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ORIGINAL CONTRIBUTION

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The status of plasma homocysteine and related B-vitamins in healthy young vegetarians and nonvegetarians

Summary *Background* Exclusion of animal products and having only plant protein in vegetarian diets may affect the status of certain B-vitamins, and further cause the elevation of plasma homocysteine concentration. *Aim* The purpose of this study was to assess the status of homocysteine and related B-vitamins in vegetarians and nonvegetarians. The effects of biochemical parameters of B-vitamins and dietary protein on plasma homocysteine were also examined. *Methods* The study was performed at the Chung Shan Medical University, Taichung, in the central part of Taiwan. Thirty-seven vegetarians (28.9 ± 5.5 y) and 32 nonvegetarians (22.9 ± 1.6 y) were recruited. Nutrient intake was recorded using 3-day dietary records. Fasting venous blood samples were obtained. Plasma homocysteine, folate and vitamin B-12 were measured. Vitamin B-6 status was assessed by direct measures [plasma pyridoxal 5'-phosphate (PLP) and urinary 4-pyridoxic acid (4-PA)] and indirect measures [erythrocyte alanine (EALT-AC) and aspartate (EAST-AC) aminotransaminase activity coefficient]. *Results* There was no significant difference in vitamin B-6 intake between the two groups, although the vegetarian group had a significantly lower vitamin B-12 intake than the nonvegetarian group. Vegetarian subjects had significantly lower mean plasma PLP and vitamin B-12 concentrations than did nonvegetarian subjects ($p < 0.05$); however, a significantly higher mean plasma folate concentration was found in the vegetarian group. Vegetarian subjects had a significantly higher mean plasma homocysteine concentration than nonvegetarian subjects (13.2 ± 7.9 vs. 9.8 ± 2.2 μmol/L). Negative correlations were seen between plasma homocysteine and vitamin B-12 concentrations in the vegetarian ($p = 0.004$), nonvegetarian ($p = 0.026$), and pooled ($p < 0.001$) groups. From best subsets regression analyses, the plasma homocysteine concentration could be significantly predicted by total protein intake ($p = 0.027$) and plasma vitamin B-12 concentration ($p = 0.005$) in the pooled group. When the intake of protein is not considered, vitamin B-12 concentration is still a strong predictor of plasma homocysteine concentration ($p = 0.012$). *Conclusions* Vitamin B-12 intake and mean plasma vitamin B-12 concentration were lower for vegetarian subjects than for nonvegetarian subjects, leading to an increase in plasma homocysteine concentration. Vitamin B-6 and folate had little effect on plasma homocysteine concentration when individuals had adequate vitamin B-6 and folate status.

Key words homocysteine – vitamin B-6 – folate – vitamin B-12 – vegetarians – nonvegetarians

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Introduction

Plasma homocysteine concentration has been shown to be an independent risk factor of coronary heart disease [1–3]. Many factors have been associated with mild and moderate hyperhomocysteinemia; of particular interests are nutritional deficiencies in the vitamin cofactors that are required for homocysteine metabolism, folate, vitamin B-6 and B-12. A large cohort of subjects from the Framingham Study indicated that folate, vitamin B-6 and B-12 were important determinants of plasma homocysteine levels in an elderly population [4]. The Nurses Health Study of 80,000 nurses studied over a 14-y period revealed that the risk of mortality and morbidity of myocardial infarction was inversely related to dietary intakes of folate and vitamin B-6 [5].

Vegetarian diets contain restricted amounts of animal products, which are the primary sources of vitamin B-12. A certain percentage of vegetarians may be at risk of having a vitamin B-12 deficiency. Vitamin B-12 is the cofactor for the methyl transfer from 5-methyltetrahydrofolate to homocysteine during remethylation. In a recent study by Hung et al. [6], it was shown that Buddhist nuns in Taiwan had a significantly lower plasma vitamin B-12 level than omnivores, and a mildly elevated fasting plasma homocysteine level due to lower levels of vitamin B-12. Thus, in vegetarians, having a lower vitamin B-12 status may increase plasma homocysteine concentration.

It is commonly thought that vegetarians have a higher fiber intake than do nonvegetarians [7–9]; however, dietary fiber might be a factor compromising the bioavailability of vitamin B-6 [10–12]. The vegetarians consumed only plant proteins which are low in tryptophan and methionine, possibly reducing the vitamin B-6 requirement [13, 14]. Since, however, the vegetarians obtained most of their vitamin B-6 from grains, cereals, nuts, legumes, vegetables and fruits, which supplied high amount of pyridoxal-5'-β-D-glucoside, vitamin B-6 status could possibly be compromised. Vitamin B-6 deficiency may impair the conversion of homocysteine to cystathionine and then to cysteine by the PLP-dependent enzyme during transsulfuration to be impaired and subsequently increase plasma homocysteine concentration.

Exclusion of animal products and having only plant protein in vegetarian diets may affect the status of certain B-vitamins, and further cause the elevation of plasma homocysteine concentration. The purpose of this study was to assess and compare the status of homocysteine and related B-vitamins between vegetarians and nonvegetarians. The effects of biochemical parameters of B-vitamins and dietary protein on plasma homocysteine were also examined.

Subjects and methods

Subjects

Vegetarian and nonvegetarian young adults were recruited from the university campus and the community in Taichung city by advertising. For the purpose of this study, a vegetarian was defined as someone who ate neither meat nor fish. Potential subjects were interviewed personally by one of the principal investigators, at which time the study was explained and interest determined. Demographic and health data (i. e., blood pressure, smoking and drinking habits) were collected and weight and height were measured. A fasting blood sample was obtained for clinical chemistry evaluation. Subjects had to meet the following criteria for inclusion in this study: 1) age < 40 y; 2) no illness or medical condition requiring a physician's supervision; 3) no history of heart diseases, kidney disorders, respiratory disorders, liver disease, diabetes, gastrointestinal disorders that have been reported to alter homocysteine, vitamin B-6, folate or vitamin B-12 status; 4) no history of hormonal therapy (i. e., oral contraceptives) or other drugs (i. e., H_2 blockers, proton pump inhibitors, metformin, phenytoin, methotrexates, etc.) which would influence homocysteine and vitamin B-6, folate and vitamin B-12 status and metabolism; 5) a history of consuming a vegetarian diet > 1 year for vegetarian subjects; 6) normal blood chemistry evaluations; 7) no weight change exceeding ± 4 kg within the last year; 8) no pregnant subjects.

Thirty-seven vegetarians (8 males, 29 females) and 32 nonvegetarians (10 males, 22 females) were recruited in this study. Informed consent was obtained from each subject. The study protocol was approved as ethical when the research proposal was reviewed by the Chung Shan Medical University.

Experimental protocol

Current body weight and height were measured. The body mass index (BMI; kg/m^2) was then calculated. All subjects were given instruction on how to complete a 3-day dietary record (2 weekdays and 1 weekend day). Subjects were asked to write down the times food was eaten, all food and beverages consumed, the amounts consumed, and methods of preparation. Nutrient composition of the 3-day records was calculated with use of Nutritionist Professional software (Kitchen Business Corporation, Taiwan), and the nutrient database was based on the Taiwan food composition table (Department of Health, 1998). The results of nutrient data were compared to the Taiwan Dietary Reference Intakes (DRI) (Department of Health, Taiwan, 2002).

Once the dietary record had been completed, fasting venous blood samples were obtained to estimate hematological and vitamin status parameters. Blood specimens were collected in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing EDTA as an anticoagulant. Plasma and erythrocytes were prepared as previously described [15] and then stored frozen (–20 °C) until analysis. Plasma homocysteine was measured by high performance liquid chromatography (HPLC) using the method of Araki and Sako [16]. The intraassay and interassay of plasma homocysteine variabilities were 1.9% (n = 6) and 1.2% (n = 6), respectively. Plasma PLP was determined by HPLC as previously described [17]. The intraassay and interassay of plasma PLP variabilities were 0.94% (n = 6) and 0.33% (n = 6), respectively. Erythrocyte alanine aminotransaminase and EAST with and without PLP stimulation in vitro were measured by the method of Woodring and Storvick [15]. All EALT and EAST activity measurements were performed by using fresh erythrocyte samples collected on the day of analysis. The intraassay of EALT and EAST activity variabilities were 2.27% (n = 5), and 2.43% (n = 3), respectively. Plasma folate and vitamin B-12 were analyzed by using standard competitive immunochemiluminometric methods on a Chiron Diagnostics ACS:180 Automated Chemiluminescence System (Chiron Diagnostics Corporation, USA).

Twenty-four hour urine collections were obtained on the third day of the dietary record. The pH of the urine samples was maintained at ~3.0 by the addition of 1 N HCl. Twenty-four h urine volumes were measured and aliquots stored frozen (–20 °C) until analyzed. Urinary 4-PA was analyzed by HPLC by the modified method described by Gregory and Kirk [18]. Urinary 4-PA intraassay and interassay variabilities were 3.92% (n = 4) and 1.93% (n = 8), respectively. Urinary creatinine concentrations were determined in all urine samples by using a colorimetric kit (Sigma Chemical Co., St. Louis, MO) to check the completeness of collection. Urinary 4-PA, plasma PLP concentrations and transaminase activity measurements were carried out under yellow light to prevent photodestruction. All analyses were performed in duplicate.

Statistical analyses

Data were analyzed by using SigmaStat statistical software (version 2.03; Jandel Scientific, San Rafael, CA). Differences in subject characteristics, nutrient intake and biochemical measurements between dietary groups were determined by use of the Student's *t* test. When the data were skewed rather than normally distributed, differences between the two sex groups were determined by using the Mann-Whitney Rank test. Spearman and Pearson correlation coefficients were performed to assess the association between plasma homocysteine and B-vitamins, and between plasma homocysteine and dietary variables. Multiple regression analyses were performed to assess the effects of biochemical parameters of B-vitamins and dietary intake on plasma homocysteine. Statistical results were considered to be significant at $p \leq 0.05$. Values presented in the text are means and 95% of confidence intervals (CI).

Results

Descriptive characteristics

Characteristics of vegetarian and nonvegetarian subjects are shown in Table 1. Vegetarian subjects were older than the nonvegetarian subjects. The difference was not expected to affect the comparison of nutrient intake and the status of vitamins. The two groups (vegetarian vs. nonvegetarian) were of comparable body size. The vegetarian group included 3 vegans, 18 lacto-vegetarians, and 16 lacto-ovo-vegetarians. These subjects had been following this diet practice for a mean of 5.6 years, ranging from 1.3 to 19 y. No subjects took any supplements, nor smoked or drank regularly.

Dietary intakes

Nutrient intake from the 3-d dietary records kept by the subjects was calculated (Table 2). The mean energy intake of both vegetarian and nonvegetarian subjects was not significantly different. Vegetarians had a significantly higher carbohydrate intake than nonvegetarian subjects. Although no significant differences were found in total protein intake between the two groups, the vegetarians had a significantly higher plant protein and lower animal protein intake than the nonvegetarians. There was no significant difference in fat intake between the two groups; however, vegetarian subjects had a significantly lower cholesterol intake than nonvegetarian subjects. When intake of the energy-providing nutrient was expressed as a percentage of total caloric consumption, the vegetarian group had a significantly higher mean percentage of carbohydrate (58% vs. 53%), but

Table 1 Descriptive characteristics of vegetarian and nonvegetarian subjects

Characteristics	Vegetarians (n = 37)	Nonvegetarians (n = 32)
Age (y)	28.9 [27.1, 30.7][a, *]	22.9 [22.3, 23.5][b]
Height (cm)	161.7 [160.0, 163.4]	163.1 [161.2, 165.0]
Weight (kg)	54.3 [52.2, 56.4]	55.9 [53.0, 58.8]
Body mass index (kg/m^2)	20.7 [20.0, 21.4]	20.9 [20.1, 21.7]

Values are means with 95 % confidence intervals in brackets.
* Values in a row with different superscript letters are significantly different, $p \leq 0.05$

Table 2 Daily nutrient intakes of vegetarian and nonvegetarian subjects

Nutrients	Vegetarians (n = 37)	Nonvegetarians (n = 32)
Energy		
(MJ)	8.9 [8.0, 9.7]	7.8 [7.2, 8.4]
(kcal)	2124.9 [1923.4, 2326.4]	1872.8 [1729.7, 2015.9]
Carbohydrate (g)	307.1 [274.5, 339.7][a,*]	245.7 [224.7, 266.6][b]
(% total energy)	57.7 [55.7, 59.8][a]	52.7 [50.3, 55.1][b]
Fat (g)	74.3 [65.8, 82.8]	71.5 [63.8, 79.3]
(% total energy)	31.5 [29.7, 33.7]	34.2 [31.9, 36.5]
Cholesterol (mg)	84.4 [51.8, 117.1][a]	293.7 [253.5, 333.8][b]
Protein (g)	60.9 [54.7, 67.0]	63.9 [57.0, 70.7]
(% total energy)	11.5 [10.8, 12.2][a]	13.6 [12.7, 14.6][b]
Plant protein (g)	56.4 [50.4, 62.4][a]	32.6 [27.1, 38.1][b]
Animal protein (g)	4.5 [2.9, 6.0][a]	31.3 [26.6, 36.0][b]
Vitamin B-6 (mg)	0.94 [0.8, 1.1]	1.05 [0.9, 1.2]
B-6 (mg)/protein (g) ratio	0.015 [0.013, 0.017]	0.017 [0.014, 0.019]
Vitamin B-12 (µg)	0.74 [0.5, 1.0][a]	3.32 [2.6, 4.0][b]
Dietary fiber (g)	19.2 [17.0, 21.5][a]	10.5 [9.1, 12.0][b]

Values are means with 95 % confidence intervals in brackets.
* Values in a row with different superscript letters are significantly different, $p \leq 0.05$

Table 3 Biochemical indices of vitamin status in vegetarian and nonvegetarian subjects

Index	Vegetarians (n = 37)	Nonvegetarians (n = 32)
Plasma homocysteine (µmol/L)	13.2 [10.6, 15.7][a,*]	9.8 [9.1, 10.6][b]
Plasma vitamin B-12 (pmol/L)	191.8 [164.0, 220.0][a]	310.9 [278.2, 343.6][b]
Vitamin B-6 indicators		
Plasma PLP (nmol/L)	58.5 [48.2, 68.7][a]	85.9 [73.4, 98.5][b]
EALT-AC	1.36 [1.23, 1.49]	1.25 [1.19, 1.32]
EAST-AC	2.10 [1.93, 2.27][a]	1.82 [1.72, 1.92][b]
Urinary 4-PA (µmol/d)	3.1 [1.3, 4.8][a]	3.5 [2.7, 4.4][b]
Plasma folate (nmol/L)	28.5 [23.6, 33.3][a]	19.6 [17.2, 22.0][b]

Values are means with 95 % confidence intervals in brackets. *PLP* pyridoxal 5′-phosphate; *EALT-AC* erythrocyte alanine aminotransaminase activity coefficient; *EAST-AC* erythrocyte aspartate aminotransaminase activity coefficient.
* Values in a row with different superscript letters are significantly different, $p \leq 0.05$

had a significantly lower mean percentage of protein (12 % vs. 14 %) than the nonvegetarian group.

There is a lack of information on the folate content for Chinese foods; therefore, the intake of folate could not be determined. There was no significant difference in vitamin B-6 intake and the ratio of vitamin B-6 to protein (mg/g) between vegetarian and nonvegetarian subjects; however, the vegetarian group had a significantly lower vitamin B-12 intake than the nonvegetarian group. The mean vitamin B-6 and B-12 intake for both vegetarians and nonvegetarians was lower than the US DRI [19] (vitamin B-6: 1.3 mg/d; vitamin B-12: 2 µg/d) and the current Taiwan DRI (vitamin B-6: 1.5 mg/d; vitamin B-12: 2.4 µg/d for both adult men and women). Vegetarian subjects had a significantly higher dietary fiber intake than nonvegetarian subjects.

Biochemical analyses

Hyperhomocysteinemia is defined as a plasma homocysteine concentration ≥ 15 µmol/L [20]. Vitamin B-12 and folate deficiency are defined as a plasma concentration of less than 125 pmol/L, and 6.8 nmol/L, respectively. Vitamin B-6 deficiency was defined as plasma PLP concentration < 20 nmol/L, the EALT-AC value ≥ 1.25, the EAST-AC value ≥ 1.8, and urinary 4-PA value < 3 µmol/d [19, 21, 22].

Plasma homocysteine concentrations are shown in Table 3. Vegetarian subjects had significantly higher mean plasma homocysteine concentration than nonvegetarian subjects. A total of 21.6 % (n = 8) of vegetarians but only 3.1 % (n = 1) of nonvegetarians had moderate hyperhomocysteinemia (≥ 15 µmol/L).

Plasma PLP, the EALT-AC and EAST-AC values are shown in Table 3. Vegetarian subjects had a significantly lower mean plasma PLP concentration than nonvegetarian subjects. None of the subjects had plasma PLP concentrations below the suggested value of 20 nmol/L for adequate vitamin B-6 status. The mean EAST-AC but not the EALT-AC value was significantly different between vegetarian and nonvegetarian groups. The mean EALT-AC was ≥ 1.25 in the two groups, which is the suggested value for inadequate vitamin B-6 status. Similarly, the mean EAST-AC value of subjects was higher than the suggested value for inadequate vitamin B-6 status. The vegetarians had a significantly lower mean urinary 4-PA level than the nonvegetarians. The value was, however, above 3.0 µmol/d which is considered to be associated with adequate vitamin B-6 status in both groups.

The vegetarian group had a significantly higher mean level of plasma folate but a lower mean level of vitamin B-12 than the nonvegetarian group (Table 3). The mean folate and vitamin B-12 concentrations were higher than the suggested values for adequate folate and vitamin B-12 status.

There was a significantly positive correlation between plasma homocysteine and animal protein ($r = 0.384, p = 0.03$) and total protein ($r = 0.408, p = 0.02$) intakes in the nonvegetarian group. Dietary fiber intake did not correlate with any biochemical status parameters in any group. No significant correlation was found between plasma homocysteine concentration and any vitamin B-6 status indicators in any group (vegetarian, nonvegetarian and pooled groups). Plasma homocysteine also did not correlate with plasma folate in the vegetarian group and pooled groups (n = 69). However, plasma homocysteine did significantly correlate with plasma folate in the nonvegetarian group ($r = –0.363$,

Table 4 Influence of different variables of plasma B-vitamins on plasma homocysteine concentrations using best subsets regression analyses

Variables	Vegetarians (n = 37)	Nonvegetarians (n = 32)	Pooled (n = 69)
Total protein intake	–0.148 (0.045)	0.034 (0.11)	–0.083 (0.027)
Vitamin B-12	–0.017 (0.417)	–0.005 (0.137)	–0.016 (0.005)
Plasma pyridoxal 5'-phosphate	–0.011 (0.788)	0.007 (0.538)	–0.01 (0.643)
Folate	–0.325 (0.178)	–0.126 (0.373)	–0.224 (0.097)

Values are regression coefficient with the *p* value in the parentheses

$p = 0.026$). A negative correlation was seen between plasma homocysteine and plasma vitamin B-12 concentrations in the vegetarian ($r = -0.482, p = 0.004$), nonvegetarian ($r = -0.394$, $p = 0.026$), and pooled ($r = -0.459$, $p < 0.001$) groups.

From best subsets regression analyses (Table 4), it was shown that the plasma homocysteine concentration was only significantly and independently influenced by total protein intake ($p = 0.045$) in the vegetarian group. The status parameters of vitamin B-6, folate and vitamin B-12 could not predict plasma homocysteine concentration in the individual group (vegetarian and nonvegetarian groups). If, however, we pooled the data (n = 69), plasma homocysteine concentration could be significantly predicted by total protein intake ($p = 0.027$) and plasma vitamin B-12 concentration ($p = 0.005$). When the intake of protein is excluded, vitamin B-12 concentration is still a strong predictor of plasma homocysteine concentration in the pooled group ($p = 0.012$).

Discussion

It has been reported that vegetarians have a lower incidence of coronary heart disease than do nonvegetarians [23]. On the other hand, hyperhomocysteinemia has been recognized as an independent risk factor of cardiovascular diseases [1–3]. Our present study and others [6, 24, 25] demonstrated that vegetarians had significantly higher plasma homocysteine concentrations than do nonvegetarians. Although the mean plasma homocysteine concentration of our vegetarians did not show hyperhomocysteinemia (≥15 μmol/L), one fourth of the vegetarians had moderate hyperhomocysteinemia. Herrmann et al. [25] also reported that 20 % of all vegetarians had moderate hyperhomocysteinemia in their study.

Dietary methionine is abundant in the omnivore diet, and methionine intake actually regulates the metabolism of homocysteine. Inadequate methionine intake and/or serum methionine concentration will lead to homocysteine preferentially remethylating to methionine. The present study showed a correlation between plasma homocysteine concentration and either dietary total protein or animal protein intake. In disagreement with the opinion of previous studies [6, 24, 26], we suggest that dietary protein intake and serum methionine concentration might affect plasma homocysteine concentration, although the amounts of dietary methionine intake and serum methionine concentrations were not measured in this study.

Vitamin B-6, folate, and vitamin B-12 deficiencies are known to cause the elevation of plasma homocysteine concentration. Among the three B-vitamins, vegetarians are particularly vulnerable to having inadequate vitamin B-12 intakes due to the lack of consumption of animal products. Previous studies [8, 27, 28] have shown that vegetarians, including lacto-ovo-vegetarians have a high prevalence of vitamin B-12 deficiency. Our study did show that vegetarian subjects had an inadequate mean vitamin B-12 intake, but that their mean plasma vitamin B-12 concentrations were not in the deficient range. It may be due to our subjects being lacto-ovo- and lacto-vegetarians who consume vitamin B-12 from egg and dairy products. In addition, vitamin B-12 is conserved in the body through enterohepatic circulation; thus it would take years to develop a vitamin B-12 deficiency [29]. Herbert [30] indicated an early manifestation of vitamin B-12 deficiency is the elevation of plasma homocysteine concentration. Looking at the individual data, although subjects with moderate hyperhomocysteinemia were not necessarily in the deficient stage of vitamin B-12, vegetarian subjects with moderate hyperhomocysteinemia did show inadequate vitamin B-12 intake (0.56 μg, n = 8). Since studies [31, 32] have shown that vitamin supplementation effectively reduces plasma homocysteine concentration, vegetarians, especially vegans, might consider vitamin B-12 supplementation to prevent the increase of plasma homocysteine concentration if their diet practice continues.

There was no significant difference in mean vitamin B-6 intake between vegetarians and nonvegetarians, yet vegetarians had a significantly lower plasma PLP concentration when compared with nonvegetarians. The major reason might be due to a lack of information on the vitamin B-6 content for some Chinese foods. Our vegetarians and nonvegetarians had an adequate vitamin B-6 status. This might be the reason that there was a lack of correlation between plasma homocysteine concentration and vitamin B-6 in both dietary groups. Previous studies [6, 33, 34] also indicated no association between vitamin B-6 and homocysteine. Pyridoxal 5'-phosphate dependent enzymes involved in the transulfuration pathway of homocysteine metabolism might not be the predominant one affecting plasma homocysteine concentration when vitamin B-6 status is adequate.

Folate and vitamin B-12 are the two important components in the remethylation of homocysteine. Studies [33, 35, 36] have shown that plasma folate and vitamin B-

12 strongly inversely correlate with plasma homocysteine. This was in contrast to the findings of Hung et al. [6], who found no correlation between plasma homocysteine and either plasma folate or vitamin B-12 in the nonvegetarians. Comparing mean plasma folate concentration, our nonvegetarians had a lower plasma folate concentration than did omnivores of Hung et al. [6]. Since plasma folate concentrations change rapidly with recent dietary folate intakes, this suggests that declining levels of plasma folate would become the determinant of plasma homocysteine concentrations. Erythrocyte folate concentration has been considered as an indicator of long-term folate status because folate is taken up only by the developing erythrocyte in the bone marrow and not by the circulating mature erythrocyte [37]. We did not measure erythrocyte folate concentration in this study; otherwise, the correlation between folate status and plasma homocysteine concentration might be estimated in the vegetarian and pooled groups.

In conclusion, vegetarian subjects had a lower vitamin B-12 intake and mean plasma vitamin B-12 concentration than did the nonvegetarian subjects leading to the elevation of plasma homocysteine concentration. Vitamin B-6 and folate had little effect on plasma homocysteine concentration when individuals were in adequate status. We, therefore, strongly suggested that adequate vitamin B-12, B-6, and folate intakes are beneficial in lowering plasma homocysteine.

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