0090-9556/88/1606-0880$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 16, No. 6
Printed in U.S.A.

MECHANISM FOR INHIBITORY EFFECT OF CANNABIDIOL ON MICROSOMAL TESTOSTERONE OXIDATION IN MALE RAT LIVER

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(Received February 23, 1988; accepted May 17, 1988)

ABSTRACT:

Effects of four cannabinoids [cannabidiol (CBD), Δ^8-tetrahydrocannabinol, Δ^9-tetrahydrocannabinol, and cannabinol] on hepatic microsomal oxidation of testosterone (17β-hydroxy-androst-4-ene-3-one) were examined in adult male rats. Only CBD (30 μM) competitively inhibited 2α-hydroxy-testosterone (2α-OH-T) and 16α-OH-T formation by hepatic microsomes but did not affect androstenedione (androst-4-ene-3,17-dione) and 7α-OH-T formation. Kinetic analyses demonstrated that the inhibitory profile of CBD for testosterone oxidation was different from those of SKF 525-A, which caused competitive inhibition for 2α- and 16α-hydroxylations and noncompetitive inhibition for 6α-hydroxylation, and of metyrapone, which inhibited only 6β-hydroxylation competitively. CBD also suppressed formation of 2α-OH-T, 16α-OH-T, and androstenedione from testosterone, catalyzed by a reconstituted system containing hepatic cytochrome P-450 purified from phenobarbital-treated rats. Pretreatment of the rat with CBD (10 mg/kg, ip, once a day for 3 days) decreased testosterone oxidation at the 2α-, 16α-, and 17-positions and increased 7α-OH-T formation, while total cytochrome P-450 content was decreased. These results suggest that CBD suppresses hepatic testosterone oxidation at the 2α-, 16α-, and 17-positions through selective inhibition of the male-specific cytochrome P-450 in the adult male rat.

CBD,[1] one of main constituents of marijuana, lacks psychotoxicity but has anticonvulsant activity, especially to seizures induced by the maximal electroshock, indicating CBD as a possible antiepileptic (1–4). Further, Burstein *et al.* (5) have recently suggested that CBD may be effective in the treatment of Niemann-Pick disease. However, CBD is known to inhibit the hepatic mixed function oxidase system as a harmful side effect (6–8). Although some knowledge (9–12) has been accumulated on the inhibitory effect of CBD on the hepatic drug metabolism, its mechanism remains to be elucidated.

Binder (13) pointed out the similarity of the structure of cannabinoids to that of steroids, indicating possible interaction between them. Among many studies on this line, Burstein *et al.* (14, 15) demonstrated that THC inhibited the activity of an esterase that releases cholesterol from acylated cholesterol in the initial step of testosterone biosynthesis, resulting in a decrease in the blood level of testosterone in the rat. List *et al.* (16) reported that chronic treatment of rats with Δ^9-THC or CBD increased testosterone hydroxylation activity in the hepatic microsomal fraction. Recently, various working groups paid much attention to hepatic testosterone oxidation and showed that some of the multiple forms of hepatic cytochrome P-450 were responsible for testosterone oxidation (17–22). Considering the inhibitory nature of CBD for the hepatic drug metabolism catalyzed by cytochrome P-450, it seems likely that CBD suppresses hepatic testosterone oxidation. Furthermore, a question arises as to whether the inhibitory effect of CBD is general for various isozymes or specific for selected isozymes of cytochrome P-450. Testosterone hydroxylations have proved useful in characterizing the individual catalytic capacity of cytochrome P-450 isozymes (23). This means that the nature of action of CBD on hepatic cytochrome P-450 can be studied using testosterone as a substrate. In the present study, the inhibitory effect of CBD on testosterone oxidation was examined with the microsomal fraction or a purified cytochrome P-450 from adult male rat liver.

Materials and Methods

Chemicals. Various chemicals and reagents were obtained as follows: testosterone, epitestosterone, 16α-OH-T, G-6-P dehydrogenase (type V, EC 1.1.1.49), dilauroylphosphatidylcholine, cholic acid, sodium deoxycholate, metyrapone, cytochrome *c*, and molecular weight markers (Dalton Mark VII L) for SDS-PAGE from Sigma Chemical Co. (St. Louis, MO); 2β-OH-T-2β,17β-diacetate, 6β-OH-T, and 7α-OH-T from Steraloids Inc. (Wilton, NH); NADP, NADPH, and G-6-P from Boehringer Mannheim GmbH. (Darmstadt, Federal Republic of Germany); silica gel for column chromatography, sodium phenobarbital, dithiothreitol, TMCS, BSTFA, and TMSI from Wako Pure Chemicals (Osaka, Japan); Sepharose 4B, 2′,5′-ADP-Sepharose 4B, DEAE-Sephacel, and CM-Sephadex C-50 from Pharmacia Fine Chemicals (Uppsala, Sweden); hydroxyapatite and DEAE-cellulose (DE-52) from Bio-Rad (Richmond, CA); Amberlite XAD-2 from Organo Co. (Tokyo, Japan); SKF 525-A from Smith Kline & French (Philadelphia, PA); and leupeptin and pepstatin from Peptide Institute Inc. (Osaka, Japan). 2α-OH-T, *d*-benzphetamine hydrochloride, and Emulgen 913 were gifts from Dr. Nakamura, Shionogi Pharmaceutical Co. (Osaka, Japan), Dr. Neal, Research Triangle Institute (Research Triangle Park, NC), and Kao-Atlas Co. (Tokyo,

This research was supported in part by a Grant-in Aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan. A preliminary account of this work was presented at the Japanese-United States Congress of Pharmaceutical Sciences, Honolulu, Hawaii, December, 1987.

[1] Abbreviations used are: CBD, cannabidiol; THC, tetrahydrocannabinol; CBN, cannabinol; Xα-OH-T, Xα-hydroxytestosterone; epitestosterone, 17α-hydroxy-androst-4-ene-3-one; Δ^4-A, androstenedione (androst-4-ene-3,17-dione); G-6-P, glucose-6-phosphate; TMCS, trimethylchlorosilane; BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; TMSI, trimethylsilylimidazole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Japan), respectively. CBD, Δ^9-THC, and CBN were isolated and purified from cannabis leaves supplied by Professor Nishioka, Kyushu University, according to the method of Aramaki *et al.* (24). Δ^8-THC was obtained by isomerization of Δ^9-THC with *p*-toluenesulfonic acid in benzene (25). Purities of these cannabinoids were found to be above 98% by GC and HPLC. Aniline hydrochloride from Nakarai Chemicals Co. (Tokyo, Japan) and cholic acid from Sigma were recrystallized from methanol before use. Other chemicals and solvents used were of the highest quality commercially available.

Microsomal Incubation. Male Sprague-Dawley rats (150–170 g of body weight) were fasted for 24 hr and liver microsomes were prepared by differential centrifugation as previously described (26), then suspended in 1.15% KCl (1 g liver equivalent per 1 ml). A typical incubation medium consisted of magnesium chloride (20 μmol), G-6-P (2 μmol), NADP (0.1 μmol), G-6-P dehydrogenase (2.5 units), microsomes (0.2 g liver equivalent), and potassium phosphate buffer (pH 7.4, 150 μmol) to make a final volume of 2.68 ml in a 40-ml volume polypropylene tube. After preincubation for 5 min at 37°C, testosterone (200 μg, 0.69 μmol) was added to the reaction mixture, which was incubated for 5 min at 37°C. After addition of ethyl acetate (25 ml), 12.5 μg of epitestosterone was then added as an internal standard, followed by shaking and centrifugation. The organic layer was evaporated under a N_2 stream, and the residue was dissolved in 2 ml of ethanol. The solution was filtered through a 2-μm membrane filter, followed by evaporation. The residue obtained was dissolved in 100 μl of ethanol, and an aliquot (2 μl) was subjected to HPLC (condition A), as described below.

Effects of Cannabinoids on Microsomal Testosterone Oxidation. Major cannabinoids (Δ^9-THC, Δ^8-THC, CBD, and CBN, each 30 μM) were added to the reaction medium just before the preincubation, and their effects on the microsomal metabolism of testosterone were studied. The effect of the dose of CBD, which showed the strongest effect among the cannabinoids examined, was compared with those of SKF 525-A and metyrapone, known inhibitors of hepatic drug metabolism (27). Moreover, kinetic analysis was performed for their inhibitory effects on hepatic microsomal testosterone oxidation, using Lineweaver-Burk plots and Dixon plots, and kinetic parameters (apparent K_m, V_{max}, and K_i values) were estimated (28).

Enzyme Preparation. Male Sprague-Dawley rats (150–170 g of body weight) were injected ip with sodium phenobarbital at a dose of 80 mg/kg/day for 4 days. After fasting for 24 hr, the rats were killed on day 5. One of the hepatic cytochrome P-450 isozymes was purified by a slight modification of the method reported by Nagata *et al.* (29). The washed microsomes were solubilized with 0.6% sodium cholate in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, and 0.1 mM DTT (buffer A). The supernatant of the solubilization was applied to an aminooctyl-Sepharose 4B column (4 × 22 cm) equilibrated with buffer A containing 0.5% sodium cholate. After washing of the column with the same buffer, cytochrome P-450 was eluted with buffer A containing 0.4% sodium cholate and 0.08% Emulgen 913. The pooled peak fractions were concentrated by ultrafiltration, followed by dialysis against 10 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, and 0.1 mM DTT (buffer B). The dialyzed solution was applied to a DE-52 column (2.6 × 20 cm) previously equilibrated with buffer B containing 0.5% sodium cholate and 0.2% Emulgen 913. After the first peak has been eluted with buffer B, elution of the main band was carried out with buffer B containing 20 mM NaCl. The eluate was applied to a hydroxyapatite column (2.6 × 15 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.2% Emulgen 913. The column was eluted stepwise with 40, 80, and 160 mM potassium phosphate buffer containing the same ingredients as in the equilibration buffer. Fractions eluted with the 40 mM buffer were pooled and concentrated by ultrafiltration, followed by dialysis against 5 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, and 0.1 mM DTT (buffer C). The solution was then applied to a column of DEAE-Sephacel (1.6 × 20 cm) previously equilibrated with buffer C containing 0.2% Emulgen 913. The column was washed with buffer C and then with 20 mM potassium phosphate buffer containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.2% Emulgen 913, and 20 mM NaCl. Cytochrome P-450-rich fractions were eluted by increasing the NaCl concentration from 20 to 40 mM. Emulgen 913 in the eluate was removed by treatment with Amberlite XAD-2, followed by hydroxyapatite chromatography as previously reported (29). The final preparation of cytochrome P-450 gave a specific content of 13.2 nmol/mg of protein (yield of 0.92% from the microsomes). NADPH-cytochrome *c* reductase (EC 1.6.2.4) was purified by the method of Yasukochi and Masters (30). The specific activity of the reductase was 36.2 units/mg of protein. Both enzyme preparations showed a single band on each SDS-PAGE.

Reconstituted System. *N*-Demethylation of *d*-benzphetamine was determined by a colorimetric method, measuring formaldehyde (31). The reconstituted system consisted of cytochrome P-450 (0.17 nmol), NADPH-cytochrome *c* reductase (0.34 units), dilauroylphosphatidylcholine (48 nmol), $MgCl_2$ (40 μmol), NADPH (1 μmol), *d*-benzphetamine (0.5 μmol), and potassium phosphate buffer (70 μmol, pH 7.4) to make a final volume of 1.053 ml. Incubation was carried out at 37°C for 10 min. Aniline hydroxylation was measured according to the method of Imai *et al.* (32). The reconstituted system was the same as that in benzphetamine *N*-demethylation, except for the substrate (aniline hydrochloride, 1 μmol). It was incubated at 37°C for 10 min. Testosterone

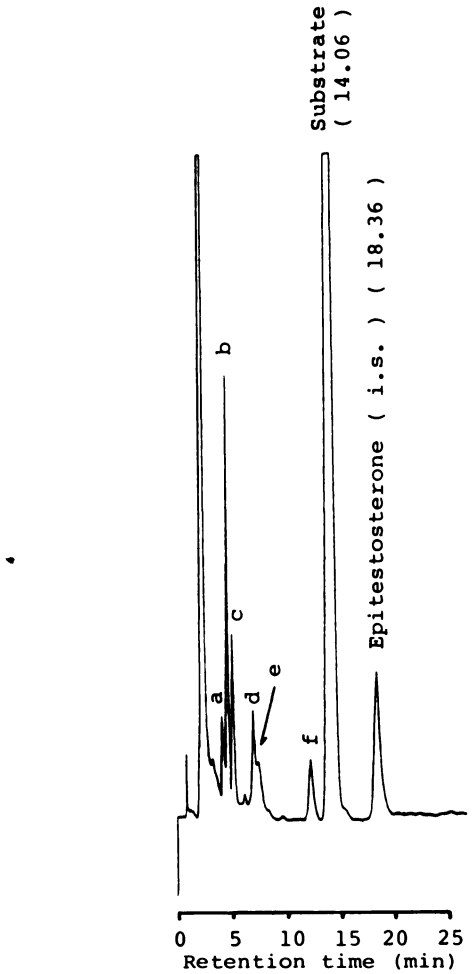

FIG. 1. *HPLC profile of testosterone metabolites formed with rat hepatic microsomes.*

A DuPont Zorbax ODS column and a mobile phase of methanol/acetonitrile/water (55:10:35) were used for the analysis of extract after 5-min incubation of the reaction mixture containing 3.2 mg of protein and 200 μM testosterone as described under *Materials and Methods. a*, 7α-OH-T; *b*, 6β-OH-T; *c*, 16α-OH-T; *d*, 2α-OH-T; *e*, 2β-OH-T; and *f*, Δ^4-A. *i.s.*, internal standard.

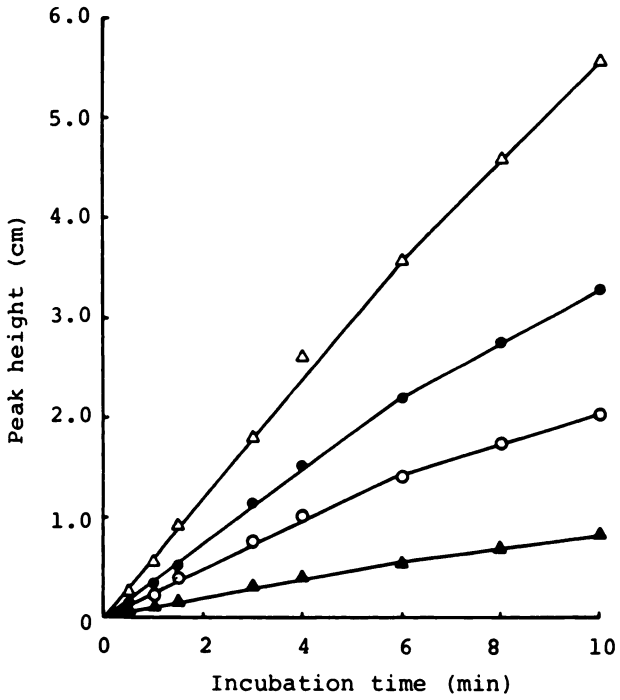

FIG. 2. *Effect of incubation time on microsomal testosterone oxidation.*

The reaction medium contained 3.2 mg of protein and 200 μM testosterone, as well as a NADPH-generating system. Each *point* represents the mean value of two determinations. Δ, 6β-OH-T; ●, 16α-OH-T; ○, 2α-OH-T; and ▲, Δ^4-A.

hydroxylation was examined under the same conditions using a substrate concentration of 25 μg (87 μM). Incubation was performed at 37°C for 20 min, and metabolites formed were extracted into 5 ml of ethyl acetate. The metabolites were then examined by HPLC in the same manner as described under *Microsomal Incubation.*

Effect of CBD Treatment of the Rat on Microsomal Testosterone Oxidation. One of the two groups of male Sprague-Dawley rats (155–170 g of body weight; $N = 4$ for each group) received three daily ip injections of CBD (10 mg/kg/day). The other group was given the vehicle (physiological saline containing 1% v/v, Tween 80). After starvation for 24 hr, the rats were killed on day 4. Hepatic microsomal fractions were prepared, and testosterone oxidation was assayed by the same method described above.

Identification of Testosterone Metabolites Formed by Microsomal Reaction and Reconstituted System. In order to isolate the metabolites, testosterone (750 μg) was incubated with microsomes in 5-fold larger reaction, as compared with that described above, at 37°C for 20 min. Metabolites formed were extracted into ethyl acetate (25 ml twice), and the organic layer was evaporated under a N_2 stream. The residue was dissolved in 50 μl of ethanol. An aliquot (2 μl) was subjected to HPLC (condition A) described later, and another aliquot (5 μl) was subjected to TLC using a precoated fluorescent silica gel plate with a solvent system of chloroform/ethyl acetate/ethanol (4:1:0.7, v/v) as described by Waxman *et al.* (18). Testosterone metabolites on the plate were located by spraying ethanolic H_2SO_4 and heating at 110°C. For identification of the metabolites, the remaining sample solution was subjected to preparative TLC using a precoated fluorescent silica gel plate and the same solvent system described above. Six bands showing the same R_F values as those of synthetic standards codeveloped on both sides of the plate were scraped, and metabolites were extracted twice with 5 ml of chloroform/ethyl acetate/ethanol (4:1:0.7, v/v). R_F values for synthetic standards were as follows: 16α-OH-T, 0.28; 7α-OH-T, 0.35; 6β-OH-T, 0.48; 2α-OH-T, 0.59; testosterone, 0.72; and Δ^4-A, 0.76. The extracts were evaporated under N_2, and each of the residues was dissolved in 20 μl of acetonitrile. After analyses by TLC and HPLC, the remaining sample solution was then heated with BSTFA (10 μl), TMSI (5 μl), and TMCS (5 μl) at 60°C for 20 min. The trimethylsilylated metabolites were analyzed by GC-MS. Testosterone metabolism in the reconstituted system was also carried out in a 5-fold larger incubation mixture, and three metabolites, which had the same R_F values as those of synthetic 16α-OH-T, 2α-OH-T, and Δ^4-A, respectively, were obtained by preparative TLC. Lastly, they were identified by HPLC and GC-MS described above.

GC-MS. A JEOL JMS D-300 mass spectrometer equipped with a JMS mass data system and a column packed with 5% SE-30 on Chromosorb

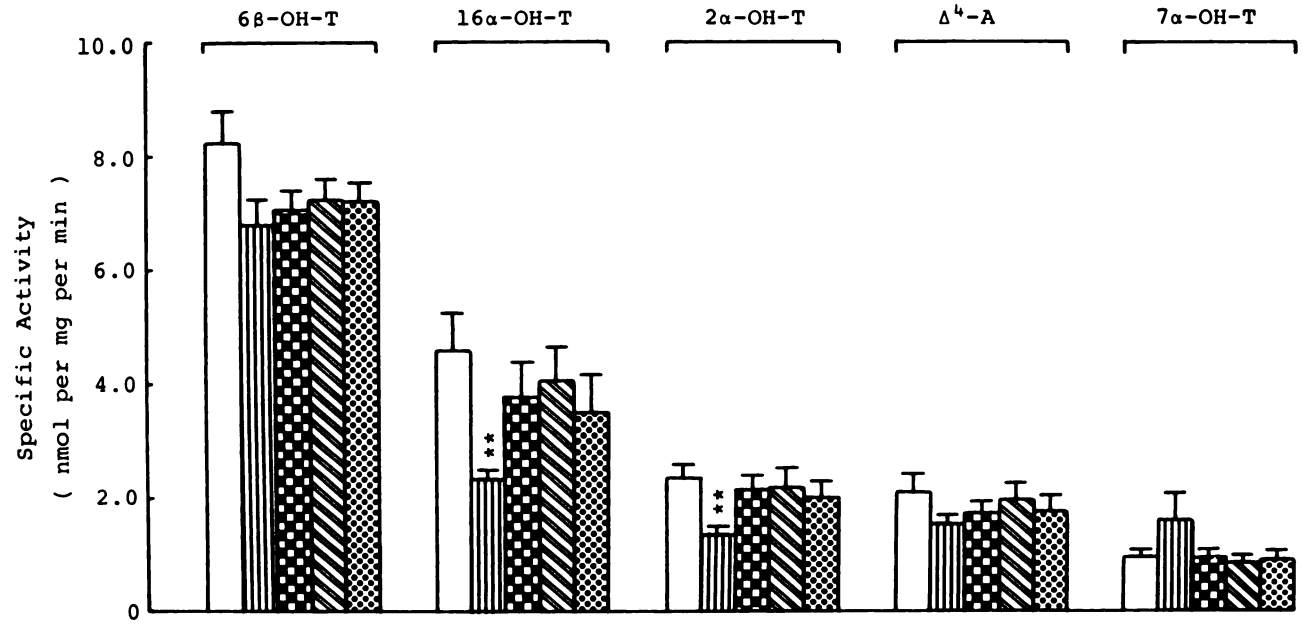

FIG. 3. *In vitro effects of cannabinoids on microsomal testosterone oxidation.*

Cannabinoids (CBD, Δ^8-THC, Δ^9-THC, and CBN, 30 μM each) dissolved in ethanol (10 mg/ml) were added to the reaction medium just before preincubation. Each *column* and *bar* represent the mean value ± SE of four determinations. ** Significantly different from the control to which only vehicle (ethanol) was added ($p < 0.01$). □, control; ▥, CBD; ▩, Δ^8-THC; ▧, Δ^9-THC; and ▨, CBN.

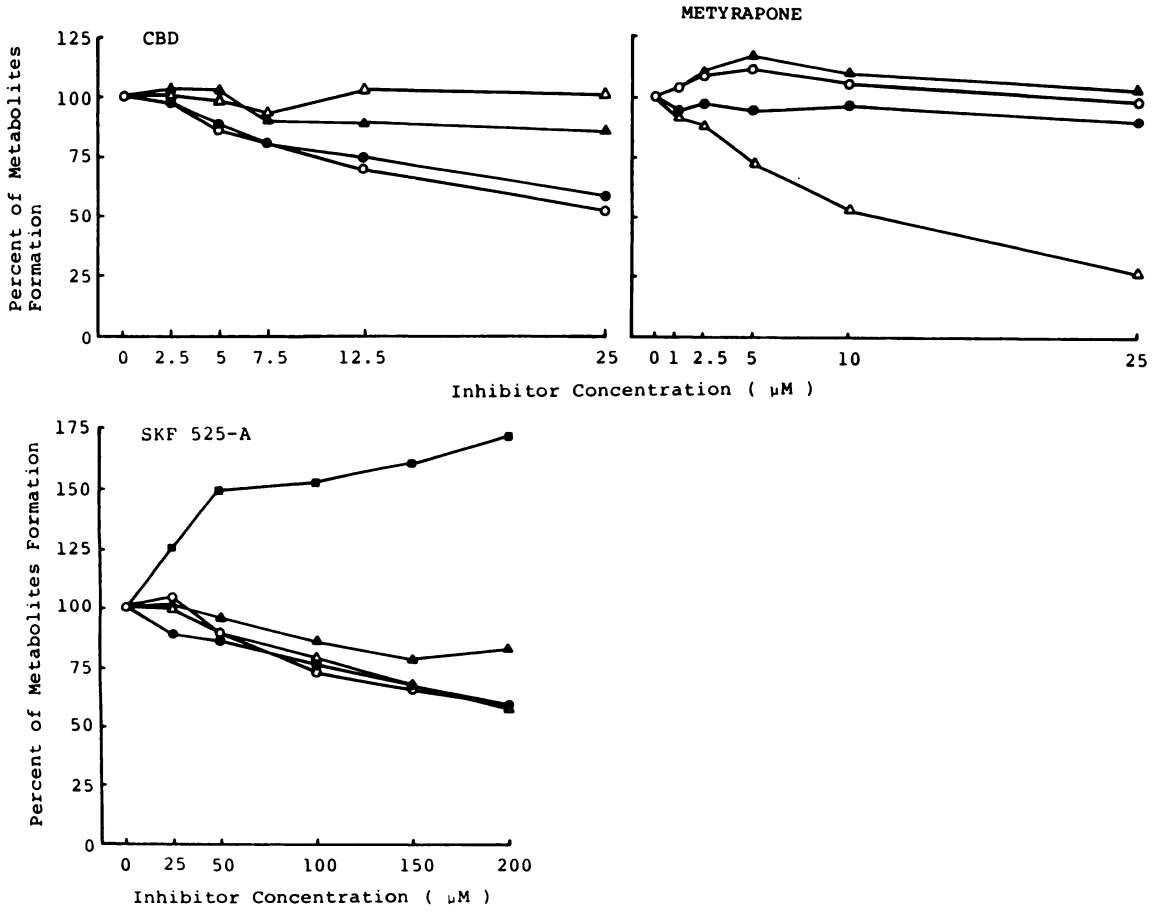

FIG. 4. *Effects of various concentrations of CBD, metyrapone, and SKF 525-A on microsomal testosterone oxidation.*

The inhibitors were added to the incubation medium just before the preincubation. In the case of SKF 525-A, the amount of 7α-OH-T formed was determined by HPLC under condition B (see *Materials and Methods*). Each *point* represents the mean value of two determinations. Δ, 6β-OH-T; ●, 16α-OH-T; O, 2α-OH-T; ▲, Δ^4-A; and ■, 7α-OH-T.

TABLE 1

Inhibition of testosterone oxidation by CBD, metyrapone, and SKF 525-A

Each value represents the mean ± SE of three determinations. Numbers in parentheses mean the percentage of the control.

Inhibitors	Concentration	6β-OH-T	16α-OH-T	2α-OH-T	Δ^4-A	7α-OH-T
	μM			*nmol/mg/min*		
Control	0	1.89 ± 0.15 (100)	0.99 ± 0.09 (100)	0.59 ± 0.04 (100)	0.49 ± 0.05 (100)	0.25 ± 0.03 (100)
CBD	25	1.82 ± 0.04 (96)	0.50 ± 0.03[a] (50)	0.32 ± 0.02[a] (54)	0.53 ± 0.06 (108)	ND[b]
Metyrapone	25	0.37 ± 0.03[a] (20)	0.91 ± 0.05 (91)	0.58 ± 0.05 (99)	0.51 ± 0.05 (104)	ND
SKF 525-A	200	1.00 ± 0.05[a] (53)	0.52 ± 0.02[a] (53)	0.34 ± 0.02[a] (58)	0.41 ± 0.04[c] (84)	0.43 ± 0.03[a] (173)

[a] Significantly different from the control value ($p < 0.01$).

[b] ND, not determined.

[c] Significantly different from the control value ($p < 0.05$).

W (60–80 mesh, 1.5 mm i.d. × 2 m) for GC were used. The conditions were as follows: column temperature, 250°C; injector temperature, 270°C; carrier gas, He, 40 ml/min; ionization current, 0.3 mA; and ionization energy, 70 eV.

HPLC. A Hitachi 655-60 type liquid chromatograph equipped with a 638-type variable wavelength UV monitor, a 655-60-type data processor, and a DuPont Zorbax ODS column (4.6 mm i.d. × 15 cm) were used. The conditions for analysis of testosterone metabolites were as follows: (condition A) mobile phase, methanol/acetonitrile/water (55:10:35, v/v); flow rate, 1 ml/min; detection wavelength, 254 nm. Retention times at ambient temperature were 4.18 min for 7α-OH-T, 4.61 min for 6β-OH-T, 5.11 min for 16α-OH-T, 6.69 min for 2α-OH-T, 6.82 min for 2β-OH-T, 12.24 min for Δ^4-A, 14.06 min for testosterone, and 18.36 min for epitestosterone. 7α-OH-T formation was analyzed using a mobile phase of tetrahydrofuran/water (3:7, v/v) (condition B). It showed a R_T of 3.89 min.

Standard curves for 2α-OH-T, 6β-OH-T, 7α-OH-T, and 16α-OH-T were made by analysis of incubation medium containing known amounts of standard hydroxytestosterones. Recoveries of the five kinds of testosterone metabolites varied in the range from 90.3 to 92.1%.

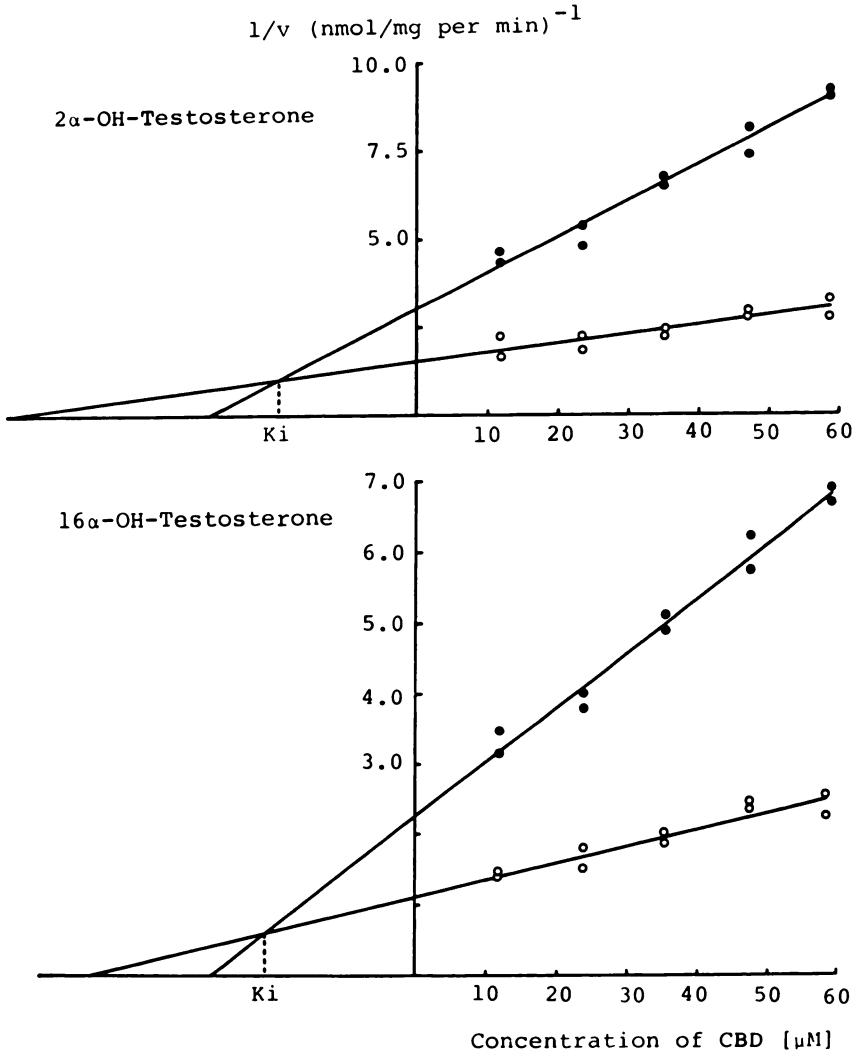

FIG. 5. *Dixon plots showing inhibition by CBD of microsomal testosterone hydroxylation.*

CBD (11.8–58.9 μM) was added, just before preincubation, to the reaction medium containing 64.3 (●) or 257.2 (○) μM of testosterone. Duplicate incubations were performed at each concentration of CBD. Regression lines were calculated by the linear least square methods using the mean values of the duplicate data.

TABLE 2

Kinetic parameters for inhibitory effects of CBD, metyrapone, and SKF 525-A on the microsomal testosterone oxidation

The kinetic parameters were the mean values of two experiments.

	K_m	V_{max}	CBD		Metyrapone		SKF 525-A	
			Type of Inhibition	K_i	Type of Inhibition	K_i	Type of Inhibition	K_i
	μM	nmol/mg/min		μM		μM		μM
6β-Hydroxylation	115.6	4.36	None		Competitive	5.1	Noncompetitive	160.8
16α-Hydroxylation	165.5	1.50	Competitive	19.9[a]	None		Competitive	97.9
2α-Hydroxylation	155.7	1.08	Competitive	21.6[a]	None		Competitive	71.2

[a] The values were calculated on the basis of the data obtained by Dixon plots. Other values were obtained by Lineweaver-Burk plots.

SDS-PAGE. SDS-PAGE was carried out using a discontinuous buffer system according to the method of Laemmli (33). The 2-mm thick slab gel (8.0%, w/v, acrylamide) was run at 10 mA/gel for 12 hr at room temperature. The gel was stained with Coomassie blue R-250 (0.25%, w/v, in methanol/acetic acid/water, 1:1:8, v/v) and destained in methanol/acetic acid/water, 1:1:8 (v/v).

Assay Methods. Protein concentration was determined by the method of Lowry *et al.* (34) using bovine serum albumin as a standard. Contents of cytochrome P-450 and cytochrome b_5 were measured by the methods of Omura and Sato (35), using extinction coefficients of 91 mM^{-1} cm^{-1} and 185 mM^{-1} cm^{-1}, respectively. NADPH-cytochrome *c* reductase activity was assayed as previously described (36). Statistical significance was calculated by Student's *t* test. Regression lines were calculated by the linear least square methods.

Results

Conditions for Microsomal Testosterone Oxidation. A typical liquid chromatogram of microsomal metabolites of testosterone

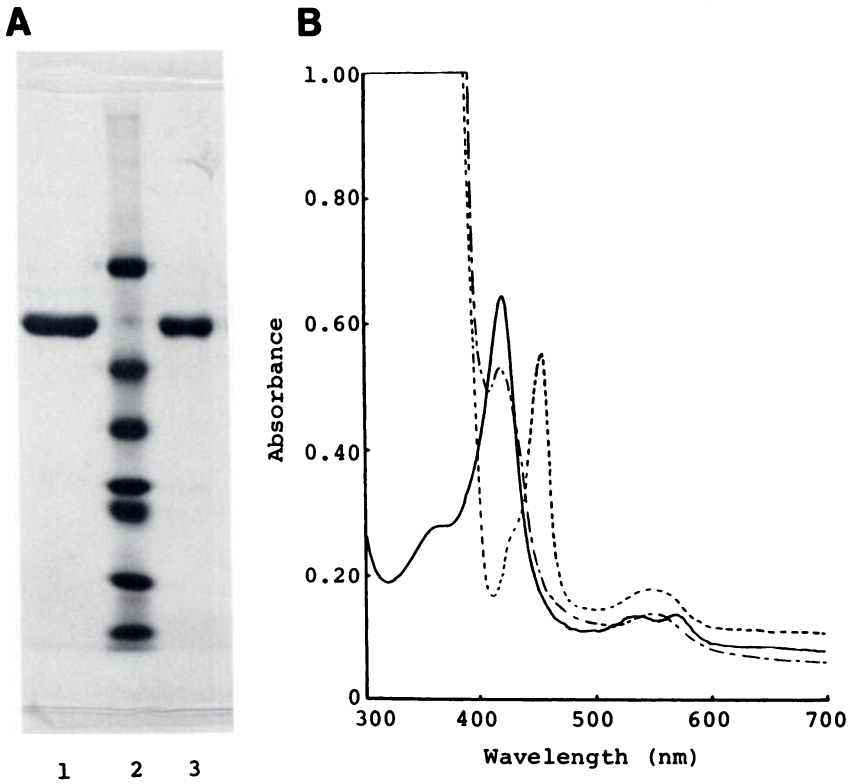

FIG. 6. *SDS-polyacrylamide gel of purified cytochrome P-450PB (A) and absolute spectra of cytochrome P-450PB (B).*

The cathode is at the *top*. Electrophoresis was toward the anode. See *Materials and Methods* for conditions. *Lanes 1* and *3* contained 20 and 10 μg, respectively, of purified cytochrome P-450PB. *A*, Standard proteins (*lane 2*) used were 5 μg each of bovine serum albumin (molecular weight, 66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,200). *B*, Spectra were measured with a sample containing 5.9 μM cytochrome P-450PB in 50 mM phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. ——, Oxidized; – · –, reduced; · · · ·, CO-reduced.

under condition A is shown in fig. 1. Analysis by HLPC and GC-MS of six fractions of testosterone metabolites obtained by preparative TLC proved that peaks a, b, c, d, e, and f corresponded to 7α-OH-T, 6β-OH-T, 16α-OH-T, 2α-OH-T, 2β-OH-T, and Δ^4-A, respectively. 6β-OH-T, 16α-OH-T, 2α-OH-T, and Δ^4-A were thus analyzed by HPLC using condition A. 7α-OH-T was determined under condition B. The effect of microsomal protein and substrate concentration on testosterone oxidation were then examined. Formations of 6β-OH-T, 16α-OH-T, 2α-OH-T, and Δ^4-A linearly increased up to 0.3 g liver equivalent (4.8 mg of protein) and to 200 μg (257 μM) of the substrate (data not shown).

Time course of the testosterone oxidation was then studied at 37°C for the incubation temperature using the protein concentration of 3.2 mg (0.2 g liver equivalent) and the substrate concentration of 200 μM. A linearity in the formation of the four testosterone metabolites was seen up to 6 min of the incubation time (fig. 2). Preincubation at 37°C for 5 min was necessary to obtain the linearity in metabolite formation. Therefore, the following conditions were used in the further studies: enzyme source, microsomes (0.2 g liver equivalent, approximately 3 mg of protein); substrate, testosterone (150 μg, 193 μM); preincubation and incubation, 37°C for 5 min.

Effects of Cannabinoids and Inhibitors on Microsomal Testosterone Oxidation. Fig. 3 illustrates the effect of the preincubation of the major cannabinoids with the reaction medium on the microsomal testosterone oxidation. Among the four major cannabinoids (Δ^9-THC, Δ^8-THC, CBD, and CBN, each 30 μM) examined, only CBD significantly inhibited 2α-OH-T and 16α-OH-T formation. But the effects of CBD on 6β-OH-T, Δ^4-A, and 7α-OH-T formation were not significant.

As shown in fig. 4, CBD showed dose-dependent inhibition for 2α-OH-T and 16α-OH-T formation, whereas 6β-OH-T- and Δ^4-A-forming activities were not affected, up to 25 μM CBD concentrations. Various amounts of SKF 525-A and metyrapone were added to the reaction medium, and their inhibitory effects on testosterone oxidation were compared with that of CBD. Fig. 4 shows that SKF 525-A suppressed the formation of 6β-OH-T, 16α-OH-T, 2α-OH-T, and Δ^4-A. However, 7α-OH-T formation was elevated with increasing concentrations of SKF 525-A added. On the other hand, the addition of metyrapone caused an inhibition only of 6β-OH-T formation, among the four metabolites measured.

These inhibitory profiles were demonstrated to be statistically significant in table 1, which lists the effects of these inhibitors, at their maximal concentrations examined, on the testosterone oxidation. As compared with the control, the inhibitory effects of CBD for 16α-OH-T and 2α-OH-T formations were significant, as was the effect of metyrapone on 6β-OH-T formation. SKF 525-A (200 μM) significantly increased 7α-OH-T-forming activity but decreased all other hydroxylation activities.

Kinetic Analysis. Effects of the inhibitors at fixed concentration (25, 12.5, and 200 μM, respectively, for CBD, metyrapone, and SKF 525-A) were kinetically analyzed by Lineweaver-Burk plots. For 2α- and 16α-hydroxylation of testosterone, the inhibition by CBD was competitive. Furthermore, the inhibitory

nature of CBD was examined by changing the concentration of CBD in the range of 11.8 to 58.9 μM, analyzed in Dixon plots (fig. 5). The concentrations of the substrate used were 64 and 257 μM of testosterone. The Dixon plots also show the inhibition of testosterone 2α- and 16α-hydroxylations by CBD was a competitive type. Kinetic parameters calculated for metyrapone and SKF 525-A, together with those for CBD, are summarized in table 2. Metyrapone was found to competitively inhibit 6β-hydroxylation with a K_i value of 5.1 μM, which is one fourth of those for CBD. Although SKF 525-A suppressed testosterone oxidation similarly at the 6β-, 16α-, and 2α-positions as shown in fig. 4, the kinetic analysis showed that the inhibition was competitive for 16α-OH-T and 2α-OH-T formations and noncompetitive for 6β-OH-T formation.

Properties of Cytochrome P-450PB. Cytochrome P-450PB that was purified from the livers of phenobarbital-treated adult male rats exhibited the minimum molecular weight of 53,000 on SDS-PAGE (fig. 6*A*). Its absolute spectra in oxidized, reduced, and CO-reduced states are shown in fig. 6*B*. The cytochrome in the oxidized state was a low-spin protein, which had Soret, β, and α peaks at 419, 538, and 570 nm, respectively. Reduction of the cytochrome with dithionite caused a slight blue shift of the Soret peak to 415 nm and changed the two peaks of α and β to a single peak. A Soret peak of CO-reduced cytochrome P-450PB was seen at 451 nm.

Reconstituted System Studies. The catalytic activity of the reconstituted system with cytochrome P-450PB was measured using aniline, *d*-benzphetamine, and testosterone as substrate. Turnover numbers for aniline hydroxylation and benzphetamine *N*-demethylation were 0.50 and 16.52 nmol/min/nmol of cytochrome P-450PB, respectively. Fig. 7 shows a typical result of HPLC of testosterone metabolites formed by the reconstituted system. Cytochrome P-450PB converted testosterone to 2α-OH-T, 16α-OH-T, and Δ^4-A. When added to the reconstituted system at final concentrations of 12.5 and 25 μM, CBD repressed the formation of the three kinds of metabolites in a dose-dependent manner (table 3).

***In Vivo* Effects of CBD on Hepatic Microsomal Testosterone Oxidation.** Fig. 8 summarizes the effects of three daily injections of CBD to the rat on the hepatic microsomal enzymes and testosterone oxidation. Pretreatment with CBD significantly decreased cytochrome P-450 content by 23% as compared with the control but was without significant effect on NADPH-cytochrome *c* reductase activity and cytochrome b_5 content. As was its *in vitro* effects on the testosterone oxidation, CBD caused a significant decrease in 16α-OH-T and 2α-OH-T formation and did not affect 6β-hydroxylation. Contrary to the *in vitro* effects (figs. 4 and 5), however, Δ^4-A-forming activity also was significantly suppressed, whereas 7α-OH-T formation was significantly increased by pretreatment with CBD.

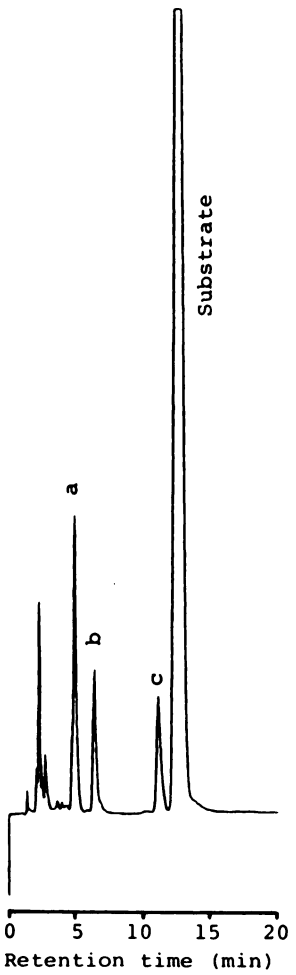

FIG. 7. *HPLC profile of testosterone metabolites formed by the reconstituted system containing cytochrome P-450PB.*

The same column and mobile phase as described in fig. 1 were used for the analysis of the extract of a 20-min incubation containing 0.17 nmol of cytochrome P-450PB and 25 μg of testosterone. *a*, 16α-OH-T; *b*, 2α-OH-T; *c*, Δ^4-A.

Discussion

It has been established that sex hormones (androgen and estrogen) are biosynthesized in the sexual organs, such as testes and ovary, and are metabolized mainly in the liver and kidney. The gonadal steroids are also known to be oxidized by the hepatic mixed function oxidase system, followed by formation of conjugates such as glucuronides and sulfates. Recently, much attention has been directed to testosterone hydroxylation, demonstrating that testosterone is hydroxylated by several isozymes of hepatic cytochrome P-450 in a regio- and stereoselective manner (17, 18, 23). On the other hand, CBD is known to inhibit hepatic drug metabolism, probably through its interaction with cytochrome P-450 (12, 37). It is thus deduced that CBD may suppress hepatic testosterone metabolism; however, limited knowledge is available on this matter. Chan and Tse (38) have observed that Δ^9- and Δ^8-THC competitively inhibited the hepatic microsomal testosterone hydroxylation in the mature male rat. List *et al.* (16) have reported that the acute and chronic treatment of adult male and female rats with Δ^9-THC or CBD increased hepatic microsomal testosterone hydroxylation, but the chronic treatment with the cannabinoids caused a decrease in the level of hepatic cytochrome P-450.

The present study has shown that, among the major cannabinoids examined, only CBD evoked a competitive inhibition for 2α- and 16α-hydroxylation of testosterone and did not show any effect on 6β- and 7α-hydroxylated metabolite formation with hepatic microsomes. SKF 525-A also exerted a competitive inhibition for testosterone hydroxylation at the 2α- and 16α-positions. However, the inhibitory nature of CBD is distinguishable from that of SKF 525-A, inasmuch as the latter exhibited a noncompetitive inhibition for testosterone hydroxylation at the 6β-position. Furthermore, on the basis of K_i values calculated, the potency of CBD to inhibit 2α-OH-T and 16α-OH-T forma-

TABLE 3

Testosterone oxidation catalyzed by purified cytochrome P-450PB

Numbers in parentheses represent the percentage of control.

	Substrate[a]	CBD added	Turnover Number		
			16α-OH-T	2α-OH-T	Δ⁴-A
	μg	μM	nmol/min/nmol of cytochrome P-450		
Control	25	0	2.07 (100)	1.02 (100)	1.08 (100)
Treated	25	12.5	1.53 (74)	0.73 (72)	0.86 (80)
Treated	25	25.0	1.10 (53)	0.56 (55)	0.66 (61)

[a] Testosterone (87 μM).

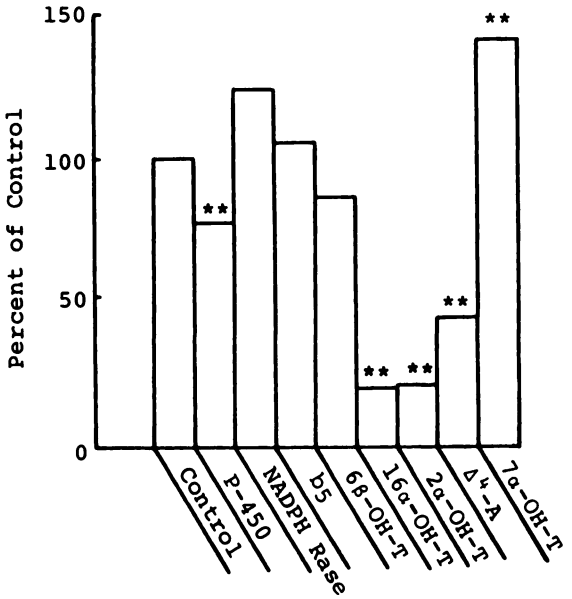

FIG. 8. *In vivo effects of CBD on microsomal drug-metabolizing enzymes and testosterone oxidation.*

Adult male rats were pretreated with CBD (10 mg/kg/day for 3 days, ip) and the hepatic microsomal enzyme content and activities were measured on day 4. *P-450, NADPH Rase,* and b_5 on the figure represent cytochrome P-450, NADPH-cytochrome *c* reductase, and cytochrome b_5, respectively. Each *column* represents the mean value of four determinations. The control value for each index was as follows: cytochrome P-450, 1.162 ± 0.038 nmol/mg; NADPH-cytochrome *c* reductase, 0.029 ± 0.01 units; cytochrome b_5, 0.348 ± 0.024 nmol/mg; 6β-OH-T, 2.06 ± 0.14 nmol/mg/min; 16α-OH-T, 1.15 ± 0.16 nmol/mg/min; 2α-OH-T, 0.59 ± 0.06 nmol/mg/min; Δ⁴-A, 0.53 ± 0.07 nmol/mg/min; 7α-OH-T, 0.30 ± 0.04 nmol/mg/min. ** Significantly different from the control value ($p < 0.01$).

tion is 3 to 4 times that of SKF 525-A. Shiverick and Neims (39) reported that SKF 525-A stimulated testosterone 7α-hydroxylation by the reconstituted system consisting of partially purified cytochrome P-450. A similar stimulation by SKF 525-A in microsomal testosterone 7α-hydroxylation was observed in the present study. This may be due to the amphipathic nature of SKF 525-A, as suggested by Shiverick and Neims (39). On the other hand, metyrapone competitively inhibited only 6β-hydroxylation and did not affect 2α- and 16α-hydroxylation of testosterone with microsomes. Hence, the inhibitory nature of CBD is distinct from that of metyrapone as well.

The reconstituted system consisting of cytochrome P-450PB catalyzed oxidation of testosterone at the 2α-, 16α-, and 17-positions, forming 2α-OH-T, 16α-OH-T, and Δ⁴-A, respectively. Waxman *et al.* (18) reported that an isozyme, termed P-450 PB-2c, which was purified from phenobarbital-treated rat livers, oxidized testosterone at the 2α-, 16α-, and 17-positions. Later, Waxman (40) demonstrated that the isozyme (P-450 PB-2c) corresponded to a male-specific cytochrome P-450, termed P-450 2c. With respect to the male-specific isozyme of hepatic cytochrome P-450 in the rat, cytochrome P-450 LM5 (17), P-450 h (40), P-450 male (19), P-450 2c (PB-2c) (40), P-450 UT-A (22), P-450$_{25}$ (41), P-450cc$_{25}$ (42), and P-450 M-1 (21) have been reported so far, and they may be the same species of cytochrome P-450, on the basis of their catalytic natures and *N*-terminal amino acid sequences. Inasmuch as a similarity exists in the catalytic profiles (testosterone hydroxylation at the 2α-, 16α-, and 17-positions) and molecular weights, the cytochrome P-450PB purified in the present study may correspond to cytochrome P-450 PB-2c reported by Waxman *et al.* (18), namely, to the male-specific cytochrome P-450 reported by the various working groups described above.

This speculation was supported by the results of the *in vivo* treatment of the rat with CBD. That is, the treatment of the rat with three daily ip administrations of CBD (10 mg/kg, once a day) caused a marked decrease in the formation of 2α-OH-T, 16α-OH-T, and Δ⁴-A with the liver microsomes. In the present study, 23% of the total microsomal cytochrome P-450 content was decreased by the pretreatment of rats with CBD. Matsumoto *et al.* (21) suggested, using the antibody preparation, that 30% of the total microsomal cytochrome P-450 might be the male-specific isozyme. If CBD selectively reduces the male-specific cytochrome P-450 content, the percentage (23%) of the decrease in the total P-450 content means that about 80% of the male-specific isozyme was decreased by the CBD pretreatment, which corresponds well to the loss of 80% in 2α-OH-T- and 16α-OH-T-forming activities in the hepatic microsomes of the CBD-treated rat. However, it is not clear at present whether the *in vivo* mechanism of a decrease in the detectable amount of hepatic cytochrome P-450 by CBD is the same as that under the *in vitro* conditions examined in the present study. It is known that various inducers cause a decrease in the male-specific cytochrome P-450, which results from a competition between constitutive and inducible apoproteins for available heme (40, 43). Recently, Horie *et al.* (44) reported that administration of aztreonam, a monobactam antibiotic, suppressed a male-specific cytochrome P-450 in the liver microsomes of male rats. On the other hand, 7α-OH-T formation was significantly increased by CBD pretreatment (143% of the control value) in the present study. This implies that CBD may suppress the male-specific cytochrome P-450-catalyzed hydroxylation on the one hand and induce other isozyme(s) such as 7α-hydroxylase on the other. Moreover, CBD did not affect the Δ⁴-A formation from testosterone with the microsomes from untreated rats, whereas the *in vivo* pretreatment of CBD significantly decreased the testosterone

oxidation at the 17-position. In the reconstituted system with cytochrome P-450PB, Δ^4-A-forming activity in the presence of CBD (25 μM) decreased by 39%, as compared with the control value. These results indicate that not only male specific P-450 but also other isozymes may contribute to the oxidation at the 17-position, as reported by Wood *et al.* (23) and Sonderfan *et al.* (45).

Together with these, the present findings that CBD suppressed hepatic testosterone oxidation at the 2α-, 16α-, or 17- (probably, 17α-) positions in the rat, both *in vivo* and *in vitro*, suggest that CBD may disturb the sex hormonal homeostasis of the mammals to some extent through selective inhibition of the male-specific cytochrome P-450 in the liver.

Acknowledgments. We thank Professor I. Nishioka and Associate Professor Y. Shoyama, Faculty of Pharmaceutical Sciences, Kyushu University, for supplying cannabis leaves.

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