Metabolism of Estradiol in Liver Cell Culture

DIFFERENTIAL RESPONSES OF C-2 AND C-16 OXIDATIONS TO DRUGS AND OTHER CHEMICALS THAT INDUCE SELECTIVE SPECIES OF CYTOCHROME P-450

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ABSTRACT The oxidative metabolism of estradiol (the natural estrogen 2,3,5(10)-estratriene-3,17β-diol) at positions C-2 and C-16 was examined in primary cultures of chick embryo liver cells using estradiol which was labeled with ^3H specifically at either the C-2 or C-16 position as the substrate. Oxidation of estradiol by the cultured liver cells was assessed by the release of ^3H which accumulated as ^3H_2O in the culture medium; both C-2 and C-16 oxidative reactions were detectable in the liver cell cultures by this technique. When incubated with a concentration of estradiol substrate close to the Michaelis constant (K_m), ~45.8 pmol [2-^{3}H]estradiol and 5.0 pmol [16-^{3}H]estradiol/mg protein per minute underwent oxidative metabolism in untreated cells. Total amounts of oxidized product formation after 2 h of incubation were 28 and 5 pmol/mg protein for C-2 and C-16 oxidation, respectively. Treatment of cultures with phenobarbital or 2-propyl-2-isopropylacetamide significantly increased oxidation at C-16 (1.9-fold and 2.6-fold greater than control values, respectively), whereas no significant change in C-16 oxidation was observed after treatment of the cultures with 3-methylcholanthrene, benzo[a]pyrene, or benz[a]anthracene. The latter chemicals, however, were found to increase the extent of oxidation at C-2 significantly (i.e., 1.5–2.2-fold increases over control values). The increase in C-2 oxidation after treatment of cultures with phenobarbital or 2-propyl-2-isopropylacetamide was significantly less than that observed for oxidation at C-16. The apparent K_m values for these oxidations in control cultures were 23.5 and 30.3 μM for C-2 and C-16 oxidation, respectively; corresponding maximum velocity (V_{max}) values were 119 and 11.7 pmol/mg protein per minute, respectively. These data indicate that the C-2 and C-16 oxidations of estradiol take place in cultured avian hepatocytes and that the extent of metabolism at these positions on the hormone molecule can be altered by chemicals, such as drugs and polycyclic aromatic hydrocarbons, which induce distinctive species of cytochrome P-450 in the liver.

INTRODUCTION

Cytochrome P-450 catalyzes the oxidative metabolism of a wide variety of structurally diverse chemicals in the liver and other tissues (1). Multiple forms of this microsomal hemeprotein exist that differ with respect to their substrate specificity and their degree of inducibility and inhibition by various agents (2–4). Steroid hormones represent a major class of endogenous compounds metabolized in the liver by cytochrome P-450-dependent monooxygenases in mammals and other species. For example, androgens, such as testosterone and androstenedione, are biotransformed not only by reductive metabolism at the C4-5 double bond, but also by hydroxylation reactions catalyzed by cytochrome P-450 in rat liver (5, 6); the substrate specificity and the regulatory mechanisms of these oxidations have been extensively examined (7, 8). Differential inducibility of P-450-dependent steroid oxidations has also been demonstrated in trout liver microsomes (9).

We describe in this paper the oxidative metabolism of estradiol (the natural estrogen 2,3,5(10)-estatriene-3,17β-diol) at the C-2 and C-16 positions in primary monolayer cultures of chick embryo liver cells incubated in a chemically defined medium. Evidence is provided that indicates that these oxidations in the

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avian embryo liver are cytochrome P-450-dependent and that the extent of the C-2 and C-16 oxidations can be selectively altered by drugs and other agents that induce different species of cytochrome P-450. These findings may have potential clinical implications, because C-2 and C-16 oxidations of estradiol represent the primary metabolic pathways of estradiol in humans and because their differential inducibility by drugs, environmental chemicals, and nutritional factors thus could alter the patterns of endogenous hormone biotransformation in man.

METHODS

Preparation of [2-^{3}H]estradiol and [16-^{3}H]estradiol. [2-^{3}H]Estradiol and [16-^{3}H]estradiol were prepared and the specificity of the label at the desired position was confirmed as previously described (10). The substrate radiohomogeneity exceeded 98% in the case of the former, and >95% of the ^{3}H on the molecule was located at the designated position. For the [16-^{3}H]estradiol, substrate radiohomogeneity exceeded 96%, with 81% of the ^{3}H being located in the α- and 15% in the β-orientation. Oxidation of estradiol labeled at both positions C-2 and C-16 proceeds without an isotope effect and a National Institutes of Health shift does not occur in the case of the C-2 reaction (10). Therefore, as recently demonstrated (11–13), there is a stoichiometric relationship between the amount of ^{3}H released and the extent of the oxidative reaction occurring at that position on the estradiol molecule. Because the stereospecificity of [16α-^{3}H]estradiol is 81%, release of ^{3}H would represent a composite of 16α- and 16β-hydroxylated metabolites. No attempt was made to separate the 16α- and 16β-epimers. Enzyme activity was expressed as picomoles of C-2 or C-16 oxidized product (i.e., as reflected in ^{3}H released) per milligram of protein per minute.

Chick embryo liver cells in culture. Chick embryo liver cell suspensions for culture were prepared as described previously (14). The erythrocyte-free cell pellet was suspended in 75 volumes of modified F12 medium (14) supplemented with 5% fetal bovine serum (FBS).[1] 5 ml of the 75-fold diluted cell suspension, equivalent to 4–5 $\times$ 10^5 cells, was then added to a flask of 25-cm^2 surface area (Falcon tissue culture flask, type 3013; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) and incubated for 3–4 h. At this time, chemicals were added to the culture medium and the cultures were incubated for a further 24 h.

All chemical agents were dissolved in an appropriate solvent, as described below. The inducer compounds employed in these studies comprised the following: phenobarbital (2 mM) and 2-propyl-2-isopropylacetamide (0.3 mM), prepared in sterile water; 3-methylcholanthrene (2 μM), β-naphthoflavone (15 μM), benzo[a]pyrene (2 μM), and benz[a]anthracene (2 μM), prepared in acetone; and Aroclor 1254 (2 μg/ml), prepared in ethanol. None of the solvents alone had significant effects on cellular viability or on estradiol C-2 and C-16 oxidations.

Assays of estradiol C-2 and C-16 oxidations in liver cell cultures. After incubation for 24 h in the presence or in the absence of an inducer chemical, cells were rinsed with Earle's buffer and 5 ml of serum-free, modified F12 medium containing insulin (1 μg/ml) and [2-^{3}H]- or [16-^{3}H]estradiol (6.8 mCi/mmol) was added. [2-^{3}H]Estradiol with a sp act of 20 Ci/mmol was used in the control set of experiments, which measured estradiol C-2 oxidation as a function of incubation period. After further incubation of the cells for 2 h, 1–2 ml aliquots of medium were collected for determination of C-2 or C-16 oxidation as reflected in the formation of ^3H_2O. The samples were quickly frozen in dry ice-acetone and then lyophilized. The ^3H_2O obtained by this procedure was collected, mixed with 5 ml of Hydrofluor liquid scintillation mixture (National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, NJ) and the radioactivity was determined to within +5% accuracy with a Packard 2650 liquid scintillation spectrometer (Packard Instrument Co., Inc., United Technologies, Downers Grove, IL).

Other assays. Cytochrome P-450 was determined according to the method of Omura and Sato (15); an Aminco-Chance DW-2a spectrophotometer (American Instrument Co., Silver Spring, MD) was used for all assays. Supernatants from a 9,000-*g* centrifugation of cell homogenates were prepared according to Sinclair et al. (16) and used for measurement of cytochrome P-450. Protein determinations were carried out by the method of Lowry et al. (17), with bovine serum albumin as a standard.

RESULTS

Time course of the oxidation of estradiol in liver cell cultures. The formation of the ^3H_2O product from both the C-2 and C-16 oxidative reactions increased in the culture medium as a function of the incubation period of the substrate. The time required for 50% maximal ^3H_2O formation was ~2 h for C-2 oxidation (Fig. 1 *A*) and 4 h for C-16 oxidation (Fig. 1 *B*). A 2-h incubation period was selected as a reasonable time point for measurements of both oxidations in subsequent experiments, unless otherwise stated. The interassay results from these experiments were highly reproducible, the standard error of product formation after incubation for 2 h being 4.2% for C-2 oxidation and 4.9% for C-16 oxidation. The standard error of ^3H_2O formation obtained upon incubation of cultures for 24 h was 8.5% (six experiments) and 7.9% (four experiments) for the C-2 and C-16 oxidations, respectively. The standard error of the mean for product formation among various assays was 1.0% for the C-2 oxidation (six experiments) and 7.1% for the C-16 oxidation (four experiments) after incubation for 2 h. Data are expressed as picomoles of ^{3}H released per milligram of protein per minute. Both estradiol oxidations were critically dependent on the presence of intact liver cells and there was no measurable activity either in the absence of cells or when cells were denatured by 10% trichloroacetic acid or 0.5 N perchloric acid/50% methanol.

Effect of cell density on estradiol oxidative metabolism at C-2 and C-16. The extent of both the C-2 and C-16 oxidations changed as a function of cell den-

[1] *Abbreviation used in this paper:* FBS, fetal bovine serum.

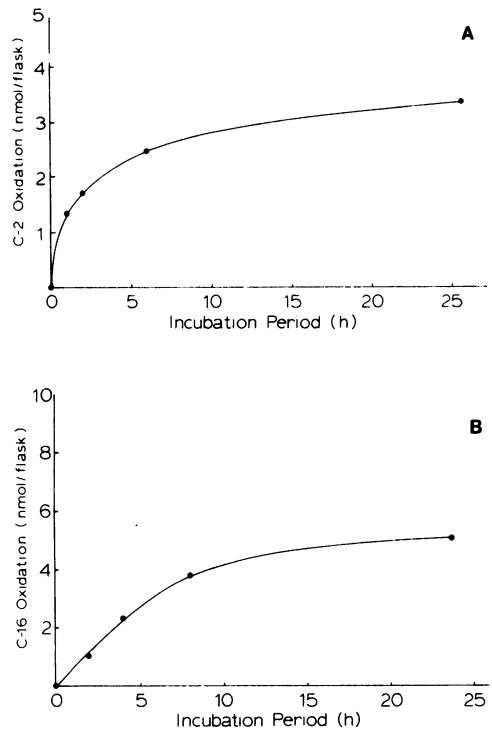

FIGURE 1 Estradiol oxidation as a function of incubation period in cultured chick embryo liver cells. Cells were prepared and incubated in a modified F12/5% FBS medium as described in Methods. The medium was discarded after a 24-h incubation, and serum-free modified F12/insulin (1 μg/ml) medium was added containing 0.5 μCi of [2-^{3}H]estradiol (*A*) or [16-^{3}H]estradiol (*B*). Each data point represents the mean value of 2–4 determinations. The sp act used was 20 Ci/mmol (*A*) and 6.8 mCi/mmol (*B*) for [2-^{3}H]- and [16-^{3}H]estradiol, respectively. The final concentrations of estradiol were 5 nM for [2-^{3}H]- and 15 μM for [16-^{3}H]estradiol.

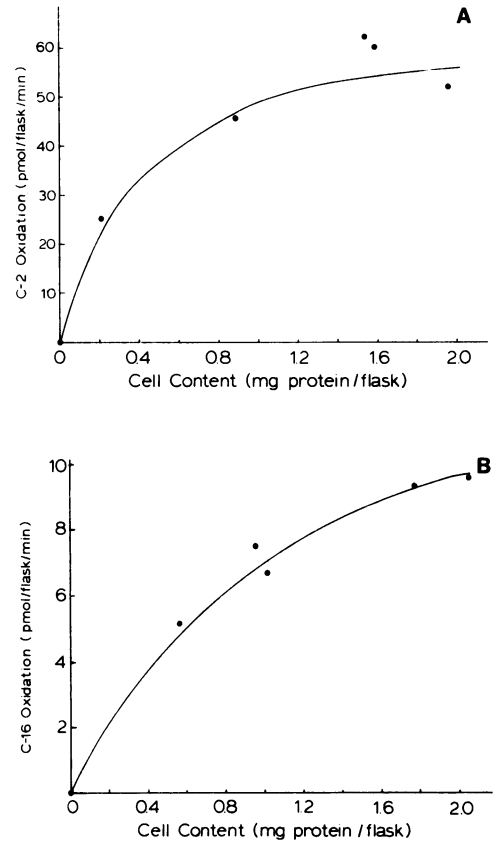

FIGURE 2 Effect of cell density on estradiol oxidation in cultured liver cells. Cell suspensions of 1:75, 1:100, 1:150, 1:200, and 1:400 (i.e., 1 vol of packed cells suspended in 400 vol of medium) were prepared and incubated under the standard assay conditions outlined in Methods. The extent of estradiol oxidation was determined as ^3H_2O released from [2-^{3}H]- (*A*) and [16-^{3}H]estradiol (*B*) (15 μM, 6.8 mCi/mmol).

sity in culture. Although an increase in product formation by these reactions was demonstrable with increasing cell density, the amount of product (^3H_2O) formed per milligram of protein tended to decrease at higher cell concentrations. A plateau of activity was reached at a cell density of ~1.5 mg protein/25-cm^2 flask for both the C-2 and C-16 reactions. These data suggest that the extent of metabolism at both positions on the steroid molecule is influenced by cell density in culture. To avoid variation in the oxidative biotransformations due to differences in cell density, experiments were performed in flasks that originally contained 5 ml of a 1:75-fold suspension. This generally yielded 2 mg of protein per flask (Fig. 2, *A* and *B*).

Effects of inducers of cytochrome P-450 on estradiol metabolism. The effects of known inducers of cytochrome P-450 on the oxidative metabolism of estradiol at the C-2 and C-16 positions were examined (Table I). Phenobarbital and 2-propyl-2-isopropylacetamide have been shown to induce cytochrome P-450 in the cultured liver cell system used in this study (16, 18). These compounds significantly stimulated C-16 oxidation, producing a 1.9- and 2.6-fold increase over control values, respectively. C-2 oxidation was stimulated to a lesser extent (1.7- and 1.2-fold greater than control values, respectively). Polycyclic aromatic hydrocarbons and other compounds, i.e., β-naphthoflavone, 3-methylcholanthrene, benz[a]anthracene, and benzo[a]pyrene, which induce a specific group of cytochrome P-450s collectively called P-448, did not cause appreciable changes in the extent of oxidation at C-16. In contrast to their lack of effect on C-16 oxidation, a significant enhancement of C-2 oxidation was observed with β-naphthoflavone, 3-methylcholanthrene, benz[a]anthracene, and benzo[a]pyrene, this resulted in 2.2-, 1.5-, 1.5-, and 2.2-fold increases over

TABLE I

Effects of Inducers of Mixed Function Oxidases on the Oxidative Metabolism of Estradiol at C-2 and C-16

Compound	Dose (concentration)	Estradiol metabolism: C-2 oxidation Mean±SEM	P	Ratio relative to control	C-16 oxidation Mean±SEM	P value	Ratio relative to control
		pmol/mg protein/min			*pmol/mg protein/min*		
Control		47.5±1.3			4.3±0.7		
Phenobarbital	2 mM	81.7±1.0	<0.001	1.7	8.0±0.1	<0.001	1.9
2-Propyl-2-isopropylacetamide	0.3 μM	56.7±0.33	<0.001	1.2	10.8±0.3	<0.001	2.6
3-Methylcholanthrene	2 μM	70.8±1.3	<0.001	1.5	4.9±0.2	NS	1.2
β-Naphthoflavone	15 μM	104.2±7.3	<0.001	2.2	5.8±0.3	<0.05	1.4
Benzo[a]pyrene	2 μM	104.2±2.3	<0.001	2.2	4.9±0.3	NS	1.2
Benz[a]anthracene	2 μM	72.5±0.3	<0.001	1.5	4.3±0.1	NS	1.0
Aroclor 1254	2 μg/ml	68.3±1.7	<0.001	1.4	5.7±0.1	NS	1.4

Cells were prepared and incubated in modified F12/5% FBS medium in the presence of a specific chemical inducers of mixed function oxidases. After incubation for 24 h, the medium was replaced with serum-free modified F12/insulin (1 μg/ml) medium containing [^{3}H]estradiol (6.81 mCi/mmol). The cells were then incubated for another 2 h, at which time aliquots of medium were obtained for evaluation of the amount of ^3H_2O released by the site-specific oxidation of estradiol as described in Methods. Data represent the mean±SEM values of four to six determinations.

The values listed for control samples correspond to 0.070±0.002 and 0.0037±0.0008 of the added substrate per milligram protein per minute for the formation of ^3H_2O via the oxidation of estradiol at C-2 and C-16, respectively. P values < 0.05 were considered to be statistically significant.

control values, respectively. The postmitochondrial fraction (9,000 *g*) from avian hepatocytes incubated with phenobarbital or 2-propyl-2-isopropylacetamide displayed an increased CO-reduced difference spectrum with a peak at 453.5 nm, the cytochrome subtype in the avian liver (16, 18) that corresponds to P-450 in rat liver. Cells treated with 3-methylcholanthrene, β-naphthoflavone, or Aroclor 1254 showed an increased peak at 451 nm, the cytochrome subtype in the avian liver that corresponds to P-448 in rat liver (Fig. 3).

Effects of inducers of cytochrome P-450 on estradiol metabolism in chick embryo liver in ovo. Experiments corresponding to those in liver cell cultures were also conducted with liver microsomes prepared from chick embryos in ovo treated with the same chemicals in order to compare the metabolic activities of whole liver with those of cultured liver cells from the same species. In this experimental system, natural steroids as well as drugs and other foreign chemicals are known to induce cytochrome P-450 in liver (19, 20). The doses of drugs used per kilogram of body weight were as follows: phenobarbital (100 mg), benzo[a]pyrene (40 mg), or 3-methylcholanthrene (40 mg). The individual chemicals were injected through the airsac of 17-d-old chick embryos 24 h before they were killed. Measurements of the extent of the estradiol C-2 and C-16 oxidations were carried out in vitro on hepatic microsomes prepared according to the method described by Numazawa et al. (12). Analysis of ^3H_2O was performed as described for the liver cell cultures. Treatment of chick embryos with 3-methylcholanthrene and benzo[a]pyrene preferentially induced C-2 oxidation, whereas the enhancement of C-16 oxidation by these two chemicals was minimal. Phenobarbital, on the other hand, preferentially induced C-16 oxidation and did not increase C-2 oxidation significantly (data not shown). These findings indicate that cultured hepatocytes and whole avian liver respond similarly by differential induction of C-2 and C-16 estradiol oxidative reactions in response to chemicals known to induce distinctive species of cytochrome P-450.

The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) of estradiol oxidations at C-2 and C-16 in cultures of chick embryo liver cells were determined. For these studies, the specific activity of the substrate estradiol was held constant (17,000 dpm/nmol) per assay. A Lineweaver-Burk plot of C-16 oxidation yielded an apparent K_m of 30.3 μM and a V_{max} of 11.7 pmol/mg protein per minute (Fig. 4 *A*). Estradiol oxidation at C-2 showed an apparent K_m 23.5

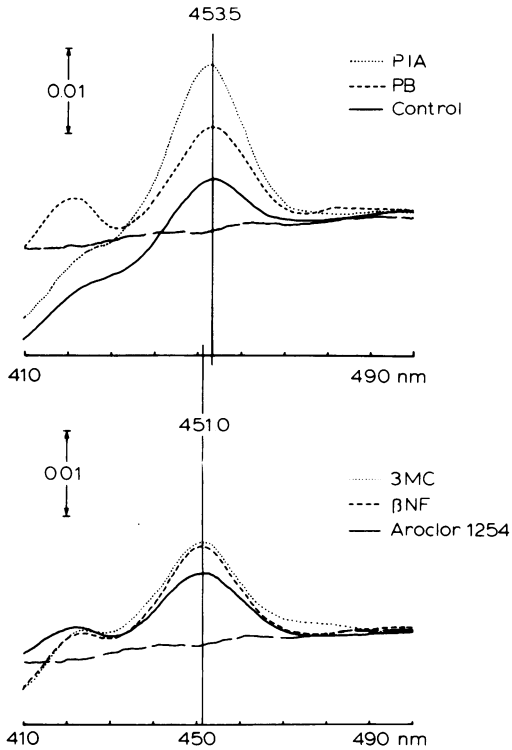

FIGURE 3 Effects of special chemical inducers of mixed function oxidases on the induction of cytochrome P-450 in cultured liver cells. Cells were prepared and incubated as described in Methods. After incubation for 24 h with chemicals, culture dishes were rinsed and cells were scraped and homogenized in 1 ml of 0.1 M phosphate buffer containing 20% glycerol/0.2% Emulgen 913/1 mM EDTA and 1 mM dithiothreitol. Homogenates were centrifuged at 10,000 *g* for 30 min and supernatants were used for cytochrome P-450 determination by the method of Omura and Sato (15). *3MC*, 3-methylcholanthrene; *βNF*, β-naphthoflavone; PB, phenobarbital; PIA, 2-propyl-2-isopropylacetamide.

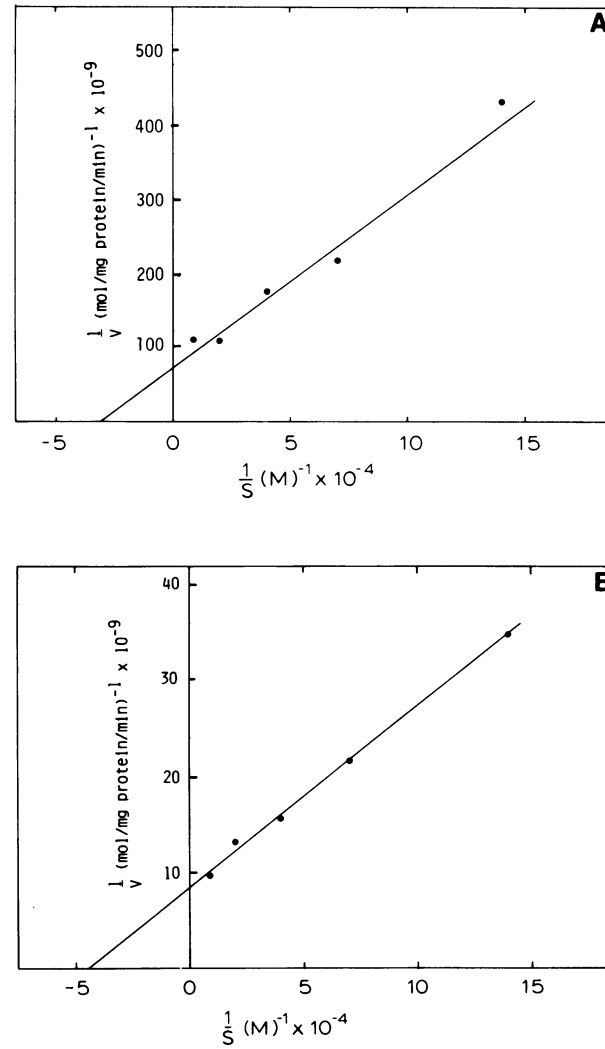

FIGURE 4 Lineweaver-Burk plot of estradiol oxidation. (*A*) C-16 oxidation; (*B*) C-2 oxidation. Cells were prepared and incubated as described in Methods. The medium was discarded after a 24-h incubation and serum-free modified F12/insulin (1 μg/ml) medium containing [^{3}H]estradiol (17,000 dpm/nmol) was added. The extent of estradiol oxidation was examined at varying concentrations of estradiol (3.7 to ~117 μM). Each point represents the mean of four determinations.

μM and a V_{max} of 119 pmol/mg protein per minute (Fig. 4 *B*).

DISCUSSION

The results of the present study demonstrate that cultured avian embryo liver cells actively oxidize estradiol at the C-2 and C-16 positions and that these reactions in avian liver cells appear to be cytochrome P-450-dependent. Furthermore, oxidation of this estrogen at either position C-2 or C-16 can be differentially stimulated by chemicals known to induce distinctive species of cytochrome P-450 in the liver. The differential induction of steroid oxidative metabolism in the cultured hepatocytes was also observed with liver microsomes from chick embryos treated in ovo with various inducers of this hemeprotein. Our findings therefore indicate that exposure to drugs and other chemical inducers of distinctive forms of cytochrome P-450 in liver can, to some extent, selectively alter estradiol metabolism towards the differential formation of C-2 or C-16 oxidized metabolites. Differential enhancement of androgen hydroxylases by chemical inducers of hepatic cytochrome P-450 has also been demonstrated by Conney (1) and Conney and Klutch (21).

The radiometric procedure used in these studies permits a ready assessment of the total in vitro metabolism of estradiol in cultured liver cells via its two principal oxidation pathways. In this method, the release of ^3H from radiolabeled estradiol (10) was used as a determinant of a site-specific oxidation on the estradiol molecule. The data obtained by the radiometric technique, including varying incubation time, protein content, or substrate concentration, were all in conformity with an enzymatic basis for the reactions measured by the release of ^3H from the steroid substrate.

Recently Ryan et al. (22), using an in vitro reconstituted mixed function oxidase system composed of purified cytochrome P-450, NADPH-cytochrome *c* reductase, and lipid, demonstrated that cytochrome P-450e, which is inducible in rat liver by Aroclor 1254 and phenobarbital, catalyzes the 2-hydroxylation of estradiol. Our data, utilizing a radiometric method to measure the site-specific oxidation at C-2, are consistent with this finding, although we were not able to quantitate the 2-hydroxylated product of estradiol because of the small amounts of tissue available for study. However, as recently shown by others using other experimental models (11–13), it is a reasonable assumption that the formation of ^3H_2O from both [2-^{3}H]estradiol and [16-^{3}H]estradiol in these liver cells reflects the formation of the expected C-2 and C-16 hydroxylated products.

The steroid oxidations demonstrated in avian liver also represent the principal metabolic biotransformations of estradiol in humans (23). The fact that they can be induced by drugs and other foreign chemicals, and that such substances may also preferentially enhance the C-2 or the C-16 reaction, suggests a possible mechanism whereby exogenous chemicals could significantly alter the profiles of endogenous hormone metabolism in humans. Alterations in these metabolic patterns may have potential biological significance since many of the metabolites of such steroid hormones as testosterone, progesterone, and estradiol (24–29) can exert potent biological actions of their own. C-2 and C-16 oxidations of estradiol have been presumed to be mediated by cytochrome P-450; the results of the present study support this view and indicate that chemicals known to induce selective species of cytochrome P-450, can preferentially enhance either C-2 or C-16 oxidation of the hormone. Thus, exposure to other types of environmental chemicals, including those derived via the diet, which are known to enhance cytochrome P-450-dependent drug oxidations (30, 31), may also result in alterations in the oxidative metabolism of estradiol. Recent studies in humans have in fact shown that alterations in the protein/carbohydrate ratio in the diet result in selective changes in the rate of C-2 oxidation of this hormone (32).

The avian embryo liver cell culture system used in this study has proved valuable for studying a wide variety of metabolic processes in isolated hepatocytes, including aspects of heme and cytochrome P-450 metabolism (14, 16, 18, 33–35), malic enzyme and fatty acid synthesis (36, 37), plasma protein production (38), and the interactions of environmental chemicals with heme pathway enzymes (39). The data in this report indicate that this experimental system, when coupled with radiometric analysis (10), is also useful for defining site-specific steroid hormone oxidations in liver cells and the effects on such biotransformations of drugs and other chemicals to which humans may be exposed.

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