STEROID 1-DEHYDROGENASE OF *NOCARDIA RESTRICTUS*

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SUMMARY

This paper describes in detail the properties of the steroid 1-dehydrogenase from *Nocardia restrictus*. The 1-dehydrogenase of this organism is an adaptive enzyme associated with intracellular particles. It has been partially purified and separated from 4(5α)-dehydrogenase. This 1-dehydrogenase can introduce double bonds into 11-oxygenated steroids; it exhibits significant enzymic activity at a neutral pH and it can utilize several oxidation–reduction dyes as electron acceptors. These properties differ from those of the 1-dehydrogenase of *Pseudomonas testosteroni*.

Evidence is presented that the 1-dehydrogenase contains a flavin prosthetic group which catalyzes a direct dehydrogenation of the steroid; the participation of hydroxylated intermediates is unlikely.

INTRODUCTION

The introduction of 1,2-double bonds into steroids attracted considerable attention since the 1-dehydro derivatives of cortisone and cortisol, prednisone and prednisolone, showed increased antirheumatic and antiallergic activity and produced less undesirable side effects[1].

Several methods have been used for the preparation of 1-dehydro steroids: selenium dioxide[2], 2,3-dichloro-5,6-dicyanobenzoquinone[3], and microbiological methods[4]. Previous studies in this laboratory have shown that cell-free extracts of *Nocardia restrictus* were capable of introducing double bonds into the 1,2 and 4,5 positions of steroids possessing either the 5α-pregnane or 5β-pregnane nucleus[5]. This observation thus provided an enzymic method for the introduction of 1,2 double bonds into steroids. In the present communication, we wish to describe the properties of 1-dehydrogenase from this organism, so that optimum conditions for the preparation of 1-dehydro steroids may be known.

EXPERIMENTAL

Materials and methods

DPN, TPN, FMN, FAD, *p*-hydroxymercuribenzoate, and cytochrome *c* were obtained from Sigma Chemical Company. Phenazine methosulfate was obtained from the Aldrich Chemical Company. All steroids used (from the Squibb stock collection)

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had been recrystallized twice. Whatman No. 1 paper from H. Reeve Angel and Co. was used for paper chromatography. Celite (diatomaceous earth) obtained from the Johns-Manville Company was acid washed before use. All solvents used were redistilled. Protein was determined by the method of GORNALL *et al.*[6]. Calcium phosphate gel was prepared by the method of TSUBOI AND HUDSON[7]. The 3-keto-4-dehydrosteroids from the column eluates were followed by reacting an aliquot with the arsenomolybdate reagent of NELSON[8] with 30 min heating.

Preparation of cell-free extracts

The organism was grown on the medium previously described[5] with the addition of progesterone (200 μg/ml), dissolved in dimethylformamide. After 48 h of growth, (stationary phase of the growth curve), the cells were harvested by centrifugation at 3000 $\times$ *g* for 10 min and washed with cold 0.03 *M* phosphate buffer pH 7.0. Cell-free extracts were prepared by placing a cell suspension in the sonic field of a 10 kc magnetostrictive oscillator for 20 min. The cell debris was removed by centrifugation at 3000 $\times$ *g* for 10 min. The supernatant was decanted and used directly as the source of crude enzyme or it was further purified by ammonium sulfate fractionation.

RESULTS

Assay of steroid dehydrogenases

Three assay methods were used throughout this work; each possesses advantages depending on the reaction system involved.

Paper-chromatographic assay

The reaction mixture consisted of 1 mg of progesterone, 500 μg of phenazine methosulfate, and various concentrations of the enzyme, in 5 ml of 0.03 *M* phosphate buffer pH 7.0. After 10 min, the reaction was terminated by the addition of 1 ml of methylisobutylketone. The two layers were thoroughly mixed and 0.1 ml of the solvent layer was spotted on Whatman No. 1 filter paper (which had been washed twice in 95% ethanol) and developed for 16 h in a methylcyclohexane–diethylene glycol monoethyl ether system[9]. The steroids were eluted from the paper with 95% ethanol and the absorption at 240 mμ was measured in a D.U. Spectrophotometer. This method was used when there were colored substances in the reaction mixture which interfered with spectrophotometric assays.

Indophenol method

Since the 1-dehydrogenase can utilize oxidation–reduction dyes such as 2,6-dichlorophenolindophenol as electron acceptors, the reduction of this dye can be followed spectrophotometrically at 600 mμ as a measurement of enzyme concentration. The assay system consisted of 30 μg of 2,6-dichlorophenol, 100 μg of testosterone and various concentrations of enzyme in a total volume of 3.0 ml of 0.03 *M* phosphate buffer pH 7.0. Under these conditions the amount of enzyme which initiates the decrease of 0.01 absorbancy unit/min is defined as 1 indophenol unit. The advantage of this assay is its simplicity and its specificity for 1-dehydrogenase under these conditions. 2,6-Dichlorophenolindophenol can also act as an electron acceptor for 4(5α)-dehydrogenase, but only at a rate considerably slower than 1-dehydrogenase. Therefore, this assay is unsuitable for 4(5α)-dehydrogenase.

Phenazine-cytochrome c method

Among the artificial electron acceptors tested, phenazine methosulfate was the best one for the 1-dehydrogenase. Since reduced phenazine methosulfate has higher affinity for mammalian cytochrome *c* than molecular oxygen[10], the change in absorption at 550 mμ (characteristic of the reduced cytochrome *c*) can be used as the measure of enzyme concentration. These reactions can be summarized as follows:

Testosterone	+	Phenazine methosulfate	⟶	Reduced phenazine methosulfate	+	1-Dehydro-testosterone
Reduced phenazine methosulfate	+	2 Cytochrome *c*	⟶	Phenazine methosulfate	+	2 Reduced cytochrome *c*
Testosterone	+	2 Cytochrome *c*	⟶	1-Dehydro-testosterone	+	Reduced 2 cytochrome *c*

The assay system consisted of 2 mg of cytochrome *c*, 100 μg of testosterone, and various concentrations of enzyme in a total volume of 3.0 ml of 0.03 *M* phosphate buffer pH 7.0. The reaction was initiated by the addition of 0.1 ml of a 0.1% phenazine methosulfate solution. Under these conditions, 1 unit is defined as the amount of enzyme which will initiate the change of 0.01 absorbancy unit/min and it is equivalent to 0.5 indophenol unit. The advantage of this method is that the reaction can be used for both the 1-dehydrogenase and the 4(5α)-dehydrogenase (using 1-androstene-3,17-dione in place of testosterone) and can be carried out either aerobically or anaerobically. Also, higher concentrations of phenazine methosulfate can be used and this method eliminates the direct measurement of phenazine methosulfate, which is only slightly soluble when reduced and may cause turbidity problems.

Induction of the 1-dehydrogenase

Preliminary experiments were designed to determine conditions which would yield an enzyme extract with the highest specific activity. Using 5β-pregnane-3,20-dione as the inducer, it was found that maximum enzyme production occurred between 24 and 48 h (log phase of the growth curve) under our conditions. Table I shows that in the presence of an added inducer, the 1-dehydrogenase activity increased about 8-fold, indicating the adaptive nature of this enzyme. Among the steroids tested, as shown in Table II, progesterone appeared to act best as inducer, whereas testosterone and androstenedione were relatively poor inducers of the 1-dehydrogenase, even though the latter steroids were metabolized more rapidly than progesterone. The optimum concentration of progesterone as the inducer was between 100–300 μg/ml of medium; at 500 μg/ml, the enzyme activity dropped off considerably from 125 to 25 indophenol units/mg of protein.

Purification of the 1-dehydrogenase

The 1-dehydrogenase was apparently associated with both small and large particles within the cell. When the cells were ruptured by grinding with levigated alumina, approx. 50% of the enzyme activity could be sedimented by centrifuging the crude enzyme extract at 144000 × *g* for 30 min. If centrifugation was continued for 2 more hours, 90% of the enzyme activity was sedimented. Several methods for the solubilization of particular enzymes were tried such as the use of solvent fractionation[11,12], and heating at 60° for various time intervals but no purification was

achieved by these methods. However, when the cells were ruptured by sonic oscillation, partial solubilization of the enzyme was obtained which could then be fractionated with ammonium sulfate.

TABLE I

INDUCTION OF THE 1-DEHYDROGENASE

The cells were grown as described with 200 μg of pregnanedione added/ml of medium as the inducer; the cells were then ruptured sonically and the enzyme extract was assayed by the indophenol method.

Time (h)	*Indophenol units/mg of Protein*	
	No steroid	*With steroid*
16	11	106
24	17	142
48	15	140
72	9	125
96	2	90

All of the following purification procedures were carried out at 4°. Dilute NH_4OH was added to maintain the pH at neutrality during the ammonium sulfate fractionation. By direct fractionation of the initial crude extract with solid ammonium sulfate,

TABLE II

STEROID INDUCERS OF THE 1-DEHYDROGENASE

The cells were grown for 48 h in the presence of $6.5 \cdot 10^{-4}$ *M* steroid inducer. The enzyme extract was assayed by the indophenol method as described in the text using testosterone as the substrate.

Inducer	*Specific activity (indophenol units/mg of protein)*
None	15
4-Androstene-3,17-dione	36
1,4-Androstadiene-3,17-dione	55
Testosterone	18
17α-Ethinyl-19-nortestosterone	15
Cyclohexanone	6
5β-Pregnane-3,20-dione	120
Progesterone	130
16-Dehydroprogesterone	110
1-Dehydroprogesterone	24
Pregnenolone	76
Cholestenone	15

a tenfold purification over the initial extract was obtained. As shown in Table III, the fraction between 50 to 70% saturation had a specific activity of 1230 indophenol units as compared to 122 indophenol units for the initial extract. This purified fraction had an activity ratio for 1-dehydrogenase to 4(5α)-dehydrogenase 17 to 1 as compared to 1 to 1 with the initial crude extract. Further purification was not achieved by means of adsorption on calcium phosphate gel although the ratio of 1-dehydrogenase activity to 4(5α)-dehydrogenase activity was further increased to 25, as the major portion of the 1-dehydrogenase activity was preferentially eluted with 10^{-4} *M* phosphate buffer pH 7.0.

TABLE III

PURIFICATION OF THE 1-DEHYDROGENASE

The enzyme activity was assayed by the indophenol method.

Initial extract	*Total units* 160 000	*mg of protein* 1300	*Specific activity* 122
$(NH_4)_2SO_4$ 0–40%	42000	510	82
$(NH_4)_2SO_4$ 40–50%	18000	65	280
$(NH_4)_2SO_4$ 50–70%	44400	36	1230

Effect of pH

As shown in Fig. 1, the 1-dehydrogenase exhibited maximum activity at an alkaline pH with either phenazine methosulfate or 2,6-dichlorophenolindophenol as the electron acceptor. Optimum pH for the 1-dehydrogenase was between 9.0 and 9.5. At pH 7.0, the 1-dehydrogenase had about 50% of the maximum activity. The paper-chromatographic method was used in obtaining these values as the extinction coefficient of 2,6-dichlorophenolindophenol varies at different pH values and at alkaline pH cytochromes *c* becomes denatured.

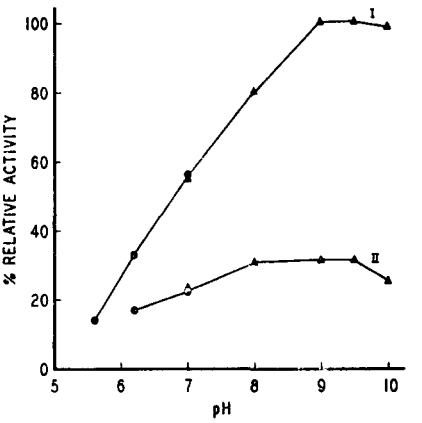

Fig. 1. Effect of pH on 1-dehydrogenase activity. The paper-chromatographic method was used to determine the activities as described in the text. I, Phenazine methosulfate; II, 2,6-Dichlorophenolindophenol. ●—●, 0.03 *M* Phosphate; ▲—▲, 0.03 *M* Tris buffer.

Substrate specificity

The reactivity of a variety of steroids were examined with partially purified preparations of 1-dehydrogenase. From preliminary kinetic studies a Michaelis constant of $1.4 \cdot 10^{-5}$ *M* was obtained for the interaction of testosterone and 1-dehydrogenase at pH 7.0 in 0.03 *M* phosphate buffer as assayed by the phenazine–cytochrome *c* method. Maximal activity was attained at a concentration of about $1 \cdot 10^{-4}$ *M*; at higher steroid concentrations, some inhibition was observed. Several conclusions can be drawn with regard to the effect of structure on activity as shown in Table IV. (a) A 3-ketone group appears to be essential for activity as no activity was observed with 4-pregnene-3β-ol-17-one or the 5α-androstene-3α-ol-17-one; (b) no activity was noted on cyclohexanone which suggests that adjacent ring systems are required for activity; (c) replacing the C_{10}-methyl group by a hydrogen decreased the dehydroge-

nase activity as 19-nortestosterone was oxidized at about 40% of the rate of testosterone; (d) the presence of a β-hydroxyl or a ketone group at the 11 position appears to reduce the rate of enzyme activity as cortisone and cortisol had only 65% and 35% the activity of cortexolone; (e) the 11α-hydroxyl does not appear to interfere with activity.

TABLE IV

SUBSTRATE SPECIFICITY OF THE 1-DEHYDROGENASE

The enzyme preparation used had a specific activity of 1230 indophenol units/mg of protein. Both the indophenol method and the phenazine–cytochrome *c* method were used and the results agree.

Steroid	*Percent relative activity*
Testosterone	100
Androsterone	0
4-Androstene-3,17-dione	86
19-Nortestosterone	43
16α-Hydroxyandrostenedione	86
Progesterone	72
1-Dehydroprogesterone	0
Pregnenolone	0
9(11)-Dehydroprogesterone	72
11α-Hydroxyprogesterone	79
17α-Hydroxyprogesterone	93
Cortexolone	86
Cortexone	86
Cortisone	57
Cortisol	29
Corticosterone	21
9α-Fluorohydroxycortisone	14
9α-Fluoro-16α-hydroxyhydrocortisone	36
Cholestenone	29
Cyclohexanone	0

The 1-dehydrogenase of *N. restrictus* is different from the 1-dehydrogenase of *Pseudomonas testosteroni* since the latter does not appear to be able to introduce a 1,2 double bond into 11β-hydroxylated steroids at any significant rate. 100 mg of 9α-fluoro-16α-hydroxyhydrocortisone was incubated with 50 mg of phenazine methosulfate and 50 ml of crude enzyme (12 mg protein/ml) in a total volume of 200 ml of 0.03 *M* phosphate buffer pH 7.0. After 12 h of incubation at 25° the reaction mixture was acidified, phenazine methosulfate was removed by the addition of Dowex-50 resin (H^+ form) and protein precipitate was removed by filtration. The steroids were then extracted into methylisobutylketone and concentrated; they were then chromatographed on a celite column according to the method of SMITH *et al.*[13]. As shown in Fig. 2, approx. 70% of 9α-fluoro-16α-hydroxyhydrocortisone was converted into 9α-fluoro-16α-hydroxyprednisolone. The latter compound was crystallized from ethanol and found to have an infrared spectrum identical to that of an authentic sample. Similar experiments were carried out using cortisone and cortisol as substrates; 1-dehydrocortisone and 1-dehydrocortisol were isolated and identified from their infrared spectra, which were identical to authentic samples. These experiments show not only the versatility of the 1-dehydrogenase of this organism but demonstrate also that the enzyme can introduce 1,2 double bonds into a variety of 11β-hydroxylated steroids.

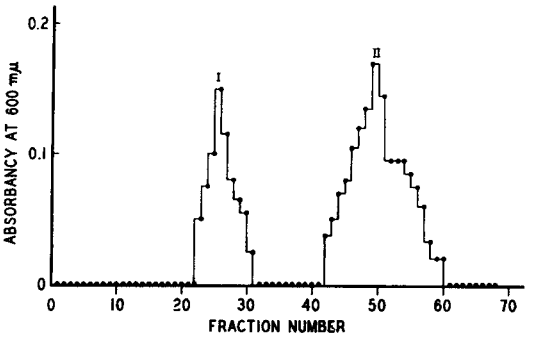

Fig. 2. Separation of 9α-fluoro-16α-hydroxyhydrocortisone and 9α-fluoro-16α-hydroxyprednisolone Adsorbent, 25 g of celite; the solvent mixture was dioxane–cyclohexane–water. The flow rate was 5 ml/min and each fraction contained 12.5 ml. Absorbancy at 600 mμ is plotted on the ordinate for each effluent fraction after treatment with arsenomolybdate reagent. I, 9α-fluoro-16α-hydroxyhydrocortisone; II, 9α-fluoro-16α-hydroxyprednisolone.

Electron acceptors

Among the artificial electron acceptors tested, only phenazine methosulfate, 2,6-dichlorophenolindophenol and resazurin exhibited high activity when added to the substrate and enzyme[5]; methylene blue was a poor electron acceptor, active only in very crude preparations; activity using ferricyanide as an electron acceptor was negligible. Thus, this 1-dehydrogenase appears to be able to use both 1- and 2-electron acceptors, and differs from the enzyme of *P. testosteroni*, for which 2,6-dichlorophenolindophenol was inactive. A number of natural electron acceptors or hydrogen carriers were tested and found to be inactive; these include FMN, FAD, DPN, TPN, mammalian cytochrome *c* and coenzyme Q_{10}.

Nature of the prothetic group

This enzyme was inhibited (30%) by acriflavin (concn. of $8.35 \cdot 10^{-4}$ *M*) and the inhibition was completely reversed by the addition of either FMN or FAD (Table V). This experiment indicates that the prothetic group of this enzyme may be a flavin although its real nature remains to be determined. Also, the fact that this enzyme can utilize either phenazine methosulfate or 2,6-dichlorophenolindophenol as electron acceptor further suggests that it is a flavoprotein since this is the only class of enzymes that have been reported to possess this property.

Inhibitors

The 1-dehydrogenase activity was inhibited by heavy metals, especially Hg and Cu salts, whereas Zn salts were less toxic. It was also inhibited by *p*-hydroxymercuribenzoate (43%) and iodoacetate (50%) at a level of $1 \cdot 10^{-3}$ *M* (Table VI). These results strongly suggest that the enzyme possesses a reactive sulfhydryl group. This property is consistent with other flavoproteins; for example, DPNH–cytochrome *c* reductase is also inhibited by sulfhydryl inhibitors[14].

Enzymic determination of steroids

TALALAY AND MARCUS[15] used the hydroxysteroid dehydrogenases to estimate steroids by measuring the reduced DPN formed at 340 mμ. The indophenol and phenazine–cytochrome *c* enzyme assays can also be used to assay steroids. As shown in Fig. 3, a linear relationship exists between the equilibrium absorption at 550 mμ and concentration of testosterone. Also, the change in absorption at 600 mμ in the indo-

TABLE V

REVERSAL OF ACRIFLAVIN INHIBITION BY FLAVIN NUCLEOTIDES

The reaction system contained 3.0 μmoles of progesterone, 500 μg of phenazine methosulfate, 0.5 ml of enzyme (specific activity of 1230 indophenol units/0.1 ml) in a total volume of 5.0 ml of 0.03 *M* phosphate buffer pH 7.0. Acriflavin was added to a final concentration of $8.35 \cdot 10^{-4}$ *M*; the concentrations of flavin nucleotides are given in the table. The 1-dehydroprogesterone was assayed by the paper-chromatographic method after the reaction system had been incubated for 10 min at 25°.

Additions	*μmoles of 1-dehydroprogesterone formed*
None	1.97
Acriflavin ($8.35 \cdot 10^{-4}$ *M*)	1.40
Acriflavin + FMN ($8.35 \cdot 10^{-5}$ *M*)	1.85
Acriflavin + FMN ($4.16 \cdot 10^{-4}$ *M*)	2.10
Acriflavin + FAD ($8.35 \cdot 10^{-5}$ *M*)	1.75
Acriflavin + FAD ($4.16 \cdot 10^{-4}$ *M*)	2.10

TABLE VI

EFFECT OF INHIBITORS ON 1-DEHYDROGENASE ACTIVITY

Both the indophenol and phenazine–cytochrome *c* assay methods were used; the results were similar. The enzyme preparation used had a specific activity of 500 indophenol units/mg of protein. 10 units of the enzyme was used with each determination. The final concentration of inhibitors was $1 \cdot 10^{-3}$ *M*.

Inhibitor	*Percent inhibition*
KCN	0
NaN_3	0
$HgCl_2$	100
$CuSO_4$	100
$ZnSO_4$	50
EDTA	0
$NaAsO_2$	0
Iodoacetate	43
Antimycin A (50 μg/ml)	0
p-Hydroxymercuribenzoate	50

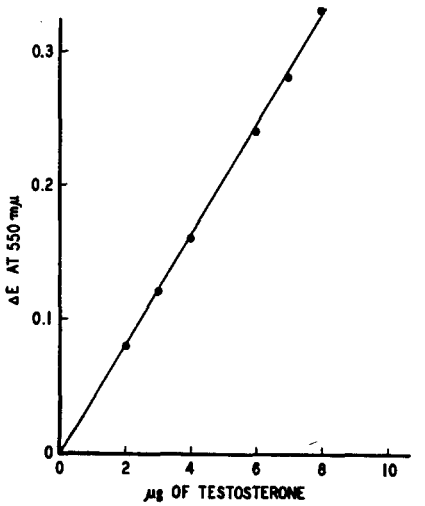

Fig. 3. Enzymic assay of testosterone by the phenazine–cytochrome *c* method.

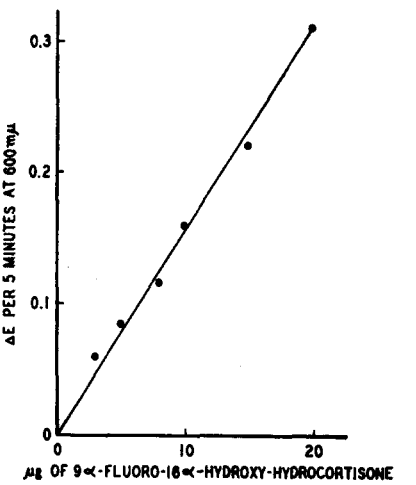

Fig. 4. Enzymic assay of 9α-fluoro-16α-hydroxy-hydrocortisone by the indophenol method.

phenol reaction can be used to assay steroids such as 9α-fluoro-16α-hydroxyhydrocortisone (Fig. 4). However, the disadvantage of these assays is that the 1-dehydrosteroids appear to be inhibitors of the enzyme; inhibition is shown by the fact that in the presence of equal concentrations of progesterone and 1-dehydroprogesterone, no linear relationship could be shown. Thus, these enzymic assays are restricted to systems virtually free of 1-dehydrosteroids.

DISCUSSION

Our findings show that the 1-dehydrogenase of this species of *Nocardia* possesses some properties similar to the 1-dehydrogenase found in *Pseudomonas testosteroni*. The 1-dehydrogenases of both organisms are adaptive enzymes associated with intracellular particles; they are sensitive to sulfhydryl inhibitors, capable of utilizing phenazine methosulfate as an electron acceptor and have an alkaline pH optimum. Both enzymes are inhibited by acriflavin and the inhibition is reversed by FAD. There are, however, three main features which differentiate the 1-dehydrogenase of these two organisms. Not only can the 1-dehydrogenase of *N. restrictus* introduce a 1,2 double bond into 11-oxygenated steroids, but it can also utilize oxidation–reduction dyes as electron acceptors. The enzyme of *P. testosteroni* does not possess these properties. Also, the 1-dehydrogenase of *N. restrictus* exhibits high activity at pH 7.0, the pH at which 1-dehydrogenase activity in *P. testosteroni* is negligible.

The 1-dehydrogenation reaction does not appear to be a hydroxylation followed by a dehydration as the reaction can be carried out anaerobically. 1α- and 1β-hydroxysteroids[16] and 2α-hydroxysteroids[17] have already been ruled out as intermediates of 1-dehydrogenation. A more probable mechanism is a direct dehydrogenation with the participation of a flavin prosthetic group in a manner similar to that with other known flavoproteins[10,18,19].

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