Effect of Dietary Supplementation with Folate on Choline Deficiency-Induced Hyperhomocysteinemia in Rats

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Summary The effects of dietary supplementation with folate (20 mg/kg diet), 2.5% serine, or both on choline deprivation-induced hyperhomocysteinemia were investigated in rats fed a 10% casein diet (10C) or 25% soybean protein diet (25S) to determine whether folate supplementation with or without serine can suppress choline deficiency-induced hyperhomocysteinemia. Choline deprivation-induced enhancement of plasma homocysteine concentration was significantly suppressed by supplementation with folate, serine, or both, but the effects of these supplements were partial or limited in the rats fed both 10C and 25S. The extents of suppression of plasma homocysteine increments by folate, serine, or both were 29.6, 37.8, and 46.2%, respectively, in rats fed 10C and 27.2, 36.6, and 42.8%, respectively, in rats fed 25S. There was no significant additive effect between folate and serine, a source of C1 units. Folate supplementation with or without serine significantly increased or tended to increase hepatic 5-methyltetrahydrofolate concentration together with methionine synthase (MS) and cystathionine β-synthase (CBS) activities and MS mRNA level in both rats fed 10C and rats fed 25S. Hepatic betaine-homocysteine S-methyltransferase activity was unaffected by folate with or without serine. Supplementation with serine alone significantly increased hepatic serine concentration and increased or tended to increase CBS activity slightly. It is thought that the suppressive effect of folate on choline deficiency-induced hyperhomocysteinemia was due to increased metabolism of homocysteine via the MS pathway and that the suppressive effect of serine was due to increased metabolism of homocysteine via cystathionine formation. One of the reasons for the insufficient effect of folate alone or in combination with serine is thought to be that the capacity of the MS pathway for homocysteine metabolism is less enhanced by supplementation with folate and serine.

Key Words choline deficiency, folate, serine, 5-methyltetrahydrofolate, plasma homocysteine

A number of studies have suggested that an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease (1–3). Plasma homocysteine is also a risk factor for the development of cognitive impairment and Alzheimer’s disease (4). Of the many factors affecting plasma homocysteine concentration, nutritional and genetic factors are thought to have a greater influence on the concentration (5). Homocysteine is remethylated to methionine or condensed with serine to cystathionine (Fig. 1) (6). Homocysteine is remethylated either by methionine synthase (MS) using the methyl group of 5-methyltetrahydrofolate (5-MTHF) or by betaine-homocysteine S-methyltransferase (BHMT) using the methyl group of betaine. Cystathionine synthesis is catalyzed by cystathionine β-synthase (CBS). It has been shown that deficiencies of some vitamins such as folate, vitamin B12, and vitamin B6 cause hyperhomocysteinemia, since folate and vitamin B12 are cofactors of MS and vitamin B6 is a cofactor of CBS (1–3). We have demonstrated that choline deprivation of low-methionine diets also causes hyperhomocysteinemia mainly due to the deficiency of betaine, which is formed from the vitamin-like compound choline (7).

When considering the metabolism of homocysteine, it is of interest to know whether impairment of one of the two remethylation pathways can be compensated by stimulating the other pathway. According to this line, we previously investigated the effect of dietary supplementation with betaine on the hyperhomocysteinemia induced by dietary folate deprivation in rats fed low (10%) casein diet (10C) or standard (20%) casein diet. The results showed that betaine significantly sup-

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Abbreviations: 10C, 10% casein diet; 10CCD, choline-deprived 10C; 25S, 25% soybean protein diet; 25SCD, choline-deprived 25S; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; MS, methionine synthase; 5-MTHF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.
pressed the hyperhomocysteinemia, but the effect was partial or limited despite the supplemented betaine level being relatively high (1%). The results suggested that folate deficiency could not be fully overcome by betaine. There have been a number of studies on the effect of folate deficiency could not be fully overcome by betaine. There have been a number of studies on the effect of folate on betaine deficiency-induced hyperhomocysteinemia. Therefore, in this study we investigated the effects of dietary supplementation with folate on choline deprivation-induced hyperhomocysteinemia in rats fed 10C and 25% soybean protein diet (25S), since choline deprivation of 10C or 25S resulted in obvious hyperhomocysteinemia (7). Serine is thought to be a main source of 5-MTHF (14). Hence, the effect of serine alone or serine in combination with folate was also investigated.

**MATERIALS AND METHODS**

**Chemicals.** Folic acid and choline bitartrate were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich and were of analytical grade. Vitamin-free casein, mineral mixture (AIN-93G), vitamin mixture (AIN-93), and cellulose powder were purchased from Oriental Yeast Co., Ltd. (Tokyo). Soybean protein isolate (SPI, Fujipro) was kindly supplied by Fuji Oil Co., Ltd. (Izumisano, Japan). Other ingredients of the diet were purchased from Wako.

**Animals and diets.** Six-week-old male rats (120–140 g) of the Wistar strain were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were individually housed in hanging stainless-steel wire cages in an isolated room kept at a controlled temperature (23–25°C) and humidity (40–60%). Lighting was maintained on a 12-h cycle (lights on from 07:00 to 19:00 h). Before starting the experiments, all rats were acclimated to the facility for 5 d and given free access to water and a 25% casein diet. In this study, two separate animal experiments were conducted. In experiment 1, 40 rats were randomly assigned to the following five diet groups: (1) 10C, (2) choline-deprived 10C (10CCD), (3) 10CCD+folate (20 mg/kg diet), (4) 10CCD+2.5% L-serine, and (5) 10CCD+folate (20 mg/kg diet)+2.5% L-serine. In experiment 2, 40 rats were randomly assigned to the following five diet groups: (1) 25S, (2) choline-deprived 25S (25SCD), (3) 25SCD+folate (20 mg/kg diet), (4) 25SCD+2.5% L-serine, and (5) 25SCD+folate (20 mg/kg diet)+2.5% L-serine. The composition of 10C was as follows (g/kg): vitamin-free casein, 100; α-cornstarch, 582.5; sucrose, 200; corn oil, 50; mineral mixture (AIN-93G), 35; vitamin mixture (AIN-93), 10; choline bitartrate, 2.5; cellulose powder, 20. In 25S, SPI was used at a level of 250 g/kg at the expense of cornstarch. In choline-deprived diets, choline bitartrate was omitted with an increase in cornstarch. The supplementation level of folate (20 mg/kg) was determined as a ten-fold level of AIN-93 (2 mg/kg). Rats were given free access to the experimental diets and water for 14 d and killed by decapitation between 10:00 and 11:00 h without prior food deprivation. Since it has been shown that non-fasting plasma homocysteine concentration was liable to be affected by dietary treatment in humans (15). This study was approved by the Animal Use Committee of Shizuoka University, and the animals were maintained in accordance with the “Guidelines for the Care and Use of Laboratory Animals” of Shizuoka University.

**Tissue collection and fractionation.** Blood plasma was separated from heparinized whole blood by centrifugation at 2,000 ×g for 15 min at 4°C and was stored at −30°C until needed for analysis. After collection of blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, cut into three portions, weighed, quickly frozen in liquid nitrogen, and stored at −80°C until needed for analysis. One portion of the liver was homogenized in 4 volumes (vol/wt) of ice-cold 0.3 M trichloroacetic acid solution and then centrifuged at 10,000 ×g for 10 min at 4°C. The supernatant of the deproteinized liver homogenate was subjected to assays for methionine metabolites, betaine and serine. Another portion of the liver was homogenized in 4 volumes (vol/wt) of a 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M KCl, and the resulting homogenate was centrifuged at 14,000 ×g for 10 min at 4°C. The supernatant was subjected to enzyme assays. The third portion of the liver was subjected to analysis of mRNA, and total mRNA was isolated using a kit, ISOGEN (Nippon Gene, Tokyo), according to manufacturer’s instructions.
Biochemical analysis. The concentrations of homocysteine and cysteine in the plasma and liver were measured by HPLC using the method of Durand et al. (16). The concentrations of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in the liver were measured by HPLC following Cook et al. (17). The betaine concentration in the liver was measured by HPLC following Laryea et al. (18). The concentrations of 5-MTHF in the plasma and liver were measured by HPLC using the method of Shimoda (19). The serine concentration in the liver was measured by an amino acid autoanalyzer. The activity of BHMT in the liver was measured following Finkelstein and Mudd (20), but HPLC was used in the assay of the reaction product, DMG, following Laryea et al. (18). The activity of MS in the liver was measured following Huang et al. (21). The activity of CBS in the liver was measured following Mudd et al. (22), but HPLC was used in the assay of the reaction product, cystathionine, following Einarsson et al. (23). The amounts of mRNA for BHMT and CBS relative to /H9252-actin in the liver were measured by quantitative real-time PCR analysis as described previously (24). The amount of mRNA for MS was also measured by the same method, where the validated probe and primer for MS (assay identification number: Rn00578368_ml) were pre-designated TaqMan Gene Expression Assay products (Applied Biosystems, Foster City, CA). The protein concentration was measured according to Lowry et al. (25) using bovine serum albumin as a standard.

Statistical analysis. Each value is expressed as the mean±SE. Data were analyzed by one-way ANOVA, and differences among the experimental groups were analyzed by the Tukey test when the F value was significant. Statistical analysis was performed with Mac Tokei-Kaiseki software (version 1.5; Esumi, Tokyo).

Table 1. Body weight gain, food intake and liver weight of rats fed the experimental diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt gain (g/14 d)</th>
<th>Food intake (g/14 d)</th>
<th>Liver wt (g/100 g body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10C</td>
<td>42±2</td>
<td>234±7</td>
<td>4.08±0.04</td>
</tr>
<tr>
<td>10CCD</td>
<td>39±3</td>
<td>219±8</td>
<td>4.15±0.06</td>
</tr>
<tr>
<td>10CCD+FA</td>
<td>43±4</td>
<td>239±7</td>
<td>4.18±0.09</td>
</tr>
<tr>
<td>10CCD+Ser</td>
<td>42±3</td>
<td>235±4</td>
<td>3.94±0.12</td>
</tr>
<tr>
<td>10CCD+FA+Ser</td>
<td>40±2</td>
<td>232±9</td>
<td>3.98±0.08</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25S</td>
<td>50±3</td>
<td>219±7</td>
<td>3.89±0.06</td>
</tr>
<tr>
<td>25SCD</td>
<td>57±2</td>
<td>230±6</td>
<td>4.17±0.07</td>
</tr>
<tr>
<td>25SCD+FA</td>
<td>53±3</td>
<td>229±6</td>
<td>4.10±0.05</td>
</tr>
<tr>
<td>25SCD+Ser</td>
<td>49±2</td>
<td>212±5</td>
<td>4.04±0.05</td>
</tr>
<tr>
<td>25SCD+FA+Ser</td>
<td>48±3</td>
<td>221±6</td>
<td>4.04±0.05</td>
</tr>
</tbody>
</table>

1 Each value is the mean±SE, n=8. Values without a common letter differ, p<0.05. 10C, 10% casein diet; 10CCD, choline-deprived 10C; 25S, 25% soybean protein diet; 25SCD, choline-deprived 25S; FA, folic acid.
2 Supplemented at a level of 20 mg/kg diet.
3 Supplemented at a level of 25 g/kg diet.

Fig. 2. Effects of supplementation of choline-deprived 10% casein diets with folate, serine or folate plus serine on plasma concentrations of homocysteine (A), cysteine (B) and 5-methyltetrahydrofolate (C) in rats (experiment 1). Each value is the mean±SE, n=8. Means in a panel without a common letter differ, p<0.05. 10C, 10% casein diet; 10CCD, choline-deprived 10C; FA, folic acid. Folic acid and serine were supplemented to the diet at levels of 20 mg/kg diet and 2.5%, respectively.
RESULTS

Effect of hyperhomocysteinemia induced by choline deprivation of 10C (experiment 1)

Body weight gain, food intake and liver weight did not differ among the experimental groups (Table 1). Plasma homocysteine concentration was significantly increased by choline deprivation from 15.75±0.38 to 34.28±0.59 μmol/L (Fig. 2A). The choline deprivation-induced elevation of plasma homocysteine was significantly suppressed by supplementation with folate alone, serine alone, and folate plus serine to levels of 28.79±0.73, 27.28±0.46, and 25.72±0.47 μmol/L, respectively. The extents of increment suppression by folate alone, serine alone, and folate plus serine were 29.6%, 37.8%, and 46.2%, respectively. Plasma cysteine concentration was slightly higher or tended to be higher in rats fed 10CCD irrespective of supplements than in rats fed 10C (Fig. 2B). Plasma 5-MTHF concentration, which is measured as an index of folate status within the body (26), was significantly increased by folate supplementation, although supplementation with serine alone also slightly increased the concentration (Fig. 2C). Choline deprivation significantly decreased hepatic SAM concentration and the SAM:SAH ratio and, conversely, increased hepatic SAH and homocysteine concentrations (Fig. 3). The increase in hepatic homocysteine concentration was significantly suppressed by supplementation with folate, serine or both, but choline deprivation-induced changes in SAM and SAH concentrations and the SAM:SAH ratio were unaffected. Choline deprivation significantly decreased hepatic activities of BHMT and CBS but not that of MS (Fig. 4A, C and E). Supplementation with folate irrespective of serine significantly increased or tended to increase these enzyme activities. Choline deprivation markedly decreased hepatic concentration of betaine, a substrate of BHMT, and this decrease was slightly suppressed by folate supplementation (Fig. 4B). Hepatic concentration of 5-MTHF, a substrate of MS, was significantly increased by folate supplementation (Fig. 4D). Hepatic concentration of serine, a substrate of CBS, was significantly increased by serine supplementation (Fig. 4F).
The relative level of mRNA for MS in the liver was significantly increased by folate supplementation; there was no significant difference in relative levels of mRNA for BHMT or CBS among the experimental groups (Fig. 5).

**Effect on hyperhomocysteinemia induced by choline deprivation of 25S (experiment 2)**

Body weight gain and food intake did not differ among the experimental groups, whereas relative liver weight was significantly higher in rats fed choline-deprived diets irrespective of supplements than in rats fed 25S (Table 1). Plasma homocysteine concentration was significantly increased by choline deprivation from 14.79 ± 0.58 to 34.61 ± 0.80 μmol/L (Fig. 6A). The choline deprivation-induced elevation of plasma homocysteine was significantly suppressed by supplementation with folate alone, serine alone, and folate plus serine to levels of 29.21 ± 0.58, 27.35 ± 0.71, and 26.12 ± 0.71 μmol/L, respectively. The extents of increment suppression by folate alone, serine alone, and folate plus serine were 27.2, 36.6, and 42.8%, respectively. The profile of plasma homocysteine concentration was similar to that in experiment 1. Plasma cysteine concentration did not differ among the experimental groups (Fig. 6B). Plasma 5-MTHF concentration was significantly increased by folate supplementation, although supplementation with serine alone slightly decreased the concentration (Fig. 6C). Similar to the results in experiment 1, choline deprivation significantly decreased hepatic SAM concentration and the SAM:SAH ratio and, conversely, increased hepatic SAH and homocysteine concentrations (Fig. 7). These changes induced by choline deprivation were not affected by supplementation with folate, serine, or both, although the increase in hepatic homocysteine concentration tended to be suppressed by supplements. Choline deprivation significantly decreased hepatic betaine concentration, but it did not affect BHMT or MS activities (Fig. 8A, C, and E). The decrease in CBS activity was slightly suppressed by supplementation with folate, serine, or both. Choline deprivation markedly decreased hepatic betaine concentration and this decrease was unaffected by supplementation with folate, serine, or both (Fig. 8B). Hepatic concentration of 5-MTHF was significantly increased by folate supplementation and hepatic serine concentration was significantly increased by serine supplementation (Fig. 8D and F).

**DISCUSSION**

Our previous study showed that choline deprivation of low-methionine diets such as 10C and 25S resulted in hyperhomocysteinemia in rats (7). In the present
study, we therefore used both 10C and 25S as basal diets to induce hyperhomocysteinemia by choline deprivation, because the supplemental effect of folate alone or in combination with serine may differ depending on the type and level of dietary protein. It appears that choline deprivation-induced hyperhomocysteinemia is mainly caused by decreased homocysteine removal via the BHMT pathway due to a decrease in hepatic betaine concentration. In the present study, choline deprivation significantly decreased hepatic BHMT activity in rats fed 10C but not in rats fed 25S. Although the reason for the discrepancy of response of BHMT to choline deprivation between rats fed 10C and rats fed 25C is currently unclear, decreased BHMT may also contribute to choline deprivation-induced hyperhomocysteinemia at least in rats fed 10C. Under the condition of choline deficiency, hepatic SAM concentration decreases and, conversely, hepatic SAH concentration increases (27). This results in depression of the synthesis of phosphatidylcholine (PC), which provides choline and further betaine endogenously, via the phosphatidylethanolamine (PE) N-methylation pathway, since the reaction of PE N-methylation depends on hepatic SAM concentration and SAH inhibits the reaction (28, 29). Thus, dietary choline deprivation accelerates betaine deficiency in the liver. The objective of the present study was to determine whether choline deprivation-induced depression in the BHMT pathway can be compensated by stimulation of the MS pathway. To stimulate the MS pathway, choline-deprived diets were supplemented with folate alone or in combination with serine, since serine is thought to be a major source of 5-MTHF (14). The results obtained in the present study demonstrated that choline deprivation-induced hyperhomocysteinemia could be significantly suppressed by dietary supplementation with folate alone, serine alone, or folate plus serine. The profiles of plasma homocysteine concentrations were similar in rats fed 10C and rats fed 25S, indicating that the effects of folate and/or serine on choline deprivation-induced hyperhomocysteinemia were not dependent on dietary protein levels. In our preliminary experiment, supplementation of 100CD with folate at levels of 5, 10, and 20 mg/kg diet suppressed choline deprivation-induced elevation of plasma homocysteine concentration in a dose-dependent manner (data not shown). This suggests that a...
supplementation level of folate higher than 20 mg/kg diet may bring about a further effect. However, it has been shown that a supraphysiological dose of folate (e.g., 20 times the requirement, 40 mg/kg diet) tended to have a harmful effect on colorectal carcinogenesis in rats, while modest doses of folate (4–10 times the requirement, 8–20 mg/kg diet) suppressed the carcinogenesis (30). Hence, in the present study, we used folate at a level of 20 mg/kg diet, which is considered to be the maximal dose within the nutritional range. On the other hand, in the present study, we used serine at a level of 2.5% according to our previous study showing that dietary supplementation with 2.5% serine completely suppressed methionine-induced hyperhomocysteinemia (31). The serine contents of 10C and 25S were estimated as 0.47 and 0.95%, respectively. Hence, it appears that supplementation with 2.5% serine is adequate for assessing the effect of serine.

Although the hypohomocysteinemic effect of folate plus serine was significantly greater than the effect of folate alone, there was no significant difference between the effects of folate plus serine and serine alone in either experiment. These results indicate that folate and serine had little additive effect on the plasma homocysteine concentration. Judging from the results shown in Figs. 4 and 8, it is probable that folate supplementation decreased plasma homocysteine concentration, though only partially, by increasing hepatic 5-MTHF concentration together with MS and CBS activities. On the other hand, serine supplementation might decrease plasma homocysteine concentration by increasing hepatic serine concentration rather than by increasing hepatic 5-MTHF concentration, since hepatic 5-MTHF concentration was not increased by supplementation with serine. However, it is uncertain whether increased serine concentration actually stimulated CBS reaction, since CBS appears to be saturated with serine even in rats fed serine-unsupplemented diets when the reported Km value of CBS for serine, about 0.7 mM (32), is taken into consideration.

It should be stressed that the effect of folate or serine was only partial or limited even in the case of the combination of folate and serine, which tended to exhibit the maximal effect. It has been shown that the activity of MS was lower than the activity of BHMT in the liver of rats (33–35), although these enzyme activities were also influenced by dietary conditions. This is also the case for the present study; supporting the concept that the capacity of the MS pathway for homocysteine metabolism is far lower than the capacity of the BHMT pathway. This might be one of the reasons for the insufficient effect of supplementation with folate alone or in combination with serine. If so, there is the question of why folate deficiency generally causes hyperhomocysteinemia despite the capacity of the MS pathway being small. Although several mechanisms for the folate deficiency-induced elevation of plasma homocysteine concentration have been proposed, the most likely mechanism is that folate deficiency might impair not only the MS pathway but also the BHMT pathway (36). This mechanism is based on the fact that folate deficiency increases the plasma concentration of N,N-dimethylcine (DMG) in human subjects (35). DMG is a product of BHMT reaction but also an inhibitor of BHMT (37). Tetrahydrololate (THF) is required for the metabolism of DMG as a methyl-group acceptor (38), indicating that activities of both the MS and BHMT pathways are influenced by folate deficiency. In our previous study, we demonstrated that folate deprivation-induced hyperhomocysteinemia could not be fully suppressed by dietary supplementation with betaine even at a relatively high level, 1%, in rats (unpublished data). One of the reasons for the insufficient effect of betaine might be that folate deficiency impaired BHMT reaction by increasing hepatic DMG concentration, based on the fact that there was a significantly positive correlation between hepatic DMG concentrations and plasma homocysteine concentrations. Thus, our previous and present studies support the notion that the two pathways for homocysteine removal by remethylation, MS and BHMT pathways, cannot be fully compensated mutually.

It has been shown that dietary addition of guanidinoacetic acid (GAA) increased plasma homocysteine concentration in rats (39, 40). At least two mechanisms are considered for the GAA-induced hyperhomocysteinemia: (i) accelerated conversion of SAM to SAH and homocysteine due to compulsive metabolism of GAA to creatine (39, 40) and (ii) betaine deficiency due to decreased PC synthesis via the PE N-methylation pathway (41, 42). The latter mechanism resembles that of choline deprivation-induced hyperhomocysteinemia. In fact, GAA-induced hyperhomocysteinemia could be effectively suppressed by dietary supplementation with choline or betaine (42), but it was not suppressed by folate supplementation (unpublished data). These results, together with the results in the present study, suggest that folate deficiency causes obvious hyperhomocysteinemia, whereas folate supplementation has no more than a partial or limited effect on several types of hyperhomocysteinemia, except for folate deficiency-induced hyperhomocysteinemia.

There have been several reports on the distinct features of MS and BHMT and the roles of the MS pathway and BHMT pathway. The most striking difference is the Km value for homocysteine. In rats, the Km value of hepatic MS for homocysteine was 1.7 μM (43), whereas the Km value of hepatic BHMT for homocysteine was 12 μM (37). Under normal conditions, the hepatic homocysteine concentration in rats is relatively low, e.g., approximately 4 nmol/g (44), which is considerably lower than the Km value of BHMT. Another difference is the response to dietary methionine level. The activity of hepatic BHMT increased as the dietary methionine level was increased in rats, although methionine restriction also increased the enzyme activity (34, 45). In contrast, the activity of hepatic MS decreased as the dietary methionine level was increased (34). Furthermore, hepatic BHMT activity increased in response to dietary levels of choline or betaine (46). Based on these facts, Finkelstein et al. (33, 34, 45, 46)...
have postulated that homocysteine remethylation by the MS pathway might contribute to maintenance of the basal methionine level and that homocysteine remethylation by the BHMT pathway might function as a pathway for catabolism of choline and betaine in addition to removal of homocysteine. Furthermore, it should not be ignored that the MS pathway regenerates THF and, conversely, the BHMT pathway provides C1 units, which are accepted by THF in the metabolism of DMG and sarcosine. These features and roles characteristic of the MS or MS pathway and the BHMT or BHMT pathway appear to be reconciled with the fact that impairment of one pathway could not be fully compensated by another pathway.

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