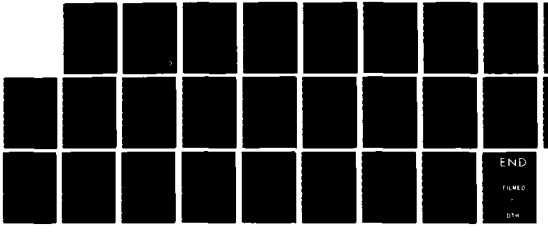
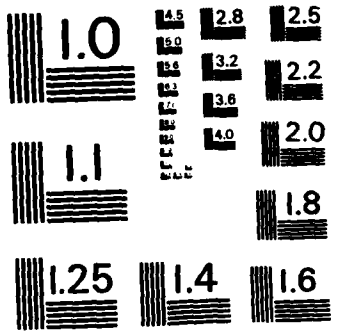
AD-A162 979 TRYPANOSOMA B RHODESIENSE (WRATAT SERODEME): PURFICATION AND CHARACTERIZA (U) COLORADO STATE UNIV FORT COLLINS COLL OF VETERINARY MEDICINE A

1/1

UNCLASSIFIED G R KEILMAN MAR 83 DAMD17-79-C-9117 F/G 6/15 NL





AD_____

REPORT NUMBER 2

Trypanosoma b. rhodesiense (WRATat Serodeme): Purification and Characterization of Surface Antigens for the Vaccine Development Program

Annual and Final Report

Gerald R. Keilman

March 1983

Supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract Number: DAMD 17-79-C-9117

College of Veterinary Medicine
and Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A162979 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Trypanosoma rhodesiense (WRATat Serodeme): Purification and characterization of surface antigens for the vaccine development program	5. TYPE OF REPORT & PERIOD COVERED *ANNUAL AND FINAL REPORT July 1979 - August 1981
	6. PERFORMING ORG. REPORT NUMBER Report Number 2
7. AUTHOR(s) Gerald R. Keilman, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9117
9. PERFORMING ORGANIZATION NAME AND ADDRESS Colorado State University Fort Collins Colorado 80523	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A.3M162770A871.AF.114
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, MD 21701	12. REPORT DATE March 1983 / 13. NUMBER OF PAGES 34
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified / 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

*Annual Report covers work done May 1980 - August 1981.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Trypanosoma b. rhodesiense WRATat serodeme; variant-specific glycoprotein; antigen purification; chemical characterization; insulin; glucose; NSILA's; MSA; septic shock; dexamethasone; streptozotocin; T.b. rhodesiense CSUT-J1; chronic infections; acute infections.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The variant-specific glycoproteins (VSG's) from some of the antigenic clones of organisms of the Trypanosoma b. rhodesiense WRATat serodeme were purified from cell lysates. Pooled eluate-fractions of lectin-affinity chromatography using concanavalin-A agarose was the primary fractionation tool followed by gel permeation chromatography on TSK, SW-3000 HPLC columns. VSG's which showed single band purity by denaturing polyacrylamide gel electrophoresis were hydrolized with 3N mercaptoethanesulfonic acid and their amino acid

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

compositions determined.

Studies were initiated to determine how to manipulate the rats and the parasites to obtain better and consistent yields of trypanosomes from the blood of the infected animals. Host responses to acute infections were measured for changes in glucose, insulin, multiplication-stimulating activity (MSA), nonsuppressible insulin-like activity (NSILA), lactate, catecholamines and others. Extremely low levels of glucose and insulin were seen when the parasitemias were elevated. MSA values were shown to decrease dramatically by the first day post-infection and stayed depressed for the remaining days of the infection. NSILA-p levels were unchanged during the acute infections. The lactate and catecholamine concentrations that were measured proved to be too variable to relate to the progress of the parasitemias. The only other parameter that showed positive correlation to the course of the acute infection was spleen weight. Gross specimen spleen weight increased about 4-fold in rats infected with Trypanosoma b. rhodesiense WELLCOME.

Attempts were made to isolate and clone acute, isoantigenic trypanosomes from the terminal waves of parasitemia initiated with one of the chronic clones of T.b. rhodesiense WRATat-5. An acute strain was isolated, cloned and designated T.b. rhodesiense CSUT-J1. The VSG from this isolate was chemically characterized and antigenic reactivity to known WRATat clones proved to be negative. Further characterization of this strain was being pursued at the termination of this contract.

ABSTRACT

The variant-specific glycoproteins (VSG's) from some of the antigenic clones of organisms of the Trypanosoma b. rhodesiense WRATat serodeme were purified from cell lysates. Pooled eluate-fractions of lectin-affinity chromatography using concanavalin-A agarose as the primary fractionation tool, was followed by gel permeation chromatography on TSK, SW-3000 HPLC columns. VSG's which showed single-band purity by denaturing polyacrylamide gel electrophoresis were hydrolized with 3N mercaptoethanesulfonic acid and their amino acid compositions determined.

Studies were initiated to determine how to manipulate the rats and the parasites to obtain better and more consistent yields of trypanosomes from the blood of infected animals. Host responses to acute infections were measured for changes in glucose, insulin, multiplication-stimulating activity (MSA), nonsuppressible insulin-like activity (NSILA), lactate, catecholamines and others. Extremely low levels of glucose and insulin were seen when parasitemias were elevated. MSA values were shown to decrease dramatically by the first day post-infection and stayed depressed for the remaining days of the infection. NSILA-p levels were unchanged during the course of the acute infections. The lactate and catecholamine concentrations that were measured proved to be too variable to relate to the progress of the parasitemias. The only other parameter that showed positive correlation to the course of the acute infection was spleen weight. Gross spleen weight increased about 4-fold in rats infected with Trypanosoma b. rhodesiense WELLCOME.

Attempts were made to isolate and clone acute, isoantigenic trypanosomes from the terminal waves of parasitemia initiated with one of the chronic clones of T.b. rhodesiense WRATat-5. An acute strain was isolated, cloned and designated Trypanosoma b. rhodesiense CSUT-J1 (Colorado State University Trypanozoan-J1). The VSG from this isolate was chemically characterized and antigenic reactivity to known WRATat clones proved to be negative. Further characterization of this strain was being pursued at the termination of this contract.

FOREWORD

In conducting the research described in this report, the investigator (s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

ITEM	PAGE
Title Page	1
Abstract	2
Foreword	3
Table of Contents	4
Personnel Employed	6
Report	
I. Statement	7
II. Experimental Approaches	9
III. Results	12
IV. Conclusions	16
References	18
Table 1. Molecular weight data of purified VSG's	19
Table 2. Amino acid composition of VSG,s	20
Table 3. NSILA, MSA, glucose, spleen and epididymal fat pad weights during an acute infection	21
Legends to Figures	22
Figure 1. Growth of T.b. rhodesiense WRATat-1 and plasma glucose levels in rats treated with dexamethsone	23
Figure 2. Parasitemia and plasma glucose levels of rats treated with streptozotocin and infected with T.b. rhodesiense WRATat-1	24
Figure 3. Concentration of parasites in the penultimate and terminal waves of infection in the rat "J1"	25
Figure 4. Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense WELLCOME	26
Figure 5. Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense CSUT-J1	27

Figure 6. Parasitemia, plasma glucose levels, insulin levels and spleen weights of rats infected with T.b. rhodesiense WELLCOME ---------- 28

Final Summary Report ---------- 29

Acknowledgements ---------- 31

Distribution List ---------- 32

PERSONNEL EMPLOYED

Laboratory Technicians

David Boon
Khalif Ahmed
Scott Sauer

Consultant

David Spencer

Hourly/Student Hourly

Kathryn Steele
Brenda Baker
Patricia Sutterlin
Gregory Reed
Amy Pettibone
Mary Weiss
Carla Cross
David Carvill

ANNUAL REPORT - 2

I. STATEMENT

These studies were a continuation of the work started in 1979 to establish a relationship between the chemical nature of the variant-specific glycoproteins (VSG's) to Trypanosoma b. rhodesiense (WRATat serodeme) and the form that active infections take in selected rodent hosts. This concept was in part prompted by the observations that trypanosomes which lacked the VSG "coat" were incapable of establishing an infection in rodents. Although other factors are most certainly necessary for this complex process, the surface antigens may well play the primary role in determining the relative virulence of trypanosome infections.

The course that an infection takes can either be acute or chronic. This is the result of numerous interactions between the genetic capabilities of the host and the parasite. It has been demonstrated that chronic trypanosome infections are often typified by the morphological transformation of the "long-slender" type to the "short-stumpy" forms. If this apparent change does not occur in the dividing population, the host will normally succumb with a fulminating infection. As cellular differentiating events have often been observed to be initiated by cell surface interaction with triggering molecules, we feel that trypanosomes displaying chronic infection profiles in a specific host possess the proper VSG type necessary for transformation. Conversely, acute infections occur when the VSG type is not correct for the signal event.

It is our observation and others, that one can develop an acute strain of trypanosome population from a normally chronic one. No one has yet to demonstrate the opposite! Work in this laboratory has clearly shown that trypanosomes harvested from the terminal wave of a chronic infection were composed of acute organisms. Immediate passage to rodents with the same genetic background, sex and age resulted in their deaths within a few days.

In order to demonstrate that the VSG's were important in determining whether or not a strain was acute or chronic, we attempted to isolate and clone a virulent population of trypanosomes from the terminal waves of parasitemia initiated with a chronic strain from the WRATat serodeme. Once this was accomplished, isoantigenic clones would be identified among the many less virulent variants. Careful chemical and gene sequence analyses would be conducted to help identify whether or not VSG's are intrinsically necessary virulence factors.

Many aspects of the infection process had to be better defined in order to clarify the role of VSG's in the overall phenomenon. One point of focus was the host's response during an acute infection. Specifically, we initiated studies that would help identify the physiological lesions that proved to be fatal. The data revealed the animals died of septic shock, a process that has yet to be clearly explained even in well-defined systems. The terminal phase of chronic infections showed similar shock profiles. We were continuing to search for the

most critical metabolic lesions when this research ended.

Undergraduate students were employed part-time to assist us in various aspects of this work. One student was encouraged to set up a system to study the effect of purified VSG's, or sera from infected animals, on the migration of PMN's in agar. This same individual sought to correlate macrophage spreading *in vitro* with stages of infection and degrees of virulence.

Another student prepared the tissue culture media, buffers, plates, etc. involved in establishing hybridoma cell lines. These efforts were being made for several reasons:

a) We wanted monoclonal antibody (McAb) probes that would assist us in defining isoantigenic types.

b) We desired to use immunoaffinity techniques to purify VSG's from isoantigenic types.

c) These McAb's would assist us in characterizing the homogeniety of our cloned stocks of trypanosomes.

d) The same McAb's could be used to establish the isoantigenic type clones by fluorescence-activated cell sorting or less costly immunoaffinity manipulations.

II. EXPERIMENTAL APPROACHES

A. Isolation and Characterization of VSG's from Trypanosoma b. rhodesiense WRATat's

1. Growth of trypanosomes from stabilates in dexamethasone-treated animals.

a. Trypanosomes that had been cloned and stabilated by WRAIR were used to establish our own stocks in Co-60-irradiated animals. Male rats with body weights in excess of 300 gm were routinely used to host the infections needed to produce large numbers of parasites for chemical extractions. These animals were treated with dexamethasone (3 mg/100 gm body weight) freshly diluted in 50% ethanol.

b. Organisms from the first peak of parasitemia were purified from heparinized-blood by ion-exchange chromatography (1), then washed with a phosphate buffer containing phenylmethylsulfonyl flouride (PMSF; 1 mM) but without glucose. The final washed pellet of trypanosomes was rapidly frozen in liquid nitrogen and stored at -70 C.

2. Purification of VSG's from frozen pellets.

a. An equal volume of Tris-acetate buffer (20 mM Tris-acetate with 500 mM NaAcetate, pH 7.3 containing 1 mM PMSF) at 4 C was added to the frozen pellet and the pellet was allowed to thaw slowly in a 4 C bath.

b. The resulting lysate was frozen and thawed again three times before it was expressed through a series of syringe needles (#18 - #27) of decreasing diameters. The material was centrifuged at 30,000 x g/30 min/4 C.

c. The supernatant fluid was applied directly to a 2 ml column of concanavalin-A-sepharose equilibrated with a fresh Tris-acetate buffer. The column was washed with the same buffer until only slight traces of 280 nm absorbing material eluted. The glycoproteins that remained absorbed to the packing was step-eluted by adding alpha-methylmannopyranoside (100 mM) to the buffer.

d. The fractions containing the eluted glycoproteins were pooled and concentrated in an ultrafiltration device (Amicon Model M3; PM-10 filter) to a final volume of 2 ml.

e. The concentrated fractions were applied to an HPLC column (TSK, SW-3000) equilibrated with Tris-acetate buffer (20 mM Tris with 100 mM NaAcetate, pH 7.3) and calibrated with bovine serum albumin (BSA). The buffer was pumped at a rate of 1 ml/min/25 C.

f. Protein-containing fractions were collected, concentrated to about 1 mg/ml and stored in the Tris-acetate eluting buffer made 50% with glycerol at -21 C.

3. The molecular weights of VSG proteins were estimated by their retention times on the calibrated gel permeation column (TSK,

SW-3000), as well as their migration in denaturing, polyacrylamide gels (2). Suitable samples were hydrolyzed for 20 h in mercaptoethanesulfonic acid (3) and subjected to automated amino acid analysis (Dionex Chemical Corporation).

B. Experiments to Increase the Level of the First Parasitemic Plateau.

1. Relationship of the plasma glucose concentration to the level of the first peak of parasitemia - An attempt was made to determine if there was a correlation of the concentration of glucose in the circulation and the level of the first wave of organisms in the blood. These studies were prompted by the observations that dexamethasone-treated rats usually showed higher primary plateaus of infection when injected with the chronic T.b. rhodesiense WRATat clones or other chronic strains of trypanosomes. Although the effect of steroids upon the host is quite diverse, elevated glucose in the plasma is one result that could metabolically benefit the parasites which use glucose as their primary carbon source.

a. One-half of the experimental rats were treated with dexamethasone as previously indicated. Venous blood was collected from the tail veins into heparinized capillary tubes before, during and at the end of the study period. All of the animals were infected with the chronic trypanosome strains by syringe injection i.p.. Parasites in the blood were enumerated by light microscopy using counting chambers.

b. Another approach to assessing the effect of circulating glucose on the growth of parasites in these animals was to induce hyperglycemic conditions comparable to those in diabetic animals. The drug streptozotocin (65 mg/kg) was given to rats that had been fasted for 16 h (4). Treated animals were allowed to feed and drink ad libitum. When glucose levels in these rats became constantly higher than 400 mg/dL, they were infected with trypanosomes.

2. Search for acute, isoantigenic trypanosomes derived from the WRATat serodeme - In order to obtain the large quantities of parasites needed for the chemical characterization of their VSG's, one could also attempt to derive acute clones from the chronic forms. Selection from the acute populations would be based on specific immunological cross-reactivity with monoclonal antibodies raised against cloned, chronic trypanosomes, i.e., isoantigenic strains. These experiments were initiated under the assumption that the terminal wave of parasitemia of a chronically-infected host would consist primarily of acute forms of the parasite.

a. Three groups of rats were infected with trypanosomes by syringe injection. Each set of animals was inoculated with the antigenically distinct clone designated WRATat 5.

b. The animals were observed and tested for the level of trypanosomes in the circulation by direct viewing of wet mounts of tail vein blood.

c. When the animals showed parasitemias greater than

1 x 10^8/ml, they were closely observed for terminal signs. Animals judged to be near death were bled by cardiac puncture. The heparinized blood containing the population of trypanosomes from the final wave of parasitemia was mixed 1:1 with glycerol.

d. The mixture was stored at -70 C until needed. The biological cloning of organisms was performed from these materials.

C. Characterization of Acute, WRATat-Derived Trypanosomes and an Established, Acute Strain of Trypanosome.

The process of establishing the acute nature of the trypanosomes that were isolated from the terminal waves of parasitemia was to compare the magnitude of the parasitemias; the length of the patency period; changes in the host's specific physiological responses; and the chemistry of each clone's VSG to the corresponding parameters of a recognized acute strain.

1. Biological cloning from the terminal populations of the "J" and "L" series of infected animals was attempted by limit-dilution techniques.

2. "Cloned" organisms were injected into rats and their growth patterns were determined by direct counting of parasites in diluted blood (tail vein) samples throughout the course of the infection.

3. Multiple samples of venous blood were collected throughout the course of the infection. Plasma was prepared and frozen rapidly to be used for later analysis of circulating levels of glucose, insulin, NSILA's, somatamedins, catecholamines, lactate, complement-fixing activity, migration-inhibitory factors, and others.

D. Monoclonal Antibodies to Trypanosomes of the WRATat Serodeme.

The ability to identify an antigenic clone with the greatest precision, as well as establishing a technique to initiate infections with a single, antigenic type of trypanosome prompted us to attempt to prepare monoclonal antibodies against the cloned, acute, WRATat-derived variant specific glycoproteins.

1. Balb/c mice were obtained from the Charles River colony.

2. Tissue culture reagents were prepared for the growth and maintenance of a mouse myeloma cell line (SP2/0 - Ag14; 5).

3. The growth characteristics of the mouse myeloma cells were determined.

4. Purified VSG's were incorporated into complete Freund's and Balb/c mice were immunized with primary doses of 300 ug/animal and boost doses of 100 ug/animal in incomplete Freund's adjuvant.

5. Fusion of immune spleen cells and the SP2/0 - Ag14 cells was performed and the resulting hybridomas were characterized.

III. RESULTS

A. Isolation and Characterization of VSG's

1. Growth of Trypanosomes - the cloned populations of organisms representing the WRATat serodeme showed variable growth in rats treated with dexamethasone. However, the drug regimen normally allowed the concentration of parasites in the blood to reach between 2×10^8 to 1×10^9 trypanosomes per ml by day 3 - day 5 post-inoculation. Once the organisms began to transform to the short-stumpy morphology, the parasites were either harvested immediately or rejected for use in VSG preparation. The timing of this transition could neither be anticipated nor controlled.

2. Purification of the VSG's - the procedure followed in this work was essentially the same as that previously documented (SEE: Report 1 of this series). The only noticeable change was the replacement of hydrochloric acid with acetic acid in preparing the buffers used to lyse the cells and purify the VSG's. Also, sodium acetate was used instead of sodium chloride. The changes were made to avoid the deterioration of the HPLC hardware caused by the high concentrations of chloride ions.

3. Table 1 contains the molecular weight data acquired from the VSG's purified from Trypanosoma b. rhodesiense WRATat clones 1-10 and 12; the acute derivative of T.b. rhodesiense WRATat 5 called T.b. rhodesiense CSUT-J1; and from the acute strain T.b. rhodesiense-WELLCOME. In all instances, the fractionation of the samples eluted from the lectin affinity column suggested that the VSG's were existant as dimers, partial dimers, monomers and degradation fragments. The highest molecular weight determined for a dimer was 2.2×10^5 (VSG-4) which also had the greatest monomeric molecular weight of 9.8×10^4 when resolved by HPLC techniques. The averaged monomeric molecular weight was 8.2×10^4.

The fractions collected from the gel permeation column were subjected to analysis by polyacrylamide gel electrophoresis (2). Even though the materials were treated with heat, SDS and DTT, some of the VSG complexes were not completely dissociated and bands of proteins could be detected with estimated molecular weights greater than the monomeric form. VSG-9 had the greatest molecular weight estimate by this technique of 7.4×10^4. The average molecular weight was calculated to be 6.6×10^4.

The amino acid compositions of 5 purified VSG's were determined and are shown in Table 2. Major quantitative differences are evident in the residues of glycine, alanine, valine, methionine, tyrosine, and phenylalanine in these glycoproteins. VSG-1 appears to lack both tyrosine and phenylalnine and has the least number of methionine residues. The surface coat proteins from T.b. rhodesiense WRATat 2 and WRATat 3 seem to have similar amino acid compositions, more so than any of the others assayed.

B. The major problem in the work remained the inconsistent levels of the first waves of parasitemia of these chronic infections. Figure 1 depicts the growth of T.b. rhodesiense WRATat 1 in rats treated with dexamethasone. Although the plasma glucose levels remained high (400 mg/dL) in the doubly-dosed rats, the concentration of parasites in the blood of

these animals was the same as that in the pretreated rodents. The latter's plasma glucose declined to normal values (100 mg/dL) by the fourth day.

To further examine the effect of plasma glucose concentration on the plateau level of a chronic infection, some rats were pretreated with streptozotocin, a drug known to induce diabetes (4). When the treated animals became hyperglycemic (500 mg/dL) they were infected with T.b. rhodesiense WRATat 1. The levels of plasma glucose and parasitemia were determined for 14 days and are shown in Figure 2. The profile of trypanosome concentration was similar to that seen in dexamethasone-treated rats, being about 10-fold higher at the first peak than was seen in untreated, infected animals. The parasites did transform to short-stumpy forms by day 6 and by day 9 were developing the second wave of long-slender forms. Plasma glucose concentrations increased by 100 mg/dL when the short-stumpy forms appeared and the parasite numbers were declining. The glucose levels remained around 600 mg/dL during the second wave of parasitemia.

2. Search for acute, isoantigenic trypanosomes - Figure 3 depicts the late stages of an infection from an animal inoculated with T.b. rhodesiense WRAT 5. On day 87 post-infection, the population of trypanosomes was harvested. The host had become semi-comatose at this stage, a sign that the animal would succumb to the parasitemia at any moment. Untreated, uninfected rats were immediately injected i.p. with 1×10^6 organisms from this population. Within 9 days, all of the rats had died from overwhelming infections, clearly a 10-fold decrease in the time required for the hosts to die from the T.b. rhodesiense WRATat strain's normally, chronic pattern.

Attempts were made to clone parasites from this population. Although no antigenic characterization was performed, biological clones were developed by the limit-dilution technique. Acute forms of other trypanosome strains have been shown to be rather homogeneous and easily cloned. Most of the mice (6/10) injected with the diluted inoculum showed infections by day 6. The parasites from one positive mouse were further treated to two additional cycles of cloning by dilution. The resulting population was designated as T.b. rhodesiense CSUT-J1 (Colorado State University Trypanosoan, isolate J1).

The acute nature of this isolate was documented by comparing the prepatent and patent periods to a known acute trypanosome strain (Trypanosoma b. rhodesiense WELLCOME; WRAIR collection). Figure 4 shows the growth profiles and plasma glucose levels of two sets of rats infected with T.b. rhodesiense WELLCOME. The streptozotocin-treated rodents died one day later than the untreated rats, but both sets showed acute hypoglycemias on the terminal days of infection.

Figure 5 indicates that the T.b. rhodesiense CSUT-J1 organisms killed untreated rats in about 8 days and streptozotocin-treated animals by day 11. The patentcy periods however were identical. As in the cases of the known acutely infected rats, the hosts died with parasitemias greater than 1×10^9 and plasma glucose levels less than 50 mg/dL.

The VSG's isolated from the Wellcome strain and the CSUT-J1 organisms were compared by gel permeation chromatography on a TSK, SW-3000 column. Table 1 includes the molecular weight values of 134,000 and 84,000 determined for the major peaks from the Wellcome and the CSUT-J1 strains, respectively.

C. Characterization of Acute Infection - Host Response:

To help establish the causal relationships between the trypanosome's intrinsic nature and the host's response, experiments were initiated to determine the utilization of plasma glucose, insulin concentrations, non-suppressible insulin-like activities, spleen weights and epidydimal fat pad weights, catecholamine and lactate levels in rats infected with T.b. rhodesiense WELLCOME. Once established, the same parameters would be examined in T.b. rhodesiense CSUT-J1-infected rats.

1. The information presented in Figure 6 shows some of the data from one of several similar experiments. Plasma glucose levels were seen to rise slightly (3-6%) in the animals on day 1 and day 2. By day 3, all of the infected animals became hypoglycemic, acutely so by day 4. Insulin concentrations in the same samples decreased somewhat by the first day of infection, showing dramatic hypoinsulinemia on days 2 and 3. The animals are almost devoid of plasma insulin on day 4, the terminal day of the infection. Spleen weight increased throughout the study.

2. Table 3 lists values obtained in another study set of rats infected with the WELLCOME strain. Insulin levels were not determined. Instead, two other factors were assayed that have been shown to possess insulin-like activities. The NSILA-p (6) activity in the peripheral circulation of these rats showed no significant variation with that seen in uninfected animals. However, the MSA (7) levels detected on days 1,3 and 4 were 3-6 times lower than the control rats. This activity seemed to be steadily decreasing during the 4 day period. The gross tissue weights of epidydimal fat pads showed some increase on day 1, but were within normal values from day 2 - day 4. Spleen weights again were seen to increase to about 3-times the average spleen weight from normal, uninfected rats.

3. Catecholamines and lactate determinations on sera from some of the animals used in these experiments were made. The levels of norepinephrine and epinephrine (8) ranged from 6,000 - 13,000 pg/mL, values almost 10-20 times greater than reported normal basal concentrations in rats (9). These concentrations were detected in both infected and uninfected animals, suggesting the need to use only catheterized, trained rats when attempting to analyze sera for these factors. Any form of handling or treating these animals was shown to stimulate them. As expected, lactate levels were quite high (65 mg/dL average) from these same sera. No pertinent data was derived from the measurements made on these samples regarding complement either.

4. The studies performed by one of our undergraduate students on the effects of acutely infected sera on the migration of normal polymorphonuclear leukocytes under agarose (10) proved to be more complicated than reported. A great deal of work revealed no conclusive information regarding the presence or absence of migration factors in these samples.

5. Peritoneal macrophage-spreading assays were performed on the cells obtained from animals sacrificed on various days after being infected with T.b. rhodesiense WELLCOME. No measureable change in this activity, an indirect indicator of interferon release, could be established during the course of an acute infection.

6. Qualitative analyses for endotoxin in the sera of WELLCOME-infected rats using Limulus amebocyte pyrogent (Mallinckrodt, St. Louis, MO) was positive on day 3 and day 4 of the infection. Samples obtained and handled in the same manner but from uninfected rats were negative, as were those gathered on days 1 and 2. No attempt was made to quantitate these findings.

D. Monoclonal Antibodies to Trypanosomes

A great deal of time and effort was spent on developing the tissue culture system that could be used to produce hybridoma cell lines secreting monoclonal antibodies to the purified VSG's from the T.b.rhodesiense WRATat serodeme. Balb/c mice were immunized with VSG-2 as shown by specific reactivity to cloned organisms of T.b. rhodesiense WRATat-2. Several fusions of the spleen cells from these mice with the SP2/0 - Ag14 mouse myeloma cells were done successfully. Although over 20 positive secretors of mouse IgG, as detected by Staphylococcus aureus protein-A, the hybrids proved to be either unstable or were contaminated. The system again proved to be too complex for routine handling by an undergraduate student, despite her conscientious efforts. To our great disappointment, no hybridoma's generated in this phase of the study survived long enough to be of any applied value.

IV. CONCLUSIONS

The procedures developed over the two year period of the contract for the purification of the variant-specific glycoproteins from organisms of the Trypanosoma b. rhodesiense WRATat serodeme proved to be efficient and lead to highly informative data. Two major difficulties involved in the program were the unpredictability of the trypanosome concentration in the first wave of parasitemia and all the conditions necessary to preserve the integrity of the purified glycoproteins. The use of the glucocorticoid, dexamethasone, helped to alleviate the former problem by usually stimulating the growth of these chronic clones 10-fold. Even so, we could not always depend upon this technique. When the host's response was favorable, large quantities of the parasites appeared in the plasma and they could be processed rapidly and efficiently. This condition resulted in VSG preparations mostly devoid of degradation products.

Once the cells were purified from the plasma by ion-exchange chromatography, they had to be treated with protease inhibitors to prevent damage to the surface glycoproteins. Lectin-affinity chromatography (ConA-Sepharose) column eluates were rapidly resolved into various molecular weight classes by gel permeation chromatography. The latter technique demonstrated quite clearly that the VSG's existed in stable monomeric or dimeric forms. The dimers were dissociated with denaturing agents and further resolved by polyacrylamide gel electrophoresis into monomeric bands.

High glycoprotein concentrations appeared to cause aggregation of the samples and loss of some material at various steps during purification. This property suggested that the VSG's probably coat the surface of the trypanosomes by associating with each other and interacting membrane components.

Using the non-destructive analytical technique of gel permeation chromatography on the TSK, SW-3000 column matrix, an HPLC device, the VSG's could be easily resolved into their respective molecular weight groups. The estimates of the average size of the monomers by this technique was 16,000 daltons greater than the molecular weights determined by polyacrylamide gel electrophoresis under denaturing conditions. As the latter procedure has been well documented to yield results that are quite near the residue molecular weights of numerous proteins, one must conclude the the VSG's had less interaction with the column matrix than did the BSA standard. The relatively high salt concentration of the carrier buffer could have contributed to the hydrophobic interactions of BSA with the matrix, causing the protein standard to elute later than the VSG's of similar molecular weight.

The amino acid composition of five of the purified VSG's were quite different. Glycoproteins from WRATat-1 and -2 lacked tyrosine while the former also lacked phenylalanine. Residues of alanine and glycine varied greatly in these surface antigens. The purified VSG's from WRATat-5,-6,-7,-8, and -9 could not be accumulated in large enough quantities to submit for amino acid analysis. This problem resulted from the loss of material by aggregation or degradation of the samples during purification. The difficulty of accumulating sufficient material also contributed to our inability to assess the type of sugar residues associated with these proteins.

Because of the problems inherent in working with the chronic forms of trypanosomes, we sought to develop approaches to reliably and economically obtain more organisms from the first parasitemic population. The initial effort was directed at interpreting the effects of dexamethasone in stimulating this process. Our use of the steroid showed that most of the WRATat clones would grow to levels 10-times higher than they would if the host were not pretreated. Among other activities, dexamethasone is a glucocorticosteroid which causes a transient increase in concentrations of glucose in the blood. If the level of plasma glucose was directly related to the peak parasite load, other manipulations of the host that increase circulating levels of glucose would verify the observation. We generated diabetes in rats using the drug streptozotocin. Although these animals had glucose levels 6-times greater than normal, the trypanosomes did not grow unchecked. The parasitemic profile was similar to that of rats pretreated with dexamethasone. The trypanosomes attained a first peak concentration of about 2×10^8/ml before they transformed into short-stumpy forms and decreased in number. From these observations, we have concluded that dexamethosone and streptozotocin act similarly to help elevate the parasitemia. However, neither drug had any effect on preventing the transformation process. We felt that it was this event that was actuated in the chronic strains that primarily limited the growth of the trypanosomes. This activity was not triggered by low plasma glucose, as transformation occurred even with abnormally high circulating glucose. In a few diabetic animals, we had even observed concentrations of short-stumpy forms greater than 7×10^8/ml which remained at that level for two days!

Stimulated by these observations and previously reported ones, we started work to determine the difference between chronic and acute strains. We chose to study the acute properties of a widely used strain Trypanosoma b. rhodesiense WELLCOME obtained from the WRAIR collection. In addition, we attempted to generate an acute strain from the WRATat serodeme. The latter was needed so we could demonstrate through genetics that the traits we sought were heritable. From our work, we can conclude that the highly acute strains of the African trypanosomes did not transform to short-stumpy types; that they fatally depressed glucose and insulin levels; that they did not change the activity of NSILA-p; but did cause a significant drop in MSA levels (MSA is known to be secreted by the hepatocytes). The animals died of irreversible septic shock, although we did not determine if the endotoxin was due to secondary bacterial endotoxemia or the parasites' own endotoxin.

Part of our endeavor to help define isoantigenic types of trypanosomes related to the production of a panel of monoclonal antibodies to a cloned, chronic strain. We had developed a great deal of data on the WRATat-2 clone and decided to immunize inbred mice with purified VSG-2. As previously mentioned, this part of our program proved to be more complex than we had thought. The concept, however, remains valid. In order for us to readily define the role that VSG's play in determining the virulence of an infection, isoantigenic clones of genetically related strains must be developed. Isoantigenicity must be specifically and thoroughly determined with a series of epitope-unique antibodies.

In summary, we feel that the second year of this study was quite successful. Not only did our group characterize many of the VSG's from the clones of the WRATat serodeme, but they assisted greatly in the research to establish the beginnings of understanding of trypanosome virulence and VSG chemistry.

REFERENCES

1. Lanham, Sheila M.: Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. Nature 218: 1273-1274 (1968).

2. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).

3. Penke, B., R. Ferenczi and K. Kovacs: A new acid hydrolysis method for determining tryptophan in peptides and proteins. Anal. Biochem. 60: 45-50 (1974).

4. Chamras, H., M. Fouchereau-Peron and G. Rosselin: The effect of streptozotocin-induced diabetes on the early steps of glucagon action in isolated rat liver cells. Diabetologia 19: 74-80 (1980).

5. Shulman, M., C.D. Wilde and G. Kohler: A better cell line for making hybridomas secreting specific antibodies. Nature 276: 269-270 (1978).

6. Poffenbarger, P.L.: Purification and partial characterization of an insulin-like protein from human serum. J. Clin. Invest. 56: 1455-1463 (1975).

7. Moses, A.C., S.P. Nissley, P.A. Short and M.M. Rechler: Immunological cross-reactivity of multiplication-stimulating activity polypeptides. Eur. J. Biochem. 103: 401-408 (1980).

8. Wagner, J., M. Palfreyman and M. Zraika: Determination of dopa, dopamine, dopac, epinephrine, norepinephrine, alpha-monofluoromethyldopa, and alpha-difluoromethyldopa in various tissues of mice and rats using reversed-phase ion-pair liquid chromatography with electrochemical detection. J. Chromat. 164: 41-54 (1979).

9. Buhler, H.U., M. DaPrada, W. Haffely and G.B. Picotti: Plasma adrenaline, noradrenaline, and dopamine in man and different animal species. J. Physiol. 276: 311-320 (1978).

10. Fallon, H.J., E. Frei, and J.D. Davidson: Leukocyte preparation from human blood: Evaluation of their morpholic and metabolic state. J. Lab. Clin. Med. 59: 779-791 (1962)

11. Cross, G.A.M.: Identification, purification, and properties of clone-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei. Parasitol. 71: 393-417 (1975).

TABLE 1

Molecular weight determinations of the glycoproteins obtained from the indicated clones of Trypanosoma b. rhodesiense WRATat serodeme, e.g. VSG-1 would designate the variant-specific glycoprotein purified from T.b. rhodesiense WRATat 1

Glycoprotein	Gel Permeation Chromatography	Polyacrylamide Gel Electrophoresis
VSG-1	89,580 ± 5,054	55,000
VSG-2	92,131 ± 2,976	66,000
VSG-3	92,381 ± 1,098	64,500
VSG-4	98,460 ± 2,008	64,000
VSG-5	90,437 ± 2,644	68,000
VSG-6	----------	73,700
VSG-7	64,437 ± 669	73,000
VSG-8	58,045 ± 987	------
VSG-9	91,330 ± ND	74,000
VSG-10	79,311 ± 2,154	58,000
VSG-12	66,000 ± 1,414	68,000
CSUT-J1	84,153 ± 2,697	73,000
WELLCOME	133,000 ± ND	73,000

TABLE 2

Amino acid composition of VSG's purified from various clones of the Trypanosoma b. rhodesiense WRATat serodeme expressed as residues per 65,000 molecular weight

T.b. rhodesiense WRATat #:	1	2	3	4	10
Amino Acid					
ASX	44	46	41	53	34
THR	34	46	34	51	36
SER	31	21	24	31	27
GLX	38	52	42	46	39
GLY	99	33	34	91	58
ALA	78	53	47	102	80
CYS	--	--	--	--	--
VAL	10	9	9	18	7
MET	2	5	4	5	8
ILE	9	12	12	16	11
LEU	27	31	28	31	30
TYR	--	--	5	7	3
PHE	--	10	8	9	4
HIS	4	6	6	8	4
LYS	26	33	28	38	22
TRP	--	--	--	--	--
ARG	5	12	11	10	4

Cysteine and tryptophan were not determined in this series.

TABLE 3

Nonsuppressible insulin-like activities and other factors determined in sera from male rats infected with Trypanosoma b. rhodesiense WELLCOME

	DAYS POST-INFECTION				
ITEM	1	2	3	4	4-CT
NSILA-p Activity (uL-eq/mL serum)	0.87 (0.15)	0.89 (0.07)	0.96 (0.07)	0.84 (0.08)	0.93 (0.02)
MSA (nG/dL)	36	NA	25	23	205 (85)
GLUCOSE (mG/dL)	118 (3)	112 (7)	21 (16)	2.5 (1.5)	112 (6)
SPLEEN WEIGHT (gm)	0.67 (0.08)	0.77 (0.04)	1.64 (0.15)	1.97 (0.12)	0.64 (0.06)
EPIDYDIMAL FAT WEIGHT (gm)	1.51 (0.20)	1.03 (0.11)	1.16 (0.17)	0.99 (0.32)	1.18 (0.11)

"4-CT" = the values obtained from uninfected animals inoculated with buffer only at zero time.
Values in parentheses are ± standard deviation.
NSILA-p is nonsuppressible insulin-like activity-p = a protein of molecular weight 88,000 (6).
MSA is multiplication-stimulating activity = a protein with a low molecular weight of 8,700 (7).

LEGENDS TO FIGURES

Figure 1: Growth of T.b. rhodesiense WRATat-1 and plasma glucose levels in rats treated with dexamethasone. Two groups of rats (300 gm) were treated with dexamethasone (20 mg/kg) and infected with T.b. rhodesiense WRATat-1 (10^6 trypanosomes/rat). A: Parasitemia and plasma glucose levels of rats injected with dexamethasone 6 hr before infection. B: Parasitemia and plasma glucose levels of rats injected with dexamethasone 6 hr before infection and again of Day-4 post-infection.

Figure 2: Parasitemia and plasma glucose levels of rats treated with streptozotocin and infected with T.b. rhodesiense WRATat-1. A group of rats (300 gm) was treated with streptozotocin (65 mg/kg) after they had been fasted for 16 hr. Four days after treatment when the plasma glucose levels reached 500 mg/dL the rats were infected with T.b. rhodesiense WRATat-1 (10^6 trypanosomes/rat). The parasitemias and the plasma glucose levels were determined for 14 days.

Figure 3: Concentration of parasites in the penultimate and terminal waves of infection in the rat "J1". The concentration of trypanosomes in the peripheral circulation of a rat (J1) was determined during the penultimate and terminal waves of parasitemia. The "X" indicates the level of trypanosomes in the blood on Day-72 post-infection of this animal and two others with T.b. rhodesiense WRATat-5. The arrow shows the day on which this animal became comatose and was sacrificed. A biological clone (CSUT-J1) was generated from this population of organisms.

Figure 4: Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense WELLCOME. Two groups of rats (300 gm) were infected with an acute strain of T.b. rhodesiense WELLCOME (10^6 trypanosomes/rat). SZ: Depiction of the parasite and plasma glucose levels in rats pretreated with streptozotocin (65 mg/kg). N: The same parameters are shown for infected, untreated rats. The streptozotocin-treated rats died on Day-5, and the normal rats died on Day-4 post-infection.

Figure 5: Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense CSUT-J1. The parameters measured and displayed in this figure are the same as those explained in Figure 4. The rats, however, were infected with trypanosomes cloned from the terminal wave of a chronic infection designated as T.b. rhodesiense CSUT-J1. The normal rats died on Day-8, and the streptozotocin-treated rats died on Day-11 post-infection.

Figure 6: Parasitemia, plasma glucose levels, insulin levels and spleen weights of rats infected with T.b. rhodesiense WELLCOME. Pooled data from 6 rats infected with T.b. rhodesiense WELLCOME (10^6 trypanosomes /rat). The parasitemias, plasma glucose and plasma insulin (immunoreactive) concentrations and spleen weights were determined over a 5 day period. Glucose and insulin levels decreased accutely. All the rats died on Day-4 post-infection with greatly enlarged spleens.

Figure 1

Growth of T.b. rhodesiense WRATat-1 and plasma glucose levels in rats treated with dexamethasone.

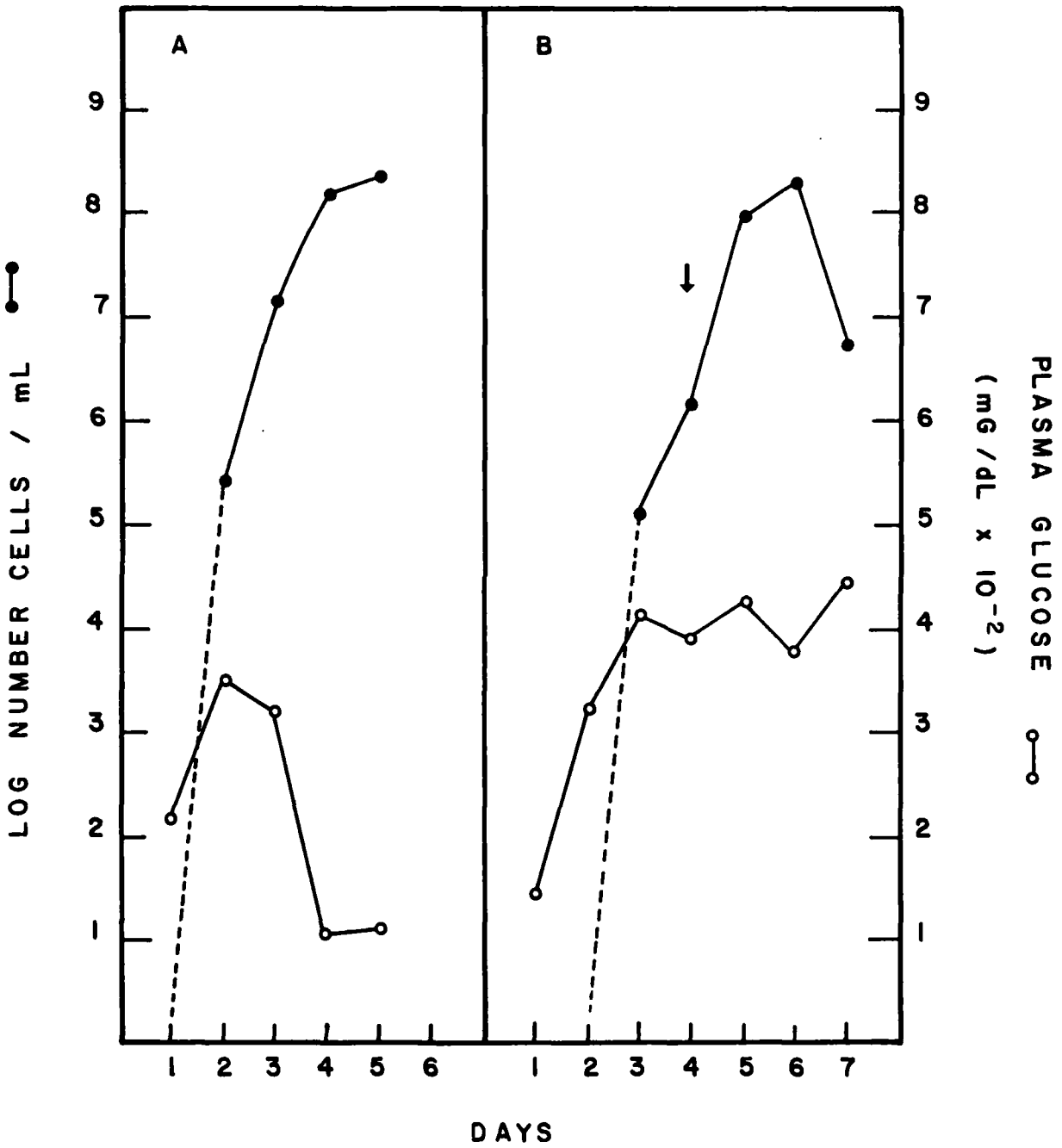


Figure 2

Parasitemia and plasma glucose levels of rats treated with streptozotocin and infected with T.b. rhodesiense WRATat-1.

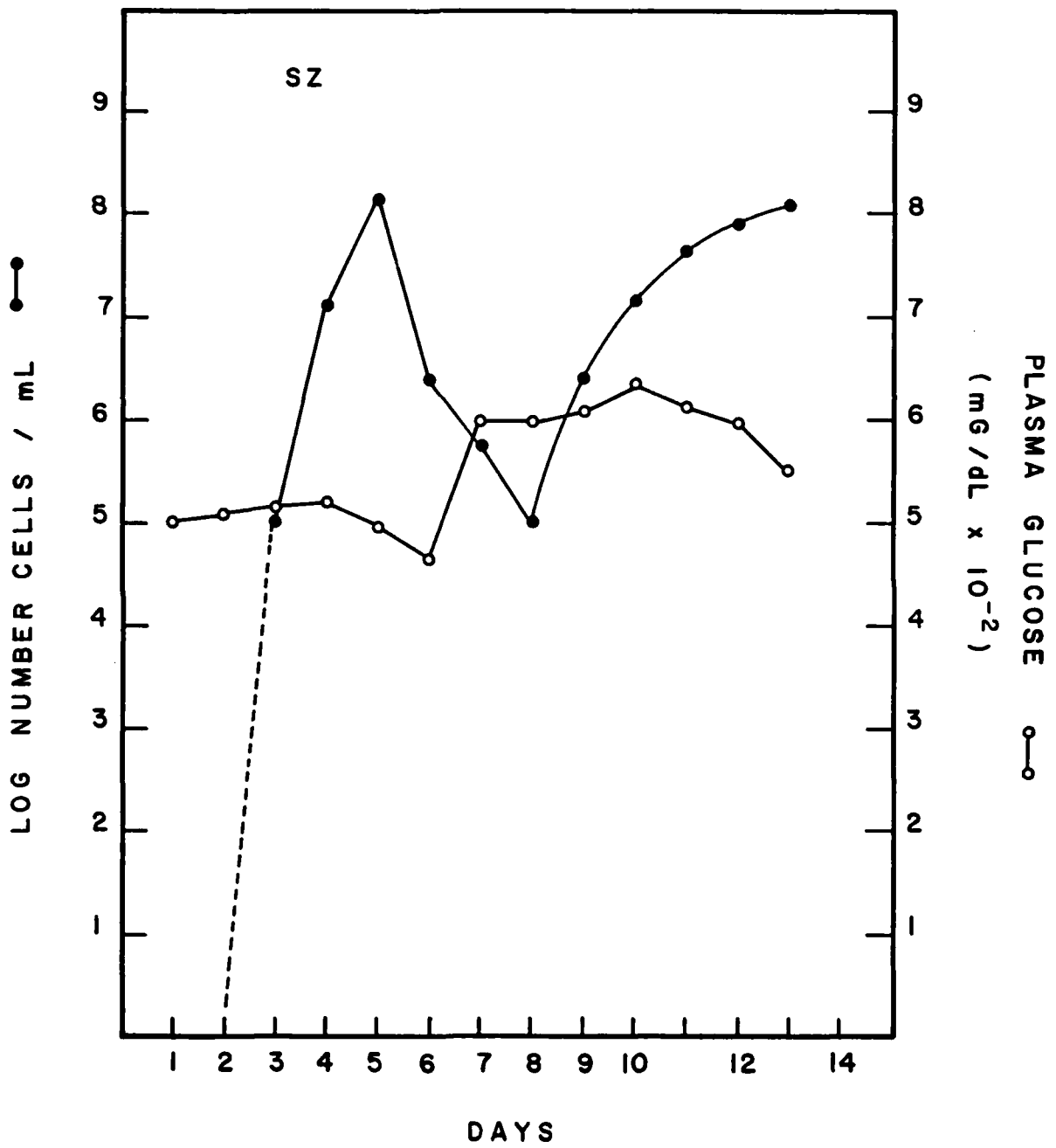


Figure 3

Concentration of parasites in the penultimate and terminal waves of infection in the rat "J1".

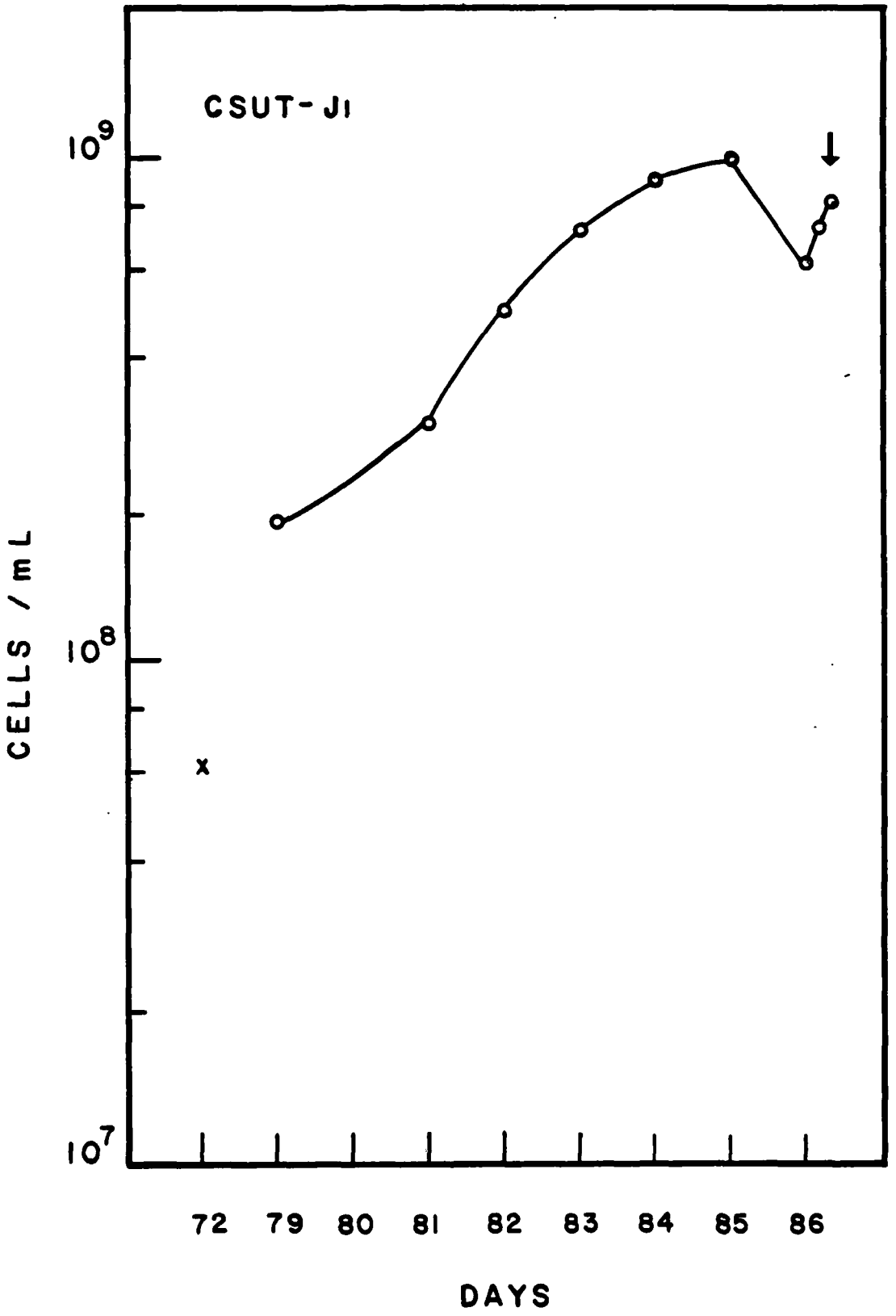


Figure 4

Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense WELLCOME.

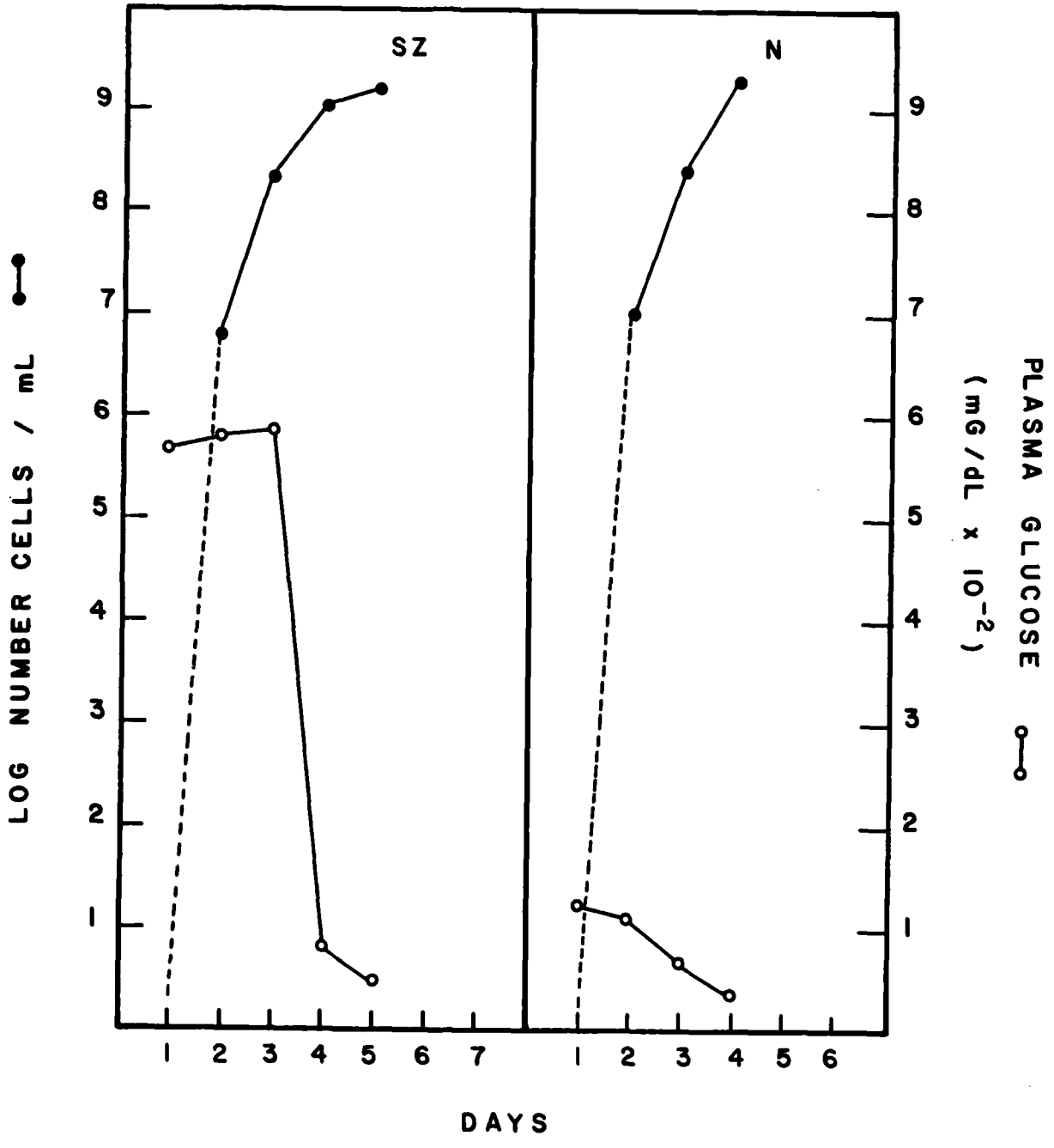


Figure 5

Parasitemia and plasma glucose concentration of untreated rats and rats treated with streptozotocin then infected with T.b. rhodesiense CSUT-J1.

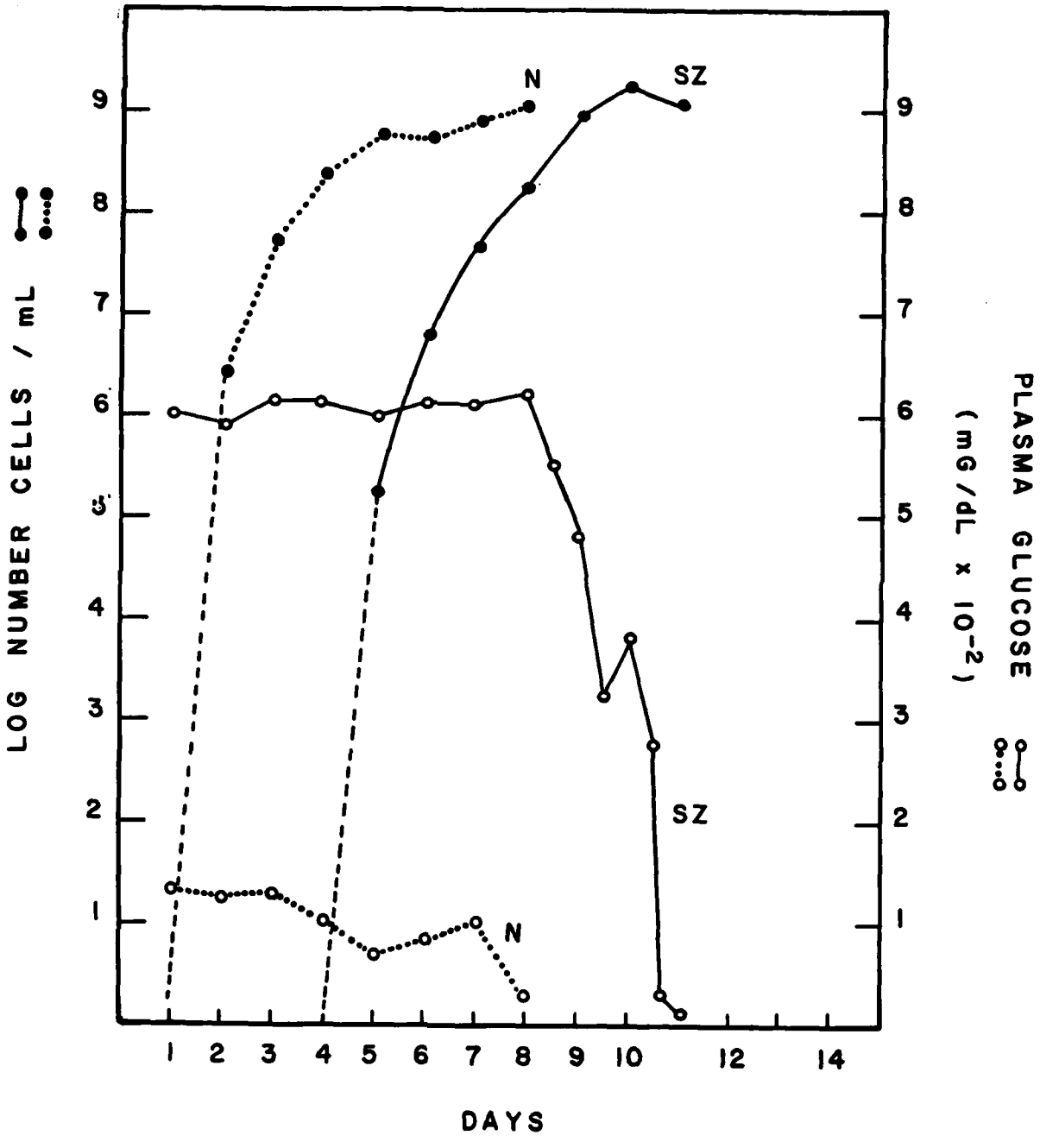
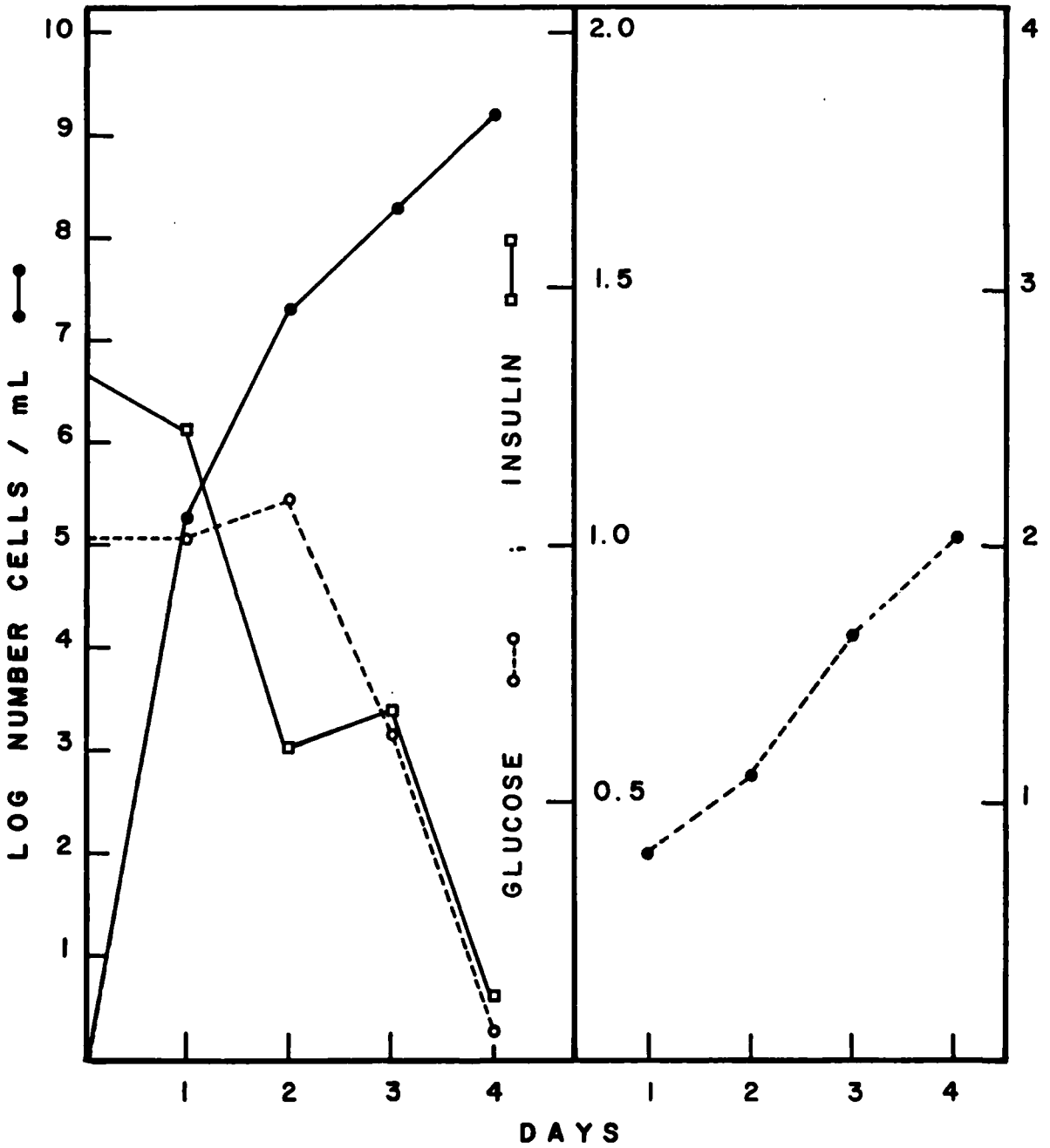


Figure 6

Parasitemia, plasma glucose levels, insulin levels and spleen weights of rats infected with T.b. rhodesiense WELLCOME.



FINAL REPORT SUMMARY

The work performed in our laboratory in the Department of Pathology at Colorado State University on the purification and characterization of the variant-specific glycoproteins from the Trypanosoma b. rhodesiense WRATat serodeme's clones was successful in many ways. We were able to demonstrate that major reactive carbohydrate residues were associated with the hydrophobic domains of these surface antigens. Although the molecular weights of the different VSG's were relatively similar, their amino acid compositions were quite unique. These results correlate well with those of Cross's group (11). In addition, the observations that the VSG's showed the tendency to form dimers, suggested that they could exist in similar associations on the surface of the parasites. Dimers appeared to be more prevalent when the buffer salt concentration was greater than 0.1 M, implying that the hydrophobic interactions were the predominant associative forces.

The biggest difficulty we faced throughout the course of this work was obtaining sufficient numbers of parasites which were needed to secure the surface antigens for analysis. Animals treated with whole-body gamma irradiation, cyclophosphamide, anti-PMN sera, or dexamethasone all permitted improved growth of the chronic strains. However, the timing and peak concentration of the trypanosomes in the first wave could not be dependably predicted. Most often these organisms began to transform to short-stumpy forms which coincided with the first immune crisis and were rapidly removed from the circulation. These observations supported the concept that an immunological event was not required to trigger the transformation process.

We felt that it was critical to this work and future endeavors to attempt to define the elements that triggered the morphological and physiological transformation of the long-slender typomastigotes to the short-stumpy forms. In the rats, the highly virulent trypanosomes do not transform, i.e. they are monomorphic. Organisms of intermediate virulence were seen to change to the short-stumpy forms on the initial wave of parasitemia, but failed to do so on the next one, resulting in the death of the rodent host. Trypanosomes displaying chronic infection profiles repeatedly transform until the proper internal conditions or parasite strain (or both) predominate, killing the host.

Two approaches were used to study this problem: 1) We needed to evaluate the host's responses to an acute infection and, 2) We felt that it was necessary to clone strains of trypanosomes that were derived from the WRATat serodeme, that were both isoantigenic and acute parasites. The former efforts would allow us to discern the elements of the host's response that obviously compromised the animal's ability to cope with the parasitic threat. We appreciated that the parasites were the source of the fatal stress and hoped that isoantigenically related chronic and acute strains would allow a precise assessment of the contribution of the variant-specific surface antigens to the determination of virulence.

The primary result of studying the host's response to an acute infection was the observation that the animals died of septic shock. The most interseting change was that related to the plasma activity of MSA (multiplication stimulating activity). MSA has been shown to be synthesized by hepatocytes. The dramatic decrease in MSA in Trypanosoma b. rhodesiense WELLCOME infected rats suggested that liver cells were

directly damaged by acute trypanosomes. Alternatively, MSA could be rapidly degraded or removed from the circulation by binding to peripheral cells. In the latter case, these tissues would be stimulated in their utilization of glucose. We did not have time to delve further into this problem before the contract period ended. However, a link to the massive consumption of glucose by the host and the parasites may have been glimpsed.

We were able to isolate a WRATat-related, acute trypanozoan by cloning organisms from the terminal wave of parasitemia initiated with Trypanosoma b. rhodesiense WRAT-5. It was not isoantigenic to WRATat-5 or to any of the other clones of the WRATat serodeme that we had in our laboratory. We designated this strain as T. b. rhodeseinse CSUT-J1 and characterized it to be acute by all criteria established for the known acute strain T.b. rhodesiense WELLCOME. We had immunized mice with purified VSG from the CSUT-J1 organisms but failed to produce monoclonal antibodies reactive to it before the contract period ended. A panel of antibodies was to be developed that would define the epitopes associated with this VSG, so organisms possessing the same regions could be identified among established chronic clones of the WRATat serodeme.

It is strongly felt that the structure and chemistry of the variant-specific surface proteins, including their sugar residues, play an important role in determining the relative virulence of a particular trypanosome strain. The only plausible approach to resolving the critical aspects of this phenomenon will be to evaluate isogenic clones of these organisms that are also isoantigenic, but show different degrees of virulence. The resulting information should simultaneously reveal methods to be used to control the pathogenicity of trypanosome-infected mammals, including humans.

ACKNOWLEDGEMENTS

The following persons and their staffs are gratefully acknowledged for their assistance in assaying plasma concentrations of insulin, nonsuppressible insulin-like activity (NSILA-p) and multiplication-stimulating activity (MSA):

Robert W. Phillips, DVM, Ph.D.
Department of Physiology and Biophysics
College of Veterinary Medicine and
Biomedical Sciences
Colorado State University
Fort Collins, Colorado

Philip L. Poffenbarger, M.D.
Departments of Medicine and Human
Biological Chemistry and Genetics
The University of Texas Medical Branch
Galveston, Texas

S. Peter Nissley, Ph.D.
Endocrine Section, Metabolism Branch
National Cancer Institute
National Institutes of Health
Bethesda, Maryland

DISTRIBUTION LIST

2 Copies	Director Walter Reed Army Institute of Research Walter Reed Army Medical Center ATTN: SGRD-UWZ-C Washington, DC 20307-5100
2 Copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, Maryland 21701-5012
1 Copy	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 Copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 Copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END

FILMED

2 0-86

DTIC