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DRUG–PROTEIN CONJUGATES–VII

DISPOSITION OF [^{3}H]-ETHINYLESTRADIOL–PROTEIN CONJUGATES IN THE RAT

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Abstract—[^{3}H]17α-Ethinylestradiol ([^{3}H]EE_2; 5 μg/kg, 98.5 μCi) was administered to a female rat. After 18 hr less than 0.02% of the dose was present per ml plasma. Approximately 60% of radioactivity present in plasma was irreversibly bound to proteins, as determined by exhaustive solvent extraction and by high performance ion exchange chromatography of proteins after removal of unbound metabolites with activated charcoal. After chronic administration of [^{3}H]EE_2 (5 μg/kg; 2 μCi per day) for 22 days, there was a three- to fourfold accumulation of radioactivity in the plasma, together with an accumulation of radioactivity in the lung, liver, kidney, spleen and brain, compared to animals receiving a single dose. The spleen showed the greatest (>tenfold) significant ($P < 0.001$) accumulation of radioactivity. There was a greater increase in radioactivity irreversibly bound to the soluble fraction than to the microsomal fraction of the liver. [^{3}H]EE_2 was conjugated to rat serum proteins by incubation with rat microsomes *in vitro*. Upon administration to female rats, the [^{3}H]EE_2–rat serum protein conjugate had a small volume of distribution (12.5 ± 0.5 ml), and its plasma concentration declined slowly ($t_{1/2}$ = 450 ± 140 min). Immunization of male New Zealand White rabbits with a chemically synthesized conjugate of 2-hydroxyethinylestradiol (2-OH-EE_2) and human serum albumin produced antibodies which bound EE_2 and 2-OH-EE_2 but not estrone. These data indicate that although reactive metabolite formation represents a minor biotransformation, drug protein conjugates may accumulate during chronic administration.

17α-Ethinylestradiol (EE_2), the estrogenic component of most combined oral contraceptive formulations, is metabolized to a reactive metabolite *in vivo* and *in vitro*, which may bind irreversibly to cellular macromolecules [1, 2]. Thus we have observed that approximately 1.5% of a 5 μg/kg dose of [^{3}H]EE_2 is irreversibly bound to liver proteins 3 hr after administration to female rats. Although this is a minor pathway of metabolism in the rat, the significance of irreversible binding of EE_2 to proteins and cell structures has yet to be established.

Beaumont *et al.* [3, 4] have reported the presence of antibodies against EE_2 in some women taking oral contraceptives, and suggest that immune complexes in the blood may be responsible for an increased incidence of vascular disease in oral contraceptive users. It is therefore possible that the irreversible binding of EE_2 metabolite(s) to proteins *in vivo* may produce an immunological reaction with subsequent toxicological implications.

Because EE_2 is rapidly excreted into bile, and because small quantities of radioactivity were used in our previous experiments [1], it was not possible to establish the presence of irreversibly bound metabolites of EE_2 in the serum of rats. We were therefore interested to see if EE_2–protein conjugates appeared in rat serum, and, if so, in their disposition. In addition, it was of interest to see if an accumulation of drug–protein conjugates occurred upon chronic administration of [^{3}H]EE_2.

This paper describes the investigation of the presence of EE_2–protein conjugates in rat serum after a single dose of [^{3}H]EE_2 (5 μg/kg; 98.5 μCi), and after chronic treatment for 22 days. We also report on the *in vivo* fate of [^{3}H]EE_2–protein conjugates prepared *in vitro*, and on the immunogenicity in rabbits of a chemically synthesized EE_2–human serum albumin conjugate.

MATERIALS AND METHODS

Materials. [6,7-^{3}H]17α-Ethinylestradiol ([^{3}H]EE_2) (sp. act. 55 Ci/mmole) and ammonium [6,7-^{3}H]-estrone sulphate (44 Ci/mmole) were obtained from New England Nuclear Corp. (F.R.G.). [^{3}H]2-Hydroxyethinylestradiol ([^{3}H]2-OH-EE_2) was prepared as previously described [5]. Unlabelled EE_2, estrone and NADPH were purchased from Sigma (London) Chemical Co. Ltd (Poole, U.K.). Unlabelled 2-OH-EE_2 was prepared by the method of Stubenrauch and Knuppen [6]. Amberlite XAD-2 resin was purchased from British Drug Houses Ltd (Poole, U.K.). NE260 scintillant was a product of Nuclear Enterprises (Edinburgh, U.K.). All other chemicals were of AnalaR grade and were obtained from British Drug Houses Ltd.

Animals. Female Wistar rats weighing 200–250 g

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and male New Zealand White rabbits weighing 2.5 kg were obtained from Bantin & Kingman Ltd. (Hull, U.K.). They were maintained on a pellet diet (Rank Hovis McDougall, Poole, U.K.) and tap water *ad libitum*.

Irreversible binding of [^{3}H]EE_2 *to plasma proteins* in vivo. A female rat (210 g) was administered [^{3}H]-EE_2 (5 μg/kg; 98.5 μCi) in 1.2 ml 0.9% saline–ethanol (9:1 v/v) by i.p. injection, at 4.30 p.m. After 18 hr, blood was collected into a heparinized container by cardiac puncture under light ether anaesthesia. The plasma was separated from the blood and the radioactive content of a 20 μl aliquot was measured in 4 ml NE260 scintillant in a Packard Tricarb 4640 liquid scintillation spectrometer.

Plasma was examined for the presence of irreversibly bound metabolites of [^{3}H]EE_2 by several methods. Plasma proteins were separated by high performance ion exchange chromatography (HPIEC). HPIEC was performed on a Gilson Gradient HPLC system comprising two 302 pumps, a Gilson 111B fixed wavelength u.v. detector monitoring at 280 nm, a Gilson 201 fraction collector, all controlled by an Apple IIe microcomputer running a Gilson Gradient Former program.

The column used was a TSK DEAE 5-PW (Toyo Soda Mfg. Co., Tokyo, Japan). The gradient was programmed according to Table 1, using the following buffers: Buffer A: 20 mM Tris/HCL, pH 7.7; Buffer B: A + 1 M NaCl. The flow rate was 1 ml/min.

In an attempt to remove any tightly bound metabolites, plasma was heated at 56° for 30 min in the presence of activated charcoal (50 mg/ml plasma). This procedure removed 98% of radioactivity from a mixture of rat plasma (0.5 ml) and [^{3}H]EE_2 (0.5 μCi). In addition, plasma was subjected to exhaustive solvent extraction of proteins as previously described [2].

Chronic treatment. Five female rats were administered [^{3}H]EE_2 (5 μg/kg per day, 2 μCi per day each, at 4.30 p.m. each day) for 22 days. On days 1, 5, 9, 12, 16 and 19 after the initial injection, animals were lightly anaesthetized with ether and 1 ml blood collected from the tail artery 18 hr after dosing. On day 21, a further five female rats were given a single dose of [^{3}H]EE_2. All ten rats were lightly anaesthetized with ether on day 22, and 5 ml of blood removed from each into heparinized containers by cardiac puncture. The animals were sacrificed by cervical dislocation and the livers, kidneys, lungs, spleens and brains removed, rapidly frozen and stored at −60° until analysed.

Table 1. Gradient profile for high performance liquid chromatography of plasma proteins

Time (min)	% B
0	0
16	20
20	20
24	30
24.5	100
30	100

Buffer A: 20 mM Tris/HCl, pH 7.7.
Buffer B: 20 mM Tris/HCl, pH 7.7 + 1 M NaCl.

The extent of irreversible binding of metabolites to plasma proteins from all samples and to microsomal and soluble protein fractions of the livers, was determined by exhaustive solvent extraction as previously described [2].

The distribution of radioactivity was determined in each tissue. Samples of tissue (50 mg) were digested in 1 ml NCS tissue solubilizer overnight at 50°. Radioactivity was determined after quenching with hydrogen peroxide (0.25 ml per aliquot) and neutralization with glacial acetic acid (40 μl per aliquot), in 10 ml NE260 scintillant in a Packard Tricarb 4640 liquid scintillation spectrometer.

Preparation of [^{3}H]EE_2*–rat serum protein conjugate.* A female rat was lightly anaesthetized with ether and exsanguinated by cardiac puncture. The serum was separated and stored at 4° until used. Pooled liver microsomes were prepared from four female rats as described previously [2]. [^{3}H]EE_2 (10 μM; 40 μCi) was added to rat liver microsomes (2 mg protein/ml) and NADPH (0.6 mM) in 0.1 M phosphate buffer, pH 7.4 (9 ml), mixed with female rat serum (3 ml) and incubated at 37° for 45 min. The incubation mixture was rapidly cooled to 4° and the microsomes removed by centrifuging at 105,000 *g* for 60 min at 4°. The supernatant containing the conjugate was passed through a column (30 × 2.5 cm) of Amberlite XAD-2 resin equilibrated with 0.1 M phosphate buffer, pH 7.4. The eluate was monitored with an LKB Uvicord® u.v. monitor at 280 nm and the first protein peak collected and concentrated on an Amicon B15 protein concentrator. A yield of 6 μCi (15%) [^{3}H]EE_2–conjugate was obtained. A 100 μl aliquot (400,000 dpm) was added to 0.9 ml bovine serum albumin as carrier (0.5% in phosphate buffer) and extracted three times with 3 ml absolute ethanol. The precipitate was dissolved in 1 ml 0.1 M NaOH and duplicate 0.25 ml aliquots neutralized with glacial acetic acid (10 μl), and the radioactivity determined in 4 ml scintillant. Ninety-six percent of the radioactivity was assessed to be irreversibly bound by this method.

Disposition of [^{3}H]EE_2*–protein conjugate* in vivo. Four female rats were anaesthetized with urethane (1.4 g/kg in isotonic saline, i.p.), and their jugular veins and carotid arteries cannulated. After heparinization of the animals (400 I.U./kg), 0.55 ml of the solution of conjugate (1 μCi; 30 mg protein) was infused over 1 min through the jugular cannula. Blood (0.5 ml) was collected from the carotid artery at 10, 30, 60, 120, 180 and 300 min after infusion, and replaced with an equal volume of isotonic saline. The plasma was separated immediately and 10 μl aliquots were mixed with 4 ml scintillant and the radioactivity determined. Duplicate 50 μl aliquots were extracted three times with absolute ethanol (3 ml total volume). The proteins were dissolved in 1 ml 0.1 M NaOH and the ethanol extracts combined, reduced in volume and the radioactivity of both fractions measured. The half-life of the conjugate was determined by least squares linear regression analysis of the plasma concentration vs time curve, and the apparent volume of distribution was calculated by dividing the administered dose by

the extrapolated value for the zero-time concentration [7].

In addition, for comparison, plasma-free EE_2 concentrations were determined by radioimmunoassay as described by Back *et al.* [8, 9], in four anaesthetized female rats which were administered 100 μg EE_2/kg.

Synthesis of human serum albumin–EE_2 conjugate and immunization of rabbits. 2-OH-EE_2 (70 mg) and [^{3}H]2-OH-EE_2 (1.5 μCi) were dissolved in 50% aqueous acetic acid (25 ml). Sodium metaperiodate (100 mg) was added in distilled H_2O (2 ml) and the mixture shaken vigorously for 30 sec. Excess periodate was quenched with ethylene glycol (1 ml) and distilled H_2O (12.5 ml). The resulting quinone was extracted twice with dichloromethane (20 ml total), and the combined extracts were washed with saturated aqueous sodium chloride, then evaporated to dryness. The quinone was redissolved in dioxan (4.2 ml). Human serum albumin (HSA; 100 mg) was dissolved in deoxygenated distilled H_2O (8.6 ml) and dioxan (5.8 ml), and a few drops of 1 M NaOH added. The solution of quinone was added and the mixture stirred for 10 min. The resulting 2-OH-EE_2–HSA conjugate was dialysed for 48 hr against distilled H_2O in the presence of 0.5 mM ascorbic acid [5], with 4 changes of water.

Three male New Zealand White rabbits weighing 2.5 kg were immunized with the 2-OH-EE_2–HSA conjugate as follows. Protein conjugate (3 mg) was dissolved in 1 ml saline (0.9% w/v) and emulsified with 1 ml Freund's complete adjuvant. This was further emulsified in 1 ml aqueous 'Tween 80' (1% w/v), and the resulting 3 ml emulsion divided between two injections (1 ml) into the thigh muscles and four dorsal subcutaneous injections (0.25 ml).

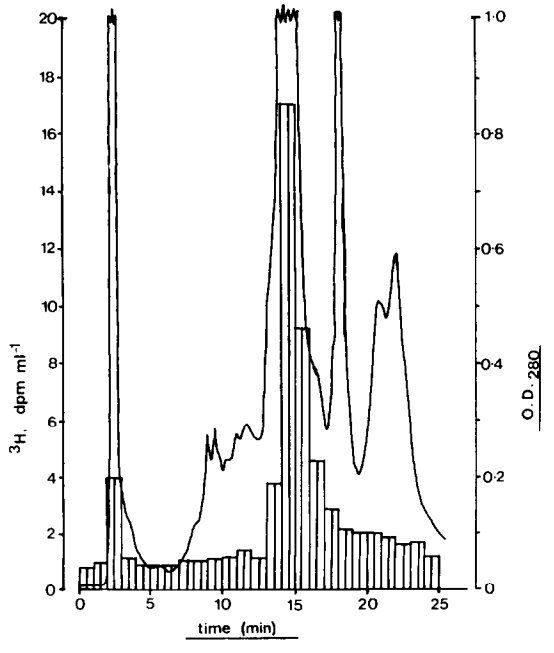

Fig. 1. Radioactivity (▥) and protein elution (—) profiles for high performance ion exchange chromatography of plasma from a rat 18 hr after administration of [^{3}H]-ethinylestradiol (5 μg/kg; 98.5 μCi).

Booster doses were administered one and two months after the initial injections, using the same schedule.

Blood was collected from the marginal ear vein at fortnightly intervals until two weeks after the final booster and allowed to clot overnight. The serum was separated and stored at −20° until assayed for antibody. The formation of antibody was determined by radioassay. Serum was diluted 1:100 in 0.1 M phosphate buffered saline (0.9% w/v; pH 7.0) containing 0.5 mM ascorbic acid (PBSA) and 0.1% (w/v) bovine serum albumin (0.1% BSA). [^{3}H]EE_2, [^{3}H]2-OH-EE_2 and [^{3}H]estrone were diluted in 0.1% BSA so that 0.05 ml contained 20,000 cpm. Unlabelled steroids were diluted in 0.1% BSA so that 0.05 ml contained 1 ng. Diluted antiserum (0.1 ml) was incubated overnight at 4° with 0.05 ml radiolabelled steroid and either 0.05 ml of 0.1% BSA or 0.05 ml of unlabelled steroid, in duplicate, for all three ligands. BSA (0.1 ml of 0.5% solution) followed by 1 ml dextran coated charcoal (2.5% w/v charcoal, 0.025 mg/ml dextran in PBSA) were added. After 10 min at 4° the charcoal was removed by centrifugation at 2000 rpm at 4° for 10 min and the bound radioactivity determined by liquid scintillation spectrometry. Specific binding was calculated as the difference in binding between incubations with and without unlabelled steroid.

RESULTS

Plasma protein conjugates of [^{3}H]EE_2 *in the rat after single dose*

Eighteen hours after administration of [^{3}H]EE_2 (5 μg/kg, 98.5 μCi) to a single female rat, less than 0.02% of the dose was present per ml plasma.

Protein conjugates of [^{3}H]EE_2 were detected in the plasma by two methods. Exhaustive solvent extraction showed that approximately 50–60% of the radioactivity in the plasma was covalently bound to proteins. HPLC of plasma proteins (Fig. 1) showed that the major proportion of the radioactivity (60%)

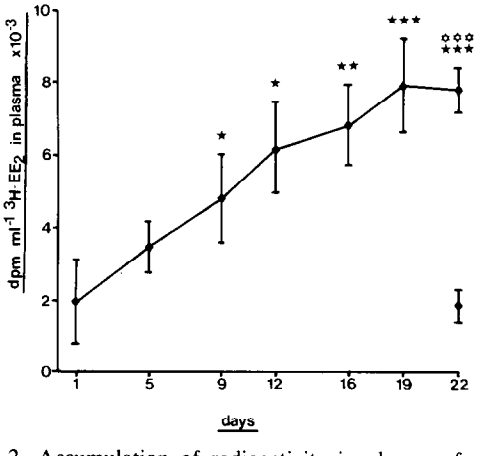

Fig. 2. Accumulation of radioactivity in plasma of rats administered [^{3}H]ethinylestradiol (5 μg/kg + 2 μCi per day) for 22 days or a single day. Plasma samples were obtained from the tail artery 18 hr after drug administration (N = 5, mean ± S.D.). ⋆ $P < 0.05$; ⋆⋆ $P < 0.005$; ⋆⋆⋆ $P < 0.001$; ☆☆☆ $P < 0.001$ (compared to single treatment group).

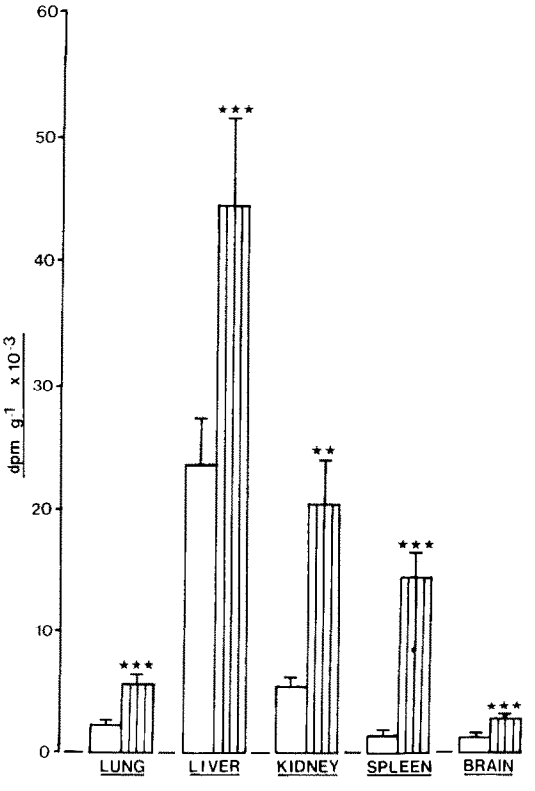

Fig. 3. Distribution of radioactivity in various organs of rats administered [^{3}H]ethinylestradiol (5 μg/kg; 2 μCi) after a single dose (□) and after chronic administration for 22 days (▥) (N = 5, mean ± S.D.). ★★ $P < 0.005$; ★★★ $P < 0.001$.

was associated with the albumin fraction, while a small quantity (<10%) eluted in the void volume. Extraction with charcoal (50 mg per ml plasma) could only remove 20% of the radioactivity, while this method removed 98% of radioactivity from plasma which was mixed with [^{3}H]EE_2 and immediately extracted.

Plasma radioactivity and irreversible binding of [^{3}H]-EE_2 *to plasma proteins after chronic administration*

Figure 2 shows the accumulation of radioactivity in plasma when female rats were administered [^{3}H]-EE_2 (5 μg/kg per day, 2 μCi per day) for 22 days, compared to radioactivity from animals treated for a single day. After 22 days, 59 ± 15% of the radioactivity in plasma was irreversibly bound to proteins, as determined by exhaustive solvent extraction.

Radioactivity in tissues after chronic treatment

Figure 3 shows the radioactivity present in the organs examined, expressed as dpm per g tissue. There was a significantly greater quantity of radioactivity in the tissues of animals which had been treated chronically with [^{3}H]EE_2 than in those which had only received a single dose. The greatest increase in content of radioactivity occurred in the spleen, where there was approximately a tenfold increase per g tissue. The livers contained the greatest quantity of radioactivity per g tissue in both sets of animals:

Table 2. Distribution of [^{3}H]ethinylestradiol irreversibly bound to liver protein fractions

Radioactivity in liver fraction (dpm/g × 10^{-3})		
	Microsomes	Soluble
Controls	263.9 ± 62.2	69.1 ± 20.8
22 days EE_2	412.0 ± 111.0	168.5 ± 11.5
P	<0.05	<0.001

Results are means ± S.D. (N = 5).

P values from comparison of data by Student's non-paired *t*-test.

Animals received [^{3}H]ethinylestradiol (5 μg/kg; 2 μCi) daily for 22 days or a single dose (controls) 18 hr before sacrifice.

there was a twofold increase in total radioactivity in the liver after chronic treatment. Examination of radioactivity in the soluble and microsomal protein fractions (Table 2) showed a highly significant increase in the quantity irreversibly bound to soluble proteins (greater than twofold increase, $P < 0.001$), with a smaller, significant increase in radioactivity irreversibly bound to microsomal proteins ($P < 0.05$). The kidneys showed a three- to fourfold increase in radioactivity, while the lungs and brain showed a two- to threefold increase.

Fate of [^{3}H]EE_2*–protein conjugate* in vivo

Figure 4 shows the plasma concentration–time curve of [^{3}H]EE_2 irreversibly bound to rat serum proteins, after administration of the [^{3}H]EE_2–rat serum protein conjugate. The plasma concentration–time curve for free EE_2 as determined by radioimmunoassay is shown for comparison. The plasma concentration of the conjugate declined monoexponentially, with an apparent half-life of 450 ± 140 min and an apparent volume of distribution of 12.5 ± 0.5 ml per animal.

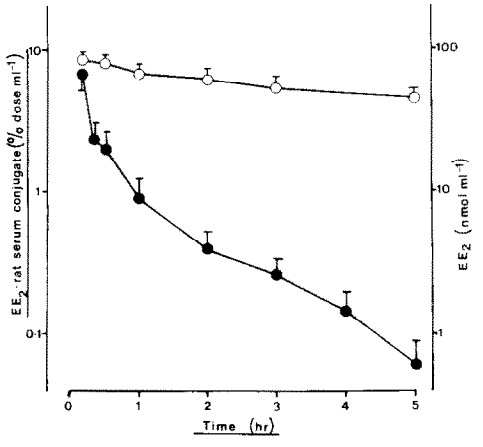

Fig. 4. Plasma concentration vs time curve for [^{3}H]-ethinylestradiol–rat serum protein conjugate (○—○) measured by liquid scintillation spectrometry after solvent extraction of plasma proteins (N = 4, mean ± S.D.). Plasma concentration vs time curve for free ethinylestradiol (●—●) measured by radioimmunoassay after administration of ethinylestradiol (100 μg/kg) (N = 4, mean ± S.D.).

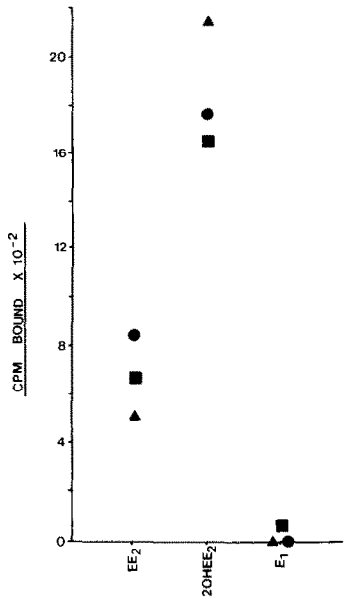

Fig. 5. Specific binding of [^{3}H]ethinylestradiol (EE_2), [^{3}H]-2-hydroxyethinylestradiol (2-OH-EE_2) and [^{3}H]estrone (E_1) by antibody raised in three rabbits against ethinylestradiol–protein conjugate, prepared by chemical oxidation of 2-hydroxyethinylestradiol

Immunization of rabbits with EE_2–HSA conjugate

Figure 5 shows the binding of EE_2, 2-OH-EE_2 and estrone to serum (1:100), from the three rabbits immunized with the EE_2–HSA conjugate. 2-OH-EE_2 was bound by the antiserum to a greater extent than was EE_2, while estrone did not bind at all.

DISCUSSION

We have confirmed the presence of [^{3}H]EE_2–protein conjugates in rat serum after i.p. administration of [^{3}H]EE_2, using several methods for removing tightly bound metabolites combined with a novel analytical tool, namely high performance liquid chromatography of proteins. Thus we have shown that approximately 60% of the radioactivity present in rat serum is irreversibly bound to proteins, 18 hr after a single dose of 98.5 μCi [^{3}H]EE_2 (5 μg/kg). The protein conjugate is a minor metabolite of [^{3}H]-EE_2, accounting for less than 0.02% of the administered dose per ml plasma.

There was an accumulation of radioactivity in the plasma of rats administered [^{3}H]EE_2 over 22 days. It was not possible to assess accurately the extent of irreversible binding in the early plasma samples from these rats due to the relatively small quantity of radioactivity present. It is not certain, therefore, whether the accumulation of radioactivity in plasma over the 22 days is due to irreversibly bound radioactivity or other metabolites of EE_2. However, at the end of this period, the proportion of radioactivity in plasma which was irreversibly bound to plasma proteins, was similar to the proportion bound after administration of the single dose of highly radiolabelled EE_2 to a female rat, described above.

In agreement with the findings of Bolt and Remmer [10] in the mouse, there was an accumulation of radioactivity over the 22-day period in several organs. Thus there was a twofold increase of radioactivity in the liver, accompanied by a two- to threefold increase in the lungs, a three- to fourfold increase in the kidneys, a twofold increase in the brain, and a greater than tenfold increase in the spleen. In the liver, this accumulation of radioactivity may at least partly be explained by an increase in the amount of radioactivity irreversibly bound to proteins. Specifically, there was a significant increase in radioactivity irreversibly bound to proteins in the microsomal and soluble fractions, the increase being greater in the soluble fraction. A differential rate of protein turnover in the liver may account for this difference in accumulation. The accumulation of radioactivity may also be due to the presence of other metabolites of EE_2 [10].

Serum protein conjugates of [^{3}H]EE_2 were prepared by incubation of [^{3}H]EE_2 and rat serum in the presence of rat liver microsomes in order to achieve as close an approximation as possible to serum protein conjugates formed *in vivo*. Also, while chemical methods may be used for generating reactive metabolite(s) of [^{3}H]EE_2 [11], we felt that as mild a method as possible should be used in the preparation, in order not to denature the proteins and thus alter their disposition inadvertently. When injected intravenously into the rats, the protein conjugate declined monoexponentially with an apparent plasma half-life of 450 min (7.5 hr). The low apparent volume of distribution, 12.5 ml, indicates that the conjugate was situated mainly in the plasma compartment. The slow but definite decline in plasma radioactivity may be due to distribution of the conjugate into extravascular spaces [12], as well as uptake into tissues such as liver and spleen, where metabolism of protein conjugates may occur (B. K. Park, unpublished observation).

The formation of a drug–protein conjugate *in vivo* may be important as the initiating step in eliciting an immune response. We therefore investigated whether a conjugate of EE_2 with human serum albumin could be immunogenic *in vivo*. In this case, a chemical method of conjugation was used to produce a conjugate with several drug molecules per protein molecule, to ensure an immune response. Antibodies were obtained in rabbits to EE_2 and 2-OH-EE_2 but not to estrone. This indicates that the antibody recognizes a difference in structure of the D-ring but not of the A-ring of the steroid nucleus, confirming the conjugation of the reactive metabolite to the protein through the A-ring. The synthetic conjugate had a steroid:protein ratio of 7.7:1. Assuming albumin to be the protein to which the reactive metabolite irreversibly binds *in vivo* in the rat, we have calculated that after a single dose of 5 μg/kg, only one protein molecule in 5×10^5 is conjugated. Immunization schedules designed to produce a hyperimmune response to haptens generally employ hapten:protein ratios much higher than this (>1:1). However, little is known about the immune response to haptens during chronic exposure, especially in the absence of immunostimulants such as Freund's adjuvant.

We have therefore shown that administration of [^{3}H]EE_2 to female rats results in the presence of irreversibly bound metabolite(s) in the plasma. The protein conjugate is largely confined intravascularly and has a long half-life compared to EE_2. On chronic administration, an accumulation of irreversibly bound metabolite(s) occurs in plasma and in the liver. The immunological consequences of such an accumulation have yet to be established, but it is possible that antibody formation may follow, directed against the protein conjugate. It is interesting to note the large increase in radioactivity in the spleen on chronic administration, an organ which has the capacity to phagocytose blood-borne particles and antigens.

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