Copper Deficiency Increases Total Protein and Apolipoprotein A-I Synthesis in the Rat Small Intestine[1,2] (44028)

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Abstract. **This study was designed to determine whether an enhanced intestinal synthesis of apolipoprotein (apo) A-I is associated with the hyperapolipoproteinemia observed in copper-deficient rats. Male weanling Sprague-Dawley rats were assigned to two dietary treatments, Cu deficient (0.6 ppm Cu) and Cu adequate (6.0 ppm Cu) for 6 weeks. *In vivo* studies were then performed after rats were injected with a flooding dose of 150 μM [^{3}H]phenylalanine (PHE, 50 μCi/ml/100 g body wt). Three rats from each treatment were sacrificed at 5, 10, 15, 30, and 60 min postinjection. The small intestine was rapidly rinsed and frozen in liquid N_2. *In vitro* studies were performed by labeling freshly isolated 6-cm segments from duodenum, jejunum, and ileum with [^{3}H]PHE (33 μCi/ml, 49.7 Ci/mmol) in PHE-free minimum essential medium for 7 and 14 min. *In vivo* and *in vitro* intestinal samples were sonicated, solubilized in 1% Triton X-100, and centrifuged to provide the detergent soluble fraction for the isolation of nascent apo A-I and total protein. Radioactivities associated with nascent apo A-I isolated by immunoprecipitation and SDS-PAGE, and with total protein precipitated by trichloroacetic acid, were measured to determine the influence of Cu deficiency on nascent apo A-I and total protein synthesis. In the Cu-deficient small intestine, the synthesis of total protein was measured only in the duodenum and was enhanced after 1 hr for the *in vivo* studies. Moreover, total protein synthesis was enhanced at both 7 and 14 min of the *in vitro* studies for all three small intestinal segments of the Cu-deficient rats. Apo A-I synthesis was measured only at the jejunum and was also enhanced by Cu deficiency in the *in vitro* studies. Thus, an increase in intestinal apo A-I synthesis may contribute to the elevated plasma apo A-I level in Cu-deficient rats.**

[P.S.E.B.M. 1996, Vol 212]

In 1973, Klevay (1) induced hypercholesterolemia in copper (Cu)–deficient rats by feeding a high ratio of zinc to Cu. Numerous laboratories have confirmed that the hypercholesterolemia can be induced in rats by Cu deficiency alone. Moreover, the hypercholesterolemia induced by Cu deficiency has also been established in studies with various species, including a study with a single human (reviewed in Ref. 2). In Cu-deficient rats, hypercholesterolemia is primarily associated with elevated high-density lipoproteins (HDL) (3–5), in which increases in both cholesterol and protein levels have been observed (5). As the major apolipoprotein in HDL, plasma apo A-I pool size was also elevated in Cu-deficient rats when it was normalized to the enlarged plasma volume (5). In an *in vivo* study, the absolute catabolic rate of labeled HDL total apolipoprotein removal from plasma was higher in Cu-deficient rats (6). The distribution of radioactivity among the major HDL apolipoproteins (apo A-I, apo Cs, apo E, apo A-II, apo A-IV) was not significantly altered during the experimental period in both Cu-deficient and control rats, indicating that the pattern of removal of HDL apolipoproteins was not spe-

[1] This study was presented in 1994 Experimental Biology Meeting (FASEB J 8:A711).

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Supported by USDA National Research Initiative Program, Human Nutrition Competitive Grants (92-37200-7579), and by funds from the University of Arizona Agricultural Experiment Station.

Received September 5, 1995. [P.S.E.B.M. 1996, Vol 212]
Accepted April 15, 1996.

0037-9727/96/2124-0369$10.50/0

cific. Thus, the clearance of apo A-I from plasma may also be increased, instead of decreased, in Cu-deficient rats. The liver is a major organ that takes up HDL particles, and the HDL taken up are associated primarily with parenchymal cells. *In vivo*, the uptake of HDL protein by the liver was not altered in Cu-deficient rats. In the *in vitro* binding studies with liver parenchymal cells (7), apo E-free-HDL was used to prevent cross-reaction between HDL and apo E receptor. The amount of surface bound apo E–free HDL was higher in the cells derived from Cu-deficient rats, while the amount of apo E–free HDL internalized was not changed. These observations suggested that the elevated plasma HDL protein (mainly apo A-I) in Cu-deficient rats was due not to a reduction in plasma clearance but rather to an increase in synthesis.

The liver and small intestine are well recognized as the most important sites of lipoprotein and apo A-I synthesis (8). The mRNA level of apo A-I is relatively high in both the small intestine and liver of humans (9), rats (10), mice (11), and rabbits (12). In the adult rats, the apo A-I mRNA ratio of liver to small intestine is 36 to 100. Wu and Windmueller (8) estimated that *in vivo* about 56% of the circulating apo A-I was contributed by small intestine in the rat. In a pulse-chase study using freshly isolated rat hepatic parenchymal cells, Hoogeveen *et al.* (13) demonstrated that the apo A-I synthesis was significantly increased in the cells derivedfrom the Cu-deficient rats. The intracellular nascent apo A-I content and the amount of labeled apo A-I secreted into the culture medium were also significantly higher in the cells derived from the Cu-deficient rats.

In view of the high capacity of the small intestine to synthesize total protein and apo A-I, the present studies were designed to investigate the effect of copper deficiency on total protein and apo A-I synthesis in the rat small intestine.

Materials and Methods

Experimental Animals and Experimental Diets. Male weanling Sprague-Dawley rats, initially weighting between 40 and 60 g, were housed individually in suspended stainless steel wire cages in an animal room with 12:12-hr light (700 to 1900 hr):dark (1900 to 700 hr) cycle and maintained at 20°–22°C. Diets and distilled-demineralized water were provided *ad libitum*.

Rats were randomly divided into two dietary treatments: Cu-deficient diet (CuD, 0.6 ppm Cu) and Cu-adequate diet (CuA, 6.0 ppm Cu). The basal diet (copper-deficient) was modified in a number of ways from the formulation of the American Institute of Nutrition (14). Modifications included the absence of Cu supplement in the mineral mix, a different source of carbohydrate and a different amount of corn oil (Table I). This basal diet contained 0.6 mg Cu/kg diet as determined by atomic absorption spectrophotometry (Model 180-70; Hitachi, Sunnyville, CA). The Cu-adequate (control) diet was prepared by adding a cupric carbonate mix (1.05 g of cupric carbonate in 998.05 g glucose) to the basal diet to obtain a final concentration of 6 mg Cu/kg diet. The animals were fed their respective diets for 6 weeks prior to the experiments. To investigate the influence of Cu repletion on *in vitro* total protein synthesis, an additional group of animals originally fed the Cu-deficient diet for 6 weeks were fed the Cu-adequate diet for 1 day just prior to the experiments. Food was withdrawn 1 hr after the beginning of the dark cycle, and rats were fasted for 10–12 hr prior to the experiments. Experimental protocol was approved by the University of Arizona Animal Use and Care Committee. The copper content of diets and tissues were determined by atomic absorption spectrophotometry (6).

Table I. Diet Composition

Ingredient	Diet	
	Copper adequate	Copper deficient
Glucose monohydrate	64	65
Casein	20	20
Fiber cellulose	5	5
Corn oil	4	4
AIN mineral mix[a]	3.5	3.5
AIN vitamin mix (water soluble)	1	1
AIN vitamin mix (fat soluble)	1	1
DL-methionine	0.3	0.3
Choline bitartrate	0.2	0.2
Cupric carbonate mix (1.05 g $CuCO_3$ in 998.95 g glucose)	1	—
Dietary Cu content (ppm)[b]	6	0.6
ME (Kcal/g)	4.11	4.11

Note. Values are expressed as percentage of total composition unless noted.
[a] Contains all minerals except copper.
[b] As determined by atomic absorption spectrophotometry.

***In Vivo* Labeling of Newly Synthesized Intestinal Protein.** To examine the rate of protein synthesis in the rat small intestine, an *in vivo* study was performed according to the method of Garlick *et al.* (15). After 6 weeks of dietary treatment, rats were anesthetized with a subcutaneous injection of sodium pentobarbital (5 mg/100 g body wt). Three rats were randomly selected from each dietary treatment and assigned to be sacrificed at 5, 10, 15, 30, and 60 min after L-[4-^{3}H]phenylalanine injection. A flooding dose of L-[4-^{3}H]phenylalanine (1 ml/100 g body wt of a 150 mM, 50 mCi/l, ^{3}H-phenylalanine solution) was injected *via* the portal vein. At various designated time periods after injection, similar to those used in previous *in vivo* studies (13), rats were anesthetized further by ether inhalation. Blood was collected in a syringe containing ethylenediamine tetracetic acid (EDTA) (1 mg/ml blood) by cardiac puncture and hematocrit was measured immediately. Hearts and livers were rapidly excised and weighted. Small intestines (SI) were quickly removed and rinsed in ice-cold phosphate-buffered saline (PBS: 10 m*M* sodium phosphate, pH 7.4, 154 m*M* sodium chloride). Then the SI were frozen with liquid N_2 and stored at −80°C until further processing.

***In Vitro* Labeling of Newly Synthesized Intestinal Protein.** The rate of intestinal protein synthesis was also examined *in vitro* by the method of organ culture (16, 17) with some modifications. At the end of 6 weeks of dietary treatment, four rats from each treatment were anesthetized with ether and sacrificed. Blood was collected, and liver and heart were excised and weighted as described in the *in vivo* studies. The small intestines were quickly removed and cut into three equal segments, representing the duodenum, jejunum, and ileum. A 6-cm segment was obtained from the proximal duodenum, the central jejunum, and the distal ileum. The gut segments were opened, washed in 37°C saline (0.15 *M* NaCl with 10 units penicillin and 10 μg streptomycin/ml), dried on pre-moistened paper towels, and cut longitudinally into two halves. Each half was cut into two 3-cm pieces, inverted to expose mucosa, and pre-incubated (5% CO_2, 95% air) in a 35 × 10–mm culture dish (Falcon, Lincoln Park, NJ) with 1 ml of phenylalanine-free MEM (generated from a MEM select-amino acids kit, containing 0.1 m*M* nonessential amino acids, 1 m*M* sodium pyruvate, 10 units penicillin and 10 μg streptomycin/ml, 20 m*M* HEPES, pH 7.4, without sodium bicarbonate, all obtained from GIBCO, Grand Island, NY) for 20 min in a 37°C incubator. Then 0.5 ml of this medium (37°C) containing 50 μCi ^{3}H-phenylalanine (49.7 Ci/mmol, NEN, Wilmington, DE) was added. After the tissues were pulsed for 7 or 14 min (one half for 7 min and the other half from the same segment for 14 min), they were rapidly rinsed in ice-cold saline, and dried. The tissue was suspended in 1 ml of ice-cold lysis buffer (150 m*M* NaCl, 150 m*M* Tris, pH 7.4, 15 m*M* EDTA, 187.5 m*M* sucrose, 1% Triton X-100, 0.5% sodium deoxycholate, and freshly added protease inhibitors [2 m*M* PMSF, 0.1 m*M* TPCK, 100 kallikrein-inactivating units/ml aprotinin, 0.01 mg/ml of leupeptin, 10 mg/ml benzamidine]), immediately frozen with liquid N_2 and stored at −80°C.

Preparation of Detergent Soluble Fraction. Frozen samples from *in vitro* study (SI segments plus 1 ml lysis buffer/sample) were left at room temperature until they were almost thawed, then placed on ice bath. In the *in vivo* study, the duodenum part of small intestine was put on ice until almost thawed. Then a 3-cm segment was excised and put into a tube containing 1 ml ice-cold lysis buffer. All samples from both *in vivo* and *in vitro* studies were sonicated for four times, 30 sec each with a 15-sec interval, by a sonicator (Sonic Dismembrator model 150; Fisher, Pittsburgh, PA) at 55% power. The sonicatant was centrifuged at maximum speed for 5 min in a centrifuge (Model 5415c; Eppendorf, Madison, WI) at 4°C to obtain a detergent-soluble supernatant fraction. Newly synthesized total protein in the detergent soluble fraction was precipitated by trichloroacetic acid (TCA). Nascent apo A-I was immunoprecipitated with monospecific anti-rat apo A-I polyclonal antibody and further isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The amount of radioactivities associated with newly synthesized total protein and apo A-I was quantitated by scintillation counting.

Total Protein Measurement. The total protein concentration of the detergent-soluble fraction was measured colorimetrically by using the method of Lowry *et al.* (18). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) served as the protein standard.

Trichloroacetic Acid Precipitation. Aliquots of 50 μl detergent-soluble samples were mixed with 1 ml ice-cold 10% trichloroacetic acid (TCA), vortexed, incubated on ice for 20 min, and then centrifuged for 4 min at 9000 rpm in an Eppendorf centrifuge. The pellet was washed twice with 1 ml 10% TCA, and digested overnight with 1 ml 0.1 *N* NaOH at 4°C. After the addition of 0.1 ml 1 *N* HCl to adjust the pH, 10 ml Ecolite (ICN, Irvine, CA) were added, and the mixture was counted in a liquid scintillation counter (Parckard Tri-carb 460CD; Packard, Downer Grove, IL).

Tissue-Free Phenylalanine Assay. The phenylalanine concentration of the detergent-soluble fraction was measured by using the Sigma phenylalanine kit (Cat. No. 60, Sigma).

Immunoprecipitation of Apo A-I. Sheep anti rat apo A-I polyclonal antisera were produced by our laboratory (13). Apo A-I was immunoprecipitated by the method described by Dixon *et al.* (19) with some modifications. Aliquot of 200 μl detergent-soluble fraction

was diluted with 200 μl NET buffer (150 m*M* NaCl, 5 m*M* EDTA, 50 m*M* Tris, pH 7.4, 0.5% Triton X-100, and 0.1% SDS). Two hundred microliters of preimmune sheep serum (control) or 200 μl anti-rat apo A-I antiserum were added to such mixture and incubated overnight at 4°C. One hundred microliters protein G (2 mg/ml) were added to precipitate IgG-antigen complex, incubated at 4°C for 1 hr. The immunoprecipitate was collected by centrifugation at 14,000 rpm for 30 sec in an Eppendorf centrifuge, the supernatant was quantitatively transferred to a new tube for secondary immunoprecipitation, and the pellet was washed three times with 0.5 ml NET buffer. The immunoprecipitate was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure described by Laemmli (20). The discontinuous system of Laemmli was a 5%–15% gradient gel with 4% stacking gel. The immunoprecipitate was dissolved in 100 μl SDS-PAGE loading buffer (8 *M* urea, 1% SDS, 0.05 *M* Tris-HCl, pH 6.8, 0.01% bromophenol blue, and 5% β-mercaptoethanol added just before use), and heated at 65°C for 1 hr followed by 100°C for 5 min. The mixture was applied to a 5%–15% SDS-PAGE gel (3.0-mm thick). After samples were loaded, electrophoresis was performed at 40-mA constant current until the blue dye reached the bottom of the gel. The low-range SDS-PAGE Molecular Standard (Bio-Rad, Richmond, CA) was used as molecular weight marker. After migration, the gel was stained for about 6 hr in staining solution (50% methanol, 10% acetic acid, 0.25% Coomassie Brilliant Blue R), and destained by several changes of destaining solution (7.5% acetic acid, 5% methanol). A 1-cm gel piece containing rat apo A-I was cut out at the place determined by previous experiments, and a gel of similar size was cut from the same place at an empty lane to serve as the background. The gel pieces were digested overnight at 70°C in the presence of 1.0 ml 30% hydrogen peroxide and counted in the presence of 10 ml Ecolite in a liquid scintillation counter.

Data Analysis. Data were analyzed by one-way (Table II and Fig. 2) or two-way (Cu and time, Fig. 1) analysis of variance (ANOVA) (21), as indicated in the legends. Apo A-I synthesis was analyzed by two-way ANOVA (Cu and time, Fig. 3). The significant level was chosen as $P \leq 0.05$.

Results

Reductions in the body weight, hematocrit and liver Cu concentration, and increases in relative liver and heart weights were observed in rats fed the Cu-deficient diet for 6 weeks (Table II). All of these observations are well-established features of Cu deficiency in rats (22), so the experimental rats were indeed deficient in Cu. In addition, the Cu concentration of the jejunum (Table II) as well as the duodenum and ileum (data not shown) were markedly reduced in the Cu-deficient rats. However, the relative weight of the small intestine was significantly higher (19%) in Cu-deficient rats. Moreover, the free phenylalanine concentration of the jejunum (Table II) as well as the duodenum and ileum (data not shown) were not changed by Cu status. Furthermore, the plasma concentration of phenylalanine and other amino acids were not altered by Cu status (13). Thus, the possibility that any alteration in intestinal protein synthesis rate might be due to a change in the intestinal free phenylalanine concentration and the plasma level of free amino acids in Cu-deficient rats may be excluded.

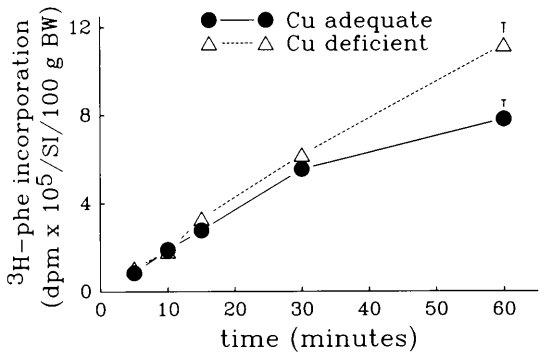

Figure 1. Effect of dietary copper on [^{3}H]phenylalanine incorporation into TCA precipitable intestinal protein in Cu-adequate and Cu-deficient rats. Value of each dietary treatment group represent mean ± SEM from three experiments. Each experiment represents one copper-deficient and one copper-adequate rat sacrificed 5, 10, 15, 30, and 60 min after [^{3}H]phenylalanine injection. Thus, three rats were randomly selected from each treatment and sacrificed at each time point. Response curves were analyzed by two-way ANOVA: Cu effect (df = 1), $P < 0.01$; time effect (df = 4), $P < 0.001$; Cu × time (df = 4), $P < 0.01$. SI, small intestine; BW, body weight.

To determine whether the Cu status may alter the intestinal protein synthesis, a pulse study was performed *in vivo*. The rate of [^{3}H]-labeled phenylalanine incorporation into TCA precipitable protein was measured to determine the rate of *in vivo* intestinal protein synthesis in the duodenal region. In order to obtain a rapidly equilibrated and relatively constant labeled phenylalanine plasma pool, rats were injected intravenously with a flooding dose of 150 μmol [^{3}H]phenylalanine/100 g body wt (15). Incorporation curves of [^{3}H]phenylalanine were shown in Fig. 1. Because the amount of [^{3}H]phenylalanine injected was based on the animal's body weight, the data were also expressed as dpm per intestine per 100 g body wt. Response curves were analyzed by two-way ANOVA and resulted in a significant interaction (Cu × time; df = 4; $P < 0.01$), which indicated that the response curves were significantly different. After 30 min, the response curve in control rats appeared to be approaching a plateau, while in Cu-deficient rats the response curve remained linear up to 60 min. The sig-

Table II. Influence of dietary copper on body weight, relative small intestine (SI), liver and heart weights, hematocrit, plasma and jejunum phenylalanine concentrations, and liver and jejunum copper concentrations

	Copper adequate	Copper deficient	ANOVA[a]
Body weight (g)[b]	302 ± 6	240 ± 7	<0.001
Liver weight (g/100 g body wt)[b]	3.49 ± 0.07	4.20 ± 0.21	<0.01
Heart weight (g/100 g body wt)[b]	0.37 ± 0.01	0.71 ± 0.05	<0.001
SI weight (g/100 g body wt)[c]	2.01 ± 0.03	2.39 ± 0.06	<0.001
Hematocrit (% PCV)[b]	50.2 ± 0.4	30.9 ± 1.8	<0.001
Plasma phenylalanine (μmol/ml plasma)[d]	0.11 ± 0.02	0.13 ± 0.01	NS
Jejunum phenylalanine (μmol/mg protein)[e]	32.1 ± 3.1	32.6 ± 2.8	NS
Liver copper (μg/g dry wt)[e]	10.9 ± 0.6	2.1 ± 0.1	<0.001
Jejunum copper (μg/g dry wt)[e]	4.13 ± 0.15	0.84 ± 0.10	<0.001

Note. Values represent mean ± SEM.
[a] *P* values from one-way ANOVA; NS, not significant ($P > 0.05$).
[b] $n = 7$ rats.
[c] $n = 30$ rats.
[d] $n = 5$ rats.
[e] $n = 4$ rats.

nificant interaction term was resulted mainly because the incorporation was markedly higher in the Cu-deficient than in the Cu-adequate rats at 60 min. Thus, these findings indicated that the rate of intestinal total protein synthesis was higher in Cu-deficient rats. Since the data were expressed as dpm per small intestine per 100 g body wt, the enlarged relative SI weight in Cu-deficient rats (19%; $P < 0.001$) may have contributed to the observed increase in protein synthesis rate. To eliminate this possibility, the data were also expressed as dpm per mg cellular detergent soluble protein, and a very similar plot was obtained (Cu effect: $P < 0.05$; time effect: $P < 0.01$; Cu × time: $P < 0.01$). Thus, Cu deficiency appeared to enhance the *in vivo* total protein synthesis in the rat duodenal intestine.

To confirm the *in vivo* observation, and also to evaluate the ability of protein synthesis along different regions of the small intestinal tract, an *in vitro* study was performed. Freshly isolated gut pieces were rapidly excised from the duodenal, jejunal, and ileal region of intestine. To eliminate the individual variance in this pulse study, each piece was cut longitudinally into two halves and subjected to 7 and 14 min of labeling. The incorporation was normalized and expressed on the basis of dpm per mg of cellular detergent soluble protein. The incorporations of [^{3}H]phenylalanine into total protein after 14-min labeling were twice as high as that after 7-min labeling in all gut pieces (data not shown), indicating that the radioactive label incorporation was linear and the current tissue culture system was performing well. In view of the linear response between 7 and 14 min of incubation, only the [^{3}H]labeled total protein data obtained at 14 min were analyzed by one-way ANOVA and depicted in Figure 2. Consistent with the *in vivo* results, the synthesis of total protein *in vitro* was significantly enhanced in the Cu-deficient rats than in the Cu-adequate rats. In the Cu-deficient rats, total protein synthesis was 3.1-, 2.4-, and 2.1-fold higher in duodenum, jejunum, and ileum, respectively, than it was in Cu-adequate rats. The influence of 1-day Cu supplementation on protein synthesis among the three different intestinal regions was found to be variable. In the duodenum, Cu repletion for 1 day rapidly restored the total protein synthesis back to the control level. While in both jejunum and ileum, such limited copper repletion failed to do so.

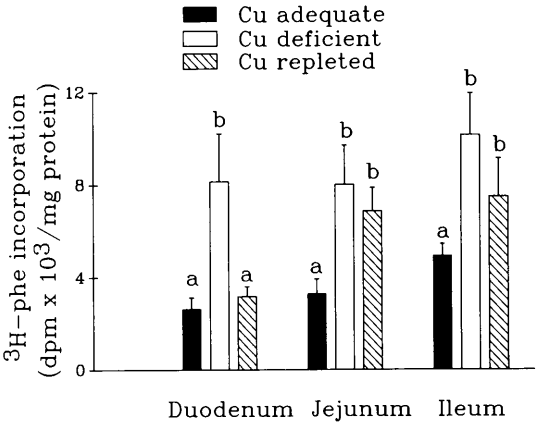

Figure 2. Effect of dietary copper on [^{3}H]phenylalanine incorporation into TCA precipitable total protein synthesized by freshly isolated rat small intestinal segments from Cu-adequate, Cu-deficient, and Cu-repleted rats. Value of each dietary group represent mean ± SEM from four experiments after 14 min of labeling. Because of H^3 incorporation was linear in all cases from 7 to 14 min, this figure only presented the responses after 14 min of labeling. Each experiment represents one Cu-deficient and one Cu-adequate rat used to provide isolated intestinal segments for *in vitro* incubation. Thus, four rats from each treatment were used. Data were analyzed by one-way ANOVA within each intestinal segment, means were ranked by least significance difference test and means with different letters were significantly different ($P < 0.05$).

To address the question of whether Cu deficiency may alter intestinal uptake of free amino acids, the radioactivity associated with TCA soluble component (free [^{3}H]phenylalanine) in detergent-soluble fraction prepared from the intestinal segments (intracellular fraction) were measured in randomly selected samples. After normalizing the data by the amount of cellular protein, which reflects the number of mucosal cells, the TCA soluble counts were found to be similar between the treatment groups, and were about 40- and 80-fold higher than that associated with TCA insoluble total protein in the same aliquot from 7- and 14-min pulse samples, respectively (data not shown). Thus, the ability of the intestine to take up amino acids and the cellular pool size of free labeled substrate in the enterocytes appeared not to be altered by Cu status.

The influence of Cu status on intestinal apo A-I synthesis was also examined in these *in vivo* and *in vitro* studies. The rate of apo A-I synthesis was estimated by the incorporation of labeled phenylalanine into nascent apo A-I which was isolated by immunoprecipitation using a sheep polyclonal antibody against rat apo A-I (13) followed by SDS-PAGE. In the *in vivo* study, we attempted to estimate the rate of nascent apo A-I synthesis in the duodenal region of the small intestine. However, because the incorporation was low, the amount of radioactivity associated with apo A-I was only slightly above the background in several time points, and an accurate estimate of the rate of apo A-I synthesis could not be attained. The effort to investigate the influence of Cu deficiency on the intestinal apo A-I synthesis was therefore concentrated in the *in vitro* study.

Previous data demonstrated that the jejunum was the most active region for intestinal apo A-I synthesis and secretion (16), although substantial amount of apo A-I mRNA was detectable along the entire intestinal tract (10). The *in vitro* apo A-I synthesis was therefore investigated in the jejunal segment. Samples from both 7- and 14-min pulse were examined, and the data were presented in Figure 3. Unlike the synthesis of total protein, the ^{3}H incorporation into nascent apo A-I appeared to have slowed down between 7 to 14 min of the pulse, which may suggest that nascent apo A-I was being secreted into the culture medium during this period of time. In a two-way ANOVA, the effect of Cu treatment was found to be significant ($P < 0.05$), while the effect of labeling time and the interaction were not significant. Thus, apo A-I synthesis was significantly higher in the jejunum of Cu-deficient rats than in that of Cu-adequate rats.

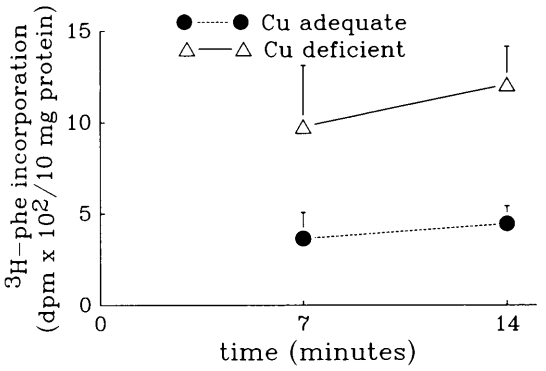

Figure 3. Effect of dietary copper on the rate of [^{3}H]phenylalanine incorporation into immunoprecipitable intestinal apo A-I in fleshly isolated jejunum from Cu-adequate and Cu-deficient rats. Value of each dietary treatment group represent mean ± SEM from four experiments. Each experiment represents one Cu-deficient and one adequate rats used to provide isolated intestinal segments for *in vitro* incubation for 7 and 14 min. Thus, four rats from each treatment were used. Response curves were analyzed by two-way ANOVA: Cu effect ($df = 1$), $P < 0.05$; time effect ($df = 1$), nonsignificant; and Cu × time ($df = 1$), nonsignificant.

Discussion

Depressed growth (23), weight gain (24), liver Cu concentration (23) and hematocrit (5), as well as increased heart weight, heart-to–body weight ratio, and liver-to–body weight ratio (23), are well established symptoms of diet induced Cu deficiency in various animal species. All of these symptoms of Cu deficiency as well as reductions in intestinal Cu level were observed in the present study.

The intestine is a tissue actively engaged in the synthesis of protein. Alpers *et al.* (25) demonstrated that the *in vitro* rate of protein synthesis observed in isotope incorporation studies and expressed as cpm incorporation per mg detergent soluble cellular protein was similar for intestinal slices (mucosal cell + muscle wall) and for intestinal mucosal scrapings, while that for intestinal muscle wall alone was quite small and could be omitted. Thus, the intestinal muscle wall contributed neither to total protein synthesis nor to the detergent-soluble cellular protein pool. Therefore, in the present studies, the total protein synthesis data were obtained directly from homogenate of whole intestinal segment and not from isolated enterocytes (26).

The overall incorporation of radioactivity into TCA precipitable protein was relatively low when compared with other studies. This was probably due to the pulsing conditions. The *in vivo* study was performed using a flooding dose labeling design. Samples from the liver had much higher incorporation rate when compared with that of the intestine from the same animals. Such observations indicated that the intestine may not be very active in the uptake of free amino acids from the circulation, since the mucosal cells are responsible for the absorption of dietary amino acids and transport them to the circulation. In other *in vivo* studies that examined intestinal protein synthesis (8, 27), the radioactive labeled amino acids were administrated into the lumen of the intestine *via*

intestinal cannula, which may reflect protein synthesis using newly absorbed amino acids during the absorptive state. Alternatively, the intestinal protein synthesis may be relatively slow in the fasting state, because the major metabolism of intestine is associated with nutrient absorption and transportation. The incorporation of the radioactive label in the *in vitro* study was also lower than findings reported by other investigators using similar culture systems (17, 28). This may be due not only to the differences in animal species but also to the differences in the amount of radioactivity and the pulsing time used. In these previous studies, up to 1–2 mCi radioactive labeled amino acids were used and the pulsing time ranged from 1 hr (16) up to 24 hr (28). Thus the amount of radioactivity used was higher and the pulsing periods used were longer than the 50 μCi and 14 min employed in our *in vitro* system which used a comparable amount of intestinal tissue. Since the intestine is an organ which actively secretes proteins, and the major objective of this study was to examine the protein synthesis only, the long pulsing time previously used appeared not to be suitable. Furthermore, the morphological study performed by Plumb *et al.* (29) demonstrated that the structural integrity of the mucosa was markedly changed at the tip of the villi after 20 min of incubation in a similar system, which implied that prolonged incubation may lead to severe structural damage. Nevertheless, the linear radioactivity incorporation response in the current *in vivo* and *in vitro* systems indicated that the systems performed well.

An increased intestinal total protein synthetic capacity was observed in the Cu-deficient rats, in the *in vivo* (Fig. 1) and *in vitro* (Fig. 2) studies, respectively. This observation was different from findings obtained from other studies, in which rats treated with high levels of dietary fat (16, 30) or different sources of protein (17), demonstrated either altered intestinal apolipoprotein synthesis or secretion but no change in the overall total protein synthesis. Thus, Cu may play a special role in the intestinal protein synthesis. Such a contention was further supported by the data from the limited Cu repletion study. When the Cu-deficient rats were repleted with only one day of Cu-containing control diet, the intestinal total protein synthesis was rapidly normalized back to the level of controls in the duodenal portion of the small intestine (Fig. 2), which may be considered to be the most active site for dietary Cu absorption. While in the jejunum and ileum, which receive nutrients that escape absorption by the duodenum and are relatively less active in Cu absorption, such limited Cu repletion did not normalize their protein synthetic capacity. These observations strongly suggested that the increased intestinal protein synthetic capacity observed in the Cu-deficient rats was truly due to the depleted Cu status.

Interestingly, the ileum appeared to have a higher ability to synthesize total protein than did the duodenum and jejunum, which are generally thought to be more active in food digestion and nutrient absorption. Since the data were expressed on the basis of per mg cellular protein, and the ileal mucosa appeared thinner than that for duodenum and jejunum, one possible explanation was that the ileal mucosa had relatively low cellular protein content and in turn projected a relatively high level of protein synthesis. This contention was supported by the observation that consistently lower amounts of detergent-soluble protein were found in samples prepared from the ileal region (data not shown), although the wet weights of the intestinal segments used were roughly identical.

An increase in the concentration of intracellular free amino acids may possibly enhance the intestinal protein synthesis in Cu deficiency. To eliminate such possibility, the current *in vivo* study employed a flooding dose of [^{3}H]phenylalanine to ensure the specific radioactivity of plasma phenylalanine was maintained. Moreover, plasma unlabeled free phenylalanine concentration was found to be similar between the two treatment groups (13). In the *in vitro* study, all intestinal segments used were similar in wet weight and the incubation media were identical. Nevertheless, it is possible that Cu deficiency may alter the intestinal uptake of free amino acids and subsequently alter the intracellular free [^{3}H]phenylalanine pool size. If this possibility does exist, it may contribute to an alteration in protein synthesis. However, the radioactivity associated with TCA soluble component (free [^{3}H]phenylalanine) in detergent soluble fraction of intestine was found to be similar between treatments and were 40- to 80-fold higher than that associated with TCA-insoluble total protein in the same aliquot from 7- and 14-min pulse samples, respectively. In addition, the intestinal free phenylalanine concentration was not altered by Cu status. These observations suggested that neither the ability of the intestine to take up free amino acids nor the cellular pool size of free labeled and unlabeled substrate in the enterocytes appeared to be altered by Cu status, and that there was no substrate limitation in the observed protein synthesis. Thus, the enhanced intestinal protein synthesis in Cu-deficient rats appeared not to be caused by a change in the availability of intracellular free amino acids. Furthermore, the enhanced intestinal protein synthesis appeared not to be caused by a reduction in food intake because a recent substrate utilization study (31) indicated that food intake was not altered but feed efficiency was significantly reduced in Cu-deficient rats treated in an identical fashion as in the present study.

The small intestine, together with the liver, are known to be the most important synthetic sites for

apolipoproteins such as apo A-I and apo B (32). Moreover, the intestinal contribution to the circulating apo A-I appears to be quantitatively important. Although the apo A-I mRNA can be detected along the entire intestinal tract, the levels of mRNA for apo A-I was highest in the jejunum. In rats, the jejunal apo A-I mRNA level was reported to be more than 2-fold higher than that of the duodenum and ileum (10). This finding was matched by the fact that the jejunal segment was the most active site for dietary fat absorption and chylomicron secretion (33). In the present *in vitro* study, the apo A-I synthesis was therefore examined only at the jejunum. Unlike the synthesis curves for total protein, the apo A-I synthesis curves lost the linearity between 7 and 14 min in both control and treatment groups, probably because newly synthesized apo A-I was being secreted into the medium (Fig. 3). The jejunal apo A-I synthesis response curve for Cu-deficient rats was significantly higher than that of control rats. However, this increase in apo A-I synthesis appeared not to be specific because a marked increase (2.4-fold) in total protein synthesis was also observed in the same intestinal segment. Whether the increase in apo A-I synthesis was a general effect of an increase in overall total protein synthesis was not apparent. Nevertheless, the observed increase in jejunal apo A-I synthesis may lead to an increase in apo A-I secretion and in turn contribute to the elevated plasma apo A-I pool size in the Cu-deficient rats.

An increased RNA to DNA ratio observed in hepatocytes from Cu-deficient rats (13), as well as in HepG2 cells (a human hepatoblastoma cell line) depleted of Cu by a cupruretic chelator tetramine (unpublished observations), may be considered as an indication that the Cu-deficient cells are metabolically more active. However, the total protein synthesis in these two hepatic systems was not altered by Cu deficiency, although the specific apo A-I synthesis and secretion were enhanced (13, 34). Thus, the intestinal and hepatic responses to changes in Cu status appeared not to be the same. At present, the exact role of Cu in intestinal protein synthesis is not apparent.

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