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ESTROGEN METABOLISM IN RAT LIVER MICROSOMAL AND ISOLATED HEPATOCYTE PREPARATIONS—I

METABOLITE FORMATION AND IRREVERSIBLE BINDING TO CELLULAR MACROMOLECULES

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Abstract—The metabolism of endogenous estrogens, estradiol and estrone, and the irreversible binding of estrogens to cellular macromolecules have been examined and compared in subcellular microsomal and in intact hepatocyte preparations. In studies with rat liver microsomal preparations containing estradiol, an NADPH-generating system, and denatured DNA, the irreversible binding of radiolabeled steroid metabolite(s) to the microsomal proteins was 3.26 nmoles/mg protein in 1 hr (S.D. 0.39; 7.9% of total steroid) while binding to DNA was found to be 0.288 nmole/mg DNA/mg protein (S.D. 0.025; 0.39% of total steroid). No significant difference was observed between microsomal preparations from untreated, phenobarbital-treated or 3-methylcholanthrene-treated rats. Irreversible binding to proteins was also demonstrated in the intact hepatocyte cell incubations. After 2-hr incubations of estradiol with hepatocytes, 5.9% (S.D. 1.4%) of the steroid(s) was irreversibly associated with cellular proteins (approximately 1.43 pmoles/mg/min). Analysis of the organic-soluble metabolites demonstrated the presence of the catechol estrogens and their metabolites, 2-hydroxyestradiol, 2-hydroxyestrone, 2-methoxyestradiol, and 2-methoxyestrone. Estrone and estriol were also identified. The aqueous-soluble materials isolated from hepatocyte incubations contained glucuronide, sulfate, and apparent thioether conjugates, as determined by liberation from estrogen metabolites by treatment with β-glucuronidase, sulfatase, and Raney nickel. Thus, extensive primary and secondary metabolism of estrogens occurs in intact hepatocyte incubations. Furthermore, irreversible binding of estrogens to cellular proteins occurs in these intact cells having demonstrated conjugative pathways of metabolism.

The metabolism of estrogens both *in vitro* and *in vivo* has been and continues to be examined extensively [reviews: 1–3]. The covalent binding of estrogens to proteins was observed over two decades ago by Szego [4] and Reigel and Mueller [5]. Moreover, recently Nelson *et al.* [6] examined the involvement of cytochrome P-450 in the irreversible binding of estrogens to proteins. The isolation of glutathione conjugates of oxidized estrogens [7–9] also suggests a metabolic oxidation. The covalent binding of estrone and estradiol to DNA *in vitro* [10–12] and *in vivo* [13] has been demonstrated. Certain metabolites of diethylstilbestrol (DES) such as dienestrol [14–18] and a catechol derivative [19, 20] are thought to arise from enzymatic activation of the parent compound. Blackburn *et al.* [21] demonstrated that DES binds covalently to DNA in the presence of microsomal enzymes and in cell culture. Similar observations of metabolism, metabolic activation(s), and irreversible binding have also been reported for the synthetic estrogen, 17α-ethinylestradiol [22–25]. The significance of the metabolic activation of estrogens and the subsequent covalent binding to cellular macromolecules with regard to cellular toxicities and/or cancer development remains to be determined.

This report describes our research efforts in examining the metabolism and covalent binding of estrogens to cellular macromolecules in microsomal and isolated hepatocyte preparations. Comparison of the degree of metabolism and irreversible binding of estrogens to cellular macromolecules between the two *in vitro* systems provides further information on the extent and significance of the oxidative activation(s) of estrogens in tissue damage.

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MATERIALS AND METHODS

Materials. Commercial steroids were obtained from Searle Laboratories, Skokie, IL, and Steraloids, Wilton, NH, and purified by recrystallizations. Chemical reagents were purchased from the Aldrich Chemical Co., Milwaukee, WI, and the purity was determined by melting point and/or thin-layer chromatography. [6, 7-^{3}H]Estradiol (Sp. Act. 53 Ci/mmole) and [6, 7-^{3}H]estrone (sp. act. 56 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA, and purified by thin-layer chromatography prior to use. All biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO. Tissue culture media (L-15) and calf serum were purchased from the Grand Island Biological Co., Grand Island, NY. 3-Methylcholanthrene was purchased from Eastman Kodak, Rochester, NY.

Instrumental methods. Reverse-phase high pressure liquid chromatography (HPLC) was performed using an Altex Ultrasphere ODS column

(4.5 × 250 mm) on a Beckman 421 gradient HPLC system. Mass spectrometry was performed on a DuPont Instruments model 21-491 mass spectrometer (for direct probe analysis) and a Finnigan model 4021 Automated Gas Chromatograph/EI-CI mass spectrometer (for EI GC/MS analysis) of the Campus Chemical Instrumentation Center of The Ohio State University. GC separations were performed on a 3% OV-17 column using a programmed linear temperature gradient from 200° to 250° at a rate of 2°/min with an injector temperature of 260° and an interface temperature of 250°. Mass spectra were compared with EPA/NIH library spectra for identification. Radioactive samples were dissolved in NEN Formula 963 and counted by liquid scintillation in a Beckman LS 6800 scintillation counter.

Preparation of microsomal fractions. Male and female adult Sprague–Dawley rats (250–300 g, 9- to 10-weeks-old) were purchased from Harlan Industries, Inc., Cumberland, IN. Treated rats were injected intraperitoneally with sodium phenobarbital (75 mg per kg per day for 5 days prior to killing) in 0.9% NaCl solution or with 3-methylcholanthrene (25 mg per kg per day for 3 days prior to killing) in corn oil. Rat liver microsomes were prepared by differential centrifugation procedures on homogenized tissues as previously described [26]. The microsomal pellet was stored at −70° and remained stable for over 6 months.

Microsomal incubations. Estrone (37 μM/flask; 50 μCi/flask) in ethanol or estradiol (37 μM/flask; 50 μCi/flask) in ethanol was added to a 25-ml Erlenmeyer flask containing 50 μl of propylene glycol. The ethanol was removed with a stream of nitrogen. $NADP^+$ (0.80 mM/flask), glucose-6-phosphate (2.0 mM/flask), and glucose-6-phosphate dehydrogenase (5 units) were dissolved in 0.2 M sodium phosphate buffer, pH 7.4 (0.5 ml/flask). Microsomes were thawed and resuspended in 0.2 M sodium phosphate buffer (5.0 mg microsomal protein/4.0 ml buffer). Calf thymus DNA (Sigma, Type I) was denatured by dissolving in 0.2 M sodium phosphate buffer (0.5 mg/0.5 ml buffer) and warmed to 100° for 15 min. The incubation began by addition of cofactors (0.5 ml/flask), nucleic acid (0.5 ml/flask), and microsomal suspension (4.0 ml/flask) to the flask and continued at 37° for 60 min. Control samples were performed in two ways; by incubating with microsomes boiled for 15 min or by omitting the NADPH-generating system in the incubation. Protein was determined using the method of Lowry *et al.* [27].

Isolation of cellular macromolecules. After 60 min, $CHCl_3$/isoamyl alcohol (24:1/5.0 ml) was added to each flask and the mixture was shaken to quench the assay. The aqueous-organic suspension was centrifuged at 1000 *g* for 5 min, resulting in a protein precipitate between the aqueous and organic layers. The aqueous layer was removed and extracted two more times with $CHCl_3$/isoamyl alcohol. The aqueous layer was then cooled to 4° and the DNA precipitated with cold ethanol (4°, 10 ml). This precipitate was twice redissolved in 0.2 M sodium phosphate buffer, pH 7.4, and precipitated with cold ethanol. The precipitate was then washed with cold ether and then dissolved in warm water (1.0 ml). The DNA content was determined by the diphenylamine colorimetric assay method [28]. Less than 0.2% protein (1.0 μg) was present in the isolated DNA fraction. An aliquot was dissolved in Formula 963 and counted by liquid scintillation. The protein precipitates from the extractions were combined and solubilized with Protosol (0.5 ml). The solution was then neutralized with acetic acid, Formula 963 was added, and the solution was counted for radioactivity by liquid scintillation.

Hepatocyte isolation. The hepatocytes were isolated by enzymatic digestion of rat liver slices with a collagenase–hyaluronidase mixture following modifications of the procedures of Fry *et al.* [29, 30]. Rats were killed by decapitation, the liver was removed immediately into 10 ml wash solution (Dulbecco's phosphate-buffered saline supplemented with glucose at 1.0 mg/ml), and the liver was sliced into 1–2 mm pieces. The sliced tissue was then divided into 3-g lots and placed in 125 ml Erlenmeyer flasks containing 10 ml wash solution. The flasks were shaken at 37° for 10 min (90 oscillations/min). The solution was removed by pouring through cheesecloth. The liver slices were washed a total of three times, followed by two washings in 10 ml chelating solution [0.5 mM ethyleneglycolbes(aminoethylether)tetra-acetate (EGTA) in the washing solution] for 10 min each.

The enzyme solution consisted of collagenase (2.5 mg), hyaluronidase (5 mg) and glucose (1.0 mg/ml) in 10 ml of Hanks' buffer. This solution (10 ml) was added and the liver slices were shaken for 20 min at 37°. The resulting suspension was then filtered through cheesecloth into a beaker containing 20 ml wash solution. After gentle mixing, this solution was centrifuged for 5 min at 500 *g*. This initial enzymatic digestion resulted in a significant amount of lysed cells and debris and was discarded. Another 10 ml of enzyme solution was added to the liver slices and incubated at 37° for 30 min. The resulting suspension was then filtered through cheesecloth into a beaker containing 20 ml wash solution. Again, this solution was centrifuged for 5 min at 500 *g*. The supernatant fraction was carefully removed with a Pasteur pipette and the pellet of cells was resuspended in 30 ml modified L-15 media containing 10% calf serum and 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES). Another 10 ml of enzyme solution was added to the liver pieces and incubated again at 37° for 30 min. The hepatocytes from this digestion were filtered, washed, and resuspended as before. The hepatocytes isolated from the last two enzymatic digestions were essentially free from tissue debris. These cell suspensions were combined, counted, and utilized in the metabolism studies. Small aliquots of the cell suspension were used for cell counting, viability tests, and protein determination. The cell viability ranged from 70 to 85%, as determined by the trypan blue exclusion method.

Hepatocyte incubations. The hepatocyte suspension (approximately 1×10^6 cells/ml modified L-15 media) was incubated with estradiol (10 μM; 10 μCi) for varying periods of time. Aliquots (2 ml) of the suspension were removed at various time periods (from 0 to 2.0 hr) from each flask. The suspension was mixed with an equal volume of acetone to lyse

the cells and precipitate the proteins. After centrifugation, the supernatant fraction was removed. The pellet was washed with acetone/water (1:1) two more times and the washings were added to the supernatant fraction. The supernatant was extracted three times with equal volumes of ethyl acetate. The ethyl acetate layers were combined, dried with Na_2SO_4, evaporated under nitrogen, and resuspended in 2.0 ml of 50% ethanol in water. The protein pellet was resuspended in 1.0 ml of sodium dodecyl sulfate (SDS)-solubilizing solution. The radioactivities present in the three fractions (aqueous layer, organic layer, and protein solution) were determined by liquid scintillation counting.

Metabolite identification. The organic-extractable estrogens were further analyzed by reverse-phase HPLC. Aliquots (250 μl) were separated on an Ultrasphere-ODS column using a linear gradient of 50% methanol in water to 90% methanol in water over 40 min at a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected, 5.0 ml of Formula 963 counting solution was added, and the samples were counted for radioactivity. Metabolites were identified by co-elution with authentic estrogen metabolite standards and by mass spectrometry (by GC/MS of TMS-derivatized fractions and by direct probe analysis) of the collected fractions from nonradiolabeled incubations. Aliquots of the aqueous-soluble estrogen metabolites were treated with β-glucuronidase, arylsulfatase, and Raney nickel [31, 32] to liberate the radiolabeled steroid metabolites from the glucuronides, sulfates and apparent thioether conjugates respectively. The ether-extractable steroids were analyzed by HPLC under the conditions described above.

RESULTS

Irreversible binding in microsomal incubations. The extent of irreversible binding of estrone and estradiol to cellular macromolecules catalyzed by rat liver microsomal preparations was first examined. The term irreversible binding refers to the amount of radiolabeled steroid present in the macromolecular fraction following organic solvent precipitation and extraction as described in Materials and Methods. Following isolation of the protein and nucleic acid fractions, aliquots of the solutions were counted for radioactivity. Since the amount of microsomal protein in the incubation mixtures and the amount of DNA isolated varied, the results are expressed as total estrogen bound per mg microsomal protein for the irreversible binding to proteins and as total estrogen bound per mg DNA per mg microsomal protein for comparison of the results from different incubations. These results are represented graphically in Fig. 1. The irreversible binding of radiolabeled metabolite(s) of the natural estrogens, estrone and estradiol, to cellular macromolecules such as DNA and protein in our microsomal incubation system is demonstrated and is consistent with earlier data [9–12]. As expected, the percentage of binding is quite low, being less than 0.5% for DNA and less than 8.0% for microsomal protein.

Hepatocyte incubations. Investigations on estrogen metabolism in isolated cell incubations were initiated to determine if this *in vitro* irreversible binding catalyzed by microsomal enzymes also occurs in the whole cell. Hepatocytes were isolated by the procedure of Fry *et al.* [29, 30] using a collagenase/hyaluronidase incubation of liver slices. The metabolism of estradiol by the hepatocytes was examined at various time periods from 0 to 2 hr. The cells were lysed with acetone, the proteins were precipitated, and the aqueous layer was extracted with ethyl acetate. The amount of radiolabeled estradiol and metabolites was determined in each fraction—the organic-soluble, water-soluble, and precipitated protein frac-

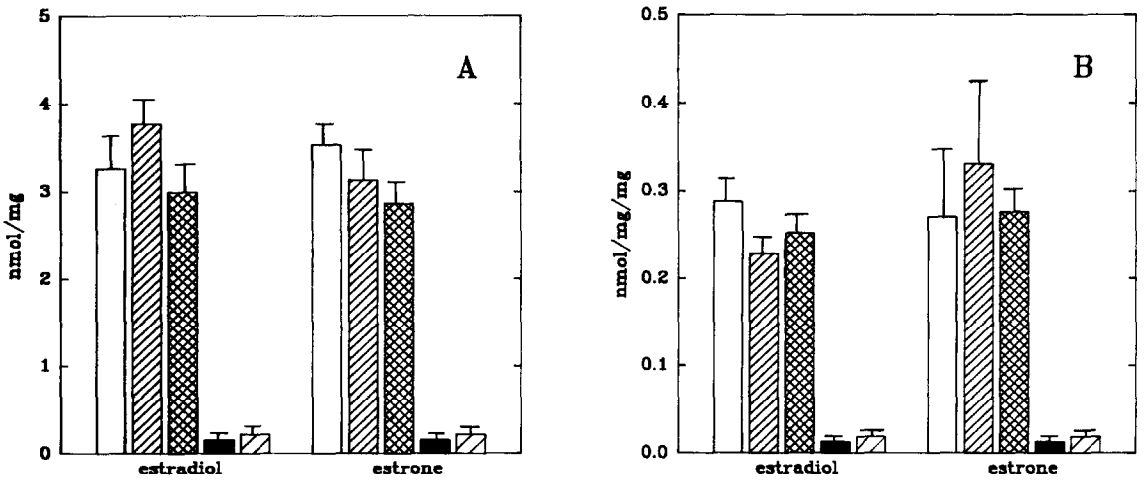

Fig. 1. Amount of radiolabeled estrogen irreversibly-bound to cellular macromolecules from microsomal incubations. (A) Irreversible binding to proteins is expressed as nmoles estrogen/mg microsomal protein. (B) Irreversible binding to DNA is expressed as nmoles estrogen/mg DNA/mg microsomal protein. Microsomal incubations were performed using liver microsomes isolated from untreated rats (□), phenobarbital-treated rats (▨), or 3-methylcholanthrene-treated rats (▩). Control incubations were performed with boiled microsomes (■) or by omitting the NADPH-generating system (▨). The values represent the average of five determinations.

tions. The time course of the presence of radiolabeled material in these three fractions is shown in Fig. 2. After 2 hr, the organic-soluble fraction contained 61.4% (S.D. 7.2%) of the radioactivity, the aqueous-soluble contained 31.0% (S.D. 3.8%) and the precipitated protein fraction 5.9% (S.D. 1.4%). Considerable metabolism of the estrogens occurred, with the organic-soluble materials decreasing with time and the aqueous-soluble and protein-bound radiolabeled materials increasing with incubation time. The plot demonstrates a time-dependent, first-order rate of metabolism.

Metabolite identification. The organic extracts from the hepatocyte incubations taken at several time periods were applied to reverse-phase HPLC chromatography and 0.5-ml fractions were collected and counted. At time 0 hr, only the radiolabeled estradiol was found by HPLC and, as expected, no metabolism occurred. As the incubation time increased, numerous radioactive peaks were observed. These metabolic peaks increased over time as the substrate estradiol decreased during the same period (Fig. 3 and Table 1). Co-elution of the radioactive peaks with metabolite standards and mass spectral confirmation of the structures by comparison with online mass spectra library and authentic steroid metabolites were used to determine the metabolite pattern. Both more polar metabolites such as 2-hydroxyestradiol, 2-hydroxyestrone and estriol and the less polar metabolites 2-methoxyestradiol and 2-methoxyestrone were identified. No radioactivity co-eluted with 4-hydroxyestradiol or 4-hydroxyestrone. These results are consistent with the reports of little 4-hydroxylation of estradiol occurring in the liver [3]. A heterogeneous mixture of radiolabeled material eluted very early in the HPLC chromatogram (retention time of 1.5 to 2.0 min). The standards 6α-hydroxyestradiol and 6α-hydroxyestriol co-elute with this heterogeneous peak; however, the presence of either of these metabolites was not confirmed by mass spectrometry. Further analysis of this mixture is underway.

Enzymatic hydrolysis and treatment with Raney nickel were performed on the aqueous-soluble metabolites from the hepatocyte incubations in order to indirectly determine the amount of estrogen present in that fraction resulting from conjugative metabolic pathways. The results in Table 2 indicate that 11.0% of the total radioactivity used in the incubation was present as glucuronide conjugates after a 2-hr incubation with hepatocytes. This indicates that 36.7% of the aqueous-soluble metabolites was present as the glucuronides. Likewise, 5.4% of the total radioactivity (18.0% of aqueous-soluble material) was present as sulfates and 5.0% (16.7% of aqueous) as thioethers. The major radioactive steroids liberated by the enzymatic treatments were identified as estradiol and estrone based on the HPLC radiochromatogram profiles. Minor amounts of the 2-methoxyestrogens, 2-hydroxyestrogens, and estriol were also identified on the radiochromatograms.

DISCUSSION

Extensive oxidative metabolism of estradiol has been demonstrated both *in vitro* and *in vivo* [1–12]. This oxidative metabolism and subsequent irreversible binding found in studies utilizing both the rat liver microsomal system and in isolated hepatocyte incubations confirms and extends these earlier observations. In the experiments described in this report, liver microsomes from phenobarbital-treated, 3-methylcholanthrene-treated, and noninduced rats were able to metabolize estrogens to intermediates which result in irreversible binding to cellular macromolecules such as proteins and nucleic acids. In the absence of the NADPH-generating system or in the presence of boiled microsomes, very little radioactivity was irreversibly bound. In our hands, no statistically significant difference exists between the

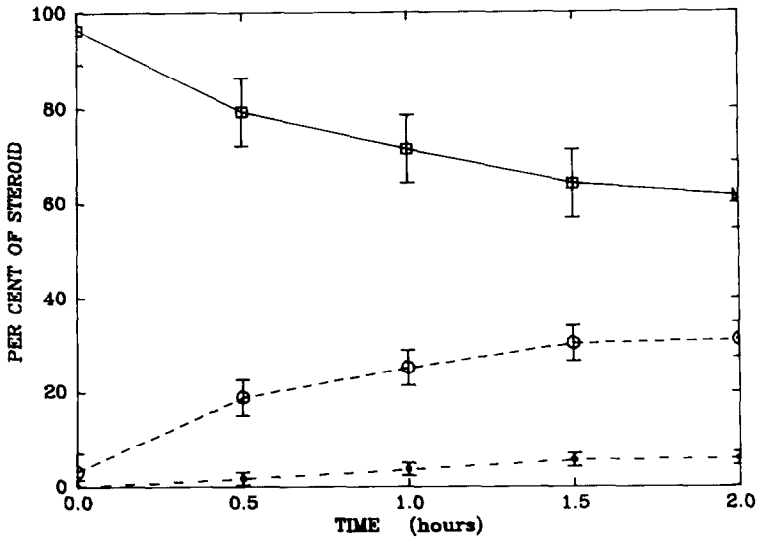

Fig. 2. Time course of the distribution of estradiol and metabolites during the hepatocyte incubations. The percentages of radioactive steroids present in the organic-soluble (—□—), aqueous-soluble (- -○- -), and precipitated protein (- -●- -) fractions were determined from 0 to 2 hr (N = 4).

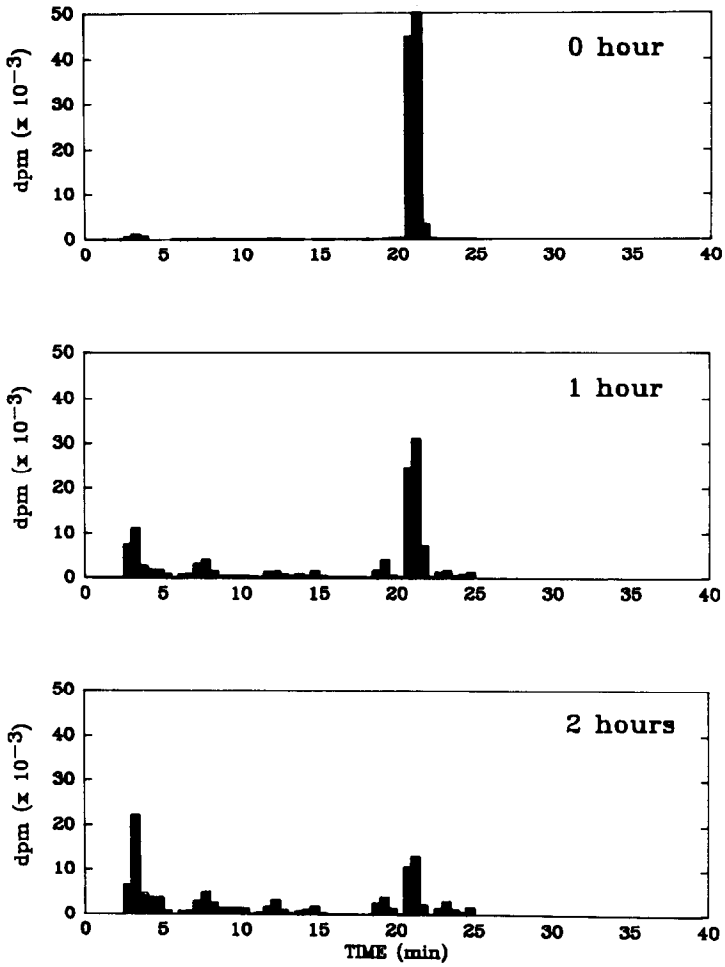

Fig. 3. Reverse-phase HPLC radiochromatograms of estradiol and metabolites present in the organic-soluble fractions. Fractions from incubations at 0 hr, 1 hr and 2 hr were analyzed by HPLC and counted by liquid scintillation as described in Materials and Methods. The peaks are the heterogenous mixture (retention time of 2.5–4.0 min), estriol (7.0–8.0), 2-hydroxyesterone (11.0–12.5), 2-hydroxyestradiol (13.5–14.5), estrone (18.0–19.0), estradiol (20.5–22.0), 2-methoxyestrone (22.5–24.0), and 2-methoxyestradiol (24.5–25.5).

Table 1. Distribution of organic-soluble metabolites of estradiol from hepatocyte incubations*

Estrogen	Radioactivity (%)		
	At 0 hr	At 1 hr	At 2 hr
Heterogenous peak	1.0 ± 0.8	21.1 ± 6.8	34.7 ± 5.8
Estriol	<0.1	8.4 ± 3.4	10.5 ± 1.6
2-Hydroxyestrone	<0.1	3.9 ± 1.8	5.6 ± 1.8
2-Hydroxyestradiol	<0.1	2.0 ± 1.4	3.1 ± 1.9
Estrone	<0.1	3.6 ± 2.1	6.1 ± 2.5
Estradiol	97.2 ± 4.2	59.3 ± 9.4	32.4 ± 8.6
2-Methoxyestrone	<0.1	2.8 ± 0.8	5.3 ± 2.4
2-Methoxyestradiol	<0.1	1.4 ± 0.6	2.8 ± 2.2

* The distributions of the radioactive estradiol metabolites in the organic extract from hepatocyte incubations at various times were determined by HPLC separation. The data are presented as the percentage of the radioactivity in the organic phase (mean % ± S.D.; N = 4).

Table 2. Distribution of aqueous-soluble metabolites of estradiol from hepatocyte incubations*

Conjugates	Radioactivity	
	% of Total	% of Aqueous-soluble
Glucuronides	11.0 ± 1.2	36.7 ± 4.0
Sulfates	5.4 ± 1.0	18.0 ± 3.3
Thioethers	5.0 ± 0.8	16.7 ± 2.9

* The distributions of the radioactive estradiol metabolites in the aqueous extract from hepatocyte incubations after 2 hr were determined by measuring the organic-soluble materials liberated by β-glucuronidase, arylsulfatase, and Raney nickel treatment of the aqueous solutions. The data are presented as the percentage of total radioactivity used in the experiment and the percentage of the radioactivity present in the aqueous phase (mean % ± S.D.; N = 4).

irreversible binding of estrogens to DNA or protein catalyzed by phenobarbital-induced and 3-methylcholanthrene-induced microsomal preparations. This suggests that the cytochrome P-450 isozyme(s) responsible for this irreversible binding is not induced by these agents. This is consistent with biochemical studies on the microsomal enzyme estrogen 2-hydroxylase, which is not inducible with phenobarbital or 3-methylcholanthrene [26, 33, 34].

The studies with isolated hepatocytes also demonstrate that extensive oxidative metabolism can occur in the intact cell. The two major pathways of primary metabolism were hydroxylations at the 2-position and the 16α-position. Subsequent metabolism of the catechol estrogens 2-hydroxyestradiol and 2-hydroxyestrone to the 2-methoxy derivatives was also observed. The total percentages of 2-hydroxyestrogens and 2-methoxyestrogens formed by hepatocytes in cell suspension after 2 hr and isolated by HPLC were found to be 8.7% (S.D. = 2.6%) and 8.1% (S.D. = 3.3%) per 1×10^6 cells respectively. Further metabolism and/or degradation of the catechol estrogen formed may also be responsible for the more polar radioactive HPLC peak consisting of a heterogeneous mixture of metabolites and for the irreversible binding to cellular macromolecules. The amount of estriol formed under the same conditions was found to be 10.5% (S.D. = 1.6%) per 1×10^6 cells.

Approximately 30% of radiolabeled estrogen metabolite(s) was found in the aqueous fractions from the hepatocyte incubations after 2 hr, suggesting that extensive conjugation occurred via known pathways of glucuronidation, sufation and glutathione formation. Incubation of the aqeuous fraction with β-glucuronidase and arysulfatase resulted in 11.0% (S.D. = 1.2%) arising from glucuronides and 5.4% (S.D. = 1.0%) from sulfates. The organic-extractable steroids liberated by the β-gluronidase and sulfatase included estradiol, 2-hydroxyestradiol, and 2-hydroxyestrone as determined by reverse-phase HPLC. Treatment of the solution with Raney nickel provides indirect evidence that 5.0% (S.D. = 0.8%) of the radioactivity was present as the glutathione (or other sulfhydryl-linked) conjugate.

Finally, irreversible binding of radiolabeled estrogens was also observed in the protein fractions of the hepatocytes. The amount of radioactivity irreversibly-bound from the hepatocyte incubation after 2 hr was found to be 4.5% (S.D. = 1.22%) per 1×10^6 cells. Thus, irreversible binding of estrogens to cellular macromolecules occurs in the intact cell. Furthermore, such irreversible binding to proteins is observed in cells that have demonstrated secondary pathways of metabolism, such as catechol methylation and glucuronidation, sulfation and glutathione conjugation reactions. In addition, the extent of irreversible binding of estradiol metabolites(s) to the proteins in the hepatocytes is approximately the same as the irreversible binding to microsomal proteins in the *in vitro* microsomal incubations (7–8% of total estradiol in the microsomal studies). Hepatocyte incubations with more cells and with estradiol of higher specific radioactivity will be necessary in order to observe the degree of irreversible binding to the nucleic acids such as DNA.

The results of estradiol metabolism by isolated hepatocytes compares very favorably with *in vivo* metabolism studies in rats and with investigations using tissue slices by other investigators [1–3, 32]. Investigations of steroid metabolism in the isolated hepatocytes thus provides an effective *in vitro* technique for examining the total metabolic pathways—oxidative, reductive, and conjugative reactions—that are functional in the liver. The hepatocyte system also enables one to examine the roles of biochemical constituents (such as glutathione or other thiol reagents), enzyme inhibitors, hormones, and enzyme regulators (particularly inducers such as phenobarbital and 3-methylcholanthrene) in directly affecting the metabolic processes and altering adverse pathways, e.g. minimizing irreversible binding to cellular macromolecules. These *in vitro* studies with intact cell incubations, along with studies with cultured cell systems, can aid in further elucidation of the importance of metabolism and bioactivation of endogenous estrogens in physiological processes, in cellular damage, and in the development of diseases such as estrogen-dependent cancers.

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