# Increase of Methionine Aminopeptidase Activity in Hyperplastic Leydig Cells of Rat Cryptorchid Testis: A Histochemical Study

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ABSTRACT. Histochemical study on the changes of the aminopeptidase activities in rat testes after surgically-induced cryptorchidism was conducted comparing them with the histochemical changes in regenerated hepatic cells of the partially hepatectomized rat liver. Methionine-aminopeptidase in Leydig cells gradually increased after cryptorchid was induced, whereas the enzyme activity in regenerated hepatic cells decreased. These histochemical observations were coincident with the data obtained by enzyme assay. The present study has indicated that in the rat cryptorchid testis the increase of methionine-aminopeptidase activity was caused by hyperplastic Leydig cells.—KEY WORDS: aminopeptidase, cryptorchidism, rat, testis.

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Aminopeptidase, an exopeptidase, which plays an important role in protein metabolism by hydrolyzing terminal peptide bonds associated with the $-NH_2$ group, is concerned with the proliferation, degeneration, or inflammation of cells [1, 6, 11].

Vanha-Perttula [15] has indicated that rat testicular aminopeptidases are categorized into four groups according to substrate specificity, aminopeptidase I, II, III, and IV. He [16] has also shown biochemically that in cryptorchid testes, there is increased activity of aminopeptidase I, which preferentially hydrolyzes methionine-$\beta$-naphthylamide. In surgically-induced cryptorchidism the shrinkage of seminiferous tubules accompanied by the degeneration of germ cells occurs while Leydig cells are hyperplastic. These facts suggest that the increase in activity of the enzyme that preferentially hydrolyzed methionine-$\beta$-naphthylamide (methionine-aminopeptidase) may be due to the hyperplastic Leydig cells, though its physiological significance remains obscure [16].

The present investigation aims to comfirm histochemically whether the increased activity of methionine-aminopeptidase in cryptorchid testes is caused by hyperplastic Leydig cells, comparing them with the histochemical changes of regenerated hepatic cells in the lobules of rats subjected to partial hepatectomy.

## MATERIALS AND METHODS

Adult rats of Wistar strain were used in the present study. Twenty-five rats were made surgically bilateral cryptorchid at 80 days of age. Cryptorchidism was induced by closing the inguinal canal after the testes with epididymides were gently pushed into the abdominal cavity through the canals. Control animals were sham-operated by repeating the push and down procedure of the testes from the scrotum to the abdominal cavity twice through the canals. In order to examine histochemical changes in the regenerated liver, partial hepatectomies were carried out in male rats (80 days old) by the method of Higgins and Anderson [7]. Sham-operations for the partial hepatectomy were also done as a control. All the operations were done from 10 A.M. to noon. Testes and livers were then removed, perfused with cold saline, homogenized with a suitable amount of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 per cent of Triton X-100 in a glass homogenizer, and centrifuged at 10,000 × g for 20 min. Both the testicular and liver supernatants obtained were employed for enzyme assay. To prepare for the cell fractions, fresh testes perfused with saline were homogenized with 0.25 M sucrose solution and subjected to differential centrifugation by the method of Tamaoki [14]. These fractions were used for enzyme assay and zymogram analysis after freeze-thawing twice, following a 5-min sonication at 200 W by cell disruptor.

For the assay of aminopeptidase activity, a modified method of Flegenhauer and Glenner by Matsuzawa [5] was employed [10].

For a histochemical demonstration of aminopeptidase activity, cold-acetone treated tissue sections with 10 $\mu$m thickness were employed by the method

of Takikawa and Matsuzawa [13]. For counting the number of Leydig cells, both toluidine blue staining and the staining method for 3 β-hydroxysteroid dehydrogenase were applied [13]. Cellulose acetate membrane electrophoresis was carried out in a continuous buffer system (0.05 M barbituate; pH 8.6) with a constant current (1 mA/cm). The method of Oya *et al.* [12] was employed to demonstrate aminopeptidase zymograms. The chromogenic substrates for the aminopeptidases were L-alanine-, L-arginine-, L-leucine-, and D.L.-methionine-β-naphthylamide (β-NA) which were purchased from Sigma Co., Ltd. (St. Louis, Mo., U.S.A.). Protein concentration was measured by the method of Lowry *et al.* [9].

## RESULTS

### *Histochemical observations*

*Cryptorchid testsis*: In the intact testes the activities of aminopeptidases with both methionine-, and leucine-β-NA as substrates (methionine- and leucine-aminopeptidase) were found in the cytoplasms of Leydig cells, spermatogonia, spermatocytes, and round spermatids, whereas aminopeptidase activity with arginine-β-NA as substrate (arginine-aminopeptidase) was observed in both the nucleus of these cells and heads of spermatozoa (Fig. 1). Some Leydig cells often showed a strong activity in the nucleolus. In the cytoplasm, methionine-aminopeptidase activity was higher than leucine-aminopeptidase activity (Fig. 2). No significant difference was observed between the intact and the sham-operated testes. Seven days after cryptorchidism was induced, histological observation revealed degeneration of seminiferous tubules and hyperplasia of Leydig cells (Figs. 1, 2; Table 1).

Despite the shrinkage of the tubules, seminiferous epithelia were rather hypertrophic. In these seminiferous epithelia only spermatocytes were observed. Invasion of a few leukocytes which showed a moderate methionine-aminopeptidase activity was found in the seminiferous tubules in the early stage but not in the later stage of cryptorchid testes. Methionine-aminopeptidase activity in the cytoplasms of these hyperplastic Leydig cells increased (Fig. 3). Both leucine- and methionine-aminopeptidase activities increased, although the activity level of leucine-aminopeptidase was lower than that of methionine-aminopeptidase (Fig. 4). Arginine-aminopeptidase activity in the nucleus of spermatocytes, spermatogonia, and hyperplastic Leydig cells seemed to be normal.

*Regenerated liver*: In intact livers, while moderate activity of methionine-aminopeptidase was distributed homogeneously in the cytoplasms of hepatic cells, it was more elevated in the cells of the central area than in those of the peripheral areas of hepatic lobules (Fig. 5). Twenty four hours after partial hepatectomy, the liver was characterized by the appearance of regenerated cells in the peripheral areas of the lobules. In these regenerated cells, there were slightly lower activities of both methionine- and leucine-aminopeptidase than the normal hepatic cells which showed moderate activities of these enzymes (Fig. 6).

### *Enzyme assay*

*Cryptorchid testis*: Arginine-aminopeptidase activity in cryptorchid testes decreased continuously for 7 days after crytorchidism was induced. At 7 days after the operation, the activity had decreased to about half level in intact testes or testes of sham-operated rats (Fig. 7). In contrast, methionine-aminopeptidase activity of cryptorchid testes increased dramatically at 5 days after the operation and reached a constant level of about 1.5-fold higher than in the intact testes (Fig. 8). The cryptorchid testes showed a slightly lower activity in leucine-aminopeptidase than in methionine-aminopeptidase activity (Fig. 9).

*Regenerated liver*: In regenerated livers, the activities of all the enzymes examined decreased at 24 hours and then recovered to approximately their normal level by 48 hours after the operation. The activities of enzymes in the livers of sham-operated rats increased continuously just after the operation (Fig. 10).

### *Intra-cellular distribution of aminopeptidase activities*

In intact testes, arginine-aminopeptidase activity was highest in the nuclear fraction while both methionine- and leucine-aminopeptidase activities were higher in the microsomal fraction than in any others (Table 2). After 14 day of cryptorchidism methionine- and leucine-aminopeptidase activities in mitochondrial fraction increased remarkably to show 4-fold in the former and 6-fold in the latter enzyme. On the other hand, arginine-aminopeptidase activity in the nuclear fraction of cryptorchid testes was about 30 percent of that in the fraction of intact testes. The activities of both methionine- and leucine-aminopeptidase did not

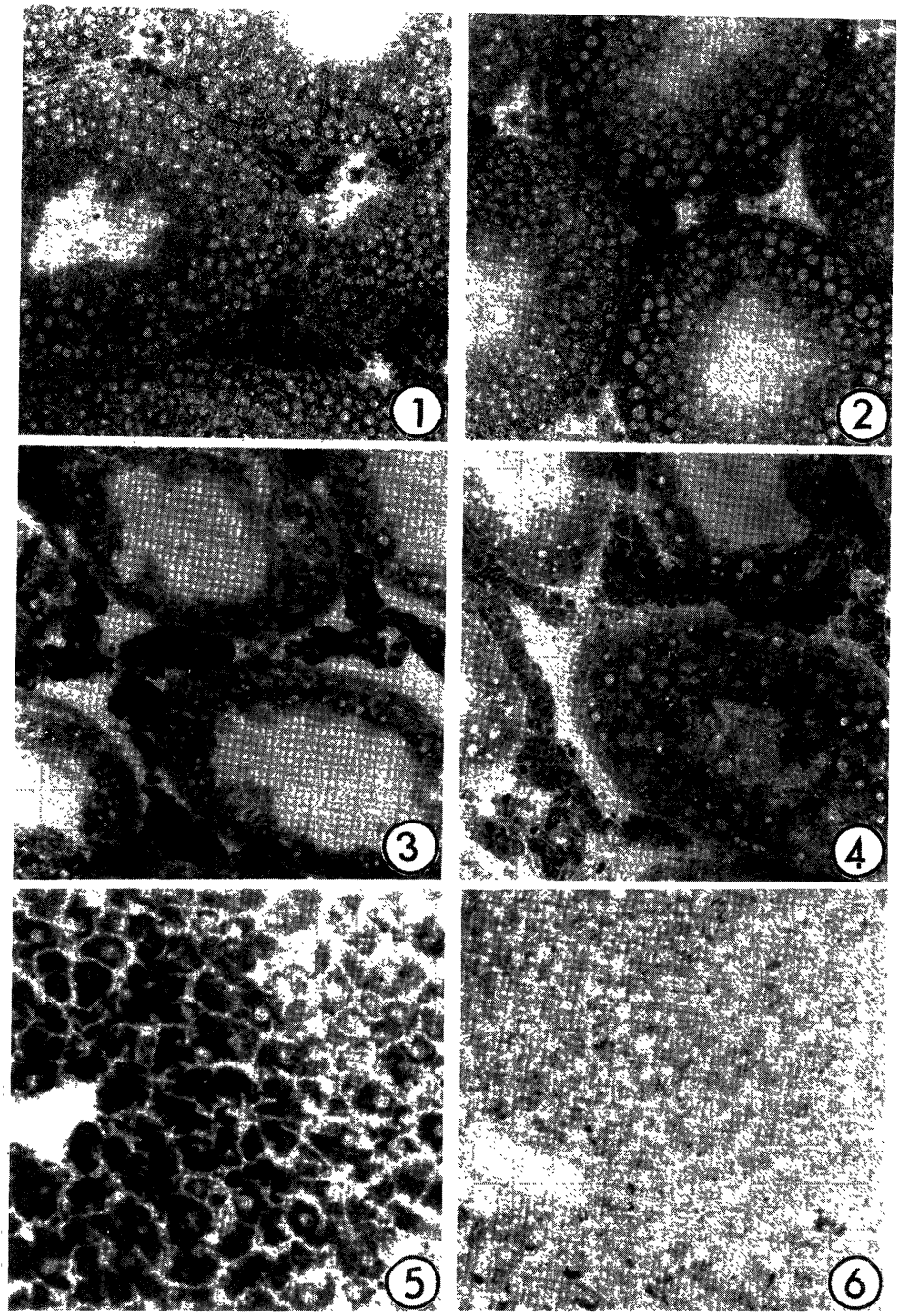


Fig. 1. Moderate methionine-aminopeptidase activity is observed in Leydig cells of the intact testis. × 120.

Fig. 2. Leucine-aminopeptidase activity demonstrated in Leydig cells of the intact testis is slightly lower than methionine-aminopeptidase activity. × 120.

Fig. 3. High methionine-aminopeptidase activity appears in hypertrophic Leydig cells of cryptorchid testis at 7 days after the operation. × 120.

Fig. 4. Leucine-aminopeptidase activity in the cryptorchid testis at 7 days after the operation. × 120.

Fig. 5. Methionine-aminopeptidase activity in the intact liver is higher in the central than the peripheral area of hepatic lobules. × 120.

Fig. 6. Methionine-aminopeptidase activity decrease in the regenerated hepatic cells. × 120.

Table 1. Number of Leydig cells surrounding one seminiferous tubule in rat testis (M±SD)

| Intact testis | Cryptorchid testis (7 days) |
|---|---|
| 70.2±13.1 (10) | 147.1±25.2 (10)* |

Numbers in parentheses indicate numbers observed. *; Significantly different ($p<0.01$) by student's *t*-test compared with the intact testis.

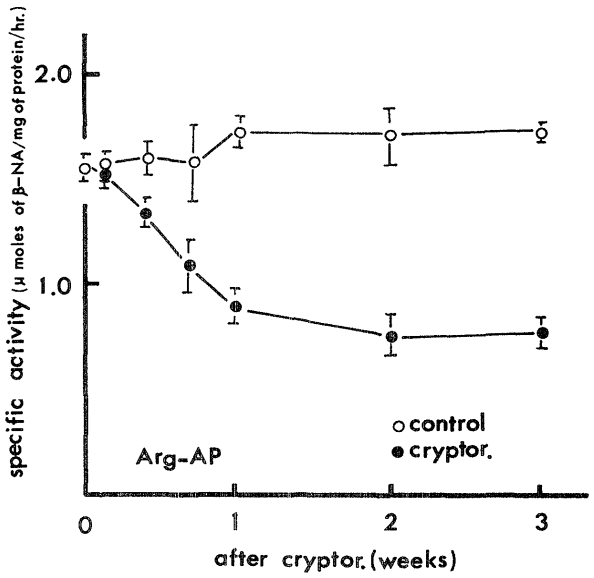


Fig. 7. Change of arginine-aminopeptidase activity in the testis after cryptorchidism was induced.
cryptor.; cryptorchidism.

change in the nuclear fraction ($p<0.01$).

*Zymograms of aminopeptidases*

A specific change was observed only in the zymogram of the mitochondrial fraction (Figs. 11a, 11b). In this fraction of intact testes, one isozyme of methionine-aminopeptidase and one arginine-aminopeptidase were demonstrated. In cryptorchid testes, a new additional isozyme which ran slower than the original enzyme was observed in both zymograms of methionine- and arginine-aminopeptidase. The change was especially prominent in the methionine-aminopeptidase zymogram, which was characterized by a higher activity of the new isozyme than the original enzyme activity (Figs. 11a, 11b).

## DISCUSSION

Based on biochemical investigation, Vanha-Perttula [16] has suggested that in the cryptorchid

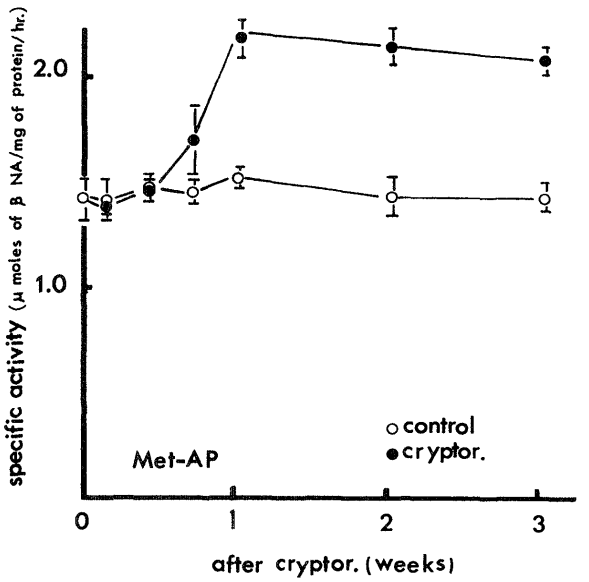


Fig. 8. Change of methionine-aminopeptidase activity in the testis after cryptorchidism was induced.
cryptor.; cryptorchidism.

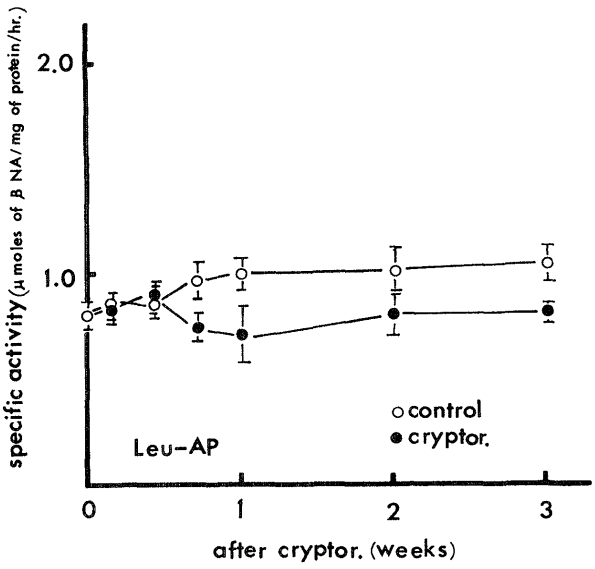


Fig. 9. Change of leucine-aminopeptidase activity in the testis after cryptorchidism was induced.
cryptor.; cryptorchidism.

testes, the increase in activity of aminopeptidase I preferentially hydrolyzing methionine-β-NA depends on the hyperplasia of Leydig cells. In the present investigation, a moderate activity of methionine-aminopeptidase in Leydig cells as well as in leukocytes was demonstrated in the early stage of cryptorchid testes which were surgically induced. In the later stages, hyperplastic Leydig cells which

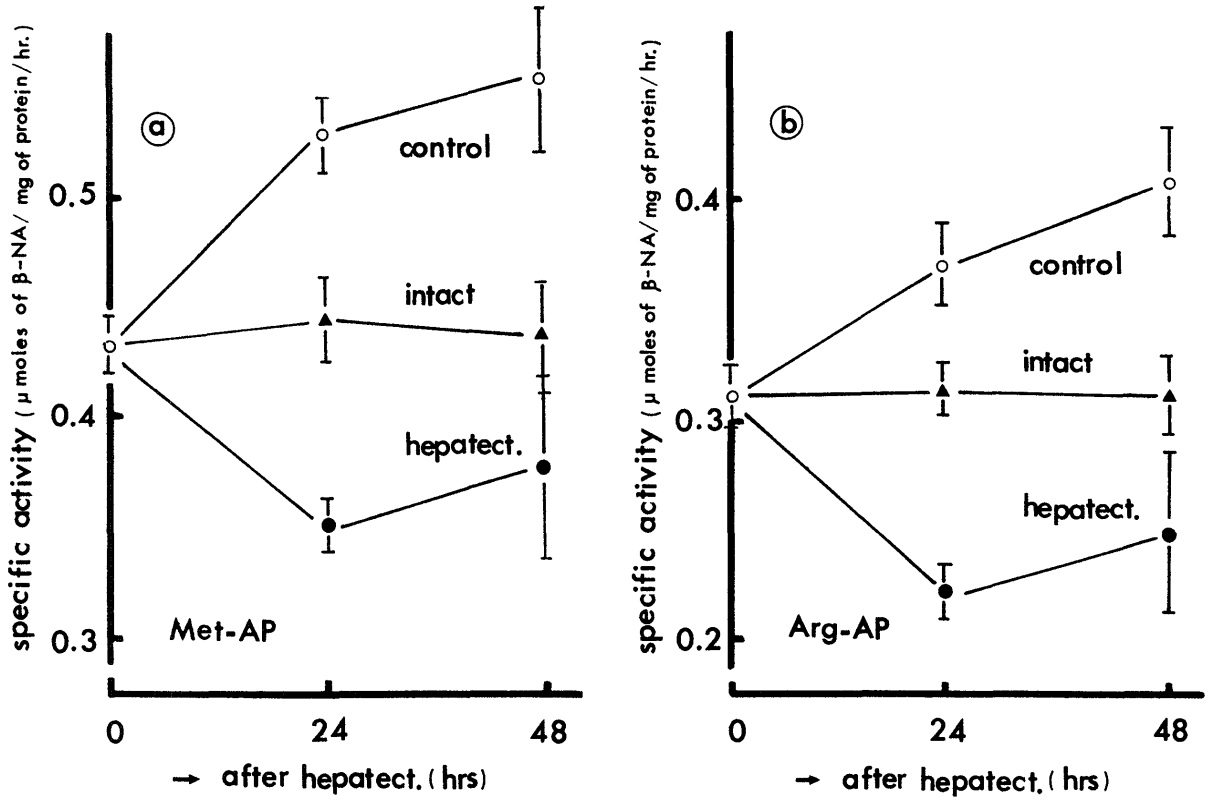


Fig. 10. Change of both methionine-aminopeptidase (a) and leucine-aminopeptidase (b) in partial hepatectomized rat liver (regenerated liver). Hepatect.; partial hepatectomy.

Table 2. Hydrolysis of substrates by each cell-fraction of rat testis ($\mu$ moles of liberated $\beta$-naphthylamide/mg of protein/hr, M±SD)

| Substrate | L-arg-$\beta$-NA | | L-leu-$\beta$-NA | | D.L-met-$\beta$-NA | |
|---|---|---|---|---|---|---|
| | Int. | Crypt. | Int. | Crypt. | Int. | Crypt. |
| Fraction | | | | | | |
| Cytosol (5) | 2.38±0.23 | 3.64±0.11* | 0.51±0.04 | 1.41±0.10* | 0.61±0.08 | 2.28±0.14* |
| Mitochondria (7) | 1.63±0.18 | 2.34±0.06* | 0.41±0.05 | 2.36±0.09* | 0.56±0.07 | 2.42±0.07* |
| Microsome (5) | 1.00±0.02 | 1.65±0.06* | 1.09±0.22 | 2.57±0.14* | 1.28±0.24 | 2.38±0.06* |
| Nucleus (5) | 7.96±0.86 | 2.50±0.16* | 0.88±0.18 | 1.09±0.17$^{ND}$ | 1.02±0.17 | 1.09±0.17$^{ND}$ |

Numbers in parentheses indicate numbers examined in 14 days of cryptorchid testis. Abbreviations used are arg, arginine; met, methionine; leu, leucine; NA, naphthylamide; Crypt., cryptorchid testis and Int., intact testis, respectively. *, Significantly different ($p<0.01$) by student's $t$-test compared with intact testis; ND, not significant.

showed a high methionine-aminopeptidase activity were prominent, although neither the continuous drastic shrinkage of seminiferous tubules nor leukocytes was observed. These histochemical changes were coincident with the data obtained by enzyme assay. The present data obtained by histochemical observation in the rat cryptorchid testis indicate that the increase in activity of methionine- aminopeptidase was due to both hyperplasia of Leydig cells and the increased enzyme activity in Leydig cells. In the data obtained from enzyme assay, the whole homogenate of regenerated livers showed a lower methionine-aminopeptidase activity than that of normal livers or sham-operated rat livers, with evidence of a high enzyme activity. Histochemical investigation showed that regenerated liver cells also had a slightly lower activity of methionine-aminopeptidase and leucine-aminopeptidase than normal cells. The hyperplastic Leydig cells in cryptorchid testes exhibited an increase of

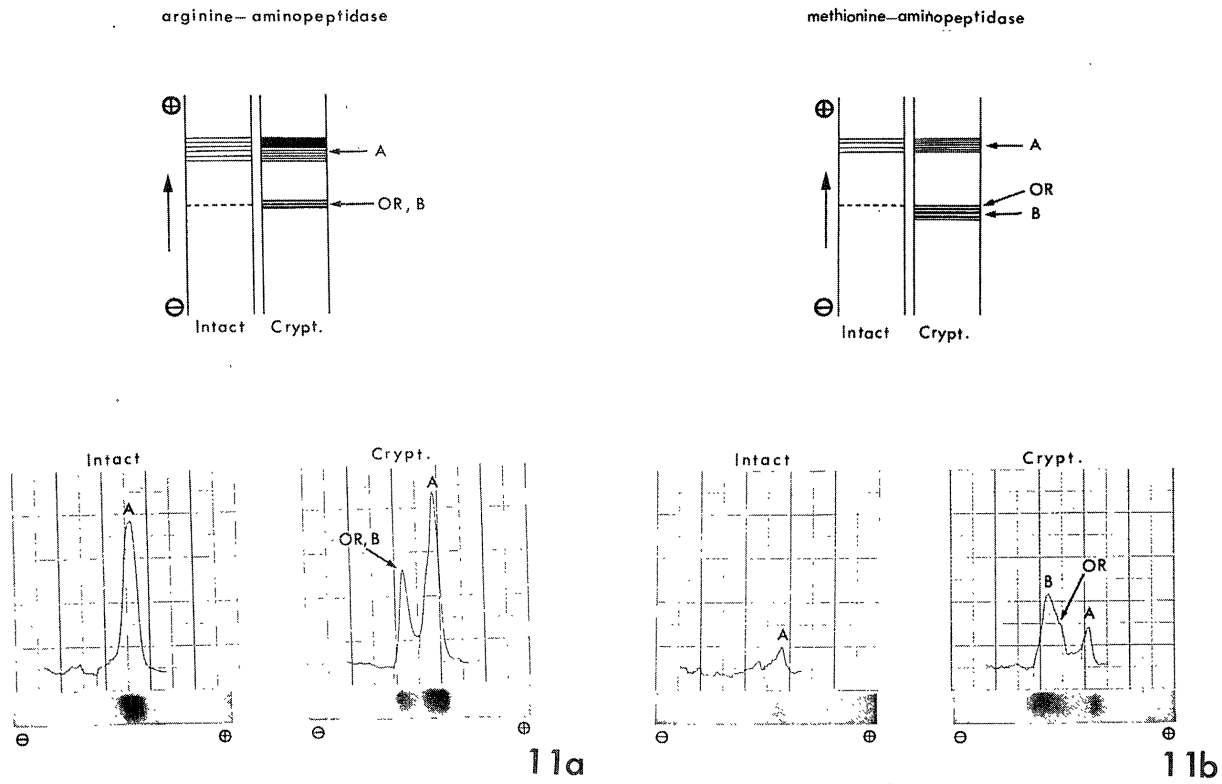


Fig. 11. Zymograms of methionine- (a) and arginine-aminopeptidase (b) in mitochondirial fraction of testicular tissue. Crypt. indicate cryptorchid testis. OR: The area in which the sample was applied.

methionine-aminopeptidase activity with a slight increase of leucine-aminopeptidase activity. In contrast, regenerated hepatic cells showed a decrease in activities of both methionine- and leucine-aminopeptidase. These findings indicate that hyperplasia of Leydig cells in the cryptorchid testis differs from that of hepatic cells in regenerated livers, and suggest that the increase of methionine-aminopeptidase activity in hyperplastic Leydig cells is a specific phenomenon in the cryptorchid testis.

Damber *et al.* [2] have indicated that blood flow to the cryptorchid testis was lower than the normal testis because the cryptorchid contained fewer vessels in the interstitum than the normal testis. DeKretser *et al.* [3] have reported that in *in vitro* experiment hyperplastic Leydig cells of cryptorchid testes produced not only testosterone but also a greater amount of both pregnenolone and estradiol than the normal Leydig cells. DeKretser *et al.* [4] also reported that at 4 weeks after rat cryptorchid testes were surgically induced, a 60 percent decrease occurred in the $^{125}$I-hCG binding of testicular homogenate which showed a true decline in hCG receptors with no change in the affinity constant. It has been also reported that in the rat with the cryptorchidism, the serum testosterone level was reduced while both the levels of serum LH and FSH increased [4]. In surgically-induced rat cryptorchidism, ultrastructural study showed that the principal changes observed were hypertrophy of Leydig cells with respect to cellular size and an increase in quantities of Leydig cell organelle particular to the mitochondria, Golgi membrane and smooth endoplasmic reticulum (ER) [3, 8]. It is well known that both mitochondria and smooth ER are the main sites for steroidogenesis in Leydig cells [14]. In the present investigation, the alteration of aminopeptidase zymogram was prominent in the mitochondrial fraction as well as in the changes of enzyme activities. From these data obtained by the present study and others, the increase in methionine aminopeptidase activity in rat hyperplastic Leydig cells of surgically-induced cryptorchid testes can likely be explained as follows: Despite the fact that the serum contains a higher concentration of LH, only small amounts of LH reach the Leydig cells due to impairment of blood vessels or smaller blood flows compared to normal testes. Then, Leydig cells

become hyperplastic, accompanied by both the structural and functional change of smooth ER and mitochondria in response to a lesser amount of LH to produce steroid hormones. These changes of cell orgenella require or induce the increase in activity of methionine-aminopeptidase.

DeKretser *et al.* [3] mention a similar possibility with the appearing of hyperplastic Leydig cells in cryptorchid testes.

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