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DRUG–PROTEIN CONJUGATES—II

AN INVESTIGATION OF THE IRREVERSIBLE BINDING AND METABOLISM OF 17α-ETHINYL ESTRADIOL *IN VIVO*

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Abstract—Irreversible binding of radiolabelled material derived from [6,7-^{3}H]17α-ethinyl estradiol ([^{3}H]EE_2) to rat liver microsomal and soluble proteins occurred *in vivo*. It was measured by exhaustive solvent extraction and equilibrium dialysis. Three hours after administration of 5.0 μg kg^{-1}, 0.27 ± 0.14% (mean ± S.D., N = 4) of the dose was irreversibly bound to hepatic microsomes and 0.24 ± 0.16% to soluble protein. Induction of hepatic microsomal cytochrome P-448 and cytochrome P-450 by β-naphthoflavone (BNF) and phenobarbitone, respectively, did not significantly alter the irreversible binding of [^{3}H]EE_2. Although enzyme induction did not affect the extent of binding, treatment with BNF, but not phenobarbitone, altered the quantitative pattern of the sulphated biliary metabolites of [^{3}H]EE_2. The sulphated metabolites excreted by BNF-dosed rats comprised a significantly ($P < 0.005$) greater proportion of 2-hydroxyEE_2 than those excreted by vehicle-dosed controls. The increase was principally due to a decline in the proportion of 2-methoxyEE_2. It was suspected that the biliary metabolites of [^{3}H]EE_2 insusceptible to hydrolysis might include conjugates formed by reactions between thiols and the reactive metabolite(s) of [^{3}H]EE_2. Therefore L-[^{35}S]cysteine was administered prior to EE_2 in an attempt to label on or more of them. However, except for an ^{35}S-labelled component also excreted by rats given only L-[^{35}S]cysteine, none of the sulphur-labelled biliary components co-eluted with a major [^{3}H]EE_2 metabolite.

17α-Ethinyl estradiol (EE_2) *in vitro* [1, 2] and *in vivo* in a number of species [3–6], including man [7], undergoes extensive metabolism. Hydroxylation at C-2 is the predominant reaction *in vitro* [8] and *in vivo* [1]. Data from *in vitro* exeriments indicate that cytochromes P-450 and P-448 are involved in EE_2 hydroxylation [2]. EE_2 is oxidized, via 2-hydroxyEE_2 (2-OHEE_2), to a reactive metabolite which becomes irreversibly bound to microsomal and soluble protein [2, 9, 10]. Kappus *et al.* [9] and Stramentinoli *et al.* [11] observed that very small amounts of ^{3}H were irreversibly bound to microsomes from the livers of rats administered [^{3}H]EE_2. Oxygenation of the ethinyl group may also be responsible for reactive metabolite formation [12, 13]. The available data is most readily explained on the basis that the reactive metabolite of EE_2 generated *in vitro* via 2-OHEE_2 is an *o*-semiquinone [10].

The *in vitro* binding is inhibited by thiols, including cysteine and glutathione, which undergo non-enzymic reaction with the metabolite, resulting in the formation of C-1 and C-4 thioether adducts [1, 9, 10, 14]. Elce and Harris [15] observed that rats given 17β-[4-^{14}C]2-hydroxyestradiol by intraperitoneal (i.p.) injection converted 3–8% of the steroid to C-1 and C-4 glutathione conjugates of estrone. The former was further metabolized to [4-^{14}C]2-hydroxyestrone-3-methyl ether-1-*S*-cysteine. The results of several studies have established the concept that irreversible binding of estrogen metabolites to proteins stems from reaction with cysteinyl sulphydryl groups [16].

It has been suggested that the elevated incidence of vascular complications amongst women taking oral contraceptives may have an immunological basis [17]. Anti-EE_2 antibodies and circulating immune complexes containing EE_2 have been detected in such women [18, 19]. Immune complex formation might be a determinant of the initiation and progression of vascular damage. An immunogen could be formed when the reactive metabolite of EE_2 irreversibly binds to cellular proteins which are subsequently transported into the blood.

In order to assess the production of potentially immunogenic EE_2–protein conjugates *in vivo*, we have examined: (1) irreversible binding of ^{3}H to liver microsomes and soluble protein isolated from rats administered [^{3}H]EE_2, (2) reactions which either regulate the formation of reactive EE_2 metabolites or deactivate them, and (3) perturbations of [^{3}H]EE_2 metabolism which might result in an increase in irreversible binding. These reactions were investigated by analysing the biliary metabolites of EE_2. We have presented a detailed study of the biliary metabolites of EE_2 in the rat elsewhere [20]. In this paper we describe studies relating to the effects of enzyme induction on the metabolism and irreversible binding of EE_2 *in vivo*, and an examination of the possibility that cysteine-containing thioether conjugates of EE_2 are formed *in vivo*. Aspects of this work have been presented in preliminary form at meetings [21, 22].

MATERIALS AND METHODS

Materials. [6,7-^{3}H]17α-Ethinyl estradiol ([^{3}H]EE_2)

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(sp. act. 55 Ci mmole^{-1}) was obtained from New England Nuclear Corp. (F.R.G.). It was repurified by the method of Bolt *et al.* [1]. L-[^{35}S]Cysteine was a product of the Radiochemical Centre (Amersham, U.K.). When used, it had a sp. act. of 64 mCi mmole^{-1}. Unlabelled EE_2, 6α-hydroxyethinyl estradiol (6-$OHEE_2$) and 16α-hydroxyethinyl estradiol (16-$OHEE_2$) were generously donated by Schering A.G. (Berlin, F.R.G.). 2-Methoxyethinyl estradiol (2-$MeOEE_2$) was kindly provided by Dr W. Slikker Jr (National Centre for Toxicological Research, Jefferson, AR). 2-Hydroxyethinyl estradiol (2-$OHEE_2$) was prepared by the method of Stubenrauch and Knuppen [23]. All other reference estrogens, β-naphthoflavone (BNF) (5,6-benzoflavone) and sodium dodecyl sulphate (SDS) were purchased from Sigma (London) Chemical Co. Ltd (Poole, U.K.). Phenobarbitone (PB), Aristar® diethyl ether and liquid chromatography grade methanol were products of BDH Ltd (Poole, U.K.).

β-Glucuronidase from beef liver (Ketodase®) was a product of W. R. Warner & Co. Ltd (Eastleigh, U.K.). Arylsulphohydrolase (type VIII from abalone) and H-1 arylsulphohydrolase–β-glucuronidase preparation were obtained from Sigma.

Animals and treatments. Male Wistar rats (250–300 g) maintained on a pellet diet [41 Labsure Animal Diets (Rank Hovis McDougall, Poole, U.K.)] and tap water *ad lib.* were used. They were anaesthetized with urethane [1 ml per 100 g of 14% (w/v) solution, by i.p. injection], and their jugular veins and common bile ducts were cannulated. The rats received intravenous (i.v.) administrations (1 ml kg^{-1}) of 5.0 μg kg^{-1} [^{3}H]EE_2 (approximately 20 μCi kg^{-1}) in 0.9% (w/v) saline : ethanol (9 : 1 w/v). Bile was collected for 3 hr, after which blood was obtained by cardiac puncture and livers were removed for the immediate preparation of subcellular fractions. Bile and blood samples and the subcellular fractions were stored at −30° unless they were immediately analysed.

Pre-treatment with phenobarbitone (PB) and BNF. Male Wistar rats were subjected to the following pre-treatment regimens: four animals received BNF (75 mg kg^{-1} i.p.) in vegetable oil every 24 hr for 3 days and five animals received PB (80 mg kg^{-1} i.p.) in 0.9% (w/v) saline every 24 hr for 4 days. Controls were given vegetable oil or saline alone. Each rat was administered 5.0 μg kg^{-1} [^{3}H]EE_2 i.v. as described earlier 24 hr after completion of the pre-treatment regimen.

Administration of L-[^{35}S]*cysteine.* Male Wistar rats received EE_2 and L-[^{35}S]cysteine (240 μCi kg^{-1} i.p.) in an attempt to label any thioether adducts formed during metabolism of the steroid.

The amino acid was administered as three equal doses (80 μCi kg^{-1}) in isotonic saline at 12 hr intervals over 24 hr. The rats were cannulated via the jugular vein and common bile duct. Five hours after the final administration of L-[^{35}S]cysteine, one group received EE_2 (500 μg kg^{-1} i.v.) in saline : ethanol (3 : 7, v/v) whilst the other received vehicle alone. Bile was collected at 30 min, 1 hr and every hour for 5 hr thereafter. It was assayed for radioactivity and analysed by high-performance liquid chromatography (HPLC).

Measurement of irreversible binding to liver microsomes and soluble protein. Rat liver microsomes and 105,000 *g* supernatant proteins were prepared as previously described [20]. Protein concentrations were measured by the method of Lowry *et al.* [24] using bovine serum albumin as a standard. Microsomal cytochrome P-450 was assayed by its carbon monoxide difference spectrum after reduction with dithionite as described by Omura and Sato [25]. Spectra were recorded on a Pye Unicam SP-8 100 spectrophotometer.

The irreversible binding of ^{3}H-labelled material to microsomes, hepatic soluble proteins and plasma proteins was determined by: (1) exhaustive solvent extraction, and (2) equilibrium dialysis in the presence of SDS; the latter was based on the method of Sun and Dent [26]. Method (1) has been recorded in detail elsewhere [20]. In brief, the protein (*ca.* 10 mg) was sequentially extracted with two volumes of ethanol (70%, v/v) (× 2), 0.1 M sodium phosphate buffer (pH 7.4) (× 2), methanol (× 2) and ethanol (70%, v/v). The final protein pellet was dissolved in 1.0 M NaOH at 50°. Aliquots (0.2 ml) were neutralized with glacial acetic acid (50 μl), and the radioactivity was measured by liquid scintillation spectrometry. In method (2), portions of microsomal and soluble fractions and plasma, containing approximately 10 mg of protein, were dissolved in 10 mM sodium phosphate buffer (pH 7.4) containing 2% (w/v) SDS, in a boiling water bath. Aliquots (1-ml) were dialysed against 1.0 l of SDS (0.1%, w/v)–phosphate buffer for at least 18 hr. Thereafter, the radioactivity (dpm ml^{-1}) in the dialysis bag and dialysis buffer was determined. Substraction of the latter from the former gave a measure of the irreversibly bound radiolabelled material.

Enzymic hydrolysis of biliary metabolites. Bile samples from rats administered 5.0 μg kg^{-1} [^{3}H]EE_2 (50–300 μl, 0.2×10^6–0.5×10^6 dpm) were either extracted with ether (2 × 4 ml) or first incubated with enzyme under conditions which effected maximum hydrolysis. The following hydrolase preparations were employed: H-1 preparation from *H. pomatia* (82 units of arylsulphohydrolase activity per 100 μl of bile, pH 5.0, 37°, 3 hr), which possesses arylsulphohydrolase and β-glucuronidase activity; arylsulphohydrolase from abalone (80 units per 100 μl of bile, pH 5.0, 37°, 3 hr); β-glucuronidase from beef liver (Ketodase®) (5000 units per 100 μl of bile, pH 5.0, 37°, 16 hr). Control incubations contained 0.1 M sodium acetate buffer (pH 5.0). The ether extracts were combined, dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen, and the residue redissolved in 30 μl of methanol for analysis by HPLC.

HPLC. The analysis of [^{3}H]EE_2 biliary metabolites by reversed-phase HPLC has been detailed elsewhere [20], and similar methods were employed here. Metabolites were separated with an Aerograph Model 8500 liquid chromatograph (Varian Instruments Division, Palo Alto, CA) linked to a Partisil® 10/25 ODS-2 column [0.46 cm i.d. × 25 cm, N = 38×10^3 plates m^{-1} (Whatman Inc., Clifton, NJ)] protected by a guard-column packed with Co : Pell ODS® (Whatman Survival Kit). Samples (10–25 μl, 20×10^3–300×10^3 dpm ^{3}H or ^{35}S) were eluted from

Table 1. Excretion and irreversible binding of ^{3}H-labelled metabolites following administration of [^{3}H]EE_2 to male rats: effects of phenobarbitone and β-naphthoflavone on cytochrome P-450 or P-448 content and ^{3}H binding in liver

Experimental group*	^{3}H excreted in bile over 3 hr (% of dose)	Cytochrome P-450 or P-448 (nmoles mg^{-1} protein)	Irreversible binding of ^{3}H to whole liver protein (% of dose)			
			Exhaustive extraction		Dialysis method	
			Microsomal	Soluble protein	Microsomal	Soluble protein
Control	53 ± 12	Not determined	0.27 ± 0.14	0.24 ± 0.16	0.99 ± 0.58	0.62 ± 0.14
Saline control	65 ± 14	0.67 ± 0.16	0.29 ± 0.12	0.30 ± 0.13	0.72 ± 0.37	0.84 ± 0.83
Phenobarbitone-dosed	53 ± 4	1.31 ± 0.28‡	0.63 ± 0.36	0.30 ± 0.16	1.06 ± 0.22	1.19 ± 1.13
Vegetable oil control	62 ± 9	0.64 ± 0.14	0.23 ± 0.10	0.17 ± 0.10	1.27 ± 0.69	1.13 ± 0.49
β-Naphthoflavone-dosed	82 ± 10†	1.00 ± 0.19†	0.15 ± 0.05	0.32 ± 0.24	0.70 ± 0.27	0.65 ± 0.45

* Means ± S.D. (N = 4) except for saline control and phenobarbitone-dosed rats (N = 5). [^{3}H]EE_2 was administered i.v. (5 μg kg^{-1}) to bile duct cannulated male rats and bile was collected for 3 hr. Irreversible binding of ^{3}H-labelled metabolites to liver microsomes and soluble protein was determined by exhaustive extraction and equilibrium dialysis (see text).

† Significantly different from vehicle-treated group ($P < 0.05$).

‡ Significantly different from vehicle-treated group ($P < 0.01$).

the column with a linear gradient of methanol in 0.5% (w/v) ammonium dihydrogen phosphate buffer (pH 3.0) (40–65% for bile, 50–65% for deconjugated metabolites); the gradient (2% min^{-1}) was preceded by a 2-min isocratic period. The flow rate was 2 ml min^{-1}. The effluent was monitored at 280 nm with a Model LC3 u.v. absorbance detector (Pye Unicam Ltd, Cambridge, U.K.). Effluent fractions were collected at 30-sec intervals into scintillation vial inserts. Scintillant [NE260 (Nuclear Enterprises, Edinburgh, U.K.)] was added for measurement of radioactivity. The ^{3}H-labelled metabolites and unmetabolized [^{3}H]EE_2 liberated by enzymic hydrolysis were identified by co-chromatography with authentic compounds [20].

Statistical analysis. The results are expressed as means ± S.D. Student's non-paired *t*-test was employed to determine the significance of differences between comparable groups.

RESULTS

Irreversible binding of ^{3}H-labelled material to microsomes and soluble protein

Three hours after the i.v. administration of 5.0 μg kg^{-1} [^{3}H]EE_2 to cannulated male rats, very small quantities of radiolabelled material were irreversibly bound to microsomes prepared from their livers (Table 1). The bound ^{3}H was estimated, by the dialysis method, to represent 0.99 ± 0.58% (mean ± S.D., N = 4) of the dose. Consistently lower estimates were obtained with the exhaustive extraction technique (0.27 ± 0.14%, N = 4). Similar amounts of ^{3}H were bound to hepatic soluble protein and immeasurably small amounts to plasma protein.

Induction of hepatic microsomal mixed-function oxygenases

Rats pretreated with PB or BNF had a significantly greater hepatic microsomal cytochrome P-450/P-448 concentration than control animals (Table 1). In accordance with BNF's reported oxygenase induction properties [27], the hepatic microsomes from rats pre-treated with this compound possessed an absorption maximum of 448 nm.

Although both compounds acted as enzyme inducers, neither had a significant effect on the irreversible binding of ^{3}H to microsomes and soluble protein (Table 1).

Analysis of biliary metabolites of [^{3}H]EE_2

The metabolites of [^{3}H]EE_2 were rapidly and extensively excreted in bile following i.v. administration to cannulated rats (Table 1). Animals pretreated with either PB or BNF and control animals excreted similar proportions of their doses over 3 hr. The biliary metabolites were in all instances largely polar conjugates: only approximately 5% of the ^{3}H was extracted by ether (Table 2). After all incubations of bile samples with a mixture of β-glucuronidase and arylsulphohydrolase, approximately 60–70% of the radioactivity was extracted (Table 3). Incubating the bile with abalone arylsulphohydrolase and beef liver β-glucuronidase, which possess little contaminating enzyme activity [20], demonstrated that about 40 and 20% of the radiolabelled material

Table 2. Enzymic hydrolysis of biliary metabolites of [^{3}H]EE_2 in male rats

Experimental group	Per cent of incubated ^{3}H extracted into ether (mean ± S.D., N = 4)*			
	Buffer (pH 5.0)	β-Glucuronidase	Arylsulphohydrolase	H-1 preparation
Control	4 ± 2	44 ± 4	25 ± 4	58 ± 5
Saline control	5 ± 3	45 ± 3	27 ± 6	60 ± 5
Phenobarbitone-dosed	5 ± 1	40 ± 8	27 ± 6	65 ± 2
Vegetable oil control	5 ± 2	39 ± 4	29 ± 5	59 ± 2
β-Naphthoflavone-dosed	6 ± 1	45 ± 3	26 ± 7	70 ± 8

* [^{3}H]EE_2 was administered i.v. (5 μg kg^{-1}) to bile duct cannulated male rats and bile collected for 3 hr. Samples of bile were either mixed with buffer (pH 5.0) and extracted with ether or first incubation with enzyme at pH 5.0. Biliary conjugates were not hydrolysed during 16 hr incubations with buffer (control = 5 ± 1% extraction). Extraction was not pH-dependent over range pH 5.0–6.8 (control: pH 6.8, 6 ± 1%).

Table 3. Reversed-phase HPLC of deconjugated biliary metabolites of [^{3}H]EE_2 in male rats*

^{3}H-labelled component	Per cent of ^{3}H in HPLC column effluent (mean ± S.D., N = 4)				
	Control	Saline control	PB†-dosed	Oil control	BNF‡-dosed
Heterogeneous peak	14 ± 4	15 ± 5	9 ± 3	13 ± 4	6 ± 4
16-$OHEE_2$	3 ± 1	3 ± 1	2 ± 1	3 ± 1	3 ± 1
2-$OHEE_2$	16 ± 6	18 ± 7	26 ± 9	11 ± 1	32 ± 10§
EE_2	14 ± 1	14 ± 1	12 ± 2	15 ± 3	17 ± 2
2-$MeOEE_2$	33 ± 4	30 ± 3	31 ± 4	36 ± 7	25 ± 5
2-OHME‖	6 ± 1	6 ± 1	5 ± 2	6 ± 1	4 ± 0

* [^{3}H]EE_2 was administered i.v. (5.0 μg kg^{-1}) to bile duct cannulated male rats. Bile samples were incubated with arylsulphohydrolase–β-glucuronidase (H-1 preparation), and the metabolites were extracted into ether (see text). A minor (< 5%) peak of ^{3}H, eluting between 2-$OHEE_2$ and EE_2, did not co-chromatograph with any available authentic standards. The heterogeneous peak appears to be comprised of three components.

† Phenobarbitone.

‡ β-Naphthoflavone.

§ Different from vehicle-treated group ($P < 0.005$).

‖ 2-Hydroxymestranol.

Table 4. Reversed-phase HPLC of deglycosylated and desulphated biliary metabolites of [^{3}H]EE_2 in male rats*

^{3}H-labelled component	Per cent of ^{3}H in HPLC column effluent (means ± S.D., N = 4)					
			β-Glucuronidase		Arylsulphohydrolase	
	Saline control	BP†-dosed	Oil control	BNF‡-dosed	Oil control	BNF-dosed
Heterogeneous peak	16 ± 3	7 ± 2	11 ± 1	6 ± 4	16 ± 5	14 ± 5
16-$OHEE_2$	4 ± 1	3 ± 1	4 ± 1	3 ± 1	6 ± 1	6 ± 1
2-$OHEE_2$	7 ± 2	8 ± 2	7 ± 3	7 ± 2	8 ± 3	22 ± 5*
EE_2	25 ± 4	28 ± 3	25 ± 3	32 ± 4	8 ± 3	14 ± 5
2-$MeOEE_2$	27 ± 5	28 ± 2	27 ± 4	29 ± 8	42 ± 8	30 ± 6
2-OHME‖	7 ± 1	8 ± 3	8 ± 1	7 ± 1	2 ± 1	2 ± 1

* [^{3}H]EE_2 was administered i.v. (5.0 μg kg^{-1}) to bile duct cannulated male rats. Bile samples were incubated with beef liver β-glucuronidase (Ketodase®) and arylsulphohydrolase from abalone (see Fig. 1).

† Phenobarbitone.

‡ β-Naphthoflavone.

§ Different from vehicle-treated group ($P < 0.005$).

‖ 2-Hydroxymestranol.

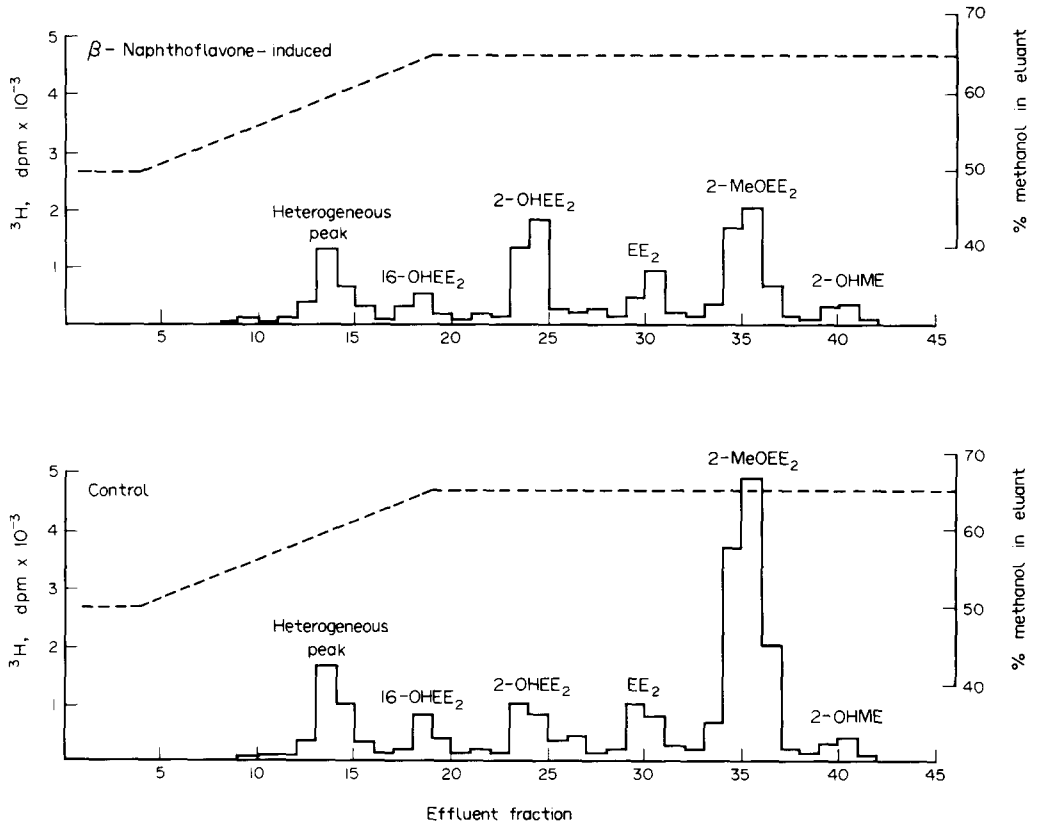

Fig. 1. Reversed-phase high-performance liquid chromatograms of desulphated biliary metabolites of [^{3}H]EE_2. Lower chromatogram, bile from a vegetable oil dosed rat. Upper chromatogram, bile from a β-napthoflavone-dosed rat. The conditions for the separations and the peak assignments are described in the text. Bile (0–3 hr) from bile duct cannulated male rats was incubated with abalone arylsulphohydrolase, and the desulphated ^{3}H steroids were extracted into ether. The solid lines show profiles of radioactivity; the broken lines the gradient of methanol in phosphate buffer.

was comprised of β-glucuronides and arylsulphate esters, respectively (Table 4). Pre-treatment of animals with either PB or BNF did not significantly alter the extents of these hydrolyses.

Chromatographic and mass spectrometric analyses have shown that the deconjugated ^{3}H-labelled material is comprised largely of EE_2, 16-$OHEE_2$, 2-$OHEE_2$, 2-$MeOEE_2$ and 2-hydroxymestranol (2-OHME) [20]. A ^{3}H peak which co-chromatographed with 6-$OHEE_2$ was also observed, but mass spectrometry suggested that it was composed of three co-eluting metabolites; none of which were unequivocally identified. The same components were excreted as biliary conjugates by rats pre-treated with either PB or BNF. In every experimental group, EE_2 and 2-$MeOEE_2$ were the principal radiolabelled aglycones (Table 4). Administration of the enzyme inducers had neither qualitative nor significant quantitative effects on the aglycone metabolite profile. In contrast, administration of BNF, but not of PB, increased the proportion of 2-$OHEE_2$ in the conjugate fraction hydrolysed by the H-1 preparation: from 11 ± 1% (N = 4) to 32 ± 10% ($P < 0.005$). By employing abalone arylsulphohydrolase, it was confirmed that this increase was confined to the arylsulphate fraction; and was largely accounted for by a decrease in the proportion of 2-$MeOEE_2$ (Table 4, Fig. 1). There were no significant alterations in the proportions of deconjugated EE_2.

Although BNF perturbed the deconjugated-metabolite profile, it did not affect the qualitative and quantitative pattern of biliary conjugates resolved by reversed-phase HPLC [20] (unpublished data).

Examination of bile for thioether metabolites of EE_2

Cannulated male rats pretreated with 240 μCi kg^{-1} L-[^{35}S]cysteine excreted 1.5 ± 0.5% (N = 3) of the 75 μCi dose via the bile during the 5 hr experiment. Intravenous administration of 500 μg kg^{-1} EE_2 to rats pre-treated with L-[^{35}S]cysteine failed to affect the excretion of radioactivity: these animals eliminated 1.8 ± 0.4% of the dose over 5 hr. The ^{35}S-labelled material in bile was analysed by HPLC. Four components, excreted by both groups of rats, eluted in the same region of the chromatogram as the biliary metabolites of [^{3}H]EE_2 [20]. Over 5 hr, no additional peaks of ^{35}S were found in bile from animals given EE_2 and L-[^{35}S]cysteine (Fig. 2); during this period, more than 50% of a 500 μg kg^{-1} dose of [^{3}H]EE_2 is excreted via the bile [20]. Only one of the ^{35}S-labelled components (retention time 13 min) had the same retention time as a major biliary metabolite of [^{3}H]EE_2. HPLC analysis of sequential

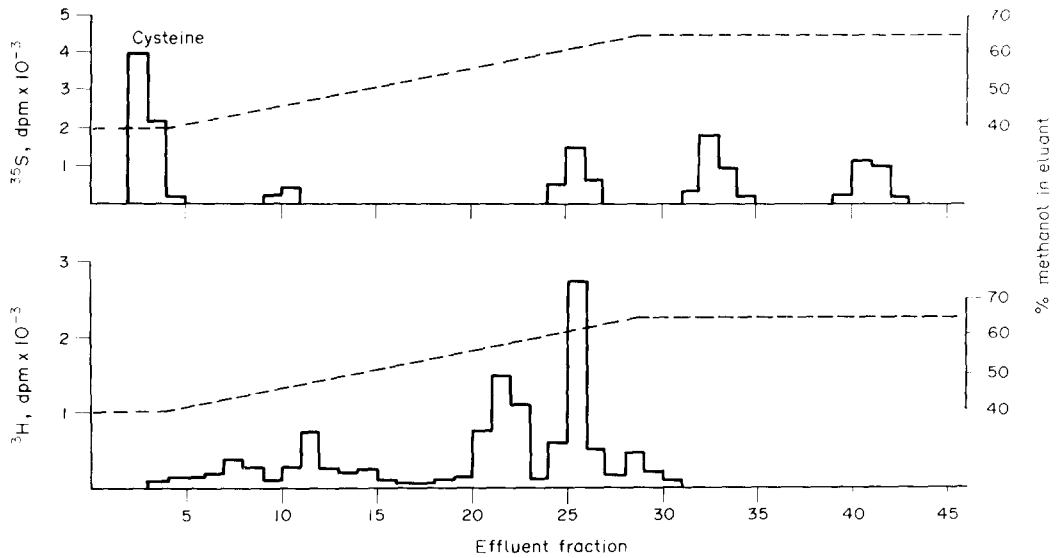

Fig. 2. Reversed-phase high-performance liquid chromatograms. Upper chromatogram: ^{35}S-labelled biliary components excreted by male rats administered either L-[^{35}S]cysteine (i.p.) or L-[^{35}S]cysteine (i.p.) followed by 500 μg kg^{-1} EE_2 (i.v.). Lower chromatogram: Biliary metabolites of [^{3}H]EE_2 excreted by male rats administered 500 μg kg^{-1} (i.v.). The solid lines show representative profiles of radioactivity; the broken lines the gradient of methanol in phosphate buffer.

bile samples failed to detect any change in the proportions of this component consequent upon administration of EE_2.

DISCUSSION

The data recorded here have confirmed and amplified previous reports [9, 11, 20] that small amounts of radiolabelled material irreversibly bind to cellular proteins *in vivo* following administration of [^{3}H]EE_2. Approximately 0.5% of the 5 μg kg^{-1} dose was bound to hepatic microsomes and soluble protein 3 hr after i.v. administration. The persistence of this material is indicated by the finding of Kappus *et al.* [9] that up to 0.5% (0.83 ± 0.19% g^{-1} protein) of an 11 μg kg^{-1} dose of [^{3}H]EE_2 is irreversibly bound to rat hepatic microsomal proteins 48 hr after dosing. These workers employed an extraction method. They established that the binding of ^{3}H from [6,7-^{3}H]estrogens to microsomal protein was not due to ^{3}H exchange processes.

The values for irreversible binding obtained using the dialysis method were approximately double those determined by the exhaustive extraction technique. Regarding their relative merits, dialysis in the presence of SDS is claimed to provide a more accurate estimate of binding [26]. With the extraction method, lower mol. wt proteins may fail to precipitate and certain precipitable proteins might be resolubilized by the extraction procedure. These factors would lead to an underestimation of irreversible binding. During this investigation it was found that soluble and microsomal protein concentrations decreased by 50% in the course of exhaustive extraction, whereas the protein concentrations were unaffected by dialysis. In contrast, when [^{3}H]EE_2 was incubated with hepatic microsomes under conditions which precluded metabolism, 14% of the ^{3}H remained bound after dialysis (unpublished data). The corresponding figure for the extraction method was 0.75%. Both techniques removed all the biliary metabolites of [^{3}H]EE_2. From this it appears that dialysis considerably overestimates irreversible binding of the reactive metabolites.

Soluble hepatic protein irreversibly bound a very small quantity of ^{3}H *in vivo*. This suggests that some of the reactive metabolites escaped from the endoplasmic reticulum. Analagous observations have been made in respect of *in vitro* experiments: serum albumin reduces irreversible binding of [^{3}H]EE_2 metabolites to microsomes and itself becomes radiolabelled [9, 10, 14]. The failure to detect any ^{3}H-labelled protein in plasma indicates that little or none of the labelled intracellular protein was rapidly secreted in blood. Nevertheless, the possibility of a slow release of antigenic hepatic protein cannot be excluded.

In agreement with the findings of Maggs *et al.* [20], hydroxylation at C-2 followed by methylation of the catechol moiety was the principal route of [^{3}H]EE_2 metabolism in all experimental groups investigated. *In vitro*, formation of 2-OHEE_2 leads to extensive irreversible binding: the methylation, glucuronidation and sulphation which occurs *in vivo* would largely preclude this. Pre-treatment of rats with BNF produced an approximately three-fold increase in the proportion of 2-OHEE_2 excreted as biliary conjugates without changing the proportion of unmetabolized EE_2. However, it did not effect a significant increase in irreversible binding. These observations contrast with those made when an *in vivo–in vitro* system is employed. Thus, pre-treatment with PB—which, as reported here, does not appear to effect the metabolism of [^{3}H]EE_2 *in vivo* in

rats—produces an increase in hepatic microsomal cytochrome P-450 content [28], and enhances the metabolism of [^{3}H]EE_2 by hepatic microsomes and irreversible binding of its metabolites [1, 9]. Induction of cytochrome P-450/P-448 during the present study did not enhance the already extensive hydroxylation of [^{3}H]EE_2 in male rats. Hoffman *et al.* [29] have shown that the estrogen-2-hydroxylase activity in male rat liver is about 6 times higher than in the female liver. The failure to detect any significant perturbation of [^{3}H]EE_2 metabolism in the rat following chronic administration of PB is noteworthy: PB has been found to enhance hydroxylation, demethylation and glucuronidation [28, 30]. However, previous reports [29, 31] have recorded that rat liver estrogen-2-hydroxylase activity is unchanged after administration of PB or 3-methylcholanthrene.

The increase in the proportion of 2-OHEE_2 was largely a product of decreased 2-*O*-methylation. There was no change in the sum of 2-OHEE_2 and its isomeric catechol *O*-methylated metabolites [vehicle-treated group 53 ± 8%, BNF-treated 61 ± 6% (Table 3)], which gives an overall measure of 2-OHEE_2 formation. The mechanism by which BNF exerts its effect on the extent of 2-OHEE_2 catechol *O*-methylation is unknown, but it may be via an enhancement of *O*-demethylation. BNF is a potent inducer of *O*-dealkylation in rats [32]. It was notable that the increase in 2-OHEE_2 was confined to the arylsulphate fraction. Since 2-OHEE_2 does not appear to be preferentially sulphated *in vivo* (Table 4, [20]), the increase may have resulted from selective demethylation of the 3-*O*-sulphate of 2-MeOEE_2. If this was the case, the greater excretion of sulphated 2-OHEE_2 in bile would not be a reflection of a higher intra-hepatic concentration of the catechol. Nor would it suggest a potential for enhanced irreversible binding to liver protein. Steroid sulphates have been shown to be substrates for a cytochrome P-450 dependent mono-oxygenase of rat liver microsomes [33]. The work of Pennings and Van Kempen [34] suggests that catechol *O*-sulphation of 2-OHEE_2 would preclude subsequent A-ring *O*-methylation.

The present investigation and previous studies [9, 11, 20] collectively demonstrate that *in vivo* there is sufficient metabolic capacity to prevent extensive irreversible binding of [^{3}H]EE_2 metabolites over the dose range 5–500 $\mu g\ kg^{-1}$. However, Stramentinoli *et al.* [11] have shown that administration of an analogue of *S*-adenosyl-L-methionine to rats reduces the binding of [^{3}H]EE_2 *in vivo*, apparently by enhancing catechol *O*-methylation of 2-OHEE_2 [35]. We have established that 2-MeOEE_2 is the principal glucuronidated and sulphated biliary metabolite of [^{3}H]EE_2 in male rats [20].

Analysis of the biliary metabolites by hydrolysis with selective enzymes as described earlier is superior to chemical degradation [22]. Estrogen glycosides are quite stable to hydrolysis by dilute acid, and under more drastic conditions there is considerable destruction of the aglycone and decreased extraction of ^{3}H into ether [36]. The biliary metabolites which remained in aqueous solution after incubation with enzymes were possibly thioether conjugates. 2-17β-Hydroxyestradiol is converted into glutathione conjugates in the rat [15, 37]. These are eliminated via the bile and urine.

The biliary excretion of ^{35}S from L-[^{35}S]cysteine was not affected by administering a large i.v. dose of unlabelled EE_2. Taken in conjuction with the fact that cannulated rats given L-[^{35}S]cysteine and EE_2 did not excrete a unique ^{35}S-labelled biliary metabolite, this suggests that thioethers do not account for a substantial fraction of the enzyme-resistant metabolites. Jellinck and Elce [38] have reported that thioether adducts of estrogens are highly susceptible to alkali degradation. However, this effect is an unlikely explanation for the apparent absence of biliary thioether metabolites of EE_2 in rats since the pH of the bile was *ca.* 6.5. This deduction agrees with the finding that only small amounts of [^{3}H]EE_2 metabolites irreversibly bind to cellular protein *in vivo*: the latter reaction is considered to involve formation of thioether linkages with cysteinyl moieties [16].

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