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Simultaneous determination of D- and L-amino acids in the nervous tissues of crustaceans using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate and reversed-phase ion-pair high-performance liquid chromatography

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Abstract

After the derivatization of D- and L-amino acids with (+)-1-(9-fluorenyl)ethyl chloroformate, nineteen amino acids were separated into their D- and L-enantiomers and from other physiological amino compounds by reversed-phase ion-pair high-performance liquid chromatography. The separation was performed by three separate runs differing in mobile phase compositions and gradient profiles. Tyrosine, tryptophan and cysteine could not be detected because of their weak reactions with the derivatization reagent. Of seven D-amino acids found in the crustacean nervous tissues and eyes, D-alanine, D-arginine and D-aspartate were the most abundant and widely distributed.

1. Introduction

Since the development of the amino acid analyser, the analyses of physiological free amino acids have been used in various areas as a convenient method for evaluating amino acid pools in biological tissues. In recent years, however, several free D-amino acids have been found even in animal tissues [1,2] and have gained increasing attention in biochemical, physiological and medical areas. Hence the chromatographic resolution of amino acid enantiomers in biological fluids and tissues is becoming increasingly important in various research fields.

Several methods have been developed for the simultaneous determination of D- and L-amino acids by using chiral-phase capillary gas chromatography [3] and high-performance liquid chromatography (HPLC) of diastereometric amino acid derivatives with some chiral reagents, such as 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide [4,5], *N-tert.*-butyloxycarbonyl-L-cysteine [6] and (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [7]. The application of these methods to physiological free amino acids is difficult, however, because various chiral or non-chiral free amino compounds are present in the tissue extracts or physiological fluids and are highly suspected to be co-chromatographed with D- and L-amino acids.

Einarsson *et al.* [7] originally reported the separation of FLEC derivatives of D- and L-

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amino acids using conventional reversed-phase HPLC. This chiral reagent is expensive but has some advantages: high stability of FLEC derivatives of amino acids, high sensitivity with fluorescence detection and a simple derivatization procedure. The well established HPLC separation of amino acid derivatives with non-chiral 9-fluorenylmethyl chloroformate (FMOC) [8–11] would lend support to the development of an HPLC method for FLEC derivatives of D- and L-amino acids because FLEC differs from FMOC only in an additional methyl group. To our knowledge, this FLEC method has never been applied to the determination of physiological free amino acids. We therefore developed a method for the simultaneous determination of FLEC derivatives of D- and L-amino acids and other physiological free amino compounds using ion-pair reversed-phase HPLC.

Free D-aspartic acid has been identified in the brain and nervous tissues of some animals such as the mollusc *Aplysia fasciata* [12], cephalopods [13–15], chick embryo [16], neonatal rat [16,17] and man [18], and is recognized as a neurotransmitter in the nervous system [19]. Other than D-aspartic acid, only D-serine and D-alanine have been found in the brain of rat [6], mouse [20] and/or man [18]. We are interested in the occurrence of these D-amino acids in the brain of marine animals and their functions in the animal brain.

In this paper, we describe the HPLC determination of FLEC derivatives of physiological free D- and L-amino acids and its application to the determination of D-amino acids in the nervous tissues and eyes of several crustaceans.

2. Experimental

2.1. Chemicals

All D- and L-amino acids and related compounds were purchased from Sigma (St. Louis, MO, USA). FLEC was obtained from Eka Nobel (Surte, Sweden). Solvents for HPLC, acetonitrile and tetrahydrofuran of HPLC grade were purchased from Wako (Osaka, Japan). All other reagents were of analytical-reagent grade and purchased from Wako. Water was purified with a Milli-Q Labo system (Millipore, Tokyo, Japan) from distilled, deionized water.

2.2. Derivatization of amino acids

Precolumn derivatization of D- and L-amino acids with FLEC was performed essentially according to Einarsson *et al.* [7]. In a 2-ml Mixxor separation vial (Lidex, Technion, Israel), 0.1 ml of sample solution was mixed with 0.4 ml of 0.3 *M* borate–NaOH buffer (pH 11.0) and 0.5 ml of 18 m*M* FLEC solution in acetone. After reaction for 45 s at room temperature, the reaction mixture was extracted twice with 2 ml of pentane to remove excess of reagent. The aqueous phase was filtered through a 0.45-μm filter (Toyo Roshi, Tokyo, Japan) and injected directly into the HPLC system.

2.3. Separation of FLEC derivatives of amino acids with HPLC

The HPLC system consisted of three LC-6A pumps (Shimadzu, Kyoto, Japan), a CTO-6A column oven (Shimadzu), a SCL-6B system controller (Shimadzu), a GT-103 degasser (Lab-Quatec, Tokyo, Japan), a SIL-6B autosampler (Shimadzu) with a WIG-7000A cooling system (Ishido, Chiba, Japan), an RF-535 fluorescence detector (Shimadzu) and a Chromatopak C-R7A data processor (Shimadzu). The analytical column was a reversed-phase Shim-pack CLC-ODS (250 × 4.6 mm I.D.) (Shimadzu) with a guard column (10 × 4.6 mm I.D.) packed with Nucleosil-C_{18} resin (Macherey–Nagel, Düren, Germany).

The mobile phase consisted of 15 m*M* citric acid containing 10 m*M* tetramethylammonium chloride (TMA) as an ion-pair reagent, acetonitrile and tetrahydrofuran (THF). After mixing these solvents, the apparent pH of the mobile phase was adjusted to a suitable value with 6 *M* NaOH or HCl. Elution was carried out with a ternary gradient using high-pressure mixing with three pumps at a flow-rate of 0.7 ml/min and at a column temperature of 55°C. Separation was

Table 1
Mobile phase compositions and gradient profiles for HPLC separation of FLEC derivatives of amino acids

	Run I			Run II			Run III		
	A (%)	B (%)	C (%)	A (%)	B (%)	C (%)	A (%)	B (%)	C (%)
Mobile phase composition									
Citrate–TMA[a]	75	70	30	73	60	30	75	60	30
Acetonitrile	10	20	60	25	35	60	10	35	60
Tetrahydrofuran	15	10	10	2	5	10	15	5	10
Final pH	2.0	5.3	6.2	2.0	5.3	6.2	2.0	5.3	6.2
Gradient profile									
0 min	100	0	0	100	0	0	100	0	0
3 min	15	85	0						
5 min				60	40	0	70	30	0
10 min							60	40	0
15 min	13	87	0						
30 min				50	50	0	50	50	0
55 min				0	70	20			
65 min							0	80	20
75 min				0	0	100			
80 min							0	0	100
85 min	0	30	70	0	0	100	0	0	100
85.1 min	0	0	100						
95 min	0	0	100						

[a] Citric acid (15 m*M*) containing 10 m*M* tetramethylammonium chloride.

conducted with three separate runs that differed in mobile phase compositions and gradient profiles (Table 1). FLEC-amino acids were monitored fluorimetrically. The excitation and emission wavelengths were set at 260 and 310 nm, respectively. The injection volume was 20 μl for authentic amino acid mixture and tissue extracts.

The capacity factor (k') and separation factor (α) of each amino acid were evaluated from the column hold-up time, which was determined by injection of water under isocratic conditions.

2.4. Animals and preparation of tissue extract

Live crustaceans used in this experiment were obtained from local fish suppliers (Tokyo and Mie, Japan). Two crab species were marine snow crab, *Chionoecetes opilio* ($n = 3$), and freshwater Japanese woolly-handed crab, *Eriocheir japonicus* ($n = 5$). Three species of lobster and prawn were marine kuruma prawn, *Penaeus japonicus* ($n = 35$), marine rock lobster, *Jasus lalandi* ($n = 5$), and freshwater crayfish, *Procambarus clarkii* ($n = 20$).

These crustaceans were dissected immediately after having been brought to the laboratory. Eyes or nervous tissues including both ganglia and nerves were collected, mixed together and homogenized with a ninefold excess of 8% perchloric acid. After centrifugation at 20 000 *g* for 10 min at 0°C, the supernatant obtained was neutralized with solid potassium hydrogen carbonate and centrifuged again to eliminate the crystals of potassium perchlorate that formed. The supernatant solution was used as a tissue extract. Before derivatization, the extract was stored at −20°C.

3. Results

Figs. 1–3 show the chromatograms of the standard amino acid mixture (20 pmol each) in three separate runs. Most of the amino acids

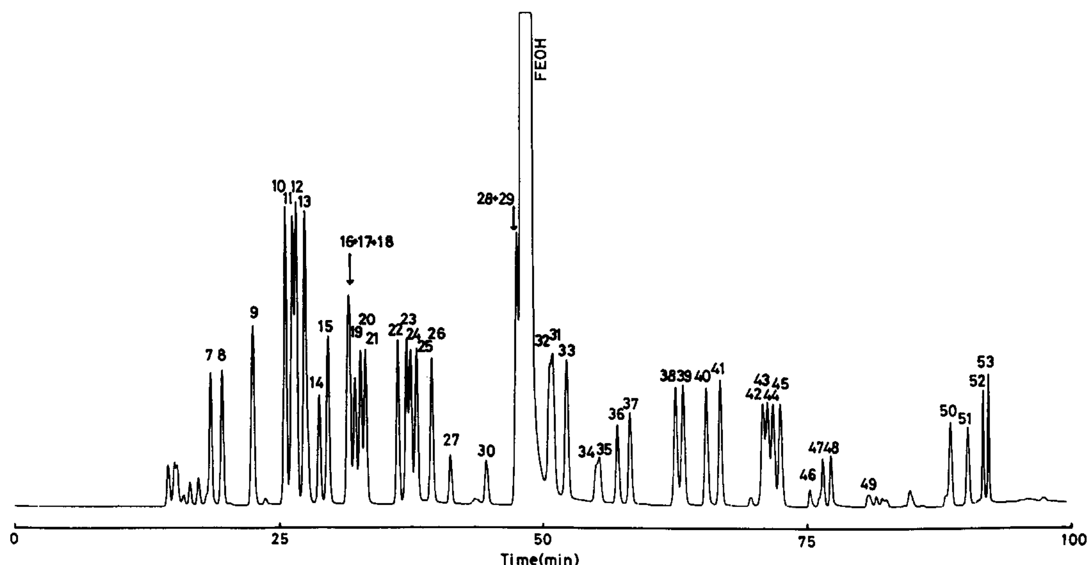

Fig. 1. Chromatogram of FLEC derivatives of standard free amino acids (20 pmol each) in run I. See Experimental and Table 1 for chromatographic conditions. Peaks: 1 = D-His; 2 = L-His; 3 = 1-methyl-L-His; 4 = 3-methyl-L-His; 5 = carnosine; 6 = anserine; 7 = D-Arg; 8 = L-Arg; 9 = taurine; 10 = D-Asn; 11 = L-Asn; 12 = D-Gln; 13 = L-Gln; 14 = D-citrulline; 15 = L-citrulline; 16 = D-Hyp; 17 = D-Ser; 18 = L-Ser; 19 = L-Hyp; 20 = D-Asp; 21 = L-Asp; 22 = D-Glu; 23 = L-Glu; 24 = D-Thr; 25 = L-Thr; 26 = Gly, 27 = L-α-aminoadipic acid; 28 = D-Ala; 29 = L-Ala; 30 = β-Ala; 31 = D-Pro; 32 = L-Pro; 33 = γ-aminobutyric acid; 34 = D,L-β-aminobutyric acid; 35 = L-α-aminobutyric acid; 36 = D-Met; 37 = L-Met; 38 = D-Val; 39 = L-Val; 40 = D-Phe; 41 = L-Phe; 42 = D-Ile; 43 = D-Leu; 44 = L-Ile, 45 = L-Leu; 46 = D-cystine; 47 = cystathionine; 48 = L-cystine; 49 = D,L-Hyl; 50 = D-ornithine; 51 = L-ortnithine; 52 = D-Lys; 53 = L-Lys; FEOH = hydrolysis compound of FLEC.

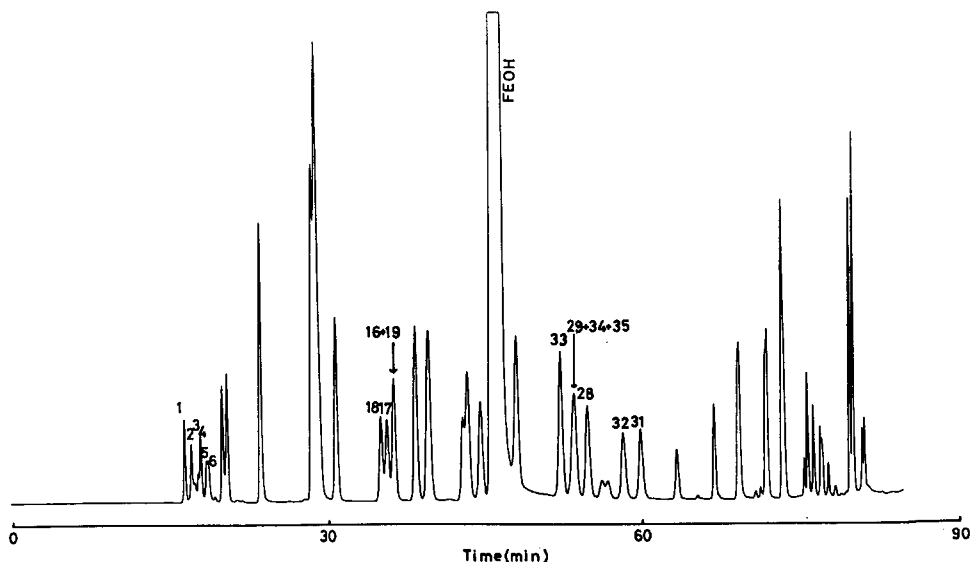

Fig. 2. Chromatogram of FLEC derivatives of authentic free amino acids in run II. See Experimental and Table 1 for chromatographic conditions. Peak numbers as in Fig. 1.

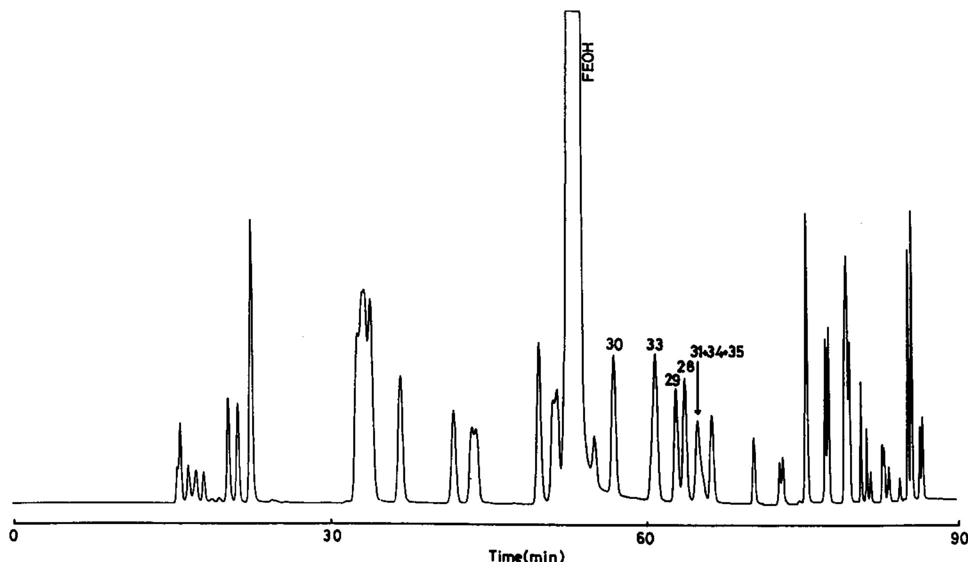

Fig. 3. Chromatogram of FLEC derivatives of authentic free amino acids in run III. See Experimental and Table 1 for chromatographic conditions. Peak numbers as in Fig. 1.

could be determined in run I, as shown in Fig. 1. Each D-enantiomer eluted before the corresponding L-enantiomer. Under these conditions, histidine (peaks 1 and 2 in Fig. 2), serine (17 and 18), proline (31 and 32) and alanine (28 and 29 in Fig. 3) were, however, not separated into D- and L-enantiomers or co-chromatographed with the other amino compounds or a large peak of a hydrolysis compound, 1-(9-fluorenyl)ethanol (FEOH), of FLEC [7]. In run II, D- and L-histidine were separated from each other and from methylated L-histidine derivatives (peaks 3 and 4 in Fig. 2). Both enantiomers of serine, and also those of proline, could also be determined in run II, as shown in Fig. 2. Even in run II, L-alanine was not separated from α- and β-aminobutyric acid. They could be separated only under the conditions of run III, as shown in Fig. 3. In runs II and III, the elution order of D- and L-enantiomer was reversed (L eluted before D), except for D- and L-histidine (Figs. 2 and 3). The 53 peaks in Figs. 1–3 were identified by the injection of each amino acid separately and the omission of single amino acid from the mixture.

The detection limit for these D- and L-amino acids was below 1 pmol at a signal-to-noise ratio of 2.6:1. The peak areas of FLEC derivatives of amino acids were linear upto 250 pmol (data not shown). Tyrosine, tryptophan and cysteine did not react sufficiently with FLEC under the conditions described above, although cystine could be derivatized and determined (peaks 46 and 48 in Fig. 1). Both D- and L-tryptophan, however, appeared between L-valine and D-phenylalanine in Fig. 1 if the amount injected exceeded 500 pmol. This was also the case with tyrosine, whereas only one peak was found for both enantiomers between glycine and L-α-aminoadipic acid. Table 2 gives the capacity factors and resolution factors of 44 amino acids determined by the present method. FLEC derivatives of amino acids were stable for at least 4 weeks at −20°C.

The method was applied to the determination of D-amino acids in the nervous tissues and eyes of several crustaceans (Table 3). The values shown are mean values for 3–35 animals from one species because each tissue was collected and mixed for economy of the expensive FLEC reagent. It can be seen in Table 3, that a D-amino acid present in less than 0.5% of total (D + L)-amino acid could be determined by the present method. Of seven D-amino acids found in these crustacean tissues, D-alanine and D-ar-

Table 2
Capacity factors (k') and separation factors (α) of FLEC derivatives of amino acids

Peak No.	Amino acid	k'			α	Run[a]
		D		L		
1,2	Histidine	2.28		2.41	1.06	II
7,8	Arginine	2.70		2.91	1.08	I
9	Taurine		3.48			I
10,11	Asparagine	4.09		4.23	1.03	I
12,13	Glutamine	4.29		4.46	1.04	I
14,15	Citrulline	4.74		4.91	1.04	I
17.18	Serine	6.15		6.03	1.02	II
20,21	Aspartic acid	5.52		5.62	1.02	I
22,23	Glutamic acid	6.23		6.40	1.03	I
24,25	Threonine	6.48		6.59	1.02	I
26	Glycine		6.87		–	I
27	α-Aminoadipic acid		7.23		–	I
28,29	Alanine	11.7		11.6	1.01	III
30	β-Alanine		10.4		–	I
31,32	Proline	11.0		10.6	1.04	II
33	γ-Aminobutyric acid		11.2		–	I
36,37	Methionine	10.4		10.6	1.02	I
38,39	Valine	11.5		11.6	1.01	I
40,41	Phenylalanine	12.1		12.3	1.02	I
42,44	Isoleucine	13.1		13.3	1.02	I
43,45	Leucine	13.2		13.5	1.02	I
47	Cystathionine		14.3		–	I
46,48	Cystine	14.0		14.4	1.03	I
50,51	Ornithine	16.7		17.0	1.02	I
52,53	Lysine	17.3		17.4	1.01	I

[a] See Table 1 for mobile phase compositions and gradient profiles in runs I–III.

ginine were the most abundant and widely distributed, whereas they were at lower levels than D-aspartate in the tissues of kuruma prawn. The level of D-alanine represented ca. 30% of the total alanine except for kuruma prawn. This percentage was much higher for D-aspartate but only a few percents for D-arginine. In all species, nervous tissue contained higher D-amino acid levels than eye. The amounts and the percentages of the D-form were low for other D-amino acids found in the crustaceans.

4. Discussion

As shown in Table 2, nineteen amino acids were separated into D- and L-enantiomers by the present HPLC method and were also separated from various physiological free amino compounds such as histidine-related compounds, taurine, glycine, hydroxyproline, α-aminoadipic acid, β-alanine, α-,β- and γ-aminobutyric acid, cystathionine and hydroxylysine. Some of these amino compounds were also determined by the present method (Table 2).

Utilization of FLEC as a derivatization reagent has several advantages: (1) FLEC derivatives of amino acids are stable for at least 1 month at −20°C; (2) the derivatization procedure is simple and needs no severe or complicated procedures; (3) the sensitivity is so high (below the pmol level) with fluorescence detection that one can detect less than 2 nmol/g wet mass of tissue D-amino acids. However, FLEC is expensive. Although we tried to decrease the

Table 3
Distribution of free D-amino acids in the nervous tissue and eye of crustaceans (μmol/g wet mass)

Tissue	D-Alanine	D-Arginine	D-Aspartate	D-Histidine	D-Serine	D-Glutamate	D-Proline
Kuruma prawn ($n = 35$)							
Nervous tissue	0.980(8.3)	0.645(2.6)	2.42(50.8)			0.077(1.5)	
Eye	0.815(5.2)	0.505(3.3)	4.61(65.3)				
Rock lobster ($n = 5$)							
Nervous tissue	3.83(35.8)	0.211(4.0)	4.59(49.4)	0.157(26.4)	0.103(11.1)	0.161(1.9)	
Eye	1.63(30.3)	0.138(4.1)	0.864(51.3)		0.017(2.9)	0.046(2.3)	
Crayfish ($n = 20$)							
Nervous tissue	3.43(35.3)	0.700(7.4)		0.231(26.0)			
Eye	2.53(36.0)	0.145(3.3)					
Snow crab ($n = 3$)							
Nervous tissue	9.74(33.9)	4.22(19.6)					
Eye	3.13(33.5)	0.472(3.5)	3.13(55.3)				
Woolly-handed crab ($n = 5$)							
Nervous tissue	9.72(24.1)	5.09(13.5)		0.068(10.0)		0.023(0.2)	0.037(0.2)
Eye	4.39(37.5)	0.197(3.7)					

Percentages of D-enantiomer relative to total (L + D)-enantiomers are given in parentheses. Blanks indicate <1.5 nmol/g wet mass.

amount of FLEC used, 0.5 ml of 18 m*M* FLEC solution was the minimum amount for the derivatization under the conditions described here. The optimum pH of borate buffer was 11 for FLEC derivatization, in contrast to the lower pH value reported [7]. Above or below pH 11, several amino acids gave two peaks, probably corresponding to mono- and disubstituted amino acids which were reported for FMOC derivatives of amino acids [10]. The reaction period required for derivatization was confirmed to be a minimum of 45 s, as reported previously for FMOC derivatization [8]. Precautions should be taken with the period of pentane extraction: if the extraction is not completed within 15 min, a significant loss of lysine will occur. During the derivatization procedure, no racemization occurred in the standard L-amino acid mixture.

The HPLC separation of FLEC derivatives of amino acids was difficult because of the numerous peaks and needed ternary gradient elution and three separate runs for complete analysis. Among various ODS columns tested in the present experiment, Shim-pack CLC-ODS (17 000 theoretical plates) gave the most satisfactory separation. Other columns, however, could also be employed if the gradient profile was changed slightly.

The present results clearly indicate the presence of several D-amino acids in the crustacean nervous system and eye. In these tissues, the D-alanine level and the ratio to total alanine were high compared with those in human brain [18] and the brain of mutant mice lacking D-amino acid oxidase [20], in which cases the D-alanine levels were below 0.05 μmol/g wet mass and the ratio to total alanine did not exceed 7%.

The presence of free D-aspartate in the brain of several animals has been well documented [12–18]. The level of D-aspartate in the brain of these animals, however, has been reported to be less than 0.5 μmol/g except for that in cephalopod brain, in which is 4–17 μmol/g [13–15]. In the brain of these animals, however, the ratio of D-aspartate to total aspartate is less than 8% in adult animals and does not exceed 30% in the brain of chick embryo and newborn rat [16]. Hence this may be the first report of a high percentage of D-aspartate in the animal brain or nervous system. This appears also to be the first report of the detection of several D-amino acids other than D-alanine, D-aspartate and D-serine in animal nervous systems. The proposed HPLC method will be useful for the survey of various D-amino acids in the nervous system as well as other tissues.

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