Evaluation of the cobalt requirement of beef cattle based on vitamin B_{12}, folate, homocysteine and methylmalonic acid

G. I. Stangl*, F. J. Schwarz, H. Müller and M. Kirchgessner

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

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This investigation was designed to estimate the Co requirement of growing cattle on the basis of plasma and liver levels of vitamin B_{12} and folate, plasma levels of homocysteine and methylmalonic acid (MMA) and haematological variables. For this purpose thirty-four male intact cattle of the German Simmental breed (236 kg) were assigned randomly to ten groups and were fed corn silage-based diets which contained 70, 90, 109, 147, 184, 257, 327, 421, 589 or 689 μg Co/kg DM for 40 weeks. One-slope broken-line model analysis and a quadratic model with plateau were used to estimate the Co requirement. The broken-line model estimated the dietary Co requirement of growing cattle to be 257 (SE 29) μg/kg dietary DM based on plasma vitamin B_{12} as response criterion. The dietary Co levels needed to maximise the liver vitamin B_{12} and liver folate were 236 (SE 8) and 190 (SE 8) μg/kg dietary DM respectively. Plasma folate did not show any response to the different Co levels. The dietary Co was inversely correlated with the plasma concentrations of homocysteine and MMA. Estimates of the dietary Co concentration required to minimise homocysteine were 161 (SE 10) μg/kg DM. When MMA was used as response criterion, the linear model yielded a Co requirement of 124 (SE 3) μg/kg dietary DM. The quadratic model did not provide a better closeness of regression fit and yielded similar requirements to the linear model. Haemoglobin concentration and haematocrit tended to have a slight response to increasing dietary Co and were only decreased in cattle on diets containing less than 100 μg Co/kg DM. On the basis of the present data, recommended levels of dietary Co for normal folate metabolism and minimum homocysteine and MMA levels can be set to be 150–200 μg/kg DM; for maximum vitamin B_{12} levels, the desired Co content in the diet seems to be 250 μg/kg DM.

Cobalt requirement: Cattle

Co-responsive disorders of ruminants that are brought about by the inability of the rumen micro-organisms to synthesise sufficient vitamin B_{12} to meet the metabolic needs of ruminant cells and tissues, have been reported in different parts of the world (Poole *et al.* 1972; Musewe & Gombe, 1980; Duncan *et al.* 1986). Field experiences have shown that species differences among ruminants in Co requirements are small (National Research Council, 1980), and early evidence indicated that dietary Co concentrations which ranged between 70 and 110 µg/kg were just adequate for sheep and cattle (e.g. Filmer & Underwood, 1937; McNaught, 1948; Marston, 1952; Andrews *et al.* 1958; Andrews, 1965; Somers & Gawthorne, 1969; Smith, 1987). However, most of these early estimations of the minimum Co requirement lacked sufficient numbers of dietary Co levels for valid statistical analysis and conclusions or have been obtained from animals producing well below industry standards or have predominantly been estimated on clinical and pathological signs of deficiency alone. Unfortunately, the dietary Co requirement, which has been reviewed in the most recent editions of the National Research Council (1996) publication on the nutrient requirement of cattle and the nutrient recommendations of the Society of Nutrition Physiology (Gesellschaft für Ernährungsphysiologie, 1995) is based on those obsolete data. Thus, dietary Co levels of about 100 µg/kg DM have been widely accepted as the minimum requirement for cattle. However, recent work from our laboratory (Kirchgessner *et al.* 1998; Stangl *et al.* 1998*a*,*b*) indicates a higher Co requirement for growing beef cattle than is currently estimated by the National Research Council (1996) or Gesellschaft für Ernährungsphysiologie (1995).

Abbreviations: Hgb, haemoglobin; MMA, methylmalonic acid.

* **Corresponding author:** Dr G. I. Stangl, fax +49 8161 715367, email stangl@weihenstephan.de

Furthermore, more recent findings from a few authors also indicate the necessity to increase the amount of dietary Co for ruminants up to a level of 300–500 μg/kg DM for optimum rumen microbial activity, fermentation and vitamin B_{12} synthesis (Paragon, 1993; Singh & Chhabra, 1995). However, we think that a re-evaluation of the Co requirement of cattle is needed, based on several biochemical variables as response criteria and by the addition of graded increments of Co that are obviously deficient and in excess of the presumed adequate Co level.

When re-evaluating the Co requirement for cattle, one must taken into consideration the fact that dietary factors other than Co, such as increasing the dietary fibre content and total nutrient intakes, have also been shown to influence slightly, but favourably, vitamin B_{12} production in the rumen (Sutton & Elliot, 1972; Hedrich *et al.* 1973). Nowadays, the production of beef cattle in large parts of Europe is based on corn silage which has recently been shown to support inadequate amounts of Co for growing cattle (Kirchgessner *et al.* 1998). This implication and the absence of an established dietary Co requirement for growing beef cattle provided the stimulus to re-evaluate the Co requirement for cattle finished on a corn silage-based diet.

Recent data from this present experiment have shown that feed intake and growth development of the male intact cattle were significantly affected by the Co concentration of the diet (Schwarz *et al.* 2000). The minimum dietary Co required to maximise feed intake and growth performance of growing cattle finished on a corn silage-based diet has been found to range between 160 and 180 μg/kg DM (Schwarz *et al.* 2000). Since trace element deficiencies are impossible to diagnose with certainty on the basis of feed intake and growth response alone, this investigation was designed to estimate the minimum Co requirement of growing beef cattle on the basis of biochemical criteria including vitamin B_{12} and folate in plasma and liver as well as homocysteine and methylmalonic acid (MMA) plasma concentrations and haematological blood variables of the relative response to the Co supplements, and to evaluate the potential use of those variables as response criteria to determine the Co requirement. The biochemical response variables were tested for regression on dietary Co by the broken-line model, and a quadratic equation with plateau, in which more than one diet was obviously deficient and in excess of the presumed adequate Co level respectively.

Materials and methods

Animals and diets

Before starting the trial, forty bull calves of the German Simmental breed with a mean live weight of 83·5 (SE 1·2) kg and a mean age of 37 d were purchased. The calves came from six sires, which enabled the genetic origin to be taken into account in the subsequent treatment allocations. Rearing was uniform according to a standardised procedure (Schwarz *et al.* 2000) which involved feeding milk replacer for 5 weeks, corn silage *ad libitum*, not more than 0·5 kg hay and concentrate in increasing amounts up to a maximum of 2·5 kg. In addition to corn grain, wheat, barley and soyabean meal, the concentrate contained 20 g commercial mineral feed/kg, which included sufficient amounts of Co. On completion of the 132 d starter period, the calves were moved to a loose barn with pens of six lying and feeding places and a fully slatted floor. As each feeding place was closed with an electronically-operated gate, individual feeding was possible despite the group housing (Schwarz *et al.* 1985). The period of adaptation to the housing system, during which the diet remained unchanged, was 28 d from the beginning of the trial. After this, thirty-four male intact cattle with an average body weight of 236 (SE 1·9) kg were assigned randomly to ten groups and were fed diets which were deficient, sufficient or in excess of Co for 40 weeks. The basal diet consisted of corn silage and a concentrate. The animals were adjusted to the corn silage-based diet for 28 d. Corn silage, which was fed *ad libitum*, provided 30 μg Co/kg DM. The concentrate contained (g/kg): soyabean meal 440, ground corn 232, barley 232, vitamin–mineral mixture 40, limestone 16, pre-mixture 40 (ground corn supplemented with different amounts of Co); this was fed in amounts of 2·5 kg/animal per d. The basal Co concentration of the concentrate was 122 μg/kg DM. For the Co-supplemented diets, Co was added as $CoSO_4.7H_2O$. The relative bioavailability of $CoSO_4.7H_2O$ based on multiple regression slope ratios of vitamin B_{12} concentrations has been shown to be 100 % (Kawashima *et al.* 1997). The amount of pre-mixture was on average 100 g per animal but ranged between 90 and 110 g depending on the amount of corn silage ingested by the animals. This was done in order to achieve a minimum variation in Co supply during the experimental period within each diet. For the different Co treatments the concentrate was formulated so that the 1 kg DM contained on average 70, 90, 109, 147, 184, 257, 327, 421, 589 or 689 μg Co. The concentrate used for the treatments was supplemented with sufficient amounts of minerals and vitamins according to recommended guidelines (Gesellschaft für Ernährungsphysiologie, 1995; National Research Council, 1996). Further details of the experimental diet have been published recently (Schwarz *et al.* 2000).

The cattle were offered the corn silage once per d and the concentrate in two equal portions per d. All animals had free access to water. The cattle were housed in pens of six animals each and were individually fed using electronically-controlled feeders (Schwarz *et al.* 1985). All cattle were treated in accordance with normal animal husbandry practices. Biochemical values for Co requirement estimation were derived from a minimum of three animals in each group. The group that was fed the diet with 70 μg Co/kg comprised five animals, the groups fed 90 and 147 μg Co/kg DM each comprised four animals, while all other groups included three animals each.

Sample collection and analyses

At week 40, 18–19 h after the last feeding, all cattle were slaughtered, and blood and liver were excised. Blood for determination of the haemoglobin (Hgb) concentration, haematocrit, red blood cell count and mean corpuscular volume, and plasma levels of vitamin B_{12}, folate, MMA and homocysteine was collected into EDTA-treated tubes. Plasma samples were obtained by centrifugation at 4°C for 10 min at 1100 ***g***. From each animal, liver samples were collected from the same region of the liver and stored at −80°C prior to analysis of vitamin B_{12} and folate.

Sample preparation for the Co analysis of the corn silage and the concentrate was done as described previously (Kirchgessner *et al.* 1998). The Co concentrations of the samples were then determined by absorbance at 240·7 nm by introduction into a pyrolytically-coated graphite tube of an atomic absorption spectrophotometer (model 5100, HGA-600 Graphite Furnace; Perkin-Elmer, Überlingen, Germany). Four aliquots of the concentrate and nine aliquots of the corn silage were used for the Co analysis. Each aliquot was analysed in duplicate. In the analysis of Co, the CV was below 5 %.

Plasma and liver concentrations of vitamin B_{12} and folate were determined using a competitive binding radioimmunoassay kit (ICN, Costa Mesa, CA, USA) that worked with an extracting reagent (containing 1 M NaOH and an organic extracting enhancer) to release vitamin B_{12} from transcobalmines. In the radioimmunoassay test kit used in this study, the non-specific vitamin B_{12}-binding R-protein was removed by affinity chromatography. Before radioimmunoassay quantification of liver vitamin B_{12} and folate, a tissue homogenate with borate buffer (pH 9·2) was prepared.

The blood variables Hgb, haematocrit, red blood cell count, and the mean corpuscular volume were determined with a Coulter Counter and a haemoglobinometer (Coulter Electronics GmbH, Krefeld, Germany).

Plasma levels of total homocysteine were determined by HPLC according to a method of Cornwell *et al.* (1993). Plasma samples were prepared for derivatisation according to the method of Ubbink *et al.* (1991) using 7-fluorbenzo-2-oxa-1,3-diazole-4-sulfonamide as derivatisation reagent. Homocysteine was separated using a 'reversed-phase' column (Nucleosil 120-5 C_{18}, 250 mm × 4·6 mm internal diameter, 5 μm film thickness; 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 M-KH_2PO_4 (adjusted to pH 2·1 with orthophosphoric acid, containing 100 ml acetonitrile/l).

Plasma MMA concentration was determined as described by McMurray *et al.* (1986) using a modified capillary GC method (HP 5790 A GC system; Hewlett-Packard, Taufkirchen, Germany). The acetyl chloride-butan-1-ol-derivatized samples were injected onto the column (DB-1701, 30 m × 0·25 mm internal diameter, 0·25 μm film thickness; J&W Scientific, Folsom, CA, USA) using the following oven temperature programme: 100°C followed by a temperature ramp of 10°C/min to 230°C; post-run, the temperature was held at 230°C for 10 min to flush the column.

Statistics

Estimates of the dietary Co requirement for maximising vitamin B_{12} and folate status and minimising the homocysteine and MMA plasma concentration were determined by fitting the data to a one-slope broken line model (Robbins *et al.* 1979; Robbins, 1986; Coma *et al.* 1995), and by calculation of the inflection point from a quadratic model with plateau (Coma *et al.* 1995). Statistical analyses were performed using the NLIN procedure of SAS (SAS/STAT® User's Guide, release 6·03, 1988; Statistical Analysis Systems Inc., Cary, NC, USA).

The general model of the one-slope, broken-line is as follows: $Y = L + U(R - X)$ if $X < R$, and $Y = L$ if $X \geq R$. In these equations, L is the *y*-coordinate and R is the *x*-coordinate of the inflection in the curve. U is the slope of the line. The dietary Co concentration (R) at which the breakpoint is achieved is defined as the Co requirement. The quadratic model with plateau is a segmented model with two theoretical hypotheses: $Y = b_0 + b_1X + b_2X^2$ if $X < R$ (*x*-coordinate of the inflection point), and $Y = P$ (where P is the plateau value) if $X \geq R$. That is, for values of $X < R$, the equation relating Y and X is quadratic, and for values of $X > R$, the equation is a horizontal line. The dietary Co requirement is also estimated to be the Co concentration (R) for which the response is the breakpoint.

When a definite relationship between the response variables and the Co supply by the two-phase regression analysis was not apparent, treatment comparisons were conducted by ANOVA and by the use of orthogonal polynominal contrasts (linear, quadratic or cubic effect of increasing dietary Co concentrations) (Lowry, 1992). Prior to the regression analysis and ANOVA, homogeneity of variances was tested by the Bartlett procedure. Results from the Bartlett procedure confirmed variance homogeneity for all variables measured.

Results

The response of vitamin B_{12} concentrations in plasma and liver to the changes in dietary concentration of Co is shown in Fig. 1. There were significant increases in plasma and liver levels of vitamin B_{12} as dietary Co increased. The broken-line method estimated the dietary Co requirement of growing cattle to be 257 (SE 29) μg/kg dietary DM based on plasma vitamin B_{12} as response criterion (Table 1). The quadratic model estimated a lower requirement with a higher asymptotic standard error than the linear model 215 (SE 29). The intake of Co needed to maximise the liver vitamin B_{12} was 236 (SE 8) μg/kg dietary DM based on the broken-line estimate, and the intersection point estimated by the quadratic model with plateau was 205 (SE 15) μg/kg dietary DM (Table 1). Analysis of liver samples collected at slaughter revealed a linear response of liver folate to additions of dietary Co up to a folate level of 57 nmol/g (Fig. 2). The one-slope broken-line model yielded a requirement of 190 (SE 8) μg Co/kg dietary DM (Table 1). The non-linear model estimated a Co requirement of 162 (SE 14) μg/kg DM when hepatic folate was used as the response variable. Similarly to the results obtained from vitamin B_{12} in plasma, and also in the case of the hepatic vitamin B_{12} and folate concentrations, the quadratic model yielded lower Co requirements than the linear model. This phenomenon resulted from the fact that the shape of the quadratic regression curve segment described by these response variables had a slight left bend. In addition, the

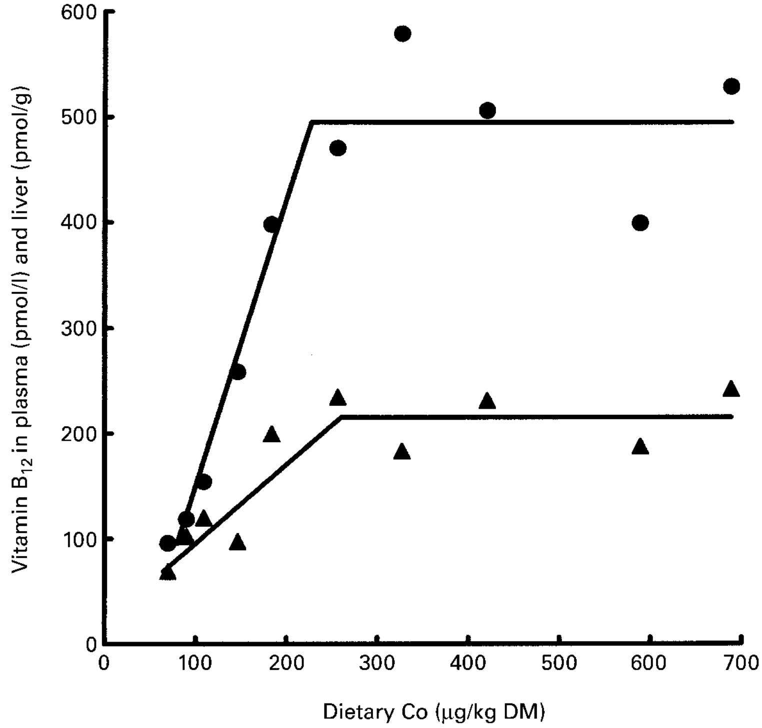

Fig. 1. Relationship between plasma (▲) and liver (●) levels of vitamin B_{12} (y) and cobalt concentration of the diet (x) and the corresponding one-slope broken-line plots. Each point (▲ and ●) represents the mean value of at least three cattle. For details of the composition of the diets see p. 646, and for procedures see pp. 646–647. For details of the model see p. 647.

Table 1. Estimation of the cobalt requirement of growing beef cattle from response variables by the one-slope broken-line regression model and the quadratic regression model with plateau*

Response variable and model†	Equation	Requirement (μg/kg DM) Mean	SE‡	R^2	RSD
Plasma vitamin B_{12}					
One-slope broken-line	$Y = 215 - 0{\cdot}75\,(257 - X)$	257	29	0·543	58
Quadratic with plateau	$Y = 93{\cdot}0 - 0{\cdot}529X + 0{\cdot}0051X^2$	215	54	0·546	59
	Plateau = 216; $X_B = 215$				
Liver vitamin B_{12}					
One-slope broken-line	$Y = 497 - 2{\cdot}53\,(236 - X)$	236	8	0·840	79
Quadratic with plateau	$Y = 104 - 1{\cdot}20X + 0{\cdot}0152X^2$	205	15	0·846	79
	Plateau = 497; $X_B = 205$				
Liver folate					
One-slope broken-line	$Y = 57{\cdot}2 - 0{\cdot}346\,(190 - X)$	190	8	0·795	9·0
Quadratic with plateau	$Y = 32{\cdot}0 - 0{\cdot}480X + 0{\cdot}0039X^2$	162	14	0·802	9·0
	Plateau = 57; $X_B = 162$				
Plasma homocysteine					
One-slope broken-line	$Y = 8{\cdot}13 + 0{\cdot}382\,(161 - X)$	161	10	0·821	6·7
Quadratic with plateau	$Y = 57{\cdot}4 - 0{\cdot}143X - 0{\cdot}00109X^2$	157	12	0·822	6·8
	Plateau = 8·1; $X_B = 157$				
Plasma methylmalonic acid					
One-slope broken-line	$Y = 0{\cdot}653 + 0{\cdot}112\,(124 - X)$	124	3	0·748	1·4
Quadratic with plateau	$Y = 17{\cdot}7 - 0{\cdot}185X + 0{\cdot}00041X^2$	129	13	0·750	1·4
	Plateau = 0·65; $X_B = 129$				

RSD, residual standard deviation; X_B, x-coordinate of the breakpoint.
* For details of the two models see p. 647.
† Based on thirty-four observations.
‡ Asymptotic standard error.

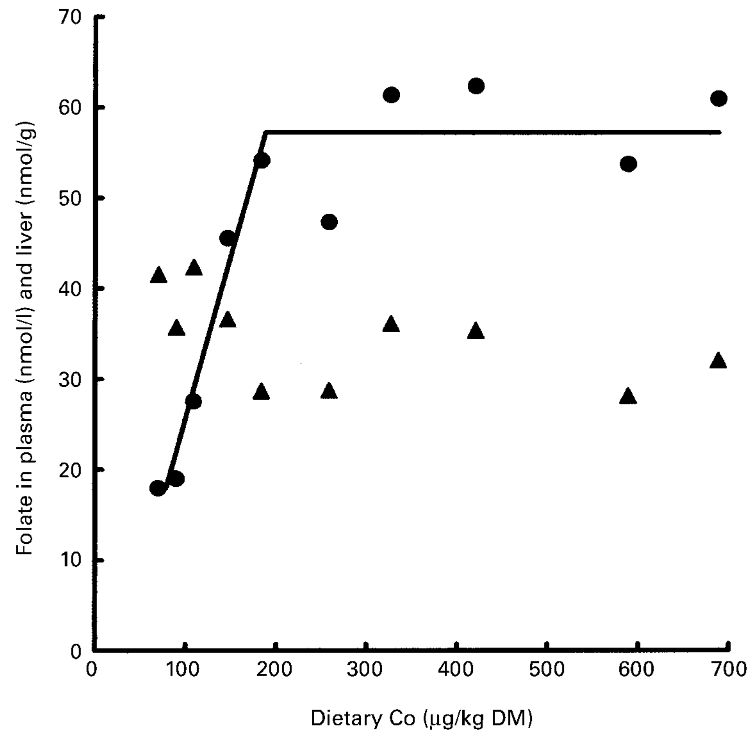

Fig. 2. Relationship between plasma (▲) and liver (●) levels of folate (*y*) and cobalt concentration of the diet (*x*) and the corresponding one-slope broken-line plot for liver folate. Each point (▲ and ●) represents the mean value of at least three cattle. For details of the composition of the diets see p. 646, and for procedures see pp. 646–647. For details of the model see p. 647.

quadratic model provided higher standard error values and did not essentially improve the R^2 and residual standard deviation values, so that the broken-line model seems to be a better model to describe vitamin B_{12} and folate responses to dietary Co than the quadratic model. In contrast, the different Co levels used in this experimental design did not significantly effect the plasma folate concentration. Fig. 2 demonstrates that plasma folate did not follow a clear-cut curve as was observed with hepatic folate and there was also no obvious breakpoint among the Co treatments indicating that plasma folate concentrations do not reflect the long-term Co supply.

Concentrations of homocysteine and MMA in plasma were inversely correlated with the dietary Co ingested (Figs. 3 and 4). Plasma profiles elucidate a dramatic fall of the homocysteine and MMA levels with increasing dietary Co concentrations up to the required level. Estimates of the dietary Co content required to minimise homocysteine by broken-line analysis and the quadratic method with plateau agreed well (Table 1). Predicted values by the broken-line and the quadratic methods were 161 (SE 10) and 157 (SE 12) µg/kg dietary DM respectively. For estimation of minimum Co required for cattle based on plasma MMA concentration, the two models yielded a Co requirement of 124 (SE 3) (broken-line) and 129 (SE 13) (quadratic) µg/kg DM respectively. Both the plasma homocysteine and the plasma MMA levels decreased in a linear manner with increasing dietary levels of Co and when the dietary Co requirement was met, both variables were minimized and reached a plateau and *vice versa*; at intakes below the requirement, both plasma variables levels increased and the magnitude of the elevation was linearly associated with the degree of deficiency.

The Hgb concentration and the haematocrit tended to have a slight response to increasing dietary Co, and therefore appeared to be less sensitive to Co status than vitamin B_{12}, folate, homocysteine and MMA (Table 2). Hgb concentration and haematocrit were decreased by week 40 only in cattle on diets containing <100 µg Co/kg DM. In addition, the response of both variables showed significant linear and cubic effects on dietary Co, but no quadratic effect. The oscillating character of the response of the Hgb concentration and haematocrit to the dietary Co concentrations, therefore, do not allow an estimation of the Co requirement. The red blood cell counts and the MCV remained totally unaffected by the dietary Co levels used in this study (Table 2).

Discussion

Ruminants normally do not have any dietary source of vitamin B_{12}. For their supply of vitamin B_{12} they are dependent on its production by bacteria that inhabit the rumen and utilise Co from the host's diet for this purpose. One approach which may be used to assess Co deficiency in ruminants is the measurement of the vitamin B_{12} status in those animals. From the present findings it was obvious that the concentrations of vitamin B_{12} in the plasma and

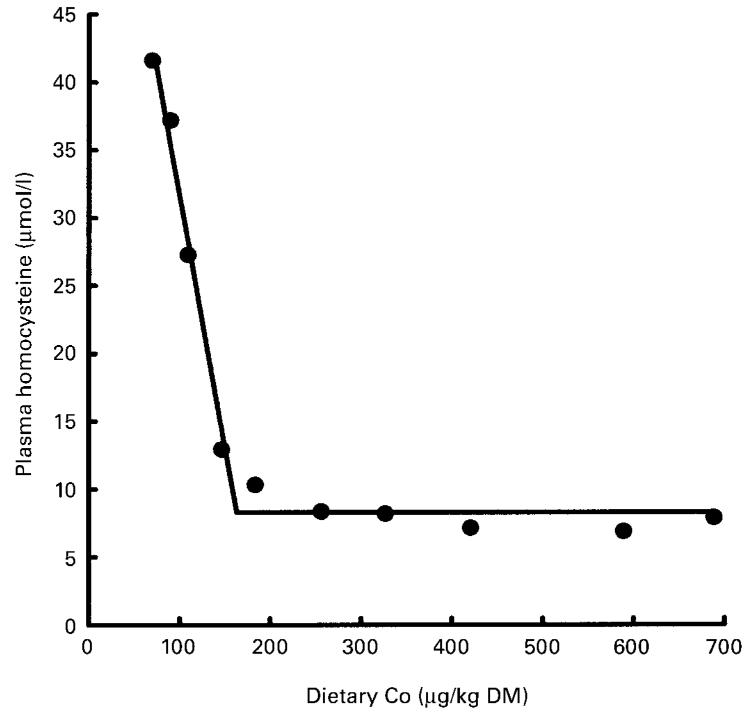

Fig. 3. Relationship between plasma levels of homocysteine (*y*) and cobalt concentration of the diet (*x*) and the corresponding one-slope broken-line plot. Each point (●) represents the mean value of at least three cattle. For details of the composition of the diets see p. 646, and for procedures see pp. 646–647. For details of the model see p. 647.

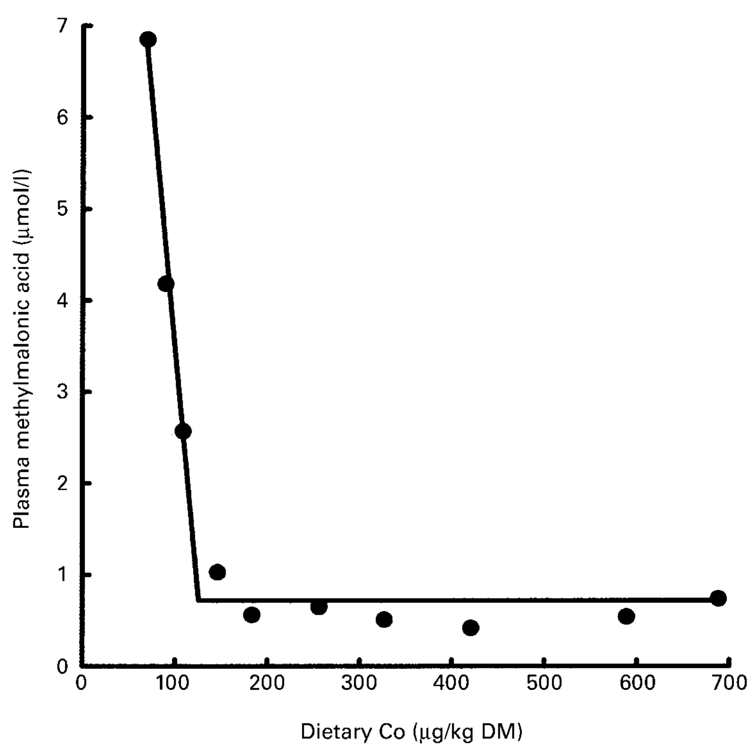

Fig. 4. Relationship between plasma levels of methylmalonic acid (*y*) and cobalt concentration of the diet (*x*) and the corresponding one-slope broken-line plot. Each point (●) represents the mean value of at least three cattle. For details of the composition of the diets see p. 646, and for procedures see pp. 646–647. For details of the model see p. 647.

Table 2. Blood variables of growing beef cattle fed graded levels of dietary cobalt*
(Mean values with pooled standard errors of the means†)

	Co concentration of the diet (μg/kg DM)												*P* value‡		
	70	90	109	147	184	257	327	421	589	689	SEM	ANOVA	L	Q	C
Hgb (g/l)	142	144	154	154	155	161	155	152	152	161	5	0·13	0·02	0·16	0·02
HCT (%)	40·4	40·6	43·6	43·8	43·9	45·4	43·9	43·3	43·3	45·8	1·4	0·08	0·01	0·15	0·02
RBCC (10^{12}/l)	8·78	8·76	9·43	9·14	9·12	9·49	9·45	8·93	9·11	10·0	0·48	0·50	0·16	0·81	0·19
MCV (μm^3)	45·7	46·6	46·4	47·7	47·7	48·3	46·2	46·1	45·3	45·8	1·5	0·91	0·56	0·53	0·38

Hgb, haemoglobin; HCT, haematocrit; RBCC, red blood cell count; MCV, mean corpuscular volume.
* For details of the composition of the diets see p. 646, and for procedures see pp. 646–647.
† Based on the harmonic mean, *n* 3·3.
‡ L, Q and C are respectively the linear, quadratic and cubic polynomial effects of the dietary cobalt concentration. For details of statistical procedures see p. 647.

liver are sufficiently responsive to changes in Co intake, and therefore seem to be useful means of diagnosing deficiency in the field. On the basis of these findings, recommended levels of dietary Co for maximum vitamin B_{12} levels can be set at about 250 μg/kg dietary DM. Our recommended value is similar to that which may be derived from the results of Singh & Chhabra (1995) for optimum rumen microbial activity, fermentation and vitamin B_{12} synthesis of crossbred calves. The main difficulty with the estimation of plasma concentrations of vitamin B_{12} in cattle has been the finding that a large proportion of the total plasma vitamin B_{12} concentration is not released by usual assay procedures from binding sites on transcobalamin 1, the principal vitamin B_{12}-carrier protein in bovine plasma (Price *et al.* 1991). The measure of vitamin B_{12} in plasma and liver which was done by a competitive binding radioimmunoassay using a specific extracting reagent containing NaOH with organic extracting enhancer provided control values equal to those reported by other authors (Kennedy *et al.* 1990, 1995; Paterson & MacPherson, 1990) who used a specific approach for estimation of ruminant plasma vitamin B_{12}. Thus, we suggest that the current analytical procedure guarantees a complete release of vitamin B_{12} from the specific bovine transcobalamins in plasma. However, it was remarkable that the control values of vitamin B_{12} in plasma analysed in this study were distinctly lower than the control values analysed in a recent experiment (Stangl *et al.* 2000) indicating large individual variations within the vitamin B_{12} plasma levels in cattle which have also been observed previously (Price *et al.* 1993). However, one must take into consideration that dietary factors other than Co, such as increasing the fibre content and total feed intakes, have also been shown to influence slightly, but favourably, vitamin B_{12} production in the rumen and the proportion of vitamin at the expense of its analogues (Sutton & Elliot, 1972; Hedrich *et al.* 1973). Thus, it is highly probable that the Co estimates would be somewhat higher or lower if the diet is based on ingredients and fibre sources other than corn silage.

Although vitamin B_{12} is required for normal liver folate metabolism (for review see Shane & Stokstad, 1985), this response variable has not hitherto been used to estimate the dietary requirement for Co. It was obvious from the present study that liver level of folate is also a valid variable to estimate the Co requirement of cattle. We conclude that at least 190 μg Co/kg dietary DM are required by growing cattle to maximise the levels of metabolic available folate in liver and that corresponds to a 77 % level of the maximum vitamin B_{12} concentration in liver. The interrelationship between these two vitamins is best explained by the methyl trap hypothesis stating that vitamin B_{12} deficiency can lead to lowered levels of methionine synthase, which results in a functional folate deficiency by trapping an increased proportion of folate as the 5-methyl derivative (Scott & Weir, 1981; Shane & Stokstad, 1985). In contrast, plasma levels of folate are not indicative of a deficient or adequate supply of Co and vitamin B_{12} respectively.

One approach which may be used to assess changes in the activities of the two vitamin B_{12}-dependent enzymes, methylmalonyl-CoA mutase and methionine synthase is the measurement of the accumulation in plasma of the enzyme substrates. Vitamin B_{12} dependency has been established for the isomerisation of methylmalonate to succinate and the methylation of homocysteine to methionine. Previous investigations from our laboratory have demonstrated that cattle fed a diet containing 83 μg Co/kg DM developed a number of metabolic perturbations compared with those fed 200 μg Co/kg DM (Stangl *et al.* 1998*a*,*b*). These were: dramatically increased concentrations of homocysteine and MMA in plasma; marked declined vitamin B_{12} and folate concentrations in the body; an accretion of Fe and Ni in liver. For the cobalamin-deficient cattle, measuring plasma metabolite concentrations proved to be a highly sensitive test of deficiency. We conclude that normal levels of both MMA and total homocysteine rule out clinically significant cobalamin deficiency with virtual certainty. MMA is elevated in the early stages of deficiency and it remains elevated as long as the ruminants are unsupplemented with Co (Rice *et al.* 1989). This suggested that an elevated plasma concentration of MMA is a comparatively early indicator of functional vitamin B_{12} deficiency (O'Harte *et al.* 1989). In our study, we conclude that at least 124 and 161 μg available Co/kg dietary DM are required by growing cattle to minimise plasma MMA and homocysteine respectively. The present results indicate that plasma MMA and homocysteine levels will increase when hepatic vitamin B_{12} concentrations fall below about 50 % of the maximum.

The present results also confirm the role of vitamin B_{12} and folate for the production of haem, since clinical pathology showed mild anaemia in Co deficiency as manifested by somewhat reduced Hgb concentration and haematocrit; this has also been found in a few Co-deficiency studies with sheep and goats (Mitchell *et al.* 1982; Mburu *et al.* 1993). Findings from this present study could demonstrate that determination of haematological variables may only be of value as a diagnostic indicator in severe Co deficiency, and are not suitable for the estimation of the Co requirement.

In conclusion, homocysteine and MMA together with the vitamin B_{12} and hepatic folate status appear to be useful predictors of the magnitude of Co–vitamin B_{12} deficiency and are a valuable tool in assessing Co requirements in cattle. The results suggest that the Co concentration required to minimise homocysteine and MMA in plasma and to maximise vitamin B_{12} and folate status was considerably greater than National Research Council (1996) and Gesellschaft für Ernährungsphysiologie (1995) recommendations. We found that plasma levels of homocysteine and hepatic folate appeared to be as sensitive to Co status as feed intake and growth development (Schwarz *et al.* 2000). We estimated the Co requirement of growing cattle to be in the range of 150 to 200 μg/kg DM based on hepatic folate and plasma levels of MMA and homocysteine: this is considerably higher than the National Research Council and Gesellschaft für Ernährungsphysiologie estimates. The Co requirement estimated from the vitamin B_{12} status was somewhat higher than from the other variables and this suggests that vitamin B_{12} level may increase as the level of dietary Co exceeds that required for minimum of homocysteine and MMA and for normal growth (Schwarz *et al.* 2000). On the basis of these findings, recommended levels for cattle of dietary Co for normal folate metabolism and minimum homocysteine and MMA can be set to be about 150–200 μg/kg; for maximum vitamin B_{12} levels, the desired Co content in the diet seems to be 250 μg/kg.

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