A new bioassay for assessment of copper availability and its application in a study of the effect of molybdenum on the distribution of available Cu in ruminant digesta

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1. Investigations were carried out on the feasibility of using an oral repletion technique in the rat to assess the bioavailability of copper in experimental sources providing no more than 250 μg Cu from any one source.

2. Preliminary studies on the response in plasma Cu of partially Cu-depleted rats given repletion doses of 20–50 μg Cu as $CuSO_4$/d on four consecutive days indicated that this index of Cu status was insufficiently sensitive to Cu dose.

3. In contrast, the activity of cytochrome c oxidase (*EC* 1.9.3.1) in the duodenal mucosa of partially Cu-depleted rats showed a measurable and uniform response to 10 μg Cu as $CuSO_4$/d given on three consecutive days. Furthermore, when the rats were given 0, 2·5, 5·0 or 10·0 μg Cu/d, the increase in cytochrome c oxidase activity above that of the unsupplemented control group was linearly related to Cu dose. The mean response in cytochrome c oxidase activity in groups of eight rats was therefore used to assess the availability of Cu from experimental sources relative to that of Cu as $CuSO_4$, only 240 μg Cu being required from each experimental material.

4. The assay was used to study the effect of the Cu-antagonist molybdenum on the distribution of available Cu in digesta from sheep given dried grass either untreated (1·6 mg Mo/kg dry matter (DM)) or treated with ammonium molybdate (11·6 mg Mo/kg DM).

5. The relative availability of Cu in untreated dried grass (75 %) was substantially higher than in rumen (12 %), duodenal (43 %) or ileal (28 %) digesta. In all cases, addition of Mo to the diet resulted in a substantial reduction in Cu availability.

6. The effects of Mo on availability of Cu are discussed with special reference to the possible involvement of thiomolybdates in the Cu–Mo antagonism.

The efficiency with which dietary copper is utilized by ruminants is influenced by a number of factors among which are ration type (Suttle, 1980), the concentration of molybdenum in the diet and the potentiation of its inhibitory effect on Cu utilization by both inorganic sulphate (Dick, 1953) and organic forms of sulphur (Suttle, 1975*a*; Mills *et al.* 1977). It has been suggested that sulphide production in the rumen may render Cu unavailable through formation of insoluble sulphide (Mills, 1960) and that many aspects of the Cu–Mo interaction could be explained by formation of thiomolybdates in the rumen (Suttle, 1974*a*; Dick *et al.* 1975; Mills *et al.* 1978). However, few attempts have been made to characterize the forms of Cu present in ruminant digesta and progress towards quantifying the effects of dietary factors influencing Cu availability is seriously hampered by a lack of understanding of the basic processes involved.

The importance of changes in the distribution and solubility of Cu within the alimentary tract of the ruminant (Bremner, 1970; Ivan *et al.* 1983) cannot be assessed until sensitive techniques are developed for measuring the availability of the small quantities of Cu present in digesta fractions. Since the biological availability of Cu to non-ruminants has frequently been assessed from the responses of initially Cu-deficient animals to repletion with different sources of the element, an attempt has been made to apply this technique, in the rat, to assess the relative availability of Cu in various fractions isolated from sheep digesta. Although oral-repletion techniques used by previous workers have measured the efficiency of utilization of Cu from various sources for functions such as caeruloplasmin (Kirchgessner & Grassmann, 1970) or haemoglobin synthesis (Mills, 1957), the quantities of Cu required

to obtain a measurable response are many times greater than those readily obtainable from centrifugal fractions of sheep digesta.

The present paper describes firstly a study of the changes in the plasma Cu concentration of Cu-depleted rats given $CuSO_4$ orally for 4 d. The low and variable responses observed have been the subject of a preliminary report (Price *et al.* 1981) and led to a search for an index more sensitive and consistent in response to repletion with small quantities of Cu. Since Dallman & Loskutoff (1967) found histological evidence of an extremely rapid recovery of mucosal cytochrome *c* oxidase (*EC* 1.9.3.1) activity in the intestine of Cu-deficient rats given $CuSO_4$ in the diet, the responses of this enzyme at different intestinal sites after administration of Cu in the diet were first assessed. Responses in the activity of cytochrome *c* oxidase in duodenal mucosa were subsequently used as the basis of a bioassay to assess the availability of Cu from different sources relative to that of $CuSO_4$.

EXPERIMENTAL

Animals and diets

Male hooded Lister rats (Rowett Institute strain) were weaned at 21 d and offered a pelleted diet (Stock Breeding Diet; Oxoid Laboratory Products, Southwark Bridge Road, London SE1) for 6 d. Thereafter the rats were individually housed in perspex cages and offered a semi-synthetic diet containing either albumen or casein as the protein source. The composition of the albumen diet (AL) was as described by Davies & Reid (1979) except that $CuSO_4$ was omitted from the diet and Zn added to it to provide 40 mg Zn/kg. In the casein-based diet (C), albumen (200 g/kg) was replaced by casein at a level of 250 g/kg with a corresponding reduction of 50 g/kg in the sucrose content. The basal diets AL and C contained 0·5–0·8 mg Cu/kg and 0·4–0·6 mg Cu/kg respectively and were used to deplete the animals of Cu. Where an intake of Cu adequate to prevent depletion was required, the basal diets were supplemented with $CuSO_4$. $5H_2O$ (Analar grade; BDH, Poole, Dorset) to provide 6 mg Cu/kg diet (+Cu diets). Diet AL was used in the plasma Cu studies and diet C in the cytochrome *c* oxidase investigations.

Plasma Cu studies

In a series of experiments, rats were offered the +Cu albumen-based diet *ad lib.* before depletion. The basal diet was then offered *ad lib.* to all rats to deplete plasma Cu and continued to be offered during the subsequent repletion phase. The depletion phase varied from 4 to 8 d, depending upon the experiment, and was followed immediately by a repletion phase in which Cu was given by gavage, as $CuSO_4$ in 1 ml distilled water, on four consecutive days. In each experiment plasma Cu concentration was determined in blood samples collected into heparinized haematocrit tubes from the tail vein at 2 or 3 d intervals during the depletion phase and immediately before and 24 h after each daily Cu depletion dose.

Expt 1. Twenty-four rats with a mean live weight of 105 g were offered the depletion diet for 4 d, then allocated at random to three groups and given 0, 25 or 50 μg Cu/d for 4 d to investigate the rates of depletion and repletion of plasma Cu.

Expt 2. To examine the effect of live weight on the rates of plasma Cu depletion and repletion, four groups of eight rats were offered the depletion diet when their mean live weights were 85, 115, 165 and 220 g respectively. The rats were maintained on this diet until the mean plasma Cu concentration for each group showed a decrease of 0·7–0·8 μg/ml and were then given 50 μg Cu/d for 4 d.

Expt 3. Thirty-two rats with a mean live weight of 120 g were used to investigate the effect of Cu depletion and repletion on liver Cu concentration. Before depletion eight rats were selected at random and killed. The remaining rats were then offered the depletion diet and,

after 5 d, ten animals with plasma Cu concentrations ranging from 0·1 to 0·7 μg/ml were killed. The fourteen rats remaining were allocated at random to groups of seven and given either 20 or 35 μg Cu/d for 4 d. The livers from all rats were retained for Cu determination.

Initial studies on cytochrome c oxidase activity in the rat intestinal mucosa

Expt 4. Twenty-five rats weighing 65 g were allocated at random to five groups and one group was killed immediately as the untreated control. The remaining animals were offered either the basal diet C (three groups) or diet C supplemented with Cu (one group). Rats given the basal diet were killed after 9, 16 and 24 d and those receiving the supplemented (+Cu) diet were killed after 24 d. The activity of cytochrome c oxidase was assayed in the duodenal mucosa immediately after killing.

Expt 5. Two groups of ten rats weighing 65 g were offered the basal depletion diet for 28 d and then given either 0 or 10 μg Cu on each of three consecutive days. In a preliminary repletion experiment there was a rapid and substantial response in mucosal cytochrome c oxidase to Cu given in the diet but not to Cu given by gavage. Because of this, doses of Cu were added to the diet rather than given by gavage in this and subsequent experiments. The Cu source ($CuSO_4$) was dispersed in milled sucrose to provide 10 μg Cu/g premix. To ensure that all of the daily Cu dose was consumed, the quantity of feed offered during repletion was reduced from 20 to 10 g/d. Thus Cu-supplemented animals were offered 9 g diet C plus 1 g Cu–sucrose premix daily; the untreated animals were offered 9 g diet C plus 1 g sucrose. At 24 h after the final Cu dose was given, the rats were killed and cytochrome c oxidase activity assayed in the mucosa from a 10 mm section from the proximal duodenum, the mid-jejunum, the terminal ileum and the proximal colon. The livers were retained for Cu determination.

Expt 6. Four groups of eight rats weighing 65 g were offered the basal depletion diet for 28 d and then given 0, 2·5, 5·0 or 10·0 μg Cu on each of three consecutive days. At 24 h after the final Cu dose was given, cytochrome c oxidase and NADH dehydrogenase (EC 1.6.99.3) activities were assayed in the duodenal mucosa.

Cytochrome c oxidase and NADH dehydrogenase assay

The rats were killed and a 30 mm length of the intestine was removed and washed with ice-cold physiological saline (9 g sodium chloride/l). A 10 mm length of the washed intestinal section was then slit open and subjected to mild sonication (50 watts) in 10 ml 0·1 M-sodium phosphate buffer, pH 7·4, for 30 s at 0 °(9 mm probe, model 180; Ultrasonics Ltd). The intact intestinal tissue remaining after this treatment was discarded and the homogenate subjected to sonication for 15 s at maximum energy input (180 watts). The homogenates were assayed for cytochrome c oxidase and NADH dehydrogenase activities immediately after preparation

The assays were based on the spectrophotometric determination of the rate of oxidation of reduced cytochrome c by the oxidase (Mills & Dalgarno, 1970) or the rate of reduction of oxidized cytochrome c by the dehydrogenase as estimated from the rate of change in absorbance at 550 nm. The following components were added to a cuvette which had a 10 mm light path: 1·0 ml reduced cytochrome c preparation, 0·05–0·2 ml of the prepared mucosal homogenate, sodium phosphate buffer (0·1 M, pH 7·4) to give a final volume of 3 ml.

The rate of decline in absorbance at 550 nm due to the oxidase activity was determined at ambient temperature. The reaction was then stopped by addition of 50 μl 10 mM-sodium cyanide solution and the following were added: 50 μl cytochrome c (oxidized form, 1·66 mM) in phosphate buffer (pH 7·4), 50 μl NADH (63 mM) in phosphate buffer (pH 7·4). The rate

of increase in absorbance at 550 nm due to the dehydrogenase activity was then determined at ambient temperature.

In the previously mentioned system a change in absorbance of 1·0 unit corresponds to the oxidation or reduction of 0·166 μmol cytochrome c. In the preliminary investigations (Expts 1 and 2) cytochrome c oxidase activity was expressed in units of μmol cytochrome c oxidized/min per mg protein, the latter being determined by the method of Lowry *et al.* (1951). Since the activity of the oxidase is Cu-dependent, while that of the dehydrogenase is not, cytochrome c oxidase activity may also be expressed as the ratio, change in absorbance due to the oxidase:change in absorbance due to the dehydrogenase. The latter basis for expression of activity has the advantage that the activity of both enzymes can be assayed sequentially in the same spectrophotometer cuvette. Thus, after substantiating that Cu status did not influence NADH dehydrogenase activity, the activity ratio method was subsequently used for expression of cytochrome c oxidase activity.

Assay of Cu availability

Because the basal cytochrome c oxidase activity after Cu depletion varied substantially between experiments, as did the sensitivity of response to a standard dose of Cu, the availability of Cu was assessed from the increase in cytochrome c oxidase activity rather than the activity itself. The following procedure was therefore used to assess the availability of Cu from an experimental source relative to that of Cu as $CuSO_4$. Groups of eight rats were partially depleted of Cu by offering the basal casein diet *ad lib.* for 28 d. Thereafter sufficient of each Cu source to provide 10 μg Cu was mixed with 9 g basal diet/d and this mixture was given on each of three consecutive days. Rats were killed 24 h after the final Cu dose. In addition to groups receiving the experimental source of Cu (exp) each assay contained two groups of eight animals given either 0 μg Cu (control) or 10 μg Cu/d as $CuSO_4$ ($CuSO_4$). The relative availabilities (A, %) of Cu from the experimental sources were calculated from the mean ratios (R) of oxidase:reductase activities for each of the groups as follows:

$$\frac{(R_{exp} - R_{control}) \times 100}{R_{CuSO_4} - R_{control}}.$$

Standard errors for the estimates of A (7 df) were derived from the group means and their variances by the method described by Kendall & Stuart (1963) for calculation of standard errors of functions of random variables assuming no covariance between groups.

Availabilities of Cu from feeds and digesta

Dried grass. Grass was cut just prior to ear emergence and dried. The dried material was chopped to give a maximum straw length of approximately 50 mm and either left untreated ($-$Mo) or sprayed with an aqueous solution of ammonium molybdate to increase the Mo content by 10 mg/kg dry matter (DM). The Cu content of the dried grass used throughout these studies varied between 6·0 and 7·0 mg/kg, the Mo content of the grass before treatment was 1·6 mg/kg and the sulphur content 2·9 g/kg. The relative availability of Cu in the dried grass was assayed in samples milled to pass a 1 mm mesh.

Digesta fractions from sheep. Five mature male castrate sheep were cannulated in the rumen and proximal duodenum (three animals) or in the rumen and terminal ileum (two animals). Dried grass, prepared as described previously, was offered *ad lib.* either untreated during the first period, or treated with Mo in the second. In order to minimize deterioration of samples during the period of storage between initial collections of digesta fractions and sufficient being accumulated for assay of Cu availability, all five sheep received the same diet in any one period. Following change-over from the $-$Mo to $+$Mo diet, an equilibration period of 3 weeks was allowed before collecton of digesta.
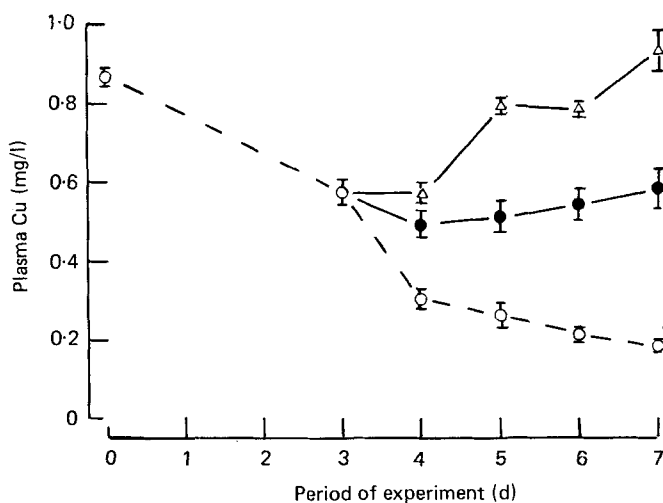
Fig. 1. Change in plasma copper concentration (mg/l) in groups of eight rats during depletion using a low-Cu diet (○) and repletion with 25 (●) or 50 (△) μg Cu/d given by gavage on four consecutive days. Points are mean values with their standard errors represented by vertical bars.

Table 1. *Effect of live weight on rate of plasma copper depletion in rats offered a low-Cu diet and the subsequent increase in plasma Cu concentraion after repletion with 50 μg Cu/d given on four consecutive days*

(Mean values with their standard errors for eight rats per group)

| Initial live weight (g) | Depletion phase | | | | | Repletion phase | |
| | Initial plasma Cu concentration (mg/l) | | Rate of decrease in plasma Cu concentration (mg/l per d) | | Depletion time (d) | Increase in plasma Cu concentration (mg/l) | |
	Mean	SE	Mean	SE		Mean	SE
85	0·85	0·024	0·109[a]	0·004	7	0·65[a]	0·155
115	1·02	0·026	0·106[a]	0·011	7	0·53[a]	0·068
165	0·91	0·014	0·067[b]	0·004	10	0·50[a]	0·066
220	1·14	0·034	0·070[b]	0·007	11	0·53[a]	0·057

[a,b] Mean values within the same column with different superscript letters were significantly different ($P < 0.05$).

Digesta (350 ml) were collected twice weekly from the rumen, duodenum or ileum approximately 2 h after feeding. Coarse, undigested material was removed from the rumen fluid by straining through muslin before fractionation of the resulting digesta. Rumen, duodenal and ileal digesta were fractionated by centrifugation at 20° in an atmosphere of oxygen-free carbon dioxide as in the following scheme: 1000 g, 1 min, large particles including protozoa (fraction 1); 2200 g, 10 min, large micro-organisms (fraction 2); 30000 g, 1 h, bacteria (fraction 3); 30000 g supernatant (fraction 4). The distribution of micro-organisms is relevant only to fractions from rumen digesta.

The fractions were freeze-dried, milled and stored under nitrogen before assay for Cu availability. Digesta were collected from two to five sheep on several occasions over a period of weeks and identical fractions were combined in order to obtain sufficient material for assay.
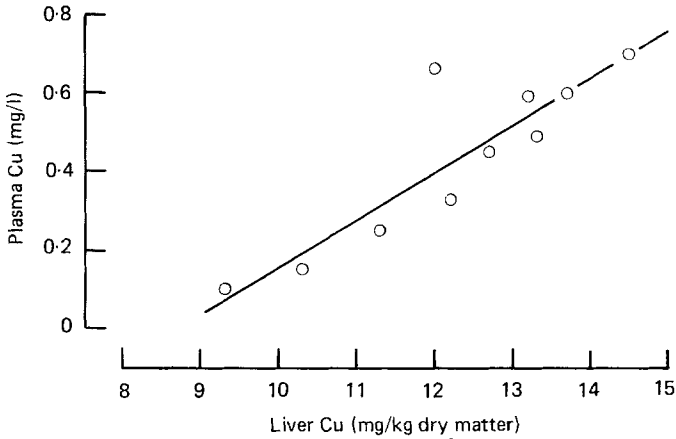
Fig. 2. Relation between plasma copper concentration and liver Cu concentration in rats offered a low-Cu diet for 5 d.
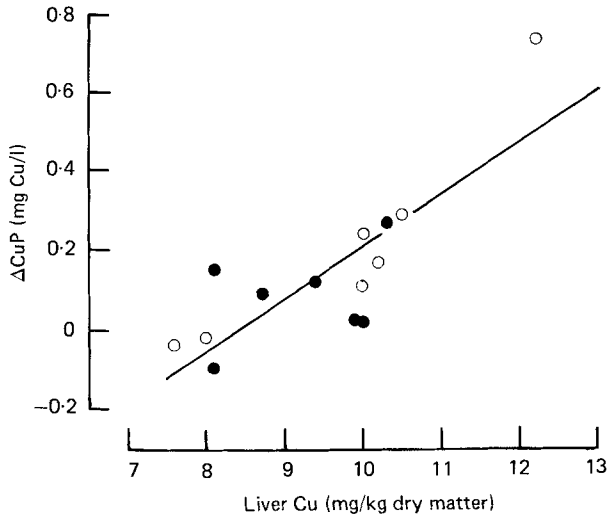
Fig. 3. Relation between the increase in plasma copper concentration (ΔCuP) and the final liver Cu concentration in groups of seven rats offered a low-Cu diet for 5 d and then given 20 (O) or 35 (●) μg Cu/d on each of four consecutive days.

Effect of tetrathiomolybdate on Cu availability. Ammonium tetrathiomolybdate was prepared by the method of Tridot & Bernard (1962). Using the assay procedure described, a group of rats was given 10 μg Cu as $CuSO_4$ and 7·5 μg Mo as thiomolybdate/d in the diet to assess the effects of the latter on the availability of Cu.

Analytical methods

Plasma (10 μl) was diluted with 10 mM–nitric acid (200 μl) and the Cu concentration determined by graphite furnace atomic absorption spectroscopy (AAS) (HGA 76 furnace, model 460 spectrophotometer; Perkin Elmer & Co. GmbH).

Duplicate 0·5 g samples of DM from rat and sheep diets, rat liver and digesta fractions from sheep were digested in a mixture of 18 M-sulphuric acid, 12 M-perchloric acid and 16 M-HNO_3 (0·5:1·0:5·0, by vol). After dilution, Cu concentration was determined by flame AAS.

For Mo determination, 1 ml 16 M-HNO_3 was added to 1 g samples of diet or digesta DM, taken to dryness and ashed at 550° for 12 h. The ash was treated with 1 ml 16 M-HNO_3, taken to dryness and ashed again at 550° for 2 h. The residue was taken up in 5 ml 10 mM-HNO_3 and Mo determined by graphite furnace AAS (Pye Unicam PU 9095 furnace, PU 9000 AAS).

RESULTS

Plasma Cu studies

Expt 1. The pattern of change in plasma Cu concentration during Cu depletion of rats with a mean initial live weight of 105 g and their subsequent repletion with 25 or 50 μg Cu/d given by gavage is shown in Fig. 1. When they were offered the low-Cu diet the mean plasma Cu concentration fell from 0·87(SE 0·01) mg/l on day 0 to 0·57 (SE 0·03) mg/l by day 3 and to 0·18 (SE 0·02) mg/l by day 7 of the experiment. Cu given from day 3 at the higher dose rate of 50 μg Cu/d increased plasma Cu concentration after 4 d to the pre-depletion level, but 25 μg Cu/d was sufficient only to prevent further depletion.

Expt 2. Relations between the initial live weight of rats and their depletion and subsequent repletion of plasma Cu are shown in Table 1. Although the rate of decline in plasma Cu concentration was greater ($P < 0.05$) in rats with initial live weights of 85 or 115 g at the start of depletion than in those at 165 or 220 g live weight, the mean increases in plasma Cu over the 4 d repletion period did not differ significantly. However, the greater variability observed in the response to repletion of animals initially weighing 85 g suggested that further work should be carried out with rats at a live weight of 115 g or greater at the start of depletion.

Expt 3. Rats maintained on the +Cu diet showed little variation in their liver Cu concentrations, the mean being 15·9 (SE 0·25) mg/kg DM. However, after being offered the −Cu diet for 5 d, their plasma Cu concentrations differed widely and a group was selected with values ranging from 0·1 to 0·7 mg/l. Within this group, liver Cu concentrations (CuL, mg/kg DM) varied from 9·3 to 14·5 mg/kg DM and were directly correlated with plasma Cu concentration (CuP, mg/l) (Fig. 2). The relation could be described by the equation:

$$CuP = 0.12 \text{ (SE 0.016) } CuL - 1.04 \text{ (residual SD 0.11, } P < 0.001, \text{ 8 df)}$$

When groups of rats which had been maintained on the low-Cu diet for 5 d were given 20 or 35 μg Cu/d respectively, the increases in plasma Cu concentration were not related to dose; mean plasma Cu concentrations were 0·21 (SE 0·04) and 0·16 (SE 0·01) mg/l before repletion and 0·42 (SE 0·12) and 0·26 (SE 0·05) mg/l after repletion for 4 d in groups given 20 and 35 μg Cu/d respectively. Furthermore, in the latter two groups there was a significant relation between the increase in plasma Cu (ΔCuP, mg/l) after repletion for 4 d and the final liver Cu concentration (CuL, mg/kg DM) (Fig. 3). This relation could be described by the equation:

$$\Delta CuP = 0.13 \text{ (SE 0.033) } CuL - 1.12 \text{ (residual SD 0.13, } P < 0.005, \text{ 12 df)}$$

Cytochrome c oxidase activity in rat intestinal mucosa

Expt 4. The mean cytochrome *c* oxidase activities in duodenal mucosa of rats maintained on the −Cu diet C (0·5 mg Cu/kg) declined from an initial value of 0·356 (SE 0·061) at day 0 to 0·234 (SE 0·040), 0·176 (SE 0·053) and 0·103 (SE 0·011) units/mg protein on days 9, 16 and 24 respectively. In rats offered the Cu-supplemented diet (6 mg Cu/kg), the oxidase activity (0·359 (SE 0·042) units/mg protein) at day 24 did not differ from that of the rats before depletion.

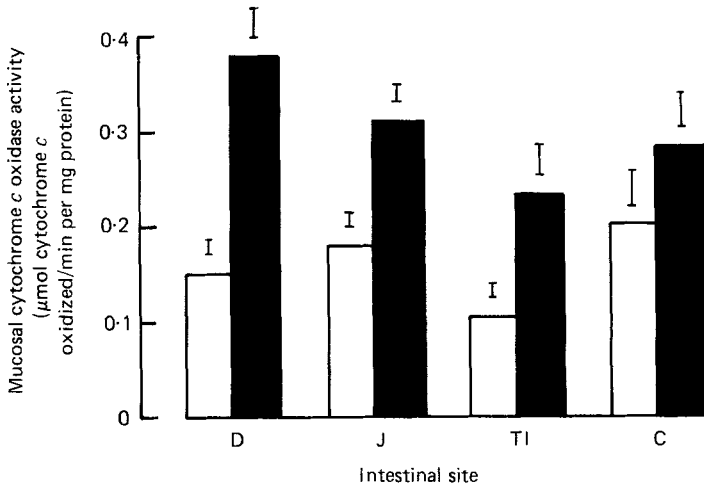*Expt 5.* In rats which had been offered the low-Cu diet for 28 d, 10 μg Cu given on each

Fig. 4. Cytochrome c oxidase (*EC* 1.9.3.1) activity in the musosa from the duodenum (D), jejunum (J), terminal ileum (TI) and colon (C) of rats offered a low-copper diet for 28 d and then given 0 (□) or 10 (■) μg Cu as $CuSO_4$/d for 3 d. Standard errors of treatment means for groups of ten rats are represented by vertical bars.
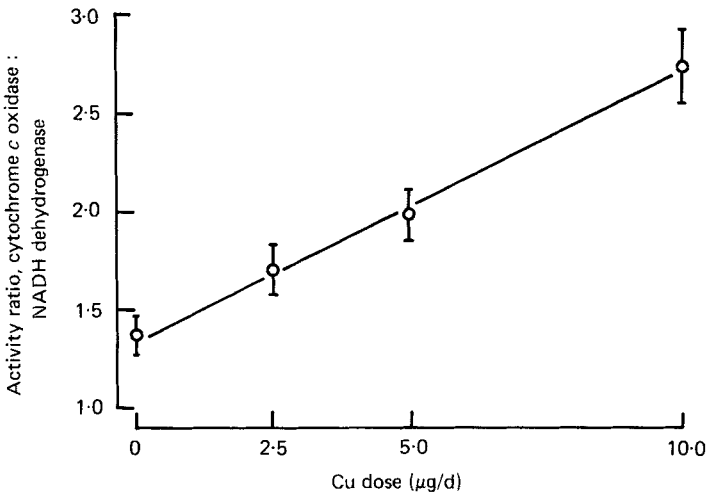

Fig. 5. Activity ratio, cytochrome *c* oxidase (*EC* 1.9.3.1):NADH dehydrogenase (*EC* 1.6.99.3) in duodenal mucosa of rats initially depleted of copper and then given 0, 2·5, 5·0 or 10·0 μg Cu as $CuSO_4$/d for three consecutive days. Points are mean values for groups of eight rats with their standard errors represented by vertical bars.

of three successive days resulted in a significant increase ($P < 0.005$) in cytochrome *c* oxidase activity above that of the untreated controls at all four intestinal sites studied (Fig. 4). Ranked according to the magnitude of the increase, the sites were duodenum > jejunum = ileum > colon. Henceforth the change in oxidase activity in the mucosa from the duodenum was used in all experiments. This level of Cu supplementation did not increase liver Cu concentration above that of the untreated controls. The mean liver Cu concentrations in animals given 0 or 10 μg Cu/d were 4·08 (SE 0·67) and 4·42 (SE 0·80) respectively.

Expt 6. The activity of mucosal cytochrome *c* oxidase increased linearly with respect to

Table 2. *Composition of pooled digesta from sheep given untreated (−Mo) or molybdenum treated (+Mo)† dried grass and the relative availability of copper in fractions isolated from this digesta*

| Cu source | Dry matter (% of whole digesta DM) | Cu concentration (mg/kg DM) | | Mo concentration (mg/kg DM) | | Relative Cu availability‡ (%) | | | | Contribution of fractions to Cu in whole digesta | | | |
| | | | | | | −Mo | | +Mo | | Total Cu (mg/kg DM whole digesta) | | Available Cu (units §/kg DM whole digesta) | |
		−Mo	+Mo	−Mo	+Mo	Mean	SE	Mean	SE	−Mo	+Mo	−Mo	+Mo
Rumen fractions (five sheep):													
1000 *g* pellet	40	14·4	13·3	4·5	17·0	7	1·0	−2	6	5·76	5·32	0·40	0·00
2200 *g* pellet	16	7·3	12·9	2·0	10·9	14	8	−8	7	1·17	2·06	0·16	0·00
30000 *g* pellet	12	10·8	10·1	0·4	11·5	11	13	−7	8	1·30	1·21	0·14	0·00
30000 *g* supernatant	32	3·4	2·3	0·6	6·9	40	22	3	9	1·09	0·74	0·44	0·02
										9·32	9·33	1·14	0·02
Duodenal fractions (three sheep):													
1000 *g* pellet	54	10·9	12·8	3·1	16·7	10	12	0	12	5·89	6·91	0·59	0·00
2200 *g* pellet	17	19·1	18·5	5·2	24·4	74	22	−11*	11	3·25	3·15	2·40	0·00
30000 *g* pellet	11	10·3	10·9	1·8	11·4	102	17	17*	13	1·13	1·20	1·16	0·20
30000*g* supernatant	18	2·3	3·9	0·5	1·7	107	17	50*	14	0·41	0·70	0·44	0·35
										10·68	11·96	4·59	0·55
Ileal fractions (two sheep):													
1000 *g* pellet	71	15·6	17·9	1·6	9·9	24	11	−5	12	11·08	12·76	2·66	0·00
2200 *g* pellet	5	31·0	37·9	0·9	8·1	20	14	6	13	1·55	1·90	0·31	0·11
30000 *g* pellet	7	52·2	50·8	1·9	22·4	34	10	10	10	3·65	3·56	1·24	0·36
30000 *g* supernatant	17	16·3	11·5	1·3	19·4	43	12	14	9	2·77	1·96	1·19	0·27
										19·05	20·13	5·40	0·74
Dried grass		6·0	7·0	1·6	11·6	75	25	43	12				

DM, dry matter.

* Relative availability values for −Mo and +Mo fractions differ significantly ($P < 0.05$).

† +Mo treatment, dried grass sprayed with a solution of $(NH_4)_6Mo_7O_{24}.4H_2O$ to increase the Mo concentration by $10\mu g/g$ DM.

‡ Relative availabilities were assessed with groups of eight rats and the mean values are presented with their standard errors calculated from the between rat differences (7 df, see p. 326).

§ 1 unit of available Cu is equivalent to that available from 1 mg Cu as $CuSO_4$.

Cu dose whether expressed on a protein basis or (Fig. 5) by the activity ratio, oxidase: dehydrogenase. Since variability tended to be lower using the ratio method, cytochrome c oxidase activity was henceforth expressed on this basis. The relation between the increase in activity (ΔE) above that of the untreated control group and daily Cu dose (x, μg Cu/d) could be described by the following equation:

$$\Delta E = 0 \cdot 124x \ (\text{SE } 0 \cdot 034) + 0 \cdot 02 \ (\text{residual SD } 0 \cdot 522, \ P < 0 \cdot 005, \ 22 \text{ df})$$

Cu availability from low-Mo dried grass and digesta fractions

Relative availability values for Cu in dried grass and digesta fractions are shown in Table 2. When sheep were offered the untreated grass, 88% of the Cu present in the rumen digesta was associated with the solid phase and was of significantly ($P < 0 \cdot 05$) lower availability than that in the diet. Although Cu in the soluble phase of rumen fluid was apparently lower in availability than that in dried grass, the difference did not achieve significance. Using values for individual fractions, the relative availability of Cu in strained whole rumen fluid was estimated to be 12% compared with 75% for Cu in dried grass. Within the rumen, fractions 1 and 4 made the major contribution to the pool of available Cu.

In duodenal digesta the availability of Cu, except for that in fraction 1, was significantly ($P < 0 \cdot 05$) greater than in the corresponding fractions from the rumen. The calculated relative availability of Cu in whole duodenal fluid was 43%, fraction 2 making the greatest contribution to the total available Cu pool followed by Cu associated with fraction 3.

Cu in fractions 3 and 4 from ileal digesta was of lower ($P < 0 \cdot 05$) availability than that in the corresponding duodenal fractions. The calculated relative availability of Cu in whole ileal fluid was 28%.

Effect of Mo and thiomolybdate

Addition of Mo to the dried grass increased the Mo content of all fractions of digesta, but did not alter the concentration of Cu in individual fractions or the distribution of Cu between fractions. The availability of Cu in Mo-treated grass was 43% compared with 75% for that in the untreated material but the difference did not reach statistical significance. Mo reduced ($P < 0 \cdot 05$) the availability of Cu in fractions 2, 3 and 4 from duodenal digesta but the reduction in availability of Cu in fractions from the rumen and ileum did not reach statistical significance. The increase in dietary Mo resulted in negative estimates for the mean availability of Cu in a number of digesta fractions, particularly in the rumen. These were observed when the extent of depletion of cytochrome c oxidase activity was greater in the group given 10 μg Cu/d from the experimental source than in the group given no supplementary Cu.

The values for 'available Cu' present in the various fractions (Table 2) further demonstrate the marked effect of Mo on Cu utilizaton. The quantity of utilizable Cu was reduced by over fifty-fold in the rumen, eight-fold in duodenal digesta and seven-fold in the ileal digesta of the sheep when the Mo concentration of the dried-grass diet was increased from 1·6 to 11·6 mg/kg DM.

Cytochrome c oxidase activity in Cu-depleted rats given 7·5 μg Mo as tetrathiomolybdate/d, but with no supplementary Cu, did not differ significantly from that of the untreated control offered the low-Cu diet alone. However, in the same assay, tetrathiomolybdate (7·5 μg Mo/d) reduced the availability of Cu (10 μg/d) given as $CuSO_4$ by 54% ($P < 0 \cdot 01$).

DISCUSSION

Oral-repletion techniques have been used previously to assess the relative availability of differing forms of Cu (Mills, 1957; Kirchgessner & Grassmann, 1970). In the present study the objectives were first to develop a similar but more sensitive technique in the rat and second to apply this technique to the assessment of the relative availability of Cu in fractions isolated from ruminant digesta. Anticipating that subsequent studies might necessitate investigation of the bioavailability of Cu from isolated components of such fractions, it was considered imperative to develop an assay that could be conducted with not more than 250 μg total Cu from any one source.

Initial investigations which centred on the possibility of using changes in the plasma Cu concentration of hypocupraemic rats given a Cu source, indicated that this index was capable of differentiating between 0, 25 or 50 μg Cu/animal per d given as $CuSO_4$ on four consecutive days. However, the initial decline in plasma Cu during repletion at the lower dose rate suggested that extension of the depletion period to reduce plasma Cu to less than 0·5 μg/ml might be advantageous in maximizing differences in the final plasma concentrations. Furthermore, one might expect that for a given dose of Cu the rate of plasma Cu repletion would be greater the lower the live weight and hence plasma volume and tissue mass of the animal. Subsequent results did not however support this hypothesis, for while the rate of depletion was greater in rats weighing 85–115 g in comparison with those at 165–220 g, the increase in plasma Cu after 4 d repletion with 50 μg Cu/d was similar for all animals.

In the present experiments the large differences observed in the rate of plasma Cu depletion and subsequent repletion rendered it impossible to differentiate between repletion doses of 20 and 35 μg Cu/d (see Fig. 3). Using a repletion technique to study the availability of Cu in sheep, Suttle (1974b) also reported a wide variation between individual animals in their plasma Cu response during repletion with Cu given orally. From the observation that these differences were no longer evident when Cu was given intravenously, it was concluded that variability in plasma Cu response to oral repletion in the sheep was due largely to differences in Cu absorption from the gut. However, despite the initial uniformity of both plasma and liver Cu concentrations in the present studies, the relation between plasma and liver Cu concentration after depletion, when little dietary Cu was available for absorption, suggests that variability in plasma Cu changes in the rat may have arisen from differences between individual animals in their ability to mobilize liver Cu stores. The question of whether mobilization of Cu from the liver was the main cause of the variability in plasma Cu during repletion as well as during depletion, or whether differences in absorption were also involved, was not investigated further, since there appeared to be little possibility of increasing the sensitivity of this index to obtain a measurable response to repletion with less than 25 μg Cu/animal given on 4 d (or 800 μg Cu/assay assuming eight replicates per test).

In comparison with these inherent limitations in the sensitivity of our plasma repletion method in rats for assessing Cu availability, subsequent investigations demonstrated that as little as 2·5 μg Cu/d given on each of 3 d resulted in a measurable and relatively uniform response in cytochrome *c* oxidase activity in the duodenum. The greater sensitivity of intestinal cytochrome *c* oxidase to increased dietary Cu supply suggests that either the synthesis of cytochrome *c* oxidase has priority over caeruloplasmin synthesis for absorbed Cu, or that Cu absorbed from the digesta may be used directly in the intestinal mucosa for synthesis of the former. However, this may well be an over-simplification of the problem since caeruloplasmin Cu has been reported to be utilized in preference to albumin-bound Cu for cytochrome *c* oxidase synthesis (Marceau & Aspin, 1973; Hsieh & Frieden, 1975).

The lower variability between individual animals in their cytochrome *c* oxidase activity compared with that for plasma Cu concentrations after the end of the depletion phase is thought to be related to the extent to which liver Cu reserves were depleted in each assay procedure. Due to the longer depletion period employed in the enzyme studies, liver Cu concentrations were reduced to a mean value of 4·08 (SE 0·67) mg/kg DM, less than half the concentration observed in the plasma investigations and considerably less variable. From the work of Evans & Abraham (1973) it is evident that liver Cu in the rat is extremely resistant to depletion beyond this concentration. It would therefore appear that, in the present enzyme studies, liver reserves had been reduced to a point beyond which further mobilization of Cu was minimal, thus removing that part of the variability in response associated with the liver. However, attempts to reduce variability in the plasma Cu assay technique by extending the depletion period from 7 to 28 d were abandoned after finding that plasma Cu concentration no longer showed a measurable response to four daily doses of 50 μg Cu after this more rigorous pre-treatment.

Although a number of factors are known to influence the utilization of dietary Cu by the ruminant, the mechanisms by which these operate are largely unknown. Suttle (1975*a*) reported a marked reduction in Cu utilization with the development of a functional rumen in the lamb and, in the present study, the relative availability of Cu in rumen fluid was found to be substantially lower than that in the dried grass given to sheep. Thus it is evident that modification of dietary components by microbial activity in the rumen is a major factor governing Cu availability. If progress is to be made towards an understanding of the processes affecting Cu utilization in the ruminant, a knowledge of the chemical and physical forms in which the element is presented to the mucosa for absorption is essential. Furthermore, since the true absorption of Cu is low, values of less than 7% having been reported in sheep maintained on fresh and conserved herbage (Suttle, 1980), information regarding the availability of the different forms of Cu present in the digesta is required. The cytochrome *c* oxidase assay allows this information to be obtained but cannot be extended to estimate directly the availability of dietary Cu to ruminants as in the repletion technique of Suttle (1974*b*) because the present assay would not take account of changes in availability occurring during passage through the rumen.

The results of the present study showed that, within the rumen, over half the total Cu was present in the large particulate material, the remainder being distributed equally between the three fractions consisting of micro-organisms and soluble material. The concentrations of Cu in the rumen-soluble DM are in agreement with those of Bremner (1970) for sheep maintained on dried grass but substantially lower than the concentrations reported when the diet consisted of hay (Mitchell & Tosic, 1949) or maize silage (Ivan *et al*. 1983).

While Cu in the soluble DM showed a markedly higher availability than that in the solid material, the contribution of this fraction to the pool of available Cu in the rumen digesta was equalled by that in the large particulate fraction, indicating that solubility may not be equated directly with availability. Furthermore, the increase in Cu availability observed on passing from the rumen to the duodenum was accompanied by a decrease in the proportion of soluble Cu in the digesta. Cu associated with the solids, probably of bacterial origin, made the greatest contribution to the pool of available Cu in the duodenum. It is tempting to speculate that this increase in availability resulted from the fall in pH of the digesta on passage through the abomasum. However, the fact that both rumen and duodenal digesta must pass through the stomach of the rat during the availability assay would point to other causes. Although there is evidence for a net secretion of Cu into digesta in the rumen–omasal–abomasal region in sheep (Grace, 1975; Stevenson & Unsworth, 1978; Ivan *et al*. 1979), it is not clear whether the quantitites of endogenous Cu involved would influence availability of Cu entering the duodenum to the extent observed in the present study.

The availability of Cu in digesta from the terminal ileum may have been influenced by passage through the acid medium of the rat's stomach but was considerably lower than that in the duodenum, as might be expected since studies with simple-stomached animals indicate a net absorption of Cu from the small intestine (Owen, 1964; Van Campen & Mitchell, 1965; O'Dell & Campbell, 1970).

The addition of Mo to dried grass resulted in a wide distribution of the element throughout the particulate and soluble fractions of rumen, duodenal and ileal digesta, and decreased the mean availability of Cu in fractions 2, 3 and 4 from the duodenum. In duodenal digesta the overall reductions in Cu availability due to increased Mo intake were in broad agreement with those reported by Suttle (1983) for sheep grazing herbage of similar Mo content to that of the Mo-treated dried grass offered in the present experiment. Negative means for Cu availability were only observed in fractions from Mo-supplemented sheep. Although not statistically significant, these may have resulted from the interaction of Mo compounds with either Cu present in the basal depletion diet (0·5 mg Cu/kg) or with Cu of endogenous origin, since considerable quantities of the element are known to be excreted into the small intestine of the rat (Owen, 1964; O'Dell & Campbell, 1970).

It has previously been suggested that formation of thiomolybdates in the rumen may reduce the availability of Cu through the production of insoluble Cu–thiomolybdate complexes (Dick *et al.* 1975; Mason, 1978). Although the thiomolybdate anions are water-soluble, they must be largely associated with the particulate matter in ruminant digesta if they are to account for the loss of availability of Cu seen in the present experiments. It is therefore interesting to note that MoS_4^{2-}, when added in solution to rumen contents, rapidly disappears from the aqueous phase (El-Gallad *et al.* 1983). The results of the present study would suggest that while the availability of Cu given to the rat simultaneously with MoS_4^{2-} in the molar ratio, 2 Cu: 1 Mo (i.e. similar to that of some rumen and duodenal fractions) was markedly reduced, conversion of the Cu to an unavailable form was far from complete.

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Printed in Great Britain