Trypanosoma congolense
I. Clinical Observations of Experimentally Infected Cattle

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Wellde, B., Löttzsch, R., Deindl, G., Sadun, E., Williams, J., and Warui, G. 1974. Trypanosoma congolense: I. Clinical observations of experimental infection in cattle. Experimental Parasitology 36, 6-19. The course of disease was studied in 8 cattle infected with Trypanosoma congolense. Although the onset of patency was dependent on the numbers of infecting organisms, the duration of the infection was not. High fevers were present on the day of or the day after initial patency. Succeeding peaks of parasitemia, and a progressive weight loss of over 30% occurred. A decrease in packed cell volume (PCV) beginning the first week after infection was observed. Early in the course of the developing anemia, many polychromatophilic erythrocytes and occasional normoblasts were found in the blood. A leucopenia persisted for the duration of the disease. Total serum protein concentrations fell sharply during the first 5 weeks of infection, then gradually increased to low normal levels. Serum albumin levels followed a similar pattern for the first 5 weeks, and remained at a relatively low level. Although gamma globulin levels also declined during the first 5 weeks, their levels gradually surpassed those of preinfection samples. No marked changes in serum glucose were noted. A mild elevation of serum urea nitrogen values occurred early during infection, but subsided. The animals dying early after infection developed elevated total bilirubin levels.

INDEX DESCRIPTORS: Trypanosoma congolense; Bovine trypanosomiasis; Clinical studies; Packed cell volume; Erythrocytes; Proteins, serum; Nitrogen, serum; Bilirubin; Cattle; Leukocytes; Enzymes; Transaminase; Glutamic oxaloacetic; Immunization.

INTRODUCTION

The extent and severity of the problems caused by trypanosomiasis of domestic animals in Africa have been well known for many years, and various aspects of the disease have received considerable attention by researchers. Although many pathological changes occurring in host animals have been attributed to trypanosomiasis, in some instances the findings appear to be of a fragmentary and inconclusive nature. This may be due partially to differences in virulence of separate species of trypanosomes and of strains within one species, as well as by host factors such as age, condition, sex, breed, and the presence of concomitant infections. Recently, the pathology of trypanosomiasis has been reviewed by Krampitz (1970), Fiennes (1970), Goodwin (1970), and Losos and Ikede (1972).

In the course of experimental immunization studies, we had an opportunity to
document the course of disease in a group of cattle infected with Trypanosoma congolense under similar conditions. Hematologic, serum chemical, and serologic procedures were conducted at regular intervals following infection, and histopathological observations were made at death.

Materials and Methods

Experimental Animals

Two Hereford steers and 9 nonpregnant Hereford heifers, weighing from 505 to 845 pounds, were selected from the Veterinary Department herd at Kabete, Kenya, and used in these studies. The age of the animals ranged from 1.3 to 2.8 yr. The cattle were stabled at the Trypanosomiasis Research Laboratory in Kabete, and grazed in a nearby pasture during the day. All animals were weighed and dipped in an acaricide weekly.

Parasites

The Trans Mara I strain of T. congolense which was isolated from an infected cow in the Trans Mara area near the Kenya-Tanzania border in 1966, was used throughout the experiment. The strain had been stored as a stabilitate in dry ice and maintained in cattle by blood passage. For inoculation, trypanosome concentrations were calculated from counts of the numbers of trypanosomes per 10,000 erythrocytes (RBC's) on thin blood smears and total RBC counts per mm³. Blood for inoculations was diluted with phosphate buffered saline (pH 7.8) containing 5% glucose, and injected into the jugular vein. Parasite concentrations of individual animals were determined daily by counting the number of trypanosomes in 50 oil immersion fields containing approximately 200 RBC's per field in thin blood smears; if no parasites were seen at this level, 200 oil immersion fields on thick blood smears were observed.

Collection of Specimens

Thick and thin blood smears were usually obtained 6 days per week at approximately 9 AM by puncturing the tip of the tail. Blood for biochemical, hematological, and serological determinations was collected (from the jugular vein) in tubes, allowed to clot overnight at 4 C and stored at -20 C until assayed. Animals found dead or moribund were transported to the Diagnostics Section of the Veterinary Research Laboratory for post mortem examinations. Impression smears were made from the cut surfaces of organs of recently killed animals. Tissue specimens were collected in buffered formalin for histopathological evaluation.

Hematology

Erythrocytes and leukocytes were counted in a Neubauer Chamber using Hayem's solution as diluent for erythrocytes, and 1% acetic acid solution for leukocytes. The microhematocrit method was used to determine packed cell volumes (PCV). Thin blood smears from each animal were fixed in methanol before staining with Giemsa's stain. Differential white cell counts were done by classifying 100 leukocytes on thin smears.

Serum Chemistry

Serum glutamic oxaloacetic transaminase (SGOT), glucose, creatinine, and total protein levels were determined by the methods described by Williams et al. (1966). Levels of urea nitrogen were determined by a modification of the urease method (Gentzkow 1942). Total serum bilirubin values were obtained using a modification of the method described by Jendrassik and Grof (1936). Serum electrophoresis on cel-
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**TABLE I**

Trypanosoma congolense Infections in Cattle

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Size of inoculation</th>
<th>1st Day of patency</th>
<th>Duration of infection (days)</th>
<th>Terminal conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I (donors)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62101, ZM</td>
<td>$5 \times 10^8$</td>
<td>1</td>
<td>80</td>
<td>Persistent parasitemia, icteric; died</td>
</tr>
<tr>
<td>1381.1F</td>
<td>$3 \times 10^8$</td>
<td>1</td>
<td>216</td>
<td>Recrudescing parasitemia, recumbent; killed</td>
</tr>
<tr>
<td>1621F</td>
<td>$2 \times 10^7$</td>
<td>2</td>
<td>213</td>
<td>Recrudescing parasitemia; died suddenly</td>
</tr>
<tr>
<td>6193M</td>
<td>$2 \times 10^7$</td>
<td>3</td>
<td>52</td>
<td>Persistent parasitemia; died</td>
</tr>
<tr>
<td>1441F</td>
<td>$1 \times 10^7$</td>
<td>3</td>
<td>204</td>
<td>Recrudescing parasitemia, recumbent; killed</td>
</tr>
<tr>
<td><strong>Group II (controls)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1401M</td>
<td>$2 \times 10^5$</td>
<td>5</td>
<td>95</td>
<td>Persistent parasitemia, died</td>
</tr>
<tr>
<td>151F</td>
<td>$2 \times 10^5$</td>
<td>5</td>
<td>Treated with Berenil on Day 196</td>
<td>Survived; no parasitemia detected after treatment.</td>
</tr>
<tr>
<td>153F</td>
<td>$2 \times 10^5$</td>
<td>5</td>
<td>170</td>
<td>Recrudescing parasitemia, recumbent; killed</td>
</tr>
<tr>
<td><strong>Group III (immunized)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>148F</td>
<td>$2 \times 10^5$</td>
<td>7</td>
<td>Treated with Berenil on Day 196</td>
<td>No parasitemia, recumbent; killed on Day 200.</td>
</tr>
<tr>
<td>145F</td>
<td>$2 \times 10^5$</td>
<td>7</td>
<td>98</td>
<td>Persistent parasitemia; died</td>
</tr>
<tr>
<td>149F</td>
<td>$2 \times 10^5$</td>
<td>7</td>
<td>19</td>
<td>Persistent parasitemia, concurrent anaplasma infection, recumbent, died</td>
</tr>
</tbody>
</table>

*1, vaccinated (foot and mouth type C); 2, vaccinated (anaplasma); M, male; F, Female.*

Lulose acetate strips was performed by the Beckman Microzone technique.

**Immunization**

An attempt was made to immunize 3 animals with irradiated trypanosomes. Sixty milliliters of blood was obtained from the donor cattle during the first peak of patent parasitemia. Tubes containing the blood and heparin were put on ice and transported to the Veterinary Faculty, Kabete, where they were irradiated in a $^{60}$Co source at a level of approximately 60 krad. The dose rate at the time of the experiment was 3.3 krad per min. The tubes were placed in a plastic rack inside the irradiator, and were not cooled during irradiation. After the desired level of radiation had been obtained, the tubes were again placed on ice and taken back to the Trypanosomiasis Research Laboratory where 20 ml of irradiated parasitized blood was injected intravenously into each of the 3 experimental animals. Four injections of irradiated parasites were given at about weekly intervals, and the number of trypanosomes per injection ranged from $1 \times 10^5$ to $8.9 \times 10^8$. A week after the fourth immunization, the 3 immunized as well as 3 control animals were challenged intravenously with $2 \times 10^5$ viable T. congolense.

*Provided by the International Atomic Energy Agency.*
RESULTS

Clinical Observations

The animals in these experiments were separated into 3 groups. Group I consisted of 5 animals which were injected with viable T. congolense and used as donors of trypanosomes for immunization and challenge. Group II contained 3 animals which served as controls for the immunized cattle which were designated as Group III (Table I). The only observable protective effect elicited by the irradiated parasites in the animals in Group III, when compared to their controls in Group II, was an increase of 2 days in the length of their prepatent periods and a corresponding delay in the development of fever. After the onset of parasitemia, the course of infection in 2 immunized animals was similar to that of the controls. The other immunized animal died relatively soon after challenge, but its death was complicated by concomitant anaplasmosis. Since the 8 cattle in Groups I and II received no preinfection treatment, the course of infection in these animals was studied closely.

The length of the prepatent periods, but not the duration of the disease, was related to the numbers of trypanosomes injected (Table I). After the onset of patency, trypanosomes were detected daily for the first 7 weeks in all 8 animals. After the 7th week of infection, however, parasites were not always found in the capillary blood, and the frequency of positive blood smears decreased as the infection progressed (Fig. 1).

Two of the 3 animals which died following acute infections had high parasitemias at the time of death (Fig. 2). Conversely, the cattle which developed chronic infections had a pattern of lower levels of parasitemia of a recrudescing nature and died when relatively few or no parasites could be detected in the peripheral blood (Fig. 3).

High fevers were detected on the day of or the day after onset of patent parasitemia, and usually subsided within 3–4 days to return intermittently accompanying peaks of parasitemia. With one exception, the highest degree of fever was always detected during the onset of parasitemia. A less distinct fever response was evident in the animals which survived for longer periods, and normal or subnormal temperatures were recorded from animals at or near the time of death.

Animals were lethargic during the onset of patent infections and at irregular inter-
vals as the disease progressed, usually coincident with febrile episodes. All animals showed profound weakness, confusion, and dyspnea during the course of infection. Although total body weights were stable for the first 3 weeks of infection, a progressive weight loss followed this initial period, and animals became extremely emaciated even though their food consumption was not obviously reduced (Fig. 4). Death was usually preceded by several days of recumbency with the animal becoming progressively weaker; all animals continued eating until a few hours before death. None of the animals appeared to have paralysis or other signs of central nervous system disorder. One animal (162) died suddenly, apparently from bloat. Another animal (6120) became icteric shortly before death. No eye involvement or external enlargement of lymph nodes were detected in any of the animals.

**Hematology**

Packed cell volumes began to decrease with the onset of patency, and continued to decrease rapidly for the first 7 weeks of infection (Fig. 5). The 3 animals which died acutely, had a more rapid decrease in packed cell volumes than did those which survived for longer periods. Low values continued in chronically infected animals although a slight increase was noted from the 11th to 15th week, whereupon a decrease again occurred. In all animals, this progressive anemia was accompanied by changes in the composition and morphology of the peripheral erythrocytes which could be recognized as early as the 3rd week of infection, and which became more prominent as the anemia became more severe. Anisocytosis and polychromatophilia were the earliest changes noted. Basophilic stippled cells and normoblasts were found later in some animals. The erythropoietic response was especially prominent during the period between the 6th and 9th week, and appeared to be most pronounced in acutely infected animals. Except for rare polychromatophilic erythrocytes, the RBC’s after the 13th week appeared to be normocytic and normochromic.

Uninfected cattle in the Kabete area had elevated leucocyte counts when compared to previously reported values. Twenty-seven determinations done on 11 animals averaged 15,200/mm³, and ranged from 11,200 to 29,400. In infected animals, a leukopenia was evident at the onset of patent parasitemia and persisted throughout the course of infection (Fig. 6). Especially low WBC counts were recorded during high levels of parasitemia. The percent of segmented neutrophils declined as a relative increase in mononuclear leukocytes occurred early in infection, and usually persisted until shortly before death, when percentages of neutrophils rose moderately in some animals. Erythrophagocytosis by medium sized mononuclear leukocytes was seen in the peripheral smears in 3 of 8 animals.
**T. Congolense INFECTION IN CATTLE**

**Fig. 3.** The chronic course of disease in animal 144.

**Fig. 4.** Total body weights.
Fig. 5. Packed cell volumes. Crosses indicate days of death. Preinfection values are noted by the shaded area (range) and solid line (mean) in all figures. Experimental values are expressed as a mean and range.

Fig. 6. Total leukocyte concentrations.

Fig. 7. Total serum protein concentrations. Brackets indicate values inclusive of these dates were pooled.
Both macrophages and neutrophils with remnants of trypanosomes in their cytoplasm were also seen in peripheral smears.

**Serum Chemical Components**

*Serum proteins.* Concentrations of total serum protein decreased markedly for the first 5 weeks of infection, after which they gradually rose to levels usually somewhat below preinfection values (Fig. 7). Both albumin and gamma globulin levels followed a similar pattern for the first 5 weeks. However, whereas albumin concentrations remained relatively low throughout the infection (Fig. 8), those of gamma globulin increased after the 5th week to reach levels equal to or greater than preinfection values (Fig. 9). Alpha globulin levels were lower in chronically infected animals, whereas beta globulins generally decreased as the infection progressed (Figs. 10 and 11).

*Transaminase.* After an early downward trend, concentrations of SGOT remained relatively stable. The exception was animal 6120 which developed increasing levels of transaminase which persisted for 3 weeks before death (Fig. 12).

*Serum urea nitrogen.* Soon after infection, values rose to a small or moderate degree over those of preinfection samples. However, after the initial rise, lower levels were found in the animals surviving for longer periods (Fig. 13).

*Serum creatinine.* Concentrations remained constant early in the course of infection and tended to be slightly lower in chronically infected animals (Fig. 14).

*Serum glucose.* Glucose levels remained relatively constant except for terminal val-

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**Fig. 8.** Serum albumin concentrations.

**Fig. 9.** Serum gamma globulin concentrations.
ues which were reduced in the acutely infected animals (Fig. 15).

Total serum bilirubin. The 3 animals with acute infections developed elevated concentrations of total serum bilirubin after the 3rd week of infection. The highest values were obtained from animal 6120. With one exception, bilirubin levels were not elevated over preinfection values in chronically infected animals (Fig. 16).

Impression Smears

Table II gives an estimate of the relative distribution of intact trypanosomes in smears from the various organs studied. In one animal (153), an extremely large number of trypanosomes was found in smears from the heart. Surprisingly few parasites were found in smears of spleen, bone marrow, or lymph nodes. A similar pattern was observed for phagocytized or degenerating forms of trypanosomes which were found
**Fig. 14.** Serum creatinine concentrations.

**Fig. 15.** Serum glucose concentrations.

**Fig. 16.** Total serum bilirubin concentrations.
TABLE II

Number of Intact Trypanosomes per 100 Oil Immersion Fields in Impression Smears from Organs of Chronically Infected Cattle

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Venous Blood</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>Spleen</th>
<th>Bone marrow</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>32</td>
<td>20</td>
<td>152</td>
<td>ND</td>
<td>30</td>
<td>156</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>153</td>
<td>10</td>
<td>1720</td>
<td>52</td>
<td>14</td>
<td>52</td>
<td>64</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>138</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Not done.

TABLE III

Response to a Curative Dose of Berenil in Animal 151

<table>
<thead>
<tr>
<th>Weeks after challenge</th>
<th>0</th>
<th>24</th>
<th>26</th>
<th>28a</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>35</th>
<th>58</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV %</td>
<td>40</td>
<td>13</td>
<td>11</td>
<td>17</td>
<td>11</td>
<td>23</td>
<td>24</td>
<td>28</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>Body weight (lbs)</td>
<td>745</td>
<td>545</td>
<td>550</td>
<td>505</td>
<td>—</td>
<td>485</td>
<td>460</td>
<td>505</td>
<td>635</td>
<td>835</td>
</tr>
</tbody>
</table>

* Berenil treatment with 1.05 g of active ingredient given intramuscularly in one injection.

primarily in smears from the brain, heart, and lung. Bone marrow taken from ribs of animals 6193, 153, 144, 138, and 148 was red in color; however, marrow obtained from femurs was yellow and fatty in consistency. A large number of normoblasts were found in the rib marrow of animal 6193. These cells were present in the marrow of the other cattle but in greatly reduced numbers. However, leucocytic precursors and megakaryocytes appeared to be present in abundance in all smears. Many plasma cells were found in smears of the bone marrow, spleen, and lymph nodes, and macrophages from these sites contained large amounts of yellowish brown pigment.

Chemotherapy

Two animals (151 and 148) which had become extremely weak, emaciated, and anemic were treated with Berenil,* on the 196th day after challenge (Table III). Four days after treatment animal 148 died. Three weeks after treatment the PCV of animal 151 had doubled but the animal continued to lose weight. Subsequently, both parameters consistently improved and eventually the animal appeared to recover fully from the infection.

Concomitant Infections

An erythrocytic parasite, presumably *Theileria mutans*, was seen occasionally at low levels in blood smears from most of the cattle. There was no increase in the level of these parasites in either acute or chronically infected animals, with the exception of 6120, who died with 1% of its RBC's parasitized. *Anaplasma marginale* contributed to the death of one of the animals in Group III.

Discussion

Hereford cattle injected with the Trans Mara I strain of *T. congolense* developed
acute and chronic diseases comparable to those described by Fiemmes (1970). We did not see the hyperacute or recovery courses of infection described by this author in any of our animals. This strain of *Trypanosoma congolense* produced a similar disease in young Holstein bulls (J. S. Anderson, unpubl.) and extremely virulent hyperacute infections developed in Beagle dogs infected with this parasite (Johnson *et al.* unpubl.).

The most striking and puzzling aspect of *T. congolense* infections in Hereford cattle was the development and persistence of anemia. An erythropoietic response to this anemia was evident early in the course of infection, but for unknown reasons subsided without having resulted in greatly elevated packed cell volumes. Chronically infected cattle continued to be anemic without any further evidence of erythropoietic response until the time of death, even though trypanosomes were being suppressed in large measure. It could be speculated that the presence of live trypanosomes was needed to perpetuate the anemic condition, since after curative chemotherapy the level of packed cell volumes gradually increased, and eventually regained preinfection levels. The finding of elevated levels of total bilirubin in animals which underwent the most severe anemia was probably due to hemolysis of erythrocytes, since even in severe liver disease in cattle, serum bilirubin levels rise only slightly. All of the cattle in these studies also had large amounts of yellowish brown iron positive pigment in macrophages of the spleen, liver, and bone marrow. This pigment, thought to be hemosiderin, was seen in both impression smears and histological sections (Kaliner 1974). The excessive accumulations of pigment have been reported by other authors and appears to be a relatively consistent finding, although Krampitz (1970) reported that only 1 of 16 cattle which died of trypanosomiasis in his studies had excessive accumulation of pigment. These apparent discrepancies may be the result of differences in severity of anemias and corresponding destruction of erythrocytes among various experiments. While the initial fall in packed cell volumes seems to be associated primarily with destruction, i.e., hemolysis of erythrocytes and erythrophagocytosis, it appears that later in the course of infection a suppression of production of erythrocytes also becomes a prominent factor. Studies of bone marrow obtained from the rib indicated that some erythropoiesis was occurring, but overall erythropoiesis appeared to be suppressed, since marrow in the long bones had not regenerated. While the etiology of the anemia has not been defined, the suggestion that it is at least in part immunologically induced has been proposed by a number of workers (see Desowitz 1970 for review).

Concentration of total serum proteins also decreased rapidly early during the infection, and may be the result of increased protein breakdown, loss by proteinuria, disturbances in metabolism or absorption, or dilution as suggested by Fienens (1970). The animals regained some of the early loss of total serum protein and in chronically infected animals the value was not severely lowered. The total body weight continued to decrease, however, and the relatively stable serum protein values found later were probably maintained at the expense of the tissue protein. The early depletion or dilution of gamma globulin is coincident with the decrease noted in the complement fixation and indirect fluorescent antibody titers of the same animals (Lotzsch and Deindl 1974). Levels of gamma globulin increased after the 5th week and may reflect a more pronounced antibody response which in chronically infected cattle apparently influences the level of parasitemia; 78 gamma globulins obtained from the chronically infected animals at the terminal stage strongly inhibited parasitemia in mice infected with the same strain of *T. congolense* (Wellde and Rodriguez unpubl.). Desowitz (1959) observed similar fluctua-
tions in serum protein concentrations in susceptible breeds of cattle infected with *T. vivax*.

Excessive catabolism of serum protein could be responsible for the elevated urea nitrogen levels, since the loss of protein and increased urea nitrogen values occurred during the same period. There was no corresponding increase in creatinine levels which would be expected if renal impairment had occurred, although some degree of kidney pathology was present (Kaliner 1974). The slightly lower levels of urea nitrogen present in chronic animals may reflect a decrease in the rate of protein metabolism.

The relatively stable levels of SGOT indicate that the liver was not severely affected by the infection. No severe histopathologic lesions were found there or in the heart or skeletal muscle (Kaliner 1974).

The spleen did not appear to be greatly involved during the course of infection, since hypertrophy of this organ was not noted in either acute or chronically infected animals in our study. It would be reasonable to assume that the spleen, acting as the major lymphatic organ of the circulatory system, would actively sequester, phagocytize, and destroy circulating trypanosomes, but evidence from our studies does not confirm this assumption. Plasma cells, however, were found in abundance in the spleen and the antibody forming role of the spleen may prove to be of an important nature. Krampitz (1970) has pointed out that much conflicting information has been published on splenic hypertrophy in bovine trypanosomiasis and the subject needs more concentrated study.

While the injections of irradiated *T. congolense* initiated a detectable antibody response (Lötzsch and Dcindl 1974), they did not produce a strong resistance. The effect of a 2 day delay in onset of patent parasitemia corresponds to an approximate one hundredfold dilution of the challenge inoculum. This is in contrast to the strong resistance produced in cattle by irradiated *T. rhodesiense* (Wellde et al. 1973). In general, *T. congolense* has proven to be a more difficult parasite to control by experimental immunization than *T. rhodesiense*, either by the use of attenuated parasites (Duxbury et al. 1973), or by infection and chemotherapeutic cure (Fulton and Lourie 1946). Whether or not this indicates a greater propensity for antigenic variation on the part of *T. congolense* (Wilson and Cunningham 1972), is not known.

**Acknowledgments**

Dr. I. E. Muriithi, Director of Veterinary Services, is thanked for inviting us to proceed with these experiments in the facilities under his supervision at Kabete, Kenya. We thank Dr. John Tremlett, Chief Veterinary Research Officer, Mr. Jan Le’Roux, Chief Zoologist, and Mr. Samuel Githatha, Zoologist, for their advice and assistance during these experiments. Dr. R. Schindler’s contribution to the planning of these experiments was also appreciated. Excellent technical assistance was provided by the workers at the Trypanosomiasis Research Laboratory, and we especially thank Samuel Gitchunge, Shem Kaiga, and Reuben Mutuaahi. We also thank Mr. Arthur Moon, Dr. Carter Diggs, Dr. Anthony Johnson, and Mrs. Genevieve Zivona, for their suggestions and help with the manuscript.

**References**


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