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EFFECT OF δ-9-TETRAHYDROCANNABINOL AND THEOPHYLLINE ON HEPATIC MICROSOMAL DRUG METABOLIZING ENZYMES

S. K. GHOSH and M. K. PODDAR*

Department of Biochemistry, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India

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Abstract—Theophylline (Th) under *in vitro* conditions stimulated the activities of rat liver microsomal aniline hydroxylase, *N*-demethylase and *O*-demethylase, while δ-9-tetrahydrocannabinol (δ-9-THC) inhibited the activities of these hepatic microsomal drug metabolizing enzymes under similar conditions. δ-9-THC-induced inhibition of hepatic microsomal drug metabolizing enzymes was significantly reduced in the presence of Th. Analysis of Lineweaver–Burk plots showed that Th-induced stimulation of hepatic microsomal drug metabolizing enzymes occurs due to an increase in substrate affinity ($1/K_m$) and of V_{max}. δ-9-THC-induced inhibition of *N*-demethylase and *O*-demethylase is probably due to competition of the drug with the substrates for a common intermediate in the microsomal electron transport chain. Non-competitive and mixed-type inhibition caused by δ-9-THC on aniline hydroxylation appears to be associated with a non-specific action of δ-9-THC. Blocking of δ-9-THC-induced inhibition or reduction of Th-induced stimulation of hepatic drug metabolizing enzymes with Th or δ-9-THC was due to an increase or decrease in either V_{max}, substrate affinity ($1/K_m$) or both with respect to the corresponding K_m and V_{max} observed with δ-9-THC or Th alone.

δ-9-Tetrahydrocannabinol (δ-9-THC†), the active ingredient of marijuana [1], is metabolized through hydroxylation and O-demethylation [2] in liver microsomes. Theophylline (Th), an essential constituent of tea and coffee [3] is also metabolized through N-demethylation and hydroxylation in liver microsomes [4]. Th has been reported to stimulate rat liver microsomal demethylation and hyroxylation processes [5–7], while the activity of these enzymes has been found to be inhibited by δ-9-THC both under *in vivo* and *in vitro* conditions [2]. It is known that inhibitors of cytochrome P450 inhibit Th metabolism under *in vitro* conditions [4]. Cohen *et al.* [8] have also shown that δ-9-THC forms a complex with cytochrome P450 (an NADPH, oxygen-dependent mixed-function oxidase system) at the time of its (δ-9-THC) biotransformation. Thus, it may be assumed that Th and δ-9-THC interact at the level of their hepatic metabolism. In the present investigation an *in vitro* effect of δ-9-THC and Th either alone or in combination on the activities of hepatic drug metabolizing enzymes has been studied to gain insight into the influence of one drug on the pharmacological response of the other through modulation of the activities of the hepatic drug detoxicating enzymes.

MATERIALS AND METHODS

Adult male albino rats (125–150 g body wt) of Charles Foster strain maintained on a standard laboratory diet and water *ad lib.* were used in the present study.

NADP, isocitrate dehydrogenase (type IV), isocitric acid, bovine serum albumin and pure crystalline Th were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). δ-9-THC was purchased from the National Narcotic Laboratory (Geneva, Switzerland). Aniline, *N,N*-dimethylaniline and *p*-nitroanisol were purchasd from BDH (Poole, U.K.). All other reagents used in the present study were of analytical grade. Aniline and *N,N*-dimethylaniline were distilled each time before use.

Preparation of rat liver microsomes. Blood-free rat liver tissue was collected and the microsomes were prepared from 0.25 M sucrose liver homogenates (1:10 w/v) by differential centrifugation as described by Schenkman *et al.* [9].

In vitro *treatment of rat liver microsomes with Th and/or δ-9-THC.* δ-9-THC in 0.01 mL of water containing 0.001% Tween-80 was mixed with the liver microcomes at concentrations of 1.6×10^{-6} and 13.33×10^{-6} M/mg protein, or Th in 0.01 mL water was mixed with the microsomes at concentrations of 110.0×10^{-6} and 550.0×10^{-6} M/mg protein, and incubated at 37° for 30 min before addition to the enzyme assay medium. Th and δ-9-THC at their respective concentrations were also mixed together with rat liver microsomes and were incubated at 37° for 30 min before addition to the enzyme assay medium. Control liver microsomes were incubated with an equivalent amount of the vehicle for δ-9-THC or Th under similar conditions. In another set of experiments (second experiment) liver microsomal protein together with δ-9-THC and/or Th, or an equivalent amount of corresponding vehicle was

* Corresponding author.

† Abbreviations: δ-9-THC, δ-9-tetrahydrocannabinol; Th, theophylline.

added without preincubation to the enzyme assay medium. Only the microsomal protein used in these experiments was preincubated for 30 min at 37°. In a third group of experiments, liver microsomes preincubated with only one drug (Th or δ-9-THC) at 37° for 30 min were added to the enzyme assay medium together with the other drug.

In vitro *assay of hepatic microsomal drug metabolizing enzyme activities.* The activities of hepatic microsomal enzymes (aniline hydroxylase, *N*-demethylase and *O*-demethylase) were determined at 37° in a medium containing 50 mM Tris–HCl buffer, pH 7.5; 5 mM $MgCl_2$; 0.33 mM $NADP^+$; 8 mM isocitrate; 15 μg/mL of isocitrate dehydrogenase (Sigma type IV); about 1 mg of liver microsomal protein/mL of incubation mixture; and the substrates *N,N'*-dimethylaniline (2.5 mM) for *O*-demethylase and aniline (4 mM) for aniline hydroxylase in a final volume of 3 mL. In kinetic studies, substrate concentrations were varied from 0.5 to 5 mM in the case of dimethylaniline, 1 to 5 mM in the case of aniline and 1 to 6 mM in the case of *p*-nitroanisole. The kinetic parameters (K_m and V_{max}) were calculated from Lineweaver–Burk plots. In all cases, the reaction was carried out for 10 min at 37° under air in a Dubnoff shaker. Aniline hydroxylation was determined by measuring *p*-aminophenol formation [9]. N-Demethylation of *N,N'*-dimethylaniline was determined by measuring formaldehyde formation using Nash's reagent B [10], and O-demethylation of *p*-nitroanisole was determined by measuring *p*-nitrophenol formation [11].

Estimation of protein. Protein content of rat liver microsomes was estimated according to the method of Lowry *et al.* [12] using bovine serum albumin as standard.

Statistical analysis. Significance of the results was assessed using Tukey test for analysis of variance [13].

RESULTS

Table 1 demonstrates the *in vitro* effects of Th and δ-9-THC either alone or in combination on the activities of hepatic microsomal aniline hydroxylase, *N*-demethylase and *O*-demethylase. It is evident from Table 1 that Th significantly increased the activities of aniline hydroxylase, *N*-demethylase and *O*-demethylase under *in vitro* conditions. It is also evident from Table 1 that δ-9-THC significantly inhibited the activities of aniline hydroxylase, *N*-demethylase and *O*-demethylase under *in vitro* conditions. Furthermore, Table 1 shows that Th at a low concentration in the presence of δ-9-THC (1.6×10^{-6} M) under *in vitro* conditions did not significantly alter the activities of aniline hyroxylase and *N*-demethylase but caused a 20% stimulation of the *O*-demethylase activity of liver microsomes. On the other hand, Th at a higher concentration in combination with 1.6×10^{-6} M δ-9-THC, significantly stimulated the activities of aniline hydroxylase, *N*-demethylase and *O*-demethylase of rat liver microsomes. Table 1 also shows that there was an inhibition of the activities of rat liver microsomal aniline hydroxylase, *N*-demethylase and *O*-demethyl-

Table 1. *In vitro* effect of Th and/or δ-9-THC on the activities of liver microsomal drug metabolizing enzymes following preincubation of microsomal protein with drug(s)

Concentration of Th and/or δ-9-THC (μM)	Aniline hydroxylase (nmol *p*-aminophenol/ hr/mg protein)	*N*-Demethylase (nmol formaldehyde/ hr/mg protein)	*O*-Demethylase (nmol *p*-nitrophenol/ hr/mg protein)
Control	22.2 ± 0.50	42.9 ± 0.36	26.8 ± 0.24
Th (110.0)	26.8 ± 0.90*	60.7 ± 1.30*	34.8 ± 0.69*
Th (550.0)	31.3 ± 0.25*	74.2 ± 0.56*	41.5 ± 1.33*
δ-9-THC (1.6)	20.4 ± 0.60*	30.6 ± 1.47*	25.1 ± 0.63*
δ-9-THC (13.33)	16.1 ± 0.35*	23.1 ± 1.20*	20.2 ± 0.88*
Th (110.0) + δ-9-THC (1.6)	23.9 ± 0.86*	45.2 ± 0.60*	32.2 ± 0.88*
Th (110.0) + δ-9-THC (13.33)	20.9 ± 0.40*	33.9 ± 1.46*	22.3 ± 0.34*
Th (550.0) + δ-9-THC (1.6)	26.3 ± 0.54*	52.8 ± 1.68*	38.0 ± 0.40*
Th (550.0) + δ-9-THC (13.33)	20.1 ± 0.83*	47.0 ± 0.44*	27.9 ± 0.44*

Results are expressed as means ± SEM of five separate determinations. Th and/or δ-9-THC was mixed with liver microsomes, and the mixture was preincubated for 30 min at 37° prior to enzyme assay. In the control experiment, an equivalent amount of vehicle for δ-9-THC and/or Th was mixed instead of drug(s).

*Significantly different from control, $^*P < 0.01$ using Tukey test for analysis of variance.

ase in the presence of a low concentration of Th and a high concentration of δ-9-THC.The combination of a higher concentration of Th with the lower concentration of δ-9-THC produced a small but significant inhibition of aniline hydroxylase (9.4%) activity, and *N*-demethylase and *O*-demethylase activities were, on the other hand, stimulated 9.6 and 4.0%, respectively, under similar *in vitro* conditions.

Table 2 shows the *in vitro* effect of Th and/or δ-9-THC on the activities of liver microsomal drug metabolizing enzymes without preincubation with drug (Th and/or δ-9-THC). Drug was added together with preincubated microsomal protein to the enzyme incubation medium. It is evident from this Table that addition of Th and/or δ-9-THC without preincubation with microsomal protein produced no appreciable change in the activities of hepatic microsomal aniline hydroxylase, *N*-demethylase and *O*-demethylase.

The *in vitro* effect of preincubation of rat liver microsomes with only one drug (Th or δ-9-THC), with the other drug being added without preincubation, on the activities of hepatic microsomal drug metabolizing enzymes are shown in Table 3. The effect of preincubation of one drug, either Th or δ-9-THC, with the liver microsomes on the activities of microsomal drug metabolizing enzymes (see Table 1) did not appreciably change with the other drug (δ-9-THC or Th) added without preincubation. The preincubation of Th or δ-9-THC with liver microsomes showed a dose-dependent significant increase or decrease, respectively, in the activities of all the microsomal drug metabolizing enzymes studied (Table 1).

Table 4 shows the K_m and V_{max} values of liver microsomal aniline hyroxylase, *N*-demethylase and *O*-demethylase (from Lineweaver–Burk plots analysis) in the presence of Th and δ-9-THC either alone or in combination *in vitro*. Th alone decreased the K_m and increased the V_{max} of all three enzymes. δ-9-THC, on the other hand, at a lower concentration, decreased the V_{max} of aniline hydroxylase without affecting its K_m; but the K_m of *N*-demethylase and *O*-demethylase was increased without affecting their corresponding V_{max}. A higher concentration of δ-9-THC increased the K_m of aniline hydroxylase, *N*-demethylase and *O*-demethylase, and decreased the V_{max} of aniline hydroxylase. There was no significant change in the V_{max} of *N*-demethylase and *O*-demethylase. δ-9-THC in combination with Th increased the V_{max} of aniline hydroxylase, *N*-demethylase and *O*-demethylase, and the K_m of *N*-demethylase. The K_m of *O*-demethylase under identical conditions was decreased with no significant change in the K_m of aniline hydroxylase. When a higher concentration of δ-9-THC was present in combination with the same concentration of Th, the K_m of aniline hydroxylase and *N*-demethylase was found to be increased by 30.8% and 13.5%, respectively, without any appreciable change in the K_m of *O*-demethylase. Under similar conditions of treatment with Th and δ-9-THC (see Table 4) the V_{max} of *N*-demethylase was increased and that of *O*-demethylase was decreased, but there was no significant change in the V_{max} of aniline hydroxylase.

Table 2. *In vitro* effect of Th and/or δ-9-THC on the activities of liver microsomal drug metabolizing enzymes without preincubation of microsomal protein with drug(s)

Concentration of Th and/or δ-9-THC (μM)	Aniline hydroxylase (nmol *p*-aminophenol/hr/mg protein)	*N*-Demethylase (nmol formaldehyde/hr/mg protein)	*O*-Demethylase (nmol *p*-nitrophenol/hr/mg protein)
Control	23.5 ± 0.38	40.8 ± 0.62	27.0 ± 0.44
Th (110.0)	24.2 ± 0.60	41.1 ± 0.50	26.9 ± 0.70
Th (550.0)	23.9 ± 0.52	40.2 ± 0.24	27.3 ± 0.36
δ-9-THC (1.6)	22.9 ± 0.32	39.9 ± 0.22	26.7 ± 0.81
δ-9-THC (13.33)	24.0 ± 0.70	41.4 ± 0.60	26.7 ± 0.28
Th (110.0) ± δ-9-THC (1.6)	25.1 ± 0.85	38.5 ± 1.10	28.2 ± 0.68
Th (110.0) ± δ-9-THC (13.33)	21.8 ± 1.20	42.0 ± 1.00	27.6 ± 0.50
Th (550.0) ± δ-9-THC (1.6)	24.6 ± 0.45	41.5 ± 0.52	26.4 ± 0.70
Th (550.0) ± δ-9-THC (13.33)	22.6 ± 0.28	40.2 ± 0.35	26.0 ± 1.10

Results are expressed as means ± SEM of five separate determinations. Th and/or δ-9-THC were added without preincubation with liver microsomes to the enzyme assay medium. Only liver micrsomes was preincubated at 37° for 30 min before addition to the enzyme assay system. In the control experiment, an equivalent amount of vehicle for Th and/or δ-9-THC was added instead of drug(s).

Table 3. *In vitro* effect of Th and δ-9-THC on the activities of liver microsomal drug metabolizing enzymes following preincubation of microsomal protein with one drug

Concentration of Th and δ-9-THC (μM)	Aniline hydroxylase (nmol *p*-aminophenol/hr/mg protein)	*N*-Demethylase (nmol formaldehyde/hr/mg protein)	*O*-Demethylase (nmol *p*-nitrophenol/hr/mg protein)
Control	20.6 ± 0.47	42.0 ± 0.27	25.9 ± 0.58
Th (550.0) + *δ-9-THC (1.6)	29.6 ± 0.22†	76.6 ± 0.81†	39.7 ± 0.92†
Th (550.0) ± *δ-9-THC (13.33)	30.8 ± 0.66†	75.2 ± 0.24†	40.7 ± 0.36†
δ-9-THC (1.6) + *Th (550.0)	17.8 ± 0.55†	30.8 ± 0.70†	23.8 ± 0.25†
δ-9-THC (13.33) + *Th (550.0)	14.4 ± 0.90†	24.6 ± 0.32†	20.3 ± 0.54†

Results are expressed as means ± SEM of five separate determinations. Liver microsomes preincubated with only one drug (Th or δ-9-THC) at 37° for 30 min were added to the enzyme assay medium together with the other drug without preincubation. In the control experiment, an equivalent amount of vehicle for Th and δ-9-THC was used instead of drug under similar conditions.

* Drugs added to the enzyme assay medium without preincubation.

Significantly different from control, †$P < 0.01$ using Tukey test for analysis of variance.

Table 4. *In vitro* effect of Th and/or δ-9-THC on the K_m and V_{max} of rat liver microsomal drug metabolizing enzymes

Concentration of Th and/or δ-9-THC (μM)	Aniline hydroxylase		*N*-Demethylase		*O*-Demethylase	
	K_m (mM)	V_{max} (nmol *p*-aminophenol/ hr/mg protein)	K_m (mM)	V_{max} (nmol formaldehyde/ hr/mg protein)	K_m (mM)	V_{max} (nmol *p*-nitrophenol/ hr/mg protein)
Control	5.88 ± 0.08	55.5 ± 2.01	2.0 ± 0.04	76.9 ± 2.52	11.1 ± 0.04	71.4 ± 2.60
Th (550.0)	4.54 ± 0.05*	62.5 ± 1.42*	1.66 ± 0.02*	111.0 ± 3.18*	9.8 ± 0.05*	100.0 ± 2.31*
δ-9-THC (1.6)	5.88 ± 0.06	45.5 ± 2.22*	4.45 ± 0.09*	83.3 ± 2.66*	14.3 ± 0.03*	71.4 ± 1.92
δ-9-THC (13.33)	8.33 ± 0.10*	45.5 ± 2.18*	6.25 ± 0.10*	83.3 ± 2.24*	18.2 ± 0.14*	71.4 ± 2.69
Th (550.0) + δ-9-THC (1.6)	5.88 ± 0.12	62.5 ± 1.08*	2.17 ± 0.02*	90.9 ± 1.00*	10.0 ± 0.02*	90.9 ± 1.98*
Th (550.0) + δ-9-THC (13.33)	7.69 ± 0.08*	55.5 ± 3.08	2.27 ± 0.06*	90.9 ± 0.92*	11.1 ± 0.06	58.8 ± 3.01*

Results are expressed as means ± SEM of three separate determinations. The K_m and V_{max} values were calculated from Lineweaver–Burk plots (see Figs 1–3).

Significantly different from control, *$P < 0.01$ using Tukey test for analysis of variance.

DISCUSSION

Both Th and δ-9-THC are metabolized through hepatic microsomal hydroxylation and demethylation [2, 4] but the effect of interaction of these drugs at the level of hepatic microsomal drug detoxicating enzymes has not been studied previously. In this study we found that Th increases the activities of hepatic microsomal aniline hydroxylase, *N*-demethylase and *O*-demethylase (Table 1). These increases may be explained by the increase in affinity ($1/K_m$) as well as an enhanced rate of enzyme catalyzed reaction (V_{max}) (Table 4). Th may bring about an allosteric modification of these drug metabolizing enzymes and hence stimulate their activities. There is a possibility that the Th-induced enhancement of the activities of the drug metabolizing enzymes may occur by the stabilization of the hepatic microsomal membrane [14]. The anionic form of Th [15] can bind to the ε-amino group of the lysine residue on the exofacial site of the microsomal membrane [16] which may stabilize the membrane by neutralizing the surface charge [14, 17]. δ-9-THC, on the other hand, inhibits the activity of all the three enzymes studied (Table 1). Competitive inhibition of *N*- and *O*-demethylase activities as observed with the different concentrations of δ-9-THC under *in vitro* conditions (Table 4 and Figs 1 and 2) indicates that this drug (δ-9-THC) alters the affinity of these enzymes for their corresponding substrates, perhaps by acting as an alternative substrate. Alternatively, the inhibition may be due to competition with these substrates for a common intermediate in the microsomal electron transport chain. Non-competitive and mixed-type inhibition by δ-9-THC of aniline hydroxylation (Table 4 and Fig. 3), on the other hand, appear to be associated with some non-specific action of δ-9-THC on the type II binding sites of the enzyme, aniline hydroxylase, rather than a specific interaction as observed in the case of inhibition of type I substrates [18]. Our observation on the inhibition of type I substrate by δ-9-THC is consistent with the observations of Cohen *et al.* [8], Dingel *et al.* [19] and Mitra *et al.* [2]. δ-9-THC-induced inhibition of hepatic microsomal drug metabolizing enzymes, such as aniline hydroxylase, *N*-demethylase and *O*-demethylase, was significantly reduced with the combination of δ-9-THC and Th (Table 1). At a very high concentration of Th together with δ-9-THC increased stimulation of all the three hepatic

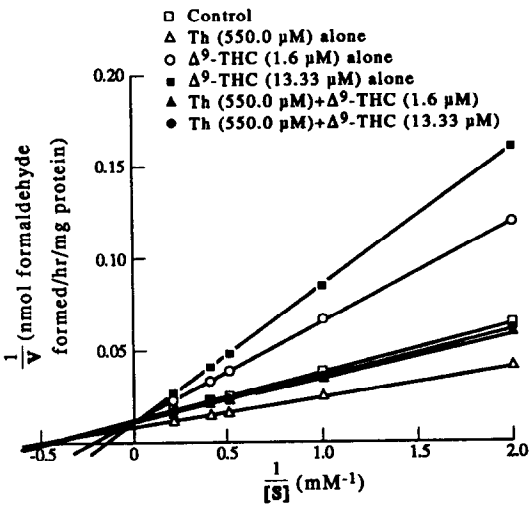

Fig. 1. Lineweaver–Burk plots of rat liver microsomal *N*-demethylase. Each point represents the mean of three separate determinations.

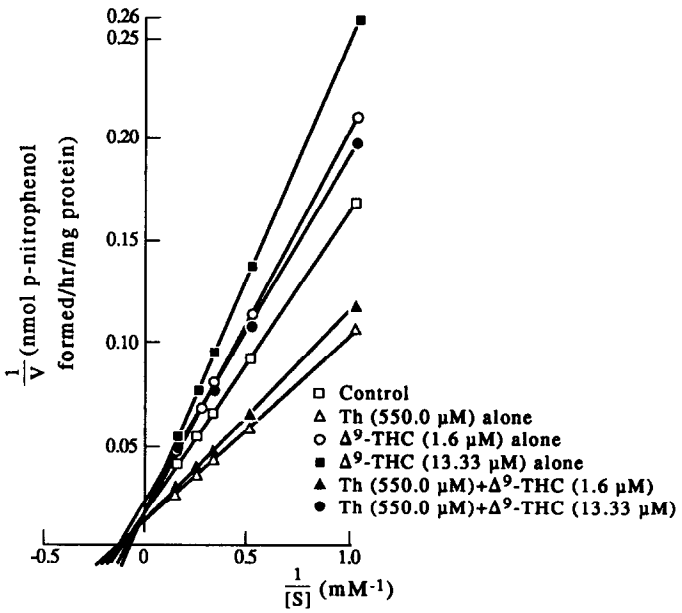

Fig. 2. Lineweaver–Burk plots of rat liver microsomal *O*-demethylase. Each point represents the mean of three separate determinations.

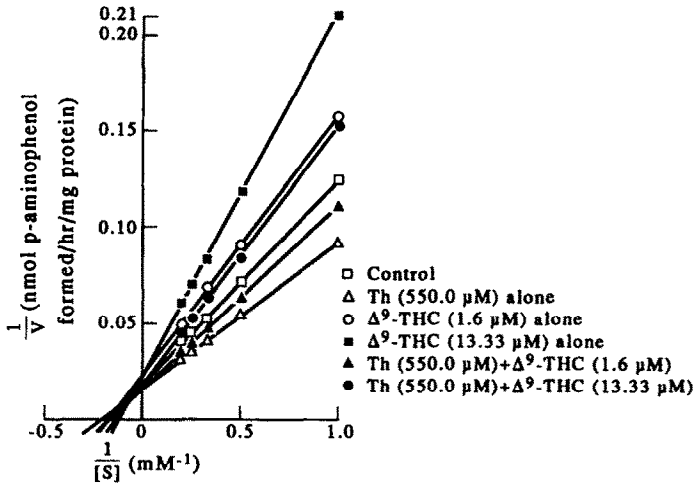

Fig. 3. Lineweaver–Burk plots of rat liver microsomal aniline hydroxylase. Each plot represents the mean of three separate determinations.

drug metabolizing enzymes was observed (Table 1) with respect to δ-9-THC alone. This may be due to the interaction of these drugs with the hepatic microsomal membrane.

Since both Th and δ-9-THC are metabolized through hydroxylation and demethylation [2, 4], and Th enhances δ-9-THC binding to the hepatic microsomal membrane [20], it is not unlikely that δ-9-THC is inactivated through enhanced oxidation in the presence of Th, and Th ultimately prevents δ-9-THC-induced inhibition at the level of hepatic drug metabolizing enzymes (Table 1). This idea is supported by the observation that: (a) Th and/or δ-9-THC without preincubation with hepatic microsomes did not appreciably change the activities of the hepatic microsomal drug metabolizing enzymes (Table 2), and (b) Th or δ-9-THC was unable to produce a considerable effect on the activities of the drug metabolizing enzymes of liver microsomes preincubated with δ-9-THC or Th (Table 3). The reduction by δ-9-THC of the Th-induced increase in hepatic microsomal drug metabolizing enzyme activities may be explained by the fact that δ-9-THC reduces the binding of Th to the hepatic microsomal membrane.* This view is supported by the insignificant effect of δ-9-THC or Th on the activities of the hepatic drug metabolizing enzymes (Tables 2 and 3), and hence the induction by Th of an enhanced liver microsomal δ-9-THC oxidation.

Although no direct evidence is available, it is not unlikely that the presence of Th and δ-9-THC in combination in the living system reduces the potency of δ-9-THC via interaction with serum albumin [21] leading to a decrease in the free (bioactive) drug concentration, and/or via an increase in the binding of δ-9-THC to hepatic microsomal membranes [20].

* Ghosh SK and Poddar MK, Delta-9-THC inhibits the binding of theophylline to mammalian neuronal and non-neuronal membranes. *Drugs Exp Clin Res*, submitted.

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