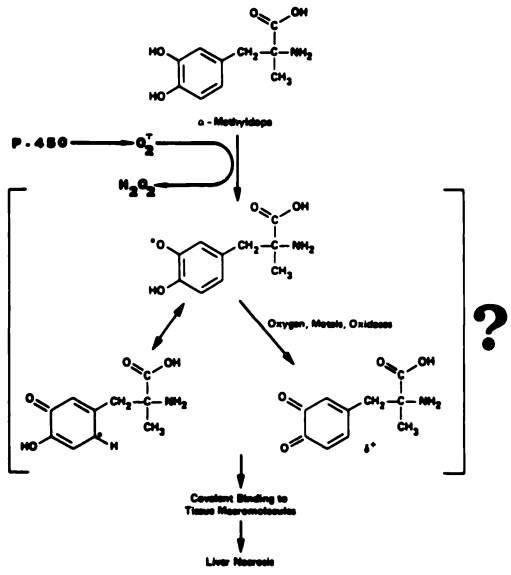

FIG. 3. *Proposed scheme for activation of α-methyldopa by liver microsomal cytochrome P-450*

A similar scheme has been proposed for the autoxidation of norepinephrine in aqueous solutions (27) and could apply to the activation of catechol estrogens, which have been shown to bind irreversibly to microsomal and other proteins (28). Remmer and colleagues (29) have concluded that the microsomal activation of catechol estrogens occurs via NADPH-cytochrome *c* reductase-generated superoxide anion. Since the initial presentation of our results with epinephrine, dopa, dopamine, and α-methyldopa (5, 6, 10), these authors have independently reported their preliminary results showing irreversible binding of dopa and dopamine to microsomal protein (29). As with the catechol estrogens, they concluded that microsomal superoxide anion was generated by NADPH-cytochrome *c* reductase and not by cytochrome P-450, because irreversible binding of these catechols occurred with partially purified preparations of the reductase. Similarly, other investigators have previously shown that cytochrome *c* reductase catalyzed the conversion of epinephrine to adrenochrome, but no direct evaluation of the P-450 system was attempted (30). In our studies, however, the activation of α-methyldopa, dopa, dopamine, epinephrine, and other catechols, including catechol estrogens such as 2-hydroxyestradiol (31), were all markedly decreased by carbon monoxide-oxygen atmospheres and by prior treatment of animals with cobaltous chloride or piperonyl butoxide. The finding that CO-O_2 inhibits the binding by more than 60% indicates that most of the superoxide formed in microsomes is mediated by cytochrome P-450 rather than NADPH-cytochrome *c* reductase. The generation of superoxide anion by one or more of the microsomal cytochrome P-450s, and not by NADPH-cytochrome *c* reductase (29), appears to be the important rate-limiting step in catechol activation by intact hepatic microsomes.

Whether the reaction mechanisms presented here play a role in the hepatotoxicity observed in patients receiving α-methyldopa is unclear at this time. It is possible that liver injury could result directly from covalent binding of the reactive semiqui-

none or quinone to vital hepatic macromolecules, or liver damage might result from peroxidative attack of tissues similar to that envisioned by Heikkila and Cohen (32) for the destruction of nerve terminals by 6-hydroxydopamine. It is interesting in this regard that 6-hydroxy-α-methyldopa produces renal tubular necrosis.[4] The production of hepatic cell injury by one or a combination of the reactive intermediates that might be generated during α-methyldopa oxidation, such as semiquinone, quinone, superoxide, hydrogen peroxide, or hydroxyl radical, would depend on several factors. These would include the concentration of α-methyldopa or other catechols, the activities of enzymes such as superoxide dismutase, cytochrome P-450, catalase, and catechol *O*-methyltransferase, and perhaps the hepatic concentrations of ascorbic acid and glutathione.

REFERENCES

1. Elkington, S. G., Schreiber, W. M. & Conn, H. O. (1969) *Circulation*, 40, 589–595.
2. Hoyumpa, A. M. & Conell, A. M. (1973) *Dig. Dis.*, 18, 213–222.
3. Goldstein, G. B., Lam, K. C. & Mistilie, S. P. (1973) *Dig. Dis.*, 18, 177–184.
4. Schweitzer, I. L. & Peters, R. L. (1974) *Gastroenterology*, 66, 1203–1211.
5. Dybing, E., Mitchell, J. R., Nelson, S. D. & Gillette, J. R. (1975) *Proc. Satellite Symp. Active Intermediates, 6th Int. Congr. Pharmacol. (Turku, Finland)*, p. 7.
6. Mitchell, J. R., Dybing, E. & Nelson, S. D. (1975) *Gastroenterology*, 69, 847.
7. Mitchell, J. R. & Jollow, D. J. (1975) *Gastroenterology*, 68, 392–410.
8. Potter, W. Z., Thorgeirsson, S. S., Jollow, D. J. & Mitchell, J. R. (1974) *Pharmacology (Basel)*, 12, 129–143.
9. Mitchell, J. R., Nelson, W. L., Potter, W. Z., Sasame, H. A. & Jollow, D. J. (1976) *J. Pharmacol. Ex. Ther.*, in press.
10. Sasame, H. A., Mitchell, J. R. & Gillette, J. R. (1975) *Fed. Proc.*, 34, 729.
11. Heacock, R. A. (1959) *Chem. Rev.*, 59, 181–237.
12. Omura, T. & Sato, R. (1964) *J. Biol. Chem.*, 239, 2370–2378.
13. Sasame, H. A., Mitchell, J. R., Thorgeirsson, S. S., & Gillette, J. R. (1973) *Drug. Metab. Disp.*,1, 150–155.
14. Nash, T. (1953) *Biochem. J.*, 55, 416–421.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
16. Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.*, 56, 658–666.
17. Bouchilloux, S. & Kodja, A. (1960) *Bull. Soc. Chim. Biol.*, 42, 1045–1064.
18. Nishikimi, M. (1975) *Biochem. Biophys. Res. Commun.*, 63, 463–468.
19. Deamer, D. W., Heikkila, R. E., Panganamela, R., Cohen, G. & Cornwell, D. G. (1971) *Physiol. Chem. Phys.*, 3, 246–430.
20. Haber, F. & Weiss, J. (1934) *Proc. R. Soc. Lond., Ser.*, 147, 332–351.
21. Fridovich, I. & Handler, P. (1958) *J. Biol. Chem.*, 233, 1581–1585.
22. Philpot, R. M. & Hodgson (1971) *Life Sci.*, 10, 503–512.
23. Agrup, G., Falck, B., Kennedy, B. M., Rorsman, H., Rosengren, A. M. & Rosengren, E. (1975) *Acta Derm.-vernereol.*, 55, 1–3.
24. Knight, R. H. & Young, L. (1958) *Biochem. J.*, 70, 111–119.
25. Jellinck, P. H. & Irwin, L. (1963). *Biochim. Biophys. Acta*, 78, 778–780.
26. Hamilton, G. A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, I., ed.), pp. 435–438, Academic Press, New York.
27. Maguire, M. E., Goldmann, P. H. & Gilman, A. G. (1974) *Mol. Pharmacol.*, 10, 563–581.
28. Kappus, H., Bolt, H. M. & Remmer, H. (1973) *Steroids*, 22, 203–225.
29. Scheulen, M., Wollenberg, P., Bolt, H. M., Kappus, H. & Remmer, H. (1975) *Biochem. Biophys. Res. Commun.*, 66, 1396–1400.
30. Aust, S. D., Roerig, D. L. & Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.*, 47, 1133–1137.
31. Nelson, S. D., Mitchell, J. R., Dybing, E. & Sasame, H. A. (1976) *Biochem. Biophys. Res. Commun.*, 70, 1157–1165.
32. Heikkila, R. E. & Cohen, G. (1973) *Science*, 181, 456–457.

[4] N. Castagnoli, Jr., and D. Mussen, personal communication.