Mechanism of the Protective Effect of Sodium Malate on Cisplatin-Induced Toxicity in Mice

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We examined the mechanism of the protective effect of sodium malate on cis-diamminedichloroplatinum(II) (cisplatin, CDDP)-induced nephrotoxicity in mice and obtained the following findings:

1) Sodium malate showed a maximum reduction of toxicity when it was administered at the same time as CDDP or at 30 min before the administration of CDDP; the reduction was significantly decreased when sodium malate was given after the administration of CDDP.

2) It is thought that diaminoplatinum(II) malate (DPM) is produced in the body following the administration of a combination of CDDP and sodium malate. DPM showed the same antitumor effect as CDDP and produced little nephrotoxicity.

3) When CDDP was combined with sodium malate, the time necessary for the elimination of platinum from the blood was prolonged, showing an intermediate value between the times for the elimination of CDDP and DPM from the blood. When the drug clearance was calculated based on this result, it was found that about 40% of the CDDP administered was converted to DPM in the blood.

4) In an experiment using l-[14C] malic acid, it was shown that sodium malate is distributed in the liver and the kidney at high concentrations, but in the tumor at only a low concentration.

5) When sodium malate was administered in combination with CDDP, the amount of platinum which accumulated in the kidney after 24 h was decreased by about 55% compared with the uncombined group, but there was no change in the amount of platinum which accumulated in the tumor.

These results suggest that the sodium malate administered may be distributed rapidly in the blood and the tissue, and about 40% is bound to CDDP and converted to DPM, thus reducing the nephrotoxicity of CDDP.

Key words sodium malate; cisplatin; diaminoplatinum(II) malate; nephrotoxicity; Kampo medicine; malic acid

cis-diamminedichloroplatinum(II) (cisplatin, CDDP) is an excellent anticancer agent used all over the world for the treatment of testicular tumors, ovarian cancer, urinary bladder cancer and uterine cancer. However, because of the severe side effects of CDDP, such as nephrotoxicity, vomiting and nausea, its usage is limited, and the development of countermeasures for its side effects is an important challenge.

We have been searching for an agent that can reduce CDDP-induced toxicity, and we isolated sodium malate as an agent for the reduction of CDDP-induced nephrotoxicity from a Kampo medicine, Juzen-taiho-to. Sodium malate almost completely inhibited CDDP-induced nephrotoxicity in mice at a dose of more than 0.25 times the equimolar amount of CDDP, without reducing the antitumor effect of CDDP.

In the present study, to clarify the mechanism of action of sodium malate, we investigated the plasma pharmacokinetics and tissue distribution of platinum in combinations of CDDP and sodium malate administered to mice. Diaminoplatinum (II) malate (DPM, Fig. 1) is a compound expected to be produced by the reaction of sodium malate and CDDP in the body. We examined its antitumor effect, toxicity and the rate of production in mice. Additionally, we examined the distribution of sodium malate in the mouse body using l-[14C] malic acid.

MATERIALS AND METHODS

Animals Five-week-old male ddY mice (average weight: 25 g) were obtained from Japan SLIC, Inc. (Hamamatsu, Japan) and kept in rooms with controlled temperature (23 ± 0.5 °C), humidity (50 ± 5%), and 12-h light/12-h dark cycles. They were fed commercial mouse chow (MF: Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum, and were used after 1 week of acclimation (average weight: 30 g).

Chemicals CDDP was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The solutions of CDDP were prepared with sterile 0.9% saline at concentrations of 0.5 and 1.0 mg/ml, respectively. Sodium malate was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo). DPM was purchased from Hodogaya Contract Lab. Co., Ltd. (Tokyo). l-[14C] Malic acid (l-[1,4,2,3,14C] malic acid) was purchased from Amersham K. K. (Tokyo) (specific radioactivity: 12.5 MBq/mg; radiochemical purity: not less than 99%).

Treatment of Animals Animal treatment using this method was a modification of the method reported previously. Briefly, the animals in the test groups, each comprised of 10 mice, were subcutaneously inoculated with sarcoma 180 (S-180) cells (10⁶/mouse) in the left thigh on day 1. CDDP (10 µmol/kg) was given intraperitoneally (i.p.) to the mice on days 3, 4, 5, 6, 7, 8, 10, 11 and 12. Sodium malate was given orally to the mice on days 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14 and 15. The mice in the control group were treated

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with water (orally) and 0.9% saline (i.p.). On day 17, the mice were anesthetized with ether, blood was collected from the inferior vena cava, and the number of red blood cells (RBC), white blood cells (WBC) and platelets was immediately counted. After centrifugation of the remaining blood, the serum was analyzed for blood urea nitrogen (BUN), serum creatinine, serum glutamic-oxaloacetic transaminase (sGOT) and serum glutamic-pyruvic transaminase (sGPT). The liver, kidneys, spleen, thymus, stomach (organ and contents) and tumor were excised and weighed. For examination of the reduction of CDDP-induced toxicity by the timing of administration, sodium malate was orally administered at 3, 1 and 0.5 h before the administration of CDDP, immediately before the administration of CDDP and at 1 and 3 h after the administration of CDDP. In the examination of the effect of DPM, DPM was administered i.p. at doses ranging from 2 to 10 μmol/kg/d instead of CDDP according to the same schedule, and 0.5 ml of water was administered orally instead of sodium malate.

**Measurement of Functions**

RBC, WBC and platelet counts were made on a Celltac 4150 (Nihon Koden, Co., Tokyo). BUN, serum creatinine, sGOT and sGPT were measured spectrophotometrically on a Cobas Fara (Baxter, Ltd., Tokyo), with the use of the following assay kits: for urea nitrogen, HR II test Wako; creatinine, HA test Wako; sGOT, FA test Wako; and sGPT, FA test Wako (Wako Pure Chemical Industries, Ltd., respectively).

**Antitumor Activity**

The inhibitory percent of the antitumor activity was calculated by the following formula:

\[
\text{inhibition (\%) = (1 - B/A) \times 100}
\]

where \( A \) is the mean tumor weight of the control group and \( B \) is that of the drug-treated groups.

**Determination of Plasma Pharmacokinetics**

In a preliminary experiment, when CDDP was administered to mice at a dose of 10 μmol/kg, platinum could not be detected in plasma. Therefore, the CDDP pharmacokinetics were studied using CDDP by a single administration at 40 μmol/kg. Briefly, sodium malate (80 μmol/kg) was orally administered to mice 30 min after an i.p. administration of CDDP (40 μmol/kg). In the CDDP alone and the DPM alone groups, CDDP or DPM was administered i.p. to mice 30 min after the oral administration of water. Subsequently, at 10, 20, 30, 60, 90 and 120 min after the administration of CDDP, blood was collected from the inferior vena cava, and the plasma was immediately separated with the use of a centrifugal ultracentrifuge (Centricon 10, Grace and Co., MA, U.S.A.) to obtain a fraction with a molecular weight of not more than 10000 (non-protein-binding platinum), in which the concentration of platinum was then determined using a flameless atomic absorption spectrophotometer (AA-880 mark II, FLA-1000, AS-301, Nippon Jarrel Ash Ltd., Tokyo). The half-life of platinum in plasma was calculated using the following 2-exponential formula:

\[
C = A \exp(-\alpha t) + B \exp(-\beta t)
\]

where \( C \) is the concentration at time \( t \), and \( A, B, \alpha \) and \( \beta \) are the constants.

**Detection of DPM in Plasma**

CDDP, CDDP + sodium malate and DPM were administered to mice under the same conditions as those used for the determination of plasma pharmacokinetics. The non-protein-binding platinum fraction was separated at 10 min after the administration of CDDP or DPM. Fractionation was conducted under the following high-performance liquid chromatography (HPLC) conditions:530 gel ODS-80Ts (5 mm × 25 cm); mobile phase: solution I (5 mm sodium dodecyl sulfate (SDS) + 10 mm sodium phosphate, which was adjusted to pH 2.6 with phosphoric acid), solution II (5 mm SDS + 60 mm sodium phosphate + 25% 2-propanol, which was adjusted to pH 2.6 with phosphoric acid); gradient condition: from 100% solution I to 100% solution II in 45 min; flow rate: 0.25 ml/min; fractionation: every 1 min for 45 min.

**Pharmacokinetics of Malic Acid**

L-[14C] malic acid was added to unlabeled sodium malate and diluted with water to 20 μmol (as sodium malate)/10 ml. At 30 min after the oral administration of this sample at 10 ml/kg (radioactivity administered: 3.7 MBq/kg), CDDP (10 μmol/kg) was administered i.p. After 0.5, 1, 2 and 6 h, mice were anesthetized with ether, and blood was collected from the abdominal vena cava, then the kidneys, liver and tumor were excised. The blood was centrifuged (3000 rpm, 4°C, 10 min, Sakura RSL-05A, Sakura, Ltd., Tokyo) to give 100 μl of plasma for the determination of radioactivity. The wet weight of each tissue was determined, and the liver, kidneys and tumor were cut into small pieces for use as samples for the determination of radioactivity. These plasma and tissue samples were lyed with 2 ml of a histolyzer (Soluene-350, Packard Instrument Co., Tokyo) and decolorized with 0.4 ml of a benzyl peroxide-saturated benzene solution if necessary. Radioactivity was determined after the addition of 13 ml of a scintillator (Hionic-Fluor 9, Packard), and the concentration of radioactivity in the tissue was determined with a liquid scintillation counter (Tri-Carb 2000, Packard) for 2 min. The blank sample was collected for each tissue from unlabeled sodium malate and CDDP-treated mice. The value determined after treatment in a similar manner was used as the background value (dpm), and the limit of detection was established at twice the background value.

**Concentration of Platinum in the Kidneys and Tumor**

In a preliminary experiment, when CDDP was administered to mice at a dose of 10 μmol/kg/d, platinum could not be detected in the kidneys and tumor. Therefore, as with the pharmacokinetics, the concentration of platinum was examined using the CDDP dose of 40 μmol/kg. Briefly, S-180 cells (105 cells/mouse) were implanted subcutaneously into the inguinal region of the foot. Two weeks later (the tumor weight was about 0.9 g), water or sodium malate (80 μmol/kg) was orally administered, and 30 min later, CDDP was i.p. administered at 40 μmol/kg. At 24 h after the administration of CDDP, mice were killed by bleeding under ether anesthesia. The kidneys and tumor were excised, their wet weight was determined, and they were ashed with nitric acid to determine the amount of platinum. Platinum concentrations in the kidneys and tumor were determined using an ICP plasma emission spectrophotometer (Seiko SPS1200A, Seiko Ltd., Tokyo).

**Statistics**

Student's t test was used to evaluate the significances of differences between the experimental groups.
RESULTS

1. Relationship between the Dosing Time of Sodium Malate and the Reduction of CDDP-Induced Toxicity

At 3, 1 and 0.5 h before, immediately before, and 1 and 3 h after the i.p. administration of CDDP (10 μmol/kg) to mice, sodium malate (20 μmol/kg) was orally administered to examine the reduction of the nephrotoxicity, bone marrow toxicity, hepatotoxicity, decrease in body weight and the effect on the antitumor activity of CDDP on day 17.

Nephrotoxicity: In the CDDP treatment group, the BUN value was increased to about 3 times the values obtained in the control group (28.5 mg/dl), showing severe nephrotoxicity. When sodium malate was administered at the same time as or at 30 min or 1 h before the administration of CDDP, the CDDP-induced nephrotoxicity was significantly reduced, but the toxicity was hardly reduced when sodium malate was administered after the administration of CDDP. Although a significant reduction was produced by the administration of sodium malate 3 h before the administration of CDDP, the reduction was relatively weak (Fig. 2A).

Bone Marrow Toxicity: In the CDDP treatment group, the WBC was decreased to about 1/3 of the control group values (6.20×10^3/mm^3), showing severe bone marrow toxicity. Because sodium malate reduced the decrease in WBC, but the reduction was weaker than that of the nephrotoxicity, a significant effect was observed with the administration of sodium malate only at the same time as or at 30 min before the administration of CDDP (Fig. 2B).

Hepatotoxicity: In the CDDP treatment group, sGOT was increased to about 3 times the values in the control group (18.5±2.2 U/l). Similarly to the nephrotoxicity, when sodium malate was administered at the same time as or before the administration of CDDP, the CDDP-induced hepatotoxicity was significantly reduced, but the toxicity was not reduced when sodium malate was administered after the administration of CDDP (Fig. 2C).

Decrease in Body Weight: In the CDDP treatment group, the body weight of the mice was decreased to about 2/3 that of the control group (35.3±1.7 g). Sodium malate treatment produced a significant reduction in body weight only when administered at the same time as or at 30 min or 1 h before the administration of CDDP (Fig. 2D).

Effect of Sodium Malate on Antitumor Activity: Sodium malate showed no effect on the antitumor activity of CDDP against S-180 (Fig. 3).

2. Detection of DPM in Plasma

The fractions of non-protein-binding platinum at 10 min after the administration of CDDP alone, after the combination of CDDP and sodium malate and after the administration of DPM alone were obtained by HPLC. The platinum content in each fraction was obtained by atomic absorption spectroscopy (Fig. 4).

In the CDDP alone group (A), a single peak was observed at the retention time of 12 min. The retention time of this peak was consistent with that of CDDP (data not shown). In the DPM alone group (B), a small peak was observed at the retention time of 12 min and a large peak was seen at 28 min. The former was consistent with the peak of CDDP and the latter was consistent with the peak of DPM (data not shown). Additionally, two peaks were observed in the CDDP—sodium malate combined group (C) at the retention times of 12 and...
28 min. These peaks were consistent with those of CDDP and DPM, respectively, with a peak ratio of 3:2.

3. Kinetics of Platinum in Plasma after the Combined Administration of Sodium Malate and CDDP and after the Administration of DPM

Sodium malate or water was orally administered to mice, and 30 min later, CDDP or DPM was i.p. administered. Blood was collected at 10, 20, 30, 60, 90 and 120 min after the administration of CDDP or DPM to determine the concentration of platinum in the plasma fraction with a molecular weight of not more than 10000 (non-protein-binding platinum). In the CDDP alone group, the non-plasma protein-binding platinum rapidly disappeared from the blood; the half-life was 13.26 min. In the combined CDDP and sodium malate group, the platinum disappeared biphasically, with a half-life of 9.24 min for phase 1 and 54.91 min for phase 2, indicating prolongation of the half-life in plasma. DPM showed a biphasic disappearance, with a half-life of 12.66 min for phase 1 and 46.26 min for phase 2. This kinetic pattern resembled that of CDDP combined with sodium malate (Fig. 5).

4. Antitumor Activity of DPM

CDDP and DPM were i.p. administered to tumor-bearing mice at doses ranging from 2 to 10 μmol/kg/d, and the tumor weight was determined on day 17 (as shown in Fig. 6). In the CDDP treatment group, the tumor weight was dose-dependently decreased, and the ED50 was 2.59 μmol/kg/d. The dose–response relationship of DPM was quite similar to that of CDDP, with an ED50 of 2.35 μmol/kg/d.

5. Toxicity of DPM

DPM was i.p. administered at doses ranging from 2 to 10 μmol/kg/d after the oral administration of 0.5 ml of water, and the toxicity was determined on day 17.

Nephrotoxicity: The BUN and creatinine levels and the relative kidney weights are shown in Fig. 7. After the administration of CDDP at a high dose (10 μmol/kg/d), the BUN value (95.6 ± 12.3 mg/dl) increased to about 3 times higher than the control value. In contrast, there was no increase in the BUN value (29.7 ± 1.1 mg/dl) after the administration of DPM at any dose. The creatinine values increased slightly after the administration of high-dose CDDP but remained near the control level (28.5 ± 0.7 mg/dl) after the administration.
tion of DPM, as in the case of BUN. The relative kidney weight was also increased by CDDP (1.54±0.21×10^{-2}) but not by DPM (1.28±0.15×10^{-2}).

Bone Marrow Toxicity: The results of the examination of WBC, platelets, relative spleen weights and relative thymus weights are shown in Fig. 8. Although all of these variables decreased after the administration of DPM compared with the non-treated controls, the decreases were mild compared with those produced by CDDP of the equimolar amount. No change in the RBC was produced by DPM at any dose (data not shown).

Hepatotoxicity: The sGOT and sGPT values increased to about 4 and 5 times higher than the non-treated control values, respectively, after the administration of CDDP at a dose of 10 μmol/kg/d. In contrast, there was no significant increase in these values after the administration of DPM (Fig. 9).

Other Toxicity: The decrease in body weight after the administration of DPM at a dose of 10 μmol/kg/d was mild, about 1/2 that produced by CDDP at an equimolar amount. It was reported that the relative stomach weight was increased by the administration of CDDP because undigested matter in the stomach was retained due to a decrease in digestive function. Although the relative stomach weight was significantly increased by CDDP, the increase after the administration of DPM at a dose of 10 μmol/kg/d was slight in comparision (Fig. 10).

6. Pharmacokinetics of Sodium Malate Radioactivity in the kidneys, liver, tumor and plasma of mice at 0.5, 1, 2 and 6 h after the administration of ^14C-radiolabeled sodium malate and CDDP was determined with a liquid scintillation counter (Fig. 11).

The results showed that sodium malate was rapidly distributed in the blood and tissue after the oral administration, with the highest radioactivity in the liver, kidneys and plasma, in that order. The radioactivity then slowly decreased and reached about 1/2 of the initial activity after 6 h. The distribution in the tumors (0.337±0.286 μg eq/g) was relatively small, observed at a low level compared with that in the liver (1.935±0.210 μg eq/g) and kidneys (1.279±0.198 μg eq/g).

7. Concentration of Platinum in the Kidneys and Tumor Water or sodium malate was orally administered to the mice at 2 weeks after the subcutaneous implantation of S-180 cells into the inguinal region of the foot (the tumor
weight was about 0.9 g), followed 30 min later by the administration of CDDP. After 24 h, the kidneys and tumor were excised to determine the concentration of platinum by the ICP method. The changes in the concentration of platinum in the kidneys and tumor are shown in Fig. 12.

The concentration of platinum in the kidneys and tumor after 24 h in the CDDP alone group was 5.48±0.68 and 1.31±0.23 μg/g tissue, respectively. When CDDP was combined with sodium malate at a dose twice the equimolar amount of CDDP, the concentration of platinum in the kid-

**DISCUSSION**

CDDP incorporated into the cell was converted to an active diaquo form (9,10) and rapidly bound to DNA, producing a DNA injury. It was previously proposed that the nature of
this cytotoxicity is via the inhibition of DNA synthesis. However, it was recently shown that the CDDP-induced inhibition of DNA synthesis and cell death are not directly linked. Rather, it has been suggested that the antitumor activity of CDDP may involve the inactivation of P34cde 2 kinase based on inhibited dephosphorylation. It has also been suggested that the diatomic form may bind to various enzymes in the cell or the cell membrane via a thio, an amino or carboxy group, to induce the inactivation of an antioxidant enzyme and ATPase, as well as destruction of the lysosomal membrane, thereby producing cytotoxicity. Despite much research, there are still many uncertainties regarding the mechanism of the toxicity of CDDP, and no consensus has yet been obtained. In particular, for nephrotoxicity, since CDDP and many of its metabolites are excreted from and reabsorbed into the immediate Segment III of the urinary tubule in the border region between the cortex and the medulla, in which various enzymes are most commonly present, it is thought that this part is likely to be injured and that this may be the main mechanism of CDDP's nephrotoxicity. The development of agents for the reduction of nephrotoxicity is therefore commonly based on this hypothesis. This line of research has thus far produced fosfomycin, which stabilizes the lysosomal membrane, and o-(β-hydroxyethyl)-rutoside, which shows an antioxidizing effect, and selenious acid, which induces metallothionein. However, none of these agents completely inhibit CDDP-induced toxicity, and thus the current anti-nephrotoxicity practice is to induce diuresis by the sufficient infusion and administration of diuretics (e.g., furosemide and mannitol). Our investigation of agents for the reduction of CDDP-induced nephrotoxicity has pointed to sodium malate, which is isolated from a Kampo medicine, Juzen-taiho-to, as an effective agent.

In the present study, sodium malate showed no effect when administered after CDDP. However, it showed a near maximum effect when administered at the same time as or before the administration of CDDP, particularly at 30 min before (Fig. 2). These findings suggest that sodium malate did not have a therapeutic effect on CDDP-induced toxicity but rather prevented the occurrence of this toxicity. We therefore propose that the presence of sodium malate in the blood or tissue before the administration of CDDP may be a requirement for the reduction of CDDP-induced toxicity.

In this regard, carboplatin (CBDCA), a derivative of CDDP obtained by replacing the chloride group of CDDP with 1,1-cyclobutane dicarboxylic acid to decrease the protein binding ability, has also been reported to produce decreased toxicity. Although it is known that the plasma half-life of CBDCA is longer than that of CDDP, since sodium malate has two carboxyl groups in its structure, it is also possible that sodium malate may bind to CDDP in the body to form a chelate compound such as CBDCA. We therefore investigated whether the reaction intermediate DMP, which is expected to be formed by the reaction of sodium malate and CDDP, is present in the blood. DMP was detected in the plasma, with a plasma CDDP to DMP ratio of 3:2.

To investigate more closely the ratio of DMP formed in the plasma, we determined the plasma kinetics of platinum with a combination of sodium malate and CDDP. The kinetics of DMP shown a biphasic disappearance, significantly prolonged compared to that of CDDP. The disappearance time of platinum with a combination of sodium malate and CDDP was approximately intermediate between that of CDDP and DPM (Fig. 5). Because CDDP has an extremely strong protein binding capacity, its rate of elimination from the blood depends on the rate of protein binding in plasma or tissues; i.e., because CDDP binds rapidly to protein after administration, its elimination from the plasma is very rapid. However, the rate of excretion into the urine is very slow, with platinum remaining in the body for a long time, leading to serious adverse events. For CBDCA of low protein binding capacity, however, the rate of elimination from plasma depends on that of its excretion into the urine. As a result, because CBDCA has a slow rate of elimination from plasma but a low protein binding capacity compared to CDDP, it is rapidly excreted from the body, and therefore shows low toxicity. When CDDP and sodium malate were used concomitantly in the present study, part of the CDDP was converted to DPM of low protein binding capacity, as CBDCA, in plasma. No apparent toxicity occurred, despite the prolonged half-life in the plasma. Additionally, the calculation of drug clearance, using the equation below and assuming that the total clearance of CDDP is the same as that of DPM and that the only metabolite of CDDP is DPM, showed that about 38% of the CDDP administered was bound to sodium malate and converted to DPM.

\[ \text{drug clearance} = \frac{\text{dose}}{\text{AUC} \times F} \]

where \( \text{AUC} \) is the area under the blood concentration–time curve and \( F \) is the rate of absorption.

We compared the antitumor effect and toxicity of DPM with those of CDDP. We found that DPM produced the same antitumor effect as CDDP but hardly induced nephrotoxicity (Figs. 6 and 7). Additionally, DPM showed almost the same bone marrow toxicity as CDDP (Fig. 8). Thus, considering both the antitumor effect and the BUN value, our results suggest that, since CDDP increased BUN at doses of 6 μmol/kg/d or more if more, than about 40% of CDDP combined with sodium malate (corresponding to CDDP at 4 μmol/kg/d) is converted to DPM, nephrotoxicity can be reduced without loss of the antitumor effect. As previously shown, this result is consistent with that of the plasma kinetics of platinum using a combination of sodium malate and CDDP. Taken together, the results suggest that sodium malate may bind to about 40% of CDDP after absorption into the body.

Our pharmacokinetic study of sodium malate using [14C] malic acid showed that sodium malate was rapidly distributed in the blood and tissues after oral administration, in large concentrations in the liver, kidneys and plasma but in smaller amounts in the tumor. Additionally, it was shown that the 24h concentration of platinum was significantly decreased in the kidneys but not decreased in the tumor. These results may explain why the combination of CDDP and sodium malate reduced nephrotoxicity and hepatotoxicity but did not reduce the antitumor effect. On the basis of these results, we concluded that sodium malate binds to CDDP not only in plasma but also in the tissue, where it forms DPM, thereby reducing CDDP's toxicity.

Based on the above findings, we suggest that sodium malate is rapidly distributed in the blood and tissues after absorption into the body, binds to about 40% of CDDP, forms
DPM, and selectively reduces toxicity without a reduction in antitumor activity. This selectivity of sodium malate for a reduction in toxicity may be attributable to the difference in the distribution of sodium malate in the tissues.

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