Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency

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1. Copper deficiency was induced in five Friesian cattle offered a semi-synthetic diet containing < 1 mg Cu/kg. Changes in blood and liver Cu contents and in the Cu-containing enzymes, ferroxidase I (caeruloplasmin; *EC* 1.16.3.1) and monoamine oxidase (*EC* 1.4.3.4) of plasma and cytochrome oxidase (*EC* 1.9.3.1) of liver and skeletal muscle were monitored during Cu depletion.

2. Rapid decreases in blood and liver Cu and plasma ferroxidase I activity were found at least 80 d before the first appearance of overt clinical signs of deficiency. Plasma monoamine oxidase and liver cytochrome oxidase activities decreased less rapidly and thus may provide useful indices of chronic Cu depletion.

3. Although results of these assays indicated that Cu depletion was occurring and metabolic defects supervening, none facilitated the early recognition of individuals that subsequently showed marked overt clinical signs of Cu deficiency compared with those less severely affected.

4. Irrespective of their clinical appearance at slaughter, Cu-depleted cattle showed gross or microscopic lesions of the skeleton and cardiovascular system and, in some instances, lesions of the ligamentum nuchae and small intestine. The aetiology of these lesions is considered with particular respect to changes in the activities of the Cu-dependent enzymes studied and to the interpretation of field surveys based solely upon determination of blood or liver Cu content.

5. A second group of five cattle was offered the same diet supplemented with Cu to provide 8 mg Cu/kg and, later, 15 mg Cu/kg. Although no pathological lesions attributable to Cu deficiency were detected at slaughter a marked reduction in liver Cu content, a decrease in plasma ferroxidase I activity and, in four animals, the development of a diarrhoea controlled by oral administration of Cu, suggested that 8 mg Cu/kg diet did not meet their requirement for Cu.

Doubt has frequently been cast upon the value of blood or plasma copper determination as a diagnostic aid for the detection of Cu deficiency in cattle. This arises partly from observations that a low blood Cu may occur in individuals or herds showing no gross clinical manifestations of deficiency (e.g. Allcroft & Parker, 1949; Field, 1957; Todd, Milne & How, 1967; Todd, 1971; Bingley & Anderson, 1972) and from instances in which ostensibly spontaneous improvements in clinical condition or rate of weight gain of hypocupraemic cattle have been unaccompanied by changes in blood Cu content (Allcroft & Parker, 1949; Jamieson & Allcroft, 1949, 1950).

It has been suggested that the commonly accepted value of 0·5 μg Cu/ml blood or plasma used for distinguishing 'normal' from 'deficient' animals may be too high (Smith & Coup, 1973) or that different criteria of normality for plasma Cu may apply to calves, yearling or adult stock and should take account of seasonal influences (Committee on Mineral Nutrition, 1973). Similar arguments surround the

interpretation of measurements of liver Cu content (Hill, Thambya, Wan & Shanta, 1962; Todd *et al.* 1967; Poole & Walshe, 1970). Thus Smith & Coup (1973) have suggested that the threshold value used to differentiate deficient from normal animals might be as low as 5 mg Cu/kg liver dry matter (DM) rather than the more commonly accepted 20–25 mg Cu/kg liver DM (van der Grift, 1955; Hill *et al.* 1962; Ammerman, 1970).

These differing opinions upon the acceptability of biochemical criteria for the recognition of Cu deficiency have all been based upon the analysis of field survey results. The periods for which animals have been under surveillance have differed greatly, and in most instances there has been no opportunity to assess the extent to which low tissue contents of Cu may reflect the current existence of pathological changes or, alternatively, merely provide warning of their later development. The assessment of adverse responses to a low Cu status has usually been restricted to observations of reduced rates of weight gain, deterioration of hair coat or other gross manifestations of deficiency. The relationship of the Cu status of cattle to the development of lesions in the skeleton, in connective tissue and other soft tissues has received little attention although there have been many reports of such pathological responses in Cu-deficient small experimental animals (e.g. Underwood, 1971). Although such changes may not be of great economic significance in cattle receiving a suboptimal Cu intake for short periods, they may well become so in animals exposed for a longer period.

The objectives of this experiment were to follow the progressive changes in blood and liver Cu content in Friesian calves offered a low-Cu semi-synthetic diet, to study the relationship of these changes to those of three Cu-dependent enzymes in plasma or liver that might offer alternative indicators for the detection of Cu deficiency, and to determine whether any of these indicators accurately reflected the extent to which either gross or microscopic pathological changes developed in Cu-depleted animals.

The Cu-enzymes selected for study were those with activities known to be reduced by a low Cu status, and which may thus play a part in the genesis of lesions of Cu deficiency; they are: cytochrome oxidase (*EC* 1.9.3.1), through its wide involvement in oxidative reactions; monoamine oxidase (*EC* 1.4.3.4), through its involvement in the maturation of collagen and elastin (Carnes, 1971); ferroxidase I (caeruloplasmin; *EC* 1.16.3.1), an enzyme probably involved in the utilization of hepatic stores of iron (Osaki, Johnson & Frieden, 1971).

The performance of calves offered a low-Cu diet was compared with that of others receiving the same diet supplemented with copper sulphate to provide 8 mg Cu/kg and, later, 15 mg Cu/kg.

EXPERIMENTAL

Ten Friesian bull calves were purchased from a commercial herd with no previous record of Cu deficiency in cattle. They were offered up to 4·5 l milk daily until 8 weeks of age. The milk was offered in plastic buckets and was supplemented with ferrous sulphate to provide 40 mg Fe/d. During the next 4 weeks the quantity of milk offered was progressively reduced and access was given to a solid diet of the composition

Table 1. *Composition of copper-deficient basal diet given to the low-Cu group of Friesian calves*

Main components (g/kg)

Skim milk, dried	230
Urea	30
Maize starch	352
Glucose	236
Cotton linters, bleached	90
Arachis oil	10
Mineral and vitamin premix*	52

Supplementary analytical data (dry matter basis): moisture content, as fed, 123 g/kg; sulphur content (g/kg) from inorganic sulphate 1·6, from dried skim milk 0·8.

Adventitious trace elements (mg/kg): Cu 0·83 ± 0·11, molybdenum 0·08 ± 0·2, barium 1·1, chromium 1·8, nickel 0·12, lead 0·3, strontium 7·3, titanium 1·7, vanadium 1·3.

* Major elements (British Pharmacopoeia (1973) grade) (g/kg): $CaHPO_4.2H_2O$ 22, $MgSO_4$. $7H_2O$ 12·4, $KHCO_3$ 7·6, NaCl 9·2. Trace elements (analytical grade) (g/kg): $FeSO_4.7H_2O$ 0·150, $CoCl_2.6H_2O$ 0·004, $ZnSO_4.7H_2O$ 0·079, $MnSO_4.4H_2O$ 0·160, KI 0·0013. Vitamins (added as Rovimix; Roche Products Ltd, Welwyn Garden City, Herts.): retinol 0·43 mg/kg, cholecalciferol 3·25 μg/kg, α-tocopherol 2·275 mg/kg.

given in Table 1. Calves were castrated at 12 weeks of age, abruptly weaned onto the solid diet and allocated at random into two groups. One group (subsequently the low-Cu group (LCu)) received the unsupplemented basal diet (mean Cu content 0·83 ± 0·11 mg Cu/kg). The second group (SCu) received the same diet supplemented with Cu as cupric sulphate. For the first 224 d of the experiment the diet of group SCu provided 8·0 ± 0·3 mg Cu/kg; on day 225 the Cu content was increased to 15 mg Cu/kg after observations (discussed later) suggesting that the Cu status of this group was becoming undesirably low. Deionized water was offered *ad lib.*; food was offered to appetite and food refusals of each group were weighed.

Animals were housed in groups of two or three in wooden pens with concrete floors and sawdust bedding material. Initially, they had no access to direct sunlight. On monitoring blood composition of animals on the 98th day of the experiment, low plasma concentrations of calcium and phosphorus (over-all means, 2·05 ± 0·14 and 2·03 ± 0·13 mmol/l respectively) were detected despite the fact that the basal diet was formulated to meet suggested requirements (Agricultural Research Council, 1964) for Ca, P and cholecalciferol. A single intramuscular injection of 25 mg cholecalciferol (Dupharal; Duphar Veterinary Products, Southampton, Hants) was given to all animals on day 112; plasma Ca and P returned to normal and remained so after animals were given access to open yards and direct sunlight.

Marked individual differences occurred in the severity and distribution of changes in hair coat pigmentation. A subjective estimate of these changes was obtained by asking five independent observers to assess the over-all extent of coat depigmentation from black-and-white photographs and to rank the results on a scale from 5 (normal pigmentation) to 1 (severe greying).

One animal of group LCu died of bloat after 96 d of treatment. As there was no evidence that this death was attributable to the experimental treatment, results for this animal have been excluded. The death of a second animal of this group (no. 834)

after 245 d was attributed to rupture of the posterior vena cava. In view of the probability that this was a consequence of Cu deficiency, results for this animal have been included.

Analytical methods

Blood samples were collected into acid-washed tubes using heparin as anticoagulant. The ferroxidase I activity of plasma was determined by the method of Houchin (1958) using Bandrowski's base as external standard (Rice, 1962); the monoamine oxidase activity of plasma was determined by the method of Tabor, Tabor & Rosenthal (1954) using twice-recrystallized benzylamine hydrochloride as substrate and 0·1–0·2 ml plasma in 3 ml final volume for the assay. Benzaldehyde produced during this reaction was measured spectrophotometrically assuming an extinction coefficient of 12×10^3 at 250 nm (Dearsden & Forbes, 1958). Cytochrome oxidase activity of liver and semitendinosus muscle samples obtained by biopsy under local anaesthesia was determined by a colorimetric method similar to that of Mills & Dalgarno (1970) except that tissue homogenates were prepared in distilled water. All enzyme assays were carried out using a Hilger–Gilford reaction-kinetics spectrophotometer (Rank-Hilger, London) at a temperature of 25°; enzyme activities are expressed as units/ml for plasma samples or units/mg protein in other tissues. One unit of enzyme activity is defined as the amount catalysing the transformation of 1 μmol substrate per min (International Union of Biochemistry, 1965). The Cu, Fe and zinc contents of diets and tissues were determined by atomic absorption spectrophotometry after wet oxidation of samples in concentrated nitric–concentrated sulphuric–concentrated perchloric acids (3:1:1, by vol.).

The four animals of group LCu that survived the experiment were slaughtered after 257–259 d of treatment. Because of financial considerations only three of the five animals of group SCu were slaughtered; after 264, 266 and 273 d of treatment.

Samples of ligamentum nuchae, aorta, cardiac muscle and bone were taken immediately after slaughter and either frozen in liquid nitrogen if required for biochemical studies or fixed in formol-saline or glutaraldehyde if required for examination by optical or electron microscopy. Details of procedures used in chemical studies of the elastin from the ligamentum nuchae of these animals have been given by Whiting, Sykes & Partridge (1974); the procedures used for examination of the small intestinal mucosa and cardiac muscle have been described by Fell, Dinsdale & Mills (1975) and Leigh (1975) respectively.

RESULTS

Overt clinical signs of Cu deficiency

The clinical condition of all animals receiving diet LCu remained normal for the first 170 d of the experiment. Thereafter, all but one of the animals of this group developed a 'stilted' gait and a 'knock-kneed' appearance when walking. This appearance was attributed to over-extension of the flexor tendon and splaying of the hooves and was accentuated by swellings surrounding the metacarpophalangeal and carpometacarpal joints (Plate 1). These swellings were caused by a thickening of

Table 2. *Gross clinical and pathological changes in Friesian cattle offered a low-copper diet* (0·83 mg Cu/kg) (LCu) or the same diet supplemented with Cu (8·0 mg Cu/kg to day 225, then 15 mg Cu/kg) (SCu)*

Group	Animal no.	Hair pigmentation scores† Day 170	Day 193	Day 225	Day 250	First appearance of diarrhoea (no. of d of experiment)	Gait affected Day 170	Day 193	Small intestine villus atrophy‡	CBI§ H	R	F	T	MC	MT	Lesions of connective tissue	Cardiac hypertrophy	Cardiac muscle lesions||
LCu	837	5	3	2	2	167	Moderate	Moderate	+	22	33	41	37	37	42	+	+	+++
	832	4	4	3	2	207	Slight	Slight	+	26	32	35	38	37	39	+	+	+++
	838	4	5	5	5	Nil	Slight	Nil	Nil	29	38	39	35	36	40	+	+	+++
	834	3	2	2	2	172	Severe	Severe	NE	20	34	38	36	35	45	+	+	+
	Significance of group mean differences in CBI : *P* <									0·05	0·01	0·05	0·01	0·01	0·05			
SCu	836	5	5	3	4	215	Nil	Nil	NE	NE	NE	NE	NE	NE	NE	Nil	NE	NE
	840	5	5	4	5	207	Nil	Nil	Nil	55	56	52	51	47	60	Nil	Nil	Nil
	846	5	4	3	5	196	Nil	Nil	Nil	30	45	42	57	45	51	Nil	Nil	Nil
	835	5	5	5	5	Nil	Nil	Nil	Nil	44	52	51	48	47	49	Nil	Nil	Nil
	839	5	5	4	5	215	Nil	Nil	NE	NE	NE	NE	NE	NE	NE	Nil	NE	NE

+, Lesion present; nil, lesion absent; NE, tissue not examined; H, humerus; R, radius; F, femur; T, tibia; MC, metacarpus; MT, metatarsus.

* For details of composition, see Table 1.

† Hair pigmentation scored on scale 5 (full black pigmentation) to 1 (severe greying); for details, see p. 311.

‡ From Fell, Dinsdale & Mills (1975) and D. Dinsdale (personal communication).

§ Determined by radiography of dissected, cleaned bones using the procedure of Barnett & Nordin (1960).

|| From Leigh (1975, and personal communication).

subcutaneous tissue surrounding the synovial capsule and a minor distortion of under-lying bone. This defect appeared to be similar to that described by Jamieson & Allcroft (1950) in Cu-deficient cattle in Caithness. It was not as marked as that described in Cu-deficient Irish cattle by Poole (1963) and attributed to substantial exostoses occurring around the metatarsal epiphyses. The lesion was not apparent in one animal (no. 838) receiving diet LCu and did not occur in calves receiving diet SCu at any stage of the experiment.

Changes in hair pigmentation appeared in three of the four animals receiving diet LCu by the 165th day of treatment, initially by the development of a grey-brown cast over the normally black areas of the coat (Plate 2). The 'spectacle eye' pattern of depigmentation described by Jamieson & Allcroft (1949) only became apparent after 225 d of treatment. One animal of this group (no. 838) retained normal coat pigmenta-tion throughout the experiment (Plate 2, Table 2). A brownish-grey cast was also apparent on the hair of three of the five animals receiving diet SCu when pigmentation was assessed on day 225 of the experiment (Table 2). The coat pigmentation of these animals rapidly returned to normal when the Cu content of their diet was increased from 8 to 15 mg/kg.

Diarrhoea

Three of the animals receiving diet LCu developed a severe diarrhoea commencing between the 167th and 207th day of treatment. Repeated attempts to control this by the oral administration of a sulphadimidine–streptomycin–kaolin preparation ('Stromez'; ICI Pharmaceuticals, Alderley Park, Macclesfield, Cheshire) were not successful. To determine whether the diarrhoea was attributable to the low Cu content of the diet, a single oral dose of 10 mg Cu (as $CuSO_4 \cdot 5H_2O$ in 100 ml water) was given to all affected animals. In every instance the diarrhoea ceased within 12 h. Initially this single small dose of Cu controlled diarrhoea for between 7 and 14 d (e.g. animal no. 837, Table 4) but, as Cu depletion occurred, control was only achieved for 3–4 d. Diarrhoea did not occur in animal no. 838.

A similar diarrhoea occurred in group SCu 206–215 d after first offering the diet providing 8 mg Cu/kg. Attempts to control the condition by sulphadimidine–streptomycin–kaolin therapy again failed and a single dose of 10 mg Cu was given orally to each affected animal. A rapid response to this treatment suggested that the diarrhoea was again related to the dietary content of Cu and, from the 225th day of the experiment, this was increased to 15 mg Cu/kg diet. Only one further instance of diarrhoea occurred subsequently, this being in one animal 2 d after the modification of the diet.

Weight gain

No significant differences in rate of weight gain were apparent between the two groups during the first 201 d (0·71 ± 0·14 and 0·77 ± 0·19 kg/d for animals receiving diets LCu and SCu respectively). The mean rate of gain of group LCu decreased significantly (0·01 < P < 0·05) to 0·21 ± 0·19 kg/d during the period from 201 d until the end of the experiment (Fig. 1). A significantly greater rate of gain was maintained in group SCu during the corresponding period (P < 0·05).
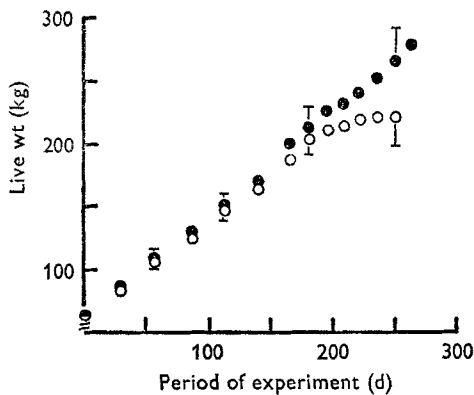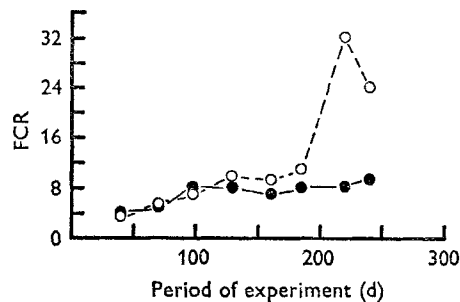
Fig. 1   Fig. 2

Fig. 1. Mean live weights (kg) of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○), or the same diet supplemented with Cu (8 mg Cu/kg to day 225, then 15 mg Cu/kg) (●). Representative SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311.

Fig. 2. Changes in the food conversion ratio (kg food intake/kg body-weight gain) (FCR) of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○--○) or the same diet supplemented with Cu (8 mg Cu/kg to day 225, then 15 mg Cu/kg) (●—●). For details of diets, see Table 1 and p. 311.

There were marked individual differences in the weight responses of the cattle in group LCu. One animal (no. 838) maintained a growth rate of 0·91 kg/d over the first 222 d of the experiment. Thereafter, its rate of gain decreased to 0·28 kg/d before the end of the experiment 4 weeks later. In contrast, animal no. 837 of the same group suffered a decreasing rate of gain after 111 d of this treatment (0·91, 0·68, 0·23 and 0·04 kg/d during the intervals 0–111, 112–167, 168–195 and 196–250 d respectively).

The rate of weight gain of group SCu during the 39 d before the change in the Cu content of their diet was 0·74 ± 0·19 kg/d; during the 37 d after this change the rate increased to 0·87 ± 0·18 kg/d. This difference was not statistically significant. The cattle were housed in groups of two or three and thus the food consumption of individuals could not be measured. However, records of the total food consumed by the two groups indicated that there was no consistent difference in consumption for the first 112 d of the experiment. Thereafter, the per capita daily consumption for the groups expressed as the ratio, LCu diet consumed:SCu diet consumed was 0·89 for the period 112–224 d and 0·82 from 224 d until the end of the experiment. The increased difference between the groups during the terminal phases of the experiment was partly attributable to a small reduction in the intake of group LCu and partly from an increase in intake of group SCu subsequent to increasing the Cu content of their diet from 8 to 15 mg/kg on day 225. Changes in the food conversion ratio (kg food consumed/kg body-weight gain) for the two groups of cattle (Fig. 2) suggested that a marked reduction in the efficiency of food utilization of animals receiving the LCu diet certainly occurred after 185 d and may have begun after 126 d of treatment.
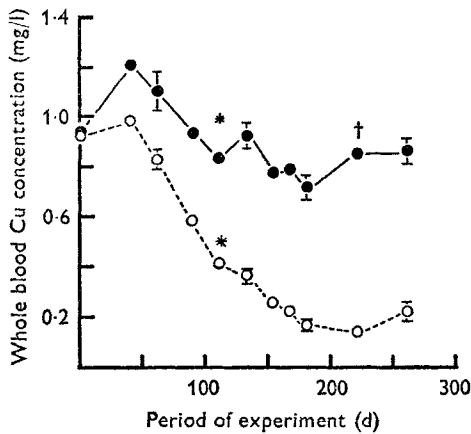
Fig. 3

Fig. 4

Fig. 3. Changes in the whole blood copper concentration (mg/l) of Friesian cattle offered a low-Cu diet (0·83 mg Cu/kg) (O--O) or the same diet supplemented with Cu (8 mg Cu/kg to day 225(†), then 15 mg Cu/kg) (●—●). All animals received a single intramuscular injection of cholecalciferol on day 112(*). Representative SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311.
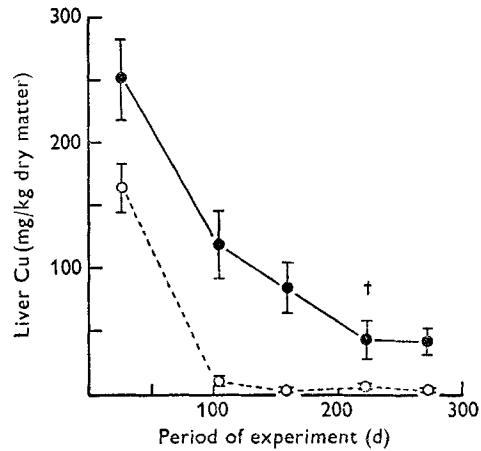
Fig. 4. Mean copper content (mg/kg dry matter) of liver biopsy samples from Friesian cattle offered a low-Cu diet (0·83 mg Cu/kg) (O--O) or the same diet supplemented with Cu (8 mg Cu/kg to day 225(†), then 15 mg Cu/kg) (●—●). SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311.

Biochemical studies

Changes in whole blood Cu concentration. Changes in blood Cu content are shown in Fig. 3. A highly significant difference between groups was apparent at the first sampling after treatment was initiated, and the difference remained significant thereafter ($P < 0.01$, 41–111 d; $P < 0.001$, 133–262 d). Interpolation between values suggests that the mean blood Cu concentration of group LCu decreased below the 'threshold value' of 0·5 mg/l by the 100th day of treatment. This rapid reduction in blood Cu continued until day 181. Thereafter, the mean values for this group were within the range 0·14–0·22 mg/l for the remainder of the experiment. The intermittent oral doses of 10 mg Cu used to control diarrhoea in animals of this group during the terminal phases of the experiment had no significant effect upon blood Cu. The blood Cu of group SCu increased during the first 41 d of treatment, decreased during the next 70 d and suffered no major change thereafter. Two additional results should be mentioned. After the intramuscular injection of cholecalciferol on day 112 a transient (but statistically non-significant) increase in blood Cu occurred (see later discussion). Increasing the dietary Cu content from 8 to 15 mg/kg on day 225 had no effect on blood Cu content. The lowest individual blood Cu value recorded in group SCu was 0·57 mg/l on day 221 of treatment, when the group mean was 0·85 ± 0·203 mg/l.

Liver Cu content. Because of their small size, liver biopsy on the calves was not attempted before they were assigned to treatments. When liver samples were taken 26 d after treatment was initiated there was already evidence of a significantly lower
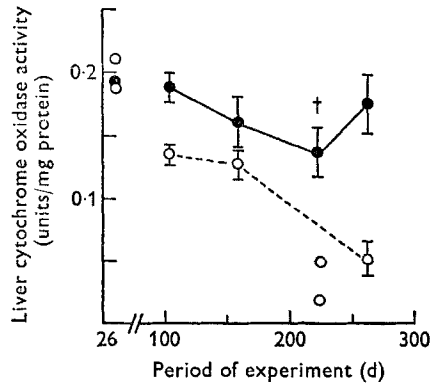
Fig. 5. Cytochrome oxidase (*EC* 1.9.3.1) activity (units/mg protein) in biopsy samples of the livers of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (O--O) or the same diet supplemented with Cu (8 mg Cu/kg to day 225(†), then 15 mg Cu/kg) (●—●). Only three samples obtained on day 26 and two samples from the low-Cu group on day 228 were sufficiently large to permit enzyme assay; these individual values are indicated. Other points represent group means ± SE. For details of diets, see Table 1 and p. 311. One unit of activity is defined as the amount catalysing the transformation of 1 μmol substrate/min.

liver Cu content in group LCu than in group SCu (mean values 163 ± 45 and 318 ± 162 mg Cu/kg liver DM respectively; $P < 0.05$) (Fig. 4). The liver Cu content of the former group decreased to 15·8 ± 10·4 mg/kg DM by day 104; relatively small changes in content occurred thereafter with a minimum mean value of 3·20 ± 1·10 mg/kg DM occurring on day 262. The liver Cu of calves receiving diet SCu decreased markedly during the 224 d that this diet provided only 8 mg Cu/kg. After this period the mean liver Cu content was 44·5 ± 33·1 (range 7–73) mg/kg DM and no longer significantly different from that of the LCu group. Increasing the Cu content of diet SCu to 15 mg/kg on day 225 reduced the variability of liver Cu within this group and restored the statistical significance of the difference in liver Cu content between the two groups ($P < 0.001$), but did not increase mean liver Cu content during the next 37 d. It is noteworthy that the liver Cu of one animal of this group (no. 836) had decreased to 7·0 mg/kg DM by day 228 of treatment and that this animal was the only one of the group to develop an episode of diarrhoea subsequent to increasing the dietary content of Cu.

Cytochrome oxidase activity in liver and other tissues. Only three of the liver biopsy samples obtained on day 26 were sufficiently large to permit determination of both trace metal content and cytochrome oxidase activity. The individual values for these animals are shown in Fig. 5. There was no suggestion of an effect of treatment on activity at this stage. By day 104 liver cytochrome oxidase activity in group LCu was significantly lower than that of group SCu ($P < 0.01$). After a slight decrease during the next 54 d, activity in LCu animals decreased more rapidly to reach 0·051 ± 0·014 units/mg protein after 262 d of treatment.

Cytochrome oxidase activity in the liver of cattle receiving supplementary Cu (SCu) slowly decreased during the period that their diet contained 8 mg Cu/kg and on day 158 the difference between groups was no longer statistically significant. The lowest
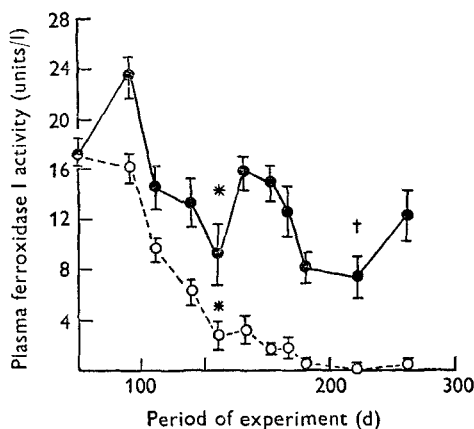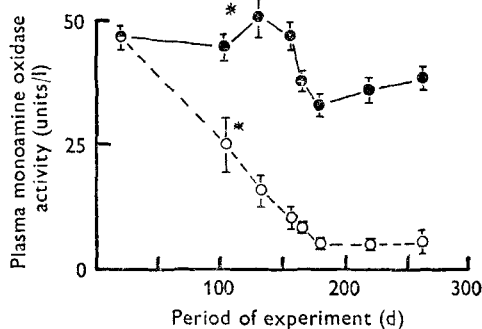
Fig. 6

Fig. 7

Fig. 6. Mean activity of ferroxidase I (caeruloplasmin; EC 1.16.3.1) (units/l) in plasma of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○--○) or the same diet supplemented with Cu (8 mg Cu/kg to day 225 (†), then 15 mg Cu/kg) (●—●). All animals received an intramuscular injection of cholecalciferol on day 112 (*). SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311. One unit of activity is defined as the amount catalysing the transformation of 1 μmol substrate/min.

Fig. 7. Mean activity of monoamine oxidase (EC 1.4.3.4) (units/l) in blood plasma of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○--○) or the same diet supplemented with Cu (8 mg Cu/kg to day 225, then 15 mg Cu/kg) (●—●). All animals received an intramuscular injection of cholecalciferol on day 112 (*). SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311. One unit of activity is defined as the amount catalysing the transformation of 1 μmol substrate/min.

mean activity found in this group was $0·137 \pm 0·020$ units/mg protein on day 221, i.e. 4 d before the Cu content of diet SCu was increased to 15 mg/kg. There was a suggestion that liver cytochrome oxidase may have increased in response to the increase in dietary Cu, but the difference in mean activity between days 221 and 262 was not statistically significant.

The cytochrome oxidase activity of samples of semitendinosus muscle obtained by core biopsy under local anaesthesia was also followed throughout the experiment. The variable content of connective tissue present in these samples created difficulty and, although from day 117 onwards the mean activity in muscle from group LCu was always lower than that of SCu, the differences were not statistically significant. At the end of the experiment the mean activity in the former group was $0·165 \pm 0·038$, and in the latter was $0·232 \pm 0·054$ units/mg protein.

The marked effects of diet LCu in reducing the cytochrome oxidase activity of the mucosa of duodenum, ileum and jejunum have been described elsewhere (Fell et al. 1975).

Plasma ferroxidase I activity. Changes in plasma ferroxidase I activity (Fig. 6) reflected, in accentuated form, the changes in blood Cu content previously described. All differences in mean values obtained after the beginning of treatment were statistically significant ($P < 0·05$ or lower). After 111 d the activity of this enzyme in group

LCu was frequently below the detection limit (approximately 1 unit/l plasma). Other notable features are that plasma ferroxidase I activity increased significantly ($P < 0.05$) in group SCu after the intramuscular administration of cholecalciferol on day 112, and that the increase in the Cu content of their diet to 15 mg/kg on day 225 produced a significant increase in activity ($P < 0.05$) during the subsequent 31 d.

Monoamine oxidase activity in plasma. An uninterrupted decrease in plasma monoamine oxidase activity occurred in group LCu during the first 180 d of treatment. By this time a mean activity of 4.75 ± 1.00 units/l was obtained (Fig. 7). Little change occurred thereafter.

Significantly higher values were maintained in group SCu throughout the experiment ($P < 0.05$ at day 109; $P < 0.001$ on all later occasions). As with plasma ferroxidase I activity, there were indications that cholecalciferol injection on day 112 provoked a transient increase in monoamine oxidase activity but, in this instance, the difference in activity before and after treatment was not significant. The minimum mean activity recorded for group SCu was 32.0 ± 2.00 units/l on day 180; thereafter a small but statistically non-significant increase in activity occurred which was not influenced by increasing the Cu content of the diet on day 225.

Changes in other blood characteristics. The mean haemoglobin (Hb) concentration of calves at the beginning of the experiment was 120 ± 7.7 g/l. Group LCu suffered a small reduction in blood Hb during the experiment to a final mean value of 92 ± 3.5 g/l. A smaller decrease occurred in the control group (final value 104 ± 3.6 g/l) and the difference between groups was statistically significant ($P < 0.05$). Values for packed cell volume decreased from an initial value of 0.37 to 0.27 in group LCu and to 0.30 in group SCu. Although this trend was maintained throughout the experiment, group differences in packed cell volume were only significant on one of eleven occasions. No significant differences in erythrocyte count or white cell count were apparent.

Changes in plasma Fe content are shown in Fig. 8. Although variability between individuals was high, it was apparent that higher concentrations of plasma Fe were maintained in group SCu animals than in group LCu during the final phases of the experiment. This difference was statistically significant on days 154, 167 ($P < 0.01$), 181 and 221 ($P < 0.05$).

The mean plasma Zn content of group LCu was consistently higher than that of group SCu from day 41 onwards but the difference only reached statistical significance ($P < 0.05$) after 180 d of treatment. When allocated to treatments the mean plasma Zn content of all animals was 1.08 ± 0.14 mg/l; at the end of the experiment the plasma of LCu animals contained 1.25 ± 0.20 mg Zn/l, and that of SCu 0.81 ± 0.05 mg Zn/l.

The trace metal content of soft tissues. The Cu, Zn and Fe contents of liver, kidney, heart and spleen of those animals slaughtered at the end of the experiment are given in Table 3. A significant difference in the mean content of liver Cu of the two groups of animals ($P < 0.01$) confirmed earlier results from biopsy samples. Smaller differences were found in the Cu content of kidney, heart and spleen. No consistent differences in the Zn content of liver, kidney, heart and spleen were detectable. A significantly higher Fe content was found in the livers of group LCu at slaughter. Determination of the Fe
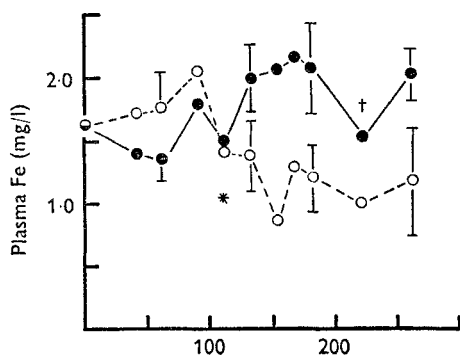
Fig. 8.   Fig. 9.

Fig. 8. Mean concentration of iron (mg/l) in plasma of Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○– –○) or the same diet supplemented with Cu (8 mg Cu/kg to day 225 (†), then 15 mg Cu/kg) (●—●). All animals received an intramuscular injection of cholecalciferol on day 112 (*). SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311.
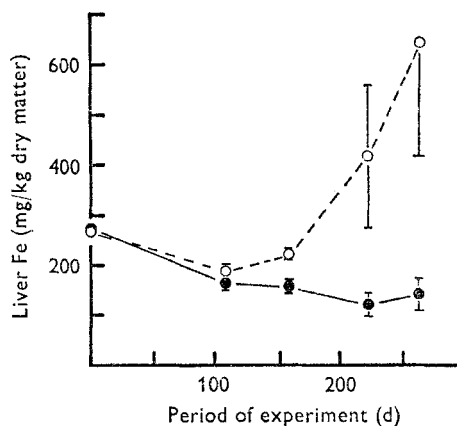
Fig. 9. Mean iron content (mg/kg dry matter) of liver biopsy samples from Friesian cattle offered a low-copper diet (0·83 mg Cu/kg) (○– –○) or the same diet supplemented with Cu (8 mg Cu/kg to day 225, then 15 mg Cu/kg) (●—●). SE of mean values are indicated by vertical bars. For details of diets, see Table 1 and p. 311.

Table 3. *The copper, iron and zinc contents (mg/kg dry matter) of liver, kidney, heart and spleen of Friesian cattle offered a low-Cu diet† (0·83 mg Cu/kg) (LCu) or the same diet supplemented with Cu (8 mg Cu/kg to day 225, then 15 mg Cu/kg) (SCu)*

(Mean values with their standard errors)

		Group LCu‡			Group SCu§	
		Mean	SE		Mean	SE
Cu	Liver	4·7	1·59	**	20·1	2·88
	Kidney	11·4	0·85	*	16·1	0·56
	Heart	9·4	0·91	**	16·0	0·65
	Spleen	1·9	0·02	***	4·3	0·01
Fe	Liver	581	208	*	116	18
	Kidney	176	39	NS	179	34
	Heart	146	7	NS	170	12
	Spleen	3312	830	NS	1784	571
Zn	Liver	116	7	NS	101	13
	Kidney	81	2	NS	81	1
	Heart	78	1	NS	80	5
	Spleen	92	2	NS	97	4

NS, not significant.

Statistical significance of differences between LCu and SCu groups determined after logarithmic transformation of results: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† For details of composition, see Table 1.

‡ Results for group LCu include that for animal no. 834 which died 12–13 d before others of this group were slaughtered.

§ Results for group SCu are for the three slaughtered of the five in the group (see p. 312).

content of liver biopsy samples indicated that a significant difference first became established by the 158th day of treatment, with marked increases in liver Fe content of group LCu occurring subsequently (Fig. 9). Liver Fe contents did not, however, reach the high values reported to occur in instances of the Cu-deficiency syndrome 'falling disease' (Bennetts, Beck & Hartley, 1948). The Fe content of the spleens of three Cu-deficient and three control animals was determined after slaughter; although the mean Fe contents were 3312 and 1784 mg/kg DM respectively this difference was not statistically significant. There were no obvious effects of treatment on the Fe content of kidney and heart tissue.

Post-mortem pathology

Leg and skeletal defects. The primary cause of the leg swellings observed in most of the LCu group was a fibrous thickening of the stratum fibrosum of the synovial capsule surrounding the carpal and tarsal joints. Haematomas were frequently present in this tissue, which was infiltrated by a pale yellow lymph-like liquid of undetermined origin. These lesions were not present in group SCu. Radiographic examination of the limb bones of four animals of group LCu and three of group SCu provided clear evidence of bone rarefaction and a reduction in the cortical bone index of humerus (Plate 3), radius, femur, tibia, metacarpus and metatarsus of all individuals of the former group (Table 2).

Conformational changes in the metacarpus and metatarsus were indicated by significantly lower ratios, bone length:midshaft breadth, andmids haft breadth:epiphyseal breadth in group LCu ($P < 0.05$) than in SCu. A similar trend was apparent in the length:epiphyseal breadth ratio, but the difference between groups was not statistically significant ($0.05 < P < 0.1$). (Full details of these measurements can be obtained from the authors.) Although no major conformational changes were apparent in ribs or vertebrae of group LCu, there were again indications of a decrease in radiographic density and, in one animal, evidence of healing of at least two rib fractures.

Histological studies indicated that the described effects on the skeleton of LCu cattle had a complex aetiology. In agreement with the previous findings of Baxter, van Wyk & Follis (1953) with Cu-deficient dogs; of Follis, Bush, Cartwright & Wintrobe (1955) with pigs and of Suttle, Angus, Nisbet & Field (1972) with lambs, osteoporosis associated with a decrease in osteoblastic size and number (Plate 4A) and abnormal (spindle-like) morphology was apparent in all animals of this group. At the epiphyses there was delayed ossification of calcified cartilage lattice and numerous islands of this tissue occurred in trabecular bone. Their presence was associated with patchy loss of birefringence when sections were examined by polarized light, but otherwise there was no evidence of defective bone matrix. Such lesions were particularly associated with cortical thinning and a deficiency of trabecular bone. Osteoid borders were not seen in sections of decalcified bone stained with haematoxylin and eosin or with the periodic acid–Schiff reagent; undecalcified sections of bone, however, were not examined.

In addition to these changes, zones of osteitis fibrosa were apparent, particularly in the cortical bone of vertebrae and the radial metaphyses of group LCu. In these

regions (Plate 4 B) there was an increase in osteoclast activity and numerous examples of trabecular fracture associated with haemorrhage and fibrosis. It is possible that a rickets-like condition was present but that other manifestations (e.g. widening of the epiphyseal growth plate, defective calcification and osteoid borders) were not seen because of a failure in matrix formation (B. F. Fell, personal communication). The above lesions were absent in skeletal tissues from group SCu.

Connective tissue. Histological examination of the aorta and posterior vena cava revealed a marked reduction of wall thickness in both these tissues in all cattle of group LCu. This was accompanied by a decrease in the quantity of collagen present between the elastic laminae. There was no evidence of defective elastogenesis in the wall of the aorta, vena cava or spleen. This situation contrasts markedly with that described by Waisman, Cancilla & Coulson (1969) in which the most significant lesion in the aortas of Cu-deficient pigs was fragmentation and focal degeneration of the elastic laminae accompanied by definite but less extensive lesions in intralaminar collagen. An additional feature possibly contributing to reduced wall thickness of the aorta and also of the splenic capsule in cattle of group LCu was a decreased content of smooth muscle dispersed throughout the matrix of collagen and elastin. This change was particularly apparent in animal no. 834 of group LCu that died subsequent to rupture of the posterior vena cava. Atrophy of smooth muscle was also apparent in the wall of the portal vein in animals of this group.

Within group LCu there were marked individual differences in the effects of treatment on the elastin and collagen of the ligamentum nuchae. Transverse sections of the ligamentum nuchae of animal no. 838, which continued to grow through the greater part of the experiment, showed clumps of densely staining elastin occluded in substantial aggregates of a material giving a much weaker staining reaction, suggestive of 'immature' elastin precursors. The surrounding collagen was disorganized and fragmented (Plate 5). Such changes were not evident in ligamentum nuchae of group SCu or in those Cu-deficient animals that had suffered a marked reduction in growth rate as a consequence of treatment. That the ligamentum nuchae of low-Cu animal no. 838 contained an appreciable proportion of elastin precursors was confirmed by Whiting *et al.* (1974) who found that the ligamentum nuchae from this animal contained salt-soluble elastin, deficient in desmosine and isodesmosine cross-links. No defective cross-linking was apparent in the aortic elastin from this animal (M. Partridge, personal communication), confirming the histological observations on the aorta reported previously.

Pathology of small intestine. No gross morphological or histological abnormalities were detected in the small intestines of group SCu. Extensive petechiation of the intestinal mucosa and partial or complete atrophy of duodenal and jejunal villi were apparent in group LCu, except in animal no. 838 whose duodenum and ileum appeared normal in these respects. Additional details of the nature of mitochondrial lesions in enterocytes and of the low cytochrome oxidase activity in duodenal, jejunal and ileal mucosas of these deficient animals have been published elsewhere (Fell *et al.* 1975).

Heart. All calves in group LCu showed myocardial hypertrophy at the termination

of the experiment. The mean weight of the hearts of low-Cu animals was $6 \cdot 03 \pm 0 \cdot 48$ g fresh tissue/kg live weight compared with $4 \cdot 15 \pm 0 \cdot 21$ g fresh tissue/kg live weight for animals receiving supplementary Cu. Details of the degenerative changes in the cardiac muscle of all animals of group LCu have been described by Leigh (1975).

DISCUSSION

The initial intention of this experiment was to study the relationship of changes in blood Cu and other possible indicators to the development of clinical signs of Cu deficiency, and to compare the performance of Cu-deficient animals with those having an adequate Cu status. The decision to use a diet containing 8 mg Cu/kg for 'control' (group SCu) animals was based on the fact that this Cu content had not induced a significant reduction in blood Cu during previous experiments with cattle maintained on a similar diet for a period of 12 weeks during studies of Zn deficiency. Although appreciating that a previous publication (Agricultural Research Council, 1964) has suggested that a dietary content of 10 mg Cu/kg may be required for cattle in contrast to 5 mg Cu/kg for sheep, we questioned whether sufficient evidence was available to justify this differentiation between species and held the view that 8 mg Cu/kg might well be adequate, particularly when using a purified diet low in molybdenum content ($0 \cdot 08$–$0 \cdot 2$ mg Mo/kg).

At an early stage of the experiment it was apparent that a rapid depletion of liver Cu was taking place in group SCu. Although this was accompanied by a decrease in whole blood Cu content the fact that a 'classical' hypocupraemia (i.e. $< 0 \cdot 5$ mg Cu/l) did not develop made it difficult to assess the biological significance of the changes in liver Cu. Furthermore both Cunningham (1946) and van der Grift (1955) have suggested that a decrease in liver Cu of calves occurs during the 1st year of life under circumstances that are not normally associated with the ultimate appearance of clinical Cu deficiency. As we were uncertain whether this decrease in liver Cu may have been merely a function of increasing age, the decision was taken to maintain the dietary Cu content at 8 mg/kg until there were clinical indications that this was inadequate. Circumstantial evidence of this became apparent after approximately 200 d of treatment, as the development of a diarrhoea was controlled by a single oral dose of a solution of $CuSO_4$ providing 10 mg Cu and was abolished by increasing the dietary Cu content to 15 mg/kg. It is of interest that Suttle (1975), in giving preliminary details of experiments to induce Cu deficiency in the calf, also reported diarrhoea without hypocupraemia in control animals receiving a diet providing $8 \cdot 8$ mg Cu/kg; in this instance the possible corrective effect of extra Cu was not studied.

Thus the probability exists that the Cu status of our control group may have become marginal when their diet provided 8 mg Cu/kg. Support for this view is added by the discoloration of hair observed in two animals of this group after 225 d on the experiment (Table 2). Also relevant may be that increasing the dietary Cu content to 15 mg/kg promoted a significant increase in plasma ferroxidase I activity and nonsignificant increases in liver cytochrome oxidase activity and rate of weight gain. Food intake also increased and hair pigmentation returned to normal.

High dietary contents of Mo and sulphur are known to antagonize the utilization of Cu by sheep and may similarly affect Cu utilization by cattle (Abdellatif, 1968). Neither element can be incriminated in our experiment as the Mo content of the basal diet was low and the S content only adequate to meet tentative estimates of requirement (for review, see Whanger, 1972). It is provisionally concluded that, even without the intervention of such antagonists, the Cu requirement of growing cattle may exceed 8 mg Cu/kg diet. Confirmation of this suggestion must await more detailed pathological studies.

The transient but significant increases in plasma ferroxidase I activity and the suggestion of similar increases in whole blood Cu content and plasma monoamine oxidase activity that occurred in response to cholecalciferol injection were unexpected. As stated previously, cholecalciferol was included in the basal diet to meet estimated requirements but during the first 112 d of the experiment animals had no access to direct sunlight. We are uncertain whether the single injection given may have promoted a transient increase in the efficiency of utilization of dietary Cu or merely caused a redistribution of Cu already retained in tissues (e.g. from the skeleton). The fact that these responses were not maintained when animals subsequently had access to sunlight and when normal plasma Ca and P concentrations became re-established without the need for cholecalciferol injection suggests that the phenomenon may have a pharmacological origin rather than being a previously undescribed consequence of cholecalciferol deficiency.

The limitations of diagnostic criteria for the detection of Cu deficiency

During the first 200 d of the experiment no statistically significant relationships were found between the weight gain of individual animals during the 4 weeks subsequent to any tissue sampling and the Cu content or enzyme activity of whole blood plasma, liver or muscle. Later, when marked differences in the weight gain of individuals of group LCu had appeared and the Cu content of the diet of group SCu had been increased to 15 mg/kg, some evidence of relationships between tissue composition and weight gain emerged.

For example, liver cytochrome oxidase activity (x) at the end of the experiment was related to weight gain during the last 35 d (y) by the expression:

$$y = 165x - 1 \cdot 7 \; (r \; 0 \cdot 83, \, P < 0 \cdot 05).$$

Correlation coefficients for relationships between final weight gain and other tissue characteristics were, for muscle cytochrome oxidase, $0 \cdot 63$ ($P = 0 \cdot 05$), and for plasma monoamine oxidase, ferroxidase I and whole blood Cu, $0 \cdot 79$, $0 \cdot 72$ and $0 \cdot 78$ respectively ($P < 0 \cdot 05$).

During the earlier part of the experiment, the time of onset of a reduced rate of weight gain, gait abnormalities and depigmentation of hair differed greatly between individuals of group LCu. This point is emphasized in results given in Table 2 and in greater detail in results given in Table 4, which show the extremes of performance of two animals of this group that survived the experiment. A comparison of the results obtained for blood and liver composition completely fails to reflect the severity of the

Table 4. *Period (d) after Friesian cattle nos. 837 and 838 were first offered a low-Cu diet* (0·83 mg Cu/kg), when they showed physical and the first biochemical signs of Cu deficiency*

Calf no.	Rate of body-wt gain less than mean rate for Cu-supplemented group	Body-wt gain < 0·5 kg/d	Diarrhoea incidence	Blood Cu < 0·5 mg/l	Plasma		Liver	
					Ferroxidase I (EC 1.16.3.1) < 10 units‡/l	Monoamine oxidase (EC 1.4.3.4) < 10 units‡/l	Cu < 20 mg/kg	Cytochrome oxidase (EC 1.9.3.1) < 0·1 units‡/mg protein
837	111–257†	111–173, 190–257†	167, 186, 205, 214, 230, 239, 246, 249, 254	80	58	132	98	222
838	222–258†	222–229, 243–258†	Nil	86	57	142	90	162

* For details of composition, see Table 1.
† Day of slaughter.
‡ One unit of activity is defined as the amount catalysing the transformation of 1 μmol substrate/min.

gross clinical response of animal no. 837 and the delayed and mild response of animal no. 838. From the results given in Tables 2 and 4 and Figs. 1–7 it is evident that, while the development of a low blood Cu, low liver Cu and low plasma ferroxidase I activity provides early warning of the possibility that overt signs of deficiency may develop, neither these indicators nor the later reductions in plasma monoamine oxidase and liver cytochrome oxidase activities appear to be closely linked to the unidentified lesions primarily responsible for the reduced rate of weight gain.

In this respect, the present experiment reflected the frequent finding in field survey work that existing biochemical techniques for the detection of Cu deficiency are of limited value for predicting the speed or extent to which an individual will develop overt signs of deficiency. However, this limitation may be less important than has been appreciated in view of the fact that pathological lesions attributable to Cu deficiency were found at the end of our experiment in every animal of group LCu irrespective of the severity, or even the absence of, external clinical signs of deficiency. It is probable that most of these lesions became established long before the experiment was terminated, but their time of onset and its relationship to tissue composition and enzyme activity at that time cannot be determined.

The origins of lesions of Cu deficiency in cattle

The validity of any biochemical procedure for the detection of Cu deficiency, or for the prediction of its later development, depends upon the existence of a close association of a compositional change in tissues accessible to sampling with the development of functional defects in tissues often remote from the sampling site. The results of this and similar experiments indicate how superficially such relationships have been studied.

(a) *Effects on the gastrointestinal tract.* Most animals of group LCu showed a marked atrophy of the villi of the small intestine (Fell *et al.* 1975), and this effect was of such a severity that the absorptive efficiency of the mucosa was likely to be markedly reduced. This lesion may be largely responsible for the ultimate decrease in weight gain and the poor utilization of food by most animals of this group. Such a suggestion is supported by the fact that animal no. 838 of this group, which maintained a good rate of growth through the greater part of the experiment and showed only mild overt signs of deficiency, did not have such mucosal damage in its small intestine.

Villus atrophy in LCu animals was accompanied by extensive mitochondrial damage and an almost complete loss of mitochondrial cytochrome oxidase activity. Regrettably, we have found only a low order of correlation between the cytochrome oxidase activity of intestinal mucosa and that of liver. Thus, even though determination of the cytochrome oxidase activity of liver biopsy samples is a feasible procedure in survey work (e.g. Poole, 1973) such an approach is unlikely to reflect the extent of pathological changes that may ultimately be attributed to a low activity of the same enzyme in the intestinal mucosa.

The effectiveness of even small doses of Cu in controlling diarrhoea in LCu animals suggests that the intestinal tract may be sensitive to small changes in the concentration of Cu in its lumen once the supply of this element from other tissues has

become restricted. As in our experiment, the incidence of diarrhoea in Cu-deficient herds is a highly variable individual characteristic (e.g. Jamieson & Allcroft, 1950), responding either to Cu administration or to a change in diet that may not promote an increase in blood Cu content (Allcroft & Parker, 1949). Whether susceptibility to the diarrhoea of Cu deficiency is heritable is not known. It is perhaps relevant that populations of Cu-deficient human infants susceptible or resistant to the development of clinical signs of deficiency and its associated diarrhoea have been described by Graham & Cordano (1969), while the results of other studies have suggested that inherited defects inducing susceptibility to Cu deficiency in children (Danks, Cartwright, Stevens & Townley, 1973) and in mice (Hunt, 1974) are attributable to defective metabolism of Cu in the intestinal mucosa. Such findings may well be relevant to the above situation in cattle.

(b) *Lesions in connective and skeletal tissues attributable to Cu deficiency.* A marked decrease in the activity of monoamine oxidase in plasma occurred in every animal of group LCu. How closely this change reflects a similar reduction in the activity of monoamine oxidase in other tissues is not known, but there were clear indications that the treatment received by this group induced defects probably attributable to defective cross-linking of collagen or elastin at a variety of sites. The death of one animal of group LCu through rupture of the posterior vena cava and the structural defects found on histological examination of the cardiovascular wall tissue of all cattle of this group (i.e. a decreased content of interlaminar collagen and, in the aorta, atrophy or degeneration of smooth muscle) may have had adverse consequences on the tensile strength of the walls of these blood vessels. Similar defects in the aortic collagen of Cu-deficient chicks have been directly attributed to defective cross-linking as a result of a low monoamine oxidase activity (Chou, Savage & O'Dell, 1968) but, as the same process is involved in the maturation of elastin, it is surprising that there was no clear evidence of defective elastogenesis in the aortas of our cattle. The origin of the accompanying lesions in the smooth muscle of the walls of aorta, portal vein and splenic capsule is obscure, but it should be noted that similar lesions have been detected in the thoracic aortas of Cu-deficient pigs (Waisman *et al.* 1969).

In contrast, there was very clear evidence of defective elastogenesis in the ligamentum nuchae of animal no. 838 of group LCu which maintained a good rate of growth although its Cu intake was low. Concurrent chemical studies on the same tissue by Whiting *et al.* (1974) have clearly indicated that this defect was attributable to partial inhibition of the cross-linking of elastin at sites which are fully consistent with the view that a low monoamine activity could have been responsible. The picture with respect to the effects of Cu depletion on collagen in the ligamentum nuchae varied between individual animals. In one there was histological evidence of a decrease in collagen content, while in another the collagen appeared to be randomly distributed rather than highly organized. At present it is difficult to assess the clinical significance of these changes, to understand why they are so variable in character and why, as in animal no. 838, they are probably exacerbated when growth continues even though tissue Cu reserves are depleted.

Defective cross-linking in the collagen matrix of bone as a consequence of a decrease in monoamine oxidase activity has been suggested as the cause of skeletal lesions arising from Cu deficiency in rats and chicks (Rucker, Parker & Rogler, 1969). The histological examination of skeletal tissues from our experiment was carried out on decalcified specimens and thus we were unable to determine whether defects in collagen structure were involved in the development of osteoporosis in group LCu. Nevertheless, it is abundantly clear that such a lesion could not account for the other effects of Cu deficiency upon the skeleton of all animals of this group. Thus, the observed decrease in osteoblastic activity and, particularly, the local increases in osteoclast activity suggest a disturbance in the balance between bone accretion and resorption which could result in the reduction of cortical bone thickness and density and alterations in gross morphology of bones found throughout this group. That such changes proceeded to the extent that skeletal strength was decreased was suggested by the finding of several healing rib fractures in one animal.

The extent to which lesions of connective tissue, probably originating from defective cross-linking of collagen and elastin, are demonstrable in Cu-deficient animals suggests that study of the relationships between the activity of monoamine oxidase in plasma and in connective tissues is warrantable. Whether or not activity in plasma is closely related to that in other tissues, our results suggest that the determination of plasma monoamine oxidase activity could be advantageous. The decrease in the activity of this enzyme during Cu depletion appears to occur later than that of whole blood Cu content and plasma ferroxidase I activity, and thus it may offer the possibility of distinguishing a relatively brief history of Cu depletion from chronic depletion more likely to result in adverse effects upon growth and health.

(c) *Effects of Cu depletion on Fe metabolism.* The decrease in blood Hb concentration and the changes in other blood characteristics resulting from Cu depletion during this experiment were, in our view, insufficiently great to suggest that the monitoring of such changes could be of appreciable diagnostic value. Such criticism does not apply to the monitoring of plasma ferroxidase I activity and plasma Fe concentration, both of which suffer a significant decrease as a consequence of Cu depletion. While it must be accepted that a decrease in plasma ferroxidase I activity may provide a fairly early warning of the development of Cu deficiency, there are clear indications from our experiment that, as Frieden (1971) has suggested, this Cu-enzyme is intimately involved in tissue Fe mobilization, with a decrease in plasma activity shortly after a decrease in plasma Fe (Fig. 8) and an excessive accumulation of Fe by the liver (Fig. 9).

Interpretation of field surveys of Cu status

Surveys based on blood and liver Cu analysis are, with increasing frequency, suggesting that the Cu status of many UK herds of cattle may be low. Nevertheless, the interpretation of such situations is often in doubt because of the occasional absence of overt signs of deficiency. Characteristic of the current response is that of Davies & Baker (1974) who, having found mean herd serum Cu concentrations of < 0·5 mg/l in 568 of 1078 beef cattle herds in Wales, comment 'The significance of

the hypocupraemia remains to be established but it will be surprising if administration of Cu did not give a growth response . . . in herds profoundly and more or less permanently hypocupraemic'.

Our own results and those of Whiting *et al.* (1974), Leigh (1975) and Fell *et al.* (1975), all based on results from the same experiment, indicate the wide range of biochemical and pathological lesions that can develop in Cu-depleted animals in addition to effects on weight gain.

Because of the limited scale of our experiment, which precluded sequential slaughter of animals for pathological studies, our description of the relationships between the chemical composition and Cu-enzyme activity of blood or other accessible tissue and the time of development of lesions in the skeleton, connective tissue, heart and small intestines is superficial. A more complete appraisal of the early consequences of a low Cu status of cattle maintained under farm conditions is warrantable, and should include field evaluation of the use of plasma ferroxidase I and monoamine oxidase assays which offer some advantages not shared by the determination of blood Cu.

Meanwhile our results and those of a similar experiment of Suttle (1975, and personal communication) strongly suggest that wider recognition of the probability that growing cattle have a higher Cu requirement than sheep (Agricultural Research Council, 1964) could do much to reduce the present high incidence of hypocuprosis in cattle.

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EXPLANATION OF PLATES

Plate 1. Copper-deficient Friesian calf no. 834 of the low-Cu group (for details, see p. 311), illustrating swellings surrounding metacarpophalangeal and carpometacarpal joints, over-extension of fore-limbs and splaying of hooves.

Plate 2. Superimposed photographs of copper-deficient Friesian cattle, nos. 837 (front) and 838 (rear) of the group given low-Cu diets (for details, see Table 1), illustrating extreme differences in clinical appearance after 193 d of treatment. Results relating to these animals are given in Tables 2 and 4. Skeletal and connective tissue lesions in animal no. 838 are illustrated in Plates 4 and 5.

Plate 3. Post-mortem radiographs of humeri of Friesian cattle: (A) animal no. 837 (low-copper diet (0·83 mg Cu/kg), LCu (for details, see p. 311)), showing severe overt signs of Cu deficiency, (B) animal no. 838 (group LCu) showing only mild overt signs of deficiency and (C) animal no. 835 (Cu-supplemented diet (8 mg Cu/kg to day 225, then 15 mg Cu/kg)). Results for cortical bone indices are given in Table 2.

Plate 4. Skeletal lesions in copper-deficient Friesian cattle; animal no. 838 (low-Cu diet (0·83 mg Cu/kg), LCu (for details, see p. 311)) which showed no overt signs of deficiency. Note fibrosis and small number and reduced size of osteoblasts lining cancellous cavities but no evidence of osteoid formation in vertebral cortex (A) (stain: haematoxylin–eosin) (× 60). Similar lesions were present in the radial diaphysis. Section of radial metaphysis (B) illustrates local increase in osteoclast activity with fibrous replacement (× 150). Such lesions were present in all animals of the LCu group.

Plate 5. Transverse sections of ligamentum nuchae of Friesian cattle, (A) animal no. 838 (low-copper diet (0·83 mg Cu/kg), LCu (for details, see p. 311)) and (B) animal no. 835 (Cu-supplemented diet

(8 mg Cu/kg to day 225, then 15 mg Cu/kg)). Section A illustrates both the reduced quantity and abnormal morphology of elastic fibres. Many fibres with a normal elastin-staining reaction are surrounded by a cuff of tissue having a poor affinity for stain. The surrounding matrix of collagen appears to be less highly organized than in (B) (stain: Weigert–Sheridan-van Geisen) (× 600).

The ligamentum nuchae of two other animals in the LCu group contained elastic fibres of small size or exhibiting a weak staining reaction but with no cuffing. Normal morphology was found in a fourth animal.