Intestinal β-carotene 15,15′-dioxygenase activity is markedly enhanced in copper-deficient rats fed on high-iron diets and fructose

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The purpose of the present work was to examine effects of the Cu–Fe interaction on intestinal β-carotene 15,15′-dioxygenase activity when a wide range of dietary Fe (deficiency to excess) was used in relation to Cu status of rats. The effect of dietary carbohydrates was also examined since they play a role in the Cu–Fe interaction *in vivo*. Weanling male Sprague-Dawley rats (*n* 72) were divided into twelve dietary groups, which were fed on either low-, normal-, or high-Fe levels (0·9, 9·0, and 90·0 mmol Fe/kg diet respectively) combined with Cu-adequate or -deficient levels (0·94 and 0·09 mmol Cu/kg diet respectively) and with starch or fructose in the diets. The data showed that both Fe concentration and β-carotene 15,15′-dioxygenase activity in small intestinal mucosa were enhanced with increasing dietary Fe and with Cu deficiency *v*. Cu adequacy. Dietary fructose did not aggravate the Fe-enhancement, related to Cu deficiency, in the small intestine; however, fructose increased the intestinal dioxygenase activity in rats fed on normal- or high-Fe diets when compared with starch controls. Thus, the highest intestinal dioxygenase activity associated with the lowest hepatic retinol (total) concentration was found in rats fed on the Cu-deficient, high-Fe, fructose-based diet. Finally, a positive linear relationship was found between the dioxygenase activity and Fe concentration in intestinal mucosa. In conclusion, the data indicate that β-carotene 15,15′-dioxygenase activity requires Fe as cofactor *in vivo* and the enzyme is modulated by the three dietary components: Cu, Fe, and fructose.

β-Carotene 15,15′-dioxygenase activity: Retinol: Iron: Copper: Fructose

Although people living in the USA are, in general, adequately nourished in vitamin A (retinol), preventing vitamin A deficiency is now a major challenge in many developing countries. Programmes to improve vitamin A status are presently in place for over sixty countries compared with only three countries two decades ago (Sommer, 1998). The most abundant provitamin A carotenoid is β-carotene which is found mainly in fruits and vegetables. β-Carotene is converted to retinal (direct precursor of retinol) by the action of a cytosolic enzyme, β-carotene 15,15′-dioxygenase, which was first identified in small intestine and liver of rats (Goodman & Huang 1965; Olson & Hayaishi, 1965). Although it is a key enzyme for the synthesis of retinol, the role that dietary components play in affecting the efficacy of β-carotene conversion is poorly understood.

Accumulating data from previous *in vitro* studies indicate that β-carotene 15,15′-dioxygenase activity is activated by Fe, particularly by ferrous iron (Fe^{2+}) (Olson & Hayaishi, 1965; Goodman *et al*. 1967; Fidge *et al*. 1969; Singh & Cama, 1974; Dmitrovskii & Ershov 1993). Furthermore, Dmitrovskii & Ershov (1993) reported an inhibition of the enzyme activity by addition of Cu *in vitro*. However, *in vivo* rat studies have failed to show an effect of those minerals on β-carotene conversion to retinal as reflected by liver vitamin A concentration (Seaborn *et al*. 1991; Swanson & Parker, 1993; Dulin *et al*. 1995).

As Fe absorption is tightly regulated (Conrad, 1993), it is difficult to alter the concentration of Fe in tissues by simply varying dietary Fe. However, Cu deficiency is capable of increasing liver Fe concentration (Owen, 1978; Williams *et al*. 1983; Fields *et al*. 1991). Indeed, we recently reported that increasing dietary Fe concentration 2-fold (16 *v*. 8 mmol Fe/kg diet) neither changed intestinal Fe concentration nor intestinal β-carotene 15,15′-dioxygenase activity in rats. However, dietary Cu deficiency resulted in an increase of both Fe concentration and the dioxygenase activity in the intestinal mucosa compared with Cu adequacy. We thus concluded that the dioxygenase activity was dependent on Cu–Fe interaction *in vivo*, even when the range of dietary Fe concentrations was relatively narrow and within recommended dietary levels (During *et al*. 1999).

A primary objective of the present investigation was to examine effects of Cu–Fe interaction on β-carotene

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dioxygenase activity when a wide range of dietary Fe was used in relation to Cu status. In addition, it has been repeatedly demonstrated that, in rat liver, the type of dietary carbohydrate affects the signs associated with Cu deficiency: dietary fructose aggravates the signs, but starch ameliorates them (Fields *et al*. 1984). To our knowledge, the effect of carbohydrates on β-carotene 15,15′-dioxygenase activity has not been previously reported. Therefore, an additional objective of the present study was to determine the effect of the source of dietary carbohydrate (fructose *v*. starch) on β-carotene conversion in relation to the Cu and Fe status of rats.

Materials and methods

Materials

All-*trans* β-carotene (type IV), all-*trans* retinal, and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). D-α-Tocopherol was obtained from Dr James Clark of Henkel (LaGrange, IL, USA). HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All-*trans* retinol was prepared by reducing all-*trans* retinal with $NaBH_4$. All-*trans* β-carotene, all-*trans* retinal, and all-*trans* retinol were purified as described previously (Nagao *et al*. 1996).

Animals and diets

Seventy-two weanling male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing 40–45 g were divided into twelve dietary groups (six rats per group) differing in levels of Cu, Fe, and the type of dietary carbohydrate. Thus, six dietary groups which consumed starch were assigned as follows: group 1 Cu-adequate, low Fe, group 2 Cu-adequate, normal Fe; group 3 Cu-adequate, high Fe; group 4 Cu-deficient, low Fe; group 5 Cu-deficient, normal Fe; and group 6 Cu-deficient, high Fe. An identical organization was applied for the six additional dietary fructose groups designated 7–12.

The basal purified diet met the requirements of laboratory rats, except for Fe and Cu which were omitted from the mineral mix (Table 1). To this basal diet, Fe (ferric citrate) was added at 0·9, 9·0, and 90·0 mmol Fe/kg respectively for low-, normal-, and high-Fe diets. The Cu-adequate diet was prepared by adding cupric carbonate to yield a final concentration of 0·94 mmol Cu/kg diet. The Cu-deficient diet contained 0·09 mmol Cu/kg diet. Concentrations of Fe and Cu in the diets were confirmed by atomic absorption analyses (Hill *et al*. 1986).

Table 1. Composition of the basal diet

	(g/kg diet)
Starch or fructose	627
Egg white	200
Fibre	30
Maize oil*	95
AIN-76 salt mix† (Cu and Fe omitted)	35
AIN-76A vitamin mix‡	10
Biotin	0·002
Choline bitartrate	2·7

* Maize oil contained only 85 pmol β-carotene/g oil (analysed by HPLC).
† Salt mix composition (g/kg): calcium phosphate, dibasic 500, sodium chloride 74, potassium citrate, monohydrate 220, potassium sulfate 52, magnesium oxide 24, manganous carbonate (430–480 g Mn/kg) powdered 3·5, zinc carbonate 1·6, potassium iodate 0·01, sodium selenite 0·007, chromium potassium sulfate powdered 0·55, sucrose fine powder 124·3.
‡ Vitamin mix composition (g/kg): thiamin HCl 0·6, riboflavin 0·6, pyridoxine HCl 0·7, niacin 3·0, calcium pantothenate 1·6, folic acid 0·2, biotin 0·02, vitamin B_{12} 1·0, dry vitamin A palmitate 0·002, dry vitamin E acetate 0·05, cholecalciferol trituration 0·025 mg, menadione sodium bisulfite complex 0·15, sucrose fine powder 981·08.

Rats were individually housed in stainless-steel cages in a room with 12 h light–dark cycle. The room was maintained at 22° and 50–60 % humidity. Rats were fed on their respective diets for 4 weeks; the diets and deionized water were provided *ad libitum*. At the end of the feeding period, after an overnight fast the rats were decapitated and liver and intestine immediately collected and placed on ice. The procedures used met the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Preparation of organ samples and tissue homogenates

The same day that the organs were harvested, the liver was cut into small pieces (about 2 g) and stored at −20° until analysed. The upper half of the intestine (about 250 mm) was rinsed free of ingesta using an ice-cold 9·0 g NaCl/l solution and the intestinal mucosa was gently scraped off and immediately frozen at −80°.

Tissue homogenates were obtained according to the procedure previously described (During *et al*. 1999). The homogenates were prepared in 1 : 5 (w/v): 50 mM-HEPES-KOH, 1 mM-EDTA, 11·5 g KCl/l, 0·1 mM-dithiothreitol buffer, pH 7·4. From a portion (about 5 ml) of intestinal mucosa homogenate, a supernatant fraction was prepared by centrifugation at 10 000 ***g*** for 30 min followed by a gel filtration using a Sephadex G-25M column equilibrated with 10 mM-HEPES-KOH, 0·1 mM-EDTA, 50 mM-KCl, 0·1 mM-dithiothreitol buffer (pH 7·4). The eluted fraction was immediately assayed for β-carotene dioxygenase activity. Finally, liver homogenate as well as the remaining intestinal mucosa homogenate and supernatant were divided into portions (0·5 to 1 ml) and frozen at −20°. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

β-Carotene 15,15′-dioxygenase assay in rat intestine

β-Carotene dioxygenase was assayed using the procedure described previously (During *et al*. 1996). Briefly, the reaction medium (0·2 ml) contained all-*trans* β-carotene (3 nmol), *N*-tris-((hydroxymethyl)methyl)glycine (Tricine)-KOH buffer, pH 8·0 (20 μmol), dithiothreitol (0·1 μmol), Tween 40 (0·3 mg), sodium cholate (0·8 μmol), α-tocopherol (20 nmol), nicotinamide (3 μmol) and the enzyme (< 0·5 mg protein). After incubation at 37° for 60 min, the reaction was stopped by adding 50 μl formaldehyde (370 g/l) and 500 μl of acetonitrile, followed by a centrifugation at 10 000 ***g*** for 2 min. The amount of retinal as the product of the reaction was determined by injecting 200 μl of the supernatant fraction directly to the HPLC system. The HPLC system was equipped with a 114M pump (Beckman Instruments, Inc., CA, USA), a UV-970 uv-vis absorbance detector

(Jasco, Tokyo, Japan), and the GOLD®software system for analyses (Beckman Instruments, Inc.). Retinal was separated on a TSK gel ODS-80Ts C18 reverse phase column (5 μm particle size, 80-Å pore size, 4·6×150 mm) (Toso-Haas, Tokyo, Japan), attached to a guard-column (2× 20 mm) of Pelliguard LC-18 (Supelco Inc., Bellefonte, PA, USA). Acetonitrile–water (90 : 10, v/v) containing 1 g ammonium acetate/l was used as mobile phase at the flow rate of 1·0 ml/min. Retinal formed in the enzyme assay was quantified from its peak area by using a standard curve of purified all-*trans* retinal (0·2–50 pmol/200 μl injected) and was proportional to the incubation time (5–60 min) and the protein concentration (0·05–0·7 mg) (During *et al*. 1996).

Extraction and analysis of retinol in rat liver

Total retinol was extracted from liver homogenate (100 μl) using the procedure of Ross (1986). Retinol was analysed by reverse phase HPLC using a Hewlett Packard system (HP 1100) equipped with a G1311A pump, a G1315A diode array detector, and a G1329A thermostated auto-sampler (Hewlett-Packard GmbH, Waldbronn, Germany). Retinol was eluted on the TSK gel ODS-80Ts C18 column, 4·6×250 mm, with acetonitrile–water (90 : 10, v/v) containing 1 g ammonium acetate/l as the mobile phase. Retinol was quantified from its peak area by use of a standard curve of purified all-*trans* retinol (0·2–200 pmol/100 μl injected).

Determination of copper and iron concentrations in liver and intestinal mucosa

Cu and Fe concentrations in diets and tissue homogenates were determined according to the method of Hill *et al*. (1986). Briefly, the samples were digested by a combination of dry heat and acid digestion procedures; then they were analysed by flame atomic absorption spectrometry (Model 5000, Perkin-Elmer Corp., Norwalk, CT, USA). Fe and Cu concentrations in the sample were determined using standard curves of each mineral. Standard reference materials (bovine liver (SRM no. 1577b) and bovine serum (SRM no. 1598) of the National Institute of Standards and Technology, Gaithersburg, MD, USA) were used to verify

Table 2. Effect of level of copper and iron in starch- or fructose-based diets on organ weights and retinol, copper, and iron concentrations in liver and intestinal mucosa of rats

(Mean values with standard errors of the means)

		Liver								Intestinal mucosa					
		Weight† (% body wt)		Retinol (nmol/g)		Copper (μmol/g)		Iron (μmol/g)		Weight (% body wt)		Copper (μmol/g)		Iron (μmol/g)	
Dietary groups*	*n*	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Starch*															
Cu-adequate*															
Low Fe	6	3·1	0·1	99	11	1·43	0·27	6	1	1·0	0·1	0·11	0·01	0·4	0·0
Normal Fe*	6	3·1	0·1	126	7	0·82	0·01	17	1	0·9	0·1	0·12	0·01	1·1	0·0
High Fe	6	3·1	0·1	125	5	0·80	0·05	35	1	1·0	0·1	0·11	0·01	5·7	0·5
Cu-deficient															
Low Fe	6	3·1	0·1	109	7	0·22	0·03	8	1	1·0	0·1	0·06	0·00	0·5	0·0
Normal Fe	6	3·7	0·1	86	6	0·17	0·01	36	2	0·9	0·1	0·05	0·00	1·4	0·2
High Fe	5	4·3	0·2	92	9	0·20	0·03	59	3	0·9	0·1	0·06	0·00	5·6	0·7
Fructose															
Cu-adequate															
Low Fe	6	3·6	0·1	99	7	0·94	0·06	5	0	1·2	0·1	0·13	0·01	0·4	0·0
Normal Fe	6	4·0	0·1	93	7	0·74	0·03	15	1	1·2	0·0	0·09	0·00	1·1	0·2
High Fe	6	3·5	0·1	90	8	0·42	0·04	46	3	1·0	0·0	0·08	0·01	6·8	0·7
Cu-deficient															
Low Fe	6	3·7	0·1	87	3	0·20	0·03	9	0	0·8	0·1	0·16	0·01	0·5	0·0
Normal Fe	6	4·7	0·1	87	7	0·17	0·01	32	2	0·9	0·1	0·11	0·00	1·4	0·2
High Fe	5	5·1	0·3	79	7	0·17	0·03	71	4	1·0	0·1	0·14	0·03	5·6	0·4
ANOVA test:‡															
Cu effect		$P < 0·0001$		$P < 0·001$		$P < 0·0001$		$P < 0·05$		$P < 0·001$		$P < 0·01$		$P < 0·001$	
Fe effect		$P < 0·0001$		NS		$P < 0·05$		$P < 0·0001$		NS		$P < 0·05$		$P < 0·0001$	
CHO effect		$P < 0·0001$		$P < 0·0001$		$P < 0·05$		$P < 0·05$		NS		$P < 0·0001$		NS	
Cu × Fe interaction		$P < 0·0001$		$P < 0·05$		$P < 0·0001$		$P < 0·0001$		NS		$P < 0·05$		$P < 0·001$	
Cu × CHO interaction		NS		NS		$P < 0·05$		NS		$P < 0·01$		$P < 0·0001$		NS	
Fe × CHO interaction		$P < 0·05$		NS		NS		$P < 0·0001$		NS		NS		NS	
Cu × Fe × CHO interaction		NS		$P < 0·05$		NS		NS		$P < 0·01$		NS		NS	

* The control diet of the study was based on starch combined with normal copper and iron levels. Cu-adequate: normal copper diet (0·94 mmol/kg); Cu-deficient: no copper added in diet (0·09 mmol/kg); Low Fe : low iron diet (0·9 mmol/kg diet); Normal Fe: normal iron diet (9·0 mmol/kg); High Fe: high iron diet (90·0 mmol/kg).

† Final body weights were: in the six starch-based diet groups: 170 (SEM 7) g (Cu-adequate, low Fe); 238 (SEM 9) g (Cu-adequate, normal Fe); 244 (SEM 6) g (Cu-adequate, normal Fe); 197 (SEM 3) g (Cu-deficient, low Fe); 224 (SEM 7) g (Cu-deficient, normal Fe); 203 (SEM 10) g (Cu-deficient, high Fe) and in the six fructose-based diet groups: 164 (SEM 7) g (Cu-adequate, low Fe); 219 (SEM 4) g (Cu-adequate, normal Fe); 229 (SEM 4) g (Cu-adequate, normal Fe); 172 (SEM 7) g (Cu-deficient, low Fe); 158 (SEM 9) g (Cu-deficient, normal Fe); 139 (SEM 5) g (Cu-deficient, high Fe).

‡ Data (or data $\log_{10}$-transformed if necessary) were tested by three-way ANOVA for main effects of dietary copper (Cu), iron (Fe), and carbohydrate (CHO) and main interactions between Cu×Fe, Cu×CHO, Fe×CHO, and Cu×Fe×CHO.

accuracy. These materials were analysed concurrently with the tissue samples, beginning at the digestion phase.

Statistical Analysis

All data are expressed as means with standard error of the mean. Data were tested for homogeneity of variances among groups using the Bartlett's test (if necessary, data were $\log_{10}$-transformed to stabilize variances). Main effects and interactions of the three independent variables dietary Cu, dietary Fe, and carbohydrate source were tested by three-way ANOVA. Relationships among variables were examined by simple regression analysis. All statistical analyses were performed using StatView, version 5·0 (SAS Institute Inc., Cary, NC, USA). Data were considered statistically significant at $P < 0{\cdot}05$.

Results

Before the conclusion of the 4-week feeding period, two rats from Cu-deficient, high-Fe groups (one rat from the starch group and the other from the fructose group) died of ruptured hearts. Cu-deficient diets resulted in a significant reduction of body weight compared with Cu-adequate diets. Similarly, rats fed on low-Fe diets weighed significantly less than rats fed on normal-Fe diets. Moreover, the combination of Cu deficiency with fructose resulted in lower body weights compared with the combination of Cu deficiency with starch (see footnote of Table 2).

Liver weight (expressed as a percentage of body weight) was greater in rats fed on Cu-deficient diets *v*. Cu-adequate diets, in Cu-deficient rats fed on increasing dietary Fe, and also in rats fed on fructose diets *v*. starch diets (Table 2). Total retinol concentration in liver was reduced with Cu deficiency and with fructose in normal- and high-Fe groups. The lowest hepatic retinol concentration was noted in the Cu-deficient, high-Fe, fructose-fed group (Table 2). As expected, Cu concentration in the liver was lowered with Cu-deficient diets. Rats fed on low-Fe diets exhibited higher hepatic Cu concentrations than those fed on normal-Fe diets, but only in Cu-adequate groups. Similarly, dietary fructose reduced Cu concentration in liver compared with starch, but only for Cu-adequate rats (Table 2). Hepatic Fe concentration was increased with increasing dietary Fe and with Cu deficiency *v*. Cu adequacy. The combination of high Fe with fructose in the diet resulted in the increase of hepatic Fe concentration compared with the combination of high Fe with starch in diet (Table 2).

Intestinal mucosa weight (expressed as a percentage of body weight) was reduced in Cu-deficient rats, but only for the fructose-fed groups. Intestinal Cu concentration was reduced in Cu-deficient rats, but only for the starch-fed

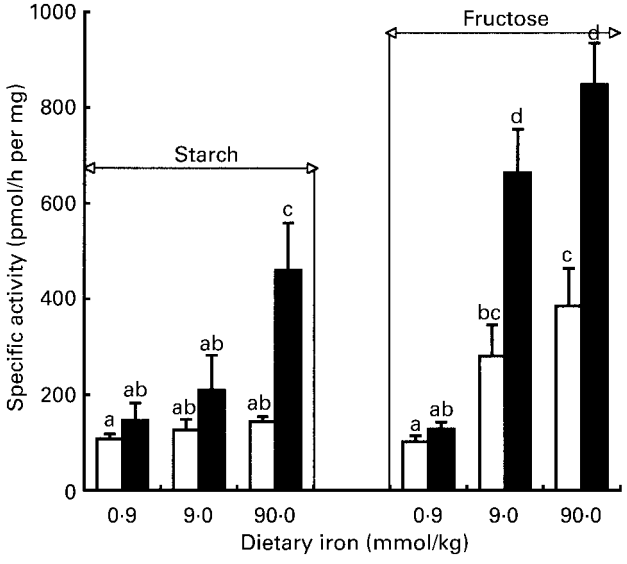

Fig. 1. Effects of three dietary iron levels in relation to the copper status and the source of carbohydrate on intestinal β-carotene 15,15′-dioxygenase activity expressed as specific activity (pmol retinal formed/h per mg protein) in the rat. (□), Cu-adequate diet; (■), Cu-deficient diet. Enzyme assays were conducted as described on pp. 118–119. Values are means with standard errors of the means represented by vertical bars (*n* 6 rats for all dietary groups, except *n* 5 rats for high Fe, Cu-deficient groups). Main effects and interactions of dietary copper, iron, and carbohydrate (CHO) on the enzyme activity were examined by three way ANOVA after $\log_{10}$-transformation of data: Cu effect $P < 0{\cdot}0001$, Fe effect, $P < 0{\cdot}0001$, CHO effect $P < 0{\cdot}0001$, Cu × Fe interaction $P < 0{\cdot}02$, Cu × CHO interaction NS, Fe × CHO interaction $P < 0{\cdot}001$, Cu × Fe × CO interaction NS. Multiple comparisons of means among groups were assessed using Fisher's test. [a,b,c,d]Mean values with different letters were significantly different ($P < 0{\cdot}05$).

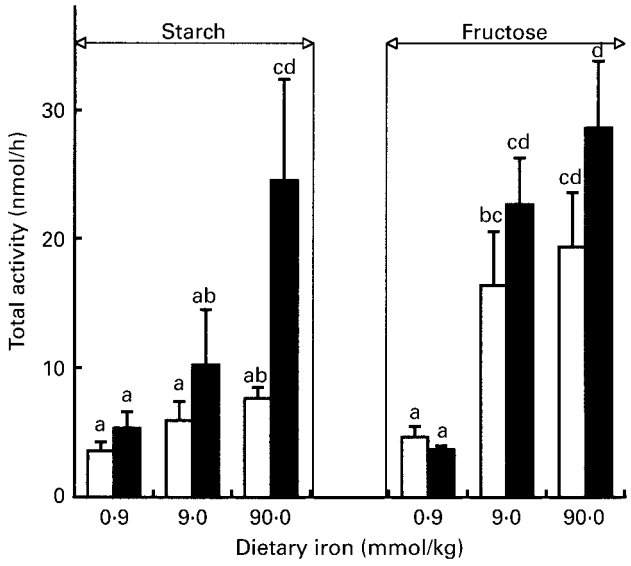

Fig. 2. Effects of three dietary iron levels in relation to the copper status and the source of carbohydrate on β-carotene 15,15′-dioxygenase activity expressed as total activity (nmol retinal formed/h) in the rat. (□), Cu-adequate diet; (■), Cu-deficient diet. Enzyme assays were conducted as described on pp. 118–119. Values are means with standard errors of the means represented by vertical bars (*n* 6 rats for all dietary groups, except *n* 5 rats for high Fe, Cu-deficient groups). Main effects and interactions of dietary copper, iron, and carbohydrate (CHO) on the enzyme activity were examined by three way ANOVA after $\log_{10}$-transformation of data: Cu effect $P < 0{\cdot}005$, Fe effect $P < 0{\cdot}0001$, CHO effect $P < 0{\cdot}0001$, Cu × Fe interaction NS, Cu × CHO interaction NS, Fe × CHO interaction $P < 0{\cdot}01$, Cu × Fe × CHO interaction NS. Multiple comparisons of means among groups were assessed using Fisher's test. [a,b,c,d]Mean values with different letters were significantly different ($P < 0{\cdot}05$).

groups (Table 2). When comparing dietary Fe intake alone, low-Fe diets resulted in an increase of Cu concentration in intestinal mucosa compared with normal-Fe diets. Intestinal Fe concentration was increased with increasing dietary Fe concentrations (10–100-fold) and with Cu deficiency *v*. Cu adequacy, but only for low- and normal-Fe groups (Table 2).

β-Carotene 15,15′-dioxygenase activity expressed as specific activity (pmol retinal formed per h and per mg protein) in small intestinal mucosa was enhanced with Cu deficiency *v*. Cu adequacy as well as with increasing dietary Fe concentration. For instance, in the starch-fed groups, the specific activity was markedly higher in the three Cu-deficient groups by 40 %, 70 %, and 220 % respectively for low-, normal-, and high-Fe diets, compared with the respective Cu-adequate groups (Fig. 1). Finally, dietary fructose enhanced the specific activity compared with starch, but only for rats fed on the normal- and high-Fe diets. The highest enzyme activity was found in the Cu-deficient, high-Fe, fructose-fed rats (Fig. 1). Because intestinal mucosa weight varied with the type of the diet (Table 2), we also expressed the enzyme activity as total activity (nmol retinal formed per h and per whole organ) (Fig. 2). Similar to the specific activity, total activity was increased with Cu deficiency as well as with increasing dietary Fe concentration in both Cu-adequate and Cu-deficient rats. Total activity was also elevated by dietary fructose, however, only for rats fed on normal- or high-Fe diets (Fig. 2).

A significant positive linear relationship between β-carotene dioxygenase activity and Fe concentration in intestinal mucosa was evident (r 0·444, $P = 0·002$, n 68 rats). However, a higher and more significant correlation was found when the two variables were log-transformed (r 0·577, $P < 0·0001$, n 68) (Fig. 3), indicating that the enzyme activity increased exponentially with intestinal Fe concentrations. Moreover, 74 % of the values corresponding to the six starch groups (open symbols) were below the linear regression and 68 % of the values corresponding to the six fructose groups (closed symbols) were above the line (Fig. 3). Finally, no significant relation was found between the enzyme activity and Cu concentration in intestinal mucosa (data not shown).

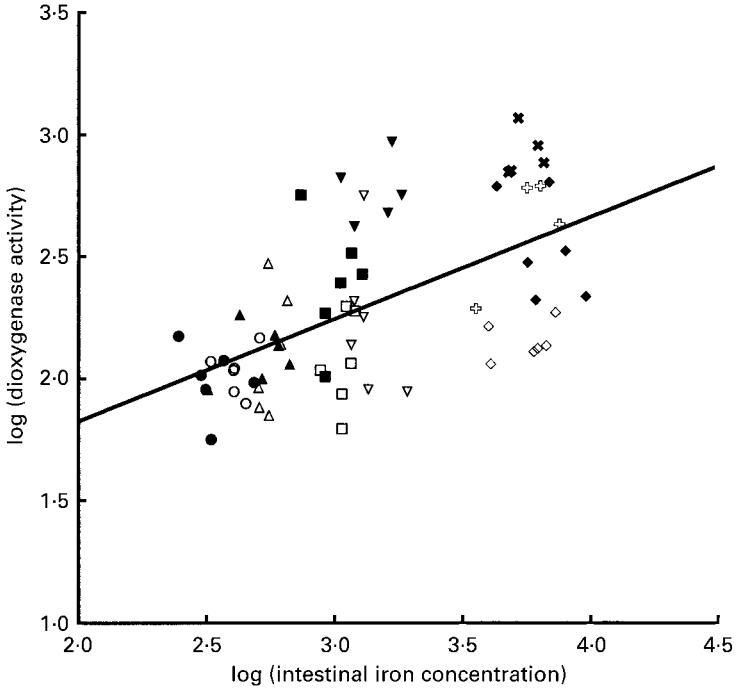

Fig. 3. Relationship between β-carotene 15,15′-dioxygenase activity (pmol/h per mg) and tissue iron concentration (μmol Fe/g wet wt) in rat intestinal mucosa. Individual values of the two variables were log-transformed before plotting on this graph for the six 'starch' groups (open symbols) and the six 'fructose' groups (closed symbols) fed on one of the three levels of iron (0·9, 9·0, 90·0 mmol/kg diet respectively for low, normal, and high Fe) combined with Cu adequacy (0·94 mmol Cu/kg) or Cu deficiency (0·09 mmol Cu/kg). ○, Low Fe, Cu adequate (starch); △, low Fe, Cu deficient (starch); □, normal Fe, Cu adequate (starch); ▽, normal Fe, Cu deficient (starch); ◇, high Fe, Cu adequate (starch); ✣, high Fe, Cu deficient; ●, low Fe, Cu adequate (fructose); ▲, low Fe, Cu deficient (fructose); ■, normal Fe, Cu adequate (fructose); ▼, normal Fe, Cu deficient (fructose); ◆, high Fe, Cu adequate (fructose); ✖, high Fe, Cu deficient (fructose). The coefficient of correlation r was obtained by simple regression analysis and significance of the linear relationship was determined by ANOVA test ($F(x) = 0·42x + 0·99$, r 0·577, $P < 0·0001$ (n 68)).

Discussion

Cu deficiency resulted in a significant increase of Fe concentrations and Fe deficiency enhanced Cu concentrations in both liver and intestinal mucosa. Thus, the Cu–Fe interaction, which has been well established in rat liver (Owen, 1978; Williams *et al*. 1983; Fields *et al*. 1991), is also present in small intestinal mucosa of rats. Several specific mechanisms of Fe transport across membranes were reported to be Cu-dependent (Yu & Wessling-Resnick, 1998; Vulpe *et al*. 1999) and may explain why Fe accumulates in tissues of Cu-deficient rats.

β-Carotene 15,15′-dioxygenase activity was positively correlated with tissue Fe concentration in rat small intestinal mucosa, independently of the three dietary factors. On the other hand, intestinal Fe concentration was increased by both a Cu-deficient status (as mentioned earlier) and by increasing dietary Fe. Thus, the data indicate clearly that the dioxygenase activity is affected by the Cu–Fe interaction in small intestinal mucosa and support the hypothesis that Fe is required as a cofactor for the dioxygenase activity *in vivo* (During *et al*. 1999). The participation of Fe in the enzymic reaction was confirmed by the inhibitory effect of different Fe chelators (such as α,α′-dipyridyl and *o*-phenanthroline) on the intestinal dioxygenase activity (Olson & Hayaishi, 1965; Goodman *et al*. 1967; Fidge *et al*. 1969). In our present study, when desferrioxamine mesylate (an Fe chelator used in clinical therapy (Olivieri & Brittenham, 1997)) was added at 0·5 mM or 2·5 mM to enzyme preparations from Cu-deficient rats fed on a high-Fe diet (90·0 mmol Fe/kg), the dioxygenase activity was inhibited by 71% and 95% respectively (data not shown). However, our enzyme preparations should be free of Fe since they were submitted to a Sephadex gel filtration procedure (see p. 118). Thus, a possible explanation of the inhibitory effect of desferrioxamine is that metal chelator acts on Fe attached structurally to the enzyme. This point is supported by the facts that, first, identical dioxygenase activities were found before and after gel filtration (if the enzyme required only Fe at free state, no (or lower) activity would be detected in the enzyme preparation after gel filtration) and, second, the addition of ferrous sulfate to the enzyme preparation after gel filtration did not increase the dioxygenase activity. However, further measurements of Fe in enzyme preparations before and after gel filtration are necessary to provide definitive evidence that Fe is attached or not to the protein.

An excess of Fe may also result in a non-enzymic oxidation of β-carotene. Mordi (1993) showed that the degradation of β-carotene, involving several reactional steps including isomerization and oxidation of the molecule, conducted to the formation of stable products called β-apocarotenals, which can be converted to retinal. Thus, chemical degradation of β-carotene may induce an overestimate error in the measurement of β-carotene 15,15′-dioxygenase activity. However, no apocarotenal was detected in both zero-time control and after a complete enzyme assay in presence of the enzyme preparation (intestinal mucosa) from a Cu-deficient rat fed on a diet overloaded with Fe (90·0 mmol Fe/kg diet).

In addition to its interaction with Fe metabolism, Cu could play a role in the enzymic cleavage of β-carotene itself. Indeed, Dmitrovskii *et al*. (1993) reported that *in vitro* Cu ions inhibit the dioxygenase activity by deactivating the enzyme–Fe^{2+} complex associated with the substrate. In spite of the fact that the dioxygenase activity was significantly higher with Cu deficiency *v*. Cu adequacy, the present study did not show a negative correlation between the enzyme activity and Cu concentration in small intestinal mucosa, as we reported recently (During *et al*. 1999). Thus, taken together, the data indicate that the enzyme is not Cu dependent *per se*.

In contrast to our data, Dulin *et al*. (1995) reported that Cu deficiency did not affect β-carotene conversion *in vivo* in rats. The major differences between the two studies were the dietary source of vitamin A (β-carotene *v*. retinol palmitate) and the assessment of β-carotene conversion (hepatic retinol concentration *v*. specific intestinal activity of β-carotene 15,15′-dioxygenase) respectively for the study of Dulin *et al*. (1995) *v*. the present study. These points raise the following question: how would Cu deficiency affect the dioxygenase activity if rats were fed on β-carotene instead of retinol palmitate? Such results are difficult to predict. Indeed, Vliet *et al*. (1996) indicated that β-carotene-supplemented diets containing low or normal amounts of retinol acetate decreased the intestinal dioxygenase activity in rats, compared with the respective control diets without β-carotene. The present data showed that Cu deficiency enhanced the intestinal dioxygenase activity when using an adequate amount of retinol palmitate but no β-carotene in the diet. Thus, possible interactive effects may occur in relation to the combination of Cu deficiency with β-carotene supplementation and could explain the results of Dulin *et al*. (1995).

Earlier, Moore (1969) drew attention to an inverse relationship between Cu and vitamin A in human plasma. However, data concerning effect of Cu deficiency on vitamin A metabolism in the literature are contradictory despite adequate amounts of vitamin A in the diet, when using rats as experimental model. For instance, Rachman *et al*. (1987) reported an increase of hepatic retinol and a decrease of plasma retinol in Cu-deficient rats, but they used high ‘Cu-adequate’ controls (about 6 mmol Cu/kg diet). In contrast, Dulin *et al*. (1992) failed to show any effect of Cu deficiency on rat serum and liver retinol concentrations. Finally, our present data showed that Cu deficiency reduced hepatic retinol concentration in rats fed on normal- and high-Fe diets.

In the present study, an inverse linear relationship between hepatic retinol concentration and intestinal β-carotene 15,15′-dioxygenase activity was established (r 0·404, $P = 0·0005$, n 68 rats), independently of the three dietary factors (data not shown). Thus, the intestinal dioxygenase activity may increase in response to a reduction of tissue retinol in the rat. Indeed, Villard & Bates (1986) have already suggested that low levels of vitamin A in tissues (plasma and liver) may feed back to increase the dioxygenase activity. A similar inverse relationship can be noted when rats were fed on diets rich in polyunsaturated fatty acids; indeed, polyunsaturated fatty acids depressed total hepatic retinol (Gronowska-Senger & Rupniewska, 1979; Furr *et al*. 1989) and enhanced both intestinal and hepatic β-carotene dioxygenase activities (During *et al*. 1998).

The highest intestinal dioxygenase activity and the lowest hepatic retinol level were found in the Cu-deficient, high-Fe, fructose-fed group. These three independent factors (Cu deficiency, high Fe, and dietary fructose) are known to promote oxidative stress *in vivo*. Thus, under oxidative conditions, the turnover of vitamin A could be enhanced, resulting in the increase of β-carotene 15,15′-dioxygenase activity in effort to replace and/or maintain retinol stores. Indeed, Gronowska-Senger & Rupniewska (1979) indicated that the reduction of retinol storage in rat liver enriched in polyunsaturated fatty acids may be due to an increase of antioxidant demand. On the other hand, recent studies suggested that retinol may be an antioxidant *in vivo* (Livrea *et al*. 1995; Swartz *et al*. 1997).

Finally, the present data showed that intestinal β-carotene 15,15′-dioxygenase activity was enhanced with dietary fructose *v*. starch. Indeed, when rats were fed on normal Cu and normal Fe diets (0·94 mmol Cu and 9·0 mmol Fe/kg diet respectively), the dioxygenase activity was increased by 123 % in the fructose group (282 (SEM 65) pmol/h per mg) compared with that of the starch group (126 (SEM 22 pmol/h per mg). A similar observation was noted with total activity of the enzyme. Moreover, the effects of fructose on β-carotene conversion were more pronounced with higher dietary Fe intake and under Cu-deficient conditions.

In conclusion, the data indicate that β-carotene 15,15′-dioxygenase activity requires Fe as a cofactor *in vivo* as well as that the enzyme is modulated by the three dietary components: Cu, Fe, and fructose (those three dietary factors have been reported to promote oxidative stress). However, further investigations are necessary to elucidate the hypothetical relation between the oxidative status and intestinal β-carotene 15,15′-dioxygenase activity *in vivo*.

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