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ISOLATION OF THE RAT TRYPANOSOMAL AGGLUTINATING

ANTIBODY AND THE TRYPANOSOMA LEWISI

AGGLUTINATING ANTIGEN

by

DAVID F. LAPP

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# TABLE OF CONTENTS

INTRODUCTION ................................................................. 1

MATERIALS AND METHODS ...................................................... 16
  Collection of sera and isolation of gamma globulin ............. 16
  Preparation of ablatic serum ....................................... 17
  Preparation of isolated agglutinating antibody ................. 18
  Isolation of trypanosomal agglutinating antigen (agglutinogen) 21

RESULTS .................................................................................. 27
  Isolation of the agglutinating antibody (the first trypanosomal antibody) ............................................. 27
  Isolation of the agglutinating antigen ................................. 37

DISCUSSION .......................................................................... 52
  The agglutinating antibody and ablassin ............................... 52
  The nature of the agglutinating antigen ............................... 62

SUMMARY ........................................................................... 71

BIBLIOGRAPHY .................................................................... 73
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Agglutinin activity of pooled immune gamma globulin and its homologous agglutinating antibody preparation</td>
<td>28</td>
</tr>
<tr>
<td>II</td>
<td>In vitro titration of the agglutinating activity of pooled immune gamma globulin and its homologous antibody preparation</td>
<td>29</td>
</tr>
<tr>
<td>III</td>
<td>Reproductive activity of inhibited (adult) T. lewisi in normal serum and in pooled immune gamma globulin, and its homologous agglutinating antibody preparation</td>
<td>33</td>
</tr>
<tr>
<td>IV</td>
<td>Isolation of the trypanosomal agglutinating antigen</td>
<td>38</td>
</tr>
<tr>
<td>V</td>
<td>Passive hemagglutination activity of sheep erythrocytes sensitized with TCA extracts and 45% phenol extracts of reproductive trypanosomes</td>
<td>39</td>
</tr>
<tr>
<td>VI</td>
<td>Passive hemagglutination activity of trypanosomal antigen extracts</td>
<td>41</td>
</tr>
<tr>
<td>VII</td>
<td>Passive hemagglutination activity of 45% phenol extracts of the particulate and soluble fractions of reproductive trypanosomes</td>
<td>42</td>
</tr>
<tr>
<td>VIII</td>
<td>Agglutination of sensitized sheep erythrocytes with adsorbed immune gamma globulin</td>
<td>44</td>
</tr>
<tr>
<td>IX</td>
<td>The effect of rat epididymal glycosidase on the agglutination of reproductive T. lewisi</td>
<td>47</td>
</tr>
<tr>
<td>X</td>
<td>Trypanosome agglutination inhibition test</td>
<td>48</td>
</tr>
<tr>
<td>XI</td>
<td>Trypanosome agglutination inhibition test</td>
<td>49</td>
</tr>
<tr>
<td>XII</td>
<td>The effect of papain on the agglutination of reproductive and adult T. lewisi</td>
<td>51</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Electropherogram of pooled immune rat serum, ammonium sulfate precipitated gamma globulin and agglutinating antibody preparations isolated with reproductive trypanosomes on cellulose acetate microzone electrophoresis in 0.01M $\beta$-mercaptoethanol barbital buffer, pH 8.6, $\mu = 0.075$</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>The influence of antibody preparations on the relative in vitro reproduction of adult trypanosome</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>In vitro titration for ablastic activity of pooled immune gamma globulin and its homologous agglutinating antibody preparation isolated with four-day postinfection (reproductive) trypanosomes</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>In vitro titration for ablastic activity of pooled immune gamma globulin and its homologous agglutinating antibody preparation isolated with fourteen-day postinfection (adult) trypanosomes</td>
<td>36</td>
</tr>
</tbody>
</table>
INTRODUCTION

_Trypanosoma lewisi_, a cosmopolitan, flagellated blood parasite of rats, was first described by Lewis in 1879. It is transmitted from host to host by the rat flea, _Nosopsyllus fasciatus_, and by the rat louse, _Polypex spinulosus_ (Laveran and Mesnil, 1901; Minchin and Thompson, 1915; and Kudo, 1954). Rats become infected by ingesting from their fur or skin freshly deposited flea feces. After a latent period of several days, the parasites appear in the blood and multiply rapidly for one or two weeks by equal and unequal fission and by multiple fission with rosette formation. Multiplication then ceases, and the number of trypanosomes declines rapidly during an initial phase (a numbers crisis), then more slowly, until, after a week to several months, the rat is free from parasites and immune to further infection. When fleas suck the blood of infected rats, they ingest trypanosomes, about 25% of which persist in the flea. These parasites do not become infective to the rat again until at least five to six days have elapsed. During this latent period in the flea, the trypanosomes enter epithelial cells of the stomach, and differentiate into rounded or oval flagellated forms which in turn give rise to many young trypanosomal forms. The latter escape into the stomach and may repeat the cycle. Eventually they transform into crithidia forms and pass into the rectum. Here they swim about or adhere to the rectal walls and multiply rapidly by longitudinal fission. Following transformation into metacyclic trypanosome forms, the protozoans which pass out with the feces are again infective to rats (Hyman, 1940; and Minchin and Thompson, 1915).
Although the rat is considered to be the chief host for *Trypanosoma lewisi*, other animals can be infected experimentally. Laveran and Mesnil (1901) and Coventry (1929) infected guinea pigs and rabbits with the bloodstream form of the parasite, but the total number of circulating trypanosomes was never as numerous as in the normal host. Lincicome (1955) succeeded in infecting mice with *T. lewisi*, providing the mice were given small, daily injections of normal rat serum. Johnson (1933) reported a natural infection of *T. lewisi* in a child who had lived in very close association with infected wild rats. The infection lasted five days, and was accompanied by anemia and a fever, both of which disappeared with the parasites.

The development of parasitemia in rats, experimentally induced by transfer of infected blood to healthy rats, has been described by Steffan (1921). The course of the experimentally-induced parasitemia closely follows that of the natural flea-transmitted infection. Intraperitoneal injection of infected blood is often followed by a prepatent period, which may last for several days. However, if sufficient numbers of parasites are injected, there may be no prepatent period. Once the organisms are seen in the blood, their numbers increase to a peak of 300,000 or more per cubic millimeter, eight to ten days after inoculation. When this population peak is reached, a numbers crisis occurs and most of the trypanosomes are destroyed. The survivors remain in the blood for an additional three to four weeks or longer, and then a second crisis terminates the infection. The rat is then refractory to reinfection by the parasite.

Several ideas, including antibodies as well as other defense mechanisms, have been advanced to explain how the rat recovers from
and becomes immune to the infection. Laveran and Mesnil (1901) considered phagocytosis to be the only mechanism involved in both active and passive immunity. They observed agglutination of the trypanosomes but it was dismissed as an insignificant mechanism of defense, since the parasites were neither immobilized nor apparently harmed in any way. They suggested that the agglutination factor and the immobilization factor represent separate entities. Manteufel (1909) believed that phagocytosis played a part in cases of weak immunity, but that lysis was the mechanism involved in highly immune animals.

MacNeal (1904) observed that six to eight days postinfection, reproducing trypanosomes tended to form rosettes while continuing to divide, and that they were able to separate and to continue individually in the blood stream for some time. He suggested that rosette formation was due to an increased difficulty of division which was associated with an antagonistic reaction on the part of the host. MacNeal was unable to observe any phagocytosis of the organisms in rats, but using immune guinea pigs, found evidence of immobilization and gradual lysis of the trypanosomes in the peritoneal fluid. He concluded that the parasites disappeared as a result of some cytolytic factor rather than phagocytosis. Regendanz and Kikuth (1927) claimed that the final disappearance of trypanosomes was due to a gradual non-specific phagocytosis throughout the infection.

As early as 1899, Rabinowitsch and Kempner reported that the blood of rats during early infection contained many dividing forms, whereas later in the infection, it contained only non-reproducing parasites. This observation was verified by subsequent workers (von Wasielewski
and Senn, 1900; Laveran and Mesnil, 1901; MacNeal, 1904; and Brown, 1915). From this observation and subsequent work, Taliaferro and his co-workers (Taliaferro et al., 1922; Taliaferro, 1924, 1932; and Coventry, 1925, 1930) presented a working hypothesis to explain the resistance of the rat to a T. lewisi infection. Taliaferro (1932a) concluded that there were three factors involved in resistance to the parasitic infection: 1) a factor which completely inhibits reproduction of the parasite by the tenth day; 2) some trypanocidal factor which kills the majority of the organisms at the time of the first numbers crisis (eighth to fourteenth day); and 3) a similar trypanocidal factor which terminates the infection at the end of a few days to several months. Inhibition of reproduction was not shown by number counts, but by methods which are not affected by trypanocidal factors. Two basic methods have been used by most workers: The first method consists of ascertaining the percentage of dividing forms found in daily blood smears (Laveran and Mesnil, 1901; MacNeal, 1904; Brown, 1914; Rabinowitsch and Kempner, 1899; and von Wasielewski and Senn, 1900); the second method, involving measurement of the coefficient of variation for the total length of the trypanosomes, was developed by W. H. Taliaferro and L. G. Taliaferro (1922). It is based on the fact that members of the growing trypanosome population vary in total length because of young and growing forms, whereas members of an adult population do not.

The immunological basis for the inhibition of reproduction of T. lewisi in the rat was first demonstrated by Taliaferro in 1924 and was later confirmed by others (Coventry, 1925; Regendanz and Kikuth, 1927). Taliaferro found that serum from an infected rat, in which trypanosomes
had ceased to reproduce, contained a passively transferable factor
which prevented adult (non-reproducing) trypanosomes from dividing in
normal, non-immune rats, but which apparently did not kill them or
affect their vitality. Coventry (1925) demonstrated that the repro-
duction-inhibiting antibody appeared suddenly on the fifth to sixth
day of infection. The titer increased gradually until the thirty-fifth
day and then slowly declined. In 1929, she showed that the same type
of antibody could be demonstrated in the evanescent infection of T.
lewi in guinea pigs, and that it was passively transferable from
from guinea pigs to rats. Neither Taliaferro (1925, 1932a, b) nor
Coventry (1925, 1930) felt that the antibody which inhibited reproduc-
tion was instrumental in causing either the numbers crisis, which
occurred early in the infection, or the termination of the infection.

Regendanz and Kikuth (1927) showed that splenectomy sometimes
markedly lowered synthesis of the reproduction-inhibiting antibody,
hence implying an involvement of reticulo-endothelial system in the
defense mechanism. Their work was confirmed by Marmorston-Gottesman
et al. (1930). The problem was reexamined by Taliaferro and co-workers
in 1931, and they concluded that the functional level of the macrophage
system was high enough in a rat to inhibit reproduction of the trypano-
somes by the eighth to tenth day. This level was lowered by splenec-
tomy, India ink blockage, Bartonella infection, paratyphoid infection
and pregnancy. When two or more of these factors (rarely one) were
simultaneously present, the functional level of the macrophage system
was too low to operate completely and effectively. Taliaferro (1932b)
fractionated immune serum from rats which had recovered from the
parasitemia and found that the reproduction-inhibiting antibody, which he termed ablastin, and the trypanocidal antibodies, were precipitated with the serum globulins. The trypanocidal antibody could be removed from the serum by adsorption with trypanosomes in vitro, and such organisms, after being in contact with immune serum, were sensitized so that they were quickly killed when they were introduced into a normal, previously uninfected rat. Unlike the trypanocidal antibody, ablastin showed no in vitro affinity for either dividing or adult trypanosomes, and it could not be removed from serum by adsorption. Similarly, neither dividing nor adult parasites were sensitized by treatment with ablasic serum. Taliaferro (1932b) did find that ablastin could be produced by immunization of rats with triturated parasites. This was the first demonstration that ablastin and the trypanocidal antibody were separate entities.

Coventry (1930) associated the first numbers crisis in the infection with a trypanocidal antibody. She found that serum taken from rats after the first numbers crisis caused disappearance of trypanosomes when injected into rats in which the parasites had just appeared. A zonal phenomenon, typical of antigen-antibody reactions was also associated with this serum. Such serum, however, was without effect if injected after the natural numbers crisis; and she postulated that the parasites which survived the first numbers crisis were resistant to this antibody but were still susceptible to the antibody which brought about the termination of the infection. Taliaferro (1938b) reported similar ablasic and trypanocidal activity against Trypanosoma duttoni in the mouse, and he established some cross-immunity of the ablastin to Trypanosoma lewisi.
A number of investigators (Laveran and Mesnil, 1901; Regendanz and Kikuth, 1927; Becker et al., 1947) observed agglutination of trypanosomes during the course of infection in rats. Barnes (1951) emphasized the importance of agglutination and recognized it as the manifestation of an antibody which is distinct from both ablastin and the terminal trypanocidal antibody. Blood samples taken at the time of the first numbers crisis revealed that most of the flagellates were agglutinated. Agglutination was a common phenomenon among parasites examined from the day of crisis until that time when all parasites were absent from the blood. Barnes believed that the agglutination in conjunction with trypanolysin played an important role in bringing T. lewisi infections to an end.

More recently, D'Alesandro (1960) fractionated immune rat serum by electrophoresis and ultracentrifugation. Ablastin and the agglutinating antibody (the first trypanocidal antibody) are low molecular weight, 7s globulins. The terminal trypanocidal antibody is a 19s macroglobulin. Electrophoresis on starch gel revealed that all three antibodies migrated between the slow moving beta globulins and the fast moving gamma globulins (the gamma-1 or T component).

D'Alesandro (1962) developed an in vitro system for demonstrating ablastic activity. All previous studies had been made in vivo and usually involved passive transfer of either immune serum or its fractions. Since the role of complement could not be established with these in vivo methods, D'Alesandro was the first investigator to demonstrate that ablastin was not complement-dependent. The trypanocidal antibodies of hyperimmune serum, however, require complement for lytic
activity (Taliaferro, 1932b, 1938b), but may act as agglutinins or opsonins without it (D'Alesandaro, 1966).

Considerable work has been done on the biochemical and metabolic changes associated with the marked morphological changes induced by ablastin. In studies using the Warburg respirometer, Moulder (1948a, b) found that ablastin-inhibited trypanosomes utilized less glucose and consumed more oxygen than those that were freely reproducing. During the course of infection in the rat, the oxygen to glucose ratio rose from about one to three. Malonate inhibition of oxygen uptake also increased with the age of infection. Since these changes were related chronologically to the appearance of ablastin in the rat, Moulder suggested that they were caused by the inhibition of ablastin of the oxidative assimilation of glucose in such a manner as to stop cell division and growth of T. lewisi. Similar studies were also made by later workers (Ryley, 1951 and Thurston, 1948). Pizzi and Taliaferro (1960) confirmed these findings using labeled glucose. Taliaferro and Pizzi (1960) using $^{35}$S-labeled amino acids and $^{14}$C-adenine, found that in the inhibited adult stage, nucleic acid synthesis is virtually stopped, and that protein synthesis is reduced to a low maintenance level. That ablastin was responsible for these marked changes in metabolism was demonstrated by experiments in which passively-transferred ablastic serum concomitantly inhibited parasite reproduction and protein and nucleic acid synthetic activity. Taliaferro and Pizzi (1960) also noted that the highest synthetic activity was found in recently released adults (i.e. adult trypanosomes which had been washed and injected into normal uninfected rats).
Since ablastin changed glucose metabolism from one of assimilation to one of maintenance (Moulder, 1948b), and lactic acid was reported to be an endproduct of glucose metabolism (Ryley, 1951). D'Alesandro and Sherman (1964) decided to investigate the changes in lactic dehydrogenase (LDH) levels of T. lewisi associated with the appearance of ablastic immunity. During the early, reproductive phase of the infection, when glucose utilization was high, the parasites contained over three times as much enzyme as the non-reproducing, ablastin-inhibited parasites found late in the infection. No qualitative difference between isozymes of adults and reproductive trypanosomes was found, and ablastic serum had no neutralizing effect when added directly to the in vitro LDH assay system.

D'Alesandro (1966) has begun to examine other trypanosomal enzymes to determine possible changes in enzyme levels, which may occur during the course of infection, and whether there is a general and consistent pattern in the levels found. To date, only those enzymes involved in carbohydrate metabolism which require DPN or TPN, have been studied. No differences in activity levels between reproductive and adult trypanosomes have been demonstrated with malic dehydrogenase, isocitric dehydrogenase or malic enzyme. However with lactic dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, the dividing organisms contained from over two to three and one half times as much enzymatic activity as in the inhibited, adult organisms. From these few measurements and the observations of Moulder (1948a, b) and Taliaferro and Pizzi (1960), it was suggested that reproducing populations of trypanosomes rely heavily on the glycolytic pathway and the hexose
monophosphate shunt, and that adult populations have a greater dependence on the tricarboxylic acid cycle.

The hypothesis that ablastin is responsible for the inhibition of reproduction of T. lewisi has not been universally accepted. Augustine (1943) studied the defense mechanisms of immune rats against reinfection with T. lewisi. He concluded that ablastin is either absent or non-operative as a defense mechanism against reinfection, which is contrary to the results reported by Taliaferro (1932a, b) for recovery from initial infection. Reinfection lasted from a few minutes to several hours and occasionally up to four days. Augustine believed that the time span during which trypanosomes remained in the circulating blood of an immune rat was determined by two factors: a) the titer of the trypanocidal antibody and b) the age of the parasite at the time of transfer (i.e. a reproductive or adult trypanosomal inoculum). Reproductive trypanosomes were almost immediately sensitized by trypanocidal antibody, being either killed or immobilized, and then removed from circulation by blood macrophages. Adult trypanosomes were more resistant to the trypanocidal antibody and were neither immobilized nor killed by it. Only when the trypanocidal antibody reached an optimum titer were they agglutinated and the living agglutinated mass then filtered out in the liver and spleen. Augustine's experiments gave no indication that ablastin functions in recovery from reinfection. Although the parasite population in an immune rat did not increase, he believed that this failure was not due to ablastin. In reinfection with adult trypanosomes, division could be initiated and could continue for some time in the circulation, but the dividing cells were particularly
susceptible to the trypanocidal antibody and were removed from the circulation as soon as they were formed. Thus, an increase in parasite population was checked, but the reproductive activity was not. Augustine also found that the trypanocidal antibody was strictly localized in the blood. An immune rat was inoculated intraperitoneally with adult trypanosomes and examined twenty-four hours later for infection. No trypanosomes were found in the blood but fluid aspirated from the peritoneal cavity teemed with trypanosomes which could produce an infection when transferred to another rat. Having also demonstrated that there was free passage of the parasites from the peritoneal cavity through the lymph nodes into the blood circulation in hyperimmune rats, Augustine concluded that the titer of antibody in this immune rat was so high that the trypanosomes were destroyed as soon as they entered the blood stream.

Ormerod (1963) proposed a "two antigen" hypothesis to explain immunity of the rat to *T. lewisi*. Like Augustine's, his hypothesis is based on selective killing and removal from circulation of dividing forms. He believed that developmental (reproductive) forms and adults each contain a separate and unrelated antigen. When developmental forms first appear in the blood, antibody against them begins to form and eventually wipes them out at the first numbers crisis. Before this has happened a proportion have been converted into adults which are insensitive to this antibody. These adults survive in the blood until at terminal crisis they are removed by the formation of a new antibody against their particular antigen. This idea differs from Augustine's hypothesis in that when adults begin to divide and are
selectively removed, the population level remains constant because the lost parasites are replaced by "low grade" reproduction occurring outside the peripheral circulation. Ormerod (1963) reported finding dividing trypanosomes in kidney smears of infected rats.

Becker and co-workers (1943, 1947, 1948) found that the reproductive phase of the *T. lewisi* infection is prolonged when the host rat is fed a pantothenic acid-deficient diet, or when sodium salicylate was administered orally. Becker and Gallagher (1947) suggested that ablastin was not an antibody, but an oxidative enzyme composed of a protein moiety in union with pantothenic acid. The apoenzyme would be a specific protein (ablastin) that accumulated in the rat's blood during the first nine days of the infection, becoming associated with the coenzyme, pantothenic acid, which is normally present in the diet. The hypothesis accounted for the failure or partial failure of the anti-reproductive process in rats on pantothenate-deficient diets. The inhibition by salicylate was explained by its displacement of the coenzyme, pantothenic acid, thus rendering the enzyme inactive. Lysenko (1951), however, has found no evidence for salicylate combining with ablastin in vivo or in vitro. He also reported that the levels of total protein and of gamma globulin were higher in normal rats than in salicylate-treated rats, suggesting that sodium salicylate inhibited general protein and antibody synthesis.

Thillet and Chandler (1957) reported that rats could be rendered completely refractory to *T. lewisi* infections by repeated injection of the soluble metabolic products of trypanosomes produced by the in vitro incubation of living organisms in a mixture of saline and normal rat
serum for twenty-four hours. Immunization with dead, triturated trypanosomes resulted in practically no agglutinating potency. It was also possible to remove most of the protective value of serum from recovered rats, including its ablasic activity, by adsorption with lyophilized, washed trypanosome bodies. These results suggested to Chandler (1958) that ablastin is an antibody directed against metabolic products, is primarily responsible for the immunity of rats to reinfection with *T. lewisi*, and acts as the agglutinating antibody which is responsible for the first numbers crisis. Inhibition of reproduction would occur when the ablastin titer was low, and would be due to neutralization of secreted extracellular enzymes of the parasite. Agglutination would occur when ablastin titer was high, and would be due to ablastin in combination with extracellular enzymes that were still in contact with trypanosomal cell surface. Complete destruction of trypanosomes at the first numbers crisis would not occur because the remaining parasites were so few in number that there would be little chance for contact and subsequent agglutination.

A hypothesis previously favored by the present author and strongly influenced by the work of Chandler (1958) and Ormerod (1963), was that the agglutinating antibody also exhibits ablasic activity. Since adult and reproductive trypanosomes differ in their susceptibility to the agglutinin it was postulated that the reproducing trypanosome population induced the formation of agglutinin. The titer of antibody would rise as the parasite population increased, and when an optimal antibody-trypansome ratio was reached (about day eight), there would be mass agglutination and a sharp decline in parasite numbers. Since
only reproductive trypanosomes would possess the agglutinating antigen, adult organisms would be left in the circulation. It was also theorized, that during development from reproducing to adult trypanosome, there was partial loss of masking of the agglutinin antigenic determinant(s) resulting in a very low affinity of the antigen for the agglutinating antibody. The adult trypanosome would then be coated by a loosely bound antibody which could inhibit uptake of metabolites and induce a switch from an assimilative type of metabolism to one of maintenance. An adult, non-reproducing trypanosomal population would thus be maintained until the appearance of the terminal trypanocidal antibody.

Throughout their work, Taliaferro (1922, 1932) and colleagues used the coefficient of variation (a measurement of deviation in percentage of the mean) of the total length of the T. lewisi population, to measure the reproductive activity of the population. This measure is valid and independent of the total number of individuals only in the sample size is adequate and if there is no destructive agent (trypanocidal antibody) that selectively removes trypanosomes of a certain age. Some investigators (Augustine, 1943; Ormerod, 1963) who do not accept ablastin as an inhibitor of reproduction, believe that Taliaferro's work has not completely eliminated the possibility that dividing parasites are selectively removed from the circulation. They believe that ablastic activity is a reflection of an antigenic change of the trypanosome and not a result of the elaboration of a non-adsorbable antibody by the host (Ormerod, 1963). The present investigation was undertaken to test whether these objections were valid and whether the agglutinating antibody and ablastin were identical. A method of isolating the
agglutinating antibody which is responsible for the first numbers crisis, was developed and tested in vitro for ablasic activity.

Since Coventry (1930) had shown that adult and reproductive T. lewisi differ in their susceptibility to the agglutinating antibody, and since this difference was postulated to result in inhibition of reproduction, the present study relates attempts to isolate the agglutinating antigen and to develop an in vitro assay system so that the differences might be studied quantitatively. The results of these attempts are reported in the following pages.
MATERIALS AND METHODS

The Rice strain of *Trypanosoma lewisi* used for this work was maintained by passage through albino rats obtained from the Holtzman Company. Rat blood was collected by cardiac puncture into 4% citrated saline, and centrifuged at 1400 Xg for twenty minutes. The white buffy coat, found layered on top of the packed erythrocytes, was collected, diluted with approximately four volumes of saline, and 0.75 ml injected intraperitoneally into uninfected rats. Uninfluenced infections usually lasted from three to four weeks.

A. Collection of sera and isolation of gamma globulin.

Normal and immune sera were collected from 12-hour fasted rats by cardiac puncture; the blood was allowed to clot, the tube was ringed and stored overnight at 4°C. Serum was then aspirated, centrifuged, and stored in the freezer until further use. D'Alesandro (1960) found that serum samples thus stored, for as long as ten months, showed little change in antibody titer.

Rat gamma globulin was isolated from serum by repeated precipitation with ammonium sulfate, between 0 and 33% saturation, following the method of Campbell et al. (1963). With constant stirring at room temperature, a saturated ammonium sulfate solution was added dropwise to a serum sample until a one-third saturated solution was obtained. The pH was then adjusted to 7.8 with 2N NaOH, and the suspension was stirred at room temperature for two hours in order to avoid mechanical trapping of serum components other than gamma globulins in the precipitate. The material was centrifuged at 1400 Xg for 30 minutes at 25°C and the
supernatant fluid discarded. This precipitate contained all of the gamma globulins plus other globulins and traces of albumin. The precipitate was dissolved in enough saline to restore the volume of the original serum sample, and purification of the gamma globulin fraction was achieved by two additional precipitations. The precipitate from the third purification step was dissolved in borate-buffered saline, pH 8.4 (Campbell et al., 1963), to a final volume of half that of the original serum sample. Ammonium sulfate was removed by dialysis of this solution against borate-buffered saline at 4°C, until no sulfate ions were detected in the dialysis medium upon addition of a few drops of 2% barium chloride to an aliquot of this buffer. The solution was centrifuged at 4°C for 30 minutes at 1400 Xg to remove all traces of precipitate, and frozen.

B. Preparation of aelastic serum.

Aelastic serum was prepared from pooled immune serum from rats, 14 or 25 days postinfection. The trypanocidal antibodies were removed by adsorption with living, reproductive trypanosomes. Rats were bled by cardiac puncture into 4% sodium citrated saline, four days after inoculation. The parasite suspension was purified by differential centrifugation, washed once with saline, and counted. This suspension was concentrated by centrifugation and the supernatant discarded. Immune serum was added slowly to the sediment with constant agitation of the mixture. Sufficient serum was used so that there was at least one to two billion trypanosomes per milliliter of serum. After mixing and incubation at 37°C for twenty minutes, the trypanosome-serum mixture was
held at 4°C overnight. Following removal of the trypanosomes by centrifugation, the adsorbed serum was ready for use, containing no trypanocidal antibody.

C. Preparation of isolated agglutinating antibody.

Rat agglutinating antibody was isolated by alkali dissociation of the pooled immune serum or gamma globulin adsorbed on trypanosomes, following the method described by Sternberger et al. (1963). Trypanosomes were collected in citrated saline by cardiac puncture and separated from erythrocytes by differential centrifugation. Once a clean suspension of parasites was obtained, it was washed three times with D'Alesandro's phosphate-buffered saline with 0.01M glucose, pH 7.4, and fixed overnight at 4°C in the same buffer with 1% formalin added. In order to remove from the trypanosomes any alkali-soluble material that could have interfered with subsequent purification of antibody, the fixed parasites, finely suspended in saline, were brought to pH 11.8 in frozen beakers at 0°C with 1.0 and 0.1N NaOH solution. The suspension was centrifuged immediately at 1400 Xg at 1°C for 20 minutes. The sediment was resuspended by addition of borate-buffered saline, pH 8.4, and immediately brought to pH 8.0 at 1°C with 1.0 and 0.1N HCl solutions. The suspension was then recentrifuged and washed three times with cold saline.

Adjustments in pH at this temperature were made following the method of Sternberger (1952). A Beckman Expandomatic pH meter with a Fisher general purpose glass electrode (for use to -5°C) was employed. The electrode was standardized at room temperature with pH 10.0 buffer and
the temperature compensator of the pH meter was then adjusted to 20°C. Failure to follow this procedure resulted in disintegration of the parasites.

The trypanosome suspension, now ready for adsorption, was counted (using an AO Spencer Bright Line hemocytometer), recentrifuged, and enough immune serum or gamma globulin added so that the number of trypanosomes per milliliter of serum exceeded 1 X 10^9. This trypanosome-antiserum suspension was shaken at 37°C for four hours at the maximum speed at which foaming did not occur, and then placed on a multipurpose rotator (5-15 rpm) for two days at 4°C. The suspension was centrifuged, the sediment washed three times with saline at 1°C, and the material finely resuspended by the addition of saline. To dissociate the antibody, the suspension was brought to pH 11.8 at 1°C with 1.0, 0.1, and 0.001N NaOH solutions and centrifuged immediately (1400 Xg for 20 minutes). The sediment was discarded and the supernatant was brought without delay to pH 8.0 at 1°C using 1.0, 0.1, and 0.01N HCl solutions. The material was then recentrifuged to removal all traces of precipitate, placed in dialysis tubing, and concentrated to the desired volume by pervaporation or with the aid of dry carbowax. The purified antibody was centrifuged and the supernatant was frozen until further use.

Agglutinating titers were determined simultaneously in vitro on the purified antibody and the pooled immune serum or the gamma globulin from which the antibody was isolated. Serial dilutions of antibody were prepared in 0.2 ml volumes; and 0.2 ml of a clean, four-day postinfection trypanosome suspension (10^7 trypanosomes per ml) was added. The tubes were shaken, incubated for two hours at room temperature, and
scored for agglutination using a dissecting microscope. The standard - to 4+ scale was employed to score trypanosomal agglutination. A ++++ indicates complete agglutination of trypanosomes, with a pellet forma-
tion in the bottom of the tube, a - signifies no agglutination, and the other symbols represent intermediate steps between 4+ and -.

Titrations for ablasic activity of serum fractions and aggluti-
nating antibody preparations were performed in vitro in 13 X 100 mm screw cap culture tubes. To each tube, 1.5 ml of D'Alesandro's (1962) mono-
phasic culture medium, and 0.2 ml of antibody, serially diluted with normal, non-immune serum, was added. The culture tubes were inoculated with 0.05 ml of an adult, inhibited trypanosome suspension in saline, containing 4-5 million parasites, and incubated at 37°C for 17 hours. Samples were removed from the culture tubes with a bacteriological loop, spread on a glass slide, and stained with Giemsa stain. Repro-
ductive activity was measured by the method of Taliaferro et al. (1931). Two hundred to three hundred parasites were examined at random, and the number of actual dividing forms and small forms less than twenty microns in total length was recorded. Reproductive activity was expressed as the percentage of dividing and small forms found among the parasites examined. Each culture tube was examined microscopically at the end of each experiment for T. lewisi viability and for contamination.

Electrophoresis of immune rat serum and its various fractions was performed on cellulose acetate strips, using the Beckman Microzone Cell, at 300 volts for 30 minutes at 5°C. The buffer used was Beckman's B-2, pH 8.6, ionic strength 0.075; this is a barbital-sodium barbital buffer with 0.01M beta-mercaptopethanol added to improve resolution of the protein
components. The strips were subsequently stained for protein with Ponceau S (Beckman) for seven to ten minutes, and destained with 5% acetic acid. Some membranes were cleared in a solution of 25% (v/v) glacial acetic acid and 75% ethanol (95%) dried for ten to fifteen minutes at 110°C, and scanned on a Spinco Analytrol Model R densitometer.

D. Isolation of the trypanosomal agglutinating antigen (Agglutinogen).

Thirty to forty rats were used for the collection of trypanosomes for the four-day and fourteen-day postinfection stages. Clean parasite suspensions were obtained by differential centrifugation, and the total number of trypanosomes was counted. The agglutinogen was extracted from whole cells, 1% formalin fixed cells, sonicated parasites (Brosonik probe Bromwill oscillator, 5 minutes, 4°C) or from a crude particulate fraction (from French press, 15,000 psi) with phenol and water, a procedure described by Kabat and Meyer (1964) for the extraction of bacterial lipopolysaccharide. The trypanosomes were suspended in a minimum of 50 ml of a homogenous mixture of 90% phenol and water (1:1 v/v) and extracted at 64-68°C for thirty minutes with constant stirring. The mixture was cooled to 5-10°C and centrifuged at 1400 Xg for thirty minutes at 4°C. The aqueous layer was decanted, and the phenol layer was again treated with an equal volume of water of 64-68°C for thirty minutes, cooled, and the second aqueous phase removed. The two aqueous phases were combined, dialyzed against running tap water for 48 hours and against glass distilled water for 24 hours, and lyophilized. The material was redissolved in distilled water and precipitated with six volumes of 100% ethanol in the presence of sodium acetate. The precipitate was redissolved in distilled water. An absorption spectrum was
run between 200 and 300 μm on a Zeiss PMQII spectrophotometer to determine the presence of nucleic acids. The solution was lyophilized and the antigenic product stored in the freezer.

Whole trypanosomes were once extracted with trichloroacetic acid (TCA), as described by Kabat and Meyer (1964). Parasites were suspended in saline, cooled to 4°C and an equal volume of ice cold 0.05N TCA added. The suspension was thoroughly mixed and kept in an ice bath for three hours. The mixture was centrifuged at 39,100 Xg for thirty minutes at 2°C and the supernatant dialyzed for 48 hours against running tap water and 24 hours against glass distilled water. Any trace of precipitate was removed by centrifugation, and the extract was lyophilized.

Both macro- and microimmunodiffusion experiments were performed to test for antigenic activity of the isolated agglutinogen. Ouchterlony plates consisted of 2% Difco Ion Agar No. 2 prepared in a borate buffered saline solution (pH 7.8). Microimmunodiffusion slides were prepared with the same medium or with 1% Ion Agar in saline containing 0.8% sodium barbital, titrated to pH 7.4 with barbituric acid. Ouchterlony plates and microimmunodiffusion slides were incubated at 37°C and observed for up to one week. Some micro-slides were stained for protein with 0.1% amidoschwarz in a solution containing equal parts of 1M acetic acid and 0.1M sodium acetate and 10% glycerol, or for lipids and lipoproteins with a saturated solution of Sudan Black in 60% ethanol.

Sheep erythrocytes were sensitized with agglutinogen following the method of Landy and Lamb (1953). Four- or fourteen-day trypanosomal agglutinogen was dissolved in saline and heated in a boiling water bath for one hour. Sheep erythrocytes were washed three times with saline
at 4°C, and a 10% red blood cell suspension was made. Equal volumes of
erthrocyte suspension and antigen extract were mixed and incubated at
37°C for two hours with shaking every thirty minutes. Enough agglu-
tinogen was used so that a final concentration of 50 micrograms of
reducing material, as measured by the phenol-sulfuric acid method (Du
Bois et al., 1956), per 10⁶ sheep erythrocytes was obtained. Sensitized
erythrocytes were centrifuged and the supernatant discarded; the cells
were washed three times in ten volumes of saline to remove unadsorbed
antigen. The cells were resuspended in enough saline to give a one
percent sensitized sheep erythrocyte suspension. Sheep erythrocytes
mixed with saline were carried through the same procedure simultaneously
and used as controls.

Serial dilutions of inactivated serum or immune gamma globulin were
prepared in 0.2 ml volumes and 0.2 ml of the 1.0% sensitized erythrocyte
suspension was added. The tubes were shaken, incubated at 37°C for two
hours and scored macroscopically for agglutination (Salk, 1944). In
some instances, the erythrocytes were resuspended, incubated overnight
at 4°C, and read again.

In order to attempt to identify partially the biochemical nature
of the agglutinating antigenic determinant(s), trypanosomes were treated
with two hydrolytic enzymes. A glycosidase preparation was isolated
from rat epididymal tissue following the procedure described by Levvy
and Conchie (1966) and the product dissolved in 3 ml of 0.85% saline.
Such a preparation was reported to be a rich source of α-mannosidase,
β-galactosidase, α-L-fucosidase and β-N-acetylglucosaminidase. Other
enzymes present in much smaller amounts are β-mannosidase, α-galactosidase,
α-glucosidase, β-glucosidase and β-glucuronidase (Conchie et al., 1959; Levvy and Conchie, 1966). To one milliliter of a 2% reproductive trypansome suspension, an equal volume of the glucosidase preparation was added; and the mixture was incubated at 37°C, for increasing periods of time up to fifteen minutes. The reaction was stopped by dilution of the sample with 7 ml of cold saline, followed by centrifugation at 1400 Xg for fifteen minutes at 4°C. The parasites were washed three times with saline and resuspended in enough saline to give a 2% trypansome suspension. Control reaction tubes contained parasite suspension: a) in saline, and b) with heat-inactivated (100°C for 20 minutes) enzyme preparation; both sets of controls were incubated for fifteen minutes at 37°C. Enzymatically altered trypanosomes were tested for agglutinating activity with fourteen-day immune serum. Immune serum (0.2 ml) was added to 0.2 ml of the 2% parasite suspension and incubated at room temperature for two hours. All tubes were microscopically examined for agglutination.

Papain reagent (J. K. and Susi L. Wadley Research Institute and Blood Bank) was activated with L-cysteine and stored at -20°C. An equal volume of activated papain (2 mg/ml) was added to 2 ml of 2% suspensions of adult or reproductive trypanosomes. The tubes were incubated for one through five minutes at 37°C, diluted with cold saline and centrifuged at 1400 Xg for fifteen minutes. The parasites were washed three times with saline and resuspended in 2 ml of saline. Papainized reproductive trypanosomes (0.2 ml) were incubated with fourteen-day immune serum (0.2 ml) and papainized adult parasites incubated with ablustin. The tubes were incubated at room temperature for two hours and checked for agglutination.
The agglutinating antigen was partially hydrolyzed with acid and the resulting mixture was chromatographed in order to identify the carbohydrate components of the antigen. Samples of the antigen preparation (100-500 μg of phenol-sulfuric positive material) were hydrolyzed with 2N HCl in sealed ampules for two hours in a boiling water bath. The liquid hydrolysates were transferred to test tubes and evaporated to dryness in vacuo. Water (5 ml) was added and the samples were redried three additional times to remove the HCl. Samples were redissolved in 0.1 ml or less of 10% n-propanol for chromatography.

Both ascending and descending paper chromatography were performed with Whatman No. 1 paper, to attempt to identify the carbohydrates. Chromatographic solvent systems used were ethyl acetate-methyl ethyl ketone-pyridine-water (5:3:6:3:6:3), n-butanol-95% ethanol-acetone-water (5:4:3:2), and ethyl acetate-acetic acid-water (3:3:1). Reagents used for identification of substances included saturated silver nitrate in acetone, and aniline diphenylamine (Block et al., 1958) for carbohydrates, 0.3% ninhydrin (Sigma) for amino sugars (and amino acids), ammonium molybdate reagent for phosphate sugars (Block et al., 1958), and triphenyl tetrazolium (Sigma) for reducing compounds. Ascending thin-layer chromatography was performed on silica gel F254 coated glass plates (Brinkman Instruments Inc.), using propanol-ethyl acetate-water (7:1:2) as the solvent system. Developing reagents included those used for paper chromatography, as well as a sulfuric acid spray with charring, and ammonical silver nitrate with U.V. illumination for reducing sugars as well as cyclic amino acids, purines, pyrimidines, and hydroxy acids (Price and Dietrich, 1956).
The sugars identified by chromatography were tested with reproductive trypanosomes, to see whether or not they were part of the antigenic determinant. Serial dilutions (in 0.2 ml volumes) of rat agglutinating antibody were made with a 2% solution of the sugar to be tested, in saline and incubated at 37°C for one hour. An equal volume of a clean, reproductive trypanosomal suspension (1 X 10^8 trypanosomes/ml) was added to each tube; these mixtures were incubated at room temperature for two hours and scored microscopically for agglutination.
I. Isolation of the agglutinating antibody (the first trypanocidal antibody).

In three initial experiments, acid dissociation of the agglutinin was attempted following the method described by Lee and Wu (1940). Assays of all the material recovered from these experiments showed no agglutinating activity. Rat T. lewisi-agglutinating antibody was isolated from pooled 26-day postinfection immune sera and from 21- to 25-day postinfection immune gamma globulin using alkali dissociation of four-day postinfection reproductive trypanosomes. The agglutinating antibody finally isolated appeared to be the same as the first agglutinating antibody described by D'Alesandro (1960), since no agglutinating activity was recorded from material dissociated from fourteen-day, postinfection inhibited trypanosomes (Table I).

Agglutinating activity was assayed by serial dilution of the isolated agglutinin preparation. Table II shows the antibody titration assay for agglutinating activity of immune gamma globulin and of the agglutinin preparation. No prozone phenomenon was found with immune serum, immune gamma globulin or purified agglutinin. This result is contrary to that reported by Coventry (1930). A graded decrease in agglutination of trypanosomes with the increase in antibody dilution and the sharp endpoint of the titration indicate that one is indeed dealing with an antibody rather than a non-specific agglutinating substance.

Approximate antibody yield (Table I) of the agglutinating antibody preparations was calculated from the relative titers of the agglutinating
Table I

Agglutinin activity of pooled immune gamma globulin and its homologous agglutinating antibody preparation
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Antibody</th>
<th>mg of protein per ml of antibody solution</th>
<th>Reciprocal of agglutination titer</th>
<th>Approximate yield (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26-day immune serum</td>
<td>N.D.*</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agglutinin isolated with 4-day trypanosomes</td>
<td>N.D.</td>
<td>4</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>25-day immune γ-globulin</td>
<td>49.00</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agglutinin isolated with 4-day trypanosomes</td>
<td>3.00</td>
<td>8</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>21-day immune γ-globulin</td>
<td>40.00</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agglutinin isolated with 4-day trypanosomes</td>
<td>1.15</td>
<td>16</td>
<td>25.0</td>
</tr>
<tr>
<td>4</td>
<td>25-day immune γ-globulin</td>
<td>34.00</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agglutinin isolated with 14-day trypanosomes</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* N.D. - Not determined
** Calculated from the relative titers of antibody preparations and pooled immune gamma globulin or antiserum.
Table II

*In vitro* titration of the agglutinating activity of pooled immune gamma globulin and its homologous antibody preparation
<table>
<thead>
<tr>
<th>Reciprocal of Antibody Dilution</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Immune γ-Globulin (40 mg/ml)</td>
<td>+++</td>
</tr>
<tr>
<td>Purified Antibody</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ = maximal agglutination  
- = no agglutination
antibody preparations and the pooled homologous antisera or immune gamma globulin solutions. Using reproductive trypanosomes, five percent of the agglutinating activity of immune serum was recovered while 12.5% and 25% of the antibody was recovered from immune gamma globulin. No agglutinating activity was recovered from pooled immune gamma globulin with fourteen-day postinfection (adult) trypanosomes. The yield of antibody from pooled antisera with reproductive trypanosomes falls within the range of yield (2.5 to 10%) reported by Sternberger et al. (1963). Agglutinin yield from pooled immune gamma globulin was two to four times higher than the average yield reported by Sternberger and coworkers.

Purity of the agglutinin preparation was determined by electrophoresis on a cellulose acetate membrane. Figure 1 shows the electrophoretic distribution pattern of pooled immune serum, pooled immune gamma globulin and the purified antibody preparations. Most of the serum proteins which were precipitated with 33% ammonium sulfate saturation migrated with the gamma and beta globulins on electrophoresis. Some traces of $\alpha_1$ and $\alpha_2$ globulins were also present in the gamma globulin preparations. The major portion of the purified agglutinating antibody preparation migrated with the faster moving gamma globulins or with the beta globulins. D'Alesandro (1960) reported that, on starch gel, both trypanocidal antibodies and ablastin migrated between the beta and gamma globulins.

Ablastic activity of the agglutinin preparation was determined in vitro using the D'Alesandro (1962) culture medium. Reproductive activity was determined by ascertaining the percentage of small and dividing forms
Figure 1

Electropherogram of pooled immune rat serum, ammonium sulfate precipitated gamma globulin and agglutinating antibody preparations isolated with reproductive trypanosomes on cellulose acetate microzone electrophoresis in 0.01M β-mercaptoethanol barbital buffer, pH 8.6, μ = 0.075
in a bacteriological smear made from the medium. The agglutinating antibody did not exhibit ablasic activity in vitro when tested with adult non-reproducing trypanosomes. Table III shows the reproductive activity of adult trypanosomes with equal concentrations of normal serum, agglutinin preparation or pooled immune gamma globulin added to the culture medium. In all four experiments, the reproductivity of adult parasites in the agglutinin preparation was essentially the same as that in normal serum. In no instance did the agglutinin preparation exhibit ablasic activity resembling that of the pooled immune gamma globulin from which it was isolated. Since reproductive activity of the trypanosomes in normal serum varied between experiments (D'Alesandro, 1962), the results were plotted so that reproductive activity in normal serum was arbitrarily counted as 100 percent and the activity of the immune gamma globulin and agglutinin preparation determined as the percentage of reproduction of adult trypanosomes in normal serum (Figure 2). A typical ablastin titration of the agglutinin preparation isolated with four- and fourteen-day postinfection trypanosomes and of the immune gamma globulin from which they were isolated is shown in Figures 3 and 4 respectively. The agglutinin isolated with four-day postinfection trypanosomes showed essentially the same percentage of reproductive activity of adult parasites with increasing protein concentration as that of the control non-immune serum. The cultures containing the agglutinin preparation from the fourteen-day postinfection trypanosomes showed a stimulation of reproductive activity above the normal serum control. Both pooled immune gamma globulin preparations showed a typical ablastin titration curve,
Table III

Reproductive activity of inhibited (adult) *T. lewisi* in normal serum and in pooled immune gamma globulin, and its homologous agglutinating antibody preparation
<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Per Cent Dividing and Small Forms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Serum</td>
</tr>
<tr>
<td></td>
<td>γ-Globulin</td>
</tr>
</tbody>
</table>

**Agglutinin Isolated with 4-day Trypanosomes**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73.0</td>
<td>30.0</td>
<td>76.5</td>
</tr>
<tr>
<td>2</td>
<td>50.5</td>
<td>14.85</td>
<td>52.75</td>
</tr>
<tr>
<td>3</td>
<td>29.0</td>
<td>3.9</td>
<td>26.85</td>
</tr>
<tr>
<td>Mean</td>
<td>50.8</td>
<td>16.3</td>
<td>49.0</td>
</tr>
</tbody>
</table>

**Agglutinin Isolated with 14-day Trypanosomes**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>68.9</td>
<td>25.2</td>
<td>70.5</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

The influence of antibody preparations on the relative in vitro reproduction of adult trypanosome
Figure 3

In vitro titration for ablasic activity of pooled immune gamma globulin and its homologous agglutinating antibody preparation isolated with four-day postinfection (reproductive) trypanosomes

Inoculum: adult T. lewisi
In vitro titration for ablastic activity
of pooled immune gamma globulin and its
homologous agglutinating antibody
preparation isolated with fourteen-day
postinfection (adult) trypanosomes

Inoculum: adult *T. lewisi*
i.e. decrease in reproductive activity of adult trypanosomes with an increase in protein concentration of the antibody solution.

II. Isolation of the agglutinating antigen.

Four- and fourteen-day postinfection trypanosomes were collected from forty to fifty rats and a clean parasite suspension was obtained by differential centrifugation. Extracts of the agglutinating antigen were prepared from whole trypanosomes and from the particulate fraction of lysed parasites. The details of the method are shown in Table IV. Extraction of whole trypanosomes with trichloroacetic acid did not yield an antigenic fraction which would react with immune gamma globulin in immunodiffusion or passive hemagglutination tests (Table V).

To obtain the largest yield of trypanosomes possible (1.0-1.3 X 10^11), rats were inoculated intracardially and bled by cardiac puncture on the day of harvest. Coventry (1925) has shown that the route of inoculation does not influence the course of infection of T. lewisi in the rat. The average yield of crude agglutinating antigen (agglutinogen) preparation, in terms of total reducing material present (Dubois et al., 1956), was 22.1 μg/10^9 trypanosomes. A large number of infected rats was used since there was a substantial loss of trypanosomes during their separation from erythrocytes and platelets. In all experiments, the final trypanosome suspension had one or fewer rat red blood cells and platelet per low power (45X) field.

In initial experiments, and at various times throughout the course of this work, development of a macro- or microimmunodiffusion assay system was attempted. In no instance, using stained and unstained agar plates or slides, were the results successful.
Table IV

Isolation of the trypanosomal agglutinating antigen
Washed trypanosome suspension

passed twice through French press (15,000 psi)

Lysed trypanosome suspension

centrifuged 31,900 Xg, 30 minutes, 2°C

Supernatant
(no passive hemagglutinating activity)

Precipitate

Frozen overnight

Washed twice with distilled water

Extracted twice with 45% phenol in water
(64°C-67°C, 30 minutes)

Aqueous phase

Phenol phase and

Protein precipitate

Dialyzed
(48 hours running tap water
24 hours distilled water)

Iyophilized.

Redissolved in distilled water

Alcohol precipitation
(6 volumes of 100% ethanol in
presence of sodium acetate)

Centrifuged 1400 Xg, 30 minutes, 25°C

Supernatant

Precipitate = antigen preparation
Table V

Passive hemagglutination activity of sheep erythrocytes sensitized with TCA extracts and 45% phenol extracts of reproductive trypanosomes
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reciprocal of Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized Sheep RBC</td>
<td>0 2 4 8 16 32 64 128 C</td>
</tr>
<tr>
<td>45% Phenol Extract</td>
<td>++ ++ ++ ++ + + † - -</td>
</tr>
<tr>
<td>Sensitized Cells</td>
<td>++ ++ ++ ++ + + † - -</td>
</tr>
<tr>
<td>TCA Extract*</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Sensitized Cells</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td>Unsensitized Cells</td>
<td>- - - - - - - -</td>
</tr>
</tbody>
</table>

0.2 ml of 25-day pooled immune γ-globulin, 0.2 ml of 1.0% erythrocyte suspension; 2 hour incubation at 37°C.
Passive hemagglutination of trypanosomal agglutinogen-sensitized sheep erythrocytes proved to be a suitable assay system to test for antigenic activity of trypanosomal extract preparations. As can be seen from Table VI, only those preparations from four-day postinfection (reproductive) parasites showed positive antigenic activity when tested with fourteen-day postinfection immune serum. Fourteen-day postinfection (adult) trypanosomal extracts prepared by the same method exhibited no agglutinating activity. All control experiments (i.e. sensitized erythrocytes with saline, sensitized cells with normal serum and normal cells with immune serum) conducted simultaneously, were also negative. The system is not complement-dependent; inactivation of immune and normal sera (60°C for thirty minutes) had no effect on the final agglutination titer when run simultaneously with homologous unheated sera.

Isolation of the agglutinating antigen with phenol and water, from whole cells, also resulted in the extraction of considerable amounts of nucleic acids. This problem was circumvented by lysis of the parasites with a French pressure cell (15,000 psi), and centrifugation (39,100 Xg, for 30 minutes) of the lysate. The pellet was frozen overnight, thawed, and then washed twice with distilled water in order to lyse any unbroken nuclei or trypanosomes before the extraction with 45% phenol. Absorption spectra (200-300 mμ) of the material isolated after phenol extraction showed no peaks at 260 mμ or 280 mμ. The agglutinating antigen activity remained with the particulate fraction (Table VII), suggesting that nucleic acid is not part of the antigenic determinant for the agglutinating antibody.
Table VI

Passive hemagglutination activity of trypanosomal antigen extracts
<table>
<thead>
<tr>
<th>Sheep Erythrocytes Sensitized with:</th>
<th>Reciprocal of Antibody Dilution</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10  20  40  80  160  320  640</td>
<td></td>
</tr>
<tr>
<td>4-day Trypanosomal Antigen</td>
<td>++  +  +  +  -  -  -</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>++  +  +  +  -  -  -</td>
<td></td>
</tr>
<tr>
<td>14-day Trypanosomal Antigen</td>
<td>-    -  -  -  -  -  -</td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td>-    -  -  -  -  -  -</td>
<td></td>
</tr>
<tr>
<td>Unsensitized Sheep Erythrocytes</td>
<td>-    -  -  -  -  -  -</td>
<td></td>
</tr>
</tbody>
</table>

1. 0.5 ml of 14-day immune serum; 0.1 ml of 1.0% erythrocyte suspension; 2 hour incubation at 37°C.

2. Inactivation of serum did not change the titer.
Table VII

Passive hemagglutination activity of 45% phenol extracts of the particulate and soluble fractions of reproductive trypanosomes
<table>
<thead>
<tr>
<th>Sheep Erythrocytes Sensitized with:</th>
<th>Reciprocal of Antibody Dilution</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Particulate Fraction</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Soluble Fraction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unsensitized Erythrocytes</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Control:

<table>
<thead>
<tr>
<th>Sheep Erythrocytes Sensitized with:</th>
<th>Reciprocal of Normal Serum Dilution</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Particulate Fraction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soluble Fraction</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

0.2 ml of 14-day pooled immune sera; 0.2 ml of 1.0% erythrocyte suspension; 2 hour incubation at 37°C.
To insure that the trypanosomal agglutinogen was not a heterophile or Forsman type antigen, fourteen-day pooled immune gamma globulin was adsorbed with reproductive trypanosomes and an equal volume of packed sheep erythrocytes. The gamma globulin was tested for agglutinating activity by passive hemagglutination and the results are shown in Table VIII. Almost all the agglutinating activity of the antiserum adsorbed with reproductive trypanosomes was removed, while the activity of the erythrocyte-adsorbed material remained unchanged.

An attempt was made to utilize the passive hemagglutination system to assay for ablastin. Sheep erythrocytes, sensitized with adult and reproductive trypanosomal antigen, were incubated with ablastic serum. No agglutination ever occurred in these experiments. In a pilot study, rats were immunized with sensitized sheep erythrocytes in an effort to stimulate the production of ablastin and/or the agglutinating antibody. Two male rats (250-300 gm) were immunized intracardially with a 10% suspension of sheep erythrocytes sensitized with 140 µg of reproductive trypanosomal antigen. Two control rats were similarly injected with a 10% suspension of unsensitized sheep erythrocytes. Rats were injected on days 1, 3, and 5 and were bled two days after the last injection. When titrated for agglutinin activity in vitro with adult trypanosomes, both the experimental and control sera gave positive agglutinin titers as high as 128. Serum adsorbed twice with sheep erythrocytes (1 ml of packed red blood cells per ml of serum) did not lose any agglutinating activity when tested with either adult or reproductive trypanosomes. Adsorption of serum with normal sheep erythrocytes followed by adsorption with adult parasites did not decrease the agglutinin titer.
Table VIII

Agglutination of sensitized sheep erythrocytes with adsorbed immune gamma globulin
<table>
<thead>
<tr>
<th>Immune γ-globulin adsorbed with:</th>
<th>Antigen</th>
<th>Reciprocal of Antibody Dilution</th>
<th>Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Sheep Erythrocytes</td>
<td>Sensitized Sheep Erythrocytes</td>
<td>+++A</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+++)*</td>
<td>(+++)</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>(+++)</td>
<td>(+++)</td>
<td>(+++)</td>
</tr>
<tr>
<td>Untreated Sheep Erythrocytes</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Reproductive Trypanosomes</td>
<td>Sensitized Sheep Erythrocytes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Untreated Sheep Erythrocytes</td>
<td>(-)</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Δ - incubated 2 hours at 37°C.
* - incubated overnight at 4°C.
In order to circumvent the problem of sheep erythrocyte and trypanosomal cross-reacting antigens, sensitization of rat erythrocytes with reproductive trypanosomal antigen was performed, following the same procedure described for the sensitization of sheep erythrocytes. The results of two experiments to date have been negative when the sensitized cells were tested for agglutinating activity. Results notwithstanding, the author feels that this line of investigation is worth pursuing. Neter et al. (1952) were able to sensitize rat erythrocytes with E. coli antigenic lipopolysaccharide.

A number of observations led the author to believe that the antigenic determinant(s) of the trypanosomal agglutinogen was a polysaccharide. Firstly, fixation of biological material in 1% formalin usually results in denaturation of protein. However, as described previously, the author isolated the agglutinating antibody with trypanosomes fixed in 1% formalin. Similarly, Culbertson and Kessler (1939) reported using formalin-fixed T. lewisi as a standardized antigen for in vitro tests of trypanocidal antibody. Although fixation of reproductive trypanosomes in this system failed to achieve the desired removal of nucleic acids from the final agglutinating antigen preparation, this material did contain agglutinating activity. Secondly, the fixation method of antigen preparation was devised for the extraction of bacterial lipopolysaccharides, and has also been used for the extraction of human erythrocyte ABO antigens. Neter (1956) reported that, in all but two instances (both of which needed confirmation), chemical analysis of the antigens which sensitized untreated erythrocytes revealed that they are polysaccharides or contain polysaccharides as the serologically
active component. Antigens other than polysaccharides, e.g. proteins, can only be adsorbed onto the altered surfaces of erythrocytes (Kwapinski, 1965).

A reproductive trypanosome suspension was incubated with a crude preparation of rat epididymal glycosidase, and then incubated with fourteen-day postinfection serum and with pooled nonimmune serum for two hours at room temperature. In all experiments, trypanosomes thus altered by the enzymes were agglutinated in both normal and immune sera (Table IX). Mostly likely, the enzymes exposed new antigenic determinants which cross-reacted with some rat antibodies produced against other naturally occurring antigens.

Chromatography of acid hydrolysates of reproductive trypanosomal agglutininogen has not consistently yielded the same results from one antigen preparation to another. However, the monosaccharides that were consistently found were galactose, mannose and glucosamine. Ribose was found in antigen preparations extracted from whole cells, and fucose appeared in a number of preparations. Similar results were obtained with the adult agglutininogen preparations.

Agglutination-inhibition tests were undertaken to determine whether or not the monosaccharides were part of the antigenic determinant reacting with the agglutinating antibody. None of the simple carbohydrates tested (galactose, mannose, glucosamine, rhamnose, fucose, ribose, cellobiose, inositol) inhibited agglutination of reproductive trypanosomes with fourteen-day postinfection immune gamma globulin (Tables X, XI).

Since attempts to date to modify the reproductive trypanosomal surface with glycosidase, or to inhibit agglutination with various
Table IX.

The effect of rat epididymal glycosidase on the agglutination of reproductive T. lewisi
<table>
<thead>
<tr>
<th>Tube</th>
<th>Treatment of T. lewisi with glycosidase</th>
<th>Agglutination Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immune Serum</td>
</tr>
<tr>
<td>1</td>
<td>1 minute</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2 minutes</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3 minutes</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>5 minutes</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>15 minutes</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>heat inactivated enzyme</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>saline control</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = agglutination
- = no agglutination
Table X:

Trypanosome agglutination inhibition test
<table>
<thead>
<tr>
<th>Test Inhibitor</th>
<th>Reciprocal of Antibody Dilution</th>
<th>Inhibitor-Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Saline</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Galactose</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>(2%)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>(2%)</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

0.2 ml of 14-day immune gamma globulin diluted with 2% test inhibitor-saline; 0.2 ml of reproductive trypanosomes (10^8/ml); incubated 2 hours at 23°C.
Table XI

Trypanosome agglutination inhibition test
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test Inhibitor</th>
<th>Reciprocal of Antibody Dilution</th>
<th>Inhibitor-Saline Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number 1</td>
<td>Saline</td>
<td>10  20  30  40  50  60  70  80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++  +  ++  +  ±  ±  ±  -</td>
<td>-</td>
</tr>
<tr>
<td>Mannose (2%)</td>
<td></td>
<td>+++  +  ++  +  ±  ±  ±  -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++  ++  ++  +  ±  ±  ±  -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Rhamnose (2%)</td>
<td>+++  ++  ++  +  ±  ±  ±  -</td>
<td>-</td>
</tr>
<tr>
<td>Number 2</td>
<td>Saline</td>
<td>10  20  30  40  50  60  70  80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++  +++  ++  ++  +  ±  ±  ±</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Fucose (2%)</td>
<td>+++  +++  +++  ++  +  +  +  +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Glucosamine (2%)</td>
<td>+++  +++  ++  ++  ++  +  +  +</td>
<td>-</td>
</tr>
</tbody>
</table>

0.2 ml of 14-day pooled immune gamma globulin diluted with 2% test inhibitor-saline; 0.2 ml of reproductive trypanosomes (10⁸/ml); incubated for 2 hours at 23°C.
monosaccharides have not been successful, protease treatment of the parasites was attempted. Reproductive trypanosomes were treated with papain and then incubated with fourteen-day postinfection immune gamma globulin. Similar to the experiments with the glycosidases, the papainized parasites were agglutinated in immune and normal sera. No agglutination was seen in saline or in heat-inactivated controls (Table XII).

In a like manner, adult trypanosomes were treated with papain and tested with ablastin for agglutination. It was hoped that this alteration of the cell surface would lead to the development of a simplified agglutination test for ablastin. However, these experiments gave the same results as were seen with papain-treated reproductive trypanosomes, i.e. agglutination with both immune and normal sera (Table XII).
Table XII

The effect of papain on the agglutination of reproductive and adult *T. lewisi*
<table>
<thead>
<tr>
<th>Tube</th>
<th>Treatment of T. lewisi with papain</th>
<th>Agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immune Serum</td>
</tr>
<tr>
<td>Reproductive Trypanosomes</td>
<td>1 minute</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>2 minutes</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>3 minutes</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>5 minutes</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>heat inactivated enzyme</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>saline control</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adult Trypanosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

N.D. = Not Determined
+ = agglutination
- = no agglutination
DISCUSSION

I. The agglutinating antibody and ablastin.

Ablastin or ablastin-like antibodies reportedly occur in response to a number of different types of parasitic invasions. Ascoli (1908) coined the term "antiblastic immunity" to describe the inhibition of capsule formation in Bacillus anthracis by immune donkey sera. He found (1906) that bacterial adsorption would not remove the antibodies from the sera, and that the organisms were not sensitized by the antibody; he later concluded (1908) that immune sera inhibited certain assimilative processes in the bacteria, resulting in the retardation of growth. Dochez and Avery (1916) described a similar antiblastic action of immune horse sera against Pneumococcus. They reported that the antisera retarded the growth of the organism and inhibited or markedly delayed bacterial fermentation of inulin and hydrolysis of protein. Blake (1917) questioned these results and attributed the antiblastic action of the antisera to agglutination. More recently, Dannis and Marston (1966) demonstrated a growth-inhibiting antibody specific for staphylococcal L-forms. Adsorption of the immune sera by the parent staphylococcus did not remove the antibody. However, unlike T. lewisi ablastin, the growth-inhibiting antibody to the L-forms could be adsorbed from antisera with an L-form membrane preparation. The protoplasmic fraction failed to adsorb or inhibit the antibody, suggesting that the antibody was not induced by bacterial metabolites.

In a number of instances, inhibition of reproduction of animal parasites by immune serum factors has also been reported. Rats that
recover from infection by the intestinal nematode *Nippostrongylus muris* are relatively immune to a second infection (Schwartz et al., 1931; Chandler, 1938). In adult female worms which are successful in reinfecting an immune rat, there is retardation of growth and development, and inhibition of egg laying, without any marked lethal effect. This immunity can be transferred passively (Sarles and Taliaferro, 1936; Chandler, 1938). Taliaferro and Sarles (1939) found that an immune precipitate forms *in vivo* with excretions and secretions from the mouth, excretory pore and the anus of the parasite. Often the mouth was capped, and the whole gut filled with the immune precipitate. Sarles (1938) demonstrated the same phenomenon in passively immunized rats.

Blacklock, Gorden and Fine (1930), working with the myasis-producing fly, *Cordylobia anthropophaga*, found immune precipitates in the gut and around the larval in the skin of immune guinea pigs. They postulated that the precipitate inhibited assimilation of food and caused death by mechanically blocking the gut.

Taliaferro and Pavlinova (1936) and Taliaferro (1938b) found inhibition of reproduction of *T. duttoni* in infected mice and guinea pigs. In mice, the population size of *T. duttoni* never reaches the peak it does with *T. lewisi* in rats, and the inhibition or reproduction is not as early or as complete as in the case of *T. lewisi*. These authors found some cross-reactivity between the ablastin formed against *T. lewisi* with *T. duttoni* and visa versa. Other than *T. lewisi*, this represents the only reported instance of an ablastic type of immunity to a protozoan infection.
The significance of ablasic immunity has been studied extensively only in rats infected with *T. lewisi*. The hypothesis of Taliaferro and coworkers, that in the rat ablasic is a separate entity, distinct from the trypanocidal antibodies, appears to be confirmed by the results of the present work. The agglutinating antibody preparation isolated by this author by alkali dissociation did not show any ablasic activity when tested *in vitro* with adult trypanosomes. To the author's knowledge, this is the first report of the complete separation of the agglutinating antibody from ablasm. These results tend to negate the criticisms of several investigators (Lincicome and Watkins, 1965; Sanchez, personal communication), who have attributed ablasic activity to residual agglutinating antibody which could not be completely removed by adsorption with *T. lewisi* during the preparation of ablasic serum.

The hypothesis of Augustine (1943) and Ormerod (1963) that the ablasic phenomenon is the result of selective removal of dividing forms by the agglutinating antibody also seems doubtful. As Taliaferro (1948) has stated, this hypothesis contains a contradiction, since a population, from which adult trypanosomes were removed as soon as they started to divide, or even after they had divided, would not remain constant in number but rather would decrease. Both Augustine (1943) and Ormerod (1963) have reported observing adult populations of constant size in immune rats. Ormerod (1963) circumvented Taliaferro's criticism by proposing that reproduction of *T. lewisi* occurred outside the peripheral circulation, particularly in the capillaries of the kidney, although he was never able to demonstrate reproduction of adult forms occurring there. However, if such reproduction did occur, the size of the adult
population in the rat would remain constant instead of decreasing, because the lost parasites would be replaced by "low grade" reproduction occurring in the capillaries.

In this laboratory, when ablasic activity of the agglutinin was studied in vitro, inhibition of reproduction did occur in the control tubes containing immune gamma globulin, but agglutination of the reproductive trypanosomes was never encountered. D'Alesandro (1962) also reported that when ablasic activity was studied in vitro where no cryptic reproduction could occur, inhibition of reproduction occurred without any marked changes in parasite numbers. He found that adsorption of immune sera with reproductive trypanosomes did not effect the titer of ablasicin. Thus Chandler's claim (1958) and the author's hypothesis, that ablasicin and the agglutinating antibody are identical, are contradicted by the results presented here, since the agglutinin preparation exhibited no evidence of ablasic activity. Thillet and Chandler (1957) reported successful ablasicin and trypanocidal antibody induction by using soluble "metabolic products" as the antigen. However, Thillet (1957) concluded that when the immunized rats were challenged, the injected trypanosomes were so rapidly destroyed that inhibition of reproduction could not easily be observed thus casting doubt upon the validity of the conclusions. In this work, there was also little evidence that the antigen would adsorb out ablasicin from immune serum. Wilson (1962) repeated the work of Thillet and Chandler and obtained equivocal results. He was doubtful that ablasicin could be adsorbed from immune sera with the T. lewisi "metabolic products" antigen. Using an in vitro culture technique, D'Alesandro (1962) tried unsuccessfully
to inhibit ablasic activity of immune serum with trypanosomal "metabolic products". This lack of success may further demonstrate that these products were not directly responsible for the induction of the ablasic antibody obtained by Thillet and Chandler (1957).

Ablastin probably plays a significant role in the final recovery of the rat from a T. lewisi infection. It can be considered the "stop-gap" antibody which protects the rat from the first trypanosomal numbers crisis until the elimination of the parasites by the terminal trypanocidal antibody. Without this protection, the disease could possibly be terminated in death, as is seen with young rats less than 25 days of age (Culbertson, 1938). The proposed role of ablastin in protecting immune rats from reinfection is questionable. The agglutinin and, particularly, the terminal trypanocidal antibody which is a lysin (Coventry, 1930), are probably the most important factors of defense against reinfection. Cox (1964) has reported that in hyperimmune rats, trypanosomes which were injected intraperitoneally never appeared in the circulating blood. Augustine (1943), using a very large reinfection inoculum of T. lewisi, found that the dividing trypanosomes which appeared in the blood were quickly killed or immobilized (agglutinated?) and removed by phagocytosis. Neither author found any obstruction by the lymphatic system to the passage of trypanosomes from the peritoneal cavity into the circulating blood. The results of both authors suggest that the trypanocidal antibodies quickly destroy the parasites as they migrate from the site of inoculation into the circulating blood. In most naturally occurring infections of T. lewisi in the rat, the number of parasites would never be large enough to overwhelm the titer of the trypanocidal antibodies;
and the presence of a serum trypanolysin could easily insure the destruction of the invading organisms.

Ablastin appears to be a unique antibody in that it is both nonadsorbable by present techniques and it inhibits the reproduction of the organism against which it is directed. There are numerous reports of nonadsorbable antibodies in the literature; e.g., human diptheria antitoxin, Kuhns and Papenheimer, 1952; rabbit anti-Salmonella typhimurium antibody, Herzberg et al., 1966, but none of these is known to inhibit reproduction. As has been reviewed previously, there are also a number of antibodies which are reported to inhibit reproduction but which are adsorbable. The unique nature of ablastin is probably not due to the properties of the antibody itself but rather to the association of a non-avid antibody; i.e., an antibody having a low affinity for the antigen, with the antigenic surface of a single cell organism. Most, if not all, of the trypanosomal surface is probably involved in the uptake of metabolites, and it is not inconceivable that the surface could be coated with ablastin, which would inhibit uptake of these metabolites. The accessibility of the "transporting surface" to the antibody, and the relatively small total surface area, would make T. lewisi easily susceptible to such an antibody. Thus the inhibition of reproduction would be due to the physical blocking of the uptake of metabolites necessary to the assimilative metabolism commonly associated with reproduction.

Three different hypotheses can be proposed to account for the non-adsorbability of ablastin. The first suggests that this characteristic can be accounted for by assuming that the specific antigens are not
present in sufficient concentration to remove the antibody. If this were so, and if it is assumed that ablastin has a high avidity for the antigen, then parasites from immune serum which had been repeatedly washed would still retain combined antibody. The results presented in this study do not support this hypothesis. The antibody preparations isolated from both reproductive and adult parasites, which had been incubated with immune serum and washed, showed agglutinating activity but no evidence of ablastic activity. It could be argued that the procedure employed destroyed the activity of the ablastin antibody. Although such a possibility cannot be definitely ruled out, selective inactivation seems improbable, since isolation of the rat trypanosomal agglutinating antibody by alkali dissociation was successful, and a number of different antibodies from rabbits have been similarly isolated (Sternberger et al., 1963). D'Alesandro (1966) points out that if ablastin were an antibody with high avidity, parasites incubated with ablastic serum should retain combined antibody, becoming permanently sensitized (as happens with the two trypanocidal antibodies) and not reproduce when transferred into normal uninfected serum. This obviously does not occur with T. lewisi since nonreproducing parasites actively divide upon injection into a new uninfected host.

The second hypothesis offered to explain the nonadsorbability of ablastin, is to assume that ablastin is an extremely non-avid antibody. This idea was first suggested by Taliaferro (1963), when he suggested that the avidity of ablastin was comparable to that of the normal Forssman haemolysin which has a very low affinity for erythrocytes. If this hypothesis is to be accepted, it is necessary then to assume that high
concentrations of the antibody are required in the surrounding medium in order to maintain an adequate amount of antibody on the trypanosome; and conversely, when low concentrations of ablastin are present in the medium, there would be dissociation of most of the antibody from the parasite. That such conditions exist, according to Taliaferro (1963), is indicated by the fact that the highest rate of incorporation of amino acids is found in recently-released adult T. lewisi; i.e., those parasites which have been removed from ablastin-containing rats three hours previously, and injected into normal uninfected hosts. "A dissociation time of a few hours for a non-avid antibody may seem a long time; however when compared to an avid antibody which can often require days to dissociate, if at all, this is a relatively short time" (D'Alesandro, 1966).

The third hypothesis, promulgated by this author, which can be invoked is that the antigen is masked in the living parasite which is used for the adsorption of immune serum. During the early part of an infection, some trypanosomes are lost because of natural death of the cells and phagocytosis by the host's macrophages. Destruction of the trypanosomes would result in the exposure of antigenic sites which are not normally completely accessible in the living parasite. These new sites would induce formation of antibodies that would only partially bind to the antigen in the living trypanosomes, resulting in a loosely bound antibody coat surrounding the organism.

Of the three hypotheses stated, the first, suggesting that ablastin is an avid antibody, can be discarded for the reasons stated earlier. Most investigators would probably agree that ablastin is a non-avid
antibody, but there is no evidence to suggest whether this characteristic is due to the nature of the antibody itself, the nature of the antigen, or both. This question will only be answered when it is determined how ablastin operates.

No evidence has been reported to date, suggesting that ablastin causes immediate changes in the metabolic activities or in the morphology of T. lewisi. D'Alesandro (1962) has shown in vitro that dividing trypanosomes require at least an overnight exposure to ablastin before the majority of the parasites are transformed into adults. This is not too surprising since the normal generation time of the organism is seven to eight hours. No substantial attempt has been made to assess the direct effects of ablastin on the metabolism of T. lewisi. Moulder, as communicated to Taliaferro (1948), found that ablastin had no effect on the oxygen consumption of reproductive parasites, as measured in the Warburg respirometer. Patton and Clark (1966) have studied the in vitro effects of ablastin on the incorporation of tritiated thymidine by dividing trypanosomes using autoradiographic techniques. Nine percent of the reproductive parasites which were cultured in the presence of normal serum, were labeled within thirty minutes, and by thirteen hours as many as eighty two percent were labeled. When normal serum was replaced by ablastic serum, the minimum time required for the label to be incorporated was 2.5 hours. At this time, between two and eight percent of the trypanosomes were labeled. Longer periods of incubation in the presence of ablastin and labeled thymidine did not increase the number of parasites labeled.
Thus, most of the evidence now available suggests that these morphological and biochemical changes (e.g., enzyme levels, rates of protein and nucleic acid synthesis) are secondary effects of ablastin upon the parasite. There is a great need for experiments designed to test the effect of ablastin on the passage of metabolites across the cell membrane. Studies of the transport of various metabolites; e.g., sugars, amino acids, nucleotides, and the effects of addition of ablastin on such transport, might yield valuable information on the nature of ablastic action. This type of study would permit one to confirm or reject the widely accepted hypothesis that ablastin does act at the surface membrane level. If ablastin acts at the surface, then examination of the effects of ablastin on the transport of a wide variety of compounds would indicate whether the inhibition was specific or general in nature.

There is a definite need for a better assay system for ablastic activity. D'Alesandro's (1962) system is adequate for some studies, but leaves much to be desired. It is not easily reproducible, and the complexity of the culture medium and a long incubation period make it difficult to use for ablastin-inhibition studies, which are necessary for the elucidation of the nature of the antigenic determinant(s). Measurement of the effect of ablastin at the cell surface (e.g., the effect of ablastin on transport) may prove to be a more suitable assay system. D'Alesandro (1966), using immunoelectroadsorption, has tried to measure the thickness of the serum protein layer adsorbed onto trypanosomes incubated with normal and ablastic serum. However, his results were ambiguous and have not been confirmed to date.
In any study of the effects of ablastin on the metabolism of *T. lewisi*, it is essential that a pure antibody preparation be used. As noted earlier, some investigators have found that the agglutinating antibody was not completely removed from immune serum by adsorption with reproductive trypanosomes. Traces of agglutinin in ablastic sera could result in coating of the parasite and could inhibit some of its normal metabolic functions. This inhibition could be falsely attributed to ablastin. Desowitz (1956) has reported that anti-*T. vivax* serum from cattle inhibits the oxygen consumption of the parasites. Zwisler and lysenko (1954) found similar results with rat anti-*T. lewisi* serum. Since this study has shown that active agglutinating antibody can be isolated free from ablastin, it now appears feasible that a rabbit or horse anti-rat agglutinin antibody can be prepared, which could be used to remove the traces of the contaminating agglutinin in the ablastic serum. Characterization of ablastin could then be more easily performed.

II. The nature of the agglutinating antigen.

Since the early studies of Taliaferro and coworkers, *T. lewisi* has been known to pass through a series of antigenic changes during the course of infection of the rat. The reproductive parasites, injected into a nonimmune host, stimulate the production of the first trypanocidal antibody (the agglutinating antibody), which results in a drastic decrease in the trypanosome population. The organisms which survive are not susceptible to the antibody, and are unaffected until they are transferred to a new host, at which time they repeat the cycle, or until the
infection is terminated. The work presented here includes the first reported isolation of the \textit{T. lewisi} agglutinating antigen, and represents an attempt to correlate changes in the living parasite with changes in the antigen.

The phenol-water method of isolation of the agglutinogen is similar to that reported by Seneca \textit{et al.} (1966) for the isolation of chagastoxin from the sonicated culture forms of \textit{T. cruzi}. Seneca and Peer (1966) reported that their preparation was a lipopolysaccharide, contained no protein and was composed of carbohydrate and lipids. It was insoluble in water, saline, acids, alkali, alcohols, ether and acetone. The lyophilized product in aqueous suspension was so insoluble that it had to be prepared immediately before use.

The agglutinogen isolated from \textit{T. lewisi}, unlike the chagastoxin was soluble in water and saline and was stable in solution for an indefinite period of time. Although it did not show an adsorption peak at 280 m\text{	extmu}m, a weak positive Lowry protein reaction was obtained in some preliminary experiments. The Lowry positive material was probably a small peptide. Bacterial lipopolysaccharide extracted with phenol-water consists of a polysaccharide component, firmly bound lipid and small amounts of peptide (Luderitz \textit{et al.}, 1966). The protein component may also be a contaminant from the insoluble layer that forms at the interface of the phenol and water layers during the last step of the extraction procedure. The agglutinin could also be a glycoprotein, since the phenol-water method has also been used for the isolation of this type of polymer from erythrocytes and mammalian cells grown in tissue culture.
The failure of the trypanosomal agglutinogen to form precipitin bands with immune rat serum in immuno gel-diffusion is not unusual. Seneca and Peer (1966) found that the lipopolysaccharide from *T. cruzi* would not react with rabbit antibody, using the same test system as that employed by the author. Neter (1956) has also reported that the reaction of some bacterial lipopolysaccharides with homologous immune serum cannot be demonstrated using immunodiffusion tests. Using Marrack's lattice hypothesis to explain precipitate and agglutinate formation in antibody-antigen reactions, one must assume that the antigen is at least bivalent. This allows cross linkage between antigen and antibody molecules, resulting in the formation of a visible antigen-antibody aggregate. If one assumes that the isolated antigen in this case is monovalent, there would be no visible precipitate when the isolated antigen was mixed with antibody. However, if a particle (i.e. sheep erythrocytes) were coated with the antigen and then mixed with antibody, the polyvalent particles would react with more than one antibody molecule, resulting in the formation of an antigen-antibody lattice structure and visible agglutination. Failure of the sheep cells sensitized with adult antigen to agglutinate may have been due to the addition or deletion of a monosaccharide(s) or amino acid(s); there may have been a shift from an α- to a β-glycosidic linkage between sugar components of the polysaccharide, or there may be some chemical or structural change at the end attaching to the erythrocyte, inhibiting sensitization. Since the whole *T. lewisi* organism can be considered a polyvalent antigen, agglutination occurs when the living parasite is mixed with immune serum.
Attempts to chemically characterize trypanosomal antigens are not extensive. The results of early workers (Kligler et al., 1940; Ikejiani, 1947) on T. lewisi, T. equiperdum and T. evansi were largely negative, due to the lack of sensitive serological techniques. More recent work on the brucei trypanosomes have shown that the extracted antigenic material is composed mainly of protein. Williamson and Brown (1964) have tentatively characterized the common antigens of this group as nucleoproteins and the variable antigens as unconjugated proteins. No polysaccharide was found in any of the isolated antigenic material. LePage (1967) has confirmed the protein nature of the variable antigens of T. brucei.

The lewisi group (including T. lewisi and T. cruzi) on the other hand, possess a polysaccharide as a major or minor component of most of their antigenic determinants. A serologically active polysaccharide-peptide complex has been extracted from culture forms of T. cruzi, and was shown to contain glucose, glucosamine, xylose, mannose, galactose and eleven amino acids (Goncalves and Yamaha, 1956). This complex also possessed precipitin activity with serum from patients with Chagas disease. Fife and Kent (1960) reported extracting two complement-fixing antigens from T. cruzi, one of which was a carbohydrate and the other a protein; both were essentially lipid-free. The immunologically active chagastoxin, as previously mentioned, is a lipopolysaccharide (Seneca and Peer, 1966).

The antigens of Leishmania are unlike those of the African trypanosomes but resemble those of the lewisi group in their chemical composition.
Bray and Lainson (1967), using passive agglutination studies, have found that a number of strains of *L. tropica* and *L. mexicana* possess a carbohydrate component in their antigenic determinants.

As might be expected, the agglutinogen of *T. lewisi* appears to be similar to those antigens extracted from the closely related *T. cruzi* rather than those of the *brucei* group. Its polysaccharide component has a composition similar to the complement-fixing antigen isolated by Goncalves and Yamaha. However, glucose and xylose detected in *T. cruzi* antigen were not found in the *T. lewisi* agglutinogen. The differing methods of isolation may account for the fact that the complement-fixing antigen will form a precipitate with immune serum, while the agglutinating antigen will not. The *T. cruzi* antigen was extracted with 33% chloral hydrate, and precipitated twice with alcohol, while the *T. lewisi* agglutinogen was isolated with phenol-water.

Individual monosaccharides were tested with the antigen to determine whether any single component could inhibit trypanosomal agglutination; the negative results of such experiments may suggest that: 1) the agglutinin is composed of several antibodies, each with a different specificity; 2) the antigenic determinant is larger than a mono- or disaccharide; or 3) this antigen is composed of amino acids.

The facts that only traces of protein are associated with the antigen, and that formalized trypanosomes agglutinate with immune sera, suggest that the third alternative is fairly improbable. It is well known that even the simplest antigen, a conjugated hapten, will elicit the formation of a number of antibodies with different specificities; and adsorption of the serum with the conjugate is necessary before inhibition
tests may be performed. The multivalent antigenic surface of the trypanosome may be too complex for agglutination to be inhibited by a single sugar. The possibility that the antigenic determinant is larger than a monosaccharide cannot be ruled out. It is quite possible that failure of the inhibition of agglutination with the individual monosaccharides may be due to a larger trypanosomal antigenic determinant and to the presence of several antibodies with different specificities.

From the data available at this time, there appears to be a distinct difference in the antigenic makeup between the brucei and lewisi trypanosomes. A polysaccharide has not been found to be associated with any of the brucei group antigens identified to date, while the lewisi group possess both protein and complexed carbohydrate antigens. This difference may be related to the divergence of the two groups, as has been demonstrated morphologically and biochemically, or it may be due in part to the "modus operandi" of the investigators working with the two groups.

One basic difference in methodology lies in the type of immune sera used to test for the antibody-combining capacity of several isolated trypanosomal fractions. Anti-brucei immune sera have been prepared by injection of trypanosome homogenates or saline extracts thereof into experimental animals. Those investigators working with T. cruzi, as well as the author, have used antisera taken from animals which were infected with the living parasite. The range of antigenic variation, however, is not as great in T. cruzi and T. lewisi as it is in the African trypanosomes, so that the collection of a standardized antigen and antibody is simplified. The method of assay for antigenicity used by
Williamson and Brown (1964) may have precluded demonstration of the complexed polysaccharide antigen. As they state in their paper, "Antigen means capacity to form a precipitate in agar gel with antitypanosom immune sera." Seneca and Peer (1966) and the author have found that the antigen extracted with phenol-water from T. cruzi and T. lewisi respectively, does not give a positive reaction using this technique. Perhaps if these investigators had sensitized untreated erythrocytes with material from the aqueous phase of the phenol-water extracts of T. brucei, polysaccharides might have been found.

As mentioned earlier, trypanosomes exhibit a wide spectrum of antigenic variation in infected animals. The African trypanosomes demonstrate the greatest antigenic plasticity. Rtiz (1916) reported that a single T. brucei can give rise to at least twenty-two different antigenic types of parasites. Other workers have demonstrated the ability of clonal strains of trypanosomes to produce many antigens (Lourie and O'Connor, 1937; Gray, 1965). In relapsing African trypanosomiasis infections, antigenic variation can be related to variations in the number of parasites in the blood and to the formation of a series of variant-specific antibodies. Gray (1962) has used specific antisera to suppress the development of selected antigens of a strain of T. brucei in rabbits. Change in antigenicity of T. lewisi in rats can be correlated with the agglutinating antibody.

There is considerable controversy as to the cause of antigenic variation. Some investigators suggest that it results from random mutation and the selection of viable mutants, while others propose that the sequence of synthesis is organized, and that new antigens are
induced by environmental stimuli. Cantrell (1958) and Watkins (1964),
estimating the mutation rate of \textit{T. equiperidum} and \textit{T. brucei} in rats and
mice, have suggested that the mutation rate and trypanosome population
size were great enough to account for the observed production of new
antigenic variants.

Taking the opposing view, Gray (1967) has cited numerous features
of antigenic variation that support the adaptive or inducible process
as the basis for antigenic variation among the African trypanosomes:

"Variants of a strain tend to revert to a 'parent' or 'basic'
antigenic type during blood-passage in experimental animals;
strains persist in a 'basic' antigenic form or tend to revert
from variants to a 'basic' antigenic form during development
in the tsetse fly; clones and variants of the same strain
produce the same antigens in different hosts, and antigenic
variation often occurs in a relatively fixed pattern."

Isolation of the \textit{T. lewisi} agglutinogen should make it possible to
study the mechanism of antigenic variation. Working with \textit{T. lewisi} in
rats has the advantage of limiting the variant population to two types,
agglutinogenic (reproductive trypanosomes) and nonagglutinogenic (adult
trypanosomes). The change from one form to the other is predictable
and easily reversible. Using the agglutinogen as the marker, chemical
and structural analysis of the reproductive and adult antigen should
reveal which changes have occurred. Isolation of the agglutinogen could
now provide the opportunity to examine the synthesis of the antigen, and
to determine whether it is inducible or mutational in nature. The same
antigenic system could also be used to study various biochemical changes
of trypanosomes which occur in their transformation from blood stream
forms to culture forms and visa versa. A number of reports have been
published on factors which induce the morphological transition and
metabolic differences between the two forms (Steinert and Moore, 1956; D'Alesandro, 1962; Deane and Deane, 1961; von Brand, 1966), but there is little information on how these changes occur, and no information on what changes occur at the cell surface. Antigenic differences between blood and culture forms may be due directly to the presence or absence of an antigenic constituent in the surrounding medium of the parasite. It is interesting to note that antigen-negative human cells grown in culture would become antigen-positive in a medium containing the individual antigenic components, i.e. the cells became A, B, or H positive upon addition of the sugars which were components of the antigen (Chessin et al., 1965).

There has been a renewal of interest in the immunological aspect of trypanosomiasis in the past few years. Workers have begun to realize that chemotherapy is not the complete answer to the problem of trypanosomiasis control. Such drugs are expensive to develop, and mass treatment is difficult to administer. In many instances, mass chemotherapy leads to the development of drug-resistant parasites. Gray (1967) has cited a number of problems involved in immunological studies of trypanosomiasis. Several species of trypanosomes are difficult to obtain in sufficient quantity for experimental work, and there is a great need for fundamental information on the nature and properties of the parasite antigen, with particular reference to the capacity of trypanosomes for antigenic variation. Since trypanosomal antigens are required for almost all research on the immunology of the disease, a method of isolating the T. lewisi agglutinating antigen (agglutinogen) as well as a suitable assay system has been developed. It is hoped that this work may stimulate interest in an area that has long been neglected.
SUMMARY

1. *T. lewisi* agglutinating antibody was isolated by alkali dissociation from formalin-fixed reproductive trypanosomes which had been incubated with fourteen-day and twenty-five-day postinfection immune rat gamma globulin or sera.

2. Electrophoretically, the agglutinin preparation migrated with the beta and gamma globulins of rat serum.

3. When tested *in vitro*, the agglutinin preparation exhibited no evidence of ablasic activity, leading the author to conclude that the agglutinating antibody and ablasis are separate entities. This confirms the hypothesis of Taliaferro (1948) while refuting those of Augustine (1943) and Ormerod (1963). The data presented here is the first report of the complete separation of an active agglutinin preparation from ablasis.

4. The data were discussed in terms of the probable nature of ablasis and its mechanism of action against *T. lewisi*. It was proposed that the agglutinating antibody could be used to prepare a "pure" ablasic serum, in order to elucidate the nature of ablasis.

5. The agglutinating antigen was isolated from reproductive *T. lewisi* by extraction with a forty-five percent phenol-water solution. Active agglutinating antigen preparations were not isolated from adult trypanosomes by the same method.

6. A passive hemagglutination test was developed for the assay of the agglutinating antigen. The lack of precipitin bands in immuno-gel diffusion tests may indicate a monovalent antigen preparation.
7. Carbohydrate components of the isolated antigen were identified by paper chromatography of the acid hydrolysates; these included galactose, mannose, glucosamine, and traces of fucose. When tested singly, none of these monosaccharides (nor some related compounds) inhibited trypanosomal agglutination, possibly suggesting that a more complex antigenic deter-
minant is involved.

8. These data were discussed in terms of the possible carbohydrate nature of the antigenic determinant, and of the relationship of \textit{T. lewisi} to the other hemoflagellates. Hypotheses were presented for the mechanism of the observed shift from one antigenic type to another.

9. For the first time, a fairly controlled system was developed for the study of the nature of the changes in antigenicity in trypanosomes.
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