Interactions in indices of vitamin A, zinc and copper status when these nutrients are fed to rats at adequate and increased levels

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The purpose of the present study was to determine the effects of feeding nutritionally adequate and increased levels of vitamin A (retinyl acetate at 1·4, 34·4, and 206·4 mg/kg diet) in combination with adequate or increased Zn (12 and 240 mg/kg) and Cu (5 and 50 mg/kg) on serum and tissue concentrations of retinol and retinyl palmitate and on indices of Cu and Zn status in female Sprague–Dawley rats, and to measure interactive effects of such nutrient imbalances. Rats fed on diets containing 34·4 and 206·4 mg vitamin A/kg had higher feed intakes and relative liver weights than those fed on diets containing 1·4 mg vitamin A/kg. An interaction between dietary Cu and Zn and an independent effect of vitamin A affected serum ceruloplasmin oxidase (*EC* 1.16.3.1) activity. Rats fed on high Zn, adequate-Cu diets (240 and 5 mg Zn and Cu/kg respectively) had lower serum ceruloplasmin oxidase levels than rats fed on adequate-Zn, adequate-Cu diets (12 and 5 mg Zn and Cu/kg respectively). This effect was not observed in rats fed on high-Zn, high-Cu diets (240 and 50 mg Zn and Cu/kg respectively). Alterations in dietary levels of Cu and vitamin A independently affected haemoglobin levels. Serum cholesterol concentration was affected by interactions between Zn and vitamin A and Cu and vitamin A. Levels of retinol and retinyl palmitate in liver and kidney were significantly higher in rats fed on diets with increased dietary vitamin A than in those fed on diets with adequate vitamin A. Three-way interactions among Cu, Zn, and vitamin A affected levels of retinol in serum and liver. Two-way interactions between Cu and vitamin A affected liver retinyl palmitate and the sum of liver retinol + retinyl palmitate. An independent effect of dietary Zn on these variables was also observed. Interactions between Cu and vitamin A affected levels of Cu in liver and kidney, while Fe and Zn in kidney were affected by interactions between Cu and Zn. This study demonstrates that differing interactions among variables of vitamin A metabolism and mineral status occur with higher dietary levels of vitamin A, Zn and Cu in the rat.

Vitamin A: Zinc: Copper: Nutrient interactions

Numerous interactions occur among and between nutrients, with mineral–mineral and mineral–vitamin interactions among those most extensively described (Hambridge *et al.* 1986; Davis & Mertz, 1987). Many studies have demonstrated the importance of Zn in the metabolism of vitamin A and its role in the mobilization of vitamin A from liver to the plasma (Brown *et al.* 1976; Sundaresan *et al.* 1977; Duncan & Hurley, 1978; Goodman, 1984; Shankar *et al.* 1986). Shankar *et al.* (1986) studied the effects of feeding laboratory chow supplemented with high vitamin A (i.e. 154 times the level recommended by the

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National Research Council (NRC)) in combination with increased Zn (thirty-three times the NRC-recommended level (NRC, 1978)) on total lipids, cholesterol, vitamin A and Zn concentrations in liver and cholesterol in serum in adult female rats. They reported that the concentrations of total liver lipids and liver cholesterol were higher in rats fed on laboratory chow diets with high vitamin A or a combination of high vitamin A and high Zn than in rats fed on a basal diet, suggesting that high vitamin A was affecting these variables regardless of the dietary Zn level. Furthermore, rats given high dietary Zn or a combination of high Zn and high vitamin A had significantly higher serum cholesterol levels, and concentrations of Zn in the liver were positively correlated with serum cholesterol. Liver and kidney vitamin A levels were significantly lower in rats fed on high-Zn–high-vitamin A diets than in those fed on adequate-Zn–high-vitamin A diets, suggesting altered vitamin A metabolism (e.g. altered absorption, transport, storage or hydrolysis) in the former group. These observations suggested that high Zn or an altered Zn:Cu ratio was influencing these tissue vitamin A levels. In the study of Shankar *et al.* (1986), the Zn:Cu ratio in the high-Zn diets was 26:1 (w/w) compared with a Zn:Cu ratio of 3·5:1 (w/w) in the control diet. Shankar *et al.* (1986) did not specifically examine whether the large decrease in Cu relative to Zn in the high-Zn-fed group influenced their results.

The present study was designed to extend the observations of Shankar *et al.* (1986) by investigating the effects of feeding purified diets containing increased levels of vitamin A in combination with increased Zn, and by determining whether increased dietary Cu, by altering the Zn:Cu ratio, affected the responses to increased Zn and vitamin A. In the present study purified diets were used instead of the chow diets used by Shankar *et al.* (1986) to allow better control of vitamin A and major and trace mineral composition.

MATERIALS

Standards

All-*trans*-retinyl acetate, all-*trans*-retinol, and all-*trans*-retinyl palmitate were gifts from Hoffmann–LaRoche (Nutley, NJ, USA). Butylated hydroxytoluene (BHT) was purchased from Sigma Chemical Co. (St Louis, MO, USA). HPLC solvents were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Diets

Diet composition was based on that of diet AIN-76A (American Institute of Nutrition, 1977, 1980). Diets, prepared in powdered form, contained (g/kg): protein (casein) 200, choline bitartrate 2, DL-methionine 3, maize starch 150, maize oil 50, fibre 50, mineral mix 35, vitamin mix 10, and glucose 500. Minerals were added to diets to provide calculated concentrations (mg/kg diet): Ca 5100, P 5200, Na 1090, K 3600, Mg 500, Mn 54, Fe 50, Zn 12 or 240, Cu 5 or 50, I 0·21, Se 0·11, and Cr 2·0. Vitamin A, as retinyl acetate, was added to diets at levels of 1·4, 34·4 or 206·4 mg/kg. These levels correspond to 4000, 100000 and 600000 IU retinyl acetate respectively. The study had a $2 \times 2 \times 3$ factorial design. Rats (n 120) were randomly assigned to the twelve experimental diets, ten rats per group.

Experimental animals

Female Sprague–Dawley rats (22 d old, weighing 46 (SD 5) g) were obtained from Harlan Sprague–Dawley, Indianapolis, IN, USA. Rats were housed singly in suspended stainless steel cages with wire-mesh flooring, fed *ad libitum*, and weighed three times weekly. Feed consumption was measured weekly. Distilled deionized water was provided *ad libitum*. Room temperature was maintained at 70–74°. Room lighting provided alternating 12 h

periods of light and dark. After 4 weeks, rats were fasted overnight and then killed by CO_2 asphyxiation. Blood was collected by cardiac puncture. Serum was prepared and stored at $< -70°$ until analysis. Soft tissues were stored at $-17°$ until analysis.

The studies reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (NRC, 1986).

METHODS

Analysis of retinol and retinyl palmitate in biological samples

Serum retinol was extracted by denaturing serum (1·0 ml, rat) with absolute ethanol (1·0 ml) containing the internal standard (3·46 nmol retinyl acetate) as described by Sundaresan *et al.* (1994). Liver vitamin A was extracted from samples of approximately 1 g, essentially as described by Sundaresan *et al.* (1994) except that the tissue homogenate (10 ml) was re-extracted twice with equal volumes (10 ml) of petroleum ether. The combined petroleum ether extracts only (approximately 18 ml) were evaporated under N_2 and dissolved in 1 ml of acetonitrile–butanol (1:1 v/v). One whole kidney (approximately 0·8 g) was extracted in the same manner as liver. Portions (20 μl) of the final extracts and standards were analysed by reverse-phase HPLC (Beckman model 346 (Beckman Instruments, Inc., Fullerton, CA, USA) equipped with an IBM PS/2 Model 502 data integration system (International Business Machines Corporation, Boca Raton, FL, USA)).

A gradient HPLC analysis was performed using a 5 μm Econosphere C18 4·6 × 250 mm column (Alltech Associates, Deerfield, IL, USA) and a 5 μm precolumn packed with reverse phase C18 (Separations Group, Hesperia, CA, USA). Retinyl acetate was used as the internal standard. The mobile phase was 100% methanol (solvent A) and isopropanol–methanol (50:50, v/v; solvent B). The gradient procedure at a flow rate of 1·5 ml/min was as follows: 100% solvent A was used for 5 min followed by a 2 min linear gradient to 100% solvent B, a 9 min hold at 100% solvent B, and then a 2 min gradient back to 100% solvent A. The ultraviolet wavelength detector, set at 325 nm, was used at the maximum sensitivity (0·001 absorbance units, full scale).

Quantitation of retinol and retinyl palmitate in biological samples was achieved from standard curves determined by HPLC of varying concentrations of standards (retinol and retinyl palmitate) and a single concentration of retinyl acetate, and from comparisons of ratios of their respective peak areas to the peak area of the internal standard. Results were corrected for recovery of the internal standard. Concentrations of standards were determined spectrophotometrically.

Linear responses with correlation coefficients of the corresponding regression lines (in parentheses) were observed from 1·36 to 10·91 μM for retinol (r 0·99979, n 12) and 1·31 to 10·50 μM for retinyl palmitate (r 0·99959, n 12). The mean recovery (%) of retinyl acetate from three independent experiments was 88 (SD 8·8). The CV for measurement of retinyl acetate, retinol, and retinyl palmitate were 9·9 (n 28), 4·4 (n 16) and 6·5% (n 13) respectively. The sum of retinol and retinyl palmitate in liver or kidney represents approximately 85–90% of the total vitamin A content of these tissues. Thus, 10–15% of total vitamin A in these tissues is present as esters other than retinyl palmitate. It was not possible to quantitate these retinyl esters due to lack of appropriate standards.

Other analyses

Haemoglobin was measured with Sigma Diagnostics Kit no. 525. Serum ceruloplasmin (*EC* 1.16.3.1) was measured by its oxidase activity with *o*-dianisidine dihydrochloride (Schosinsky *et al.* 1974). Serum cholesterol was measured enzymically using Sigma

Diagnostics Kit no. 352. Superoxide dismutase activity (Zn–CuSOD, *EC* 1.15.1.1) in liver homogenates was measured as described by Misra & Fridovich (1977).

Analysis of minerals in diets and tissues

Portions of tissues and diets were weighed and wet-digested in mixtures of HNO_3, $HClO_4$ (diets) or HNO_3, $HClO_4$, and H_2SO_4 (liver, kidney) (Rader *et al.* 1986) and analysed by inductively coupled argon plasma–atomic emission spectrometry (ICP–AES) using a sequential Plasma II system (Perkin-Elmer, Norwalk, CT, USA). Analytical wavelengths used for analyses have been reported previously (Rader *et al.* 1984). Portions of National Institute of Standards and Technology (NIST) Standard Reference Material Bovine Liver 1577 were digested and analysed together with the samples. Values fell within $\pm 5\%$ of certified values.

Statistical analysis

Data were subjected to a $2 \times 2 \times 3$-way factorial ANOVA using the SAS general linear models procedure (Statistical Analysis Systems Institute, Cary, NC, USA, version 6.08). All of the hypothesis tests in such a design use a common pooled estimate of the error variance. Thus, it is important that the treatment variances are approximately equal and that there is not a strong association between the variance of the response variable and its mean (Miller, 1986). In the present study the data for vitamin A in liver and kidney showed profound increases in variances with increased dietary vitamin A and with the response means. For example, for the raw data for the variable liver retinol + retinyl palmitate, the ratio of the variance of the 206·4 mg retinyl acetate/kg group to that of the 1·4 mg retinyl acetate/kg group was almost 15000. The results of a regression analysis of the relationship between the response mean and the variance suggested that it would be appropriate to analyse the natural logarithms (ln) of the liver vitamin A data. The ratio of the variances of the two groups mentioned above was reduced to 1·5 after the log transformation. The variances of data for liver retinol and retinyl palmitate (see Table 2) were similarly stabilized with the log transformation and the data were then suitable for ANOVA.

The raw data for kidney retinol, retinyl palmitate, and retinol + retinyl palmitate were also not suitable for ANOVA due to the several hundred-fold increase in variance with vitamin A treatment. In contrast to the raw data for vitamin A in liver, however, the natural logarithm was not an appropriate transformation. Consequently, separate two-way ANOVA were performed at each vitamin A treatment level. Comparisons were made between treatment groups only in the absence of any higher level interactions. Hypothesis tests on individual comparisons were performed using the least significant difference *t* test option in the general linear models procedure. For all tests, P values $< 0{\cdot}05$ were considered to be statistically significant.

RESULTS

Body-weight gains among diet groups were affected by a two-way interaction between dietary Zn and vitamin A (Table 1). Weight gain was slightly lower (6·4%; $P = 0{\cdot}0206$) with 240 mg Zn/kg than with 12 mg Zn/kg. The main effect on feed intake was that of dietary vitamin A: mean intake by rats given 206·4 mg retinyl acetate/kg was 5·5% higher than that of rats given 1·4 mg retinyl acetate/kg ($P = 0{\cdot}0014$). Haemoglobin values in rats given 34·4 mg retinyl acetate/kg were about 7% higher than haemoglobin values in rats given 1·4 mg retinyl acetate/kg ($P = 0{\cdot}0113$). Haemoglobin values were also about 5% higher in rats given 50 mg Cu/kg than in those given 5 mg Cu/kg ($P = 0{\cdot}0221$). Mean

Table 1. *Weight gain, feed consumption, haemoglobin (HGB), relative liver weight, serum ceruloplasmin and cholesterol, and liver zinc–copper superoxide dismutase (EC 1.15.1.1) (Zn-CuSOD) in rats fed on diets containing various levels of retinyl acetate, zinc, and copper**

(Mean values and standard deviations for ten rats per group)

Dietary level (mg/kg)			Weight gain (g)		Feed intake (g)		HGB (g/l)		Liver weight (% of body wt)		Serum ceruloplasmin (Units/l)		Serum cholesterol (mmol/l)		Liver Zn-CuSOD (mg/g)	
Retinyl acetate	Zinc	Copper	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1·4	12	5	123	10	401	25	139	22	4·0	0·3	79	15	2·41	0·26	1·16	0·25
1·4	12	50	125	10	403	21	148	33	4·0	0·3	85	11	2·41	0·18	1·13	0·32
1·4	240	5	119	11	394	36	136	17	3·9	0·3	43	31	2·90	0·36	0·90	0·29
1·4	240	50	114	6	374	17	159	14	4·0	0·2	104	13	2·85	0·47	1·08	0·28
34·4	12	5	121	12	403	34	161	12	4·1	0·3	100	8	2·82	0·28	1·16	0·32
34·4	12	50	118	8	398	31	159	20	3·9	0·2	113	17	3·21	0·57	1·14	0·30
34·4	240	5	125	6	406	26	151	13	4·0	0·3	59	49	3·39	0·49	0·85	0·33
34·4	240	50	125	11	411	34	151	9	3·9	0·2	109	18	3·18	0·59	1·02	0·22
206·4	12	5	120	8	413	35	144	12	4·2	0·2	162	24	3·34	0·59	1·10	0·28
206·4	12	50	116	11	411	19	157	6	4·1	0·3	185	15	3·31	0·52	1·14	0·25
206·4	240	5	118	8	410	32	147	9	4·2	0·2	126	53	2·95	0·39	1·01	0·17
206·4	240	50	116	14	424	38	146	10	4·2	0·2	159	16	3·00	0·59	1·15	0·26
Analysis of variance (2 × 2 × 3): *P* =																
Copper			NS		NS		0·0221		NS		0·0001		NS		NS	
Zinc			NS		NS		NS		NS		0·0001		NS		0·0075	
Copper × zinc interaction			NS		NS		NS		NS		0·0006		NS		NS	
Vitamin A			NS		0·0059		0·0285		0·0002		0·0001		0·0001		NS	
Copper × vitamin A interaction			NS		NS		NS		NS		NS		0·041		NS	
Zinc × vitamin A interaction			0·0234		NS		NS		NS		NS		0·0007		NS	
Copper × zinc × vitamin A interaction			NS		NS		NS		NS		NS		NS		NS	

* For details of diets and procedures, see pp. 916–918.

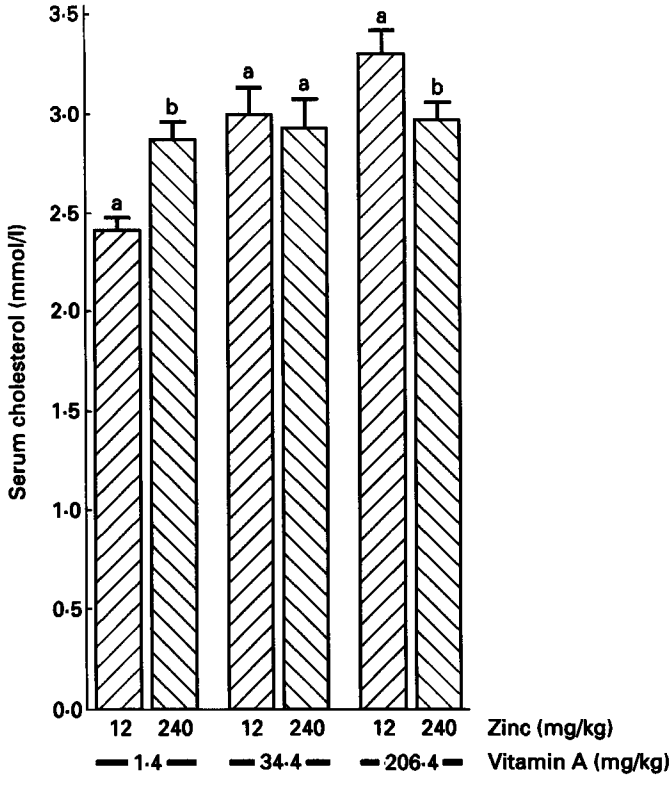

Fig. 1. Graphic representation of the effect of a two-way interaction between zinc and vitamin A on serum cholesterol concentration in rats. Comparisons between means in the zinc groups were made within each level of dietary vitamin A. The effects of two levels of zinc (12 and 240 mg/kg) on serum cholesterol are shown separately for each of the three levels of retinyl acetate tested (1·4, 34·4 and 206·4 mg/kg). [a,b] Mean values within each dietary vitamin A group not sharing a common letter were significantly different ($P < 0·05$) based on the least significant difference t test using the pooled error variance from the complete model.

relative liver weight was 5·3% higher in rats given 206·4 mg retinyl acetate/kg than in those given 1·4 mg retinyl acetate/kg ($P = 0·002$). All of these changes are quite modest and may not be biologically significant in this 4-week study.

Serum ceruloplasmin was significantly higher in rats fed on diets with increased dietary vitamin A (Table 1). A two-way interaction between Cu and Zn also affected serum ceruloplasmin activity. Serum ceruloplasmin in rats fed on the 5 mg Cu/kg diet was higher with dietary Zn at 12 mg/kg than with dietary Zn at 240 mg/kg ($P = 0·0001$). There was no significant difference between Zn treatments when Cu was given at 50 mg/kg.

Serum cholesterol was affected by interactions between Cu and vitamin A and Zn and vitamin A. When retinyl acetate was given at 34·4 mg/kg, serum cholesterol was about 16% higher in rats given 50 mg Cu/kg than in those given 5 mg Cu/kg ($P = 0·0026$). No effects of increased Cu were observed at dietary levels of retinyl acetate of 1·4 or 206·4 mg/kg. When retinyl acetate was given at 1·4 mg/kg, serum cholesterol was 18·7% higher in rats given 240 mg Zn/kg diet than in those given 12 mg Zn/kg diet ($P = 0·0024$). There was no effect on serum cholesterol of increased dietary Zn in rats given 34·4 mg retinyl acetate/kg diet. At dietary retinyl acetate levels of 206·4 mg/kg, serum cholesterol was about 12% lower in rats given 240 mg Zn/kg than in those given 12 mg Zn/kg ($P = 0·0203$). The complexity of this interaction is shown graphically in Fig. 1.

Table 2. *Retinol and retinyl palmitate concentrations in serum and liver of rats fed on diets containing various levels of retinyl acetate, zinc, and copper**
(Mean values and standard deviations for ten rats per group)

Dietary level (mg/kg)			Serum retinol (μmol/l)		Liver level (μmol/g)					
					Retinol		Retinyl palmitate		Retinol + retinyl palmitate	
Retinyl acetate	Zinc	Copper	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1·4	12	5	3·0	0·5	0·02†	0·004	0·21	0·02	0·23	0·02
1·4	12	50	2·0	0·3	0·01	0·001	0·18	0·03	0·19	0·03
1·4	240	5	1·4	0·3	0·01	0·002	0·23	0·05	0·24	0·05
1·4	240	50	1·8	0·6	0·03	0·005	0·18	0·02	0·21	0·03
34·4	12	5	1·7	0·4	0·16	0·03	5·42	1·03	5·58	1·03
34·4	12	50	1·5	0·4	0·04	0·01	6·00	1·41	6·04	1·40
34·4	240	5	1·8	0·4	0·12	0·08	5·67	0·78	5·80	0·82
34·4	240	50	1·8	0·3	0·05	0·02	7·16	1·13	7·21	0·81
206·4	12	5	1·7	0·3	0·09	0·01	24·4	4·5	24·5	4·5
206·4	12	50	1·6	0·2	0·39	0·19	23·1	4·9	23·3	5·0
206·4	240	5	1·5	0·3	0·27	0·18	25·3	1·5	25·6	1·6
206·4	240	50	1·7	0·3	0·22	0·04	25·0	3·9	25·2	3·9
Analysis of variance‡ (2 × 2 × 3): *P* =										
Copper			NS		NS		NS		NS	
Zinc			0·0018		0·0029		0·0217		0·0089	
Copper × zinc interaction			0·0001		0·0056		NS		NS	
Vitamin A			0·0001		0·0001		0·0001		0·0001	
Copper × vitamin A interaction			NS		0·0001		0·0001		0·0005	
Zinc × vitamin A interaction			0·0001		0·0026		NS		NS	
Copper × zinc × vitamin A interaction			0·0005		0·0001		NS		NS	

* For details of diets and procedures, see pp. 916–918.
† *n* 9.
‡ The analysis of variance was performed on log-transformed data for all variables above except serum retinol.

The activity of liver Zn–CuSOD was affected only by changes in dietary Zn level, with the enzyme activity lower by about 14% when Zn was increased from 12 to 240 mg/kg diet (Table 1).

Three-way interactions among vitamin A, Cu, and Zn affected serum and liver retinol concentrations (Table 2). These interactions are shown graphically in Figs. 2 and 3 respectively. Effects of dietary Cu and Zn on serum retinol were much more pronounced in rats given 1·4 mg retinyl acetate/kg diet than in those given the higher levels of vitamin A (Fig. 2). In the case of liver retinol, effects of dietary Cu and Zn were more pronounced in rats fed on the two lower levels of vitamin A. Two-way interactions between Cu and vitamin A and a main effect of Zn were responsible for changes in liver retinyl palmitate and the sum of liver retinol + retinyl palmitate (Table 2).

Separate two-way ANOVA were performed for vitamin A variables in kidney at the three levels of dietary vitamin A tested (Table 3). When rats were fed on the 1·4 mg retinyl acetate/kg diet, kidney retinol, retinyl palmitate, and the sum of retinol + retinyl palmitate

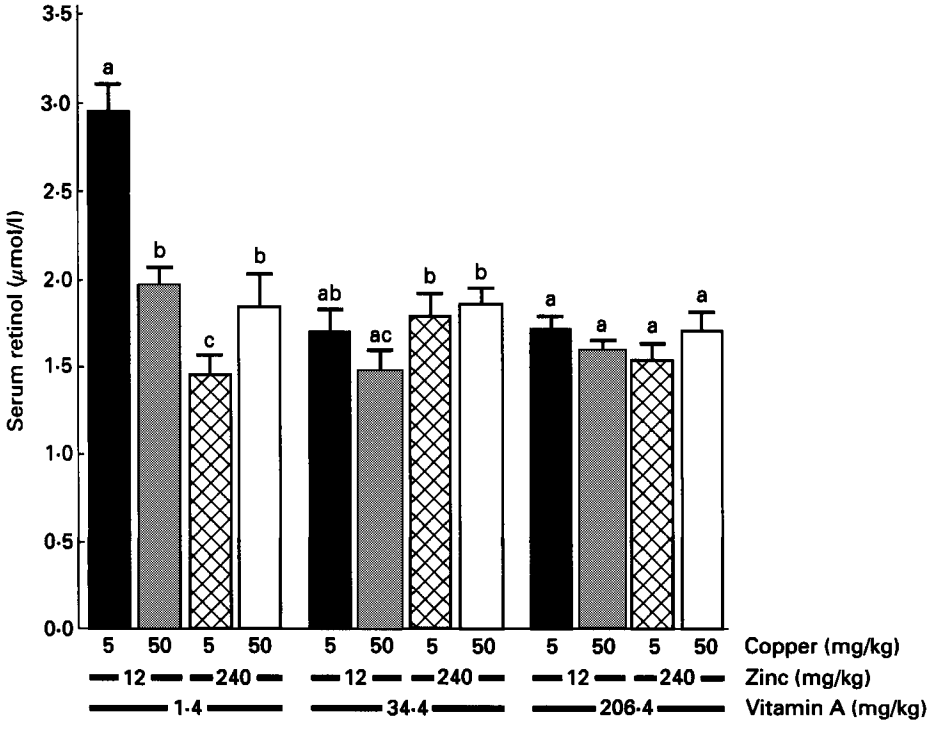

Fig. 2. Graphic representation of the effect of a three-way interaction among copper, zinc and vitamin A on serum retinol concentration in rats. Comparisons between means in the copper and zinc groups were made within each level of dietary vitamin A. The effects of two levels of copper (5 and 50 mg/kg) and two levels of zinc (12 and 240 mg/kg) on serum retinol are shown in separate panels for each of the three levels of retinyl acetate tested (1·4, 34·4 and 206·4 mg/kg). [a, b, c] Mean values within each dietary vitamin A group not sharing a common letter were significantly different ($P < 0·05$) based on the least significant difference t test using the pooled error variance from the complete model.

were significantly lower with 240 mg Zn/kg diet than with 12 mg Zn/kg diet. This effect of increased dietary Zn was not observed at the higher levels of vitamin A tested.

Concentrations of Fe in liver were affected by levels of dietary vitamin A, Cu, and Zn. An interaction between Cu and Zn affected Fe levels in kidney (Table 4). In rats given 5 mg Cu/kg diet, kidney Fe was lower by about 9% when dietary Zn was increased from 12 to 240 mg/kg ($P = 0·0005$). This effect of Zn was not observed in rats given 50 mg Cu/kg diet.

Cu levels in liver were significantly affected by two-way interactions between Cu and vitamin A and Zn and vitamin A. When diets contained 1·4 or 34·4 mg retinyl acetate/kg, liver Cu levels were about 31 and 29% higher respectively in rats given 50 mg Cu/kg than in those given 5 mg Cu/kg ($P = 0·0001$ for both comparisons). At the highest level of vitamin A tested, however, the difference between liver Cu values in rats given 50 *v.* 5 mg Cu/kg was considerably smaller (13%, $P = 0·0039$).

Kidney Cu was affected by a Cu × vitamin A interaction similar to that observed for liver Cu. When diets contained 1·4 or 34·4 mg retinyl acetate/kg, kidney Cu levels were about 32 and 27% higher respectively in rats given 50 mg Cu/kg than in those given 5 mg Cu/kg ($P = 0·0001$ for both comparisons). At the highest level of vitamin A tested, however, the difference between kidney Cu values in rats given 50 *v.* 5 mg Cu/kg was smaller (12%,

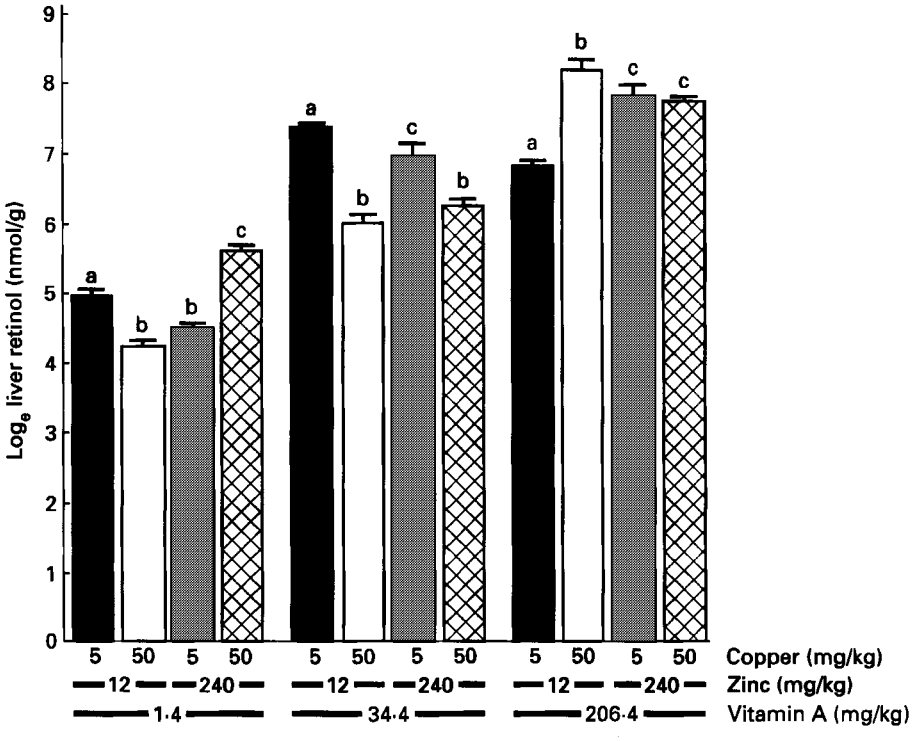

Fig. 3. Graphic representation of the effect of a three-way interaction among copper, zinc, and vitamin A on liver retinol concentration in rats. Comparisons between means in the copper and zinc groups were made within each level of dietary vitamin A. The effects of two levels of copper (5 and 50 mg/kg) and two levels of zinc (12 and 240 mg/kg) on liver retinol (log scale) are shown in separate panels for each of the three levels of retinyl acetate tested (1·4, 34·4 and 206·4 mg/kg). ^{a,b,c} Mean values within each dietary vitamin A group not sharing a common letter were significantly different ($P < 0·05$) based on the least significant difference t test using the pooled error variance from the complete model.

$P = 0·021$). A main effect of dietary Zn on kidney Cu ($P = 0·0012$) was observed (Table 4).

Zn in liver was sensitive to a main effect of dietary Cu as well as to a Zn × vitamin A interaction (Table 4). In contrast, Zn in kidney was not affected by dietary vitamin A but was affected by an interaction between Zn and Cu. Kidney Ca was about 30% lower in rats given 240 mg Zn/kg diet than in those given 12 mg Zn/kg diet ($P = 0·006$) (Table 4).

DISCUSSION

There is increasing evidence that significant interactions occur between and among essential nutrients at levels that are not considered to be toxic. The NRC-recommended dietary levels of vitamin A (as retinyl acetate), Zn, and Cu for rats are 1·38, 12 and 5 mg/kg respectively (NRC, 1978). Levels of retinyl acetate (34·4 and 206·4 mg/kg), Zn (240 mg/kg) and Cu (50 mg/kg) in the present study were chosen to provide significantly increased but not toxic intakes. Dietary levels were 25- and 150- (retinyl acetate), 20- (Zn), and 10- (Cu) fold higher than NRC recommendations. No signs of hypervitaminosis A were observed in our study at the levels of vitamin A fed.

Pronounced Cu depletion has been reported in weanling rats fed on diets containing very low levels of dietary Cu (< 0·5 mg Cu/kg diet) for about 4 weeks (Rader *et al.* 1991). In

Table 3. *Retinol and retinyl palmitate concentrations in kidney of rats fed on diets containing various levels of retinyl acetate, zinc, and copper**
(Mean values and standard deviations for ten rats per group)

Dietary level (mg/kg)			Kidney level (μmol/g)					
			Retinol		Retinyl palmitate		Retinol + retinyl palmitate	
Retinyl acetate	Zinc	Copper	Mean	SD	Mean	SD	Mean	SD
1·4	12	5	0·006	0·001	0·002	0·001	0·008	0·002
1·4	12	50	0·007	0·001	0·003	0·008	0·100	0·009
1·4	240	5	0·003	0·002	ND†	—	0·003	0·003
1·4	240	50	0·003	0·002	ND†	—	0·003	0·002
ANOVA‡: *P* =	Copper		NS		NS		NS	
	Zinc		0·0001		0·0471		0·0004	
	Copper × zinc		NS		NS		NS	
34·4	12	5	0·008	0·001	0·010	0·001	0·019	0·002
34·4	12	50	0·007	0·001	0·002	0·001	0·009	0·002
34·4	240	5	0·008	0·002	0·003	0·001	0·011	0·002
34·4	240	50	0·008	0·001	0·003	0·001	0·010	0·002
ANOVA‡: *P* =	Copper		NS		0·0404		NS	
	Zinc		NS		NS		NS	
	Copper × zinc		NS		NS		NS	
206·4	12	5	0·16	0·04	0·17	0·06	0·33	0·09
206·4	12	50	0·16	0·04	0·14	0·06	0·30	0·10
206·4	240	5	0·15	0·03	0·17	0·05	0·32	0·08
206·4	240	50	0·14	0·03	0·17	0·03	0·31	0·06
ANOVA‡: *P* =	Copper		NS		NS		NS	
	Zinc		NS		NS		NS	
	Copper × zinc		NS		NS		NS	

ND, not detected.
* For details of diets and procedures, see pp. 916–918.
† The limit of detection for the analysis of retinyl palmitate is 0·44 μM.
‡ The analysis of variance was performed on raw data.

the present study we observed changes consistent with Cu depletion (e.g. lower levels of serum ceruloplasmin, kidney Cu, and of liver Zn–CuSOD) in rats given 240 mg Zn/kg diet even when adequate Cu (5 mg/kg) was included in the diets. Serum ceruloplasmin oxidase activity was clearly influenced by a Cu × Zn interaction. Serum ceruloplasmin oxidase levels were significantly higher in rats fed on diets containing 240 mg Zn and 50 mg Cu/kg than in those fed on diets with 240 mg Zn and 5 mg Cu/kg.

Several studies have examined the effects of changes in dietary Zn alone or in combination with changes in other nutrients on plasma cholesterol levels. Klevay (1973) reported an increase in plasma cholesterol concentration in rats provided with drinking water in which the Zn:Cu ratio was 40:1 (w/w) for 45–151 d, and Koo & Williams (1981) reported that acute dietary Zn depletion (dietary Zn < 0·4 mg/kg) caused a reduction in total serum cholesterol concentration in rats. We observed interactions between Cu and vitamin A and between Zn and vitamin A in their effect on serum cholesterol levels under the conditions of the present study, but we did not observe an interaction between Zn and Cu. The interactive effect of Zn and vitamin A was the predominant influence on serum

Table 4. *Concentrations of iron, copper and zinc in livers of rats fed on diets containing various levels of retinyl acetate, zinc, and copper**

(Mean values and standard deviations for ten rats per group)

Dietary level (mg/kg)			Liver level (μmol/g)						Kidney level (μmol/g)							
			Iron		Copper		Zinc		Iron		Copper		Zinc		Calcium	
Retinyl acetate	Zinc	Copper	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1·4	12	5	2·92	0·34	0·061	0·008	0·42	0·05	0·80	0·06	0·071	0·006	0·37	0·01	7·52	6·66
1·4	12	50	2·79	0·68	0·080	0·013	0·45	0·02	0·77	0·05	0·088	0·017	0·35	0·02	3·99	2·49
1·4	240	5	2·86	0·56	0·050	0·009	0·50	0·03	0·70	0·06	0·058	0·011	0·38	0·02	2·48	1·26
1·4	240	50	2·40	0·25	0·066	0·005	0·51	0·04	0·77	0·06	0·085	0·011	0·41	0·03	1·97	0·19
34·4	12	5	2·78	0·41	0·063	0·005	0·44	0·03	0·76	0·05	0·063	0·006	0·37	0·01	4·59	3·37
34·4	12	50	2·51	0·29	0·080	0·011	0·46	0·02	0·74	0·04	0·079	0·011	0·35	0·01	4·08	1·39
34·4	240	5	2·40	0·45	0·049	0·014	0·43	0·02	0·71	0·05	0·055	0·008	0·37	0·03	1·98	0·22
34·4	240	50	2·33	0·14	0·065	0·006	0·46	0·02	0·77	0·05	0·071	0·009	0·38	0·02	2·08	0·49
206·4	12	5	2·24	0·18	0·061	0·005	0·44	0·02	0·73	0·11	0·065	0·011	0·35	0·04	5·69	5·96
206·4	12	50	2·10	0·23	0·068	0·006	0·43	0·02	0·76	0·10	0·066	0·009	0·36	0·04	5·25	3·27
206·4	240	5	2·04	0·20	0·058	0·008	0·45	0·03	0·69	0·04	0·057	0·005	0·37	0·02	2·08	0·42
206·4	240	50	1·95	0·25	0·068	0·006	0·46	0·02	0·76	0·07	0·069	0·005	0·38	0·01	2·98	1·26
Analysis of variance (2 × 2 × 3): *P* =																
Copper			0·0057		0·0001		0·0029		0·0175		0·0001		NS		NS	
Zinc			0·0012		0·0001		0·0001		0·0343		0·0012		0·0001		0·0001	
Copper × zinc interaction			NS		NS		NS		0·0035		NS		0·0073		NS	
Vitamin A			0·0001		NS		0·001		NS		0·0001		NS		NS	
Copper × vitamin A interaction			NS		0·0234		NS		NS		0·0071		NS		NS	
Zinc × vitamin A interaction			NS		0·0022		0·0001		NS		NS		NS		NS	
Copper × zinc × vitamin A interaction			NS		NS		NS		NS		NS		NS		NS	

* For details of diets and procedures, see pp. 916–918.

cholesterol (Table 1). At the highest level of vitamin A tested, serum cholesterol concentration was significantly higher in rats given 240 mg Zn/kg diet than in those given 12 mg Zn/kg diet. This finding confirms the observations of Shankar *et al.* (1986) on the effects of high dietary Zn on serum cholesterol levels. It is clear from the graphical presentation in Fig. 1 that effects that occur at lower levels of nutrient intake are not necessarily the same as those that occur with increased intakes of the same nutrients.

We measured liver superoxide dismutase activity because of its sensitivity to changes in Cu status (Fields *et al.* 1984*a*, *b*; Reicks & Rader, 1990). Like serum ceruloplasmin oxidase activity, liver superoxide dismutase activity was significantly lower in rats given 240 mg Zn/kg diet than in those given 12 mg Zn/kg diet. We observed no other main effects or interactive effects on the activity of this enzyme.

Although interactions between Zn and Cu and between Zn and vitamin A have been well described, less is known about interactions between Cu and vitamin A. Retinol and Cu are stored preferentially in liver and carried in blood plasma bound to retinol binding protein and ceruloplasmin respectively (Goodman, 1984; Cousins, 1985). Complex interactions between retinol and Cu have been reported in chronic Cu poisoning in sheep (Moore *et al.* 1972), but little is known about interactions between Cu and vitamin A under more usual circumstances.

The effect of a three-way interaction among Cu, Zn, and vitamin A on serum retinol level is presented graphically in Fig. 2. The three panels depict the relationship between Cu and Zn separately for each dietary level of vitamin A. The strong interaction is reflected by marked differences in effects of these minerals at different dietary levels of vitamin A. In general, effects of both minerals on serum retinol are more pronounced in rats fed on the lowest level of vitamin A tested. A similar three-way interaction among Cu, Zn, and vitamin A on liver retinol is shown in Fig. 3. Profound effects of changes in levels of dietary Zn and Cu occurred in rats fed on the two lower doses of vitamin A tested.

We observed higher serum ceruloplasmin oxidase activity with higher vitamin A contents in the diets. These findings are in agreement with results reported by Barber & Cousins (1987), who described the induction of serum ceruloplasmin oxidase activity by single high doses of 13-*cis*-retinoic acid (150 mg/kg) in vitamin A-repleted rats. The same authors also demonstrated the induction of ceruloplasmin oxidase activity in vitamin A-deficient rats repleted with 100 μg 13-*cis*-retinoic acid given orally every day for up to 5 d or 100 μg retinyl acetate given orally every day for 5 d.

About 90% of plasma Cu is found in ceruloplasmin in normal mammals and highly significant correlations have been demonstrated between ceruloplasmin levels and plasma, serum, and whole-blood Cu levels (Davis & Mertz, 1987). Our finding that a 10-fold increase in dietary Cu had no significant effect on serum ceruloplasmin oxidase activity is consistent with observations that moderate additions of Cu to normal diets have little effect on blood Cu concentration. For example, Milne & Weswig (1968) found no significant differences in ceruloplasmin activity in rats receiving diets containing 10, 50, 100 or 200 mg Cu/kg, and reported no increase in plasma Cu level in rats when the Cu content of the diet was increased from 10 to 50 mg/kg. Plasma Cu, however, doubled from 1·16 to 2·34 μg/ml when dietary Cu was increased from 50 to 100 mg/kg.

In the present study, serum ceruloplasmin oxidase activity was strongly influenced by an interaction between Zn and Cu. Serum ceruloplasmin oxidase activity was almost 50% higher in rats given adequate-Cu, adequate-Zn diets (5 and 12 mg Cu and Zn respectively) than in those fed on diets with adequate Cu and high Zn (5 and 240 mg Cu and Zn respectively). However, when Cu was included at 50 mg/kg the effect of high dietary Zn on serum ceruloplasmin oxidase activity was not observed. Elements such as Zn or Cd can depress Cu absorption and can reduce plasma Cu concentrations when ingested at high

dietary levels. Campbell & Mills (1974) reported that weanling rats given supplemental Zn at levels as low as 300 mg/kg in diets marginally adequate in Cu (e.g. 2·6 mg Cu/kg) had levels of plasma ceruloplasmin oxidase activity that were 63% of those of rats fed on the control diet (e.g. without supplemental Zn). Rats given supplemental Zn at levels of 1000 mg/kg diet had plasma ceruloplasmin levels that were 22% of those of rats fed on the control diet. Diets containing 1000 mg Zn/kg also reduced growth and reduced plasma Cu concentrations by 89% and liver Cu by 47%. Thus, levels of dietary Zn at which toxic effects are evident depend on the dietary Zn:Cu ratio.

In the present study, effects of Zn, as evidenced by lower levels of Cu in liver and kidney, were observed at a Zn level of 240 mg/kg when dietary Cu level was 5 mg/kg. In addition, at the highest level of retinyl acetate tested (206·4 mg/kg) the effect of increased dietary Zn on Cu levels was less pronounced both in liver and in kidney.

Concentrations of Fe in liver were sensitive to changes in dietary vitamin A levels and were significantly lower in rats receiving high dietary vitamin A than in those given adequate dietary vitamin A (Table 4). Similar results were reported by Staab *et al.* (1984), who demonstrated that a high vitamin A intake (21·9 mg retinyl palmitate/kg; 40000 IU/kg) was associated with significantly lower liver Fe levels in weanling rats. The authors proposed that vitamin A was involved in the regulation of Fe release from liver.

Liver Fe was also lower when dietary Cu was 50 mg/kg than when it was 5 mg/kg and when dietary Zn was 240 mg/kg than when it was 12 mg/kg regardless of the level of dietary vitamin A. The effects of vitamin A, Zn, and Cu on liver Fe appeared to represent independent actions of these nutrients. These results appear to be tissue-specific as well, since a Cu–Zn interaction was observed with kidney Fe but not with liver Fe. Kidney Ca was significantly lower in rats fed on diets with 240 mg Zn/kg than in those fed on diets with 12 mg Zn/kg at all levels of vitamin A tested.

Conclusions

This study has demonstrated that, in the rat, when several components of the diet are increased simultaneously, interactions occur that affect a wide range of variables related to status for specific nutrients. In addition, different effects on status indicators occur as dietary levels of the nutrients are increased, making it difficult to predict the effects of changes in concentrations of nutrients.

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