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METHYLCHOLANTHRENE: A POSSIBLE PSEUDOSUBSTRATE FOR ADRENOCORTICAL 17α-HYDROXYLASE AND ARYL HYDROCARBON HYDROXYLASE

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Abstract—In cultured bovine adrenocortical cells, loss of 17α-hydroxylase activity was observed after incubation with 3-methylcholanthrene (3-MC). The suppression of 17α-hydroxylase by 3-MC was rapid (50% loss of activity in 10 hr at 1 μM 3-MC), did not exhibit a lag period, and was not affected by cycloheximide. Direct effects of 3-MC on 17α-hydroxylase were observed only at high concentrations, but the concentration for 50% loss of activity was 0.3 μM when 3-MC was added for 24 hr prior to assay of 17α-hydroxylase. High concentrations (to 40 μM) of substrate (progesterone), did not affect the loss of activity due to 3-MC. Loss of 17α-hydroxylase activity was specific; 11β-hydroxylase was unaffected and cell growth was unaltered. However, 22-amino-23,24-bisnorchol-5-en-3β-ol, an inhibitor of 17α-hydroxylase, partially prevented the loss of 17α-hydroxylase at 1–30 nM. 3-MC is thought to induce cytochrome P-450s via a receptor with high affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD was without effect on 17α-hydroxylase over the range of 10 nM to 10 μM. Benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene, chrysene, and methylphenanthrenes suppressed 17α-hydroxylase at high concentrations (10–50 μM for 50% loss of activity). Some steroids that lack a substituent at position 17 also caused loss of 17α-hydroxylase. Like 17α-hydroxylase, bovine adrenocortical cell AHH was found to be suppressed by exposure to 3-MC. Compounds that caused loss of 17α-hydroxylase caused loss of AHH, with a similar order of potency and at similar concentrations. Suppression of AHH by 3-MC did not require protein synthesis and was prevented by an inhibitor of enzymatic activity, α-naphthoflavone. This implies a degree of similarity of the cytochrome P-450s for 17α-hydroxylase and adrenal AHH, but the activities were shown to be likely due to different enzymes. The suppression of 17α-hydroxylase and AHH by 3-MC appears not to occur by a receptor-mediated mechanism but to be similar to the suppression of 11β-hydroxylase and 21-hydroxylase by steroid pseudosubstrates previously observed.

3-Methylcholanthrene (3-MC) has been used extensively as an inducer of a class of xenobiotic-metabolising cytochrome P-450s that are particularly known for their abilities to activate precarcinogens to their ultimate carcinogenic forms [1, 2]. Methylcholanthrene is thought to act via a cytosolic receptor that recognizes several xenobiotic inducers of cytochrome P-450s, with particularly high affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [1–4]. Expression of this receptor and consequent sensitivity of cells to cytochrome P-450 induction by TCDD and 3-MC are regulated by the *Ah* locus in mice [1, 2]. Several cell types in culture have been shown to exhibit cytochrome P-450 induction when exposed to either 3-MC or TCDD [5–7]. During an investigation of the regulation of aryl hydrocarbon hydroxylase (AHH) in cultured adrenocortical cells, we observed unexpectedly that 3-methylcholanthrene suppressed aryl hydrocarbon hydroxylase activity, and investigation of the specificity of this effect revealed a more striking loss of steroid 17α-hydroxylase after incubation of cells with 3-MC. The present experiments investigate this phenomenon. It does not appear that 3-MC affects 17α-hydroxylase and AHH through a receptor-mediated mechanism. The loss of activity appears very similar to the previously described loss of activity of 11β-hydroxylase and 21-hydroxylase on interaction with steroid pseudosubstrates [8–11]. Testicular 17α-hydroxylase, but not adrenal 17α-hydroxylase, was shown to be suppressed by steroid pseudosubstrates [9, 12–14]. The currently described pseudosubstrate effect differs from the pseudosubstrate effects on 11β-hydroxylase and 21-hydroxylase in that the suppressor is not an adrenal steroid but a xenobiotic polycyclic hydrocarbon, thus suggesting that pseudosubstrate effects on cytochrome P-450s are not confined to steroids.

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MATERIALS AND METHODS

Preparation of third passage bovine adrenocortical cell cultures. Cells dispersed from bovine adrenal cortex tissue were prepared as previously described [15], and stored frozen in 5% dimethyl sulfoxide until required for experiments. On thawing, cells were plated in 10% fetal bovine serum in a 1:1 mixture of Ham's F-12 medium and Dulbecco's

modified Eagle's medium, which was found to be optimal for adrenocortical cell growth [16]. Plates were pre-coated with fibronectin to improve cell attachment and growth [16]. The dishes were then incubated at 37° in a humidified atmosphere of 5% O_2, 85% N_2 and 10% CO_2 in a Queue Systems (Parkersburg, WV) three-gas incubator. Cells were grown in fetal bovine serum that had been processed to remove lipids by extraction with 20% butanol in diisopropyl ether at 4° [17]. Lipids removed by this procedure include cholesterol, fatty acids, and triglycerides, but not phospholipids [17]. After extraction, the serum was dialyzed against 100 vol. of water (Milli-Q grade) at 4° for 24 hr. Third passage cultures of bovine adrenocortical cells were prepared by growth through three passages in this extracted, dialyzed serum in Dulbecco's Eagle's medium/F-12 medium; 1:1, with 100 ng/ml partially purified bovine brain fibroblast growth factor (FGF), supplied by Dr. Denis Gospodarowicz of the University of California, San Francisco, as previously described [14]. For experiments, cells were subcultured into 24-well plates (2 cm^2 area per well) (Falcon, Cockeysville, MD). Each well contained approximately 10^4 cells. Cell numbers were measured using a Coulter Counter (Coulter Electronics, Hialeah, FL).

Assay of 17α-hydroxylase. Cultures were washed by incubation with medium and 10% ether-extracted serum for 1 hr prior to the assay for 17α-hydroxylase. For assay, cells were incubated for 30 min or 1 hr with 10 μM progesterone as substrate in medium with 10% ether-extracted serum. Ten micromolar is close to the K_m for progesterone on 17α-hydroxylase in this system [14, 18, 19]. The products, 17α-hydroxyprogesterone, deoxycortisol, and cortisol, were separated by high performance liquid chromatography (HPLC) as previously described [19] and were summed for calculation of 17α-hydroxylase activity. The medium was extracted with 4 vol. dichloromethane (HPLC grade). The dichloromethane phase containing unconjugated steroids was evaporated, redissolved in 20% methanol, and chromatographed using an Altex 320 high performance liquid chromatograph on an octadecylsilyl column (Regis, Morton Grove, IL). Elution was with various concentrations of methanol as follows: a linear gradient of 20 to 62.5% over 10 min; held at 62.5% for 10 min; a linear gradient of 62.5 to 100% over 10 min [19]. Detection was by absorbance at 240 nm. Conversion rates for products were calculated by comparing peak heights with peak heights of known amounts of standards, calculating percent conversion to each product, and converting percent conversion to pmoles from the amount of precursor added. Conversion rates were expressed as pmoles/10^4 per cells per hr.

Assay of 11β-hydroxylase. 11β-Hydroxylase was assayed as for 17α-hydroxylase using medium containing 10 μM deoxycorticosterone as 11β-hydroxylase substrate. Ten micromolar is close to the K_m for DOC on 11β-hydroxylase in this system [10]. A gradient of 20 to 100% acetonitrile over 10 min was used to separate DOC from corticosterone, monitored by absorbance at 240 nm [19].

Assay of aryl hydrocarbon hydroxylase. Aryl hydrocarbon hydroxylase was assayed with [^{3}H]-benzo[*a*]pyrene as substrate. Before incubation with benzo[*a*]pyrene, cells were washed by incubation for 1 hr in medium with 10% ether-extracted serum. [^{3}H]Benzo[*a*]pyrene was obtained from New England Nuclear and was stored in hexane under N_2 in the absence of light until required. Periodically, the purity of the stock solution was examined by HPLC. Just before use, the hexane solution was evaporated under N_2 and a solution of benzo[*a*]pyrene in acetone was added to a concentration of 4 mM. This was then added to medium with 10% ether-extracted serum to a final benzo[*a*]pyrene concentration of 4 μM together with 1 μCi/ml [^{3}H]benzo[*a*]pyrene (final specific activity, 250 Ci/mole). The use of ether-extracted serum in the incubation medium rather than serum albumin alone was found to greatly improve the recovery of polar benzo[*a*]pyrene metabolites. The incubation was normally for 24 hr. Shorter incubation times yielded a lower production of metabolites, but the same pattern of products as 24 hr incubations. Samples of benzo[*a*]pyrene-containing medium without cells were also incubated as controls. No breakdown of [^{3}H]benzo[*a*]pyrene was observed in these control incubations.

The complete medium was assayed without solvent extraction, to avoid loss of polar compounds. The medium was deproteinized prior to analysis by addition of acetonitrile to 80% v/v, then filtered through a 0.45 μm Zetapor filter (AMF, Seguin, TX), diluted with water to 20% acetonitrile v/v, and concentrated by loading on a octadecylsilyl loop column. The loop column was then switched in line with a separation column, and gradient elution from 20 to 100% acetonitrile in 17 mM NaCl was performed. NaCl was used in the aqueous phase to increase the retention of the polar products.* Radioactive products were collected using a programmable fraction collector with 0.1-min sample collection during peaks and 1-min sample collection between peaks. Products were evaluated as pmoles produced per 10^4 cells per 24 hr. Total AHH activity was calculated as the sum of phenol, diol, tetrol and conjugate products formed from benzo[*a*]pyrene. The pattern of metabolism of benzo[*a*]pyrene by these cells will be presented in detail elsewhere.* Benzo[*a*]pyrene metabolite standards were obtained from the Chemical Repository of the National Cancer Institute.

Addition of non-water-soluble compounds. Non-water-soluble compounds, e.g. 3-methylcholanthrene, benzo[*a*]pyrene, and steroids, were added from 1000-fold concentrates in acetone. Lower concentrations of TCDD in tissue-culture medium (10–100 nM) were prepared from 1000-fold concentrates in acetone. A 1 μM concentration of TCDD in medium was prepared from a 100-fold concentrate. The 10 μM concentration was prepared from a 100 μM acetone concentrate by evaporating ~90% of the acetone and then adding medium with homogenization. In the latter case, TCDD may not be completely solubilized. Acetone at 0.1 or 1% is

* P. J. Hornsby, K. A. Aldern and S. E. Harris, unpublished observations.

non-toxic and has not been observed to have any effects in a variety of different experiments.

Materials. Steroids were obtained from Sigma (St. Louis, MO). Polycyclic aromatic hydrocarbons were obtained from Sigma, except for methylphenanthrenes and 4-methylcholanthrene (22-methylcholanthrene in sterol numbering), which were from ICN-K & K Laboratories (Plainview, NY). Solvents were from J. T. Baker (Phillipsburg, NJ), except for dichloromethane (Fisher, Fair Lawn, NJ). Other chemicals were from Sigma. Sera were purchased from Irvine Scientific (Irvine, CA). Synthetic adrenocorticotropic hormone [ACTH(1–24); Cortrosyn] was from Organon (West Orange, NJ) and when required was added from a 1000-fold concentrate in phosphate-buffered saline with 1% bovine serum albumin.

RESULTS

Loss and recovery of 17α-hydroxylase activity in cultured adrenal cells incubated with 3-methylcholanthrene. Third passage bovine adrenocortical cells have low 17α-hydroxylase activity before reinduction with ACTH. When 1 μM ACTH was added to cultures, 17α-hydroxylase activity was reinduced over a period of 48 hr (Fig. 1). The increase in activity was followed by a slow decline over the following 4 days. When 1 μM 3-methylcholanthrene was added to induced cultures, 17α-hydroxylase activity was lost and remained very low, despite removal of 3-methylcholanthrene after 24 hr (Fig. 1). 17α-Hydroxylase in cultures that had lost activity after exposure to 3-MC remained re-inducible by fresh addition of ACTH, however. No effects of 3-MC were seen in non-ACTH-induced cultures with the low basal level of 17α-hydroxylase.

Lack of direct inhibition of 17α-hydroxylase by 3-MC. The suppression of 17α-hydroxylase by 3-MC would be most readily explained by direct inhibition of the enzyme by 3-MC. However, when 3-MC was added directly to cells during assay of 17α-hydroxylase, inhibition was observed only at >3 μM, and even 100 μM inhibited only to 35% (Fig. 2). In contrast, when various concentrations of 3-MC were added to cultures for 24 hr and then removed prior to assay of 17α-hydroxylase, the EC_{50} for 3-MC suppression of activity was 0.3 μM (Fig. 2).

Specificity of 3-MC suppression of 17α-hydroxylase: Effects on 11β-hydroxylase and cell growth. The loss of 17α-hydroxylase activity was specific for that enzyme and did not extend to another steroidogenic enzyme activity, 11β-hydroxylase (Fig. 2). Concentrations up to 10 μM also had no effect on cellular growth rate (Fig. 2). However, 100 μM 3-MC did inhibit cell growth and also caused loss of 11β-hydroxylase activity.

Time-course of suppression of 17α-hydroxylase by 3-MC. 3-MC appeared not to suppress 17α-hydroxylase during a 30-min assay period but was effective after being incubated for 24 hr. When the time-course of the suppression effect was examined in more detail, it was found that 1 μM 3-MC caused 50% loss of activity in 10 hr and 10 μM 3-MC caused 50% loss after 1.8 hr (followed by a 1-hr recovery period to wash out 3-MC) (Fig. 3).

Lack of effect of inhibition of protein synthesis. The time-course of loss of 17α-hydroxylase did not

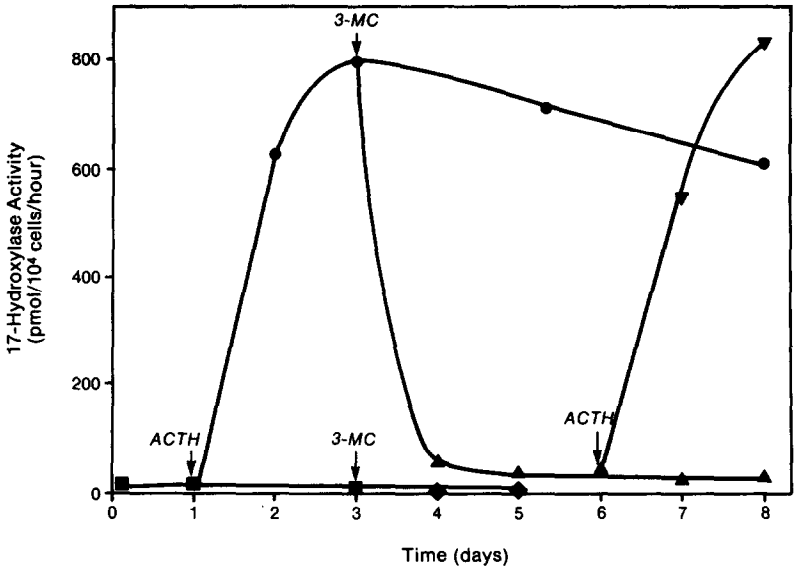

Fig. 1. Suppression of 17α-hydroxylase by 3-methylcholanthrene. Third passage bovine adrenocortical cells were cultured in medium with 10% ether-extracted serum and 17α-hydroxylase was measured at 24-hr intervals, as described in Materials and Methods. 1 μM concentration of ACTH was added to some cultures (●) to re-induce 17α-hydroxylase. ACTH was added for a 24-hr period. Forty-eight hours after exposure to ACTH, when 17α-hydroxylase activity was maximal, some ACTH-treated (▲) and control (◆) cultures received 1 μM 3-MC. 3-MC was removed from these cultures after an additional 24 hr. Additionally, some cultures previously exposed to ACTH and 3-MC were restimulated with ACTH at the indicated time (▼).

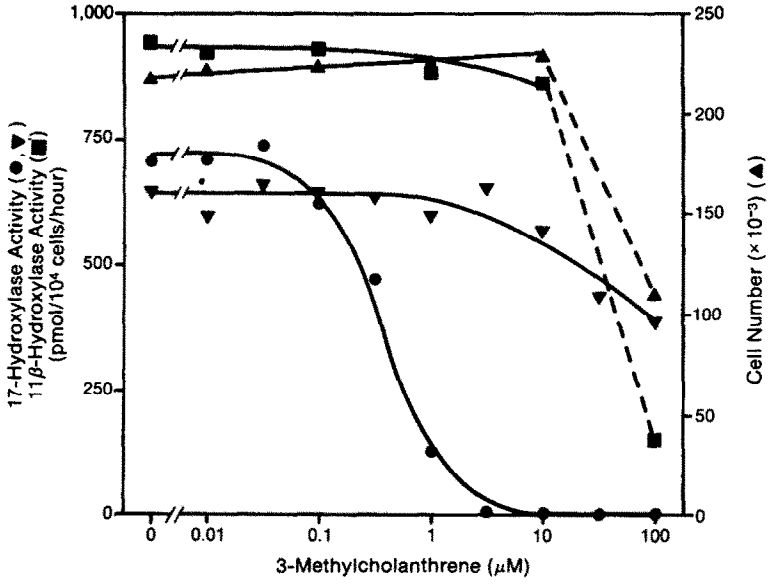

Fig. 2. Dose-response curves for 3-methylcholanthrene effects on 17α-hydroxylase, 11β-hydroxylase, and cell growth. Third passage bovine adrenocortical cells were cultured in medium with 10% ether-extracted serum and 17α-hydroxylase was re-induced as described in the legend to Fig. 1. For some cultures (▼) 3-MC at the indicated concentrations was included in the 30-min incubation with substrate progesterone for assay for 17α-hydroxylase. For others (●), the indicated concentrations of 3-MC were added for 24 hr and then removed prior to assay of 17α-hydroxylase, as described in Materials and Methods. A further set of cultures (■), after incubation with 3-MC for 24 hr, was assayed for 11β-hydroxylase activity. Additionally, cells were plated at low density and after 24 hr changed to medium containing the indicated concentrations of 3-MC. After 4 days, cell numbers were estimated as described (▲).

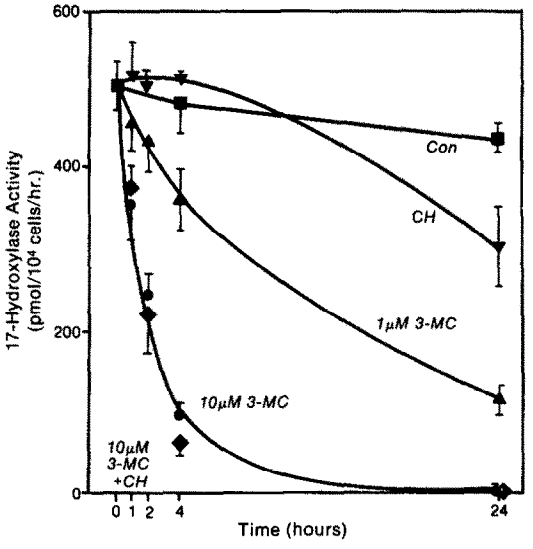

Fig. 3. Time-course of suppression of 17α-hydroxylase by 3-methylcholanthrene. 17α-Hydroxylase was re-induced in third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum as described in the legend to Fig. 1. Some cultures were then incubated with 1 μM 3-MC (▲); 10 μM 3-MC (●); 10 μg/ml cycloheximide (▼); 10 μM 3-MC plus 10 μg/ml cycloheximide (◆); or received no treatment (■). 17α-Hydroxylase was measured at the indicated times. Data are means from duplicate wells ± standard deviation.

appear to be compatible with induction of new protein products for the suppression effect to occur. This was confirmed by inclusion of cycloheximide at a concentration that inhibits amino acid incorporation by ~90% [20]. The time-course of 10 μM 3-MC suppression of 17α-hydroxylase was unaltered (Fig. 3). Cycloheximide alone caused about 40% loss of 17α-hydroxylase in 24 hr (Fig. 3).

Prevention of 17α-hydroxylase suppression by an inhibitor. Previously, two other adrenocortical cytochrome P-450 activities, 11β-hydroxylase and corticosterone methyl oxidase, were found to be stabilized in the presence of the pseudosubstrate cortisol by metyrapone, an inhibitor of mitochondrial adrenocortical cytochrome P-450s [9, 21]. 22-Amino-23,24-bisnorchol-5-en-3β-ol is a potent inhibitor of 17α-hydroxylase [22]. The half-maximal effective concentration for inhibition of 17α-hydroxylase by this compound was 5 nM in the present system (Fig. 4). When 22-amino-23,24-bisnorchol-5-en-3β-ol was added with 1 μM 3-MC, the loss of 17α-hydroxylase was partially prevented at concentrations of 22-amino-23,24-bisnorchol-5-en-3β-ol from 1 to 30 nM (Fig. 4). Higher concentrations did not prevent the loss by 3-MC and were themselves inhibitory. This appeared to result from failure to wash out all of the inhibitor in the incubation prior to the assay of 17α-hydroxylase. Control experiments with preincubation of cells with higher concentrations of 22-amino-23,24-bisnorchol-5-en-3β-ol followed by 17α-

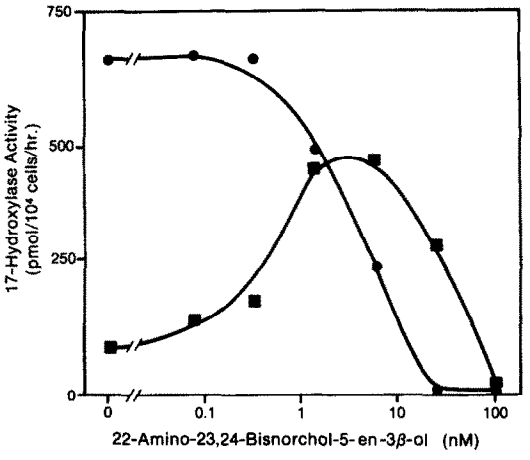

Fig. 4. Partial prevention by an inhibitor of loss of 17α-hydroxylase. 17α-Hydroxylase was re-induced in third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum as described in the legend to Fig. 1. For some cultures (●), the 17α-hydroxylase inhibitor, 22-amino-23,24-bisnorchol-5-en-3β-ol at the indicated concentrations was included in the 30-min incubation with substrate progesterone for assay of 17α-hydroxylase. For others (■), the indicated concentrations of inhibitor were added for 24 hr together with 1 μM 3-MC, and then both 3-MC and inhibitor were removed prior to assay of 17α-hydroxylase.

hydroxylase assay also showed carryover of the inhibitor.

Lack of effect of substrate on suppression by 3-MC. When cells were incubated with the substrate, progesterone, at 0.1 to 40 μM, together with 1 μM 3-MC, no effect on the suppression of 17α-hydroxylase was observed, when added either together with 3-MC or after removal of 3-MC (Table 1).

Lack of effects of oxygen and antioxidants. The loss of 11β-hydroxylase activity in the presence of the pseudosubstrate cortisol is prevented by lowered oxygen [8, 14], phenol and phenolic compounds such as butylated hydroxyanisole (BHA) [8, 23], sulfoxides such as dibenzyl and dimethyl sulfoxides [8, 24], and ascorbate [8, 14]. These substances had no effect on the loss of 17α-hydroxylase in the presence of 3-MC (Table 1).

Comparison of 3-MC and other xenobiotics on suppression of 17α-hydroxylase. The molecular structure required for suppression of 17α-hydroxylase was examined by comparison of 3-MC with other compounds. 4-MC was similar in effect to 3-MC (Fig. 5). The methyl group and its position may not be critical to activity. Other polycyclic aromatic hydrocarbons, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene (DMBA), benzo[*a*]pyrene, chrysene, and methylphenathrenes were all much less effective, with EC_{50} values from 10 to 50 μM (Fig. 5 and Table 2). Our observations on the effects of DMBA on 17α-hydroxylase are similar to those of Dibartolomeis and Jefcoate [25]; however, these authors investigated the long-term direct effect of DMBA on 17α-hydroxylase, rather than the effect of prior exposure followed by assay of activity. 2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD), which has effects on cytochrome P-450 induction similar to those of 3-MC, did not suppress 17α-hydroxylase when used over the range of 10 nM to 10 μM.

Effects of steroids on 17α-hydroxylase. For other adrenocortical cytochrome P-450s, with 11β-hydroxylase, corticosterone methyl oxidase, and 21-hydroxylase activities, we previously documented suppression of activity by various steroids [8–10]. 17α-Hydroxylase was noted to be relatively resistant to suppression by steroids [9, 14]. This was confirmed here; the major secreted adrenocortical steroids, cortisol, androstenedione, 17α-hydroxyprogesterone, and progesterone (Table 2) and also deoxycortisol, corticosterone, and deoxycorticosterone, had little effect on 17α-hydroxylase. Some steroids lacking a substituent at the 17 position on the steroid nucleus, androst-16-ene and estra-1,3,5(10)-triene

Table 1. Lack of effect of substrate, oxygen, or antioxidants on suppression of 17α-hydroxylase by 3-methylcholanthrene

Additions	17α-Hydroxylase activity (pmoles/10^4 cells/hr)
None	680 ± 90
1 μM 3-Methylcholanthrene	90 ± 30
1 μM 3-MC + 40 μM progesterone	70 ± 40
1 μM 3-MC; 40 μM progesterone added for 24 hr after 3-MC removed	120 ± 40
None; 2% oxygen	790 ± 70
1 μM 3-MC at 2% oxygen	110 ± 20
1 μM 3-MC at 2% oxygen + 100 μM BHA	120 ± 40
1 μM 3-MC at 2% oxygen + 100 μM dibenzyl sulfoxide	110 ± 20
1 μM 3-MC at 2% oxygen + 100 mM dimethyl sulfoxide	90 ± 10
1 μM 3-MC at 2% oxygen + 5 mM ascorbate	80 ± 20

3-Methylcholanthrene (1 μM) was added to third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum, after re-induction of 17α-hydroxylase as described in Fig. 1, together with the indicated compounds at 5% oxygen (standard) or 2% oxygen. After 24 hr, compounds were removed and 17α-hydroxylase activity was measured as described in Materials and Methods. The assays of 17α-hydroxylase were all performed at 5% O_2. Data ± S.D. are from triplicate wells.

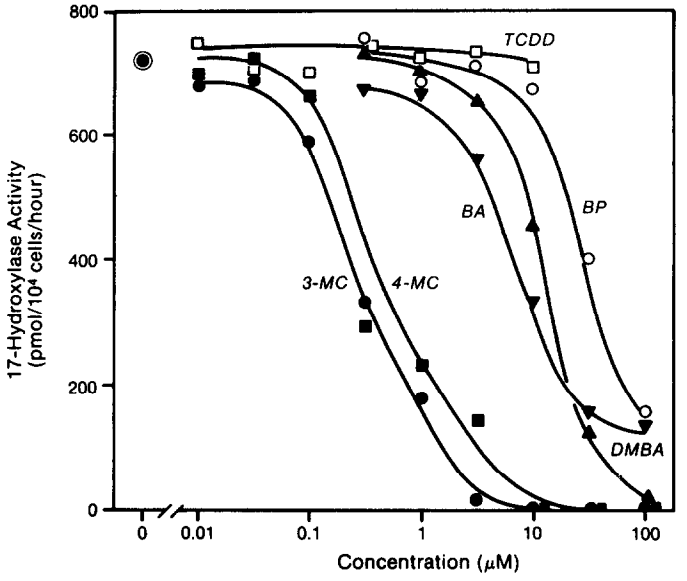

Fig. 5. Dose–response curves for suppression of 17α-hydroxylase by 3-methylcholanthrene and other compounds. Third passage bovine adrenocortical cells were cultured in medium with 10% ether-extracted serum and 17α-hydroxylase was re-induced as described in the legend to Fig. 1. Some cultures were then incubated with the indicated concentrations of 3-methylcholanthrene (●); 4-methylcholanthrene (4-MC; ■); benz[*a*]anthracene (BA; ▼); 7,12-dimethylbenz[*a*]anthracene (DMBA; ▲); benzo[*a*]pyrene (BP; ○); or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; □). 17α-Hydroxylase was measured after 24 hr.

derivatives, did cause loss of 17α-hydroxylase to ~50% when added at 10 μM.

Concomitant loss of aryl hydrocarbon hydroxylase and 17α-hydroxylase. We compared the effects of 3-MC on 17α-hydroxylase with its effects on AHH activity, assayed with benzo[*a*]pyrene as substrate. Aryl hydrocarbon hydroxylase was present at much lower levels than 17α-hydroxylase (~1.5% of re-induced 17α-hydroxylase activity). Incubation of cultures with 1 μM 3-MC for 24 hr suppressed aryl hydrocarbon hydroxylase activity as well as 17α-hydroxylase (Fig. 6). Unlike 17α-hydroxylase, AHH spontaneously increased to original levels 3–4 days after 3-MC was removed from the culture (Fig. 6).

Table 2. Suppression of 17α-hydroxylase: Comparison of effects of 3-methylcholanthrene and other compounds

Additions	17α-Hydroxylase activity (pmoles/10⁴ cells/hr)
None	770 ± 30
Polycyclic aromatic hydrocarbons (see also Fig. 5)	
3-Methylcholanthrene	0
Chrysene	560 ± 110
1-Methylphenanthrene	720 ± 70
2-Methylphenanthrene	730 ± 30
Steroids	
Progesterone	810 ± 40
17-Hydroxyprogesterone	840 ± 70
Cortisol	790 ± 60
Androstenedione	700 ± 10
5α-Androst-16-en-3α-ol	470 ± 90
5α-Androst-16-en-3β-ol	390 ± 140
5α-Androst-16-en-3-one	550 ± 40
Estra-1,3,5(10)-triene-3,16β-diol	430 ± 30

A 10 μM concentration of the indicated compounds was added to third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum, after re-induction of 17α-hydroxylase as described in Fig. 1. After 24 hr, compounds were removed, and 17α-hydroxylase activity was measured as described in Materials and Methods. Data ± S.D. are from triplicate wells.

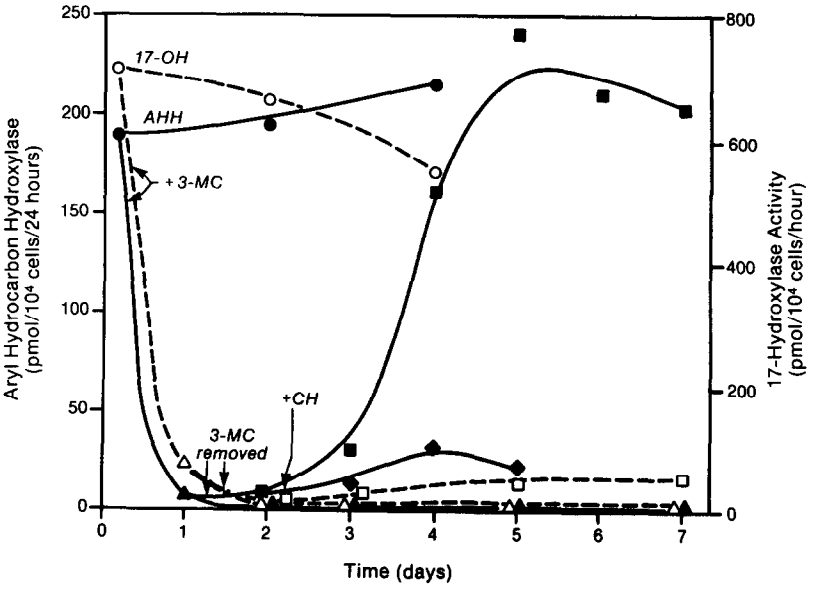

Fig. 6. Comparison of effects of 3-MC on 17α-hydroxylase and aryl hydrocarbon hydroxylase. Third passage bovine adrenocortical cells were cultured in medium with 10% ether-extracted serum and 17α-hydroxylase and aryl hydrocarbon hydroxylase were measured at 24-hr intervals, as described in Materials and Methods. Open symbols are 17α-hydroxylase and solid symbols are AHH. 3-MC (1 μM) was added to some cultures (△, ▲) while others were left untreated (○, ●). 3-MC was removed from some cultures after 24 hr (□, ■); in the others it was re-added at each medium change at 48-hr intervals. Some of the cultures from which 3-MC was removed were also incubated with 10 μg/ml cycloheximide (◆).

This increase was largely inhibited by the simultaneous presence of cycloheximide, added 24 hr after removal of 3-MC. When 3-MC was not removed from the culture, activity remained low. Examination of benzo[*a*]pyrene products by HPLC showed no change in the pattern, but a general depression of all metabolites.

17α-Hydroxylase and aryl hydrocarbon hydroxylase as activities of different cytochrome P-450s. The similarity of the effects of 3-MC on 17α-hydroxylase and AHH raised the possibility that these activities may be due to the same cytochrome P-450. To examine this possibility, we examined the effects of progesterone and 17α-hydroxylase inhibitors on AHH and the effects of benzo[*a*]pyrene and AHH inhibitors on 17α-hydroxylase.

Table 3 shows that the 17α-hydroxylase substrate, progesterone, did not inhibit AHH, and that benzo[*a*]pyrene did not directly inhibit 17α-hydroxylase (when included in the assay medium). The 17α-hydroxylase inhibitor 22-amino-23,24-bisnorcholen-5-en-3β-ol had no effect on AHH, while completely inhibiting 17α-hydroxylase, whereas two compounds that completely inhibited AHH, α-naphthoflavone and ellipticine, inhibited 17α-hydroxylase by 30–40%. These compounds have been shown to inhibit rat adrenal AHH [26].

Additional evidence that AHH and 17α-hydroxyl-

Table 3. Comparison of effects of substrates and inhibitors on 17α-hydroxylase and aryl hydrocarbon hydroxylase

	17α-Hydroxylase activity	Aryl hydrocarbon hydroxylase
Additions	(pmoles/10⁴ cellls/hr)	(pmoles/10⁴ cells/24 hr)
None	860 ± 80	160 ± 40
10 μM Progesterone		190 ± 40
10 μM Benzo[*a*]pyrene	810 ± 90	
1 μM 22-Amino-23,24-bisnor-chol-5-en-3β-ol	0	220 ± 30
10 μM α-Naphthoflavone	690 ± 70	0
10 μM Ellipticine	580 ± 100	0

The indicated compounds were added to third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum, after re-induction of 17α-hydroxylase as described in Fig. 1. Compounds were included in the assays for 17α-hydroxylase and aryl hydrocarbon hydroxylase described in Materials and Methods. Data ± S.D. are from triplicate wells.

Table 4. Comparison of effects of ACTH on 17α-hydroxylase and aryl hydrocarbon hydroxylase

Addition	17α-Hydroxylase activity (pmoles/10^4 cells/hr)	Aryl hydrocarbon hydroxylase (pmoles/10^4 cells/24 hr)
None (uninduced culture)	10	290 ± 40
1 μM ACTH, 48 hr	810 ± 50	230 ± 50

ACTH 1 μM was added to third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum. After 48 hr, ACTH was removed and 17α-hydroxylase activity and aryl hydrocarbon hydroxylase activity were measured as described in Materials and Methods. Data ± S.D. are from triplicate wells.

ase are separate cytochrome P-450 species was obtained by examining the effect of induction by ACTH. Table 4 shows that ACTH, while strongly increasing 17α-hydroxylase, had no effect on AHH. Similar results were obtained with other compounds that stimulate intracellular cAMP production (cholera toxin and forskolin) and by added monobutyryl cAMP (not shown). Thus, bovine adrenocortical cells differ from human adrenocortical cells, in which AHH is stimulated by ACTH and cAMP [27].

Comparison of 3-MC and other xenobiotics on AHH. We examined whether the suppression of AHH by 3-MC was specific for this compound or extended to other polycyclic aromatic hydrocarbons. Figure 7 shows that, although benz[*a*]anthracene and benzo[*a*]pyrene did cause some loss of AHH after 24 hr of incubation, much higher concentrations (10–100 μM) were required than for 3-MC (EC_{50} of 0.3 μM). TCDD also caused no loss of AHH when used at concentrations from 10 nM to 10 μM. There was no significant induction of AHH by TCDD, at least over the 24-hr incubation period used here.

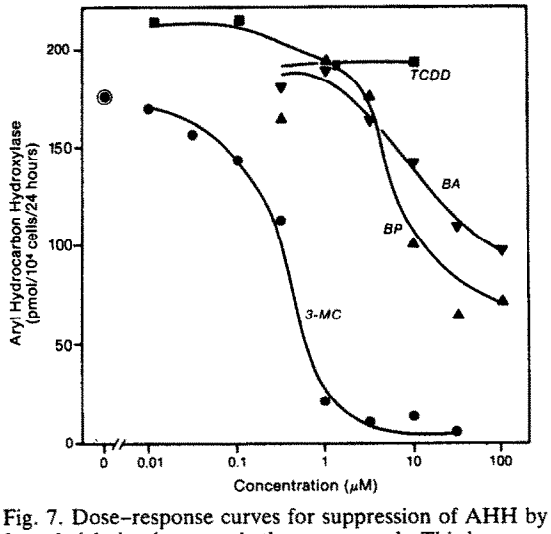

Fig. 7. Dose–response curves for suppression of AHH by 3-methylcholanthrene and other compounds. Third passage bovine adrenocortical cells were cultured in medium with 10% ether-extracted serum. Some cultures were incubated with the indicated concentrations of 3-methylcholanthrene (●); benz[*a*]anthracene (BA; ▼); benzo[*a*]pyrene (BP; ▲); or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); ■). (●) equals control. AHH was measured after 24 hr.

The other compounds listed in Table 2 were also examined for suppression of AHH; none had effects greater than that of benzo[*a*]pyrene shown in Fig. 7.

Comparison of 3-MC suppression of AHH and 17α-hydroxylase. Suppression of 17α-hydroxylase by 3-MC did not require protein synthesis and was prevented by an inhibitor of enzymatic activity. Similar results were obtained for AHH (Table 5). Inclusion of cycloheximide during the incubation with 3-MC did not prevent the loss of AHH activity. Inclusion of the inhibitor α-naphthoflavone completely prevented the suppression of AHH by 3-MC. α-Naphthoflavone did not prevent suppression of 17α-hydroxylase by methylcholanthrene (not shown).

DISCUSSION

When investigating the effects of 3-MC on cultured bovine adrenocortical cells, we unexpectedly found that exposure of cells to 3-MC caused loss of 17α-hydroxylase activity. The present experiments investigate this effect, which appears to involve an unusual interaction between a polycyclic aromatic hydrocarbon and a steroidogenic cytochrome P-450.

The suppression of 17α-hydroxylase by 3-MC apparently was not mediated by a receptor mechanism. TCDD, which exhibits high affinity binding to receptors to which 3-MC also binds, was without effect on 17α-hydroxylase at any concentration tested. The time-course of the suppression of 17α-hydroxylase by 3-MC, with no perceptible lag period, appears incompatible with a receptor-mediated induction of new proteins. Additionally, inhibition of protein synthesis by cycloheximide did not prevent the suppression. Bovine adrenocortical tissue does not appear to have been examined for the presence of receptors for 3-MC or TCDD. Syrian hamster adrenal tissue has been shown to bind TCDD [28], whereas no binding for TCDD has been detected in rat adrenocortical tissue [29].

The suppressive effect of 3-MC on 17α-hydroxylase was not one of nonspecific cytotoxicity. 3-MC showed no growth inhibition at concentrations which caused complete loss of 17α-hydroxylase activity. Additionally, specificity was demonstrated by showing no loss of another steroidogenic cytochrome P-450 activity, 11β-hydroxylase. The specificity for 17α-hydroxylase indicates that the effect of 3-MC could not be simply due to inhibition of cyclic AMP production. Although synthesis of 17α-hydroxylase is known to be induced by cyclic AMP [30], the other

Table 5. Effects of cycloheximide and α-naphthoflavone on suppression of aryl hydrocarbon hydroxylase by 3-methylcholanthrene

Additions	AHH activity (pmoles/10^4 cells/24 hr)
None	150 ± 20
1 μM 3-Methylcholanthrene	20 ± 0
10 μg/ml Cycloheximide	120 ± 40
1 μM 3-MC + 10 μg/ml cycloheximide	20 ± 10
10 μM α-Naphthoflavone	170 ± 30
1 μM 3-MC + 10 μM α-naphthoflavone	140 ± 50

The indicated compounds were added to third passage bovine adrenocortical cells cultured in medium with 10% ether-extracted serum. After 24 hr, compounds were removed, and aryl hydrocarbon hydroxylase activity was assayed as described in Materials and Methods. Data ± S.D. are from triplicate wells.

steroid hydroxylases are also under cyclic AMP regulation. Additionally, removal of cyclic AMP, e.g. by withdrawal of ACTH, results in a considerably slower decline in 17α-hydroxylase activity than that seen with addition of methylcholanthrene (Fig. 1).

The loss of activity of 17α-hydroxylase appeared not to result from reversible inhibition of the enzyme. When 3-MC was included in the 17α-hydroxylase assay medium, no inhibition of activity was seen except at very high concentrations. No recovery of 17α-hydroxylase activity was seen when 3-MC was removed from cultures after a 24-hr exposure; enzyme activity remained at very low levels until cells were re-exposed to ACTH, when normal reinduction of the enzyme activity occurred. Thus, there was no evidence for tight but reversible binding of 3-MC to the enzyme which might cause inhibition. High amounts of substrate, progesterone, did not affect the suppression of activity by 3-MC and also did not cause recovery of activity when added after removal of 3-MC as might be expected if 3-MC were simply bound to the enzyme substrate site and would compete with substrate. Thus, 3-MC is unlikely to act by a mechanism involving some form of binding to the enzyme which leaves the enzyme in a form from which it can recover activity. If there was interaction with the enzyme, it was irreversibly altered by the interaction.

It is possible that reactive metabolites of 3-MC could irreversibly inactivate the enzyme. The protective effect of a specific 17α-hydroxylase inhibitor (22-amino-23,24-bisnorchol-5-en-3β-ol) implies that any such reactive metabolites must be produced by 17α-hydroxylase itself, since AHH activity was not affected by this compound. Although such metabolism is possible, it would be unlikely, in view of the high degree of substrate specificity of the adrenal hydroxylases.

There are striking similarities between the suppression of 17α-hydroxylase by 3-MC and the previously described suppression of 11β-hydroxylase, corticosterone methyl oxidase, and 21-hydroxylase by steroid pseudosubstrates [8–11]. The time-course of loss of activity—no apparent lag period and requiring 4–24 hr for completion, depending on the concentration used—is very similar to those for 11β-hydroxylase loss in the presence of cortisol [8, 9] and for 21-hydroxylase loss in the presence of androstenedione [9]. 11β-Hydroxylase and corticosterone methyl oxidase activities have been found to be protected by inhibitors, principally metyrapone [9, 10, 21] and also by phenols [23] and sulfoxides [24], which act partially by inhibition. 3-MC suppression of 17α-hydroxylase is partially prevented by low levels of a potent inhibitor of this enzyme, 22-amino-23,24-bisnorchol-5-en-3β-ol [22]. The failure of higher concentrations of this inhibitor to provide further protection of 17α-hydroxylase activity may result from difficulty in washing out all of the inhibitor from the cells prior to assay of 17α-hydroxylase. For both 11β-hydroxylase and 21-hydroxylase, examination of the molecular structure involved in suppression suggested an interaction at the substrate site of the enzyme [8, 9, 11]. There was some evidence also in the present experiments for a common structure of compounds that could cause suppression. 3-Methylcholanthrene has a molecular structure that is similar to that of steroids (Fig. 8); 3-MC can be synthesized from sterols [31]. No compounds other than 4-MC were as active as 3-MC. Polycyclic hydrocarbons lacking the cyclopentane ring, such as benzo[*a*]pyrene, were much less active (Fig. 8). For polycyclic hydrocarbons, therefore, a steroid-like structure is required, suggesting that interaction at the substrate site of 17α-hydroxylase might be required. Some activity was shown by steroids that lack a substituent at C_{17}, such as 5α-androst-16-en-3-one (Fig. 8). Several other steroids, including those that are normal secretory products of the adrenocortical cell, were without activity. Thus, it may be that 3-MC is a pseudosubstrate for 17α-hydroxylase as certain steroids are for 11β-hydroxylase and 21-hydroxylase.

Such a pseudosubstrate-stimulated loss of activity could occur by several different mechanisms, as we have discussed elsewhere [32]. If 3-MC binds to the enzyme substrate site, the presence of a carbon at position 17* that is not hydroxylatable may result in activation of the enzyme, as for a normal cytochrome P-450 substrate, but in this case without alteration of the bound hydrocarbon. Activation of the enzyme

* When 3-methylcholanthrene is numbered as a sterol.

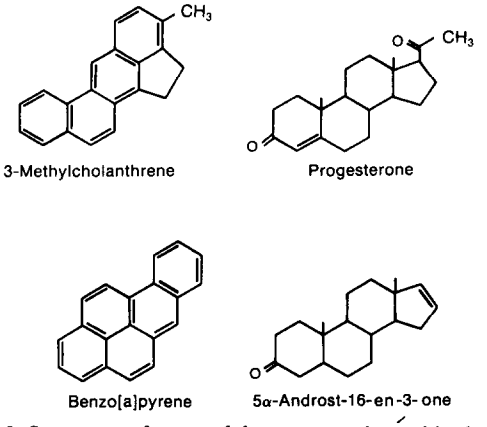

Fig. 8. Structures of some of the compounds used in these studies.

by a non-metabolizable compound may result in loss of enzyme activity by uncoupling, as in the interaction of non-metabolizable fluorocarbons with liver microsomal cytochrome P-450s [33–36]. In the adrenocortical mitochondrial cytochrome P-450, 11β-hydroxylase/corticosterone methyl oxidase, pseudosubstrate interactions appear to produce destructive oxidants that can be scavenged, as evidenced by protection of the enzyme activities by suitable antioxidants and by a lowered concentration of oxygen [8, 11, 14, 23, 32]. The microsomal adrenocortical cytochrome P-450s, 21-hydroxylase and 17α-hydroxylase, appear to differ, in that no effect of oxygen and antioxidants on the pseudosubstrate effects are observed [9].

We originally observed the loss of 17α-hydroxylase in the presence of 3-MC in an investigation of the regulation of AHH in cultured adrenocortical cells. 3-MC, rather than inducing AHH activity, caused the rapid loss of AHH activity as it was subsequently observed to do for 17α-hydroxylase. AHH has been studied in adrenocortical tissue from several species, including bovine and human [25, 27, 37, 38]. At least in some species (e.g. human, rat) adrenal AHH is unresponsive to xenobiotic inducers, including 3-MC and TCDD, but is induced by ACTH [27, 39, 40]; in the mouse; however, 3-MC does induce AHH in the adrenal [41]. Whether 3-MC induces bovine adrenal AHH is not known; any induction of AHH in the present experiments was concealed by the suppression of AHH by 3-MC. The suppression has several similarities to the suppression of 17α-hydroxylase. The compounds that were active in causing loss of 17α-hydroxylase also caused loss of AHH and did so with a similar order of potency. The concentration of 3-MC required for half-maximum suppression of AHH was close to that for half-maximum suppression of AHH. Despite the similarity in the behaviours of AHH and 17α-hydroxylase, it is clear that these activities result from different enzymes in the adrenocortical cell. Aryl hydrocarbon hydroxylase in the adrenal cortex is likely due to cytochrome P-450s that differ from the steroidogenic enzymes; a form of cytochrome P-450 metabolizing polycyclic aromatic hydrocarbons, distinct from the steroidogenic cytochrome P-450s, has been characterized and partially purified from the rat adrenal cortex [40, 42]. Data from the present experiments also show that 17α-hydroxylase and AHH are due to separate cytochrome P-450s. On removal of 3-MC, AHH activity "spontaneously" recovered to initial values within a period of about 72 hr. It is not clear what is present in the culture environment which maintains AHH in this system. On the other hand, synthesis of 17α-hydroxylase is known to be highly cAMP-dependent [30], and levels of 17α-hydroxylase were extremely low in the absence of ACTH. The enzymes also appear to be different by the criterion of susceptibility to different inhibitors. AHH was unaffected by the potent 17α-hydroxylase inhibitor, 22-amino-23,24-bisnorchol-5-en-3β-ol, whereas 17α-hydroxylase was only weakly inhibited by two compounds found to inhibit adrenal AHH, naphthoflavone and ellipticine [26].

Although there are some similarities in the suppression of 17α-hydroxylase and AHH by 3-MC, suggesting a common mechanism, there are some differences that preclude a definite conclusion. Whereas 17α-hydroxylase and AHH are likely due to different enzymes, 3-MC is quite likely to be metabolized by the same enzymes as benzo[*a*]pyrene; investigation of the metabolism of 3-MC by adrenocortical cells* shows competition with benzo[*a*]pyrene, as well as competition by 3-MC for metabolism of benzo[*a*]pyrene. Because AHH "spontaneously" recovers from suppression with 3-MC, it is not ruled out that there is tight binding of 3-MC to the enzyme, with slow release and recovery of activity. However, the inhibition of this recovery by cycloheximide suggests that, like 17α-hydroxylase, AHH requires new protein synthesis and recovery does not represent mainly pre-existing enzyme. The similarities of suppression of the two enzymes in effective concentrations of 3-MC and similarities in effective substances suggest that loss of AHH may also be a pseudosubstrate effect. This may imply a degree of similarity between the cytochrome P-450s for 17α-hydroxylase and adrenal AHH.

The adrenal cortex *in vivo* is known to be damaged by a number of xenobiotics, by mechanisms which have been only partially elucidated [43]. Some polycyclic hydrocarbons, such as dimethylbenz[*a*]anthracene, cause extensive adrenal necrosis [44]. Although an adrenolytic effect of 3-MC does not seem to have been observed, the present results suggest that toxic effects of this compound on the adrenal *in vivo* are possible.

The present studies extend the list of adrenal cytochrome P-450s that are affected by pseudosubstrate effects, and suggest that this form of interaction is not limited to steroids. Additionally, 3-MC may have effects on levels of cytochrome P-450 activity by mechanisms other than activation of *Ah*-like receptors.

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* P. J. Hornsby, K. A. Aldern and S. E. Harris, unpublished observations.

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