Acta Chemica Scandinavica B 28 (1974) 832—840

Transformation of Steroids by Cell-free Preparations of *Penicillium lilacinum* NRRL 895. V. Properties of 20-Oxopregnane Side Chain Cleavage and 20(α+β)-Oxidoreductase Activities

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Assay conditions for the 20-oxopregnane side chain cleavage and the 20(α + β)-oxidoreduction in cell-free preparations from *P. lilacinum* have been determined. Progesterone and 17α-hydroxyprogesterone but not 17α-acetoxyprogesterone were substrates for the two types of enzymes. Side chain cleavage showed an absolute requirement for NADPH and may involve participation of flavins and iron-sulphur protein. The reaction was intensitive to cyanide, azide, fluoride, and carbon monoxide, indicating that no cytochromes take part in the oxygenation of the substrate. Reduction at C-20 required NADH or NADPH with a preference for NADH. NADPH favoured formation of the 20α-alcohol while NADH favoured formation of the 20β-epimer. Clomiphene citrate selectively inhibited the 20α-reduction.

Oxidation of ketones to esters (Baeyer-Villiger oxidations) are well known in microbial metabolism. Long chain aliphatic ketones, simple cyclic ketones, monoterpenes and steroids serve as substrates for this reaction.[1] While the electron transport system in the monoterpene biooxidation has been thoroughly studied,[2-6] little is known about the corresponding systems utilized in steroid oxidations. Prairie and Talalay studied the lactonization of ring D in 17-oxosteroids by a partially purified enzyme from *P. lilacinum*.[7] They found that atmospheric oxygen was incorporated in the testololactone formed from 4-androstene-3,17-dione. The reaction showed an absolute requirement for NADPH and was insensitive towards cyanide, chelating agents and several metal ions. The C_{17-20}-lyase and steroid esterase activities in purified enzyme preparations from *Cylindrocarpon radicicola* have been studied by Rahim and Sih.[8] Esterase-free lyase preparations transformed progesterone into testosterone acetate in good yield and Nakano and co-workers demonstrated that atmospheric oxygen was incorporated in the 17β-position.[9] The reaction required NADPH and the effects of metal ions and inhibitors were similar to those observed for the ring D lactonization.

The 20-oxopregnane side chain cleavage in cell-free extracts from *P. lilacinum* has recently been shown to proceed *via* the same pathway as in *C. radicicola* and other microorganisms.[8,10-15] This reaction is accompanied by reduction of the 20-oxo group, and it has been suggested that the 20-reductase(s) compete with the side chain cleaving enzyme for the C_{21} steroid substrate.[16,17] The present paper describes assay conditions for the 20-oxopregnane side chain cleavage as well as 20(α + β)-reduction. Effects of metal ions, inhibitors, and substrate structure have also been studied.

MATERIALS AND METHODS

Abbreviations and trivial names. GLC: gas liquid chromatography; GC-MS: gas chromatography-mass spectrometry; silyl: trimethylsilyl; TLC: thin layer chromatography; UV: ultra-

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violet. Clomiphene citrate: 1(*p*-2-diethylaminoethoxyphenyl)-1,2-diphenyl-2-chloroethylene dihydrogen citrate; progesterone: 4-pregnene-3,20-dione; 17α-acetoxyprogesterone: 17α-acetoxy-4-pregnene-3,20-dione; 17α-hydroxyprogesterone: 17α-hydroxy-4-pregnene-3,20-dione; testololactone: 17α-oxa-4-androstene-3,17-dione; testosterone: 17β-hydroxy-4-androsten-3-one.

Radioactive steroids. [7-^{3}H]Progesterone (specific activity 16 Ci/mmol) and [21-^{14}C] progesterone (specific activity 0.050 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass, They were purified by TLC before use.

[7-^{3}H,21-^{14}C]17α-Hydroxyprogesterone was prepared by enzymatic 17α-hydroxylation of labelled progesterone.[18] The following procedure was used: Five male Sprague-Dawley rats (200 – 230 g) were killed by a blow to the head. The testes were removed, decapsulated and washed with ice cold 0.06 M Tris-HCl pH 7.2, containing 5 g of NaCl, 0.3 g of KCl, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4.7H_2O$, and 2 g of glucose per litre. The testes were homogenized with one volume of the medium in a Turmix household mixer for 60 s at +4 °C. Twenty ml of the homogenate were transferred into a beaker and 50 μCi of [7-^{3}H]progesterone + 20 μCi of [21-^{14}C]-progesterone in 30 μl of ethanol and 20 mg of NADPH were added. The mixture was stirred in air at 37° for 45 min. The reaction was terminated by the addition of 1 ml acetic acid. After addition of 50 μg each of unlabelled progesterone, 4-androsten-3,17-dione, 17α-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one, and testosterone, the mixture was extracted three times with one volume of ethyl acetate. The ethyl acetate extract was washed with 1 M NaOH, 8 % $NaHCO_3$ and water and was evaporated to dryness.

The extract was defatted by partition between 70 % methanol and hexane and subjected to TLC on Silica gel GF_{254} (system I). The zone containing 17α-hydroxyprogesterone + 20α-hydroxy-4-pregnen-3-one was isolated, acetylated with acetic anhydride in pyridine and subjected to a second TLC in the same system. The ^{3}H radioactivity in the 17α-hydroxyprogesterone zone corresponded to a yield of 25 %. The 17α-hydroxyprogesterone was rechromatographed on Al_2O_3 GF_{254} 0.5 % ethanol in benzene as solvent. It moved as a single homogenous band. Its radiochemical homogeneity was established by crystallization to constant specific activity.

In the substrate solutions the radioactive compounds were diluted with unlabelled steroids dissolved in ethanol. For progesterone the final radioactivity corresponded to 18 000 – 100 000 cpm ^{3}H per 0.319 μmol steroid in 10 μl of ethanol. The corresponding figure for 17α-hydroxyprogesterone was 2 500 cpm ^{3}H 0.319 μmol steroid in 10 μl of ethanol.

Other reagents. Progesterone, 20α-hydroxy-4-pregnen-3-one, and 20β-hydroxy-4-pregnen-3-one were purchased from Ikapharm Ltd, Ramat-Gan, Israel; 5α-cholestane, 17α-hydroxyprogesterone, testosterone, and 4-androstene-3,17-dione from Sigma Chemical Co, St Louis, Mo, and 17α-acetoxyprogesterone, 17α,20α-dihydroxy-4-pregnen-3-one, and 17α,20β-dihydroxy-4-pregnen-3-one from Steraloids Inc., Pawling, N. Y. All steroids were checked for purity by TLC and/or GLC.

Clomiphene citrate was kindly donated by Draco, AB, Lund, Sweden. It contained 60 % of the *cis* and 40 % of the *trans* epimer.

Table 1. Variation between individual samples in duplicate incubations expressed as S. D. $=\sqrt{\sum d^2/2N}$. N = number of duplicate incubations.

Steroid	Mol-% in sample mean range	N	S.D., mol-%
Progesterone	68.7 (54.4 – 75.8)	26	± 1.5
»	88.5 (77.9 – 98.6)	31	± 0.9
20(α+β)-Hydroxy-4-pregnen-3-one	3.1 (0.4 – 5.0)	20	± 0.5
»	7.3 (5.1 – 9.6)	15	± 0.7
»	15.8 (10.1 – 27.0)	30	± 1.8
Testosterone	1.9 (0.1 – 5.0)	24	± 0.6
»	7.4 (5.3 – 9.4)	21	± 0.6
»	14.9 (10.6 – 30.0)	11	± 1.3
4-Androstene-3,17-dione	2.4 (0.9 – 5.0)	36	± 0.4
»	7.2 (5.2 – 11.9)	13	± 0.4
Testosterone acetate	1.4 (0.3 – 7.1)	16	± 0.4
Testololactone	1.0 (0.2 – 1.9)	32	± 0.2
Total C_{19} steroids	3.0 (1.5 – 5.0)	28	± 0.5
»	7.4 (5.1 – 9.9)	27	± 0.6
»	15.1 (10.1 – 32.2)	33	± 0.9

Other chemicals were of reagent grade and were treated as described previously.[19]

Chromatographic systems. TLC on Silica gel GF_{254} (system I) and on Al_2O_3 GF_{254} (0.5 % ethanol in benzene as solvent); GLC on OV-17 and GC-MS were carried out as previously described.[19] Steroid silyl ether mixtures were quantitatively analyzed by GLC using 2.5 % XE-60 as stationary phase.

Growth of organism and preparation of cell-free extracts. P. lilacinum NRRL 895 was grown on Czapek-Dox medium, induced with progesterone, washed and frozen as described previously.[19] Cell-free extracts were prepared by high speed grinding with glass beads followed by centrifugation at 100 000 *g*.[20,21] The crude 100 000 *g* supernatant contained endogenous cofactors sufficient for a limited degree of C_{17-20}-lyase activity and minor amounts of testosterone. Extracts free from cofactors and steroids were prepared by gel filtration through a 300 mm × 15 mm i.d. Sephadex G-25 column connected to a LKB Uvicord II UV recorder and a fraction collector. Extracts free from steroids were prepared by passing the sample through an Amberlite XAD-2 column.

Incubations of steroids with cell-free extracts. The procedures for incubation and extraction of the reaction mixture have been described previously.[10] The steroid substrates were added in 10 µl of ethanol per ml of enzyme preparation. Inhibitors and metal salts were added as solutions in 0.06 M Tris-HCl adjusted to pH 7.2. If not otherwise stated, the transformations were carried out using crude 100 000 *g* supernatants with [7-^{3}H,21-^{14}C]progesterone as substrate for 60 min at 26 – 28 °C.

Steroid analysis. Quantitative analysis was made by TLC and liquid scintillation counting as previously described.[10] The variation between individual samples in duplicate incubations is given in Table 1. When [7-^{3}H,21-^{14}C] progesterone was the substrate, blank values due to tailing or non-enzymatic reactions were rather small. Thus, after incubation with boiled extracts + NADPH, 98.0 % of the total ^{3}H radioactivity on the TLC plate was found in the progesterone fraction, 0.9 % in the 4-androstene-3,17-dione fraction, 0.3 % in the 20($\alpha+\beta$)-hydroxy-4-pregnen-3-one fraction, 0.2 % in the testosterone fraction, and 0.6 % in the "testololactone" fraction. However, with [7-^{3}H,21-^{14}C]17α-hydroxyprogesterone as substrate there were considerable blank values, especially in the zone more polar than 17α-hydroxyprogesterone. When unacetylated blank samples were chromatographed, 0.9 % of the ^{3}H radioactivity was found in the 4-androstene-3,17-dione fraction, 88.4 % in the 17α-hydroxyprogesterone fraction, 6.6 % in the testosterone fraction, 3.4 % in the 17α,20($\alpha+\beta$)-dihydroxy-4-pregnen-3-one fraction and 0.6 % in the most polar fraction. Due to contamination of the testosterone fraction with substrate, samples from incubations with [7-^{3}H,21-^{14}C]17α-hydroxyprogesterone were analyzed before and after acetylation. Blank corrections were made in all assays.

The reactions were terminated at a stage when still large amounts of C_{21} steroids remained in the mixture. This will explain the low or insignificant amounts of testololactone detected.[15]

Protein assays were made by the biuret method.[22]

Identifications. The steroid metabolites were identified by their chromatographic and mass spectrometric properties as free steroids, acetates, and silyl ethers and by their ^{3}H/^{14}C ratios.[10,19] In the GC-MS, 17α,20α-dihydroxy-4-pregnen-3-one and its 20β-epimer were analyzed as 20-acetates and as *O*-methyloxime silyl ether derivatives.

RESULTS

Assay conditions. The C_{17-20}-lyase activity had its optimum between pH 7.2 and 7.6. 20-Reduction was maximal at pH 6.4 (Fig. 1). The time-course of the transformation of progesterone is shown in Fig. 2. The concentration of 20($\alpha+\beta$)-hydroxy-4-pregnen-3-one was maximal at about 60 min and then decreased. The formation of C_{19} steroids still continued after 180 min.

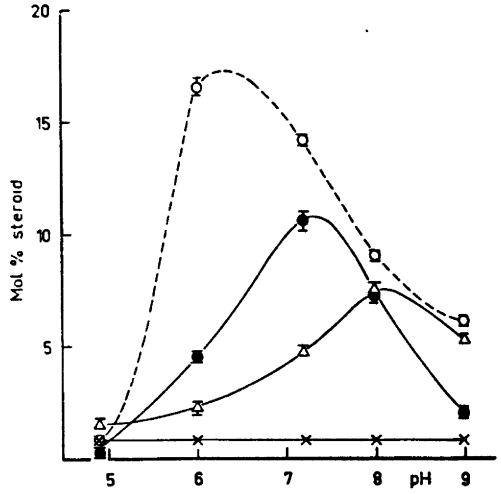

Fig. 1. pH/activity relationship for the side chain cleavage and the 20($\alpha+\beta$)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatants. Initial concentration of progesterone 2.90×10^{-4} M and of exogenous NADPH 1.21×10^{-3} M. 20($\alpha+\beta$)-Hydroxy-4-pregnen-3-one ○; testosterone ●; 4-androstene-3,17-dione△; "Compound T" (tentatively identified as testololactone) ×.

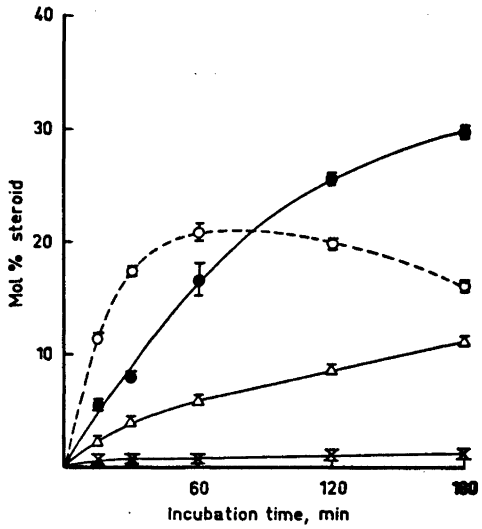

Fig. 2. Time-course of the side chain cleavage and the 20(α+β)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatant. Initial concentration of progesterone 2.90×10^{-4} M and of exogenous NADPH 1.34×10^{-3} M. 20(α+β)-Hydroxy-4-pregnen-3-one ○; testosterone ●; 4-androstene-3,17-dione △; "Compound T" (tentatively identified as testololactone) ×.

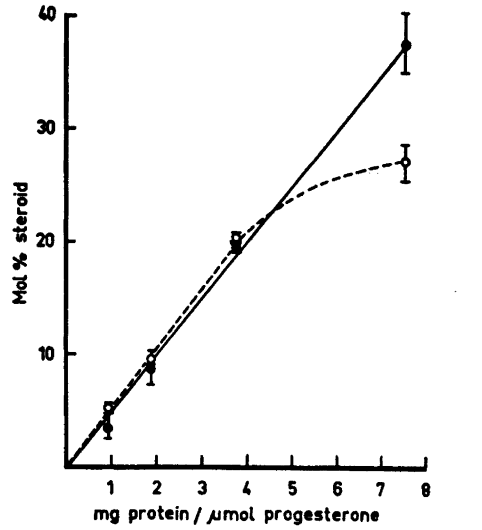

Fig. 3. Effect of the enzyme/substrate ratio on the side chain cleavage and the 20(α+β)-reduction of progesterone by cell-free preparations from *P. lilacinum*. Crude 100 000 *g* supernatant. Initial concentration of exogenous NADPH 1.10×10^{-3} M and of progesterone 2.66×10^{-4} M in the first three points and 1.33×10^{-4} M in the last point (7.52 mg protein/μmol progesterone). Total side chain cleavage ●; total 20-reduction ○.

The side chain cleavage as well as the 20-reduction was linear with the enzyme/substrate ratio up to about 4 mg/μmol (Fig. 3).

Substrate specificity. Progesterone and 17α-hydroxyprogesterone were substrates for the C_{17-20}-lyase and the 20(α+β)-oxidoreductase activities (Table 2). 17α-Acetoxyprogesterone yielded no detectable metabolites (by GLC and TLC).

The metabolites of 17α-hydroxyprogesterone were identified after isolation by TLC in system I (triple run). One half of the 17α,20-dihydroxy-

Table 2. Transformation of progesterone and 17α-hydroxyprogesterone by cell-free preparations from *P. lilacinum*.

Experiment	Steroid composition of reaction mixture (mol %)							
	Progesterone	20(α+β)-Hydroxy-4-pregnen-3-one	17α-Hydroxyprogesterone	17α20(α+β)-Dihydroxy-4-pregnen-3-one	Testosterone	4-Androstene-3,17-dione	Testololactone	Total C_{19}steroids
1/P[b]	73.1	11.7	—	—	9.6	5.1	0.5	15.2
1/17[b]	—	—	77.5	7.8	1.8	12.9	n.s.	14.7
2/P[a]	81.8	3.9	—	—	11.6	2.2	0.5	14.3
2/17[a]	—	—	70.6	19.1	2.0	8.3	n.s.	10.3
3/P[a]	82.5	7.8	—	—	7.1	2.1	0.5	9.7
3/17[a]	—	—	82.4	10.1	2.1	5.8	n.s.	7.9

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. P indicates progesterone as substrate, 17 17α-hydroxyprogesterone. Initial concentrations of progesterone and 17α-hydroxyprogesterone 2.90×10^{-4} M and of exogenous NADPH 1.33×10^{-3} M.

Table 3. R_F- and t_R values for the 20-epimeric 17α,20-dihydroxy-4-pregnen-3-ones as free alcohols, 20-acetates and O-methyloxime silyl ether derivatives.

	R_F-value, system I		t_R-value (5α-cholestane = 1.00)
	Double run	Triple run	1.5 % SE-30, column temp. 240 °C, carrier gas flow 32 ml/min
17α,20α-Dihydroxy-4-pregnen-3-one	0.12	0.20	
17α-Hydroxy,20α-acetoxy-4-pregnen-3-one	0.37		1.47
O-Methyloxime silyl ether derivative of 17α,20α-dihydroxy-4-pregnen-3-one			1.49
17α,20β-Dihydroxy-4-pregnen-3-one	0.16	0.28	
17α-Hydroxy,-20β-acetoxy-4-pregnen-3-one	0.29		1.39
O-Methyloxime silyl ether derivative of 17α,20β-dihydroxy-4-pregnen-3-one			1.41

4-pregnen-3-one fraction was acetylated and rechromatographed in the same system (double run). R_F-Values and t_R-values are given in Table 3. The "atypical" behaviour of the 20α-acetate with greater chromatographic mobility than the 20β-epimer is noteworthy. TLC and GLC behaviour and mass spectra of the metabolites were in close agreement with those of authentic reference compounds.

Cofactor requirements. The crude 100 000 *g* supernatant contained endogenous cofactors sufficient for a certain degree of C_{17-20}-lyase activity. In such extracts this activity was stimulated by NADPH as well as by NADH (Table 4). Extracts purified by gel filtration were inactive without cofactors. In such extracts the C_{17-20}-lyase activity showed an absolute requirement for NADPH. Addition of FMN

Table 4. Effect of exogenous cofactors on the transformation of progesterone by cell-free preparations of *P. lilacinum*.

Experiment	Initial concentration of exogenous cofactors, mM			Initial concentration of progesterone, mM	Mol % total side chain cleavage	Mol % 20 (α+β)-reduction
	NADPH	NADH	FMN			
4[a]	–	–	–	0.266	6.7	n.s.
4	0.55	–	–	»	15.9	12.7
4	1.10	–	–	»	17.9	19.8
4	2.20	–	–	»	16.9	21.9
5[b]	–	–	–	0.228	2.3	n.s.
5	–	0.50	–	»	3.3	10.7
5	–	1.00	–	»	4.7	18.2
5	0.52	–	–	»	4.5	7.0
5	1.04	–	–	»	4.2	10.8
5	1.04	–	0.90	»	6.4	7.6
6[c]	–	–	–	0.266	n.s.	n.s.
6	–	1.06	–	»	n.s.	21.3
6	1.10	–	–	»	4.8	11.2
6	1.10	–	1.04	»	6.2	8.9
7[c]	1.10	–	–	0.266	7.1	8.8
7	1.10	–	0.11	»	8.6	7.4
7	1.10	–	1.10	»	8.8	7.2

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. [c] Gel filtrated 100 000 *g* supernatant.

Table 5. Influence of cofactors and of clomiphene citrate on the 20α and 20β reduction of progesterone by cell-free preparations from *P. lilacinum*.

Experiment	Initial concentration of exogenous cofactors, mM		Clomiphene citrate added, mM	Steroid composition of reaction mixture (mol-%)			
	NADPH	NADH		20α-Hydroxy-4-pregnen-3-one	20β-Hydroxy-4-pregnen-3-one	Total 20-reduction	Total side chain cleavage
5[b]	0.52	—	None	4.5	2.5	7.0	4.5
5	1.04	—	»	7.3	3.5	10.8	4.2
5	—	0.50	»	4.3	6.4	10.7	3.3
5	—	1.00	»	6.9	11.3	18.2	4.7
6[c]	1.10	—	»	6.2	5.0	11.2	4.8
6	—	1.06	»	4.9	16.4	21.3	n.s.
8[a]	1.21	—	»	7.2	6.1	13.3	6.7
8[d]	1.21	—	»	5.1	2.7	7.8	n.s.
8[d]	1.21	—	3	n.s.	6.6	6.6	n.s.
8[d]	—	1.25	None	8.2	20.0	28.2	n.s.
8[d]	—	1.25	3	n.s.	18.5	18.5	n.s.

[a] Crude 100 000 *g* supernatant. [b] Crude 100 000 *g* supernatant after passing Amberlite XAD-2. [c] Gel filtrated 100 000 *g* supernatant. [d] Protein fraction obtained from 100 000 g supernatant by precipitation with 80 % saturated ammonium sulphate, redissolving in Tris buffer and dialysis. Initial concentration of progesterone in experiment 5 2.28×10^{-4} M, in experiments 6 and 8 2.66×10^{-4} M.

caused a slight increase in the stimulatory effect of NADPH (Table 4).

The 20-reduction in crude 100 000 *g* supernatants and in purified extracts required NADPH or NADH (Tables 4 and 5). NADH was more effective in stimulating the 20-reduction than was NADPH. NADH favoured formation of the 20β-epimer in all types of extracts. Clomiphene citrate selectivity inhibited the 20α-reduction.

Effects of metal ions. The C_{17-20}-lyase activity was strongly suppressed by Hg^{2+}, Cu^{2+} and

Table 6. Effect of metal ions on the transformation of progesterone by cell-free preparations from *P lilacinum*.

Experiment	Metal added	Mol-% total side chain cleavage	Mol-% 20(α+β)-reduction
9[a]	None	17.0	5.3
9	10^{-3} M Hg^{2+}	n.s.	n.s.
10[a]	None	14.5	13.9
10	10^{-3} M Fe^{2+}	14.5	10.8
10	10^{-3} M Fe^{3+}	12.4	11.0
10	10^{-3} M Ni^{2+}	10.5	13.8
10	10^{-3} M Co^{2+}	11.2	8.9
10	10^{-3} M Mn^{2+}	14.2	10.8
10	10^{-3} M Cu^{2+}	1.5	3.4
10	10^{-3} M Zn^{2+}	3.3	5.5
11[b]	None	5.8	12.3
11	10^{-4} M Fe^{2+}	6.2	11.4
11	10^{-3} M Fe^{2+}	8.2	10.1
11	10^{-3} M Fe^{3+}	6.1	11.0
11	10^{-3} M Ni^{2+}	3.4	8.0
11	10^{-3} M Co^{2+}	5.7	7.5
11	10^{-3} M Mn^{2+}	6.9	8.0

[a] Crude 100 000 *g* supernatant. [b] Gel filtrated 100 000 *g* supernatant. Initial concentrations of progesterone 2.66×10^{-4} M and of exogenous NADPH 1.21×10^{-3} M.

Table 7. Effect of inhibitors on the transformation of progesterone by cell-free preparations of *P. lilacinum*.

Experiment	Inhibitor (10^{-3} M)	Mol % total side chain cleavage	% inhibition of side chain cleavage	Mol % 20 ($\alpha+\beta$)-reduction	% inhibition of 20($\alpha+\beta$)-reduction
4	None	17.9	–	19.8	–
4	Ferron	3.5	80	8.3	58
4	1,10-phenantroline	9.7	46	17.0	n.s.
4	Phenazine metosulphate	n.s.	>99	2.2	89
4	10^{-3} M KCN	13.3	26	11.9	40
12	None	12.9	–	15.2	–
12	Ferron	8.4	35	9.8	35
12	*p*-Hydroxymercuribenzoate	2.0	85	6.2	59
12	Methylene blue	6.2	51	4.2	72
13	4 % O_2+96 % N_2	13.3	–	18.3	–
13	40 % CO+4 % O_2+56 % N_2	14.2	n.s.	14.6	21

10^{-3}M concentrations of KCN, NaN_3, NaF, EDTA, and 8-hydroxyquinoline had no significant effects on side chain cleavage or 20-reduction. Crude 100 000 *g* supernatants were used. Initial concentration of progesterone was 2.66×10^{-4}M and of exogenous NADPH 1.10×10^{-3} M. The incubations in experiment 13 were performed in darkness.

Zn^{2+} and to a lesser degree also by Ni^{2+} (Table 6). Fe^{2+} slightly stimulated the activity in extracts purified by gel filtration.

The 20($\alpha+\beta$)-reduction was strongly inhibited by Hg^{2+}, Cu^{2+} and Zn^{2+}. A slight suppression of the activity was achieved by Ni^{2+}, Co^{2+} and Mn^{2+} ions.

Effects of inhibitors. The enzyme preparation was preincubated with inhibitors 15 min before the addition of steroid and NADPH. The effects of inhibitors are shown in Table 7. The C_{17-20}-lyase activity was insensitive towards CO and 10^{-3} M concentrations of CN^-, N_3^-, and F^-. Methylene blue, phenazine metosulphate, and *p*-hydroxymercuri benzoate were strong inhibitors. Ferron and 1,10-phenantroline had a significant inhibitory effect whereas EDTA and 8-hydroxyquinoline were inactive.

The reduction at C-20 was inhibited by 10^{-3} M CN^- and by ferron, phenazine metosulphate, methylene blue, and *p*-hydroxymercuri benzoate, whereas the other inhibitors tested were without effects.

DISCUSSION

Progesterone and 17α-hydroxyprogesterone but not 17α-acetoxyprogesterone were substrates for the side chain cleavage in *P. lilacinum*. Side chain splitting enzymes from microorganisms in general have a very broad substrate specificity. It has previously been reported that whole cells of *P. lilacinum* split the side chain from different 20-oxosteroids in the 5α-pregnane, 5β-pregnane, 4-pregnene, 5-pregnene and 1,4-pregnadiene series.[11,23]

Crude 100 000 *g* supernatants contained endogenous cofactors sufficient for a limited degree of side chain cleavage, and addition of exogenous NADPH or NADH further increased the activity. In contrast to crude extracts, extracts purified by gel filtration showed an absolute requirement for NADPH. The stimulatory effect of NADH in crude extracts (see also paper I in this series [19]) might be explained by hydrogen transfer to endogenous $NADP^+$, thereby increasing the NADPH levels. The occurrence of transhydrogenases in microorganisms is well known.[24] It should be mentioned that stimulation by NADH due to transhydrogenation has been reported for the side chain cleavage of cholesterol by adrenal mitochondria.[25]

From a wealth of information on microbial and mammalian oxygenases it might be assumed that flavin participates in the side chain cleavage in *P. lilacinum*, at least in the oxidation of NADPH. In accordance with this assumption FMN caused a slight increase in the stimulatory effect of NADPH. It should be mentioned that participation of FMN in the oxidation of NADH as well as in the oxygenation of the substrate has also been established in the NADH-de-

pendent lactonization of camphor by highly purified enzymes from *Pseudomonas putida*.[6]

An iron-sulphur protein might also participate in the side chain cleavage studied. Thus heavy metals known to combine with the sulphur moiety as well as substances reacting with Fe^{2+} inhibited the reaction. Ni^{2+} was also inhibitory, which might be due to a similarity in the ionic radii of Ni^{2+} and Fe^{2+}.[26] A similar effect can be expected from Co^{2+} and in fact inhibition by Co^{2+} was observed in one experiment. Addition of Fe^{2+} slightly stimulated the side chain cleavage in purified extracts but was ineffective in crude 100 000 *g* supernatants. It is noteworthy that the effects of Fe^{2+} and Fe-reacting agents differ from those presented for the ring D lactonization in *P. lilacinum* enzyme preparations and the side chain cleavage in *C. radicicola*.[7,8] In those studies Fe^{2+} and 1,10-phenantroline were inactive. An explanation for the discrepancy might be found in the higher degree of purity of the enzymes used in those studies. Early studies by Gunsalus and co-workers indicated the participation of a non-heme iron protein in the camphor lactonization.[3] Thus addition of Fe^{2+} generally stimulated and bipyridine inhibited the reaction. The effect of Fe^{2+} was, however, less reproducible. In a later study using a reconstituted, extensively purified system devoid of iron, added Fe^{2+} and bipyridine were without effect.[6] The lack of effect of Fe^{2+} in the purified system could be due to the "ferredoxin-flavodoxin replacement phenomenon", *i.e.* replacement of the non-heme iron protein in the electron transport chain by an iron-free flavoprotein such as flavodoxin.[6,27]

Cytochrome P-450 has been shown to be the terminal oxidase protein in the mammalian 20-oxopregnane side chain cleavage as well as in a large number of mammalian and microbial hydroxylations.[28–30] However, the insensitivity to heme-reacting inhibitors excludes participation of cytochromes in the side chain cleavage of *P. lilacinum*. In the camphor lactonizing system no heme-proteins have been found and the reaction catalyzed by this system was insensitive to CO, CN^- and N_3^-.[3] Insensitivity to CN^- and N_3^- has also been demonstrated for 20-oxopregnane side chain cleavage by whole cells of *Nocardia restrictus*[31] and by enzyme preparations from *C. radicicola*.[8] D-Ring lactonization in enzyme preparations from *P. lilacinum*[7] and lactonization of cyclohexanone in cell-free extracts from *Nocardia opaca*[32] are also known to be insensitive towards CN^- and N_3^-. Obviously the "biochemical Baeyer-Villiger oxidations" belong to a class of oxygenases which do not utilize cytochromes as terminal oxidase.

The side chain cleavage occurred with a concomitant and efficient reduction in the C-20 position. Crude 100 000 *g* supernatants contained endogenous cofactors sufficient for a limited degree of side chain cleavage but not for a simultaneous 20-reduction (Table 4, see also paper I in this series[19]). Addition of exogenous NADH of NADPH increased the side chain cleavage and simultaneously 20-reduced metabolites appeared. Initial exogenous cofactor levels higher than those necessary for a maximal side chain cleavage did not affect that reaction but further stimulated the 20-reduction. In the early stages of incubations excess of NADPH was present and the amounts of 20-alcohols and C_{19} steroids increased simultaneously (Fig. 2). When a larger part of the NADPH was oxidized, the concentration of 20-alcohols levelled off and later even decreased while the C_{19} steroid concentration still increased. El-Tayeb and co-workers suggested a competition between C_{17-20}-lyase and 20-oxidoreductase enzymes for the 20-oxo-C_{21} steroids.[16,17] The results given above show that the level of reduced pyridine nucleotide is an important limiting factor for the 20-reduction. With an excess of NADPH or NADH also "good" lyase substrates such as progesterone yield considerable amounts of 20-alcohols together with the side chain cleavage products.

Purified microbial 20-oxidoreductases are known to possess a pronounced pyridine nucleotide specificity. NADPH is the specific cofactor for the 20α-oxidoreductase in *Actinomyces roseochromogenus* and the 20β-oxidoreductase in *Curvularia lunata*, while the crystallized 20β-oxidoreductase from *Streptomyces hydrogenans* is NADH-specific.[33–35] The 20(α + β)-reduction in *P. lilacinum* was more effeciently stimulated by NADH than NADPH. NADH favoured formation of the 20β-epimer. Interestingly clomiphene citrate selectively inhibited the 20α-reduction.

Acknowledgement. The GC-MS analysis was carried out at the Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden.

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Received March 7, 1974.