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Difference spectra of rat hepatic microsomes induced by cannabinoids and related compounds

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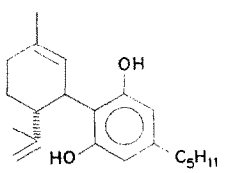
STUDIES OF Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-THC and cannabinol (CBN) show that metabolism of these compounds *in vivo* and *in vitro* using liver microsomal preparations is largely via hydroxylations at allylic and benzylic positions and in the side chain.[1,2] Cytochrome P-450, the terminal oxidase of the microsomal mono-oxygenase system, was suggested to be involved in these hydroxylations.[3,4] The binding of various substrates of the system to cytochrome P-450 is associated with characteristic absorbance changes in the difference spectra of the microsomes.[5] The interactions of cannabidiol (CBD), CBN, Δ^8- and Δ^9-THC and of Δ^8-THC homologues carrying a methyl or propyl group in place of the amyl group with microsomal cytochrome P-450 were, therefore, investigated by measuring the microsomal difference spectra. For convenience, the last two substrates will be referred to as methyl-Δ^8-THC and propyl-Δ^8-THC respectively[6] (Fig. 1). The difference spectra of several resorcinol derivatives, which are structurally similar to but simpler than cannabinoids, and of hexobarbital were measured also. While our work was in progress, White and Hine[7] reported that THC gave neither a type I nor a type II spectrum with cytochrome P-450, but Cohen *et al.*[8] found a type I interaction. Very recently, Kupfer *et al.*[9] published K_s values for CBN, Δ^8- and Δ^9-THC which differ from those reported here. Reports of metabolic interaction *in vivo* between Δ^9-THC and CBD,[10] of prolongation of barbiturate sleeping times by cannabinoids[10–12] and of the low inhibition of hydroxylation of THC by hexobarbital[3,4] prompted us to investigate also the inhibition of the binding of hexobarbital to P-450 by cannabinoids and of Δ^9-THC binding by hexobarbital and cannabinoids.



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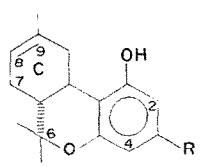

Δ^8-THC; R = C_5H_{11}
methyl-Δ^8-THC; R = CH_3
propyl-Δ^8-THC; R = C_3H_7
CBN; ring C aromatic, R = C_5H_{11}

FIG. 1. Structures of some cannabinoids and homologues.

Orcinol, resorcinol, 3-methoxyphenol and 1,3-dimethoxybenzene were commercially available samples. Olivetol, CBN, CBD and Δ^9-THC (93%) were authentic samples from this branch. Δ^8-THC and its homologues were prepared by the method of Petrzilka *et al.*,[13] and Δ^8-THC methyl ether by the method of Wildes *et al.*[14] 2,6-Dimethoxyallylbenzene and 2,6-dimethoxypropenylbenzene were prepared by Claisen rearrangement of 3-methoxyallyloxybenzene,[15] followed by methylation and double bond isomerization.[16] 3,5-Dimethoxypropylbenzene and 3,5-dimethoxyamylbenzene were prepared by treatment of 3,4,5-trimethoxybenzaldehyde with ethyl magnesium bromide and butyl magnesium bromide, respectively, followed by reduction of the resulting trimethoxyphenyl alkyl carbinol with sodium in ethanol.[17] All other reagents used were analytical reagent grade. Male albino Wistar rats of 170–220 g were starved overnight and decapitated. The livers were perfused *in situ* with 0·9% NaCl, and were then quickly placed in ice-cold Sorensen's buffer (0·1 N, pH 7·4), dried between gauze sponges and weighed. The tissue was homogenized in the buffer at 0°, using a Potter–

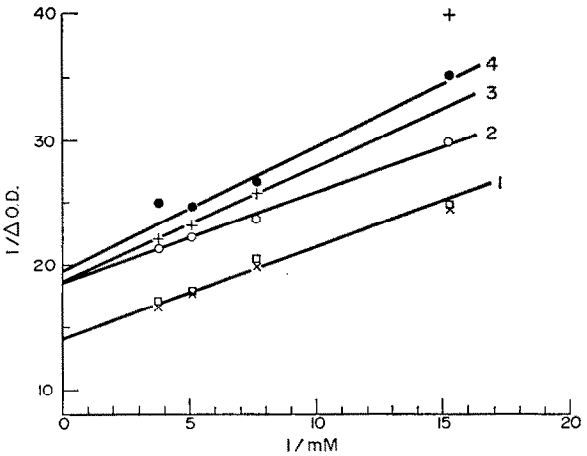

FIG. 2. Double reciprocal plots of Δ O.D. vs. Δ^9-THC concentration. Curve 1: experiments 1, □, and 2, ×, are repetitions of the results for Δ^9-THC with no microsomal modifier added; curve 2: ○, Δ^9-THC, microsomes containing hexobarbital (0·05 mM concentration in sample and reference cuvettes) as modifier; curve 3: +, Δ^9-THC, microsomes modified with CBN (0·054 mM); and curve 4: ●, Δ^9-THC, microsomes modified with CBD (0·013 mM). P-450: 0·88 nmole/mg of protein; protein: 2·98 mg/ml throughout.

Elvehjem homogenizer fitted with a Teflon pestle, and the homogenate was centrifuged at 12,000 *g* for 20 min. The supernatant was centrifuged at 105,000 *g* for 60 min. The pellet was rinsed, resuspended in buffer, and centrifuged again at 105,000 *g*. The pellet was again rinsed, resuspended in buffer, and adjusted to a concentration equivalent to 1 g of fresh liver tissue/ml. Protein concentration was determined by the method of Lowry *et al.*,[18] and the P-450 content by the method of Omura and Sato.[19]

The difference spectra were measured at 25° (except when indicated) with a Cary model 15 double beam spectrophotometer fitted with a high-intensity light source using cuvettes of 1 cm path length and microsomes diluted to approximately 2 mg of protein/ml, 3 ml/cuvette. The baseline was recorded from 350–500 nm, and then increments of substrate solution (in ethanol) to a total of 30 μl were added to the sample cuvette and equal volumes of solvent to the reference cuvette. The maximum substrate concentrations were about 0·25 mM. The spectrum was scanned repeatedly after each addition until the maximally developed spectrum was obtained. The lines drawn for the double reciprocal plots shown in Figs. 2 and 3 were not obtained by calculation, but the values of K_s for substrates in the absence of modifiers, presented in Table 1, were derived by the method of Orrenius *et al.*[20] using linear regression analysis of Lineweaver–Burk plots with a Hewlett–Packard 9810A calculator.

Difference spectra were not detectable with resorcinol or its mono- and dimethyl ethers, or with orcinol. Typical type I difference spectra[5] were seen for all of the compounds (Table 1), all having maximum absorption at 385 nm and minimum at 420 nm. The maxima of the simpler resorcinol derivatives were broader than those of the cannabinoids. The positions of the maxima and minima were not observed to change with changing substrate concentration. The K_s values for CBN, Δ^9-THC and Δ^8-THC (Table 1) are comparable to those published for hexobarbital (50 μM[20] and 80 μm[21]). The K_s found for ethanolic solutions of hexobarbital (measured in the concentration range 0·05 to 0·25 mM) was 59 (± 10) μM. Concentrations of the substrates lower than 0·05 mM gave Δ O.D. values (the difference in optical density between maximum and minimum) considered too small for accurate measurement. Working in the concentration range *ca.* 0·005 to 0·05 mM, others have found K_s values for these cannabinoids in the range 12–20 μM.[8,9] Di Augustine and Fouts[22] pointed out that water-insoluble, strongly protein-bound substrates may give K_s values greater than the true values.

We noticed that ethanolic solutions of Δ^9-THC (*ca.* 30 mM) produced an initial slight turbidity when aliquots (5 μl) were added to the microsomal preparation (3 ml) and that the difference spectrum required about 15 min for maximum development. These phenomena were not apparent with any of the other compounds, all of which were examined under the same conditions.

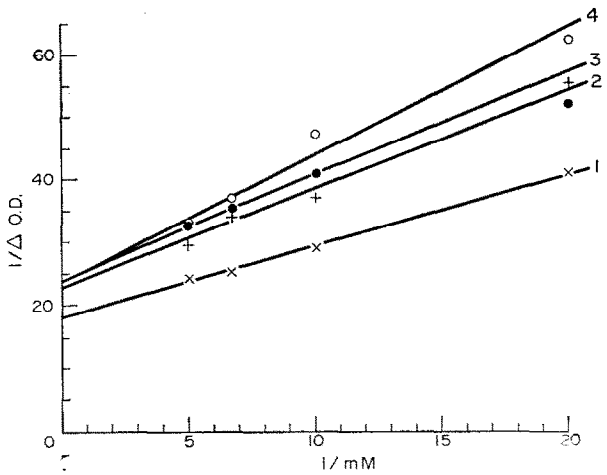

FIG. 3. Double reciprocal plots of Δ O.D. vs. hexobarbital concentration. Curve 1: ×, hexobarbital with no microsomal modifier added; curve 2: +, hexobarbital, microsomes modified with CBN (0·054 mM); curve 3: ●, hexobarbital, microsomes modified with CBD (0·013 mM); and curve 4: ○, hexobarbital, microsomes modified with Δ^9-THC (0·065 mM). P-450: 0·88 nmol/mg of protein; protein: 2·98 mg/ml throughout.

Comparatively large Δ O.D. values were obtained for CBD in the same concentration range, and the low K_s obtained (*ca.* 13 μM) is indicative of very strong binding of CBD to the microsomal system. This is interesting, since Paton and Pertwee[12] showed CBD to be a much stronger inhibitor of hepatic drug metabolism than Δ^9-THC.

TABLE 1. K_s VALUES FOR CANNABINOIDS AND RESORCINOL DERIVATIVES*

Compound	K_s†	K_s‡	K_s§
CBD	13 (±6)		
CBN	47 (±15)		
Δ^9-THC	50 (±11)		
Δ^8-THC	52 (±10)	60	79
Propyl-Δ^8-THC		42	
Methyl-Δ^8-THC		42	
Δ^8-THC methyl ether			88
3,5-Dimethoxpropylbenzene		66	92
3,5-Dimethoxyamylbenzene		63	65
3,5-Dihydroxyamylbenzene		48	79
2,6-Dimethoxyallylbenzene		101	140
2,6-Dimethoxypropenylbenzene		408	243

* K_s in μM, obtained for substrates in the concentration range *ca.* 0·05 to 0·25 mM; divarinol (3,5-dihydroxypropylbenzene) at these concentrations gave values of Δ O.D. too small for accurate measurement.

† Mean (± standard deviation) of at least four experiments using at least three different microsomal preparations in which the P-450 level varied between 0·79 and 0·88 nmole/mg of protein and the protein level varied between 1·82 and 2·98 mg/ml. Spectra measured at 25°.

‡ One determination, P-450 level: 0·79 nmol/mg of protein; protein level: 1·82 mg/ml. Spectra measured at 25°.

§ One determination, P-450 level: 1·09 nmole/mg of protein; protein level: 2·20 mg/ml. Spectra measured at ambient temperature.

CBD apparently inhibits the metabolism of Δ^9-THC *in vivo*,[10] CBN blocks some of its actions,[23] and there is some inhibition of hydroxylation of THC by hexobarbital.[3,4] It was, therefore, of interest to examine the difference spectrum of Δ^9-THC in the presence of these compounds. The results presented in Fig. 2 suggest that, in the presence of CBD, CBN or hexobarbital, there are complex interactions which inhibit the binding of Δ^9-THC to the microsomal system. Both K_s and maximum Δ O.D. change, and simple competitive inhibition, which might have been expected between the cannabinoids at least, are not observed.

Prolongation of barbiturate sleeping time* and the inhibition of liver microsomal activity are properties shared by many cannabinoids and CBD is particularly potent in these respects.[12] Experimental data presented in Fig. 3 indicate that hexobarbital binding is inhibited by cannabinoids, and especially by low concentrations of CBD (concentrations of the order used for THC and CBN effectively destroyed the binding spectrum). As before, simple competition between the substrates for a binding site is not observed, and the nature of the interactions remains to be clarified.

Paton and Pertwee[12] surmised that the two phenolic groups of CBD confer a specific affinity for the hydroxylating system of the microsomes. The limited data presented in Table 1 suggest that the location of the terminal double bond in CBD may exert a considerable effect (thus, 2,6-dimethoxyallylbenzene is more strongly bound than the propenyl analogue) and that etherification of hydroxyl groups is less important.

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REFERENCES

1. M. E. WALL, *Ann. N.Y. Acad. Sci.* **191,** 23 (1971).
2. D. E. MAYNARD, O. GURNY, R. G. PITCHER and R. W. KIERSTEAD, *Experientia* **27,** 1154 (1971).
3. S. H. BURSTEIN and D. KUPFER, *Chem. Biol Interac.* **3,** 316 (1971).
4. S. H. BURSTEIN and D. KUPFER, *Ann. N.Y. Acad. Sci.* **191,** 61 (1971).
5. J. B. SCHENKMAN, *Biochemistry N.Y.* **9,** 2081 (1970).
6. R. A. DE ZEEUW, J. WIJSBEEK, D. D. BREIMER, T. B. VREE, C. A. M. VAN GINNEKEN and J. M. VAN ROSSUM, *Science, N.Y.* **175,** 778 (1972).
7. E. R. WHITE and C. H. HINE, *Abst. Fifth Int. Congress on Pharmacology*, San Francisco, California (July 23–28, 1972).
8. G. M. COHEN, D. W. PETERSON and G. J. MANNERING, *Life Sci.* **10,** 1207 (1971).
9. D. KUPFER, I. JANSSON and S. ORRENIUS, *Chem. Biol Interact.* **5,** 201 (1972).
10. G. JONES and R. G. PERTWEE, *Br. J. Pharmac. Chemother.* **45,** 375 (1972).
11. J. C. GARRIOT, L. J. KING, R. B. FORNEY and F. W. HUGHES, *Life Sci.* **6,** 2119 (1967).
12. W. D. M. PATON and R. G. PERTWEE, *Br. J. Pharmac. Chemother.* **44,** 250 (1972).
13. T. PETRZILKA, W. HAEFLIGER and C. SIKEMEIR, *Helv. chim. Acta* **52,** 1102 (1969).
14. J. W. WILDES, N. H. MARTIN, C. G. PITT and M. E. WALL, *J. org. Chem.* **36,** 721 (1971).
15. W. N. WHITE and C. D. SLATER, *J. org. Chem.* **26,** 3631 (1961).
16. A. T. SHULGIN, *Can. J. Chem.* **43,** 3437 (1965).
17. Y. ASAHINA, *Ber. dt. Chem. Ges.* **69,** 1643 (1936).
18. O. H. LOWRY, M. L. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
19. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
20. S. ORRENIUS, D. KUPFER and L. ERNSTER, *Fedn Eur. Biochem. Soc. Lett.* **6,** 249 (1970).
21. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3,** 113 (1967).
22. R. P. DI AUGUSTINE and J. R. FOUTS, *Biochem. J.* **115,** 547 (1969).
23. B. L. WELCH, A. S. WELCH, F. S. MENIHA and H. J. BERGER, *Res. Commun. Chem. Path. Pharmac.* **2,** 382 (1971).
24. L. LEMBERGER, N. R. TAMERKIN, J. AXELROD and I. J. KOPIN, *Science, N.Y.* **173,** 72 (1971).
25. W. L. DEWEY, J. S. KENNEDY and J. F. HOWES, *Fedn Proc.* **29,** 650 (1970).
26. E. B. TRUITT, *Ann. N.Y. Acad. Sci.* **191,** 80 (1971).

* It has been suggested that Δ^9-THC induces its own metabolic enzyme.[24] An increase in cytochrome P-450 levels induced by THC (but see Ref. 25) might explain the reported[26] shortening of barbiturate sleeping times when rats were pretreated for 3 days with low doses of THC.