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Differences between Serum and Plasma Ceruloplasmin Activities and Copper Concentrations: Investigation of Possible Contributing Factors

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Abstract

Values for ceruloplasmin activities and copper concentrations were found to be lower in serum than in paired samples of plasma in both sheep and cattle. Ceruloplasmin activities in serum were 13–40% lower relative to plasma for nine different groups of animals, and 10–65% lower for individual animals ($n = 112$). As the values are not directly interchangeable, plasma rather than serum should be used when estimating copper nutrition in these animals. Maximum effects in serum were apparent 3–4 h after collection, the earliest time at which serum could be obtained. Lower ceruloplasmin and copper values in serum could not be attributed to the type of blood collection vessel used, subsequent storage of samples, the methods used for measuring ceruloplasmin activities and copper concentrations, the formation of fibrin in blood, or to the effects of dietary molybdenum. In contrast, the addition of neuraminidase to whole blood before clotting decreased the differences between serum and plasma ceruloplasmin activity and copper concentration in a dose-dependent manner. Of the two major copper-containing enzymes present in blood, effects of clotting were only evident with ceruloplasmin. Cu–Zn-containing superoxide dismutase activity in erythrocytes was unaffected by clot formation. The results indicate that ceruloplasmin and the copper associated with this protein are sequestered into the clot during clot formation by attachment of the enzyme to the blood cellular fraction. The minimizing of this effect by the addition of neuraminidase suggests that this attachment may be through sialic acid residues.

Introduction

In the plasma fraction of blood, 70–90% of the copper is accounted for by the copper-containing enzyme ceruloplasmin (ferroxidase; iron(II):oxygen oxidoreductase, EC 1.16.3.1), a glycoprotein containing six copper atoms and nine sialic acid (*N*-acetylneuraminic acid) residues per molecule (Frieden 1980). The site of synthesis of this enzyme appears to be the liver (Markowitz *et al.* 1955) and plasma ceruloplasmin activities are dependent on liver copper reserves, with lowered activities reflecting a depletion of these reserves.

In studies measuring the activity of ceruloplasmin and other copper-containing enzymes present in the blood of various species (Paynter 1980; Paynter and Allen 1981), ceruloplasmin activities and copper concentrations were observed to be consistently lower in samples of serum than in corresponding samples of plasma. A preliminary account of these differences has been given (Paynter 1981). Differences in serum and plasma ceruloplasmin activities and copper concentrations have also been reported by McMurray (1980).

In the present study, possible factors that could contribute to these serum–plasma differences were investigated. These included the effects of collection vessels, sample storage and analytical methods. As thiomolybdates, which may be formed from

dietary molybdenum in ruminants (Dick *et al.* 1975; Suttle 1975), are known to affect both ceruloplasmin activity (Kelleher and Mason 1979) and the partitioning of copper within blood (Dick *et al.* 1975), the possible contribution of dietary molybdenum to these differences was investigated in sheep. Effects of fibrin formation and the addition of neuraminidase (EC 3.2.1.18) to blood before clot formation were also investigated. A mechanism to account for the differences observed between serum and plasma is proposed.

Materials and Methods

Sampling

Blood was routinely sampled from the jugular vein, or, in some samples from cattle, the coccygeal vein, using evacuated blood-collecting tubes. Unless otherwise specified, tubes containing lithium heparin as anticoagulant were routinely used for the collection of whole blood, and silicon-coated tubes for the collection of serum. These tubes and their needles had negligible copper contamination, as measured by flame atomic absorption spectrometry.

Mineral Assays

Copper concentrations were determined by flame atomic absorption spectrometry. Total copper concentrations in serum or plasma were routinely measured following the addition of an equal volume of 20% (w/v) trichloroacetic acid (TCA) containing 0·5 M HCl, to the samples. After 20 min at room temperature, the mixtures were centrifuged and copper concentrations determined in the supernatant fractions (Smeyers-Verbeke *et al.* 1973).

In experiments investigating the effects of dietary molybdenum, the TCA-soluble copper fraction of the total plasma copper was determined by adding an equal volume of 10% (w/v) TCA to samples of plasma, followed by centrifugation and measurement of copper in the supernatant fraction (Smith and Wright 1975). Plasma TCA-insoluble copper concentration was determined by subtraction of the plasma TCA-soluble copper from total plasma copper, determined after complete wet ashing of the plasma. Complete wet ashing was accomplished by the addition of 3·0 ml of nitric acid (70% w/w), 0·2 ml of perchloric acid (70% w/w), boiling chips and 0·025 ml of kerosene to 1·0-ml samples of plasma, followed by heating to 210°C until white fumes were evolved. The digest volumes were adjusted to 1·5 ml with water and copper concentration determined in this final solution.

Copper concentration in feed samples was determined after samples (0·5 g) were wet-ashed by the addition of 9·0 ml of nitric acid, 1·0 ml of perchloric acid, boiling chips and 0·05 ml of kerosene. After heating to 210°C until white fumes were evolved, the digest volume was adjusted to 5·0 ml with water, and copper determined in this final solution.

Molybdenum concentrations in feed samples were determined by the colorimetric method of Bingley (1963).

Enzyme Assays

Ceruloplasmin activity in plasma and serum was measured using *o*-dianisidine as substrate (Schosinsky *et al.* 1974), in assays optimized for each species (Paynter 1980). One unit of activity is equivalent to 1 μmol of *o*-dianisidine oxidized per minute at 30°C. In some samples, ceruloplasmin was also measured using *p*-phenylenediamine as substrate in the assay described by Bingley and Dick (1969).

Activity of Cu–Zn-containing superoxide dismutase in erythrocytes was measured as described previously (Paynter and Allen 1981). Activity of this enzyme is expressed relative to the blood haemoglobin concentration, and one unit of activity is that activity equivalent to 1 μg of bovine Cu–Zn-containing superoxide dismutase purified by the method of McCord and Fridovich (1969).

Dietary Molybdenum Treatments

Eight mature, non-pregnant, non-lactating crossbred ewes, previously grazing pasture adequate in available copper, were used in this experiment. The sheep were individually penned on slatted floors and fed 800 g of an appropriately supplemented commercial diet (Barastoc Stud Sheep Starter Ration) once daily. Water was available *ad libitum*.

The commercial diet, containing 16·7 μg of copper and 0·3 μg of molybdenum per gram, was mixed with 0·2% (w/w) of powdered elemental sulfur to give the − Mo diet, and with 0·2% (w/w) of powdered elemental sulfur and 16 μg of molybdenum (as an aqueous solution of ammonium molybdate) per gram of diet to give the +Mo diet. All sheep were fed the − Mo diet for 7 days, after which four of the eight sheep were changed to the +Mo diet. Blood samples were obtained immediately before the sheep were fed at 0900 h.

Effect of Fibrin Formation and Addition of Neuraminidase to Blood

To investigate the effects of fibrin formation on ceruloplasmin activity, blood from two sheep was collected using heparin and/or EDTA (disodium salt, 4 mM final concentration in blood) as anticoagulants. Plasma was separated and fibrin formation induced in samples of this plasma by the addition of $CaCl_2$ (0·01 ml of 0–900 mM $CaCl_2$ per millilitre of plasma). Any fibrin subsequently formed was removed by centrifugation 2 h after addition of the $CaCl_2$, and the supernatant assayed for ceruloplasmin activity. These activities were compared with those for serum and for plasma collected from whole blood shaken with defibrinating beads immediately after collection.

Effects of addition of neuraminidase activity to the difference in copper concentrations and ceruloplasmin activities between serum and plasma were also investigated using sheep blood. In this experiment, paired samples of blood were collected from each of three sheep into vessels containing known volumes (final volumes 0–0·14 ml per millilitre of blood) of an isotonic solution of neuraminidase from *V. cholerae* (Wellcome Laboratories, Kent, England) with and without heparin as anticoagulant. Samples were centrifuged after 3 h at 25°C and the serum and plasma obtained then assayed for ceruloplasmin activity and copper concentration.

Table 1. Mean ceruloplasmin activities and copper concentrations in paired serum and plasma samples from sheep and cattle

Values for animal groups A–E are means for at least 10 cows or calves per group. Values for animal groups F–H are means for at least eight sheep per group. For all groups, values for serum were significantly lower ($P<0·05$) than values for paired samples of plasma

Animal group	In plasma:		In serum:	
	Ceruloplasmin activity (mU/ml)	Copper concn (μg/ml)	Ceruloplasmin activity (mU/ml)	Copper concn (μg/ml)
A	94·4	1·37	70·2	1·07
B	56·2	0·79	36·9	0·60
C	44·1	0·76	32·1	0·63
D	27·9	0·52	16·7	0·40
E	18·3	0·37	13·0	0·31
F	80·5	1·07	60·7	0·84
G	64·6	0·99	54·5	0·81
H	29·7	0·53	23·2	0·46

Results

Mean ceruloplasmin activities and copper concentrations for paired samples of plasma and serum obtained from sheep and cattle on a total of eight properties are shown in Table 1. For all pairs, values for serum were significantly reduced ($P<0·05$) compared with values obtained for plasma. This reduction in serum values relative to plasma, which ranged from 13 to 40% for ceruloplasmin activity between different groups of animals, was apparent for samples containing normal or low ceruloplasmin activities and copper concentrations, for both sheep and cattle and for all age groups of animals investigated. Ceruloplasmin activities in serum were lowered by 10–65% between individual bovine samples ($n = 112$). Similar results were apparent for ovine samples. These decreased ceruloplasmin activities were obtained using either

o-dianisidine or *p*-phenylenediamine as substrate in the ceruloplasmin assays, and activities measured using both substrates were highly correlated ($r = 0{\cdot}90$, $n = 20$). Similar decreases in serum copper concentrations relative to plasma were observed when copper was measured by either the TCA/HCl precipitation method or by the complete wet-digestion method.

The relative decrease in ceruloplasmin activities were greater than those for copper concentrations. Linear regression analysis of the values shown in Table 1 demonstrated that in bovine plasma, ceruloplasmin activities (x) were directly correlated with copper concentrations (y), according to the equation $y = 0{\cdot}0129\,x + 0{\cdot}147$ ($r = 0{\cdot}993$, $P < 0{\cdot}001$). The corresponding equation for these values in serum ($y = 0{\cdot}0129\,x + 0{\cdot}168$, $r = 0{\cdot}993$, $P < 0{\cdot}001$) indicates that although values in serum are decreased relative to those in plasma, the slopes and intercepts for the individual correlations are highly similar. A similar conclusion was evident in values for ovine serum and plasma, and slopes and intercepts were 0·0110 and 0·220, respectively, for plasma and 0·0105 and 0·220, respectively, for serum. It is apparent that at zero-extrapolated ceruloplasmin activity, the copper concentration remaining (approximately 0·15 μg/ml for bovine samples and 0·22 μg/ml for ovine samples) was not affected by serum or plasma collection. This indicates that the non-ceruloplasmin copper fraction of blood plasma is unaffected by clot formation, and effects of clotting are associated primarily with the ceruloplasmin copper fraction.

Table 2. Effect of blood collection vessel and subsequent storage on ceruloplasmin activities in paired serum and plasma samples from cattle

Each value is the mean for five cows. Only sample type (serum or plasma) significantly affected ($P < 0{\cdot}05$) mean values

Sample storage[A]	Blood collection vessel	Ceruloplasmin activity (mU/ml) In plasma	In serum
H	Evacuated tube 1[B]	55·6	36·6
	Evacuated tube 2[C]	56·0	36·7
	Plastic syringe[D]	56·7	36·6
V	Evacuated tube 1[B]	57·0	35·7
	Evacuated tube 2[C]	55·8	36·1

[A] After collection, bloods were stored overnight in either a horizontal (H) or vertical (V) position (blood with and without contact with rubber tube stoppers).
[B] Becton Dickinson 'Vacutainer' tube (Rutherford, New Jersey.)
[C] Jintan Terumo 'Venoject' tube (Tokyo, Japan.)
[D] Scientific Products 'Peel-A-Way' sampler (Longbeach, California.)

Differences between serum and plasma could not be attributed to either the type of collection vessel used or the method of sample storage (Tables 2 and 3). Essentially the same values were obtained if samples were collected using either evacuated sampling tubes or plastic syringe samplers; contact of serum or plasma with the rubber stoppers of evacuated collection tubes for prolonged periods also had no effect (Table 2). Effects of clot formation on ceruloplasmin activity were not attributable to temperature inactivation or precipitation of ceruloplasmin with storage of blood at room temperature during clot formation. Effects of clotting on ceruloplasmin activity were evident within 3 h of blood collection, or the earliest time at which serum was separated from the clot fraction (Table 3). Storage of heparinized

whole blood at either 25 or 4°C, for up to 48 h before separation of serum or plasma, had only minor effects on ceruloplasmin activity when compared with effects of clotting (Table 3).

Table 3. Effect of blood storage time and temperature on ceruloplasmin activities in paired serum and plasma samples from sheep

Each value is the mean for four sheep fed a commercial diet

Sample	Storage temp. (°C)	Ceruloplasmin activity (mU/ml) after storage for: 0·5 h	4 h	21 h	48 h[A]
		In plasma			
Whole blood	4	76·8	77·4	76·0	76·1
	25		74·2	69·7	70·8
Plasma[B]	4				77·8
		In serum			
Whole blood	25		61·1	58·9	60·6

[A] Time before separation of serum or plasma from whole blood.
[B] Plasma separated at 0–5 h and stored at 4°C before being assayed.

No effects of clotting were apparent on erythrocyte Cu–Zn-containing superoxide dismutase activity. Activity of this enzyme in chloroform/ethanol extracts of heparinized whole blood of sheep did not differ significantly from activity measured in whole blood that had been allowed to clot for 3–24 h and the clot and serum homogenized in a glass tissue homogenizer before being extracted with chloroform/ethanol (mean Cu–Zn-containing superoxide dismutase activities, 568 and 554 U per gram of haemoglobin, respectively, $n = 2$).

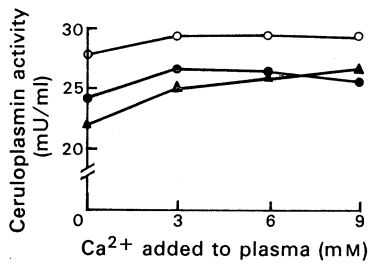

Fig. 1. Effect of Ca^{2+}-induced fibrin formation in plasma on ceruloplasmin activity. Whole blood was collected from two sheep, using as anticoagulants 12·5 i.u. of heparin per millilitre (○), 4 mM EDTA (●) or 12·5 i.u. of heparin per millilitre + 4 mM EDTA (▲). Partial or complete fibrin formation occurred only with EDTA anticoagulant, at additions of 6 and 9 mM Ca^{2+}, respectively. Mean ceruloplasmin activities for defibrinated plasma, collected by agitation of whole blood with defibrinating beads, and for serum, were 29·2 and 21·9 mU/ml, respectively.

Fibrin formation alone did not appear to affect ceruloplasmin activity. Defibrinated plasma, obtained after agitation of whole blood with defibrinating beads or from plasma collected using EDTA and heparin as anticoagulants with fibrin formation subsequently induced by titration with Ca^{2+}, had activities comparable to those of the respective heparinized or heparin plus EDTA control plasmas containing fibrinogen (Fig. 1).

Differences in both copper concentration and ceruloplasmin activity between serum and plasma were unchanged by dietary molybdenum treatment (Fig. 2). Sheep fed diets high in molybdenum had a rapid increase in the TCA-insoluble copper fraction in plasma, compared with sheep fed diets low in molybdenum (Fig. 2). This increase in TCA-insoluble copper was reflected in a corresponding increase in

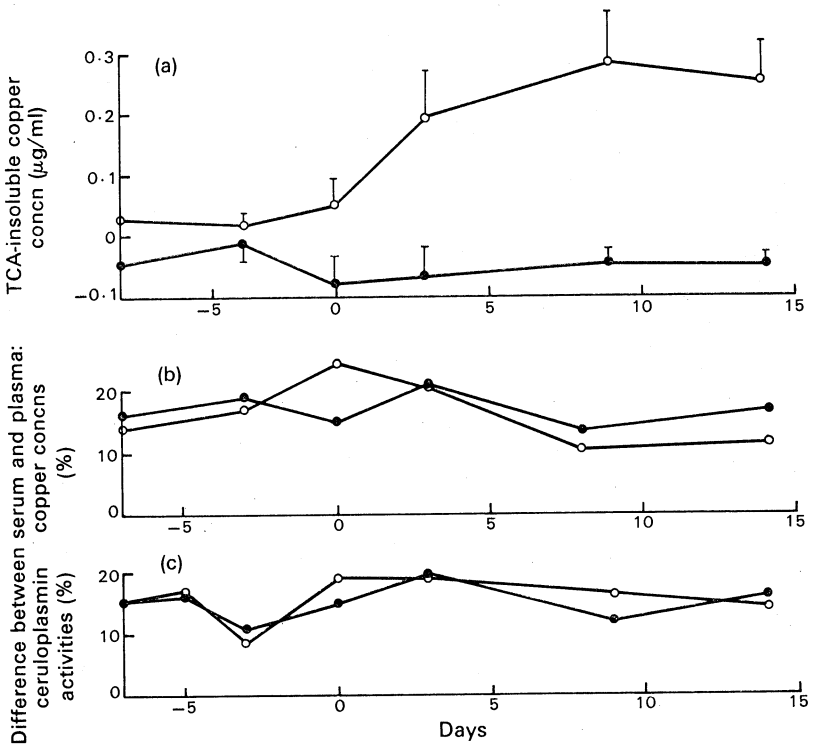

Fig. 2. Effect of dietary molybdenum on plasma TCA-insoluble copper (*a*), the difference between serum and plasma copper concentrations relative to those of plasma (*b*), and the difference between serum and plasma ceruloplasmin activities relative to those of plasma (*c*). Feeding of the + Mo diets (○) and the − Mo diets (●) was begun at day 0, after a period when all sheep were fed the − Mo diet. Values shown are means (+s.e.m.) for four sheep.

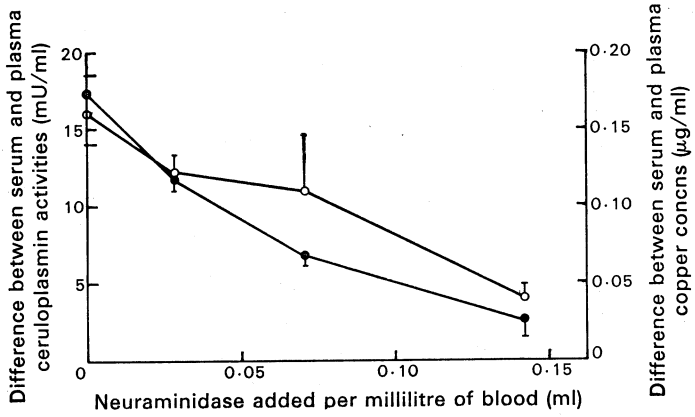

Fig. 3. Effect of addition of neuraminidase to paired samples of whole blood and heparinized whole blood on the difference between serum and plasma ceruloplasmin activities (●) and copper concentrations (○). Each point represents the mean value (+s.e.m.) of three sheep. Least significant differences ($P < 0{\cdot}05$) between means were 3·5 and 0·08 for ceruloplasmin and copper values, respectively. Mean plasma ceruloplasmin activities of these sheep, without neuraminidase treatment, was 68·2±6·17 mU/ml.

total copper in serum and plasma of sheep fed the +Mo diets. Activities of serum and plasma ceruloplasmin and erythrocyte Cu–Zn-containing superoxide dismutase were largely unaffected by this relatively short period (14 days) of dietary molybdenum treatment. Plasma ceruloplasmin activities in sheep fed the −Mo and +Mo diets were $62 \cdot 5 \pm 4 \cdot 04$ and $55 \cdot 2 \pm 3 \cdot 90$, respectively, at the start of the dietary treatment period and $59 \cdot 3 \pm 2 \cdot 15$ and $60 \cdot 7 \pm 5 \cdot 50$, respectively, after 14 days of dietary treatment (means ± s.e.m.). Corresponding activities of Cu–Zn-containing superoxide dismutase were $663 \pm 28 \cdot 8$ and $643 \pm 46 \cdot 2$ at the start of dietary treatment and $630 \pm 43 \cdot 8$ and $643 \pm 27 \cdot 3$ at the end of this period.

Addition of neuraminidase activity to whole blood before clotting had a marked effect on the relative differences between serum and plasma ceruloplasmin activities and copper concentrations (Fig. 3). Addition of neuraminidase to heparinized whole blood at the time of collection resulted in a small decrease in both plasma ceruloplasmin activities and copper concentrations. However, when neuraminidase was added to non-heparinized whole blood before clotting and serum formation, the effect apparent with heparinized blood appeared to be overridden and serum values increased relative to those for plasma at comparable neuraminidase addition. Differences between serum and plasma ceruloplasmin activities and copper concentrations were reduced in direct proportion to the amount of neuraminidase added to whole blood, and this reduction was highly reproducible between individual animals (Fig. 3).

Discussion

Copper concentrations and ceruloplasmin activities of serum or plasma are widely used as indicators of copper nutrition in domestic animals and values obtained for serum are often directly compared with those for plasma. It is apparent from this study that values obtained for serum are significantly lower than those for plasma and the extent that serum values are decreased between individual animals is highly variable. The results strongly indicate that estimates of copper nutrition will be far more reliable when based on copper and ceruloplasmin in plasma than in serum.

Differences in ceruloplasmin activity and copper concentration between serum and plasma were not due to *in vitro* factors operating with the collection of blood samples or during subsequent analysis. Healy *et al.* (1978) and Healy and Turvey (1978) reported decreased serum copper concentrations, but not ceruloplasmin activities, when whole blood was subjected to prolonged contact with the rubber stoppers of certain evacuated blood collection tubes. These effects were not apparent in the present studies and could not be implicated as a mechanism for the differences observed.

Dietary molybdenum does not appear to be involved in the difference between serum and plasma ceruloplasmin activities or copper concentrations or in the large variation observed for these between individual animals. Thus, effects of thiomolybdates, particularly tetrathiomolybdates, which have been reported to increase plasma TCA-insoluble copper *in vivo* (Dick *et al.* 1975) and to decrease ceruloplasmin activity *in vitro* (Kelleher and Mason 1979), and may be formed from dietary molybdenum and sulfur in the rumen (Dick *et al.* 1975; Suttle 1975), do not appear to be involved in the mechanism of these differences. Similarly, increases in plasma TCA-insoluble copper, reported to occur with storage of whole blood samples and attributed to the generation of sulfides (Dick *et al.* 1975), does not appear to be involved. Whole blood obtained from sheep fed a commercial diet (Table 3) or sheep fed the +Mo and −Mo diets for 14 days, had similar activities of plasma ceruloplasmin when

assayed immediately or when stored for up to 24 h as whole blood, whereas major decreases in ceruloplasmin in sera of these bloods were apparent after only 3–4 h.

That effects of clotting on ceruloplasmin are not those affecting copper generally, such as sulfide generation or thiomolybdate compounds, is also supported by the finding that, of the major copper enzymes present in blood, only ceruloplasmin is affected. Neither clotting nor storage of heparinized whole blood affected erythrocyte Cu–Zn-containing superoxide dismutase activity, even though copper is known to be essential for activity of this enzyme (McCord and Fridovich 1969).

The results of the present study demonstrate that ceruloplasmin is removed from serum during clot formation. The simultaneous removal of copper and the finding that a fraction of the plasma copper, of similar concentration to non-ceruloplasmin copper, is unaffected by clot formation, suggest that the copper removed from plasma is that specifically associated with the ceruloplasmin protein. The question remains as to how ceruloplasmin and copper associated with it are sequestered and apparently bound to the clot fraction during its formation. The finding that neuraminidase activity added to blood at the time of collection minimizes these effects indicates that sialic acid residues, which would be specifically removed by neuraminidase treatment, may be intimately involved in this sequestering reaction.

Ceruloplasmin may be bound by sialic acid residues to the blood cellular fraction as the formation and subsequent removal of fibrin from plasma had no apparent effect on ceruloplasmin (Fig. 1). That fibrin formation had no effect indicates that although the trigger for the binding of ceruloplasmin must involve the clotting mechanism, the removal of ceruloplasmin from the plasma during clotting is dependent on cellular inclusion into the contracting clot. The trigger involved may be one of the proteases formed in the clotting cascade (Linman 1975), activating sialic acid residues or their receptors to induce binding. Binding of ceruloplasmin may be through attachment of the sialic acid residues present on ceruloplasmin and essential for its survival in the circulation (Van den Hamer *et al.* 1970; Frieden 1980), to receptors on erythrocytes or platelets. Alternatively, the sequestering of ceruloplasmin may involve the sialic acid residues of platelets, or to a lesser extent the erythrocytes, which contain considerably less sialic acid than platelets (Madoff *et al.* 1964).

Ceruloplasmin in plasma is well established as a multifunctional enzyme, able to catalyse a variety of oxidase-type reactions (Frieden 1980). Its activity is known to be highly dependent on dietary copper intake, with rapid changes in activity occurring with dietary copper depletion and repletion relative to changes observed in other copper-dependent enzymes (Underwood 1977; Paynter *et al.* 1979). Decreases in blood packed-cell volume, rupture of blood vessels and haemorrhages have all been associated with dietary copper deficiency (Underwood 1977). The possibility that copper, through ceruloplasmin activity, could be directly involved in the blood clotting process, does not appear to have been considered.

Whether ceruloplasmin retains any of its multifunctional activities when bound into the clot fraction, and the contribution, if any, that these activities may make to the overall mechanism of clot formation remain to be determined.

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