Secretion of D-aspartic acid by the rat testis and its role in endocrinology of the testis and spermatogenesis

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Abstract The D-isomer of aspartic acid (D-Asp) has been found in rat testes. In the present study, samples of testicular venous blood plasma, rete testis fluid, interstitial extracellular fluid, luminal fluid from the seminiferous tubules, testicular parenchymal cells, epididymal spermatozoa and peripheral blood plasma were collected and analyzed for D-Asp by two methods, an enzymatic and a chromatographic HPLC method. The two methods gave very similar results for all samples. The highest concentrations of D-Asp (about 120 nmol/ml) were found in testicular venous blood plasma, with slightly lower concentrations in rete testis fluid (95 nmol/ml) and epididymal spermatozoa (80 nmol/g wet weight). Lower levels were found in testicular parenchymal cells (which would comprise mostly spermatids and spermatocytes), luminal fluid from the seminiferous tubules and interstitial extracellular fluid (26, 23 and 11 nmol/ml respectively). However, these values were all higher than those for peripheral blood plasma (6 nmol/ml). It would appear that D-Asp is being secreted by the testis mostly into the venous blood, passing thence into the rete testis fluid and being incorporated into the spermatozoa at the time or after they leave the testis. The distribution of D-Asp is thus quite different from that of testosterone, and its role and the reason for its high concentration in the male reproductive tract remain to be elucidated.

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1. Introduction

It has been demonstrated that D-amino acids (D-AAs), and in particular D-aspartic acid (D-Asp) occur in living organisms, although in minute quantity compared to their optical isomers. D-Asp was measured in the brain, stellate ganglia, and in the axoplasmic fluid of cephalopods *Octopus vulgaris*, *Loligo vulgaris* and *Sepia officinalis* [1,2]. Since these first studies, this amino acid has been found in the nervous and endocrine systems of many invertebrates [3,4] and vertebrates. In vertebrates, this amino acid occurs in the nervous tissues of chicken [5], rat [6–8] and man [9,10]. In the human brain, it is present in embryos [9], and in adults [10]. In addition to nervous tissues, in mammals it is also present in the pituitary gland and the gonads [7,11].

In the central nervous system, D-Asp occurs at high levels in embryos, whereas in adult animals it nearly disappears, only to increase in endocrine glands and in particular the pituitary [7,11]. Interestingly, this amino acid has also been found in epinephrine cells of the adrenal medulla, and in high quantities in rat pineal gland where it is hypothesized to play an important role as a novel messenger [12].

It has been shown that testosterone synthesis in the rat shows two active phases: immediately before birth and during sexual maturity [13,14]. More recently, we demonstrated that D-Asp is present at elevated concentration in rat testis. Immunocytochemical studies give us indications that this enantiomer was localized in Leydig and Sertoli cells. In vitro experiments showed that D-Asp induced the release of testosterone and was involved in rat steroidogenesis [11]. Finally, D-Asp has also been reported to occur in rat spermatozoa [15]. In addition, this amino acid was detected in the ovary of *Rana esculenta* and was correlated to the different sexual phases of reproductive cycle [16].

It has been known for many years that there are high concentrations of certain L-amino acids in semen and rete testis fluid from rams and rats, and that these substances are largely formed inside the seminiferous tubules from glucose [17,18] and that there appears to be a saturable transport system for L-leucine in the endothelial cells of the testicular blood vessels in the rat [19,20]. All the above data induced us to establish more clearly whether D-Asp was formed by the interstitial tissue in association with steroid production or inside the seminiferous tubules of the testis, in response to changing testosterone levels.

2. Materials and methods

2.1. Sample collection

Adult male rats, Sprague Dawley strain, obtained from B and K Universal, Sollentuna, Sweden, weighing between 346 and 375 g were used. Three were anaesthetized with pentobarbital sodium (Apoteksbolaget, Umea, 35 mg/kg body weight i.p.) and the efferent ducts of the left testis ligated as described by Setchell [21]. About 20 h later, the rats were anaesthetized again and rete testis fluid (33 ± 2.9 μl) collected from the left testis of each rat into a haematocrit tube by the technique described by Setchell and Main [22]. Then testicular venous blood was collected from both testes, as described by Galil and Setchell [23]. The testes were then removed from the animal, decapsulated and the cells of the parenchyma dispersed by forcing through a 20 gauge needle with a 5 ml syringe; the cell dispersion was centrifuged ($10\,000 \times g$ for 5 min) and separated into a cell fraction and a fluid fraction, as described by Setchell et al. [24–26]. A peripheral venous blood sample was then removed from the posterior vena cava. Three other similar rats were anaesthetized, testicular venous blood collected from both testes as before, and then the left testis was removed, decapsulated and the parenchyma irrigated with 4 ml 300 mM mannitol, to remove the interstitial extracellular fluid. The cells were then dispersed and fluid and cell fractions separated as before. The right testis was then removed, decapsulated and the pa-

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renchyma dispersed without irrigating with mannitol, and fluid and cell fractions separated by centrifugation. Blood was then collected from the posterior vena cava. The volumes of the rete testis fluid samples were estimated from the length of the column of fluid in the haematocrit tube, and the samples were then diluted with 0.5 ml 0.9% sodium chloride, and centrifuged (10 000×*g* for 5 min) to remove the spermatozoa. The blood samples were centrifuged (10 000×*g* for 10 min for the testicular venous samples and 3000×*g* for 20 min for the peripheral samples) and plasma separated. The mannitol samples were also centrifuged (3000×*g* for 20 min) to remove any cells. All samples were put immediately on ice, and kept cool throughout all subsequent procedures. As the extracellular interstitial fluid was diluted with 4 ml mannitol, the sodium concentration was determined by flame photometry to calculate how many μl of fluid was removed from each testis, assuming that the interstitial extracellular fluid contained the same concentration of sodium as blood plasma [27]. The cell fraction samples from the six unligated testes, without mannitol irrigation weighed 1005 ± 30 mg, those from the three ligated testes 1047 ± 49 mg and from the mannitol irrigated testes 977 ± 33 mg. The fluid fraction samples weighed 356 ± 16 mg, 581 ± 21 mg and 408 ± 16 mg, respectively. The fluid fraction consists largely of fluid from the lumina of the seminiferous tubules, and there is considerably more fluid from the ligated testis, because the ligature traps inside the tubules the fluid secreted by the testis [24–26].

The spermatozoa were collected from cauda epididymidis of three different rats and suspended in 0.9% NaCl. The suspension was allowed to settle for 10 min so that large pieces of tissue could be separated. The supernatant was centrifuged at 3000×*g* for 10 min to concentrate the spermatozoa. The pellet was resuspended in a small volume of 0.9% NaCl and put in a swim up system in order to separate the spermatozoa from other cells. Then, the purified spermatozoa were counted and reduced to a small volume by centrifugation as described.

2.2. Sample preparation for amino acid analyses

The samples as soon as collected, were homogenized with 0.2 M perchloric acid (PCA) in a ratio of 1:10, except for the mannitol samples which were mixed with 0.6 M PCA in a ratio of 2.8:1. After homogenization, the samples were centrifuged at 15 000×*g* for 30 min and the supernatant was purified on a cation exchange resin AG 50-WX 8 (Bio-Rad), H^+ form, 100–200 mesh (previously regenerated by treatment with an excess of 2 M NaOH for 10 min, washed with distilled water until neutral, then treated with an excess of 2 M HCl for 10 min followed by distilled water and finally equilibrated with 0.01 M HCl) using a bed volume of the settled resin of 2 ml. After the samples were absorbed on the column, the column was washed twice with 5 ml 0.01 M HCl. The eluent was discarded. The resin was eluted with 8 ml of 4 M NH_4OH and this last eluent was evaporated by using a rotoevaporator at the temperature below 30°C, or allowed to evaporate in a Petri dish on a hot plate at 30–40°C under a hood. The residue was dissolved in 0.3 ml 0.05 M phosphate buffer, pH 8.2.

2.3. Enzymatic fluorimetric method for the specific determination of D-aspartic acid

This method is based on the fact that when D-Asp is treated with D-aspartate oxidase (D-AspO) it is oxidized to oxaloacetate [28–30]. Then, the oxaloacetate reacts with malate dehydrogenase (MDH) in presence of NADH to give malate and oxidized NAD. The NADH that remains in excess can be destroyed by HCl, and the NAD generated by the reaction will become fluorescent with strong NaOH.

The reactions are the following:

(1) $\text{D-Asp} + O_2 + H_2O \xrightarrow{\text{D-AspO}} \alpha - \text{oxaloacetate} + H_2O_2 + NH_3$,

(2) $\alpha\text{-oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + NAD^+$,

(3) NADH unreacted + HCl is destroyed,

(4) NAD^+ generated by the reaction + NaOH becomes fluorescent.

The method is specific for the determination of D-Asp because D-AspO is an enzyme which specifically oxidizes only D-Asp and D-Glu [28–30], and only α-oxaloacetate reacts specifically with MDH and NADH to generate NAD^+.

For the assay, the method from Perna et al. [31], with minor modifications was used. In brief, to two Eppendorf tubes (sample and sample blank), is added 5–50 μl of the sample and distilled water for a final volume of 50 μl. Then to each tube, is added 10 μl of 1 M Tris-HCl, pH 8.2 and 10 μl of a solution prepared by mixing in proportion 1 ml 10 mM NADH (7 mg/ml) in 0.2 M Tris-HCl, pH 8.2 with 500 μl MDH (5500 U/ml). After that, 2 μl of D-AspO purified from *Octopus vulgaris* [32] at a concentration of about 5 mg/ml (30–40 U/ml) or purified from beef kidney [30] at the same concentration are added to the sample and the tubes are incubated at 37°C for 30 min. After the incubation period, 50 μl of 6 M HCl are added to each tube and the tubes are incubated for 30 min at 37°C. After this last incubation, 900 μl of 5 M NaOH are added to each tube which are then incubated at 37°C for 30 min. Finally the fluorescence of the sample and the sample blank is read at 340 nm excitation and 440 nm emission against a reagent blank prepared as the sample, but using water in place of the sample.

Under the same assay conditions, a standard curve, consisting of 5, 10, 20 and 40 μl of D-Asp at the concentration of 0.1 mM and the other component as used in the sample was carried out. The fluorescence was read against the reagent blank as prepared above.

In order to determine the D-Asp content, the fluorescence of each sample blank was subtracted from that of the respective sample and the net fluorescence was compared with that of the standard curve.

2.3.1. Note to the method. Both NADH and NAD^+ become fluorescent when treated with NaOH, but only NADH is destroyed by HCl, whereas NAD^+ remains intact. Therefore, after the treatment with the oxidative enzyme, HCl is added to the assay mixture in order to completely destroy the NADH that has not reacted. Then, after this last incubation NaOH is added in order to make the NAD^+ generated fluorescent.

Using this method it was possible to determine reliably a minimum amount of D-Asp or NMDA corresponding to 10 pmol/assay mixture. Recovery studies were performed by adding D-Asp at different concentrations to a sample and determining its concentration in the sample before and after addition of D-Asp. The percent (%) recovery obtained was between 95–105%. Inter-assay coefficient of variation (C.V.) was evaluated at several points along the standard curve in five different assays. The % C.V. was of: 4–7% in the range 10–100 pmol of D-Asp. The data were statistically analyzed using the program STATVIEW version 4, ABASCUS Concepts.

2.4. Specific determination of D-aspartic acid by HPLC chromatography

This was based on the diasteroisomeric separation of D-Asp from L-Asp and other amino acids according to the Aswad method [33] slightly modified as follows: 5–20 μl of the sample as above prepared were mixed with 5–20 μl of 0.1 M NaOH (to bring the pH to between 8.2 to 8.6), then with distilled water to obtain a final volume of 360 μl and with 40 μl of OPA-NAC buffer (*o*-phthaldialdehyde/N-acetyl-L-cysteine-sodium borate) prepared by mixing 10 mg of OPA plus 10 mg of N-acetyl-L-cysteine/1 ml methanol and 1.0 ml of 0.5 M sodium borate buffer, pH 8.5, plus 5–20 μl of 1 M NaOH until pH 8.5). After 2 min, 20 μl of this mixture was injected onto a C-18 Supelcosil HPLC column (0.45×25 cm, Supelco, Inc. Belafonte, PA, USA). The column was eluted with a gradient consisting of solvent A (5% acetonitrile in 30 mM sodium acetate buffer, pH 5.5) and solvent B (70% acetonitrile in 30 mM sodium acetate buffer, pH 5.5) starting with 0% B to 30% B over 20 min, then 30% B to 100% over 5 min, and 100% B for 3 min. The flow rate was 1.2 ml/min. The amino acid derivatives were detected fluorometrically using an excitation wavelength of 330 nm and an emission wavelength of 450 nm. For the standard curve, a mixture of 17 amino acids in the L-form each at the concentration of 0.1 mM plus D-Asp at a concentration between 0.005 to 0.02 mM were used instead of the sample.

Under these conditions, D-Asp was eluted in about 6 min and separated from L-Asp by about 0.5 min (Fig. 1). Using this method it was possible to determine reliable amounts of D-Asp up to 10 pmol in the 20 μl of the injected mixture.

Recovery studies were performed by adding D-Asp at different concentration to a sample and D-Asp was determined before and after addition of D-Asp. The % recovery obtained was between 94–108%. The inter-assay coefficient of variation (C.V.) was evaluated at several points along the standard curve in five different assays. The % C.V. was of 4.5–8.5% in the range between 10–100 pmol of D-Asp. The data were statistically analyzed using the program STATVIEW, version 4, ABASCUS Concepts.

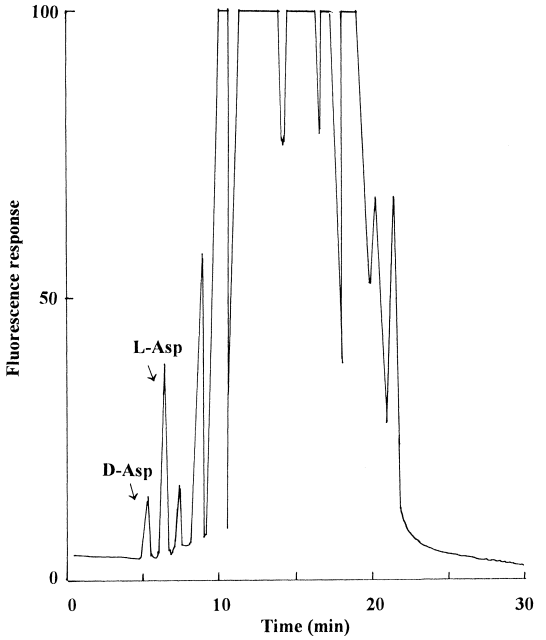

Fig. 1. Typical example of HPLC analysis for the determination of D-Asp in testicular venous plasma. The analysis is carried out as described in Section 2. In 20 μl of derivatized sample are contained an amount of D-Asp and other amino acids corresponding to that contained in 3 μl of original sample. The figure reports the elution time of peaks corresponding to D- and L-Asp. The remaining amino acids (eluted following L-Asp) are at a strong concentration compared to the previous.

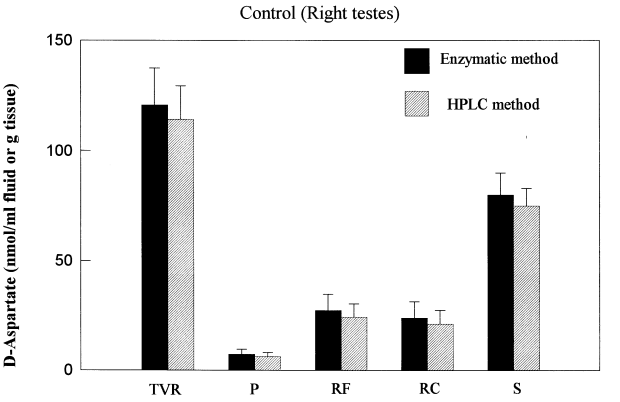

Fig. 2. Endogenous occurrence of free D-Asp in rat testis compartments. The data refer to the mean ± S.D. obtained from six right rat testes. Black bars are the values determined with enzymatic method and grey bars with HPLC method. TVR, testicular venous plasma from right side; P, plasma from posterior vena cava; RF, fluid fractions from right testes; RC, cell fractions from right side; S, spermatozoa.

3. Results

In this study using an enzymatic fluorimetric method and a chromatographic HPLC method (Fig. 1) for the measurement of D-Asp in biological tissues, we have determined the content of this amino acid in various compartment of the testis. With the two methods, we obtained very similar results, which are presented in Figs. 2 and 3. As is shown, in all fractions examined, D-Asp was present in concentrations between 10 and 130 nmol/g tissue or ml fluid. The highest concentrations of D-Asp were found in venous blood plasma from a vein on the surface of the testis (TVR, about 118 nmol/ml), followed by spermatozoa from the cauda epididymidis (S, 80 nmol/g wet weight), which corresponds to a mean value of 1.5×10^6 pmol/cell. Furthermore, the fluid fraction (RF) and the cells (RC) from the control unligated or unirrigated testes also contained significant amounts of this amino acid (about 26 nmol/ml and 23 nmol/g, respectively), but the testicular cells contained much less than epididymal spermatozoa. Plasma from the posterior vena cava (P) contained the lowest concentrations (about 6 nmol/ml, Fig. 2).

Rete testis fluid was also collected from the other testes of three rats, after efferent duct ligation, and in the other rats, interstitial extracellular fluid was collected by irrigating the other testes with isotonic mannitol (Fig. 3). Again, testicular venous blood contained the highest concentration of D-Asp (136 and 108 nmol/ml, this difference between these values and those from the control testes were not significant), but there were almost as high concentrations in rete testis fluid (95 nmol/ml). Surprisingly, testicular interstitial extracellular fluid contained only about 11 nmol/ml, much less than testicular venous blood, and less even than the cell and fluid fractions of the testis, but still higher than peripheral blood plasma.

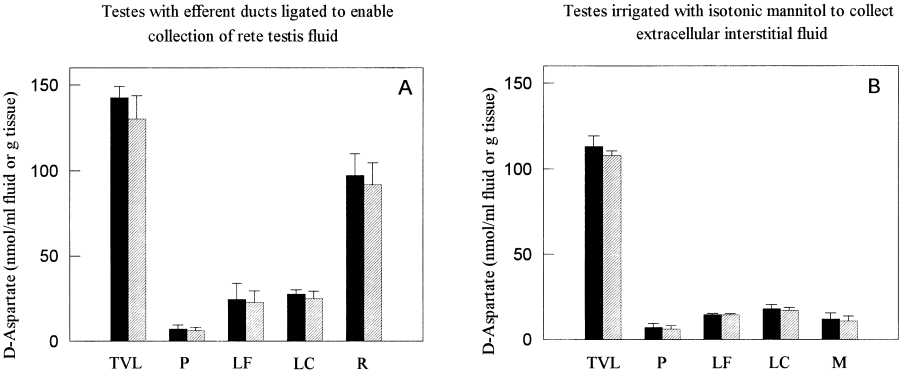

Fig. 3. Endogenous occurrence of free D-Asp in rat testis compartments. The data refer to values obtained using samples from left rat testes with efferent ducts ligated (A) and testes irrigated with 4 ml isotonic mannitol to collect interstitial extracellular fluid (B). Enzymatic (black bars) and HPLC (grey bars) methods for the determination of D-Asp were used. The results represent the mean ± S.D. obtained from three adult male rats for each treatment. TVL, testicular venous plasma from left side; P, plasma from posterior vena cava; LF, fluid fractions from left testes; LC, cell fractions from left testes; R, rete testis fluid; M, interstitial extracellular fluid collected by irrigation of the testis with mannitol.

From the concentrations in the fluid fraction from the ligated and unligated testes, and the volumes of these fluids, it is possible to calculate a value for the concentration in the additional fluid secreted by the testis and trapped in the tubules by the ligature between the time of ligation and sampling, from the formula: Concentration in secreted fluid = (concentration in fluid from ligated testis × volume of this fluid − concentration in fluid from unligated testis × volume of this fluid)/(volume of fluid from ligated testis − volume of fluid from unligated testis). These calculated values were 13.9 ± 17.4 nmol/ml for the enzymatic method and 12.5 ± 14.3 nmol/ml for the HPLC method.

4. Discussion

The present results indicate clearly that considerable quantities of D-aspartic acid (D-Asp) are secreted by the rat testis into the bloodstream, while smaller, but still appreciable amounts pass from the testis into the epididymis with the rete testis fluid. From the known values [34] for testicular blood flow (approx. 500 μl/min) and fluid secretion (0.3 μl/min), and the fact that the concentrations are comparable in the venous blood and rete testis fluid, the magnitude of the difference can be appreciated.

The high concentration in testicular venous blood plasma, and the lower values in interstitial extracellular fluid, seminiferous tubular fluid and in the testis cells indicate that either the D-Asp is being secreted preferentially by the Leydig cells into the circulation, as has been suggested to occur under some circumstances with testosterone [27], or that the endothelial cells of the testicular blood vessels are involved. These cells do have a number of unusual characteristics [35,36] and have been identified as the site for a specific saturable transport system for L-leucine [19,20]. The fact that the concentration in the tubular fluid is no higher than that outside or indeed in the testicular cells would make it unlikely that the Sertoli cells are involved in secreting D-Asp into the luminal fluid, as they do for L-glutamate, aspartate, alanine and glycine in the ram [17].

In view of the lower concentration in the tubular fluid, it is perhaps surprising that there is such a high concentration in rete testis fluid, which is believed to be derived from tubular fluid, although its composition does show some significant differences [34]. The concentration of potassium is much lower [18,26] and there is somewhat less total protein [37,38], although the concentrations of androgen-binding protein [39] and inositol [26,40] are comparable. There is evidence that the peptide hormone inhibin passes in significant amounts from rete testis fluid into venous blood in the vessels overlying the rete [41,42], but the present results are the first to suggest that substances may pass from the venous blood into rete testis fluid.

In rat brain, it has been suggested that D-Asp may have a neuronal and neuroendocrine role [12] and it may be relevant that there is now evidence that the Leydig cells should be considered as members of the diffuse neuroendocrine system [43]. In the present work we found two significant results which give credence that D-Asp is a molecule having a direct role in the steroidogenesis: (1) a very significant amount of this amino acid in the spermatic vein, where it is well known that many steroid hormones occur in this vein and (2) a significant amount of D-Asp also occurs in the spermatozoa (Fig. 2).

The cell fraction from the testis would consist largely of spermatids and spermatocytes, with probably about 4 times as many of the smaller spermatids. It is therefore likely that the high concentration of D-Asp in spermatozoa from the cauda epididymidis and the lower concentration in the testicular cells would indicate that D-Asp is being added to the sperm as they leave the testis, probably in the epididymis from the rete testis and epididymal luminal fluid. It would be very interesting to measure the concentrations of D-Asp in isolated spermatocytes and spermatids, in spermatozoa from the rete testis fluid, which were discarded in the present experiments, and in epididymal tissue and luminal fluids.

However, while the difference in concentration of D-Asp between testicular venous and peripheral blood plasma (21 times) is comparable to that seen with testosterone (3 to 70 times, see [23,26,34,39,44]), the concentrations of testosterone in testicular venous blood, interstitial extracellular fluid and the fluid fractions from unligated and ligated testes, as well as the calculated concentration in secreted tubular fluid are all similar ([26,39,44,45]), quite different from the situation for D-Asp as reported in the present paper.

Although the significance of the D-Asp in the testis is still unclear, the present results provide strong evidence that this unusual amino acid plays an important role in male reproduction.

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