Studies on the fate of D-aspartic acid in pineal and pituitary glands of rats after intravenous administration

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Abstract: The present study was intended to confirm the idea that D-aspartic acid (Asp) in pineal and pituitary glands could originate from diet as suggested by our previous report [Imai *et al.* (1997) Proc. Japan Acad. **73B**, 48–52]. When the rats were kept under fasting for 2 or 3 days, the levels of D-Asp, but not L-Asp in these glands were significantly lower as compared with those in the rats with diet. The fact suggests that D-Asp in these glands is derived from the exogenous origin. Next, we determined the D- or L-Asp concentration in these glands (at 30 min, 3 hr and 24 hr) and plasma (2 min - 5 hr) following intravenous administration of D- or L-Asp (50 mg/kg) into rats. D-Asp concentrations remarkably increased after administration of D-Asp, and the levels were almost constant from 30 min to 24 hr after administration, indicating that D-Asp was accumulated and deposited in these glands. In contrast, no accumulation of L-Asp was observed in case of L-Asp or D-Asp administration. In plasma, either D- or L-Asp concentration decreased to the normal level at 3 hr after the administration of the respective enantiomers, although different half-lives for D- and L-Asp in plasma were obtained; 7.84 min for D- and 3.75 min for L-Asp. These results indicate that D-Asp incorporated from extracellular fluids into pineal and pituitary glands is difficult to be metabolized and eliminated, and strongly support the idea that the D-Asp in these glands *in vivo* is derived from the exogenous source, such as dietary D-Asp.

Key words: D-aspartic acid; intravenous administration; fasting; rat; pineal gland; pituitary gland.

Introduction. Although D-amino acid has long been recognized as an unnatural type of amino acid,¹⁾ the recent advances in chromatographic techniques of enantiomeric separation and sensitive detection have allowed the occurrence of D-amino acids in tissues or biological fluids of mammals²⁾ to be found and accurately measured. Among them, D-aspartic acid (Asp)^{3)–8)} and D-serine (Ser),^{7)–10)} have been well studied because of their selective distribution in the mammalian tissues. D-Ser has a potent binding activity to the NMDA strychnine-insensitive glycine binding site in brain and is now considered to be an intrinsic ligand for the NMDA receptor.^{8),9)} Recently, the biosynthesis of D-Ser in rat brain through racemization of L-Ser was also suggested.¹⁰⁾

As for D-Asp, there are some reports on the occurrence in endocrine organs⁵⁾ such as pituitary gland, testis and adrenal gland, and its distinctive pattern of emergence in the life cycle of each respective tissues of

the central nervous system and periphery.⁸⁾ We found an extremely high concentration of D-Asp in rat pineal gland,^{11),12)} and the change of concentration with age was also observed. Hence, D-Asp is considered to be closely related to the genesis or maturation of the mammalian tissues or organs. Recently, Schell *et al.* also reported the occurrence of D-Asp in rat pineal gland,¹³⁾ and considered that D-Asp functions as a novel neurotransmitter, or a modulator of steroid biosynthesis.

However, the origin of D-Asp in several tissues mentioned above is still ambiguous. D'Aniello *et al.* reported that radioactivity was detected in the whole brain of mice fed with the diet containing ³H-D-Asp.⁴⁾ We administered radiolabelled D-Asp (¹⁴C-D-Asp) to rats intravenously, and investigated the distribution of radioactivity by whole body autoradiography (ARG) to identify the tissues concerned.¹⁴⁾ It was found that a high level of radioactivity was incorporated and accumulated in the rat pineal gland, followed by pituitary gland and other peripheral tissues, adrenal gland and testis,¹⁴⁾ where the

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amounts of D-Asp were high *in vivo*.^{5),11),12)} Thus, it was considered that D-Asp present *in vivo* in those tissues is originated from the daily diet containing D-Asp (a rat chow, CE-2, contains 50 nmol D-Asp/g).¹⁵⁾

To further clarify this issue, since there is a possibility that the radioactivity detected might include that of the metabolite, in the present work, we administer cold D- or L-Asp instead of radiolabelled Asp to male rats to investigate the fate of the intact D- or L-Asp in pineal and pituitary glands adopting high-performance liquid chromatographic determination and identification. We also investigate the effect of fasting on the concentration of D-Asp in these tissues *in vivo* to search for its origin. Discussion will be made on the accumulation and elimination of D- or L-Asp in these glands.

Materials and method. *Chemicals.* 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was purchased from Tokyo Kasei, Co., Ltd. (Tokyo, Japan). Sumichiral OA-2500 (S) (250×4.6 mm i.d., 5 μ m, Lot. No. OA251877) and (R) (Lot. No. OA251773) were kindly supplied by Sumika Chemical Analysis Service, Ltd. (Osaka, Japan). A TSK guardgel ODS-80Ts (1.5×3.2 mm i.d., 5 μ m) was from Tosoh, Co., Ltd. (Tokyo, Japan). Methanol (MeOH) and acetonitrile (CH₃CN) were of HPLC grade, and purchased from Kanto Pure Chemicals. Co., Ltd. (Tokyo, Japan). D-Asp was purchased from Sigma, Co., Ltd. (St. Louis, MO, U.S.A.), and L-Asp was from Wako Pure Chemicals. (Tokyo, Japan). Citrate monohydrate used was an analytical grade from Kanto Pure Chemicals.

Animal experiment. Male Sprague-Dawley rats (7-week-old, Nihon Charles River, Kanagawa, Japan) were housed in an environmentally controlled room with a 12 hr light/dark cycle, and provided with tap water and a commercial chow diet (type CE-2, Clea Japan, Tokyo, Japan) *ad libitum*. D- or L-Asp dissolved in saline (Otsuka Pharmaceuticals, Co., Ltd., Tokushima, Japan) was administered via a tail vein (50 mg/10 ml/kg) using a disposable syringe (Terumo, Tokyo, Japan) with a 25 gauge needle. The saline instead of D- or L-Asp solution was administered (10 ml/kg) as a control experiment.

Analysis in pineal and pituitary glands of rats under non-fasting and fasting. Twelve rats were divided into 3 groups, which were non-fasting, fasting for 2 days and 3 days. The rats were anesthetized with diethyl ether, and the blood was drawn from the abdominal aorta, and then, pineal and pituitary glands were removed quickly, and frozen at -20°C until analysis. The glands dissected were homogenized in MeOH with cooling on ice, and then, the homogenate obtained was centrifuged at 3,000 rpm for 5 min. One hundred μ L for pituitary gland and 200 μ L for

pineal gland of the resultant supernatant was evaporated to dryness using a centrifugal evaporator, SPE-200 (Shimadzu, Osaka, Japan). The residue was dissolved in 20 μ L of 0.2 M borate buffer (pH 8.0) containing 4 mM EDTA.2Na, and 30 μ L of 50 mM NBD-F in CH₃CN was added. The reaction mixture was heated at 60°C for 5 min, and 950 μ L of 20 mM citrate in MeOH was added. Five μ L of the solution was injected into the HPLC with Sumichiral OA-2500 (S) or (R).

Analysis in pineal and pituitary glands of rats after administration of D- or L-Asp. In this experiment, all rats used were under fasting for 2 days, and 3 rats were sacrificed at time points of 30 min, 3 hr and 24 hr after administration. The rats were anesthetized with diethyl ether, and the blood was drawn from the abdominal aorta and then, pineal gland and pituitary gland were removed quickly, and frozen at -20°C until analysis. The glands dissected were treated in a similar manner described above.

Analysis in plasma of rats after administration of D- or L-Asp. At each time point of 2, 15, 30, 60 min, 2, 3 and 5 hr for D-Asp, and of 2, 15, 30 min and 3 hr for L-Asp administration, 0.5 ml of blood was drawn from a jugular vein using a heparinized syringe with a 25 gauge needle (N=3). Subsequently, the plasma sample was obtained by centrifugation of the blood at 4,000 rpm for 15 min. Fifty μ L of plasma was added to 450 μ L of MeOH, and mixed vigorously. One hundred μ L of the resultant supernatant was evaporated to dryness using SPE-200, and the residue was treated in a similar manner described above.

HPLC apparatus. HPLC used in this study consists of an intelligent pump, BIP-1 (Jasco, Tokyo, Japan), a fluorometric detector, L-7480 (Hitachi, Tokyo, Japan) and a chromatointegrator, D-7500 (Hitachi, Tokyo, Japan). A TSK guardgel ODS-80Ts was connected to the top of the chiral column [Sumichiral OA-2500 (S) or (R)]. The mobile phase employed was 8.0 mM citric acid in MeOH for the analysis of pineal gland and 6.5 mM for pituitary gland and plasma. The flow rate was at 1.0 ml/min, and the column temperature was ambient. The fluorometric detection was made at 530 nm with 470 nm as an excitation wavelength.

Calibration curve. Standard solutions of D- and L-Asp were prepared at the concentration of 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 μ M in 0.2 M borate buffer (pH 8.0) containing 4 mM EDTA.2Na. Twenty μ L of the solution was treated in the same way as for the sample from the biological materials.

Statistical analysis. All data cited in the text were expressed as mean $\pm$ SD, and significant differences between the groups were determined by Duncan's multiple

range test. Half-life of D- or L-Asp in plasma was calculated by a computer software, MULTI using damping Gauss-Newton method (1-compartment model).

Results. As shown in Table I, the D-Asp concentrations in pineal and pituitary glands decreased significantly under fasting for 2 or 3 days, as did the body weights. In contrast, L-Asp concentration did not change even at 2 or 3 days. These data support the idea that D-Asp in pineal and pituitary glands occurring *in vivo* may be derived from external source, since the daily diet contains D-Asp,¹⁵⁾ whereas L-Asp may be from the product of *in vivo* biosynthesis as well as from diet.

Considering the results, the next experiment of the intravenous administration of D-Asp or L-Asp into the rat fasted for 2 days was performed in order to augment the incorporation. When 5 mg of D-Asp / kg was administered, no difference from the basal level of D-Asp concentration in these glands were detected. Thus, a dose of 50 mg of D- or L-Asp / kg was administered intravenously via the tail vein. Fig. 1 shows the typical HPLC [Sumichiral OA-2500(S)] chromatograms of the methanol extracts obtained from pineal gland at 30 min after the administration of saline (control) (a), D-Asp (b) and L-Asp (c), respectively. The increased large peak corresponding to D-Asp derivative was clearly observed in the extract from pineal gland of rat to which D-Asp was administered (b). The peak areas for L-Asp in the chromatograms obtained from every rats to which D-Asp or L-Asp was administered were similar to those obtained from the control rats. The peak was identified as NBD-D-Asp or -L-Asp because of the same retention times as the standard NBD-D-Asp or -L-Asp and also the same retention times (48 min and 44 min, respectively) as the standard by using the chiral column having the opposite chiral center [Sumichiral OA-2500(R)].

The amounts of D- and L-Asp obtained in these glands are summarized in Fig.2. As shown in Fig.2 (upper part), D-Asp concentrations in pineal gland increased extraordinarily from the control level ($p < 0.01$), and continued to be

high at the time points examined (3 and 24 hr). The same trend was also true for D-Asp concentration in pituitary gland although the level slightly decreased at 24 hr (Fig.2, lower part). The data indicate that the D-Asp incorporated was stable for a long period of time in these glands. In the case of L-Asp administration, the accumulation of L-Asp was not observed at any time points examined in the glands. The data also show that the administration of D- or L-Asp did not affect so much the concentration of L- or D-Asp, respectively, in both the glands. Although D'Aniello *et al.* mentioned the increase and long deposit of D-Asp in pituitary gland and testis of rats with an intraperitoneal administration of D-Asp (0.5–4.0 $\mu\text{mol/g}$ body weight), no quantitative data on time-course were reported in the text.⁶⁾

The time-course studies on D- or L-Asp in plasma revealed that the elimination rate of D-Asp in plasma was apparently slower than that of L-Asp, whereas the levels of both Asp at 2 min were almost identical (Fig. 3). The half-life of D-Asp in plasma was 7.84 min and its concentration continued to decrease gradually at least until 24 hr to reach the basal level (almost null). In contrast, the half-life of L-Asp was 3.75 min and its concentration reached at a steady state from 30 min, which was within the normal range of its concentration of 6.90 ± 2.75 nmol/ml plasma obtained by the control experiment with saline administration. Although D-Asp concentrations in plasma after 3 hr were already very low as compared to those at 30 min, those in pineal and pituitary glands at 3 and 24 hr were quite similar to those at 30 min (Fig. 2). The data suggest that D-Asp was incorporated from plasma into these glands and stayed for a long period of time in them.

Discussion. In the previous study, we administered ^{14}C -D-Asp into rats intravenously and investigated its distribution into the tissues by whole body ARG. The results showed that radioactivity was accumulated and deposited in peripheral tissues such as adrenal and testis and the distinct parts of brain, pineal and pituitary glands.¹⁴⁾ Considering the data, it was assumed that a part

Table I. D- and L-Asp concentration in pineal and pituitary glands as well as body weight of rats under fasting for 2 and 3 days

	Pineal gland		Pituitary gland		Body Weight (g)
	D-Asp	L-Asp	D-Asp	L-Asp	
	(nmol/glands)		(nmol/glands)		
Control	6.70 $\pm$ 3.58	3.81 $\pm$ 0.78	2.45 $\pm$ 1.05	13.98 $\pm$ 3.76	210 $\pm$ 10
Fasting for 2 days	2.53 $\pm$ 0.90*	5.70 $\pm$ 1.71	0.14 $\pm$ 0.07**	13.82 $\pm$ 2.60	168 $\pm$ 6**
Fasting for 3 days	2.38 $\pm$ 1.00*	1.79 $\pm$ 0.76	0.58 $\pm$ 0.18**	12.20 $\pm$ 3.71	127 $\pm$ 5**

All values are expressed as mean $\pm$ SD (N = 4). *: $p < 0.05$, **: $p < 0.01$ VS. control group.

Fig. 1. Chromatograms of MeOH extracts obtained from pineal glands of rats at 30 min after administration of saline (10 ml/kg) (a), D-Asp (50 mg/kg) (b) and L-Asp (50 mg/kg) (c), respectively.

Fig. 2. D- and L-Asp concentration in pineal (upper) and pituitary glands (lower) of rats at 30 min, 3 hr and 24 hr after intravenous administration of saline (10 ml/kg), D-Asp (50 mg/kg) and L-Asp (50 mg/kg). **; $p < 0.01$ versus control.

of D-Asp present *in vivo* in these tissues originated from an exogenous source, such as the daily food intake which contained D-Asp. In order to further explore the origin of D-Asp, in the present work, the effect of fasting was first investigated on the concentration of D-Asp present *in vivo* in pineal and pituitary glands.

The decrease of D-Asp concentration was clearly observed by fasting since the values 6.70 ± 3.58 decreased to 2.38 ± 1.00 nmol/pineal gland ($p < 0.05$), and 2.45 ± 1.05 to 0.58 ± 0.18 nmol/pituitary gland ($p < 0.01$, Table I), supporting further the idea that D-Asp in the diet is one of the supply source of D-Asp in pineal and pituitary glands.

Fig. 3. D- and L-Asp concentration in plasma of rats after intravenous administration of D-Asp (50 mg/kg) (a) and L-Asp (50 mg/kg) (b).

However, the fact that the level did not reach to null even after 3 days fasting suggests that the possibility of biosynthesis *in vivo* could not be excluded. Although Dunlop and Neidle¹⁰⁾ reported recently that D-Ser was produced from L-Ser in the rat forebrain *in vivo*, to our knowledge, there is no report on the biosynthesis of D-Asp in mammals. Further studies have to be taken to clarify the issue of biosynthesis.

No remarkable change of L-Asp concentration observed under fasting may be attributed to the biosynthesis of L-Asp *in vivo*, or the release of L-Asp from a storage site in the other tissues such as liver. In the previous work, it was not clarified whether the long deposit of radioactivities in pineal and pituitary glands were derived from ¹⁴C-D-Asp itself or from the radioactive metabolite(s).¹⁴⁾ Thus, in this experiment, the fate of D-Asp itself was examined by HPLC after intravenous administration of cold D-Asp. As was observed in the previous experiment, a large amount of D-Asp already appeared and accumulated in pineal and pituitary glands at 30 min after the administration. The chemical identification clearly demonstrated the long deposit of D-Asp in both the glands. The levels in these glands hardly changed from 30 min to 24 hr (16.7–16.5 nmol/pineal glands, 14.0–7.22 nmol/pituitary gland), although the plasma concentration of D-Asp approached to the control level from 3 hr after administration, indicating that D-Asp once incorporated from an extracellular fluid to both the glands was difficult to be metabolized and eliminated.

As for the incorporation of D-Asp from extracellular fluids to pineal and pituitary glands, it was suggested recently that it was mediated by the transporter for L-glutamate (Glu) in plasma membrane of pinealocyte cultured in the medium.¹⁶⁾ The *in vivo* incorporation of D-

Asp from extracellular fluids into both the glands might be attributed to the same transporter.

In the case of L-Asp administration, no accumulation in these glands were observed. This is mainly due to that L-Asp is metabolized instantaneously to the other substances in mammalian body. Moreover, the administration of L-Asp gave no detectable change of D-Asp concentration in pineal and pituitary glands, indicating that the possibility of *in vivo* epimerization of L-Asp to D-Asp in the glands could be excluded.

These results obtained in the present study indicate that D-Asp once incorporated from extracellular fluids to pineal and pituitary glands is difficult to be metabolized and eliminated, whereas L-Asp is immediately metabolized to the other compounds. The resistance to the leak of the incorporated D-Asp was also verified by *in vitro* experiment where only 50 μ M digitonin which permeabilize the plasma membrane allowed the release of D-Asp from pinealocyte to the outer medium.¹⁶⁾

Further studies are now underway on the metabolism of D-Asp in pineal and pituitary glands and the details will appear elsewhere.

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