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17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CANINE PANCREAS

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Summary: The mitochondrial fraction of the dog pancreas showed NAD(H)-dependent enzyme activity of 17β-hydroxysteroid dehydrogenase. The enzyme catalyzes oxidoreduction between androstenedione and testosterone. The apparent Km value of the enzyme for androstenedione was 9.5 ± 0.9 μM, the apparent Vmax was determined as 0.4 nmol mg^{-1} min^{-1}, and the optimal pH was 6.5. In phosphate buffer, pH 7.0, maximal rate of androstenedione reduction was observed at 37°C. The oxidation of testosterone by the enzyme proceeded at the same rate as the reduction of the androstenedione at a pH of 6.8-7.0. The apparent Km value and the optimal pH of the enzyme for testosterone were 3.5 ± 0.5 μM and 7.5, respectively.

Over the last few years a number of interesting observations have provided evidence suggesting the involvement of the pancreas in the biosynthesis and metabolism of steroid hormones. Estrogen binding globulins and steroid receptors have been reported in normal and malignant pancreatic tissue (1-4). Aromatase and 5α-reductase activity has been measured in cell-free homogenates of human pancreatic tissue (5). Several changes in the serum androgen profile, mainly low testosterone levels, have been found to be related to human pancreatic carcinoma (6-8), and recent studies on experimental pancreatic carcinoma in rat suggested that this tumor could be hormone-responsive (9,10). Despite the importance of steroid metabolism in the pancreas, few studies have been performed and little information on the pancreas ability to synthesize steroids is available.

To obtain more information on the steroidal functions of the pancreas, it appeared of interest to study the enzymatic activ-

Abbreviations: EDTA, ethylene diamine N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; (SE), standard error; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-en-3-one.

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ities involved in the metabolic transformations of steroid hormones in this gland. The present paper reports an active 17β-hydroxysteroid dehydrogenase in the mitochondrial fraction of the dog pancreas, which catalyzes oxidoreduction between androstenedione and testosterone. This finding supports the hypothesis that the pancreas could be an extragonadal site of steroid hormone metabolism.

MATERIALS AND METHODS

Reagents. [1,2-^{3}H] Androstenedione (specific activity, 46.1 Ci/mmol) and [1,2-^{3}H] testosterone (specific activity, 49.0 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.), and rechromatographed on a Sephadex LH-20 column, equilibrated and developed with the solvent system toluene:methanol (85:15, v/v). The radioactive purity of the steroids was confirmed by thin-layer chromatography in a system of methylene dichloride: ethyl acetate (80:20, v/v) prior to use. The radioactive steroids were diluted with the corresponding authentic non-radioactive steroids purchased from Steraloids Inc. (Wilton, N.H.) to obtain appropriate specific radioactivity for the enzyme assays.

Pyridine nucleotides (NAD^+, NADH, $NADP^+$, NADPH), PMSF and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo.), thin-layer chromatography sheets and analytical-grade solvents were obtained from E. Merck (Darmstadt, Germany). All the chemical reagents used were of analytical grade. Deionized water was used for all aqueous solutions.

Tissue preparation. Pancreas of male mongrel dogs were obtained from the Instituto Nacional de Cardiología (México, D. F.). After transportation in ice to the laboratory, tissues were dissected away from obvious fat and connective tissue, weighed, minced with scissors, and homogenized in ice-cold 0.25 M sucrose solution containing 0.05 M potassium phosphate buffer, 1 mM EDTA, 5 mM $MgCl_2$, 10 μM PMSF, and 7 mM 2-mercaptoethanol, pH 7.0, with a weight/volume ratio of 1:10. Homogenization was performed in a Polytron apparatus with three 20 sec bursts at 3000 rpm. Subcellular fractions were prepared by differential centrifugation (11). All operations were conducted at 0-4°C. The homogenate was subjected to a 600 x g, 10-min centrifugation to eliminate nuclei, unbroken cells, and heavy debris. The resulting supernatant was centrifuged at 1000 x g for 10 min to sediment the zymogen granules. The mitochondrial fraction was sedimented by centrifugation for 15 min at 8700 x g. The pellet was washed three times with homogenization buffer and sedimented at 12,000 x g. The initial supernatant from the mitochondrial fraction was then centrifuged at 109,000 x g for 60 min, the resulting pellet and supernatant were employed conventionally as the microsomal and cytosol fractions of dog pancreas, respectively. The subcellular fractions were kept at -20°C until incubations were started.

Enzyme assays. 17β-hydroxysteroid dehydrogenase was determined by measuring both the reduction of androstenedione to testosterone and androstenedione formation from testosterone. Unless otherwise noted, the assay mixture contained, in a total volume of 1 ml, steroid substrate (50 nmol; 200,000 cpm) added in 0.05 ml

of 95% ethanol, 1.35 µmol of NAD^+ or 1.35 µmol of NADH, dissolved in 5 mM potassium phosphate buffer, containing 1 mM EDTA, 5 mM $MgCl_2$, 10 µM PMSF, and 7 mM 2-mercaptoethanol, pH 7.0. The reactions were initiated by the addition of appropriate quantities of the enzyme preparation. The assays were conducted at 37°C for 10 min in air under constant shaking in a Dubnoff-type metabolic incubator. Blank incubations were prepared without nucleotide cosubstrate as well as using enzyme preparations inactivated by heating at 98°C for 2 min.

Enzyme reactions were stopped by the addition of 10 volumes of diethyl ether; androstenedione and testosterone (50 µg each per flask) were added to the mixture as carrier steroids, and the extraction was repeated twice with 10 volumes of diethyl ether. The extracts were pooled and evaporated to dryness under reduced pressure. The final residue was redissolved in 0.2 ml of ethanol. An aliquot of 0.05 ml was used to quantitate recovery and 0.05 ml was chromatographed on a 0.2 mm thin-layer sheet of silica gel 60 F_{254}, in a solvent system of methylene dichloride:ethyl acetate (80:20, v/v). The spots of the carrier steroids were visualized under ultraviolet light at 254 nm. The silica gel containing the identified compounds was scraped out of the thin-layer sheet and transferred into scintillation vials.

For scintillation counting, 10 ml of a toluene solution, containing 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene, was added to each vial. Radioactivity was determined using a Packard Tri-carb Liquid Scintillation Spectrometer (Model 3255). The counting efficiency for tritium was of 60%.

The amount of product was expressed in nmoles by dividing its radioactivity by the specific activity of the initial substrate. 17β-hydroxysteroid dehydrogenase activity was expressed as nmoles of product formed per mg of protein per min.

Protein concentrations were determined by the Coomassie blue G dye-binding technique of Bradford (12), with bovine serum albumin used as standard.

Kinetic data were fitted to the Michaelis-Menten equation and the corresponding parameters calculated according to Wilkinson (13).

RESULTS AND DISCUSSION

Identification of Products. Only one radiolabeled product which comigrated with testosterone was formed from [^{3}H] androstenedione in incubation mixtures containing cell-free homogenates of dog pancreas and NADH. This compound was identified as authentic testosterone demonstrating constant specific radioactivity through recrystallization with non-radioactive testosterone. Similarly, the only radioactive product obtained from [^{3}H]testosterone in the presence of NAD^+ was identified as androstenedione by its mobility on thin-layer chromatography and recrystallization with authentic non-radioactive androstenedione. The radioactivity recovered as androstenedione and testosterone on the thin-layer chromatograms averaged 93% under the conditions used in the pres-

ent study. No detectable radioactive compounds were formed when heat-denatured homogenates of dog pancreas were employed.

Subcellular Localization. The results obtained on subcellular fractionation of the homogenate are summarized in Table I. More than 86% of both oxidative and reductive activities of the 17β-hydroxysteroid dehydrogenase, found in the whole homogenate, were concentrated in the mitochondrial fraction with a 4-fold increase in the specific activity. The remaining activity was found in the particulate sediment of centrifugation at 600 x g. Treatment of the mitochondrial fraction with phospholipase A resulted in a 90% decrease of the enzyme activity and attemps to solubilize the enzyme, using sonication as reported for the testicular microsomal 17β-hydroxysteroid dehydrogenase (14), were unsuccessful. These observations suggest that the enzyme is tightly bound to the membrane and its activity related to the membrane structure.

Cofactor Requirement. 17β-hydroxysteroid dehydrogenase of dog pancreas appeared to be a NAD(H)-specific enzyme. Androstenedione was not reduced by the enzyme when NADPH at the high concentration of 2.0 mM was used as cosubstrate. Similarly, $NADP^+$ could not substitute for NAD^+ in the reverse enzyme reaction for testosterone. At a high fixed concentration of the corresponding steroid substrate (80 μM), the apparent Michaelis constants (Km) for NADH and NAD^+ were determined to be 157 μM and 315 μM, respectively. Bioconversion of steroid substrates could not be detected in the absence of added pyridine nucleotide.

The mitochondrial localization and the NAD(H)-specificity of this enzyme are in contrast to the microsomal localization and

TABLE I

INTRACELLULAR DISTRIBUTION OF THE 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CANINE PANCREATIC TISSUE

Subcellular Fraction	Protein mg.ml	Volume ml	Androstenedione Reduction			Testosterone Oxidation		
			Specific activity ($nmol.min^{-1}mg^{-1}$)	Total activity (μmol.min)	Activity recovered (%)	Specific activity ($nmol.min^{-1}mg^{-1}$)	Total activity (μmol.min)	Activity recovered (%)
Homogenate	14.8	505.0	0.081	0.605	100.0	0.111	0.829	100.0
Nuclei	14.5	50.0	0.104	0.075	12.4	0.150	0.108	13.0
Mitochondria	37.8	45.0	0.317	0.539	89.0	0.420	0.714	86.1
Microsome	38.2	11.0	0.027	0.011	1.8	0.051	0.021	2.5
Cytosol	11.3	380.0	0.000	0.000	00.0	0.003	0.012	1.4

the NADP(H) of the testicular 17β-hydroxysteroid dehydrogenases (14-16).

Kinetics. The amount of either testosterone or androstenedione produced from the corresponding substrates by the mitochondrial fraction of dog pancreas increased proportionately to the protein concentrations of the tissue preparation (Fig.1a). In time-course studies of the reductase and oxidase functions of 17β-hydroxysteroid dehydrogenase with a fixed protein concentration of the pancreatic mitochondrial fraction, the amount of the respective steroid product increased linearly with time of incubation for at least 30 min (Fig.1b).

pH Dependency. The pH dependence of both, reductase and oxidase activities of the pancreatic enzyme was examined using three different buffer systems; 0.05 M sodium acetate, pH between 4.0 and 6.0; 0.05 M Tris-HCl, pH from 6.5 to 9.0; and 0.05 M glycine-NaOH, pH from 9.5 to 12.0. The optimal pH of the enzyme for androstenedione reduction was found to be at 6.0-6.5, whereas for oxidation of testosterone it was 7.5. The enzyme reaction was found to be reversible at a pH between 5.5 and 8.5, with oxidation and reduction ocurring at approximately the same rate at a pH of around 7.0 (Fig.2a).

Temperature Dependency. Initial velocities of the enzyme reaction were determined at an incubation temperature between 20°C and 70°C

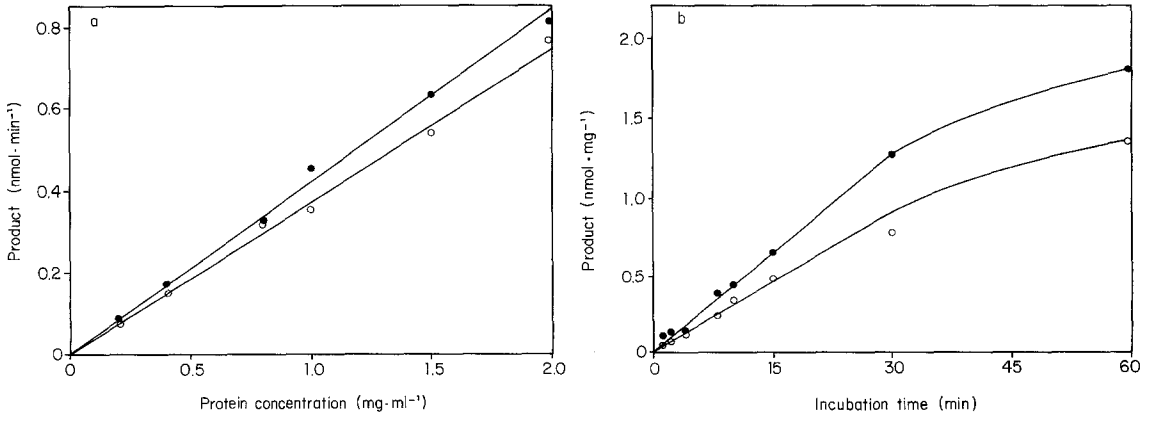

Fig.1. (a): *Effect of protein concentrations of the pancreatic tissue preparation on the product formation*. Enzyme activity was determined as described in the text. The amount of testosterone (o—o) or androstenedione (●—●) formed by the mitochondrial fraction was plotted against protein concentration. (b): *Time-course of product formation with the pancreatic tissue preparation*. Incubations were performed with the mitochondrial fraction (1.0 mg of protein per flask) as described in the text, and the amount of testosterone (o—o) or androstenedione (●—●) formed was plotted against incubation time.

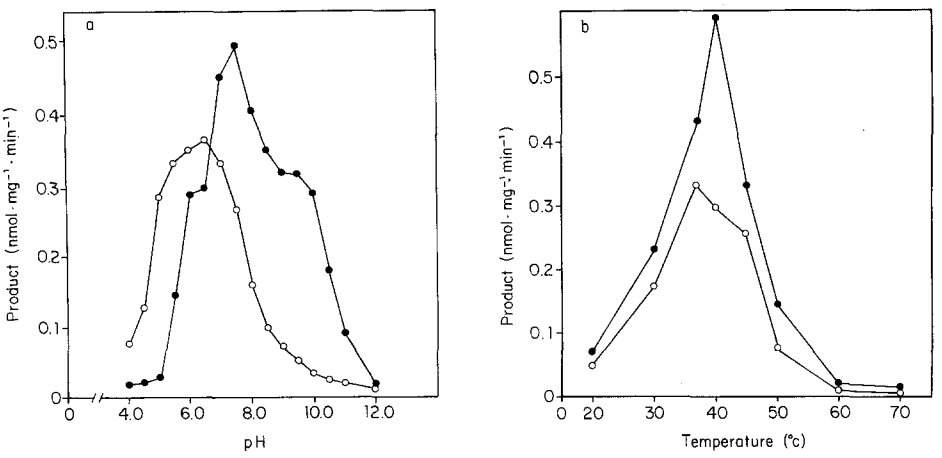

Fig. 2. *17β-hydroxysteroid dehydrogenase activity as a function of* (a) *pH and* (b) *temperature.* Incubations were performed with the pancreatic mitochondrial preparation containing 0.4 mg of protein per flask. The amount of testosterone (o—o) or androstenedione (●—●) formed was plotted.

(Fig.2b). The apparent maximal reductive activity for androstenedione was attained at 37°C, and maximal rate for testosterone oxidation was achieved at 40°C. Both, reductive and oxidative activities rapidly decreased at temperatures over 40°C until no enzymatic conversion could be measured.

Substrate Saturation Kinetics. The effect of increasing concentrations of androstenedione and testosterone (in the range of 2.0 to 80.0 μM), incubated separately, upon the initial velocity of pancreatic mitochondrial 17β-hydroxysteroid dehydrogenase is illustrated in Fig.3. The apparent Km for androstenedione was determined to be 9.5 ± 0.9 μM (SE) in phosphate buffer, pH 7.0 and 37°C.

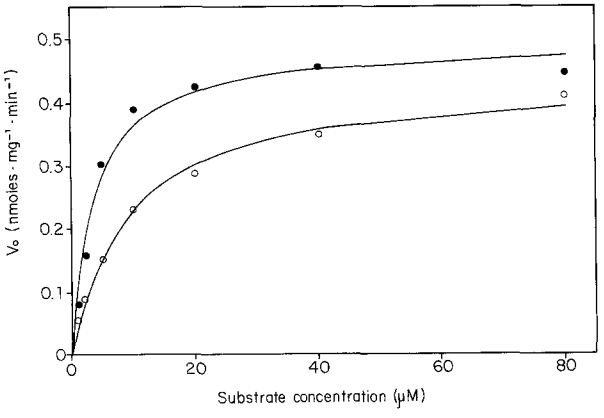

Fig. 3. *Direct plot of initial velocities as a function of steroid substrate concentration.* Androstenedione (o—o); testosterone (●—●). Protein concentration of the mitochondrial enzyme preparation was 0.4 mg ml^{-1}. Full lines are corrected curves given by the computer. Each point represents the average of at least two assays.

Using testosterone as a substrate, the apparent Km value was 3.5 ± 0.5 μM (SE) under the same conditions. Both, androstenedione and testosterone yielded an identical Vmax value of 0.4 nmol mg^{-1} min^{-1}, agreeing with the results obtained in the pH studies, in which oxidation and reduction occur at the same rate at a pH of around 7.0 (Fig.2a).

The Km values estimated in the present experiments were of the same order of magnitude as those of the 17β-hydroxysteroid dehydrogenases of porcine, rat, and human testes, which are physiologically involved in the biosynthesis of testosterone (14-16).

Although the physiological role of pancreatic 17β-hydroxysteroid dehydrogenase remains to be determined, the presence of steroid-transforming enzymes supports the hypothesis that the pancreas could be an extragonadal site of steroid hormones biosynthesis.

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