て相関が低いことについては、立証はできないが、主食に比べて主菜・副菜の摂取量を重要視していないため、記憶が曖昧になっているという可能性が考えられる。これは、思い出しによる目測の汁や特に漬物の相関が低かったことからも推測できる。漬物の目測については、その場での目測および思い出しによる目測の両者共に相関があまり高くなかった。漬物の目測の相関が低いことについては、1人分の盛り付け量が少なく、残量を目測することが難しいからということも要因として推察される。また、漬物のその場での目測について、有意な相関がみられなかったことについてはN数が少ないことが要因の1つであると考えられる。漬物の思い出しによる目測については、有意な相関があるものの相関係数が低いことに関しては、少量のため残量を目測することが難しいことに加えて、前述したとおり、漬物の摂取量を重要視していないため、ほとんど記憶に残っていないということが考えられる。現に、思い出しによる目測の値は「全部食べた ＝10」か「全部食べない ＝0」に偏っているが、実際には様々な摂取量であった。

今回は、異なる施設で、異なる方法での検討であったため、今後は条件を整え、実験的に問題点や妥当性を検討していく必要がある。

E. 結論

今回の結果から、①その場での目測では相関が非常に高いこと、②思い出しによる目測も散布図ではばらつきが大きいものの相関は比較的高かった。ただし今回の結果だけでは、この目測の値を栄養摂取量算出に活用させてよいかは不明である。今後は、この目測値を活用して栄養摂取量を算出することで、個人の栄養摂取量を評価することが可能か、その妥当性を検討することが必要である。

F. 研究発表

1. 発表論文

   なし

2. 学会発表

   なし

G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

   なし

2. 実用新案登録

   なし

3. その他

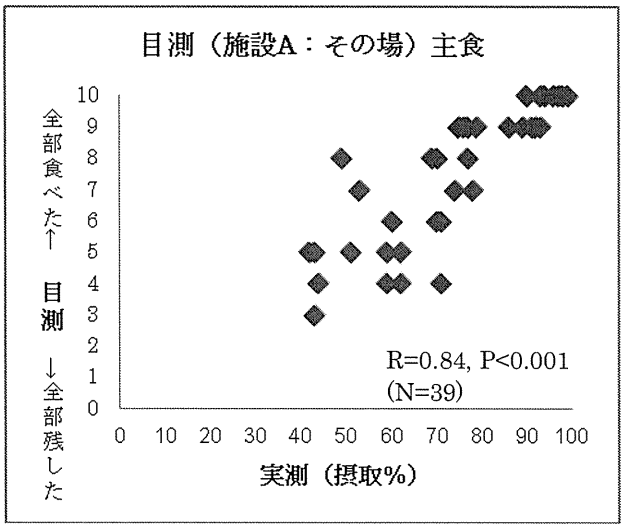
   なし

H. 引用文献

1. 独立行政法人 国立健康・栄養研究所. 日本人の食事摂取基準の改定と活用に資する総合的研究 平成 22 年度 総括・分担研究報告書. (2011).
2. 厚生労働省策定 日本人の食事摂取基準 2010 年版. (2009).

表 1. 料理個数（総数）と残菜状況

	施設 A			施設 B		
	料理総数（n）	残菜があった料理の数（n）	残菜があった料理の割合（%）	料理総数（n）	残菜があった料理の数（n）	残菜があった料理の割合（%）
主食	105	39	37.1	140	40	28.6
主菜	71	20	28.2	132	77	58.3
副菜	165	34	20.6	143	84	58.7
汁	38	15	39.5	144	45	31.3
漬物	27	10	37.4	91	38	41.8
乳製品	35	1	2.9	46	15	32.6
デザート	41	9	22.0	48	6	12.5
間食	0	0	0.0	48	13	27.1



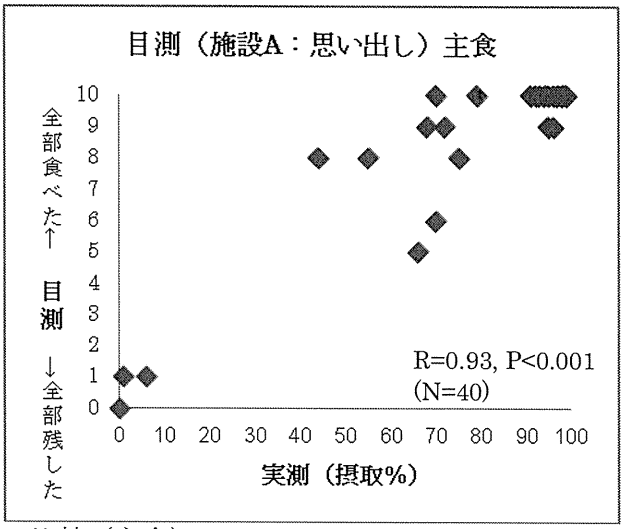
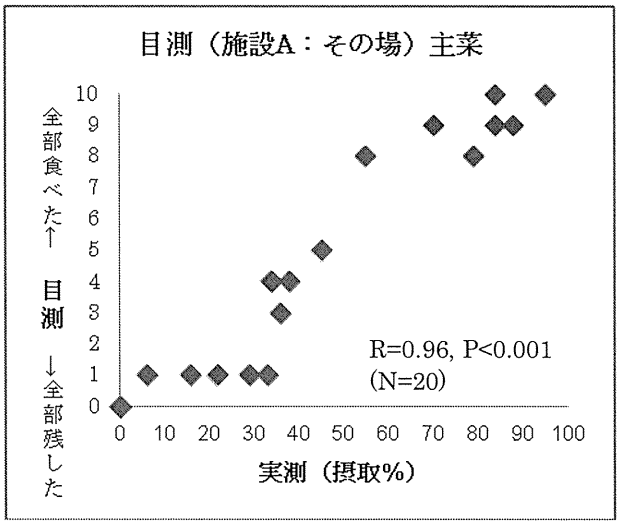


図 1. 目測と実測の比較（主食）



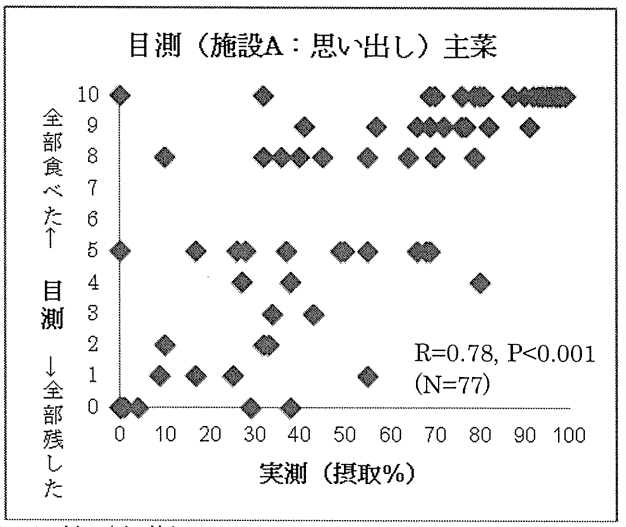
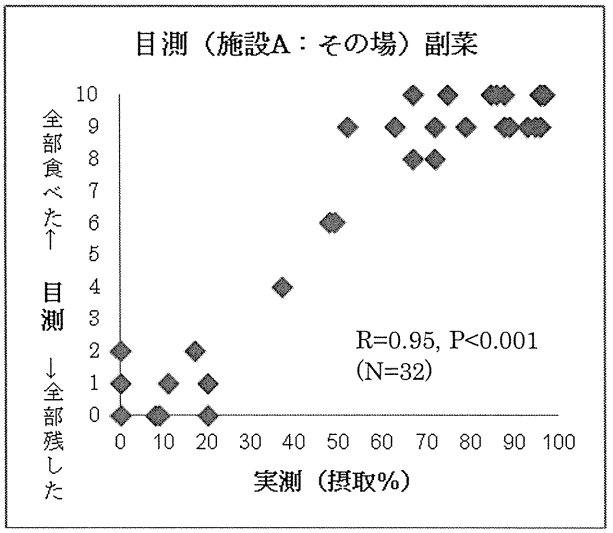


図 2. 目測と実測の比較（主菜）



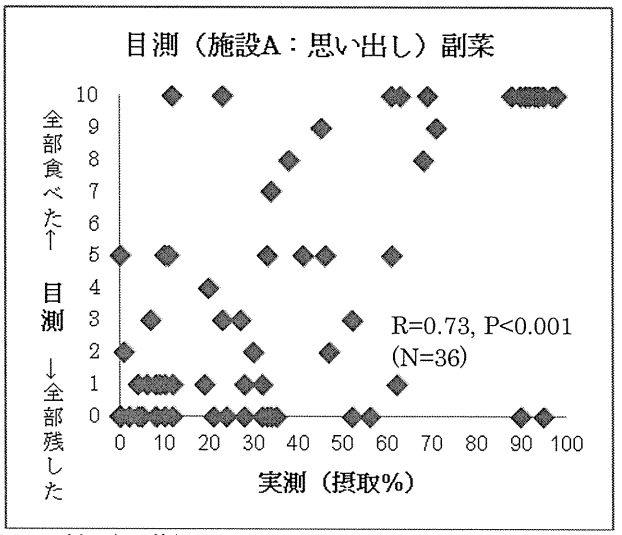


図 3. 目測と実測の比較（副菜）

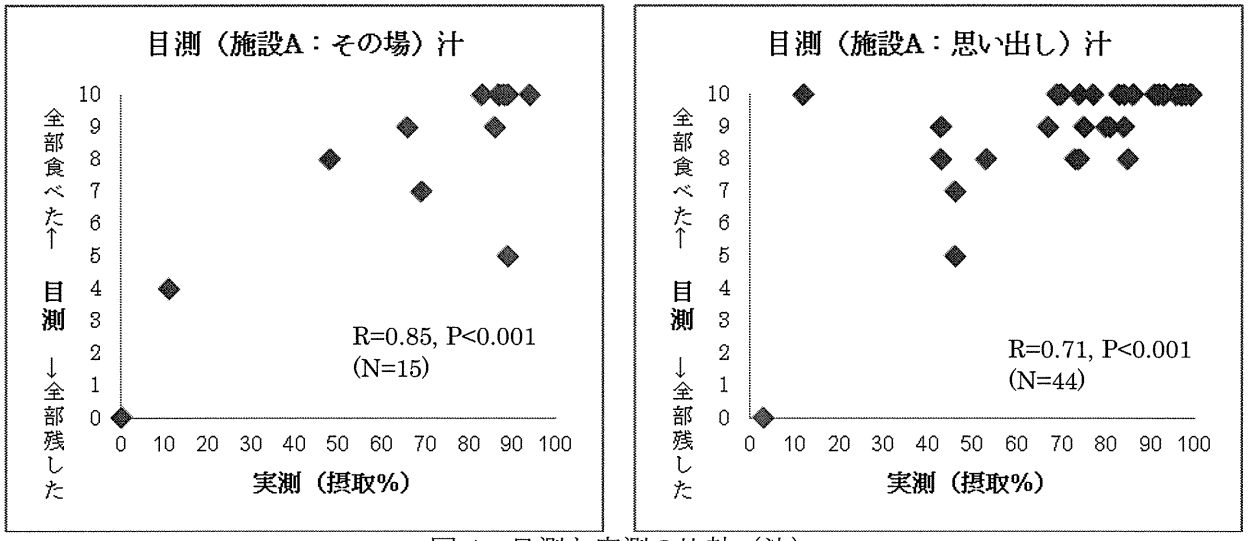


図 4. 目測と実測の比較（汁）

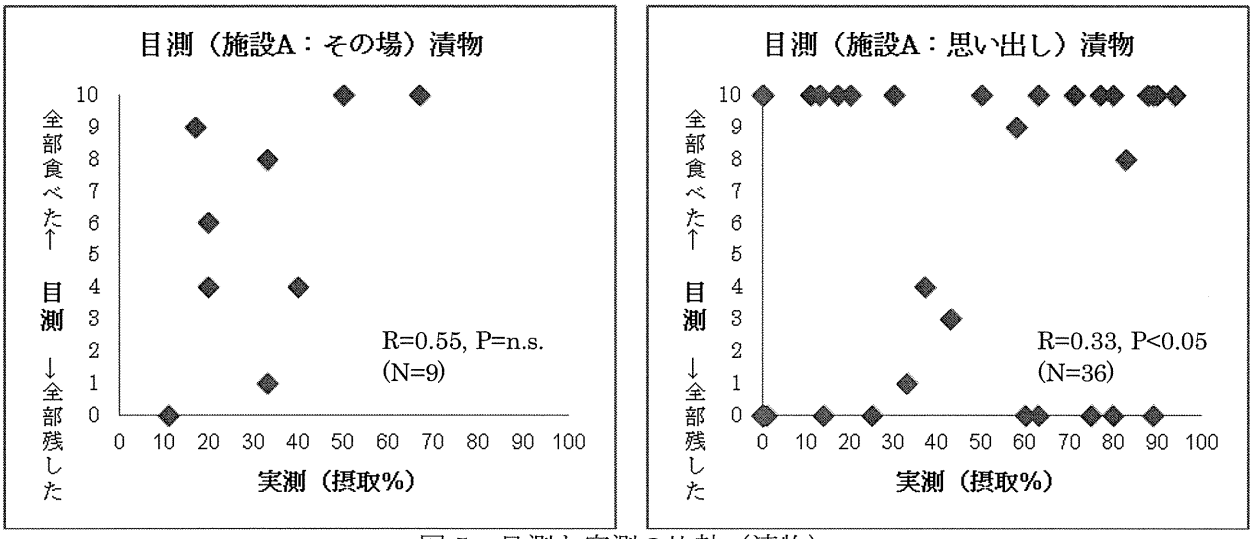


図 5. 目測と実測の比較（漬物）

Ⅳ. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yoh K, Uzawa T, Orito T, Tanaka K.	Improvement of quality of life (QOL) in osteoporotic patients by elcatonin treatment: a trial taking the participants' preference into account.	Japanese Clinical Medicine		In press	2012
Saito K, Yokoyama T, Yoshida H, Kim H, Shimada H, Yoshida Y, Iwasa H, Shimizu Y, Yoshitaka K, Handa S, Maruyama N, Ishigami A, Suzuki T.	A Significant Relationship between Plasma Vitamin C Concentration and Physical Performance among Japanese Elderly Women.	J Gerontol A Biol Sci Med Sci	67	295-301	2012
Ogawa A, Naruse Y, Shigemura Y, Kobayashi Y, Suzuki I, Wada S, Hayamizu K, Kuwahata M, Kido Y.	An evaluation of protein intake for metabolic demands and the quality of dietary protein in rats using an indicator amino acid oxidation method.	J Nutr Sci Vitaminol	57	418-425	2011
Ezaki O.	The optimal dietary fat to carbohydrate ratio to prevent obesity in Japanese population: a review of the epidemiological, physiological and molecular evidence.	J Nutr Sci Vitaminol	57	383-393	2011
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坪田（宇津木）恵.	欧米の循環器疾患予防のための食事ガイドラインの現状.	循環器内科	70	607-614	2011
笠岡（坪山）宜代, 桑木泰子, 瀧沢あす香, 田中律子, 藤生惠子, 斎藤トシ子, 恩田理恵, 山岸博之, 江田節子, 木村祐子, 小谷一子, 小田光子, 田代晶子, 池本真二.	諸外国における栄養士養成のための臨地・校外実習の現状に関する調査研究.	日本栄養士会雑誌	54	556-565	2011
田中清、桑原晶子.	日本人の食事摂取基準における目安量は健康人の摂取の中央値でよいのか？	ビタミン	85	608-609	2011

Ⅴ．研究成果の刊行物・別刷

Journal of Gerontology: MEDICAL SCIENCES
Cite journal as: J Gerontol A Biol Sci Med Sci
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A Significant Relationship between Plasma Vitamin C Concentration and Physical Performance among Japanese Elderly Women

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Background. Maintenance of physical performance could improve the quality of life in old age. Recent studies suggested a beneficial relationship between antioxidant vitamin (eg, vitamin C) intake and physical performance in elderly people. The purpose of this study was to examine the relationship between plasma vitamin C concentration and physical performance among Japanese community-dwelling elderly women.

Methods. This is a cross-sectional study involving elderly females residing in an urban area in Tokyo, Japan, in October 2006. We examined anthropometric measurements, physical performance, lifestyles, and plasma vitamin C concentration of participants.

Results. A total of 655 subjects who did not take supplements were analyzed. The mean age (±standard deviation) of participants was 75.7 ± 4.1 years in this study. The geometric mean (geometric standard deviation) of plasma vitamin C concentration was 8.9 (1.5) μg/mL. The plasma vitamin C concentration was positively correlated with handgrip strength, length of time standing on one leg with eyes open and walking speed, and inversely correlated with body mass index. After adjusting for the confounding factors, the quartile plasma vitamin C level was significantly correlated with the subject's handgrip strength (p for trend = .0004) and ability to stand on one leg with eyes open (p for trend = .049).

Conclusions. In community-dwelling elderly women, the concentration of plasma vitamin C related well to their muscle strength and physical performance.

Key Words: Plasma vitamin C—Physical performance—Elderly women—Japanese.

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PHYSICAL performance and physical ability are the most important indicators of health status in elderly people and are also closely related to the quality of life. Declines in physical performance and physical activity, whether from specific disease, fall, fracture, poor nutrition, or aging itself, are associated with future disability, morbidity, and death (1,2).

In recent years, many studies have examined the roles of diet, protein, and vitamins in physical performance and physical activity(3–5). Several studies have associated low serum albumin concentration with deteriorated muscle strength and function (6,7). Some other studies have examined the relationship between serum vitamin D level and physical performance such as muscle mass, muscle strength, handgrip, walking speed, and functional capacity (8,9). Cesari et al. (3) examined the relationship between antioxidant vitamin intake (vitamin C, vitamin E, β-carotene, and retinol) and physical performance in elderly people and showed significant positive correlations between most antioxidants, especially vitamin C, and higher skeletal muscular strength in this group of people.

There are a number of mechanistic hypotheses about the potential beneficial effects of antioxidant vitamins(10–12). Vitamin C, vitamin E, β-carotene, and retinol are important antioxidants that are not synthesized by humans and, therefore, are mainly supplied via dietary intake. Vitamin C

(ascorbic acid) is a water-soluble antioxidant present in the cytosol and extracellular fluid and can directly react with free radicals such as superoxide ($O_2 \cdot^-$) and hydroxyl radicals ($\cdot OH$) (13,14). Each one of these oxygen-derived intermediates is considered highly reactive because of their unstable electron configurations, which could attract electrons from other molecules, resulting in another free radical that is capable of reacting with yet another molecule. This chain reaction is thought to contribute to lipid peroxidation, DNA damage, and protein degradation during oxidative stress. Oxidative damage is thought to play an important role in the age-related decline of functional activity in human skeletal muscle (15). Concentration of plasma vitamin C, which has potent antioxidant activity, is known to increase after exercise (4).

An increase in the amount of blood vitamin C content has been used as an indicator of increased oxidative reaction (11). Previous studies have examined the effects of vitamin C supplementation on physical performance and exercise (4,11). Although findings from some of the previous studies do not support any beneficial effect of increased antioxidant intake on physical performance, other studies have shown improved recovery from exercise with antioxidant intake and have also shown a preventive role of antioxidant supplementation against oxidative damage. These studies were carried out on athletes after heavy exercise. So far, however, there has been no study examining the relationship between physical performance and blood levels of vitamin C, which may be a more direct marker of the antioxidative ability of the human body.

The present study, to the best of our knowledge, is the first report that examines the relationship between plasma vitamin C concentration and physical performance in Japanese community-dwelling elderly women.

Subjects and Methods

Study Subjects

The present cross-sectional study was carried out as part of a project involving mass health examination of community-dwelling people ("Otasha-kenshin" in Japanese) aged 70 years and older living in Itabashi-ku, Tokyo. "Otasha-kenshin," which literally means "health examination for successful aging," is a comprehensive health examination program for community-dwelling older adults aimed at preventing geriatric syndromes including falls and fractures, incontinence, mild cognitive impairment, depression, and undernutrition (16).

The eligible subjects were all female residents, aged between 70 and 84 years, living in the Itabashi area, an urban part of Itabashi-ku, Tokyo, Japan in October 2006. The population of women belonging to this age range and residing in the Itabashi area was 5937, and they were recruited by invitation through postal mail. Of them, 1,112 women applied for admission and 957 women ultimately participated in this study. The participants who were taking vitamin C supplements (n = 238) were excluded from the primary analyses for examination of the relationship between plasma vitamin C and physical performance because intake of supplements could strongly influence the plasma vitamin C level. Thus, data from 655 subjects were ultimately used for the primary analysis. However, data from the 238 supplement users were also used for subanalysis to determine whether any relationship exists between vitamin C supplementation and physical performance.

All participants were examined at the Tokyo Metropolitan Institute of Gerontology's hall. Physical performance, blood examinations, lifestyle assessments, and anthropometric measurements were performed as described below (9).

The present study was approved by the ethics review committee of the Tokyo Metropolitan Institute of Gerontology. All subjects gave written informed consent.

Anthropometric Measurements

Height and weight of each participant were measured, and body mass index was defined as weight/height^2 (kg/m^2). Body composition measurements (percent body fat) were obtained by segmental bioelectrical impedance using eight tactile electrodes according to the manufacturer's instructions (In Body 3.0; Biospace, Seoul, Korea). Measurements for the triceps surae muscles were taken between the knee and the ankle, at the level of maximum circumference of the medial and anterior calf of the left leg of each participant at sitting position.

Physical Performance

Physical performance was assessed by muscle strength (handgrip strength), balance capability, and usual and maximal walking speeds, without prior practice before the actual measurements. These assessments are routinely conducted for the elderly community as described previously (9). Handgrip strength (kg) was measured once for the dominant hand with the subjects in a standing position using a Smedley's Hand Dynamometer (Yagami, Tokyo, Japan). Grip devices were calibrated with known weights. Subjects held the dynamometer at thigh level and were encouraged to exert the strongest possible force. Balance capability was measured in terms of the length of time standing on one leg, that is, we asked the subjects to look straight ahead at a dot 1 m in front of them and to stand on the preferred leg with their eyes open and hands down alongside the trunk. The time until balance was lost (or maximum 60 seconds) was recorded. We used the better of two trials in the analysis. To determine the walking speed, participants were asked to walk on a flat surface at their "usual and maximum walking speeds." Two marks were used to delineate the start and end of a 5-m path. The start mark was preceded by a 3-m approach to ensure that the participants achieved their pace of usual or maximum before entering the test path. The participants were also instructed to continue walking past the end of the 5-m path for a further 3 m to ensure that their walking pace was maintained

throughout the test path. The time taken to complete the 5-m walk was measured by an investigator and used for analysis. Walking test at maximum speed was repeated twice, and the faster speed was recorded for the test.

All physical performance tests were performed between 9 AM and 4 PM during the day. We have no data on the reproducibility of the measurements. To reduce interexaminer variation, each test was conducted by the same staff member specifically trained for this study.

Blood Examinations

Blood samples (nonfasting) were collected from the subjects between 9 am and 4 pm during the day. There was no difference in mean plasma vitamin C concentration with regard to the time of collection (data not shown). Venous blood samples were drawn into Ethylene diamine tetraacetic acid tubes. Plasma was then obtained by centrifugation at 3,000 rpm for 15 min at 4°C and subsequently used for biochemical assays. Plasma was treated with Ethylene diamine tetraacetic acid to prevent the spontaneous vitamin C degradation. Next, 100 μl of the plasma was dispensed into storage tubes, to which 450 μl of 3% metaphosphoric acid solution was added, and the mixture was stored at −80°C until further use. Vitamin C concentration was determined by an High performance liquid chromatography-electrochemical detection–based method (17). The analysis was carried out centrally in our laboratory. Serum albumin concentration was measured by the Bromocresol Green method (Special Reference Laboratories Inc., Tokyo, Japan). The coefficient of variation for serum albumin found using this method was less than 1% (9).

Lifestyle Assessment

Information regarding the participants' general health (such as medical history, smoking habits, alcohol drinking habits, regular exercise habits, vegetable intake, fruit intake and use of vitamin C supplement) was collected by interview, and history of medical conditions including hypertension, stroke, heart attack, diabetes mellitus, and hyperlipidemia was self-reported.

Alcohol drinking habits of the subjects were classified as nondrinker, current drinker, or ex-drinker. Smoking habits of the subjects were classified using three categories: never smokers, current smokers, and ex-smokers. The frequency of vegetable and fruit intake was asked using four categories: almost every day, once every two days, once or twice per week, and almost never. Subsequently, for analysis, the categories were summarized as almost every day and others.

Statistical Analysis

Data were summarized as mean and standard deviation or percentage values. The data of plasma vitamin C concentration was logarithmically transformed to approximate a normal distribution and was summarized as the geometric mean and geometric standard deviation.

Table 1. Characteristics of Study Subjects ($N = 655$)

Characteristic	Mean (*SD*)
Age (y)	75.7 (4.1)
Height (cm)	149.1 (5.7)
Weight (kg)	51.0 (8.3)
Body mass index (kg/m^2)	22.9 (3.4)
Triceps surae muscle (cm)	33.1 (2.8)
Plasma vitamin C (μg/ml)*	8.9 (1.5)
Serum albumin (mg/dL)	4.3 (0.2)
Body composition	
Percent body fat (%)	32.2 (7.0)
Physical performance tests	
Handgrip strength (kg)	18.7 (4.4)
One leg standing with eyes open (s)	35.2 (23.5)
Usual walking speed (m/s)	1.2 (0.3)
Maximal walking speed (m/s)	1.8 (0.4)
	%
Medical history	
Hypertension	50.7
Stroke	6.6
Heart attack	21.2
Diabetes mellitus	9.0
Hyperlipidemia	34.7
Alcohol drinking habit	
Current	25.3
Former	5.0
Never	69.6
Smoking habit	
Current	3.7
Former	5.7
Never	90.7
Regular exercise habit	
Yes	69.2
No	30.8
Vegetable intake	
Everyday	84.2
Others†	15.8
Fruit intake	
Everyday	81.8
Others†	18.2

Notes: Data of vitamin C supplement users were excluded.
*The geometric mean and geometric *SD*.
†Including participants taking vegetables/fruits not everyday or almost never.

The age-adjusted Pearson's correlation coefficient between the plasma vitamin C concentration and other factors were calculated. The least square means and SEs adjusted for potential confounders were calculated and compared between categories by analysis of covariance. To examine the relationship between plasma vitamin C concentration and physical performance, statistical adjustment was done by analysis of covariance for variables (except for other physical performance variables) that were correlated to plasma vitamin C concentration with $p < .20$. The same analyses were repeated for the 238 users of vitamin C supplement. All statistical analyses were performed using the SAS (version 9.0; SAS Institute Inc., NC).

RESULTS

Table 1 summarizes the basic characteristics of the subjects. As shown, the mean age (±standard deviation) of the

Table 2. Correlation between Plasma Vitamin C Concentration and Selected Factors ($N = 655$)

Factor	Correlation*	
	r	p
Age	−0.004	.91
Height	0.04	.27
Weight	−0.05	.19
Body mass index	−0.08	.054
Triceps surae muscle	0.001	.98
Serum albumin	−0.04	.33
Percent body fat	−0.12	.002
Handgrip strength	0.16	<.001
One leg standing with eyes open	0.15	<.001
Usual walking speed	0.14	<.001
Maximal walking speed	0.09	.036

Notes: Number of subjects is slightly different for the selected factors because of missing values.

*Age-adjusted Pearson's correlation coefficient between logarithm of vitamin C concentration and each factor.

subjects was 75.7 ± 4.1 years. The geometric mean (geometric standard deviation) of plasma vitamin C concentration was 8.9 (1.5) μg/mL. The prevalence of women eating vegetables everyday was 84.2% and those eating fruits everyday was 81.8%.

The age-adjusted geometric mean of plasma vitamin C concentration was significantly lower in subjects who had a medical history of hypertension (8.53 vs 9.22, $p = .0015$) and diabetes mellitus (7.59 vs 9.00, $p = .002$) as compared with those who did not. A history of stroke, heart attack, or hyperlipidemia was not associated with plasma vitamin C concentration. Subjects who took fruits every day had a significantly higher concentration of vitamin C than those who did not (9.14 vs 7.78, $p < .0001$). Vegetable intake, alcohol drinking habit and smoking habit were not related to plasma vitamin C concentration (not shown in table).

Table 2 shows the age-adjusted correlations between the plasma vitamin C concentration and selected factors. As shown, the plasma vitamin C concentration was positively but modestly correlated with handgrip strength, length of time standing on one leg with eyes open, as well as usual walking speed and maximal walking speed, and modestly inversely correlated with body mass index and percent body fat of the subjects.

Table 3 shows the relationship between plasma vitamin C concentration and each physical performance after adjusting for confounding factors. Results obtained after the adjustment for potential confounders confirmed that the plasma vitamin C concentration was correlated with the handgrip strength independently from the other factors (eg, p for trend = .0004 after adjusting for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake; Table 3). There was also a significant relationship between the plasma vitamin C level and the subject's length of time standing on one leg with eyes open after adjustments for age, body mass index, percent body fat, hypertension, diabetes mellitus, and fruit intake (Table 3; p for trend = .049). We did not observe any significant association between the plasma vitamin C level and the usual or the maximal walking speed of the subjects.

A subanalysis using data from the 238 vitamin C supplement users showed almost null relationship between handgrip strength and plasma vitamin C concentration (data not shown).

Discussion

A previous study has shown an association between higher daily dietary intake of vitamin C and skeletal muscle strength in elderly people (3). Results described in the present study indicated that plasma vitamin C concentration was positively related with muscle and physical performance in community-dwelling elderly women. To the best of our knowledge, this is the first study showing a significant

Table 3. Relationship between Plasma Vitamin C Concentration and Physical Performance Adjusted for Potential Confounder

Physical performance	Quartile of plasma vitamin C level				
	Q1	Q2	Q3	Q4	
	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	Mean ± *SE*	p for trend
Handgrip strength (kg), *N*	154	159	154	152	
Age adjusted	17.70 ± 0.34	18.75 ± 0.33	18.75 ± 0.34	19.60 ± 0.34	.0001
Multivariate adjusted*	17.83 ± 0.34	18.83 ± 0.32	18.89 ± 0.33	19.60 ± 0.33	.0004
One leg standing with eyes open† (s), *N*	162	163	164	161	
Age adjusted	31.44 ± 1.71	33.98 ± 1.70	37.70 ± 1.70	37.83 ± 1.71	.003
Multivariate adjusted*	33.39 ± 1.74	34.08 ± 1.67	37.63 ± 1.67	37.50 ± 1.70	.049
Usual walking speed (m/s), *N*	146	154	145	147	
Age adjusted	1.13 ± 0.02	1.19 ± 0.02	1.23 ± 0.02	1.21 ± 0.02	.008
Multivariate adjusted*	1.18 ± 0.02	1.19 ± 0.02	1.22 ± 0.02	1.21 ± 0.02	.23
Maximal walking speed (m/s), *N*	146	154	154	147	
Age adjusted	1.70 ± 0.03	1.76 ± 0.03	1.82 ± 0.03	1.76 ± 0.03	.15
Multivariate adjusted*	1.76 ± 0.03	1.77 ± 0.03	1.80 ± 0.03	1.75 ± 0.03	.94

Notes: Values are least squares mean and *SE* adjusted for the factors by analysis of covariance. Q1–Q4: first to fourth quartile groups of plasma vitamin C concentration, respectively.

*Adjusted for age, body mass index, percent body fat, hypertension, diabetes mellitus and **fruit intake**.

†Length of time standing on one leg with eyes open.

correlation between plasma vitamin C concentration and handgrip strength and ability to stand on one leg with eyes open. We, however, were unable to find any relationship between skeletal muscle mass and plasma vitamin C concentration. Handgrip strength has been found to correlate well with the strength of other muscle groups and is thus a good indicator of overall strength (18). Consistent with this idea, handgrip strength was found to be a strong and consistent predictor of all-cause mortality and morbidity of Activities of Daily Living in middle-aged people (19). The handgrip test is considered an easy and inexpensive screening tool to identify elderly people at risk of disability. Handgrip strength, an indicator of overall muscle strength, is thought to predict mortality through mechanisms other than underlying disease that could cause muscle impairment (18,19). The one leg standing test is one of the balance tests (20). The test is a clinical tool to assess postural steadiness in a static position by quantitative measurement. Many studies have shown that the decreased one leg standing time is associated with declines in Activities of Daily Living and increases in other morbidities including osteoporosis and fall (20).

Our findings suggest that vitamin C may play an important role in maintaining physical performance and thereby may help to improve healthy life expectancy in the elderly. However, the usual and maximal walking speeds did not relate to plasma vitamin C concentration. Walking speed test may be an efficient tool in screening older persons with higher risk of mortality and may easily identify high-risk groups in the community (21). Walking is a rhythmic, dynamic, and aerobic activity of the large skeletal muscles that confers multifarious benefits with minimal adverse effects. Muscles of the legs, limbs, and lower trunk are strengthened, and the flexibility of their joints are preserved (22). One of the reasons why walking speed was not related to vitamin C concentration may be because walking requires coordinated movements of arms, legs, and many parts of the body rather than a simple muscle and balance function. Previous reports showed that walking balance function did not correlate with standing balance function (23). Although we did not find any clear association between walking and plasma vitamin C concentration in this study, vitamin C may still have effects on relatively simple strength and balance functions.

One of the possible explanations for the observed relationship between vitamin C and physical performance, especially handgrip strength and the ability to stand on one leg with eyes open, may be the potential protective effects of the antioxidant vitamins against muscle damage (4,11). Vitamin C is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not in humans (12). Vitamin C is an antioxidant because, by donating its electrons, it prevents other compounds from being oxidized (12). Thus, vitamin C readily scavenges reactive oxygen and nitrogen species, thereby effectively protects other substrates from oxidative damage (10,24). Although habitual exercise reduces systemic inflammation and oxidative stress as the production of endogenous antioxidants are enhanced, acute exercise increases the generation of oxygen-free radicals and lipid peroxidation (4,25). Strenuous physical performance can increase oxygen consumption by 10- to 15-folds over the resting state to meet the energy demands and results in muscle injury (26). Prolonged submaximal exercise was shown to increase the amount of both whole-body and skeletal muscle lipid peroxidation by-products; in the case of the former, the increase was indicated by greater exhalation of pentane but not of ethane (4,27,28). Supplementation with vitamin C was shown to decrease the exercise-induced increase in the rate of lipid peroxidation (27,28). Several studies suggested that oxidative damage may play a crucial role in the decline of functional activity in human skeletal muscle with normal aging (15). Consistent with this idea, several studies showed significantly lower plasma vitamin C level in the elderly population than in the younger adult population (29–31). Because the plasma vitamin C levels in these apparently healthy elderly persons rose markedly after an oral dose of vitamin C, their initially low plasma levels can be attributed to the low intake rather than to an age-related physiological defect.

In fact, the relationship between handgrip strength and plasma vitamin C concentration was significantly different between supplement users and nonusers, that is, an almost null relationship in the former and a positive relationship in the latter (data not shown). This finding suggested that vitamin C supplementation did not have any beneficial effect on the physical performance and muscle strength despite the increased plasma level of vitamin C. A number of studies reported that vitamin C supplement users had significantly higher blood vitamin C concentration than non-users (29, 32, 33). Several studies have examined the effects of exercise on changes in the serum vitamin C concentration (34–36). Some other experimental studies have shown that vitamin C supplementation can reduce symptoms or indicators of exercise-induced oxidative stress (37–40). However, the results regarding vitamin C supplementation are equivocal, and most well-controlled intervention studies report no beneficial effect of vitamin C supplementation on either endurance or strength performance (41,42). Likewise, vitamin C restriction studies showed that a marginal vitamin C deficiency did not affect the physical performance (43). Although evidence from a number of studies show that vitamin C is a powerful antioxidant in biological systems in vitro, its antioxidant role in humans has not been supported by currently available clinical studies.

Vitamin C is especially plentiful in fresh fruits and vegetables. Plasma vitamin C concentration may be merely a marker for intake of other nutrients that are abundant in fruits and vegetables. However, the statistical adjustment for fruit intake did not attenuate the relationship between plasma vitamin C and physical performance (Table 3), suggesting that vitamin C did have some beneficial effects

independently of other nutrients. A number of biochemical, clinical, and observational epidemiologic studies have indicated that diets rich in fruits, vegetables, and vitamin C may be of benefit for the prevention of chronic diseases such as cardiovascular disease and cancer (44,45). Several cohort studies have examined associations between plasma vitamin C concentration and mortality from stroke or coronary heart disease (30,46,47). The effects of vitamin C supplementation are, however, still unclear. A pooled study suggested reduced incidences of coronary heart disease events with higher intake of vitamin C supplement (48), while another study showed that a high intake of vitamin C supplement is associated with an increased risk of mortality due to cardiovascular diseases in postmenopausal women with diabetes (49). A randomized placebo controlled 5-year trial, however, did not show any significant reduction in the mortality from, or incidence of, any type of vascular disease or cancer (50). These studies, in fact, have failed to demonstrate any benefit from such supplementation.

There are a number of potential weaknesses in our study that should be mentioned here. The subjects used in this study were not selected randomly from the study population, and they may be relatively healthy elderly women who were able to come to the health examination hall from their homes. A previous study assessed the correlation of antioxidants with physical performance and muscular strength (3) and demonstrated that a higher daily intake of vitamin C and carotene associated with skeletal muscle strength. However, we have no data regarding the presence of other dietary antioxidants in blood such as vitamin E, retinol, and carotene. In our questionnaire, participants were asked to respond "Yes" or "No" to whether they took supplements, and not about the frequency and quantity of intake of the supplements. Thus, we were unable to examine the reason why plasma vitamin C was not related to the handgrip strength in the supplement users by considering the dose of vitamin C they took.

This study was a cross-sectional study and, therefore, does not provide cause/effect relationships, although we demonstrated a significant correlation between physical performance and concentration of plasma vitamin C. Therefore, longitudinal follow-up studies and controlled clinical trials are necessary to confirm the role of plasma vitamin C and physical performance of the elderly women. These limitations should be considered in future studies.

In conclusion, we found a strong correlation of a higher plasma vitamin C concentration with handgrip strength and one leg standing time in community-dwelling elderly women. Although the elderly are prone to vitamin C deficiency, and they appear to have a higher dietary requirement for vitamin C, the beneficial effects of vitamin C supplementation to maintain physical performance in elderly people are equivocal and thus, need further in-depth studies.

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An Evaluation of Protein Intake for Metabolic Demands and the Quality of Dietary Protein in Rats Using an Indicator Amino Acid Oxidation Method

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Summary Currently, protein requirements are generally determined based on nitrogen balance studies, but there are a variety of limitations associated with this method. The indicator amino acid oxidation (IAAO) method, with a theoretical base that differs widely from the nitrogen balance method, was developed as an alternative method for humans. The objective of the present study was to evaluate protein intakes for metabolic demands and protein quality, using protein itself, in rats employing the IAAO technique with L-[1-^{13}C]phenylalanine. Male Wistar/ST rats (5–6 wk old) received a graded casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%), or a wheat gluten (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet, along with L-[1-^{13}C]phenylalanine. An isotopic plateau in breath was achieved 210 min after the start of the ^{13}C ingestion. The protein intakes for metabolic demands were calculated by applying a mixed-effect change-point regression model to breath $^{13}CO_2$ data, which identified a breakpoint at minimal breath $^{13}CO_2$ in response to graded protein intake. The protein intakes for metabolic demands determined by the IAAO method were 13.1 g/kg BW/d for casein and 18.1 g/kg BW/d for wheat gluten, showing a tendency similar to that determined by the nitrogen balance method. These results demonstrated that the IAAO method could be employed to evaluate not only the protein intakes for metabolic demands, but the dietary protein quality in freely living rats, suggesting that this method might be viable in a clinical setting.

Key Words protein metabolic demand, protein quality, indicator amino acid oxidation, rats

The nitrogen balance method is normally employed to determine protein requirements, as specified in the 2007 WHO/FAO/UNU (*1*). However, the limitations of the nitrogen balance method, which can result in considerable error in the prediction of balance (*2*, *3*), have been well described (*4–6*). In the nitrogen balance method, after the diet has been changed, a period of time is usually allowed for adaptation to be complete during the first 5–7 d (*7*). Therefore, employing the nitrogen balance method, the metabolic demand for protein cannot be assessed in patients with a widely varying metabolic demand. The indicator amino acid oxidation (IAAO) method was originally employed to study amino acid requirements in pigs (*8*), and thereafter it has been widely used for studies on pigs (*9–11*) and humans (*12–17*). Since the IAAO method does not require prior dietary adaptation (*18*) to each of the varying protein intake levels, it could be available when an assessment of the metabolic demand for protein is required for post-operative patients or patients with injuries or infections.

In 2007, Humayun et al. (*19*) applied the IAAO method and conducted a reevaluation study on the protein requirements in healthy young men by feeding the subjects graded protein intake as a crystalline amino acid mixture and measuring changes in the oxidation of orally administered L-[1-^{13}C]phenylalanine. However, no studies have previously been conducted on determining the protein requirement using protein itself in animals or humans employing the IAAO method. Therefore, sufficient evidence has not been gathered showing that the IAAO method is viable for measurements of the protein requirement, and it has not been sufficiently validated in studies employing experimental rats up to the present. We should consider that the mechanism of the assimilation of the amino acid mixture differed from that of the protein. Amino acid mix-

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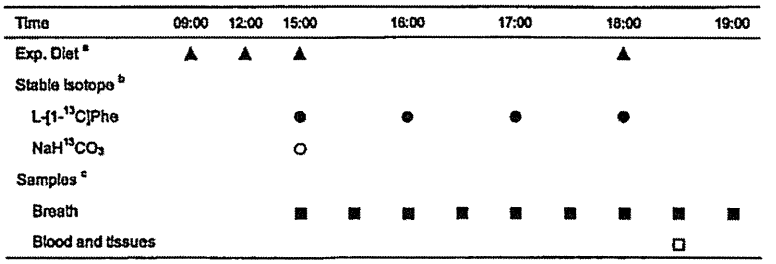

Fig. 1. The protocols employed for each IAAO study day. [a]The experimental diet was either a 4.3% or 17.2% casein diet. The diet was provided every 3 h (9:00–18:00). Each meal represented one-eighth of each rat's daily intake. [b]Isotope: Priming doses of L-[1-^{13}C]phenylalanine and $NaH^{13}CO_3$ were started with the third meal at 15:00, and the infusion of L-[1-^{13}C]phenylalanine was continued hourly until the end of the study. [c]Sample collection: Baseline breath sample was collected before the isotope protocol began. Nine breath samples were collected every 30 min after the initiation of the isotope protocol. Samples of blood, liver, and gastrocnemius muscle were collected at 18:30.

tures will be absorbed very rapidly, and protein utilization will show a higher efficiency, compared with slow proteins such as casein (*20*). Incidentally, a previous study by Moehn et al. (*21*) evaluated the metabolic availability of amino acids in peas, and they indicated the applicability of using IAAO for intact protein sources.

Measurements of the quality and quantity of the dietary protein employed can be used to facilitate adjustments to the diet to ensure that the metabolic demands for protein can be met sufficiently. Poor protein quality compromises the nutritional status and increases the protein requirement. In the 1991 FAO/WHO/UNU report (*22*), the protein digestibility corrected amino acid score (PDCAAS) value for casein is 1.00, compared with 0.25 for wheat gluten. Therefore, the protein requirement calculated for rats fed a wheat gluten diet is higher than that for rats fed a casein diet. In a clinical setting, the adequate quality and quantity of protein or amino acid for each disease might be estimated using the IAAO method.

The objective of the present study was to establish whether or not the IAAO method is viable for determining the metabolic demand for protein and to evaluate protein quality using protein itself, employing casein and wheat gluten as protein sources in experimental diets and using the IAAO method with L-[1-^{13}C]phenylalanine.

MATERIALS AND METHODS

Animals. This study was performed in accordance with the guidelines for animal experimentation at Kyoto Prefectural University, Japan. Male Wistar/ST rats (4 wk old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The rats were housed in individual mesh cages under controlled temperature (22±2°C) and lighting (lights on from 08:00 to 20:00) conditions. The rats were given free access to water and a 17.2% casein maintenance diet, and they were allowed to adapt to the laboratory environment for at least 1 wk before starting the experiment. After adaptation, 5- to 6-wk-old rats (initial BW=130.1±2.3 g) were used for the experiment. The amount of feed available and any feed not eaten were recorded for each rat for 3 d before the first study day, and the total daily intake for each rat, equivalent to the 24-h dietary intake, was calculated on the basis of the average intake during the previous 3 d.

Experiment 1. The objective of Experiment 1 was to examine the effect of L-[1-^{13}C]phenylalanine administration on breath $^{13}CO_2$ enrichment, and to evaluate whether the protein metabolism could be measured by the IAAO method in rats consuming different protein level diets. All of the eight rats were included in two IAAO studies, consuming both 4.3% and 17.2% casein diets (N×6.38) (*23*) with a time period of more than 2 d between the studies. The 17.2% casein maintenance diet employed for all of the studies was provided for at least 24 h. Then, the rats fasted overnight for 13 h from 20:00 on the day before the study day, but had free access to drinking water. The study protocol for all of the IAAO studies is depicted in Fig. 1. On the study day, the rats were weighed in the morning before feeding. Then, they received either 4.3% or 17.2% casein diets (Table 1). The study-day diet was provided in 4 isoenergetic, isonitrogenous diets, and each meal accounted for one-eighth of the rat's total daily intake. Specifically, the casein diet was consumed beginning at 09:00 and continued at each 3-h interval until 18:00 for a total of 4 meals. The rats were allowed free access to drinking water during the experiment period. The rats were fed the remaining half of the daily ration in the evening. The tracer protocol was started with the third meal at 15:00 to measure the phenylalanine kinetics with the use of L-[1-^{13}C]phenylalanine, and continued hourly until 18:00. The rats were placed in the chamber immediately after the oral administration of the ^{13}C substance. Breath samples were collected, and the $^{13}CO_2$ level in breath CO_2 was measured at 30-min intervals from 15:00 to 19:00. Baseline breath samples were collected before the isotope protocol began at 15:00. On a later day, the rats were dissected at 18:30; blood, liver and gastrocnemius muscle samples were collected for subsequent analysis of amino acid concentration in plasma and tissues.

Experiment 2. The protein intake for metabolic demands was measured using the IAAO method for rats fed the casein diets, and also for rats fed diets based on

Table 1. Composition of experimental diets.

Protein	Casein diet						Wheat gluten diet					
	4.3%	8.6%	12.9%	17.2%	21.5%	25.8%	7.2%	10.8%	14.4%	18.0%	21.6%	25.2%
	g/kg diet						g/kg diet					
Casein[1,2]	50	100	150	200	250	300	—	—	—	—	—	—
Wheat gluten[3,4]	—	—	—	—	—	—	100	150	200	250	300	350
Cornstarch[1]	557	523	490	457	423	390	527	498	470	440	411	383
Sucrose[1]	278	262	245	228	212	195	265	250	235	221	206	190
Rapeseed oil[5]	35	35	35	35	35	35	31	27	22	18	14	9
Soy bean oil[6]	15	15	15	15	15	15	12	10	8	6	4	3
Vitamins[1,7]	10	10	10	10	10	10	10	10	10	10	10	10
Minerals[1,8]	35	35	35	35	35	35	35	35	35	35	35	35
Cellulose[1]	20	20	20	20	20	20	20	20	20	20	20	20
L-Phenylalanine[9]	11	9	7	5	2	—	9	7	5	3	1	—
L-Tyrosine[10]	13	10	8	5	3	—	13	11	10	9	8	6
Energy (kJ/g)	15.4	15.4	15.5	15.5	15.5	15.6	15.5	15.5	15.5	15.5	15.6	15.6

[1] Oriental Yeast Co., Ltd., Japan.

[2] Protein, 86.2% (N×6.38). Amino acid (mg/100 g Casein): L-alanine, 2,700; L-arginine, 3,300; L-aspartic acid, 6,300; L-cysteine, 430; L-glutamic acid, 19,000; L-glycine, 1,600; L-histidine, 2,700; L-isoleucine, 4,900; L-leucine, 8,400; L-lysine, 7,100; L-methionine, 2,600; L-phenylalanine, 4,500; L-proline, 10,000; L-serine, 4,600; L-threonine, 3,700; L-tryptophan, 1,100; L-tyrosine, 5,000; L-valine, 6,000; total, 93,930.

[3] Weston Bioproducts Ltd., Queens land, Australia.

[4] Protein, 72.0% (N×5.70). Amino acid (mg/100 g wheat gluten): L-alanine, 2,100; L-arginine, 2,700; L-aspartic acid, 2,700; L-cysteine, 1,600; L-glutamic acid, 29,000; L-glycine, 2,700; L-histidine, 1,800; L-isoleucine, 3,000; L-leucine, 5,400; L-lysine, 1,400; L-methionine, 1,300; L-phenylalanine, 4,100; L-proline, 11,000; L-serine, 3,600; L-threonine, 2,000; L-tryptophan, 780; L-tyrosine, 2,500; L-valine, 3,300; total, 80,980.

[5] Nisshin Oillio Ltd., Japan.

[6] Wako Pure Chemical Industries, Ltd., Japan.

[7] AIN-76™ vitamin mixture (per g mixture): vitamin A, 400 IU; vitamin D_3, 100 IU; vitamin E, 5 mg; vitamin K_3, 0.005 mg; vitamin B_1, 0.6 mg; vitamin B_2, 0.6 mg; vitamin B_6, 0.7 mg; vitamin B_{12}, 0.001 mg; D-biotin, 0.02 mg; folic acid, 0.2 mg; calcium pantothenate, 1.6 mg; nicotinic acid, 3 mg; choline chloride, 200 mg; sucrose, 0.968 g.

[8] AIN-76™ mineral mixture (g/kg mixture): calcium phosphate dibasic, 500.0; sodium chloride, 74.0; potassium citrate, 220.0; potassium sulfate, 52.0; magnesium oxide, 24.0; manganese carbonate, 3.5; ferric citrate, 6.0; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.0066; chromium potassium sulfate, 0.55; sucrose, 118.03.

[9] L-Phenylalanine content was kept constant at 13,500 mg/kg diet in all diets, except the 25.2% wheat gluten diet (14,350 mg/kg diet).

[10] L-Tyrosine content was kept constant at 15,000 mg/kg diet in all diets.

wheat gluten instead of casein to determine whether it was important to consider the effects of the source of the protein in the diet. Sixteen rats were used, and even when they were measured for the wheat gluten diets, the 17.2% casein diet was provided as a maintenance diet for the 2 d before the study day for all of the IAAO studies. On the study day, eight rats received, in random order without repeats, one of six levels of the casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%) diet (N×6.38) (*23*), and the other eight rats received one of six levels of the wheat gluten (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet (N×5.70) (*23*). The tracer protocol employed was the same as that employed in Experiment 1, and ^{13}C substance administration was performed for a total of four times at 15:00, 16:00, 17:00, and 18:00. However, breath samples were collected and the $^{13}CO_2$ level in the breath was measured only twice at 15:00 and 18:30. The experimental design was a completely randomized crossover design. Eight rats consumed the casein diet at all six levels, and the other eight rats consumed the wheat gluten diet at all six levels. Each IAAO study day was separated by 2 d, and the six IAAO studies were completed within 2 wk. Except for these points, all of the protocols were the same as those employed in Experiment 1.

Tracer administration protocol. L-[1-^{13}C]Phenylalanine (Cambridge Isotope Laboratories, Andover, MA) and $NaH^{13}CO_3$ (Cambridge Isotope Laboratories) were used as tracers. Labeled compounds were dissolved in saline and stored at 4°C. Isotopic solutions were prepared and administered in a volume of 2.5 mL/kg BW. Oral priming doses of 0.88 mg/kg BW $NaH^{13}CO_3$ and 7.92 mg/kg BW $NaHCO_3$ were given with the third meal at 15:00. An oral dosing protocol of 3.3 mg/kg BW L-[1-^{13}C]phenylalanine and 29.7 mg/kg BW phenylalanine was commenced simultaneously with the third meal, and administration of 6.0 mg/kg BW L-[1-^{13}C]phenylalanine and 54.0 mg/kg BW phenylalanine

was performed hourly until the end of the study.

Experimental diets. The composition and source of the powdery experimental casein and wheat gluten diets are shown in Table 1. Casein and wheat gluten provided the sole source of protein in the casein and wheat gluten diets, respectively. The compositions of the amino acids in the casein and wheat gluten are shown in the footnote to Table 1 (*23*). L-Phenylalanine and L-tyrosine were added to the diets to achieve an equal content of these amino acids in all diets. In the present study, L-phenylalanine (13.5 g/kg diet) and L-tyrosine (15.0 g/kg diet) were consumed in excess of these amino acid requirements for rodents (L-phenylalanine, 8.8 g/kg diet; L-tyrosine, 9.3 g/kg diet) (*24*), in order to minimize the net hydroxylation of phenylalanine to tyrosine. Each casein diet with varying protein content was kept at an identical energy level by varying the levels of sugar and starch. The oil levels in the wheat gluten diet were decreased because the energy level of wheat gluten is higher than those of casein. Thus, all of the diets had a similar energy level (15.4–15.6 kJ/g).

Breath sample collection and analysis. The instruments used for the collection of breath samples in the rats consisted of an acrylic chamber (10.6 L) fitted with a drinker, an aspiration pump (Columbus Instruments, Columbus, OH) and an air flow meter (Columbus Instruments). The chambers were continuously charged with fresh room air through the aspiration tube by a pump. The rats were moved outside the chamber for the administration of the ^{13}C substance, and thereafter moved back into the same chamber. Because the chambers filled with expired air were necessary in order to collect the breath samples, rats were placed in separate compartments for 30 min before the collection of the breath samples.

Breath samples of 200 mL volume drawn into a 200 mL syringe were injected into breath-sampling bags (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The $^{13}CO_2$ concentration in the expired air was measured by attaching the breath-sampling bags to the sampling joint of an infrared spectrometer (POCone; Otsuka Electronics Co., Ltd., Tokyo, Japan). Using the measurement system provided by POCone, the concentration of CO_2 in the aspirated air in the breath sampling bags was at least more than 0.5%. Therefore fresh room air was drawn through the system at comparatively low rates of approximately 0.4 L/min, and the CO_2 concentration within the chamber was stabilized at 0.8–1.2%. The $^{13}CO_2$ rate was measured as the $^{13}CO_2/^{12}CO_2$ ratio, and followed by a pulse of mixed gas composed of 5% CO_2, 12% O_2 and the rest of the mixture was N_2 for the control. Isotopic abundances were expressed relative to the international Vienna Pee Dee Belemnite standard (‰) as over the baseline ($\Delta-\Delta_0$) value, further normalized by each rat's weight.

Blood and tissue samples collection and analysis. Blood samples drawn from the inferior vena cava were collected in tubes with heparin, and plasma was separated from the blood samples by centrifugation at 1,500 $\times g$ for 5 min. The plasma was stored at −20°C until it was analyzed. The liver and gastrocnemius muscle were rapidly removed and snap-frozen in liquid nitrogen and stored at −80°C for analysis. Approximately 0.5 g of liver and muscle were homogenized in 4.5 mL of saline, centrifuged at 1,000 $\times g$ for 10 min.

A 100 μL plasma sample and the supernatant of liver and muscle obtained as described above were deproteinized with 300 mL ethanol and centrifuged at 1,500 $\times g$ for 10 min. A 200 mL sample of the supernatant fluid was cleared of contamination by using a strong cation exchanger (AG 50W-X8, Bio-Rad Laboratories, Hercules, CA), dried under a vacuum, derived to its 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivative using the Waters AccQ, Fluor Reagent Kit (Waters Corp., Milford, MA) and dried. Then the supernatant fluid was reconstituted in 200 μL of 0.1% formic acid. Phenylalanine and tyrosine concentrations were measured by an HPLC system. The individual amino acids were separated by an Inertsil ODS-3 column (250×4.6 mm, GL Sciences, Tokyo, Japan) with a binary LC gradient (0–60% aqueous acetonitrile containing 0.1% formic acid). The areas under the peaks were integrated using Peak Net 5.1c (Dionex Corp., Osaka, Japan). L-[1-^{13}C]Phenylanine and L-[1-^{13}C]tyrosine enrichment in the plasma and tissue samples was analyzed with a MS (LCQ Fleet, Thermo Scientific, Waltham, MA) coupled to the HPLC system. Selected ion chromatograms were obtained by monitoring ions *m/z* 336 and 337 for L-phenylalanine and L-[1-^{13}C]phenylalanine, *m/z* 352 and 353 for L-tyrosine and L-[1-^{13}C]tyrosine, respectively.

Statistical analysis. Data analysis was performed using Statcel2 software (Oms Publishing Inc., Tokyo, Japan). All results were presented as the mean±SE. Values of $p<0.05$ were considered statistically significant. Student's *t* test was used to analyze differences between two different groups, such as the protein intake. Statistical analysis for multiple comparisons was performed using one-way analysis of variance (ANOVA) with repeated measures followed by a Tukey-Kramer post hoc test.

The protein intake for metabolic demands was derived by applying a mixed-effect change-point model to breath $^{13}CO_2$ data (*25*), and the regression oxidation rate of the dietary protein contents. The first regression line showed a downward slope and the second line was horizontal with minimal or no slope. The breakpoint, the protein intake with a plateau in oxidation, was regarded as the protein intake for metabolic demand.

RESULTS

Experiment 1

The rats were given free access to a 17.2% casein diet as a maintenance diet for 3 d before the first study day, and the total daily intake for each rat was 16.5±0.5 g/d (calorie, 255.9±7.8 kJ/d; protein, 2.8±0.1 g/d). The body weights for the rats used for the 4.3% and 17.2% casein diet experiments were 144.1±5.7 g and 143.5±5.0 g, respectively.

Complete data sets of 9 breath samples were obtained

in only 7 of the rats fed the 17.2% casein diet. One rat did not consume its feed completely at 18:00, which affected the $^{13}CO_2$ values thereafter. Regardless of the protein intake and the 4.3% or 17.2% casein diets, breath $^{13}CO_2$ enrichment gradually increased after the initiation of the isotope protocol (Fig. 2). The plateau breath samples were collected during the isotopic steady state every 30 min during the period from 16:30 to 19:00 in rats fed the 17.2% casein diet, and from 17:30 to 19:00 in rats fed the 4.3% casein diet. This isotope protocol had been shown to achieve a satisfactory isotopic steady state 2.5 h after the start of L-[1-^{13}C]phenylalanine isotope administration. In addition, when the 4.3% casein diet was employed, the enrichment of breath $^{13}CO_2$ was greater than that achieved with the 17.2% casein diet, and during the period from 17:30 to 19:00, significant differences were shown between the 4.3% and 17.2% casein diets on breath $^{13}CO_2$ enrichment at 18:30 ($p<0.01$) and 19:00 ($p<0.01$).

The amino acid concentrations of plasma, liver and gastrocnemius muscle obtained at 18:30 on the IAAO study day are shown in Table 2. In both phenylalanine and tyrosine, ^{13}C-amino acid concentrations, ^{12}C-amino acid concentrations, and the total of these concentrations in the plasma and tissues of rats fed the 4.3% casein diet were similar to those of rats fed the 17.2% casein diet, and there were no significant differences.

Experiment 2

The rats were given free access to a 17.2% casein maintenance diet for 3 d before the first study day. The total daily intake for each rat used for the casein and wheat gluten diet experiments employing the IAAO method were 16.7±0.3 g/d (calorie, 258.9±4.3 kJ/d; protein, 2.9±0.1 g/d) and 17.3±0.5 g/d (calorie, 268.2±7.0 kJ/d; protein, 3.0±0.1 g/d), respectively. The body weights for the rats used for the 4.3, 8.6, 12.9, 17.2, 21.5, and 25.8% casein diet experiments were 149.4±5.7, 141.0±9.9, 155.2±10.8, 147.4±3.5, 158.0±2.4, and 184.7±3.1 g, respectively. The body weights for the rats used for the 7.2, 10.8, 14.4, 18.0, 21.6, and 25.2% wheat gluten diet experiments were 130.7±3.5, 148.8± 4.8, 157.1±5.4, 160.8±10.0, 134.4±2.8, and 142.4±3.0 g, respectively.

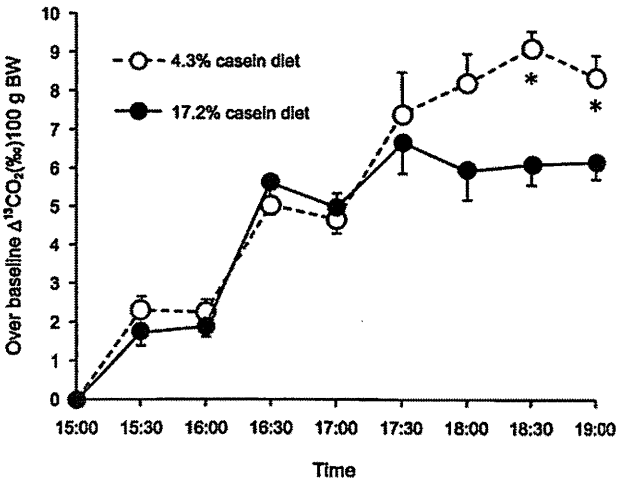

Fig. 2. The effect of L-[1-^{13}C]phenylalanine infusion on $^{13}CO_2$ enrichment of the breath. Values are mean±SE for the 4.3% (n=8 per mean) and 17.2% (n=7 per mean) casein diets. On the study day, the rats received either a 4.3% or 17.2% casein diet every 3 h from 09:00. Each meal represented one-eighth of the rat's total daily intake. The administrations of L-[1-^{13}C]phenylalanine were performed at 15:00, 16:00, 17:00, and 18:00. The establishment of a plateau in the breath samples on the basis of no significant differences among the timed samples was confirmed using repeated-measures ANOVA. The isotopic steady state was confirmed at 16:30–19:00 in rats fed the 17.2% casein diet, and at 17:30–19:00 in rats fed the 4.3% casein diet. *The asterisk marks shown significant differences ($p<0.01$) between the 4.3% and 17.2% casein diets at 18:30 and 19:00.

Table 2. The concentrations of phenylalanine and tyrosine in the plasma, liver and gastrocnemius muscle in rats fed 4.3% or 17.2% casein diets.

Diet	Phenylalanine			Tyrosine		
	^{13}C-Phe	^{12}C-Phe	Total	^{13}C-Tyr	^{12}C-Tyr	Total
			Plasma (nmol/mL)			
4.3% casein	13.2±2.9	47.2±4.3	60.4±7.0	7.5±2.0	113.0±29.4	120.6±30.7
17.2% casein	12.1±2.5	50.8±10.0	62.9±11.8	8.5±1.4	119.8±15.2	128.3±16.2
			Liver (nmol/g)			
4.3% casein	10.6±0.4	40.9±5.1	51.5±4.9	7.4±1.3	99.4±32.0	106.9±33.2
17.2% casein	10.4±2.1	43.1±10.5	53.6±12.3	8.8±2.8	92.5±7.5	101.4±9.2
			Gastrocnemius muscle (nmol/g)			
4.3% casein	13.0±1.7	46.6±4.5	59.6±5.7	8.4±0.8	91.9±8.7	100.3±8.5
17.2% casein	11.6±1.9	48.2±2.5	59.8±3.6	7.0±1.1	84.7±5.8	91.7±5.0

Values are shown as mean±SE for the 4.3% (n=5) and 17.2% (n=5) casein diets. Student's t test was performed to assess the effect of protein intake. No significant differences were demonstrated in the plasma and tissues phenylalanine or tyrosine concentrations between the 4.3% and 17.2% casein diets.

^{13}C-Phe, L-[1-^{13}C]phenylalanine; ^{12}C-Phe, L-[1–^{12}C]phenylalanine; ^{13}C-Tyr, L-[1-^{13}C]tyrosine; ^{12}C-Tyr, L-[1–^{12}C]tyrosine.

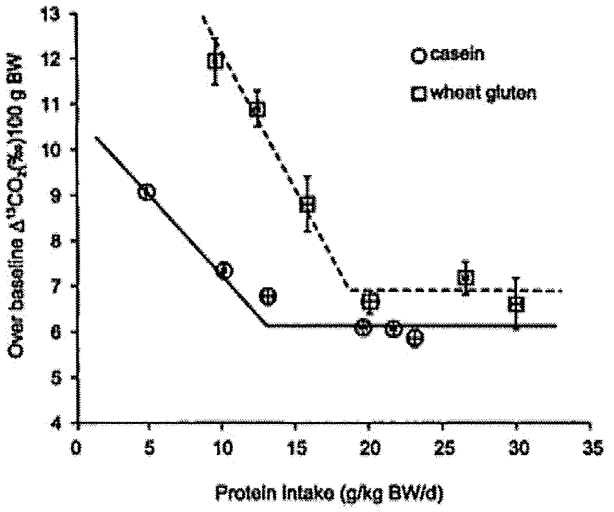

Fig. 3. The relationship between the intake of various proteins and the production of $^{13}CO_2$ from the oxidation of L-[1-^{13}C]phenylalanine when the rats were fed a casein diet (n=8 per mean) or a wheat gluten diet (n=8 per mean). Values are shown as mean±SE. The breakpoint estimates the mean protein intake for metabolic demands. The linear regression equation for the estimated protein intake for metabolic demands for the casein diet is as follows: $y=10.73-0.35x$ and $y=6.17$, for the wheat gluten diet is as follows: $y=18.87-0.66x$ and $y=6.92$, for the downward slope of the line and the level part of the line, respectively. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat's body weight. The mean protein intakes for metabolic demands for the casein and wheat gluten diets were estimated to be 13.1 g/kg BW/d and 18.1 g/kg BW/d, respectively.

Figure 3 shows the mean breakpoints illustrated in the breath $^{13}CO_2$ data, which were representative of the mean protein intake for metabolic demands. As the protein intake increased, breath $^{13}CO_2$ decreased steadily until the breakpoints were reached. There was no further decrease in breath $^{13}CO_2$ with the increase in protein intake. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat's body weight. Application of a mixed-effect change-point regression models to the breath $^{13}CO_2$ data resulted in the identification of a breakpoint at a dietary casein intake of 13.1 g/kg BW/d and a dietary wheat gluten intake of 18.1 g/kg BW/d. The enrichment of breath $^{13}CO_2$ was consistently higher in rats fed the wheat gluten diet, compared with the casein diet.

DISCUSSION

In the current IAAO study on rats, the protein intake for metabolic demands was estimated to be covered by a 13.1 g/kg BW/d for casein. This result was similar to the value recommended by the AIN-93G diet for laboratory rodents (a purified 20% casein (≥85% protein)), which was developed based on nitrogen balance studies. According to procedures recommended by the AIN, values were converted to dietary content by assuming a dietary intake of 15 g/rat/d for growing rats, and also for the rats fed 16.7±0.3 g/rat/d in the present study. This is the first study conducted that employed the IAAO method to determine the protein intake for metabolic demands using protein itself in rats, and the determined the protein intake for metabolic demands should be considered provisional.

Temperature, age, and physical activity influence the energy requirements of rats. It is difficult to estimate the energy requirement for growth due to variations in the composition of weight gain (*26–30*) and variations in the energetic efficiency of net protein and fat synthesis. However, it has been suggested that rats will generally consume enough food to meet their energy requirements (*31, 32*). The AIN-93 specifications indicate that a diet containing at least 15.0 kJ/g will meet the energy requirement for maintenance and growth if the rats are allowed free access to food and the diet is not deficient in other nutrients. In the present study, the rats accepted a 15.5 kJ/g diet containing 17.2% casein as a maintenance diet. Furthermore, the rats were given free access to this diet and the 24-h dietary intake was regarded as an individual rat's energy requirement.

Humayun et al. (*19*) reevaluated the protein requirement in young men employing the IAAO method, and the protein source of the experimental diet was consumed hourly in small meals consisting of a crystalline amino acid mixture. In the present study, casein and wheat gluten were employed as the protein source, and therefore, as the rats consumed the experimental diet at 3-h intervals, it can be considered that the mechanism of assimilation differed from that of the amino acid mixture. Amino acid mixtures will be absorbed very rapidly, and protein utilization will show a lower efficiency, compared with slow proteins such as casein (*20*).

The phenylalanine and L-[1-^{13}C]phenylalanine concentrations in the plasma, liver and gastrocnemius muscle were not affected by the amount of protein intake in the 4.3% or 17.2% casein diets, suggesting that the precursor pool for indicator oxidation did not change in size in response to the test protein intake. After phenylalanine is hydroxylated, conversion to tyrosine takes place, so the tyrosine concentration was also examined. In comparison with the ratio of L-[1-^{13}C]phenylalanine to the total phenylalanine concentration, only a trace of L-[1-^{13}C]tyrosine to the total tyrosine occurred in the plasma and tissues, regardless of the protein intake, suggesting that there was no tyrosine deficiency. In previous studies, the loss of the ^{13}C into the protein-bound tyrosine pool or tyrosine metabolites was minimized by providing a high-tyrosine diet before the study (*19*).

$^{13}CO_2$ breath tests are normally performed in the presence of a large background of naturally occurring isotope of approximately 1.1% ^{13}C (*33*). The ^{13}C rate of any unlabeled substrate ingested during a $^{13}CO_2$ breath test must be considered in order to eliminate artifacts that may reduce the sensitivity of the breath test and produce erroneous results (*33*). In our preliminary