Plasma proline kinetics and concentrations in young men in response to dietary proline deprivation[1-3]

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ABSTRACT This study examined plasma proline concentration, flux, oxidation, and endogenous biosynthesis in five healthy young men given three isocaloric, isonitrogenous diets for 1 wk [a complete egg-pattern amino acid diet (diet 1), an amino acid mixture devoid of proline (diet 2), and a diet composed solely of indispensable amino acids (diet 3)]. At the end of each dietary period, a 360-min postabsorptive, primed, continuous stable-isotope-tracer infusion of L-[1-^{13}C]proline and L-[methyl-$^{2}H_3$]leucine was performed in all subjects. Plasma proline concentrations declined by 22% on diet 2 ($p < 0.02$) and by 29% on diet 3 ($p < 0.01$). No statistically significant ($p > 0.2$) changes were observed for proline oxidation, endogenous biosynthesis, or flux. The data suggest that the absence of proline in the human diet does not trigger changes in proline dynamics during the postabsorptive state. The metabolic significance of the reduction of plasma proline concentrations requires elucidation. *Am J Clin Nutr* 1990;52:307-12.

KEY WORDS Proline, kinetics, plasma concentrations, diet, humans

Introduction

Investigations of certain avian (1) and mammalian (2) species indicated that proline is required for optimal growth, despite the presence of an endogenous biosynthetic pathway (3). This implies that a conditional essentiality may exist for this amino acid. Stable-isotope studies in humans (4) demonstrated that proline synthetic rates, though homeostatically altered in response to the short-term intravenous infusion of L-proline, were only ~10% of those reported for other dispensable amino acids measured by similar methodology (5, 6). Hence, we undertook a series of stable-isotope-tracer experiments in healthy young adults to explore the effects of dietary proline restriction on plasma proline concentration, flux, oxidation, and endogenous biosynthesis.

Methods

Materials

L-[1-^{13}C]proline (95 mol % excess 1-^{13}C) and L-[methyl-$^{2}H_3$]-leucine were purchased from MSD Isotopes, Pointe-Claire, Durval, Quebec. Isotope and chemical purities of the labeled amino acids were verified by gas chromatography–mass spectroscopy (GCMS) (7). Stock solutions of tracers were prepared with sterile 9 g NaCl/L and certified to be sterile and pyrogen-free before use. Samples were aseptically removed from the stock solutions under a laminar-flow hood and further diluted with sterile saline immediately prior to administration to human subjects. The L-amino acids used in the formulation of experimental diets were kindly donated by Ajinomoto USA, Inc, Teaneck, NJ.

Subjects

Five young men (**Table 1**) aged 19.6 ± 0.2 y ($\bar{x}$ ± SEM) with an average height of 173.7 ± 1.9 cm and weight of 73.8 ± 4.8 kg were recruited for a series of three diet and infusion studies. All were in good health as determined by medical history and complete physical examination and had normal urinalysis, complete blood count, and renal- and hepatic-function tests. They were taking no medications either on an acute or chronic basis. Written consent was obtained from each subject and experimental protocols and procedures were approved by the Massachusetts Institute of Technology (MIT) Committee on the Use of Humans as Experimental Subjects as well as the Executive and Policy Committees of the MIT Clinical Research Center. Payment was provided for participating in the investigation.

Diet

Energy requirements were established for each individual by a research dietitian who compiled a complete dietary history, including food intake and level of physical activity. Dietary protein was supplied as one of three isonitrogenous L-amino acid mixtures for a 7-d period. Diet 1 consisted of a complete egg-pattern L-amino acid composite that included proline (**Table 2**). Diet 2 contained all the amino acids except proline and was made isonitrogenous by the addition of L-alanine and L-aspartate (Table 2). Diets 1 and 2 were given to provide 0.6 g

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TABLE 1
Characteristics of subjects

Subject	Age	Height	Weight
	y	*cm*	*kg*
1	19.7	179	65.9
2	19.1	169	63.7
3	19.7	171	70.2
4	20.1	172	79.3
5	19.5	178	90.0

amino acid mixture·kg body $wt^{-1}\cdot d^{-1}$. The rationale for this level of total amino acid intake was that the total nitrogen and dispensable amino acid content would not be in excess of physiologic need and, thus, a limitation in the synthetic capacity for proline might be accentuated. In clinical terms the level of total nitrogen intake approximates that recommended for patients with progressive renal failure (8). A third diet composed only of indispensable (essential) amino acids was also administered (Table 2). The ratios of indispensable amino acids to each other in diet 3 were nearly identical to those for diets 1 and 2. Total amino acid intake with diet 3 was 0.73 g·kg body $wt^{-1}\cdot d^{-1}$ to obtain a nitrogen intake equivalent to that in diets 1 and 2. The diets were given as citrus-flavored mixtures of identical appearance. Protein-free cookies and sweetened beverages were added in quantities sufficient to meet each subject's energy needs. All diets were prepared in the diet kitchen at the MIT Clinical Research Center and they were served as four isocaloric, isonitrogenous portions at 0800, 1200, 1700, and 2000. The diets were supplemented with minerals and vitamins to meet or exceed estimated requirements (9). All subjects maintained their initial body weights over the study periods. A minimum free-choice period of 3 wk was allowed between each experimental dietary period. Subjects 1–3 progressed through diets 1, 2, and 3 sequentially whereas subjects 4 and 5 were given diet 3 before diet 2.

Experimental design

The subjects received the diets, as described above, for a period of 7 d. They then fasted overnight and a 5-cm indwelling catheter was inserted in the antecubital vein of the right and left arms. One of the catheters was used for isotope infusion and the other for the withdrawal of blood samples. $NaH^{13}CO_2$ (0.11 mg/kg) (10), priming doses of L-[methyl-$^2H_3$]leucine (2.0 μmol/kg), and L-[1-^{13}C]proline (2.0 μmol/kg) were administered at the start of the tracer experiment. This was followed immediately by a continuous, 240-min infusion of L-[methyl-$^2H_3$]leucine (2.0 $\mu mol\cdot kg^{-1}\cdot h^{-1}$) and L-[1-^{13}C]proline (2.0 $\mu mol\cdot kg^{-1}\cdot h^{-1}$) by use of calibrated syringe pumps (Harvard Apparatus, Millis, MA). The leucine-tracer technique (11) and the proline methodology (4) were based on previous studies.

Sampling procedure

Two baseline venous blood samples were obtained before the start of the isotope infusion and five plateau samples were drawn at 20-min intervals commencing at 140 min and ending at 240 min. Blood was collected in heparinized tubes and placed on ice until the plasma was separated in a refrigerated centrifuge. All plasma was transferred into several 1.8-mL Nunc cryotubes (Thomas Scientific, Swedesboro, NJ) and stored at −20 °C at the termination of the infusion. Expired-air samples for ^{13}C enrichment of CO_2 were obtained in a time sequence identical to that of the blood specimens by means of a Rudolph valve attached to a 3-L anesthesia bag. The air was then transferred without exposure to ambient atmosphere into 15-mL nonsilicone-coated, nonsterile evacuated tubes. A minimum of six Douglas-bag collections was taken during the infusion (two shortly after the start of the infusion and four at the plateau period). CO_2 concentrations were determined immediately by means of an LB2 medical gas analyzer (Beckman Instruments, Inc, Fullerton, CA) after passage of the expired breath through a drying tube filled with calcium sulfate. The minute ventilation was calculated with the aid of a Collins gasometer (Warren Collins Co, Braintree, MA). All respiratory measurements were made in a quiet room with subjects awake and recumbent in bed and the data were adjusted to standard temperature and pressure. From the two baseline and five plateau blood and air samples, kinetic estimates of proline metabolism were calculated for each infusion, as described below.

Analytic methods

An automated amino acid analyzer (model 119CL, Beckman Instruments, Inc) was used to determine free plasma proline and leucine concentrations from samples deproteinized with sulfosalicylic acid. Infusates were also analyzed for proline and leucine concentrations.

A sample of free amino acids isolated from plasma was prepared as the N-acetyl, *n*-propyl derivative by use of previously described methods (12). L-[1-^{13}C]proline enrichment in plasma was measured by GCMS in the chemical-ionization mode with methane as the reagent gas. A Hewlett-Packard (Palo Alto, CA) 5985B quadrupole instrument was used in the selected ion-monitoring mode, monitoring m/z = 228 and 229 for unlabeled and labeled proline, respectively. A 12-m × 0.20-mm-ID, OV-1 capillary column was used in the gas chromatograph.

TABLE 2
Composition of L-amino acid mixtures

Amino acids	Diet 1	Diet 2	Diet 3
		g/kg mixture	
L-Trp	15.48	15.48	30.96
L-Thr	46.73	46.73	93.46
L-Ile	62.35	62.35	124.70
L-Leu	82.46	82.46	165.28
L-Lys·HCl	75.12	75.12	150.24
L-Met	29.42	29.42	58.84
L-Cys	21.94	21.94	43.88
L-Phe	54.24	54.24	108.48
L-Tyr	40.42	40.42	84.72
L-Val	69.72	69.72	139.44
L-Arg·HCl	74.54	74.54	—
L-His·HCl·H_2O	30.43	30.43	—
L-Ala	61.10	74.17	—
L-Asp	65.80	94.35	—
L-Gly	112.39	112.39	—
L-Glu	33.00	33.00	—
L-Pro	41.62	—	—
L-Ser	83.24	83.24	—

The deuterated-leucine enrichment was determined with a Hewlett-Packard 5970 mass spectrometer. The isotope was detected by electron-impact ionization with selected ion monitoring at m/z = 128 and 131 for unlabeled and labeled leucine, respectively. A 12-m × 0.20-mm-ID, OV-1 capillary column was used in the gas chromatograph.

Enrichments of all compounds were calculated as atom % excess (APE) from natural background concentrations of isotope. All samples were run in duplicate and reproduced within 5%. If analyses were not reproduced within 5% after the first duplicate run, they were reinjected twice more.

Air samples were analyzed for $^{13}CO_2$ enrichment with a dual-collector isotope-ratio mass spectrometer (Nuclide Corp, State College, PA). APE of $^{13}CO_2$ was determined relative to the reference-gas CO_2 derived from potassium carbonate. The baseline $^{13}CO_2$ was determined relative to the reference-gas CO_2 derived from potassium carbonate.

Calculations

As described in detail elsewhere (4), the analysis of whole-body proline kinetics was based on models previously described by us for glycine (5, 13) and alanine (5, 6, 13, 14), utilizing the principles of Waterlow et al (15). The estimation of plasma amino acid flux was made by use of conventional steady-state dilution principles. An estimate of proline derived from protein breakdown can be obtained by assuming that the release of proline from body proteins occurs in a molar proportion to the liberation of leucine, with the latter being assessed by L-[methyl-$^2H_3$]leucine flux. Total proline flux (measured by L-[1-^{13}C]proline) minus the amount of proline entering the free amino acid pool from protein breakdown provides an estimate of the rate of endogenous-proline biosynthesis (4).

Proline oxidation was calculated with standard amino acid kinetic equations (11). The term *proline oxidation* refers to that quantity of proline converted to α-ketoglutarate and then irreversibly decarboxylated by the multienzyme complex α-ketoglutarate dehydrogenase. The recovery of $^{13}CO_2$ in expired air was assumed to be 0.81 of the total $^{13}CO_2$ released from proline oxidation to account for retention of CO_2 through fixation (10, 11).

Our estimate of proline oxidation was based on plasma [^{13}C]-proline enrichment whereas the desirable measure to use for this purpose is the enrichment of proline at the intracellular site(s) of proline oxidation. Because we do not have an estimate of isotope enrichment at the intracellular site(s), which would be anticipated to be less than that in the plasma compartment, it is likely that proline oxidation was underestimated. Furthermore, the possibility exists that the ratios of intracellular [^{13}C]-proline to plasma [^{13}C]proline are altered by changing dietary proline intake, leading to a variable underestimate of proline oxidation under differing dietary and/or metabolic conditions. Hence, the results for proline oxidation discussed below should be considered with these limitations in mind.

Statistical analysis

The data were analyzed with the aid of a statistical software package (*ABSTAT*, release 4, Anderson-Bell, Parker, CO) run on an IBM personal computer and expressed as $\bar{x} \pm$ SEM. Comparisons were made by two-way analysis of variance (ANOVA) for data without replication (16). Any statistically significant differences were further evaluated by Duncan multiple-range tests (17).

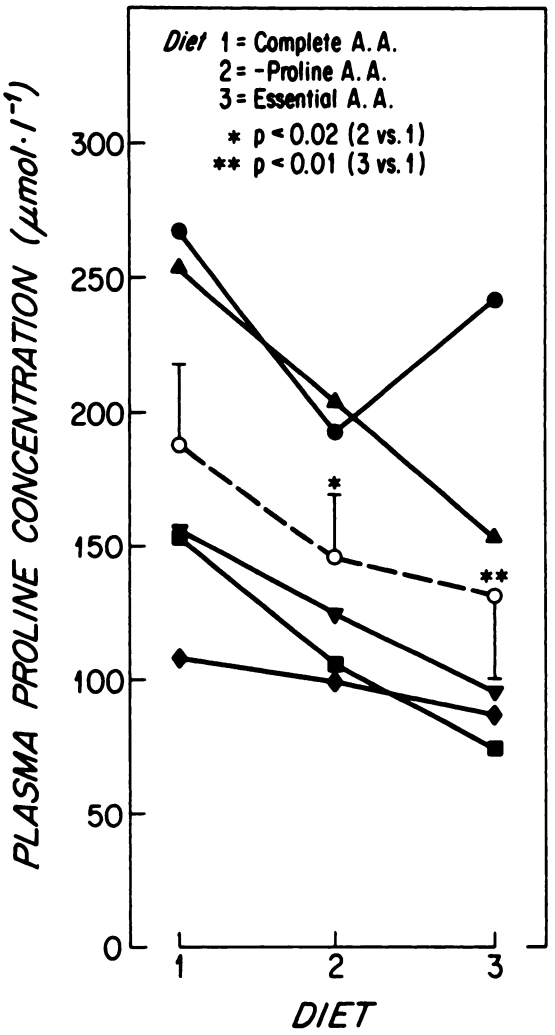

FIG 1. Plasma proline concentrations on the three experimental diets. ♦, subject 1; ●, subject 2; ▼, subject 3, ■, subject 4; ▲, subject 5; and ○, $\bar{x} \pm$ SEM. *Significantly different from diet 1, $p < 0.02$. **Significantly different from diet 1, $p < 0.01$.

Results

Figure 1 depicts the individual and average plasma proline concentrations obtained when subjects were given the complete amino acid diet (diet 1), an amino acid diet devoid of proline (diet 2), and an essential amino acid diet (diet 3). All individual values are the average of two determinations on blood taken at 0800 on the morning of the seventh day of the experimental diet and after an overnight fast. The plasma proline concentration for subjects during the period with diet 1 was 187 ± 30 μmol/L ($\bar{x} \pm$ SEM). The value obtained with diet 2 was 146 ± 22.2 μmol/L. A two-way ANOVA revealed that plasma proline concentrations were dependent on the dietary intake of proline ($F = 6.71$, $p < 0.02$). Duncan multiple-range tests demonstrated that plasma proline concentrations during diet 2 were significantly lower than those measured during diet 1 ($F = 6.88$, $p < 0.02$); serum proline concentrations for diet 3

TABLE 3
Plasma leucine concentrations in young men receiving the three experimental diets

Subject	Diet 1	Diet 2	Diet 3
		μmol/L	
1	95.0	120.5	108.5
2	149.0	110.5	114.0
3	118.5	113.5	116.0
4	103.5	147.0	123.5
5	161.5	180.0	178.5
$\bar{x}$ ± SEM	125.5 ± 12.9	134.3 ± 13.1	128.1 ± 12.8

were reduced to a further extent when compared with diet 1 (F = 12.44, $p < 0.01$). Four of five subjects showed their lowest proline concentrations after 1 wk of consuming the essential amino acid diet (diet 3). However, a comparison of plasma proline concentrations by Duncan multiple-range testing on diet 2 vs diet 3, did not yield a statistically significant difference. Fourteen of the 15 observations were consistent with the hypothesis that plasma proline concentrations on diet 1 were greater than on diet 2 and that these, in turn, were greater than for diet 3. Subject 2 on diet 3 was the only exception. Subject 4 demonstrated the largest decrease in plasma proline concentrations (50%). Statistical analysis also disclosed that different subjects varied significantly with respect to plasma proline concentrations (F = 18.40, $p < 0.001$).

Table 3 provides a summary of the plasma leucine concentrations for periods during diets 1, 2, and 3. A two-way ANOVA determined that plasma leucine concentrations were not significantly different between any of the diets (F = 0.37, $p > 0.70$).

The kinetic data for proline and leucine metabolism are shown in **Table 4.** Proline flux was not significantly affected by the dietary manipulations (F = 1.35, $p > 0.31$). Leucine flux was also unchanged by the dietary manipulations (F = 1.65, $p > 0.25$).

As shown in **Table 5,** the estimated rates of proline endogenous biosynthesis did not change (F = 0.05, $p > 0.95$) with the alterations in amino acid content of the diets despite reductions in plasma proline concentration. Although proline oxidation was slightly lower for the two proline-free diets (diets 2 and 3), the difference was not statistically significant (F = 1.84, $p > 0.21$).

Discussion

The view that the amino acid proline is not a necessary component of the human diet has been based on data obtained from brief nitrogen-balance studies. Rose et al (18–21) found that all amino acids synthesized within the body (including proline) could be removed from the diet for a period of 7 d without creating a negative nitrogen balance. The subsequent work of Kopple and Swendseid (22) showed a prompt reduction in plasma histidine concentrations within 4 wk of following a histidine-free diet. Although Rose et al (18–21), considered histidine a dispensible amino acid, a progressive deterioration in nitrogen balance and a decrease in hematocrit occurred in both normal subjects and in chronic-renal-failure patients on the histidine-free diet. Amino acid formulations have been recommended for the treatment of patients with renal failure (23) and such formulas (now with the addition of histidine) are available commercially.

Animal investigations have indicated that proline is a conditionally essential dietary component in certain species (1, 2). Although experiments undertaken in mammalian cell lines (24) and in vivo in man (4) established that proline biosynthesis and oxidation were tightly regulated, the whole-body proline kinetic data also suggested that absolute rates of proline endogenous biosynthesis and oxidation were relatively low (4).

The 7-d isocaloric, isonitrogenous dietary experiments described above, conducted in normal young men, demonstrated that dietary deprivation of proline was associated with a 22% decrease in plasma proline concentration ($p < 0.02$), and the withdrawal of proline plus the other nonessential amino acids resulted in a 29% decrease in plasma proline concentration ($p < 0.01$) (Fig 1). Considering the relatively brief dietary periods used in our study, these changes appear to be quite marked. The distribution ratios of free-proline concentrations between plasma and tissues are about 1:3 for human and rat muscle and are 1:1 for rat liver (25, 26). In reference to our earlier study (4) in which it was determined that plasma proline tracers equilibrated with a plasma volume of distribution of ~45 L for a 70-kg subject, the present plasma proline data imply that the decrease in proline concentrations reflects a significant reduction in the whole-body free-proline pool.

The 1-wk absence of proline in the diet did not result in an increase in leucine flux, which indicates that whole-body protein breakdown was unchanged under the conditions of the experiment. The present methodology, however, does not permit an isotopic assessment of net whole-body protein balance be-

TABLE 4
Proline and leucine fluxes in subjects receiving the three experimental diets

	Proline flux			Leucine flux		
Subject	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
	μmol·kg⁻¹·h⁻¹			*μmol·kg⁻¹·h⁻¹*		
1	60.0	59.2	76.2	94.3	94.9	102.0
2	81.6	85.2	100.8	97.4	103.9	102.6
3	80.0	74.4	74.2	109.1	109.1	100.2
4	66.1	58.9	67.4	87.1	77.0	103.2
5	89.6	82.2	77.7	103.7	83.3	107.2
$\bar{x}$ ± SEM	75.5 ± 5.4	71.9 ± 5.4	79.3 ± 3.9	98.4 ± 3.9	93.6 ± 6.0	103.0 ± 1.2

TABLE 5
Proline de novo synthesis and oxidation in young men receiving the three experimental diets

Subject	Proline synthesis			Proline oxidation		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
	$\mu mol \cdot kg^{-1} \cdot h^{-1}$			$\mu mol \cdot kg^{-1} \cdot h^{-1}$		
1	2.5	1.3	14.0	7.8	6.5	4.3
2	22.2	21.8	38.2	19.1	6.6	14.1
3	13.1	7.8	13.1	9.6	11.1	12.1
4	13.0	11.9	4.5	11.0	7.4	13.4
5	26.3	31.4	12.3	14.8	14.0	8.9
$\bar{x} \pm$ SEM	15.4 ± 4.1	14.8 ± 5.3	16.4 ± 5.7	12.4 ± 2.0	9.1 ± 1.5	10.6 ± 1.8

cause the tracer studies were conducted during only a portion of the 24-h d, under postabsorptive conditions, and we could not estimate rates of leucine disappearance into body proteins with the isotope tracer used.

Further kinetic data revealed that proline flux (Table 4), proline oxidation (Table 5), and proline endogenous biosynthesis (Table 5) were not measurably altered by dietary manipulations examined. Nonetheless, the substantial decrease in plasma proline concentrations accompanying dietary proline deprivation suggests the possibility that proline may well be a conditionally essential amino acid in adult humans, depending on the metabolic and functional significance of the lowered proline concentrations, which remain to be determined.

The question can be raised as to the purpose of conducting an evaluation of proline kinetics while subjects were in the postabsorptive state. Before carrying out this study it was considered possible that the lack of dietary proline, alone or in combination with its immediate amino acid precursors, might result in changes in body protein turnover and/or differences in proline oxidation and de novo proline synthesis during the postabsorptive period and in such a way as to achieve a maximum conservation of proline. We observed previously (5) a reduced rate of de novo glycine synthesis during the postabsorptive condition when healthy adult men were receiving a diet low in both total nitrogen and total dispensable amino acid content. However, the fact that we were unable to detect any significant differences in proline flux and its components under the present conditions now makes it more important to carry out experiments in subjects during the absorption of diets varying in their content of proline and of other dispensable amino acids.

In addition, it appears desirable to carry out more prolonged, experimental dietary periods, as was found necessary in the case of histidine (22), to explore whether a lack of proline and its precursors, such as glutamate, in the diet is associated with further reductions in plasma proline concentrations and alterations in proline kinetics and whole-body proline balance, which might be reversed by proline supplementation. In view of the technical and conceptual limitations of the nitrogen balance method (27, 28) and the need to better understand the metabolic and nutritional aspects of amino acids that previously were thought to be dispensable (29), the present approach, involving an assessment of proline flux, endogenous synthesis, and oxidation rates, would appear to offer a viable means for examining more thoroughly the role of proline in the maintenance of the amino acid and nitrogen economy of the intact human subject.

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