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EPIMERIZATION OF AN INTERMEDIARY METABOLITE OF NORETHYNODREL BY A 3β-HYDROXY-$\Delta^{5(10)}$-STEROID EPIMERASE*

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Abstract—3β,17β-Dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene, an intermediary metabolite formed during the biotransformation *in vitro* of norethynodrel, is converted to 3α,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene by a rat liver 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase. The reaction mechanism for the irreversible epimerization appears to involve the formation and subsequent reduction of an enzyme-bound 3-keto intermediate.

THE SYNTHETIC progestational steroid, norethynodrel (17α-ethynyl-17β-hydroxy-estra-5(10)-en-3-one), is used in a number of oral contraceptive preparations. It has recently been shown that both 3α,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene and 3β,17β-dihydroxy-17α ethynyl-$\Delta^{5(10)}$-estrene are intermediary metabolites formed during the biotransformation *in vitro* of norethynodrel by rat liver preparations.[1] Although initial studies showed the presence of essentially only the 3α-hydroxy metabolite after a 60-min norethynodrel incubation with rat liver,[2] it has more recently been found that norethynodrel is initially converted to both the 3α- and 3β-hydroxy metabolites in almost equal proportion. However, the concentration of the 3β-hydroxy epimer decreases rapidly during the first 10 min of the incubation, whereas the 3α-hydroxy steroid concentration decreases very little during this time.[1]

When the 3β-hydroxy metabolite is used as the substrate and is incubated with the 10,000 *g* supernatant from rat liver, it is converted to as yet unidentified polyhydroxylated end products and to the 3α-hydroxy epimer. Centrifugation of the 10,000 *g* supernatant at 105,000 *g* for 60 min results in a separation of enzymes responsible for the biotransformation reactions. The microsomal pellet thus obtained yields only the polyhydroxylated steroid end products, whereas the epimerization of the 3β-hydroxy steroid to its 3α-hydroxy epimer is limited exclusively to the corresponding supernatant.

The conversion of the 3β-hydroxy steroid to the 3α-hydroxy epimer could be catalyzed either by one enzyme, a 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase, or by the sequential action of two oxidoreductase enzymes, in which case a free 3-ketone intermediate is formed during the conversion. Should the reaction be catalysed by the steroid epimerase, it is most probable that the reaction mechanism involves an enzyme-bound intermediate. The experiments to be described below were designed to elucidate the mechanism by which the 3β-hydroxy steroid is biotransformed to the 3α-hydroxy epimer.

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MATERIALS AND METHODS

Male, Charles River CD strain rats (150–250 g) were used in all experiments and were allowed free access to laboratory chow and water up to the time of sacrifice. 3α,17β-Dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene and 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene were synthesized by the method of Palmer *et al.*[3] Both 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene-4-^{14}C and -3α-^{3}H were synthesized under the supervision of Dr. J. A. Kepler of this laboratory's radiochemical synthesis group. The radiolabeled steroids were at least 98 per cent radiochemically pure as checked by thin-layer chromatography (TLC) using two different solvent systems and scanning the developed plate with a radiochromatogram scanner (Packard Instrument Company, model 7201). Norethynodrel (lot No. V-55) was a gift from G. D. Searle & Company. Crystalline bovine serum albumin, NADP, NADPH, NAD, NADH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (type XV) were obtained from Sigma Chemical Company. Precoated silica gel F-254 TLC plates having a layer thickness of 0·25 mm and a BN-Chamber were purchased from Brinkmann Instruments, Inc. ChromAR 500 chromatographic medium was purchased from Mallinckrodt.

Isolation of liver fraction. After the animals were killed by decapitation, the livers were quickly removed and homogenized in 0·1 M potassium phosphate buffer, pH 7·4, using a Potter–Elvehjem homogenizer. A standard ratio of 10 ml buffer to 1 g rat liver was used throughout. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant obtained by centrifugation of the 10,000 *g* supernatant in a Beckman L2-65B at 105,000 *g* for 60 min was used as the enzyme source.

Standard incubation conditions. A constant ratio of 1 mg substrate per g of rat liver was used in all experiments. Unless otherwise specified, 2 mg of the 3β-hydroxy steroid substrate was incubated with the 105,000 *g* supernatant from 2 g rat liver for 30 min in a 125-ml Erlenmeyer flask using a Dubnoff metabolic shaker (37°). NADP (1·0 mM) was added to each incubation flask. The metabolic conversion *in vitro* was linear for at least 30 min. The reaction was stopped by the addition of 40 ml of redistilled ethyl acetate to the incubation flask.

Steroid extraction procedure. The incubate was extracted four times with redistilled ethyl acetate, the extracts were combined and evaporated to dryness. To each round-bottom flask containing the dry extract were added equal parts of previously equilibrated hexane and aqueous (10%) methanol. The aqueous methanol fraction which contained the metabolites was evaporated to dryness. This resulted in a recovery of at least 98·5 per cent as determined by counting the radiolabeled steroid before adding it to the incubation and again after extraction.

Determination of coenzyme requirement. Each of the following coenzymes was separately incubated, in triplicate, using the standard incubation conditions described above with the omission of NADP. The conversion rate was determined in the presence of 1·0 mM NAD, NADP, NADPH and an NADPH regenerating system consisting of 1·0 mM NADP, 2·0 mM glucose 6-phosphate and 10 units of glucose 6-phosphate dehydrogenase.

Determination of the number of enzymes involved in the epimerization reaction. If the epimerization reaction occurs via two enzymes, a free ketone intermediate must be formed by the first enzyme using the 3β-hydroxy steroid as substrate. Since under the standard incubation and extraction procedures no keto-steroid intermediate has ever been isolated from an incubation, the keto-steroid, if formed, must immediately be

reduced to the 3α-hydroxy steroid by a second enzyme. Should a ketone intermediate be formed during the incubation, its structure would have to be 17α-ethynyl-17β-hydroxy-$\Delta^{5(10)}$-estrene-3-one (norethynodrel).

Using the standard incubation conditions, 2 mg of ^{14}C-3β-hydroxy steroid (452,000 dis./min) was incubated in the presence of 20 mg of unlabeled norethynodrel. The large excess of norethynodrel was added to the incubation mixture to trap a considerable amount of the proposed radiolabeled norethynodrel intermediate by an exchange with the large excess of norethynodrel. Under these conditions, it is expected that more radiolabeled steroid will be found in the ketone pool than in the 3α-hydroxy product, should a free ketone be formed during the epimerization. After extraction, one-tenth (45,200 dis./min) of the steroidal extract was separated by TLC in a BN-chamber, a continuous flow horizontal TLC system, using a solvent system containing 5% ethyl acetate in benzene. The per cent metabolism was determined from this plate. The remaining nine-tenths (406,800 dis./min) of the extracted steroidal material was separated on ChromAR 500 and the exact position of each of the radiolabeled steroids was determined using a radiochromatogram scanner. The section of the ChromAR 500 in the area corresponding to the norethynodrel R_f was eluted using ethyl acetate and sonication for 20 min.

The radiolabeled material eluted from the norethynodrel zone of the TLC plate (on which 406,800 dis./min were spotted) was recrystallized six times using carrier addition techniques in an attempt to purify it to a constant specific activity. After obtaining a constant specific activity of 130 dis./min/mg of norethynodrel, this value was multiplied by the total amount of norethynodrel present after the sixth recrystallization (51·1 mg). The 6508 dis./min obtained by this procedure represented 1·6 per cent of the initial radiolabeled steroid (406,800 dis./min).

Method for obtaining evidence for the formation of an enzyme-bound intermediate. Although the incubation described above can be used to determine the presence or absence of a free ketone intermediate, it would give no evidence for the presence of an enzyme-bound intermediate, should one be formed during the epimerization reaction. If the reaction is catalyzed by one enzyme, a 3β-hydroxy-$\Delta^{5(10)}$-epimerase, then it is most probable that an enzyme-bound keto-intermediate is formed.

An incubation was performed in replicates of six, using double-labeled 3β-hydroxy steroid as substrate, the tritium being located exclusively in the 3α-position. The formation of a 3-keto intermediate requires the loss of the 3α-tritium. The ^{3}H:^{14}C ratio was determined before the substrate was added to the incubation flask and again after the extraction procedure. Substrate containing 3,752,630 dis./min-^{3}H and 687,200 dis./min-^{14}C, representing a ^{3}H:^{14}C ratio of 5·46, was added to each incubation flask. The incubations were terminated and extracted as previously described and the radiolabeled epimers were separated by TLC in a BN-chamber using a solvent system containing 5% ethyl acetate in benzene. The area of silica gel representing each of the epimers was scraped from the TLC plate and eluted with redistilled ethyl acetate. The ^{3}H:^{14}C ratio of the eluted radiolabeled steroid was determined using a Packard Tri-Carb liquid scintillation spectrometer, model 574. A control incubation was performed in the absence of enzyme to determine whether any chemical exchange occurs between the 3α-^{3}H and the aqueous media.

Identification of steroids. Initially both gas–liquid chromatography (GLC) and GLC-mass spectrometry (GLC-MS) were carried out using the methods described by

Palmer *et al.*[2] Absolute separation of the 3α- and 3β-hydroxy epimers by GLC techniques can be obtained by first forming the trimethylsilyl (TMS) ethers by reacting the steroids with hexamethyldisilazane and trimethylchlorosilane in pyridine at 100°. Once the absolute structure of each of the steroids was confirmed, the steroidal extract from every incubation was routinely silylated and the extent of epimerization determined by GLC techniques. The routine GLC analysis was carried out on a column of 1·9% OV-17 on acid-washed, silanized Chromosorb W at 215° in a Varian Aerograph series 1200 flame ionization chromatograph.

RESULTS

When the 105,000 *g* supernatant is incubated separately with 1·0 mM of either NAD, NADH, or NADPH, there is no significant increase in the reaction rate over that of the control incubation to which no coenzyme is added. However, a 30 per cent increase in the reaction rate is obtained when NADP is added to the incubation (Table 1). For this reason 1·0 mM NADP was routinely added to each incubation. Increasing the NADP concentration above 1·0 mM does not further increase the conversion rate.

In the radiolabeled ketone trapping experiment, the radiolabeled material obtained from the norethynodrel R_f region of the TLC plate was recrystallized using the carrier addition techniques previously described. A constant specific activity was obtained and calculations (see Methods) showed that the radiolabeled norethynodrel represented *ca.* 1·6 per cent of the total radiolabeled steroid added to the incubation.

TABLE 1. EFFECT OF DIFFERENT COFACTORS ON THE RATE OF EPIMERIZATION OF 3β,17β-DIHYDROXY-17α-ETHYNYL-$\Delta^{5(10)}$-ESTRENE TO THE 3α-HYDROXY EPIMER

Cofactor (1·0 mM)	Epimerization* (%)
None	63
NADH	26
NAD	72
NADPH	67
NADP	90

* Each value represents the average of three determinations. For experimental detail, see Methods.

When double-labeled (3α-^{3}H and 4-^{14}C) substrate, having a ^{3}H:^{14}C ratio of 5·46 was used as substrate in an incubation, 39 per cent of the 3β-hydroxy steroid was converted to its 3α-hydroxy epimer and the ^{3}H:^{14}C ratio of the extracted steroidal material changed from 5·46 to 3·35. The final ^{3}H:^{14}C ratio of 3·35 is the expected ratio, if the epimerization is stoichiometric (Table 2). The ^{3}H:^{14}C ratio of the control incubation (which contained no enzyme) indicated that no removal of the 3α-tritium occurred in the absence of enzyme. GLC determinations of the steroidal extract from the control incubation showed that no epimerization occurred.

The ^{3}H:^{14}C ratios of the steroidal material extracted from the six incubations are presented in Table 2. When the epimers were completely separated on TLC plates in a BN-chamber, eluted and then counted, no tritium was found in the 3α-hydroxy steroid product. This is direct evidence that the reaction goes through a 3-keto intermediate.

TABLE 2. CHANGE IN THE ^3H:^{14}C RATIO RESULTING FROM THE EPIMERIZATION OF 3β,17β-DIHYDROXY-17α-ETHYNYL-Δ$^{5(10)}$-ESTRENE TO ITS 3α-HYDROXY EPIMER

Sample	% Composition from TLC bands		^3H:^{14}C obtained from aliquot of sample			Theoretical ^3H:^{14}C for combined steroid*
	β	α	Combined	β	α	
Before incubation	100	0	5·46	5·46	0	
Control incubation	98·5	1·5	5·60	5·70	0	5·38
Incubation 1	60·7	39·3	3·56	8·0	0	3·31
2	44·5	55·5	3·12	6·5	0	2·43
3	71·2	28·8	3·86	6·6	0	3·89
4	65·0	35·0	3·30	3·3	0	3·55
5	68·1	31·9	3·12	3·1	0	3·72
6	59·0	41·0	3·18	4·4	0	3·22
Average (1–6)	60·4	39·6	3·356	5·3	0	3·353

* The theoretical ^3H:^{14}C, obtained by multiplying the fraction of substrate remaining at the end of the incubation with the initial ^3H:^{14}C ratio (5·46), is the expected value if the ^3H abstraction is stoichiometric with respect to amount of epimerization. For experimental details, see Methods.

DISCUSSION

Epimerization reactions have been shown to exist in both bacterial and mammalian systems.[4,5] Examples of biotransformation reactions involving catalysis by an epimerase include the conversion of UDP-glucose to UDP-galactose by UDPglucose-4-epimerase (EC 5.1.3.2),[6,7] L-threonine to D-threonine by threonine epimerase (EC 5.1.1.6)[8] and 2,6-LL-diaminopimelate to *meso*-diaminopimelate by 2,6-LL-diaminopimelate-2-epimerase (EC 5.1.1.7).[9] Present in the literature are two recent studies showing the interconversion of 16α- and 16β-hydroxyestrone[10] and the Δ^5-7-hydroxyandrostenes.[11] Both studies conclude that the specific steroid epimerization reaction studied occurs without intermediary formation of a ketone.

The present studies demonstrate that rat liver contains an epimerase which can irreversibly transform the 3β-hydroxy steroid to its 3α-epimer. The fact that the 3α-hydrogen is removed stoichiometrically during the epimerization demonstrates that the reaction proceeds through a ketonic type of intermediate. Since adding a 10-fold excess of norethynodrel to the incubation resulted in trapping only a relatively small amount of radiolabel as the free ketone, it appears most probable that the steroid is bound to a single enzyme throughout the epimerization.

The epimerization of the 3β-hydroxy steroid to its 3α-hydroxy epimer is irreversible and requires the addition of NADP for maximum activity. However, a substantial reaction rate (70 per cent of maximum rate) occurs in the absence of added NADP. If under normal physiological conditions NADP is strongly bound to the epimerase, the differential centrifugation required to obtain the 105,000 *g* supernatant will not dissociate the bound NADP from the NADP binding site on the enzyme. At the normal intracellular NADP concentration, a portion of the existing epimerase would have NADP bound, while some epimerase would contain no bound NADP. Thus, the rate of steroid epimerization in the absence of exogenous NADP would be directly proportional to the amount of existing enzyme–coenzyme complex. When 1·0 mM NADP is added to the incubation, all of the epimerase not containing NADP now binds cofactor and the result is seen as a 30 per cent increase in the reaction rate. Although this

explanation can only be considered speculation until an NADP titration of free enzyme is performed in the presence of excess substrate, there are a number of examples in the literature describing enzymatic reactions in which the active (catalytic) form of the enzyme is an enzyme–coenzyme complex.[12,13]

Further studies now in progress indicate that the epimerization of the 3β-hydroxy metabolite of norethynodrel to the corresponding 3α-hydroxy epimer is species specific, occurring chiefly in the rat.

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