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Mémoire.

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CATIONS IN BODY FLUIDS OF *TRYPANOSOMA BRUCEI* IN INFECTED RABBITS

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SUMMARY

Serum copper, magnesium, zinc, calcium and ionized calcium (Ca^{++}) concentrations were compared in 6 rabbits infected with *Trypanosoma brucei brucei* and 5 uninfected rabbits. There was a significant depletion of Mg and Zn and a significant increase in Cu from about day 10 of infection to the end. There was no change in plasma total calcium or free diffusible calcium. There was a development of kidney damage as shown clinically by proteinuria and urinary loss of magnesium and zinc, and histologically by the observation of hypercellularity in the glomeruli and tubular degeneration. Our findings thus indicate that trypanosomiasis causes kidney damage which may be responsible for the depletion of the cations seen in the study. Some of the clinical manifestations associated with African trypanosomiasis such as convulsions, anaemia, electrocardiographic changes and splenomegaly may therefore be related to these cation changes.

RÉSUMÉ : **Cations dans les liquides somatiques de *Trypanosoma brucei* chez le lapin.**

Une comparaison de concentrations sériques du magnésium, du zinc et du calcium total et ionisé a été faite entre six lapins infectés par *Trypanosoma brucei brucei* et cinq lapins non infectés. On a pu montrer une baisse significative du magnésium et du zinc et une augmentation significative du cuivre à partir du dixième jour. Il n'y avait pas de modification du calcium total plamatique ni du calcium libre. Une atteinte rénale s'est traduite cliniquement, proétinurie associée à une perte urinaire du magnésium et du zinc, et histologiquement, hypercellularité des glomérules et dégénération tubulaire. Ces observations indiquent que la trypanosomiase provoque des lésions rénales responsables de la baisse des cations sériques rapportée ici. Il est donc possible que des manifestations cliniques de la trypanosomiase humaine comme les convulsions, l'anémie, les perturbations électrocardiographiques et la splénomégalie puissent être rattachées, au moins partiellement, aux variations de ces cations.

INTRODUCTION

The protozoan parasites *Trypanosoma brucei gambiense* and *T. b. rhodesiense* are the causative agents of a human disease, African sleeping sickness, and the closely related *T. b. brucei* is one of several trypanosomes which cause a similar disease in animals. Ionized calcium (Ca^{++}) has been shown to be responsible for the synergistic action in blood and serum of three trypanocidal agents: SHAM/glycerol, melarsoprol and iodoacetamide (Clarkson and Amole, 1982). In view of this fact, we have thus attempted to quantify plasma ionized calcium levels in rabbits infected with *T. b. brucei.*

An opportunity was taken to measure the concentration of other metal ions since many studies have shown changes in the serum levels of some of these metals during a variety of viral and bacterial infections (Beisel, 1977; Solomon and Keusch, 1981; Pekarek, 1972; Beisel *et al.*, 1974).

In this study, the serum levels of copper, zinc, magnesium, calcium and plasma levels of ionized calcium were measured in rabbits infected with *T. b. brucei* and uninfected rabbits.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

All experiments were performed on 11 adult male New Zealand white (NZW) rabbits, weighing between 1.6 and 2.3 kg at the beginning of each experiment. The rabbits were bred in the animal house and given high protein rabbit pellets ad lib. The animals were divided into two groups as follows: Group A, uninfected controls; and Group B, infected with trypanosomes. For the period

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of the experiment, all rabbits were given intramuscular injections of broad-spectrum penicilin-streptomycin antibiotic (Hoechst Pharmaceutical Co. Nig.) to limit the occurrence of secondary bacterial infection (Ikede, 1979).

Maintenance of parasites

Parasites were maintained in the laboratory by tail blood transfer in mice.

Infection of rabbits

Trypanosoma brucei brucei (NSUKKA/62/NITR/8 strain) was used for infection. This strain produces a chronic infection in rabbits lasting 4 weeks and is usually characterized by a terminal high parasitemia. Rabbits were infected by intraperitoneal (IP) infection of 10×10^6 trypanosomes.

Hematological determinations

Parasitemia was determined everyday by counting the number of trypanosomes in 100 fields ($\times$ 40 objective) of Giemsa stained blood smears. Packed cell volume (PCV) was determined by the capillary microhematocrit method (Dacie and Lewis, 1984).

Blood collection

10 ml of blood samples from the ear vein were taken without anaesthesia and the blood allowed to clot for one hour at room temperature. Serum was then obtained after centrifugation of clotted blood at 3,000 r. p. m. for 10 minutes in a portable centrifuge (Gallenkamp, UK). Blood for plasma studies were collected, using heparin (approximately 125 U/ml of blood) as an anticoagulant. Blood samples were collected on day 0, 10, 20 and 30 post-infection and were stored in sterile glass tubes at $-$ 20° C.

Urine collection

24 hours urine samples were collected from infected and uninfected control rabbits in metabolic cages at day 0, 10, 20 and 30 post-infection. Urine samples for measurement of metal ions were centrifuged at 3,000 r. p. m. for 10 minutes at room temperature, and the supernatants were stored in sterile glass tubes at $-20°$ C. Urine samples were also collected for total protein, albumin, urea and creatinine determinations which were first clarified with an equal volume 1.0 N HCl.

Measurement of magnesium, calcium, zinc and copper in serum and urine

Serum and urine concentrations of all the elements were determined in infected and uninfected rabbits using a Perkin-Elmer 305B atomic absorption spectrophotometer. In the determination of calcium and magnesium, samples were diluted 1 : 10 with deionized water and penthanium oxide was added to correct for phosphate interference with magnesium and calcium. The other trace elements, zinc and copper, were determined in undiluted samples.

Measurement of ionized calcium (Ca^{++}) in fresh whole blood

Physiologically active ionized calcium concentrations were measured in fresh heparinized whole blood using a calcium analyzer (Ionetics inc.). This is an ion-specific electrode system designed esxclusively for determination of ionized calcium. Samples were measured immediately after collection to avoid post-collection fall in pH which could cause a shift in the ability of albumin to bind calcium ions.

Estimation of total protein in serum and urine

Total protein in serum samples was estimated by the Biuret method (Kingsley, 1972) and by the turbidimetric method (Meuleman, 1960) in urine samples.

Estimation of albumin in serum and urine

Total albumin concentration in serum saples was estimated by the bromo cresol-green (BCG) method (Doumas and Biggs, 1972). Albumin in urine was estimated by Manuel's method (Manuel *et al.*, 1970; Flaichare *et al.*, 1983).

Estimation of globulin

Globulin concentration in serum samples was calculated by total protein minus albumin difference.

Estimation of plasma creatinine

Plasma creatinine was estimated by the alkaline picrate method of Jaffe as described in Varley (1969).

Estimation of urine, urea and creatinine

Urea was estimated after diluting urine 1 : 100 with distilled water by the diacetyl monoxime method as described in Henry *et al.* (1974). Creatinine, similarly diluted 1 : 100 in distilled water, was determined by the alkaline picrate method as described in Varley (1962).

Histopathological examination

Kidney tissue was fixed at the end of experiment in buffered formalin and examined after hematoxylin and eosin staining of the tissues.

Statistics

All data were represented by the means ($\pm$ standard error of the mean) for that group. Statistical significance was evaluated using Student's *t*-test for unpaired data and difference were considered significant when $P < 0.05$.

RESULTS

Course of parasitemia

Figure 1 shows the parasitemia of infected rabbits. The parasites first appeared in the blood stream at day 13 post-infection, and the course of infection showed that the parasitemia reached a peak after 16 days of infection, followed by the first parasitemic crisis. A low parasitemia was then maintained from day 19 through to day 23. The second peak was observed at the 26th day of infection, followed by another crisis, which occurred at day 28. The highest level of parasitemia which was the third peak was observed on day 30, followed immediately by death.

Packed cell volume

The infected rabbits became anemic from the 10th day of infection through to the 20th day when the most signi-

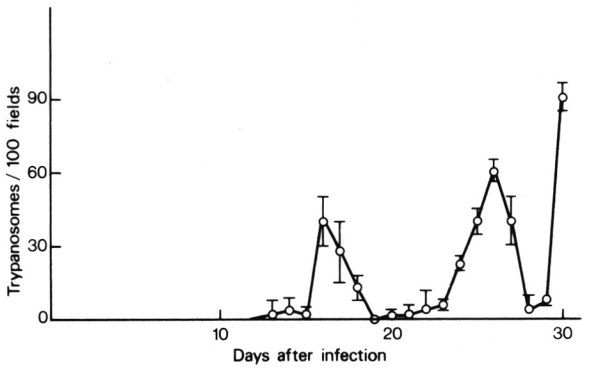

FIG. 1. — Course of parasitemia during *T. b. brucei* infection. Mean ± SE.

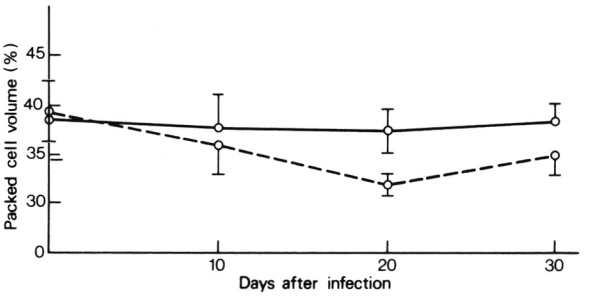

FIG. 2. — Packed cell volume during *T. b. brucei* infection. Mean ± SE for uninfected control rabbits (0 0) and infected rabbits (0---0).

ficant anemia occurred *(fig. 2)*. Though the packed cell volume improved between days 21 and 30, it was still significantly decreased.

Body weight

There was a mean increase of about 140 ± 0.069 or 7 % in body weight after 30 days of infection, while uninfected control rabbits increased in mean body weight by about 220 ± 0.11 g or 11 % during the course of the experiment.

Serum proteins

In infected rabbits, serum total proteins became markedly increased as from the 10th day of injection as shown in *table I.* There was however, no significant change in serum albumin levels during the course of infection. There was also a steep increase in globulin concentration (as determined by total protein minus albumin difference) also beginning at about the 10th day of infection.

TABLE IA. — *Total serum protein, albumin and globulin levels in infected and uninfected rabbits.*

	Total Protein (g/l)			
	Day 0	*Day 10*	*Day 20*	*Day 30*
Uninfected Controls (n = 5)	60.5 ± 3.50	61.4 ± 2.40	61.8 ± 3.40	62.7 ± 4.80
Infected (n = 6)	62.1 ± 1.90 (*a*)	70.1 ± 3.50 (*a*)	75.4 ± 10.1 (*a*)	81.5 ± 5.20 (*a*)
	Albumin (g/l)			
Uninfected Controls (n = 5)	47.5 ± 8.50	47.1 ± 4.90	46.5 ± 2.60	50.1 ± 4.40
Infected (n = 6)	48.6 ± 4.80 (*c*)	49.4 ± 4.90 (*c*)	47.1 ± 3.60 (*c*)	46.6 ± 6.20 (*c*)
	Globulin (g/l)			
Uninfected Controls (n = 5)	13.0 ± 5.30	13.0 ± 4.50	15.3 ± 3.80	12.6 ± 3.70
Infected (n = 6)	13.5 ± 5.80 (*c*)	20.6 ± 6.90 (*a*)	31.1 ± 12.0 (*a*)	28.6 ± 15.10 (*a*)

TABLE IB. — *Plasma creatinine levels in infected and uninfected rabbits.*

	Créatinine (mg per 100 ml)			
Uninfected Controls (n = 5)	0.82 ± 0.20	0.90 ± 0.22	0.98 ± 0.23	0.84 ± 0.21
Infected (n = 6)	0.80 ± 0.18 (*c*)	0.87 ± 0.17 (*c*)	0.86 ± 0.19 (*c*)	1.56 ± 0.28 (*b*)

Mean ± SE, (*a*) t-test to control, P 0.01; (*b*) t-test to control, P 0.05; (*c*) No significant difference to control.

TABLE II. — *Urine, protein, urea and creatinine levels in infected and uninfected rabbits.*

	Volume (ml/24 hrs)	
	Day 20	*Day 30*
Uninfected Controls (n = 5)	37.5 ± 29.53	20.2 ± 5.42
Infected (n = 6)	19.7 ± 17.58 (*a*)	13,63 ± 14.38 (*b*)
	Protein (mg/24 hrs. volume)	
Uninfected Controls (n = 5)	6.9 ± 7.2	4.8 ± 1.2
Infected (n = 6)	36.3 ± 30.2 (*a*)	12.7 ± 8.07 (*b*)
	Albumin (mg/24 hrs. Volume)	
Uninfected Controls (n = 5)	0.17 ± 0.06	0.14 ± 0.05
Infected (n = 6)	0.15 ± 0.03 (*c*)	0.13 ± 0.02 (*c*)
	Urea (gr per 24 hrs. Volume)	
Uninfected Controls (n = 5)	0.56 ± 0.32	1.05 ± 0.95
Infected (n = 6)	5.56 ± 3.28 (*a*)	2.66 ± 2.26 (*c*)
	Creatinine (gr/24 hrs. Volume)	
Uninfected Controls (n = 5)	0.12 ± 0.05	0.13 ± 0.06
Infected (n = 6)	0.13 ± 0.14 (*c*)	0.05 ± 0.04 (*b*)

Mean ± SE; (*a*) t-test to control, P 0.01; (*b*) t-test to control, P 0.05; (*c*) No significant difference to control.

Plasma creatinine

Plasma creatinine concentration was not significantly different in both the infected and uninfected groups until at the end of infection. At day 30, the plasma creatinine was significantly higher in the infected rabbits than in the uninfected rabbits.

Urine proteins, urea and creatinine

There was a significant reduction in the mean 24 hour urine volume of the infected rabbits as compared with the uninfected rabbits on day 20 and 30 *(table II)*. The 24 hour urine protein concentration was significantly higher in the infected than uninfected rabbits on both days, even though the albumin concentration was very low in both groups of rabbits *(table II)*. The 24 hour urine urea concentration was significantly higher at day 20 in the infected than the uninfected rabbits. The day 30 urine urea values were however not significantly different *(table II)*. The 24 hour urine creatinine concentration showed no difference between the two groups at day 20, but it was significantly lower for the infected than the uninfected rabbits at day 30.

Alterations in serum levels of cations

The concentrations of cations in sera of normal and infected animals are shown in *tables III and IV*. Significant depletion of Mg and Zn were first detected at about day 10 and which continued throughout the infection *(table III)*. Unlike the declines in Mg and Zn concentrations, serum copper levels increased during the course of infection *(table III)*. There was no significant change in the serum concentration of total calcium (Ca) or in the plasma concentration of ionized calcium (Ca^{++}), as shown on *table IV*.

TABLE III. — *Serum magnesium, zinc and copper in infected and uninfected rabbits.*

	Magnesium (mg/dl)			
	Day 0	*Day 10*	*Day 20*	*Day 30*
Uninfected Controls (n = 5)	2.07 ± 0.18	2.07 ± 0.16	1.99 ± 0.00	2.05 ± 0.15
Infected (n = 6)	2.09 ± 0.09 (*c*)	1.52 ± 0.20 (*a*)	1.57 ± 0.19 (*a*)	1.56 ± 0.15 (*a*)
	Zinc (μg/dl)			
Uninfected Controls (n = 5)	165 ± 24.00	165 ± 22.00	166 ± 18.0	165 ± 20.0
Infected (n = 6)	167 ± 22.00 (*c*)	129 ± 15.00 (*a*)	122 ± 13.0 (*a*)	142 ± 20.00 (*a*)
	Copper (μg/dl)			
Uninfected Controls (n = 5)	104 ± 5.00	110 ± 4.00	121 ± 6.00	128 ± 6.00
Infected (n = 6)	102 ± 7.00 (*c*)	199 ± 72.00 (*a*)	197 ± 63.00 (*a*)	187 ± 56.00 (*a*)

Mean ± SE; (*a*) t-test to control, P 0.01; (*b*) t-test to control, P 0.05; (*c*) No significant difference to control.

TABLE IV. — *Serum calcium (total and ionized fractions) in infected and uninfected rabbits.*

	Total calcium (mg(dl) (mean ± SE)			
	Day 0	*Day 10*	*Day 20*	*Day 30*
Uninfected Control (n = 5)	8.35 ± 0.50	8.70 ± 1.20	8.67 ± 1.70	8.54 ± 2.06
Infected (n = 6)	8.81 ± 0.97 (*c*)	9.83 ± 1.94 (*c*)	9.64 ± 0.89 (*c*)	9.72 ± 1.20 (*c*)
	Ionized calcium (mg/dl) (mean ± SE)			
Uninfected Controls (n = 5)	3.16 ± 0.24	3.24 ± 0.44	3.28 ± 0.60	3.20 ± 0.36
Infected (n = 6)	3.04 ± 0.68 (*c*)	3.64 ± 0.44 (*c*)	4.04 ± 0.52 (*c*)	4.00 ± 0.32 (*c*)

Mean ± SE. (*c*) No significant difference to control.

TABLE V. — *Urinary excretion of magnesium, zinc and copper by infected and uninfected rabbits.*

	Magnesium (mg/24 hrs)			
	Day 0	*Day 10*	*Day 20*	*Day 30*
Uninfected Controls (n = 5)	0.52 ± 0.18	0.67 ± 0.21	0.87 ± 0.32	0.59 ± 0.22
Infected (n = 6)	0.57 ± 0.26 (*c*)	1.98 ± 1.55 (*a*)	1.37 ± 1.40 (*b*)	0.933 ± 0.77 (*c*)
	Zinc (μg/24 hrs)			
Uninfected Controls (n = 5)	139 ± 41.0	133.2 ± 84	124 ± 89.19	127 ± 76.20
Infected (n = 6)	141 ± 52.0 (*c*)	601 ± 5.0 (*a*)	176.2 ± 91.4 (*a*)	180.6 ± 53.47 (*a*)
	Copper (μg/24 hrs)			
Uninfected Controls (n = 5)	10.95 ± 12.70	12.12 ± 7.60	13.21 ± 8.2	11.01 ± 8.70
Infected (n = 6)	11.52 ± 9.60 (*c*)	15.1 ± 8.20 (*c*)	19.23 ± 16.19 (*b*)	12.30 ± 6.56 (*c*)

Mean ± SE; (*a*) t-test to control, P 0.01; (*b*) t-test to control, P 0.05; (*c*) No significant difference to control.

Urinary excretion of magnesium, zinc and copper by infected rabbits

Excretion of magnesium and zinc in the urine by infected rabbits was greater than that of uninfected rabbits *(table V)*. Excretion of both magnesium and zinc were greatest on day 10 of infection, but significant rate of excretion continued throughout the course of infection. There was no evidence of greater excretion of copper by infected rabbits, as the difference in levels of urinary copper in both infected and uninfected rabbits was not statistically significant *(table V)*. There was no trace of Ca or Ca^{++} in the urine of both infected and uninfected rabbits.

Histopathological examination of organs

Figure 3 shows a section of the renal tissue showing glomeruli with prominent basement membrane and mesangial hypercellularity. The proximal and distal tubules are poorly

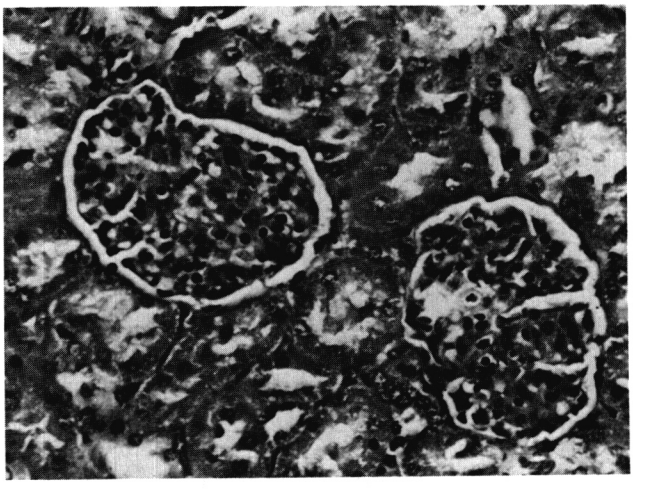

FIG. 3. — Kidney section from a rabbit infected with *T. b. brucei* after 30 days of infection. Renal glomeruli with widened tufts and mesangial hypercellularity. × 200.

preserved. The interstices contain congested vessels and chronic inflammatory cells. This picture is consistent with glomerulonephritis.

DISCUSSION

The changes observed in the infected rabbits revealed a complex picture which provided evidence of increased synthetic activity of the liver and a development of kidney damage. Clinical evidence of probable renal damage is provided by the significant proteinuria observed in infected rabbits by day 20. The lack of significance in the urine and plasma creatinine concentrations between the infected and uninfected rabbits suggest that if there is kidney damage at this stage it may have been tubular or from an insignificant number. However, by day 30, the significant fall in urine creatinine and the significant rise in plasma creatinine concentrations are clearly indicative of acute glomerulonephritis. This is strongly supported histologically by glomerular damage. There was also histological evidence of hepatomegaly (liver cell hypertrophy not shown on the result), a consistent finding in African trypanosomiasis and may be responsible for the marked globulinaemia. Although most of these are gamma globulins, probably immunoglobulins of the IgM class (Neilsen *et al.,* 1978), alpha and possibly beta globulin fractions may also be involved because high plasma ceruloplasmin levels (alpha, globulin) are believed to be responsible for the hypercupremia seen in many infections diseases (Beisel *et al.,* 1974). It is therefore possible that the hypercupremia observed in our injected animals may be partly due to increased plasma ceruloplasmin levels. There was a relatively uniform and consistent pattern of increased copper in conjunction with proportionately low urinary copper excretion. Work is currently going on in our laboratory to measure plasma levels of ceruloplasmin in trypanosomiasis.

Since past studies have shown that renal damage such as glomerulonephritis, renal insufficiency and tubular lesions can lead to hypomagnesemia and hypozincemia (June *et al.,* 1986) and since all these causes have been presented in this study and previously been associated with African trypanosomiasis (Rickman and Cox, 1979), it is thus not too unreasonable to hypothesize from the present study that hypomagnesemia and hypozincemia may have been partially derived from kidney damage. The majority of the magnesium and zinc lost may be the diffusible, free, most physiologically active forms since there was no evidence of loss of serum albumin during infection, indicating that the albumin-bound cations were not lost. The proteinuria observed during infection may thus be due to leakage from renal tubular damage as well as glomerular basement membrane fragments (Baron, 1982).

Among the most prominent clinical features of hypomagnesemia in man and animals are neurologic disturbances brought about by dysfunction of the central nervous system as manifested by convulsions (Wong *et al.,* 1983). Other clinical manifestations of hypomagnesemia include electrocardiographic changes and arythmias (Tackett, 1986), myocardial infarction (Speich *et al.,* 1981); vascular changes (Kulka and Gale, 1963); eosinophilia, degranulation of mast cells, release of histamine and splenomegaly (Hungerford, 1964). Most of these manifestations have also been seen in human and animal African trypanosomiases. These include convulsions (Markell and Voge, 1981); electrocardiographic changes (Murray *et al.,* 1974); release of pharmacologically active substances (Wright, 1979); and increased vascular permeability (Banks, 1980). Some of these manifestations such as splenomegaly, eosinophilia, and dermatologic lesions of the eyelids, nares and testicles were observed in the present study. It is therefore reasonable to assume that these symptoms in our infected animals may be partly associated with hypomagnesemia.

The serum zinc depletion in this study has been partially attributed to renal damage. In addition, depletion may have also resulted from hemolysis, a trypanosomiasis-induced hemolytic anemia has been found as a consistent and significant finding in humans (Woodruff *et al.,* 1973) and animals (Amole *et al.,* 1982). The notion that continued hemolysis can be responsible for hypozincemia is supported by the work of Prasad *et al.* (1975) who reported that continued hemolysis in patients with sickle cell disease led to a zinc deficient state. In their study, plasma zinc was decreased and urinary zinc excretion was increased in sickle cell anemia patients as compared with controls. Another evidence to support this view is the report that parasitic diseases such as hookworm infections that cause blood loss do contribute to deficiency of zinc (Prasad *et al.,* 1963). Impairment of immune functions (Fraker *et al.,* 1982), testicular degeneration, iron deficiency anemia and hepatosplenomegaly (Prasad, 1983) are however, among the most important consequence of zinc depletion. This hypozincemia we have discovered in this study may have contributed to the various symptomatologies that have long been reported in African trypanosomiasis such as immunosuppression (Amole *et al.,* 1982), testicular degeneration (Ikede, 1979) and hepatosplenomegaly (Amole *et al.,* 1982). Furthermore, we would like to state that a similar finding of immune status impairment due to hypozincemia has also been reported during *Trypanosoma cruzi* infection (Fraker *et al.,* 1982).

There was a uniform pattern of unalteration in the metabolism of calcium (total and ionized) during infection in this study. The concentration of both total and ionized fractions remained unchanged in the serum and plasma throughout the infection. Moreover, there was no evidence of calcium (total and ionized) in the urine of both infected and uninfected rabbits.

In summary, this paper shows that *T. brucei* infection

results in hypomagnesemia, hypozincemia and hypercupremia but no change in plasma calcium levels. There was also evidence of tubular damage and terminal acute glomerulonephritis. Our findings would suggest that the hypercupremia observed may be due to increased hepatic synthesis of ceruloplasmin and that the some of the clinical manifestations often associated with African trypanosomiasis such as convulsions, anemia, electrocardiographic changes and pharmacologically active substances may be associated with observed hypozincemia and hypomagnesemia.

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