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Conversion of Catalase to the Secondary Catalase-Peroxide Complex (Compound II) by α-Methyldopa

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SUMMARY

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α-Methyldopa stimulates the conversion of the primary catalase-peroxide complex (Compound I) to the catalytically inactive secondary catalase-peroxide complex (Compound II) in solutions of purified catalase as well as in suspensions of isolated hepatocytes. The rate of Compound II formation is dependent upon both the rate of H_2O_2 generation and the concentration of α-methyldopa. The catalase-dependent oxidation of α-methyldopa to the corresponding dihydroxyindole nucleus occurs much more rapidly than the formation of Compound II and suggests that the catalase-dependent one-electron oxidation of α-methyldopa may initiate a free radical process. These reactions may be important in α-methyldopa-induced hepatotoxicity.

INTRODUCTION

Catalase is a peroxisomal hemoprotein that functions along with glutathione peroxidase as primary cellular defenses against oxidative damage due to endogenously produced hydrogen peroxide. Chance (1) was able to resolve the catalase reaction cycle into two distinct processes by study of spectral changes during catalysis. In the first step, the native ferric hemoprotein ("free" catalase) reacts with H_2O_2 to form a spectrally distinct form, Compound I (for discussion, see ref. 2). In the second step, two electrons are transferred from an electron donor to form water and an oxidized product. The electron donor can be either a second molecule of H_2O_2 (catalatic mode) or another substance such as methanol, ethanol, or formic acid (peroxidatic mode).

With certain substrates, e.g., phenols, the transfer of electrons occurs in two steps (3). The rate of transfer of the second electron is slow relative to the transfer of the first and results in a steady-state accumulation of the one-electron reduced form of Compound I, which is termed Compound II (4, 5). Compound II is spectrally distinct from free catalase and from Compound I, and is an inactive form of the enzyme (for discussion, see ref. 6).

Oshino *et al.* (7) studied the formation of Compound II in perfused rat liver. They found that ascorbate, which rapidly converts the free enzyme to Compound II, does not induce Compound II formation in perfused liver. However, *p*-cresol was found to stimulate formation of Compound II in this system, and the authors concluded that since Compound II is inactive, the results indicated that *p*-cresol inactivates catalase in intact tissue.

After studies of drug-induced hydrogen peroxide formation in isolated hepatocytes (8), we sought to determine whether α-methyldopa, a widely used antiarrhythmic agent which frequently causes abnormal liver function (9), stimulates H_2O_2 production. In the course of these studies, we discovered that α-methyldopa induces a spectral change in isolated hepatocytes. In this report, we identify this change as due to the formation of the secondary catalase-peroxide complex, Compound II, and examine the characteristics of its formation in the presence of α-methyldopa.

EXPERIMENTAL PROCEDURES

Materials. Distilled and deionized water was used throughout. Chemicals were at least of reagent grade and purchased locally. α-Methyldopa [2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine], glucose oxidase, and collagenase were purchased from Sigma Chemical Company (St. Louis, Mo.).

Glucose plus glucose oxidase was used as an H_2O_2 generating system and calibrated relative to O_2 consumption as measured by a galvanic oxygen electrode (10), standardized with respect to O_2 concentration as previously described (11).

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Preparations. Isolated hepatocytes were prepared from male Sprague-Dawley rats (180–220 g) by the recirculating perfusion method of Moldeus *et al.* (12). Rat cardiac myocytes were isolated by the method of Rajs *et al.* (13). Rat liver microsomes were prepared by the method of Ernster *et al.* (14).

Twice-recrystallized catalase (prepared from bovine blood) stored in a 0.2% thymol solution was purchased from Sigma Chemical Company. The enzyme was diluted to 4 mg/ml and dialyzed against two changes of 50 mM phosphate buffer, pH 7.4, for 4 hr at 0°. This preparation was not reducible by sodium dithionite and had an A_{280}: A_{405} ratio of 1.10. Heme content was analyzed by spectroscopy of the reduced pyridine hemochromagen (15). The A_{405} corresponded to the reported extinction of catalase and indicated that little free heme or denatured, heme-containing protein was present.

Difference spectroscopy of cells as described by Jones *et al.* (16), and dual wavelength spectroscopy as described by Jones *et al.* (8) were performed. Spectra were recorded at room temperature on either an Aminco DW-2 or an Aminco DW-2a spectrophotometer.

Incubations were performed as described by Moldeus *et al.* (12). Cells were incubated in Krebs-Henseleit buffer, pH 7.4, which was supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to control pH.

RESULTS

Identification of Compound II. Conversion of catalase to Compound II by ascorbate is shown in Fig. 1A. Absorbance maxima occur at 426, 536, and 568 nm (4). The conversion occurs first by autoxidation of ascorbate to generate H_2O_2, which binds to catalase to form Compound I. Compound I is distinguished by a hypochromicity in the Soret band and an increased absorbance at 660 nm (see Table 1). Conversion to Compound II then occurs with a shift of the isosbestic point from 430 to 418 nm. With ascorbate, Compound II exists in equilibrium with Compound I, since addition of methanol causes a very rapid elimination of residual Compound I by stimulation of the peroxidatic reaction mode. This is seen as an immediate increase in absorbance in the 405-nm region with no change at 426 nm (Fig. 1B). Subsequently, Compound II undergoes conversion directly to free catalase with an isosbestic point at 418 nm.

Catalase also binds ligands, such as HCN and HCOOH, with resultant absorbance changes (for reference, see Table 1). Addition of α-methyldopa to catalase produces a complex mixture of spectral changes which prevent simple interpretation. However, subsequent analyses (below) indicate that the changes include conversion of free catalase to Compound I, conversion of Compound I to Compound II, oxidation of α-methyldopa to a species that absorbs at 475 nm, and development of nonspecific absorbance in the visible region due to polymerization of oxidation products of α-methyldopa, presumably also including reaction with functional groups on catalase.

Since Compound II is converted back to free catalase by addition of methanol (Fig. 1B), a difference spectrum was constructed in which both sample and reference cuvettes were incubated with α-methyldopa to preform

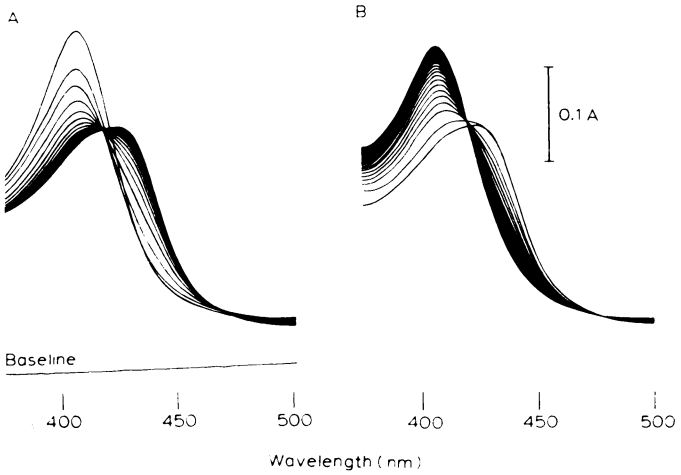

FIG. 1. *Compound II formation induced by addition of ascorbate to purified catalase*

Spectra were recorded at 37° with 3.6 μM catalase heme and 10 mM ascorbate.

A. A rapid decrease at 406 nm occurred due to Compound I formation following addition of ascorbate. Compound I was then converted at a slower rate to Compound II (426 nm). It should be noted that the curve for free catalase does not pass through the apparent isosbestic point that is established by conversion of Compound I to Compound II.

B. Addition of methanol (26 mM) resulted in a rapid conversion of the steady-state level of Compound I back to free catalase, as evidenced by the rapid increase in absorbance at 406 nm without change at 426 nm. In subsequent tracings (2.5 min/scan), an isosbestic point occurred at 418 nm.

the spectral species, and methanol was added to the reference cuvette to allow conversion of Compound II back to free catalase (Fig. 2A). The products of α-methyldopa oxidation are essentially equivalent in the two cuvettes, and the spectrum obtained is a difference spectrum of Compound II minus free catalase, i.e., the same as that formed with ascorbate (Fig. 2B), and therefore identifiable as Compound II. Results from incubation under anaerobic conditions (prepurified N_2) supported this conclusion as no spectral change was observed following addition of α-methyldopa.

Spectra of isolated hepatocytes were obtained in the same fashion (Fig. 3). Cells were incubated at least 15 min with α-methyldopa, and 2 ml were divided equally between the sample and reference cuvettes. One microliter of methanol was then added to the reference cuvette and 1 μl of water was added to the sample cuvette. The spectrum of Compound II was recognizable within minutes, but the maximal change was not detectable until

TABLE 1

Absorbance maxima of different forms of catalase[a]

Form	Absorbance maxima			
	nm			
Free catalase	405 (103)	504 (11.1)	537 (9.7)	627.5 (7.0)
Compound I	398 (44)	583 (6.8)	662 (9.4)	
Compound II	428 (77)	530 (12.5)	568 (18)	
Compound III	416 (84)	545 (13)	585 (10.5)	
Catalase-formate	408 (95)	500	540	616 (13.4)
Catalase-cyanide	426 (94)	425 (79)	555 (14.5)	585

[a] Taken from Brill (4), and Nicholls and Schonbaum (5). Millimolar extinction values are given in parentheses.

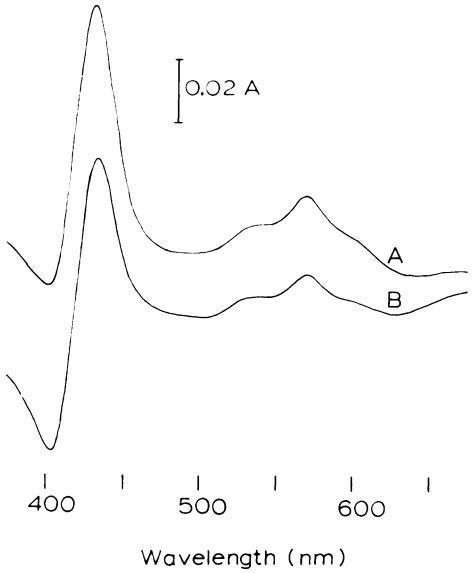

FIG. 2. *α-Methyldopa- and ascorbate-induced formation of Compound II*

A. Purified catalase (1.9 μM heme) was incubated for 1.5 hr at 37° in 50 mM potassium phosphate buffer (pH 7.4) containing 2.5 mM α-methyldopa. Two milliliters were removed and divided equally into sample and reference cuvettes. Buffer (0.5 μl) was added to the sample cuvette and absolute methanol (0.5 μl) was added to the reference cuvette. After 30 min, the spectra were recorded. Baseline spectra were recorded but were flat in the regions of interest and are omitted for clarity.

B. Difference spectrum obtained in the same manner with 10 mM sodium ascorbate. The differences in magnitudes of the absorbance changes of the 568 band relative to the Soret region appear to be due to the differences in concentrations of free catalase and Compound I in the different systems.

after 30 min. Additional studies were performed to further verify the conclusions reached on the basis of the above experiments. The first derivative of the α-methyldopa-induced difference spectrum showed the same inflections as that with ascorbate. Addition of 3-amino-1,2,4-triazole to isolated liver cells that had been incubated with α-methyldopa resulted in loss of the 568-nm absorbance maximum. Since aminotriazole reacts with Compound II to regenerate free catalase (17), this further supports identification as given above. Aminotriazole also reacts with Compound I and inactivates catalase. Destruction of catalase (>80%) in liver cells by incubation for 45 min with 5 mM aminotriazole and 10 mM glycolate prevented formation of the difference spectrum as shown in Fig. 3. In studies of isolated cardiac myocytes, which contain a relatively small amount of catalase (18, 19) but a high concentration of mitochondrial cytochromes and myoglobin, α-methyldopa did not produce a difference spectrum. A study of the spectral changes due to α-methyldopa addition to liver cells from rats that had been treated with phenobarbital to increase the content of cytochrome P-450 gave the same results as obtained with control cells. Furthermore, incubations of rat liver microsomes with α-methyldopa did not result in formation of the difference spectrum. These results indicate that the protein(s) responsible for the spectral changes are not the mitochondrial cytochromes (as found in heart) or the microsomal cytochromes, and are therefore consistent with the conclusion that the spectral changes observed in the liver cells are due to interaction of α-methyldopa with catalase.

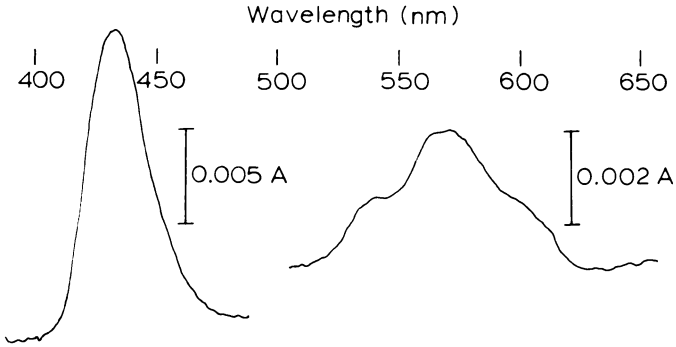

FIG. 3. *Spectrum of catalase Compound II due to α-methyldopa in isolated hepatocytes*

Isolated hepatocytes (approximately 1.4 × 10^6 cells/ml) were incubated for 30 min at 37° in Krebs-Henseleit buffer (supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) containing 2.5 mM α-methyldopa. Two milliliters were removed and divided equally into sample and reference cuvettes. Additions (1 μl of methanol to reference and 1 μl of water to sample) were made to allow visualization of Compound II and, after 30 min, the spectrum was recorded. Catalase heme, as determined by absorbance change at 660–630 nm following KCN addition to methanol-treated cells (8), was 0.23 nmol/10^6 cells.

Characteristics of Compound II formation. Determination of the rate of formation of Compound II with α-methyldopa is difficult in both the isolated cellular system and the purified enzyme system due to the presence of at least three spectral forms of catalase (free catalase, Compound I and Compound II), the multiple nonenzymic oxidation processes that occur, and the development of specific and nonspecific absorbance changes due to oxidation of α-methyldopa. In addition, the rate of H_2O_2 generation in isolated cells is not accurately controlled, and, with the reconstituted system, the H_2O_2-generating system causes conversion of Compound I to Compound II without α-methyldopa. Furthermore, studies with isolated cells require high sensitivity and are complicated by changes in turbidity due to settling of cells from suspension. However, these latter problems have been considered previously (8, 16).

Extinction coefficients were calculated for the changes in absorbance differences at 426 − 405 nm (77 $mM^{-1} \cdot cm^{-1}$) and 568 − 605 nm (10.5 $mM^{-1} \cdot cm^{-1}$) following additions of methanol to the preformed Compound II using reported extinction values (4–6). This longer wavelength pair was considered more suitable for the succeeding experiments because they minimize changes due to Compound I and oxidation products of α-methyldopa. The Soret maximum in the difference spectrum shifted from a value as high as 432 nm down to 426 nm, depending upon the fraction of the total catalase that was in the form of Compound II. This shift is apparently due to the change in the steady-state level of Compound I that occurs as the fraction of Compound II increases, since both free catalase and Compound I contribute to the absorbance in the region of 426–432 nm, but to different extents. Thus, the difference in spectral properties found by Oshino *et al.* (7) and those reported by Brill (4) may

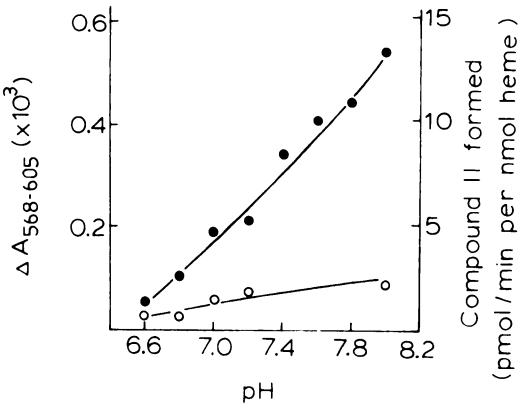

FIG. 4. *Dependence upon pH of Compound II formation with α-methyldopa*

The change in absorbance at 568 – 605 nm was measured following addition of 2.5 mM α-methyldopa to catalase (3.9 μM heme) (●). A portion of this spectral change is due to autoxidation of α-methyldopa (○) and an additional fraction is due to catalase-dependent oxidation of α-methyldopa (see Fig. 7).

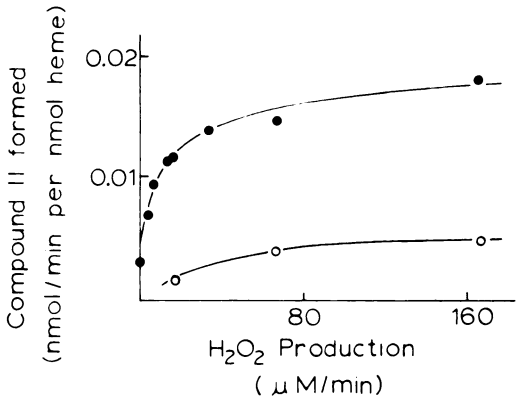

FIG. 6. *Dependence of Compound II formation in presence of α-methyldopa upon H_2O_2 production rate*

Catalase (3.9 μM heme) was incubated at room temperature with 2.5 mM α-methyldopa in the presence of glucose (10 mM) plus glucose oxidase to give the desired H_2O_2 production rate. Compound II formation was measured during the 1st min of incubation as the $\Delta A_{568-605}$. ●, + α-Methyldopa; ○, without α-methyldopa. Incubations were performed with 50 mM potassium phosphate, pH 7.4.

be a consequence of the relative equilibria of the three forms of catalase. Also, because of this shift in concentrations of free catalase and Compound I as Compound II is formed, no isosbestic point occurs. A region of relatively minor change in absorbance occurs around 605 nm and was used as the reference wavelength. The extinction coefficient of Compound II is not affected by pH over the range pH 6.6–8.0, as indicated by the early studies of Stern (20) in which the formation of the "red" complex was found to be independent of pH over the range 4–11, by the studies of Chance (21) in which reported extinction coefficients at pH 5.4 and 7.0 were essentially the same, and by studies in the authors' laboratory in which measured extinction values for catalase and Compound II were in agreement with previously reported values and independent of pH over the range of 6.6–8.0.

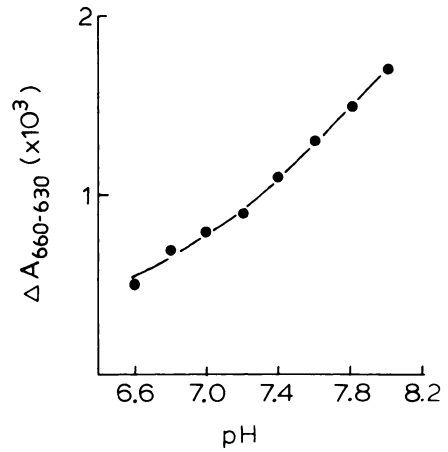

FIG. 5. *Steady-state level of Compound I as a function of pH*

The steady-state level of Compound I was measured as the $\Delta A_{660-630}$ during the 45 sec following addition of 2.5 mM α-methyldopa to solutions of catalase (3.5 μM heme) at the designated pH values. The maximal change was observed in about 30 sec and was unchanged for at least 2 min. Addition of methanol (26 mM) resulted in a reversal of the absorbance changes back to the original values (not shown) during this time, but addition of methanol at subsequent times did not give complete return to baseline.

With purified catalase, Compound II was formed with α-methyldopa even without an H_2O_2-generating system. The rate of formation is highly pH dependent (Fig. 4), increasing as pH is increased over the range of 6.6–8.0. This increased rate parallels the steady-state level of Compound II closely (Fig. 5), and suggests that in the presence of α-methyldopa, the rate of Compound II formation is limited by the steady-state level of Compound I, which is dependent upon the rate of H_2O_2 generation by the autoxidation of α-methyldopa.

The rate of Compound II formation is enhanced by the inclusion of the H_2O_2-generating system, glucose plus glucose oxidase (Fig. 6). A direct comparison is difficult because this latter system also causes conversion of Compound I to Compound II. After subtraction of this rate, the rate of Compound II formation is about 0.01 nmol/min/nmol of catalase heme when the H_2O_2 production rate is greater than 10 nmol/ml/min. The rate of Compound II formation was also measured in isolated hepatocytes in the pressence of 2.5 mM α-methyldopa. Since no evidence was obtained for changes in oxidation-reduction state of mitochondrial cytochromes in the presence of α-methyldopa, studies were performed using the same wavelength pair, 568 – 605 nm. The estimated rate was 0.013–0.016 nmol/min/10^6 cells, which corresponds to a rate of 0.074 nmol/min/nmol of catalase heme. This rate is much higher than that which is observed with the isolated enzyme incubated with an H_2O_2-generation rate similar to that estimated for isolated hepatocytes [2 nmol/min/10^6 cells (8)].

Qualitative studies on the oxidation of α-methyldopa, which were performed as described by Mason (22) for the nonenzymic oxidation of dopa[3], indicated that the reaction sequence is analogous to that for dopa. The rate of oxidation of α-methyldopa was therefore estimated from the absorbance increase at 475 nm (due to the 2,3-

[3] The abbreviation used is: dopa, 3,4-dihydroxyphenylalanine.

dihydroxyindole nucleus) as is also found in the oxidation of dopa to dopachrome (22) and epinephrine to adrenochrome (23). The extinction value for dopachrome is 3.7 $mM^{-1} \cdot cm^{-1}$ and for adrenochrome is 4.02 $mM^{-1} \cdot cm^{-1}$; therefore, an estimated extinction value of 4 $mM^{-1} \cdot cm^{-1}$ was used. The estimated rate of formation of the dihydroxyindole nucleus, termed here methyldopachrome (Fig. 7), is far greater than the rate of Compound II formation (see Fig. 6). This comparison suggests that either catalase catalyzes the peroxidatic oxidation of α-methyldopa in addition to the one-electron oxidation, or that the one-electron oxidation initiates a free-radical process that results in a substantially increased rate of α-methyldopa oxidation relative to the rate of Compound II formation.

Results from two experiments suggest that a peroxidatic reaction with α-methyldopa does not occur. If α-methyldopa acted as an efficient two-electron donor to Compound I, then addition of a high concentration should result in a rapid conversion of Compound I back to free catalase. This does not occur. Instead, a high concentration of α-methyldopa stimulates formation of Compound II. With concentrations as high as 25 mM, no stimulation of conversion of either Compound I or Compound II back to free catalase was observed. The $t_{1/2}$ for conversion of Compound II (2.5 mM α-methyldopa) back to free catalase following addition of methanol was 12 min. These data suggest that α-methyldopa may be a Group I donor according to the classification of Keilin and Nicholls (3). The second piece of evidence is based upon the establishment of a steady-state concentration of Compound I in the presence of a two-electron donor. Oshino *et al.* (24) have shown that in the presence of increasing concentrations of methanol, a higher rate of H_2O_2 generation is necessary to maintain the same steady-state concentration of Compound I. Experiments in which the steady-state concentration of Compound I was measured as a function of the rate of H_2O_2 generation in the presence and absence of α-methyldopa indicate that the ratio of Compound I/free catalase is unchanged by α-methyldopa (data not shown).

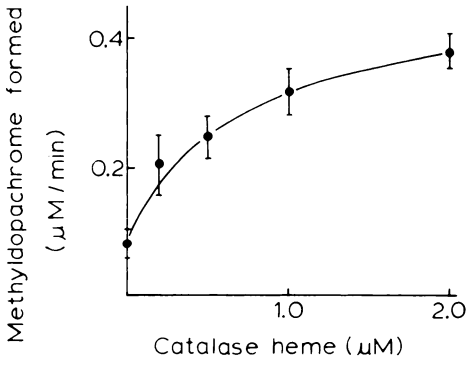

FIG. 7. *Oxidation of α-methyldopa as a function of catalase concentration*

The change in absorbance at 475 nm was used to measure the formation of the dihydroxyindole nucleus of methyldopachrome with an estimated extinction value of 4 $mM^{-1} \cdot cm^{-1}$. Incubations were performed at room temperature with 50 mM sodium phosphate buffer, pH 7.4.

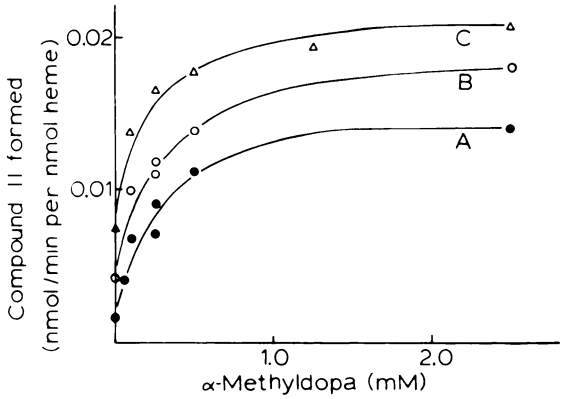

FIG. 8. *Compound II formation as a function of α-methyldopa concentration*

Incubations were performed as described in Fig. 7, with α-methyldopa concentrations as indicated and with glucose (10 mM) plus glucose oxidase. H_2O_2 production rates were 15 nmol/ml/min (●), 60 nmol/ml/min (○), and 300 nmol/ml per min (△).

The dependence of Compound II formation upon α-methyldopa concentration (Fig. 8) indicates that even 50–100 μM results in a substantial stimulation of Compound II formation. Detailed studies on the concentration dependence in isolated hepatocytes must await more detailed studies upon uptake by the cells; however, simple calculations of expected concentrations due to normal therapeutic doses provide concentrations in the 50–100 μM range.

The fraction of catalase present in the form of Compound II is of interest because this form of the enzyme is inactive in the decomposition of H_2O_2. Using the estimated extinction values for Compound II as discussed above, 40–60% of the total catalase is converted to Compound II in either the purified enzyme or isolated hepatocyte system. With the purified enzyme, it is possible to directly measure the effect of Compound II formation upon the rate of H_2O_2 metabolism (Fig. 9). However, two

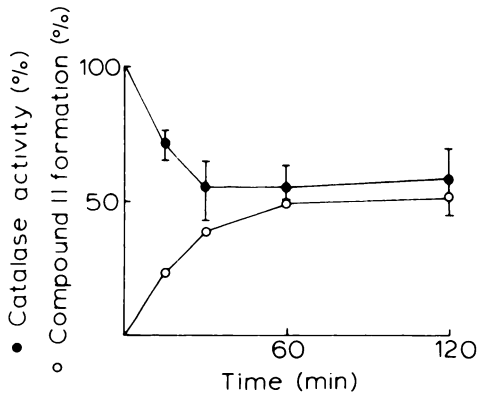

FIG. 9. *Formation of Compound II and loss of catalase activity as a function of time*

Catalase (1.9 μM heme) was incubated at 37° in 50 mM sodium phosphate buffer, pH 7.4, with 2.5 mM α-methyldopa. Catalase activity was measured spectrophotometrically at 37° by using 1-μl additions of enzyme incubation to 1-ml H_2O_2 solutions at the indicated times and measuring absorbance loss at 240 nm. Connected *points* are mean values for at least three determinations; *bars* indicate the range of values. Compound II formation was measured as described in Fig. 7.

limitations to this experiment must be recognized. The oxidation product of α-methyldopa is a melanin-like polymer which is readily bleached by H_2O_2. Consequently, a portion of the measured catalase activity is probably due to this nonenzymic reaction. Secondly, the quinones generated by oxidation of α-methyldopa can be expected to be nonspecific in their reactivity and may thus react with catalase and result in loss of activity. This "tanning" reaction results in a loss in total cyanide-detectable catalase following 1- to 2-hr incubations (data not shown).

DISCUSSION

The results presented demonstrate the conversion of catalase to the secondary catalase-peroxide complex, Compound II, in the presence of α-methyldopa. This process is a one-electron transfer that results in formation of a free radical. The rate of reaction of the free radical with Compound II is slow, as evidenced by the relatively slow conversion of Compound II back to free catalase after addition of methanol. This $t_{1/2}$ is comparable to previously reported values of 6–10 min (7). The free radical formed by this one-electron oxidation appears to initiate a free radical process that results in substantial oxidation of α-methyldopa. Although details of this process are unknown, they may be similar to that proposed by Misra and Fridovich (23) for the involvement of superoxide in the autoxidation of epinephrine. Preliminary experiments[4] indicate that superoxide dismutase inhibits the rapid oxidation of α-methyldopa that occurs in the presence of catalase.

The toxicological implications of this reaction sequence are complex. The results suggest that in addition to catalyzing the formation of a free radical, the process initiates formation of superoxide and hydrogen peroxide. In addition, the formation of Compound II represents conversion of one of the systems for decomposing H_2O_2 to an inactive form, thus exacerbating any potential toxicity due to peroxide formation. Furthermore, the methyldopachrome is a dihydroxyindole that typically undergoes rearrangement and further oxidation to an indolequinone. Both of these structures are highly reactive electrophiles that are nonspecific with regard to their reactivity to functional groups in proteins (25). Finally, the catalysis of free radical formation by catalase could be an important initiating event in α-methyldopa toxicity. The high concentration of catalase in hepatic tissue (16) relative to other tissues (18, 19), may be a predisposing factor for the abnormal liver function that occurs among patients taking α-methyldopa. However, further delineation of the reactions involved, including quantitative assessment of the reactions in perfused tissues and *in vivo*, will be necessary to substantiate this. In addition, a more detailed analysis of the possible involvement of cytochrome P-450 in the toxicity mechanism, as proposed by Dybing *et al.* (26), will be necessary.

[4] D. P. Jones and D. B. Meyer, unpublished data.

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