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Whole body autoradiographic study on the distribution of ^{14}C-D-serine administered intravenously to rats

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Summary. The distribution of radioactivities in rats following intravenous administration of ^{14}C-D- or -L-serine was investigated by whole body autoradiography. The radioactivities were distributed throughout the whole body in both cases with the greatest amount being found in the pancreas. D- and L- Serine levels in the pancreas were determined by high-performance liquid chromatography with a chiral column which revealed, for the first time, the existence of D-serine in the rat pancreas (12.6 ± 7.90 nmol/g wet tissue) together with a much higher concentration (924 ± 116 nmol/g) of L-serine. The results suggested that exogenous D-serine of dietary origin contributed at least in part to the D-serine levels found in mammalian tissues.

The accumulation of radioactivity in the kidney, especially in the corticomedullary area, even at 24 hr after administration of ^{14}C-D-serine suggested a possible link between acute necrosis of the renal proximal tubules and the administration of a large dose of D-serine [Am J Pathol 77: 269–282 (1974)].

Keywords: Amino acids – ^{14}C-D-Serine – Rat – Whole body autoradiography – Accumulation – Kidney

Introduction

D-Amino acids are found widely in mammalian tissues and body fluids. D-Aspartic acid and D-serine, which localize in the distinct parts of the brain and peripheral tissues, are now considered to have some biological significance (refer to Imai et al., 1996). The transient emergence of D-aspartic acid observed in the rat brain and peripheral tissues suggested its relation to the morphological and functional development of the tissues (Dunlop et al., 1986; Neidle et al., 1990; Hashimoto et al., 1990; Imai et al., 1995; Hamase et al., 1997). D-Serine was believed to serve as an intrinsic ligand for the N-methyl-

D-aspartate (NMDA) receptor (allosteric effect) in the forebrain (Hashimoto et al., 1993; Hashimoto et al., 1995a; Schell et al., 1995).

The origin of the localized D-amino acids in the mammalian tissues, however, is not yet fully understood. Some of the D-aspartic acid present *in vivo* in rat tissues were considered to be of dietary origin because, 1) the diet contains D-aspartic acid (50 ± 1 nmol/g) (Kera et al., 1995), 2) the orally administered ^{3}H-D-aspartic acid in the animal (mice in this case) was absorbed from the digestive tract, appeared in the blood stream and distributed in the whole brain, liver and kidney (D'Aniello et al., 1993), and 3) the radioactivity derived from ^{14}C-D-aspartic acid administered intravenously (i.v.) in the rat was incorporated and accumulated into the distinct tissues such as pineal and pituitary glands which contain a large amount of D-aspartic acid *in vivo* (Imai et al., 1997). So far there have been no reports on the *de novo* biosynthesis of D-aspartic acid in the mammals.

On the other hand, although D-serine is present *in vivo* in the mammalian tissues (cerebrum, cerebellum, pituitary, kidney, liver, adrenal, testis, lung, heart and thymus) (Hashimoto et al., 1995b), its origin has not yet been clarified. Recently, D-serine in the brain was reported to be *de novo* synthesized through L-phosphoserine (Wood et al., 1996), by a specific racemase (Dunlop and Neidle, 1997) or by the combination with glycine cleavage system (Schell et al., 1995; Iwama et al., 1997). However, sufficient evidences to support this hypothesis have not yet been accumulated. Considering these, in this paper, we investigate the radioactivity distribution of ^{14}C-labelled D-serine into rat tissues after i.v. administration using a whole body autoradiography as reported in the previous paper (Imai et al., 1997).

Materials and methods

Chemicals

1-^{14}C-D-Serine and 1-^{14}C-L-serine were purchased from American Radiolabelled Chemicals Inc. (MO, USA). Both of the specific activities were 2.04 GBq/mmol. Their radiochemical purities checked by paper chromatography were greater than 99%. Carboxymethylcellulose (CMC) was of special grade from Katayama Chemical Co., Ltd. (Osaka, Japan). 4-Fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). A TSK guardgel ODS-80 Tm was from Tosoh Co., Ltd. (Tokyo, Japan). Sumichiral OA-2500 (R) was a gift from Sumika Analytical Center, Co., Ltd. (Osaka, Japan). Methanol and acetonitrile were of HPLC grade from Kanto Chemical Co., Ltd. (Tokyo, Japan). The other reagents used were of analytical grade.

Administration to rats

The male Sprague-Dawley rats (7-week-old, 265–275 g, Charles River Japan Inc.) were allowed to feed (CE-2, Clea Japan Inc., Tokyo, Japan) and drink *ad libitum* in an environmentally controlled room with 12-hour light/dark cycle. The 0.01 N hydrochloric acid solution of ^{14}C-D-serine or ^{14}C-L-serine was neutralized with 0.01 N sodium hydroxide and diluted to 0.4 μmol/ml with physiological saline. The intravenous administration was made into the right femoral vein (740 KBq/kg) under anesthesia with diethyl ether.

Whole body autoradiography

At 30 min and 24 hours after administration, the rats were anesthetized with diethyl ether and immediately frozen by immersion into a mixture of dry ice-acetone (−70°C) and stored at −20°C for 2 days. Then, they were embedded in 3% CMC in H_2O and placed on a microtome stage. The sagittal sections of 40 μm in thickness were prepared by an autocryotome (Cryomacrocut, Leica Instruments GmbH, Nussloch, Germany) with an adhesive tape (Sumitomo 3M Co. Ltd., Tokyo, Japan). The sections were freeze-dried, wrapt in Milar film (Nakagawa, Tokyo, Japan), and in contact with the imaging plates made from the photo-stimulable phosphor, BaFBr: Eu^{2+} (Fuji Photo Co., Ltd., Tokyo, Japan). They were exposed for 3 days at 4°C in a lead case without lightening. The imaging plates were then analyzed using a Bioimaging analyzer (Bas-2000, Fuji Photo Film Co., Ltd., Tokyo, Japan). The autoradiograms of the whole body thus obtained were quantitated with the calibration curve of the standard of ^{14}C using the Bio-imaging analyzer.

Identification and quantification of D-*serine in pancreas in vivo*

The identification and determination of D-serine in pancreas were carried out in a similar way as described in the previous reports (Imai et al., 1995; Imai et al., 1997; Hamase et al., 1997). The male Sprague-Dawley rats (7-week-old) were anesthetized with diethyl ether and sacrificed by withdrawing blood from the abdominal aorta. Then, pancreas was immediately removed, and homogenized in 0.15 M KCl (10 ml/g) under ice cooling after weighing and mincing. Free amino acids in the homogenate were extracted with the addition of 9 volumes of methanol. After centrifugation at 10,000 rpm for 5 min, the supernatant (200 μl) was transferred to a microtube and evaporated to dryness by a centrifugal evaporator, SPE-200 (Shimadzu, Tokyo, Japan). The residue was dissolved in 40 μl of 0.2 M borate buffer (pH 8.0), added with 30 μl of 50 mM NBD-F in acetonitrile and heated at 60°C for 2 min. The reaction mixture was then mixed with 70 μl of 1.0% acetic acid in methanol and filtered through a 0.5 μm membrane filter (Column guard LCR 4, Nihon Millipore, Tokyo, Japan). The filtrate (20 μl) was injected into the following HPLC.

The HPLC system consisted of a Hitachi L-6200 Intelligent Pump, a 7161 injector (Rheodyne, Cotati, CA, USA), an F-1000 Fluorometric Detector and a D-2500 Chromato-Integrator. The fluorescence detection was made at 530 nm with 470 nm as an excitation wavelength. The flow rate was at 1.0 ml/min, and the column temperature was ambient. The NBD-serine was identified and quantified by reversed-phase (TSKgel ODS-80Ts, Tosoh Co., Ltd., Tokyo, Japan) HPLC (Hamase et al., 1997), and the corresponding fluorescent peak fraction (retention time; 18.9 min) was collected and dried by the centrifugal evaporator. The residue obtained was dissolved in the mobile phase (25 mM ammonium acetate in methanol) for the enantiomeric separation of D, L-serine (retention time; 19.2 min for L-serine and 22.0 min for D-serine) to obtain its D/L ratio on a Pirkle type stationary phase column (Sumichiral OA-2500(R)).

Results and discussion

In the previous experiment in which ^{14}C-D-Asp was administered intravenously into the rats and whole body autoradiograms were obtained at 30 min, 3 hr and 24 hr after the administration (Imai et al., 1997). A great incorporation of radioactivity was observed at 30 min, and the radioactivity was reduced greatly at 3 hr followed by a slight reduction at 24 hr. Therefore, in this experiment, we took the periods for investigation at 30 min and 24 hr for comparison. The whole body autoradiograms obtained at 30 min and 24 hr after i.v. administration of ^{14}C-D-serine into 7-week-old rats are shown in Fig. 1a and Fig. 1b. Those obtained after identical treatment with ^{14}C-L-serine are used as references (Fig. 2a and Fig. 2b). The radioactivities obtained at 30 min

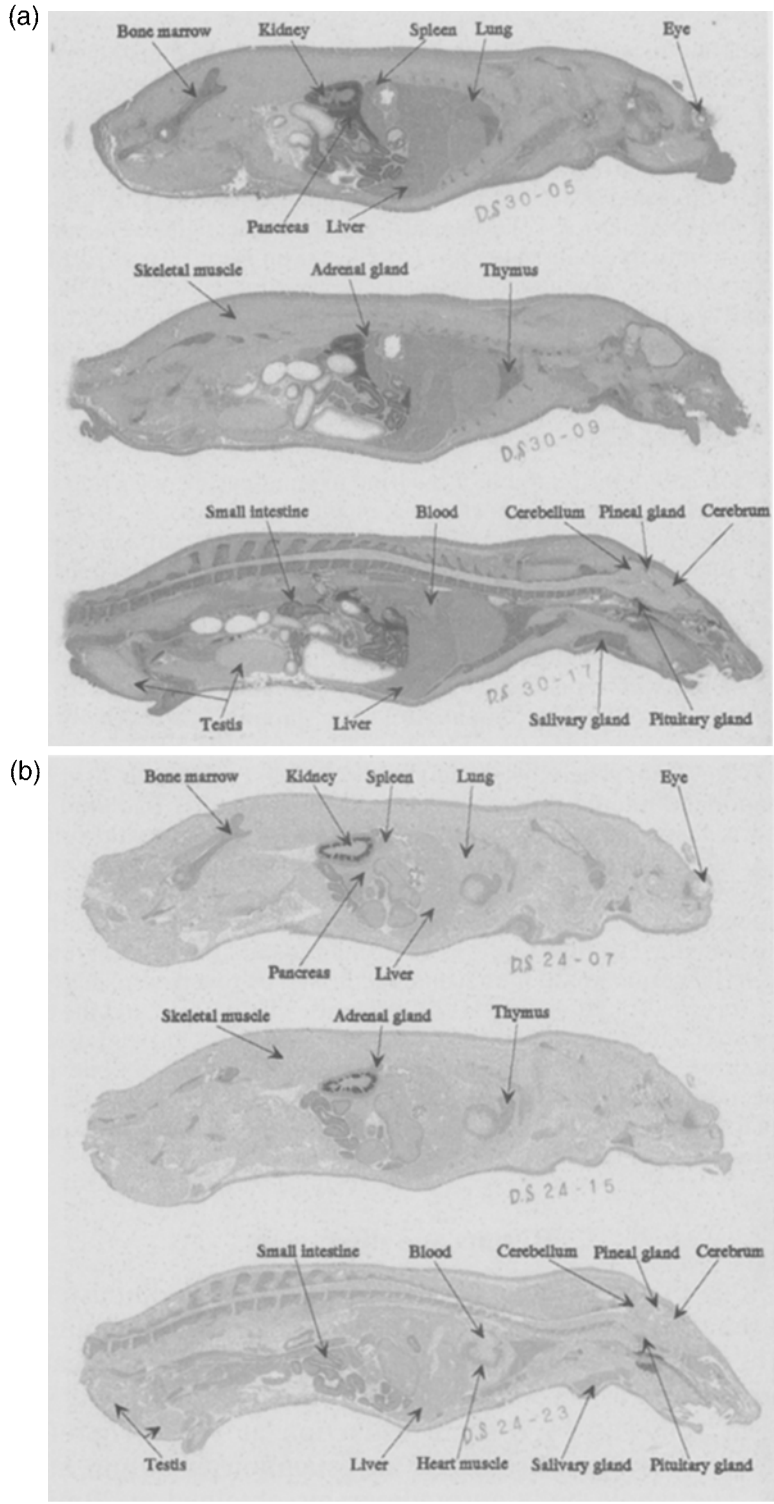

Fig. 1. Whole body autoradiograms of rats (Sprague-Dawley, 7-week-old, male) at a time point of 30 min (**a**) and 24 hr (**b**) after intravenous administration of ^{14}C-D-serine

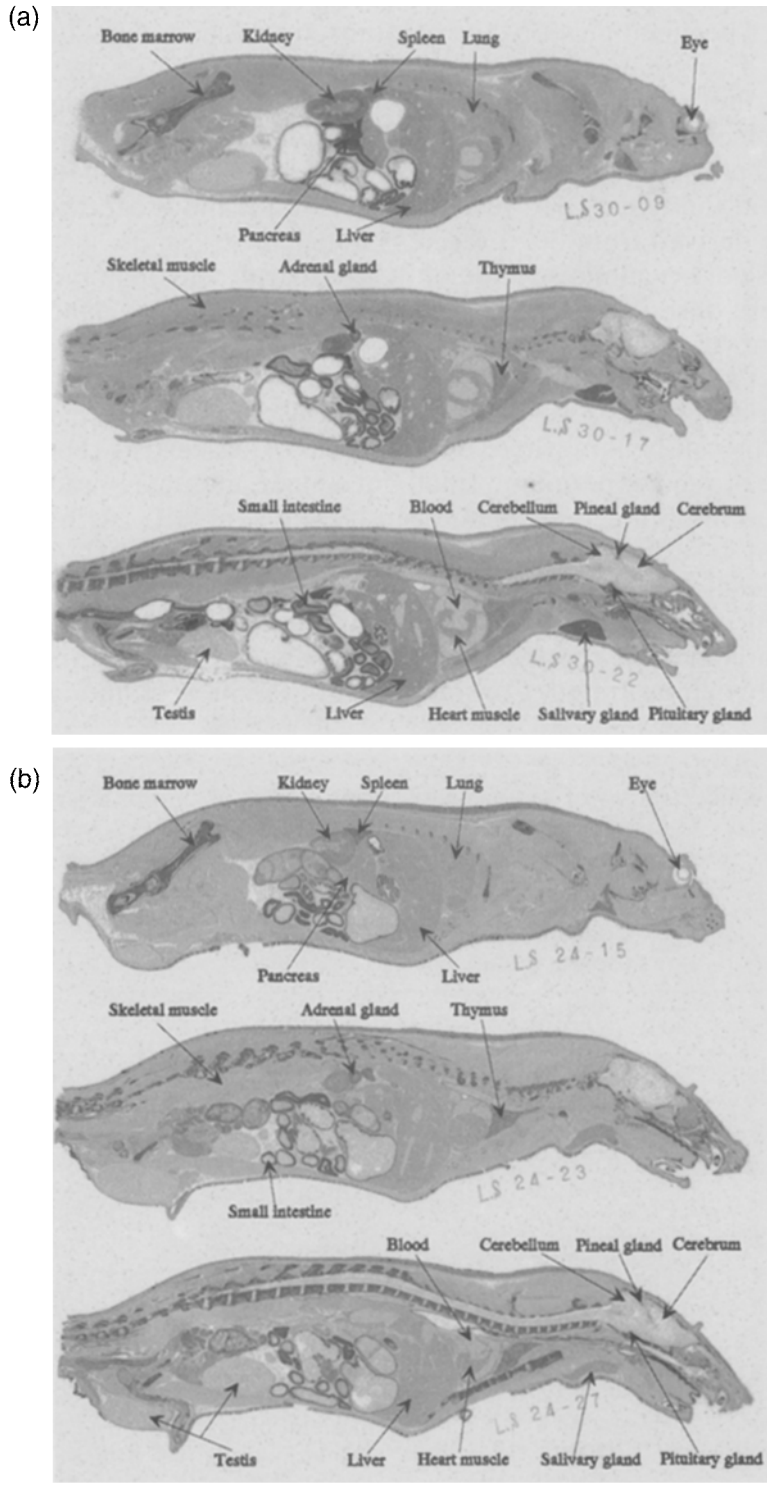

Fig. 2. Whole body autoradiograms of rats (Sprague-Dawley, 7-week-old, male) at a time point of 30 min (**a**) and 24 hr (**b**) after intravenous administration of ^{14}C-L-serine

after i.v. administration of both the ^{14}C-serine were distributed to the whole body of rats. The quantification data of radioactivities in each tissue are shown in Table 1. The greatest distribution of the radioactivities at 30 min was observed in the pancreas in both cases (Table 1). Since L-serine is a well known precursor for proteins, as well as other amino acids, such as glycine and cysteine, and phosphatidylserine, it is expected to be effectively incorporated and utilized in the tissues. However, the reason for the great distribution of radioactivity derived from ^{14}C-D-serine is not clear.

In the case of i.v. administration of ^{14}C-D-aspartic acid, high radioactivities at 30 min were observed in the tissues (pineal and pituitary glands) where a large amount of D-aspartic acid is present *in vivo* (Imai et al., 1997). Hashimoto et al. (1995b) reported the presence of a large amount of D-serine in the cerebrum (277 ng/g wet weight for 7-week) *in vivo*, whereas it was not detected in the cerebellum where D-amino acid oxidase exists (Horiike et al., 1994). In the present experiment, small but similar amounts of radioactivities were detected in both cerebrum and cerebellum (Table 1). Although it was reported that D-serine was located in the proximity of NMDA receptors (Hashimoto et al., 1993) we could not identify the exact location of D-serine in the cerebrum in the present experiment. If we observe the brain tissue by micro-autoradiography, the location of radiolabelled D-serine incorporated will be clearly demonstrated. Considering both the present and the previous

Table 1. The concentration of radioactivity in rat tissues at 30 min after intravenous administration of ^{14}C-D-serine or -L-serine to rats (Sprague-Dawley, 7-week-old, male)

14C-D-Serine		14C-L-Serine	
Tissue	Concentration (dpm/g tissue)	Tissue	Concentration (dpm/g tissue)
Blood	51287.0	Blood	14140.3
Pineal gland	87328.6	Pineal gland	93133.5
Pituitary gland	151491.4	Pituitary gland	148829.8
Salivary gland	87045.2	Salivary gland	174171.8
Liver	58444.6	Liver	60649.9
Lung	65223.2	Lung	47745.0
Adrenal gland	75744.6	Adrenal gland	130654.1
Pancreas	317109.2	Pancreas	384385.1
Testis	15120.6	Testis	13592.0
Spleen	79750.1	Spleen	115465.6
Cerebrum	22597.4	Cerebrum	7139.6
Cerebellum	21161.7	Cerebellum	6943.9
Bone marrow	102390.4	Bone marrow	149594.8
Thymus	90368.4	Thymus	80562.4
Skeletal muscle	21254.9	Skeletal muscle	51078.0
Renal medulla	70776.1	Renal medulla	74236.6
Renal cortico-medullary area	315927.9	Renal cortico-medullary area	124434.6
Renal cortex	172324.3	Renal cortex	116516.1
Intestine	126678.9	Intestine	193297.9

data (Imai et al., 1997) would suggest the possible existence of different mechanisms for the incorporation into tissues for each D-amino acid, D-aspartic acid and D-serine in rats. In fact, it was reported that D-aspartic acid was transported to astrocytes and granule cells by the same uptake system as L-aspartic acid and L-glutamic acid which are neurotransmitters of excitatory neurons (Drejer et al., 1983). In the case of D-serine, however, there have been no reports on its incorporation into the tissues so far, especially the brain tissues.

Since radioactivity was much distributed into the rat pancreas and hitherto there are no reports on the occurrence of D-serine in this tissue, we investigated the *in vivo* existence of D-serine in the rat pancreas (7-week-old) by extraction with methanol, derivatization into the fluorescent derivative, identification and quantification on a reversed-phase HPLC, and enantiomeric determination on a chiral phase HPLC. We found, for the first time, D-serine in the rat pancreas *in vivo* in the range of 12.6 ± 7.9 nmol/g wet tissue (versus 924 ± 116 nmol/g for L-serine), which was comparable to that of adrenal gland, testis, spleen and thymus (10, 12, 13.7 and 15 nmol/g, respectively) (Hashimoto et al., 1995b).

The quantification data of radioactivities in each tissue at 24 hr after the i.v. administration are shown in Table 2. The radioactivities retained in the tissues were slightly different from each other in both cases; the greatest

Table 2. The concentration of radioactivity in rat tissues at 24 hr after intravenous administration of ^{14}C-D-serine or -L-serine to rats (Sprague-Dawley, 7-week-old, male)

14C-D-Serine		14C-L-Serine	
Tissue	Concentration (dpm/g tissue)	Tissue	Concentration (dpm/g tissue)
Blood	5629.1	Blood	18785.5
Pineal gland	13548.4	Pineal gland	53959.9
Pituitary gland	20951.1	Pituitary gland	102786.4
Salivary gland	15538.5	Salivary gland	44593.9
Liver	10540.4	Liver	33061.9
Lung	9093.9	Lung	35914.8
Adrenal gland	29951.3	Adrenal gland	82560.9
Pancreas	10468.3	Pancreas	38216.7
Testis	7005.5	Testis	13174.8
Spleen	20039.6	Spleen	98375.7
Cerebrum	7824.2	Cerebrum	5490.7
Cerebellum	7448.9	Cerebellum	7331.6
Bone marrow	33139.8	Bone marrow	152476.5
Thymus	25275.4	Thymus	79500.2
Skeletal muscle	8276.7	Skeletal muscle	29848.8
Renal medulla	13791.3	Renal medulla	29681.1
Renal cortico-medullary area	128906.4	Renal cortico-medullary area	53350.8
Renal cortex	31128.9	Renal cortex	61992.2
Intestine	36814.3	Intestine	109331.6

in the bone marrow followed by the pituitary and adrenal glands for L-isomer, whereas in the renal cortico-medullary area, bone marrow, adrenal gland and pituitary glands for D-isomer (Table 2). In most tissues, the radioactivities from ^{14}C-L-serine were higher than those from ^{14}C-D-serine, with the exception of cerebrum, cerebellum and renal cortico-medullary area. The elimination rate of D-serine from the whole body of rats was faster than that of L-serine (Fig. 1b and 2b, Table 2). The latter may be incorporated into proteins and converted to the other biological compounds as mentioned above. In contrast, the D-serine, in some part, might also be utilized into the other compounds through oxidation with D-amino acid oxidase (Schell et al., 1995; Horiike et al., 1994) in tissues.

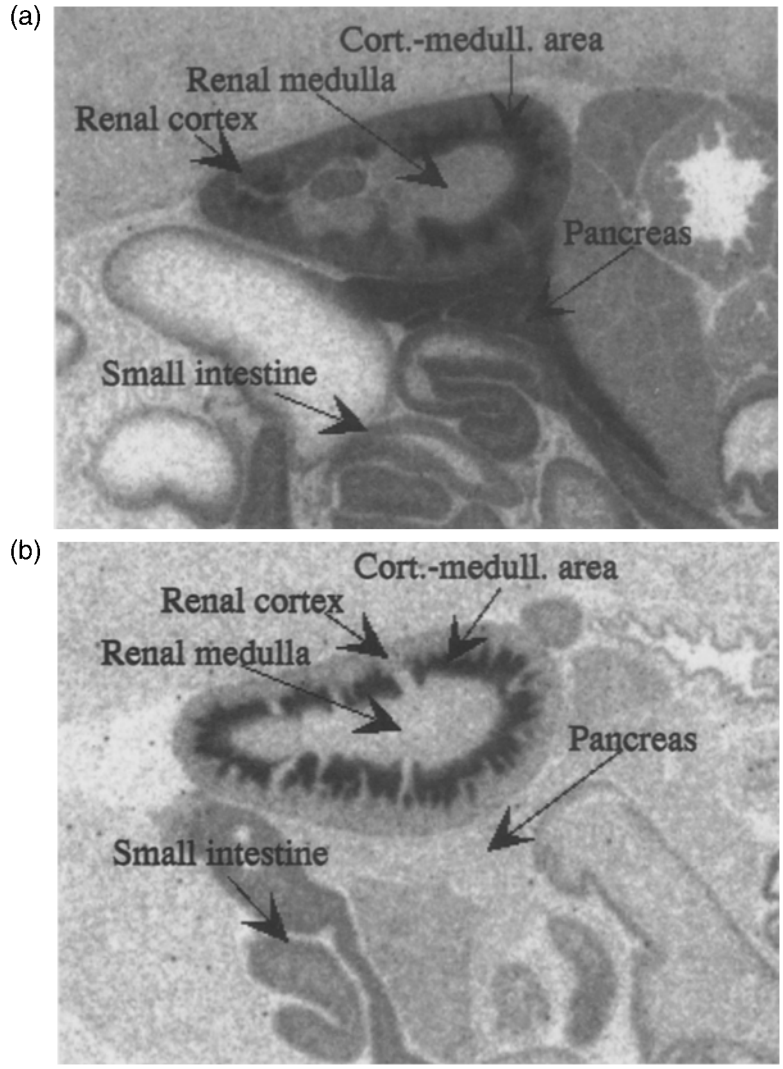

Fig. 3. Autoradiograms of the rat kidney, pancreas and small intestine etc. with magnification (×400) at a time point of 30 min (**a**) and 24 hr (**b**) after intravenous administration of ^{14}C-D-serine

Among the tissues, the distribution of the radioactivity of ^{14}C-D-serine in kidney was quite different from that of ^{14}C-L-serine (Fig. 3 and Fig. 4), where a large amount of radioactivity was observed in the renal cortico-medullary area in the kidney between the cortex and the medulla, perhaps proximal tubule (Table 1). According to the previous papers (Ganote et al., 1974; Kaltenbach et al., 1974; Carone et al., 1985), acute necrosis of proximal tubules was observed with the high dose administration of D-serine (700 mg/kg, i.p.) into rats. The amount of D-serine excreted in 9-week-old rat urine (13 μM) was much lower compared with those in the human (141 μM) and dog (229 μM) urine (Huang et al., 1998), suggesting the more extensive re-absorption of D-serine in the proximal tubule in rat than in human and dog. These

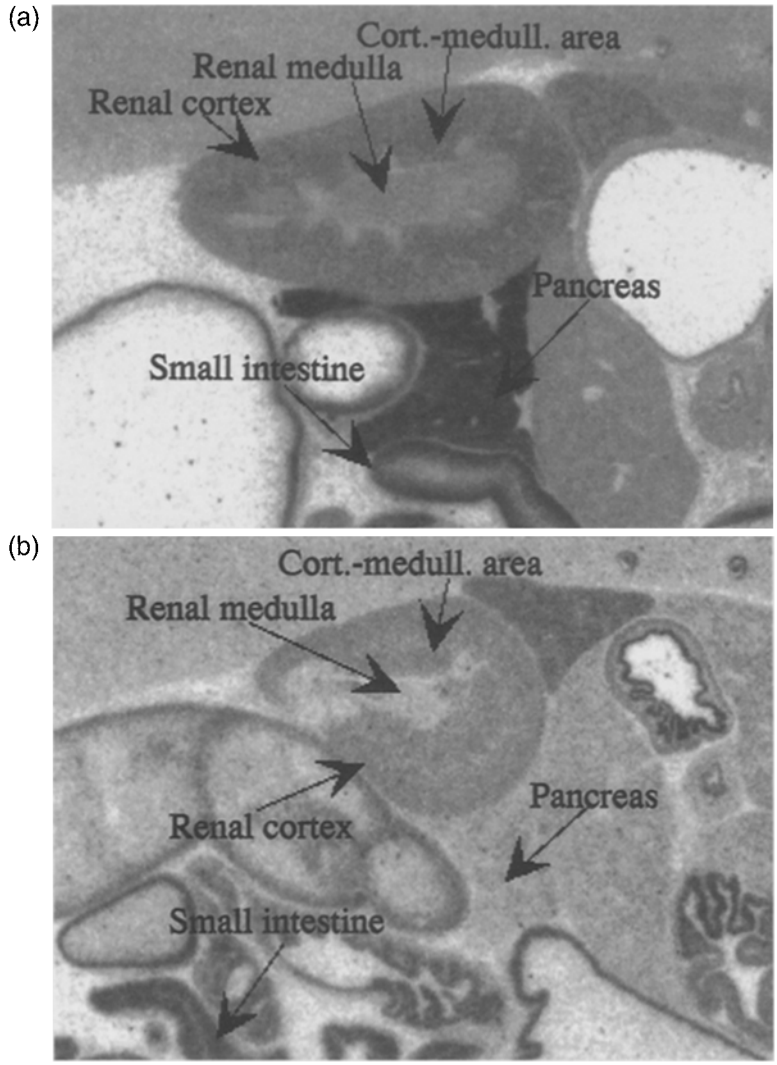

Fig. 4. Autoradiograms of the rat kidney, pancreas and small intestine etc. with magnification (×400) at a time point of 30 min (**a**) and 24 hr (**b**) after intravenous administration of ^{14}C-L-serine

data might support the idea that the acute necrosis is related to the extensive re-absorption in the rat proximal tubules (Ganote et al., 1974).

A very recent report (Dunlop and Neidle, 1997) indicated the *in vivo* synthesis of D-serine from L-serine by a racemase in the brain. The D-serine concentration was monitored in the effluent from a microdialysis probe set in the rat and mouse striatum after intraperitoneal administration of radiolabelled L-serine and glycine. They ruled out the exogenous origin of D-serine because the ^{3}H-labelled D-serine administered orally was not detected in the brain of mice. However, the present experiment suggested that the majority of the circulating D-serine was distributed into the pancreas, pituitary gland and other peripheral tissues, whereas only a small proportion was distributed into cerebrum and cerebellum. Moreover, the radioactivities in those tissues were retained for 24 hr. The present results together with the previous data that orally administered D-serine was absorbed into the rat body and excreted into the urine (Huang et al., 1998) suggest that at least part of the D-serine in the brain was derived from an exogenous source and utilized as a precursor for some components in the tissue. The incorporation of D-serine or the metabolites into various tissues, especially pancreas, pituitary gland, adrenal gland and bone marrow, might be of crucial importance for the living of the mammals.

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