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THE EFFECT OF ADENOSINE 3′,5′-CYCLIC-MONOPHOSPHORIC ACID ON THE 17β-HYDROXYSTEROID DEHYDROGENASE OF RAT TESTIS

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SUMMARY

The effect of adenosine 3′,5′-cyclic-monophosphoric acid on the conversion of 4-androstene-3,17 dione (4-androstenedione) to 17β-hydroxy-4-androsten-3-one (testosterone) catalyzed by a 17β-hydroxysteroid dehydrogenase (EC 1.1.1.64) was studied.

Mitochondrial and microsomal fractions isolated from rat testicular tissue served as the source of enzyme employed in the present experiments.

The conversion of testosterone to 4-androstenedione, in the presence of NAD^+, was inhibited by adenosine 3′,5′-cyclic-monophosphoric acid. However, the cyclic nucleotide had no effect on this conversion when $NADP^+$ was used as co-factor. Furthermore, the transformation of 4-androstenedione to testosterone in the presence of NADPH was not influenced by the cyclic nucleotide. The inhibitory effect of the cyclic nucleotide on the NAD^+ dependent conversion of testosterone to 4-androstenedione was counteracted by increasing the concentration of exogenous NAD^+. The inhibition of NAD^+ dependent 17β-hydroxysteroid dehydrogenase by the cyclic nucleotide confirms early reports on the inhibitory effect of this nucleotide on the NAD^+ dependent 5-ene-3β-hydroxysteroid dehydrogenase (EC 1.1.1.51). The results also indicate that steroid dehydrogenase which utilizes $NADP^+$ for the transfer of electrons is not influenced by adenosine 3′,5′-cyclic-monophosphoric acid.

INTRODUCTION

PREVIOUS studies have demonstrated that adenosine 3′,5′-cyclic monophosphoric acid (3′,5′-AMP) inhibits the conversion of 5-ene-3β-hydroxysteroids to 4-ene-3-ketosteroids in the adrenal cortex[1, 2] and ovarian tissue[3, 4]. This reaction is catalyzed in steroidogenic tissues by a NAD^+ dependent 5-ene-3β-hydroxysteroid dehydrogenase and a 5-ene-3-ketosteroid isomerase[5]. The inhibitory effect of the cyclic nucleotide was related to its competition for the NAD^+ binding site[1, 2, 4] and was apparently localized at the dehydrogenation step[1, 2].

The effect of 3′,5′-AMP on $NADP^+$ dependent steroid dehydrogenases has not been investigated. This report is concerned with the effect of 3′,5′-AMP on 17β-hydroxysteroid dehydrogenase of rat testis. This enzyme utilizes NADPH to convert 4-androstenedione to testosterone and $NADP^+$ as well as NAD^+ for the reverse reaction. Portions of these studies have appeared in abstract form[6].

EXPERIMENTAL

Steroids substrates

Testosterone and 4-androstenedione used for incubation purposes were purchased from Ikapharm (Israel) and were assayed for purity by thin-layer and gas liquid chromatography.

(7α-^{3}H) 4-androstenedione (3·23 Ci/mM) and (4-^{14}C) testosterone 58·2 m Ci/mM. were obtained from the Radiochemical Centre (Amersham, England). The radiochemical purity of the radioactive steroids were verified by thin-layer chromatography on silica gel-G, using benzene–ethyl acetate (4 : 1 v/v) together with standard reference samples.

Other chemicals

Nicotinamide adenine-dinucleotide phosphate ($NADP^+$), nicotinamide adenine dinucleotide (NAD^+), and reduced nicotinamide adenine–dinucleotide phosphate (NADPH) were obtained from Sigma (U.S.A.). D-glucose-6-phosphate disodium salt (Glc-6-P) and glucose-6-phosphate dehydrogenase (Glc-6-P dehydrogenase) were acquired from C. F. Boehringer and Soehne GmbH (Germany). Adenosine 3′,5′-cyclic-monophosphoric acid was purchased from Serva (Germany). Other chemicals and organic solvents were of analytical reagent grade. The acetone, methanol and carbon disulphide were distilled before use.

Preparation of the subcellular fractions

Mature male rats (Charles River Colony), about 300 g body weight (average age 2–3 months) were killed by a sharp blow on the head. The testes were removed, decapsulated, weighed and kept in ice until the next stage of the preparation. Within 15 min of killing the animals, the testicular tissue was homogenized in 5 vol. sucrose (0·25 M) with a homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 600 *g* for 15 min to sediment the nuclei and cell debris. The supernatant was decanted and retained while the sediment was washed with 1 vol. sucrose (0·25 M) and again centrifuged at the same speed for a further 15 min. The combined supernatants were centrifuged at 10,000 *g* for 20 min to sediment the mitochondria which were resuspended in 0·154 M KCl by hand homogenization and centrifuged at 10,000 *g* for 15 min. This procedure was repeated three times. The 10,000 *g* supernatant was further centrifuged at 105,000 *g* for 60 min to sediment the microsomes. The microsomal fraction was then resuspended in 0·154 M KCl and centrifuged again at 105,000 *g* for 60 min.

Incubation procedure

The washed mitochondrial or microsomal fractions were suspended in 0·154 M KCl and incubated in the presence of 0·1 M phosphate buffer pH 7·4, magnesium sulphate 50 μmoles, a NADPH generating system ($NADP^+$ 6 μmoles, Glc-6-P 30 μmoles, Glc-6-P dehydrogenase 1 IU) and 4-androstenedione 0·17 μmoles as substrate to a total volume of 3 ml. When testosterone (0·17 μmoles) was the substrate both subcellular fractions suspended in 0·154 KCl were incubated separately in the presence of 0·1 M phosphate buffer pH 7·4, magnesium sulphate 50 μmoles, and $NADP^+$ 0·15 μmoles or NAD^+ 0·75 μmoles, to a final volume of 3 ml. The steroid substrates were added last to initiate the reaction which was performed at 34°C in air for 60 min using a Dubnoff incubator, with constant shaking. Identical incubations containing the testicular enzymes only and blank samples (water) were included in each experiment and carried through the entire procedure. The incubations were stopped by the addition of 0·2 ml acetic acid and freezing.

Extraction and separation of the products of the reaction

Known amounts of [7α-^{3}H] 4-androstenedione or [4-^{14}C] testosterone were

added to the incubation mixture prior to the extraction. The mixture was then extracted three times with 10 ml ethyl acetate followed by an extraction with 5 ml ethyl acetate. The combined extracts were evaporated to dryness under nitrogen or at reduced pressure. The dried extract was dissolved in a small amount of chloroform and chromatographed on silica-gel thin layer plates. The thin-layer plates were chromatographed in benzene-ethyl acetate (6:4, v/v) together with standard reference. This solvent system separated 4-androstenedione (R_F 0·56) from testosterone (R_F 0·32). In the experiments in which 4-androstenedione was the substrate, the area on the sample lanes corresponding in chromatographic mobility to authentic testosterone, was scraped from the plate and eluted with methanol (3 × 4 ml), whereas in the experiments in which testosterone was employed as the substrate, the 4-androstenedione was scraped from the thin-layer plate and eluted in the same manner. The samples were dried and the residue dissolved in carbon disulphide and aliquots were taken for analysis by gas–liquid chromatography. An aliquot was also used to measure the recovery of radioactive testosterone or 4-androstenedione. A Packard Tricarb Model 2002 liquid scintillation spectrometer was used to determine the radioactivity. At this stage 85–90% of the added [7α-^{3}H] 4-androstenedione or [4-^{14}C] testosterone were recovered. All testosterone or 4-androstenedione values reported in this paper have been corrected for losses during the extraction and separation with thin-layer chromatography.

Gas–liquid chromatography

A Packard Model 871 gas chromatograph equipped with a flame ionization detector was used for quantification of testosterone and 4-androstenedione. The column was 196 cm long by 0·4 cm in dia. and the stationary phase was 3% SE/30 on 100–120 mesh Gas Chrom Q. The column temperature was kept at 235°C with the detector at 245°C. Samples were introduced into the column with a 10 μl Hamilton syringe.

Estimation of testosterone

After separation on thin-layer chromatography the area corresponding to testosterone was eluted from the thin-layer plate with methanol and evaporated to dryness. The testosterone samples were then placed in a water bath at 55°C and acetylation was carried out in pyridine (0·2 ml) and acetic anhydride (0·2 ml) for 1 h. After evaporation the acetylated testosterone was mixed with a known amount of an internal standard (pregnenolone acetate) dissolved in 100 μl carbon disulphide and injected into the gas-chromatograph. Pregnenolone acetate, which has a retention time (t_R) of 25 min, was found to be an adequate internal standard for this assay because it emerged from the column after testosterone acetate (t_R 20 min). Calibration curves for testosterone acetate and pregnenolone acetate were established and the response of the flame ionization detector was linear for both acetylated compounds. Peak areas were quantitated by triangulation (peak height × width at ½ height) and were plotted against mass of steroid injected. The amount of testosterone present in the sample injected was determined from the standard curve and corrected for losses during injection.

Estimation of 4-androstenedione

Following incubation with testosterone, samples were separated by thin-layer

chromatography as previously described. The area corresponding to the R_F of 4-androstenedione was eluted from the thin-layer plates with methanol and evaporated to dryness. The dry residue was mixed with a known amount of pregnenolone acetate (internal standard) and injected into the gas chromatograph. The conditions of operation were the same as described for testosterone. Under these conditions the retention time of 4-androstenedione was 15 min. The mass of 4-androstenedione was measured by triangulations of peak areas and calculated from standard curves established for 4-androstenedione and pregnenolone acetate.

Calculation of enzymic activity

17β-hydroxysteroid dehydrogenase activity was estimated from the interconversion of testosterone and 4-androstenedione. This was measured from the rate of formation of testosterone from 4-androstenedione substrate and in the reverse reaction from the rate of formation of 4-androstenedione following incubation with testosterone. In both cases the results were expressed as mμmoles testosterone or 4-androstenedione formed per min/mg mitochondrial or microsomal protein. The technique of Lowry *et al.* [7] was employed for protein estimation and crystalline bovine serum albumin was used as standard.

RESULTS

a. *Conversion of testosterone to 4-androstenedione*

The conversion of testosterone to 4-androstenedione by mitochondrial and microsomal preparations from rat testicular tissue followed a linear time course for at least 70 min when concentrations of NAD^+ or $NADP^+$ were not limiting (Fig. 1). It can be seen that 60 min incubation of both subcellular fractions resulted in values which fell on the linear part of the curves, hence this interval was chosen as the incubation period for the subsequent experiments. Figure 2 illustrates the

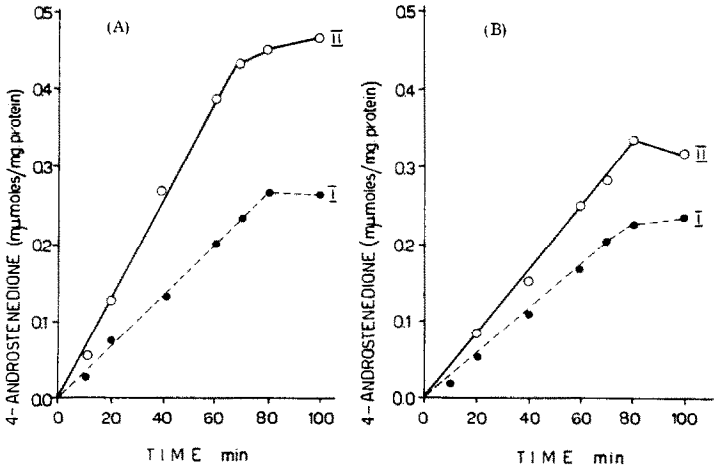

Fig. 1. Time course of the mitochondrial (I) and microsomal (II) conversion of testosterone to 4-androstenedione in the presence of $NADP^+$ (A) and NAD^+ (B). Incubation mixture: 5 mg mitochondrial or microsomal protein in 0·1 M phosphate buffer pH 7·4. 50 μmoles magnesium sulphate 0·12 μmoles $NADP^+$ or 0·60 μmoles NAD^+ and 0·17 μmoles testosterone to a total volume of 3 ml. Incubations were carried out in air at 34°C for different times.

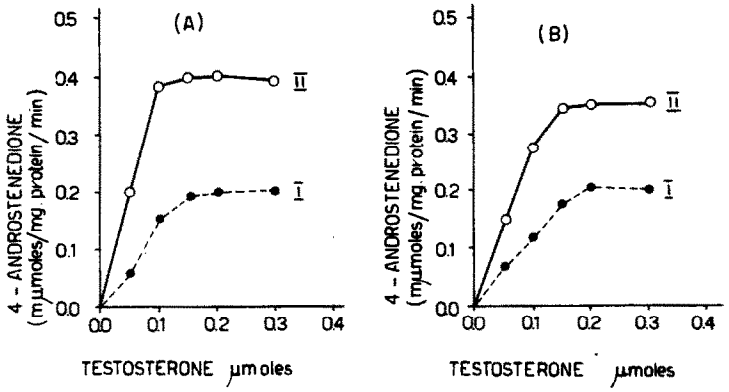

Fig. 2. Effect of varying concentration of substrate (testosterone) on the rate of 17β-hydroxysteroid dehydrogenase in mitochondrial (I) and microsomal (II) fractions of rat testis in the presence of $NADP^+$ (A) and NAD^+ (B). Incubation mixture: 5 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 0·15 μmoles $NADP^+$ or 0·75 μmoles NAD^+ and increasing amounts of testosterone. The total volume was 3 ml and the incubations were carried out at 34°C in air for 60 min.

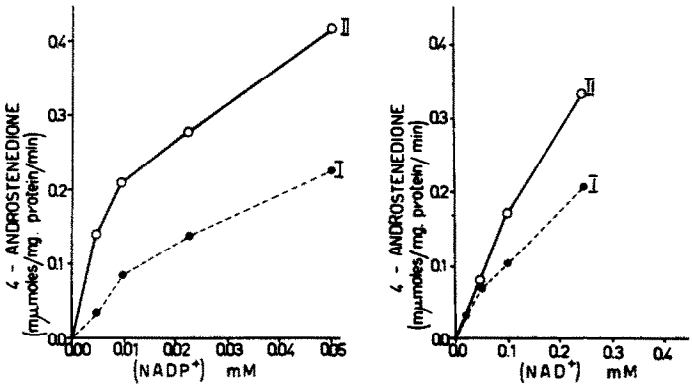

Fig. 3. Effect of $NADP^+$ and of NAD^+ on the conversion of testosterone to 4-androstenedione in mitochondria (I) and microsomal (II) fractions of rat testis. Incubation mixture: 5 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, increasing amounts of $NADP^+$ or NAD^+ and 0·17 μmoles testosterone to a total volume of 3 ml. Incubations were carried out at 34°C in air for 60 min.

effect of varying concentrations of testosterone on 17β-hydroxysteroid dehydrogenase activity. Apparent saturation of enzyme with substrate occurs at 0·15 μmoles testosterone.

Figure 3 shows the results of the experiments in which rat testis mitochondrial and microsomal preparations were incubated with testosterone as substrate and with different amounts of $NADP^+$ or NAD^+. Smaller amounts of $NADP^+$ than NAD^+ were necessary to achieve conversion, demonstrating that $NADP^+$ was the preferred co-factor for this reaction. It should be emphasized that when either coenzyme was used, the dehydrogenase activity was greater in the microsomal fraction.

b. *Conversion of 4-androstenedione to testosterone*

A time course of the conversion of 4-androstenedione to testosterone is shown in Fig. 4. The reaction proceeds in a linear manner up to 80 min. Saturation of the

enzyme with the substrate was achieved at a concentration of 0·15 μmoles 4-androstenedione per incubation.

The conversion of 4-androstenedione to testosterone in mitochondrial and microsomal fractions of rat testis as a function of enzyme concentration is illustrated in Fig. 5. Both subcellular fractions were capable of enzymically reducing 4-androstenedione to testosterone and the enzymic activity was found to be greater in microsomes. The reaction was NADPH dependent, and the conversion of 4-androstenedione to testosterone was higher when a NADPH generating system (Glc-6-P-$NADP^+$-Glc-6-P-dehydrogenase) was employed.

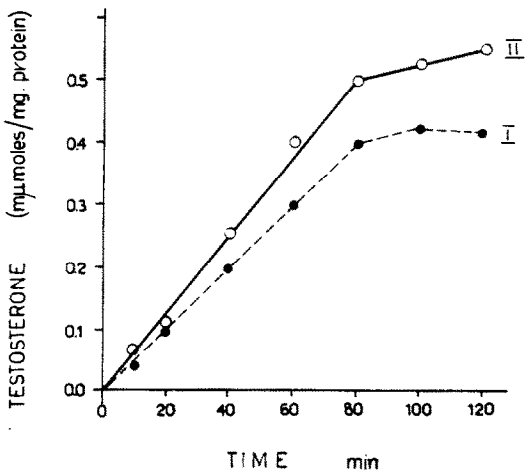

Fig. 4. Effect of time on the formation of testosterone from 4-androstenedione in mitochondria (I) and microsomal (II) fractions of rat testis. Incubation mixture: 5 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 6 μmoles $NADP^+$, 30 μmoles Glc-6-P, 1 IU Glc-6-P-dehydrogenase and 0·17 μmoles 4-androstenedione. The total volume was 3 ml and the incubations were carried out at 34°C in air for 60 min.

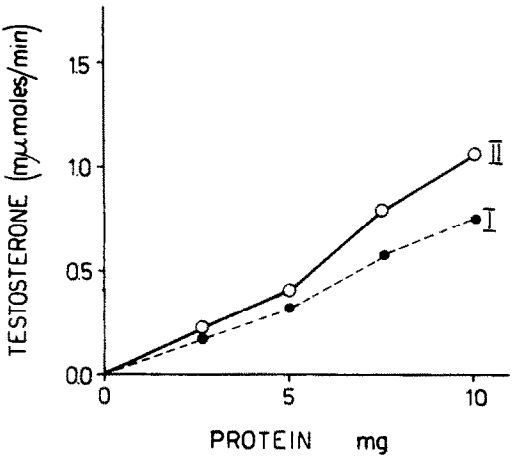

Fig. 5. Conversion of 4-androstenedione to testosterone in mitochondrial (I) and microsomal (II) fractions of rat testis as a function of enzyme concentrations. Incubation mixture: Increasing amounts of mitochondrial or microsomal protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 6 μmoles $NADP^+$, 30 μmoles, Glc-6-P, 1 IU Glc-6-P-dehydrogenase and 0·17 μmoles 4-androstenedione. The total volume was 3 ml and the incubations were carried out at 34°C in air for 60 min.

c. *Effect of 3',5'-AMP*

The transformation of testosterone to 4-androstenedione in the presence of NAD^+ was inhibited in both subcellular fractions by 3',5'-AMP (0·2–5 μmoles/ml incubation), whereas when $NADP^+$ was employed as co-factor, the cyclic nucleotide, used at the same concentrations, had no effect on this conversion (Fig. 6).

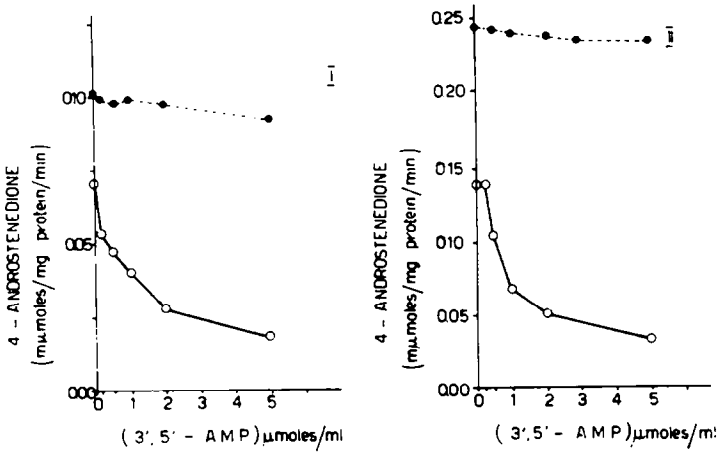

Fig. 6. Effect of 3',5'-AMP on the NAD^+ (O---O) and $NADP^+$ (●---●) dependent conversion of testosterone to 4-androstenedione in mitochondria (I) and microsomal (II) fractions of rat testis. Incubation mixtures: 5 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 0·75 μmoles NAD^+ or 0·06 μmoles $NADP^+$, 0·17 μmoles testosterone and different concentrations of 3',5'-AMP to a final volume of 3 ml. The incubations were carried out at 34°C in air for 60 min.

Furthermore, 3',5'-AMP was found to have no effect on the mitochondrial and microsomal conversion of 4-androstenedione to testosterone in the presence of NADPH.

In order to establish if NAD^+, added exogenously, would counteract the inhibitory effect of 3',5'-AMP on the conversion of testosterone to 4-androstenedione, micochondrial and microsomal preparations were incubated with increasing amounts of NAD^+ and a constant concentration of 3',5'-AMP. The concentrations of the cyclic nucleotide was 1 μmole per incubation volume for both subcellular fractions. These concentrations were found to inhibit significantly the transformation of testosterone to 4-androstenedione in mitochondrial and microsomal preparations (see Fig. 6). The results illustrated in Fig. 7 demonstrate that with increasing concentrations of exogenous NAD^+, the inhibitory effect of 3',5'-AMP was removed in both subcellular fractions. A double reciprocal plot[8] of the effect of 3',5'-AMP on the mitochondrial conversion of testosterone to 4-androstenedione in the presence of varying amounts of NAD^+ is shown in Fig. 8. Thus, the cyclic nucleotide competes with the co-factor and selectively inhibits the NAD^+ dependent conversion of testosterone to 4-androstenedione.

DISCUSSION

17β-hydroxysteroid dehydrogenase activity measured by the *in vitro* interconversion of 4-androstenedione and testosterone was found to be easily demon-

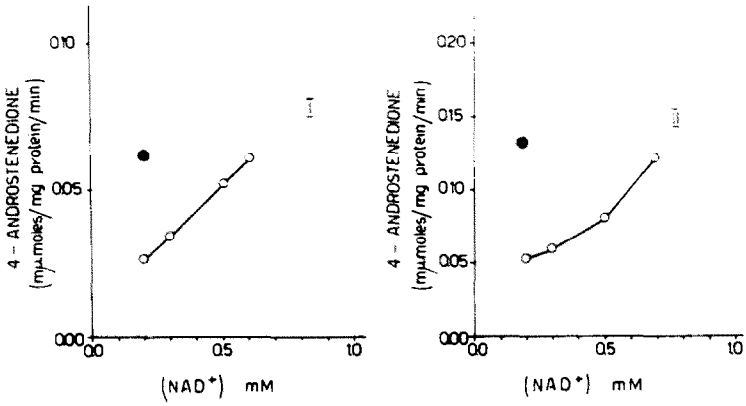

Fig. 7. Effect of increasing amounts of NAD^+ on the conversion of testosterone to 4-androstenedione in the presence of a constant amount of 3',5'-AMP (1 μmole/ml) in rat testis mitochondria (I) and microsomes (II). Incubation mixtures: 5 mg mitochondrial or microsomal protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate 0·75 μmoles NAD^+, 0·17 μmoles testosterone in the absence of 3',5'-AMP (●) and in the presence of 1 μmole/ml 3',5'-AMP (○---○) and different concentrations of NAD^+. The final volume was 3 ml and incubations were carried out at 34°C in air for 60 min.

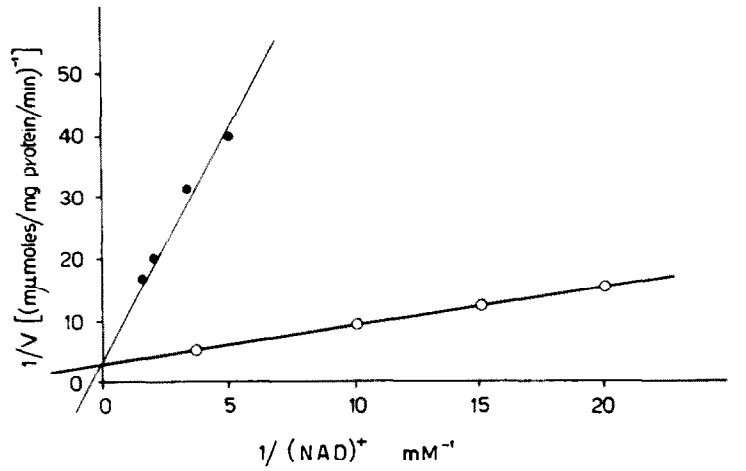

Fig. 8. The inhibition by 3',5'-AMP with respect to NAD^+ of the mitochondrial transformation of testosterone to 4-androstenedione. Velocity is expressed as mμmoles 4-androstenedione formed/mg protein/min. NAD^+ concentration was varied from 0·2–0·6 mM. The concentration of testosterone was 0·17 μmoles/3 ml incubation. The amount of mitochondrial protein present in each sample was 5 mg and the incubations were carried out at 34°C in air for 60 min. ○ No 3',5'-AMP. ● 1 μmoles 3',5'-AMP/ml incubation.

strable in the testis of mature rats. The enzyme was located in both mitochondrial and microsomal fractions and its activity was higher in microsomes. These observations confirmed previous findings which established the presence of 17β-hydroxysteroid dehydrogenase in rat testicular tissue associated with both subcellular fractions [9].

Most peptide hormones have been shown to effect their target organs by activating a membrane bound enzyme, adenyl cyclase, which produces 3',5'-AMP from ATP. Studies with adrenocorticotropin (ACTH) in adrenal cortex

[10], luteinizing hormone (LH) in the corpus luteum[11] and follicle stimulating hormone (FSH) and LH in the testis[12, 13] have demonstrated the stimulatory effect of these hormones on 3′,5′-AMP accumulation. Furthermore, 3′,5′-AMP has been shown to mimic the steroidogenic effect of LH on the testis by increasing testosterone production[14, 15]. Evidence has been presented that the cyclic nucleotide stimulates steroidogenesis by increasing the conversion of cholesterol to pregnenolone[16].

Recent studies have shown that 3′,5′-AMP inhibits the NAD^+ dependent 5-ene-3β-hydroxysteroid dehydrogenase in adrenal cortex[1, 2] and ovarian tissues[3, 4].

In the present experiments the effect of 3′,5′-AMP on the 17β-hydroxysteroid dehydrogenase was tested. This enzyme utilizes either $NADP^+$ or NAD^+ for the conversion of testosterone to 4-androstenedione and NADPH for the reverse reaction. The cyclic nucleotide added *in vitro* had no effect on the reduction of 4-androstenedione to testosterone in the presence of NADPH and on the oxidation of testosterone to 4-androstenedione when $NADP^+$ was the electron acceptor. However, when NAD^+ was used as co-factor for the latter reaction 3′,5′-AMP competed with NAD^+ and inhibited the reaction. These results suggest a direct relationship between the cyclic nucleotide and NAD^+ in this system. The concentration of 3′,5′-AMP required to produce inhibition *in vitro* of 17β-hydroxysteroid dehydrogenase (0·5 mM), appeared to be much higher than that found endogenously in other endocrine tissues[17, 18] but lower than that found necessary to stimulate testosterone production from endogenous substrate in rabbit testis slices[15]. The present data demonstrates the inhibitory effect of the cyclic nucleotide on testicular 17β-hydroxysteroid dehydrogenase when NAD^+ was used as co-factor. The mechanism of this inhibition was found to be analogous to 3′,5′-AMP inhibition of NAD^+ dependent 5-ene-3β-hydroxysteroid dehydrogenase. These observations as well as the reported inhibitory effect of 3′,5′-AMP on yeast glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)[19], and on NAD^+ isocitrate dehydrogenase (EC 1.1.1.41) and malate dehydrogenase (EC 1.1.1.37) in rat testis[20] suggest a possible role of this nucleotide in controlling the NAD^+-linked dehydrogenases.

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