An investigation of the effects of intravenous administration of thiomolybdate on copper metabolism in chronic Cu-poisoned sheep

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1. Fourteen sheep were dosed repeatedly with a solution of copper sulphate (2 g/l) in order to induce chronic copper poisoning and four similar undosed animals acted as controls.

2. Thiomolybdate (TM) was intravenously administered to all control sheep and to all except two of the test sheep. A variety of biochemical factors were studied before and after injections of TM.

3. The direct-reacting Cu, whole-blood Cu and plasma Cu concentrations were elevated in animals given TM injections and at the 'haemolytic crisis' of untreated chronic Cu-poisoned animals. But most of the increased Cu observed on injecting TM was insoluble in trichloroacetic acid (TCA) and did not enter erythrocytes. The results indicate that uptake of Cu by erythrocytes is essential for haemolysis to occur and that for this to happen the Cu must be in a direct reacting, TCA-soluble form.

4. Increased amounts of Cu were excreted in the urine at haemolysis and at the commencement of TM injections. High levels of direct-reacting Cu were found in plasma at these times.

5. Marked changes were not found in caeruloplasmin activity, packed cell volume or the osmotic fragility of erythrocytes except at haemolysis. TM injections did not alter these factors in any of the sheep studied.

Haemolysis is the most characteristic symptom of chronic copper poisoning in sheep and is known to be associated with increased blood and tissue Cu levels, and with liver and kidney damage (Todd, 1969; Ishmael *et al.* 1971, 1972; Howell, 1978). Several workers (Ross, 1966; Hogan *et al.* 1968; Kline *et al.* 1971) have used dietary supplements of molybdenum and sulphate as Cu antagonists to reduce the toxicity of Cu to sheep. It has been shown that thiomolybdate (TM), an inorganic compound containing Mo and sulphur, when injected intravenously lowered liver Cu levels, prevented chronic Cu poisoning and was of value in the treatment of animals that had developed a haemolytic crisis (Gooneratne *et al.* 1981). TM did not appear to cause any harmful effects on animal tissues. Although TM appears to be antagonistic to Cu utilization and is capable of lowering liver Cu levels the mechanism by which it prevents haemolysis in sheep is not understood.

It is difficult to identify specific sites for the Cu–Mo–S interaction because interactions located in the gut and those in the tissues are related (Dick *et al.* 1975). El Gallad *et al.* (1977) discussed the changes in trichloroacetic acid (TCA)-soluble and -insoluble fractions of Cu in the plasma of sheep given TM intravenously. They observed all Cu to be in the TCA-insoluble fraction 15 min after injection of TM but the amount of Cu in the TCA-soluble fraction increased with time until after 144 h all Cu in plasma was TCA-soluble. There is at present insufficient experimental evidence with regard to the influence of TM on the Cu metabolism of ruminants but a considerable amount of work has been done in animals given dietary Mo supplements in which, presumably, TM was formed in the rumen (Dick *et al.* 1975). Several workers (Smith *et al.* 1968; Marcilese *et al.* 1969) have reported a decrease in caeruloplasmin (Cp) synthesis in sheep given oral Mo supplements. This could result from a decrease in the supply of absorbable Cu due to its interaction with TM in the rumen rather than due to a direct effect on Cp synthesis in the liver. Mo supplementation increased the direct-reacting (DR) fraction of plasma Cu (Suttle & Field, 1968; Marcilese *et al.* 1969) but Cp and DR Cu do not account for all of the Cu in the blood of these animals

Table 1. *Details of experimental animals*

TM treatment		Group description	No. of sheep	Days on expt (mean)	Animal status	Days to haemolysis (mean)	Days in haemolysis (mean)	Days to commencement of TM injections (mean)	No. of TM injections/ sheep	Vol. TM/ injection (ml)	Total amount of TM injected/ sheep during expt (mg)	Total amount of Cu (mg) administered/ sheep (mean)
−	1A	Died at haemolysis.	2	81	D	80	2	—	—	—	—	7350
	1B	At haemolysis; stop Cu dosing. Killed or died during the 11 weeks after haemolysis.	2	152	D K	102	4	—	—	—	—	8653
+	2A	At haemolysis; one injection of TM. Died at haemolysis.	2	67	D	66	2	66	1	1	50	6235
	2B	Haemolysis; stop Cu dosing. Start TM injections.	4	158	K	80	4·5	80	22	1	1100	7120
	3	Second rise of SD; start TM injections, continue Cu dosing.	4	118	K	—	—	40	22	2	2200	9725
	4	Control group, no Cu, only TM injections.	4	131	K	—	—	55	22	1	1100	—

TM, thiomolybdate; D, died; K, killed.

(Smith *et al.* 1968; Suttle & Field, 1968). In addition to the changes in blood and plasma, the urinary excretion of Cu was also increased in animals given Mo supplementation (Rys *et al.* 1963; Smith *et al.* 1968; Marcilese *et al.* 1970).

The changes in liver Cu, plasma Cu, Cp synthesis and urinary excretion of Cu reported in the literature indicate that body Cu stores are mobilized and excreted in sheep given dietary Mo supplements. The effects of Mo may be mediated via the formation of TM in the rumen and its absorption into the bloodstream (Dick *et al.* 1975). If so, similar effects might be observed in animals given TM intravenously.

In the present investigation a variety of biochemical factors were studied before and after injections of TM to control sheep and to sheep in various stages of chronic Cu poisoning. It was hoped that the results might provide an understanding of the possible mechanisms associated with Cu transport before and at haemolysis, and the mechanisms involved in the prevention of haemolysis by intravenous administration of TM.

MATERIALS AND METHODS

Animals

A total of eighteen animals were used in this study. The procedures for housing, feeding and treating these animals have previously been reported (Gooneratne *et al.* 1981).

The animals were divided into four groups with four to six animals in each group. All sheep except those in group 4 were dosed with a solution of $CuSO_4 . 5H_2O$ (2 g/l). The dosage of TM to be injected into sheep of varying Cu status had been determined in a previous study (Gooneratne *et al.* 1981). The details of the animals used in the present study are given in Table 1 and in Gooneratne *et al.* (1981).

Six of the eighteen sheep, one each from groups 1A, 1B and 2A, 2 each from groups 2B, 3 and 4 were housed individually in fibre-glass metabolism cages for collection of urine.

Collection of blood

Blood samples were collected from the jugular vein into heparinized containers, 2 weeks before and at the start of the experiment, then twice weekly until all sheep in group 3 had a second rise of plasma sorbitol dehydrogenase (SD) level and thereafter at weekly intervals. At haemolysis blood was collected more frequently. Blood was collected before and 24 h after each of the first three injections of TM and thereafter before and after every other (odd number) injection. A 24 h post-injection period for collection of blood was selected as described by El Gallad *et al.* (1977); the highest concentration of Cu in plasma after a TM injection was found at this time.

Collection of urine

Faecal collection bags were attached to the sheep in metabolism cages to prevent contamination of urine which was collected into washed polyethylene bottles placed at the 'flow out' opening in the bottom of the cage. Samples of urine were taken once every week starting from the first day of Cu dosing, until sheep had either a 'haemolytic crisis' or the first injection of TM. From then onwards 24 h samples were taken throughout the period of haemolysis or after each of the first two injections of TM or both and thereafter 24 h samples were taken after each 'odd-numbered' injection.

Analytical techniques

Cu. Cu analysis was performed on whole blood (WB) from all sheep as described by Ishmael *et al.* (1972). Plasma Cu was estimated by the method of Suttle (1974). Cu in the TCA-soluble fraction of plasma was determined as described by Smith & Wright (1975*a*). DR Cu was measured by the method of Suttle & Field (1968).

Table 2. *Copper levels in whole-blood, plasma, and in TCA-soluble and direct-reacting fractions of plasma for one sheep from each of the six subgroups of the experiment*

(Value in parentheses is day of sampling from start of experiment)

Measurement	Copper levels (μg/ml)																			
				On days of haemolysis (groups 1A and 1B), Days					Prior to (P) and 24 h after (A) the first three injections of TM											On day died (D) or killed (K)
									Injection 1		Injection 2				Injection 3					
	Day −14	Day 0	1st inject.*	1	2	3	4	5	P	A	P−2 Days	P−1 Days	P	A	P	A	2 wks†	3–6 wks‡	7–11 wks§	
Group 1A, sheep no. 233																				
				D‖																D‖
WB Cu	1·00	1·02	0·86 ±1·04	4·49‖ (80)	1·94 (81)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·94 (81)
Pl. Cu	1·27	1·17	1·02 ±0·04	2·90	1·99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·99
TCA-sol. Cu	1·65	1·27	1·00 ±0·05	2·51	1·77	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·17
DR Cu	0·11	0·12	0·34 ±0·05	2·04	1·19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·19
Group 1B, sheep no. 238																				
																				D
WB Cu	1·00	0·97	1·26 ±0·04	7·03‖ (119)	3·13‖ (120)	2·28‖ (121)	2·17‖ (122)	1·94‖ (123)	—	—	—	—	—	—	—	—	1·81±0·07	—	—	1·91 (140)
Pl. Cu	1·48	1·36	1·87 ±0·09	4·45	2·91	2·91	2·50	2·39	—	—	—	—	—	—	—	—	2·69±0·11	—	—	2·93
TCA-sol. Cu	1·59	1·46	1·72 ±0·07	3·94	2·28	2·16	1·93	2·06	—	—	—	—	—	—	—	—	2·49±0·16	—	—	1·97
DR Cu	0·08	0·11	0·70 ±0·08	2·58	1·62	1·48	1·34	1·23	—	—	—	—	—	—	—	—	1·08±0·08	—	—	1·04
Group 2A, sheep no. 226																				
																	P	A	P	A	P	A	
									D‖														D‖
WB Cu	0·78	0·97	0·94 ±0·05	—	—	—	—	—	9·21‖ (66)	5·39 (67)	—	—	—	—	—	—	—	—	—	—	—	—	5·39 (67)
Pl. Cu	1·21	0·97	1·28 ±0·16	—	—	—	—	—	8·00	5·27	—	—	—	—	—	—	—	—	—	—	—	—	5·27
TCA-sol. Cu	1·05	1·24	1·29 ±0·14	—	—	—	—	—	5·76	3·64	—	—	—	—	—	—	—	—	—	—	—	—	3·64
DR Cu	0·18	0·14	0·40 ±0·15	—	—	—	—	—	4·96	3·36	—	—	—	—	—	—	—	—	—	—	—	—	3·36

Table 2 *cont.*

Group 2B, sheep no. 249																						
																						K
WB Cu	0·94	1·02	0·92 ±0·03	—	—	—	—	—	4·15‖ (82)	2·46‖ (83)	1·65‖ (84)	—	1·71‖ (85)	2·28‖ (86)	1·44 (89)	2·71 (90)	2·14 ±0·24	3·04 ±0·14	1·73 ±0·17	2·12 ±0·14	1·36 ±0·03	1·76 ±0·11	1·34 (161)
Pl. Cu	1·38	1·36	1·19 ±0·05	—	—	—	—	—	2·96	3·47	1·94	—	1·88	2·96	2·08	3·92	3·46 ±0·49	4·73 ±0·35	2·77 ±0·19	3·38 ±0·21	2·18 ±0·11	2·65 ±0·06	1·85
TCA-sol. Cu	1·54	1·36	1·16 ±0·06	—	—	—	—	—	1·82	1·71	1·48	—	1·60	1·03	1·87	1·39	1·75 ±0·06	1·34 ±0·09	1·37 ±0·03	1·04 ±0·11	1·18 ±0·10	0·80 ±0·03	0·89
DR Cu	0·12	0·15	0·23 +0·03	—	—	—	—	—	1·94	2·20	0·99	—	0·84	1·71	0·85	2·08	1·35 ±0·18	2·11 ±0·02	1·07 ±0·03	1·56 ±0·03	0·96 ±0·03	1·32 ±0·04	0·83
Group 3, sheep no. 223																						
																						K
WB Cu	1·10	1·07	1·02 ±0·05	—	—	—	—	—	0·88 (49)	1·90 (50)	—	—	1·76 (52)	1·98 (53)	1·60 (56)	2·83 (57)	2·38 ±0·16	2·91 ±0·05	2·58 ±0·05	3·26 ±0·18	2·62 ±0·19	3·64 ±0·10	2·85 (126)
Pl. Cu	1·59	1·17	1·12 ±0·10	—	—	—	—	—	1·00	3·82	—	—	2·24	2·67	2·08	4·17	3·23 ±0·15	4·61 ±0·07	4·12 ±0·20	5·24 ±0·25	4·40 ±0·18	5·80 ±0·13	4·20
TCA-sol. Cu	1·59	1·27	1·14 ±0·06	—	—	—	—	—	0·83	0·66	—	—	1·01	0·69	1·07	0·58	1·11 ±0·14	0·85 ±0·19	1·49 ±0·06	1·16 ±0·17	1·72 ±0·10	1·39 ±0·02	1·38
DR Cu	0·08	0·08	0·12 ±0·02	—	—	—	—	—	0·16	2·32	—	—	1·11	1·48	1·07	2·34	1·39 ±0·10	2·41 ±0·09	1·37 ±0·16	2·35 ±0·14	1·49 ±0·04	2·36 ±0·23	1·53
Group 4, sheep no. 253																						
																						K
WB Cu	1·22	1·61	0·90 ±0·10	—	—	—	—	—	0·79 (63)	0·84 (64)	—	—	0·61 (66)	0·86 (67)	0·76 (70)	0·82 (71)	0·70 ±0·04	0·84 ±0·04	0·67 ±0·07	0·94 ±0·07	0·69 ±0·06	0·83 ±0·02	0·64 (140)
Pl. Cu	1·54	1·44	1·29 ±0·18	—	—	—	—	—	0·91	1·15	—	—	1·04	1·28	1·10	1·28	1·14 ±0·05	1·33 ±0·03	1·30 ±0·05	1·51 ±0·10	1·11 ±0·03	1·26 ±0·04	1·11
TCA-sol. Cu	1·43	1·50	1·16 ±0·17	—	—	—	—	—	0·78	0·59	—	—	0·92	0·68	0·76	0·72	0·90 ±0·08	0·80 ±0·06	1·04 ±0·03	0·94 ±0·07	1·00 ±0·01	0·89 ±0·06	1·06
DR Cu	0·12	0·18	0·11 ±0·01	—	—	—	—	—	0·07	0·45	—	—	0·25	0·46	0·30	0·49	0·31 ±0·01	0·46 ±0·02	0·32 ±0·02	0·48 ±0·03	0·27 ±0·05	0·46 ±0·04	0·29

D, died; K, killed.
* Mean ± SE until first injection of TM (groups 2A, 2B, 3, 4) or haemolysis (groups 1A, 1B).
† Mean ± SE during first 2 weeks after haemolysis (group 1B) or prior to (P) and 24 h after (A) TM injections 5, 7 and 9 (groups 2B, 3, 4).
‡ Mean ± SE during 3–6 weeks after haemolysis (group 1B) or prior to (P) and 24 h after (A) TM injections 11, 13 and 15 (groups 2B, 3, 4).
§ Mean ± SE during 7–11 weeks after haemolysis (group 1B) or prior to (P) and 24 h after (A) TM injections 17, 19 and 21 (groups 2B, 3, 4).
‖ Denotes haemolysis.

Urine. Cu analysis was carried out on the urine of all six sheep housed in metabolism cages. Urine (20 ml) was digested with 5 ml concentrated nitric acid, 1 ml concentrated sulphuric acid and 1 ml perchloric acid and the Cu content of the digest measured using atomic absorption spectrophotometry. The digest mixture of samples collected at haemolysis became frothy and charred with the deposition of a black sediment at the bottom and sides of the flask due to incomplete digestion. Therefore, at haemolysis a lesser volume (10 ml) of urine was taken for digestion and an extra 5 ml concentrated HNO_3 was added for complete digestion.

Cp. Cp estimations were carried out on the six sheep housed in metabolism cages. Cp was determined by its oxidase activity towards ρ-phenylenediamine as described by Smith & Wright (1974). The results were expressed as International Units.

SD. SD activity was measured in plasma as described by Ford (1967).

Packed cell volume (PCV). PCV was determined using a microhaematocrit centrifuge (Hawksley).

Erythrocyte osmotic fragility. Erythrocyte osmotic fragility estimations were carried out on heparinized blood samples from the six sheep housed in metabolism cages by the method described by Schalm *et al.* (1975). The results were expressed as the concentration of sodium chloride in which 50% haemolysis occurred.

Urine analysis. Urine was tested for pH, protein, glucose, ketones, bilirubin and haemoglobin using reagent strips (Bili-Labstix; Ames Co.).

Preparation of TM. TM was prepared as described by Gooneratne *et al.* (1981).

RESULTS

Clinical findings

The details of individual sheep are given in Table 1.

The details of clinical, postmortem and histological findings have already been published (Gooneratne *et al.* 1981).

Cu levels in WB, plasma, TCA-soluble and DR fractions of plasma

The results for selected sheep are given in Table 2 and Figs. 1–3. The results for all sheep are available elsewhere (Gooneratne, 1979). Until haemolysis or the administration of thiomolybdate, plasma Cu, TCA-soluble Cu and WB Cu remained within normal limits with plasma Cu $\simeq$ to TCA-soluble Cu > WB Cu. In sheep dosed with Cu, beginning at approximately the fourth week of dosing, DR Cu showed a gradual increase. This increase was most marked one week before haemolysis. In all but one of these sheep that were allowed to proceed to haemolysis (group 1 and 2) the Cu level in WB and in all fractions of plasma increased sharply at and during haemolysis. In the sheep that were not given TM at haemolysis the increased Cu levels were in the following descending order WB Cu > plasma Cu > TCA-soluble Cu > DR Cu. Since WB Cu > plasma Cu it could be inferred that erythrocyte Cu was increased during haemolysis. Most of the plasma Cu was TCA-soluble. Once TM was injected a change in this pattern of distribution of Cu occurred and the increased Cu levels in the blood stream were in the following descending order, plasma Cu > WB Cu > DR Cu > TCA-soluble Cu, indicating that erythrocytes Cu was lower than plasma Cu and that some of the DR Cu was TCA-insoluble. There was also a marked decrease of Cu in the TCA-soluble fractions. The pattern of distribution of Cu within the blood stream was similar with each injection of TM irrespective of the experimental group to which the sheep belonged. In samples taken 24 h after each injection of TM the Cu levels increased in WB, and in all fractions of plasma except the TCA-soluble fraction in which the Cu level decreased. Thereafter the level of Cu in WB and in fractions of plasma except TCA-soluble, decreased slowly, only to rise again at each TM injection.

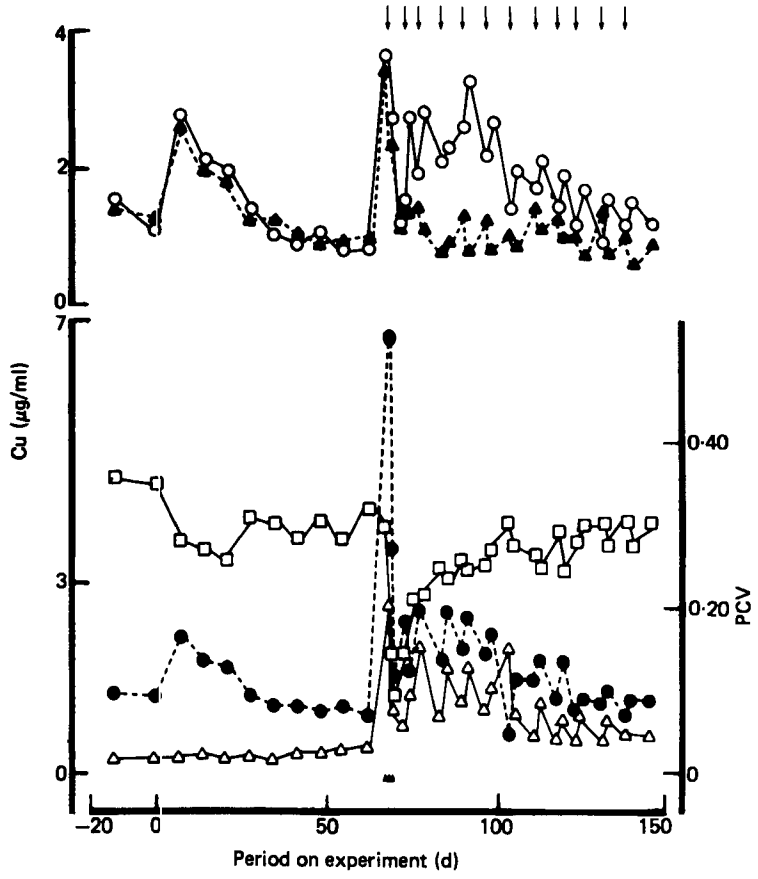

Fig. 1. Whole-blood (●- - -●), plasma (○—○), plasma trichloroacetic acid(TCA)-soluble (▲- - -▲) and direct-reacting (△—△) copper levels and packed cell volume (PCV) (□—□) of sheep no. 230 (group 2B) measured throughout the course of the experiment. On the first day of haemolysis before the start of TM injections, Cu in all fractions increased with the peak level in WB. ↓, TM injections. ▲▲▲, days of haemolysis.

In the latter part of the experiment Cu levels in blood were gradually declining and this was most marked in the sheep which received TM injections but not Cu (group 4).

Cp

The levels of Cp were most variable with a range of 55–174 IU. Marked fluctuations were seen even between consecutive measurements but these could not be related to the TM injections. The only consistent change observed was the gradual decline in Cp at a fairly regular rate in sheep of group 4 and this was quite marked in sheep number 229.

PCV

In all groups of sheep a decline in PCV was recorded during the first 3–4 weeks of the experiment (Figs. 1, 2, 3). From then onwards the levels tended to fluctuate throughout the course of the experiment except at haemolysis when a marked fall in PCV was recorded (Fig. 1). Following the haemolytic crisis PCV slowly returned to normal levels irrespective of whether the animals received TM or not.

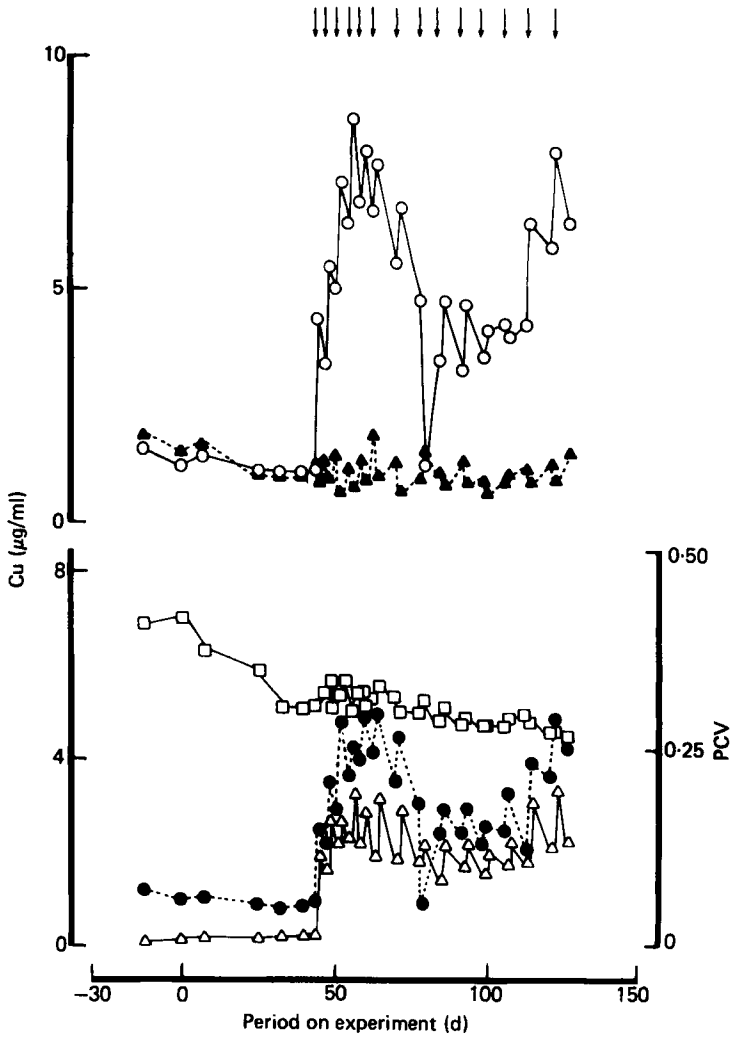

Fig. 2. Whole-blood (●- - -●), plasma (○—○), plasma trichloroacetic acid(TCA)-soluble (△- - -△) and direct-reacting (▲—▲) copper levels and packed cell volume (PCV) (□—□) of sheep no. 225 (group 3) measured throughout the course of the experiment. ↓, TM injections.

Erythrocyte osmotic fragility

The measurements of the 50% lysis point of erythrocytes of sheep at the commencement of the experiment, varied from a concentration of 6·1–7·6 g NaCl/l. At the onset of haemolysis osmotic fragility increased appreciably. Measurements taken 1 week after the cessation of haemolysis indicated a decrease of osmotic fragility. These levels were even lower than those observed before the start of the experiment. The TM injections did not appear to alter the osmotic fragility in any of the sheep studied.

Urinary Cu

The most marked change observed was a 4–7-fold increase in concentration of Cu as well as an increase in Cu excreted/24 h in the urine collected during the period before haemolysis.

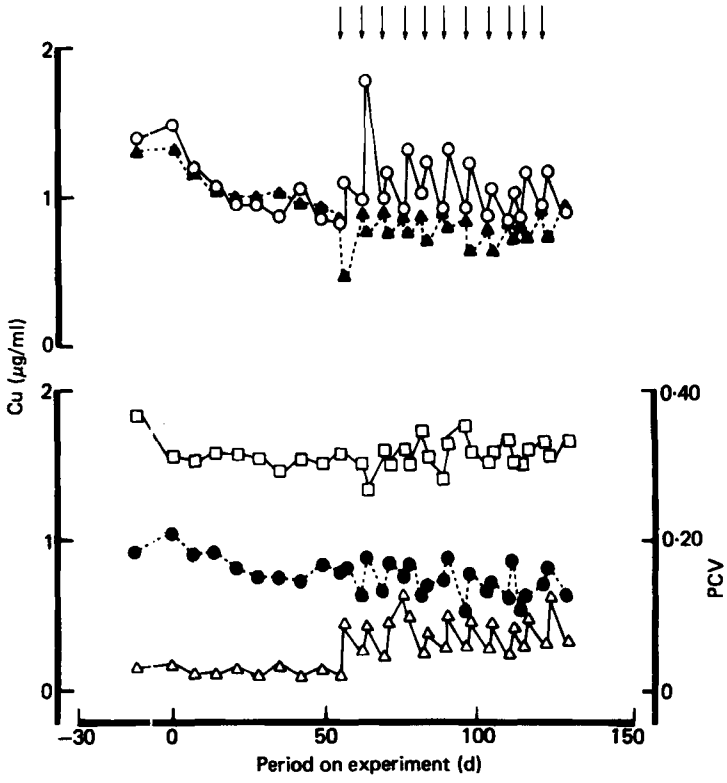

Fig. 3. Whole-blood (●- - -◗), plasma (○—○), plasma trichloroacetic acid(TCA)-soluble (△- - -△) and direct-reacting (▲—▲) copper levels and packed cell volume (PCV) (□—□) of sheep no. 229 (group 4) measured throughout the course of the experiment. ↓, TM injections.

These rose from a mean of 0·45 μg/ml and 180 μg/24 h respectively in the first week of the experiment to 1·16 μg/ml and 1067 μg/24 h in the week prior to haemolysis. Urinary Cu was markedly increased during the periods of haemolysis with a mean of 2315 μg/24 h. These increases were mostly due to an increase in the concentration of Cu in urine (mean 4·71 μg/ml) although at times the volume excreted was also greater. TM injections only increased the Cu concentration in urine in sheep which were continuously dosed with Cu (group 3). These levels rose from 0·30 μg/ml at the start of the experiment to 1·63 μg/ml at its termination.

Urine analysis

pH. Values for sheep in all groups varied from 6 to 8, until haemolysis when it became more acidic (pH 5–6). The pH returned to normal once the crisis was over.

Glucose and ketones. These compounds were not detected in any of the samples of urine collected.

Protein. Never more than a trace of protein was found in urine of any sheep until the 2nd or 3rd day of haemolysis when the levels rose to as much as 1000 mg/l but returned to normal once the crisis was over.

Bilirubin. Bilirubin was absent in samples of urine taken before haemolysis but appeared

during the periods of haemolysis. Bilirubin was detected only in red-tinged urine and could not be detected in urine of normal colour.

Haemoglobin. The presence of haemoglobin in urine, could not be detected until, or, in one sheep, on the day before haemolysis. In the latter sheep (no. 238) a sample of urine taken 1 d before haemolysis was of normal colour but was positive for blood.

DISCUSSION

The changes of Cu levels in blood and plasma presented here are similar to those reported in sheep given dietary supplements of Mo and SO_4 (Dick, 1956; Suttle & Field, 1968; Smith *et al.* 1968; Marcilese *et al.* 1969, 1970; Smith & Wright, 1975*a*, *b*). Hence it could be assumed that jugular infusion of TM adequately simulates the post-absorptive supply of Mo and S possibly in the form of TM (Dick *et al.* 1975). But since a large dose was rapidly administered directly into the systemic circulation a much greater response than previously recorded was seen. This was especially so in the concentration of Cu in WB and different fractions of plasma.

Before the start of injections of TM, the levels of Cu in WB, plasma, and TCA-soluble fraction were within normal limits in all sheep. But the level of DR Cu of sheep dosed with Cu was at least twice that of the animals in group 4 which did not receive Cu. Before the injection of TM the Cu excreted in the urine was much greater in sheep dosed with Cu than in the control animals of group 4, this difference being most marked in sheep near to the haemolytic crisis, indicating the possibility that at least a part of the DR Cu was ultrafilterable. Several groups of workers have reported that Cu in the DR fraction is in a loosely-bound form and some of it may be ultrafilterable (Gubler *et al.* 1953; Gaballah *et al.* 1965; Smith *et al.* 1968; Marcilese *et al.* 1969). If this is so the increased amounts of Cu excreted in urine at haemolysis and after commencement of TM injections could also be due to the very high levels of DR Cu found in plasma during this time. The increase in the amount of Cu excreted in urine after the commencement of TM injections was due to an increase in the concentration of Cu in urine as well as an increase in the volume of urine. Similar results were observed in a preliminary investigation, where we observed that TM injections increased excretion of Cu in urine but not in faeces (Gooneratne, 1979). This result is in agreement with the findings of Marcilese *et al.* (1970) who gave sheep Mo and SO_4 in the diet.

It has been shown that for 1 or 2 d before the haemolytic crisis plasma Cu is greatly increased and far exceeds WB Cu and erythrocyte Cu levels (Ishmael *et al.* 1972). It has also been shown that an increase in erythrocyte Cu occurs at least 24 h before haemolysis (Ishmael *et al.* 1972; Gooneratne & Howell, 1980) indicating that the excess Cu that was present in plasma before haemolysis had entered erythrocytes. This excess of Cu in the erythrocytes directly contributes to haemolysis. In the present study, irrespective of this group to which the sheep belonged, each injection of TM increased plasma Cu to very high levels, but this Cu did not enter the erythrocytes possibly because most of it was TCA-insoluble. It has been shown by Bremner (1976) that Cu is firmly bound to this novel protein fraction, in which Cu and Mo are closely associated. It has been proposed by several workers (McCosker, 1968; Ishmael *et al.* 1972) that in chronic Cu poisoning the release of Cu from the liver into the bloodstream is followed by an uptake of Cu by the erythrocytes, and that it is necessary for a high concentration of blood Cu to be maintained for 24–48 h in order to cause haemolysis (Todd & Thompson, 1964). Howell & Gopinath (1977) found that haemolysis occurred after four daily injections of $CuSO_4$ giving the daily equivalent of 20 mg Cu. This is a similar result to that obtained by Todd & Thompson (1964) using Cu acetate.

However, Ishmael & Gopinath (1972) found that haemolysis occurred within 24 h of a single intravenous injection of 50 mg Cu given as $CuSO_4$. The WB and plasma Cu content of these sheep was quickly and markedly elevated. Erythrocyte Cu was not measured but 3 h after injection WB Cu was greater than plasma Cu thus indicating an uptake of Cu by erythrocytes. In our sheep given TM injections, very high concentrations of Cu in WB and plasma were recorded for long periods of time without haemolysis occurring but this Cu was not taken up by the erythrocytes. We presume that the presence of excess DR Cu in a TCA-soluble form in plasma rather than an increase in total plasma Cu would be responsible for the increased uptake of Cu by erythrocytes that occurs in untreated sheep at the time of the haemolytic crisis. The ability of TM to increase WB and plasma Cu without initiating haemolysis simulates the in vitro studies of Goldberg *et al.* (1956) that were confirmed by Thompson & Todd (1976) who found that haemolysis only occurred when large amounts of Cu (750 mg/l) were added to erythrocytes in plasma. Hence it could be assumed that it is not the increase in WB Cu *per se* but the increase in DR Cu present in a TCA-soluble form which predisposes erythrocytes to take up more Cu and undergo lysis.

In the present study Cp levels were variable. It has been shown in rabbits (Gahallah *et al.* 1965) that high levels of Mo and SO_4 in the diet resulted in a decreased uptake of Cu and an inhibition of Cp synthesis. Mills *et al.* (1978) observed similar results when giving TM to rats; 2 μg Mo, as TM/g diet was offered to rats for 5 weeks in a diet containing 3 μg Cu/g. In these animals liver Cu stores were decreased by 30% and plasma Cp by 60%. On increasing the amount of Mo to 4 μg/g diet, liver Cu was decreased by 50% and this concentration of Mo completely abolished the Cp activity in plasma. Although the Cu levels in the livers of sheep given TM in the present experiment were decreased by up to 60% (Gooneratne *et al.* 1981), its effect on plasma Cp was minimal. Similar results have been published for sheep by Smith & Wright (1975*b*) who did not observe a change in the absolute concentration of Cp when the diet was supplemented by Mo and SO_4. It appears from the present study that in sheep, liver Cu stores have to be greatly reduced before Cp synthesis is affected. However, Marcilese *et al.* (1969) observed a decrease in the Cp Cu levels in sheep given Mo and SO_4, but the levels reported by these authors may have been an underestimation as they assumed Cp Cu to represent the difference between total plasma Cu and DR Cu. Studies of the distribution of Cu in the plasma of Mo-supplemented sheep and guinea-pigs have shown that Cp and DR Cu do not account for all of the plasma Cu (Suttle & Field, 1968; Smith *et al.* 1968). The occurrence of a non-Cp and non-albumin Cu has been demonstrated (Bremner & Young, 1978). This residual Cu fraction precipitated by TCA contains both Mo (Smith & Wright, 1975*b*) and protein (Smith & Wright, 1974, 1975*a*). The origin and metabolic significance of this fraction is yet to be established.

Marked changes in PCV were not seen in any sheep except at haemolysis when levels as low as 9% were observed. PCV of sheep in groups 1B and 2B returned to normal levels 2–3 weeks after haemolysis. TM did not appear to have any adverse effect on the erythropoetic activity.

The osmotic fragility measurements on erythrocytes give an assessment of the permeability and stability of erythrocyte membranes and also reflect their ability to take up water without lysis. It has been known for many years that heavy metals affect erythrocyte fragility (Lessler & Walters, 1973). The only report on the osmotic fragility measurements on chronic Cu-poisoned sheep (Froslie & Norheim, 1976) gave levels 20 d before and at haemolysis but failed to report on the levels during the most critical period immediately before haemolysis. In the present study, although weekly measurements were made, marked changes were not observed except at haemolysis, when osmotic fragility was increased. Osmotic fragility was still within normal limits 1 week before haemolysis. Adams *et al.* (1979)

observed a similar increase in osmotic fragility without haemolysis on incubating erythrocytes in 0·60 mM-$CuSO_4$ solution. The administration of TM did not appear to affect the permeability of erythrocytes.

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