Eur J Nutr (2007) 46:446–452
DOI 10.1007/s00394-007-0684-5

ORIGINAL CONTRIBUTION

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Effects of orange juice and proline betaine on glycine betaine and homocysteine in healthy male subjects

Received: 3 May 2007
Accepted: 9 October 2007
Published online: 4 December 2007

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Abstract *Background* Proline betaine (PB), a glycine betaine (GB) analogue found in citrus foods, increases urinary GB loss and plasma total homocysteine (tHcy) concentrations in rats. Its presence in human plasma is associated with increased GB excretion. *Aim* To compare the effects of dietary levels of PB on GB excretion, and on plasma tHcy and GB concentrations in healthy volunteers. *Methods* In a randomized crossover study, eight healthy males (18–50 years) ingested either 750 mL orange juice (containing 0.545 g PB), a PB supplement (0.545 g PB dissolved in 750 mL apple juice), or 750 mL apple juice (control). Plasma PB, GB and tHcy, and urine PB, GB and creatinine concentrations were measured hourly for 6 h and at 24 h post-treatment. *Results* Plasma tHcy concentrations were not increased (relative to control) following ingestion of either orange juice or PB supplement. Both treatments produced a significant increase in plasma PB concentrations ($P < 0.001$), this effect being greater following orange juice compared with PB supplement ($P < 0.05$, 1–2 h). Urinary excretion of PB was greater than the control following both orange juice ($P < 0.001$) and PB supplement ($P < 0.001$), from 2 to 24 h post-treatment. This increase in PB excretion was significantly greater following orange juice compared with PB supplement with higher peak excretion (C_{max} difference, $P = 0.008$). GB excretion was significantly greater following ingestion of orange juice compared with PB in apple juice ($P = 0.007$) and apple juice control ($P < 0.001$) in the first 2 h post-ingestion. *Conclusions* PB administered in dietary doses had little effect on plasma tHcy concentrations in healthy humans. Ingestion of PB in orange juice compared with PB alone resulted in greater increases in the urinary excretion of PB and GB.

Key words proline betaine (PB) – glycine betaine (GB) – total homocysteine (tHcy) – orange juice – methionine load test

Introduction

Proline betaine (PB) (*N*,*N*-dimethylproline or stachydrine) is a glycine betaine (GB) analogue. It is synthesized by many plants [3], accumulated in the cytoplasm of cells, protecting against osmotic stress [18]. PB was found in human urine [4] where it was shown to function as an osmoprotectant for bacteria in urinary tract infections, suggesting a similar role in

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mammals, although (in contrast to GB) this is not the case [13]. It is a normal component of the human diet, being found in particularly high concentrations in citrus fruit and in some legumes (~500 and 240 μg/g, respectively), and may be present in sufficient quantities to be a physiologically significant dietary component [20, 24]. PB is associated with an increased urinary loss of GB, and GB loss is associated with a higher incidence of vascular disease and elevated plasma homocysteine (Hcy) [11, 12]. Elevated Hcy is also associated with vascular disease, though whether this increased risk is causal is debated [16], and a loss of GB (a major tissue osmolyte) may be independently pathogenic [11, 12]. The aim of this study was to investigate the acute effects of a dietary load of PB, administered through increased dietary intake or oral supplementation, on GB in normal human subjects. Normally, little GB appears in the urine [13, 14]. It is metabolized by conversion to *N,N*-dimethylglycine (DMG), the methyl group being transferred to Hcy to give methionine, and therefore the effects of PB on DMG and Hcy were also studied.

Subjects and methods

Subjects

Healthy Caucasian male volunteers aged 18–50 years (n = 8) were recruited by advertisement. All subjects provided written informed consent following ethical approval of the study by the Canterbury Ethics Committee (Christchurch, New Zealand). Subjects had no current or previous history of vascular or renal disease, were free from acute or chronic illness requiring prescription medication, were non-diabetic, did not take dietary or vitamin supplements, were non-smokers and in good health based on medical history and physical examination. Subjects were excluded if they displayed any of the following; abnormal hemoglobin concentration (<130 g/L males), abnormal tHcy concentration (<5 or >15 μmol/L), deficiencies of Vitamin B_{12} (<120 pmol/L), B_6 (<35 nmol/L) or folate (<445 nmol/L), or were positive for the 677C → T polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene. Only one volunteer was rejected (because of abnormally high Hcy and low B_{12}). The baseline characteristics of the subjects are shown in Table 1.

Study design

A randomized crossover study was performed with three treatments: (a) orange juice (750 mL 'Charlies' pure orange juice, Charlies Trading Company Ltd., Auckland, New Zealand, containing 0.545 g PB estimated by analysis before the study), (b) PB supplement (0.545 g of PB dissolved in 750 mL 'Fresh Up' apple juice, Frucor Beverages Ltd., Auckland, New Zealand), and (c) apple juice (750 mL 'Fresh Up' apple juice, control). Subjects were required to consume each treatment within 15 min, and all complied well within this limit. The study was conducted over 3 consecutive weeks, with 1 day per week the treatment day and the rest of the week a wash-out period. Subjects received treatments following a 12-h overnight fast and consumed 1 treatment per day, with each subject consuming all three treatments by completion of the study. Subjects abstained from foods known to be high in dietary betaines or choline [20, 24], caffeine and alcohol for 2 days prior to treatment. Food intake was controlled during each study day, with a standard breakfast and lunch provided, immediately following treatment ingestion and 3 h post-treatment, respectively. Both standardized study meals were estimated to be low in dietary betaines, choline and methionine from food databases [20, 24]. Subjects received 100 mL water hourly throughout each study day to standardize fluid intake.

Table 1 Baseline characteristics of subjects

Age (years)	27.4 ± 1.5	(23.0–35.0)
Weight (kg)	78.8 ± 4.0	(66.0–98.0)
BMI (kg/m^2)	23.1 ± 0.5	(21.3–26.0)
Plasma tHcy (μmol/L)	9.7 ± 0.8	(6.4–13.7)
Serum B_{12} (pmol/L)	256 ± 21	(185–355)
Red cell folate (nmol/L)	682 ± 23	(574–854)
Hemoglobin (g/L)	148.1 ± 3.6	(134.0–165.0)

Data are mean ± SEM (range) on eight subjects

Blood collection and biochemical analyses

Venous blood samples were collected via an indwelling cannula in the antecubital fossa, into EDTA tubes and immediately placed on ice. Samples were collected at baseline and 20, 40, 60, 90, 120, 180, 240, 300, 360 min and 24 h post-treatment. A fasting urine sample was obtained at baseline, with subsequent sampling at 2, 4, 6, 8 h and from 8 to 24 h post-treatment.

Plasma was separated by centrifugation at 2000×g for 10 min within 2 h of blood collection. Urine sample volumes were recorded and a 10 mL aliquot removed. All samples were stored at −20 °C prior to analysis. Plasma concentrations of PB, GB, DMG and tHcy, and urine concentrations of PB, GB, DMG and creatinine were measured. Betaines and were measured in plasma and urine by high performance liquid chromatography (HPLC) after derivatization with 2-naphthacyl triflate [22, 23]. Plasma tHcy was mea-

sured by fluorescence polarization on an Abbott IM_X Analyzer (Abbott Diagnostic Division; Abbott Laboratories, USA). Urine creatinine was measured using the Jaffé reaction on an Abbott Aeroset Analyzer (Abbott Laboratories, Abbott Park, IL 60064, USA), and urine betaines and DMG excretion calculated as a ratio to creatinine. Serum Vitamin B_{12} and red blood cell (RBC) folate concentrations were measured by separate competitive immunoassays on a Chemiluminescence ACS:180 Analyzer (Chiron Diagnostics Corporation, USA). Duplicates of all foods and beverages consumed by each subject on all treatment days were assayed for dietary betaine content as described previously [20, 24].

Data analyses

Differences in plasma concentrations of PB, GB, DMG, tHcy, and urine concentrations of PB, GB and DMG, pre- and post-treatment were calculated for each subject for each treatment, and the data plotted. The statistical significance of the differences between treatments was first studied using two-way repeated measures analysis of variance (RM-ANOVA). If the RM-ANOVA indicated an overall treatment effect ($P < 0.05$), a post-hoc comparison of the means was carried out, with Tukey's test to determine significance. Urine data were log-transformed prior to analysis.

Standard pharmacokinetic parameters were calculated. The ratio to fasting baseline area under the postprandial concentration/time curve (AUC ratio) was calculated for all plasma data obtained from 0 to 6 h as the area under the postprandial concentration curve versus time, divided by fasting concentration multiplied by 6 [8]. The peak concentration (C_{max}), and the time taken to reach this peak concentration (T_{max}, not reported), were estimated for each individual subject directly from the data. The significance in differences between pharmacological parameters was assessed using one-way repeated measure analysis of variance, with the significance of post-hoc comparisons tested by the Tukey test. Data analysis was by SigmaStat version 3.1 (Systat Software Inc., San Jose, CA) and SPSS 14.0 for Windows (SPSS Inc, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$ unless otherwise stated.

Results

Analysis of study treatments

GB was not detected in any of the juices used in the study (Table 2). Aside from proline betaine, the only other betaines present were betonicine (4-hydroxyproline betaine) at about 10% of the PB content, and a trace of trigonelline. The measured proline betaine in a sample of the juice after the study was nearly 2% higher than the pre-study sample (Table 2), within the 4.5% CV of the assay used. No betaines were detected in the apple juice. Analysis of the apple juice with added PB recovered approximately 97% of the expected PB. Post-study analysis thus confirmed that the designed doses were achieved, within analytical error.

Table 2 Analysis of betaines in study treatments

Concentrations (mg/L)	Orange juice (a)	Proline betaine (b)	Apple juice (c)
Proline betaine	740	710	Nil
Glycine betaine	Nil	Nil	Nil
Betonicine	80	Nil	Nil
Pipecolobetaine	Nil	Nil	Nil
Trigonelline	15	Nil	Nil

HPLC analyses (CV 4.5%) for betaines reported to be present in some citrus fruits, conducted after study on (a) the orange juice, (b) the apple juice containing added proline betaine, and (c) the apple juice, used as treatments

Plasma proline betaine

For plasma PB concentrations, there was a highly significant interaction between treatment and time ($F = 14.4$, d.f. = 20,140, $P < 0.001$). Plasma concentrations of PB were significantly higher than the control treatment following both orange juice and PB supplement (in apple juice) throughout the study period; $P < 0.001$ from 1 to 6 h for orange juice and from 1 to 5 h for PB supplement (at 6 h $P = 0.006$), all compared with the apple juice control (Fig. 1A). When orange juice and PB supplement were compared, the trend was for a greater change in plasma PB concentrations following orange juice compared with PB supplement alone, significant ($P < 0.05$) at 1, 1.5 and 2 h post-treatment.

The peak plasma PB concentrations and the AUC ratio are higher following both treatments compared with control ($P < 0.001$ for both) (Table 3).

Plasma glycine betaine and dimethylglycine

For plasma GB, there was not a statistically significant interaction between treatment and time ($P = 0.394$). There appeared (Fig. 1B) to be a raised plasma GB in the treatments compared with the control in the first 2 h. Although a one-way RM ANOVA (on these selected time-points) suggested that this is significant ($P < 0.001$), in view of the overall interaction statistic the result is inconclusive. The pharmacological parameters were not different between treatments and controls.

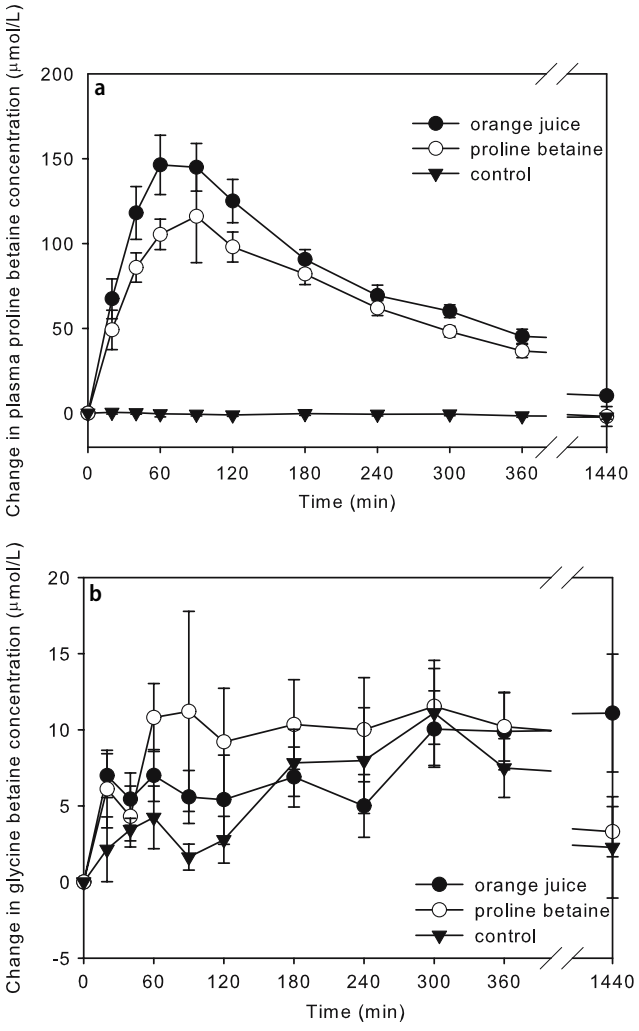

Fig. 1 Changes in plasma betaine concentrations from baseline following study treatments. Treatments are: orange juice (a) – filled circles, proline betaine in apple juice (b) – open circles, apple juice control (c) – filled inverted triangles. Data plotted are mean and standard error from eight subjects. Upper graph A: changes in plasma proline betaine concentrations; significance compared to control: $P < 0.001$ from 1 to 6 h for orange juice and from 1 to 5 h for proline betaine supplement (at 6 h $P = 0.006$). Lower graph B: changes in plasma glycine betaine concentrations; differences between treatments not significant

The interaction between treatment and time was not significant for plasma DMG ($P = 0.936$) and no notable trends were observed.

Plasma homocysteine

For plasma homocysteine, the interaction between treatment and time ($P = 0.364$) was not significant, and there were no observable trends for differences between the treatments and the control.

Table 3 Plasma and urine betaines and dimethylglycine following study treatments

	Orange juice (a)	Proline betaine (b)	Apple juice (c)
Plasma concentrations			
Proline betaine			
Baseline (μmol/L)	9.2 (±1.9)	4.4 (±4.7)	11.3 (±2.5)
AUC ratio	13.7 (±2.3)*	9.6 (±1.8)**	0.87 (0.08)
C_{max} (μmol/L)	167.0 (±13.8)*	149.6 (±23.6)*	14.7 (±3.0)
Glycine betaine			
Baseline (μmol/L)	31.1 (±2.0)	36.5 (±2.2)	36.4 (±1.9)
AUC ratio	1.26 (±0.06)	1.29 (±0.07)	1.20 (±0.07)
C_{max} (μmol/L)	47.9 (±2.3)	57.7 (±5.9)	52.2 (±3.8)
Dimethylglycine			
Baseline (μmol/L)	2.7 (±0.5)	4.8 (±1.6)	4.5 (±1.2)
AUC ratio	0.91 (±0.08)	1.00 (±0.13)	1.00 (±0.09)
C_{max} (μmol/L)	3.7 (±0.6)	7.5 (±2.3)	5.5 (±1.2)
Urinary excretions			
Proline betaine			
Baseline (mmol/mol)	47.9 (±34.0)	9.7 (±1.7)	15.4 (±4.5)
AUC ratio	55.1 (±16.5)*	37.1 (±5.6)*	1.9 (±0.4)
C_{max} (mmol/mol)	935 (±180)*,†	437 (±67)***,†	30 (±7)
Glycine betaine			
Baseline (mmol/mol)	8.4 (±1.8)	4.2 (±0.6)	6.0 (±2.2)
AUC ratio	4.08 (±1.05)	3.54 (±0.45)	2.42 (±0.28)
C_{max} (mmol/mol)	37.6 (±6.9)**,†	16.9 (±2.9)†	16.4 (±3.9)
Dimethylglycine			
Baseline (mmol/mol)	2.8 (±1.1)	0.7 (±0.2)	2.4 (±1.0)
AUC ratio	15.9 (±11.3)	7.1 (±2.5)	2.7 (±0.5)
C_{max} (mmol/mol)	26.7 (±9.7)	3.8 (±0.4)	9.4 (±4.3)

Values are mean (±SEM) for estimates on eight subjects. Treatments were (a) orange juice, (b) apple juice containing added proline betaine, and (c) apple juice control. For AUC ratio, 1.0 = no change relative to fasting baseline
*$P \le 0.001$, **$P \le 0.01$, ***$P \le 0.05$ for treatments compared with control treatment (c, apple juice). †$P < 0.01$ for differences between treatments (a) and (b)

Urinary proline betaine excretion

Urinary PB excretion showed a highly significant interaction between treatment and time ($F = 24.4$, d.f. = 8,55, $P < 0.001$). Urinary PB excretion was significantly raised by both orange juice and PB supplement ($P < 0.001$) at all time points from 2 to 24 h (Fig. 2A). Peak urinary excretion (C_{max}) and the AUC ratio were greater following both treatments ($P < 0.001$) (Table 3). There was no difference in the time taken to reach peak PB excretion following orange juice ($P = 0.705$) or PB supplement alone ($P = 0.176$). When treatments were compared, the increase in urinary excretion of PB was significantly greater following orange juice compared with PB supplement ($P < 0.05$), reaching higher maximal excretion (C_{max} difference, $P = 0.008$).

Urinary glycine betaine and dimethylglycine excretions

Overall, for urinary GB excretion there was a significant interaction between treatment and time

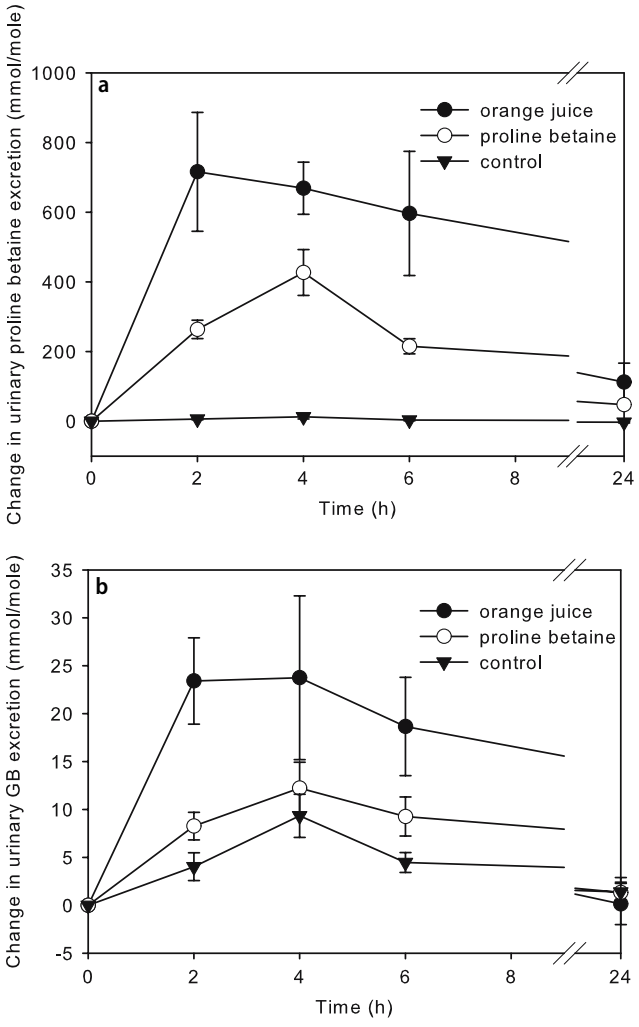

Fig. 2 Change in urinary betaine excretions (mmol/mol creatinine) from baseline following study treatments. Treatments are: orange juice (a) – filled circles, proline betaine in apple juice (b) – open circles, apple juice control (c) – filled inverted triangles. Data plotted are mean and standard error from eight subjects. Upper graph A: changes in proline betaine excretions; both supplement and orange juice significantly greater than control ($P < 0.001$) at all time points. Lower graph B: changes in glycine betaine excretions; compared to control, increase significantly greater ($P < 0.001$) at 2 h following orange juice, $P = 0.059$ at 4 h and 0.017 at 6 h. Orange juice greater ($P = 0.007$) compared with PB supplement at 2 h

($P = 0.037$). The increase in urinary GB excretion was significantly greater following orange juice compared with PB supplement ($P = 0.007$) and control ($P < 0.001$) in the first 2 h post-ingestion (Fig. 2B). The peak urinary excretion was also significantly higher following orange juice compared with both PB supplement alone ($P = 0.007$) and control ($P = 0.006$) (Table 3). After 2 h the trends were weaker (compared with control, $P = 0.059$ at 4 h and 0.017 at 6 h). There were no significant differences in urinary GB excretion following PB supplementation.

The single dose of orange juice resulted in five of the eight subjects having an abnormal GB excretion [14], and four were still abnormal 6 h after the dose.

For DMG, there was no significant interaction between treatment and time ($P = 0.157$), though trends towards greater urinary DMG excretion following orange juice compared with the PB supplement and control were observed ($P = 0.053$ for comparisons of C_{max}).

Discussion

Proline betaine (PB, stachydrine) is an abundant dietary betaine analogue [20, 24]. An acute physiological dose of PB, administered in dietary form or as an oral supplement, had little effect on plasma Hcy concentrations in a group of healthy male subjects, despite significant increases in plasma concentrations of PB. Interestingly, the urinary excretion of PB and GB differed following ingestion of PB in orange juice compared with a PB supplement in apple juice. Previously we have observed that the presence of PB in blood and urine is associated with a markedly increased urinary excretion of GB in both normal subjects [15] and diabetic patients [6]. In rats injected with a supraphysiological dose of PB there was increased excretion of GB and a prolonged elevation of circulating Hcy [21]. The results reported here suggest that the effects of an oral dose of PB, of the order that might occur within a normal human diet, are less dramatic. Whether supplied as orange juice, or dissolved in betaine-free apple juice, PB had little effect on Hcy, with no significant change in plasma concentrations recorded in the 24 h post-ingestion, despite significant increases in plasma concentrations of PB. These elevated PB concentrations were <3% of the plasma concentrations reached in the rat studies, and are of a similar order to the concentrations observed in some non-fasting normal subjects [13, 15]. If anything, PB ingestion may have a small acute effect on circulating GB (not observed in the rat studies [21]), and interestingly, the urinary excretion of PB and GB was greater following ingestion of orange juice compared with a PB supplement in apple juice. Thus although PB affects GB homeostasis, its presence does not explain all of the effects of orange juice. No other betaine analogue was found in the orange juice at concentrations that are likely to be physiologically significant, unless betonicine (4-hydroxyproline betaine), present at about 10% of the concentration of PB, is unexpectedly potent. There is clearly more to be elucidated about biochemically active components of citrus-based foods, and the results reported here leave open the possibility that repeated consumption of

citrus juices may have more significant long term effects on GB and homocysteine homoeostasis.

Circulating GB is probably controlled by the liver enzyme betaine homocysteine methyl transferase (BHMT) which catalyses the conversion of GB and Hcy to methionine and DMG [5, 11]. However, PB has been reported to be neither a substrate nor inhibitor of BHMT [9], though it has previously been claimed to be a poor substrate [17], which would suggest it could be a competitive inhibitor. In this study, PB had little or no effect on either Hcy or DMG, consistent with its having no effect on BHMT. Given that most tissues have higher concentrations of GB (accumulated as an osmolyte) than are found in the circulation, interference with transport is a plausible alternative mechanism for an effect on betaine homeostasis. Although PB is a poor substrate for osmoregulated betaine transport by BGT-1 [19] there are other mammalian betaine transporters [1, 2] that may be inhibited by PB, including those involved in the renal tubular resorption of GB [13]. However, other than the one report on accumulation by MDCK cells [19], that is, by BGT-1, there are no data on the specificity of betaine transporters for PB.

A practical consequence of the effect of PB, and other constituents of citrus foods, is its significance for the methionine load test. This is frequently used as a dynamic function test to assess Hcy metabolism. Most investigators use orange juice as the vehicle through which to administer the dose of L-methionine to patients undergoing this assessment. However, given the significant effect of orange juice on GB excretion, we question the choice of orange juice in the methionine load test. Betaine has a greater effect on post-methionine load Hcy than on fasting Hcy [7], therefore apple juice might be a more suitable choice through which to administer the methionine load. However, the effect of orange juice is minimal and is not sufficient to invalidate previous studies of the effects of methionine loading.

In conclusion, normal dietary loads of PB have little direct effect on plasma Hcy concentrations in healthy humans, but do influence the urinary excretion of GB. Orange juice had a greater effect than could be explained by its PB content. The effect on urinary GB excretion may be significant for patients who are already losing excessive amounts of GB [6, 12, 14], in some cases persistently for years [10]. These patients have an increased risk of vascular disease [12]. In several subjects in this study the urine GB excretion, after treatment with orange juice or PB, exceeded the upper limit of normal (~30 mmol/mol creatinine) after 2 or 4 h, but this was not sustained. Thus when measuring urine GB for diagnostic purposes, PB should also be measured so that abnormal excretion can be distinguished from a fondness for citrus foods and drinks. However, the effect on GB excretion is large enough to suggest that patients who are already losing GB should be cautious about frequently consuming large amounts of citrus fruit or juice. If GB replacement is being considered (whether by supplementation or dietary manipulation) then the increasing the intake of citrus-based foods (and other foods containing proline betaine, e.g. legume seedlings) should be avoided.

Acknowledgments We thank Chris McEntyre for synthesizing the proline betaine and assisting with HPLC analysis, Linda Pike for tHcy analysis, Professor Richard Robson for the use of the Christchurch Clinical Studies Trust facilities and the volunteers for their participation in the study. This study was supported by a grant from the National Heart Foundation of New Zealand, and used equipment funded by the Lottery Grants Board of New Zealand.

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