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THE PRESENCE OF FREE D-ASPARTIC ACID IN RODENTS AND MAN

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Free D-aspartic acid is present in appreciable quantities in the brain and other tissues of rodents and in human blood. In the newborn rat, the highest concentration of D-aspartic acid was found in cerebral hemispheres, where, at 164 nmol/g (8.4% of the total aspartic acid), the level of D-aspartic acid exceeds that of many essential L-amino acids. The highest ratio of D- to total aspartic acid (38%) occurred in neonatal blood cells. In the adult rat, the highest concentration was present in the pituitary gland (127 nmol/g, 3.8%). Within the central nervous system marked regional differences are present and characteristic changes with development take place. In general, the levels of D-aspartic acid fall rapidly with increasing age. In cerebral hemispheres adult values (13 nmol/g, 0.43%) are approached within one week. D-aspartic acid concentrations may also be higher in young humans since fetal blood, taken from placental cord, contains 2.6 nmol/g (4.9%) of D-aspartic acid, a value five times that of adult human blood. These distributional patterns and developmental changes may be the result of differences in the ability of various tissues to dispose of an extraneous metabolite, or, reflect alterations in a specific functional requirement for D-aspartic acid.

D-Asparty1 residues have been found in long-lived proteins (1) (including white matter of human brain (2)), where they presumably arise by racemization in situ. As part of a study of the pathological changes in protein bound D-aspartic acid that might occur during aging, the concentrations of the free D-isomer were measured in human and animal tissues. For this purpose a highly sensitive method for the resolution of D,L-aspartic acid, based on the preparation of fluorescent, asymmetric derivatives, and their separation by HPLC, was developed. In all of the tissues examined, we found what appeared to be significant concentrations of D-aspartic acid, with some exceptionally high levels in those from very young animals and in the pituitary from adult rats. The values found greatly exceeded those that might result from

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degradation of long-lived proteins. These results were unexpected since D-amino acids in general are found only rarely in animal tissues (3) and the occurrence of free D-aspartic acid has not previously been reported. We now provide evidence which supports our identification of this widely distributed tissue component as D-aspartic acid, describe its localization, and provide data on changes in its concentration that occur during development.

METHODS AND MATERIALS

Preparation and assay of naphthylethyl carbamoyl amino acids. Details of this and the following procedures will be published elsewhere. In brief, the effluent from ion-exchange columns containing amino acids in lithium citrate buffers was neutralized and mixed with an equal volume of 0.4 M sodium borate, pH 9.0. Two volumes of naphthylethyl isocyanate (16 mg/ml in dry acetone) were added and after five minutes the precipitate of symmetrical naphthylethyl urea was removed by centrifugation. The supernatant was extracted three times with cyclohexane-ether (1:1), and the aqueous layer was frozen until used for HPLC analysis. The naphthylethyl carbamoyl D- and L-aspartic acids were resolved on a reversed phase C_{18} column (Serva Si 100 Polyol) in 0.1 M acetic acid with a flow rate of 1 ml/min. A fluorescence monitor was used for quantitation (excitation, 229 nm; emission, 320 nm and above).

Preparation and assay of naphthyl carbamoyl aspartic acids. Tissues were homogenized in 9 volumes of 3% $HClO_4$. After centrifugation to remove the protein, the supernatant was neutralized with 5 M KOH, chilled and centrifuged. To 200 µl of the supernatant, 50 µl of pH 6.2 Na borate (1 M) was added followed by 250 µl of dry acetone containing 2.5 µl of naphthyl isocyanate per ml. The solution was mixed thoroughly and after 45 sec extracted three times with 2 ml of heptane. An acidified portion was chromatographed on a C_{18} column (Serva Si 100 Polyol) in 7.5 mM NaH_2PO_4 buffer, pH 6.4, 1 ml/min. The naphthyl carbamoyl aspartic acid, eluted in 15 minutes, was detected by fluorescence (229 nm excitation, 320 nm emission). A fraction of the collected derivative was acidified and rechromatographed on a C_{18} column with an eluent containing 3 mg/ml of L-asparty1-L-phenylalanine methyl ester in 50 mM Na acetate, pH 6.1. The column becomes saturated with peptide in 1 h at 1 ml/min and will then resolve the D- and L-naphthyl carbamoyl aspartic acids.

Sprague Dawley rats and BALB/cBy mice were used in these experiments.

RESULTS AND DISCUSSION

In our initial experiments, amino acids were extracted from tissues, separated by conventional ion exchange techniques, and derivatized with (+)-1-(1-naphthyl)ethyl isocyanate. The resulting carbamoyl diastereoisomers were resolved by HPLC and detected fluorometrically. About 8% of the derivatized aspartic acid from newborn rat brain coeluted with naphthylethyl carbamoyl D-aspartic acid. Other D-amino acids including glutamic acid, valine, isoleucine, and phenylalanine were present only in trace amounts (<0.2%).

In order to confirm the identity of naphthylethyl carbamoyl D-aspartic acid, the (-) isomer of naphthylethyl isocyanate was used for preparing the derivatives. As expected, the elution times of the D- and L-isomers were interchanged. In addition, derivatives were prepared with optically inactive l-naphthyl isocyanate, either from perchloric acid extracts of tissues, or from aspartic acid first separated by ion-exchange chromatography. Naphthyl carbamoyl aspartic acid was isolated by reversed phase HPLC and then resolved on a C_{18} column saturated with L-aspartyl-L-phenylalanine methyl ester. This procedure, in which the separation is independent of the purity of the reagents, also confirms the identity of D-aspartic acid, as does the orthophthalaldehyde-N-acetyl-L-cysteine procedure of Aswad (4).

A more detailed study of D-aspartic acid distribution in young and adult rats was then carried out (Table 1). Levels ranging from 2-128 nmol/g were found. In the young rat the highest concentrations were found in the brain, with decreasing levels in kidney, liver, and blood. Within the brain,

Table 1
The concentration of D-aspartic acid and the percent D isomer of total aspartic acid in various tissues of young and adult rats

	Blood	Cerebral Hemisphere	Cerebellum	Spinal Cord	Pituitary	Liver	Kidney
	Young (36 hours old)						
D-aspartic acid nmol/g*	8.9 ± 1.0	100 ± 22	28 ± 1	38 ± 1	19 ± 5	28 ± 4	49 ± 1
% D	16.0 ± 2.2	5.1 ± 1.1	1.9 ± 0.5	2.0 ± 0.4	3.8 ± 0.6	0.9 ± 0.2	1.4 ± 0.5
Tissue conc. / Blood conc.	-	11.2	3.1	4.2	2.1	3.1	5.5
	Adult (55 days old)						
D-aspartic acid nmol/g	2.2 ± 0.3	13 ± 2	22 ± 5	3.3 ± 0.8	127 ±45	11 ± 3	12 ± 2
% D	4.5 ± 0.5	0.43 ± 0.08	0.79 ± 0.08	0.13 ± 0.03	3.8 ± 1.3	1.0 ± 0.4	0.5 ± 0.1
Tissue conc. / Blood conc.	-	5.9	10	1.5	57.7	5.0	5.4

*Assayed as naphthyl carbamoyl aspartic acid. Means ± standard deviations, N>3.

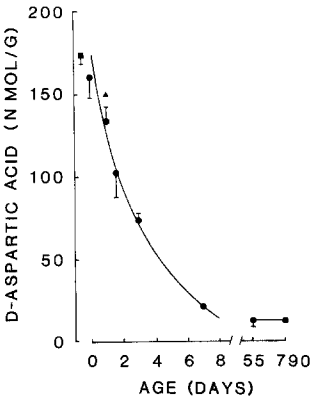

Fig. 1. Changes with age in the D- aspartic acid concentration of rat cerebral hemisphere. Rat, ●; fetal rat, approximately 19 days gestation, ■ ; mouse, ▲ . Bars indicate the standard deviation.

D-aspartic acid was not uniformly distributed, the major portion being present in the cerebral hemispheres (100 nmol/g). Significant concentrations were also found in the cerebellum, spinal cord, and pituitary. All of these values greatly exceeded the blood level. In general, D-aspartic acid concentrations decrease with increasing age. For example, in cerebral hemispheres, the concentration approaches the adult value within one week (Fig. 1). There are, however, large regional differences within the CNS in age-related changes. The decrease in the D-aspartic acid concentration in the cerebral hemisphere contrasts with the dramatic increase in the pituitary and with the relatively constant level present in the cerebellum.

Regardless of the source of tissue D-aspartic acid, cellular levels are likely to be influenced by the concentration in blood. Levels in plasma and packed cells were therefore determined in newborn and adult rats (Table 2). Both D- and L-aspartic acid were found to be sequestered in the packed cells, D-aspartic acid to a greater extent than the L-isomer. As a result, higher D/L ratios were found in packed cells then in whole blood. In blood cells from newborn rats the fraction of D exceeded 37%. Since the ratio of the plasma concentration of D-aspartic acid to that of whole blood is approximately 1 to 4, tissue-to-plasma ratios of about 40 to 1 are present in newborn cerebral hemisphere and 200 to 1 in adult pituitary. These values suggest the

Table 2
Distribution of D-aspartic acid in rat blood

	Newborn*			Adult**		
	Whole blood	Plasma	Packed cells	Whole blood	Plasma	Packed cells
D-Aspartic Acid nmol/g	19.3 ± 4.4	3.8 ± 1.1	35.5 ± 9.2	3.2 ± 0.9	0.9 ± 0.4	6.5 ± 2.4
% D	29.3 ± 1.3	10.3 ± 3.8	37.7 ± 4.3	6.2 ± 0.3	2.6 ± 0.1	7.2 ± 0.3

*5-10 hours old. **Female, 150-187 g.
Values are the means ± the standard deviation of 3 or more determinations.
The hematocrits were 0.503 for young and 0.453 for adult rat blood.

presence of various transport and compartmentation mechanisms analogous to those which regulate the concentrations of the L dicarboxylic amino acids.

To determine whether these developmental changes might also be relevant to humans we measured D-aspartic acid in adult and fetal blood obtained from placental umbilical cord (Table 3). Although the concentration of the D-isomer in fetal blood was lower than found in the rat, it nevertheless was five times higher than that of adult human blood. The human fetus may therefore also develop with appreciable concentrations of D-aspartic acid present in the nervous system. In a single sample of amniotic fluid (19 weeks gestation) 2.8% of the aspartic acid was found to be D, indicating that elevated levels of D-aspartic acid may be present during a large portion of the human gestation period.

These observations, that D-aspartic acid is present in significant quantities in mammalian tissues and that it exhibits characteristic developmental

Table 3
D-aspartic acid in human blood

	Fetus*	Pregnant female+	Adult male
D-aspartic acid nmol/g	2.6 ± 1.1	0.6 ± 0.2	0.4 ± 0.2
% D	4.9 ± 1.8	0.85 ± 0.19	0.57 ± 0.12

*Collected from the placental cord after birth.
+Taken from the corresponding mothers upon admission to hospital for delivery.
Mean ± standard deviation. N > 3.

and regional profiles, raise questions regarding its metabolism and function. Metabolic questions concern whether the D-aspartic acid arises from the diet or intestinal bacteria or is synthesized within the organism and whether the specific D-aspartic acid oxidase present in brain (5) plays a significant role in its degradation. As to its possible function, a decade ago Davies and Johnston (6) suggested that D-aspartic acid could be a natural neurotransmitter. They found, however, that, if present in the cat or rat CNS, the level of D-aspartic acid was less than one percent of that of the L-isomer. We find that this upper limit is essentially correct for whole brain from adult rats, but the concentration is much higher in newborn brain, and even in the adult, D-aspartic acid can be concentrated in particular brain areas, such as the pituitary. D-aspartic acid, because of its relatively low metabolic rate, has often been used as a marker for studying the high-affinity binding and transport of excitatory amino acids (7,8,9). The endogenous ligands of these systems have been assumed to be L-glutamic or L-aspartic acid. It seems possible now that D-aspartic acid itself could play a role in some of these processes.

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