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METABOLISM OF NORETHYNODREL BY RAT LIVER*

R. I. FREUDENTHAL, C. E. COOK, M. TWINE, R. ROSENFELD and M. E. WALL
Chemistry and Life Sciences Laboratory, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, North Carolina 27709, U.S.A.

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Abstract—The incubation *in vitro* of norethynodrel with the postmitochondrial supernatant from rat liver results in the formation of three intermediary metabolites, namely the 3α- and 3β-hydroxy derivatives and norethindrone. These three steroids are further metabolized to more polar compounds thought to be polyhydroxylated end-products. Incubation of the polar products with glucuronidase and sulfatase suggests that these compounds exist as free steroids rather than as conjugates.

THE SYNTHETIC progestational steroid 17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one (norethynodrel) is used in a number of oral contraceptive preparations. Previous studies at this laboratory[1] have shown that a number of rodent liver homogenates are capable of metabolizing norethynodrel. In these experiments 3α- and 3β,17β-dihydroxy-17α-ethynyl-estra-5(10)-ene were identified as major metabolites. In addition, norethindrone (17α-ethynyl,17β-hydroxy-estra-4-en-3-one) and a 10-hydroxy-steroid (17α-ethynyl-10β,17β-dihydroxy-4-en-3-one) were isolated as well as a number of more polar hydroxylated steroids whose structures are presently under investigation. It has also been shown at this laboratory that norethynodrel *in vivo* gives a qualitatively similar pattern.[1]

As a result of these studies it was realized that the metabolism of norethynodrel followed a complex pattern. In particular it was not certain from the above studies which steroids were intermediary metabolites and which were end-products.

A study was undertaken to determine the total pattern of the metabolism *in vitro* of norethynodrel by rat liver using time-sequence incubation procedures. It will be shown that norethynodrel is first converted to three intermediary metabolites which are then biotransformed to more polar polyhydroxylated metabolites.

MATERIALS AND METHODS

NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type XV), sulfatase from Helix Pomatia and Limpets and glucuronidase from bovine liver and *E. coli* were obtained from Sigma. Norethynodrel-6,7-^{3}H was synthesized by J. A. Kepler of this laboratory's radiochemical synthesis group. Norethynodrel and norethindrone (17α-ethynylestr-4-en-3-on-17β-ol) were supplied by G. D. Searle & Co. The 3α- and 3β-hydroxy derivatives were prepared by reduction of norethynodrel.[2] Brinkmann precoated silica gel F-254 and ChromAR 500 TLC plates were used for steroid separation. Charles River CD male rats (180–220 g) were used in these studies.

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Enzyme preparation. When induced livers were desired, 37·5 mg/kg sodium phenobarbital was given twice daily for 3 days prior to sacrifice. Immediately upon exsanguination the livers were removed and 5 g was homogenized in 50 ml of 0·1 M potassium phosphate buffer, pH 7·4. The postmitochondrial supernatant obtained by centrifuging the liver homogenate at 10,000 *g* for 20 min was used as the enzyme source. When the microsomal pellet was used it was obtained by centrifuging the 10,000 *g* supernatant at 105,000 *g* for 60 min.

Drug incubation. In addition to the enzyme preparation and steroid (3 μc), the reaction mixture (50 ml), incubated in 125-ml Erlenmeyer flasks in a Dubnoff metabolic shaker at 37°, contained an NADPH regenerating system consisting of 1·0 mM NADP, 2·0 mM glucose 6-phosphate and 10 units of glucose 6-phosphate dehydrogenase. The rationale for the type and ratio of cofactors used has been presented in a recent paper from this laboratory.[3] A high substrate to enzyme ratio (5 mg/5 g) was used to recover intermediary metabolites. A control experiment was performed in which norethynodrel was incubated with the 10,000 *g* supernatant from heat inactivated rat liver. No norethindrone was found at the end of the 60-min incubation.

Aliquots were removed from the incubation at specific times (0·25, 0·5, 1, 2, 3, 5, 10, 20, 40, 60 min) after the introduction of the substrate, norethynodrel. The 5 ml aliquots were immediately pipetted into 10 ml of redistilled ethyl acetate to stop the biotransformation. The procedure was carried out in duplicate.

Steroid extraction procedure. The steroids were extracted from the aqueous phase with redistilled ethyl acetate, dried and then partitioned between hexane–aqueous methanol. The aqueous methanol fraction containing the metabolites and any remaining substrate was dried, redissolved in ethyl acetate, transferred to a 2-dram vial and dried under a stream of nitrogen. The residue was redissolved in 0·5 ml of redistilled ethyl acetate and 150 μl was spotted on TLC plates. The plates developed in a solvent system containing 25 per cent ethyl acetate in chloroform were studied using a Packard model 7210 radiochromatogram scanner. When ChromAR was used in place of Brinkmann TLC plates the per cent ethyl acetate in the solvent system was changed to 10 per cent.

Metabolite identification. Gas–liquid chromatography (GLC) was carried out on 3·8 per cent OV-17 on acid-washed, silanized Chromosorb W using an F & M Model 402 gas chromatograph with flame ionization detector. GLC-mass spectrometry (GLC-MS) was carried out on a Chromosorb W column containing 1 per cent OV-17 using an LKB Model 9000 instrument. For positive identification of compounds, TLC zones were scraped off and eluted with ethyl acetate. The retention times and mass spectra of the eluted substances were compared with those of genuine samples. Alcohols III and IV were converted to the trimethylsilyl ethers by heating with hexamethyldisilazane and trimethylchlorosilane in pyridine before GLC and GLC-MS. Norethindrone (II) was injected directly.

Product incubation. The relatively polar steroid metabolites produced as end-products in the incubation *in vitro* of norethynodrel were incubated for 3 hr with both *E. coli* and bovine liver glucuronidase and with sulfatases from helix pomatia and limpets to determine whether sulfate or glucuronide conjugates were formed during the drug incubation. The incubation conditions were those supplied on the data sheet received with the crude enzyme preparations.

RESULTS

Norethynodrel (I) is initially converted to the 3α- and 3β-hydroxy metabolites (III and IV, Fig. 1) by a $\delta^{5(10)}$-3-keto steroid reductase located both in the microsomal pellet and the soluble cell fraction (105,000 *g* supernatant) and to norethindrone (II) by an isomerase located in the microsomal pellet (Table 1). Within 5 min after the introduction of substrate to an incubation containing noninduced liver enzyme, a considerable amount of both the 3α- and 3β-hydroxy metabolites is seen in addition

TABLE 1. INTRACELLULAR LOCATION OF ENZYMES INVOLVED IN THE METABOLISM OF NORETHYNODREL AND ITS INTERMEDIARY METABOLITES*

Substrate	Product	10,000 *g* Pellet	Microsomal pellet	105,000 *g* Supernatant
Norethynodrel	Norethindrone	0	+	0
Norethynodrel	3α-Hydroxy steriod	0	+	+
Norethynodrel	3β-Hydroxy steroid	0	+	+
Norethynodrel	Polyhydroxylated steroids	0	+	0
Norethindrone	Polyhydroxylated steroids	0	+	0
3β-Hydroxy steroid	3α-Hydroxy steroid	0	0	+
3β-Hydroxy steroid	Polyhydroxylated steroids	0	+	0
3α-Hydroxy steroid	Polyhydroxylated steroids	0	+	0

* In addition to the cell fractions from 5 g male rat liver prepared in 0·1 M potassium phosphate buffer, pH 7·4, the standard incubation contained 5 mg substrate and an NADPH regenerating system consisting of 2·0 mM G6-P, 1·0 mM NADP and 10 units G6-P dehydrogenase.

to norethindrone. Figure 2 is a radiochromatogram scan from a typical incubation showing the relative amounts of the four steroids after incubating for 5 min (non-induced liver). The three intermediates are metabolized further, the concentration of the 3β-hydroxy metabolite decreasing fastest followed by that of norethindrone. The concentration of the 3α-hydroxy metabolite in the incubation decreases very slowly,

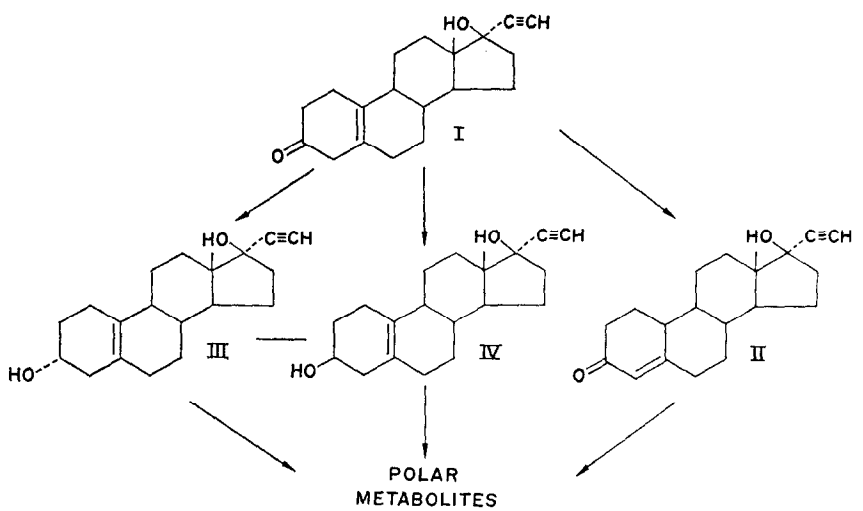

FIG. 1. Pathway *in vitro* for norethynodrel biotransformation in the rat.

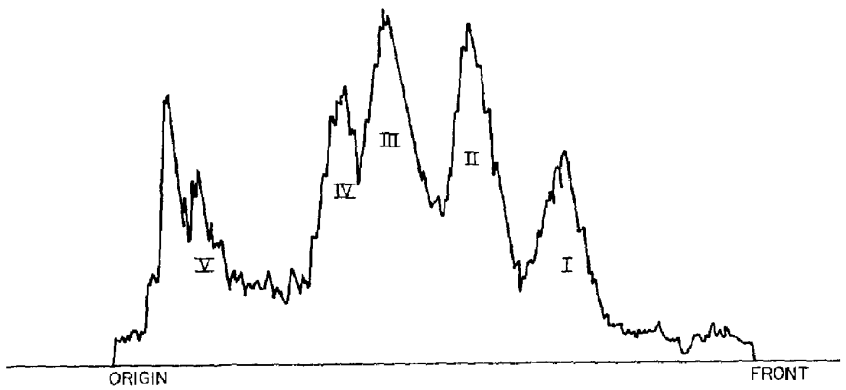

FIG. 2. Radiochromatogram scan showing the extent of norethynodrel metabolism by noninduced rat liver incubated for 5 min. The peaks represent I, norethynodrel; II, norethindrone; III, 3α-hydroxy metabolite; IV, 3β-hydroxy metabolite; V, polyhydroxylated end-products.

an appreciable amount remaining at the end of the 60-min incubation. When the 3β-hydroxy metabolite is incubated under the standard conditions described above it is both metabolized further to the more polar end-products and epimerized enzymatically to the 3α-hydroxy steroid. This accounts in part for the apparent slower conversion of the 3α-metabolite to the polar end-products. The incubation of the 3α-metabolite results only in its conversion to the polar end-products.

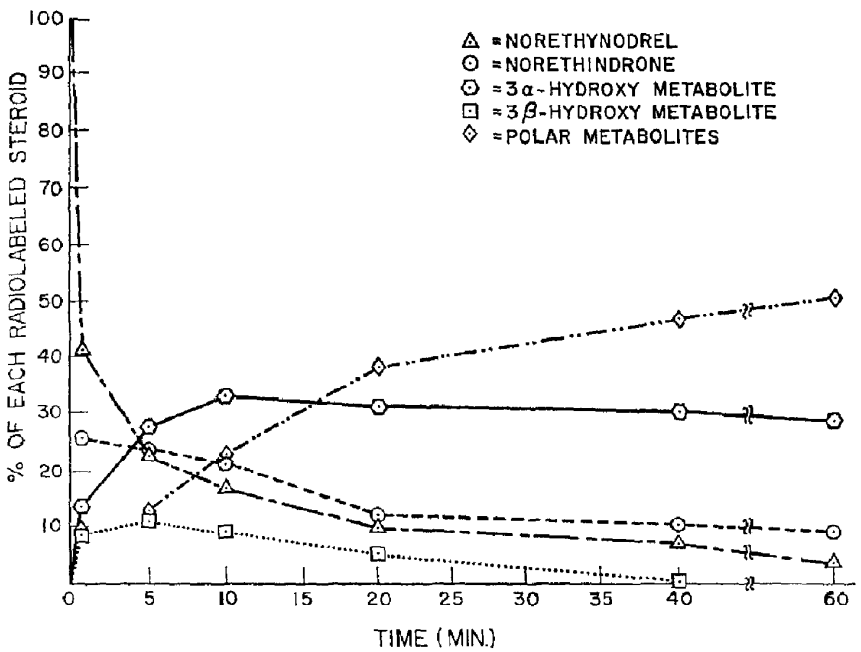

FIG. 3. Relative concentrations of norethynodrel and its metabolites during a 60-min incubation with the 10,000 *g* fraction from noninduced rat liver.

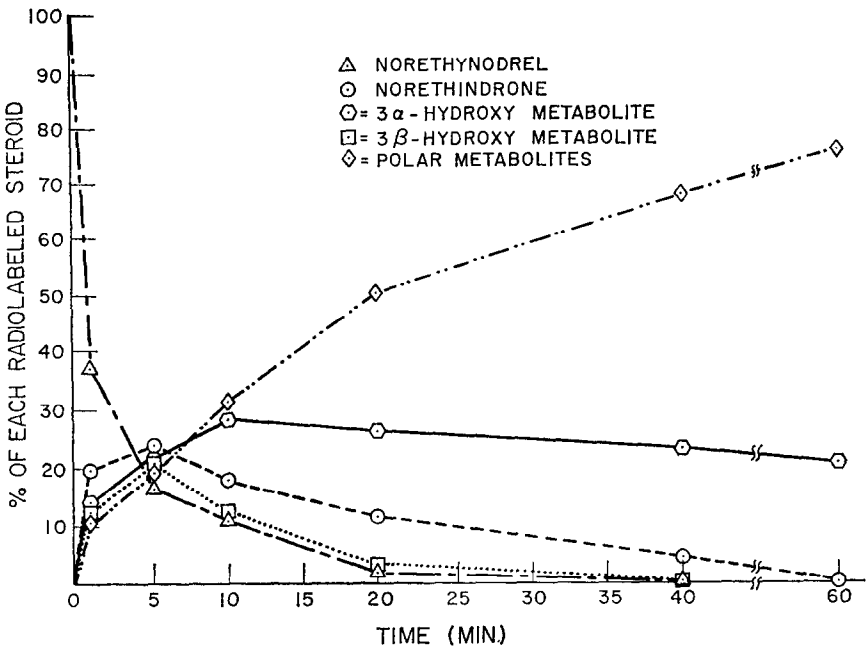

FIG. 4. Relative concentrations of norethynodrel and its metabolites during a 60-min incubation with the 10,000 *g* fraction from phenobarbital induced rat liver.

While the overall pattern of norethynodrel metabolism with either phenobarbital-induced or noninduced rat liver is essentially the same in that the same metabolites are biosynthesized, the rate of product formation by the induced liver preparations is increased more than 2-fold over that of the noninduced liver.

Figure 3 is presented to show the relative concentration of norethynodrel, norethindrone, the 3α- and 3β-hydroxy metabolites and the polar fraction in a typical norethynodrel incubation with the 10,000 *g* fraction from noninduced rat liver.

Figure 4 shows the relative concentrations of norethynodrel, norethindrone, the 3α- and 3β-hydroxy metabolites and the more polar fraction during a 60-min incubation with the 10,000 *g* supernatant from phenobarbital induced rat liver.

The relatively polar steroid metabolites (Fig. 2, peak V) from the norethynodrel incubation could be either polyhydroxylated steroids or conjugated intermediary metabolites. It was therefore necessary to incubate these end-products in the presence of either β-glucuronidase or sulfatase. The extracted steroidal material from these incubations was subjected to TLC using the conditions described in the Methods section. Had conjugate hydrolysis occurred, the free intermediary metabolites would have been seen as radiolabeled bands on the TLC plate (Fig. 2, II, III and IV) when the plate was scanned with a radiochromatogram scanner. The scan of the TLC plate revealed only peak V, thus demonstrating that no conjugate hydrolysis occurred.

DISCUSSION

The incubation *in vitro* of norethynodrel with rat liver postmitochondrial supernatant results in a complex metabolic pattern. It has previously been shown that

norethynodrel is converted to the 3α- and 3β-hydroxy derivatives.[1] 3-Keto-steroid reductases capable of 3-keto reduction have been found both in the microsomal pellet[4] and the 105,000 *g* supernatant of the rat.[5] The conversion of norethynodrel to norethindrone is not surprising in view of the fact that an isomerase capable of converting 17β-hydroxyestr-5(10)-en-3-one steroids to the corresponding δ^4-steroid has been prepared in crystalline form.[6]

It is apparent from the foregoing discussion that the metabolism *in vitro* of norethynodrel is complex and dynamic. We have demonstrated that several metabolic pathways are involved in the biotransformation of norethynodrel. It is of interest to note that norethindrone itself has progestational activity and is used in certain oral contraceptive preparations. Since recent studies in man show similarities in the pattern of norethynodrel metabolism, the absolute structure of all the metabolites and studies of their physiological activities will be continuing projects at this laboratory.

It is of considerable interest that the 3β-hydroxy alcohol is rapidly converted to the corresponding 3α-hydroxy epimer by an enzyme system located only in the 105,000 *g* supernatant. The reaction is not reversible and represents the transformation of the less stable 3β, axial-alcohol to the more stable 3α, equitorial epimer. Although the epimerization of steroidal alcohols has been infrequently cited in the literature, studies showing the interconversion of the epimeric 3β, 7α- and 3β, 7β-dihydroxy-5-androsten-17-ones[7] and of 16α- and 16β-hydroxy-estrone[8] have recently appeared.

Incubation of norethindrone and the 3α- and 3β-hydroxy metabolites with the microsomal pellet results in their conversion to more polar steroids. However, incubation of the intermediary metabolites with the 105,000 *g* supernatant does not result in a biotransformation to the polar end-products. Since this conversion to more polar steroids is limited to the microsomal pellet and since no hydrolysis occurs when these polar compounds are incubated in the presence of sulfatases and glucuronidases, it is most likely that these relatively polar steroids result from microsomal hydroxylation. The separation and structural characterization of these polar end-products are now in progress.

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