AMINOPEPTIDASES OF RAT TESTIS

II. EFFECTS OF PUBERTY, CRYPTORCHIDISM AND CADMIUM CHLORIDE TREATMENT

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Summary. Four aminopeptidases of rat testicular tissue were quantified during pubertal maturation, after cryptorchidism, and following cadmium chloride treatment. The assay conditions were based on differential substrate and modifier characteristics outlined previously.

Aminopeptidase I with Val- β -naphthylamide (β -NA) and aminopeptidase II with Ala- β -NA and EDTA as activator showed a high activity in the prepubertal testis. A marked increase in activity after cryptorchidism suggested a Leydig cell origin for these enzymes. Aminopeptidase III was characterized as aminopeptidase B and quantified with Arg- β -NA as substrate. The activity of this enzyme closely followed the maturation of spermatogenesis during puberty and showed a rapid decrease after cryptorchidism. The differential quantification of Aminopeptidase IV was unsuccessful but there was evidence to suggest that its origin was in the seminiferous tubules.

There was a suppression of the activity of all four enzymes after cadmium chloride treatment following the necrotic changes in the testis.

INTRODUCTION

In a previous study (Vanha-Perttula, 1973), four distinct enzymes hydrolysing different aminoacyl naphthylamides were separated by DEAE-cellulose chromatography and sucrose density gradient centrifugation.

In the present study, a differential quantification of the four enzymes was carried out during the testicular development of puberty, after cryptorchidism, and following cadmium chloride treatment, using assay methods outlined in the previous study for each of the separate enzymes. The changes in enzyme activities were correlated with the testicular histology as shown in another communication (Vanha-Perttula & Nikkanen, 1972).

MATERIALS AND METHODS

Experimental material

Sprague-Dawley albino rats subjected to the same experimental conditions

as those described by Vanha-Perttula & Nikkanen (1972) were used. The pubertal groups contained four animals each and were killed at 10-day intervals between 10 and 70 days of age. The cryptorchid groups (2, 5, 8, 10, 15 and 20 days of artificial cryptorchidism) consisted of four animals per group and a control group of eight rats which were all killed on the same day. Cadmium chloride (0.5 mg/100 g) was given as a single subcutaneous injection 1, 2, 4, 6, 10, 15 and 20 days before autopsy to four adult animals in each group and the enzyme activities were compared to those of a control group of eight animals.

In each experiment, one testis was immediately fixed in Bouin-Hollande fixative and prepared for light microscopy. The other testis was deprived of its capsule and homogenized in 0.25 M-sucrose prepared in 0.02 M-tris-HCl, pH 7.5, with a glass homogenizer and a Teflon pestle. An aliquot of this homogenate was further diluted in 0.02 M-tris-HCl buffer, pH 7.5, sonicated with an MSE Ultrasonic Disintegrator and used for enzyme assays. The rest of the homogenate in 0.25 M-sucrose was centrifuged with an MSE Ultraspeed 50 centrifuge at 105,000 g for 60 min. The supernatant was used in a parallel assay for quantification of Enzyme IV.

Determination of enzyme activities

For the determination of Enzyme I activity, valyl- β -naphthylamide (Val- β -NA, Mann Research Laboratories) was used as substrate at a final concentration of 0.25 mM. The buffer was 0.1 M-tris-HCl, pH 7.0, and the incubation time was 60 min at 37° C. The reaction was terminated with the p-dimethylaminobenzaldehyde reagent according to the method of Takenaka (1964). The absorbance was measured at 460 nm with a Beckman DB spectrophotometer.

Enzyme II activity was determined with alanyl- β -naphthylamide (Ala- β -NA, Mann Research Laboratories) as substrate at a final concentration of 0.25 mM. The buffer was 0.1 M-tris-HCl, pH 7.0, to which EDTA was added. During a 30-min preincubation period, the EDTA concentration was 1.5 mM but during the incubation period of 60 min, it was reduced to 1 mM, following the addition of the substrate.

Enzyme III activity was measured using arginyl- β -naphthylamide (Arg- β -NA, Mann Research Laboratories) as substrate at 0.25 mM concentration in 0.1 M-tris-HCl buffer, pH 6.5, which also contained 0.2 M-NaCl. The incubation time was 30 min at 37° C.

Enzyme IV activity was determined with leucyl- β -naphthylamide (Leu- β -NA, Sigma Chemical Company) as substrate at a final concentration of 0.25 mM in 0.1 M-tris-HCl buffer, pH 8.0, which also contained cysteine to give a 1.5-mM concentration during the 30-min preincubation period and a final concentration of 1 mM. The incubation time was 30 min at 37° C.

In all enzyme assays, the reaction was followed under zero order kinetics (hydrolysis rate was linear to time and enzyme concentration) and all the assays were in duplicate. The protein of the samples was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. A standard curve with aqueous solutions of β -naphthylamine HCl was prepared for calibration. The unit of enzyme activity is given

as nmol of β -naphthylamine liberated/min/mg protein or in the fractionation studies as nmol/min/ml enzyme solution. The mean and the standard deviation were calculated for each group and in the statistical correlation, Student's *t* test was utilized.

Samples of testicular tissue from 10-day, 20-day and 60-day-old rats were

TEXT-FIG. 1. Hydrolysis of Val- β -NA at pH 7.0 by testicular homogenate under varying experimental conditions. O, Enzyme activity during puberty; the age in days is given in the upper abscissa. ●, Enzyme activity after cryptorchidism; the time after operation is given in the lower abscissa. Δ , Enzyme activity after cadmium chloride treatment; the time after treatment is given in the lower abscissa.

homogenized, in 0.02 M-tris-HCl, pH 7.5, and sonicated for 30 sec. The homogenate was applied to a DEAE-cellulose (DE 23, Whatman) column (2 $\times$ 30 cm) and eluted with a linear NaCl gradient (0 to 0.35 M) as outlined previously (Vanha-Perttula, 1973). The enzyme activities in the fractions were determined using Val- β -NA, Leu- β -NA with cysteine (1 mM) and Arg- β -NA as substrates in 0.1 M-tris-HCl buffer, pH 7.0.

RESULTS

The histology of the testis

The histological changes during testicular maturation in the Sprague-Dawley rat have been described earlier (Knorr, Vanha-Perttula & Lipsett, 1970; Vanha-Perttula & Nikkanen, 1972) and the present observations were closely similar. The time-related changes in cryptorchid and cadmium chloride-damaged testes have also been recently published (Vanha-Perttula & Nikkanen, 1972).

TEXT-FIG. 2. Hydrolysis of Ala- β -NA at pH 7.0 in the presence of EDTA (1 mmol/litre) under varying experimental conditions. ○, Enzyme activity during puberty. ●, Enzyme activity after cryptorchidism. Δ, Enzyme activity after cadmium chloride treatment.

Aminopeptidase I activity (Text-fig. 1)

During the pubertal maturation of the testis, the hydrolysis rate of Val- β -NA was high at 10 and 20 days of age, though these values were not significantly different from the control value at 70 days of age, which was regarded as the

adult control level. At 30 and 40 days, however, significantly lower ($P < 0.01$) values were obtained.

After the induction of cryptorchidism, an initial significant ($P < 0.05$) decrease was found on the 2nd day. This was followed by a rapid increase, which was highly significant ($P < 0.001$) after 8 days.

Treatment with cadmium chloride resulted in an initial short activation on the 1st and 2nd days but subsequently a rapid decline ensued, reaching a low basal level by the 6th day.

TEXT-FIG. 3. Hydrolysis of Arg- β -NA at pH 6.5 in the presence of NaCl (0.2 mol/litre) under varying experimental conditions. ○, Enzyme activity during puberty. ●, Enzyme activity after cryptorchidism. △, Enzyme activity after cadmium chloride treatment.

Aminopeptidase II activity (Text-fig. 2)

The hydrolysis rate of Ala- β -NA with EDTA (1 mM) showed the highest hydrolysis rates in the early stages of testicular maturation. Subsequently, a rapid decline of enzyme activity was recorded. All time points before 50 days of age were significantly different from the 70-day adult control value. Cryptorchidism resulted in a gradual increase in enzyme activity with a more rapid

rise on the 8th day, after which the hydrolysis rates remained significantly above the control level ($P < 0.01$).

After treatment with cadmium chloride, a significant suppression of Enzyme II activity was found on the 4th day, but the later values remained reasonably high when compared to the activities of Aminopeptidase I.

Aminopeptidase III activity (Text-fig. 3)

Very low activities were found with Arg- β -NA at 10 and 20 days of age, but

TEXT-FIG. 4. Hydrolysis of Leu- β -NA at pH 8.0 in the presence of cysteine (1 mmol/litre) under varying experimental conditions. O, Enzyme activity during puberty. ●, Enzyme activity after cryptorchidism. Δ, Enzyme activity after cadmium chloride treatment.

subsequently the enzyme activity showed a remarkably rapid rise. The adult level was reached at 50 days of age, whereas all the previous values were significantly ($P < 0.001$) different from the 70-day control value.

After cryptorchidism, an initial increase ($P < 0.05$) was found on the 2nd day, but subsequently a rapid decline in activity followed, reaching the values of the immature testis 15 to 20 days after the induction of cryptorchidism.

Treatment with cadmium chloride caused a similar but more precipitous decrease in Aminopeptidase III activity. The suppression was already highly

significant 4 days after treatment and very low basal values were reached by the 10th day.

Aminopeptidase IV activity (Text-fig. 4)

When Leu- β -NA was used as substrate to test the activity of aminopeptidase IV in the total testicular homogenate, the pattern was essentially similar to that obtained with Ala- β -NA with EDTA (Text-fig. 2). There was no remarkable difference, whether the enzyme determination was carried out with or without cysteine.

TEXT-FIG. 5. Elution pattern of aminoacyl aminopeptidases from the DEAE-cellulose chromatography of homogenates of total testis at different ages. O, Hydrolysis of Leu- β -NA. ●, Hydrolysis of Arg- β -NA.

Attempts were made to measure the Aminopeptidase IV activity of the supernatant after high-speed centrifugation. This resulted in a slightly different pattern. During pubertal maturation, a slight increase in activity was observed after 30 days of age. After cryptorchidism, no significant changes were encountered at any time point. However, treatment with cadmium chloride caused a marked and rapid decline which was much more pronounced than that found with the total homogenate.

Chromatography of testicular tissue at various ages (Text-fig. 5)

The elution patterns of the aminopeptidases after DEAE-cellulose chromato-

graphy of total testicular homogenates of 10-, 20- and 60-day age groups are depicted in Text-fig. 5. All four aminopeptidases were present in all age groups, but relative differences in their activities were clearly demonstrated. Enzymes I and II were relatively more active in the early age groups, whereas Enzyme III was very low at 10 days of age but showed a steady increase during testicular maturation. Enzyme IV activity showed only a slight relative augmentation with increasing age.

DISCUSSION

The present studies confirm the earlier findings of Vanha-Perttula (1973). The solubility of Enzymes III and IV as well as the sensitivity of Enzymes I and II to diazo-coupling agents in the histochemical reaction have rendered their cellular site inaccurate using the conventional methods. The present quantitative results give at least some information of their tissue distribution in the testis, although the accurate cellular site still remains unknown.

Aminopeptidase I, which has already been located mainly in the interstitial tissue (Vanha-Perttula, 1973), showed a moderate decrease during pubertal maturation and a permanent increase after cryptorchidism. The peak of activity was reached at 10 to 20 days, when the testicular tissue consisted almost exclusively of Leydig and Sertoli cells. There is, thus, strong evidence that the Leydig cells may be the major site for this enzyme.

The quantification of Aminopeptidase II with Ala- β -NA, using a chelator for the testicular trace metals which are inhibitory to this enzyme, resulted in a pattern closely similar to that of Enzyme I activity. This indicates that both enzymes may be dependent on the integrity of the same cell population, which for Enzyme II may also be the Leydig cells. A slightly different subcellular distribution in the sucrose density gradient centrifugation (Vanha-Perttula, 1973) suggests, however, that these enzymes are not necessarily functionally connected.

In a previous study, Blech (1968) reported a two- to three-fold increase in leucyl-glycyl-glycine, leucyl-glycine, leucinamide, glycyl-leucine and glycyl-glycyl-glycine-splitting peptidase activities in the cryptorchid testis at 2 weeks. The enzymes hydrolysing these substrates have not been characterized from the rat testis, but there is previous evidence that di- and tripeptides are hydrolysed by the same enzymes as the aminoacyl naphthylamides (Vanha-Perttula, Hopsu & Glenner, 1966). Thus, the increased activity found by Blech (1968) may be due to Aminopeptidase I or II, or to both enzymes. The results obtained in these studies do not presume a *de novo* synthesis of these enzymes. The increase in the hydrolytic activity could very well be derived from the decrease of seminiferous tubule protein low in enzyme activity.

Enzyme III represents a typical aminopeptidase B, which has also been described in many other tissues (Hopsu & Mäkinen, 1966a, b, c; Mäkinen & Hopsu-Havu, 1967). A special feature characteristic of this enzyme in the testicular tissue was its seminiferous tubule origin (Vanha-Perttula, 1973). The present quantitative results are consistent with this localization and the rapid increase during the maturing spermatogenesis at 30 to 50 days of age, as well

as the precipitous decline after cryptorchidism and treatment with cadmium chloride support a spermatogenic origin for this enzyme. Closely similar changes in these experimental situations have been encountered for a specific testicular Acid phosphatase IV (Vanha-Perttula & Nikkanen, 1972). The only difference was the total absence of Acid phosphatase IV after fractionation of testicular tissue from animals at 10 days of age, whereas some Aminopeptidase III activity was already present at this age. Both these enzymes may be used as markers for the control of spermatogenic maturation, e.g. after hormonal stimulation in immature animals.

The quantification of Aminopeptidase IV, which has been located within the seminiferous tubules by fractionation of isolated tubular material (Vanha-Perttula, 1973), proved to be a difficult task due to overlapping in substrate specificity with other enzymes. The exclusive intratubular site cannot therefore be regarded as confirmed, and the contribution of the Sertoli cells remains to be assessed. The enzyme was also present in high concentration in immature rat testes.

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