Effects of Vitamin E on Toxicity by Minute Amounts of Paraquat Fed Continuously to Rats

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Summary

The effects of vitamin E on toxicity by minute amounts of paraquat fed continuously for some period to rats were investigated. Two experiments were carried out as experiments 1 and 2. In both experiments, weaning rats were divided at first into two groups; one group was given a vitamin E-deficient diet, and the other a vitamin E-supplemented control diet (50 mg α-tocopherol/kg of diet). They were fed on these diets for 40 days. After that, in both experiments, the rats that had been fed the vitamin E-deficient diet were further divided into two groups, which were either given a paraquat-added diet (+PQ-E) or continuously fed the same vitamin E-deficient diet (−E). The amount of paraquat added was 250 mg of methyl viologen per kg of diet. After the addition of paraquat, these two groups were pair-fed. In experiment 1, paraquat was given to all the rats fed the vitamin E-supplemented control diet (+PQ+E). In experiment 2, rats fed the control diet were divided into paraquat-added (+PQ+E) and non-paraquat-added (+E) groups, similar to those of vitamin E-deficient rats. These two groups were also pair-fed thereafter. In both experiments, about 35 days after paraquat addition, they were sacrificed. Plasma and liver α-tocopherol contents were measured by HPLC, and liver peroxidation value was measured by chemiluminescence and the TBA method. And, as parameters of vitamin E deficiency, plasma pyruvate kinase and GOT activities and α-cysteine proteinase inhibitor (α-CPI) level were measured. When the analyzed values were compared between paraquat-added and the corresponding not-added control groups (+PQ−E vs. −E, +PQ+E vs. +E), the following results were obtained. In experiment 1, the values of plasma and liver α-tocopherol levels were significantly lower in the +PQ−E group than those of the −E group; however, liver peroxidation values and values of the three parameters of vitamin E deficiency were not different significantly. In
experiment 2, the value of liver \( \alpha \)-tocopherol level was significantly lower in the \(+PQ+E\) group than that of the \(+E\) group. As to liver peroxidation values, in the case of TBA reactive substance (TBA-RS), the value of the \(+PQ-E\) group was significantly higher than that of the \(-E\) group, and in the case of chemiluminescence, the value of the \(+PQ+E\) group was significantly higher than that of the \(+E\) group. As to the three parameters of vitamin E deficiency, the values of all three parameters were significantly higher in the \(+PQ-E\) group than those of the \(-E\) group, and the values of pyruvate kinase activity and \( \alpha \)-CPI level were significantly higher in the \(+PQ+E\) group than those of the \(+E\) group. But, when ratios of weights of lung, kidney and liver to body weight were compared, these values increased significantly in both experiments by paraquat addition irrespective of the presence of vitamin E. Moreover, in experiment 2, occurrence of gross pathological signs were noticed in the paraquat-fed two groups.

Key Words  paraquat, vitamin E, lipid peroxide, pyruvate kinase, glutamic oxalacetic transaminase, proteinase inhibitor

Paraquat is a widely used herbicide in our country, but it has various toxic effects on human beings, and many cases of illness or death resulting from its toxicity have been reported by the Ministry of Welfare (1).

As a mechanism of paraquat toxicity, a paraquat free radical is easily formed from a paraquat ion by accepting an electron from NADPH, and the formed radical has a strong potential to reduce oxygen to produce a superoxide anion (2, 3). The large amounts of superoxide anion produced would not be scavenged completely by existing superoxide dismutase activity, and it would have a strong damaging action for living organisms. Furthermore, a more toxic singlet oxygen would be formed, and cell damage would be accelerated (4, 5). Vitamin E is a well-known antioxidant, and it would effectively reduce the toxic effect of paraquat by blocking free radical formation.

However, conflicting results were reported concerning the effects of vitamin E on acute paraquat toxicity. Morisaki et al. (6) reported that vitamin E had a protective effect against toxicity of paraquat given to rats in large amounts at one time. It was shown further that vitamin E was also effective in in vitro experiment using cultured rat hepatocytes (7). But, in other reports, in acute paraquat toxicity, no protective effects of vitamin E were reported (8–10).

In our country, many farmers are handling paraquat throughout the weeding season. During this period, they are constantly exposed to minute amounts of this toxic compound. Further, many golf links are present in Japan now, and in these places large amounts of herbicides including paraquat are often used. Constant exposure to minute amounts of paraquat could damage living organisms by continually producing free radical in the body, although the effects would not be so
drastic as those induced by giving large amounts at one time. In a vitamin E-deficient dietary condition, such effects would be magnified. Block (11) reported that acute paraquat toxicity was enhanced by vitamin E deficiency. However, to vitamin E-deficient subjects, the effects of minute amounts of paraquat fed continuously are not clear yet. Therefore, it would be important to investigate the effects of vitamin E deficiency on toxicity by minute amounts of paraquat fed continuously, and further to make clear the protective effects of vitamin E.

Considering these facts, in the present experiment, an amount of paraquat supposed to be the maximum non-toxic level by a joint committee of FAO and WHO (12) was continuously given for about 35 days to rats fed vitamin E-deficient or-supplemented diets. Simultaneously, as a control, other rats were fed on these diets with no addition of paraquat. Then, the effects of paraquat addition on liver peroxidation values, liver and plasma \( \alpha \)-tocopherol levels, and on the parameters of vitamin E deficiency symptoms were analyzed, and occurrence of gross pathological signs was observed.

From the results of these analyses and observations, the poisonous effects of minute amounts of paraquat fed continuously, and the detoxifying effects of added vitamin E are discussed.

MATERIALS AND METHODS

Materials. Solvents used for chromatography were high performance liquid chromatography (HPLC) grade from Kanto Chemical (Tokyo). \( \alpha \)-Tocopherol standard was obtained from Wako Pure Chemical (Osaka). Methyl viologen (as paraquat) and 1,1,3,3-tetraethoxypropane were purchased from Sigma Chemical (St. Louis, U.S.A.). Thiobarbituric acid was from Nacalai Tesque Inc. (Kyoto). Reagents for measurement of pyruvate kinase activity were all purchased from Boehringer Mannheim Yamanouchi (Tokyo). The kit for measurement of GOT activity was purchased from Amco (Tokyo). All other chemicals were of special grade commercially.

Experimental design. Male rats of Wistar strain (Nihon SLC, Hamamatsu) were purchased after weaning. They were housed in individual cages in a temperature-controlled room (22°C) with equal light and dark cycles daily.

Composition of vitamin E-deficient basal diet is shown in Table 1. Salt mixture made according to that of AIN-76 was used. Vitamin mixture was also made according to that of AIN-76, except vitamin E. In vitamin E-containing control diet, 50 mg of all-rac-\( \alpha \)-tocopherol per kg of diet was added.

The experiments were carried out in two phases as experiments 1 and 2. In experiment 1, at first, rats were divided into two groups; one group was given a vitamin E-deficient basal diet (14 rats), and the other a vitamin E-supplemented control diet (7 rats). They were pre-fed on these test diets ad libitum for 40 days, and after that, vitamin E-deficient basal diet fed rats were further divided into the following two groups: one, vitamin E-deficient and paraquat-added diet fed group
Table 1. Composition of basal diet.

| Ingredient                          | Amount (g/kg diet) |
|-------------------------------------|--------------------|
| Vitamin-free casein<sup>1</sup>      | 250                |
| α-Starch<sup>2</sup>                | 303.5              |
| Sucrose<sup>3</sup>                 | 300                |
| Stripped corn oil<sup>4</sup>       | 100                |
| Salt mixture (AIN-76)<sup>5</sup>   | 35                 |
| Vitamin mixture (AIN-76)<sup>6</sup>| 10                 |
| Choline chloride                    | 1.5                |

<sup>1</sup>Purchased from Oriental Yeast (Tokyo).  <sup>2</sup>Vitamin E-free α-starch purchased from Nihon Nosan Kogyo (Yokohama).  <sup>3</sup>Purchased from Oriental Yeast (Tokyo).  <sup>4</sup>Purchased from Eastman Kodak (Rochester, N.Y.).  <sup>5</sup>Purchased from Nihon Nosan Kogyo (Yokohama).  <sup>6</sup>Made according to AIN-76 except vitamin E. All vitamins used were extra pure grade reagent of Nagoya Katayama Chemical (Nagoya).

(+)PQ − E); and the other, vitamin E-deficient non-paraquat-added diet fed group (− E). And, these two groups were pair-fed thereafter (7 rats per each group). The amount of paraquat added to diet was 250 mg of methyl viologen per kg of diet, which was the amount supposed to be the maximum non-toxic level to rats by a joint committee of FAO and WHO (12). At the same time, the vitamin E-supplemented group was also added the same amount of paraquat, and they were fed ad libitum (+PQ + E).

About 35 days after giving paraquat, rats were killed by heart puncture under light nembutal anesthesia and blood was collected by heparinized syringe, and plasma was separated by centrifugation. Then, liver kidney, lung, heart, and spleen were separated and weighed. After that, liver peroxidation values and plasma and liver α-tocopherol levels were measured on frozen samples stored in a freezer at −80°C. As a parameter of vitamin E deficiency, plasma pyruvate kinase and GOT activities were measured during the same day rats were sacrificed, and later plasma α-cysteine proteinase inhibitor level was measured on frozen samples.

In experiment 2, rats were at first divided into two groups of equal number, i.e., vitamin E-deficient basal diet fed and vitamin E-supplemented control diet fed groups (12 rats each). And, after 40 days pre-feeding on these diets, vitamin E-deficient basal diet fed rats were divided into two groups similarly as in experiment 1 (+PQ − E, − E; 6 rats each). This time, the vitamin E-supplemented diet fed rats were also divided into paraquat-added and non-paraquat-added groups (+PQ + E, + E; 6 rats each). And these two pairs of groups were pair-fed thereafter for about 35 days. All the other treatments were the same as those of experiment 1.

The determination of α-tocopherol content in plasma and liver. In rats fed the vitamin E-deficient diets, α-tocopherol content in plasma and liver was very low, therefore, some devices were made in the procedure.

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The extraction of \( \alpha \)-tocopherol in plasma was adapted from the procedure described by Abe and Katsui (13), except for the utilization of \( n \)-hexane containing 0.025\% BHT (w/v). The extraction of \( \alpha \)-tocopherol in liver was a modification of the procedure developed for rat liver by Zaspel and Csallany (14). Briefly, the liver (approx. 200 mg) was homogenized in 20 vol. of acetone containing 0.05\% BHT (w/v), for 15 s at a rate of 6.5 setting with a polytron homogenizer equipped with a PTS-10 probe generator (Kinematica, Kriens, Switzerland). The homogenate was centrifuged at 1,200 × g for 10 min, and the supernatant was transferred to a test tube. The pellet was reextracted two more times with 20 vol. of acetone containing 0.05\% BHT. All of the supernatants were combined and evaporated at reduced pressure. The residue was redissolved in 1 ml of ethanol and 1.1 ml of water was added followed by 5 ml of \( n \)-hexane. \( \alpha \)-Tocopherol was extracted into the \( n \)-hexane phase by vortexing for 2 min. Four milliliters of the \( n \)-hexane phase was pipetted into a test tube and the solvent was evaporated off at reduced pressure. The residue was redissolved in 0.2 ml of \( n \)-hexane and used for \( \alpha \)-tocopherol determination. \( \alpha \)-Tocopherol determination was carried out fluorometrically by high performance liquid chromatography (HPLC). Instrumentation used for HPLC was a Shimadzu Model LC-4 A (solvent-metering pump) (Shimadzu, Kyoto) with fluorescence detector, a JASCO Model FP-550A fluorescence spectrophotometer equipped with a 15 \( \mu \)l capacity quartz cell (JASCO, Hachiouji). The analytical column used was a Shim-pack CLC-NH2 (Shimadzu, Kyoto), 6.0 × 150 mm, preceded by a guarded column, Amino spheri-5 (Brownlee Labs Santa Clara, U.S.A.), 4.6 × 30 mm. The mobile phase was \( n \)-hexane/isopropanol (97 : 3, v/v) at a flow rate of 1.5 ml/min. The \( \alpha \)-tocopherol was detected with excitation at 295 nm and emission at 340 nm. \( \alpha \)-Tocopherol content in experimental samples was calculated from a standard peak-area response curve of varying amounts of pure \( \alpha \)-tocopherol.

**Measurement of liver peroxidation value.** Liver peroxidation value was measured by two methods, namely, by measurement of chemiluminescence and by TBA test.

The chemiluminescence of liver homogenate was measured essentially as described by Miyazawa and Kaneda (15). A Hamamatsu photonics photon counting apparatus (Hamamatsu) was used. A model R-1333 photomultiplier, responsive in the range of 300–900 nm, was connected to a model C-1230 photon counter. The photomultiplier was cooled to −30°C by model C-2761 thermoelectric cooler and used with a potential of −1590 V. The sample homogenate was placed on stainless-steel plate, and chemiluminescence was measured at 30°C in a special light tight box as close as possible to the end of the light guide. Photon counts were recorded every 10 s for 10 min and expressed as average counts per second after correction for background counts of dark current.

The measurement of TBA reactive substance (TBA-RS) in liver was by the method of Masugi and Nakamura (16). Malondialdehyde (MDA) standard was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane by the method of Lee and
Various concentrations of MDA standard solution were subjected to the identical TBA test procedure, and standard curve for TBA reactivity as MDA equivalents was prepared. The molar amounts of TBA-RS in the sample tissue were calculated as MDA equivalents from the standard curve.

**Determination of parameters of vitamin E deficiency.** Plasma pyruvate kinase and GOT activities were shown by Machlin et al. (18) as good indices for representation of vitamin E deficiency. And, it was shown by the authors previously (19) that plasma α-cysteine proteinase inhibitor (α-CPI) level was increased by vitamin E deficiency, and good correlation was found between GOT activity. Therefore, in the present experiments, plasma pyruvate kinase and GOT activities and α-CPI level were measured as parameters of vitamin E deficiency.

Plasma pyruvate kinase activity was determined according to the method described by Gutmann and Bernt (20) from the decrease of absorbance of NADH at 340 nm. Plasma GOT activity was determined by using the kit system of Amco (Tokyo), which is based on the method of Reitman-Frankel. Plasma α-CPI level was determined by the method of Minakata et al. (21).

**Statistical analysis.** The significance of the difference between two groups of values was determined by Student’s t-test (22).

**RESULTS**

**Gross pathological observations of animals**

In paraquat-fed rats, gross pathological signs started to appear at about 10 days after paraquat feeding began, particularly in experiment 2. Many rats manifested one or more of the following signs: diarrhea, decreased food intake, rough coat, porphyrin secretion from the nose and porphyrinuria. There was no difference in appearance of these signs in the two paraquat-added groups, indicating vitamin E was not effective to prevent occurrence of these pathological signs. In the +PQ−E group, one rat suffered these symptoms to a severe degree, and food consumption was zero for several days before sacrifice. In the +PQ+E group, one rat suffered severe melena, and fell probably into a coma. Therefore, data of these two rats were excluded from all calculations. In experiment 1, occurrence of these pathological symptoms was less severe.

**Body and organ weights and gross pathological observations of isolated organs**

Figure 1 shows the final body weight, and Fig. 2 shows the percentage of various organ weights to body weight. There was not a great difference between groups in final body weight. However, in experiment 1, the value of the +PQ−E group was slightly lower than that of −E group. On the contrary, among the organs, in lung, kidney and liver, the increase of percentage of these organs to body weight in paraquat-added groups (+PQ−E, +PQ+E) compared with those of not-added control groups (−E, +E) was evident irrespective of whether vitamin E was present or not. For these three organs, the differences between paraquat-added...
Fig. 1. Final body weight of the three and the four diet groups in experiments 1 and 2. Values are expressed as means±SD. Asterisks (*) represent significant differences between two groups indicated by vertical line; **p<0.025.

Fig. 2. Percentage of weights of liver, kidney (sum of both sides), lung, heart and spleen to body weight in the three and the four diet groups in experiments 1 and 2. Values are expressed as means±SD. Asterisks (*) represent significant differences between two groups indicated by vertical line; *p<0.05, **p<0.025, ****p<0.005, *****p<0.001.

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and corresponding not-added control groups (+PQ-E vs. -E, +PQ+E vs. +E) were always significant in two experiments. The difference was most evident in the lung. On the contrary, percentage of heart and spleen weights did not seem to be affected by paraquat addition.

In experiment 2, among isolated organs, apparent pathological signs were seen in the lung and kidney in some rats in the paraquat-fed two groups, indicating also that the presence of vitamin E was not protective. In these rats, the kidney revealed a whitish color, and mottled appearance. In the lung, red spots were often observed, and in a few rats massive reddish degeneration of the tissue was observed. In experiment 1, these pathological signs were not clearly evident in paraquat-added diet fed rats. In experiment 2, in the other isolated organs apparent pathological signs were not noticed.

**Plasma and liver α-tocopherol levels**

Figure 3 shows the plasma and liver α-tocopherol levels. In experiment 1, in the vitamin E-deficient two groups, both in plasma and liver, α-tocopherol levels were significantly lower in the paraquat-added group (+PQ-E) than those of the non-paraquat (-E) group. In experiment 2, the same tendency was observed. However, standard deviations were large, and the differences were not significant.

Values of the vitamin E-added groups (+PQ+E, +E) were very high, especially in the liver, in both experiments. In experiment 2, when the vitamin E-added two groups were compared, both in plasma and liver, α-tocopherol levels were lower in the paraquat-added group (+PQ+E) than those of the non-paraquat group (+E), but the difference was significant only in the case of liver.

**Fig. 3.** Plasma and liver α-tocopherol levels in the three and the four diet groups in experiments 1 and 2. Values are expressed as means±SD. Asterisks (*) represent significant differences between two groups indicated by vertical line; **p<0.025, ***p<0.01, *****p<0.001.
Fig. 4. Peroxidation value of liver in the three and the four diet groups in experiments 1 and 2 measured by chemiluminescence and TBA method. Values are expressed as means±SD. Asterisks (*) represent significant differences between two groups indicated by vertical line; ***p<0.01, ****p<0.005, *****p<0.001.

Liver peroxidation value

Figure 4 shows the peroxidation value of the liver measured by the two different methods. In experiment 1, in the case of chemiluminescence, the peroxidation value of the vitamin E-deficient two groups (+PQ−E, −E) were almost the same. In the case of TBA-RS, the value of the paraquat-added group (+PQ−E) was higher than that of the non-paraquat group (−E), but the standard deviation was large, and the difference was not significant. The value of the vitamin E-added group (+PQ+E) was significantly lower than the other two groups, irrespective of method of analysis. However, the difference from the other two groups was larger in the case of chemiluminescence than in TBA-RS.

In experiment 2, in the case of chemiluminescence, values of paraquat-added groups were higher than those of corresponding non-paraquat groups (+PQ−E vs. −E and +PQ+E vs. +E), but the difference was significant only when the vitamin E-added two groups were compared (+PQ+E vs. +E). In the case of TBA-RS, values of the paraquat-added two groups were also higher than those of corresponding control groups; but, contrary to the case of chemiluminescence, this time the difference was significant when the vitamin E-deficient two groups were compared (+PQ−E vs. −E).

Parameters of vitamin E deficiency

Figure 5 shows the values of the three parameters of vitamin E deficiency. In experiment 1, in all three parameters, values of the paraquat-added group (+PQ−E) were higher than those of the non-paraquat group (−E). However, differences between the two groups were not significant.

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In experiment 2, in all three parameters, values of paraquat-added groups were higher than those of corresponding non-paraquat groups (+PQ-E vs. -E and +PQ+E vs. +E), and this time, the differences between paraquat-added and corresponding control groups were all significant except in one case (GOT, +PQ+E vs. +E). These results of experiment 2 would mean that paraquat accelerates vitamin E-deficiency symptoms not only in a vitamin E-deficient dietary condition, but also in vitamin E-sufficient condition.

DISCUSSION

In a monograph issued jointly by FAO and WHO (12), it was reported that a diet containing 250 ppm paraquat had no adverse effects on growth, survival, behavior, tumour incidence, hematologic studies, urinalysis, organ weights, ratios of organ to brain or organ to body weight and gross pathologic examination. However, in the present experiments, despite the fact that the same amount of paraquat was used, the increase of ratios of lung, kidney, and liver weights to body weight was clear, indicating that some pathological changes were occurring in these organs (Fig. 2). Moreover, in experiment 2, as mentioned before, many rats in paraquat-fed groups manifested various pathological signs; particularly, two of them fell into severe illness. Further, in this experiment, in observation of isolated organs, lung and kidney showed clearly an abnormal appearance in some paraquat-fed rats. These facts mean that minute amounts of paraquat, supposed to be a non-toxic level by a joint committee of FAO and WHO (12), clearly had harmful injurious effects on animals.

When +PQ-E and -E groups were compared on plasma and liver ox-

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tocopherol levels, in experiment 1, both values decreased significantly in the +PQ −E group, but in experiment 2, although the same tendency was observed, the differences between the two groups were not significant (Fig. 3). However, when compared on liver peroxidation values, in experiment 2, the values of the +PQ−E group were higher than those of the −E group. In the case of TBA-RS, the difference between the two groups was significant (Fig. 4). In experiment 1, in both cases, the differences were not significant. Further, when compared on the three parameters of vitamin E deficiency, in experiment 2, the values of the +PQ−E group were higher than those of the −E group. In all three cases, the differences between the two groups were all significant (Fig. 5). But in experiment 1, although the same tendency can be observed, the differences were not significant. These facts mean that in experiment 2, progression of vitamin E-deficiency symptoms and liver peroxidation were accelerated by paraquat addition. In experiment 2, although plasma and liver α-tocopherol levels were not decreased significantly by paraquat addition, paraquat-fed rats were failing in health more severely than those of experiment 1, and would be subjected more easily to the cell-damaging action of the oxidant.

Next, in experiment 2, when the +PQ+E and +E groups were compared on plasma and liver α-tocopherol levels, the liver α-tocopherol level decreased more significantly in the +PQ+E group than that of the +E group. In the case of plasma, the same tendency can be observed, but the difference was not significant (Fig. 3). When compared on the liver peroxidation values, the values increased more in the +PQ+E group than those of the +E group in both chemiluminescence and TBA-RS, but the difference between the two groups was significant only in the case of the former (Fig. 4). When compared on the three parameters of vitamin E deficiency, all the values were higher in the +PQ+E group than those of the +E group, and the differences between the two groups were significant in the cases of pyruvate kinase activity and α-CPI level except in the case of GOT activity (Fig. 5). These facts mean that paraquat addition accelerates liver peroxidation and vitamin E-deficiency symptoms in α-tocopherol-fed rats as well as in vitamin E-deficient rats.

However, both in experiments 1 and 2, when values of liver peroxidation and parameters of vitamin E deficiency in α-tocopherol-fed groups were compared to those of vitamin E-deficient groups, these values were much lower in the former groups than those in the latter. Despite this, as mentioned before, when paraquat was added to the diet, there seemed to be no difference in the appearance of abnormal pathological signs between α-tocopherol-fed and not-fed groups. Moreover, weights of the lung, kidney and liver increased similarly in all paraquat-fed groups irrespective of the presence of α-tocopherol (Fig. 2). These facts mean that there is no direct relationship between induction of a pathological state by paraquat addition and the increase of lipid peroxidation and acceleration of vitamin E deficiency.

Concerning the effects of added vitamin E on acute paraquat toxicity, as
mentioned before, no protective effects of vitamin E were reported by the other investigators (8–10). The results of the present experiments indicate also that vitamin E is not protective when minute amounts of paraquat are fed repeatedly. When paraquat is given to the living organism, a large amount of \( \text{O}_2^- \) is produced, and superoxide dismutase activity (SOD) must be induced to remove this toxic compound. In paraquat-resistant \( \text{Escherichia coli} \), it was reported that resistance to paraquat was proportional to SOD activity (23). And, paraquat-resistant tobacco is reported to have a higher level of SOD activity (24). When large amounts of water-soluble \( \text{O}_2^- \) is produced by paraquat addition, vitamin E would not be effective in scavenging this kind of superoxide, and could not protect an animal from its injurious effects. In the present experiment, in time-course observation of paraquat-fed rats, some rats recovered from their pathologic states during continued paraquat feeding. In these rats, SOD activity would be induced eventually. Therefore, the relationship between \( \text{O}_2^- \) production and induction of SOD activity in paraquat-fed animals should be investigated in the future.

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