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EFFECT OF OESTRADIOL ON THE *IN VITRO* METABOLISM OF 7,12-DIMETHYLBENZ[a]ANTHRACENE AND ITS HYDROXYMETHYL DERIVATIVES

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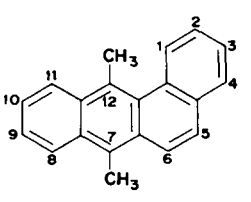
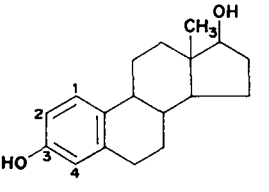
Abstract It is known that epoxides are intermediates in the conversion of aromatic hydrocarbons into dihydrodiols, phenolic metabolites and GSH conjugates. The effect of oestradiol, *in vitro*, on the metabolic conversion of 7,12-dimethylbenz[a]anthracene, 7-hydroxymethyl-12-methylbenz[a]anthracene and 12-hydroxymethyl-7-methylbenz[a]-anthracene into these derivatives by a system containing NADPH, GSH and the microsomal and dialysed soluble fractions of female rat-liver has been investigated. The amounts of all three types of metabolites formed are much reduced by the presence of oestradiol and it is likely that the steroid acts by reducing the amounts of epoxide intermediates formed rather than by inhibiting the various reactions concerned in their further metabolism. This is supported by the finding that oestradiol also reduces the total amount of each substrate metabolized. Cyclohexene oxide, an inhibitor of the conversion of epoxides into dihydrodiols by the enzyme "epoxide hydrase", greatly reduces the amount of dihydrodiols formed, but is without effect on the amounts of substrate metabolized. Furthermore, although the omission of GSH and soluble liver fraction from reaction mixtures prevents the synthesis of GSH conjugates, it does not reduce the amount of substrate metabolized, an effect that differs from that produced by oestradiol.

A SINGLE feeding of 7,12-dimethylbenz[a]anthracene (I) to 50–60-day-old female Sprague–Dawley rats causes mammary tumours to develop in all the animals.[1] The properties of these tumours are influenced by the hormonal status of the animal. Thus, the rate of growth of the tumours is accelerated by pregnancy, or by the injection of progesterone, and their appearance is delayed by the administration of oestradiol [1,3,5(10)-oestratrien-3,17 β-diol (II)].[2] Other evidence such as the inhibition of mammary cancer induction by ovariectomy immediately after the administration of a polycyclic hydrocarbon and the failure to induce mammary tumours with hydrocarbons in male rats, indicates that these neoplastic transformations cannot take place in the absence of ovarian hormones.[3,4] The role played by the hormones is not yet clear.



7, 12-Dimethylbenz[a]anthracene
(I)



Oestradiol
(II)

7,12-Dimethylbenz[a]anthracene is converted by rat-liver homogenate and NADPH into 7-hydroxymethyl-12-methylbenz[a]anthracene and 12-hydroxymethyl-7-methylbenz[a]anthracene.[5] The dihydrodiols and phenolic metabolites that are formed from the hydrocarbon and from its hydroxymethyl derivatives have been described.[6,8] The metabolic synthesis of water-soluble, as distinct from ethyl acetate-soluble, metabolites of 7,12-dimethylbenz[a]anthracene has also been reported[9-13] and one of these, *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)-glutathione, has been identified as a metabolite that is formed by homogenates of livers from rats that were pretreated with 3-methylcholanthrene.[14] The phenols, dihydrodiols and GSH conjugates are the end products of at least two reactions, since each metabolite is derived from an epoxide intermediate that is itself formed from the substrate by the action of the NADPH-dependent microsomal mixed function oxidase.[15,16] Epoxides have been detected as metabolic intermediates in the metabolism of 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene and their 7-hydroxymethyl derivatives by this system.[17] The epoxide intermediates are converted into dihydrodiols by microsomal epoxide hydrase,[18-20] into GSH conjugates[21] by glutathione *S*-epoxide transferase[22] and isomerised, probably non-enzymically, into phenols.[18,23]

The present paper describes the effect of oestradiol on the *in vitro* metabolism of 7,12-dimethylbenz[a]anthracene and of its hydroxymethyl derivatives by sub-cellular fractions of female rat-liver.

EXPERIMENTAL

Materials. ^{3}H-Labelled 7,12-dimethylbenz[a]anthracene (sp. act. 81·5 mCi/m-mole) was prepared by diluting a more highly labelled sample of the hydrocarbon (Radiochemical Centre, Amersham, Bucks) with unlabelled carrier 7,12-dimethylbenz-[a]anthracene. The purification of the ^{3}H-labelled hydrocarbon and also the preparation of 7-hydroxymethyl-12-methylbenz[a]anthracene and 12-hydroxy-methyl-7-methylbenz[a]anthracene, both generally labelled with tritium, have been described.[8] The specific activity of the hydroxymethyl derivatives was 106 mCi/m-mole. Samples of unlabelled dihydrodiols and phenolic metabolites for use as reference compounds were prepared enzymically.[14] *S*-(5,6-Dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione and *S*-(5,6-dihydro-6-hydroxy-7-hydroxymethyl-12-methylbenz[a]anthracen-5-yl) glutathione were prepared by published methods.[21]

Chromatography. Thin-layer chromatography (TLC) was carried out on glass plates coated with layers (0·25 mm thick) of Silica gel G (E. Merck A.–G. Darmstadt, Germany). Chromatograms were developed for 15 cm with solvent (a), benzene–ethanol (85:15, v/v). The compounds were located by examining the chromatograms in u.v. light, both before and after exposure to NH_3.[6]

Paper chromatography was carried out on Whatman No. 1 paper and descending chromatograms were developed for approx. 25 cm with solvent (b), butan-1-ol–acetic acid–water (12:3·5, v/v). Ascending chromatograms were developed for 15 cm with solvent (c), butan-1-ol–propan-1-ol–aqueous 2 M-NH_4OH (2:1:1, v/v). The dried chromatograms were examined in u.v. light and either sprayed with a solution of

ninhydrin (0·5%) in butan-1-ol and heated at 100° for 5 min, or sprayed with $K_2Cr_2O_7$–$AgNO_3$ reagent.[24]

Incubations with cell fractions of female rat-liver. Microsomal and soluble fractions were prepared from the livers of Wistar strain adult female rats essentially as described,[25] except that the livers from five animals were homogenized in 3 vol. of isotonic KCl (1·15% w/v). The microsomal pellets were stored at −5° for not more than 10 days and suspended in 0·1 M-phosphate buffer (NaH_2PO_4–Na_2HPO_4), pH 7·4, immediately before use.

In order to remove endogenous GSH, soluble fraction was dialysed against 100 vol. of distilled water for 24 hr and the precipitate that formed during dialysis removed by centrifugation at 1000 *g* for 10 min. The supernatant (dialysed soluble fraction) was stored frozen and thawed immediately before use. Standard reaction mixtures (10 ml) in 50-mM-phosphate buffer, pH 7·4, contained microsomal fraction (equivalent to 1 g wet wt liver), dialysed soluble fraction (1 ml) and GSH (3 mg). An NADPH generating system was supplied by the addition of $NADP^-$ (3 mg), glucose 6-phosphate (15 mg) and glucose 6-phosphate dehydrogenase (12 units). After warming the mixture to 37°, the reactions were started by the addition, either of substrate (0·8 μmole) dissolved in ethanol (0·3 ml) or of a mixture of substrate (0·8 μmole) and inhibitor dissolved in ethanol (0·3 ml). Incubations with inhibitors contained either oestradiol (0·8 μmole) or cyclohexene oxide (4 μmole). The amount of each substrate added in experiments to determine the total amount metabolized was 0·08 μmole.

Preliminary experiments using standard reaction mixtures containing any of the three substrates, showed that the amounts of all the metabolites formed and estimated were proportional both to the time of incubation over a period of 20 min, using microsomal fraction from 1 g liver, and to the amount of microsomal fraction present up to an amount equivalent to 1 g liver using an incubation time of 20 min. All subsequent experiments were therefore carried out with incubation times of 20 min and with microsomal fractions equivalent to 1 g liver.

Estimation of metabolites. At the end of the incubation period, reaction mixtures were shaken with ethyl acetate (15 ml) and centrifuged to separate the phases. In each experiment, zero time values were obtained by shaking reaction mixtures with ethyl acetate before the addition of substrate. After addition of substrate, shaking was repeated and both the ethyl acetate and aqueous phases were treated as described below. In each experiment the zero time values were subtracted from those obtained after 20 min incubation. A portion (12 ml) of the ethyl acetate phase was filtered through Whatman No. 1 paper and evaporated to dryness under reduced pressure. The residue was dissolved in ethanol (60 μl) and 40 μl of the solution was applied to a thin layer chromatogram with a "Microcap", (Shandon Southern Instruments Ltd. Camberley, Surrey). Unlabelled reference compounds were also applied to each chromatogram to facilitate the location of metabolites in u.v. light. The chromatograms were developed with solvent (a). The areas of Silica gel containing the metabolites were removed from the plate and transferred to vials for the estimation of radioactivity by liquid scintillation counting with a Packard Tri-Carb spectrometer (model 3320 or 3375). The scintillation liquid consisted of a mixture of toluene–dioxan–ethanol (5:5:3, by vol.) containing 7 g butyl-PBO and 80 g naphthaline/l.

The amounts of water-soluble metabolites remaining in the aqueous phases after extractions with ethyl acetate were estimated by the method used in a study of the GSH conjugates of oestradiol.[26] Aqueous phase (2 ml) was added to boiling ethanol (12 ml) and, after cooling, the precipitated protein was removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure and the residue washed with ethanol (2 ml). The washed residue was dissolved in water (1 ml) and a portion of the solution (0·5 ml) transferred to a vial for the estimation of radioactivity as before. All determinations are the mean values of at least three experiments.

Qualitative examination of the water soluble metabolites. Each substrate (7,12-dimethylbenz[a]anthracene, 7-hydroxymethyl-12-methylbenz[a]anthracene or 12-hydroxymethyl-7-methylbenz[a]anthracene) was incubated for 20 min in reaction mixtures (30 ml) containing all constituents in the proportions described for standard mixtures. The water-soluble metabolites were treated as described[26] and the purified material applied as a band to the base of a paper chromatogram that was developed with solvent (b). The metabolites were located by marking the chromatograms into twenty horizontal bands and removing a small portion of each for radioactivity determinations. The band of paper associated with radioactivity was cut out and the metabolites eluted with water. After concentration to approx. 0·2 ml under reduced pressure the solutions of the metabolites were applied to paper chromatograms that were developed with solvent (c).

RESULTS

Description of metabolites estimated. ^{3}H-Labelled 7,12-dimethylbenz[a]anthracene, 7-hydroxymethyl-12-methylbenz[a]anthracene or 12-hydroxymethyl-7-methylbenz-[a]anthracene were incubated in standard reaction mixtures and the amounts of the three types of metabolites that were synthesized during the incubation were estimated. When the reaction mixtures were extracted with ethyl acetate, the organic solvent phase contained dihydrodiols and phenolic metabolites that were separated by TLC, and the aqueous phase contained water soluble metabolites. The 8,9-dihydrodiols of these three substrates and the 10,11-dihydrodiol of 7-hydroxy-methyl-12-methylbenz[a]anthracene, which have all been reported as metabolic products that are synthesized by rat liver,[7,8] were located and the amounts present estimated as described. The phenolic metabolites of the two hydroxymethyl derivatives of 7,12-dimethylbenz[a]anthracene have been tentatively identified as the 3- and 4-hydroxy derivatives.[8] Since these compounds have very similar chromatographic properties, they were estimated together and are referred as "phenolic metabolites". Estimations of the corresponding derivatives of 7,12-dimethylbenz[a]anthracene were not attempted since they were not completely separated from other metabolites by the chromatographic system employed.

Evidence that the purified water soluble metabolites were GSH conjugates was obtained from paper chromatograms developed with solvent (c). The metabolites from each of the three substrates showed one area of radioactivity corresponding with an area that gave a purple colour with ninhydrin and a positive reaction for divalent sulphur when chromatograms were sprayed with the $K_2Cr_2O_7$–$AgNO_3$ reagent.[24]

Furthermore, the chromatographic properties of the water-soluble metabolite from 7,12-dimethylbenz[a]anthracene were identical to that of synthetic *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione (R_f 0·21) and the corresponding metabolites of the two hydroxymethyl derivatives of 7,12-dimethylbenz-[a]anthracene had the same R_f-values (0·17) as synthetic *S*-(5,6-dihydro-6-hydroxy-7-hydroxymethyl-12-methylbenz[a]anthracen-5-yl)glutathione. These mobilities are also characteristic of GSH conjugates that are formed in the metabolism of other aromatic hydrocarbons.[27] Further evidence of the participation of GSH in the formation of some of these metabolites is summarized in Table 1. These results show that both GSH and the dialysed soluble fraction of rat-liver are required for the formation of maximum amounts of the water-soluble metabolite of 7-hydroxymethyl-12-methylbenz[a]anthracene. These metabolites are referred to as GSH conjugates but the double bond of the substrate molecules involved in their formation have not yet been determined.

Table 1 summarizes the effects of the various constituents of the complete reaction mixtures on the formation of 7-hydroxymethyl-12-methylbenz[a]anthracene metabolites. This table shows that both the microsomal fraction and NADPH are essential for the synthesis of all the derivatives, since either omission of the cofactor or heating the microsomal fraction before incubation prevents all metabolism. These are the only constituents needed for the formation of dihydrodiols and phenolic metabolites, but the synthesis of maximum amounts of GSH conjugates also needs GSH and dialysed liver fraction. Although the presence of these two constituents caused a slight decrease in dihydrodiol formation they were included in standard reaction mixtures. This enabled the amounts of all types of metabolites to be determined in the same mixture.

Effect of oestradiol on the metabolism of 7,12-*dimethylbenz*[*a*]*anthracene and its hydroxymethyl derivatives.* The effect of the addition of oestradiol on the amounts of the various metabolites of 7,12-dimethylbenz[a]anthracene and its hydroxymethyl derivatives synthesized in standard reaction mixtures is summarized in Table 2. Considerable differences in the ratio of the various types of metabolite formed from these substrates is apparent. The largest amount of 8,9-dihydrodiol and the smallest amount of GSH conjugate are synthesized from 7,12-dimethylbenz[a]-anthracene but the reverse situation applies when 7-hydroxymethyl-12-methylbenz-[a]anthracene was used as substrate. In each case the relative amounts of 12-hydroxymethyl-7-methylbenz[a]anthracene metabolites fall in an intermediate position. The addition of oestradiol in concentrations equal to that of the substrates inhibits the synthesis of all types of metabolite. This suggests that the steroid may be preventing the formation of the epoxide intermediates rather than the three types of reaction that are involved in their further metabolism.

Further evidence that oestradiol inhibits epoxide formation was obtained from estimations of the total amount of substrate (7-hydroxymethyl-12-methylbenz-[a]anthracene) metabolized as well as the amounts of metabolites formed. The effect on these values of the addition of oestradiol or of cyclohexene oxide and the omission of GSH and dialysed soluble fraction from reaction mixtures are summarized in Fig. 1. The results are expressed as percentages of the comparable values obtained with standard reaction mixtures. Incubations which included cyclohexene oxide, an inhibitor of 'epoxide hydrase',[28] showed that although the amounts of the 10,11-

Table 1. Metabolism of 7-hydroxymethyl-12-methylbenz[a]anthracene by subcellular fractions of female rat-liver

Modification to standard reaction mixture	Radioactivity (dis/min × 10^{-5}) associated with metabolites			
	10,11-Dihydrodiol	8,9-Dihydrodiol	Phenolic metabolites	GSH conjugates
None	3·3	5·6	52·8	31·5
Microsomal fraction heated*	0	0	0·8	1·0
NADPH-generating system omitted	0	0·1	7·7	1·3
Dialysed soluble fraction omitted	3·4	6·4	57·9	13·5
GSH omitted	3·2	5·9	57·9	13·3
Dialysed soluble fraction and GSH omitted	3·9	6·2	53·4	1·9

* Microsomal fraction heated at 100° for 5 min and cooled before incubation. Standard reaction mixtures contained microsomal fraction from 1 g liver; dialysed soluble fraction (1 ml); $NADP^+$ (3 mg); glucose 6-phosphate (15 mg); glucose 6-phosphate dehydrogenase (12 units); GSH (3 mg); 50 mM-phosphate buffer, pH 7·4 (10 ml) and 7-hydroxymethyl-12-methylbenz[a]anthracene (0·8 μmole) dissolved in ethanol (0·3 ml). After being incubated for 20 min at 37° the mixtures were extracted with ethyl acetate (15 ml) and the metabolites estimated as described in the text.

TABLE 2. THE INHIBITION OF THE METABOLISM OF 7,12-DIMETHYLBENZ[a]ANTHRACENE AND ITS HYDROXYMETHYL DERIVATIVES BY OESTRADIOL

Substrate	Metabolite	Amounts (nmole) of metabolites formed when oestradiol was		Inhibition %
		Absent	Present	
7,12-Dimethylbenz[a]anthracene	8,9-Dihydrodiol	7·7	3·9	49
	GSH conjugates	5·5	4·0	27
7-Hydroxymethyl-12-methylbenz[a]anthracene	10,11-Dihydrodiol	1·4	0·5	64
	8,9-Dihydrodiol	2·4	0·4	83
	Phenolic metabolites	22·8	7·3	68
	GSH conjugates	13·6	5·3	61
12-Hydroxymethyl-7-methylbenz[a]anthracene	8,9-Dihydrodiol	4·3	2·4	44
	Phenolic metabolites	11·1	5·8	48
	GSH conjugates	6·6	4·6	30

Each substrate was incubated in standard mixtures either in the absence or in the presence of oestradiol for 20 min. After the mixtures were warmed to 37°, the reactions were started by the addition of substrate (0·8 μmole) in ethanol (0·3 ml) or by a mixture of substrate (0·8 μmole) and oestradiol (0·8 μmole) in ethanol (0·3 ml). After the incubation, mixtures were extracted with ethyl acetate (15 ml) and the metabolites estimated as described in the text.

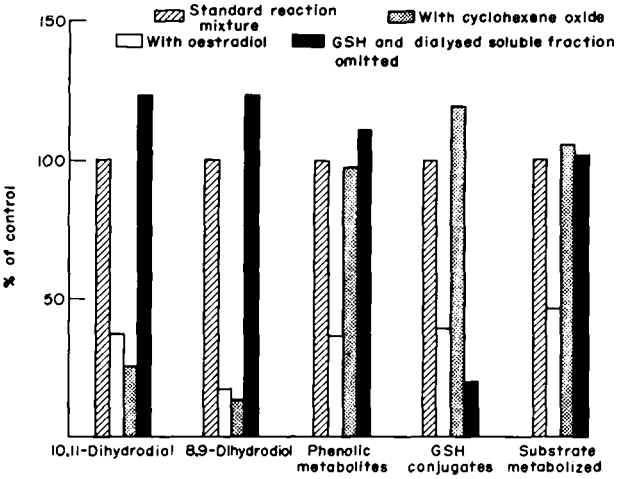


FIG. 1. Effect of inhibitors on the metabolism of 7-hydroxymethyl-12-methylbenz[a]anthracene. Standard reaction mixtures described in the text were incubated with 7-hydroxymethyl-12-methylbenz-[a]anthracene with modifications as indicated. Reactions were started by addition of substrate (0·8 μmole) or a mixture of substrate and inhibitor. Inhibitors consisted of oestradiol (0·8 μmole) or cyclohexene oxide (4 μmole). In experiments to determine the amount of substrate lost, reactions were started by the addition of 7-hydroxymethyl-12-methylbenz[a]anthracene (0·08 μmole). Results are expressed as percentages of the amounts of products formed in control experiments in which complete reaction mixtures were incubated with the substrate in the absence of inhibitors.

and 8,9-dihydrodiols were reduced, neither the synthesis of the other metabolites nor the total amount of substrate metabolized was affected. On the other hand, oestradiol reduced both the amounts of all the metabolites formed and the amount of substrate lost during the incubations. This indicates that these two inhibitors of 10,11- and 8,9-dihydrodiol synthesis are effective by different mechanisms, the conversion of 7-hydroxymethyl-12-methylbenz [a]anthracene into the corresponding epoxide intermediates being inhibited by oestradiol and the conversion of the epoxides into the dihydrodiols being inhibited by cyclohexene oxide. Similarly, the amount of substrate metabolized was not affected when the synthesis of GSH conjugates was prevented. Hence, in the complete mixtures, the inhibitory effect of oestradiol on the synthesis of the GSH conjugates is considered to be due to the inhibition of the formation of the epoxide intermediate rather than the inhibition of the conjugation of the epoxide with GSH.

DISCUSSION

The NADPH-dependent microsomal mixed function oxidase responsible for the biological transformations of many foreign compounds also catalyses the oxidative metabolism of naturally occurring steroids such as testosterone[29] and oestradiol.[30] Steroid hydroxylase is inhibited by the *in vitro* addition of hexobarbital or chlorpromazine, and since the enzymes that metabolize both drugs and steroids have many similar properties, it has been suggested that steroids are the naturally occurring substrates for drug metabolizing enzymes.[31] Furthermore, steroid hormones are competitive inhibitors of ethyl morphine and hexobarbital oxidation by

rat-liver microsomal fractions[32] and of "benzo[a]pyrene hydroxylase" by mouse-liver microsomes.[33] The progestational steroids, norethynodrel and progesterone, inhibit numerous types of reaction involved in drug metabolism and the metabolic pathways most affected are those in which hydroxylated or oxidized products are formed.[34]

The inhibition of the metabolism of 7,12-dimethylbenz[a]anthracene and its hydroxymethyl derivatives by oestradiol is another example of an apparent competition between a foreign compound and a steroid for the microsomal mixed function oxidases of liver. Since the amounts of three different types of hydrocarbon derivatives, the dihydrodiols, the phenolic metabolites and the GSH conjugates, are all decreased by oestradiol and all arise via epoxide intermediates, the steroid probably acts by inhibiting epoxide synthesis. Furthermore, cyclohexene oxide, which decreases dihydrodiol formation by inhibiting the conversion of epoxides into dihydrodiols by "epoxide hydrase",[28,35] has no effect on the amount of substrate metabolized. On the other hand, oestradiol decreases both the amounts of dihydrodiols formed and the amount of substrate metabolized and is therefore likely to exert its effect by reducing the amount of epoxide intermediate formed. The prevention of the formation of GSH conjugates, by omission of GSH and soluble liver fraction, also had no effect on the amount of substrate metabolized but produced small increases in dihydrodiol synthesis.

Furthermore, since phenolic derivatives probably arise by the non-enzymic isomerization of epoxides,[18,23] it is more likely that the steroid prevents the formation of epoxides by competition for substrate than by inhibiting their chemical rearrangement to phenolic metabolites. Similarly, the amounts of naphthol and dihydrodiol produced when naphthalene is metabolized by rat-liver microsomal fraction were decreased by the addition of GSH.[15] The double bonds of the hydrocarbon molecules that are involved in the formation of GSH conjugates is not known. However, both 7,12-dimethylbenz[a]anthracene[14] and 7-hydroxymethyl-12-methylbenz[a]anthracene* are converted into conjugates of the 5,6-oxides by livers of rats that were pretreated with 3-methylcholanthrene.

The probable inhibition of the epoxidation of 7,12-dimethylbenz[a]anthracene and its derivatives by oestradiol suggests the possibility that epoxides may also be intermediates in the metabolism of the steroid, since similarities exist between the metabolic pathways involved in the biological transformations of the two compounds. Thus the oestrogen aromatic ring is hydroxylated, chiefly at C-2, and converted into water-soluble and protein bound metabolites.[36] The water soluble metabolites from incubations of oestradiol with liver microsomal fractions, NADPH and GSH have been identified as the 1- and 4-glutathione-mono and dithioethers of 2-hydroxyoestradiol and 2-hydroxyoestrone.[26,37] Although it has generally been considered that these metabolites are formed via an *o*-semiquinone intermediate,[26,38] epoxidation of the aromatic ring of oestradiol followed by conjugation with GSH might be considered as a possible alternate pathway.

If apparently spontaneous mammary tumours were, in fact, induced by aromatic hydrocarbons present in the environment, oestradiol may exert its anti-carcinogenic effect by competing for binding sites on the mixed-function oxidase enzyme complex thus interfering with the metabolism of the hydrocarbon. This mode of

* G. R. Keysell, unpublished observation.

action has also been proposed to explain the inhibition of "aryl hydrocarbon hydroxylase' by oestradiol and of oestrogen hydroxylase by benzo[a]pyrene.[39]

The marked reduction in the amounts of the 8,9- and 10,11-dihydrodiols formed when the "epoxide hydrase" inhibitor, cyclohexene oxide was present in the incubation mixtures containing 7-hydroxymethyl-12-methylbenz[a]anthracene provides indirect evidence for the metabolic formation of the related 8,9- and 10,11-epoxides. Although it has been possible to demonstrate directly that the 5,6-epoxide, the "K-region" epoxide, of the hydroxymethyl derivative is formed in microsomal systems,[17] direct evidence for the formation of non "K-region" epoxides is so far lacking.

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