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Purification and Properties of a Presynaptically Acting Neurotoxin, Mandaratoxin, from Hornet (*Vespa mandarinia*)†

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ABSTRACT: A hornet (*Vespa mandarinia*) neurotoxin, mandaratoxin (MDTX), was purified by simple procedures with column chromatography made on Sephadex G-50 and CM-Sephadex by using an acetate buffer. The molecular weight of homogeneous MDTX was calculated to be approximately 20000 by gel filtration, $NaDodSO_4$ disc gel electrophoresis, and amino acid analysis. MDTX is a single-chain polypeptide. MDTX did not migrate electrophoretically in a basic buffer at pH 8.3 but did so when the buffer was acidic, at pH 4.3. The isoelectric point of the toxin was determined at 9.1 by isoelectric focusing. A relatively high amount of lysine was found in the amino acid analysis. $A^{1\%}_{280nm}$ was 15.1. Glucosamine and galactosamine were not detectable by amino acid analysis. MDTX had neither hemolytic nor enzymatic activity. The toxin was heat labile. By use of neuromuscular junctions of a lobster walking leg, it was found that the nanomole range of MDTX irreversibly blocked the excitatory postsynaptic potential without appreciable change in the resting conductance of the postsynaptic membrane. Intracellular recording from the presynaptic nerve fiber showed that MDTX blocked the action potential mainly by reducing the sodium current.

Much research has been carried out by using neurotoxins which block neuromuscular transmission in order to obtain information on the specific active sites and mechanisms involved in transmitter release (Ceccarelli & Clementi, 1979; Narahashi, 1974). Studies on the venom of *Hymenoptera* such as bees, wasps, and hornets have shown that the venom contains a variety of neurotoxic substances (Habermann, 1972; Piek, 1980; Kawai et al., 1980; Hori et al., 1977). However, due to the difficulty in collecting a sufficient amount of venom, research has not been carried out appreciably on the purification of neurotoxin or determination of active sites.

The giant hornet, *Vespa mandarinia*, which inhabits east and south Asia, is known as a very harmful insect since even one sting can result in death to man or domestic animals. A relatively large portion of the venom sac (ca. 20 mg fresh weight of the sac) of this species has facilitated the separation of the neuroactive components and elucidation of its action on a cellular level (Hori et al., 1977; Abe et al., 1979, 1980a).

The present study deals with a new polypeptide neurotoxin isolated from the venom of this hornet. We report the physical and chemical properties of this toxin and the specific mode of its action on the nerve membrane in blocking neuromuscular transmission. A preliminary account of this report has appeared elsewhere (Abe et al., 1980b).

Materials and Methods

Materials. The Sephadex G-50 (medium) and CM-Sephadex C-50 were purchased from Pharmacia Chemical Co. Sources of the standard proteins of sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide disc gel electrophoresis are as follows: bacitracin from Schwarz/Mann; ribonuclease from Boehringer; bovine serum albumin, ovalbumin, and chymotrypsinogen A from Miles Chemical Co. For isoelectric focusing, p*I* marker proteins containing cytochrome *c* from horse muscle and acetylated cytochrome *c* of several p*I* values, that is, 10.6, 9.7, 8.3, 6.4, 4.9, and 4.1, were purchased from Oriental Yeast Co. (Osaka). Iminodiacetic acid and ethylenediamine for the isoelectric focusing buffer were obtained from Tokyo Kasei Chemical Co. and Kishida Chemical Co., re-

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spectively. Coomassie Brilliant Blue R and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co. Sulfosalicylic acid was obtained from Wako Chemical Co. For amino acid analysis, standard amino acids were obtained from Takara Kosan Co. *o*-Phthalaldehyde was provided by Tokyo Kasei Chemical Co. Sodium tetrathionate was purchased from Pierce Chemical Co. For the enzyme assays, *p*-nitrophenyl acetate and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. and Wako Chemical Co., respectively. Egg yolk lecithin and casein were obtained from Merck Co.

Venom Sacs. Giant hornets (*V. mandarinia*) were collected from the middle part of Japan. The venom sacs were removed from anesthetized hornets and frozen immediately in liquid nitrogen (−197 °C). These sacs were stored until use.

Column Chromatography. All procedures for column chromatography were performed in a cold room at 5 °C. The Sephadex G-50 and CM-Sephadex C-50 were equilibrated with 50 mM acetate buffer, pH 5.6. The details appear in the figure legends. The protein eluted from the columns was measured for absorbance at 280 nm by using a Pharmacia dual path monitor, UV-2, and recorder, REC-2.

Disc Electrophoresis. Polyacrylamide disc gel electrophoresis was performed in 30 mM β-alanine–acetic acid buffer, pH 4.3 (Gabriel, 1971). The separating gel was prepared with 7.5% acrylamide and the stacking gel was 2.5% acrylamide. A total of 50–100 μg of the sample was mixed with sucrose (final concentration, 20%) and methyl green (final concentration, 0.02%). This mixture was applied to the top of the disc gel (i.d. 5 mm × 6 cm). For electrophoresis, a constant current of 4 mA/tube was applied. Staining and destaining were in accordance with the method of Weber & Osborn (1969). $NaDodSO_4$–polyacrylamide gel disc electrophoresis was carried out according to a slightly modified method of Weber and Osborn. The sample was reduced by 1% mercaptoethanol and also unreduced. The separating gel was prepared with 10% acrylamide, and the gel size was 5 mm × 8 cm. Electrophoresis was carried out at a constant current of 4 mA overnight. The relative mobilities of different proteins were plotted against the logarithm of their molecular weights.

Isoelectric Focusing. A carrier ampholyte for isoelectric focusing used pharmalyte ranging in pH from 3 to 10. The polyacrylamide gel rods (i.d. 5 mm × 6 cm) were prepared with 5% acrylamide. Focusing was carried out in an anode solution of 10 mM iminodiacetic acid and a cathode solution of 20 mM ethylenediamine at a constant voltage of 500 V for 5 h. After focusing, the gel rods were fixed overnight with a solution of 5% sulfosalicylic acid in 15% trichloroacetic acid and then stained with 0.05% Coomassie Brilliant Blue G-250 in 2.5% perchloric acid (Oversterberg et al., 1977). The standard p*I* value was determined by a protein kit of a known isoelectric point.

Amino Acid Analysis. A total of 0.1 mg and 10 μg of lyophilized protein sample was hydrolyzed in 0.5 or 0.1 mL, respectively, of 4 N methanesulfonic acid with 0.2% 3-(2-aminoethyl)indole at 115 °C for 24, 48, and 72 h. For estimation of the cystine, the hydrolyzed samples were treated with sodium tetrathionate (Simpson et al., 1976). Analyses on amino acids and amino sugars were carried out by using an automated amino acid analyzer (JEOL Model JLC-6AH). Each amino acid (and amino sugar) was detected by ninhydrin and *o*-phthalaldehyde (Roth, 1971).

Neuromuscular Preparation. For analysis of the action of MDTX, the neuromuscular junctions of a lobster (*Palinurus japonicus*) walking leg were used. Dissection of the preparation was essentially the same as described before (Grundfest et al., 1959; Kawai et al., 1972). The reason for this choice of preparation was that the neuromuscular transmission of Crustacea is similar to that of insects which are the prey of *Vespa*. This preparation also has an advantage of making possible an analysis of both pre- and postsynaptic events using intracellular recording from the presynaptic axon and postsynaptic membrane (Kawai & Niwa, 1977). The excitatory nerve to the stretcher muscle was isolated at the meropodite and stimulated with a pair of silver wires. By cutting off the exoskeleton and the connective tissue, we exposed the ventral stretcher muscle at the carpopodite bordering on the meropodite. KCl (3 M) electrodes, each having a resistance of 10–20 MΩ, were used for intracellular recordings from the axon and also from the stretcher muscle membrane. An intraaxonal recording was made at the border of the meropodite and carpopodite, a few millimeters from the recording electrode for the postsynaptic responses. The axonal membrane potential was differentiated by an operational amplifier. The normal solution consisted of 468 mM NaCl, 10 mM KCl, 20 mM $CaCl_2$, 8 mM $MgCl_2$ and 2 mM Tris-HCl buffer, pH 7.4.

The solution containing MDTX was prepared by diluting an appropriate amount of the toxin with the normal solution just before running the experiment which was carried out at room temperature (20–25 °C).

Enzyme Assays. Esterase activity was assayed by *p*-nitrophenyl acetate (Abe et al., 1977). Phosphatase was measured by *p*-nitrophenyl phosphate with a 0.1 M Tris-HCl buffer, pH 8.0 (Garen & Levinthal, 1960). The proteinase activity was assayed by the casein method (Arnon, 1970). The activity of phospholipase A was measured by titrating the free fatty acids released from lecithin with 0.01 N NaOH (DeHaas et al., 1968). For the hemolytic activity assay, blood was taken from the vena cava of an anesthetized mouse and washed 3 times in 10 volumes of saline. The washed blood cells were diluted to an appropriate concentration, and 4 mL of the resulting blood cell solution was incubated with 0.1 mL of the toxin solution for 10 min at 37 °C. After centrifugation at 3000 rpm, the optical density at 410 nm of the supernatant was measured.

Other Procedures. The protein was assayed by the method of Lowry et al. (1951) for crude venom protein and by the fluorescamine method (Howard & Gundersen, 1980) for purified MDTX which was used for analyses of physical, chemical, and physiological properties. For calculation of $A^{1\%}_{280nm}$ of MDTX, a measurement was made of the dry weight of the toxin (1 mg) which, when visking tubing was used, dialyzed in distilled water.

Results

Purification of Mandaratoxin from Vespa mandarinia. The presynaptically acting neurotoxin (mandaratoxin; MDTX) was purified by the following procedure. Fourteen grams of the venom sacs frozen in liquid nitrogen was homogenized in 60 mL of 50 mM acetate buffer, pH 5.6, by Ultra-Turrax for 1 min at 0 °C. The homogenate was centrifuged at 11000*g* for 60 min, and then 69 mL of the supernatant was applied on a Sephadex G-50 column equilibrated with the same buffer. The elution was separated into four peaks (A, B, C, and D) as shown in Figure 1. Peaks A and B electrophoretically identified several proteins with molecular weights exceeding 40000 and approximately 20000 corresponding to peak A and B, respectively. Peak C consists mainly of amino acids and slightly of several biogenic amines. The largest peak, peak D, contained serotonin as a major component.

The neurotoxic activity of MDTX was found in the peak B (fractions 43–60). About 270 mL of these fractions com-

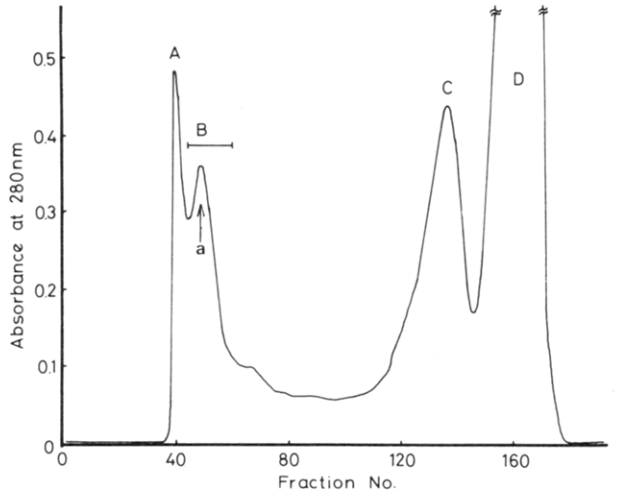

FIGURE 1: Column chromatography on Sephadex G-50 of hornet venom supernatant. 69 mL of hornet venom supernatant was applied to a column (i.d. 4.1 × 98 cm) of Sephadex G-50 equilibrated with 50 mM acetate buffer, pH 5.6. Each fraction collected was at 15 mL/tube, and the flow rate was 189 mL/h. MDTX activity appeared in peak B (fractions 43–60). The fractions were pooled and concentrated for further purification. (a) is a eluting position of chymotrypsinogen A on the same column.

Table I: Purification of MDTX from Hornet (*Vespa mandarinia*) Venom with Column Chromatography

fraction	protein concn (mg) [a]	sp act. (1/μg) [b]
venom supernatant	655.5	*c*
Sephadex G-50	128.2	$(2.5 \pm 0.5) \times 10^{-3}$
CM-Sephadex C-50	4.45	0.5 ± 0.25

[a] Protein was measured by the method of Lowry et al. (1951). [b] Specific activity is defined by the reciprocal of the toxin (micrograms) which is required for blockage of lobster neuromuscular transmission within 60 min. [c] The venom supernatant contains several kinds of neurotoxic substances, so that comparison of the specific activity is impossible.

bined was ultrafiltrated to approximately 10 mL by using a DE-5 membrane (Amicon). The concentrated sample was applied to CM-Sephadex C-50 chromatography equilibrated with the same buffer and fractionated under a rigid linear gradient of NaCl from 0 to 0.8 M. Six peaks (E–J) were obtained when the NaCl concentration was increased (see Figure 2). MDTX activity was found for peak H. The purification procedure of MDTX is summarized in Table I. The purified MDTX showed a single band when subjected to polyacrylamide disc gel electrophoresis at pH 4.3 (Figure 2, inset a) and to $NaDodSO_4$ disc gel electrophoresis in both its reduced and unreduced forms (Figure 2, inset b). These suggest that purified MDTX is a homogeneous protein.

Physical and Chemical Properties of MDTX. The molecular weight of MDTX was estimated to be 21 000 by using reduced and unreduced $NaDodSO_4$–polyacrylamide disc gel electrophoresis (see Table II). MDTX in crude venom analyzed by gel filtration of Sephadex G-50 (peak B in Figure 1) had a molecular weight similar to that found in a disc electrophoretical study. In this study, an amino acid analysis was carried out for determination of the molecular weight of MDTX which was found to be 19 000 (see Table II). It was assumed that the molecular weight was from 19 000 to 21 000 and that the protein molecule consisted of a single polypeptide chain. The polyacrylamide gel electrophoresis experiments suggest that MDTX is a basic protein since it migrated more in an acidic buffer than in a basic buffer. The isoelectric point of MDTX was focused on a single band and was estimated

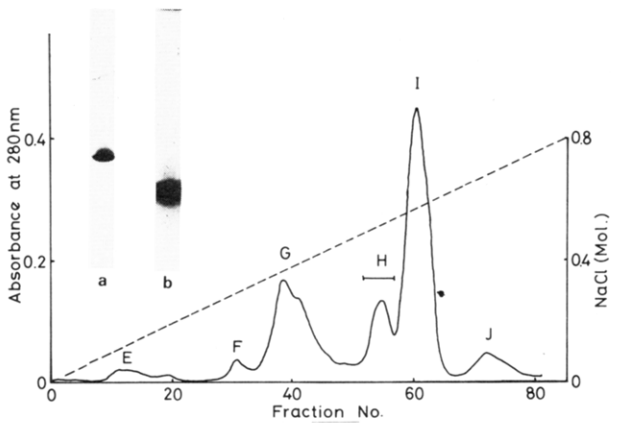

FIGURE 2: CM-Sephadex column chromatography of the peak B obtained after the Sephadex G-50 chromatography (see Figure 1). The concentrated sample (ca. 10 mL) was applied to a column (i.d. 2 × 33 cm) of CM-Sephadex equilibrated with 50 mM acetate buffer, pH 5.6. Elution was performed with a rigid linear gradient of NaCl from 0 to 0.8 M. Fractions of 4 mL were collected at a flow rate of 30 mL/h. The MDTX activity was found on peak H, and the active fractions (fractions 50–55) were combined and then concentrated for further studies. The inset is polyacrylamide disc gel electrophoresis of MDTX in β-alanine–glycine buffer at pH 4.3 (a) and in $NaDodSO_4$ with reduced conditions (b). Experimental conditions are given under Materials and Methods.

Table II: Chemical and Physical Properties of MDTX [a]

Amino Acid Composition		
amino acid	mol %	nearest integer
Cys	2.75	5
Asx	10.08	17
Thr	5.41	9
Ser	5.05	9
Glx	10.82	19
Pro	5.23	9
Gly	9.42	16
Ala	6.87	12
Val	5.77	10
Met	1.76	3
Ile	5.87	10
Leu	5.18	9
Tyr	4.82	8
Phe	2.12	4
Lys	10.18	17
His	3.09	5
Trp	2.49	4
Arg	3.09	5
total	100.05	171
glucosamine	ND [b]	
galactosamine	ND	

Molecular Weight	
by gel filtration (Sephadex G-50)	20 000
by $NaDodSO_4$–polyacrylamide disc gel electrophoresis	21 000
by amino acid analysis	19 000

Other Properties	
isoelectric point (p*I*)	9.1
$A^{1\%}_{280nm}$	15.1

[a] MDTX was hydrolyzed for 48 h and detected by ninhydrin. [b] Not detectable.

to be pH 9.1 by polyacrylamide gel rod isoelectric focusing. This value corresponds well with the results from the electrophoretical study and amino acid composition described below.

The amino acid composition of MDTX is shown in Table II. It should be noted that toxin has a high lysine residue

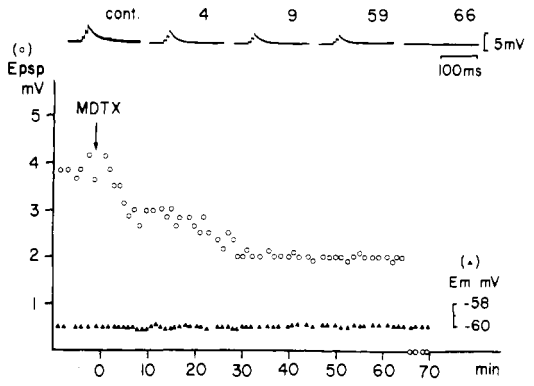

FIGURE 3: Effect of MDTX on neuromuscular junctions of lobster walking leg. Ordinate: Peak amplitudes of excitatory postsynaptic potentials (O) and the resting membrane potential (▲) in the stretcher muscle. Abscissa: Time after applying 0.1 μg of MDTX at 0 min. Specimen records: Excitatory postsynaptic potentials by three consecutive stimulations on the excitatory nerve (12 Hz). Numerals indicate time after MDTX in minutes.

content comparable to that of aspartic acid and glutamic acid. The high lysine content perhaps reflects the basic nature of MDTX. The $A^{1\%}_{280nm}$ value, owing to aromatic amino acid residues, was 15.1. Glucosamine and galactosamine were not detectable in the amino acid analysis. The neurotoxic activity of MDTX was reduced to about half that of the control after being heated to 60 °C for 30 min. This activity was lost on heating to 80 °C for 5 min. Thus the toxin is heat labile.

Enzyme Activity. A check was made for possible concomitant properties of the toxin such as enzymatic or hemolytic activity. MDTX showed no activity in the following tests: esterase activity for *p*-nitrophenyl acetate, phosphatase activity for *p*-nitrophenyl phosphate, phospholipase activity for lecithin of egg yolk, proteinase activity for casein, and hemolytic activity for mouse blood cell.

Effect of MDTX on Neuromuscular Transmission. A typical effect of MDTX on the neuromuscular junction of a lobster walking leg is shown in Figure 3. When a small amount (0.1–0.25 μg) of MDTX was applied to the lobster neuromuscular junction, the amplitude of the excitatory postsynaptic potentials (EPSP's) began to decline within a few minutes, gradually decreased, and finally disappeared. The time required to block the EPSP varied according to the preparation, ranging from several minutes to about 1 h. Larger doses of MDTX tended to shorten the time for blocking, but the decline always started within a few minutes. The resting membrane resistance of the postsynaptic membrane showed little change before and after toxin application. The action of MDTX was irreversible, and there was no recovery of EPSP after washing the preparation. Since the resting conductance channels of the postsynaptic membrane were not affected by the toxin, the active site of MDTX is considered to be presynaptic.

For verification of this, intracellular recordings were made from the axon and postsynaptic membrane. As shown in Figure 4, 10 min after MDTX was applied, the amplitude of the spike in the axon and its maximal rate of rise decreased (Figure 4b). Thereafter, the size of the axon potential and its maximal rate of rise continued to decrease. EPSP also decreased but the degree of reduction was not proportional to the axonal potential (see Figure 4c–e). Even when the amplitude of the axonal potential was reduced to 7–8 mV, EPSP still existed (Figure 4d). The data suggest that a small axonal potential propagates to the nerve terminal following transmitter release (Kawai & Niwa, 1980). Thirty-one minutes after MDTX application, there was no longer any

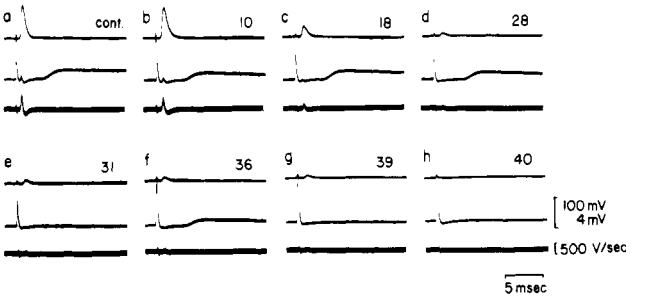

FIGURE 4: Intracellular recordings from the presynaptic axon (upper beam) and the postsynaptic membrane (middle beam) after applying MDTX (0.5 μg). The differentiated membrane potential of the axon is shown in the bottom beam. Numerals indicate time (minutes) after toxin. Further description is given under Materials and Methods.

EPSP (Figure 4e). When the intensity of the stimulus current was raised, EPSP appeared again (Figure 4f) but soon disappeared (Figure 4g). After 40 min, there was no response in the axonal recording even with supramaximal strength of stimulation. The effect of MDTX was found to be irreversible; the toxin binding to the neuromuscular junctions appeared to be short since the blockage of transmission could not be prevented by washing the preparation following the first 5 min of toxin application.

Discussion

The present study has shown that chemically and physiologically a new neurotoxin (MDTX) can be purified from a hornet (*V. mandarinia*). After an extended trial, we succeeded in devising a simple technique (two steps) for purification of MDTX in high yield. The homogeneity of MDTX was confirmed by polyacrylamide disc gel electrophoresis and also by isoelectric focusing on a polyacrylamide gel rod (Figures 1 and 2). Estimations of the molecular weight of purified MDTX in its reduced and unreduced forms with denaturing solvents and its molecular weight in the native form are nearly the same. It is concluded that the toxin is a single polypeptide chain of approximately 20 000 daltons. Thus, the toxin acts on nerve membranes as a monomer protein of similar molecular weight.

Another physical property is the basic nature of the toxin, as shown by electrophoresis under acidic conditions and isoelectric focusing (pI = 9.1). The basic nature of MDTX is perhaps reflected by its high lysine content.

Among neurotoxins which behave presynaptically, snake venom toxins such as β-bungarotoxin, crotoxin, and notoxin act through the enzymatic activity of phospholipase A (Howard & Gundersen, 1980). The toxicity of phospholipase A is characterized by loss of transmitter release followed by depression resulting from an irreversible blockage of synaptic transmission (Chang et al., 1973). It has already been reported that the venom of bees, wasps, and hornets contains enzymes with phospholipase A activity (Habermann, 1972). Thus, it might be argued that MDTX affects the nerve membrane through phospholipase activity. However, no phospholipase or proteinase activity was found in MDTX. Furthermore, an electrophysiological study showed that there was no facilitatory phase of transmitter release in the process of MDTX action. Thus, it seems unlikely that MDTX is the neurotoxin counterpart of an enzyme which affects the nerve membrane.

The intraaxonal recordings made it clear that MDTX reduced the amplitude of the action potentials and their maximum rate of rise (Figure 4). Since the rising phase of the action potential in this axon is contributed by the sodium current (Kawai & Niwa, 1980), MDTX may block the action potential at least primarily by reducing the regenerative sodium

current. The mode of action, therefore, appeared to resemble that of tetrodotoxin (TTX). The effective concentration of MDTX (10^{-6} M) was comparable to that of TTX. It is unlikely, however, that MDTX is bound to TTX since MDTX is heat labile and has an irreversible nature. Furthermore, the finding that the TTX-resistant current is blocked by MDTX in insect larva (unpublished experiments) indicates that MDTX acts differently from TTX toward the nerve membrane. This feature of MDTX supports the notion described above that the toxin may act as a binding protein with the specific structure of a nerve membrane with little catalytic activity. The high binding affinity and irreversible nature of MDTX might be useful as a marker in carrying out further study on ionic channels in a nerve membrane.

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