Studies on the roles of apotransferrin and caeruloplasmin (*EC* 1.16.3.1) on iron absorption in copper-deficient rats using an isolated vascularly- and luminally-perfused intestinal preparation

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1. Studies have been made on the effects of dietary copper on the iron and Cu distribution in rats and on the metabolic activity and absorptive capacity of intestines perfused both vascularly and luminally.

2. Rats maintained for 4–5 weeks on a Cu-deficient diet (0·4 μg Cu/kg) had significantly lower plasma, liver and intestinal Cu concentrations and significantly reduced plasma caeruloplasmin and liver cytochrome *c* oxidase (*EC* 1.9.3.1) activity compared with controls receiving a Cu-supplemented diet (5 μg Cu/kg). Disturbances in Fe metabolism in Cu-deficient rats were evident as shown by a mild anaemia, significantly elevated hepatic Fe concentrations and hypoferraemia.

3. Intestinal glucose uptake from both the luminal perfusion medium (LPM) and vascular perfusion medium (VPM) was unaffected by Cu deficiency despite a significant (25–30 %) reduction in oxygen consumption. This was associated with a 40 % decline in mucosal cytochrome *c* oxidase activity.

4. In studies of Fe absorption, Fe uptake from the LPM was unaffected by Cu deficiency while transfer of Fe to VPM was significantly reduced (50 %) compared with control preparations. Addition of apotransferrin (1 g/l) to the VPM was without effect in preparations from control rats but significantly increased the transfer of Fe to the VPM in preparations from Cu-deficient rats without affecting Fe uptake from the LPM.

5. The addition of either human or porcine caeruloplasmin (together with apotransferrin) to the VPM, such that the resultant ferroxidase (*EC* 1.16.3.1) activity of the VPM supernatant fraction was four to five times that of normal rat plasma, was without effect on either Fe uptake, tissue retention or Fe transfer to the VPM by preparations from either Cu-deficient or control rats.

6. These findings offer no evidence in support of the proposed role for caeruloplasmin with its associated ferroxidase activity in Fe absorption in the rat.

In 1928 it was observed that rats maintained on a milk diet developed a microcytic, hypochromic anaemia that was corrected only in part by the addition of iron to the diet. It was completely corrected when both copper and Fe supplements were given (Hart *et al.* 1928). These findings led to the proposal that Cu deficiency impairs dietary Fe absorption. Subsequent studies by Cartwright's group (Chase *et al.* 1952; Gubler *et al.* 1952) demonstrated reduced Fe absorption in both rats and pigs maintained on Cu-deficient diets. However, the role of impaired Fe absorption in the production of the anaemia of Cu deficiency is unclear since Cu-deficient animals can accumulate large amounts of hepatic Fe (Elvehjam & Sherman, 1932; Marston & Allen, 1967; Evans & Abraham, 1973).

Caeruloplasmin is a Cu-containing plasma protein that displays oxidase activity towards a variety of substrates including *p*-phenylenediamine (PPD), catecholamines and phenothiazines (Uriel, 1957; Løvstad, 1972). Curzon & O'Reilly (1960) reported that caeruloplasmin greatly accelerated the rate of oxidation of ferrous ions to ferric ions by oxygen. Subsequent studies (McDermott *et al.* 1968) demonstrated that of all substrates tested, Fe^{2+} had the highest V_{max} and lowest K_m.

Fe is transported in the blood plasma bound to transferrin (Holmberg & Laurell, 1947). This protein is capable of carrying two Fe atoms per molecule with the Fe being in its

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oxidized state (Warner & Weber, 1953). Following the observations that in the isolated perfused dog liver, Fe was bound to perfused apotransferrin only in the presence of added caeruloplasmin, Osaki & Johnson (1969) proposed the ferroxidase hypothesis of caeruloplasmin action. It postulates that caeruloplasmin is required for the oxidation of Fe at the cell surface before this Fe can be bound to transferrin. Accordingly, it has been proposed that caeruloplasmin should be renamed as a ferroxidase (ferro-O_2-oxido-reductase). Thus in Cu deficiency the reduced circulating levels of caeruloplasmin might result in impaired mobilization of Fe both from the absorbing cells of the intestinal mucosa as well as from liver and reticulo-endothelial Fe stores.

Direct proof for the requirement of caeruloplasmin with its associated ferroxidase activity in mucosal Fe release has not been demonstrated. Accordingly we have studied Fe absorption using isolated vascularly and luminally perfused rat intestinal preparations from Cu-deficient and Cu-adequate rats and investigated the possible roles of apo-transferrin and caeruloplasmin in intestinal Fe absorption.

MATERIALS AND METHODS
Animals and diets

In all experiments Hooded Lister rats of the Rowett Research Institute were used. Rats of between 125 and 200 g were transferred from a commercial diet (Oxiod; H. C. Styles, Bewdley, Worcs) to a semi-synthetic diet similar to that described by Williams & Mills (1970), as modified by Davies & Reid (1979). The basal diet contained (g/kg): spray-dried egg albumin 200, arachis oil 10, sucrose 660, vitamins, minerals and trace elements. Rats allocated to the Cu-deficient diet received the basal diet without supplementary Cu. On analysis by flame atomic absorption spectrophotometry this diet contained 0·4 mg Cu/kg. In addition the diet was supplemented with $ZnSO_4.7H_2O$ to provide 200 mg Zn/kg. Control rats were pair-fed to the Cu-deficient rats and were offered the basal diet supplemented with $CuSO_4.5H_2O$ to provide 5 mg Cu/kg. All rats were singly housed in cages constructed of polypropylene and stainless steel and offered deionized water *ad lib.* Body-weights were recorded weekly. After a 4–5 week period rats were either taken for perfusion studies, or blood and tissues were sampled for analysis while the rats were anaesthetized with diethyl ether.

Perfusion apparatus and surgical procedures

The apparatus and surgical and experimental procedures used for perfusion of the isolated intestine were as previously described by Coppen & Davies (1988). The rat was anaesthetized by injection of sodium pentobarbitone (60 mg/kg), its abdomen opened by a mid-line incision, the intestines exteriorized to the right and covered with gauze soaked in saline (9 g sodium chloride/l). The middle and right colic, left renal, testicular and lower limb veins and arteries were all ligated. The lumen of the small intesine was gently flushed with warmed M199 cell culture medium (Gibco Biocult Laboratories, Paisley, Scotland) to remove digesta and food residues, and then cannulated by the lumen perfusion inlet cannula, just distal to the duodeno-jejunal flexure. The outlet drainage cannula was inserted into the lumen of the ileum approximately 50 mm proximal to the ileo-caecal junction. Perfusion through the lumen of the intestine with the lumen perfusion medium (LPM) was started at a rate of 0·55 ml/min. The duodenum was not perfused, since the superior mesenteric artery only supplies blood to half the duodenum (Greene, 1955). Rather than perfuse part of the small intestine which may not be viable, it was decided to perfuse the jejunum and ileum only, even though the duodenum is normally regarded as one of the main sites of Fe absorption (Dowdle *et al.* 1960). Heparin (250 IU) was injected

into the femoral vein, and all other arteries and veins draining the lower gastrointestinal tract and the kidneys were ligated, as was the coeliac axis. The aorta was cannulated with the vascular-perfusion inlet cannula, the tip of which was passed up the aorta until it was opposite the superior mesenteric branch. The catheter was tied in place and the needle withdrawn. Perfusion commenced immediately so that the intestine suffered minimal loss of vascular flow. The aorta was rapidly ligated caudal to the superior mesenteric branch and the vena cava was also ligated. The vascular outlet cannula was inserted into the portal vein. The animal was killed by an intra-cardiac injection of sodium pentobarbitone and the intestines were covered with gauze soaked in liquid paraffin and the preparation left to stabilize for a 15 min recovery period before the experimental period was commenced. The surgical procedure took 25–35 min. After the start of the experimental treatments lumen and vascular effluent samples were collected at 10 min intervals over the 60 min perfusion. At the end of perfusion, the ligated section of intestine was removed, flushed with saline (25–30 ml) and blotted dry. The wet weight of the intestine was measured before freeze-drying.

Composition of the perfusion media

LPM was a commercially available cell culture medium containing glucose (5·5 mM, pH 4·5). Such a medium was used since it was thought that this represented conditions in vivo better than a buffered saline solution. This solution was not oxygenated with 95% O_2:5% CO_2 for the same reasons. It was supplemented with ferrous chloride (final concentration of Fe 50 μg/ml), ascorbate (100 μg/ml) and ^{59}Fe (approximately 0·03 μCi/ml; Amersham International plc, Amersham, Bucks). The vascular perfusion medium (VPM) contained washed and dialysed bovine erythrocytes resuspended in Krebs Ringer bicarbonate (pH 7·4) to give a packed cell volume (PCV) of 0·20. The medium also contained glucose (5·5 mM) and bovine serum albumin (30 g/l, Pentex, fraction V; Miles Laboratories, Stoke Poges, Slough, Bucks). Where indicated, human apotransferrin (Sigma, Poole, Dorset) was added to the VPM to give a final concentration of 1 mg/ml. Human caeruloplasmin (Sigma) or pig caeruloplasmin prepared in this laboratory (see p. 364) was added to the VPM to give a final ferroxidase activity in the range 0·4–0·7 U/μg Cu. The O_2 tension and the pH of the vascular perfusate was estimated on a Blood Microanalyser System (Radiometer, Copenhagen, Denmark) before and after perfusion through the mesenteric circulation. O_2 consumption was estimated by the method of Windmueller *et al.* (1970) taking into account the O_2 loss from the haemoglobin and medium in which the erythrocytes were suspended. It was assumed that at 37°, 100 ml of the medium would dissolve 2·3 ml O_2 at a gas pressure of 760 mm Hg (Bell *et al.* 1959). The percentage saturation of haemoglobin was estimated from nomograms (Kelman & Nunn, 1966).

Sample analysis

Plasma Cu and Fe concentrations were measured by atomic absorption spectrometry (AAS) using a Varian 1000 atomic absorption spectrophotometer (Varian Pty, Melbourne, Australia) on the supernatant fraction after protein had been precipatated by mixing equal volumes of plasma with trichloroacetic acid (100 g/l). Liver samples were wet ashed using concentrated nitric–sulphuric–perchloric acids (4:0·5:1, by vol.). After the ashing procedure, the remaining 0·5 ml H_2SO_4 containing the digested sample was diluted with distilled water to give a final concentration of 50 ml H_2SO_4/l. Cu and Fe were then analysed by AAS, using suitably prepared standards and blanks. Blood haemoglobin was determined by the standard cyan-met technique (Dacie & Lewis, 1975). Cytochrome *c* oxidase (*EC* 1.9.3.1) activity in the liver and intestinal mucosal homogenates was determined by the method of Mills & Dalgarno (1970). Protein content of the homogenates was determined by the method of Lowry *et al.* (1951). The oxidase activity of caeruloplasmin in plasma or

the supernatant fraction of the VPM was determined using either PPD as substrate (Houchin, 1958, as modified by Rice, 1960) or Fe^{2+} as substrate (Johnson *et al.* 1967). Glucose concentrations of the fluids draining the vasculature and lumen of the preparations were estimated by the method of Trinder (1969) after deproteinization with barium hydroxide–zinc sulphate (0·15 M:0·15 M). Radioiron (^{59}Fe) in samples of perfusion media and the intestinal tissue after perfusion was measured in a Tracerlab Gamma Counter (Tracerlab Ltd, Twickenham, Middlesex).

Isolation of caeruloplasmin

Caeruloplasmin was isolated from pig blood plasma by the method of Bannister (1979) using a column (25 mm by 300 mm) packed with DEAE-Sephadex (Sigma) which had been previously equilibrated with 0·1 M-sodium acetate buffer, pH 5·7. Caeruloplasmin was seen as a pale blue band. It was eluted from the column with 0·5 M-acetate buffer and the purity estimated by comparing the absorbance at 610 and 280 nm by spectrophotometry (Bannister, 1979), a ratio $A_{610}:A_{280}$ of 0·044 being equivalent to 98% purity. The Cu content of the caeruloplasmin was estimated by AAS. The mean percentage purity of the porcine caeruloplasmin prepared as described was 40% and that of the human caeruloplasmin obtained commercially was 95%.

Statistical methods

Results are expressed as means with their standard errors. The significance of difference between means was tested by an unpaired Student's *t* test after analysis of variance to determine significance of overall treatment effects.

RESULTS
Nutritional Cu status of the rats

The results of the studies to determine the Cu status of the rats maintained for 4–5 weeks on the experimental diets are shown in Table 1. While there was no difference in the final weights of the rats in the two groups, defects in Fe metabolism were evident in those receiving the Cu-deficient diet. Thus Cu-deficient rats had significantly reduced PCV and haemoglobin concentrations, reduced plasma Fe concentrations but elevated hepatic Fe contents. These were associated with significant reductions in plasma Cu concentration and PPD oxidase activity (caeruloplasmin), liver and intestinal Cu contents and significant increases in liver and mucosal cytochrome *c* oxidase activities. Cu deficiency was without significant effect on intestinal Fe concentration.

Viability of the intestinal preparations

A more detailed description of the tests of viability of the perfused preparation can be found elsewhere (Coppen & Davies, 1988). In brief, during the course of successful perfusions, vascular perfusion flow-rate and perfusion pressure remained constant over the 1 h period. Hypersecretion was not evident as assessed from failure to observe changes in concentration of a non-absorbable marker (phenol red) in the LPM during passage through the intestine. The pH of the vascular drainage remained the same as the original pH of the VPM. Histological examination of the tissue after perfusion showed that most preparations retained normal histology over a 1 h experimental perfusion period. Absorption values from the few preparations exhibiting gross histological damage were discarded.

Table 1. *The effects of copper deficiency on final body-weight, blood haemoglobin (Hb) concentration (g/l), packed cell volume (PCV), copper and iron concentrations in plasma, intestinal mucosa and liver, caeruloplasmin activity (Cp) in plasma, and cytochrome* c *oxidase activity (EC 1.9.3.1) in liver and intestinal mucosa*

(Results are means with their standard errors for five to eight rats except for body-weight where values are for thirty-six rats)

	Cu-adequate		Cu-deficient	
	Mean	SE	Mean	SE
Final body-wt (g)	295	4	296 NS	3
Whole blood				
Hb (g/l)	148	2	123**	7
PCV	0·45	0·08	0·39*	0·13
Plasma				
Cu (mg/l)	1·02	0·04	0·11***	0·02
Fe (mg/l)	2·03	0·13	1·17***	0·12
Cp (U/l)	32·1	2·4	1·57***	0·09
Intestinal mucosa				
Cu (mg/kg)	7·39	0·80	4·00*	0·40
Fe (mg/kg)	131·1	20·6	109·7 NS	12·9
Cytochrome *c* oxidase activity†	0·19	0·02	0·11***	0·01
Liver				
Cu (mg/kg)	14·8	1·0	5·1***	0·7
Fe (mg/kg)	336·0	20·6	500·5***	30·7
Cytochrome *c* oxidase activity †	0·4	0·1	0·2*	0·0

NS, not significant ($P > 0.05$).
Mean values for Cu-deficient group were significantly different from Cu-adequate controls (Student's *t* test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
† μmol cytochrome *c* oxidized/min per mg protein.

The effects of Cu deficiency on glucose uptake and O_2 consumption by isolated perfused rat intestine

The effects of addition of Fe and ascorbate to the LPM, and apotransferrin and caeruloplasmin to the VPM, on glucose uptake from the VPM and LPM and O_2 consumption by isolated preparations from Cu-deficient and control rats are shown in Table 2. Analysis of variance demonstrated that none of the additions to the perfusion media affected glucose uptake from either the LPM or VPM or O_2 consumption. However, in all treatment groups, preparations from Cu-deficient rats exhibited significantly lower O_2 consumption ($P < 0.05$) than those of controls. In contrast, no effects due to Cu deficiency were observed on glucose uptake.

The effects of Cu deficiency on Fe absorption by isolated perfused rat intestine
The effects of apotransferrin. The effects of addition of apotransferrin (1 g/l) to the VPM of isolated perfused intestines from Cu-deficient and control rats on total Fe uptake from the LPM, and the amounts of Fe retained by the intestine and transferred to the VPM during the course of a 1 h perfusion, are shown in Table 3. In the absence of added apotransferrin, preparations from Cu-deficient rats transferred approximately 50% ($P < 0.05$) of the amount of Fe to the VPM compared with controls. This defect in Fe absorption was apparently reversed by the addition of apotransferrin to the VPM. Thus,

Table 2. *The effect of additions of iron (50 µg/ml) and ascorbate (100 µg/ml) to the LPM, and apotransferrin (1 g/l) and caeruloplasmin (0·4–0·7 U/µg Cu) to the VPM, on glucose uptake and oxygen consumption over the perfusion period in preparations from Cu-deficient (−Cu) or Cu-adequate (+Cu) rats*

(Results are means with their standard errors for three to twelve preparations. For full details, see text)

| | | | Glucose uptake (µmol/g per h) | | | | O_2 uptake (µmol/g per min) | |
| | | | VPM | | LPM | | | |
Preparation	LPM	VPM	Mean	SE	Mean	SE	Mean	SE
+Cu	—	—	69·2	1·66	38·7	1·21	1·13	0·04
−Cu	—	—	67·1	2·79	40·0	2·44	0·81***	0·05
+Cu	Fe+AA	—	69·1	2·34	36·3	8·62	0·99	0·03
−Cu	Fe+AA	—	67·4	5·14	40·3	9·51	0·79*	0·05
+Cu	Fe+AA	Apotf	72·4	4·85	39·2	4·95	1·18	0·02
−Cu	Fe+AA	Apotf	67·6	2·36	39·3	2·19	0·84***	0·04
+Cu	Fe+AA	Apotf+Cp	68·2	3·75	39·6	2·50	1·21	0·07
−Cu	Fe+AA	Apotf+Cp	65·3	3·73	40·4	2·76	0·87**	0·08

AA, ascorbate; LPM, luminal perfusion medium; VPM, vascular perfusion medium; Apotf, apotransferrin; Cp, caeruloplasmin.

Mean values for −Cu preparation were significantly different from +Cu preparation (Student's *t* test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. *The effects of copper deficiency on the uptake of iron from the LPM, its retention by the intestine and transfer to the VPM with or without apotransferrin (Apotf) (1 g/l) in the VPM*

(The LPM contained Fe (50 g/l), ascorbate (100 g/l) and [59]Fe. Results are means with their standard errors for three to six rats. For full details, see text)

| | | Fe (µg/g dry wt) | | | | | |
| | | Total uptake | | Retained | | Transferred to VPM | |
Preparation	VPM	Mean	SE	Mean	SE	Mean	SE
+Cu	—	94·9	18·0	74·9	15·9	20·0	2·3
−Cu	—	76·9	9·9	66·1	10·6	10·8*	1·2
+Cu	Apotf	78·7	11·1	54·0	8·6	24·7	4·9
−Cu	Apotf	63·3	10·1	36·5	7·9	26·8††	3·6

+Cu, preparations from Cu-adequate rats; −Cu, preparations from Cu-deficient rats; LPM, luminal perfusion medium; VPM, vascular perfusion medium.

Mean values for −Cu group were significantly different from +Cu group (Student's *t* test): * $P < 0.05$.

Mean values for −Cu group with Apotf were significantly different from −Cu group without Apotf: †† $P < 0.01$.

while apotransferrin was without significant effect on either the total Fe uptake, tissue Fe retention or Fe transfer to the VPM by control preparations, it significantly increased transfer to the VPM by intestines from Cu-deficient rats. These effects are reflected in the

Table 4. *The effect of Apotf (1 g/l) in the VPM on the distribution of iron taken up from the LPM and the specific activity ratio, ^{59}Fe initially present in the LPM: that recovered in the VPM in preparations from copper-deficient rats or Cu-adequate rats over a 1 h perfusion period*

(Results are means with their standard errors for three to six preparations)

| | | Distribution of Fe taken up (%) | | | | Specific activity ratio, ^{59}Fe LPM:VPM | |
| | | Tissue | | VPM | | | |
Preparation	VPM	Mean	SE	Mean	SE	Mean	SE
+Cu	—	78·0	2·5	22·0	2·6	0·99	0·08
−Cu	—	84·8	3·0	15·2	3·2	1·06	0·12
+Cu	Apotf	68·8	5·7	31·2	5·7	1·13	0·07
−Cu	Apotf	55·6**	5·0	44·4**	5·0	0·98	0·10

LPM, luminal perfusion medium; VPM, vascular perfusion medium; +Cu, preparations from Cu-adequate rats; −Cu, preparations from Cu-deficient rats; Apotf, apotransferrin.
The effect of Apotf within the Cu-deficient preparation was significant: ** $P < 0.01$.

Table 5. *A comparison of p-phenylenediamine (PPD) oxidase and ferroxidase activity of normal rat plasma and of the supernatant fraction of the VPM supplemented with either human or porcine caeruloplasmin, used in perfusion studies with preparations from copper-deficient (− Cu) or Cu-adequate (+ Cu) rats*

(Results are means with their standard errors for four to five determinations.
For full details, see text)

| | | PPD oxidase (U/l) | | Ferroxidase (U/l) | | Ferroxidase (U/μg Cu) | | Ferroxidase: PPD oxidase |
Preparation	VPM	Mean	SE	Mean	SE	Mean	SE	
+Cu	HCp	8·82	0·96	220	24	0·53	0·005	
−Cu	HCp	8·54	0·73	206	30	0·51	0·047	24·9
+Cu	PCp	5·85	0·36	323	21	0·59	0·03	
−Cu	PCp	5·25	0·13	298	8	0·55	0·02	56·0
Plasma		32·1	2·4	45·9	3·34	0·036	0·003	1·43

VPM, vascular perfusion medium; HCp, human caeruloplasmin, PCp, porcine caeruloplasmin; U, μmol PPD or Fe^{2+} oxidized/min per l.
The VPM contained apotransferrin (1 g/l).

findings in Table 4, in which the percentage distribution of the absorbed Fe between the intestinal tissue and that appearing in the VPM after 1 h perfusion is shown. Apotransferrin was without effect in control preparations yet significantly ($P < 0.01$) decreased the percentage of Fe retained by the tissue and increased that transferred to the VPM of Cu-deficient preparations. However, the specific activity ratio, ^{59}Fe initially present in the LPM: that appearing in the VPM, was approximately unity and not significantly different between any group (Table 4).

These results suggest that neither the effects of Cu deficiency alone, nor the effects of
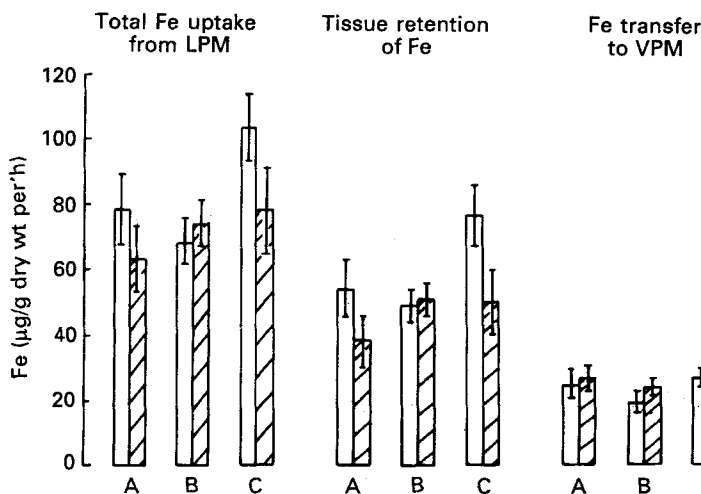
Fig. 1. Uptake and transfer of Fe from the luminal perfusion medium (LPM) to the vascular perfusion medium (VPM) during the perfusion period in preparations from control (□) and copper-deficient (▨) rats. Additions to the VPM were: A, apotransferrin; B, apotransferrin and human caeruloplasmin; C, apotransferrin and porcine caeruloplasmin. For full details, see text. Results are means with their standard errors for four to six rats.

Table 6. *The effect of human (HCp) or porcine (PCp) caeruloplasmin on the distribution of iron taken up from the LPM and the specific activity ratio,* ^{59}Fe *initially in LPM: that recovered in the VPM from preparations from copper-deficient (−Cu) or Cu-adequate (+Cu) rats*

(Results are means with their standard errors for four to six preparations. For full details, see text)

| | | Distribution of Fe taken up (%) | | | | Specific activity ratio, ^{59}Fe LPM:VPM | |
| | | Tissue | | VPM | | | |
Preparation	VPM	Mean	SE	Mean	SE	Mean	SE
+Cu	Apotf	68·8	5·7	31·2	5·7	1·129	0·07
−Cu	Apotf	55·6	5·0	44·4	5·0	0·98	0·10
+Cu	Apotf+HCp	73·0	3·7	27·0	3·8	1·09	0·08
−Cu	Apotf+HCp	67·5	1·9	32·5	1·9	0·99	0·09
+Cu	Apotf+PCp	73·5	2·5	26·5	2·5	1·06	0·12
−Cu	Apotf+PCp	64·8	5·9	35·2	6·0	1·16	0·08

Analysis of variance showed no significant difference ($P > 0.05$) between −Cu and +Cu groups or effects due to the addition of HCp or PCp.

apotransferrin in Cu-deficient preparations resulted from actions on different endogenous intestinal tissue Fe pools since in all treatment groups essentially all the Fe appearing in the VPM must have been derived from the LPM.

The effects of caeruloplasmin. In studies on the possible effects of caeruloplasmin on Fe absorption by Cu-deficient and control intestinal preparations, two sources of protein were used. Batches of human caeruloplasmin were obtained commercially while crude porcine

caeruloplasmin was prepared in this laboratory. The PPD oxidase (U/l plasma) and ferroxidase activities (U/l plasma and U/μg Cu) and the activity ratio, ferroxidase: PPD oxidase of normal rat plasma and the same activities of the supernatant fraction of the VPM used in studies on Fe absorption are shown in Table 5. The activity ratio ferroxidase: PPD oxidase of porcine caeruloplasmin was greatest and that of rats the lowest of the three species. The ferroxidase activity of the porcine and human caeruloplasmin added to the VPM in the perfusion studies was at least four times that of normal rat plasma.

The addition to the VPM of porcine or human caeruloplasmin (together with apotransferrin) had no significant effect on total Fe uptake from the LPM, Fe retention by intestinal tissue, or Fe transfer to the VPM (Fig. 1). Thus the percentage distribution of Fe between that retained by the tissue and that transferred to the VPM was similarly unaffected as was the specific activity ratio, ^{59}Fe initially in the LPM: that appearing in the VPM (Table 6).

DISCUSSION

The reduced liver and intestinal mucosal Cu content and cytochrome *c* oxidase activity, and low plasma Cu concentration and caeruloplasmin PPD oxidase activity, demonstrate that the rats receiving the Cu-deficient diet were of low Cu status. Furthermore, the reduced plasma Fe concentration, marked hepatic Fe accumulation and the development of an anaemia, confirm the findings of many other groups of workers (e.g. Roeser *et al.* 1970; Evans & Abraham, 1973; Williams *et al.* 1983) that Cu-deficient rats exhibit disturbances in Fe metabolism.

Discussion of the validity of the technique used, including histological examination of the intestinal tissue post-perfusion, can be found elsewhere (Coppen & Davies, 1988). Details of the experimental design and comparison with other workers is given. In short a perfusion speed of 0.55 ml/min was chosen as this was similar to speeds used by other workers (Windmueller & Spaeth, 1980; Hoadley & Cousins, 1985 who used speeds of 0.1–0.2 ml/min and Hanson & Parsons, 1976 and Steel, 1981, who both used speeds of 1 ml/min). Furthermore, it was felt that slow speeds represented the situation in vivo far better than faster speeds such as 10 ml/min used by Nicholls *et al.* (1983), even if at this faster speed the effect of 'unstirred layers' is overcome. Similarly, the LPM was not gassed with 95 % O_2: 5 % CO_2 as this was considered unphysiologic. This lack of O_2 from the LPM did not apparently adversely affect the viability of the preparation as assessed by histological examination of the tissue post-perfusion (Coppen & Davies, 1988). Windmueller & Spaeth (1980) reported that in their preparation in vivo, 97 % of glucose from the luminal perfusion medium was found unchanged in the venous blood with an initial glucose concentration of 70 mM (i.e. fifteen times higher than that used here). That is to say, 2.1 mM glucose was metabolized. Bearing in mind their flow rate was 0.1–0.2 ml/min and their perfusion period was 30 min, that represents 30–60 μmol/h, similar to the rates found here. Likewise the amounts of glucose taken up from the VPM and LPM during the course of a 1 h perfusion were similar to those previously reported by Kavin *et al.* (1967) and Steel (1981) using similar preparations perfused under similar conditions. Neither dietary Cu deficiency nor addition of Fe (and ascorbate) to the LPM, and apotransferrin and caeruloplasmin to the VPM, affected glucose uptake from either of the perfusion media. In contrast, and under all perfusion conditions tested, O_2 consumption by intestinal preparations from Cu-deficient rats was significantly lower than that by control intestines.

The absolute values of O_2 consumption (0.8–1.9 μmol/g dry weight per min) observed in these studies tended to be lower than those reported by other workers using similar

preparations (Kavin *et al.* 1967; Dubois *et al.* 1968; Hanson & Parsons, 1976). The differences probably arose from the method of estimation adopted. In the present study the O_2 tension of the vascular perfusate was measured with an O_2 electrode from which the O_2 saturation of haemoglobin in the bovine erythrocytes was estimated from nomograms prepared by Kelman & Nunn (1966) for fresh human whole blood. Since the O_2 affinity of bovine haemoglobin differs from that of man (Bunn, 1971), the bovine erythrocytes used were not fresh but had been repeatedly washed and dialysed and the PCV of the VPM was lower than that normally encountered in human blood, the results may have been subject to a number of biases. Nevertheless, the relative differences between Cu-deficient and control preparations remain clear. Whether the 25–30 % reduction in O_2 consumption observed was related directly to the 42 % reduction in mucosal cytochrome *c* oxidase activity was not ascertained in the present study. However, recent studies with isolated rat liver mitochondria have demonstrated that in Cu deficiency, reductions in state 3 respiration and respiratory control ratios are associated with declines in both cytochrome *c* oxidase and adenine nucleotide translocase activity, with the latter probably being of greater quantitative significance (Davies & Lawrence, 1986).

The results of the present study have shown that in the absence of apotransferrin in the VPM, the transfer of Fe to the VPM by preparations from Cu-deficient rats was reduced by 50 % compared with controls, whilst only slight differences were observed in the amount of Fe retained by the intestinal tissue after a 1 h perfusion. These findings indicate a defect in Fe absorption in Cu deficiency either in the uptake of Fe from the lumen or transfer of Fe from the intestinal tissue into the mesenteric circulation, or both.

Fe absorption by isolated everted sacs of rat duodenum was shown to be inhibited by anaerobic incubation and by respiratory inhibitors and uncouplers (Dowdle *et al.* 1960). Similarly, Fe absorption by isolated loops of rat duodenum in vivo is inhibited by the addition of azide or cyanide to the luminal fluid, both of which are potent inhibitors of cytochrome *c* oxidase. Accordingly it has been suggested that Fe is absorbed by an active transport process requiring expenditure of high-energy phosphate bonds (Manis & Schacter, 1962). However, for a number of reasons, it is unlikely that the impairment of Fe transfer to the VPM in isolated intestinal preparations from Cu-deficient rats observed in the present study was associated directly with the reduced activity of mucosal cytochrome *c* oxidase activity, lower O_2 consumption and impaired oxidase metabolism. First, the dependence of Fe absorption by everted intestinal sacs on oxidase metabolism is apparently restricted to the duodenum whereas absorption in the jejunum and ileum is supported by anaerobic gycolosis (Manis & Schacter, 1962; Forth & Rummel, 1973). In the present study, the luminal input cannula was inserted distal to the duodeno-jejunal flexure and hence the duodenum was not perfused. Second, glucose uptake from the LPM (and VPM) was unaffected by Cu deficiency whereas glucose utilization by other intestinal preparations in vitro is markedly affected by either anaerobic conditions or inhibitors of respiration and oxidative phosphorylation (for review, see Crane, 1968). Finally, we found that addition of apotransferrin to the VPM, while being without effect in preparations from Cu-adequate rats, more than doubled the amount of Fe transferred to the VPM of Cu-deficient preparations without affecting metabolic activity of the intestine as assessed from glucose uptake and O_2 consumption. This argues strongly against an involvement of the reduction of cytochrome *c* oxidase activity or other respiratory processes in the defect in Fe absorption.

The failure to observe an effect of apotransferrin on Fe absorption by intestines of Cu-adequate rats supports the proposal of Schade *et al.* (1969) that it is not directly involved in the control of Fe absorption. These workers reported normal Fe absorption in rats rendered hypertransferrinaemic. A similar lack of direct involvement of transferrin in Fe

absorption can be inferred from the excessive tissue Fe loading found in subjects suffering a genetic defect, atransferrinaemia, in whom transferrin is virtually absent from the plasma (Goya *et al.* 1972).

The addition of apotransferrin to the VPM of intestinal preparations from Cu-deficient rats was without effect on the total amount of Fe taken up from the LPM. However, it resulted in a marked change in the distribution of absorbed Fe with 44% being transferred to the VPM in the presence of apotransferrin compared with 15% in its absence. Furthermore, in all perfusions the specific activity ratio, ^{59}Fe initially present in the LPM : that appearing in the VPM, was close to unity, demonstrating that essentially all the Fe appearing in the VPM was derived from the LPM. No biochemical explanation can be offered to account for these findings other than to suggest that in Cu deficiency Fe taken up from the intestinal lumen enters a tissue Fe transport pool with different properties from that of the intestine of Cu-adequate rats, the major difference being a requirement for apotransferrin in the vascular perfusate for subsequent Fe transfer across the basolateral pole of the absorbing cells. It is tempting to speculate that due to impaired oxidative metabolism the intracellular environment of the Cu-deficient intestine may be in a more-reduced state compared with Cu-adequate intestines, thus favouring the maintenance of a greater fraction of absorbed Fe in the reduced (ferrous) state. If Fe were required to be in the ferric state for transport across the basolateral membrane of the absorbing cells, the addition of apotransferrin to the VPM of Cu-deficient preparations may have promoted a shift in valence equilibrium from ferrous to ferric due to preferential binding of ferric, thereby acting as a pseudo-ferroxidase (Bates *et al.* 1973; Frieden, 1980).

The addition of either human or porcine caeruloplasmin (together with apotransferrin as a ferric acceptor) to the VPM of Cu-deficient and control intestinal preparations, such that the ferroxidase activity was at least four times that of normal rat plasma, was without effect on Fe uptake from the LPM, Fe retention by the intestinal tissue or release of Fe to the VPM. It is unlikely that potential actions of caeruloplasmin may have been masked by a high rate of non-enzymic ferrous oxidation due to the high O_2 tension (200 μM) or the pseudo-ferroxidase activity of transferrin, since Osaki *et al.* (1966) observed that the rate of ferrous oxidation in vitro was more than twice as high in the presence of (human) caeruloplasmin when the assay was carried out under conditions of similar O_2 tension (220 μM) yet with an apotransferrin concentration of four times that used in the present study.

In agreement with the findings of Williams *et al.* (1974) the ferroxidase activity of rat caeruloplasmin was low compared with that of the other species investigated. Thus in a preliminary study, we found the ferroxidase activity (U/μg Cu) of partially purified rat caeruloplasmin (mean of three determinations 0·204 (SE 0·003)) was two-thirds that of man and one-sixth that of pig.

The low ferroxidase activity of rat caeruloplasmin, together with the findings of normal Fe metabolism in patients with Wilson's disease (Williams & Lee, 1978) and Menkes' Syndrome (Danks *et al.* 1972), despite low levels or complete absence of caeruloplasmin, lead to doubts as to the adequacy of the ferroxidase hypothesis of the role of caeruloplasmin in Fe mobilization.

In the present study no evidence was obtained that the ferroxidase activity associated with caeruloplasmin plays any part in intestinal Fe absorption in either normal or Cu-deficient rats. A similar conclusion was reached by Brittin & Chee (1969) who found no correlation between serum ferroxidase activity and Fe absorption in rats of different Fe status. Furthermore, previous injection of purified (human) caeruloplasmin failed to increase either intestinal uptake or transfer of Fe by perfused loops of rat intestine in vivo.

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