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Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino-acid oxidase activity

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Abstract

A simple and precise method for the simultaneous determination of free D-aspartic acid, D-serine and D-alanine in mouse brain tissues was established, using a reversed-phase HPLC system with widely used pre-column derivatizing reagents, *o*-phthaldialdehyde and *N*-*t*-butyloxycarbonyl-L-cysteine. With the present method, the contents of these three D-amino acids in hippocampus, hypothalamus, pituitary gland, pineal gland and medulla oblongata as well as cerebrum and cerebellum of mutant mice lacking D-amino-acid oxidase activity were determined and compared with those obtained for control mice. In both mice, extremely high contents of D-serine were observed in forebrain (100–400 nmol/g wet tissue), and the contents were small in pituitary and pineal glands. While, D-serine contents in cerebellum and medulla oblongata of mutant mice were about ten times higher than those in control mice. In contrast, D-alanine contents in mutant mice were higher than those in control mice in all brain regions and serum.

Keywords: Enantiomer separation; D-Amino-acid oxidase activity; Aspartic acid; Serine; Alanine

1. Introduction

Recently, along with the progress in analytical methods, free D-amino acids have been reported in various creatures including mammals [1,2], and their biological functions and regulation mechanisms are matters of interest. Among these free D-amino acids, D-aspartic acid (D-Asp), D-serine (D-Ser) and D-alanine (D-Ala) are especially well investigated in higher animals [1,2]. D-Asp is observed in many neuroendocrine and endocrine organs [3] and is closely related to the secretion of hormones such as melatonin or testosterone [4,5]. D-Ser, synthesized by serine racemase in the forebrain [6], is widely located in the central nervous system and is thought to be an intrinsic modulator of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate (Glu) receptor [2,3,6]. As for D-Ala, increase in its content in the brain and the plasma was observed in the cases of Alzheimer [7] and renal disease [8]. These lines of evidence indicate that the D-amino acids play significant roles in mammals. Although, these D-amino acids are widely observed in the central nervous system and peripheral tissues [1–3], the physiological roles, metabolism and regulation remain to be studied. For the enantiomer determination of amino

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acids in biological samples, a variety of chromatographic methods including GC and LC using diastereomers and chiral stationary phases have been reported [1,9–15]. However, the determination of D-amino acids in the biological samples is often interfered with by large amounts of their enantiomers, L-amino acids (major components of amino acids in mammals). In addition, the determination is also interfered with by a large number of peptides and non-chiral amino acids such as taurine, β-Ala or γ-aminobutyric acid. Therefore, a simple, sensitive and validated method for the simultaneous determination of D-Asp, D-Ser and D-Ala in tissue extracts of mammals has not been reported, and an appropriate method is required. Considering that the amounts of endogenous D-amino acids are small in many cases, D-Asp, D-Ser and D-Ala must be completely separated from other endogenous substances. Therefore, the analytical method should be established using the appropriate tissue extract itself, and the quantitative value also should be validated using the tissue extract.

In the present investigation, we improved a pre-column derivatization reversed-phase HPLC method with *o*-phthaldialdehyde (OPA) and *N*-*t*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) described by Hashimoto et al. [12], which is a useful method for the determination of D-amino acids in mammalian tissues [12,16,17], and a validated simultaneous determination method of D-Asp, D-Ser and D-Ala with a sufficient sensitivity and simplicity was developed. Because the mammalian cerebellum was reported to contain small amounts of D-amino acids [2,3,13], the mouse cerebellum was used for the investigation of separation conditions, and for the validation of quantitative values. Using the method established in the present research, we investigated in detail the distribution of D-Asp, D-Ser and D-Ala in mouse brain. Because D-amino-acid oxidase (DAO) is a major metabolic enzyme of neutral D-amino acids, the mutant mouse lacking DAO activity (ddY/DAO$^-$) is useful for the detailed study of the regulation mechanism of D-amino acids in the mammalian brain, and the contents of D-amino acids in cerebrum and cerebellum were already reported [18,19]. In the present investigation, to obtain further information on D-amino acids, the contents of D-Asp, D-Ser and D-Ala in six brain regions of ddY/DAO$^-$ mice in addition to cerebrum and cerebellum were determined, and compared with those obtained for control ddY/DAO$^+$ mice.

2. Experimental

2.1. Materials

Amino acids (D- and L-enantiomers and racemic mixtures of Asp, Ser and Ala) of guaranteed grade, methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). OPA, sodium hydroxide, acetic acid and boric acid were obtained from Wako (Osaka, Japan). Boc-L-Cys was a product of Novabiochem (Läufelfingen, Switzerland). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents and solvents were of reagent grade.

2.2. Animals

Male ddY/DAO$^+$ and ddY/DAO$^-$ mice [20] (9 weeks of age, specific pathogen-free) were used. The animals were housed under a 12-h light–dark cycle (light on at 07:00 h) and were fed food and water freely.

2.3. Sample preparation

Mice were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta, and the brain was quickly excised and separated into eight regions (cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland, pineal gland and others). In this paper, the other regions such as the midbrain and pons were excised together and analyzed as ''others''. These tissues were weighed and stored at −80°C until the time of analysis. Because the pineal gland was too small to be weighed accurately, the gland was not weighed, and the contents of D-amino acids were expressed as pmol/whole gland. The brain tissues were homogenized in 20× volumes of MeOH on ice (in the case of the hypothalamus, pituitary gland and pineal gland, each tissue was homogenized in 500 μl of MeOH). The homogenates were centrifuged at 4500 *g* for 5 min, and 20 μl of supernatant (100 μl

was used for the hypothalamus, pituitary gland and pineal gland) was evaporated to dryness under reduced pressure at 40°C. To the residue, 40 μl of 0.4 *M* sodium-borate buffer (pH 9.0) and 10 μl of the OPA reagent solution (prepared daily by dissolving 2 mg of OPA and 2 mg of Boc-L-Cys in 200 μl of MeOH) were added, and the amino acids were derivatized at 25°C for 2 min as described by Hashimoto et al. [12]. One μl of the reaction mixture was then immediately subjected to HPLC. In the case of blood samples, the obtained blood was centrifuged at 4500 *g* for 15 min at 4°C, and the serum were collected. After MeOH (20× volumes) was added, the solution was mixed for 1 min and centrifuged at 4500 *g* for 5 min. Then 100 μl of the supernatant was dried, derivatized, and analyzed by HPLC as described for brain tissues.

2.4. HPLC system

The HPLC system consisted of a DG 980-50 degasser (Jasco, Tokyo, Japan), an LG-980-02 gradient unit (Jasco), a PU-980 pump (Jasco), a 7725i injector (Rheodyne, Cotati, CA, USA), a CO-960 column oven (Jasco), an FP-920S fluorescence detector (Jasco) and an 807-IT integrator (Jasco). The analytical column was an ODS-80TsQA (250×4.6 mm I.D., Tosoh, Tokyo, Japan) maintained at 35°C. Mobile phase A was MeCN–0.1 *M* sodium acetate buffer (pH 6.0) (9:91, v/v), and mobile phase B was MeCN–0.1 *M* sodium acetate buffer (pH 6.0) (16:84, v/v). A linear gradient was applied for 35 min from mobile phase A to B; then the elution was carried out with mobile phase B alone. The flow-rate was 1.4 ml/min. Fluorescence detection of the derivative of each amino acid was carried out at 443 nm with excitation at 344 nm.

3. Results and discussion

3.1. HPLC determination of D-Asp, D-Ser and D-Ala

For the simultaneous determination of free D-Asp, D-Ser and D-Ala in mouse brain tissues, the separation conditions of HPLC were investigated. The three D-amino acids and their enantiomers (L-amino acids) were derivatized with OPA and Boc-L-Cys as described by Hashimoto et al. [12] and separated on a C_{18} reversed-phase column. As a result, gradient elution of acetonitrile from 9 to 16% was adopted for the baseline separation of diastereomers of Asp, Ser and Ala within 80 min. Under these conditions, only trace amounts of these D-amino acids were detected in the mouse cerebellum and medulla oblongata, and no overlapped peaks were observed with the peaks of D-Asp, D-Ser and D-Ala. Because these tissues were reported to have small amounts of endogenous D-amino acids [2,3,13], the present method is suitable for the determination of minute amounts of D-Asp, D-Ser and D-Ala without the interference of other endogenous substances in the brain. Compared with two-step chromatography often used for the determination of D-amino acids in biological samples [13,14], D-Asp, D-Ser and D-Ala could be determined much simpler by a single chromatographic separation. The content of each D-amino acid in the cerebrum of ddY/DAO$^+$ mouse was determined using other separation conditions, and compared with that obtained in the present investigation. D-Asp and D-Ser were determined using the 0.1 *M* sodium-acetate buffer (pH 6.0) containing THF (4%) and MeCN. The concentration of MeCN was as follows: 0–20 min, 4%; 20–35 min, linear gradient from 4 to 8%; 35–40 min, 8%. The obtained values were 56.1 nmol/g wet tissue for D-Asp, and 426.5 nmol/g wet tissue for D-Ser. D-Ala was determined using the two step chromatography [13], and the obtained value was 7.1 nmol/g wet tissue. In addition, the values obtained in the present investigation were consistent with those obtained in the previous study (D-Asp, 50 nmol/g wet tissue; D-Ser, 310 nmol/g wet tissue; D-Ala, not detectable [18]) (D-Ser, 353 nmol/g wet tissue; D-Ala, 3.5 nmol/g wet tissue [19]).

Calibration curves, within-day precision and day-to-day precision of the determination of D-Asp, D-Ser and D-Ala in the mouse cerebellum were investigated. The calibration curve of each D-amino acid was constructed by the addition of each D-amino acid (25 pmol, 250 pmol, 2.5 nmol and 25 nmol) to 20 μl of the supernatant of mouse cerebellum. The obtained calibration curves were linear from 500 fmol to 500 pmol (injection amount/1 μl) with the correlation coefficients higher than 0.999. The equations were as follows: $y = 0.381 + 0.566x$ for D-Asp,

$y = -0.199 + 0.576x$ for D-Ser and $y = 0.405 + 0.352x$ for D-Ala, where x = amounts of D-amino acids injected (pmol) and y = fluorescence response (mV). Compared with the wide calibration range, the intrinsic amounts of D-AAs in the cerebellum are too small. Therefore, the y-intercepts around zero were obtained. The lower limits of quantitation of 500 fmol are comparable or better than those obtained with the other methods [12,14,15] used for the determination of D-amino acids in mammalian tissues. Within-day precision and day-to-day precision were also investigated using a mouse cerebellum sample spiked with 10 pmol of each D-amino acid. The same sample was derivatized five times within a day and the obtained within-day precision of D-Asp, D-Ser and D-Ala was 2.5, 1.6 and 0.9% (RSD), respectively, and day-to-day precision (5 days) was 4.4, 5.7 and 6.1% (RSD), respectively. The peak heights and enantiomeric ratios of the standard racemic mixtures of Asp, Ser and Ala did not change for 5 min after derivatization. In addition, D-amino acids were not detected in some samples of cerebellum and medulla oblongata of ddY/DAO$^+$ mouse, excluding the possibility of racemization as well as kinetic resolution. These results indicate that the present method is suitable for the simultaneous determination of free D-Asp, D-Ser and D-Ala in the mouse brain tissues.

3.2. Distribution of free D-Asp, D-Ser and D-Ala in the brain of ddY/DAO$^+$ and ddY/DAO$^-$ mice

The contents of free D-Asp, D-Ser and D-Ala were determined in various brain regions and the serum of the mutant mice lacking DAO activity (ddY/DAO$^-$) and compared with those obtained in control ddY/DAO$^+$ mice. The present method was sensitive and each amino acid was completely separated from other endogenous substances. With the present method, the contents of D-Asp, D-Ser and D-Ala were successfully determined in eight regions of mouse brain (cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland, pineal gland and others). Our preliminary experiments using the method described previously [12] failed to determine the contents of minute amount of D-Ala in various brain regions. Typical chromatograms obtained for DAO$^+$ and DAO$^-$ mice using our present method are shown in Fig. 1. These chromatograms were obtained for the cerebellum of both strains. In the cerebellun of DAO$^+$ mice, small amounts of D-Ser and D-Ala were observed (Fig. 1a), while large

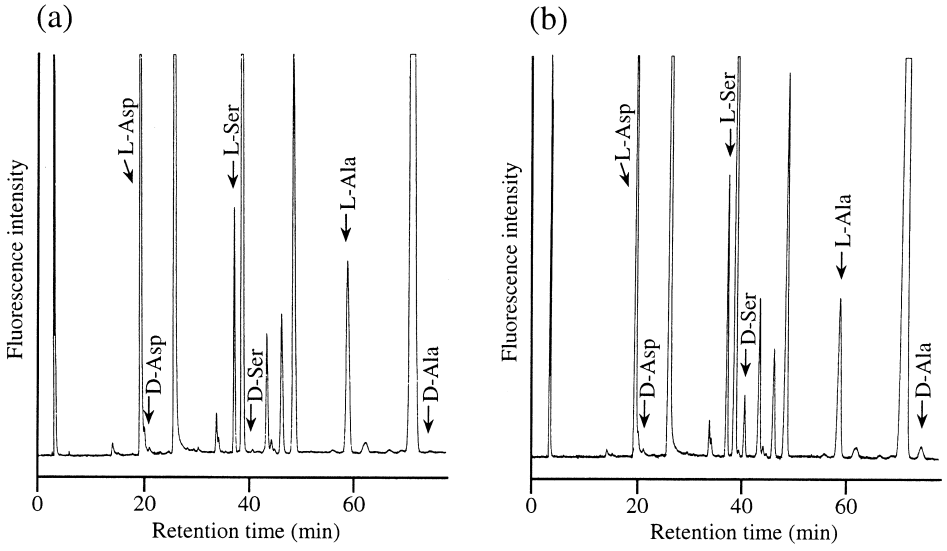

Fig. 1. Chromatograms of the derivatives of D,L-Asp, -Ser and -Ala in the cerebellum of ddY/DAO$^+$ (a) and ddY/DAO$^-$ (b) mouse. The HPLC conditions are described in the text.

amounts of these D-amino acids were detected in the cerebellum of DAO^- mice, although the contents of L-enantiomers were almost the same in both strains (Fig. 1b). Because strong DAO activity is generally observed in the mammalian cerebellum [21], the neutral D-amino acids such as D-Ser and D-Ala were thought to be metabolized by the enzyme in the cerebellum of DAO^+ mice. However, in the cerebellum of DAO^- mice, DAO activity was not observed and neutral D-amino acids are not metabolized. Therefore, the content of D-Ser and D-Ala should be higher in the cerebellum of DAO^- mice than those in DAO^+ mice, which was consistent with the present results. The distribution of D-Asp, D-Ser and D-Ala in the brains of DAO^+ and DAO^- mice and their serum levels were summarized in Tables 1–3. For the determination of small amounts of D-amino acids, standard addition method in small amount (0.5 and 5 pmol of each D-amino acid was added/injection 1 μl) was used. The slopes for three D-amino acids were 0.598 for D-Asp, 0.542 for D-Ser and 0.383 for D-Ala, which were almost the same as those of equations described in the Section 3.1.

Table 1 shows the D-Asp contents in the eight brain regions and serum of DAO^+ and DAO^- mice. The distribution of D-Asp was almost the same for both strains, and a significant difference was not observed at any region. Acidic D-amino acids (D-Asp and D-Glu) are not oxidized by DAO but are metabolized by D-aspartic acid oxidase (DAspO) [22]. Nagasaki et al. [23] reported that the activity of DAspO is not different in both strains; the present results are consistent with their results.

The contents of D-Ser are given in Table 2. In the frontal brain (cerebrum, hippocampus, others, and hypothalamus), extremely high contents of D-Ser (100–400 nmol/g wet tissue) were observed in both DAO^+ and DAO^- strains, and there was no signifi-

Table 1
D-Asp contents in the brain and serum of ddY/DAO^+ and ddY/DAO^- mice

Sample	ddY/DAO^+	ddY/DAO^-
Cerebrum	42.6±5.9 (0.9)	51.1±5.9 (1.2)
Hippocampus	32.6±2.5 (1.0)	29.2±5.7 (0.9)
Hypothalamus	9.6±1.2 (0.2)	8.3±1.4 (0.2)
Pituitary gland	15.3±2.7 (0.4)	17.5±2.3 (0.9)
Pineal gland	43.0±7.5 (4.8)	84.5±16.4 (13.8)
Cerebellum	20.1±3.6 (0.4)	13.8±2.7 (0.4)
Medulla oblongata	3.4±1.6 (0.1)	3.6±1.8 (0.1)
Others	23.4±3.2 (0.5)	24.4±4.5 (0.6)
Serum	1.0±0.1 (7.1)	1.7±0.3 (11.9)

Values represent means±SEM (nmol/g wet tissue) of four mice. The contents in pituitary gland were determined for five mice. The values for pineal glands are expressed as pmol/whole pineal gland, and those for serum are expressed as nmol/ml serum. Values in parentheses are the proportions of D-amino acids (D/(L+D)×100).

Table 2
D-Ser contents in the brain and serum of ddY/DAO^+ and ddY/DAO^- mice

Sample	ddY/DAO^+	ddY/DAO^-
Cerebrum	423.2±21.5 (32.9)	424.5±28.5 (32.0)
Hippocampus	341.5±28.6 (27.1)	300.3±50.2 (28.4)
Hypothalamus	105.2±9.7 (15.1)	147.1±4.1 (18.8)
Pituitary gland	3.4±0.8 (0.9)	10.0±1.9 (3.4)
Pineal gland	23.5±2.1 (4.4)	18.9±3.8 (5.3)
Cerebellum	11.7±2.4 (1.5)	168.9±9.8** (16.2)
Medulla oblongata	8.7±1.4 (1.4)	102.8±8.1** (13.4)
Others	225.2±18.0 (22.0)	251.6±29.9 (24.7)
Serum	2.1±0.1 (1.1)	11.6±1.2** (6.1)

Values represented means±SEM (nmol/g wet tissue) of four mice. The contents in pituitary gland were determined for five mice. The values for pineal glands are expressed as pmol/whole pineal gland, and those for serum are expressed as nmol/ml serum. Values in parentheses are the proportions of D-amino acids (D/(L+D)×100). ** $P<0.01$, significant difference from the values of DAO^+ mice.

Table 3
D-Ala contents in the brain and serum of ddY/DAO^+ and ddY/DAO^- mice

Sample	ddY/DAO^+	ddY/DAO^-
Cerebrum	12.4±2.1 (0.7)	63.7±6.7** (3.6)
Hippocampus	11.4±3.5 (0.6)	61.7±7.6** (4.5)
Hypothalamus	9.0±1.8 (0.6)	52.6±4.8** (4.2)
Pituitary gland	29.1±6.0 (1.9)	81.2±6.6** (7.2)
Pineal gland	36.5±4.4 (2.4)	60.1±10.6 (7.8)
Cerebellum	10.9±2.3 (1.2)	79.6±7.0** (6.6)
Medulla oblongata	4.6±2.2 (0.5)	59.4±9.2** (6.5)
Others	6.3±3.5 (0.5)	49.8±7.7** (4.3)
Serum	8.8±1.4 (1.2)	134.6±16.5** (15.0)

Values represented means±SEM (nmol/g wet tissue) of four mice. The contents in pituitary gland were determined for five mice. The values for pineal glands are expressed as pmol/whole pineal gland, and those for serum are expressed as nmol/ml serum. Values in parentheses are the proportions of D-amino acids (D/(L+D)×100). ** $P<0.01$, significant difference from the values of DAO^+ mice.

cant difference between the two strains. On the other hand, only small amounts of D-Ser (about 10 nmol/g wet tissue) were detected in the cerebellum and medulla oblongata of DAO^+ mice. This is because DAO is present in the cerebellum and medulla oblongata but not in the frontal brain. In contrast, the contents of D-Ser in the cerebellum and medulla oblongata of the DAO^- mice were ten times higher than those in DAO^+ mice ($P<0.01$). In the pituitary and pineal glands, small amounts of D-Ser were observed in both strains. The content of D-Ser in the serum of DAO^- mice were five times higher than that in DAO^+ mice ($P<0.01$). The contents of D-Ser in the cerebrum, cerebellum, and serum of DAO^+ and DAO^- mice were already investigated by Hashimoto et al. [18], and their results agreed with the values obtained in our present research. In the present investigation, D-Ser contents were investigated in both eight brain regions of DAO^+ and those of DAO^- mice. In the brain of DAO^- mice, extremely high contents of D-Ser were observed in the forebrain, cerebellum, and medulla oblongata, while, small amounts of D-Ser were detected in the pituitary and pineal glands. The mutant DAO^- mice could not metabolize D-Ser by DAO because of the lack of enzymatic activity, and the contents should indicate those without oxidation by DAO. Therefore, these results suggest that accumulation of D-Ser occurs in widespread areas of the mouse brain except for pituitary and pineal glands. Recently, D-Ser is reported to be de novo synthesized by serine racemase in the forebrain and cerebellum of rat [6], therefore, D-Ser observed in widespread areas of mouse brain should be synthesized by the same enzyme. In the brain of DAO^+ mice, however, high content of D-Ser was observed only in the forebrain, suggesting that the synthesized D-Ser was simultaneously metabolized by DAO in the cerebrum and medulla oblongata.

The distribution of D-Ala is shown in Table 3. In the control DAO^+ mice, relatively high contents of D-Ala were observed in the pituitary gland and the pineal gland. In other brain regions, the contents of D-Ala were about 5–10 nmol/g wet tissue. Although D-Ala is one of the substrates of DAO as well as D-Ser, the distribution was different from that of D-Ser, and similar contents of D-Ala were observed in the forebrain (for example, cerebrum and hippocampus) and cerebellum. Whereas, the content of D-Ser in the cerebellum of DAO^+ mice was approximately 1/40 of that observed in the forebrain. D-Ser should be synthesized by serine racemase in widespread brain areas and observed at high contents. Therefore, the decrease of D-Ser contents by DAO was clearly observed in the cerebellum and medulla oblongata. While, the contents of D-Ala are small in the forebrain as well as in the cerebellum and medulla oblongata, thus, the difference of D-Ala contents between brain areas was not observed. In the DAO^- mice, the D-Ala contents (50–80 nmol/g wet tissue) were higher than those of the DAO^+ mice in all brain regions. The content of D-Ala in the serum of DAO^- mice were significantly higher than that in DAO^+ mice. Hashimoto et al. [18] showed that the content of D-Ala in the serum of the mutant DAO^- mice was eight times that of normal DAO^+ mice and that the D-Ala contents of the cerebrum and cerebellum were also higher in DAO^- mice [18], which is consistent with the present results. There are some reports showing the origin and transport of D-Ala in the rodents. Konno et al. [24] reported that a large part of D-Ala in ddY/DAO^- mice was derived from the cell wall of intestinal bacteria, and Nagata et al. [19] reported that the D-Ala contents in the cerebrum, cerebellum and serum of mice orally administered D-Ala were higher than those of control mice. These results should indicate that the exogenous D-Ala (derived from intestinal bacteria or contained in their diet) was absorbed from the intestine and some part of the D-Ala was transported to mammalian brain by the blood flow. In the present investigation, large amount of D-Ala was observed in all brain areas and the serum of DAO^- mice, suggesting that the exogenous D-Ala distributes to widespread brain regions, not only to the cerebrum and cerebellum. While, in the DAO^+ mice, the absorbed D-Ala should be metabolized by DAO expressing in the liver and kidney, and only small amount of D-Ala was observed in the brain and serum.

4. Conclusions

In the present investigation, a simple, sensitive and precise method for the simultaneous determination of

D-Asp, D-Ser and D-Ala in the mouse brain tissue is established, and the distribution of these D-amino acids in the brains of DAO^+ and DAO^- mice was investigated in detail. Because these D-amino acids are widely occurring in the mammalian brain and periphery, the present method should be a valuable tool for the precise determination of them and contribute to the research on D-amino acids.

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