Journal of Chromatography A, 655 (1993) 77–83
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 042

Separation and characterization of rat kidney isometallothioneins induced by exposure to inorganic mercury

M.A. Morcillo and J. Santamaría*

División de Isótopos, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, 28040 Madrid (Spain)

ABSTRACT

High-performance liquid chromatography (HPLC) was applied to the separation of metallothionein (MT) isoforms from different tissues from a variety of eukaryotic species. Recently we reported an analytical method for ^{203}Hg–metallothionein, which detects the radioisotope bound to each iso-MT after separation by HPLC on a size-exclusion column coupled with on-line radioactivity flow detection. The MTs can be separated as distinct isoprotein peaks by elution with alkaline buffer solution owing to cation-exchange chromatographic action. In the present work, renal MT from rats exposed to inorganic mercury was separated into four peaks by UV and ^{203}Hg detection. Moreover, it was resolved into four components by non-denaturing polyacrylamide gel electrophoresis. The two major components correspond to MT-1 and MT-2, which were characterized by amino acid analysis. Finally, Hg induces and binds to both iso-MTs.

INTRODUCTION

Mercury is one of the non-essential elements that presents a high risk to human health. Following chronic exposure of animals to inorganic mercury the highest concentration of metal is found in the kidney [1] where it is mainly associated with metallothionein (MT) [2–4], a small protein rich in cysteinyl residues and heavy metals [5,6].

High-performance liquid chromatography (HPLC) has been applied to the separation of MT isoforms from different tissues and a variety of eukaryotic species. MT can be separated into different isoprotein peaks by HPLC on a size-exclusion column (HPSEC) by elution with an alkaline buffer solution due to a cation-exchange chromatographic action [7]. Thus, we have reported that the rat renal ^{203}Hg-MT induced by exposure to inorganic mercury can be resolved into at least three peaks by HPSEC connected on-line with a radioactivity detector, in order to obtain the simultaneous HPLC–UV and HPLC–radioactivity detection chromatograms [8].

In summary, the aim of this work was, first, to characterize by amino acid analysis the isometallothioneins (iso-MTs) induced in rat kidney by exposure to Hg(II) and, second, to determine whether the same number of components resolved by HPSEC can be obtained by polyacrylamide gel electrophoresis (PAGE). An attempt was made to correlate the change in the chromatographic elution profile after injection of rat renal Hg-MT on HPSEC and the mercury exposure dose.

EXPERIMENTAL

Reagents

Mercuric chloride, sucrose, hydrochloric acid,

* Corresponding author.

0021-9673/93/$06.00

β-mercaptoethanol and tris(hydroxymethyl)-aminomethane were obtained from Merck (Darmstadt, Germany), $^{203}HgCl_2$ from Amersham International (Amersham, UK), ammonium formate and ammonia solution 30% from Carlo Erba (Milan, Italy), rabbit liver metallothionein from Sigma (St. Louis, MO, USA), reagents for electrophoresis from LKB (Bromma, Sweden), Sephadex G-75 from Pharmacia (Uppsala, Sweden) and Atomflow from DuPont (Boston, MA, USA).

Preparation of rat kidney supernatant

Twenty-four male Sprague–Dawley rats (Cijisa, Madrid, Spain), weighing about 135 g, were randomly divided into four groups of six animals each. The groups were exposed to 1, 10 and 100 μg Hg as $HgCl_2$ per ml of drinking water for 8 weeks with food and water *ad libitum*. Following a single intraperitoneal dose of 23 μg of Hg per kg body mass (5–10 μCi of $^{203}HgCl_2$), the rats were sacrificed 3 h after dosing by exsanguination under light ether anaesthesia.

The kidneys were removed and homogenized in two volumes of 10 m*M* Tris–HCl buffer (pH 8.6)–0.25 *M* sucrose–5 m*M* 2-mercaptoethanol (bubbled with nitrogen before use) and the homogenate was centrifuged at 105 000 *g* for 90 min in a Centrikon T-1055 ultracentrifuge with a TFT 65.13 rotor (Kontron Instruments, Milan, Italy) at 4°C.

Separation of metallothionein on a Sephadex G-75 column

The renal supernatant was applied to a column (70 × 2.6 cm; Pharmacia, Uppsala, Sweden) of Sephadex G-75 that had been pre-equilibrated with 20 m*M* ammonium formate buffer, pH 7.65 (adjusted with ammonia solution), and the column was eluted with the same buffer (bubbled with nitrogen before use). Fractions (4.2 ml) were collected and the radioactivity in each fraction was measured in an LKB 1275 Minigamma solid scintillation spectrometer.

HPSEC

An aliquot of 100 μl of MT peak central fraction eluted from the Sephadex G-75 column was applied to a Protein-Pack 125 column (300 × 7.8 mm; Waters, Milford, MA, USA), which was eluted with 20 m*M* ammonium formate buffer (pH 7.65 at 25°C) at a flow-rate of 1 ml/min. Molecular absorbance at 254 nm was continuously monitored with a Model 484 ultraviolet detector (Waters) and the signal was registered by a Model 730 data module (Waters).

For simultaneous determination of the MT-associated radioactivity, the column outlet tube was connected on-line with a LKB Model 1208 Betacord radioactivity monitor. An aliquot of 1 ml of the MT peak eluted from the Sephadex G-75 column was dried on a Model SVC 100H SpeedVac concentrator (Savant, Farmingdale, NY, USA) and redissolved in 100 μl of 20 m*M* ammonium formate buffer (pH 7.65). The sample was subjected to HPLC analysis with a Protein-Pack 125 column and the same buffer at a flow-rate of 0.6 ml/min. A liquid scintillation cocktail, Atomflow, was added to the HPLC eluent at a flow-rate of 1.8 ml/min before the eluent passed through the flow cell of the radioactivity detector, and the presence of ^{203}Hg was simultaneously monitored.

Amino acid analysis

Samples of isometallothioneins were hydrolysed for 24 h at 116°C and subjected to amino acid analysis by the Pico.Tag method (Waters). For determination of cysteine, samples were oxidized with performic acid [9] before acid hydrolysis.

PAGE

For native PAGE the method described by Davis [10] was performed on an LKB 2001 vertical slab gel apparatus. The stacking gel contained 4.5% acrylamide, and the separation gel a gradient of 5–15% acrylamide. The gel was stained with Coomassie Blue R-250 and was scanned by an MIP-Interdens System densitometer (Microm, Barcelona, Spain).

RESULTS AND DISCUSSION

Fig. 1 shows typical elution profiles of the radioactivity associated to proteins in mercury-exposed rat kidney supernatant on a Sephadex

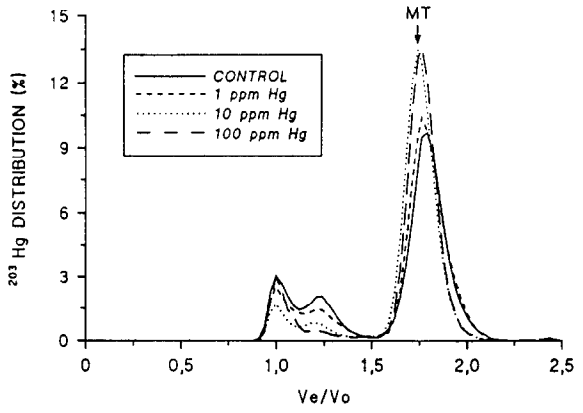

Fig. 1. Rat kidney supernatants were applied to a Sephadex G-75 column (70 × 2.6 cm) and eluted with 0.02 *M* ammonium formate buffer (pH 7.65). Fractions of 4.2 ml were collected and the radioactivity content in each fraction was determined.

G-75 column. The chromatographic profiles were different for each Hg dose studied. Thus, in the control group, 72% of the eluted cytosolic ^{203}Hg was associated with a broad band showing a peak with a ratio elution volume (V_e)/void volume (V_0) 1.6–2.1. Since metallothionein was shown to elute in this region, this peak was assumed to represent a ^{203}Hg–MT complex. The dietary mercury stimulated significantly the uptake of ^{203}Hg in renal MT, since around 85–90% is bound to MT from rats exposed to 10 and 100 ppm Hg(II).

A two-step, low-pressure, column chromatographic procedure generally involving a combination of size-exclusion and anion-exchange modes has been widely used to isolate MT from tissue extracts and continues to be the method of choice for the purification of MT on a preparative scale [11]. Whanger and Deagen [12] purified rat renal ^{203}Hg–MT by SEC (Sephadex G-75) and isolated three mercury–MT species from a DEAE-cellulose column. However, this approach is time consuming and requires relatively large amounts of sample. Recently, we reported that rat kidney ^{203}Hg–MT can be separated as distinct isoprotein peaks by HPSEC [8]; the separation of Hg–MT species on a HPSEC column revealed that they can be purified and characterized with a small sample size in a short time. Although in our above-mentioned study ^{203}Hg–MT was separated into three major peaks, in the present work an aliquot of MT (from the group exposed to 100 ppm) isolated on a Sephadex G-75 column and subjected to HPLC analysis on an SEC yielded four peaks (Fig. 2). These peaks were resolved by both UV (Fig. 2A) and ^{203}Hg detection (Fig. 2B). The radioactivity of the central fraction (number 60) was predominantly associated with the major peaks (2 and 3). Reversed-phase (RP) HPLC is a powerful technique for effectively separating peptides that differ in their composition by a single amino acid [13]. We have reported that rat renal ^{203}Hg–MT is separated into four peaks by RP-HPLC [8], therefore rat kidney mercury-induced MT is resolved into four peaks by both HPSEC and RP-HPLC.

The MT isoforms found in rat tissues can also be successfully separated by non-denaturing PAGE [14]. However, no success was achieved in isolating MTs containing variable amounts of copper (rat renal mercury-induced MT is a protein rich in copper [3,4]). The presence of copper appears to consistently affect the mobility of MT such that the lane of gel is generally a

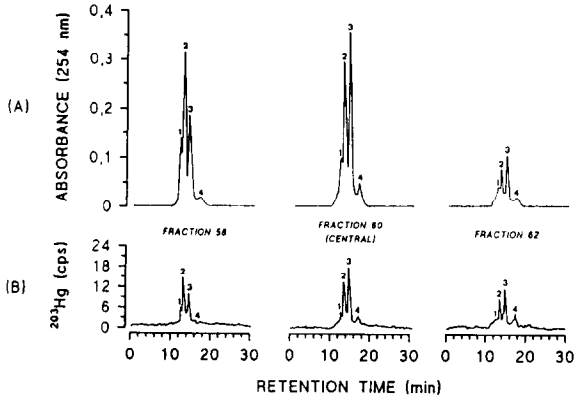

Fig. 2. An aliquot (1 ml) of mercury-exposed renal MT (100 ppm Hg group) isolated on a Sephadex G-75 column was concentrated and redissolved in 100 μl of 0.02 *M* ammonium formate buffer (pH 7.65). This sample was subjected to HPLC on a Protein-Pack 125 column (300 × 7.8 mm) and cluted with a 0.02 *M* ammonium formate buffer (pH 7.65) at a flow-rate of 0.6 ml/min. A liquid scintillation cocktail was added to the HPLC eluent at a flow-rate of 1.8 ml/min and the presence of radioactivity was continuously monitored. (A) UV chromatogram at 254 nm and (B) radioactivity detection chromatogram.

Fig. 3. A sample of mercury-exposed renal MT (100 ppm Hg group) was subjected to non-denaturing polyacrylamide gel electrophoresis. The stacking gel contained 4.5% acrylamide and the separation gel a gradient of 5–15% acrylamide. The gel was stained with Coomassie Blue R-250 and was scanned by a densitometer.

smear. Nevertheless, in the present work, Hg–MT was resolved into four components by non-denaturing PAGE (Fig. 3), which corroborates the results obtained by HPLC.

Suzuki *et al.* [15] have reported that SW and Sephadex columns are similar with respect to separation mechanisms; molecules of different molecular masses are separated by different elution rates through microspheres of hydrophilic polymer gels, and molecules of different isoelectric points are separated in a cation-exchange mode by ionized hydroxyl groups in alkaline solution. As the MT-1 and MT-2 isoforms possess two and three negative charges, respectively, between pH 7.5 and 9.5, a faster eluting metallothionein fraction on a Sephadex G-75 column is rich in iso-MT-2 and a slower eluting MT fraction is rich in iso-MT-1. Therefore, the former fraction gives lower, and the latter fraction higher, ratios of MT-1 to MT-2. In order to characterize the different peaks resolved by HPLC, the fractions of the rat kidney Hg–MT peak isolated on a Sephadex G-75 were analysed by HPSEC. Peak heights of iso-MTs were plotted against number fraction, as shown in Fig. 4. Peak 2 predominates in the faster eluting MT fractions, which indicates that peaks 2 and 3 presumably correspond to MT-2 and MT-1, respectively.

The absorption spectrum of MT in the range 240–300 nm results primarily from metal–ligand charge-transfer transitions. The shape of the absorption spectrum and the extinction coeffi-

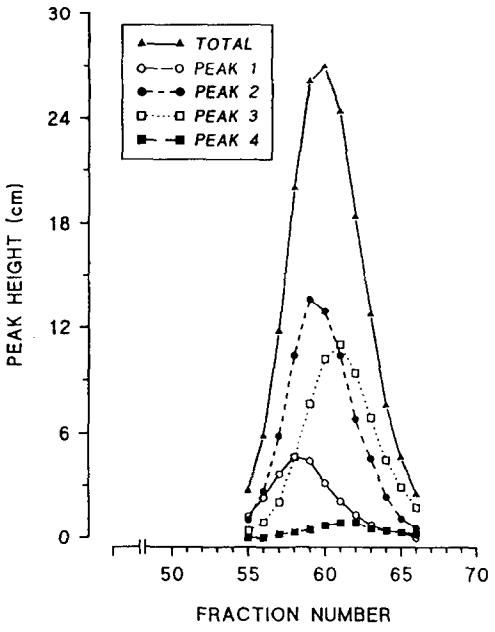

Fig. 4. Rat kidney supernatant (100 ppm Hg group) was applied to a Sephadex G-75 column (70 × 2.6 cm) and eluted with 0.02 *M* ammonium formate buffer solution, pH 7.65. Metallothionein was eluted at around tube 60. An aliquot (100 μl) of tubes 55–66 eluted from the Sephadex G-75 column was subjected to HPLC on a Protein-Pack 125 column (300 × 7.8 mm) and eluted with 0.02 *M* ammonium formate buffer solution (pH 7.65) at a flow-rate of 1 ml/min. Peak heights of the four components resolved by HPLC were plotted against fraction number. Peak 2 = MT-2; peak 3 = MT-1.

cients at various wavelengths are characteristic of metals bound to the thionein [16]. Thus, Hg–MT has a maximum absorption around 265 nm [17]. As shown in Fig. 5, peaks 2 and 3 have a maximum absorption at this wavelength.

In addition, rat kidney MT exhibits a third peak which eluted at the slowest rate on the SW column [15] owing to the presence of copper and intramolecular oxidation [18]. Oxidation of SH groups results in a denser and more globular monomer. This causes a decrease in the size of the protein molecule and the Stokes radius, and, as a consequence, an apparent loss in mass, determined by gel chromatography [19]. We have previously reported that replacement *in vitro* of metals in rabbit liver Cd,Zn–thionein with Cu^{2+} and Hg^{2+} produces additional peaks on HPSEC, which elute with longer retention time than the

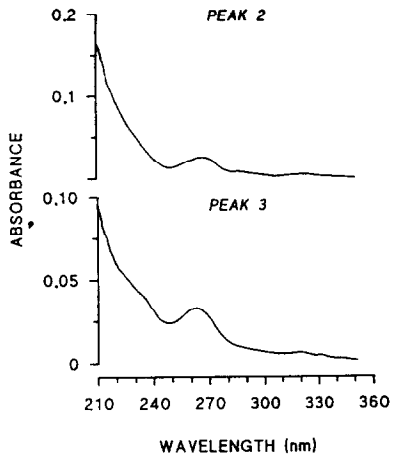

Fig. 5. A sample of mercury-exposed renal MT (100 ppm Hg group) isolated on a Sephadex G-75 column was subjected to HPLC on a Protein-Pack 125 column (300 × 7.8 mm) and eluted with 0.02 *M* ammonium formate buffer (pH 7.65) at a flow-rate of 1 ml/min. Peaks 2 and 3 were collected and an absorption spectrum was obtained by a UV–Vis 8450A spectrophotometer (Hewlett-Packard).

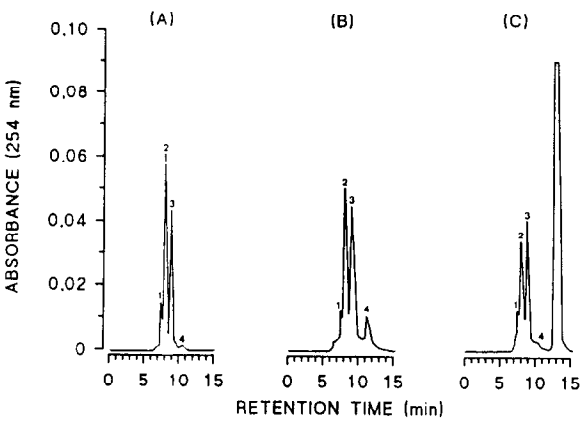

Fig. 6. A sample of mercury-exposed renal MT (100 ppm Hg group) isolated on a Sephadex G-75 column was maintained at room temperature in order to help its oxidation. Immediately, the sample was incubated in presence of 10 m*M* 2-mercaptoethanol and subjected to HPLC on a Protein-Pack 125 column (300 × 7.8 mm) and eluted with 0.02 *M* ammonium formate buffer (pH 7.65) at a flow-rate of 1 ml/min. (A) Original MT; (B) oxidized MT and (C) MT + 2-mercaptoethanol.

native molecule [4]. In order to determine whether peak resolved by HPSEC correspond to oxidized MT, an aliquot of the MT peak isolated on a Sephadex column was kept at room temperature for 1 week in order to help its oxidation. Afterwards, the sample was incubated in the presence of 10 m*M* 2-mercaptoethanol for 30 min at 37°C and subjected to HPSEC. The original Hg–MT gives the elution profile shown in Fig. 6A. The oxidized MT is characterized by a remarkable increment in peak 4 (Fig. 6B), which almost disappeared when the sample was incubated in the presence of 2-mercaptoethanol (Fig. 6C). Therefore, peak 4 may correspond to oxidized MT molecules resulting from the presence of copper/mercury and intramolecular oxidation.

A typical characteristic of MTs is their extremely high content of cysteine (about 33%); in addition to cysteine, most MTs also contain a relatively large proportion of serine (approximately 14%) and basic amino acids, especially lysine and, occasionally, arginine (lysine and arginine being around 13%) [20]. The amino acid composition of the different isoprotein peaks of rat kidney Hg–MT is shown in Table I. The results indicate that peaks 2 and 3 are

TABLE I

AMINO ACID COMPOSITION OF ISOMETALLOTHIONEINS

The values are expressed as percentages of the total number of residues in the molecule. Values were obtained from duplicate 24-h hydrolysates.

Amino acid	Rat kidney mercury-exposed MT				Rabbit liver MT	
	Peak 1	Peak 2	Peak 3	Peak 4	MT-1	MT-2
Asp	9.9	10.9	7.4	5.2	7.3	7.0
Glu	10.5	8.0	4.8	6.6	4.4	4.9
Ser	4.7	9.6	13.3	10.8	11.5	10.1
Gly	9.7	10.2	11.2	15.6	7.3	9.4
His	1.4	0.8	0.3	0.1	–	0.4
Arg	5.7	3.2	1.6	3.9	1.3	0.9
Thr	6.6	5.9	6.2	5.5	5.5	–
Ala	6.4	3.6	6.7	8.6	12.6	15.6
Pro	2.2	2.9	3.4	8.3	4.2	6.1
Tyr	–	0.5	–	–	–	–
Val	6.5	0.9	3.7	4.5	0.3	1.6
Cys	6.0	20.6	26.7	14.5	28.9	22.6
Ile	7.1	3.5	1.0	3.4	2.0	2.6
Leu	8.7	4.3	1.4	3.9	0.8	1.4
Phe	5.5	2.7	0.8	1.4	–	0.5
Lys	9.2	12.4	11.4	7.7	14.1	11.1

metallothionein-type proteins, with a characteristic cysteine content of around 21–27%, which is similar to the cysteine content of rabbit liver iso-MTs (Sigma). However, this cysteine content was lower than the cysteine content determined from DNA sequence data reported by Winge *et al.* [21]. The very confusing fact which has made impossible the unequivocal identification of several copper-proteins as MTs has been their low content of cysteine [22]. This low cysteine content has been explained by some authors [23] as a probable artefact on the basis that, in the presence of significant amounts of copper, cysteine contents are much lower. This is especially true when the method used for the determination of cysteine is based on its oxidation to cysteic acid. Therefore, the discrepancy observed between the cysteine content determined from amino acid analysis (Table I) and from DNA sequence analysis [21] could be explained by oxidative changes catalysed by Cu^{2+}. Peak 4 shows a lower content of cysteine (about 15%) than the others, and this phenomenon could be also caused by the circumstances mentioned above. Another characteristic of MTs is that they are lacking in aromatic amino acids. However, peak 2 isolated from HPSEC contained a small amount of phenylalanine. This indicates that the peak 2 sample might have been contaminated with a small amount of peak 1 (the Phe content of peak 1 is about 6%) and that the phenylalanine derived from this peak.

Winge *et al.* [22] initially reported the characterization of a distinct, non-metallothionein copper-rich protein from the livers of copper-injected rats. This putative species, called copper–chelatin, which is now generally considered to be artifactual, appeared to have a lower Cys content and to elute later than MT-2 on DEAE ion-exchange chromatography. Provided peak 1 elutes earlier than peak 2 (MT-2) on HPSEC (with this technique the iso-MTs are eluted in reverse order from an anion-exchange column) and its Cys content is 6%, it can be an artefact produced in the rat renal mercury-induced metallothionein purification.

On the other hand, we have applied HPSEC to determine whether some isoform of rat kidney Hg–MT is preferentially expressed in response to induction by different doses of mercury. Fig. 7 shows the chromatograms obtained after subjecting to HPSEC an aliquot of rat kidney MT induced by different doses of mercury. In response to mercury, both isoforms increased in a

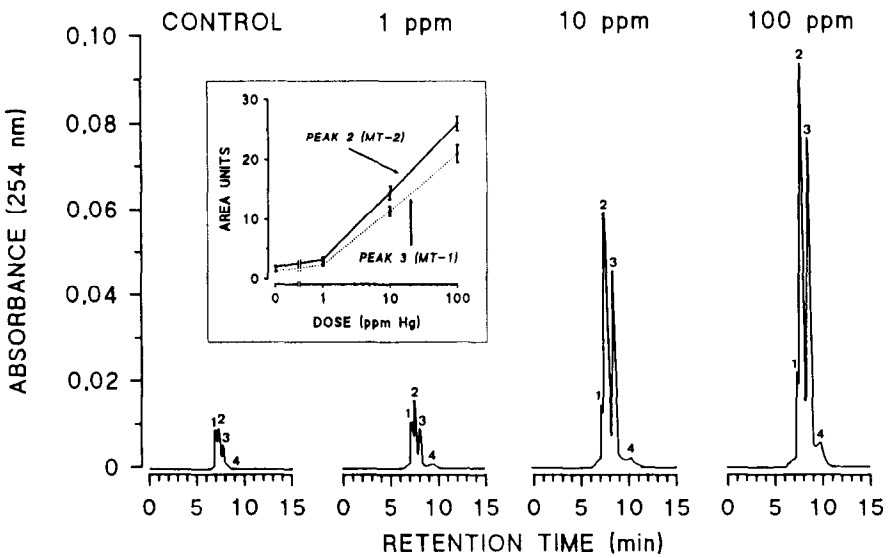

Fig. 7. An aliquot (100 μl) of mercury-exposed renal MT isolated on a Sephadex G-75 column was subjected to HPLC on a Protein-Pack 125 column (300 × 7.8 mm) and eluted with 0.02 *M* ammonium formate buffer solution (pH 7.65) at a flow-rate of 1 ml/min. Insert: Area units of MT-1 and MT-2 plotted against mercury dose.

similar way, since MT-1 and MT-2 increased thirteen- and sixteen-fold, respectively, compared with a control group (insert of Fig. 7).

In summary, rat kidney Hg–thionein yields four peaks on HPSEC and PAGE. Two of these, peaks 2 and 3, correspond to MT-2 and MT-1, respectively; the other two, peaks 1 and 4, correspond to an artifact and oxidized MT molecules, respectively. This corroborates previous evidence that there are only two functional MT isotypes in the rat, the MT-1 and MT-2 proteins [24]. Finally, Hg induces and binds to both isoforms.

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