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Covalent Binding of Norethynodrel to Proteins and Glutathione Initiated by Rat Liver Oxygenase

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SUMMARY

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When the labeled synthetic progestin, [4-^{14}C]- or [6,7-^{3}H]norethynodrel, was incubated with rat liver microsomes, the steroid became tightly bound to proteins. The binding process required oxygen and NADPH, and was inhibited by carbon monoxide and SKF 525-A. Microsomes from animals treated with phenobarbital were more effective in causing binding. Precipitation of the proteins with trichloracetic acid, washing with organic solvents, dialysis against water, and filtration through Sephadex G-200 all failed to dissociate the radioactivity from the proteins. Unlabeled steroid added after the initiation of the incubation was unable to displace radioactivity from the proteins. Disc gel electrophoresis of the solubilized proteins indicated that the steriod was bound randomly to all proteins. Reduced glutathione and compounds that react with thiol groups inhibited radiolabeling of the proteins. Moreover, glutathione increased the water-soluble radioactivity at the expense of the protein bound radioactivity. Experiments using tritiated glutathione and [4-^{14}C]norethynodrel demonstrated the formation of norethynodrel-glutathione conjugates. It was suggested that liver cytochrome P-450 oxygenase metabolized norethynodrel to a reactive intermediate(s) which could react with and bind covalently to proteins and glutathione. The implication of these findings is discussed.

INTRODUCTION

The oxygenase system in the endoplasmic reticulum of hepatocytes is capable of oxygenating a great variety of xenobiotics and endogenous materials (1). This broad spectrum of substrate specificity is ideally suited for the study of the mechanism, the disposition of substrates, and the implications *in vivo* of the oxygenation reactions of selected compounds.

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Previous work in our laboratory identified hydroperoxides as intermediates in microsomal hydroxylation reactions (2-4). In addition, evidence that epoxide and *N*-hydroxide are the initial products of oxygenation has been reported (5, 6). Since some of these oxygenated metabolites and/or their conversion products are highly reactive, one might expect them to react with cellular constituents such as proteins and nucleic acids (7-9). It has been inferred that occasional hepatonecrotic effects of some inert compounds are medi-

ated by chemically active metabolites formed by this liver microsomal oxygenase system (7).

In this communication we report a steroid-protein binding complex of the synthetic progestin, norethynodrel[1] and liver microsomes.

Norethynodrel is the first and most frequently prescribed progestin in oral contraceptives (10). Its metabolism (11–13) and its untoward effects in oral contraceptive preparations (14, 15) have been described.

MATERIALS AND METHODS

Animals. Male Charles River Sprague-Dawley rats weighing 200–250 g were used. The rats were allowed free access to food (Purina laboratory chow) and water. When phenobarbital treatment was desired, the rats were supplied with a 0.1% aqueous solution of sodium phenobarbital as drinking water for 10 days (16).

Chemicals. Norethynodrel, norethindrone, ethynylestradiol, and mestranol were supplied by G. D. Searle and Company. Estr-5(10)-en-17β-ol-3-one was a gift from Eli Lilly and Company. SKF 525-A[2] was a gift from Smith Kline & French Laboratories. All the steriods were purified by recrystallization before use.

NADPH, NADH, $NADP^+$, glucose-6-phosphate dehydrogenase (type XV), ribonuclease b, *N*-ethylamaleimide, *p*-chloromercuribenzoate, 2-mercaptoethanol, iodoacetamide, dithiothreitol, glutathione, estradiol-17β, and 19-nortestosterone were purchased from Sigma Chemical Company. Coomassie brilliant blue was obtained from Mann Research Laboratories. Guanidine HCl was purchased from Eastman Kodak. Instagel was obtained from Packard, and chicken ovalbumin, from Miles Laboratories.

L-Glutathione-([2-^{3}H]glycine) (specific radioactivity, 285 mCi/mmole) which contained some of the oxidized form was purchased from New England Nuclear Corporation. [6, 7-^{3}H]Norethynodrel (105 mCi/mmole), purity 99%, and [4-^{14}C]norethynodrel (5.4 mCi/mmole), purity 95%, were generously supplied by G. D. Searle and Company. The purity was checked by thin-layer chromatography (Brinkmann coated silica gel) using solvent systems of ethyl acetate–cyclohexane (1:1, v/v) (17) and benzene–ethyl acetate (85:15, v/v). Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) were purchased from Amersham/Searle.

Sephadex G-200 and G-50 were purchased from Pharmacia Fine Chemicals. Acrylamide, *N*,*N*′-methylenebis(acrylamine), *N*,*N*,*N*′,*N*′-tetramethylenediamine, and ammonium persulfate were obtained from Canal Industrial Corporation. Sodium dodecyl sulfate and Pyronine Y were purchased from Matheson, Coleman, and Bell. Amberlite XAD-2 beads were Rohm and Haas products.

Preparation of rat liver subcellular fractions. The rat was decapitated. The liver was perfused thoroughly with ice-cold 1.15% KCl via the portal vein before removal, cut into small pieces, and passed through a garlic press. One part of the liver slurry in 2 parts of 1.15% KCl (w/v) was homogenized in a Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle. Cellular fractions were prepared by differential centrifugation in a Sorvall centrifuge (model RC2-B) and a Spinco model L preparative ultracentrifuge.

Nuclei, mitochondria, and cell debris were removed by centrifuging the homogenate for 20 min at 10,000 $\times$ *g*. The microsomal fraction was obtained from this supernatant fraction by centrifugation at 105,000 $\times$ *g* for 1 hr. The supernatant (cytosol fraction) was removed. The microsomal pellets obtained were rehomogenized in the same volume of fresh KCl solution and centrifuged again at 105,000 $\times$ *g* for another hour. The supernatant was dis-

[1] Systematic nomenclature for compounds given trivial names in the text includes: 17α-ethynyl-5(10)-estren-17β-ol-3-one for norethynodrel; 17α-ethynylestr-4-en-17β-ol-3-one for norethindrone; 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol for ethynylestradiol; 17α-ethynyl-3-methoxy-1,3,5(10)-estratrien-17β-ol for mestranol; 1,3,5-estratriene-3,17β-diol for estradiol-17β; estr-4-en-17β-ol-3-one for 19-nortestosterone.

[2] The abbreviation used is: SKF 525-A, β-diethylaminoethyldiphenylpropyl acetate.

carded, and the tubes containing the microsomal pellets were stored at −20° and used within 4 days. All the preparations were done at 0–4°. The difference spectrum of norethynodrel with liver microsomes from rats that had been treated with phenobarbital was determined according to Schenkman *et al.* (18) on a Cary 14 spectrophotometer.

Incubation and fractionation of incubation mixture. The frozen microsomal pellets were thawed and homogenized gently in 0.1 M phosphate buffer, pH 7.4. Incubation was carried out in a Dubnoff metabolic shaker under air at 37° unless otherwise specified. The standard incubation mixture contained [6,7-^{3}H]norethynodrel or [4-^{14}C]norethynodrel in benzene solution (benzene was removed by a gentle stream of nitrogen gas), 8 mM $MgSO_4$, 10 mM nicotinamide, a NADPH-generating system (0.6 mM $NADP^+$, 6 mM glucose-6-P, and 0.5 Kornberg unit of glucose-6-P dehydrogenase), and 10 mg of microsomal protein. The final mixture was suspended in 5 ml of 0.1 M phosphate buffer, pH 7.4. In the control blank the NADPH-generating system was omitted. Protein concentration was determined by the biuret method (19), using crystalline bovine serum albumin as the standard.

The reaction was stopped by extracting the incubation mixture twice with peroxide-free ether. After drying over anhydrous Na_2SO_4, the ethereal fraction was evaporated to dryness and the residue was redissolved in 2 ml of methanol–ethyl acetate (1:1, v/v) for radioactivity assay.

Precipitation of the microsomal proteins from the aqueous fraction was facilitated by the addition of trichloracetic acid (to a final concentration of 5%) and by centrifuging the turbid solution at 1000 × *g* for 15 min. The supernatant obtained was designated the aqueous supernatant.

The protein precipitate was washed twice with 5 ml each of ethanol (95°), ethyl acetate, trichloracetic acid, and H_2O by resuspension and centrifugation to remove nucleic acids and lipids (20). The final residue was transferred to filter paper, dried, and combusted in a Packard sample oxidizer. Tritiated water and/or $^{14}CO_2$ resulting from combustion were trapped and counted.

Gel filtration. The ether-extracted aqueous fraction was dialyzed according to the procedure of Zaffaroni (21). Methanol (2 ml) was added to the protein solution, which was then dialyzed against 20 ml of 60% aqueous methanol and 10 ml of chloroform. After 22 hr the protein residues in the dialysis bag were made soluble by the addition of guanidine HCl (to a final concentration of 5 M).

Sephadex G-200 was allowed to swell for several days in 0.05 M phosphate buffer, pH 7.4, containing 5 M guanidine HCl. A column, 0.9 × 30 cm, was then prepared and allowed to equilibrate with the same phosphate–guanidine HCl buffer. Two milliliters of the solubilized microsomal protein were applied to the top of the column and eluted with 0.05 M phosphate–5 M guanidine HCl buffer. Fractions of 1.5 ml were collected. The ultraviolet absorption at 280 nm of each fraction was determined. In addition, portions were taken from each tube for radioactivity assay. The void volume of the column was 4.5 ml, and all the gel filtration procedures were done at room temperature (22).

Gel electrophoresis. For gel electrophoresis, [6,7-^{3}H]norethynodrel (0.5 μCi) was incubated with NADPH (0.6 mM), $MgSO_4$ (10 mM), nicotinamide (8 mM), and microsomal proteins (10 mg) under air at 37° for 30 min. After ether extraction, the aqueous fraction was dialyzed against 0.05 M phosphate buffer, pH 7.4, for 24 hr. The contents in the dialysis bag were made soluble by the addition of an equal volume of a reagent mixture [6% sodium dodecyl sulfate, 10 mM EDTA, 10 mM 2-mercaptoethanol, 4 M urea, and 30% (w/v) sucrose] and brief boiling. To each disc electrophoretic tube, 0.1–0.15 ml of this solubilized protein solution was applied.

The conditions for acrylamide disc gel electrophoresis, described in the legend of Fig. 4, were the same as those employed by Lenard (23) except that the pH of the phosphate buffer was 7.4 instead of 7.0, and 2 M urea was present in the 6.25% acrylamide gel.

Protein fixation and staining were done

with propan-2-ol and Coomassie brilliant blue (24). The gel was scanned with a Photovolt densitometer. Several gel pieces were combined, sliced, and combusted in the Packard oxidizer for the detection of radioactivity distribution.

Conjugation with glutathione. L-Glutathione-([2-^{3}H]glycine) (1.2 μCi) and [4-^{14}C]norethynodrel (0.5 μCi), or L-glutathione (5 mM) and norethynodrel (1 mM), were incubated in the standard incubation mixture. After shaking at 37° for 30 min, the incubation mixtures, containing either radioactive or nonradioactive substrates, were pooled and the trichloroacetic acid-soluble fraction was obtained as described earlier. This aqueous fraction was then passed through a 37 × 2 cm XAD-2 column that had been washed with ethanol and water (25). The column was then washed with 300 ml of water and 200 ml of ethanol. In this manner, the bulk of unreacted reduced glutathione was eluted with water. There was 95% recovery of ^{14}C counts in the ethanolic wash.

The ethanol fraction was concentrated to a small volume under reduced pressure. A portion was spotted on Whatman No. 3MM paper for descending chromatography. 1-Butanol–acetic acid–water (25:4:10, v/v) and 1-propanol–water (70:30, v/v) were used as the developing solvents (26). The chromatogram was run for 18 hr, dried, and sprayed with ninhydrin (Sigma) or with divalent sulfur reagent ($K_2Cr_2O_7$-$AgNO_3$) (27). The paper strips were cut into 1 × 2 cm pieces and counted in scintillation vials. An external standard curve was constructed with $^{133}BaSO_4$ for calculation of the disintegrations per minute of ^{14}C and ^{3}H.

Photo-oxygenation-induced binding. The incubation condition was the same as that used in the chemical synthesis of 17α-ethynyl-10β-hydroperoxy-19-nortestosterone from norethynodrel by Shapiro and co-workers (28). [4-^{14}C]Norethynodrel (0.6 μCi), chicken ovalbumin (20 mg), and eosin (2 mg) in 20 ml of water were stirred with or without 7 M urea in an atmosphere of oxygen. A desk-top fluorescent lamp with two 15-W Sylvania white light tubes was placed within 1 in. of the reaction flask to provide illumination. Samples of 2 ml were removed at various intervals and extracted twice with ethyl acetate. The aqueous fraction was dialyzed against distilled water. The radioactivity remaining in the bag was measured. In addition, the bag contents were subjected to gel filtration on a Sephadex G-50 column. The column (2 × 32 cm) was eluted with water. Fractions of 3 ml were collected and assayed for radioactivity and protein content. The latter was determined by method of Lowry *et al.* (29).

Measurement of radioactivity. A Nuclear-Chicago mark I scintillation spectrometer was used for counting. A toluene-based scintillator (0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene) was used for the organic extract, while a 1,4-dioxane-based scintillator (0.004% 1,4-bis[2-(5-oxazolyl)]benzene, 1.4% 2,5-diphenyloxazole, and 12.5% naphthalene) was used for aqueous and ethanol fractions, and Instagel for Sephadex G-200 eluents.

Combustion of insoluble proteins and gel slices was carried out in a Packard Tri-Carb sample oxidizer (model 305). $^{14}CO_2$-trapping reagent and scintillation mixture were prepared as described in the Packard manual.

RESULTS

Formation of protein-bound metabolite. After aerobic incubation of [6,7-^{3}H]norethynodrel or [4-^{14}C]norethynodrel with NADPH and rat liver microsomes, 23.8% of the radioactivity was recovered in the washed, trichloracetic acid-precipitated proteins (Table 1). The formation of this metabolite proceeded rapidly in the complete oxygenase system. It started at a linear rate and reached a plateau within 10 min (Fig. 1), with an apparent K_m of 3.33×10^{-5} (Fig. 2). The reaction had a pH optimum of 7.5–8.0. It required oxygen as well as NADPH. Neither NADH or $NADP^+$, nor ferrous ion or hemoglobin (30), was effective. Phenobarbital treatment of the rats doubled the radioactivity incorporation (Table 2). Carbon monoxide and SKF 525-A, both inhibitors of the NADPH-dependent microsomal oxygenase

TABLE 1

Recoveries of radioactivity after incubation of [6,7-^{3}H]norethynodrel with rat liver microsomes

Incubation was carried out at 37° for 15 min; conditions were the same as described in Fig. 1. Each fraction was obtained as described in MATERIALS AND METHODS. The radioactivity added to the incubation mixture (0.2 μCi) was taken as 100%. A similar distribution of radioactivity was found when [4-^{14}C] norethynodrel was used as the substrate.

Fraction	Recovery
	% total
Ether-soluble fraction	52.7
Trichloracetic acid-soluble supernatant	12.4
Trichloracetic acid precipitate	23.8
Hot ethanol wash	0.9
Ethyl acetate wash	1.3
Hot trichloracetic acid wash	0.5
Final insoluble residues	21.1

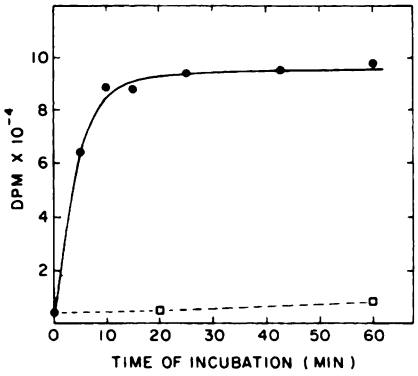

FIG. 1. *Kinetics of labeling of liver microsomal proteins*

The standard incubation mixture (●——●) contained 0.1 M phosphate buffer (pH 7.4), nicotinamide (8 mM), $MgSO_4$ (10 mM), an NADPH-generating system, and microsomal protein (10 mg) in a total volume of 5 ml. [6,7-^{3}H]Norethynodrel (0.2 μCi) was added as the substrate. Incubation was conducted at 37° under air. Protein-bound radioactivity was assayed as described in the text. The NADPH-generating system was omitted from the control mixture (□- - -□).

(1), inhibited the radioactivity binding to a significant degree (Table 3). In addition, norethynodrel elicited a typical type I spectral change in rat liver microsomes. The difference spectrum had an absorption maximum at 385 nm, a trough at 420 nm, and an isosbestic point at 400 nm.

The enzyme system was located in the microsomal fraction. The cytosol fraction (105,000 × *g* supernatant) alone and bovine serum albumin were inactive. When

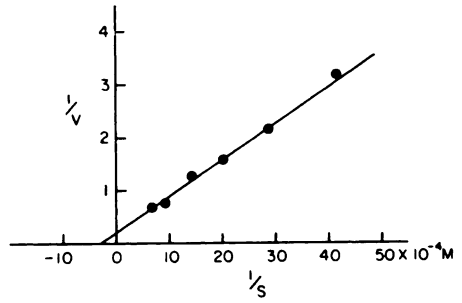

FIG. 2. *Michaelis-Menten plot of formation of protein-bound metabolite of [4-^{14}C]norethynodrel*

Reaction conditions were the same as described in Fig. 1, except that varying amounts of [4-^{14}C]norethynodrel were used. The velocity was expressed as nanomoles of [4-^{14}C]norethynodrel bound per minute. The K_m of the reaction was found to be 3.33×10^{-5} M.

TABLE 2

Requirement of rat liver microsomes and NADPH for formation of protein-bound metabolite of norethynodrel

Incubations were carried out at 37° for 30 min. The incubation mixture contained [6,7-^{3}H]norethynodrel (0.2 μCi), $MgSO_4$ (10 mM), and nicotinamide (8 mM) in 5 ml of 0.1 M phosphate buffer, pH 7.4. Proteins (10 mg) and cofactors were added as indicated.

Protein	Cofactor	Protein-bound radioactivity
		cpm/mg
Microsomal protein, phenobarbital-induced[a]	NADPH-generating system	7,543
Microsomal protein	NADPH-generating system	3,540
Microsomal protein	NADPH, 0.6 mM	3,315
Microsomal protein	NADH, 0.6 mM	300
Microsomal protein	None	310
Microsomal protein	$FeSO_4$, 0.1 mM	258
Microsomal protein	Hemoglobin, 0.1 mM	265
10,000 × *g* supernatant	NADPH-generating system	330
105,000 × *g* supernatant	NADPH-generating system	50
Bovine serum albumin	NADPH-generating system	62

[a] Microsomal proteins were prepared from phenobarbital-treated rats (16).

the 10,000 × *g* supernatant, which contained the cytosol fraction in addition to microsomes, was used as the enzyme source, the radioactivity bound to proteins was greatly reduced (Table 2). It seems that some components of the cytosol fractions are able to decrease binding of the radioactive steroid to proteins (see DISCUSSION).

Nature of binding. Labeling of the microsomal proteins after incubation with radioactive norethynodrel could be reduced by unlabeled norethynodrel. This effect could be seen only when the latter was added at the beginning of the reaction, but not after 10 min of incubation. This further suggested that metabolism of norethynodrel preceded binding and that binding was irreversible. At 0.1 mM, nonradioactive norethynodrel added at zero time gave a 70% reduction of protein labeling. The extent of inhibition by other steriods at the same concentration, such as norlutin, ethynylestradiol, testosterone, and 19-nortestosterone, ranged from 45% to 5% (Table 4).

The labeled microsomal protein was dialyzed, solubilized, and subjected to Sephadex G-200 gel filtration in 5 M guanidine HCl. As illustrated in Fig. 3, two major ultraviolet-absorbing peaks were obtained. Peak A was totally excluded. Peak B was smaller than Peak A and represented low molecular weight materials. The bulk (83%) of the radioactivity applied to the column was eluted with peak A.

The result of disc gel electrophoresis of the microsomal proteins in 1% sodium dodecyl sulfate and urea is shown in Fig. 4. Eight to 10 protein bands were discernible after staining. Radioactivity was found associated with the protein bands. There was no specific distribution pattern.

TABLE 3

Inhibition of labeling of microsomal proteins

Incubation was carried out at 37° for 30 min. Conditions were the same as described in Fig. 1, using [4-^{14}C]norethynodrel (0.05 μCi) as the substrate. In experiments 2 and 3 the gas phases were CO plus O_2 and nitrogen, respectively.

Inhibitor	Inhibition
	%
1. SKF 525-A, 0.5 mM	75
2. CO + O_2(80:20)	40
3. Anaerobic condition (nitrogen)	92

Formation of norethynodrel-glutathione

TABLE 4

Effects of nonradioactive steroids on labeling of microsomal proteins incubated with [4-^{14}C]norethynodrel

Incubation was carried out at 37° for 30 min. Conditions were the same as described in Fig. 1, with the nonradioactive steroids (0.1 mM) and [4-^{14}C]norethynodrel (3.7 μM, 0.1 μCi) added at zero incubation time.

Steroid added (0.1 mM)	Inhibition
	%
Norethynodrel	69
Norethynodrel (10 min)[a]	0
Norethindrone	45
Ethynylestradiol	34
Estradiol-17β	29
Estr-5(10)-en-17β-ol-3-one	18
19-Nortestosterone	6
Mestranol	0

[a] Unlabeled norethynodrel was added 10 min after commencement of the incubation and incubated for another 20 min.

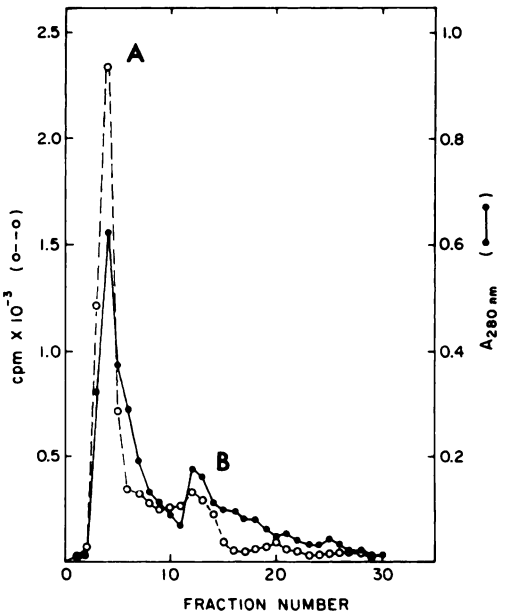

FIG. 3. *Sephadex G-200 gel filtration in 5 M guanidine HCl of microsomal proteins incubated with [6,7-^{3}H]norethynodrel (0.1 μCi)*

For details, see MATERIALS AND METHODS. Peak A, protein fraction; peak B, low molecular weight material.

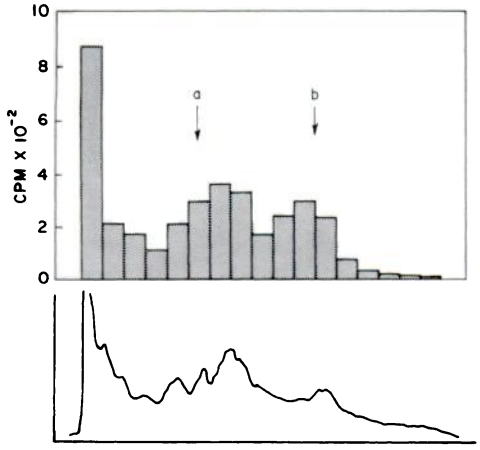

FIG. 4. *Polyacrylamide gel electrophoresis of rat liver microsomes incubated with [6,7-³H]norethynodrel*

The bar graph (upper) represents radioactivity distribution. The lower graph is the densitometer tracing on the gel. The gel contained 6.25% acrylamide, 1% sodium dodecyl sulfate, 5 mM EDTA, 5 mM 2-mercaptoethanol, and 2 M urea in 0.05 M phosphate buffer, pH 7.4. The proteins were fixed with propan-2-ol and stained with Coomassie brilliant blue. The direction of electrophoretic migration is from left to right. The marker proteins are bovine serum albumin (a) and ribonuclease (b).

conjugate. The formation of the protein-bound metabolite of norethynodrel was sensitive to sulfhydryl inhibitors such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, 2-mercaptoethanol, and dithiothreitol, as well as to glutathione and cysteine. These compounds decreased the protein-bound radioactivity to various degrees (Table 5). It was further noted that when glutathione, cysteine, and *N*-ethylmaleimide were added, the radioactivity in the aqueous supernatant increased to 247%, 148%, and 127% of the control, respectively (Table 5). The details of the effect of glutathione are shown in Fig. 5. When 0.5 M glutathione was added, the radioactivity incorporated into the protein precipitate decreased by 60%, while that in the aqueous supernatant doubled. At higher concentrations of glutahione, similar distributions of radioactivity were found; i.e., the radioactivity in the aqueous supernatant increased while that in the protein precipitate decreased. When NADPH was omitted from the incubation, very little radioactivity was recovered both in the protein precipitate and in the aqueous supernatant. Hence the accumulation of radioactivity in the aqueous supernatant was also the result of an enzymatic process.

L-Glutathione-([2-³H]glycine) was used in combination with [4-¹⁴C]norethynodrel

TABLE 5

Effects of sulfhydryl compounds and sulfhydryl-reactive compounds on distribution of radioactivity

Compounds were added to the standard incubation mixture containing [4-¹⁴C]norethynodrel (0.05 μCi). NADPH (0.6 mM) was used instead of an NADPH-generating system to eliminate any effects of these compounds on the activity of glucose 6-phosphate dehydrogenase.

Compound added (0.4 mM)	Radioactivity	
	Protein precipitate	Aqueous supernatant
	%	%
None	100	100
Dithiothreitol	83	93
2-Mercaptoethanol	44	101
Cysteine hydrochloride	32	148
Glutathione	31	247
p-Chloromercuribenzoate	12	7
N-Ethylmaleimide	54	127

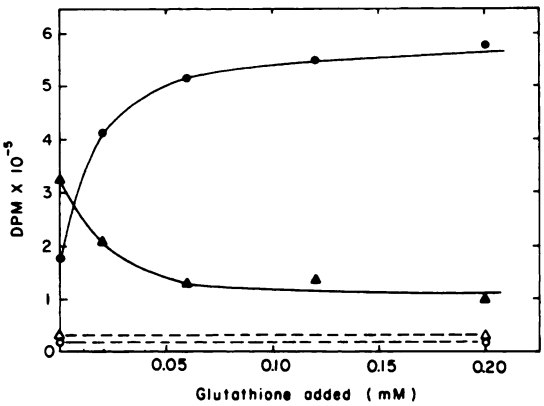

FIG. 5. *Effect of glutathione on distribution of radioactivity*

[4-¹⁴C]Norethynodrel (0.5 μCi) was incubated with rat liver microsomes, an NADPH-generating system, and glutathione for 3 min at 37°. Radioactivities in the aqueous supernatant (●——●) and protein precipitate (▲——▲) were measured. O and Δ, counts obtained from the aqueous supernatant and the protein precipitate, respectively, when the NADPH-generating system was omitted from the incubation mixture.

in the incubation. Procedures were carried out in an attempt to identify the norethynodrel-glutathione conjugate. The aqueous supernatant was passed through an Amberlite XAD-2 column. The ethanolic wash from this column was concentrated and subjected to descending paper chromatography. Several radioisotope-labeled and ninhydrin-positive spots were found. Figure 6A illustrates the radioactivity distribution when the paper was developed in butan-1-ol-acetic acid-water. Peak I was identified as oxidized glutathione, and peak III as reduced glutathione. Peaks II and IV contained both ^{14}C and ^{3}H and were positive to ninhydrin spray and divalent sulfur reagent. Similarly, in Fig. 6B, using propan-1-ol-water as the developing solvent, peaks I and III were identified as oxidized and reduced glutathione, respectively. Peaks II, IV, V, and VI all contained ^{14}C and ^{3}H. These double-labeled peaks could represent norethynodrel-glutathione conjugates. The nature of the conjugates was not investigated.

Binding induced by photo-oxidation. After the incubation of [4-^{14}C]norethynodrel with chicken ovalbumin under an atmosphere of oxygen and fluorescent light in 7 M urea, a portion of the radioactivity amounting to 11% of the total addition could not be removed from the protein by either extraction or dialysis (Fig. 7). When this labeled ovalbumin was subjected to gel filtration on Sephadex G-50, both the radioactivity and the protein were excluded

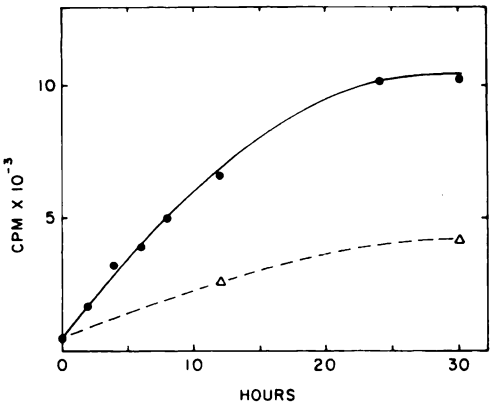

FIG. 7. *Photo-oxidation-induced binding of [4-^{14}C]norethynodrel to chicken ovalbumin*

[4-^{14}C]Norethynodrel (0.6 μCi), chicken ovalbumin (20 mg), and eosin (2 mg) in 20 ml of water were stirred with (●——●) or without (Δ- - -Δ) urea (7 M) under an atmosphere of oxygen and fluorescent light. Samples (2 ml) were removed at various intervals, and protein-bound radioactivity was measured after extraction and dialysis. For details, see MATERIALS AND METHODS.

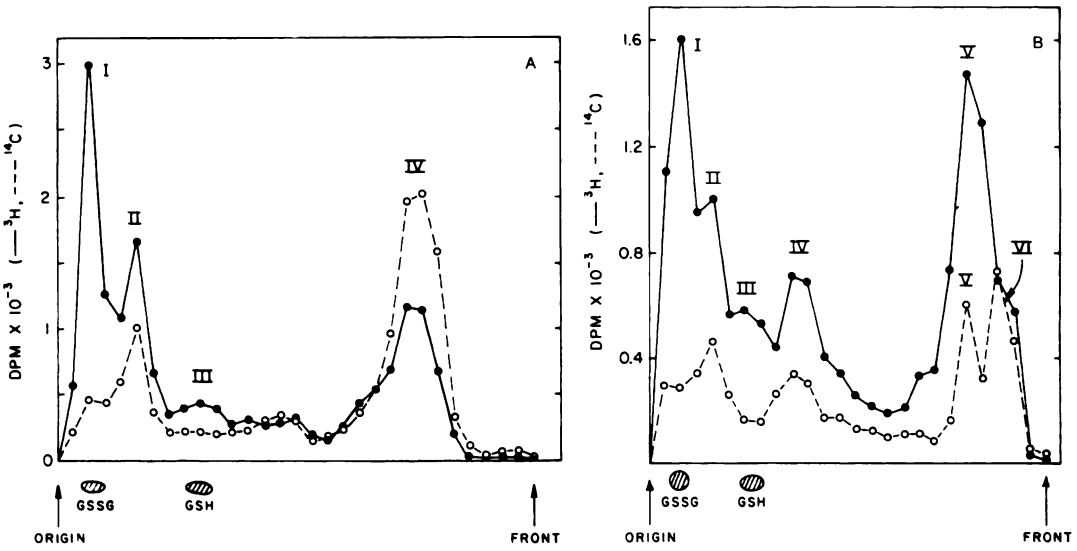

FIG. 6. *Paper chromatography of norethynodrel-glutathione conjugate*

The developing solvents were butan-1-ol-acetic acid-H_2O (25:4:10, v/v) (A) and propan-1-ol-H_2O (70:30, v/v) (B).

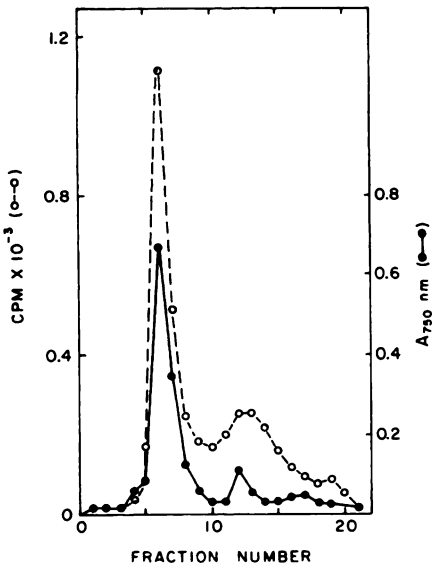

FIG. 8. *Gel filtration of labeled chicken ovalbumin*
Labeled chicken ovalbumin was obtained as described in Fig. 7. One milliliter of the 24-hr labeled protein solution was applied to a Sephadex G-50 column (2 × 32 cm; void volume, 16 ml). The column was eluted with water. Fractions of 3 ml were collected and assayed for radioactivity (O- - -O) and protein content (●——●).

in the void volume (Fig. 8). Photo-oxygenation in 7 M urea seemed to unfold the ovalbumin and to expose amino acid residues of the protein for binding, as only one-third as much of the radioactivity was bound to protein when urea was omitted (Fig. 7).

DISCUSSION

A number of interactions between steroid hormones and proteins have been described. These include the transport of steroids in plasma by transcortin or other plasma proteins (31) and hormone-receptor binding of the steroids at target organs (32). The present study has disclosed still another type of steroid-protein complex which was formed after incubation of norethynodrel with the rat liver microsomal oxygenase system *in vitro*. The bond between the radioactive steroid and the proteins is possibly covalent in nature, since the binding was irreversible and could not be dissociated by trichloracetic acid, ethanol, guanidine HCl, sodium dodecyl sulfate, gel filtration (Fig. 3), or gel electrophoresis (Fig. 4). Neither was it possible to displace the attachment with large amounts of unlabeled norethynodrel after the initial reaction (Table 4).

The following evidence suggests that norethynodrel is a substrate for liver microsomal drug-metabolizing enzymes and that metabolic oxidation of norethynodrel by this oxygenase system is a necessary step before the binding process. Norethynodrel elicited a type I spectral change with isolated liver microsomes. The formation of the protein-bound metabolite required NADPH and oxygen and was stimulated by phenobarbital treatment (Table 2). It was inhibited by SKF 525-A and carbon monoxide (Table 3). Added glutathione competed with the proteins for radioactivity (Fig. 5). Norethynodrel was probably metabolized to a "reactive intermediate" which was able to bind covalently to the proteins present in the incubation mixture. Alternatively, the same intermediate could react with glutathione, forming a norethynodrel-glutathione conjugate. Results from the experiments using tritiated glutathione and [4-^{14}C]norethynodrel suggested that more than one such conjugate was formed. The chemical nature of these conjugates remains to be established. Inhibition of protein binding by glutathione and other sulfhydryl inhibitors (Table 5) indicated that sulfhydryl groups were necessary for the viable enzyme system and also could serve as acceptor sites for the reactive intermediates. Since gel filtration and gel electrophoresis (Figs. 3 and 4) failed to relate isotope labeling to any specific protein band, a random attachment of the active norethynodrel metabolite to the sulfhydryl groups on the proteins is probable.

The inhibition of radioactivity binding to proteins by unlabeled norethynodrel was evidently caused by a dilution effect (Table 4). However, even with relatively high concentrations of nonradioactive norethynodrel, which presumably should have displaced 96% of the radioactivity, 30% residual counts in the proteins remained. The reason for this was not immediately obvious. It seems reasonable to

assume that the oxygenated metabolites of norethynodrel have a high degree of affinity for the lipoproteins of the endoplasmic reticulum membrane, and some of these strongly adsorbed steroids cannot be removed by ether extraction, trichloracetic acid precipitation or washing. Attempts to remove the unbound steroids with Amberlite XAD-2 column chromatography, which separates polar from nonpolar compounds, were unsuccessful.

When the steroid-protein complex was dialyzed according to the procedure of Zaffaroni (21) in chloroform and methanol, the radioactivity remaining in the proteins was 81% of that obtained by trichloracetic acid precipitation. Upon gel filtration of the dialyzed protein on Sephadex G-200 in 5 M guanidine HCl, the bulk of the radioactivity emerged with an ultraviolet-absorbing peak that was excluded at the void volume (peak A, Fig. 3). A residual 8% of the counts was washed out at a position corresponding to lower molecular weight materials and free steroids. Thus the residual counts that unlabeled norethynodrel failed to displace could represent the free polar steroids that were adsorbed to the microsomal lipoproteins. The same reasoning can be applied to the inhibition of protein binding by high concentrations of glutathione, in which only a maximum of 68% inhibition was observed (Fig. 5).

The metabolism of norethynodrel *in vivo* and *in vitro* has been studied in a number of laboratories (11-13). In all cases an extensive and rapid metabolism of norethynodrel was reported. Reduction of 3-ketonic to 3α- and 3β-ols was found to be the major pathway. Since most of these studies were done with the 9000 $\times$ *g* supernatant of the liver homogenate as the enzyme source, and no quantitative recoveries were reported, it is difficult to compare them with the present study. In our work, when the 10,000 $\times$ *g* supernatant was used instead of the 105,000 $\times$ *g* microsomal pellet, very little radioactivity was recovered in the proteins (Table 2). Several explanations are possible. The cytosol fraction is rich in glutathione and other sulfhydryl-containing compounds (33), which could compete with the microsomal proteins for the reactive intermediates. This conjugation process could be aided by cytosol glutathione transferase, which catalyzes the conjugation of glutathione with foreign compounds (26). Alternatively, the cytosol fraction may contain enzymes, such as peroxidase, that decompose the reactive intermediate (34), or enzymes that metabolize norethynodrel to products that do not bind to proteins. Either mechanism could lower the protein-bound radioactivity. The microsomal pellets used in the present study were washed in order to minimize contaminations from the cytosol. Yet 12% of the total counts was recovered in the aqueous supernatant, even when no glutathione was added. Part of this could have been relatively polar metabolites and norethynodrel-glutathione conjugates. When this fraction was pooled, concentrated, and subjected to paper chromatography, a radioactive band that reacted with ninhydrin and divalent sulfur reagent was located. This suggested that norethynodrel formed a conjugate with residual glutathione or cysteine. These sulfhydryl compounds could be either physically adsorbed or covalently bound as disulfides to microsomal proteins. The disulfide linkages would have been reduced during incubation.

The question whether the covalent protein binding of norethynodrel *in vitro* has any significance *in vivo* cannot be answered at this moment. Two hours after the intravenous injection of [4-^{14}C]norethynodrel (1 μCi) into rats, about 3% of the administered dose was found in the liver, 90% of which was located in the cytosol fraction. When this cytosol fraction was subjected to Sephadex G-200 gel filtration, an isotope-labeled, ultraviolet-absorbing, high molecular weight peak was found (35). This labeled, soluble protein peak was probably formed by microsomal oxygenation or itself was derived from the microsomal fractions. Further studies along this line will have to wait until more radioactive norethynodrel becomes available. Nevertheless, the results presented in this communication do suggest that norethynodrel could be metabolized to a potential alkylating agent that could react with

tissue macromolecules. There have been reports correlating the degree of macromolecular binding of hepatotoxins, such as bromogenzene and acetaminophen, with the severity of their hepatonecrotic effects (7, 36). It has also been reported that prolonged use of contraceptive steroids can produce hyperplasia, thromboembolism, cancer, and liver dysfunction (37). Although the reason or reasons are unknown, covalent binding of these steroids to cellular constituents may well play a role in such adverse effects.

The identity of the active steroid intermediate is uncertain. Several possibilities can be proposed: a hydroperoxide derivative (17α-ethynyl-10β-hydroperoxy-19-nortestosterone), an epoxide intermediate (17α-ethynyl-5α, 10α-oxidoestran-17β-ol-3-one), or ethynylestradiol. The hydroperoxide has been suggested as an intermediate in steroid metabolism (38). Shapiro *et al.* (28) reported the production of 17α-ethynyl-10β-hydroperoxy-19-nortestosterone with greater than 40% yield from norethynodrel by photosensitized oxygenation. Using the same photo-oxidation procedure, we found that chicken ovalbumin became radiolabeled after incubation with [4-^{14}C]norethynodrel (Figs. 7 and 8). These results indicated that the steroid hydroperoxide could be a reactive species. Furthermore, labeled 17α-ethynyl-10β-hydroperoxy-19-nortestosterone was isolated by the reverse isotope dilution technique from the incubation products of [6,7-^{3}H]-norethynodrel with the microsomal oxygenase system (39). That unsaturated fatty acid hydroperoxides bound and destroyed cytochromes has been shown by Desai and Tappel (9). Thus the 10-hydroperoxide of norethynodrel could be the active species involved in the binding process. The 5,10-oxide of norethynodrel was synthesized according to Ruelas *et al.* (40), but was unstable for isolation and identification. The possibility of its being the reactive species cannot be ruled out. Finally, it is also possible that norethynodrel was aromatized to ethynylestradiol and bound to proteins and glutathione via a mechanism similar to that of estradiol-17β (41). However, little if any phenolic material (less than 5%) was found during studies of the metabolism of norethynodrel (11, 13). Moreover, the result of dilution of the protein-bound radioactivity, in which ethynylestradiol was less effective than norethynodrel (Table 4), and the rapid kinetics of the binding reaction (Fig. 1) do not favor this possibility.

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