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A Facile Assay for 2-Hydroxylation of Estradiol by Liver Microsomes

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Various studies have demonstrated that hydroxylation at C-2 is a major pathway of metabolism of estrogens in man and rat (1–4,38–40). Among the tissues examined in the rat, the liver was found to contain the highest 2-hydroxylating activity, followed by the brain, with other tissues having lower activity (5). 2-Hydroxylase activity was also observed in benign and malignant human breast tumors (31). The major portion of the 2-hydroxylase activity in the liver and brain (6,7,30) and kidney (27) was found to be present in the microsomal fraction. By contrast, 2-hydroxylase in the lung was highest in the mitochondrial fraction (27). Additionally, though the liver and brain 2-hydroxylase appears to be cytochrome *P*-450 mediated (17,29,30), it does not respond significantly to certain typical inducers of monooxygenase such as 3-methylcholanthrene and phenobarbital (5,7). Recent findings also showed the presence of 2-hydroxyestrogens in adult rat brain and in human fetus brain, pituitary, and liver (5,8,9). Certain findings (10–17) on the biological activity of catechol estrogens have suggested that 2-hydroxylation is probably not entirely a catabolic pathway and that catechol estrogens may be biologically active. Until recently, the assessment of 2-hydroxylation in animal tissues has involved relatively laborious procedures, among these, thin-layer chromatography (18), radioimmunoassays (19), and an enzymatic assay utilizing catechol *O*-methyl transferase (6,8). Additionally, a procedure utilizing distillation of ^3H_2O evolved in hydroxylation of ^3H-estradiol has been available for some time (28). These procedures, though useful, hampered investigations requiring analysis of large numbers of samples. A relatively simple radiometric assay has been developed (20), which also involves the determination of ^3H_2O released during the hydroxylation of [2-^{3}H]estradiol (^{3}H-E_2).[1] This method is based on the separation of ^3H_2O from residual ^{3}H-E_2 by the preferential elution of the ^3H_2O off an Amberlite XAD-2 resin column. This method, though simple and accurate, is nevertheless time consuming, since it requires, in every instance, to establish that the columns function well and that there is no channeling of the residual substrate (^{3}H-E_2), which if it occurs unnoticeably would render a given analysis invalid. Also, this assay requires that the mixture be acidified after the incubation and left standing overnight prior to protein removal and analysis.

We hereby describe a method that utilizes a similar radiometric approach but that does not involve column separation nor an overnight waiting period. Briefly, after the incubation, the microsomal proteins and the

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[1] Abbreviations used: E_2, Estradiol-17β; ^{3}H-E_2, [2-^{3}H]estradiol-17β; 2-Iodo-E_2, 2-Iodoestradiol-17β; 2-OH-E_2, 2-hydroxy-estradiol-17β; 4-OH-E_2, 4-hydroxy-estradiol-17β; DCC, dextran-coated charcoal; DDE, 2,2-bis-[*p*-chlorophenyl]-1,1-dichloroethylene; hplc, high-pressure liquid chromatography; hpf, highly polar fraction.

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residual substrate-^{3}H-E_2 are simultaneously removed by a sequential addition of $CaCl_2$, which aggregates the microsomes (21–23), and dextran-coated charcoal, which adsorbs the residual substrate. Following brief centrifugation, the radioactivity (^3H_2O) in the supernatant liquid is determined by scintillation spectrometry.[2]

Experimental Methods

Animals. Male albino Sprague-Dawley rats were purchased from Charles River Breeding Laboratories. When so specified, to induce microsomal monooxygenase activity, rats were injected with DDE (100 mg/kg/day in corn oil) ip for 3 days and the rats were decapitated 48 h later. Control rats were either not treated or were injected with the vehicle-corn oil (regimen as above). Livers were excised and microsomes were prepared as previously described (26). The microsomal pellet was suspended in 1.15% KCl at a protein concentration specified in the text; protein determinations were carried out by a modified procedure (34) of Lowry *et al.* (35) using bovine serum albumin as a reference protein.

Materials. Estradiol-17β was obtained from Steraloids. Glucose-6-phosphate, NADPH, and glucose-6-phosphate dehydrogenase were purchased from Sigma. [2-^{3}H]Estradiol-17β, provided by Dr. K. I. Williams, was prepared as described below. [6,7-^{3}H]Estradiol-17β (46 Ci/mmol) was obtained from Amersham Corporation. Aquasol was purchased from New England Nuclear, Boston, Massachusetts. Dextran-coated charcoal (DCC) was composed of 1% charcoal (activated charcoal untreated powder, Sigma Chemical Co.), 0.05% dextran (grade C, Schwarz/Mann), and 10 mM (pH 8) Tris–HCl buffer [Tris(hydroxymethyl)aminomethane, ultrapure grade] as previously described (36,37) and was usually used within 2 weeks. DDE (2,2-bis-[*p*-chlorophenyl]-1,1-dichloroethylene) was obtained from Aldrich Chemical Company.

Prior to the development of the assay of 2-hydroxylation, the following preliminary studies were performed.

1. Availability of Specifically Labeled [2-^{3}H]Estradiol in Pure Form

[2-^{3}H]Estradiol was kindly provided by Dr. K. I. Williams. The [2-^{3}H]E_2 was prepared by New England Nuclear from 2-Iodo-E_2 synthesized by Dr. Williams as previously described (25,32). The crude [2-^{3}H]E_2 purified by Dr. Williams (32) was further purified by us by thin-layer chromatography on ITLC-SA media (Gelman Instrument Co., Ann Arbor, Mich.) using benzene:ethyl acetate (2:1). Peak ^{3}H-E_2 was detected on a Vanguard radioactivity scanner. Occasionally, unlabeled E_2 was chromatographed alongside the ^{3}H-E_2 to ascertain that the major radioactive zone represented ^{3}H-E_2. The unlabeled E_2 was visualized by spraying with Turnbull's blue [a 1:1 mixture of aqueous solutions of 8% $FeCl_3$ and 1% $K_3Fe(CN)_6$]. The major radioactive zone corresponding to E_2 was eluted by several washings with absolute ethanol, and the solution containing the ^{3}H-E_2 was kept at 0°C. Additionally, we have been advised that New England Nuclear (Boston, Mass.), Amersham Corporation (Arlington Heights, Ill.), and Moravek Biochemicals (City of Industry, Calif.) could prepare [2-^{3}H]E_2, provided the customer would supply the precursor, 2-Iodo-E_2 or 2-Bromo-E_2; Amersham Corporation could provide [2-^{3}H]E_2 on a custom basis.

2. Determination of Whether Metabolism of E_2 by Rat Liver Microsomes, Under our Conditions, Yields Primarily 2-Hydroxylation

a. Separation of 2-OH-E_2 and 4-OH-E_2. We first established conditions that would

[2] This method was developed in response to our need to determine whether the inhibition of the E_2-mediated induction of ornithine decarboxylase by antiestrogens (24) may have been due to stimulatory effects by the antiestrogens on the hepatic metabolism of E_2.

separate 2-OH-E_2 and 4-OH-E_2 by high-pressure liquid chromatography (hplc).[3] The following conditions were set up: using a C_8 Whatman column 4.6 mm × 25 cm (reversed phase), the eluting solvent [46% methanol:54% H_2O (1% acetic acid)] was run at 2 ml/min and the compounds were monitored at 280 nm. Under these conditions, 4-OH-E_2 and 2-OH-E_2 had retention times of 28.8 and 30.9 min, respectively.

b. Identification of hydroxylation site. To detect radiolabeled metabolic products, we incubated [6,7-^{3}H]E_2 instead of [2-^{3}H]E_2, which if it were 2-hydroxylated would have lost the radiomarker. Thus, [6,7-^{3}H]E_2 (0.6 μCi; 100 nmol) was incubated with rat liver microsomes and NADPH-generating system for 20 min (see below for description of incubation conditions). The contents of 10 incubations were combined, acidified to pH 2–3, and extracted three times with 2 volumes of ether. The ether phase was washed with H_2O to neutrality and was evaporated under a stream of nitrogen, and the residue was dissolved in ethanol. Chromatography of an aliquot on hplc (Whatman C_8 column) with 46% methanol/54% H_2O (containing 1% acetic acid) demonstrated that a major portion of the radioactive product (10.3%) co-chromatographed with 2-OH-E_2; under these conditions, 4-OH-E_2 has a shorter retention time (see paragraph *a*) and there was no significant amount of radioactivity in this region. Also, 76% of the radioactivity had the retention of residual substrate-E_2. In addition, there was radioactivity in two early fractions (retention times of 5 to 10 min and 10 to 15 min) which together represented 9.6% radioactivity. These highly polar fractions most probably represent compounds resulting from multiple hydroxylations and/or conjugations of 2-OH-E_2. Multiple hydroxylations of E_2 are the most likely explanation for formation of the polar metabolites, since when an incubation was conducted with extremely low substrate concentration, using [6,7-^{3}H]E_2 without the addition of 100 nmol of unlabeled E_2, most of the products were in the highly polar fraction (hpf); i.e., an hplc analysis of the radiolabeled products demonstrated residual E_2 (8.2%), 2-OH-E_2 (0.6%), and hpf (85.1%). A similar observation by Jacobson *et al.* (41) with testosterone indicated multihydroxylation of testosterone without rupture of ring *A* by rat liver microsomes in the presence of low testosterone concentrations. Also, because in a subsequent study with [2-^{3}H]E_2 incubations all of the radioactivity was accounted for as ^3H_2O (see paragraph *c*), it could be surmised that all the products, including the highly polar compounds, were derivatives of 2-OH-E_2.

c. Identification of the radioactive product of incubation of [2-^{3}H]E_2 as ^3H_2O. After incubation of [2-^{3}H]E_2 with rat liver microsomes, the residual substrate was removed with dextran-coated charcoal (DCC) as described under Assay and the supernate was lyophilized. All of the radioactivity in the supernatant after DCC treatment was accounted for as ^3H_2O in the lyophilized sample. Namely, in two experiments the respective levels of radioactivity in the DCC supernatant and lyophilized distilate were 150,300 and 151,930 dpm (Exp. 1) and 21,510 and 21,990 dpm (Exp. 2). Radioactive monitoring was carried out in vials containing Aquasol with the use of a Packard Tri-Carb scintillation spectrometer model 3330, and dpm determinations were made by the channels' ratio procedure.

[3] Dr. K. Williams kindly provided 2-OH-E_2 and 4-OH-estrone (4-OH-E_1) (33). We prepared 4-OH-E_2 as follows: 4-OH-E_1 (2 mg) and $NaBH_4$ (2 mg) were dissolved in 1 ml methanol and allowed to react for 90 min. Two milliliters of degassed H_2O was added and the mixture was extracted with ether. The ether phase was evaporated under N_2. Whereas the parent compound, 4-OH-E_1, had a major peak at 1730 to 1740 cm^{-1}, the crude product from the above reaction contained no residual ketonic function (no significant peaks at 1700 to 2500 cm^{-1}) as indicated by infrared spectroscopy of the product as a film on NaCl crystal. Also, as expected, the resulting compound was more polar (9.2 min retention) than 4-OH-E_1 (13.3 min retention) on reversed-phase hplc with 30% CH_3CN:70% H_2O (1% acetic acid), using Waters Associates "fatty acid" column.

3. *Examination of Conditions for Elimination of Residual Substrate*

Prior to attempting to optimize conditions for assay of 2-hydroxylation of E_2 by liver microsomes, we found it necessary to establish conditions that would efficiently remove the residual substrate so that the product ^3H_2O could be assayed accurately. It was observed that, whereas DCC was able to remove essentially all of the substrate (^3H-E_2) from buffer, substantial amounts of radioactivity were retained in solutions containing microsomes. Therefore, following incubation, it was essential to eliminate the microsomes prior to the addition of DCC. We observed that the addition of $CaCl_2$, previously shown to be able to aggregate microsomes (21–23), facilitated the removal of microsomes at low centrifugation speed and thus provided suitable conditions for the assay. In fact, it was observed that the incubation could be terminated by ice-cold $CaCl_2$ (final concentration 0.008 M) followed by the addition of the resulting solution to a tube containing a DCC pellet. After vortexing and vigorous shaking at 0 to 4°C for 15 min with DCC, the suspension was centrifuged at 5000*g* for 10 min and the radioactivity representing ^3H_2O in an aliquot of the supernate (devoid of microsomes, charcoal, and substrate) was determined. These findings established the feasibility of the proposed assay as described below.

Assay

Incubation. Each incubation is conducted in a glass scintillation vial containing in 1 ml volume the following constituents: sodium phosphate buffer (0.1 M, pH 7.4), 0.4 ml; $MgCl_2$ (0.1 M), 0.1 ml; microsomal suspension, 0.1 ml (50–175 μg protein); 0.1 ml NADPH-generating system in phosphate buffer (glucose-6 phosphate, 4.5 μmol; NADPH, 0.36 μmol; glucose-6-phosphate dehydrogenase, 1 IU); 0.1 ml of [2-^{3}H]E_2 solution (0.1–0.15 μCi) in buffer; 5 μl of ethanolic solution of E_2 (100 nmol),[4] EDTA, 1 μmol[5]; H_2O, 0.2 ml.

The incubation mixture is kept on ice while the components (except for the NADPH-generating system) are added. The vials are placed in the incubator at 37° for 2 min and the reaction is started by adding 0.1 ml of the NADPH-generating system. After 20 min[6] of shaking in the incubator, 1 ml of ice-cold $CaCl_2$ (0.016 M) is added and the vials are placed on ice; zero-time controls were kept on ice throughout. All the samples are transferred into "culture" tubes containing charcoal (DCC) pellets (equivalent to 1 ml DCC) and the contents are vortexed and subsequently shaken for 15 min in the cold.

The tubes are centrifuged for 10 min at about 5000*g*. An aliquot (1 ml) of the supernatant is placed into a scintillation vial and is supplemented by 0.5 ml H_2O and 5 ml Aquasol (this mixture forms a single phase) and the radioactivity (dpm) representing ^3H_2O is determined by the channels' ratio in a scintillation spectrometer. These values are corrected for radioactivity remaining in the zero-time control and multiplied by two, since the aliquot taken represents half the sample.[7] The amount of products (2-hydroxy-E_2 and 2-hydroxy-E_2

[4] With microsomes of low 2-hydroxylase activity, a higher specific radioactivity of ^{3}H-E_2 should be utilized.

[5] Though exhaustive studies were not conducted, it appears that a slightly higher (~8%) rate of 2-hydroxylation was observed in the presence of EDTA. The requirement for EDTA in incubations with microsomes from other species should be examined prior to use. We observed that the hydroxylation of prostaglandins was dramatically enhanced by EDTA with rat, but not with rabbit and guinea pig, liver microsomes (Theoharides and Kupfer, unpublished).

[6] Longer linearity of product formation with time has been observed with microsomes of low enzymic activity.

[7] In 2 typical incubations (20 min, 100 μg microsomal protein) the radioactivity in the ^3H_2O was 44,710 dpm versus 1278 dpm (0-time control) and 54,850 dpm versus 1720 dpm (0-time control). Similar control blank values were obtained in incubations lacking NADPH. There was usually low variability within an assay; e.g., for identical quadruplicates the mean ± S.E. was 43,020 ± 207 dpm.

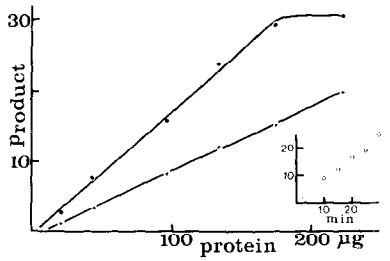

FIG. 1. Linearity of 2-hydroxylation of [2-^{3}H]E_2 with time and varying protein concentration. Ordinate depicts the product 2-OH-E_2 (nmol) formed per 10 min (▲) and per 20 min (●) incubation. The inset depicts nanomole product formation at varying time intervals using 115 μg of microsomal protein.

derivatives) formed is calculated as follows:

$$\frac{\text{dpm } {}^3H_2O \times \text{nmol } [{}^3\text{H-}E_2 + E_2] \text{ (added)}}{\text{dpm } {}^3\text{H-}E_2 \text{ (added)}} = \text{nmol product}$$

RESULTS AND DISCUSSION

To establish optimal incubation conditions for assaying 2-hydroxylation of E_2, we examined the ability of liver microsomes to catalyze this reaction in two buffers at 0.1 M, pH 7.4–7.5.[8] Rates of hydroxylation in sodium phosphate and Tris in 20-min incubations were 3.4 and 2.7 nmol of product, respectively. In all subsequent experiments we used phosphate buffer. NADPH was required for the reaction, and linearity of product formation up to 20 min was observed with NADPH (0.3 mM) or with NADPH-generating system containing NADPH (0.3 mM), glucose 6-phosphate (4.5 mM), and glucose 6-phosphate dehydrogenase (1 IU). The inclusion of EDTA, known to suppress microsomal lipid peroxidation, in the incubations with microsomes from control or *p,p'*DDE-treated rats appeared to yield a slight increase (~8%) in product formation and thus was routinely added. Linearity of product formation with time was observed for 20 min with microsomal protein up to 175 μg and for 10 min with protein amounts up to 225 μg (possibly higher) (Fig. 1). On using 43 μg (not shown) or 115 μg of microsomal protein and 0.1 mM ^{3}H-E_2, linearity of product formation to at least 30 min was observed (Fig. 1, inset). With 115 μg of the microsomal protein preparation and 20-min incubation time, a double reciprocal plot of velocity of product formation versus substrate concentration was constructed (Fig. 2). The K_m and V_{max} values determined by a linear regression analysis were 39.2 μM and 231.3 nmol/mg protein/20 min. Using a saturating concentration of ^{3}H-E_2 (100 μM) with liver microsomes (0.1 mg/incubation) for control rats (n = 4), the rate of 2-hydroxylation was 111.1 ± 10.7 nmol/mg protein/20 min. The above K_m value appears somewhat higher than the K_m (11 μM) in adult rats reported by Hoffman *et al.* (5) using their assay method involving catechol-*O*-methyltransferase (30). The lower 2-hydroxylating activity by liver microsomes observed with the radioenzymatic method (5) might be due to the absence of or diminished methylation of the putative multihydroxylated catechol estrogens.

Under the conditions described in our assay, the charcoal only partially adsorbed 2-

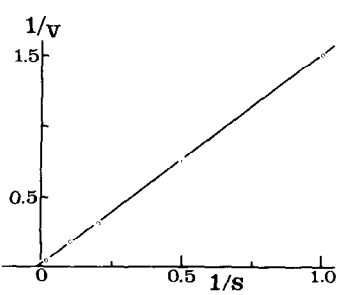

FIG. 2. A double reciprocal plot of velocity of 2-hydroxylation of [2-^{3}H]E_2 versus substrate (E_2) concentration. *V*, nmol of 2-OH-E_2 formed on incubation of ^{3}H-E_2 with 115 μg of microsomal protein and 20 min incubation time; *S*, ^{3}H-E_2 concentration (μM).

[8] Unless otherwise indicated, microsomes were usually prepared from livers of DDE-treated rats; we observed that DDE significantly increases the hepatic monooxygenase activities toward various xenobiotics and 2-hydroxylation of E_2 (Bulger and Kupfer, unpublished observations). Microsomes from control rats had lower activity (about 60% of the DDE values).

hydroxy-E_2.[9] This we concluded from the observation that incubations of [6,7-^{3}H]E_2 with liver microsomes yielded radioactivity which was not totally removable by DCC; whereas, in the absence of NADPH, essentially all of the [6,7-^{3}H]E_2 was eliminated by DCC.[10] Therefore, prior to utilizing the described assay for 2-hydroxylation of [2-^{3}H]E_2 with other tissues, with other species, or under novel conditions, it is *essential* to establish by other means, such as lyophilization or hplc (see above), that the radioactivity represents solely ^3H_2O and not hydroxylated [2-^{3}H]E_2 at sites other than C-2 or the occurrence of an NIH shift (42). Unfortunately, the study of Numazawa *et al.* (20) did not establish whether 2-hydroxy-E_2 or 4-hydroxy-E_2, similar to E_2, is also retained on the column; if it were so, then their method could have been helpful for resolving the above problem.

The absence of an NIH shift in E_2-hydroxylation has been demonstrated *in vivo* by Fishman *et al.* (43). Obviously, this does not exclude an NIH shift *in vitro*. Our observation that there is congruency in the amount of radioactivity in the aqueous phase after DCC treatment and the radioactivity in the lyophilized sample demonstrates that essentially all the radioactivity we measured was due to ^3H_2O. We also observed with hplc that an ether extract from an incubation of [2-^{3}H]E_2 with rat liver microsomes in the presence of NADPH and 0.33 mM ascorbate, which did not undergo the usual $CaCl_2$ and DCC treatment, exhibited essentially no radioactivity under the uv absorbing peak with retention time of 2-OH-E_2 and most of the radioactivity was as expected under the E_2 peak. This finding further substantiated the absence of an NIH shift in hydroxylation of E_2.

[9] Occasionally, however, a batch of charcoal totally removed 2-OH-E_2; nevertheless, it is risky to assume that each charcoal preparation will be as efficient.

[10] Alternatively, the radioactivity may have been formed by hydroxylation at C-6 or C-7.

The possibility that the described procedure underestimates the level of 2-hydroxylation because of a significant isotope effect of $^3H/^1H$ at the 2-position has been excluded. Since the values we observed for the hepatic 2-hydroxylation using the radiometric procedure were substantially higher than those obtained in the procedure described by Hoffman *et al.* (5), which does not involve a displacement of ^3H versus ^1H at C_2, a marked isotope effect was considered unlikely. Additionally, if there was a significant isotope effect, a decrease in the apparent rate of hydroxylation would be expected when the concentration of *non*labeled E_2 is increased passed the saturation level while maintaining the ^3H-E_2 constant. This decrease, however, did not occur; namely, similar rates of 2-hydroxylation were observed with the same amounts of radioactivity, albeit at 250 and 100 μM of E_2. Finally, conclusive evidence for congruency of formation of 2-OH-E_2 and ^3H_2O and for the lack of isotope effect during hydroxylation was obtained. Ether extracts of incubations of [2-^{3}H]E_2 with liver microsomes, in the presence of NADPH and 0.33 mM ascorbate, but which did not undergo DCC treatment, demonstrated the formation of 5.1 nmol of 2-OH-E_2 (analyzed by uv absorbance with hplc and corrected for 67.3% recovery of 2-OH-E_2); determination of 2-OH-E_2 by analyzing ^3H_2O formed in the same incubations, after DCC treatment, demonstrated 6.1 nmol of 2-OH-E_2 formed.

In conclusion, the described procedure demonstrates a simple and rapid method for assaying the hepatic microsomal NADPH-dependent 2-hydroxylation of estradiol. The congruency of product formation by analysis on hplc and by the measurement of the evolved ^3H_2O validates the radiometric assay. Also, this observation demonstrates that there is no significant isotope effect between displacement of ^1H versus ^3H during 2-hydroxylation of E_2, indicating that cleavage of the C_2-H bond is not the rate determining step in this monooxygenase-catalyzed hy-

droxylation. Though it has not been explored, it is expected that this method should be applicable with ease to assays involving this enzymatic activity in other tissues as well. In fact, one could achieve extremely high sensitivity, which would be needed for assaying low 2-hydroxylase activity, by merely increasing the specific radioactivity of the substrate [2-^{3}H]E_2. The finding of Jellinck *et al.* (44) that uterine *mitochondrial* peroxidase releases ^3H_2O from [2-^{3}H]E_2 indicates that caution must be exercised when applying our method to other tissues or to different subfractions. Also, if analysis (e.g., hplc) indicates that in certain tissues 4-hydroxylation is a significant pathway, this method could assess the contribution of this pathway by using [4-^{3}H]-E_2 in incubations in parallel with [2-^{3}H]E_2.

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