

# Dietary Proteins Contribute Little to Glucose Production, Even Under Optimal Gluconeogenic Conditions in Healthy Humans

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Dietary proteins are believed to participate significantly in maintaining blood glucose levels, but their contribution to endogenous glucose production (EGP) remains unclear. We investigated this question using multiple stable isotopes. After overnight fasting, eight healthy volunteers received an intravenous infusion of [6,6-<sup>2</sup>H<sub>2</sub>]-glucose. Two hours later, they ingested four eggs containing 23 g of intrinsically, uniformly, and doubly [<sup>15</sup>N]-[<sup>13</sup>C]-labeled proteins. Gas exchanges, expired CO<sub>2</sub>, blood, and urine were collected over the 8 h following egg ingestion. The cumulative amount of dietary amino acids (AAs) deaminated over this 8-h period was 18.1 ± 3.5%, 17.5% of them being oxidized. The EGP remained stable for 6 h but fell thereafter, concomitantly with blood glucose levels. During the 8 h after egg ingestion, 50.4 ± 7.7 g of glucose was produced, but only 3.9 ± 0.7 g originated from dietary AA. Our results show that the total postprandial contribution of dietary AA to EGP was small in humans habituated to a diet medium-rich in proteins, even after an overnight fast and in the absence of carbohydrates from the meal. These findings question the respective roles of dietary proteins and endogenous sources in generating significant amounts of glucose in order to maintain blood glucose levels in healthy subjects. *Diabetes* 62:1435–1442, 2013

**P**roteins are a source of gluconeogenic substrates and can be used to produce glucose under fasting or a low-carbohydrate intake. High-protein (HP) diets are generally low in carbohydrates and assumed to promote postprandial gluconeogenesis. Previous studies showed that the ingestion of an HP diet led to an increase in the postprandial expression of gluconeogenic enzymes in rats (1–3) as well as fasting fractional gluconeogenesis in humans (4), and amino acid (AA) infusions enhanced endogenous glucose production (EGP) (5). This glucostatic role of dietary proteins has been hypothesized as being involved in their greater satiating power when compared with other macronutrients (6,7).

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See accompanying commentary, p. 1371.

Under HP feeding, AAs in excess during the postprandial phase are deaminated (8–10) and generate a large quantity of carbon skeletons that are supposed to be oxidized or transformed into glucose. However, the specific postprandial fate of dietary and endogenous AA carbon skeletons has been the subject of very little study. In rats, we recently revealed that only half of postprandially deaminated dietary AAs had been oxidized 4 h after the ingestion of an HP diet (50% of energy as protein) (11), and we assessed that <5% of AA-derived carbon skeletons were used for glucose synthesis. This was achieved by the <sup>15</sup>N and <sup>13</sup>C labeling of dietary AA and following the tracers in the final products and in glucose. However, we were not able to determine this contribution precisely because we did not measure EGP, and the dietary proteins were traced using extrinsic labeling.

The true contribution of dietary AA to EGP, compared with that of endogenous AA and other gluconeogenic substrates, has not been previously addressed, except by an indirect method in humans (12). The objective of the current study was to precisely measure the contribution of dietary proteins to glucose production in humans by using egg proteins intrinsically labeled with <sup>15</sup>N and <sup>13</sup>C (13), with the concomitant infusion of deuterated glucose. This original study enabled a precise determination of the contribution of dietary proteins to EGP.

## RESEARCH DESIGN AND METHODS

**Subjects.** All participants were certified as being in good health after a thorough examination performed by medical staff at the Volunteer Research Centre based in Hôtel-Dieu Hôpital and routine biochemical tests. The purpose and potential risks of the study were fully explained to the subjects. Written informed consent was obtained from participants, and the protocol was approved by the Ethics Committee Saint-Germain-en-Laye Hospital (St. Germain en Laye, France). The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01154582).

Eight healthy subjects (three women and five men) were enrolled in this study. Their average age and BMI were 24 ± 3 years and 20.5 ± 1.1 kg/m<sup>2</sup>, respectively.

**Standardization diet and experimental meals.** During the 5 days before the study, the subjects were asked to follow a standard diet adjusted to their body weight and supplying daily 134 kJ/kg in the form of 1.4 g protein/kg, 4.5 g carbohydrate/kg, and 1 g fat/kg. Dietary notebooks containing daily menus and the specific quantities of food to be consumed at each meal were delivered, as were daily record sheets. The subjects were asked to ensure strict compliance with their respective diets, but were allowed some equivalent food exchanges. Compliance was assessed by analyzing the quality of food record.

After the standardization period, they came to the hospital on the morning of the study day, after fasting overnight for 12 h. The test meal was made up of four hen's eggs (i.e., 210 g of liquid whole eggs supplying 23 g protein, 19 g fat, and a negligible amount of carbohydrates). The four eggs were cooked in an omelet without the addition of fat. The egg proteins were intrinsically and doubly labeled with <sup>15</sup>N and <sup>13</sup>C. These [<sup>15</sup>N]-[<sup>13</sup>C]-labeled eggs were produced by the Agence Nationale de Sécurité Sanitaire de L'alimentation, de L'environnement et du Travail (Fougères Laboratory) using a technique adapted from the work of Evenepoel et al. (14), as previously described (13). Briefly,

the diet of a laying hen was supplemented with 0.3% of a mixture of 20 uniformly and doubly labeled [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-AA (CortecNet, Paris, France) for 16 days. The  $^{15}\text{N}$  and  $^{13}\text{C}$  enrichments of the egg proteins were 1.07 atom percent (AP) (i.e., 0.71 AP excess [APE] and 1.67 AP [i.e., 0.55 APE]), respectively. The  $^{13}\text{C}$  enrichment of neoglucogenic AA (alanine, serine, glycine, threonine, aspartate, glutamate), which was calculated as the sum of each enrichment (1.19–1.52 AP) weighted by the relative abundance of AA in egg proteins, was 1.28 AP.

**Protocol.** Soon after their arrival at the hospital, a venous catheter was inserted into both forearms of the subjects. After the collection of a baseline blood sample, a primed dose ( $22 \mu\text{mol} \cdot \text{kg}^{-1}$ ) and a constant infusion of [ $6,6\text{-}^2\text{H}_2$ ]-glucose ( $0.22 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) were started through the second catheter. Ninety minutes after starting the infusion and collection of a second baseline blood sample, the subjects were placed under a canopy for 30 min to measure fasting gas exchanges. At the end of this 30-min period, blood, urine, and expired gas were sampled, and the subjects were given the test meal, after which they were not allowed any other foods until the end of the experiment, although they were given 100 mL water every hour. During the 8 h following meal ingestion, blood and urine samples were collected every 30 min and 2 h, respectively. Expired air was collected every 30 min, and gas exchanges were measured over discontinuous periods of 30 min. After total blood centrifugation, aliquots of serum or plasma were collected and stored at  $-20$  or  $-80^\circ\text{C}$  (for hormones) until analysis. After measuring the volume of urine excreted, urine samples were either preserved by adding thymol and paraffin and stored at  $4$  or  $-20^\circ\text{C}$  until analysis.

**Analyses.** Urea concentrations in plasma and urine, and glucose levels in plasma, were determined using different commercial kits (Bio-Mérieux, Marcy l'Étoile, France) and a spectrophotometer (Molecular Devices, Sunnyvale, CA). Plasma AA were analyzed by ion-exchange chromatography, with the addition of norleucine as an internal standard (Biotech Instruments, St. Quentin-en-Yvelines, France). Plasma insulin and glucagon were analyzed using a human endocrine panel (Milliplex; Millipore, Billerica, MA) on a Bio-plex 200 system (Bio-Rad, Hercules, CA).

For isotopic determinations, plasma proteins were precipitated from 2 mL of plasma with a solution of 5-sulfo-salicylic acid (1 g/mL). The supernatant was titrated to pH 7, transferred on 0.5 mL cation exchange resin (Dowex AG50 X8, mesh 100–200; Bio-Rad), and the urea was hydrolyzed with 8  $\mu\text{L}$  of urease for 2 h at  $30^\circ\text{C}$ . The supernatant containing serum-free AA was collected and dried. Urinary urea was similarly extracted on the cation resin, as previously described (15).  $^{15}\text{N}$  enrichments, as well as  $^{13}\text{C}$  for plasma proteins, were determined using an isotopic ratio mass spectrometer (Isoprime; GV Instruments, Manchester, U.K.) coupled to an elemental analyzer (Euro Elemental Analyzer 3000; EuroVector, Milan, Italy). Total nitrogen and carbon in plasma proteins were measured using the elemental analyzer system, with atropine and cyclohexanone, respectively (CE Instruments, Milan, Italy) as standards.

Plasma glucose was extracted using successive exclusion on anion and cation Dowex resins (Bio-Rad) (16,17). Briefly, after precipitation of proteins from 0.5 mL plasma with 0.5 mL perchloric acid 1.2 mol, the supernatant was neutralized to pH 5.5–6.2 by the addition of 110  $\mu\text{L}$  potassium carbonate 3.2 mol. After storage overnight without a cap at  $4^\circ\text{C}$ , the samples were centrifuged, and the pH of the resulting supernatant was adjusted to between 5.5 and 7 by adding  $\text{K}_2\text{CO}_3$  or  $\text{HClO}_4$ . After centrifugation, the supernatant was filtered through a 0.22- $\mu\text{m}$  filter. Glucose in the residue was then passed through an anion (AG1-X8: formate form) and a cation (AG50-X8: hydrogen form) exchange column in order to trap organic acids and AAs, successively. Free glucose was eluted with 6 mL water in a Pyrex tube and then dried overnight.

For the determination of  $^{13}\text{C}$  enrichment, which was expected to be low, glucose extract was derivatized with methylboronic acid to limit the number of additional carbons. Five hundred microliters of a solution of 3 mg methylboronic acid in 0.5 mL of pyridine was added on the glucose extract. The solution was mixed, kept for 2 h at room temperature, and then heated at  $60^\circ\text{C}$  for 60 min. After cooling, 30  $\mu\text{L}$  *N,O*-bis(trimethylsilyl)trifluoroacetamide was added, and the sample was heated at  $60^\circ\text{C}$  for 30 min. Analysis of  $^{13}\text{C}$  enrichment was performed on an Isotope Ratio Mass Spectrometer in continuous flow (Isoprime; Micromass, Villeurbanne, France) coupled online with an HP 5890 series II gas chromatograph (Fisons Instruments, Arcueil, France) via a combustion interface (Fisons Instruments) using a 25m-HP5 column (HP5MS; Hewlett-Packard, Palo Alto, CA), as described previously (17).

Aldonitrile pentaacetate derivatization was performed for measurement of  $^2\text{H}$  enrichment (18). One hundred microliters of a solution of 20 mg hydroxylamine hydrochloride per milliliter of pyridine was added to glucose extract, vortex mixed, and microwaved for 2 min at 200 W. After cooling, 100  $\mu\text{L}$  of acetic anhydride was added and microwaved at 200 W for 6 min. Analysis of the  $^2\text{H}$  enrichment of the derivative was performed on a Gas Chromatography Mass Spectrometer (6890 N; Hewlett-Packard) using a 25m-HP5 column

(Hewlett-Packard), with electron-impact ionization and selected ion monitoring of 187 and 189 charge/mass ratio, as described elsewhere (19).

After delipidation of egg proteins, hydrolysis, and derivation of AAs with ethyl chloroformate, as previously described (13), the individual enrichments of AAs were measured by gas chromatography combustion-isotopic ratio mass spectrometry, using a Carlo Erba GC 8130 chromatograph interfaced with a VG Isochrom mass spectrometer (Fisons Instruments) and a 30 m-RTX1701 column (Restek, Evry, France).

#### Calculations

**Dietary nitrogen transfer in different metabolic pools (11,20,21).** The dietary N present in the samples was expressed in percentage of ingested nitrogen and calculated as follows:

For the nitrogen part:

$$\text{Dietary N} = N_{\text{tot-mmol}} \times 100 \times (\text{APE}_{\text{sample}}/\text{APE}_{\text{meal}})/\text{ingested N}$$

in which  $N_{\text{tot-mmol}}$  is the amount of total nitrogen (N) in the sample;  $\text{APE}_{\text{sample}}$  is [ $^{15}\text{N}$ ]-enrichment percent excess of the sample = enrichment of the sample – basal enrichment, in AP; and  $\text{APE}_{\text{meal}}$  is [ $^{15}\text{N}$ ]-enrichment percent excess of the meal = enrichment of the meal – basal enrichment of the diet. The [ $^{15}\text{N}$ ]-enrichment of the meal was 1.07 AP.

For the carbon part:

$$\text{Dietary C} = C_{\text{tot-mmol}} \times (\text{APE}_{\text{sample}}/\text{APE}_{\text{meal}})/\text{ingested C}$$

in which  $C_{\text{tot-mmol}}$  is the amount of total carbon (C) in the sample;  $\text{APE}_{\text{sample}}$  is [ $^{13}\text{C}$ ]-enrichment percent excess of the sample = enrichment of the sample – basal enrichment; and  $\text{APE}_{\text{meal}}$  is [ $^{13}\text{C}$ ]-enrichment percent excess of the meal = enrichment of the meal – basal enrichment of the diet. The conversion factor from C to protein was assumed to be 2 (50% of C in proteins). The [ $^{13}\text{C}$ ]-enrichment of the meal was 1.67 AP.

**Postprandial deamination and oxidation of dietary AA.** The recovery of dietary AA in the urea body pool 8 h after the meal, expressed in percentage of ingested N, was calculated according to the following formula:

$$N_{\text{exo-urea}} = [\text{urea}] \times 2 \times \text{TBW}/0.92 \times (\text{APE}_{\text{urea}}/\text{APE}_{\text{meal}})$$

in which [urea] is the concentration of urea in the plasma; TBW is total body water, calculated with Watson equations; and 0.92 is the water percentage in plasma.

Total deamination of dietary AA was calculated as the sum of the dietary protein recovered in urinary urea and the body urea pool during the 8 h after the meal. The carbon from dietary proteins recovered in expired air was calculated by combining [ $^{13}\text{C}$ CO<sub>2</sub>] enrichment and CO<sub>2</sub> production, as follows: Dietary carbon recovery =  $[(\text{CO}_2/22.4) \times (\text{APE}_{\text{expired air}}/\text{APE}_{\text{meal}})] \times 100/\text{Ingested C}$  from proteins.

Finally, the total oxidation of dietary AA was calculated as the sum of dietary carbon recovery in expired air at each 30-min period, as described below:

$$\text{Total oxidation} = \sum_{t=0}^{t=8} \sum_t \text{dietary C recovery} \times 2$$

in which 2 is the factor to take in account that CO<sub>2</sub> has been measured only 30 min/h.

**Whole-body glucose production and the contribution of dietary AA.** The flux of EGP was calculated from the  $^2\text{H}$  enrichment (E) in plasma glucose from steady-state equation:

$$R_{\text{endogenous}} = (V_1 \times \text{BW} \times 99)/(E_{\text{sample}} - E_{\text{baseline}})$$

in which  $R_{\text{endogenous}}$  is the flux of EGP ( $\mu\text{mol} \cdot \text{min}^{-1}$ ); and  $V_1$  is the [ $6,6\text{-}^2\text{H}_2$ ]-glucose infusion rate ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ). The infused [ $6,6\text{-}^2\text{H}_2$ ]-glucose was 99% enriched.

The appearance flux of plasma glucose produced from dietary AA ( $R_{\text{exogenous}}$  in  $\mu\text{mol} \cdot \text{min}^{-1}$ ) was calculated from the results of  $^{13}\text{C}$  enrichment (E') in plasma glucose, as described below:

$$R_{\text{exogenous}} = ([E'_{\text{sample}} - E'_{\text{baseline}}]/[E'_{\text{neoAAmeal}} - E'_{\text{baseline}}]) \times R_{\text{endogenous}}$$

in which  $E'_{\text{neoAAmeal}}$  is the  $^{13}\text{C}$  enrichment of gluconeogenic AA in the meal proteins (i.e., 1.28 AP).

**Nutrient oxidation.** Substrate oxidation was calculated using the classical stoichiometric formulae (22).

**Statistics.** Results were expressed as means  $\pm$  SD. Differences between the initial value and the values determined during the 8-h postprandial period were tested within the mixed model by contrast analysis (SAS 9.1; SAS Institute, Cary, NC), with time as a repeated factor. Differences were considered to be statistically significant at  $P < 0.05$ .

## RESULTS

**Plasma levels of urea and AAs.** Plasma levels of urea were  $4 \pm 0.5$  mmol/L at the baseline and stable on the postprandial window (not shown). Plasma concentrations of total and indispensable AA rose significantly during the first 6 h after meal ingestion (Fig. 1). Plasma levels of indispensable AA were doubled during the first 3 h, reaching  $1,360 \pm 308$   $\mu\text{mol/L}$ , and had not completely returned to their initial levels after 8 h. Plasma concentrations of non-indispensable AA (not shown) and gluconeogenic AA rose by 40% during the first 3 h and then decreased sharply; they were no longer significantly different from baseline at 4 h. **Transfer of dietary AAs to nitrogen pools.** The transfer of dietary AAs into plasma AAs increased sharply during the first 3 h after meal ingestion, peaked at between 3 and 4 h, and then decreased (Fig. 2A). The contribution of dietary AA to plasma protein increased steadily for 7 h after meal ingestion and then reached a plateau (Fig. 2B). The incorporation of  $^{15}\text{N}$  in plasma proteins was always lower than that of  $^{13}\text{C}$ . The maximum participation of dietary AA in plasma protein synthesis over the 8-h postprandial period was  $7.1 \pm 0.5$  and  $8.5 \pm 1.4\%$  of ingested AA, respectively, depending on the nitrogen and carbon tracers. **Dietary AA deamination and oxidation.** The cumulative transfer of both dietary AA  $\alpha$ -amino groups to urinary urea and of carbon skeletons to expired  $\text{CO}_2$  increased significantly during the 8 h following meal ingestion (Fig. 3). At the end of the 8-h postprandial period,  $316 \pm 100$  mmol of urea has been excreted,  $17.5 \pm 2.3\%$  of carbons from dietary protein had been transferred into expired  $\text{CO}_2$ , and  $8.6 \pm 2.7\%$  of dietary nitrogen had been excreted into urinary urea. At the same time,  $9.5 \pm 2.2\%$  of dietary nitrogen was transferred into plasma urea (data not shown), which implied that the total deamination of dietary AA involved  $18.1 \pm 3.5\%$  of dietary nitrogen. As a consequence, the oxidation of dietary AA did not differ significantly from their total deamination during the 8 h

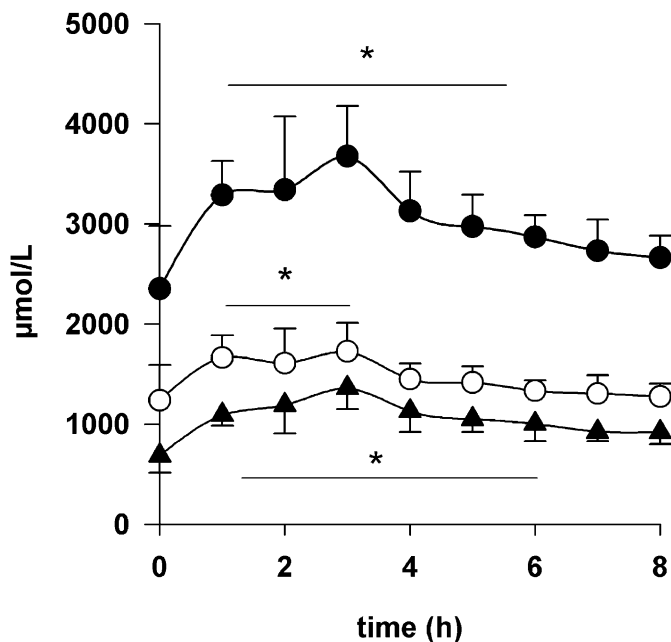
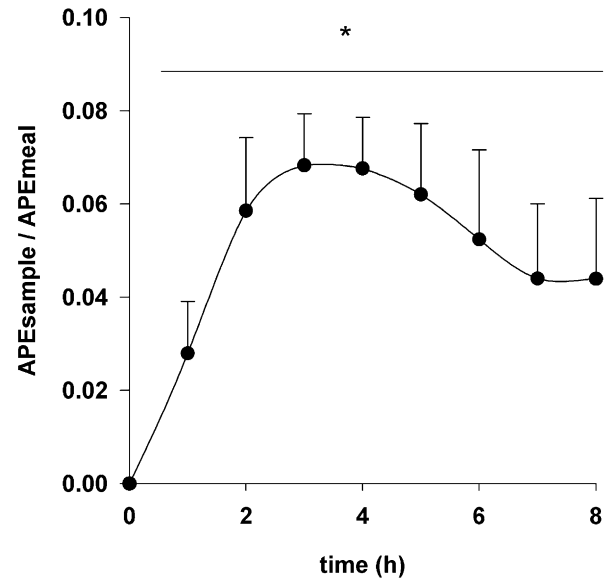


FIG. 1. Plasma levels of AAs: total (black circles), indispensable (black triangles), and gluconeogenic (open circles) during the 8 h after ingesting four eggs containing 23 g of doubly [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-labeled proteins. Data are means  $\pm$  SD ( $n = 8$  at each time point). \* indicates a significant difference from the initial value:  $*P < 0.05$ .

## A Transfer of dietary AA to plasma amino acids



## B Transfer of dietary AA to plasma protein

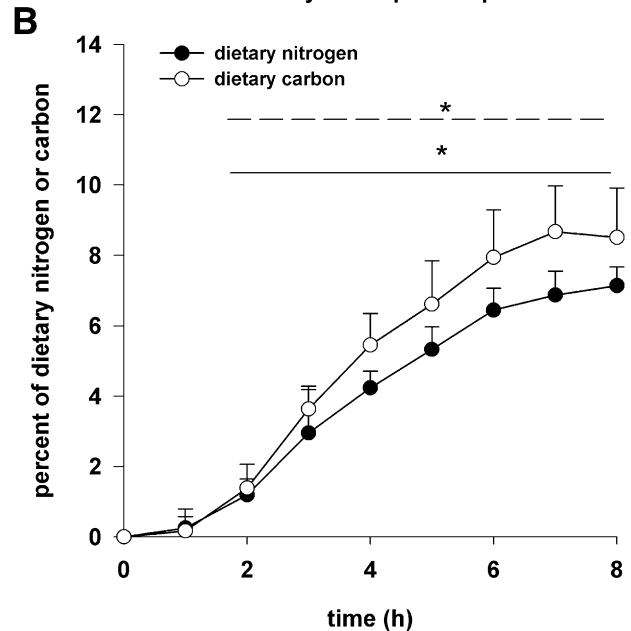
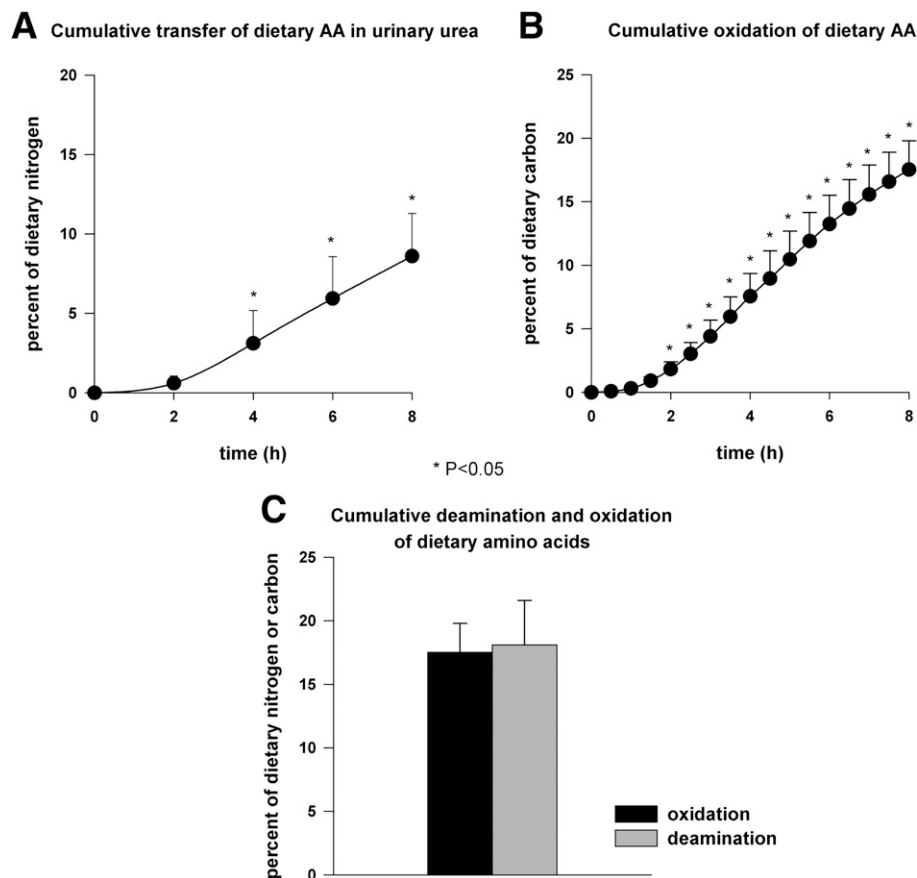


FIG. 2. Transfer of dietary AAs into plasma AAs (A) and proteins (B) in human subjects during the 8 h after ingesting four eggs containing 23 g of doubly [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-labeled proteins. Data are means  $\pm$  SD ( $n = 8$  at each time point). \* indicates a significant difference from the initial value:  $*P < 0.05$ .

after meal ingestion. By contrast, at 4 h after the meal, deamination was significantly higher than oxidation, reaching  $14 \pm 4$  and  $7.6 \pm 1.8\%$  of dietary AA, respectively. **Plasma glucose and EGP.** Plasma glucose concentrations did not change significantly for the first 6 h after meal ingestion (Fig. 4A) and then decreased significantly.

EGP did not change significantly after meal ingestion and remained constant until 4.5 h, fluctuating between 0.6 and  $0.7 \text{ mmol} \cdot \text{min}^{-1}$  (i.e.,  $8\text{--}10 \mu\text{mol} \cdot \text{kg} \cdot \text{min}^{-1}$ ) (Fig. 4B). The level started to fall off from 5 h after meal ingestion to reach  $0.5 \pm 0.1 \text{ mmol} \cdot \text{min}^{-1}$  at 8 h. Total glucose production over the 8-h postprandial period was



**FIG. 3.** Cumulative transfer of dietary AAs to urinary urea (A) and expired CO<sub>2</sub> (B) and dietary AA total deamination and oxidation in human subjects (C) after ingesting four eggs containing 23 g of doubly [<sup>15</sup>N]-[<sup>13</sup>C]-labeled proteins. Data are means ± SD (*n* = 8 at each time point). \* indicates a significant difference from the initial value: \**P* < 0.05.

50.4 ± 7.7 g glucose. The contribution of dietary AA to this glucose production rose sharply until 3 h after meal ingestion and peaked between 3.5 and 5 h, the maximum contribution being 12.1 ± 3.3% of total glucose production at 4.5 h after the meal (Fig. 4B). From 5–8 h after the meal, this contribution decreased to reach 8 ± 2% at the end of the postmeal period. Finally, only 3.9 ± 0.7 g of EGP originated from dietary AAs.

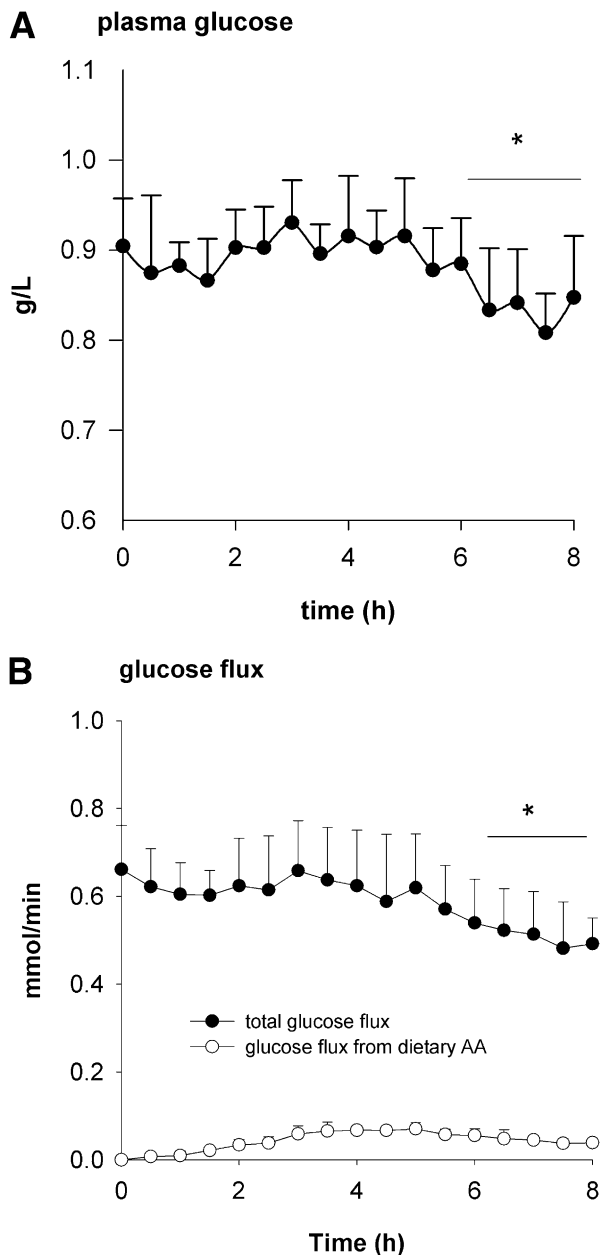
Plasma insulin levels increased up to 30 min after meal ingestion and then remained constant until 2.5 h, at ~50 pmol/L (Fig. 5A). During this period, the levels were significantly higher than baseline at each time point. After 3 h, plasma insulin levels started to fall, reaching values that were significantly lower than the initial value at 7 and 8 h after meal. Plasma glucagon levels tended to rise (*P* < 0.1) after meal ingestion and peaked at 1.5 h (Fig. 5B). Between 1.5 and 8 h after the meal, plasma glucagon concentrations did not differ significantly from baseline.

**Postprandial nutrient oxidation.** The oxidation of carbohydrates and lipids varied significantly during the postprandial window, in contrast to proteins (Fig. 6). Carbohydrate oxidation increased 2 h after the meal and then decreased after 4 h, whereas lipid oxidation increased during the same period. Over the 8-h postprandial period, 26 g of lipids were oxidized, contributing to the production of 977 ± 427 kJ. Despite the negligible amount of energy ingested as carbohydrate, 52 g of carbohydrates were oxidized, supplying 885 ± 317 kJ. Lastly, total protein oxidation was 31 g, producing 530 ± 155 kJ.

## DISCUSSION

The objective of this study was to determine the metabolic conversion of dietary AAs into energy and glucose metabolism, using multi-isotope methods that included the uniform double labeling of dietary AAs and a peripheral infusion of deuterated glucose. After the ingestion of a lipid-protein meal (four eggs), 18% of AAs were deaminated, producing carbon skeletons that were entirely oxidized within 8 h of the meal. EGP did not increase after the meal but remained stable for 6 h and then fell. Of the 50 g glucose produced over the 8-h period, 4 g originated from dietary AAs, indicating that under our experimental conditions, dietary protein-derived AAs contributed to only 8% of EGP.

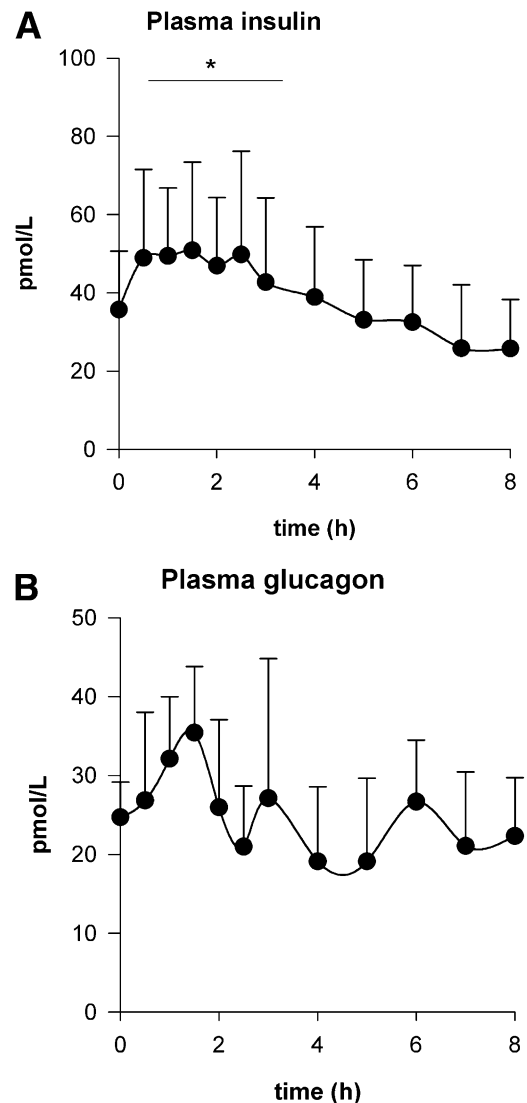
The ability of dietary proteins to generate carbon skeletons from deamination depends on the source of the protein and the habitual protein intake of the subjects, as deamination losses ranging from 18–30% have been seen in studies using a similar methodology (8,9,23–25). During the current study, the losses obtained with egg proteins were 18% of intake, which is comparable to that observed after the ingestion of total milk proteins, indicating a good retention in protein pools. Interestingly, we found that all deaminated AAs were oxidized within 8 h of meal ingestion, whereas after 4 h, only half of them were being used as an energy substrate. This compliments our previous suggestion that the use of carbon skeletons in energy pathways was delayed after the deamination process because of the saturation of carbon cycles and a lack of



**FIG. 4.** Plasma glucose concentrations (**A**), total EGP rate (**B**, black circles), and contribution of dietary AAs to this production (**B**, open circles) in human subjects during the 8 h after the ingestion of four eggs containing 23 g of doubly [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-labeled proteins. Data are means  $\pm$  SD ( $n = 8$  at each time point). \* indicates a significant difference from the initial value: \* $P < 0.05$ .

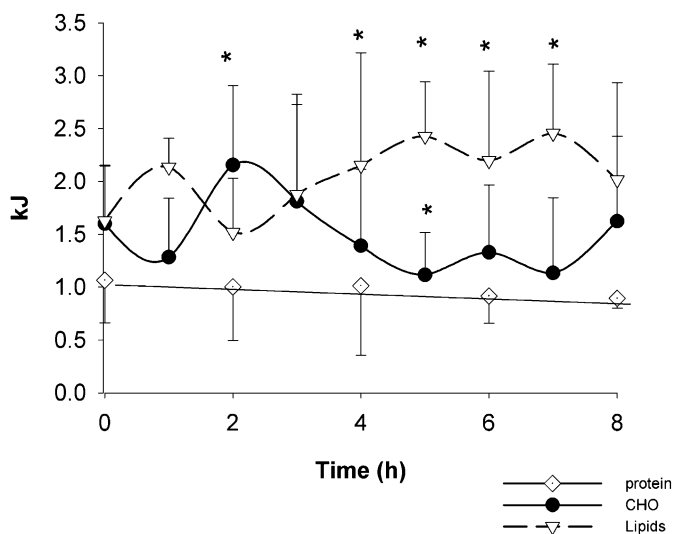
energy storage by these substrates (11). Although the dietary AA oxidation was only approximated because of the variable  $^{13}\text{C}$  enrichment among AAs, the result at 4 h was very consistent with that obtained in rats using extrinsic tracers (11), suggesting that the error was marginal. The retention in tricarboxylic acid pool could have been a source of underestimation, but this should be of limited extent due to the labeling on all carbons instead of a unique position (26).

Under optimum gluconeogenic conditions, the 8-h postprandial production of glucose was  $\sim 50$  g, and the average flux of EGP ranged from  $\sim 10 \mu\text{mol} \cdot \text{min} \cdot \text{kg}^{-1}$  before meal ingestion to  $7 \mu\text{mol} \cdot \text{min} \cdot \text{kg}^{-1}$  8 h after the meal, which is consistent with other studies performed after the



**FIG. 5.** Plasma levels of insulin (**A**) and glucagon (**B**) in human subjects during the 8 h after ingesting four eggs containing 23 g of doubly [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-labeled proteins. Data are means  $\pm$  SD ( $n = 8$  at each time point for insulin and  $n = 3$  at 1.5 h;  $n = 4$  at 5 h;  $n = 5$  at 0, 1, 6, and 7 h;  $n = 6$  at 0.5, 4, and 8 h;  $n = 7$  at 3 h; and  $n = 8$  at 2 and 2.5 h for glucagon). \* indicates a significant difference from the initial value: \* $P < 0.05$ .

ingestion of meals with varying compositions (27–29). Our results also showed that the ingestion of four eggs did not induce an increase in EGP, as the level remained constant until 5 h after the meal, decreasing significantly thereafter. Interestingly, we observed that this decrease was concomitant with the fall in circulating glucose levels. Some studies had previously focused on EGP following the ingestion of an HP meal or the infusion of AAs. Two studies in healthy humans showed that an alanine infusion induced an increase in gluconeogenesis from alanine but no change in EGP. This could be explained by either an inhibition of gluconeogenesis from other substrates (30) or a decrease in glycogenolysis (31). Indeed, the stimulation of one pathway for glucose production may be counteracted by a downregulation of other pathways in order to maintain stable global production (27,32). It is, however, important to emphasize the fact that pancreatic hormones (insulin and glucagon) were clamped at basal levels in these two experiments, and this must have influenced the



**FIG. 6.** Nutrient oxidation during the 8 h after the ingestion of four eggs containing 23 g of doubly [ $^{15}\text{N}$ ]-[ $^{13}\text{C}$ ]-labeled proteins. Lipids (open triangles), carbohydrates (CHO; black circles), and proteins (open diamonds). Data are means  $\pm$  SD ( $n = 8$ ). The effect of time was significant for lipids ( $P = 0.01$ ) and carbohydrates ( $P = 0.007$ ). \* indicates a significant difference from the initial value: \* $P < 0.05$ .

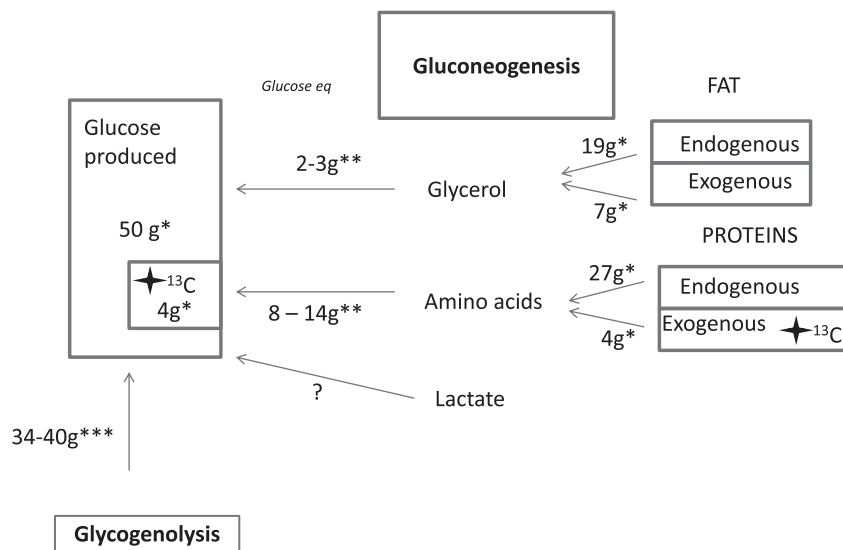
results. Indeed, another study performed in healthy subjects but without any control of the plasma hormone levels demonstrated that an infusion of AA led to a twofold rise in EGP, together with a threefold increase in gluconeogenesis (5). Our experimental design was different from that work because we were studying the effects of meal ingestion rather than a peripheral and continuous infusion of AAs. As for the effects of ingesting an HP meal, one study pointed out that the ingestion of 50 g meat proteins (without carbohydrates) led to a slight but steady decrease in EGP in subjects with type 2 diabetes (33). EGP is upregulated in diabetic patients, but this impairment is especially marked when insulin secretion is stimulated (34–36). Thus, in the case of a carbohydrate-free meal, and despite a slight stimulation of insulin secretion, it is likely that the EGP response will be similar in healthy and diabetic patients. In healthy subjects, Khan et al. (12) showed that ingesting  $\sim$ 50 g protein from cottage cheese with a small quantity of carbohydrates and lipids induced an increase in EGP during the first 3 h, followed by a marked decrease from 3 to 5 h. The results have diverged among studies, because of either the experimental conditions (infusion vs. meal) or variations in subject status (healthy vs. diabetic) or regarding the composition of the meal (presence or absence of carbohydrates, amount of proteins, and energy supply).

The use of intrinsically labeled egg proteins enabled a quantification of the contribution of dietary protein to this production, which to our knowledge had never previously been determined. We showed that after a normal intake of protein (20–25 g), the contribution of dietary protein to glucose production was small and did not exceed 10% of the total flux during the 8-h postprandial period, contributing the production of 4 g glucose to 50 g of total glucose production. As 18% of dietary AAs were deaminated, this represented about 4 g AA, among which 43% were gluconeogenic in both egg-white and egg-yolk proteins (37), resulting in 1.7 g of AA that could contribute to glucose production. Considering that three carbons in each AA would be transferred to a glucose molecule, we

can assume that 1 mol of AA would contribute to 1/2 mol glucose. However, the dilution of dietary AA in the endogenous pools results in a low probability that two labeled AAs are incorporated in the same glucose molecule. If the three remaining carbons are supplied by precursors other than dietary AAs, 1.7 g of gluconeogenic AA might thus participate in the production of 3 g glucose, a theoretical value that is comparable to our findings. This hypothesis that 1 mol of glucose contains only three carbons from dietary AAs needs to be confirmed with an isotopomer analysis, but this will require the administration of highly enriched gluconeogenic AAs. To our knowledge, the study of Khan et al. (12) is only one to have assessed postprandial gluconeogenesis due to dietary proteins in humans. The results pointed out that  $\sim$ 10 g of glucose was produced from proteins during the 8 h after the meal, which is consistent with our findings regarding the amount of protein ingested (50 g). This strongly suggests that the participation of dietary protein to glucose production mainly depends on the amount of gluconeogenic AA available, in the context of a low-carbohydrate meal. Altogether, we conclude to the robustness of our result, despite methodological limits, such as the heterogeneous  $^{13}\text{C}$  labeling of AA, the possible loss of carbons in tricarboxylic acid cycle, and the absence of access to the precursor pool (i.e., intrahepatic AAs).

The fact that the contribution of dietary AAs was low does not preclude any high participation of proteins during fasting. Gluconeogenesis rate during fasting and after exhaustion of glycogen store has been shown to be higher in subjects fed an HP than normal-protein diet (4), suggesting a stronger use of body protein in glucose production during the catabolic phase. In our study, 31 g of protein was oxidized, whereas only 4 g was of dietary origin. Postulating that 45% of endogenous AAs are gluconeogenic (based on basal circulating AA profiles),  $\sim$ 6–12 g of endogenous AAs might therefore be used as glucose precursors, depending on the source of the complementary three carbons in the molecule.

In addition to AAs, other potential sources for glucose production included other exogenous (glycerol from dietary and body fat) or endogenous (mainly residual hepatic glycogen stores but also lactate or glycerol) sources. As the test meal contained no carbohydrate but 19 g lipids, the glycerol released by the postprandial catabolism of these lipids might be the precursor of EGP. However, considering that egg lipids comprise 66% triglycerides and 33% phospholipids (38), 28 mmol of glycerol would be released if they were fully catabolized, which is likely to have occurred since 26 g of lipids were oxidized. This would produce 2.5 g of glucose. In consequence, another endogenous source must be the major source of glucose precursors, and hepatic glycogen is a good candidate because the stores that remain after fasting overnight are still significant in normal subjects. Using  $^{13}\text{C}$  nuclear magnetic resonance, it has been shown that liver glycogen concentrations reached 207 mmol/L with a liver volume of 1.44 L in humans fasted overnight (39), corresponding to  $\sim$ 70 g. Such glycogen stores could alone account for the entire 8-h postprandial endogenous production of glucose (50 g). It has been shown that after overnight fasting, glucose production from gluconeogenesis was  $1.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ , corresponding to 34 g glucose (40), a value that is also consistent with the predictions of the modeling study performed by König et al. (41). In our study, a correlation analysis between glucose flux and nutrient oxidation



**FIG. 7.** Possible sources of the glucose produced. We observed that 50 g of glucose was produced but that only 4 g was synthesized from dietary AAs. Endogenous AAs could have significantly contributed to glucose production since 27 g was oxidized. The complete dietary fat oxidation would generate 28 mmol of glycerol and lipolysis 8 mmol. Fat oxidation could then not be a major contributor. Finally, residual glycogen could have been the major contributor under our experimental conditions. \*, measured; \*\*, calculated; \*\*\*, speculated from the literature.

showed that protein and lipid oxidation were not correlated to EGP, in contrast to carbohydrate oxidation ( $R = 0.24$ ;  $P = 0.04$ ), thus supporting the hypothesis that glycogen was the principal contributor to glucose flux under our experimental conditions. Fig. 7 depicts the possible precursors of glucose production based on our results and on the hypothesis for nonobserved values.

In conclusion, this study highlighted the fact that a significant proportion of the carbon skeletons released from dietary AA deamination was converted into glucose during the 8 h following the ingestion of a protein-lipid and carbohydrate-free meal. In the context of a low-calorie meal, gluconeogenesis is likely to be maximal compared with carbohydrate-containing meals. Despite this, the contribution of dietary AAs to glucose was 8%, depending mainly on the availability of gluconeogenic AAs, whereas that of endogenous AAs could reach 20%. It is likely that most of this production, ~70%, was due to the degradation of residual liver glycogen, which could be sufficient, even after an overnight fast. We can thus suppose that the participation of dietary proteins will be negligible in the presence of carbohydrates in the meal. We provided the first direct evidence that under optimal gluconeogenic conditions and in a realistic nutritional situation, dietary proteins only make a relatively modest contribution to the maintenance of blood glucose levels.

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