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BIOTRANSFORMATION OF 7,12-DIMETHYLBENZ(a)ANTHRACENE(DMBA): ON THE GENESIS OF DMBA-trans-DIHYDRODIOLS AND DMBA-7- and 12-METHYL HYDROXYLATED METABOLITES.

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SUMMARY

Biotransformation of DMBA in various induced and uninduced S-10 fractions in the presence and absence of hepatic mixed function oxidase inhibitors (α-naphthoflavone, metyrapone, SKF-525A and CS_2) is described. Particular attention is paid to the production of dihydrodiols (DHD) and DMBA-7- and 12-methyl hydroxylated metabolites. It is suggested that DMBA-3,4-DHD, 8,9-DHD and 5,6-DHD formation and DMBA-7- and 12-methyl hydroxylations proceed predominantly via different enzyme systems.

INTRODUCTION

Considerable effort has been directed towards developing an understanding of the bioactivation of DMBA (1-12) and the subsequent covalent binding of the active metabolites to cellular macromolecules (9,13). Incubation in vitro of DMBA with phenobarbital-induced rat liver microsomes results in ring A activation to furnish DMBA-trans-3,4-DHD, 7-hydroxymethyl(OHM), 12-methylbenz(a) anthracene(MBA)-trans-3,4-DHD, 12OHM,7MBA-trans-3,4-DHD, and 7OHM,12OHMBA-trans-3,4-DHD. These 3,4-DHD have higher mutagenic and DNA binding activities (1,12,13) than DMBA. Similarly, DMBA-trans-3,4-DHD and DMBA-trans-5,6-DHD were as active as DMBA in a tumor frequency assay whereas the related 8,9- and 10,11-DHD were virtually inactive (3). 2-, 3-, and 4-Hydroxy metabolites of DMBA and their 7- and 12-hydroxymethyl derivatives have also been reported (12).

In this article we discuss the results of our work on the comparative metabolism of DMBA in induced and uninduced Sprague Dawley rat liver S-10 fractions. Through use of various mixed function oxidase inhibitors (α-naphthoflavone, metyrapone, SKF-525A and CS_2) we now provide evidence which suggests

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that DMBA-3,4-DHD, 8,9-DHD, and 5,6-DHD formation as well as DMBA-7- and 12-methyl hydroxylations proceed predominantly via different enzyme complexes. Thus, 3 4-DHD forming activities are distinctly different from those enzymatic activities responsible for formation of DMBA-trans-5,6- and 8,9-DHD and marked differences are observed in their production dependent upon the nature of the inducer or inhibitor. Similarly, one can influence the relative hydroxylation of the 7- and 12-methyl groups in DMBA by changing the nature of the inducer and inhibitor. These results reinforce the multiplicity of P450's relative to their structural (14,15) and functional (16-18) differences.

MATERIALS AND METHODS

Hydrocarbons - DMBA was purchased from Eastman Chemical Company and was checked for purity by high pressure liquid chromatography (hplc) prior to use and when necessary purified by column chromatography over silica gel (Silica Gel-60; E. Merck) using hexane-benzene (1:1) as eluent. The 2- and 4-OH-DMBA derivatives were gifts of Professor Melvin S. Newman, Department of Chemistry, The Ohio State University. 3-OH-DMBA was independently converted to DMBA-trans-3,4-DHD by a method similar to the one published by Sukumaran and Harvey (19).

Generation of Metabolites - Liver S-10 fractions were obtained from pre-treated male Sprague Dawley rats weighing 150-200g (ip dose of phenobarbital was 100 mg/kg/day for 4 days; 3MC was 20 mg/kg/day for 2 days; β-naphthoflavone was 75 mg/kg/day for 5 days).

Metabolites of DMBA were obtained by incubation in vitro of the substrate with liver S-10 fraction prepared by homogenizing rat liver with an equal weight of 0.1M phosphate buffer, pH7.3 and centrifuging the homogenate at 9,000 and 10,000 g, respectively, for a period of 1 hr. each. The S-10 fractions were frozen and stored at -70°C. All incuabtions were carried out at 37°C in 6 mls of buffer (0.1M Tris + 5.0mM $MgCl_2$ + 5.0μM $MnCl_2$ + 0.012M (DL)-isocitrate) containing 4.4 units of isocitrate dehydrogenase, 50 μmoles NADP, 1.0 ml S-10 fraction and 1 mg of DMBA in 0.3 ml acetone, for a period of 2.5 hr with constant shaking. Inhibitors were preincubated for a period of 10 min in incubation buffer containing S-10 fraction. After addition of DMBA incubation was continued for a period of 2.5 hr. At the end of the incubation periods, acetone (10 ml) and ethyl acetate (20 ml) were added. The mixture was vortexed, centrifuged, and the supernatant dried over sodium sulfate (15 g) and evaporated under reduced pressure. The residue was dissolved in a mixture of acetone: ethyl acetate (1:2;0.5 ml) with warming and analyzed by hplc.

We have found that phenobarbital- and β-naphthoflavone-induced and uninduced S-10 fractions are stable at -70°C for over 3 months. However, storage of phenobarbital-induced S-10 fractions for one week at 0°C resulted in loss of 3,4-DHD forming activity and stimulation of 7-methyl hydroxylation.

For all inhibitor studies two controls were utilized; one which did not contain inhibitor and one which did not contain substrate but had a maximum inhibitor concentration present. We have not found substantial variation in metabolic profiles for substrate concentrations of 0.7 to 1.3 mg per incu-

bation. In addition DMBA metabolic profiles were insensitive to the highest concentration of acetone and also a NADP concentration range of 10 to 50 μmoles employed for incubation.

Isolation of Metabolites - Laboratory Data Control (LDC) chromatography accessory module containing LDC gradient master, constrametric pumps, spectormonitor III and equipped with Whatman Partisil PXS 10/25 ODS column (length= 25cm; dia=4.6mm) and a Whatman precolumn (length=7cm; dia=4.6mm packed with CoPell^{Tm} ODS 10μ) was employed for hplc purposes. The column was eluted at ambient temperature with methanol in water (25% v/v) to 100% methanol at a solvent flow of 1.2 ml/min. Chromatographic peaks were collected and combined from 5 to 6 runs. The solvent was removed by evaporation with nitrogen and the residue examined spectrophotometrically. Generally tritiated DMBA was employed for inhibitor studies. The hplc peaks were collected and counted (dpm) for the quantification of various metabolites. DMBA-3,4-DHD (R.T. 47.5 min in our hplc system) was quantified by area measurement of hplc peak using a UV detector at 271 nm. Inhibition and stimulation above 10% of controls are significant.

Characterization of Metabolites - Ultraviolet and visible absorption spectra of the metabolites were measured in methanol on a Beckman UV 526 spectrophotometer. Fluorescence spectra of the metabolites were measured in methanol using an Aminco SPF-500 Spectrofluorometer. Data obtained were compared with reported (1,10,13,19-22) physical constants for DMBA and their corresponding metabolites.

RESULTS AND DISCUSSION

DMBA Metabolism - Comparative hplc DMBA metabolite profiles derived from phenobarbital-, β-naphthoflavone-, and 3MC-induced, and uninduced S-10 fractions are shown in Figure 1. The order of total diol production after 2.5 hr. incubation with the various S-10 fractions was 3MC-induced > β-naphthoflavone-induced > phenobarbital-induced > uninduced (Table 1). Only in the case of β-naphthoflavone-induced S-10 rat liver fraction was a substantial quantity

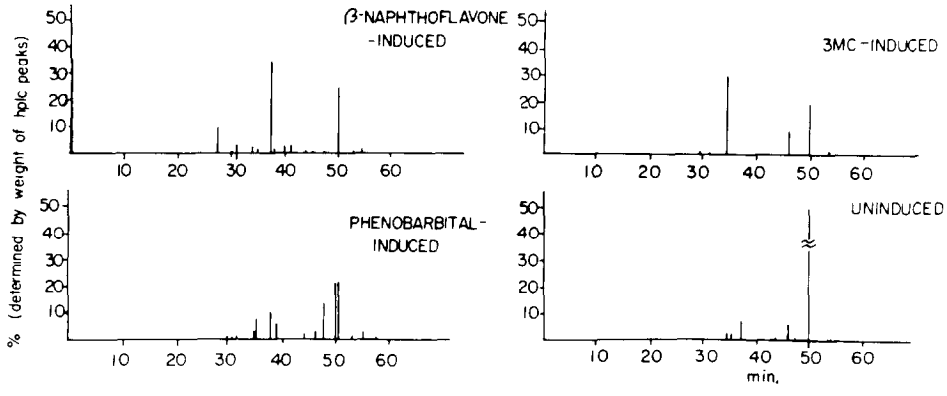


Fig. 1. Comparative metabolic profiles of DMBA in uninduced and induced S-10 fractions.

Table 1: Generation of Various Dihydrodiol Metabolites on Incubation of DMBA with Rat Liver Induced and Uninduced S-10 Fractions.

METABOLITES	Retention Time (min.)	%[a] of Total Metabolites Uninduced	3MC-Induced	Phenobarbital-induced	β-Naphthoflavone-induced
DMBA-trans-3,4-Dihydrodiol	47.4	2	ND[b]	15.0	ND
DMBA-trans-5,6-Dihydrodiol	34.75	ND	20	4.0	2.5
DMBA-trans-8,9-Dihydrodiol	37.75	13.5	30	10.1	35
DMBA-trans-10,11-Dihydrodiol	38.75	ND	ND	ND	ND
7-OHM,12-M-BA-trans-3,4-Dihydrodiol and/or 12-OHM isomer	35.30	3	ND	9.1	ND
7-OHM,12-M-BA-trans-5,6-Dihydrodiol	29.05	ND	ND	ND	ND
12-OHM isomer	25.0?	ND	ND	ND	ND
7-OHM,12-M-BA-trans-8,9-Dihydrodiol	30.05	ND	2	ND	ND
12-OHM isomer	31.20	ND	ND	<1	<1
7-OHM,12-M-BA-trans-10,11-Dihydrodiol	28.10	ND	<1	ND	10

[a]error of determination ~±6% of number shown; [b]ND=not detected

(~10%) of 7-OHM,12MBA-trans-10,11-DHD detected. β-naphthoflavone- and 3MC-induced S-10 fractions most closely resembled each other relative to DMBA metabolism. These data are consistent with the observation that β-naphthoflavone resembles 3MC-induction of cytochromes P-450 and epoxide hydratase (23,24). Of considerable importance is the observation that phenobarbital-induced S-10 fraction afforded relatively large amounts of DMBA-trans-3,4-DHD (~15%) and either a mixture (~9%) or one or the other of 7-OHM,12MBA-trans-3,4-DHD and 12OHM,7MBA-trans-3,4-DHD, whereas 3MC- and β-naphthoflavone-induced S-10 fraction produced no detectable quantities of these metabolites. Equally significant is the observation that uninduced S-10 fraction afforded small amounts of these presumed precarcinogenic 3,4-DHD metabolites. DMBA-trans-8,9-DHD was consistently detected in relatively large amounts (10-35%) with all S-10 preparations.

Table 2: Percent Transformation of DMBA to its Metabolites in the Presence and Absence of Inhibitors in Uninduced and Induced Sprague Dawley Rat Liver S-10 Fractions.

S-10 Fraction	Inhibitors[a] α-Naphthoflavone (0.42 μmole)	CS_2	SKF-525A (0.42 μmole)	Metyrapone (0.42 μmole)	No Inhibitor
Uninduced	9.0	-	13.4	-	23.4
Phenobarbital-induced	21.2	16.5[b]	14.0	-	35.2
β-Naphthoflavone-induced	15.4	15.0[c]	-	18.0	27.3

[a]Inhibitor concentration is expressed in μmoles/ml incubation medium; [b]0.74 μmoles/ml CS_2; [c]0.42 μmoles/ml CS_2

Effect of Inhibitors on DMBA-DHD Formation - As expected all AHH inhibitors (CS_2, α-naphthoflavone, metyrapone and SKF-525A) blocked formation of total DMBA metabolites in all S-10 fractions studied (Table 2). However, these inhibitors exhibited marked differences on the relative percent production of the total of the various metabolites. Metyrapone (0-0.71 μmole/ml), CS_2 (0-1.54 μmole/ml) and SKF-525A (0-0.28 μmole/ml) inhibit total DHD [sum of 3,4-5,6, 8,9, and 10,11-DHDs plus 7,12-bis-(hydroxymethyl)-BA] formation in phenobarbital S-10 fraction in a dose related fashion (Figure 2A). On the other

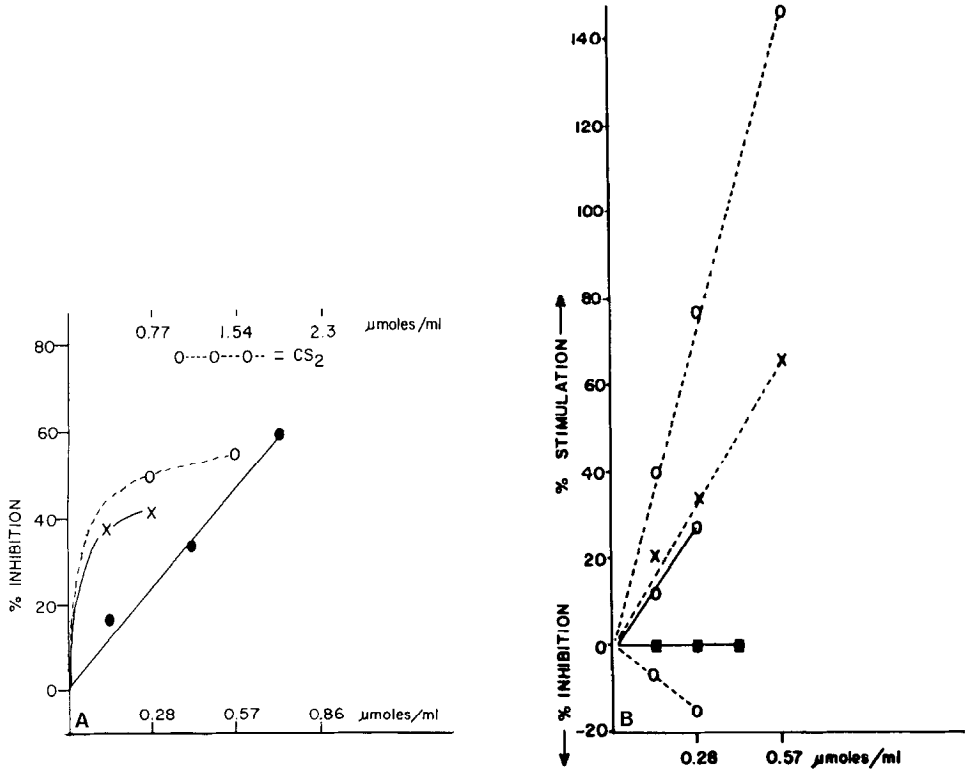
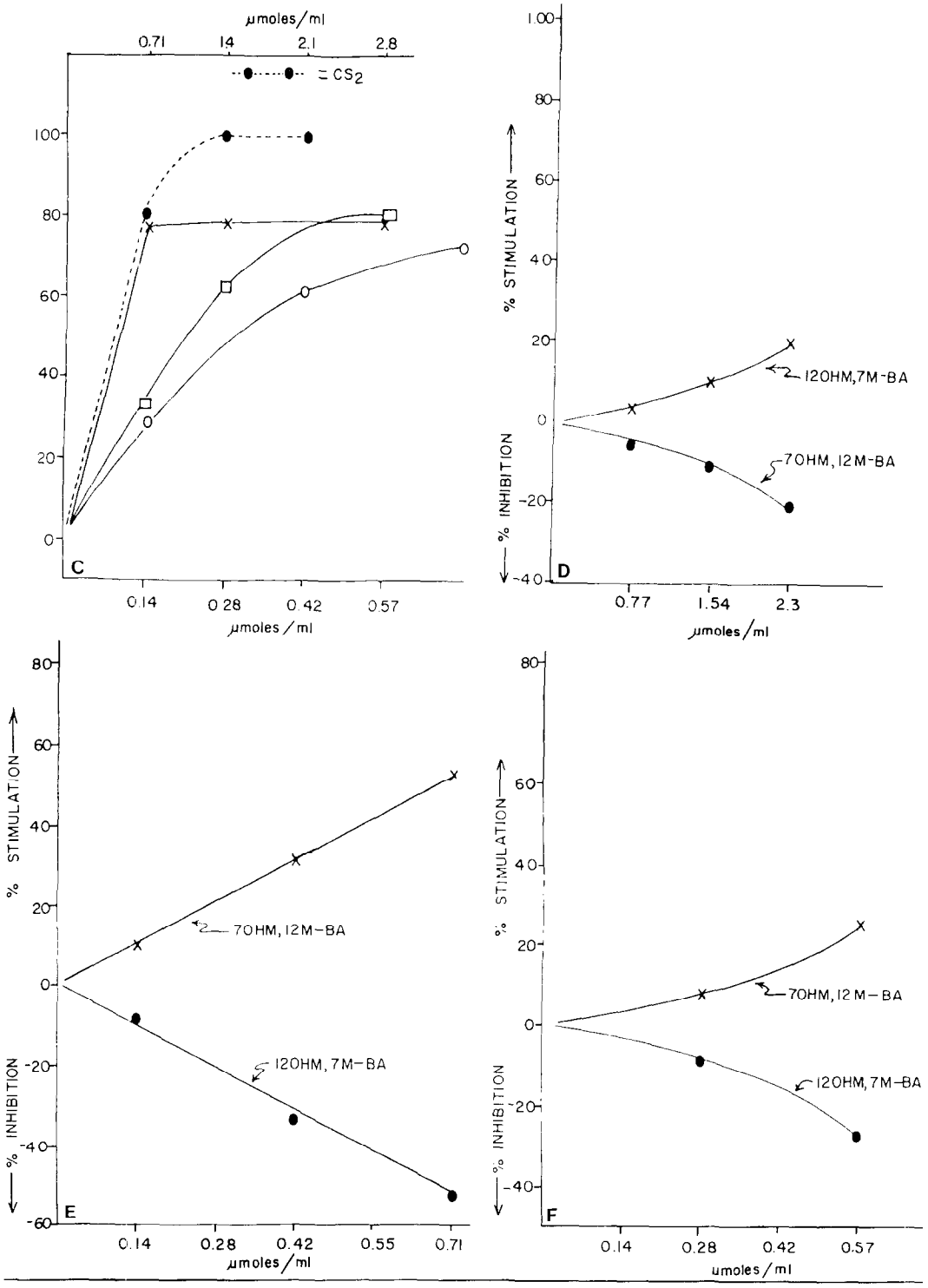


Fig. 2. (A) Effect of various inhibitors (O--O--O=CS_2); (X-X-X=SKF-525A); (●—●—● =Metyrapone) on the production of total DMBA dihydrodiols (3,4-; 5,6-; 8,9-; 10,11-) and 7,12-bis-hydroxy methylbenz(a)anthracene in phenobarbital-induced S-10 fraction. (B) Effect of various inhibitors on the production of total DMBA diols in β-Naphthoflavone-induced (X-X-X=SKF-525A); (O—O—O =Metyrapone); (●—●—● =α-Naphthoflavone) and Uninduced (X--X--X=SKF-525A); (O- - O- - O =Metyrapone); (●- -●- -● =α-Naphthoflavone) rat liver S-10 fractions. (C) Effect of inhibitors (●- -●- -●=CS_2); (O—O—O =Metyrapone); (X-X-X=SKF-525A); (□—□—□ =α-Naphthoflavone) on the formation of DMBA-trans-3,4-dihydrodiol or the total of 7OHM,12MBA- and 12OHM,7MBA-trans-3,4-dihydrodiols. (Respective curves are virtually superimposable. Only the curve for DMBA-trans-3,4-dihydrodiol is shown.) (D) Effect of CS_2 on the relative production of 12OHM,7MBA (X-X-X) in relation to 7OHM,12MBA (●—●—●). All data obtained employing phenobarbital-induced S-10 fractions. (E) Effect of metyrapone on the relative production of 7OHM,12MBA (X-X-X) in relation to 12OHM,7MBA (●—●—●). All data obtained employing phenobarbital-induced S-10



fractions. (F) Effect of SKF-525A on the relative production of 7OHM,12MBA (X-X-X) in relation to 12OHM,7MBA (●—●—●). All data obtained employing phenobarbital-induced S-10 fractions.

Table 3: Percent Inhibition (-) or Stimulation (+) of the Production of DMBA-8,9-dihydrodiol Relative to the Sum of 7-OHM,12M-BA and 12-OHM,7M-BA Metabolites, in Induced and Uninduced S-10 Fractions.

S-10 Fraction	Inhibitors[a]			
	α-Naphthoflavone (0.42 μmole)	CS_2	SKF-525A (0.42 μmole)	Metyrapone (0.42 μmole)
Phenobarbital	-16	-23[b]	-80	+22
β-Naphthoflavone	-68	-23[c]	+5.0	-16.5
Uninduced	+30	-	+30	+43

[a]Inhibitor concentration is expressed in μmoles/ml incubation medium; [b]0.74 μmoles/ml CS_2; [c]0.42 μmoles/ml CS_2

hand, α-naphthoflavone (0-0.29 μmole/ml), an inhibitor of cytochrome P-450 (16,23), stimulated total diol formation in β-naphthoflavone-induced S-10 fraction whereas metyrapone and SKF-525A had no effect (Figure 2B). Furthermore, in the case of uninduced S-10 fraction α-naphthoflavone (0-0.57 μmole/ml) and SKF-525A (0-0.57 μmole/ml) produced a marked stimulation of total diol formation in a dose related manner (Figure 2B).

All inhibitors [CS_2 (0-2.14 μmole/ml); metyrapone (0-0.71 μmole/ml); SKF-525A (0-0.57 μmole/ml); α-naphthoflavone (0-0.57 μmole/ml)] provided a marked decrease (65-100%) in the production of DMBA-*trans*-3,4-DHD and 7- or 12-methyl hydroxylated DMBA-*trans*-3,4-DHDs in phenobarbital-induced S-10 fraction (Figure 2C). Whereas CS_2 stimulated production of the 12OHM,7MBA relative to the 7OHM,12MBA metabolite (Figure 2D), the converse was observed for both metyrapone (Figure 2E) and SKF-525A (Figure 2F). Thus, the enzyme complexes responsible for the formation of 3,4-DHD and hydroxylation of the 7- and 12-methyl groups of DMBA are likely different. The observation that DMBA-*trans*-8,9-DHD is a major metabolite whereas DMBA-*trans*-3,4-DHD is not detected from DMBA in β-naphthoflavone-induced S-10 fractions suggests involvement of different enzyme systems for the origin of these dihydrodiols (Figure 1, Table 1). It is noteworthy that the production of DMBA-*trans*-8,9-DHD is affected dissimilarly by various inhibitors in phenobarbital- and β-naphthoflavone-induced and uninduced S-10 fractions (Table 3). α-Naphthoflavone, SKF-525A and metyrapone stimulated the production of DMBA-*trans*-8,9-DHD in uninduced S-10 fractions. The formation of this metabolite was inhibited by α-naphthoflavone, CS_2 and metyrapone and not affected by SKF-525A

Table 4: Percent Inhibition of Production of DMBA-5,6-Dihydrodiol in Phenobarbital- and β-Naphthoflavone-induced S-10 Fractions in the Presence of Hepatic Mixed Function Oxygenase Inhibitors.

Inhibitors	Percent Inhibition In	
	Phenobarbital induced S-10 fraction	β-Naphthoflavone-induced S-10 fraction
α-Naphthoflavone (0.42 μmoles)[a]	-30	-100
Metyrapone (0.42 μmoles)	-30	-41
SKF-525A (0.42 μmoles)	-40	-
CS_2	-63[b]	-30[c]

[a]Inhibitor concentration is expressed in μmoles/ml incubation medium; [b]0.74 μmoles/ml CS_2; [c]0.42 μmoles/ml CS_2

in β-naphthoflavone-induced S-10 fractions. In contrast DMBA-trans,8,9-DHD formation was inhibited by α-naphthoflavone, CS_2, and SKF-525A and stimulated by metyrapone in phenobarbital-induced S-10 fractions. It thus appears that DMBA-trans-8,9-DHD, in part, originates differently in phenobarbital- and β-naphthoflavone-induced and uninduced S-10 fractions. It is of interest that the production of DMBA-trans-5,6-DHD in both phenobarbital- and β-naphthoflavone-induced S-10 fractions is inhibited substantially by all inhibitors employed (Table 4) in the present study. It thus appears that trans-3,4- and trans-5,6-DHD may also arise by independent processes. Both metyrapone (0-0.57 μmole/ml) and SKF-525A (0-0.57 μmole/ml) inhibited 7OHM,12MBA-trans-10,11-DHD formation in β-naphthoflavone-induced S-10 fractions. SKF-525A also inhibited formation of 7OHM,12MBA-trans-8,9-DHD in this fraction (Figure 3A and B).

In summary, aryl hydrocarbon hydroxylase complexes in various S-10 fractions are inhibited to different degrees dependent upon the nature of the S-10 fraction and the inhibitor employed. Differential inhibition reveals that DMBA-trans-3,4-DHD, 5,6-DHD, and 8,9-DHD formation and DMBA 7- and 12-methyl hydroxylations are mediated by different enzyme complexes. In addition, DMBA-trans-8,9-DHD appears to arise, in part, differently in phenobarbital- and β-naphthoflavone-induced and uninduced S-10 fractions. We are hopeful that the present studies will allow us to modulate in vivo the production of various DHDs and their subsequent binding to DNA and thereby enable us to further define critical events in carcinogenesis.

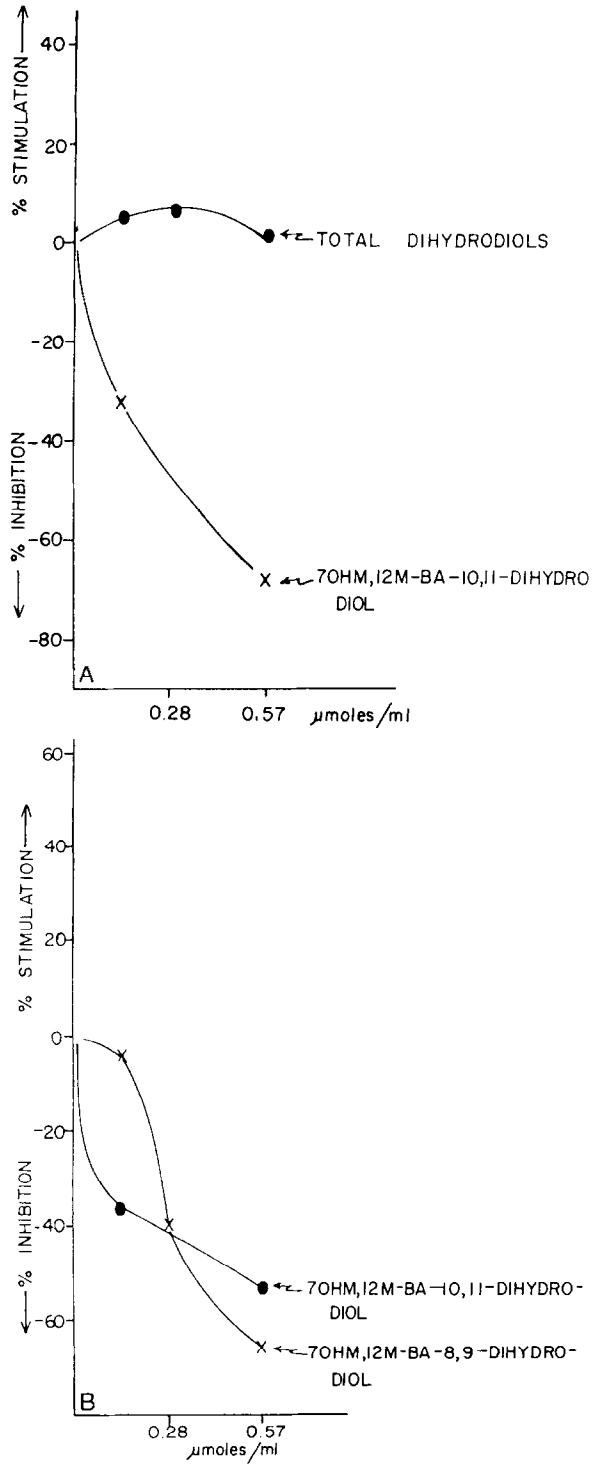


Fig. 3. (A) Effect of metyrapone on the production of total dihydrodiols (●—●—●), and 7OHM,12MBA-trans-10,11-dihydrodiol (X-X-X) in β-Naphthoflavone-induced S-10 fractions. (B) Effect of SKF-525A on the production of 7OHM,12MBA-trans-10,11-dihydrodiol (●—●—●) and 7OHM,12MBA-trans-8,9-dihydrodiol (X-X-X) in β-Naphthoflavone-induced S-10 fractions.

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REFERENCES

1. Chou, M.W., and Yang, S.K. (1978) Proc. Natl. Acad. Sci. U.S.A., 75(11), 5466-5470.
2. Dipple, A., Tomaszewski, Moschel, R.C., Bigger, C.A.H., Nebzydoski, J.A., and Egan, M. (1979) Cancer Res., 39, 1154-1158.
3. Grover, P.L., and Chouroulinkov, I. (1979) Proc. Am. Assoc. Cancer Res., 20, 66.
4. Huberman, E., Chou, M.W., and Yang, S.K. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 862-866.
5. Ivanovic, V., Geacintov, N.E., Jeffrey, A.M., Fu, P.P., Harvey, R.G., and Weinstein, I.B. (1978) Cancer Lett., 4, 131-140.
6. Inbasekaran, M.N., Witiak, D.T., Barone, K., and Loper, J.C. (1979) J. Med. Chem. in press.
7. Malaveille, C., Bartsch, H., Tierney, B., Grover, P.L., and Sims, P. (1978) Biochem. Biophys. Res. Commun., 83, 1468-1473.
8. Marquardt, H., Baker, S., Tierney, B., Grover, P.L., and Sims, P. (1978) Biochem. Biophys. Res. Commun., 85, 357-362.
9. Moschel, R.C., Baird, W.M., and Dipple, A. (1977) Biochem. Biophys. Res. Commun., 76, 1092-1098.
10. Tierney, B., Hewer, A., MacNicoll, A.D., Gervasi, P.G., Rattle, H., Walsh, C., Grover, P.L., and Sims, P. (1978) Chem. Biol. Interactions 23, 243-257.
11. Vigny, P., Duquesne, M., and Coulomb, H. (1977) FEBS Letters 82(2) 278-282.
12. Yang,S.K., Chou, M.W., and Roller, P.P. (1979) J. Amer. Chem. Soc., 101, 237-239.
13. Chou, M.W., Easton, G.D., and Yang, S.K. (1979) Biochem. Biophys. Res. Commun., 88(3) 1085-1091.
14. Ryan, D.E., Thomas, P.E. Korzeniowski, D., and Levin, W. (1979) J. Biol. Chem., 254(4) 1365-1374.
15. Botelho, L.H., Ryan, D.E., and Levin, W. (1979) J. Biol. Chem., 254(13), 5635-5640.
16. Atlas, S.A., and Nebert, D.W. (1976) Arch. Biochem. Biophys., 175, 495-506.
17. Oesch, F. (1972) Xenobiotica 3, 305-340.
18. Thorgeisson, S.S., Atlas, S.A., Boobis, A.R., and Felton, J.S. (1979) Biochemical Pharmacol., 8, 217-226.
19. Sukumaran, K.B., and Harvey, R.G. (1979) J. Am. Chem. Soc., 101, 1353.
20. Daniel, F.B., Cazer, F.D., D'Ambrosia, S.M., Hart, R.W., Kim, W.H. and Witiak, D.T. (1979) Cancer Lett., 6, 263-272.
21. Tierney, B., Hewer, A., MacNicholl, A.D., Gervasi, P.G., Rattle, H., Walsh, C., Grover, P.L., and Sims, P. (1978) Chem. Biol. Interact., 23,243-257.
22. Yang, S.K., and Dower, W.V. (1975) Proc. Natl. Acad. Sci. U.S.A., 72, 2601-2605.
23. Gelboin, H.V., Wieble, F.J. and Kinoshita, N. (1972) in Biological Hydroxylation Mechanism (G.S. Boyd and R.M.S. Smellie, eds.), Academic Press, Inc., New York, 103-133.
24. Hildebrandt, A.G. (1972) in Biological Hydroxylation Mechanism (G.S.Boyd) and R.M.S. Smellie, eds.), Academic Press, Inc., New York, 79-102.