Characterization of a corticosteroid 21-dehydroxylase from the intestinal anaerobic bacterium, *Eubacterium lentum*

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Abstract An oxygen-sensitive corticosteroid 21-dehydroxylase has been characterized in cell extracts of *Eubacterium lentum*. The enzyme was highly specific for corticosteroids containing an α-ketol structure and required $FMNH_2$ or reduced benzyl viologen for activity. The enzyme used deoxycorticosterone, deoxycortisol, dehydrocorticosterone, and corticosterone as substrates. Substrate saturation kinetics using [^{3}H]corticosterone yielded an apparent Km of 7.35 μM and a Vmax of 15.4 nmol (11 β-[^{3}H]hydroxyprogesterone) formed per hr·mg protein^{-1}. 21-Dehydroxylase activity was inhibited by both water-soluble and lipophilic metal ion chelators. NADH: flavin oxidoreductase and 21-dehydroxylase activities were separated by anaerobic DEAE-cellulose and Sepharose 6B chromatography. 21-Dehydroxylase had a relative molecular weight of 582,000 as determined by Sepharose 6B chromatography. There was a 7-fold increase in the rate of 21-dehydroxylation of [^{3}H]deoxycorticosterone in whole cell suspensions of *E. lentum* sparged with H_2 as compared to argon gas.—**Feighner, S. D., and P. B. Hylemon.** Characterization of a corticosteroid 21-dehydroxylase from the intestinal anaerobic bacterium, *Eubacterium lentum*. *J. Lipid Res.* 1980. **21:** 585–593.

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21-Dehydroxylation of corticosteroids is restricted to those steroid hormones which undergo enterohepatic circulation (1, 2). Deoxycorticosterone, dehydrocorticosterone, and corticosterone are excreted in bile (2, 3) and metabolized in the intestines. Deoxycorticosterone has been shown to be a precursor of urinary 3α,20α,dihydroxy-5β-pregnane glucuronide (4), and dehydrocorticosterone and corticosterone have been demonstrated to be precursors of 3α,20α-dihydroxy-5β-pregnane-11-one glucuronide excreted in urine (5, 6).

The involvement of intestinal bacteria in 21-dehydroxylation reactions was first demonstrated by Gustafsson (7) and Gustafsson and Sjövall (8). These studies established the absence of 21-hydroxylated C-21 steroids in feces from conventional rats whereas 3α,15α,21-trihydroxy-5α-pregnane-11, 20-dione and 3α,11β,15α,21-tetrahydroxy-5α-pregnane-20-one were the predominant fecal steroids in germ-free rats. These in vivo results, implying a role for intestinal bacteria in 21-dehydroxylation reactions of corticosteroids, were later confirmed by in vitro experiments where fecal contents from conventional rats were incubated anaerobically with 3β,21-dihydroxy-5α-pregnane-20-one with the production of 3,20-dihydroxypregnane derivatives (9). Thus, taken together, the 21-dehydroxylation of corticosteroids by intestinal bacteria causes a shift from biliary to renal excretion (5, 6).

Eriksson and Gustafsson (10) studied steroid biotransformation reactions catalyzed by fecal contents recovered from the ileostoma of a colectomized patient and observed the 21-dehydroxylation of 3β,21-dihydroxy-5α-pregnane-20-one yielding 3β-hydroxy-5α,17β-pregnane-20-one. Bokkenheuser et al. (11, 12) described 21-dehydroxylation of deoxycorticosterone in cultures of mixed fecal flora of normal individuals on a "Western diet." In more recent studies Bokkenheuser et al. (13) succeeded in isolating from human feces an obligate anaerobe capable of 21-

Abbreviations: Systematic names of steroids referred to in the text by their trivial names are as follows: deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; dehydrocorticosterone, 21-hydroxy-4-pregnene-3,11,20-trione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20; 11-ketoprogesterone, 4-pregnene-3,11,20-trione. NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; FMN, flavin mononucleotide; $FMNH_2$, flavin mononucleotide, reduced form; KPG, potassium phosphate glutathione (-SH) buffer; EDTA, ethylenediaminetetraacetic acid.

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dehydroxylating corticosteroids with an α-ketol side chain (14). The precise identity of the bacterium has not yet been established although it has been classified as an *Eubacterium lentum*-like organism (15).

We previously reported that 21-dehydroxylase activity in crude cell extracts of *E. lentum* required a reduced pyridine nucleotide and a flavin coenzyme (16). We also detected NAD(P)H:flavin oxidoreductase activity in these extracts and hypothesized that this enzyme may be providing reduced free flavins for 21-dehydroxylase. In this report the characteristics of 21-dehydroxylase are described.

EXPERIMENTAL PROCEDURES

Preparation of cell extracts

Cells of *Eubacterium lentum* (V.P.I. 11122, formerly culture 116) were grown, harvested, and cell extracts prepared as previously described (16). Maintenance of anaerobic conditions during ultracentrifugation was achieved by wrapping Parafilm around screw-capped ultracentrifuge tubes. All manipulations were performed under a continuous stream of oxygen-free argon. Molecular oxygen was removed from all gases by passage through a column of hot reduced copper filings.

Enzyme assays and product identification

21-dehydroxylase and NADH: FMN oxidoreductase assays in cell extracts were performed anaerobically as described previously (16). The standard reaction mixture for 21-dehydroxylase contained (total volume 1 ml): 42.5 mM potassium phosphate buffer (pH 6.2), 1 mM NADH, 1 mM flavin, 2.5 μCi [^{3}H]deoxycorticosterone in CH_3OH (sp act 46.8 Ci/mmol), 50 μM unlabeled deoxycorticosterone in CH_3OH, and enzyme preparation. Enzyme activity was measured (37°C) where initial reaction velocity was proportional to protein concentration. Reaction rates were linear up to 60 min using protein concentrations from 0.57 to 2.27 mg of protein/ml. 21-Dehydroxylation activity in washed cell suspensions was determined anaerobically in reaction mixtures containing whole cells suspended in 0.05 M potassium phosphate buffer (pH 6.2); 250 pmol of unlabeled deoxycorticosterone plus 2.5 μCi [^{3}H]deoxycorticosterone in CH_3OH were added to initiate the reaction.

Progesterone was identified as the 21-dehydroxylated product of deoxycorticosterone by mass spectrometry as described by Winter, Bokkenheuser, and Ponticorvo (14). Radiolabeled steroids were located on thin-layer chromatography strips by using a Packard radiochromatogram model 7201 scanner (Packard Instrument Co., Downers Grove, IL). For substrate specificity studies, steroids bearing 21-hydroxy groups (S) and expected 21-dehydroxylated product (P) were detected on thin-layer chromatograms by spraying with sulfuric acid–methanol 50:50 (v/v) and heating for 20–30 min at 160°C. Reaction products were chromatographed separately and as mixtures with authentic standards.

Methods

The procedure for generating photochemically reduced FMN has been described (16). Chemical reduction of FMN and viologen dyes was performed with either sodium dithionite or H_2 plus palladium catalysts. Sodium dithionite (0.04 M) and FMN (0.01 M) stock solutions were prepared in anaerobic 0.05 M potassium phosphate buffer (pH 6.2) and flushed with argon. Equal volumes of sodium dithionite and FMN were sequentially added to 21-dehydroxylase reaction mixtures maintaining the 4:1 molar ratio of sodium dithionite to FMN (final concentration 4mM and 1 mM, respectively). Reduced FMN, benzyl, and methyl viologen dyes were also prepared with H_2 as the source of reducing equivalents using palladium pellets as catalysts. Solutions (10 mM) of FMN, methyl viologen, and benzyl viologen were prepared in anaerobic 0.05 M potassium phosphate buffer (pH 6.2) containing 4 mM reduced glutathione (KPG). Four to eight palladium pellets (GasPak catalyst replacement charges, BBL, Cockeysville, MD) were added to the solutions in 1 × 8.5-cm test tubes, sealed with serum stoppers, and oxygen-free H_2 was bubbled over the palladium pellets for 60 min. Extreme precautions were followed during the preparations of reduced FMN to keep the systems absolutely free of molecular oxygen as reduced FMN is rapidly autooxidized with the formation of hydrogen peroxide and other oxygen radicals (17). Transfer of solutions of reduced FMN and viologen dyes to 21-dehydroxylase reaction mixtures was carried out with tuberculin syringes that had been flushed several times with oxygen-free argon.

Stock solutions of NaN_3, NaCN, and EDTA were prepared in anaerobic 0.05 M KPG buffer (pH 6.2). Concentrated solutions of mersalyl, o-phenanthroline, 8-hydroxyquinoline, and α,α'-dipyridyl were prepared in absolute CH_3OH. All inhibitor solutions were flushed with oxygen-free argon to maintain anaerobiosis. Inhibitors were preincubated with cell extract in reaction mixtures for 15 min before the initiation of the reaction by the addition of deoxycorticosterone and $FMNH_2$.

TABLE 1. Cellular location of 21-dehydroxylase activity in *E. lentum*

Fraction	Expt. #	21-Dehydroxylase Activity[a] (−) Triton X-100 S.A.	Total Units	%	(+) Triton X-100 S.A.	Total Units	%
Crude extract	A.[b]	18.7	485	100	18.7	485	100
	B.	20.7	1154	100	11.9	1348	100
	C.	5.4	601	100	15.5	1070	100
Mean ± S.D.		14.9 ± 8.4	747 ± 357		15.4 ± 3.4	968 ± 441	
105,000 × *g* Supernatant fluid	A.	12.2	282	58	18.9	357	74
	B.	18.7	751	65	11.7	1075	80
	C.	4.6	414	69	12.7	779	73
Mean ± S.D.		11.8 ± 7.1	482 ± 242	64 ± 5.6	14.4 ± 3.9	737 ± 361	76 ± 4.0
105,000 × *g* Pellet	A.	18.5	125	26	5.6	26	5
	B.	33.3	240	21	2.8	49	4
	C.	4.9	94	16	6.2	68	6
Mean ± S.D.		18.9 ± 14.2	153 ± 77	21 ± 5	4.9 ± 1.9	48 ± 21	5 ± 1.0

[a] Cell extracts were prepared in potassium phosphate buffer, pH 6.2. Extracts (where indicated) were treated anaerobically at 4°C with 1% (final concentration) Triton X-100 for 30 min. Particulate material was removed by ultracentrifugation for 2 hr. Extracts were assayed as described in Materials and Methods using NADH and FMN as cofactors. S.A. is specific activity and is defined as nmol progesterone formed per hr. mg protein^{-1}.

[b] Extract A was divided into two equal portions and one treated with Triton X-100. All other extracts were prepared independently.

Solubilization of 21-dehydroxylase

Initial studies showed that 21-dehydroxylase activity was found in both the supernatant fluid and pellet fraction after ultracentrifugation at 105,000 *g* for 2 hr. In order to obtain maximal enzyme units for anaerobic column chromatography, broken cell suspensions were centrifuged at 12,000 *g* for 10 min and the supernatant fluid was treated with 1% Triton X-100 for 30 min at 4°C. The Triton-treated cell extract was fractionated into soluble and particulate fractions by centrifugation at 105,000 *g* for 2 hr at 4°C. Treatment of the cell extracts with Triton X-100 enriched the supernatant fraction with 21-dehydroxylase activity with a concomitant reduction of enzyme activity in the particulate fraction (**Table 1**). The supernatant fluid was then subjected to anaerobic column chromatography.

Column chromatography

Anaerobic ion-exchange chromatography was carried out on a column (1.6 × 25 cm) of DEAE-cellulose at 4°C. The column packing was made anaerobic by boiling (10 min) and cooling under argon. The column was washed with several volumes anaerobic 0.05 M KPG buffer (pH 6.2). Protein was eluted with a linear KCl gradient formed from mixing 75 ml anaerobic 0.05 M KPG buffer (pH 6.2) with 75 ml KPG buffer which was 0.6 M in KCl. Fractions (5 ml) were collected by hand in anaerobe tubes and sealed with rubber stoppers.

Anaerobic gel filtration was carried out on a column (1.6 × 74 cm) of Sepharose 6B at 4°C. Protein was eluted by gravity flow (8 ml/h) with anaerobic 0.05 M KPG buffer (pH 6.2). Fractions (5 ml) were collected by hand in anaerobe tubes sealed with rubber stoppers. Anaerobic conditions for all column chromatographic techniques were assured by continuous flushing of the eluant reservoirs with oxygen-free argon.

Protein concentrations were estimated according to the procedure of Lowry et al. (18) using bovine serum albumin as a standard or by the spectrophotometric procedure of Kalb and Bernlohr (19).

Chemicals

[^{3}H]Deoxycorticosterone (46.8 Ci/mmol), [^{3}H]corticosterone (58.9 Ci/mmol), and [^{3}H]deoxycortisol (54.5 Ci/mmol) were obtained from New England Nuclear. Unlabeled deoxycorticosterone, progesterone, FMN, FAD, α,α′-dipyridyl, 8-hydroxyquinoline, o-phenanthroline, NaCN, and NaN_3 were purchased from Sigma Chemical Co. All other steroids were obtained from Steraloids. Methyl and benzyl viologen were from K and K Laboratories, Inc.

RESULTS

Preliminary experiments with crude cell extracts of *E. lentum* indicated that 21-dehydroxylase activity was rapidly (within 10 sec) and irreversibly inhibited by exposure to air. Moreover, cell extracts stored anaerobically under an argon gas atmosphere at 4°C

TABLE 2. Effect of reducing agents and glycerol on the stability of 21-dehydroxylase activity in cell extracts

Additions	Final Conc.	$10^2 \cdot$Specific Activity[a] 0hr	48hr	120hr
None		2281	1317	<1
Glycerol	10% v/v	1668	1409	<1
Glutathione (-SH)	4 mM	1950	1711	<1
2-Mercaptoethanol	4 mM	2652	1503	<1
L-Cysteine	4 mM	2482	1416	<1
Dithionite	2 mM	4463	1895	<1

[a] Duplicate values of a single experiment.

Cell extracts were prepared and stored anaerobically at 0–4°C in stoppered tubes. Aliquots were removed at indicated times and assayed under standard conditions for 21-dehydroxylase activity using [^{3}H]deoxycorticosterone as substrate. Specific activity is defined as nmol [^{3}H]progesterone formed per hr. mg protein^{-1}. Extracts contained 6.1 mg protein per ml.

for 48 hr lost over 40% of the initial 21-dehydroxylase activity (**Table 2**). However, the addition of glycerol (10%) or reduced glutathione (4 mM) to crude cell extract resulted in a partial stabilization of activity up to 48 hr. Neither 2-mercaptoethanol nor L-cysteine stabilized 21-dehydroxylase activity when added to crude cell extracts. Sodium dithionite (2 mM) initially stimulated 21-dehydroxylase activity about twofold compared to control activity. However, after 48 hr, extracts containing sodium dithionite had only 43% of the initial 21-dehydroxylase activity. All detectable 21-dehydroxylase activity was lost after 5 days storage regardless of the presence of reducing agents or glycerol. None of the reducing agents tested served as electron donors for 21-dehydroxylase.

Enzyme specificity studies

Enzyme specificity for steroid substrates was determined by two different approaches. First, by mixed substrate competition experiments and more directly by thin-layer chromatographic analysis of steroid products generated by individually incubating selected steroids with crude cell extracts containing added NADH and FMN. Mixed competition assays were performed by measuring the effect of varying concentrations of different unlabeled steroids on the rate of 21-dehydroxylation of [^{3}H]deoxycorticosterone under standard assay conditions. A concentration dependent reduction in the rate of 21-dehydroxyla-

TABLE 3. Effect of selected steroids on the rate of 21-dehydroxylation of [^{3}H]deoxycorticosterone in cell extracts of *E. lentum*

Steroid Added		Final Conc. (μM)	% Control Activity[a] Expt. 1	Expt. 2
Deoxycorticosterone (control)	(S)	50	100	100
Progesterone	(P)	100	98	98
Deoxycortisol	(S)	10	93	87
		50	71	53
		100	56	47
17α-Hydroxyprogesterone	(P)	100	67	67
Dehydrocorticosterone	(S)	10	85	ND[b]
		50	55	ND
		100	39	ND
11-Ketoprogesterone	(P)	100	103	ND
Corticosterone	(S)	10	46	ND
		50	39	ND
		100	16	ND
11β-Hydroxyprogesterone	(P)	100	100	ND
20β,21-Dihydroxy-4-pregnene-3-one	(S)	10	106	101
		50	119	110
		100	114	105
20β-Hydroxy-4-pregnene-3-one	(P)	100	105	98

[a] Average of duplicate values.

[b] ND = Not determined.

21-Dehydroxylase activity was determined as described in Experimental Procedures using NADH and FMN as cofactors. 21-Hydroxylated steroids (S) and expected 21-dehydroxylated products (P) were individually added to reaction mixtures along with [^{3}H]deoxycorticosterone. Control activity was 16.3 nmol [^{3}H]progesterone formed per hr·mg protein^{-1}.

TABLE 4. Substrate specificity of 21-dehydroxylase in cell extracts of *E. lentum*

Steroid		Incubation with Cell Extract	R_f Solvent A	R_f System B
Deoxycorticosterone	(S)	−	0.35	0.68
		+	0.63	0.84
Progesterone	(P)	−	0.63	0.84
		+	0.62	0.84
Deoxycortisol	(S)	−	0.24	0.63
		+	0.49	0.81
17α-Hydroxyprogesterone	(P)	−	0.48	0.79
		+	0.48	0.80
Dehydrocorticosterone	(S)	−	0.11	0.42
		+	0.33	0.68
11-Ketoprogesterone	(P)	−	0.33	0.69
		+	0.34	0.69
Corticosterone	(S)	−	0.11	0.45
		+	0.24	0.67
11β-Hydroxyprogesterone	(P)	−	0.25	0.67
		+	0.24	0.66
20β,21-Dihydroxy-4-pregnene-3-one	(S)	−	0.12	0.42
		+	0.13	0.42
20β-Hydroxy-4-prognene-3-one	(P)	−	0.51	0.83
		+	0.51	0.83

Steroids bearing 21-hydroxy groups (S) and expected 21-dehydroxylated product (P) were detected on thin-layer chromatograms by spraying with sulfuric acid–methanol 50:50-(v/v) and heating for 20–30 min at 160°C. Solvent system A contained ethyl acetate–cyclohexane 50:50(v/v) and B contained ethyl acetate–2,2,4-trimethylpentane–acetic acid 25:5:0.2(v/v/v). R_f refers to the ratio of the mobility of the steroid to the solvent front (18 cm).

tion of [^{3}H]deoxycorticosterone occurred when unlabeled corticosterone, dehydrocorticosterone, deoxycortisol and 17α-progesterone were individually added to standard reaction mixtures (**Table 3**). A single steroid metabolite was formed from corticosterone, dehydrocorticosterone and deoxycortisol when incubated under standard assay conditions without [^{3}H]-deoxycorticosterone. Moreover, steroid metabolites formed under these conditions co-migrated in two different thin-layer chromatography solvent systems with the expected 21-dehydroxylated steroid product (Table 3). No detectable products were formed from 17α-hydroxyprogesterone even though this compound inhibited the 21-dehydroxylation of [^{3}H]-deoxycorticosterone (**Table 4**). No detectable products were formed from progesterone, 11-ketoprogesterone, 11β-hydroxyprogesterone, 20β, 21-dihydroxy-4-pregnene-3-one or 20β-hydroxy-4-pregnene-3-one when individually incubated with cell extracts containing added NADH and FMN (Table 4).

Enzyme kinetic studies

The data presented in **Fig. 1** show the effect of increasing corticosterone concentration on the initial velocity of 21-dehydroxylase activity. A Lineweaver-Burk plot yielded an apparent Km of 7.35 μM and a Vmax of 15.4 nmol 11β-hydroxyprogesterone formed per hr·mg protein^{-1}. The maximal velocity of 21-dehydroxylase activity was about 50% greater with corticosterone compared to deoxycorticosterone (16). The enzyme in crude extracts exhibited zero-order kinetics with both corticosteroids at concentrations above 30 μM.

Electron donor systems for 21-dehydroxylase in cell extracts and whole cells

21-Dehydroxylase activity was measured by using photochemically reduced FMN in lieu of the standard NADH plus FMN electron donor system (**Table 5**). Moreover, sodium dithionite-reduced FMN or H_2 plus palladium-reduced FMN could function as electron donors for 21-dehydroxylase. Reduced benzyl viologen in combination with FMN increased 21-dehydroxylase activity twofold under standard assay conditions. Reduced or oxidized methyl viologen failed to stimulate 21-dehydroxylase activity.

The effect of various gases on the rate of 21-dehydroxylation of [^{3}H]deoxycorticosterone was measured using washed whole cell suspensions. There was

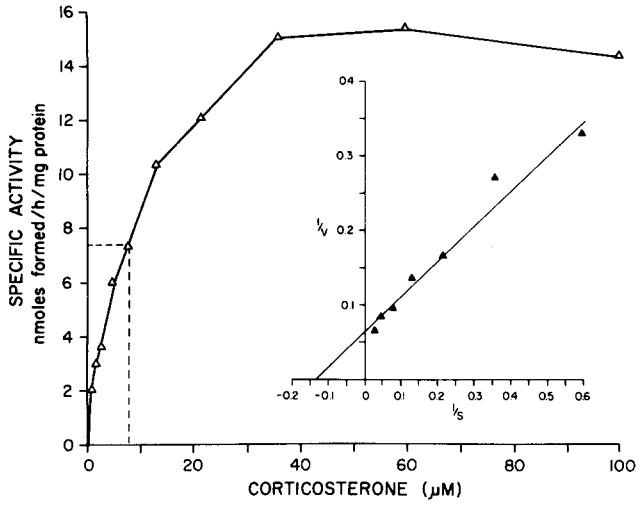

Fig. 1. The effect of corticosterone concentration on the initial velocity of 21-dehydroxylase activity in *E. lentum* cell extracts. Reactions were initiated by the addition of NADH, FMN and [^{3}H]-corticosterone.

a 7- and 35-fold increase in 21-dehydroxylation activity when whole cells were sparged with hydrogen gas as compared to argon and air, respectively (**Table 6**). We have obtained preliminary evidence for hydrogenase activity (based on methylene blue reduction) in whole cells and cell extracts. Hydrogenase activity was sensitive to pronase treatment, liable to molecular oxygen, and had an absolute requirement for molecular hydrogen.[3]

Metalloenzyme inhibitors

The effect of metalloenzyme inhibitors on 21-dehydroxylase activity was determined using H_2 plus palladium-reduced FMN. Both lipophilic and water-soluble metal ion chelating agents inhibited 21-dehydroxylase activity. The water-soluble metal ion chelating agents NaCN, NaN_3, and EDTA (all at 1 mM) inhibited 21-dehydroxylase activity by 34, 24, and 34%, respectively. Moreover, the lipophilic metal ion chelating agents mersalyl, o-phenanthroline, 8-hydroxyquinoline and α,α'-dipyridyl (all at 1 mM) inhibited enzyme activity by 45, 78, 62, and 54%, respectively.

[3] P. B. Hylemon. Unpublished data.

TABLE 5. Effect of various electron donating systems on 21-dehydroxylase activity in cell extracts from *E. lentum*

Electron-donor System	10^2·Specific Activity ($\bar{X}$ ± SD)
NADH + FMN	961 ± 372
FMN	66[a]
$FMNH_2$ (Pd + H_2)	1273 ± 362
$FMNH_2$ ($Na_2S_2O_4$)	373[a]
NADH + FMN + $Na_2S_2O_4$	995[a]
$Na_2S_2O_4$	66[a]
$FMNH_2$ (photochemical reduction)	724 ± 111
NADH + FMN + EDTA	995 ± 31
Benzyl viologen (reduced)	803 ± 245
Benzyl viologen (reduced) + FMN	1649[a]

[a] Average of duplicate values.

All electron donors were present at 1 mM final concentration and reduced forms were prepared as described in Experimental Procedures. [^{3}H]Deoxycorticosterone was used as the steroid substrate in all experiments.

TABLE 6. Effect of various gases on the rate of 21-dehydroxylation of [^{3}H]deoxycorticosterone by washed whole cell suspensions of *E. lentum*

Gas	Specific Activity[a] 10^2·pmol progesterone formed per hr·mg protein^{-1} Sparged
H_2	2956
Argon	419
Air	85

[a] Average of duplicate values.

Whole cells of *E. lentum* were harvested and washed once in anaerobic potassium phosphate buffer (pH 6.8) and the turbidity adjusted to approximately 400 Klett units. Reaction mixtures (1.0 ml) contained 0.9 ml whole cells and [^{3}H]deoxycorticosterone dissolved in CH_3OH. Initial rates of conversion were determined over a time course of 10 min and specific activities were calculated from the 5-min time point.

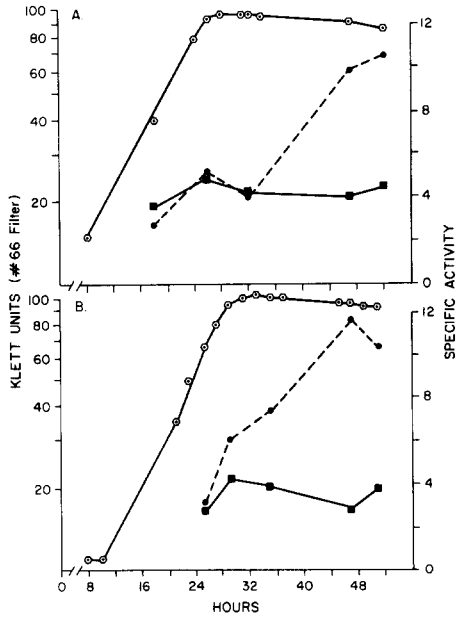

Fig. 2. 21-Dehydroxylase and NADH:FMN oxidoreductase activities as a function of growth (⊙) of *E. lentum* in the absence (A) and presence (B) of 20 μM deoxycorticosterone. Growth of cells, preparation of extracts, and enzyme assays were performed as described in Experimental Procedures. Specific activities for NADH:FMN oxidoreductase (■) and 21-dehydroxylase (●) are defined as nmol NADH oxidized per min·mg protein^{-1} and nmole progesterone formed per h·mg protein^{-1}, respectively.

21-Dehydroxylase as a function of culture age

21-Dehydroxylase and NADH:FMN oxidoreductase activities were measured in cell extracts prepared from cells grown either in the presence or absence of 20 μM deoxycorticosterone and harvested at various times thoughout the growth curve (**Fig. 2**). Under either condition, 21-dehydroxylase activity was maximal only after cultures were well into the stationary phase of growth. NADH:FMN oxidoreductase activity remained constant throughout the different phases of growth in cell extracts prepared from cells cultured in both media.

Anaerobic column chromatography

Elution profiles of $FMNH_2$-dependent 21-dehydroxylase and NADH:FMN oxidoreductase activities from DEAE-cellulose and Sepharose 6B columns are shown in **Figs. 3** and **4,** respectively. 21-Dehydroxylase activity eluted as a single peak from both columns. NADH:FMN oxidoreductase activity eluted in several fractions from the DEAE-cellulose column. The Sepharose 6B column was calibrated for molecular weight determination using Blue dextran 2000, thyroglobulin (669,000), ferritin (450,000), aldolase (158,000) and bovine serum albumin (68,000). The elution volume for 21-dehydroxylase activity corresponded to a relative molecular weight of 582,000.

DISCUSSION

We previously reported the development of a quantitative radiochromatographic assay system for measuring 21-dehydroxylase activity in cell extracts prepared from *E. lentum* (16). Anaerobically dialyzed cell extracts had an absolute requirement for both a reduced pyridine nucleotide (either NADH or NADPH) and an oxidized flavin (FAD or FMN) for the 21-dehydroxylation of deoxycorticosterone. Moreover, photochemically reduced FMN ($FMNH_2$) could replace the requirement for a reduced pyridine nucleotide and flavin. These results lead us to propose a pathway for electron transfer from reduced pyridine nucleotides to deoxycorticosterone. This pathway includes a NAD(P)H:flavin oxidoreductase for generating reduced free flavins which are reoxidized by 21-dehydroxylase during the biotransformation of deoxycorticosterone to progesterone.

In the present study, we have demonstrated by several independent methods that 21-dehydroxylase requires a reduced free flavin for activity (Table 5). Moreover, the present data indicate that NADH:FMN oxidoreductase and 21-dehydroxylase are not physically associated (Figs. 3 and 4) and these enzymes appear to be under independent control during growth (Fig. 2).

The substrate specificity of 21-dehydroxylase for corticosteroids in cell extracts was identical to the results obtained by Winter et al. (14) using growing cultures of *E. lentum.* These results suggest either a membrane location for 21-dehydroxylase or the presence of a neutral steroid transport system in this bac-

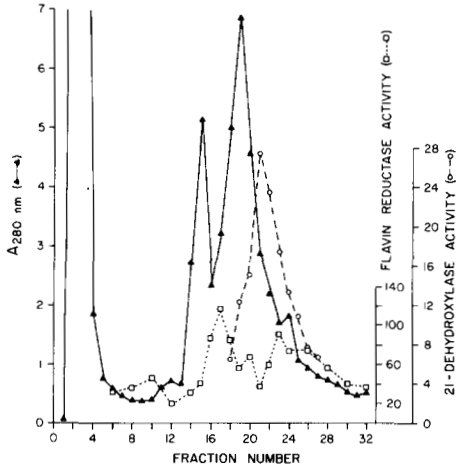

Fig. 3. Anaerobic DE-52 column chromatography of Triton X-100 solubilized 21-dehydroxylase from *E. lentum.* The absorbance at 280 nm in fractions 1 to 5 is due to Triton X-100. 21-Dehydroxylase activity was assayed with [^{3}H]deoxycorticosterone and $FMNH_2$ as substrates.

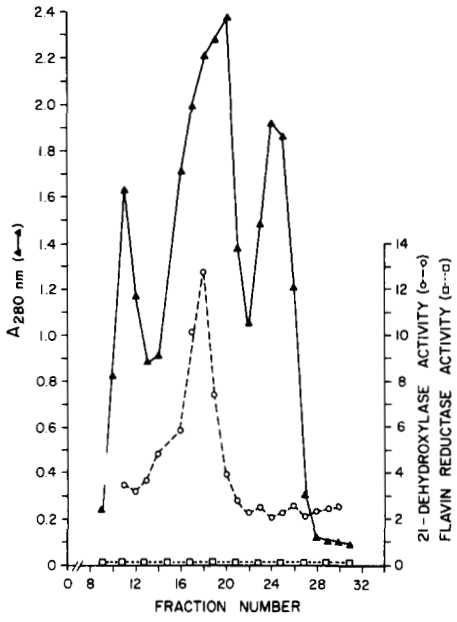

Fig. 4. Anaerobic Sepharose 6B Chromatography of Triton X-100 solubilized 21-dehydroxylase from *E. lentum.* 21-Dehydroxylase activity was assayed with [^{3}H]deoxycorticosterone and $FMNH_2$ as substrates.

terium. However, conclusive evidence for either of these hypotheses is not yet available.

17α-Hydroxyprogesterone was the only 21-dehydroxylated product which inhibited the 21-dehydroxylation of [^{3}H]deoxycorticosterone in the mixed substrate competition experiments (Table 3). A possible explanation for this inhibition was that 21-dehydroxylase might also have 17α-dehydroxylase activity. However, the results presented in Table 4 did not support this hypothesis as no 17α-dehydroxylated metabolite (i.e., progesterone) was formed following incubation of 17α-hydroxyprogesterone with cell extracts. Moreover, only one radioactive metabolite (17α-[^{3}H]hydroxyprogesterone) was detected after incubating [^{3}H]deoxycortisol with cell extracts under standard assay conditions. Winter et al. (14) also reported that growing cultures of *E. lentum* could not carry out 17α-dehydroxylation of steroid substrates. Therefore, the explanation for inhibition by 17α-hydroxyprogesterone must await further experimentation.

The importance of an α-ketol structure ($R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2\text{-}OH$) for 21-dehydroxylation was demonstrated by the absence of a 21-dehydroxylated product from 20β,21-dihydroxy-4-pregnene-3-one (Table 4) and by the lack of inhibition by this compound in mixed substrate competition experiments (Table 3). Fructose and dihydroxyacetone did not inhibit the 21-dehydroxylation of [^{3}H]deoxycorticosterone when present in reaction mixtures at 10-fold molar excess.[3] These results suggest that 21-dehydroxylase may be restricted to compounds which also contain the perhydropentanophenanthrene nucleus. Kelly et al. (20) added deoxycorticosterone labeled with ^{14}C at C-4 and ^{3}H at both C-21 hydrogens to a mixed fecal flora and found that more than half of the tritium at C-21 was always exchanged during 21-dehydroxylation. These investigators suggested that the 21-dehydroxylation of deoxycorticosterone probably involves an enolization at C-20,21.

The optimal conditions under which whole cells of *E. lentum* carry out 21-dehydroxylation of corticoids are not yet clear. However, Winter and Bokkenheuser (21) have suggested that the rate of 21-dehydroxylation by growing cultures of *E. lentum* is a function of the medium Eh. This interpretation was based on the observation that the rate of conversion of deoxycorticosterone to progesterone was greatly stimulated when *Escherichia coli* was grown as a mixed culture with *E. lentum* with subsequent lowering of the medium Eh from −80 mV to −280 mV. Our results (Table 6) demonstrated a 7-fold increase in the rate of conversion of deoxycorticosterone to progesterone in whole cell suspensions of *E. lentum* when H_2 was sparged through the reaction mixture as compared to argon gas. These results suggest that H_2 (via hydrogenase might provide reducing equivalents for 21-dehydroxylation of deoxycorticosterone. Since *E. coli* is capable of H_2 formation via formic hydrogenlyase this may account for the stimulation of 21-dehydroxylation of deoxycorticosterone in mixed cultures. Experiments designed to distinguish the stimulatory effect of oxidation-reduction potential and/or H_2 on 21-dehydroxylation activity in whole cells of *E. lentum* are in progress.

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