Biotransformation of 16α-hydroxyprogesterone by *Eubacterium* sp. 144: non-enzymatic addition of L-cysteine to Δ^{16}-progesterone

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Abstract *Eubacterium* sp. 144 dehydroxylated 16α-hydroxyprogesterone; however, the expected intermediate, Δ^{16}-progesterone, did not accumulate to significant concentrations in the culture medium. Moreover, the final end product of this biotransformation, 17α-progesterone, was produced at a very slow rate. It was discovered that, under our culture conditions, Δ^{16}-progesterone reacted chemically with L-cysteine to form a highly water-soluble derivative. The ability of Δ^{16}-progesterone to react with L-cysteine in culture media was considerably reduced when L-cysteine was autoclaved in the presence of complex medium components. Δ^{16}-progesterone also reacted chemically with D-cysteine, L-homocysteine, glutathione, and 2-mercaptoethylamine. The reaction was favored by alkaline pH (≥pH 8.0) and required both an unhindered thiol group and a proximal amino group on the mercapto compound. Chromatography of the putative Δ^{16}-progesterone L-[U-^{14}C]-cysteine reaction product by HPLC showed a single UV-absorbing, radioactive peak (RT 4.31 min).—**Glass, T. L., J. Winter, V. D. Bokkenheuser, and P. B. Hylemon.** Biotransformation of 16α-hydroxyprogesterone by *Eubacterium* sp. 144: non-enzymatic addition of L-cysteine to Δ^{16}-progesterone. *J. Lipid Res.* 1982. **23:** 352–356.

Supplementary key words nucleophilic addition • 16α-dehydroxylation

The 16α-dehydroxylation of neutral steroids has been shown to be catalyzed exclusively by the intestinal flora of man and animals (1–5). Recently, Bokkenheuser, et al. (6) isolated two strains of bacteria from rat feces, *Eubacterium* sp. 144 and 146, that catalyzed the production of 17α-progesterone from 16α-hydroxyprogesterone. Additional studies by Winter et al. (7) showed that *Eubacterium* sp. 144 dehydroxylated 16α-hydroxyprogesterone yielding Δ^{16}-progesterone. This intermediate steroid accumulated initially in the culture medium and was subsequently reduced to 17α-progesterone. In a collaborative study, we (TLG and PBH) could not detect the accumulation of Δ^{16}-progesterone from 16α-hydroxyprogesterone in cultures of this bacterium. Upon further investigation, evidence was obtained that showed that Δ^{16}-progesterone reacted chemically with L-cysteine to form a water-soluble derivative under our culture conditions.

MATERIALS AND METHODS

Cultivation of bacteria

Eubacterium sp. 144 (6) was grown in the TYA medium described by Feighner et al. (8) with the following modifications. Tryptic Soy Broth, w/o Dextrose (Difco) was used (27.5 g/l) instead of Trypticase Soy Broth (BBL), arginine · HCl was increased to 1% (wt/vol), $FeSO_4$ (4 mg/l) was added, and 11-deoxycorticosterone was omitted from the medium. Prior to autoclaving, the pH of the medium was adjusted to 7.3–7.4 with KOH. The medium was prepared anaerobically under 100% CO_2 using modifications of the Hungate procedure (9) and autoclaved. L-Cysteine · HCl and Na_2CO_3 solutions, autoclaved separately, were added to the sterile cooled medium (8). After equilibration under CO_2, the final medium pH was 7.0–7.2 and further adjustments were not made (8). The medium (10 ml) was tubed under 100% CO_2 (9). BHIC medium (3) was prepared using the same anaerobic procedures.

16α-Hydroxyprogesterone and Δ^{16}-progesterone were prepared as stock solutions in CH_3OH. For biotransformation experiments, steroids were added to the medium to give final concentrations of 20 μg/ml (0.064

Abbreviations: 16α-hydroxyprogesterone, 16-α-hydroxy-4-pregnene,3,20-dione; Δ^{16}-progesterone, 4,16-pregnadiene-3,20-dione; 17α-progesterone, 17α-4-pregnene-3,20-dione; 17β-progesterone, 4-pregnene-3,20-dione; TYA, tryptic soy broth + yeast extract + arginine medium; BHIC, brain heart infusion + cysteine medium; HPLC, high performance liquid chromatography.

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mM) steroid and 4% (v/v) CH_3OH. The tubes were inoculated with 0.5 ml of an overnight culture of *Eubacterium* sp. 144 and incubated at 37°C. Growth of the culture was followed at 660 nm with a Bausch and Lomb Spectronic 20.

Steroid determination

Medium samples (0.5 ml) were removed and added to Teflon-lined screw-cap tubes containing 0.5 ml of 0.5 N HCl. The samples were extracted twice with 2.0 ml of ether. The ether phases were combined, evaporated to dryness and the residue was dissolved in 0.25 ml of CH_3OH. The samples were then filtered using 0.6 μm porosity BDWP filters (Millipore Corp.). The steroids were separated and quantitated by HPLC using a Beckman Model 332 HPLC, equipped with a Hitachi UV-VIS variable wavelength detector and an Altex C-RIA integrator/recorder. The steroids (20 μl injection) were resolved on a Beckman C-18 Ultrasphere ODS reverse phase column (4.6 mm × 15 cm; 5μ particle packing) using CH_3OH–H_2O 78:22 (v/v) at a flow rate of 1.25 ml/min. The analytical column was protected with a Whatman Co. Pell ODS guard column. The steroids were detected by monitoring the UV-absorbance of the effluent at 240 nm and the concentrations were calculated based on external standardization of the C-RIA computer with steroid solutions of known concentration.

Steroid-cysteine conjugation

The reaction of L-cysteine with Δ^{16}-progesterone was studied with reaction mixtures containing in a final volume of 0.5 ml:50 mM potassium phosphate buffer, pH 8.1; 0.064 mM Δ^{16}-progesterone; 8.25 mM cysteine (as either the free base or L-cysteine · HCl, adjusted to pH 8.1) and CH_3OH (4% v/v). The reactions were incubated for 10 min at 37°C and were stopped by extracting 0.25-ml samples twice with 2.0 ml of ether. After evaporating, the samples were reconstituted in CH_3OH (0.25 ml) and unreacted Δ^{16}-progesterone was determined by HPLC.

Preparation of radiolabeled cysteine-progesterone conjugate

The reaction mixture, modified to contain Δ^{16}-progesterone (3.2 mM), L-cysteine · HCl (48 mM), CH_3OH (50% v/v), and 50 mM potassium phosphate buffer, pH 8.1, in a final volume of 1.0 ml was supplemented with 1 μCi of L-[U-^{14}C]-cysteine · HCl and incubated at 37°C for 60 min. The mixture was then extracted twice with 3.0 ml of ether to remove unreacted Δ^{16}-progesterone. Five ml of $CHCl_3$–CH_3OH 1:1 (v/v) was then added to the aqueous phase and it was allowed to sit overnight. Precipitated material (presumably cystine) was removed by centrifugation. The $CHCl_3$–CH_3OH solution was removed and evaporated to dryness with N_2. The residue was redissolved in 0.5 ml of CH_3OH–H_2O 65:35 (v/v) and acidified with two drops of 0.5 N H_3PO_4. The preparation was then filtered using a 0.6 μm porosity BDWP filter. The cysteine-progesterone conjugate preparation was chromatographed by HPLC as above with CH_3OH–H_2O 65:35 (v/v) at 1.1 ml/min. Fractions were collected at 1-min intervals and the radioactivity was determined by liquid scintillation spectrometry.

Chemicals

16α-Hydroxyprogesterone and Δ^{16}-progesterone were obtained from Steraloids, Inc. L-Cysteine · HCl was from J. T. Baker, Inc. and the other thiols were from Sigma. L-[U-^{14}C]-Cysteine · HCl (30.2 mCi/mol) was obtained from Amersham Corp. Chloroform and methanol were HPLC grade quality and purchased from Burdick-Jackson Laboratories.

RESULTS

Steroid biotransformation

The biotransformation of 16α-hydroxyprogesterone by a growing culture of *Eubacterium* sp. 144 is shown in **Fig. 1.** 16α-Hydroxyprogesterone was dehydroxylated within the first 2 hr of incubation. However, we could not demonstrate the accumulation of the expected intermediate, Δ^{16}-progesterone. Rather, only a small amount of Δ^{16}-progesterone was detected. The end product, 17α-progesterone, was produced very slowly with prolonged incubation. When this experiment was repeated using

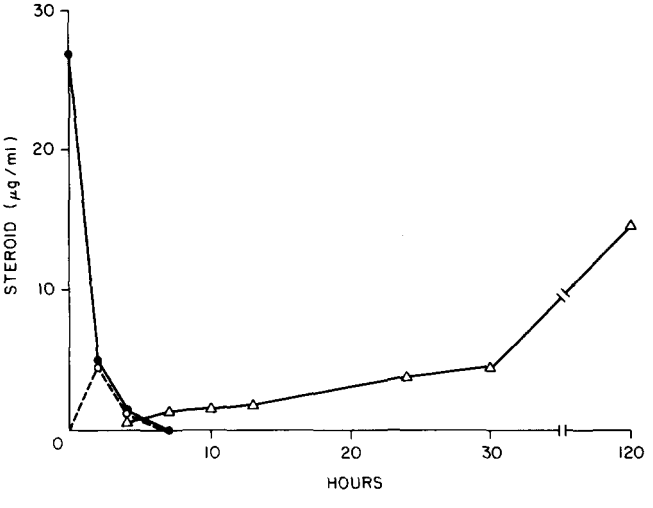

Fig. 1. Time course of steroid biotransformation by growing *Eubacterium* sp. 144. 16α-Hydroxyprogesterone (● —— ●) was added just prior to inoculation. Maximum growth of the culture occurred at 24 hr (O.D.$_{660}$ = 0.620). Δ^{16}-progesterone (O - - - O) and 17α-progesterone (Δ —— Δ).

TABLE 1. Recovery of Δ^{16}-progesterone from uninoculated media sterilized by autoclaving with and without L-cysteine

Media[a]	Hours	% Δ^{16}-Progesterone Recovered[b]	
		Cysteine (+)	Cysteine (−)
BHIC	0	100	100
	4	82	31
	9	52	9
	24	45	0
TYA	0	100	100
	4	90	57
	9	49	20
	24	27	2

[a] BHIC and TYA media were prepared by autoclaving in the presence (+) or absence (−) of L-cysteine. In the latter case, cysteine was prepared as a separately sterilized solution and then added to the media. The L-cysteine concentration for all conditions was 0.05% (wt/vol). The atmosphere was 100% CO_2.

[b] Δ^{16}-Progesterone was added to the media (0.070 mM) at time zero, prior to taking the first sample. At the times indicated, 0.5-ml samples of media were extracted with ether and unreacted Δ^{16}-progesterone was determined. The incubation temperature was 37°C.

Δ^{16}-progesterone as the initial substrate, a small amount of 16α-hydroxyprogesterone was formed during the first 2 hr of incubation. However, as with the experiment of Fig. 1, most of the Δ^{16}-progesterone was rapidly lost and the slow accumulation of 17α-progesterone was observed.

These results suggested that the inability to account for the steroids described in Fig. 1 resided in the chemical properties of Δ^{16}-progesterone. Bacterial involvement in this anomaly was eliminated when it was found that Δ^{16}-progesterone added to uninoculated media and incubated at 37°C for 1 hr could not be recovered by ether extraction. Thus, Δ^{16}-progesterone appeared to be chemically converted to a water-soluble derivative that was not extracted with organic solvents. Prolonged incubation of Δ^{16}-progesterone with uninoculated media (1 week, 37°C) did not result in the appearance of 17α-progesterone or reappearance Δ^{16}-progesterone. Therefore, the production of 17α-progesterone (Fig. 1) required the presence of *Eubacterium* sp. 144.

Further experiments were designed to identify the medium component(s) reacting with Δ^{16}-progesterone. Media were prepared lacking the various constituents and incubated with Δ^{16}-progesterone. In this manner, it was found that L-cysteine was the reactive substance. L-Cysteine is a standard medium ingredient for the cultivation of anaerobic bacteria, serving both to lower the redox potential of the medium and as a sulfur source.

The fact that L-cysteine could react with Δ^{16}-progesterone suggested that the difference between our results (Fig. 1) and those of Winter et al., (7) might reside in the method of media preparation. In the present study, media (TYA) were prepared by autoclaving a cysteine solution and adding it to separately autoclaved media. In contrast, Winter et al. (7) prepared their media (BHIC) by autoclaving with L-cysteine. To test this hypothesis, the stability of Δ^{16}-progesterone was examined in TYA and BHIC media prepared by these two procedures. The results of this experiment are shown in **Table 1.** Δ^{16}-Progesterone was more stable when added to media prepared by autoclaving in the presence of cysteine. However, a slow conversion of Δ^{16}-progesterone to an ether-insoluble form(s) still occurred.

Δ^{16}-Progesterone-cysteine conjugate

In experiments not illustrated, we found that the pH optimum for Δ^{16}-progesterone-cysteine conjugation was ≥8.0 using potassium phosphate or sodium cyclohexylaminoethanesulfonate buffers. However, a significant conversion of Δ^{16}-progesterone (~45%) still occurred at pH 6.8 in potassium phosphate buffer.

Table 2 shows the relative ability of several sulfur-containing compounds to react with Δ^{16}-progesterone. L-cysteine, D-cysteine, 2-mercaptoethylamine, and L-homocysteine showed significant reactivity. A requirement for a free sulfhydryl group was indicated by the fact that S-methyl-L-cysteine and L-methionine were unreactive. Moreover, it was also apparent that an amino group located close to the sulfhydryl group was required for reactivity. This was indicated by the nonreactivity of 3-mercaptopropionic acid and mercaptoethanol. In addition, L-homocysteine was only 25% as reactive as L-cysteine.

The results of Table 2 were obtained at a high molar ratio of sulfhydryl compound to steroid (129:1). This ratio reflected that of L-cysteine to steroid in the TYA culture medium (Fig. 1). The molar ratio of L-cysteine to Δ^{16}-progesterone could be reduced about 10 to 20-fold while still yielding 80–90% conversion of Δ^{16}-progesterone to the water-soluble conjugate under aerobic conditions in 10 min at 37°C (data not shown).

Radiolabeled Δ^{16}-progesterone-cysteine conjugate was

TABLE 2. Relative reactivity of sulfur-containing compounds with Δ^{16}-progesterone

Compound[a]	% Conversion
None	0
L-Cysteine	96
D-Cysteine	79
2-Mercaptoethylamine	81
3-Mercaptopropionic acid	1
S-Methyl-L-cysteine	2
L-Homocysteine	22
Glutathione	13

[a] The reaction contained, in 0.5 ml, 50 mM potassium phosphate buffer, pH 8.1, 0.064 mM Δ^{16}-progesterone, and the compounds tested at 8.25 mM. Stock solutions of thiols were adjusted to pH 8.1 prior to use. The mixtures were incubated for 10 min at 37°C and unreacted Δ^{16}-progesterone was extracted with ether. The following compounds were tested, but did not react with Δ^{16}-progesterone: L-serine, L-threonine, L-arginine, L-methionine, 2-mercaptoethanol, and sodium thioglycollate.

prepared in the presence of L-[U-^{14}C]-cysteine as described in the Methods. When analyzed by HPLC (**Fig. 2**), only one UV-absorbing, radioactive peak (4.31 min) was detected. Fig. 2 also shows a radioactive, non-UV-absorbing peak that probably represented L-[U-^{14}C]-cysteine and a UV-absorbing non-radioactive peak (15.01 min) that was identified as unreacted Δ^{16}-progesterone.

DISCUSSION

Winter et al. (7) showed that *Eubacterium* sp. 144 dehydroxylated 16α-hydroxyprogesterone with transient accumulation of Δ^{16}-progesterone. Δ^{16}-Progesterone was subsequently reduced to 17α-progesterone. However, in the present study, we were unable to detect comparable transformation kinetics. We found this difference was due to the fact that Δ^{16}-progesterone reacted chemically with L-cysteine and was converted to a water-soluble form that could not be recovered with organic solvents. The reaction rate was dependent on the cysteine concentration of the medium, which could be influenced by the medium preparation method. Thus, loss of L-cysteine in the presence of complex medium components at high temperature can reduce the rate of reaction with Δ^{16}-progesterone. However, other medium factors may also influence this process.

Δ^{16}-Progesterone possesses a highly reactive α,β-unsaturated ketone associated with the D-ring of the steroid. The ability of this compound and similar steroids to undergo nucleophilic addition reactions has been established (10–14). Moreover, many of these derivatives have been prepared with sulfhydryl compounds yielding 16-substituted products containing thioester or thioether linkages. Likewise, the reactivity of L-cysteine towards α,β-unsaturated functionalities has also been noted previously (15, 16). However, to our knowledge, the reaction between L-cysteine and Δ^{16}-progesterone has not been described. Furthermore, the conditions under which this reaction was detected are considerably milder than those generally used to prepare 16-thio derivatives of Δ^{16}-unsaturated steroids, but appear to be characteristic of similar reactions involving L-cysteine (16). Based on this chemistry, the Δ^{16}-progesterone-cysteine conjugate would be expected to have a 16-thioether bond (16-S-cysteinylprogesterone) and the properties of a zwitterionic detergent.

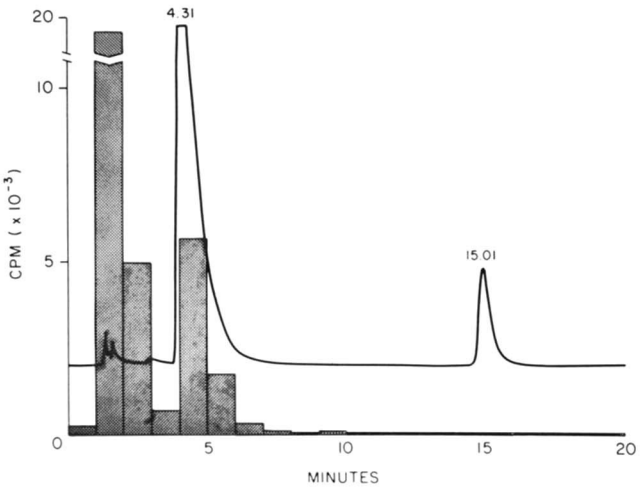

Fig 2. HPLC of the ^{14}C-labeled Δ^{16}-progesterone cysteine conjugate. The radiolabeled conjugate was prepared using L-[U-^{14}C]-cysteine as described in the procedures. The cysteine/Δ^{16}-progesterone molar ratio was 15:1. The injection volume was 20 μl and the UV-detector was operated at 2.0 AUFS (240 nm). The recorder speed was 1 cm/min. The stippled bars represent the radioactivity recovered in each 1.1-ml fraction and the solid line is the UV-absorption trace.

The ionized α-amino group of L-cysteine is known to increase the acidity of the thio group (pKa 8.53) facilitating the generation of the thiolate anion (17, 18), a reactive species for nucleophilic addition to α,β-unsaturated compounds (15, 19). These properties can account for the effect of pH and the amino-thiol requirements observed for the reaction of Δ^{16}-progesterone with L-cysteine and related compounds. While the un-ionized amino group (pKa 8.86) is a potential nucleophile in this reaction, studies by Friedman, Cavins, and Wall (15) showed that the thiolate anion of L-cysteine was about 300 times more reactive in such circumstances.

It is not known whether the cysteine-Δ^{16}-progesterone conjugate is formed in vivo. However, Calvin and Liberman (4) reported that 16α-hydroxyprogesterone could be converted to Δ^{16}-pregnanolone which was recovered from the urine of female subjects. Eriksson, Gustafsson, Sjövall (1) detected 3α-hydroxy-5α-pregn-16-en-20-one in the feces of normal rats, but did not find Δ^{16}-unsaturated steroids in human feces (2). The fact that Δ^{16}-unsaturated steroids can be detected in vivo does at least indicate the potential for non-enzymatic conjugation with L-cysteine or other nucleophiles. In this regard, it is of interest that cysteine does react by nucleophilic addition with the tyrosinase- or peroxidase-catalyzed oxidation products of L-DOPA (dopaquinones) to yield cysteine conjugates in vitro (20) and in vivo (21).

This investigation was supported by grants AM 26262 and 25324 from the National Institute of Arthritis, Metabolism and Digestive Diseases and by grant CA 25763 from the National Cancer Institute, DHHS. We would like to thank Dr. Ajay Bhatnagar, Department of Obstetrics and Gynecology, MCV-VCU for helpful discussions during this study.

Manuscript received 12 June 1981 and in revised form 13 October 1981.

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