Vitamin B-12 Deficiency and Hyperhomocysteinemia Are Partly Ameliorated by Cobalt and Nickel Supplementation in Pigs

G. I. Stangl, D. A. Roth-Maier and M. Kirchgessner

Institute of Nutritional Sciences, University of Technology of Munich, 85350 Freising-Weihenstephan, Germany.

ABSTRACT Vitamin B-12 deficiency and hyperhomocysteinemia alter the metabolism of trace elements. This study tested the hypothesis that there is a reverse relationship in which diets high in iron, copper, nickel and cobalt would influence vitamin B-12 deficiency outcomes including hyperhomocysteinemia. Piglets were assigned to six groups of 8 and fed one of the following diets for 166 d: a vitamin B-12-adequate and folate-fortified diet (30 μg/kg vitamin B-12 and 0.5 mg/kg folate) with normal trace element concentrations or one of five vitamin B-12–free, folate nonsupplemented diets (0.36 mg/kg), with either normal trace element concentrations or high concentrations of iron (300 mg/kg), copper (30 mg/kg), cobalt (1 mg/kg) or nickel (6 mg/kg). Feed intake and weight gain did not differ significantly among the groups. Vitamin B-12–deficient pigs developed diminished serum and liver concentrations of vitamin B-12 and folate, an accumulation of iron in the liver and hyperhomocysteinemia. The magnitude of changes differed among vitamin B-12–deficient groups. Vitamin B-12–deficient pigs fed 6 mg/kg nickel had distinctly higher vitamin B-12 concentrations in liver and serum and 45% lower serum concentration of homocysteine than the corresponding deficiency group fed 1 mg/kg nickel; iron concentration in liver was completely normalized. Vitamin B-12–deficient pigs fed 1 mg/kg cobalt had 47% lower homocysteine concentrations in serum than the vitamin B-12–deficient group fed 0.13 mg/kg cobalt, but the vitamin B-12 status was unaffected. Supplementation of iron and cobalt did not affect these variables. The dietary manipulations had no detrimental effects on variables symptomatic of oxidative stress. The findings indicate a collaborative relationship between vitamin B-12 metabolism and the trace elements nickel and cobalt.

KEY WORDS: vitamin B-12 deficiency hyperhomocysteinemia high trace element diets pigs
in the progression of atherothrombotic disease (Oitinen et al. 1999). The ability of excessive homocysteine to generate hydrogen peroxide has been implicated as a potential mechanism leading to endothelial dysfunction, which was observed in subjects with hyperhomocysteinemia (Kang et al. 1986). This study therefore also focused on the measurement of variables indicative of imbalances in oxidative and antioxidative status. These included the concentrations of circulating serum tocopherols and thiols, liver concentrations of oxidized glutathione (GSSG) and the antioxidant enzymes glutathione peroxidase (GSHPx, cofactors selenium, copper, EC 1.11.1.9) and catalase (EC 1.11.1.6).

Experimental vitamin B-12 deficiency can be produced in two different ways, either nutritionally or by exposure to the Co (I) vitamin B-12–inactivating agent nitrous oxide, which has often been used as a model for vitamin B-12 deficiency responses in pigs (e.g., Scott et al. 1994, Weir et al. 1992). However, metabolism of nitrous oxide could produce toxic intermediates and could also interfere with pesticide C oxidase and Ca2+-ATPase, side effects that are unrelated to cobalamin inactivation (Einarsdottir and Caughey 1988, Hong et al. 1980, Horn et al. 1999). Because of these side effects, and with a view to simulating situations as they occur with completely vegetarian diets or trophic changes at vitamin B-12 absorption sites, we decided to induce vitamin B-12 deficiency by dietary factors alone. This was done by prolonged feeding of a vitamin B-12-free diet without additional folate for 166 d, combined with a relatively high dietary methionine concentration because methionine-loading has been shown to exacerbate hyperhomocysteinemia in miniature pigs and rabbits (Jourdheuil-Rahmani et al. 1995, Koyama 1995).

**MATERIALS AND METHODS**

**Animals and diets.** In this experiment, 48 piglets (German Landrace x Pietrain) from multiple litters of commercial crossbred sows with a body weight of 8.61 ± 0.14 (mean ± SEM) kg were assigned to one of six treatment groups. Their allocation to dietary treatments was random from blocks based on litter, sex and initial weight. Each group comprised 8 piglets with 4 male castrates and 4 females. The dietary treatments were as follows: one control diet, in which pigs were fed a vitamin B-12–adequate and folate-supplemented diet with normal trace element concentrations (B12-C), or one of five vitamin B-12–free, folate nonsupplemented diets, with either normal trace element concentrations (B12-D), high iron concentrations (B12-D + Fe), high copper concentrations (B12-D + Cu) or high nickel concentrations (B12-D + Ni). The experiment lasted 166 d. Each group received the basal diet to limit observed differences in the actions of vitamin B-12 and the trace elements with the diets alone. From d 1 to 42 all pigs received basal diet 1 that was formulated to meet the nutrient requirements of piglets (5–25 kg; Gesellschaft für Ernährungsphysiologie 1987, National Research Council 1998). From d 43 to 166, a grower diet 2 was administered for all groups whose nutrient composition was designed to satisfy the mean requirement of pigs weighing 25–90 kg (Gesellschaft für Ernährungsphysiologie 1987, National Research Council 1998). The diet and nutrient compositions are shown in **Table 1**. The basal nonsupplemented diet that was offered from d 1 to 42 contained (per kg dry matter, DM) no vitamin B-12 and 0.35 mg folate/kg DM, 100 mg iron/kg DM, 0.13 mg cobalt/kg DM and 1.01 mg nickel/kg DM.

![Table 1](https://academic.oup.com/jn/article-abstract/130/12/3038/4686236)
the basal diets that were offered to the vitamin B12–adequate group and the vitamin B12–deficient groups were supplemented with taurine to contain 12 g/kg DM and 7.5 g/kg DM methionine, respectively. The energy density was 13.1 MJ metabolizable energy/kg feed for all diets. Except for vitamin B12 and folate, all diets were fortified with recommended amounts of vitamins and minerals (Geellschaft für Ernährungsphysiologie 1987; National Research Council 1998).

Pigs were fed twice a day and weighed weekly. The food intake of the groups fed the vitamin B12–free diets was adjusted to match that of the control group on the basis of body weight. The piglets were housed individually in an environmentally regulated room in cages and then were kept in pens of one animal each. The cages and pens were cleaned twice daily to minimize possible coprophagy. All pigs had free access to water. The experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the official veterinary office in Munich, Germany. One pig from the vitamin B12–deficient group died during the experiment.

**Analyses.** Five days before slaughter, on d 166 of the experiment, 12 h after the last feeding, venous blood samples were collected by puncture of the jugular vein for determination of the blood cell count and measurement of serum concentrations of vitamin B12, folate, homocysteine, clinico-chemical variables, enzymes and tocopherols. Blood cell counts and hematological variables were determined in EDTA-treated blood. Blood for determination of the serum metabolites was collected into untreated tubes. On d 166, 18 h after the last feeding, all pigs were slaughtered and livers excised for analysis of vitamin B12, folate and trace elements. Serum and liver samples for measurements were stored at −80°C pending analysis.

Serum and liver concentrations of vitamin B12 and folate were determined using a competitive binding RIA kit (ICN, Costa Mesa, CA) that worked with an extracting reagent (containing 1 mol/L sodium hydroxide and an organic extracting enhancer) to release vitamin B12 from transcobalamines. The RIA test kit used in this study served to remove the nonspecific vitamin B12–binding R-protein by affinity chromatography. Prior to the RIA quantification, vitamin B12 and folate were released from liver binders by papain proteolysis using the double extraction method of Van Tonder et al. (1975).

Serum concentrations of total homocysteine (free, bound to proteins or as mixed disulfide) were determined by HPLC (Cournwel et al. 1993). Serum samples were prepared for derivatization according to the method of Ubbink et al. (1991) using 7-fluorenyl-2-oxa-1,3-diazole-4-sulfonamide as derivatization reagent. Homocysteine was separated using a reverse-phase column (Nucleosil 120–5 C18; 250 × 4.6 mm internal diameter, 5-μm-thick film; Machery & Nagel, Düren, Germany). The fluorescence spectrophotometer was operated at an excitation wavelength of 385 nm and an emission wavelength of 515 nm. The mobile phase, pumped at 1.5 mL/min, consisted of 0.1 mol/L potassium dehydrogenephosphate (adjusted to pH 2.1 with orthophosphoric acid, containing 2.4 mol/L acetonitrile).

Hematological variables such as blood cell counts, hemoglobin concentration, hematocrit and mean corpuscular volume (MCV) were determined with a Coulter counter and a hemoglobinometer (Coulter Electronics GmbH, Krefeld, Germany). Total protein, albumin, creatinine, urea, glucose and the lipids cholesterol, triglyceride and phosphatidylcholine in serum were determined by standard procedures using an auto analyzer (model 704; Hitachi, Tokyo, Japan) and Boehringer kit reagents (Boehringer, Mannheim, Germany).

For trace element analysis of the liver, 10-g aliquots were dry-ashed for 3 d at 480°C. The dry-ashed samples were then dissolved in 0.6 mol/L hydrochloric acid. One blank was included for each drying stage. The cobalt and nickel concentrations of the samples were then determined by absorbance at 240.7 and 232.0 nm, respectively, after the specimens were loaded into a pyrolytically coated graphite tube of an atomic absorption spectrophotometer (model 5100, HGA-600 Graphite Furnace; Perkin–Elmer, Überlingen, Germany). Iron and copper concentrations of the liver were measured directly by their absorbance at 248.3 and 327.4 nm by aspirating the dry-ashed samples dissolved in hydrochloric acid into the flame of the atomic absorption spectrophotometer. All specimens were analyzed in duplicate. In the analysis of iron and copper, the CV for duplicate analyses were typically <5%, and in the analysis of cobalt and nickel, the CV for duplicate analyses was <5%. All trace element concentrations were expressed on a fresh weight basis.

Liver concentration of oxidized GSSG was determined by HPLC (Aseni et al. 1994). To 0.5 g of freeze-dried liver 3 mL of 1.2 mol/L ice-cold perchloric acid containing 40 mmol/L N-ethylmaleimide and 2 mmol/L bathophenanthrolineisulfonic acid was added. N-ethylmaleimide was used to prevent GSH oxidation during sample preparation. Then samples were centrifuged for 5 min at 15,000 × g (4°C), and supernatant was used for derivatization. 1-Fluoro-2,4-dinitrobenzene was used as derivatization reagent (Asensi et al. 1994). GSSG was separated using a Spherisorb aminopropyl column (250 × 4.6 mm internal diameter, 5-μm-thick film; Sigma Chemical, St. Louis, MO). The UV spectrophotometer was operated at a wavelength of 365 nm. The flow rate was 1.0 mL/min during the procedure. The mobile phases and the gradient were used as described by Aseni et al. (1994).

For determination of hepatic catalase (EC 1.11.1.6), liver homogenate (1:100, 20 g/mL) with 200 mmol/L sucrose (containing 0.1 mol/L ethanol) were centrifuged at 700 × g for 10 min to remove nuclei, unbroken cells and cell debris. Then supernatant was used for the determination of the catalase activity. Total catalase activity was measured spectrophotometrically (Aebi 1970), after pretreatment of the enzyme source with Triton X-100 to a final concentration of 1% to disrupt the peroxisomal membranes. The determination of the enzyme activity was based on the measurement of the rate of converting hydrogen peroxide at 240 nm and a temperature of 25°C in the presence of the enzyme. Protein in 700 × g supernatant used for enzyme determination was measured by a method of Smith and co-workers (1975) using bicinchoninic acid and bovine serum albumin as a standard. The activity of GSHPx (EC 1.11.1.9) in serum was determined by a spectrophotometric method (Paglia and Valentine 1967). The determination of total thiol groups in serum (from protein and glutathione) was done by a spectrophotometric method of Hug (1994). Prior to measuring the thiol the serum was analyzed for its protein concentration by a standardized procedure using an auto analyzer (model 704; Hitachi) and Boehringer kit reagents.

Concentrations of individual tocopherols in serum were determined by HPLC (Balz et al. 1993). Serum samples (100 μL) were mixed with 1 mL of a 80 mmol/L pyrogallol solution (in ethanol, absolute) and 150 μL saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4-dioxane (96:4, v/v) as mobile phase and a LiChrosorb Si 60 column (5-μm particle size, 250-mm length, 4-mm internal diameter; Merck, Darmstadt, Germany) and detected by fluorescence (excitation wavelength, 295 nm; emission wavelength, 320 nm).

**Statistical analysis.** Single classification ANOVA was used for analysis of serum and liver concentrations of vitamin B12 and folate, blood cell counts, the concentration of circulating homocysteine, the response of the antioxidant system and the concentrations of trace elements in liver. Data were subjected to logarthmic transformations where necessary to achieve homogeneity of variances. Differences between dietary treatment groups were compared by the Student-Newman-Keuls’s (SNK) test. Significant difference was assigned at P < 0.05. Data are presented as mean ± SEM.

**RESULTS**

At the end of the experiment, on d 166, the feed intake (B12-C: 228 ± 18, B12-D: 176 ± 20, B12-D + Fe: 204 ± 14, B12-D + Cu: 208 ± 17, B12-D + Co: 221 ± 6 and B12-D + Ni: 211 ± 17 kg), weight gain (B12-C: 81.4 ± 6.1 kg, B12-D: 66.9 ± 9.3, B12-D + Fe: 80.2 ± 7.1, B12-D + Cu: 81.3 ± 8.5, B12-D + Co: 87.8 ± 2.5 and B12-D + Ni: 83.4 ± 9.2 kg) and body weights (B12-C: 91.1 ± 6.7, B12-D: 70.5 ± 9.4, B12-D + Fe: 84.8 ± 7.5, B12-D + Cu: 85.9 ± 8.7, B12-D + Co: 92.5 ± 3.6 and B12-D + Ni: 87.9 ± 10.1 kg) of pigs were not
significantly different. Feed efficiency, defined as kilograms of feed required per kilogram of weight gain, also did not differ significantly among the groups (B12-C: 2.66 ± 0.07, B12-D: 2.72 ± 0.19, B12-D + Fe: 2.59 ± 0.09, B12-D + Cu: 2.63 ± 0.11, B12-D + Co: 2.52 ± 0.06 and B12-D + Ni: 2.66 ± 0.19).

The removal of vitamin B-12 from the diets generally resulted in a significant reduction in serum and liver vitamin B-12 concentrations compared with concentrations found in pigs receiving the corresponding vitamin B-12–adequate diet (Table 2). However, the magnitude of the decrease was different among the vitamin B-12–deficient groups. Among pigs receiving no vitamin B-12, the group fed the high nickel diet had significantly higher serum and liver concentrations of vitamin B-12 than pigs fed normal amounts of trace elements or diets fortified with iron, copper and cobalt. As was the case with vitamin B-12, the concentration of folate in liver was lower in pigs fed the vitamin B-12-deficient diets than in pigs fed the vitamin B-12–adequate control diet. The serum concentration of folate was significantly reduced only in the vitamin B-12-deficient group fed normal amounts of trace elements compared with all the other groups. Vitamin B-12-deficient pigs fed the iron-, copper-, cobalt- and nickel-fortified diets had serum folate concentrations not different from those observed in the vitamin B-12–adequate group.

The lowest homocysteine concentration was found in serum of pigs fed the vitamin B-12–adequate diet (Fig. 1). The highest homocysteine concentrations, which differed significantly from those of the vitamin B-12–adequate pigs, were found in serum of vitamin B-12–deficient pigs fed normal trace element amounts and the iron- and copper-fortified diets. Intermediate values were observed with the vitamin B-12–free high cobalt or high nickel diets. Vitamin B-12–deficient pigs fed the high cobalt or nickel diets had about 40% lower (P < 0.05) serum concentrations of homocysteine than the corresponding vitamin B-12–deficient pigs fed the normal trace element amounts or the iron- and copper-fortified diets.

Irrespective of the trace element supplementation, pigs receiving no vitamin B-12 had lower erythrocyte counts and lower platelet counts than pigs fed adequate amounts of dietary vitamin B-12 (Table 2). MCV values were significantly influenced by the dietary treatments. Pigs fed the vitamin B-12–deficient, high-iron diet had higher MCV values than the vitamin B-12–adequate control group. MCV values of the other vitamin B-12–deficient groups did not significantly differ from those of the control group. White blood cell counts (B12-C: 18.0 ± 0.6, B12-D: 18.5 ± 1.9, B12-D + Fe: 15.1 ± 1.7, B12-D + Cu: 15.4 ± 1.7, B12-D + Co: 17.6 ± 1.2 and B12-D + Ni: 17.7 ± 1.0 (10^9/L), hemoglobin concentrations (B12-C: 130 ± 8, B12-D: 113 ± 7, B12-D + Fe: 128 ± 7, B12-D + Cu: 131 ± 6, B12-D + Co: 130 ± 4 and B12-D + Ni: 121 ± 8 g/L) and hematocrit (B12-C: 0.423 ± 0.017, B12-D: 0.365 ± 0.017, B12-D + Fe: 0.404 ± 0.014, B12-D + Cu: 0.416 ± 0.014, B12-D + Co: 0.411 ± 0.007 and B12-D + Ni: 0.388 ± 0.019) did not differ among the groups.

Iron concentration in liver was twice as high in pigs fed the vitamin B-12–deficient diets with normal or high amounts of iron and copper as in the vitamin B-12–adequate group (Table 3). Intermediate values were observed for the vitamin B-12–deficient group fed the high cobalt diet. The iron concentration in livers of vitamin B-12–deficient pigs fed the high nickel diet reached values not different from those in the vitamin B-12–adequate group. The copper concentration in liver was highest in the vitamin B-12–deficient group fed the high copper diet. Additionally, the vitamin B-12–deficient group fed the high cobalt diet also had significantly higher copper concentrations in liver than the control group. Copper concentration in livers of pigs fed the vitamin B-12–free diets was comparable to those in the control group.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>B12-C</th>
<th>B12-D</th>
<th>B12-D + Fe</th>
<th>B12-D + Cu</th>
<th>B12-D + Co</th>
<th>B12-D + Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum B-12, pmol/L</td>
<td>221 ± 28a</td>
<td>55.3 ± 9.7c</td>
<td>49.8 ± 5.1c</td>
<td>74.3 ± 10.5c</td>
<td>60.2 ± 4.0c</td>
<td>133 ± 21b</td>
</tr>
<tr>
<td>Liver B-12, nmol/kg</td>
<td>329 ± 20a</td>
<td>78.4 ± 7.6c</td>
<td>78.1 ± 2.6c</td>
<td>82.0 ± 4.7bc</td>
<td>78.1 ± 4.5c</td>
<td>110 ± 7.1b</td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>51.7 ± 4.1a</td>
<td>30.0 ± 2.5b</td>
<td>47.6 ± 4.6a</td>
<td>49.1 ± 3.2a</td>
<td>49.4 ± 5.2a</td>
<td>47.1 ± 4.5a</td>
</tr>
<tr>
<td>Liver folate, μmol/L</td>
<td>13.3 ± 1.4a</td>
<td>6.93 ± 0.71b</td>
<td>7.28 ± 0.75b</td>
<td>6.84 ± 0.97b</td>
<td>8.45 ± 0.87b</td>
<td>7.96 ± 0.97b</td>
</tr>
<tr>
<td>Platelets, 10^9/L</td>
<td>8.39 ± 0.29a</td>
<td>7.01 ± 0.34b</td>
<td>6.76 ± 0.24b</td>
<td>7.49 ± 0.22ab</td>
<td>7.52 ± 0.09ab</td>
<td>7.53 ± 0.44ab</td>
</tr>
<tr>
<td>MCV, μm</td>
<td>50.5 ± 1.6b</td>
<td>54.3 ± 1.4ab</td>
<td>57.9 ± 0.9a</td>
<td>55.0 ± 1.0ab</td>
<td>54.6 ± 0.6ab</td>
<td>53.9 ± 2.5ab</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 8; values in a row with different superscripts differ significantly, P < 0.05 (SNK test). BRC, red blood cells; MCV, mean corpuscular volume.
2 B12-C, vitamin B-12–adequate control group; B12-D, vitamin B-12–deficient group fed normal concentrations of iron, copper, cobalt and nickel; B12-D + Fe, vitamin B-12–deficient, high-iron group; B12-D + Cu, vitamin B-12–deficient, high-copper group; B12-D + Co, vitamin B-12–deficient, high-cobalt group; B12-D + Ni, vitamin B-12–deficient, high-nickel group.

**FIGURE 1** Serum homocysteine concentration of vitamin B-12–deficient pigs fed iron, copper, cobalt or nickel. Values with different superscripts differ significantly, P < 0.05 (SNK test). Mean ± SEM, n = 8. B12-C, vitamin B-12–adequate control group; B12-D, vitamin B-12–deficient group fed normal concentrations of iron, copper, cobalt and nickel; B12-D + Fe, vitamin B-12–deficient, high-iron group; B12-D + Cu, vitamin B-12–deficient, high-copper group; B12-D + Co, vitamin B-12–deficient, high-cobalt group; B12-D + Ni, vitamin B-12–deficient, high-nickel group.
with normal trace element amounts or excessive iron or nickel was not significantly different from that of the vitamin B-12–adequate pigs. Vitamin B-12–adequate pigs and vitamin B-12–deficient pigs fed the high cobalt diet had much higher liver concentrations of cobalt than the other groups. The dietary treatments had no significant influence on the nickel concentration of those livers.

The dietary manipulations did not have significant detrimental effects on variables associated with the antioxidant defense system (Table 3). The serum concentrations of α-tocopherol (B12-C: 2.68 ± 0.16, B12-D: 2.87 ± 0.19, B12-D + Fe: 2.77 ± 0.31, B12-D + Cu: 2.88 ± 0.18, B12-D + Co: 2.78 ± 0.16 and B12-D + Ni: 2.68 ± 0.19 μmol/L) and thiols (B12-C: 58.4 ± 6.6, B12-D: 74.3 ± 9.7, B12-D + Fe: 73.5 ± 5.1, B12-D + Cu: 83.5 ± 6.3, B12-D + Co: 68.4 ± 4.3 and B12-D + Ni: 68.8 ± 7.6 μmol/L) were not influenced by the dietary treatment. The serum concentration of γ-tocopherol was diminished in all groups fed the vitamin B-12–deficient diets compared with the vitamin B-12–adequate control group (P < 0.05). The dietary treatment had significant effects on the activities of serum GSHPx and liver catalase. Vitamin B-12–adequate pigs and vitamin B-12–deficient pigs fed excessive iron, copper, cobalt and nickel had similar GSHPx activities in serum. The vitamin B-12–deficient pigs fed normal amounts of trace elements had the lowest activity of GSHPx, although this difference was only significant in comparison with pigs fed the high cobalt diet (Table 3). Vitamin B-12–deficient pigs fed normal trace element amounts had a greater activity of liver catalase than the control group. Catalase activities in the other groups did not differ from that of the control group. The liver concentrations of GSSG did not differ among the groups (B12-C: 1.18 ± 0.18, B12-D: 1.16 ± 0.24, B12-D + Fe: 1.23 ± 0.20, B12-D + Cu: 1.04 ± 0.10, B12-D + Co: 1.12 ± 0.17 and B12-D + Ni: 0.87 ± 0.12 mmol/kg).

Serum concentrations of the clinico-chemical variables (including total protein, albumin, creatinine, urea, and glucose) were not influenced by the dietary treatments (data not shown). The serum concentrations of cholesterol, triacylglycerols and phosphatidylcholine also did not differ among the groups (data not shown).

### DISCUSSION

In this study, the principal clinical signs of prolonged vitamin B-12 deficiency in pigs included lower serum and liver concentrations of vitamin B-12 and folate, hyperhomocysteinemia, an accumulation of iron in liver and mild forms of megaloblastosis and thrombocytopenia, which is a reflection of defective DNA synthesis (Sarode et al. 1989). However, the magnitude of changes differed among the vitamin B-12–deficient groups. The data demonstrate clearly that cobalt and nickel influence the response of the pig to vitamin B-12 deficiency when labile methyl group metabolism is compromised. The findings further indicate that there is a collaborative relationship between the vitamins B-12 and folate and the trace elements nickel and cobalt, although the responses of nickel and cobalt to vitamin B-12 deficiency differed in some respects. Nickel was more effective than cobalt at improving vitamin B-12 deficiency outcomes. The main effects of nickel were an improvement of the vitamin B-12 status and a decline of the homocysteine concentration in serum, although these variables did not reach the values observed in the control group, and a complete normalization of the iron concentration in liver. In contrast, cobalt supplementation only improved homocysteine accretion in serum and partly diminished abnormal iron accretion in liver, whereas the vitamin B-12 status remained completely unchanged.

First, the interaction between nickel and vitamin B-12 could be the result of nickel being used to synthesize vitamin B-12 by bacteria in the animals’ gut, which would result in a greater intake of vitamin B-12 through coprophagy. Nickel is an essential trace element for many microorganisms, and bacteria require nickel for the synthesis of enzymes such as urease, carbon monoxide dehydrogenase, many hydrogenases and methyl-coenzyme-M reductase (Ankel-Fuchs and Thauer 1988). Enterobacteriaceae, which have been proved to be capable of synthesizing vitamin B-12 (Vorob’eva et al. 1987), require the activity of nickel-containing hydrogenase for their anaerobic growth (Ankel-Fuchs and Thauer 1988). Because nickel is known to be poorly absorbed from ordinary diets and is excreted mostly in the feces, the high nickel diet used in this study might have contributed to improved bacterial growth and vitamin B-12 synthesis in the gut. The fecal bacterial flora

### TABLE 3

**Hepatic trace elements, antioxidant enzymes and serum γ-tocopherol concentrations of vitamin B-12-deficient pigs fed iron, copper, cobalt or nickel**

<table>
<thead>
<tr>
<th></th>
<th>B12-C</th>
<th>B12-D</th>
<th>B12-D + Fe</th>
<th>B12-D + Cu</th>
<th>B12-D + Co</th>
<th>B12-D + Ni</th>
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<tbody>
<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>Iron, μmol/kg</td>
<td>556 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1003 ± 154&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1024 ± 118&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1065 ± 176&lt;sup&gt;a&lt;/sup&gt;</td>
<td>852 ± 102&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>589 ± 31&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Copper, μmol/kg</td>
<td>68.1 ± 12.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70.5 ± 8.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>102.1 ± 20.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>232.4 ± 33.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>123.4 ± 20.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88.5 ± 9.3&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td>Cobalt, μmol/kg</td>
<td>17.9 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.71 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.81 ± 2.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.51 ± 3.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.5 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.67 ± 1.86&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Nickel, μmol/kg</td>
<td>31.7 ± 7.6</td>
<td>58.8 ± 15.6</td>
<td>32.9 ± 14.2</td>
<td>38.2 ± 13.6</td>
<td>29.7 ± 10.5</td>
<td>44.8 ± 12.0</td>
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<tr>
<td>Catalase, U/L</td>
<td>298 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>433 ± 29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>364 ± 34&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>349 ± 29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>284 ± 33&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>GSHPx, U/L</td>
<td>404 ± 44&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>244 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>359 ± 41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>417 ± 66&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>458 ± 42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>374 ± 51&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>γ-Tcp, μmol/L</td>
<td>0.37 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

1 Values are mean ± SEM, n = 8; values in a row with different superscripts differ significantly, P < 0.05 (SNK test).
2 B12-C, vitamin B-12-adequate control group; B12-D, vitamin B-12-deficient group fed normal concentrations of iron, copper, cobalt and nickel; B12-D + Fe, vitamin B-12-deficient, high-iron group; B12-D + Cu, vitamin B-12-deficient, high-copper group; B12-D + Co, vitamin B-12-deficient, high-cobalt group; B12-D + Ni, vitamin B-12-deficient, high-nickel group; GSHPx, glutathione peroxidase; γ-Tcp, γ-tocopherol.
3 The trace element concentrations are expressed on a fresh weight basis.
4 One unit of catalase is defined as 1 μmol H<sub>2</sub>O<sub>2</sub> decomposed (min · mg protein) at 25°C.
5 One unit of GSHPx is defined as 1 μmol NADPH oxidized per min at 25°C.
is a major source of vitamins for coprophagic animals. Coprophagy is a phenomenon mainly known from rodents, but not so much from pigs, although it cannot be excluded that pigs also may coprophagy in states of nutritional imbalance. However, the efforts that were made to remove feces from cages to prevent coprophagy together with failure to demonstrate any effect of cobalt supplementation on vitamin B-12 status, although cobalt is an integral compound of vitamin B-12 and could be used for vitamin B-12 synthesis by bacteria, indicate that mechanisms other than intestinal synthesis and coprophagy must be responsible for the attenuation of vitamin B-12 deficiency in nickel- and cobalt-supplemented pigs.

In any event, there is experimental evidence of a metabolic interaction between vitamin B-12 and trace elements. As early as 1952 Leccq reported that nickel acted in synergy with vitamin B-12 in stimulating hematopoiesis (Leccq et al. 1952). It was subsequently suggested that nickel has a physiologic function related to vitamin B-12 metabolism, possibly one-carbon metabolism (Nielsen et al. 1989, Poellot et al. 1990, Uthus and Poellot 1996). The present study shows that vitamin B-12–free diets with nickel concentrations of 6 mg/kg can improve several clinical outcomes of vitamin B-12 deficiency compared with vitamin B-12–deficient diets containing 1 mg nickel/kg diet, a nickel concentration that has been considered ample for animal growth (Schneeg and Kirchgessner 1975). In addition to the statement of Nielsen and co-workers (1993) to the effect that vitamin B-12 is necessary for the optimal expression of the biological role of nickel, we maintain that nickel has a vitamin B-12–sparking effect in animals deprived of vitamin B-12. Moreover, cobalt was found to act in a manner similar to that of nickel on the serum concentration of homocysteine, without influencing vitamin B-12. The reason for this phenomenon is unclear, but previous studies have established that cobalt ions induce a series of metabolic changes in experimental animals such as inhibition of cytochrome P-450 metabolism (Zhang et al. 1998), greatly reduced zinc output in urine (Rosenberg and Kappas 1989), stimulation of hepatic concentrations of reduced glutathione (Sasame and Boyd 1978) and regulation of renal tubular reabsorption processes (Goncharevskaia et al. 1985). Although this study does not show any specific site of action of cobalt in these processes, it is conceivable, for example, that cobalt may lower serum homocysteine concentration via the promotion of the renal clearance of this amino acid. Whether cobalt and nickel will interfere the betaine-dependent reaction, mediated by the zinc-containing betaine-homocysteine methyltransferase (EC 2.1.1.5; Millian and Garrow 1998), which supplements to a small extent the activity of the methylcobalamin-dependent methionine synthetase when the latter is impaired in severe vitamin B-12 deficiency (Maree et al. 1990), remains speculative.

Additionally, an interesting observation may be made from the serum concentration of folate in the vitamin B-12–deficient groups. The vitamin B-12–deficient groups fed either iron, copper, cobalt or nickel, like the vitamin B-12–adequate groups, had higher serum concentrations of folate than the vitamin B-12–deficient group fed normal amounts of trace elements. At present, we do not have a mechanistic explanation for why trace element diets lead to different responses in serum concentration of folate. The liver exerts substantial regulatory effect in folate homeostasis because of its large and rapid folate turnover, the large flux through the enterohepatic cycle, and clearance (Lashkaria and Bumji 1981). However, there is no evidence to date that trace elements may interfere in these processes, and we think that there is an obvious need for more information about the mode of action of metals on folate metabolism.

In this study we failed to demonstrate that homocysteine exhibits a distinct prooxidant potential. A reduction of the γ-tocopherol concentration in serum was the only indication for diminished antioxidant status caused by vitamin B-12 deficiency, although this observation is of minor importance because of the low γ-tocopherol concentration relative to α-tocopherol. In contrast, other studies have unequivocally demonstrated that hyperhomocysteinemia compromised plasma redox thiol status (Ueland et al. 1996) and led to increased lipid peroxidation (Brown and Strain 1990, Young et al. 1997). However, there are also in vivo studies that have failed to demonstrate marked changes in the oxidative/antioxidative balance through hyperhomocysteinemia (Dudman et al. 1993, Mele and Meucci 1996, Stangl et al. 2000). The ability of homocysteine to trigger oxidative procedure in vivo is therefore a subject of some controversy. Preibisch et al. (1993) were able to demonstrate in vitro that homocysteine exhibits a significant prooxidant potential only in the presence of either copper or iron as transition-metal ions. Our results indicate clearly that the combined effect of vitamin B-12–deficiency–induced hyperhomocysteinemia and administration of high cationic concentrations did not induce a greater loss of antioxidant potential than hyperhomocysteinemia alone.

It can be concluded from these observations that vitamin B-12–deficiency symptoms, including an accumulation of serum homocysteine, can be attenuated by nickel and cobalt, although the mode of action of these elements seems to differ. If the effects of nickel and cobalt are indeed mediated by metabolic processes, then the relevance to human nutrition is quite plausible.

LITERATURE CITED


INTERRELATION OF TRACE ELEMENTS AND VITAMIN B-12 METABOLISM


