Enzymic dehydrogenation of steroid A ring

Cell-free extracts of a *Nocardia sp.** have been found capable of introducing double bonds into the 1,2- and 4,5-positions of steroids possessing either the 5β-pregnane or 5α-pregnane nucleus.

Cells of this organism were grown on the following medium: corn steep liquor, 0.6 %; $NH_4H_2PO_4$, 0.3 %; $CaCO_3$, 0.25 %; soybean oil, 0.22 %; yeast extract, 0.25 % and glucose, 1 %. After 72-h growth, the cells were harvested by centrifugation and washed with cold 0.03 *M* phosphate buffer (pH 7.0). Cell-free extracts were prepared by placing a suspension of cells in the sonic field of a Raytheon 10 kc magnetostrictive oscillator for 30 min. The cell debris was removed by centrifugation at 100,000 $\times$ *g* for 30 min; the supernatant containing about 6 mg protein/ml was used as the source of enzymes.

In the presence of a suitable electron acceptor such as phenazine methosulfate, the cell-free extracts of this organism carried out efficient conversions of pregnane-3,20-dione, 5α-pregnane-3,20-dione and progesterone to $\Delta^{1,4}$-pregnadiene-3,20-dione. In a typical experiment, 200 mg pregnane-3,20-dione in 5 ml dimethyl formamide were incubated at 25° with 150 mg phenazine methosulfate and 200 ml of enzyme solution in a total volume of 400 ml in 0.03 *M* phosphate buffer, pH 7.0. After 10-h incubation, the reaction mixture was extracted with methyl isobutyl ketone and the extract chromatographed on a cellulose-powder column (1.6·20 cm) using the hexane-propylene glycol system; 3.0-ml fractions were collected every 2.5 min. The Δ^4-3 keto-steroids were detected using the arsenomolybdate reagent of NELSON[1] with 30-min heating. Fractions 6–15 were pooled and twice rechromatographed to obtain fractions free of phenazine methosulfate. Crystallization from hexane gave about 5 mg of a substance, m.p. 122° and 129°; λ_{max} (ethanol), 241 mμ (Σ, 16,500) and an infrared spectrum identical with that of an authentic sample of progesterone. Mixed chromatography with progesterone showed a single spot. Fractions 18–25 were also pooled and crystallization from ethyl acetate-hexane afforded 90 mg of a substance m.p. 150–152°; λ_{max} (ethanol), 244 mμ (Σ, 16,500). Its infrared spectrum in Nujol exhibited bands at 5.80 μ (20 ketone), 6.04, 6.17 and 6.26 μ ($\Delta^{1,4}$, 3-ketone) identical with that of an authentic sample of pregna-1,4-diene-3,20-dione. On mixed paper chromatography, the compound had the same mobility as pregna-1,4-diene-3,20-dione.

Similar experiments were carried out with progesterone and α-pregnane-3,20-dione; in each case pregna-1,4-diene-3,20-dione was isolated and identified as described. From the evidence obtained, it appears that this organism probably contains at least 5β- and 5α-Δ^4-steroid dehydrogenases as well as a Δ^1-steroid dehydrogenase similar to the system in *Pseudonomas testosteroni* as reported by LEVY AND TALALAY[2].

A convenient and rapid method of demonstrating the enzymic activity of the Δ^1-steroid dehydrogenase is shown in Fig. 1. A reaction mixture of a Δ^4-3-oxosteroid and 2,6-dichlorophenol indophenol was incubated with the cell-free extract in a 3 ml cuvette at room temperature and the absorbancy at 600 mμ was measured with a Beckman D.U. spectrophotometer. Fig. 1 shows the rapid decrease in absorbancy by the steroid–dye mixture. Di- and triphosphopyridine nucleotides, mammalian

* This organism was isolated from a sample of corn silage by Dr. A. LASKIN of our laboratory using cholesterol as the sole source of carbon.

cytochrome *c* and coenzyme Q_{10} did not act as hydrogen or electron acceptors for the Δ^1-steroid dehydrogenase.

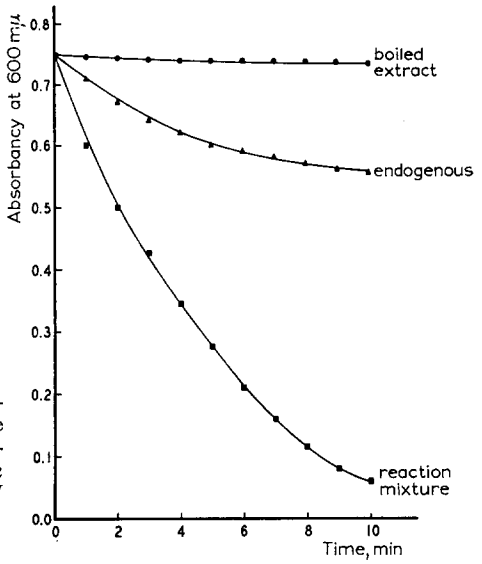

Fig. 1. The reduction of 2,6-dichlorophenol indophenol by the Δ^1-steroid dehydrogenase. The reaction mixture contained 60 μg 2,6-dichlorophenol indophenol, 100 μg progesterone and 0.2 ml enzyme in a total volume 3.0 ml in 0.03 *M* phosphate buffer, pH 7.0.

TABLE I

REACTION VELOCITIES OF Δ^1-STEROID DEHYDROGENASE WITH VARIOUS ELECTRON ACCEPTORS

The cells were broken sonically as described followed by centrifugation at 2000 × *g* for 10 min. The supernatant containing 20 mg protein/ml was the source of enzyme. The assay system consisted of 1 mg progesterone in 0.1 ml dimethyl formamide, 1 ml of enzyme solution and various concentrations of different electron acceptors in a total volume of 5.0 ml 0.03 *M* phosphate buffer, pH 7.0. After 10 min, the reaction was terminated by extraction with 1 ml methyl isobutyl ketone and chromatographed on Whatman No. 1 paper; developed in the methyl cyclohexane-carbitol system. Pregna-1,4-diene-3,20-dione was eluted with 95% ethanol and determined by absorbancy at 240 mμ. The values given here are relative maximum velocities.

Electron acceptor	*Activity* %
Phenazine methosulfate	100
2,6-dichlorophenol indophenol	50
Resazurin	40
Methylene blue	< 20
Ferricyanide	< 20

Table I shows the relative maximum velocities (V_{max}) determined by the method of LINEWEAVER AND BURK[3] of a few electron acceptors with a crude enzyme preparation. Phenazine methosulfate seems to act as the best electron acceptor whereas methylene blue and ferricyanide are poor electron acceptors for the Δ^1-steroid dehydrogenase.

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