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THE RICE INSTITUTE

**IMMUNIZATION AGAINST TRYPANOSOMA LEWISI IN
RATS BY INJECTIONS OF METABOLIC PRODUCTS**

by

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A THESIS

**SUBMITTED TO THE FACULTY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

*Approved
Asa C. Chandler*

**Houston, Texas
May, 1957**

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ACKNOWLEDGMENTS

The author is indebted to Dr. Asa C. Chandler, under whose supervision this work was carried out, for his helpful criticisms and suggestions throughout the study and in the preparation of the manuscript. The author also wishes to express his sincere appreciation to Mrs. Evelyn K. Hake for her valuable technical assistance and moral support throughout.

TABLE OF CONTENTS

I.	INTRODUCTION.....	1
II.	MATERIALS AND METHODS.....	11
III.	EXPERIMENTS WITH CULTURE AND INCUBATING MEDIA FOR <u>T. LEWISI</u>	17
IV.	PRELIMINARY EXPERIMENT. Inoculation of rats using metabolic products collected in Seneca and Henderson's medium and modifications of it, after one week of incubation.....	25
V.	EXPERIMENT NO. I. Attempted protection of rats by inoculation with metabolic products of <u>T. levisi</u> incubated 72 hours in a medium consist- ing of equal parts of saline and normal rat serum.....	29
VI.	EXPERIMENT NO. II. Inoculation of rats with metabolic products and with triturated trypano- somes collected at the end of 24 hours of in- cubation in a mixture of saline and rat serum..	35
VII.	EXPERIMENT NO. III. Inoculation of rats with metabolic products and with triturated trypano- somes collected at the end of 24 hours of incu- bation in a mixture of saline and rat serum; smaller dosage, fewer injections, and larger challenge than in Experiment II.....	43
VIII.	EXPERIMENT NO. IV. Agglutination Tests.....	48
IX.	EXPERIMENT NO. V. Protective effects of pas- sively transferred immune serum from recovered rats before and after absorption with metabolic products and with triturated trypanosomes.....	54
X.	DISCUSSION AND CONCLUSIONS.....	64
XI.	SUMMARY.....	88
XII.	BIBLIOGRAPHY.....	91

I. INTRODUCTION

Trypanosoma lewisi, a very common blood flagellate parasitic in the rat, was first found by Lewis in 1879. The parasite is transmitted from rat to rat by the rat flea, Nosopsyllus fasciatus, and by the rat louse, Polypx spinulosus. (See Laveran and Mesnil, 1901; Salvin-Moore, Breinl and Hindle, 1908; Breinl and Hindle, 1910; Minchin and Thomson, 1915; and Noble, 1955). In the midgut of the flea or louse the parasites multiply in crithidial and leptomonad stages, and eventually produce large numbers of metacyclic trypanosomes in the hindgut. Infection of the rat occurs by fecal contamination of the minute wound produced by the bite of the invertebrate host. T. lewisi is normally found in the rat only, but guinea-pigs and rabbits can be experimentally infected. In the guinea pigs and rabbits the parasites are never as numerous in the blood as in the normal host. (Laveran and Mesnil, 1901 and Coventry, 1929). Recently, Lincicome (1955) has succeeded in infecting mice with T. lewisi by providing the mice with small daily doses of rat serum.

Laveran and Mesnil (1901); Taliaferro (1924, 1929); Taliaferro and Taliaferro (1922) and Coventry (1925, 1930) have described the course of the infection with T. lewisi in the rat as follows: During the first five or six days after injection into the rat, the trypanosomes divide rapidly, but by the sixth to the tenth day reproduction

ceases and after that only adult undividing trypanosomes are found in the blood. Usually around the tenth day the trypanosomes in the blood decrease suddenly. Those that remain may survive for days, weeks and some times even for months; but eventually they also will disappear and the rat recovers completely. However, fatal infections of T. levisi in young rats have been reported by Brown (1915), Duca (1939), Culbertson and Wotton (1939) and others.

After the rat recovers from an infection with T. levisi it is refractory to a second infection by the parasite. This has been a well established fact since Kanthack, Durham and Blandford (1898) first observed the phenomenon.

Several investigators have postulated different antibodies and other defense mechanisms to explain how the rats recover and are then immune to re-infections.

Laveran and Mesnil (1901, 1912) considered phagocytosis as the only mechanism involved in both active and passive immunity. They were able to observe agglutination of the parasites but did not consider it an important factor in the defense mechanism since the agglutination or "agglomeration" of the parasites did not immobilize the flagellates, nor were they harmed in any other way. Laveran and Mesnil (1901) suggested that the substances responsible for agglutination and those for immobilization were different. Manteufel (1909) concluded that phago-

cytosis played a part in cases of weak immunity, but that lysis was the chief mechanism involved in highly immune animals.

MacNeal (1904) observed that about the sixth to the eighth day the reproducing trypanosomes tend to remain attached by their posterior ends while continuing to divide, thus forming rosettes. He advanced the opinion that this phenomenon was due to an increased difficulty of division which he associated with an antagonistic reaction on the part of the host. The trypanosomes were observed by MacNeal to be able to separate and to continue single in the blood for some time. MacNeal was unable to observe phagocytosis of the parasites. However, using immune guinea pigs he found evidence of immobilization and gradual solution of the trypanosomes in the peritoneal fluid. Thus, he concluded that the parasites disappear as the result of some cytolytic factor rather than phagocytosis. According to Regendanz and Kikuth (1927) the final disappearance of the trypanosomes is due to a gradual non-specific phagocytosis throughout the infection.

It was Taliaferro and his co-workers (1922, 1924, 1932) who first presented a working hypothesis to explain the resistance against a T. lewisi infection. According to Taliaferro (1932) this resistance is manifested in three ways during the course of an infection: first, there is a retardation and finally an inhibition of the reproduc-

tion of the parasites occurring about the fifth to the tenth day; second, between the ninth and fifteenth day there occurs a sudden crisis in the number of trypanosomes circulating in the blood; and third, the infection terminates with the total disappearance of the parasites.

By measuring the coefficient of variation (i.e., the difference in the size of the individuals in a given sample, calculating the deviation in percentages of the mean) Taliaferro and Taliaferro (1922) were able to calculate the rate of reproduction of the parasites independent of the numbers killed. Taliaferro (1924) presented evidence for the formation of a specific reaction product in the serum of infected rats which completely inhibited the reproduction of T. lewisi without harming the trypanosomes in any other way. Regendanz and Kikuth (1927) corroborated these original observations; they observed no lytic properties in the serum of rats after recovery from an infection. They first demonstrated that the reaction product which inhibited the reproduction of T. lewisi in the rat is formed chiefly in the spleen. Regendanz and Kikuth showed that after removal of the spleen the infection became heavier and they observed that splenectomy caused the reproduction period of the trypanosomes to last two to five days longer than normally. However, they did not consider the spleen to be the only site for the production of this substance since in splenectomized rats the reproduction of T. lewisi will eventually stop. Talia-

ferro, Cannon and Goodlee (1931) corroborated the conclusions of Regendanz and Kikuth. They found that the reproduction-inhibiting substance was only temporarily reduced by splenectomy and concluded thereby, that proliferation and activation of the reticulo-endothelial system in other parts of the body, particularly the Kupffer cells of the liver, probably, at least in part, restore the functional level of defense.

Further evidence for a specific acquired factor that inhibits reproduction was presented by Coventry (1925, 1930) and by Taliaferro (1932) who considered this specific reaction product to be an antibody, which he termed ablastin. Like other antibodies ablastin is precipitated with the globulin fraction of the serum, sometimes with the euglobulin alone and sometimes with both the eu- and pseudo-globulin. However, ablastin was believed to show no persistent in vitro affinity for either dividing or adult trypanosomes, and it could not be removed from immune serum by absorption with living trypanosomes.

Coventry (1925) found that the titer of ablastin increases sharply during the fifth and sixth days, when the trypanosomes are approaching a peak, and are actively metabolizing. He observed further increase in reproduction-inhibiting capacity of serums of infected rats up to 20 to 30 days after infection, followed by a tendency to decrease after 35 days, this becoming very marked by the 46th day. Neither Taliaferro (1924, 1932) nor Coventry (1925, 1930) felt that

the factor (later accepted as an antibody) which inhibited reproduction was instrumental in causing the number crisis that occurred on the ninth to fifteenth day of an infection, or the final elimination of the trypanosomes from the host some weeks later. These phenomena they believed to be due to an entirely distinct trypanocidal antibody.

Coventry (1930) found that immune or "hyperimmune" serum, injected into rats showing numerous trypanosomes in their blood on the third or fourth day of infection, had curative effects, causing disappearance or decrease in numbers of the trypanosomes from the blood in from one to five hours, with evidence of a zoning phenomenon. This is considered in more detail in the "Discussion." She interpreted this as evidence of trypanocidal action of the serum, and postulated that the appearance of this trypanocidal substance in the serum was correlated with the first number crisis. The curative effect of immune serum tended to be lost within a few weeks after the termination of an infection. Taliaferro (1932) accepted Coventry's work as evidence of a trypanocidal antibody which, in some cases but not consistently, he was able to absorb from immune serum by means of very large doses of parasites. He felt that ablastin and this trypanocidal antibody could, theoretically at least, be separated by absorption. He believed that the flagellates were sensitized by the trypanocidal antibody, since after being kept in contact with immune serum they

were quickly killed when introduced into a normal, previously-uninfected rat.

Taliaferro (1938 b) reported the presence of similar ablasic and trypanocidal action against Trypanosoma duttoni in the mouse. Augustine (1943), studying the reaction of rats to reinfection with T. lewisi, obtained results which led him to believe that a trypanocidal antibody plays the predominant role in protection against reinfection, and to question the existence of ablastin. Further details of Augustine's work will be given in the "Discussion."

Laveran and Mesnil (1901), Regendanz and Kikuth (1927), Augustine (1943), Taylor and Becker (1947), and others had called attention to agglutination of trypanosomes in the course of infections or in reinfections in rats. Barnes (1951) emphasized its importance, and recognized it as a third type of antibody, distinct from both ablastin and the trypanocidal antibodies; she believed that the agglutinin plays an important part in conjunction with a trypanolysin in bringing infections to an end. She observed agglutinated masses of parasites at the time of the first decrease in the number of trypanosomes; after its first appearance, agglutination continued throughout the period of decline of the infection.

In more recent years (1947-1953), Becker and his colleagues have published a number of papers dealing with the effects of deficiency of pantothenic acid, administration

of salicylates, and treatment with benzene on the course of T. lewisi infections, and have attempted to interpret their results on the basis of differential effects of these treatments on three antibodies--ablastin, trypanocidal antibodies and agglutinins--which they, like most others, have hitherto accepted. This matter, too, will be considered further in the "Discussion."

Considerable work has been done by various authors on the physiology and metabolic requirements of T. lewisi as well as of other trypanosomes, e.g., by von Brand and his colleagues (see von Brand, 1952); Reiner, Smythe and Pedlow (1936); Ryley (1951, 1956); and Seaman (1953). M. Lwoff (1933) found that certain trypanosomids of insects (Strigomonas fasciculata and Leptomonas ctenocephali), but not certain others, failed to multiply in culture in the absence of blood, and she later found that the blood could be substituted for by hematin, protohematin or protoporphyrin. The flagellates are apparently unable to synthesize protoporphyrin, although this compound is necessary for respiration of the organisms. Later Lwoff (1938, 1939) found that hematin was necessary for reproduction in culture of Trypanosoma cruzi and species of Leishmania, also. Further work on the need of hematin or its precursors was done by M. Lwoff (1951). Moulder (1948 a, 1948 b) studied the oxidative metabolism of T. lewisi and the metabolism of glucose in this parasite at different stages in the course

of an infection, and correlated his findings with the appearance of ablastin. The relations of some of these studies on physiology and nutritional requirements to the defense mechanism in T. lewisi infections will be dealt with further in the "Discussion."

In the present work we have attempted to determine the general nature and source of the specific antigen or antigens stimulating the formation of the antibody (ablastin) which inhibits the reproduction of T. lewisi, and to study the relations of the antibody thus stimulated to the course of infection with T. lewisi in rats, and to the various phenomena associated with immunity and resistance of rats to this parasite. We have also analyzed and discussed results and interpretations of other workers who have postulated the involvement of several functionally distinct antibodies, and we have suggested that, on the basis of our findings, ablastin may account for all or nearly all of the phenomena observed, and may be the only important antibody involved in the defense mechanism of rats against this parasite.

Data obtained in the course of our experiments support the hypothesis first proposed by Chandler (1935) that in the case of Trypanosoma lewisi, as in the case of Nippostrongylus muris and probably a great many other parasites, true functional immunity is dependent upon antibodies directed against excreted metabolic products of the parasites, most probably enzymes, as a consequence of which the

nutrition or metabolism of the parasites is interfered with in such a way as to retard growth, development and reproduction.

II. MATERIALS AND METHODS

The strain of Trypanosoma lewisi used in these studies was kindly provided by Professor A. Packchanian from the University of Texas Medical School at Galveston, Texas. The strain was continuously maintained in our laboratory by weekly passage through rats. All rats employed were male albinos obtained from Sprague-Dawley, Inc.

The normal rat serum used for the incubation of the parasites for obtaining metabolic products was procured in the following manner: large rats, previously uninfected with T. lewisi, were etherized. A piece of skin was quickly removed from the region around the neck. The carotid arteries and jugular veins were severed with a sharp razor blade and the animals aseptically bled to death into a large, sterile open container. The blood from several rats was pooled, allowed to clot, and then stored overnight in the refrigerator. Next day the serum was removed with a pipette. The few erythrocytes collected were removed by centrifugation, and the serum was then filtered through a Seitz filter. This method provided a fairly large amount of normal rat serum and we never had difficulties with bacterial contamination. The sterility of the serum was tested by inoculating 0.5 cc. of the serum in 5 cc. of nutrient broth and incubating it for 24 hours at 37°C. No bacterial growth ever occurred.

Trypanosomes for incubation for procuring metabolic

products were obtained from rats, weighing 100 to 200 grams, which had been infected three to four days previously by the intraperitoneal injection of 2 cc. of heavily infected blood. The parasitized blood was obtained directly from the heart of the infected rats, using extreme sterile precautions, by means of a 5 cc. syringe fitted with a 20-gauge hypodermic needle, one inch long. The blood was transferred to a sterile centrifuge tube and diluted with an equal volume of sterile citrated saline. It was then centrifuged for thirty minutes at 3,000 r.p.m. At the end of this centrifugation the erythrocytes were at the bottom of the tube and the trypanosomes formed a very thick layer just above the buffy coat formed by the white blood corpuscles. The trypanosomes, mixed with some erythrocytes and white blood corpuscles, were collected with a sterile capillary pipette and mixed with 5 cc. of sterile saline. This saline suspension of the parasites was washed twice by centrifugation, and after the last washing the trypanosomes were resuspended in sterile simple saline. At this point the flagellates were counted, using a haemocytometer, and the desired number of them was then introduced into a 50 cc. Erlenmeyer flask containing 5 cc. of normal rat serum. The volume was then brought up to 10 cc. by the addition of sterile saline, thus providing a 50% rat serum. Care was taken to keep the concentration of trypanosomes in the Erlenmeyer flasks below 200,000 per cmm. since it was noted that as

the concentration of parasites increased above this point early death and decomposition of the organisms began to develop.

All counts of trypanosomes were made with the help of a Spencer AO Bright-line haemocytometer and always in duplicate. A .01 cc. drop of the medium containing the trypanosomes was taken and diluted one hundred times in a blood-diluting pipette with normal saline. The living trypanosomes were slowed down by applying a moderate amount of heat from the substage lamp of the microscope to the counting chamber. This method was adequate to immobilize the trypanosomes, thus facilitating the counts.

The trypanosomes were counted immediately after introduction into the incubating medium. They were then incubated at room temperature for 24 hours and counted again at the end of this period. Since it was noticed that the number of trypanosomes remained amazingly constant (at times even slight increases in numbers) and since no deaths were recorded, it was decided to count the trypanosomes only once, midway during the incubation period; this was done in order to reduce the possibilities of bacterial contamination of the medium to a very minimum. Twelve hours after the incubation had started, glucose was added to the medium to make approximately 0.25% glucose solution.

After 24 hours of incubation the trypanosomes were separated from the incubating medium by centrifugation at

3,000 r.p.m. for ten minutes followed by filtration through a fritted-glass bacteriological filter of fine porosity. Care was taken to prevent injuring the trypanosomes during this process as a result of excess pressure from filtering to dryness. The filtrate thus recovered, which supposedly contained the accumulated metabolic products of the parasites, was then stored in the deep freezer until further use.

The incubated trypanosomes were resuspended in saline and stored in the deep freezer. Both these trypanosomes and their metabolic products, thus collected, were used later for inoculation of rats without any further treatment except that the trypanosomes resuspended in saline had to be killed by quick freezing and thawing the suspension several times. The death of all trypanosomes was confirmed by injecting previously uninfected rats. Since no infections developed, it is logical to conclude that all the trypanosomes were killed.

The metabolic products suspended in the medium of incubation were inoculated into the rats for immunization without any further treatment. Since no infection ever developed from these inoculations, it is evident that all the trypanosomes had been removed.

All rats used for immunization weighed approximately 100 grams at the time of the first immunizing injection, the actual range being from 95 g. to 115 g. All injections were given intraperitoneally and at three-day intervals.

Sera used for serological in vivo and in vitro tests were obtained by heart puncture and stored in the deep freezer until ready to use. No preservative was ever added. Three types of immune sera were thus obtained: (1) serum from rats that had recovered from an infection with T. lewisi, (2) serum collected from rats inoculated with dead triturated trypanosomes that had been thoroughly washed, and (3) serum from rats inoculated with a 24-hour accumulation of metabolic products freed from trypanosomes by centrifugation. Sera from rats artificially immunized were collected eight days after the last immunizing injection, and again after 6½ months in the case of the rats immunized with metabolic products. Sera from rats that had recovered from an infection were obtained from rats infected six weeks previously, after no trypanosomes were any longer present in the blood.

All agglutination tests were performed on hanging drop slides and examined under the low and high powers of the microscope. All dilutions of the immune sera were made in small precipitin tubes or in the wells of the slides, since we were dealing with very small amounts of sera. Sterile saline was used for all dilutions. The trypanosomes used in these tests were adult trypanosomes obtained by heart puncture from rats injected a week previously. All were alive and carefully washed in saline; finally they were suspended in sterile saline and added

to the various sera, at the rate of .05 cc. drops to two or three drops of the diluted sera. Approximately 500,000 T. lewisi were used in each case. The wells containing the drops of sera mixed with trypanosomes were covered with a cover slip and sealed with vaseline to prevent desiccation. Also, when not being examined, the slides were kept in a humid chamber.

All permanent blood smears for microscopic examinations were stained in Giemsa stain prepared with a buffer at pH 6.8. All blood for fresh and stained smears was taken from the tail of the experimental animals.

Trypanosomal counts to trace the course of an infection in control and experimental rats were made by the usual haemocytometer method and always in duplicate; 0.01 cc. of blood was taken directly from the tail of the rats and diluted one hundred times with a blood-diluting pipette.

All antigens used for the absorption of antibodies from the immune sera were prepared by lyophilizing the whole medium containing the metabolic products or by lyophilizing the suspension of triturated trypanosomes. The antigens thus prepared were kept in contact with the immune serum for 12 hours in a sterile centrifuge tube and kept inside a refrigerator. After this period, the antigen-antibody combination was removed from suspension by centrifugation at 4,000 r.p.m. followed by filtration through a Seitz micro-filter.

III. EXPERIMENTS WITH CULTURE AND INCUBATING MEDIA FOR T. LEWISI

The first problem with which we were confronted was to find an adequate culture medium for Trypanosoma lewisi which would facilitate the collection of the metabolic products of the parasite in great concentrations and which would be simple enough in its protein composition so as to reduce to a minimum the chances of complications with foreign antigens. T. lewisi grows very well in NN and MNM medium (Novy and MacNeal, 1903; Novy, Perkins and Chambers, 1912; Packchianian, 1934, 1943) but because this is a semi-solid preparation the question immediately arose as to how the parasites and their metabolic products were going to be separated from it. A liquid medium was therefore desirable because, first, it would be a very simple matter to separate the organisms by either centrifugation or filtration and, second, their metabolic products would remain in solution or suspension in the medium and could be inoculated into the experimental rats without any mechanical difficulties.

All cultures and incubated media described below were seeded with adult T. lewisi by the following method: Rats infected with T. lewisi a week previously were bled aseptically from the heart. The blood thus obtained was centrifuged at 3,000 r.p.m. for ten minutes. The trypanosomes were then collected from above the buffy coat with

the help of a sterile capillary pipette, suspended in sterile saline, and washed twice in saline. After the second washing they were again suspended in 0.85% saline. Approximately 1 cc. of the final suspension of trypanosomes was inoculated into each culture or incubating medium. After inoculation all tubes or flasks were incubated at room temperature (28°C.).

The first medium used was one described by Seneca and Henderson (1951) for the culture of Trypanosoma cruzi, consisting of 50 g. Bacto Beef (Difco), 20 g. Neopeptone (Difco) and 5 g. of NaCl (C.P.) in one liter of distilled water. The pH of the medium was adjusted to 7.3. Seneca and Henderson fortified their medium with sterile rabbit serum added in a ratio of one part of serum for each nine parts of stock liquid. In our experiments we used different kinds of sera (rat, horse and human) in the same proportions. The T. lewisi were inoculated into sterile dialysis bags containing 9 cc. of the stock medium to 1 cc. of serum. This bag was then introduced into a large sterile chamber containing 90 cc. of the stock medium. The purpose of the outside chamber was to allow waste products of the trypanosomes to escape from the small bag, in which the non-dialyzable products would accumulate. The outside chamber and medium was changed every 24 hours to remove the dialyzed products and to give the trypanosomes access to fresh nutrients, thus favoring continued reproduction.

Seneca and Henderson obtained excellent results by this method in culturing T. cruzi, but our results with T. lewisi were very inconsistent. In some instances we were able to observe some multiplication of the T. lewisi but more often the numbers of trypanosomes originally introduced would gradually decrease, and the parasites seemed to become deformed and to disintegrate very rapidly. Several modifications of Seneca and Henderson's medium were tried but with the same unsatisfactory results. These modifications consisted in using different proportions of serum and stock medium inside the dialysis bag and different proportions of medium inside and outside of the dialysis bag.

Attempts were made to grow the trypanosomes in culture media consisting of (1) dialyzed Bacto Veal in distilled water fortified with horse serum or by dialyzing horse serum into the medium, and (2) of dialyzed Bacto Veal fortified with Neopeptone and with other kinds of sera substituted for the horse serum. All were unsuccessful. In these media there were, generally, strong evidences of clumping, death and disintegration of the parasites in 48 hours or less. However, sometimes the trypanosomes would survive and even multiply, but never in great numbers. The greatest concentration of parasites that we were able to obtain in any of these culture media was 1,425 trypanosomes per cmm. in 10 cc. of medium, which was not adequate for

our experiments. Transfers of these cultures were not successful.

Culture media consisting of dialysates of Bacto Beef into Locke's solution were also completely unsatisfactory. The same very disturbing results were obtained when we attempted to use mixtures of different amino acids and vitamins in the same proportions described by Little and Oleson (1951) for T. cruzi. In none of various combinations used was there any increase in the number; on the contrary, there was rapid decrease.

Thinking that possibly some degree of oxygenation would help, we tried rotating the tubes containing some of the above media, or shaking the cultures at different speeds, but with no more success than before.

After these failures we thought that perhaps a solid substrate was necessary for the parasites to live in vitro. We thought that perhaps the function of the blood agar in the MNN medium was not only nutritional but also that it functions by absorbing some of the waste products that are toxic to the parasites, in a manner similar to the function of the outside chamber in Seneca and Henderson's medium for T. cruzi. We, therefore, attempted to grow them in a culture medium described by Tobie and Rees (1948) for culturing T. cruzi. This medium was prepared in loops of cellophane tubing by suspending the loops containing Locke's solution in the overlay of a diphasic medium. The Locke's solution

of the loops and for the overlay was composed of 8 g. NaCl, 0.2 g. KCl, 0.2 g. CaCl_2 , 0.3 g. KH_2PO_4 and 2.5 g. dextrose per 1000 cc. distilled H_2O . The solid phase consisted of blood agar containing the same ingredients present in Seneca and Henderson's medium and in the same proportions, but with the addition of 20 g. of agar, and whole citrated rat blood was used in proportions of one part of blood to three parts of base. The trypanosomes were inoculated into the suspended cellophane sac. This technic proved to be as unsatisfactory as all the others tried so far.

We also thought that part of our troubles was possibly due to the fact that a liquid medium lacks a surface to hold the trypanosomes in the part of the culture where oxygen is freely supplied. This idea had been previously suggested by Little and Oleson (1951) working with T. cruzi. Thinking that this could be the function of the solid substrate, we attempted to grow T. lewisi in the culture medium described by Tobie, et al. (1950) for T. rhodesiense and T. gambiense. This is a diphasic medium, the solid blood agar slant of which contains 1.5 g. Bacto Beef, 2.5 g. Bacto Peptone, 4 g. of NaCl and 7.5 g. of Bacto Agar per 500 cc. of distilled H_2O . Rat blood was used (instead of rabbit blood as used by Tobie, et al.) in proportions of one part of blood to three parts of agar base. The liquid overlay consists of Locke's solution. We assumed that the metabolic products could be collected in this overlying

Locke's solution.

The parasites survived in this medium fairly well. We obtained a peak of 1790 trypanosomes per cmm. during the eleventh day after inoculation. There was some indication of reproduction. We also tried a modification of this medium in which blood was replaced by an equivalent amount of dehydrated hemoglobin in the form of a 2% solution. However, none of these media proved satisfactory, since none supplied the large number of trypanosomes we should need for our experiments, and also because in these cultures it was impossible to determine how many, if any, of the organisms had died and possibly disintegrated before we collected the trypanosomes for our experiments.

In view of these failures, we turned our attention to a search for an incubation medium in which large numbers of the trypanosomes would survive in good, active condition for the longest time possible.

Since the diphasic medium in which we had some slightly encouraging results consisted of an overlay of Locke's solution, the first incubation medium used by us consisted of the same Locke's solution as prepared before. Besides the Locke's solution, a Ringer phosphate solution as described by Ryley (1951), consisting of 0.094 M of NaCl, 0.005 M of KCl, 0.001 M of $MgSO_4$ and 0.025 M phosphate buffer at pH 7.3 was used. Different concentrations of trypanosomes and different volumes of solution were used.

These two solutions were apparently satisfactory in part, but although great numbers of trypanosomes survived for 48 hours or more, still many organisms were seen to be dead and in process of degradation.

Following these partially successful attempts we employed a modification of the solution described by Moulder (1948 a) for his studies of the metabolism of T. levisi, which consists essentially of a phosphate-saline containing 0.01 M glucose and 2% bovine serum albumin. We substituted 2% whole normal rat serum for the 2% bovine serum albumin. This solution showed some improvement over the others but still there were too many deaths of the flagellates.

We next tried incubating the trypanosomes for a few hours in plain, normal rat serum. It was logical that the trypanosomes would survive for a few hours in rat serum since that is their normal environment. To incubate the trypanosomes in undiluted normal rat serum would have been an extremely expensive procedure, therefore we decided to dilute the serum with Locke's solution, Ringer phosphate, or normal physiological saline. Comparisons of these three solutions demonstrated that there was no appreciable difference between the three, so we decided to use a dilution of one part of sterile 0.85% saline and one part of normal rat serum. This simple combination proved to be the most favorable for our purposes. Large numbers of trypanosomes, up to 200,000 per cmm., could be incubated in this medium

for 24 hours or more. It was observed, however, as a result of careful trypanosomal counts, that although the numbers of trypanosomes did not decrease in 24 hours, after this period there were sharp decreases in their number and a rapid disintegration and decomposition of the flagellates became evident.

It was noted that after the first 12 hours of incubation the parasites were non-mobile. It was interesting to observe that for the first few seconds after the parasites were taken from the medium and placed on a slide for examination under the microscope they were immobile, but very slowly, probably because of access to increased oxygen, they became active again. This reminded us of Ryley's observation (1951) that under aerobic conditions in the absence of nutrients T. lewisi would lose its motility, but that this could be restored rapidly by the addition of glucose. We therefore decided to add glucose to our incubating solution in sufficient amount to make a total glucose concentration of 0.25%, which is the same concentration present in Locke's solution. This addition of glucose after the first 12 hours of incubation reactivated the trypanosomes, and they then remained active for at least 12 more hours.

IV. PRELIMINARY EXPERIMENT

Inoculation of rats using metabolic products of T. lewisi collected in Seneca and Henderson's medium, after one week of incubation

In a preliminary experiment, a series of cultures employing modifications of Seneca and Henderson's medium for T. cruzi (1951) were inoculated with 1 cc. of a saline suspension of T. lewisi (approximately 300,000 trypanosomes). The parasites were obtained from rats infected eight days previously. Cultures Nos. 1 and 2 were prepared exactly as described by Seneca and Henderson (1951), except that human plasma was used in the preparation instead of rabbit serum. Cultures Nos. 3 and 4 consisted of 20 cc. of Seneca and Henderson's medium in 50 cc. test tubes, fortified with human plasma at a ratio of 1 cc. of plasma to 9 cc. of the stock medium, to which glucose was added to make a final concentration of 0.5% of glucose. Cultures Nos. 5 and 6 were prepared similarly to cultures Nos. 3 and 4 except that glucose was omitted from them. Cultures Nos. 7 and 8 were similar to Nos. 5 and 6 in preparation except that rat serum was used instead of human plasma.

The cultures were incubated for a week at room temperature and examined every other day. This was one of the few instances in which we had some success in sustaining moderate numbers of T. lewisi for some time in either Seneca and Henderson's medium, modifications of it, or any

other types of culture media. Our examinations always showed large numbers of trypanosomes to be alive and fairly active, particularly in cultures Nos. 1 and 2 (See Table I.). Motility of the organisms was used as an index of the normality of the flagellates. We observed that in these cultures some death of the trypanosomes occurred. We could see dead and disintegrating trypanosomes each time the cultures were examined, and frequently we observed the formation or appearance of several "rosettes" which could have been formed as a result of incomplete division of the parasites (see MacNeal, 1904) or as a result of an agglutinating phenomenon due to some unfavorable condition in the cultures.

On the eighth day of incubation the trypanosomes were separated from the culture media by filtering the cultures through a fritted-glass bacteriological filter. The filtrates were examined several times for living trypanosomes and none was ever found. In these filtrates we assumed that some metabolic products (products of intermediary metabolism, excreted enzymes, etc.) of the incubated parasites were present in dilution or suspension. Presumably, the filtrate could also contain products liberated after death of some of the organisms. The filtrates of cultures Nos. 1 and 2 were mixed and will be referred to as filtrate A; those of cultures Nos. 3, 4, 5 and 6 were also mixed and will be referred to as filtrate B; and those of cultures Nos. 7 and 8 will be referred to as filtrate C.

TABLE I

Examination and handling of cultures used for collection of metabolic products of *T. levisi* in Seneca and Henderson's medium and modifications of it for inoculation into rats. Collected on 8th day of incubation.

Cult. No.	Examination of Cultures (Days of Incubation)			Fil- trate	Remarks
	4th	6th	8th		
1	Many active trypano- somes	Many active trypano- somes	Many active trypano- somes		Outer chamber changed on 4th and 6th days. Mixed with No. 2
2	Many active trypano- somes	Many active trypano- somes	Many active trypano- somes	A	Outer chamber changed on 4th and 6th days. Mixed with No. 1
3	Many active trypano- somes. Some non-motile	Many active trypano- somes. Some non-motile	Many active trypano- somes. Some non-motile		Mixed with 4, 5 and 6.
4	Few active trypano- somes. Clumps	Few trypanosomes. Not very active	Few trypanosomes. Not very active	B	Mixed with 3, 5 and 6.
5	Few active trypano- somes	Many active trypano- somes. Some non- motile	Many active trypano- somes. Some non- motile		Mixed with 3, 4 and 6.
6	Many active trypan- osomes. Some non- motile	Many active trypan- osomes. Some non- motile	Many active trypan- osomes. Some non- motile		Mixed with 3, 4 and 5.

(Table I continued next page)

(TABLE I Continued)

Cult. No.	Examination of Cultures (Days of Incubation)			Fil- trate	Remarks
	4th	6th	8th		
7	Few active trypano- somes. Some de- formed	Many active trypano- somes. Some non- motile	Many active trypano- somes		Mixed with 8
8	Many active trypano- somes. Some non- motile	Many active trypano- somes. Some non- motile	Many active trypano- somes	C	Mixed with 7

These three resulting combinations were utilized to immunize rats.

Twelve male albino rats of about 100 g. weight each were treated as follows: Rats Nos. 1-4 were kept for control and were not inoculated at all. Rats Nos. 5 and 6 were given four injections, at four to six day intervals, of filtrate A (See Table II); Rats Nos. 7, 8 and 9 were inoculated in a similar manner with filtrate B; and rats Nos. 10, 11 and 12 were similarly injected with filtrate C.

Ten days after the last injection all the inoculated rats and also the control rats were injected intraperitoneally with approximately 250,000 T. lewisi obtained from a rat infected a week before. This is approximately the same number that was customarily given to normal rats for maintenance of the parasite strain and a number which had always been adequate to infect the rats. We thought that this amount of trypanosomes would be so diluted by the circulating blood that the parasites should have been seen in the exposed animals only if reproduction of the parasites had occurred. We calculated that the rats we used had a blood volume of approximately 10 cc. Thus, the injection of 250,000 T. lewisi would give an initial concentration of approximately 25 trypanosomes per mm. of blood. In our experience we could find trypanosomes easily in a blood smear only if the concentration was close to 500 T. lewisi per mm.

TABLE II

Results of inoculation of rats with filtrates A, B and C, collected on 8th day of incubation as shown in Table I.

Rat No.	Filtrate Used	Amount of Injection			Examin. of tail blood (Days after inf.)				Remarks
		0	5 days	5 days	6 days	2	4	7	
1	-	-	-	-	-	F	M	M	Control Rats
2	-	-	-	-	-	F	M	M	
3	-	-	-	-	-	F	M	M	
4	-	-	-	-	-	F	M	M	
5	A	1cc	1cc	1cc	1cc	0	0	0	Always Negative
6	A	1cc	1cc	1cc	1cc	0	0	0	
7	B	1cc	1cc	2cc	2cc	F	F	F	Peak never as high as in control rats
8	B	1cc	1cc	2cc	2cc	F	F	F	
9	B	1cc	1cc	2cc	2cc	F	F	F	
10	C	1cc	1cc	2cc	2cc	F	F	F	
11	C	1cc	1cc	2cc	2cc	F	F	F	
12	C	1cc	1cc	2cc	2cc	F	F	F	

Note F Few trypanosomes per microscopic field
M Many trypanosomes per microscopic field
O No trypanosomes seen

Injection with 1 cc of filtrate

A fresh drop of blood from the tip of the tail of each one of the rats was examined every other day under the low and high powers of the microscope. The results shown in Table II are, of course, not significant because the experiment was not properly controlled. However, the case of rats Nos. 5 and 6 should be considered. These two rats represent the first instance in our experience in which we failed to infect a previously uninfected rat of any age with any number of trypanosomes. We failed to see trypanosomes in the tail blood of these animals even though we examined several smears for ten minutes each. We did not consider it a mere coincidence that these two rats were inoculated with filtrate A (of cultures Nos. 1 and 2, which were the two best cultures in the group used.). We also observed that even in rats Nos. 7 to 12, although reproduction had obviously occurred, the peak of the parasite population was never as high as it was in the control rats. In each of these rats we could observe that in any one microscopic field, at any time, the trypanosomes were not as numerous as in the case of control rats. Unfortunately actual counts were not taken. These results, meager as they were, stimulated us to continue with our experiments. It was still necessary to find an adequate culture or incubating medium, since we knew that it would be necessary to inoculate the rats with greater amounts of metabolic products and that, therefore, greater concentration of trypanosomes was necessary.

V. EXPERIMENT NO. I

Attempted protection of rats by inoculation with metabolic products of T. lewisi incubated 72 hours in a medium consisting of equal parts of saline and normal rat serum

Approximately 10,000 million T. lewisi, obtained from rats infected three days before, were incubated for three days in an incubating medium consisting simply of equal parts of 0.85% saline solution and normal rat serum as described under "Materials and Methods." The trypanosomes were incubated in a series of five 50 cc. Erlenmeyer flasks, each containing from 10 to 12 cc. of the medium. Extreme care was taken to avoid contamination of the flasks by bacteria. It was necessary to incubate the 10,000 million trypanosomes in approximately 52 cc. of medium because, as discussed under the section on "Experiments with Culture and Incubating Media," we had observed that in this medium concentrations of the organisms larger than 200,000 per cmm. did not do well.

Trypanosomes from a three-day-old infection were selected for incubation in order to prevent the possible contamination of the incubate by reproduction-inhibiting antibody already present in the blood of infected rats. Since Coventry (1925) was unable to demonstrate the antibodies in the circulation of rats with four-day infections, we decided that three days was a safe age to use. Besides having taken

this precaution, we were certain that even if some antibody (ablastin) was already present in the rat's blood, the method used by us of washing the trypanosomes in saline at least three times would satisfactorily eliminate interference from this source, since any small traces of antibodies that could remain after this process would be so diluted in the medium and after injection into normal rats as to be insignificant.

With the number of trypanosomes (10,000 million) employed and the long period of incubation (72 hours), we hoped that the concentration of metabolic products of the parasites might be adequate for stimulating the production of antibodies if any antigenic components were present. During the incubation period the trypanosomes were counted by the usual haemocytometer method. For the first 24 hours the number of trypanosomes remained constant but after that period we could observe death and disintegration of some of the parasites.

After the 72 hours of incubation at room temperature the trypanosomes were separated from the incubate by the process of centrifugation and filtration previously described. Approximately 50 cc. of the medium, containing only the metabolic products of the parasites, were recovered, 2 cc. having been lost during centrifugation and filtration. The filtrate thus obtained was later utilized for inoculation into rats in a series of injections, to

determine whether any protection against infections with T. lewisi would result from this procedure.

Each of six rats was given seven intraperitoneal injections of the filtrate at intervals varying from three to seven days, for a total final volume of injections of 8 cc. (See Table IV). Therefore, each inoculated rat was supposedly given the metabolic products of approximately 1,600 million T. lewisi, collected at the end of a three-day period. After each injection the rats were examined to be sure that no infection had developed as a result of any of the injections. Five additional rats, of approximately the same ages and weights as the six rats mentioned above, were set aside as controls.

Two weeks after the last immunizing injection, a period that should have been sufficient for the titer of any antibody produced to have reached a peak, all the experimental rats (i.e., those inoculated with metabolic products) and also the five previously uninfected, untreated control rats were challenged by an intraperitoneal inoculation with approximately 50,000 T. lewisi in 1 cc. of saline.

This dosage was probably in large excess of the number of trypanosomes that would normally be transmitted from rat to rat in nature by the normal method of contamination of the bites of rat fleas or lice by feces of these insects (Laveran and Mesnil, 1901). In a preliminary

experiment we were successful in infecting rats with only ten trypanosomes. A few trypanosomes were taken, with a minute capillary pipette, from a drop of very diluted infected blood. At this point, it was very hard to count the trypanosomes under the dissecting microscope so that it was necessary to further dilute them by a series of transfers to drops of saline until a concentration was reached in which ten trypanosomes could easily be picked up with a capillary pipette under the dissecting microscope. These trypanosomes were re-suspended in another drop of saline and again counted. This final drop was drawn up from the slide into a small tuberculin syringe (1 cc.) with a No. 20 needle, one and one-half inches in length. Before collecting the trypanosomes the needle was filled by drawing about 0.5 cc. of saline into the syringe, then the drop containing the trypanosomes was drawn in. The trypanosomes were then injected intraperitoneally into a rat and the procedure was repeated to infect each of three rats. All rats used were of approximately the same size and weight that we have used in our experiments (100 g.). These rats were successfully infected in this manner; not only did they develop an infection, but these infections were so heavy that two of the rats died, but not as quickly as did two controls (See Table III.). These results make it evident that our dosage of 50,000 trypanosomes was more than sufficient to infect any normally susceptible rat.

TABLE III

Infection of rats (100 g. in wt.) with 10 T. lewisi.

Rat No.	Infection with <u>T. lewisi</u>	Trypanosomal counts (Tryps./cmm. Days after infection			
		2	4	6	8
1	Massive dose		dead	-	-
2	Massive dose		dead	-	-
3	10 Tryps.	100,750	died	-	-
4	10 Tryps.	65,000	450,000	-	-
5	10 Tryps.	30,000	750,000	died	-

Notes:

Massive dose lec of heavily infected blood,
 trypanosomes not counted

trypanosomes seen in fresh drop
 of tail blood

After the inoculation of all the rats with 50,000 trypanosomes, drops of blood from the tip of tail were examined from each rat every other day. Our results, which appear in Table V., were as follows: of all the rats inoculated with metabolic products, none ever developed an infection, or at least we were not able to observe any living trypanosomes in any of the blood samples taken, even though several were taken from each rat and each was examined for 10 minutes when the first one did not show trypanosomes. Blood smears taken from each rat and stained in Giemsa proved equally negative upon examination under the microscope. On the other hand all five of the control rats, simultaneously exposed to the trypanosomes, developed normal infections. Examination of all rats continued until the infection in the control rats had died out.

Since both experimental and control rats were each inoculated with 1 cc. of the same suspension of parasites, containing 50,000 trypanosomes per cc., and experimental and control rats were alternated (i.e., one experimental rat would be injected and then one control rat, etc.), there seems no reasonable chance that all of the rats would not have become infected if susceptible.

As a result of this experiment we concluded that some substance or substances present in the medium where the trypanosomes had been incubated was antigenic in nature, giving, apparently, complete protection to rats against the

TABLE IV

Immunization of rats with metabolic products of T. lewisi collected after 3 days of incubation in equal parts of normal rat serum and saline.

Rat No.	Amount and interval between injections (in days)							Total No. of Inject.	Total Vol. of Injec.
	0	4	3	4	3	4	7		
1	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
2	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
3	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
4	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
5	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
6	1cc	1cc	1cc	1cc	1cc	1cc	2cc	7	8cc
7	-	-	-	-	-	-	-	0	0
8	-	-	-	-	-	-	-	0	0
9	-	-	-	-	-	-	-	0	0
10	-	-	-	-	-	-	-	0	0
11	-	-	-	-	-	-	-	0	0

Notes:

Total vol. of injection (8cc) given to each of the experimental rats represented the metabolic products of approximately 1,600 million T. lewisi, collected at the end of 72 hours of incubation.

TABLE V

Results of challenge infections in 6 rats (Nos. 1-6) treated as shown in Table IV by injections of metabolic products of T. lewisi, and in 5 untreated controls (Nos. 7-11). All rats challenged with 50,000 trypanosomes 17 days after last immunizing injection to experimental rats.

Rat No.		Examination of Blood for <u>T. lewisi</u> .								
		Days after infection								
		2	4	6	9	11	13	16	18	20
1	T	0	0	0	0	0	0	0	0	0
2	T	0	0	0	0	0	0	0	0	0
3	T	0	0	0	0	0	0	0	0	0
4	T	0	0	0	0	0	0	0	0	0
5	T	0	0	0	0	0	0	0	0	0
6	T	0	0	0	0	0	0	0	0	0
7	C	0	+++	+++	+++	+++	+++	+++	+	0
8	C	0	+++	+++	+++	+++	+++	+++	+	0
9	C	+++	+++	+++	+++	+++	+++	+++	+++	0
10	C	+++	+++	+++	+++	+++	+++	+	0	0
11	C	+++	+++	+++	+++	+++	+++	+	0	0

Notes:

T treated rats
 C control rats
 +++ many trypanosomes seen in drop of tail blood
 + few trypanosomes seen in drop of tail blood
 0 no trypanosomes seen in drop of tail blood

number of trypanosomes inoculated. The evidence favored the view that this substance or substances were metabolic products of some kind, either intermediary decomposition products or enzymes, secreted or excreted by the parasites.

It could perhaps be argued that the substances producing the immunity or protection in our rats were the decomposition products of the trypanosomes that inevitably died in the medium during the 72 hours of incubation, some of which, at least, had probably disintegrated. That this was almost certainly not the explanation will be demonstrated by Experiment No. II, that follows. In the present experiment about 80% of the trypanosomes were alive at the end of 72 hours' incubation period; the other 20% appeared to be dead, and some had disintegrated.

The possibility also existed that the rat serum used in the incubating medium could possibly have undergone changes over the three-day period that could make it antigenic when injected into rats. This possibility also was eliminated in later experiments.

VI. EXPERIMENT NO. II

Inoculation of rats with metabolic products and triturated trypanosomes collected at the end of 24 hours of incubation in a mixture of saline and rat serum

This new experiment was planned in order to eliminate some of the possible objections raised in the discussion of Experiment No. I.

It was necessary to shorten the length of incubation to minimize the death of the flagellates. We noticed that at the end of 24 hours apparently no deaths of trypanosomes had occurred in the medium. The number of trypanosomes at the beginning of the incubation period and 24 hours later was approximately the same, except on a few cases in which apparently there was a slight increase in numbers. Since these parasites were collected during the third day of infection and some were dividing forms it may be presumed that most of these finished body divisions that had already started. However, no further divisions in the medium would be expected. We did not think it likely that the constancy in numbers was due to replacement of dead organisms by new forms resulting from divisions. At the end of the 24 hours' period the trypanosomes were actively motile with very few exceptions. There were a few organisms that appeared non-motile but they were normal in form and their refraction was normal. Occasionally, in some cultures, a few organisms would be found dead and disintegrating; whenever this

happened, the culture was discarded.

In a shorter incubation period there would be a smaller total quantity of metabolic products collected although, on the other hand, there would be less probability of loss by chemical alteration or decomposition. According to Coventry (1925), the time at which the reproduction-inhibiting antibody becomes evident in the circulation of an infected rat corresponds with the peak of the infection. We concluded, therefore, that a satisfactory amount of metabolic products to use would be, theoretically, that produced by a number of trypanosomes approximately equal to the total population of T. lewisi in the blood at the peak of an infection. In our experience, and as reported by Taliaferro (1924) and Coventry (1925), the concentration of trypanosomes at the peak of an infection with T. lewisi ranges from 200,000 to 600,000 T. lewisi per cmm. of blood. Calculating the total volume of blood in rats the size and weight of those used in our experiments as close to 10 cc., we figured that in a rat that size, the number of trypanosomes stimulating the production of ablastin at the peak of an infection, on the basis of 400,000 per cmm., would be approximately 4,000 million. It was, therefore, decided to inoculate each rat with the 24-hour accumulation of that number of trypanosomes, although we realized that at the time of stimulation of ablastin the blood might contain the metabolic products that had gradually built up over a period

of four days or more. Quite possibly, however, these products might be decomposed or eliminated almost as fast as they were produced. In any case, it did not seem feasible, and it was possibly unnecessary, to attempt to imitate natural conditions, quantitatively, any closer.

Approximately 24,000 million T. lewisi were incubated for 24 hours at room temperature (about 25-27°C) in a medium consisting of 50% normal rat serum as described previously. After 24 hours the trypanosomes were centrifuged and filtered out of the medium. Again, care was taken not to break the organisms by excess pressure and manipulation. Presumably each cc. of the medium now contained the metabolic products or other secreted or excreted substances of approximately 333,000,000 T. lewisi. These metabolic products and the trypanosomes from which they were collected were used later to immunize rats.

Each of six young rats in Group A (Table VI.) was given the metabolic products of 4,000 million trypanosomes contained in 12 cc. of the medium in which they had accumulated. The total of 12 cc. was divided into six 2 cc. injections given intraperitoneally at three-day intervals.

The six rats in Group B (Table VII.) were inoculated with the triturated bodies of the incubated trypanosomes in order to compare the effects of inoculations of rats with metabolic products and the inoculation of rats with dead trypanosomes. All the T. lewisi that had been incubated to

collect metabolic products were washed twice in sterile saline and preserved in the deep freezer for this purpose. It is interesting to note at this time that these trypanosomes had been kept in the deep freezer for a period of several weeks, yet, when some of them were inoculated into two rats that had never been infected with T. lewisi, a normal infection developed, which indicates that at least some of the organisms, after being frozen for such a long time, were not only alive, but also infective to rats. It was, therefore, necessary to kill the trypanosomes before attempting immunization of rats with their body substances. The flagellates were triturated by a series of quick freezing in a mixture of absolute alcohol and dry ice followed by thawing. This was done ten consecutive times. To be sure that the organisms had been killed, fresh previously uninfected rats were inoculated with them. Since no infection developed in these rats it was evident that we had succeeded in killing the trypanosomes. The six rats in this group (B) were each given a total of 12 cc. of a saline suspension containing the triturated trypanosome bodies from which the metabolic products given the rats in Group A had been obtained (4,000 million). As with the metabolic products, the 12 cc. was divided into six 2 cc. injections given intraperitoneally at three-day intervals.

The six rats in Group C (Table VIII.) were controls which were similarly inoculated with six 2-cc injections

of 50% normal rat serum. This Group C was treated in this manner in order to check on the possibility, brought out in the discussion of Experiment No. I., that the rats might be protected by the inoculation of serum that might have been antigenically altered as a result of long freezing, and standing at room temperature for 24 hours. The serum used to inject these rats was from the same lot of serum used for the incubation of trypanosomes. It had been kept in the deep freezer for the same length of time, and had been standing for 24 hours just as the incubating medium had been. Each rat received the same amount of this serum as those rats in Group A (i.e. 12 cc. of 50% serum).

The six rats in Group D (Table IX.) were set apart as controls which did not receive any injections. Ten days after the experimental rats had received their last immunizing injection these control rats and the experimental rats of Groups A and B, and also control Group C, received a challenging dose of 50,000 T. lewisi intraperitoneally. Every day after this exposure a drop of blood was taken from the tip of tail of each rat and examined fresh under the microscope. Permanent preparations stained in Giemsa were also taken every day, and counts of the trypanosomes were made in a haemocytometer as described under "Materials and Methods."

The results of these different examinations appear on Tables VI to IX. The rats in Group A, immunized with

metabolic products, apparently were completely protected by the injections. No trypanosomes were ever found in the fresh preparations even though several drops were examined from each rat, and each sample was examined for ten minutes. Examination of the stained preparations likewise failed to show trypanosomes in the blood. On the other hand, in all of the rats in Group B, injected with the suspension of triturated trypanosome bodies, as well as those in the two control Groups, C and D, living trypanosomes were observed in the fresh preparations on the second or third day after exposure to T. lewisi. Trypanosomes were also seen on these days in the stained blood smears of rats from these three groups and sometimes on the first day also.

The trypanosome counts were very revealing. No counts, of course, were possible in rats from Group A in which no evidence of infection ever appeared. No difference was observed in the magnitude of infections in rats of Group C (inoculated with normal rat serum) as compared with those in control Group D (uninoculated). In both these groups the blood trypanosomes reached peaks of from a little over 200,000 to nearly 600,000 per cmm., and the blood remained positive for 18 days or more. Evidently the normal rat serum had no protective effect. Not only were the infections in these rats equally heavy but the duration of the infection was approximately the same.

Group B, inoculated with triturated trypanosomes, was

very interesting. Four of these rats (Nos. 7, 8, 9 and 12) showed living trypanosomes on the third day, and rats 10 and 11 on the second day, after being given the challenge infection. The infection in rats Nos. 7 and 9 was of very short duration; in these two rats trypanosomes were seen only on the third and fourth day after infection, and the trypanosomes were so scarce that they could not be counted in the dilution used by us. In rat No. 10 the infection lasted 11 days, but the trypanosomes never became numerous enough to count by the dilution method. In rat No. 11 the infection lasted 11 days and the peak was only 5500 trypanosomes per cmm. The trypanosomal population in rat No. 12 reached a peak of 7500 trypanosomes per cmm. on the fifth day after infection, but the infection lasted only nine days. Rat No. 8 developed a heavy infection, reaching a peak of 202,500 T. lewisi per cmm. of blood on the sixth day after infection, and the infection lasted 16 days. Some dividing forms of T. lewisi were seen in the stained preparations from rats Nos. 8, 11 and 12. In rat No. 12 dividing forms were seen from the fourth to the seventh day. In rats Nos. 11 and 12 few dividing forms were observed on the third to the seventh day, and third to sixth, respectively.

From the data obtained in this experiment it is evident that the medium in which the trypanosomes were incubated for 24 hours, and from which the trypanosomes themselves had been completely removed, contained secretions or excretions

(metabolic products of some kind), which when injected into rats in a series of inoculations, gives them complete protection against subsequent challenge infections with 50,000 trypanosomes inoculated intraperitoneally.

It is apparent that the body substances of the trypanosomes also provide the rats with some degree of protection, but this is not nearly as complete as in the case of the metabolic products. The protection given by body substances in the rats is manifested by the much shorter and lighter character of the infections in the immunized rats. Although the trypanosomes were washed carefully before being prepared for injection, it is quite possible that the protection afforded is actually due to the same substances as were present in the filtered medium, present in relatively small amount inside the trypanosomes and not yet excreted.

Six additional rats were given six injections with the metabolic products of a total of 2,000 million trypanosomes and, with three controls, were inoculated with 20 million trypanosomes ten days after the last infection. In the control rats, the blood became positive within one hour, and the infections ran a normal course, but in the injected rats again there was complete protection, since no trypanosomes were ever seen in the blood smears.

TABLE VI

Results of challenge infections with 50,000 trypanosomes in Group A, rats injected with metabolic products of approximately 4,000 million T. lewisi, 10 days after the last injection.

Rat No. No.	Total Amt. of Inj.	Inter-vals between Inj.	Examination of blood smears; Trypanosomal counts in Tryps./cmm.												
			1	2	3	4	5	6	7	8	9	10	11	12th - 22nd day	
1	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day
2	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day
3	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day
4	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day
5	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day
6	6	1200	3 days	0	0	0	0	0	0	0	0	0	0	0	every day

Notes:

0 - No trypanosomes seen in either fresh blood smears or in stained slides.

TABLE VII

Results of challenge infections with 50,000 trypanosomes in Group B, rats injected with the body substance of approximately 4,000 million triturated T. lewisi, 10 days after last injection

Treatment				Examination of blood smears; Trypanosome counts in Tryps./cmm											
Rat No.	No. of Inj.	Tot. Amt. of Inj.	Inter-val between Inj.	1	2	3	4	5	6	7	8	9	10	11	12th - 22nd day
7	6	12cc	3 days	0	0	F	F	0	0	0	0	0	0	0	0 every day
8	6	12cc	3 days	S	0	F	12, 500	50, 000	202, 000	46, 000	33, 250	2, 500	1, 000	3, 500	+ to 16th day
9	6	12cc	3 days	0	S	F	F	0	0	0	0	0	0	0	0 every day
10	6	12cc	3 days	S	F	F	F	F	F	F	F	F	F	F	0 every day
11	6	12cc	3 days	S	F	1, 500	2, 500	5, 000	1, 000	5, 500	F	F	F	F	0 every day
12	6	12cc	3 days	S	S	1, 000	3, 000	7, 500	5, 000	1, 500	F	F	0	0	0 every day

Notes:

- F too few to count in dilution used, but seen in fresh blood and in stained slides
 + trypanosomes seen in fresh drop of blood - not counted because population in control rats was already decreasing
 0 no trypanosomes seen in fresh blood examinations or in stained slides
 S trypanosomes seen in stained smears but not in fresh preparation

TABLE VIII

Results of challenge infections with 50,000 trypanosomes in Group C, rats injected with 50% normal rat serum, 10 days after last injection of serum

Treatment				Examination of blood smears; Trypanosome counts in Tryps./cmm											
Rat No.	No. of Inj.	Tot. Amt. of Inj.	Inter-val between Inj.	1	2	3	4	5	6	7	8	9	10	11	12th - 22nd day
13	6	1200	3 days	S	S	F	5, 000	54, 000	252, 500	212, 500	285, 000	325, 000	270, 000	295, 000	+ every day
14	6	1200	3 days	S	S	F	10, 000	165, 000	220, 000	165, 000	270, 000	202, 500	157, 500	220, 000	+ to 19th day
15	6	1200	3 days	S	S	F	3, 000	55, 000	110, 500	255, 000	227, 000	195, 000	177, 000	150, 000	+ every day
16	6	1200	3 days	S	F	10, 000	50, 000	87, 500	150, 000	433, 500	300, 000	250, 000	265, 000	150, 000	+ to 21st day
17	6	1200	3 days	S	F	3, 500	60, 000	300, 000	400, 500	577, 500	died				
18	6	1200	3 days	S	F	2, 000	25, 000	132, 000	136, 500	147, 000	350, 000	300, 000	200, 000	135, 000	+ to 18th day

Notes:

Symbols and abbreviations same as in Table VII

TABLE IX

Results of challenge infections with 50,000 trypanosomes in Group D, normal, untreated control rats infected at same time as rats in Groups A, B and C

Rat No.	Examination of blood smears; Trypanosome counts in Tryps./cmm											
	Number days after infection											
	1	2	3	4	5	6	7	8	9	10	11	12th - 22nd day
19	S	S	F	6, 500	25, 000	125, 000	407, 500	580, 000	320, 000	377, 000	182, 000	+ every day
20	S	S	F	10, 000	122, 000	150, 000	317, 500	520, 000	280, 000	215, 000	142, 000	+ every day
21	S	S	F	18, 000	107, 500	200, 500	435, 500	362, 000	265, 000	167, 500	167, 500	+ to 21st day
22	S	F	3, 500	50, 000	117, 000	207, 500	137, 500	136, 000	150, 000	100, 500	72, 500	+ to 19th day
23	S	F	*	25, 000	165, 000	265, 000	210, 000	200, 000	200, 000	136, 500	112, 500	+ to 19th day

Notes:

Symbols and abbreviations same as in Table VII
* not counted (broken pipette)

TABLE X

Infection of rats (100 g. wt.) intracardially or intraperitoneally with different dosages of T. levisi

Rat No.	Inf. Dose	Method of Infection	Examination of tail blood - Hours after infection															
			1	2	3	4	5	6	7	8	9	10	11	12	24	48	48+	
1	20 mill.	1.o.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
2	"	1.o.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
3	"	1.p.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
4	10 mill.	1.o.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
5	"	1.o.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
6	"	1.p.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
7	200,000	1.o.	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	
8	"	1.o.	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	
9	"	1.p.	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	
10	"	1.p.	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	
11	100,000	1.o.	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	
12	"	1.o.	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	

Notes:

i.o. intracardially
 i.p. intraperitoneally
 + trypanosomes seen in fresh drop of blood
 0 no trypanosomes seen in fresh drop of blood

TABLE XI

Results of challenge infections with 20,000,000 trypanosomes in rats injected with metabolic products (M.P.) or triturated bodies (T.B.) of 1,000 million trypanosomes, and in control rats (C), 10 days after last injection.

Antigen	TREATMENT		Total Vol. of Inj.	Examination of tail blood						Re-marks
	No. of Inj.	Interval		1 hr	2-12 hrs	24 hrs	48 hrs	72 hrs		
1	M.P.	3	3 days	6cc	0	0	0	0	0	No inf.
2	M.P.	3	3 days	6cc	0	0	0	0	0	"
3	M.P.	3	3 days	6cc	0	0	0	0	0	"
4	M.P.	3	3 days	6cc	0	0	0	0	0	"
5	T.B.	3	3 days	6cc	+	+	+	+	+	Norm. inf.
6	T.B.	3	3 days	6cc	+	+	+	+	+	"
7	C	0	0	0	+	+	+	+	+	"
8	C	0	0	0	+	+	+	+	+	"

Notes:

- 0 no trypanosomes seen in either fresh or stained tail-blood smears
- + trypanosomes seen in fresh and stained tail-blood smears

VII. EXPERIMENT NO. III

Inoculation of rats with metabolic products and triturated trypanosomes collected at the end of 24 hours of incubation in a mixture of saline and rat serum; smaller dosage, fewer injections, and larger challenge than in Experiment II

Since the dosage of metabolic products used for injections in Experiment No. II gave such complete protection, we were interested in finding out whether protection would be afforded by smaller doses. In Experiment No. III a smaller concentration of the metabolic-product antigen was used and fewer injections given, after which the protective effect was determined as in Experiment No. II.

Trypanosomes from three-day-old infections were incubated for 24 hours in 50% rat serum. After this incubation period the trypanosomes and their metabolic products were separated by the usual method. Each of four rats (Nos. 1-4 in Table XI) were injected with the metabolic products of a total of 1,000 million trypanosomes, divided into three 2 cc. injections given at three-day intervals. This final total dose was approximately one-fourth of that given to the rats in Experiment No. II, and the number of injections was reduced to a half. Rats Nos. 5 and 6 were given three 2 cc. injections of a saline suspension containing the triturated bodies of a number of trypanosomes equivalent to that from which the metabolic products used for each of the rats Nos. 1 to 4 had been collected. Rats Nos. 7 and 8,

uninjected, were set aside as controls.

Since the protective effect of the metabolic products as demonstrated in Experiments No. I and II is so complete, we thought it desirable to determine, if possible, what happened to the trypanosomes that were inoculated into the protected rats. For this purpose we planned to inoculate the rats used in Experiment III, both experimentals and controls, with a massive dose of trypanosomes such as would appear rapidly in the blood of previously uninfected rats. It was our purpose to examine the rats as soon as possible after infection to see if trypanosomes were demonstrable in the blood at any time. Augustine (1943) had succeeded in re-infecting rats 27 to 68 days after they had recovered from a first infection with T. lewisi by inoculating them with large numbers of adult trypanosomes.

We ran some preliminary experiments in order to determine which was the best method of infection and what was the most desirable dosage for this purpose. Two normal rats were inoculated with 20 million adult T. lewisi directly into the heart, and one normal rat was inoculated with 20 million T. lewisi intraperitoneally. One hour later drops of blood from the tails of all three rats showed trypanosomes already in the circulation. These rats were examined daily for a week and it was observed that they developed a normal infection. Once the trypanosomes had appeared in the blood they continued to divide.

Two new rats were injected with 10 million T. lewisi directly into the heart and one rat was inoculated with the same amount of trypanosomes intraperitoneally. Again trypanosomes were demonstrable in the three rats as early as one hour after exposure and the infection developed normally. In another group of rats the infective dose was reduced drastically; two rats were inoculated with 200,000 trypanosomes and two other with 100,000 directly into their hearts, and two rats were inoculated with 200,000 intraperitoneally. Examination of these rats showed that the trypanosomes when inoculated into the rats in such dosage were not demonstrable in the circulation before 12 hours. At the end of 24 hours, however, all the rats showed trypanosomes in their tail blood. The results of these preliminary experiments are shown in Table X.

It was decided, therefore, that an infection of rats with 20 million T. lewisi inoculated intraperitoneally would be adequate. Since the trypanosomes made their appearance in the blood at the end of one hour, no matter which of the two methods of inoculation was used, we did not want to risk the death of the rats by attempting to inoculate them into the heart, so all challenge infections were given intraperitoneally. Augustine (1943) had previously demonstrated that adult trypanosomes inoculated into the peritoneum of immune rats were not harmed at all and were filtered safely through the lymphatic system.

Ten days after the last immunizing injection of the rats in Experiment III, all experimental rats (Nos. 1-6) and the two control rats (Nos. 7 and 8) were inoculated intraperitoneally with 20 million T. lewisi obtained from rats infected a week previously. Fresh and stained preparations of tail blood were taken from each rat hourly for a period of at least six hours. The results are shown in Table XI. Rats Nos. 1 through 4, injected with metabolic products, never showed trypanosomes in the blood. We especially looked for evidence of agglutinated masses of trypanosomes or recognizable fragments of the parasites in the circulation of these rats, but at no time were these observed. It should be mentioned that our infective dose was well under than used by Augustine (1943). He used 400 million trypanosomes while we used only 20 million. This could explain the difference. On the other hand, the rats inoculated with triturated trypanosomes (Nos. 5 and 6), as well as the control rats, showed trypanosomes in the circulation as early as one hour after infection, and the infection developed normally in all four of these rats. Since the rats immunized with metabolic products did not show trypanosomes in the blood at any time, we did not consider it necessary to make daily counts of the other rats as had been done during Experiment No. II.

These results provide still further and stronger evidence for the protective effect of the metabolic products

of T. lewisi against infection with that parasite.

The two rats inoculated with triturated trypanosomes apparently developed normal infections. It is possible that the antibodies produced by the body substance of the dead trypanosomes, shown in Experiment II to be relatively small in amount, were all absorbed by the large number of inoculated living trypanosomes.

VIII. EXPERIMENT NO. IV

Agglutination Tests

Sera for these tests were obtained from the rats used in Experiment No. II. Rats of Group A, immunized with metabolic products, and rats from Group B, immunized with triturated trypanosomes, were bled from the heart eight days after they had received the last immunizing injection, and before they were challenged with an infective dose of T. lewisi. Approximately 1 to 1.5 cc. of blood was taken from each rat. Rats of Group A were again bled about 6½ months after the last injection with metabolic products; this serum will be designated Serum A'. The uninfected rats of control Group D in Experiment II were bled in a similar manner. Since rats of control Group C, injected with 50% normal rat serum, behaved in the same way as rats of Group D, it was not considered necessary to use serum from this group.

The blood from the rats in each group was pooled and allowed to clot overnight; next morning the sera thus obtained were collected by centrifugation and stored in the deep freezer until used. To compare the effects of these sera with serum obtained from rats which had recovered from an infection with T. lewisi, serum from rats infected six weeks previously, after no trypanosomes were any longer present in the blood, was collected and pooled in a similar manner, this constituting Serum E. These various sera (ex-

cept A') were stored in the deep freezer for a period of approximately one month before they were used for the agglutination tests.

All tests were done using welled slides. After the trypanosomes were mixed with the desired dilution of the serum, the slide was covered with a coverslip and sealed with vaseline to prevent desiccation. Observations were made under both the low and high powers of the microscope, and at intervals of from five minutes to an hour, for six hours. In some instances observations were made for 24 hours or more.

For Test No. 1, 1:3 dilutions of sera A, B and D were used; These were prepared as follows: One 0.05 cc. drop of each of the undiluted sera was placed in the wells of the slides; to each of these 0.05 cc. of saline was added. The sera and saline were then mixed carefully and a 0.05 cc. drop of saline suspension of washed trypanosomes from a seven-day-old infection, containing approximately 500,000 T. lewisi, was added to give the final dilution of 1:3 of serum. Observations were started five minutes after the trypanosomes were added.

As seen in Table XII, agglutination of the parasites was observed for the first time in serum A (from rats immunized with metabolic products) after ten minutes of contact with the serum. In this serum the agglutination was strong; in about 45 minutes practically all the parasites

TABLE XII

Agglutination of adult *T. lewisi* in serum from rats injected with metabolic products (Serum A), in serum from rats injected with triturated trypanosomes (Serum B), and in normal rat serum (Serum D).

TIME	Serum A	Serum B	Serum D
	Dilution 1:3	Dilution 1:3	Dilution 1:3
10:00 A.M.	Start	Start	Start
10:05	++	0	0
10:15	+++	++	0
10:30	++++	++	0
10:45	++++	+ *	0
11:00	++++	(+) *	0
11:15	++++	0	0
11:30	++++	0	0
12:30 P.M.	++++	0	0
1:30	++++	0	0
2:30	++++	0	0
3:30	++++	0	0
4:30	++++	0	0
9:30 A.M.	most dead	most dead	most dead
4:00 P.M.	most dead	most dead	most dead
9:00 A.M.	few still alive	few still alive	few still alive

Notes:

* clumps breaking up
 +++ nearly all agglutinated
 (heavy)
 ++ many agglutinated (good)
 ++ some agglutinated (light)

+ few agglutinated (very
 light)
 (+) very, very few aggluti-
 nated (almost no aggluti-
 nation)
 0 no agglutination

were agglutinated, forming complete rosettes which would gradually clump together to form very large agglutinated masses. In serum B (from rats immunized with triturated trypanosomes) there was very slight agglutination at 10 minutes and at 15 minutes, but by 45 minutes the parasites in the few small groups were dispersing, and after one hour were unagglutinated and normal in appearance and motility. In contrast, trypanosomes in normal serum D remained active and unagglutinated throughout. In all cases agglutination was accompanied by a marked loss of swimming ability, but the motility continued. The parasites in the agglutinating sera tended to adhere to each other by their posterior ends until several would combine to form rosettes. Occasionally in serum A, but very frequently in serum B, two parasites would come in contact for fractions of a second but would separate again. The detailed results of these agglutination reactions appear in Table XII.

In Test No. 2 different concentrations of serum A ("metabolic products" serum) were tested and compared with equal concentrations of normal rat serum D. All dilutions of sera were prepared in small precipitin tubes. Dilutions were made with 0.85% saline. Three drops of each dilution were placed in the wells of the slides, and to these a drop containing about 500,000 T. lewisi was added. The results are shown in Table XIII. In dilutions of serum A of 1:1 to 1:5, agglutinations of the parasites occurred with-

TABLE XIII

Agglutination of adult T. lewisi in different dilutions of serum A (from rats injected with metabolic products).

TIME	Dilutions of serum *				
	1:1	1:5	1:25	1:125	1:625
4:00 P.M.	Start	Start	Start	Start	Start
4:05	+++	++	0	0	0
4:10	++++	++++	0	0	0
4:15	++++	++++	+	0	0
4:20	++++	++++	+	+	0
4:30	++++	++++	++	+	0
4:45	++++	++++	(+)	(+)	0
5:00	++++	++++	+	(+)	0
6:00	++++	++++	+++	(+)	0
7:00	++++	++++	+++	(+)	0
8:00	++++	++++	+++	(+) some dead many dead	
9:00	++++	++++	+++	almost all dead	all dead
8:00 A.M.				all dead	all dead
11:00	massed; still alive	massed; still alive	all dead	-	-
5:00 P.M.	some alive	some alive	-	-	-
3:00 P.M.	some alive	some alive	-	-	-
9:30 A.M.	all dead	all dead	-	-	-

Notes:

- * Trypanosomes in same dilutions of normal serum did not agglutinate.
Symbols same as in Table XII

in five minutes. In a dilution of 1:25 it occurred in about 15 minutes, and in the 1:125 dilution it occurred in 20 minutes. No agglutination occurred in a dilution of 1:625. In dilutions 1:1 and 1:5 the agglutination was heavy; at 1:25 it was light; and at 1:125, very light. Agglutination did not occur at all in any of the same dilutions of normal serum D. These series of dilutions were examined at intervals for a period of 48 hours, at the end of which time the trypanosomes in the 1:1 dilutions of the two sera were still alive. The parasites in all other dilutions of the sera, however, had gradually died; those in the more highly diluted sera died sooner than those in the more concentrated sera. We think that this is significant, since it demonstrates that, at least in vitro, the antibody does not kill the parasites.

In Test No. 3, agglutination of trypanosomes was tested at various dilutions of serum D (normal rat serum), serum A ("metabolic products" serum collected 8 days after last injection of metabolic products), serum A' ("metabolic products" serum collected 6½ months after last injection), and serum E, from recovered rats (collected 6 weeks after infection). The dilutions used for each serum were: 1:1, 1:10, 1:100, 1:200, 1:400, and 1:800. Observations were made at intervals for from 4 to 5 hours as shown in Tables XIV to XVI.

The parasites in normal serum D did not agglutinate,

and remained active for the full time in dilutions up to 1:200. The results with serum A are shown in Table XIV. Most of the trypanosomes in the 1:1 and 1:10 dilutions of serum A were agglutinated in ten minutes. In dilutions of 1:100 and 1:200, agglutination occurred somewhat later, and no agglutination was observed in higher dilutions. Agglutination was heavy in dilutions 1:1, 1:10, and 1:100, but light in the 1:200 dilution. The results with serum E (from recovered rats) are shown in Table XV. In dilutions of 1:1 and 1:10, this serum caused agglutination to occur within 10 minutes. In dilutions 1:100, 1:200, and 1:400 of the same serum, agglutinated parasites were observed after about one hour. Agglutination was heavy in dilutions 1:1 and 1:10; good in dilutions 1:100; and very light in dilutions 1:400 (See Table XV). It appears, therefore, that the immune serum from rats that had recovered from an infection had approximately the same agglutinating power as the serum from rats immunized with metabolic products; if anything, it was very slightly less. At the end of five hours, the trypanosomes were still alive in dilutions 1:1 to 1:200 of the three sera. In the 1:200 dilutions, the parasites appeared somewhat sluggish in movement, and in larger dilutions all parasites were dead.

The results with serum A' are shown in Table XVI. This serum, as noted above, was obtained from the rats of Group A in Experiment II, collected 6½ months after the

TABLE XIV

Agglutination of adult T. lewisi in serum A (from rats immunized with metabolic products).

TIME	Dilutions of serum *					
	1:1	1:10	1:100	1:200	1:400	1:800
4:40	Start	Start	Start	Start	Start	Start
4:50	++	++	0	0	0	0
5:00	++++	+++	++	0 few close together	0	0
6:00	++++	++++	++++	(+)	0	0
7:00	++++	++++	++++	+	0	0
9:00	++++	++++	++++	+	most dead	all dead

Notes:

- * Trypanosomes in same dilutions of normal rat serum did not agglutinate.
Symbols same as in Table XII

TABLE XV

Agglutination of adult T. lewisi in serum E collected from rats 6 weeks after infection.

TIME	Dilutions of serum *					
	1:1	1:10	1:100	1:200	1:400	1:800
3:50	Start +	Start 0	Start 0	Start 0	Start 0 (slow)	Start 0 (slow)
4:00	+++	++	0	0	0	0 most non- motile
5:00	++++	++++	+++	+	(+)	0 some dead
6:00	++++	++++	+++	+ most slow	(+) most slow	many dead
8:30	++++	++++	+++	very slow +	most dead	most dead

Notes:

- * No agglutination in same dilutions of normal rat serum
Symbols same as in Table XII

last injection of the rats with metabolic products. In this serum there was a very marked loss of titer as compared with serum A (Table XIV), although the 1:1 dilution was still fully potent. In dilutions of 1:10 and 1:100, agglutination was much delayed and also much lighter than in the tests with the A serum.

It is evident that the metabolic products of T. lewisii stimulate the production of an antibody which agglutinates that parasite in vitro. It is significant that the results with immune serum from rats immunized with metabolic products are practically identical in this respect with the results with immune serum from rats that have recovered from an infection. As noted in the "Introduction," agglutination is a process that has been observed in connection with T. lewisii by a number of workers, some of whom, e.g. Augustine (1943) and Barnes (1951), have called attention to its importance in connection with elimination of T. lewisii from rats, but hitherto no authors have associated this phenomenon with ablastin.

IX. EXPERIMENT NO. V

Protective effects of passively transferred immune serum from recovered rats before and after absorption with metabolic products and with triturated trypanosomes

Approximately 5,000 million T. lewisi were incubated by the usual method for collection of their metabolic products. After the trypanosomes were removed from the incubating medium, the whole medium containing the suspended metabolic products of the parasites was lyophilized by the procedure described by Flosdorf and Mudd (1935) for lyophilizing serum. The trypanosomes that were incubated, after repeated washings, were likewise lyophilized. Lyophilization is known to be the best way to preserve proteins in the native state for long periods of time (Fruton and Simmonds, 1953). These two "lyophiles" were later used to absorb immune serum.

Immune serum was obtained from six rats, by the usual method of direct bleeding from the heart, three weeks after they had recovered from an infection with T. lewisi, i.e., had completely eliminated the trypanosomes. This immune serum was pooled and stored for approximately one month in the deep freezer before it was used in these tests. A 5 cc. portion of the immune serum was absorbed with the lyophilized metabolic products of the 5,000 million trypanosomes mentioned above. An additional 5 cc. portion of the serum was absorbed with the lyophilized

bodies of the same trypanosomes. This ratio of metabolic products and trypanosomes of 1,000 million per cc. of serum was selected because it had been suggested by Taliaferro (1932) that enormous numbers of trypanosomes were of utmost importance; he observed that at least 1,000,000 parasites were needed to absorb one cmm. of serum completely. For absorption, the antigens were kept in contact with the immune serum for a 12-hour period in the refrigerator. After this time the sera were centrifuged vigorously to separate the precipitated combination of antigens and antibodies presumably formed, and later they were filtered through a micro Seitz filter to eliminate any very fine precipitate and possible contaminating bacteria.

The protective effect of the two absorbed immune sera, as compared with that of the unabsorbed serum, was tested by inoculating rats intraperitoneally with approximately 1 cc. of the serum to be tested per 100 g. of body weight, one hour before challenging the rats with a dose of approximately 20,000,000 T. levisi. This dosage of immune serum was considered adequate, since Rabinowitsch and Kempner (1899) found that 1 cc. of homologous immune serum could protect rats against infections with T. levisi. Three rats (Nos. 1, 2 and 3) weighing 105, 110 and 115 g. respectively, were each injected with 1.2 cc. of the immune serum absorbed with triturated trypanosomes. Three rats (Nos. 4, 5 and 6) were injected with the serum absorbed

with metabolic products; two of them, weighing 85 and 105 g. respectively, were given 1 cc., whereas the other (No. 5) weighing 115 g., was given 1.2 cc. Two rats (Nos. 7 and 8) were each injected with 1 cc. of the unabsorbed immune serum. Rats Nos. 9 through 12 were not treated at all before injection with 20,000,000 T. lewisi. After infection all rats were examined daily, or every other day, for presence of trypanosomes and, when present, counts were made by the usual haemocytometer method. Fresh and Giemsa-stained blood smears were prepared daily.

Examination of the data presented in Table XVII shows that in rats Nos. 1, 2 and 3, which were protected with immune serum absorbed with triturated trypanosomes, the parasites appeared in the blood one or two days after infection. However, the peaks of the infections in these rats were very low, in fact even lower than in the control rats given unabsorbed serum. Although some multiplication had obviously occurred, this must have been considerably inhibited, since the total number of trypanosomes was so low, and also because no dividing forms of the trypanosomes were observed in any of the stained preparations. There was, therefore, no indication that absorption of immune serum with the triturated trypanosomes reduced the protective power of immune serum at all.

The results obtained in the rats protected with immune serum absorbed with metabolic products were strikingly

different. Two of these rats (Nos. 4 and 6) developed good infections (see Table XVIII). The infection in rat No. 4 reached a peak of 390,000 trypanosomes per cmm., which is within the range of a normal uninfluenced infection. The peak in rat No. 6 was not as high, only 62,000 trypanosomes per cmm., but it was still many times higher than in any of the three rats inoculated with serum absorbed with triturated trypanosomes. Actual dividing forms of the parasite were demonstrable in these two rats on the second to the ninth day, and second to the sixth days after infections, respectively. Also in these two rats, a greater variation was observed in the size of the trypanosomes than in rats Nos. 1, 2 and 3; while there were many non-reproducing adult forms measuring 28μ to 32μ in length and 2.5μ in width, a large number of the total population was composed of small forms that were broader and shorter than the adults. In rat No. 5 the infection reached a peak of only 5,000 trypanosomes per cmm., but dividing forms were seen on the second to the fifth days after infection, and also a greater variation was observed in the sizes of the parasites during these same days than in the case of rats injected with serum absorbed with triturated trypanosomes. (See Table XVIII).

In rats Nos. 7 and 8, which were protected with unabsorbed immune serum (see Table XIX), one of the rats (No. 7) failed to respond to the serum injection in a

normal manner. In this rat the infection reached a peak of 172,000 T. lewisi per cmm., which is unexpectedly high, although below what occurs in uninfluenced infections. Dividing forms were numerous, and also there was a great variation in size among the population. The results with rat No. 8, however, were as expected. The peak of the infection (4,000 T. lewisi per cmm.) occurred on the second day after the infection; only one actual dividing form was ever seen, that on the second day after infection, and the variation in the size of the parasite was not as great as in normal rats or rats inoculated with serum absorbed with metabolic products.

Of the four untreated control rats (see Table XX), one died while being anesthetized and the other three developed normal infections. In these rats dividing forms were present, and the variation in the population was as expected. Rat No. 10 developed a very high infection with a peak of 900,000 trypanosomes per cmm. on the eighth day after infection. This rat, apparently, never was able to arrest the reproduction of the parasites, and died on the ninth day after infection.

Although the evidence is based on only a few animals, of which two did not react as expected, the data presented here indicate that absorption with lyophilized metabolic products markedly reduces the protective power of the immune serum. In contrast, immune serum absorbed

with trypanosome body substance lost comparatively little of its protective power and, as when immune serum is used, dividing forms were rarely if ever observed.

An additional group of rats passively protected with absorbed and unabsorbed immune serum added still further evidence of the antigenic properties of the metabolic products of T. lewisi, and their ability to absorb the protective antibodies from immune serum. The immune serum for this experiment was obtained from three rats approximately two months after they had recovered from an infection with T. lewisi; 5 cc. of this serum were absorbed with approximately 3,000 million lyophilized T. lewisi (600,000 per cmm. as compared with 1,000,000 in the experiments described above) by the procedure previously described; an additional 5 cc. of the serum were absorbed with the lyophilized metabolic products of an equal number of trypanosomes. Since the number of trypanosomes and amount of metabolic products was smaller than that previously used, we expected that the absorption of antibodies might not be complete in this experiment.

Rats Nos. 13 and 14, weighing 159 and 127 g. respectively, were inoculated intraperitoneally with 2 cc. of serum absorbed with triturated trypanosomes. Rats Nos. 15 and 16 (123 and 126 g. respectively) were likewise inoculated with 2 cc. of serum absorbed with metabolic products. Rats Nos. 17 and 18, weighing 123 and 162 g. respectively,

were inoculated with 2 cc. of unabsorbed immune serum. One hour after the injection with the serum the rats were challenged with the intraperitoneal injection of 6,500,000 washed, adult T. lewisi suspended in 1 cc. of saline. An untreated control rat (No. 19), 132 g. in weight, was simultaneously injected with an equal number of trypanosomes.

After infection, all rats were examined daily and blood smears and parasite counts were made as before. Rats Nos. 13 and 14 (protected with immune serum absorbed with triturated trypanosomes) unfortunately both died on the fifth day after infection, apparently from a bacterial infection. The trypanosomes had reached a peak of 81,000 per cmm. in rat No. 13, and 87,000 per cmm. on the fifth day, before they died (see Table XXI). Examination of Giemsa-stained smears showed that in these rats, although division of the parasite had occurred, the percentage of dividing and small forms, generally, was lower than in the control rat and than in the rats protected with serum absorbed with metabolic products, indicating incomplete absorption of protective antibodies from the serum.

As shown in Table XXII, in rats Nos. 15 and 16, protected with immune serum absorbed by metabolic products, the population reached a peak of 114,000 and 115,000 trypanosomes per cmm., respectively, on the fifth day after infection. In rat No. 15 the highest percentage of divid-

ing and small forms found in the Giemsa preparations was 71%, and in rat No. 16, the highest percentage was 72%.

In contrast, in rats Nos. 17 and 18 (injected with unabsorbed serum) the peaks were only 64,000 and 68,000 trypanosomes, respectively. (See Table XXIII.) The percentage of dividing and small forms was lower in these rats than in rats injected with serum absorbed with metabolic products and than in the control rat No. 19. In the control rat, the population reached a peak of 200,000 T. lewisi per cmm. on the third day, and the percentage of dividing and small forms was larger than in any of the other rats.

The data from this last experiment indicate that the serum absorbed with metabolic products lost much of its protective effect, whereas serum absorbed with the triturated trypanosomes lost little of its protective action. The latter can not be considered conclusive since the rats died after counts were made on the fifth day. However, in all the other rats in this experiment, the peaks were reached on or before the fifth day. The antibody titer of the immune serum used in this experiment apparently was not as high as in the previous experiment. This was probably due to its having been obtained two months instead of three weeks after the termination of the infections in the donor rats.

Comparison of these results with absorption tests

done by Taliaferro (1932) will be made in the section on
"Discussion and Conclusions."

Explanation of symbols, abbreviations, etc., in Tables XVII to XXIV.

Wt. in Gms. -- Weight in grams.

D -- Days after challenge infection.

Bl. ex. -- Examination of tail blood for living trypanosomes

0 -- No trypanosomes

+ -- Trypanosomes present by direct examination of a drop of blood from the tail.

F -- Too few trypanosomes present to count with haemocytometer dilution used.

Ad. -- Non-reproducing trypanosomes, 28-32 "mu" in length, 2.5 "mu" broad.

Sm. -- Small, i.e., broader and shorter than adult trypanosomes.

Div. -- In process of division.

Tot. -- Total number of trypanosomes counted in Giemsa-stained smear.

In counts in Giemsa-stained slides, numbers in each category are based on examination of 100 trypanosomes and therefore represent percentages, except when there were less than 100 parasites on a slide, in which case the numbers represent complete counts on one slide.

TABLE XVI

Agglutination of adult T. lewisi in serum A' (from rats injected with metabolic products approximately 6½ months previously).

TIME	Dilutions of serum *					
	1:1	1:10	1:100	1:200	1:400	1:800
11:50	Start +++	Start 0	Start 0	Start 0	Start 0	Start 0
12:00	++++	+	(+)	0	0 (slow)	0 (slow)
1:00	++++	+	(+)	0 (slow)	0 (slow)	0 (slow)
2:00	++++	+	(+) (slow)	0 (slow)	0 (slow)	0 (slow)
3:00	++++	+	(+)	0 (slow)	0 (slow)	0 (slow)
4:00	++++	+	(+) non- motile	most dead	most dead	most dead

Notes:

- * No agglutination occurred in same dilutions of normal serum.
- ++++ most agglutinated (heavy)
- +++ many agglutinated (good)
- ++ some agglutinated (light)
- + few agglutinated (very light)
- (+) very, very few agglutinated (almost no agglutination)
- 0 no agglutination
- 500,000 T. lewisi used

TABLE XVII

Infections with T. lewisi in rats protected with immune serum absorbed with lyophilized trypanosomes (1,000,000 per cmm.)
Challenge infection, 20,000,000 T. lewisi.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/cmm.	Ad.	Giemsa Sm.	Stain Div.	Tot.
1	105	1.2cc	0	0	-	5	0	0	5
			1	+	F	4	0	0	4
			2	+	500	27	9	0	36
			4	+	500	18	0	0	18
			5	+	1000	40	0	0	40
			7	+	500	6	0	0	6
			9	+	F	2	0	0	2
			11	0	0	0	0	0	0
			13	0	0	0	0	0	0
			15	0	0	0	0	0	0
2	110	1.2cc	0	+	F	0	0	0	0
			1	+	1000	5	0	0	5
			2	+	F	37	1	0	38
			4	+	F	3	0	0	3
			5	+	F	8	0	0	8
			7	+	F	2	0	0	2
			9	+	F	1	0	0	1
			11	0	0	0	0	0	0
			13	0	0	0	0	0	0
			15	0	0	0	0	0	0
3	115	1.2cc	0	+	-	0	0	0	0
			1	+	F	26	0	0	26
			2	+	2000	45	10	0	55
			4	+	2000	-	-	-	-
			5	+	2000	45	7	1	53
			7	+	F	53	0	0	53
			9	+	500	5	0	0	5
			11	+	F	7	0	0	7
			13	0	0	0	0	0	0
			15	0	0	0	0	0	0

See explanation of symbols and figures on previous page.

TABLE XVIII

Infections with T. lewisi in rats protected with immune serum absorbed with lyophilized metabolic products (from 1,000,000 trypanosomes per cmm.). Challenge infection, 20,000,000 T. lewisi.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/cmm.	Ad.	Giemsa Sm.	Stain Div.	Tot.
4	85	1cc	0	+	-	0	0	0	0
			1	+	1,000	5	0	0	5
			2	+	2,000	41	18	1	60
			4	+	230,000	50	19	31	100
			5	+	390,000	55	27	18	100
			7	+	85,000	63	16	21	100
			9	+	175,000	99	0	1	100
			11	+	78,000	100	0	0	100
			13	+	37,000	100	0	0	100
			15	+	13,000	100	0	0	100
5	115	1.2cc	0	+	-	0	0	0	0
			1	+	2,000	14	0	0	14
			2	+	500	29	22	1	52
			4	+	5,000	61	24	15	100
			5	+	4,500	83	11	6	100
			7	+	1,500	50	0	0	50
			9	+	1,500	41	0	0	41
			11	+	F	4	0	0	4
			13	+	F	0	0	0	0
			15	0	0	0	0	0	0
6	105	1cc	0	0	-	0	0	0	0
			1	+	F	7	0	0	7
			2	+	1,000	16	27	2	45
			4	+	16,000	48	36	16	100
			5	+	62,000	62	22	16	100
			7	+	40,000	93	2	5	100
			9	+	9,500	100	0	0	100
			11	+	7,000	100	0	0	100
			13	+	4,000	100	0	0	100
			15	+	3,000	100	0	0	100

See explanation of symbols and figures on page preceding Table XVII.

TABLE XIX

Infections with T. lewisi in rats protected with unabsorbed immune serum. Challenge infection, 20,000,000 T. lewisi.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/cmm.	Giemsa Stain			
						Ad.	Sm.	Divs.	Tot.
7	115	1cc	0	0	-	1	0	0	1
			1	+	6,000	50	0	0	50
			2	+	8,000	60	32	8	100
			4	+	152,000	48	37	15	100
			5	+	172,000	55	12	33	100
			7	+	132,000	100	0	0	100
			9	+	135,000	100	0	0	100
			11	+	72,000	100	0	0	100
			13	+	67,000	100	0	0	100
			15	+	47,000	100	0	0	100
8	100	1cc	0	0	-	0	0	0	0
			1	+	F	30	0	0	30
			2	+	4,000	53	10	0	63
			4	+	1,500	50	2	1	53
			5	+	2,000	50	0	0	50
			7	+	1,000	13	0	0	13
			9	+	F	0	0	0	0
			11	0	0	0	0	0	0
			13	0	0	0	0	0	0
			15	0	0	0	0	0	0

See explanation of symbols and figures on page preceding Table XVII.

TABLE XX

Infections with T. lewisi in untreated control rats. Chal-
lenge infection, 20,000,000 T. lewisi.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/ cmm.	Ad.	Giemsa Stain Sm.	Div.	Tot.
9	90	None	0	+	-	1	0	0	1
			1	+	6,000	144	0	0	144
			2	+	59,000	57	28	15	100
			4	+	145,000	38	37	26	101
			5	+	447,000	54	24	22	100
			7	+	235,000	89	3	8	100
			9	+	242,000	100	0	0	100
			11	+	257,000	100	0	0	100
			13	+	102,000	100	0	0	100
			15	+	72,000	100	0	0	100
10	75	None	0	0	-	0	0	0	0
			1	+	2,000	14	0	0	14
			2	+	5,500	65	28	8	101
			4	+	467,500	34	42	24	100
			5	+	540,000	45	21	34	100
			7	+	990,000	66	20	14	100
			9	died	-	-	-	-	-
11	90	None	0	+	-	1	0	0	1
			1	+	F	59	0	0	59
			2	died	-	-	-	-	-
12	105	None	0	+	-	0	0	0	0
			1	+	3,000	26	0	0	26
			2	+	36,500	76	23	1	100
			4	+	350,000	40	21	39	100
			5	+	255,000	48	21	31	100
			7	+	145,000	92	2	6	100
			9	+	125,000	100	0	0	100
			11	+	85,000	100	0	0	100
			13	+	55,000	100	0	0	100
			15	+	22,500	100	0	0	100

See explanation of symbols and figures on page preceding Table XVII.

TABLE XXI

Infections with T. lewisi in rats protected with immune serum absorbed with lyophilized trypanosomes (600,000 per cmm.).
Challenge infection, 6,500,000 T. lewisi.

Rat. No.	Wt. in Gms	Serum Given	D	Bl. ex.	Tryps/ cmm.	Giemsa Stain			
						Ad.	Sm.	Div.	Div. and Sm. %
13	159	2cc	0	0	-	0	0	0	0
			1	+	-	60	13	27	40
			2	+	3,500	50	15	35	50
			3	+	11,500	54	15	31	46
			5	+	81,000	88	2	10	12
			6	dead	-	-	-	-	-
14	127	2cc	0	0	-	0	0	0	0
			1	+	-	64	18	18	36
			2	+	10,000	55	29	16	45
			3	+	31,000	62	20	18	38
			5	+	87,000	90	0	10	10
			6	dead	-	-	-	-	-

See explanation of symbols and figures on page preceding Table XVII.

TABLE XXII

Infections with T. lewisi in rats protected with immune serum absorbed with lyophilized metabolic products (from 600,000 trypanosomes per cmm. Challenge infection, 6,500,000 T. lewisi).

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/cmm.	Ad.	Giemsa Sm.	Stain Div.	Div. and Sm. %
15	123	2cc	0	0	-	0	0	0	0
			1	+	-	27	8	16	47
			2	+	7,000	52	17	31	48
			3	+	19,000	29	45	26	71
			5	+	114,000	64	4	32	36
			6	+	95,000	95	0	5	5
			7	+	27,500	98	1	1	2
			8	+	28,000	100	0	0	0
			9	+	18,500	100	0	0	0
16	126	2cc	0	0	-	0	0	0	0
			1	+	-	46	20	34	54
			2	+	4,500	10	25	65	90
			3	+	21,000	28	22	50	72
			5	+	115,000	92	1	7	8
			6	+	73,000	100	0	0	0
			7	+	47,000	100	0	0	0
			8	+	25,000	100	0	0	0
			9	+	13,000	100	0	0	0

See explanation of symbols and figures on page preceding Table XVII.

TABLE XXIII

Infections with T. lewisi in rats protected with unabsorbed immune serum. Challenge infection, 6,500,000 T. lewisi.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/cmm.	Giemsa Stain			
						Ad.	Sm.	Div.	Div. and Sm. %
17	123	2cc	0	0	-	0	0	0	0
			1	+	-	59	17	24	41
			2	+	7,000	45	14	41	55
			3	+	64,000	66	14	20	34
			5	+	57,000	96	0	4	4
			6	+	12,000	100	0	0	0
			7	+	10,000	100	0	0	0
			8	+	10,500	100	0	0	0
			9	+	5,000	100	0	0	0
18	162	2cc	0	0	-	0	0	0	0
			1	+	-	63	15	22	37
			2	+	3,500	45	11	44	55
			3	+	16,500	62	12	26	38
			5	+	68,000	97	0	3	3
			6	+	11,500	100	0	0	0
			7	+	11,500	100	0	0	0
			8	+	9,500	100	0	0	0
			9	+	6,500	100	0	0	0

See explanation of symbols and figures on page preceding Table XVII.

TABLE XXIV

Infection with *T. levisi* in a normal untreated control rat.
 Challenge infection, 6,500,000 *T. levisi*.

Rat No.	Wt. in Gms.	Serum Given	D	Bl. ex.	Tryps/ cmm.	Ad.	Giemsa Stain Sm. Div.	Div. and Sm. %
19	132	none	0	0	-	1	0	0
			1	+	4,000	40	9	51
			2	+	45,000	27	12	61
			3	+	200,000	51	8	41
			5	+	190,000	96	0	4
			6	+	32,000	98	0	2
			7	+	6,000	100	0	0
			8	+	3,000	100	0	0
			9	+	1,000	100	0	0

See explanation of symbols and figures on page preceding Table XVII.

X. DISCUSSION AND CONCLUSIONS

As pointed out in the introduction to this thesis, Taliaferro (1924, 1932) believed that there are dual mechanisms of immunity, ablasic and trypanocidal, against Trypanosoma lewisi, and this view has received wide acceptance. Augustine (1943), on the other hand, working on resistance to reinfection rather than development of resistance to primary infections, believed that the disposal of the trypanosomes in reinfections depends solely on a trypanocidal antibody which agglutinates the parasites and renders them susceptible to phagocytosis. He was able to reinfect rats 27 to 68 days after they recovered from primary infections. He observed that when very large numbers of adult or dividing parasites were injected into the peritoneal cavity of these immune rats, they filtered through the lymph nodes as readily as in non-immune, previously uninfected rats, and appeared in the circulation without apparently having been injured. They persisted in the peritoneal cavity for 48 hours, and showed no evidence of destruction. This new infection lasted from a few minutes to five days (usually not more than four), and the trypanosomes were then agglutinated and mechanically removed from the blood by phagocytosis. Whenever the infection lasted for as long as two days, reproduction invariably started, but in spite of this there was no appreciable increase in numbers in the blood. This course of events occurred even when the reinfections were

made 6 to 18 months after termination of the primary infection.

Augustine concluded that the trypanocidal antibody is definitely located in the blood and has no effect upon trypanosomes remaining in the peritoneal cavity, but he did not take into consideration the possibility that the very large numbers of parasites in the peritoneal cavity would quickly neutralize the limited amount of antibody present in it, or entering it from the general circulation. According to our view there would not be enough ablastin available in the body cavity to prevent reproduction from beginning, even though there might be enough in the blood to agglutinate the parasites as they entered it. In later experiments Augustine injected actively multiplying trypanosomes from a four-day-old infection (number not stated) directly into the heart, and found that agglutination occurred almost at once and the parasites were removed from the circulation within 15 minutes. He attributed this to a greater vulnerability of dividing parasites to the "trypanocidal" antibody so that they were susceptible to lower titers, but we think the difference could very well be explained by the intracardial instead of intraperitoneal inoculation, with possibly smaller numbers of trypanosomes.

Augustine suggested that the trypanocidal antibody acts as an opsonin, sensitizing the reproducing trypano-

somes to the action of phagocytic cells, and that it agglutinates the adults which are then mechanically removed from the circulation. Augustine thought it was possible that there were two distinct antibodies, an opsonin and an agglutinin, but preferred to regard the trypanocidal antibody as a single sensitizing agent.

Taliaferro (1948) accepted Augustine's results, but did not believe they could be interpreted as showing there is no such antibody as ablastin. He, like Augustine, obtained static populations containing dividing forms of trypanosomes in passive transfer of immune serum. He believed that such static populations can occur in cases of superinfections because, as shown by Coventry (1925), ablastin decreases in titer after about the 35th day of infection. Taliaferro (1948) also did not accept Augustine's view that a static population of adult trypanosomes is the result of the selective removal of dividing forms of the trypanosomes. If that were the case, Taliaferro stated, the population would not remain stationary but it would decrease.

As noted in the introduction, Barnes (1951) postulated three antibodies to be concerned in the development of resistance in rats against T. lewisi; she suggested that besides ablastin and the trypanocidal antibody, or trypanolysin, there is an agglutinin present which works in conjunction with the trypanolysin to terminate the infection. She observed agglutinated masses of parasites at the time

of the first number crisis, and found that after its first appearance, agglutination continued throughout the period of decline of the infection. Other authors have considered agglutination as a function of the trypanocidal antibody, but our results clearly indicate that it is a function of ablastin.

On the basis of the results of the experiments herein presented, it can be asserted that soluble substances, secreted or excreted by T. lewisi, when inoculated into previously uninfected, normal rats strongly protect these animals against infections with T. lewisi. Our evidence indicates that this protection is due to the production in rats of an antibody against these secretions and/or excretions, which we believe to be identical with ablastin. Furthermore, it can be tentatively suggested that ablastin alone might account for all the observed phenomena of immunity to Trypanosoma lewisi.

We are certain that the medium in which the trypanosomes were incubated, which afforded protection when injected into rats, contained only substances secreted or excreted by the parasites. As discussed previously, great pains were taken to avoid the contamination of this medium by antibodies already formed, by disintegrating bodies of the parasites, by changes in the antigenic nature of the serum, and by bacteria. We are convinced, therefore, that excreted or secreted substances were alone responsible for

the complete protection afforded. We believe that these substances are probably enzymes, or possibly intermediary products of the metabolism of the parasite resulting from enzyme action, and that the antibody produced can be considered as an anti-enzyme, as suggested by Chandler (1935, 1937).

It is very significant that, in our experiments, serum obtained from rats immunized with metabolic products was strongly agglutinating. The same was true of serum obtained from rats that had recovered from an infection with the parasite, confirming in vitro observations made by Laveran and Mesnil (1901, 1912), who found that immune serum agglutinates (or "agglomerates") the parasites and slows them down. Our results indicate that this agglutination and consequent reduced locomotion, attributed to trypanocidal or other antibodies by Augustine (1943) and Barnes (1951), and which was shown by Augustine to render the parasites more susceptible to being filtered out of the blood and phagocytized, are really produced by ablastin. A striking feature of the process, as originally observed by Laveran and Mesnil, is that the individual trypanosomes in the agglutinated masses exhibit a definite orientation of their bodies; at the beginning of the process two trypanosomes adhere by their posterior ends; later, large masses are formed with the same orientation. It is interesting that Laveran and Mesnil further found that

sera which agglutinated living T. lewisi also agglutinated them when killed with formalin, but in this case there was no orientation of their bodies. Manteufel (1909) found that when the trypanosomes were killed by heating to 45°C. they were not agglutinated by specific antisera.

We believe that the agglutination observed by these investigators and in our experiments might be due to the combination of the antibody with enzymes or other metabolic substances being excreted at the posterior end, possibly by some activity of the kinetoplast, although this, at present, is purely speculative. The agglutination of trypanosomes killed with formalin, without the typical orientation characteristic of living trypanosomes, could possibly be due to some change in permeability of the body wall permitting the secretions and excretions of the parasites to be discharged through all the body surface, or, since this has been observed only with serum from recovered animals, it may indicate an entirely different antigen-antibody reaction which is not operative when the parasites are alive. Failure to agglutinate parasites killed by heat suggests alteration in the character or combining power of the ablastin-stimulating antigen.

In our experiments on absorption of immune serum by lyophilized metabolic products, in four of five rats we removed a large part of the protective power of the serum, when the latter was injected into rats prior to

challenge inoculations with 20,000,000 trypanosomes; in these rats good infections developed in four of five rats, and evidence of multiplication, based on percentage of dividing and small forms and on duration of the period during which dividing forms were observed, approximated that in control rats given no serum. In contrast, in 5 rats inoculated with serum absorbed with lyophilized washed and triturated trypanosomes, there was no evidence of reduction in protective power of the immune serum.

Taliaferro (1932) considered it possible to differentiate between a trypanocidal and a reproduction-inhibiting antibody by absorption of immune serum by means of large numbers of living trypanosomes (at least 1,000,000 per cmm.). The trypanosomes were left in the serum for six hours on ice followed by one hour at 37°C. In contrast to our findings, this resulted in complete loss of curative power of the serum, and also of protective power in all but one of six rats. Taliaferro interpreted this as indicating absorption of antibody by the trypanosomes, and since he found that ablastin was not absorbed by trypanosomes and did not kill them, since they resumed multiplication when transferred to a fresh rat, he concluded that this was clear indication of a separate trypanocidal antibody.

We think it more likely that the time during which the living trypanosomes were left in contact with the serum

was sufficient to allow them to excrete enough ablastin-producing antigen to combine with the ablastin that was present, and eliminate the curative and protective potency of the serum in this manner; our absorption experiments showed that substances produced by the living parasites absorb the protective antibodies from immune serum, although the triturated bodies of the trypanosomes do so to a very small extent.

Taliaferro did not succeed in absorbing the immune serum taken from rats 20 days after infection, when the rats had not yet recovered from the infection, but when the parasites had ceased reproduction and had gone through the first number crisis. It is possible that, since at this time ablastin would be at its highest titer, the number of trypanosomes used by Taliaferro was not sufficient to produce enough metabolic products, in the time in which they were immersed in the serum, to absorb the ablastin completely. Coventry (1930) had shown that the curative effect of immune serum tends to be lost within a few weeks after termination of an infection and, in our own experiment V, we found the potency of serum taken 3 weeks after termination of an infection to be greater than that of serum taken 2 months after termination.

Taliaferro (1932) also reported successful suppression of reproduction of trypanosomes in rats injected one to three times with massive doses of triturated bodies of

trypanosomes seven to nine days prior to infection. In our own experiments with rats injected with similar material, we failed to get more than partial protection; in rats thus immunized there was some division of the trypanosomes for a few days, but reproduction was inhibited earlier and thus peaks of parasite numbers were much lower than in untreated rats. This was in contrast to the complete inhibition of reproduction and absolute protection against infection which we obtained in rats injected with metabolic products. In Taliaferro's experiments, however, much larger doses of trypanosome bodies were used than in our experiments, and on consecutive days instead of being spaced out (six injections at three-day intervals).

We believe that the partial protection we obtained might have been due to ablastin-stimulating material contained in the bodies of the parasites and not yet excreted. Possibly the massive 5 cc. doses of heavy suspensions of trypanosomes used in Taliaferro's experiments provided enough antigen to stimulate a protective level of ablastin within a period of seven days. The same explanation may account for Novy's (1907) successful immunization of rats with plasmolyzed cultures of T. lewisi. Of course, the possibility cannot be ruled out that if large enough quantities of trypanosome body substance is injected, greatly exceeding the parasite burden in a normal infection, other liberated antigens might stimulate the forma-

tion of additional trypanocidal antibodies. Taliaferro's observation that a lysin that is active in vitro is sometimes produced in rats gives some support to this view.

Coventry (1930) believed that the termination of a T. levisi infection in rats was associated with a curative trypanocidal property of serum, distinct from ablastin, that was passively transferable. She observed that passively transferred immune serum had much less curative effect when larger numbers of trypanosomes were present in the infected rats. When very few trypanosomes were present the serum was effective in all doses. When the trypanosomes were slightly more numerous the serum appeared to be less effective, and when trypanosomes were very numerous the serum appeared to be ineffective. She attributed these differences to a zonal phenomenon. Taliaferro (1932) considered Coventry's work to be conclusive evidence of the presence of a trypanocidal antibody. To us it seems possible that when large numbers of trypanosomes are present there may not be enough ablastin to go around. It is possible that in Coventry's experiments the ablastin in the immune serum was neutralized before all of the trypanosomes were agglutinated and prevented from reproducing.

Taliaferro later (1938 a) found what he considered to be differences in "ablastic" and "trypanocidal" serum in experiments on the effect of passive transfer of these

sera in normal rats and in rats that had been splenectomized and had had the reticulo-endothelial system blocked by India ink. "Ablastic" serum was obtained from rats 20 to 25 days after a primary infection when living trypanosomes were still present in small numbers, and which, therefore, was considered to be free of "trypanocidal" antibody. In addition, in some experiments this serum was "absorbed" by living trypanosomes as in his earlier (1932) experiments described above. "Trypanocidal" antibody was obtained from rats that had completely cleared out the parasites from a primary infection and were then given an additional large dose of trypanosomes intravenously. If these disappeared within 24 hours the supposedly trypanocidal serum was collected a week later.

The "ablasic" serum was found to be equally effective in preventing reproduction of the parasites in normal and in splenectomized and blocked rats for the first few days (usually six), after which the parasites began to reproduce actively in the experimental rats, but not in the normal rats, in which active production of ablastin is presumed to have occurred. On the other hand, when "trypanocidal" serum was transferred to normal rats the challenge trypanosomes disappeared on the day following their injection, except in two that received very small doses of serum, whereas in the splenectomized and blockaded rats 8 out of 18 did not completely lose their trypanosomes,

and after four or five days some multiplication was evident. Later in these rats, as in those given "ablastic" serum, there was evidence of active production of ablastin in the normal rats but not in the splenectomized and blockaded ones. These results were interpreted to indicate that macrophages do not play an important role in the passive transfer of "ablastic" serum but do when "trypanocidal" serum is transferred. However, there were two important differences in the experiments with these two supposedly different kinds of serum: (1) the "trypanocidal" serum was obtained from more highly immunized rats and may be presumed to have contained a higher titer of antibody, and (2) the rats given "trypanocidal" serum were challenged with only about one-tenth as many trypanosomes as were the rats given "ablastic" serum. We believe, therefore, that the observed differences can be accounted for by quantitative rather than qualitative differences in the sera used for passive transfer.

In this same paper Taliaferro reported that when trypanosomes "sensitized" by exposure to inactivated "trypanocidal" serum for an hour on ice, after being washed and centrifuged, were injected into either normal or splenectomized and blockaded rats, they disappeared rapidly. In some they could not be found even immediately after injection. We believe it possible that these trypanosomes, even if not agglutinated when injected, might have had

enough combination of ablastin with the ablastin-producing excretion (concentrated at the posterior end) to cause their agglutination after injection and consequent rapid removal, by clumping in capillaries or by phagocytosis. The evidence for non-absorption of ablastin by trypanosomes is based on the normal multiplication of trypanosomes transferred from rats in which they have ceased reproduction but obviously have not yet become agglutinated, and on failure of living or dead trypanosomes to remove appreciable quantities of ablastin from immune serum in which they have been immersed.

A number of substances have been found to modify the course of T. lewisi infections in rats, including prolongation of reproduction activity and, therefore, presumed interference with ablastin production. Becker, Manresa, and Johnson (1943) and Becker, Taylor and Fuhrmeister (1947) proved that deficiency of pantothenic acid in rats increased parasite peaks and prolonged reproductive activity of T. lewisi, and concluded that this was due to a deficiency in the production of ablastin.

Becker and Gallagher (1947) reported similar effects from administration of sodium salicylate. They postulated that ablastin might function as an oxidative enzyme, using pantothenic acid as a co-enzyme, and that the salicylate acted as a non-functional substitute for pantothenic acid, thus inhibiting the enzyme (ablastin). The evidence, how-

ever, indicated that the salicylate affected only the formation of ablastin and did not affect already-formed ablastin. Becker and Lysenko (1948) were able to reinfect recovered or nearly recovered rats with T. lewisi if both recipient and donor rats were treated with sodium salicylate, and the inoculation was made with large numbers of dividing trypanosomes. They concluded from this that ablastin, like many enzymes, can be broken down after it is formed. Taylor and Becker (1948) observed agglutinated trypanosomes in the blood and liver smear of normal infected rats, but not in pantothenate-deficient rats, the implication being that the pantothenate is needed for the production of agglutinin. Lysenko (1951), however, found no evidence of a combination between the salicylates and the ablastin molecule, nor did he find any evidence that pantothenic acid formed part of the molecule. He found that there was a drop in the total serum nitrogen and of gamma globulin in the salicylate-treated rats, which he considered to indicate either that the production of ablastin was inhibited, or that there was an increase in its break-down. Saul and Becker (1949) concluded that the effect of pantothenate deficiency or administration of salicylate on reproduction was due to the interference either with the formation or the action of ablastin, and that decrease in number of parasites in spite of continued reproduction was due to the trypanocidal antibody being

unaffected. Barnes (1951) observed an increase in the rate of reproduction and found no evidence of agglutination in salicylate-treated rats, but she did find remnants of disintegrated parasites in the blood. From this she, too, concluded that ablastin was affected but not trypanolysin. Meyers and Lysenko (1953) believed that the action of the salicylate could be due to an increased output of ACTH, which results in a reduction of the total antibody concentration.

It is obvious that the discussions and conclusions of these various authors are based on the assumption, which was practically universal, that they were dealing with several distinct protective substances--ablastin, considered in some of these papers as an antibody, and in others as an oxidative enzyme; trypanolysin, a trypanocidal antibody; and an agglutinin. If, as our results suggest is possible, the protective mechanisms in rats infected with T. lewisi are all manifestations of a single antibody produced in response to stimulation by metabolic products, and therefore to be considered as ablastin, the results described above might be interpreted in quite different ways.

Pantothenic acid is known to be an important factor in protein synthesis and therefore in antibody production (see Axelrod et al, 1947; Ludovici and Axelrod, 1951), and salicylate is an antagonist of it. It is logical to suppose,

therefore, that the effect of deficiency of pantothenic acid or administration of salicylate suppresses the synthesis of the ablastic antibody, which we have seen to be responsible not only for inhibition of reproduction but also for agglutination, and thus indirectly for the elimination of the parasites by slowing of locomotion and clogging of capillaries, and consequently phagocytic destruction. The possibility exists, however, that pantothenic acid or salicylate may somehow be involved either in the secretions or excretions of the parasites that stimulate the production of ablastin, or perhaps in the need of the parasites for these excreted substances. Since salicylate was found to produce anemia, the possibility exists that it may be instrumental in releasing hematin from destroyed blood corpuscles, thus providing this necessary metabolite without the intervention of a special enzyme, the inhibition of which, as we postulate below, though thus far without experimental evidence, may be the mode of action of ablastin.

Barnes (1951) found that in rats treated with benzene the trypanosome population at the peak of the infection was larger, the parasites were removed from the blood less efficiently, and the duration of the infection was prolonged. She ascribed these changes to an effect of benzene upon trypanolysin. However, since benzene destroys leucocytes, producing a leucopenia, and may affect other

phagocytic cells, the effects she observed and attributed to action upon the trypanolysin may well have been due, in part at least, to interference with phagocytic activity.

The substance, or substances, responsible for the production of ablastin has not yet been determined, although it, or they, is evidently needed for the nutrition or metabolism of the parasites. Since the material is excreted, it evidently enables the parasites to utilize some constituent or constituents of the environment not available when this material, antigenic in character, is neutralized by combination with the specific antibody, ablastin, which is formed in response to its presence. It is suggested as a possibility that the material principally involved is an enzyme by means of which the parasites are able to release hematin or protoporphyrin from hemoglobin. The need for these compounds by certain flagellates of the family Trypanosomidae was mentioned in the "Introduction." It is an attractive speculation, therefore, that ablastin might interfere with enzymatic liberation of hematin from hemoglobin, so that it would not be available to the parasites in adequate amount. Since the hematin or its precursors appears to be needed to build the flagellates' iron-catalyzed respiratory enzyme system, such interference would interfere with respiration and consequently with multiplication and development of the parasites. At present we are experimenting

with this particular aspect of the problem.

This proposed interference of ablastin with the respiratory enzyme has some experimental basis to support it. Moulder (1948 a, b) has shown some relation between the appearance of ablastin and a decrease in the utilization of glucose by the developing trypanosomes. During the course of an infection, the RQ rises from about 0.75 to 0.90. These observations were confirmed by Zwisler and Lysenko (1954). Moulder (1948 b) suggested that the shift in the glucose metabolism is caused by an interference in the oxidative assimilation of glucose by ablastin, and that ablastin probably acts in a manner similar to such metabolic inhibitors as malonates, sodium cyanide, sodium azide, atabrine, 2, 4 dinitro phenol, and sodium iodoacetate.

It is possible that differences, both quantitative and qualitative, in the enzyme systems of the different groups of trypanosomes might explain the presence or absence of the ablastic action. Recent works on the metabolism of the different trypanosomes, particularly work with therapeutic inhibitors, clearly demonstrate that such differences do exist. (See works of von Brand, 1938, 1950, 1951 a, 1951 b, 1953; von Brand and Johnson, 1947; von Brand and Tobie, 1947; von Brand, Tobie, and Mehlman, 1950; Ryley, 1951, 1956; Seaman, 1953; Reiner, Smythe and Pedlow, 1936).

We believe that this production of functional im-

munity through the agency of antibodies directed against metabolic products, particularly enzymes, which results in interference with nutrition and consequently with metabolism, will be found to be a widespread phenomenon among infective organisms. Taliaferro (1938 b) and Taliaferro and Pavlinova (1936) observed the presence of a similar ablasic action against Trypanosoma duttoni in mice. The reproduction of T. duttoni never reaches the peak it does in T. lewisi, and although it is inhibited during the early days of the infection, the inhibition is not as early nor as complete as in the case of T. lewisi. Taliaferro demonstrated some degree of group reaction between antibodies (which he considered to be both ablasic and trypanocidal) formed against T. duttoni and T. lewisi.

It is possible that ablasic action occurs in other trypanosomes also, and perhaps in other Protozoa and in bacteria as well, but that it is not readily demonstrable. In the case of T. lewisi and T. duttoni the interference with nutrition is so drastic that the inhibition of growth and reproduction becomes evident before the organisms begin dying in large numbers. In other organisms, particularly bacteria, antibodies against surface and body antigens, causing agglutination and often lysis, may develop earlier and thus mask the effect of possible ablasic action. In the cases of intracellular parasites, e. g., Leishmania, Trypanosoma cruzi, Toxoplasma, and Rickettsia, and even

malaria, the possibility of ablastic reaction might still be present, the antigens being released when the parasitized cells break up. In this case the ablastic action would probably only be effective during the brief extracellular life of the parasites.

Ascoli (1906) found that the protective properties of anti-anthrax serum were not absorbed by the bacteria and that the bacteria were not sensitized by the serum. He later (1908) concluded that the serum acted by inhibition of certain assimilative processes, stopping growth and multiplication. This he called antiblastic immunity. The death of the non-multiplying organisms he thought to be due to non-specific factors connected with the normal defense mechanism of the host, and autolysis. Dochez and Avery (1916) postulated similar antiblastic action against pneumococci; Blake (1917) questioned this and attributed the inhibition of metabolic processes to agglutination. In view of our finding that ablastin may cause agglutination both authors may have been correct.

The functional importance of ablastic action is particularly well demonstrated in nematode infections, in which effects attributable to interference with nutrition are easily distinguished from antibody action against dead worm substance. Chandler (1935) suggested that the retardation of growth, development and reproduction manifested in intestinal immunity against Nippostrongylus muris might

be caused by a failure of nutrition due to the formation by the host of anti-enzymes that would interfere with the assimilative processes of the parasites. This author (1936) demonstrated that the reaction against Nippostrongylus muris in the intestine is not due to a permanent effect on the worms themselves, but to an unfavorable environmental condition. When the worms are removed to a non-immune host, they resume normal growth and reproduction. He suggested that a similar mechanism might be of wide application in parasitic infections and be the true explanation of the inhibition of reproduction of T. lewisi. Sarles and Taliaferro (1936) also considered it possible that the stunting of growth and decreased egg production of N. muris in rats might not be due to the antibody involved in killing the worms, but to an ablastin.

Sarles (1938) demonstrated that precipitates formed at the orifices and in the alimentary canal of larvae of N. muris immersed in immune serum (serum from repeatedly infected rats), and Taliaferro and Sarles (1939) showed that this precipitin reaction does not involve the general body surface, but only the excretions and secretions that flow out of the orifices. Blacklock, Gordon and Fine (1930), working on the larvae of the myasis-producing fly, Cordylobia anthropophaga, had previously described precipitates in the gut and around the larvae in the skin of immune guinea pigs. They assumed that the precipitate prevented

the assimilation of food and led to the death of the larvae by mechanically blocking the gut. Subsequently similar precipitin reactions were demonstrated for larvae of many helminths, and in some cases adult worms also, e.g., Ancylostoma caninum (Otto, 1940); Trichinella (Oliver-Gonzalez, 1940, 1941); Ascaridia (Sadun, 1949); Litosomoides (Scott and MacDonald, 1951), and cercariae (Papirmeister and Bang, 1948). Oliver-Gonzalez (1941) stated that the precipitate forming around the vulva of adult Trichinella appeared to interfere with the exit of embryos.

Thorson (1951, 1953) succeeded in obtaining precipitating antibodies and in producing some degree of immunity by injecting serum in which Nippostrongylus larvae had been living in vitro. He presented (1953) a view similar to Chandler's: "That antibodies produced by the hosts against enzymes in the secretions and excretions of the worms would react against the enzymes of the invading larvae to slow down their migration and, by adversely affecting their nutrition, would interfere with their normal development." Thorson later (1956) demonstrated that the proteolytic activity of the adult Ancylostoma caninum could be inhibited by serum from a dog refractory to infections with the parasite. He concluded that substances containing this proteolytic activity serve as antigen.

Campbell (1955) demonstrated that injections into mice of the excretions and secretions of Trichinella spiralis

stimulated an immune response manifested by a decreased number of larvae recovered 30 days after infection, by a more rapid loss of adults, and by a smaller size of the adult female worms. Chute (1956) confirmed the antigenicity of secretions and excretions of trichina larvae. He demonstrated a direct effect of the antibody upon the larvae; the larvae were not reduced in number but their growth was retarded. His procedure was very similar to ours with T. levisi except that he incubated the organisms in a non-nutrient saline solution and collected the metabolic products over a period of six days (which might have allowed for decomposition of the products secreted early in the incubation).

On the basis of these results obtained by various authors, and our own successful results with Trypanosoma levisi in rats, we believe that immunization with metabolic products, and consequent production of ablastin, may be applicable to a great variety of both microorganisms and Metazoa, and may possibly have practical therapeutic or preventive potentialities. Chandler (1953) remarked that much emphasis has been given in recent years to the knocking out of enzyme systems by chemotherapeutics, particularly antibiotics, but that little attention has been given to knocking out these enzymes by immunological processes. We hope that the present work will lay the foundation for further work on antimetabolite immunity in other infections,

-87-

and for the possible consequent development of helpful vaccines.

XI. SUMMARY

Trypanosoma lewisi was incubated in normal rat serum diluted with an equal volume of saline, at the rate of 200,000 trypanosomes per cmm. of medium, for a period of 24 hours. The trypanosomes were then separated from the medium, so that the latter contained only the accumulated metabolic products. The trypanosomes, washed and killed and triturated by freezing and thawing, were injected into rats at the rate of 4,000 million trypanosomes per rat (the approximate number of trypanosomes in a rat at the peak of a normal infection), divided into 6 injections at 3-day intervals. The accumulated metabolic products of these same trypanosomes, contained in the medium after removal of the trypanosomes, were likewise injected into rats in a series of 6 injections at 3-day intervals. The rats injected with metabolic products showed complete protection against challenge infections with 20,000,000 trypanosomes, whereas the rats injected with the washed, triturated trypanosomes showed some degree of protection, but it was never complete. The same results were obtained when rats were injected with the bodies or metabolic products of 1,000 million trypanosomes, given in 3 injections.

Serum from rats immunized by injections of metabolic products, taken 8 days after the last injection, and serum taken from rats that had recovered from a natural infection

6 weeks earlier, caused strong agglutination of trypanosomes immersed in them; this occurred in about ten minutes or less in serum diluted up to 1:10, and in an hour or two at higher dilutions, up to 1:100. "Metabolic products serum" taken from rats infected about 6½ months earlier still caused agglutination -- strongly at a dilution of 1:1, but only very weakly at dilutions of 1:10 or higher. In contrast, the serum of rats immunized by injections of triturated trypanosomes, at a dilution of 1:3, caused light agglutinations at 15 and 30 minutes, after which the clumps broke up, so no agglutination was evident after about an hour. Normal rat serum never caused agglutination.

The protective effect of immune serum from recovered rats is markedly reduced after absorption with lyophilized metabolic products. In rats injected with serum thus absorbed (1 cc. per 100 g. weight) one hour previous to injection with a challenging infective dose of 20,000,000 T. lewisi, the parasites reproduced normally, and produced a normal infection. In contrast, immune serum absorbed with lyophilized T. lewisi lost little of its protective power.

The data presented indicate that the antigenic substances stimulating the production of ablastin in T. lewisi infections are excreted metabolic products, presumably enzymes or intermediate products produced by them, and that ablastin is the antibody responsible for agglutination of

the trypanosomes, and is the chief factor involved in protective and curative action of immune serum.

It is tentatively suggested that ablastin by itself may be bringing about (1) inhibition of reproduction, (2) the number crisis following this, (3) the eventual elimination of all the parasites as the titer increases, and (4) protection against reinfection. Although it is probable that antibodies may also be formed against the trypanosome protoplasm or other constituents of the trypanosome bodies, it seems questionable that these play any important part in functional immunity of rats against T. levisi.

Suggestions, based both on our own work and on that of others, are made concerning the exact mechanism by which ablastin brings about inhibition of reproduction, and by which the non-reproducing trypanosomes in a primary infection, and both dividing and adult trypanosomes in natural infections or in challenge infections, are eliminated. Further work on this phase of the problem is contemplated.

The possibility of ablatic immunity being a widespread phenomenon among infective organisms, including both microorganisms and Metazoa, is discussed and instances of its occurrence are cited. It is suggested that artificial immunization with metabolic products may be found to have practical therapeutic or prophylactic potentialities in some cases.

BIBLIOGRAPHY

- Ascoli, A. 1906. Zur Kenntnis der aktiven Substanz des Milzbrandserums. Ztschr. f. physiol. Chem. 48, 315-330.
- Ascoli, A. 1908. Ueber den Wirkungsmechanismus des Milzbrandserums: Antiblastische Immunität. Centralbl. f. Bakt. Parasitenk. 1 Abt. (Orig.) 46, 178-188.
- Augustine, D. L. 1943. Some factors in the defense mechanism against reinfections with Trypanosoma lewisi. Proc. Amer. Acad. Arts Sci. 75, 85-93.
- Axelrod, A. E., Carter, B. B., McCoy, R. H., and Geisinger, R. 1947. Circulating antibodies in vitamin-deficiency states; I. Pyridoxin, riboflavin and pantothenic acid deficiencies. Proc. Soc. Exptl. Biol. Med. 66, 137-140.
- Barnes, E. A. 1951. Effect of benzene and six selected salicylates on the development of immunity in Trypanosoma lewisi infections and on various aspects of the blood picture. Iowa State Coll. J. Sci. 26, 1-17.
- Becker, E. R., and Gallagher, P. L. 1947. Prolongment of the reproductive phase of Trypanosoma lewisi by the administration of sodium salicylate. Iowa State Coll. J. Sci. 21, 351-362.
- Becker, E. R., and Lysenko, M. G. 1948. Reinfection with Trypanosoma lewisi and recurrence of reproduction in recovered and near-recovered rats. Iowa State Coll. J. Sci. 22, 239-255.
- Becker, E. R., Manresa, M., and Johnson, E. M. 1943. Reduction in the efficiency of ablastic action in Trypanosoma lewisi infection by withholding pantothenic acid from the host's diet. Iowa State Coll. J. Sci. 17, 431-441.
- Becker, E. R., Taylor, J., and Fuhrmeister, C. 1947. The effect of pantothenate deficiency on Trypanosoma lewisi infection in the rat. Iowa State Coll. J. Sci. 21, 237-243.
- Blacklock, D. B., Gordon, R. M., and Fine, J. 1930. Metazoan immunity: a report on recent investigations. Ann. Trop. Med. Hyg. 24, 5-54.

- Blake, F. G. 1917. Studies on antiblastic immunity. J. Exptl. Med. 26, 563-580.
- von Brand, T. 1938. The metabolism of pathogenic trypanosomes and the carbohydrate metabolism of their hosts. Quart. Rev. Biol. 13, 41-50.
- von Brand, T. 1950. The carbohydrate metabolism of parasites. J. Parasitol. 36, 178-192.
- von Brand, T. 1951. In Parasitic infections in man, H. Most, ed., Columbia Univ. Press, New York.
- von Brand, T. 1951. In Biochemistry and physiology of Protozoa, A. Lwoff, ed., Acad. Press, Inc.
- von Brand, T. 1952. Chemical physiology of endoparasitic animals, Acad. Press, Inc., New York.
- von Brand, T. 1953. Discussion of papers on haemoflagellates. Ann. N. Y. Acad. Sci. 56, 1004-1005.
- von Brand, T., and Johnson, E. M. 1947. A comparative study of the effect of cyanide on the respiration of some Trypanosomidae. J. Cell. Comp. Physiol. 29, 33-49.
- von Brand, T., and Tobie, E. J. 1948. Further observations on the influence of cyanide on some trypanosomes. J. Cell. Comp. Physiol. 31, 49-68.
- von Brand, T., Tobie, E. J., and Mehlman, B. 1950. The influence of some sulfhydryl inhibitors and of fluoroacetate on the oxygen consumption of some trypanosomes. J. Cell. Comp. Physiol. 35, 273-300.
- Breinl, A., and Hindle, E. 1910. Observations on the life history of Trypanosoma lewisi in the rat louse (Haematopinus spinulosus). Ann. Trop. Med. Parasitol. 3, 553-562.
- Brown, W. H. 1915. Concerning changes in the biological properties of Trypanosoma lewisi produced by experimental means, with especial reference to virulence. J. Exptl. Med. 21, 345-364.
- Campbell, C. H. 1955. The antigenic role of excretions and secretions of Trichinella spiralis in the production of immunity in mice. J. Parasitol. 41, 483-491.
- Chandler, A. C. 1935. Studies on the nature of immunity to intestinal helminths. I. The local nature of the im-

- munity of white rats to Nippostrongylus infections. Am. J. Hyg. 22, 157-167.
- Chandler, A. C. 1936. Studies on the nature of immunity to intestinal helminths. III. Renewal of growth and egg production in Nippostrongylus after transfer from immune to non-immune rats. Am. J. Hyg. 23, 46-54.
- Chandler, A. C. 1937. Studies on the nature of immunity to intestinal helminths. VI. General resumé and discussion. Am. J. Hyg. 26, 309-321.
- Chandler, A. C. 1953. Immunity in parasitic diseases. J. Egyptian Med. Assoc. 36, 811-834.
- Chute, R. M. 1956. The dual antibody response to experimental trichinosis. Proc. Helm. Soc. Wash. 23, 49-58.
- Coventry, F. A. 1925. The reaction product which inhibits reproduction of the trypanosomes in infections with Trypanosoma levisi, with special reference to its change in titer throughout the course of the infection. Am. J. Hyg. 5, 127-144.
- Coventry, F. A. 1929. Experimental infections with Trypanosoma levisi in the guinea pig. Am. J. Hyg. 9, 247-259.
- Coventry, F. A. 1930. The trypanocidal action of specific antisera on Trypanosoma levisi in vivo. Am. J. Hyg. 12, 366-380.
- Culbertson, J. T., and Wotton, R. M. 1939. Studies on age resistance against trypanosome infections. VI. Production of ablastin in rats of different age groups after infection with Trypanosoma levisi. Am. J. Hyg. 30, 101-113.
- Dochez, A. R., and Avery, O. T. 1916. Antiblastic immunity. J. Exptl. Med. 23, 61-68.
- Duca, C. J. 1939. Studies on age resistance against trypanosome infections. II. The resistance of rats of different age groups to Trypanosoma levisi, and the blood response of rats infected with this parasite. Am. J. Hyg. 29, 25-32.
- Floedorf, E. W., and Mudd, S. 1935. Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. J. Immunol. 29, 389-425.
- Frutton, J. S., and Simmonds, S. 1953. General Biochemistry, John Wiley and Sons Inc., New York.

- Kanthack, A. A., Durham, H. E., and Blandford, W. F. H. 1898. On Nagana, or Tsetse-fly disease. Proc. Roy. Soc. London, 64, 100-118.
- Laveran, A., and Mesnil, P. 1901. Recherches morphologiques et experimentales sur le trypanosome des rats (Trypanosoma levisi, Kent). Ann. Inst. Pasteur 15, 673-714.
- Laveran, A., and Mesnil, P. 1912. Trypanosomes et trypanosomiasis, Masson, Paris.
- Lewis, T. R. 1879. Flagellated organisms in the blood of healthy rats. Quart. J. Micro. Sci. 19, 109-114.
- Lincicome, D. R. 1955. Growth factor in normal rat serum for Trypanosoma levisi. J. Parasitol. 41 (Suppl.), 15.
- Little, P. A., and Oleson, J. J. 1951. The cultivation of Trypanosoma cruzi. J. Bacteriol. 61, 709-714.
- Ludovici, P. P., and Axelrod, A. E. 1951. Relative effectiveness of pantothenic acid and panthenol in stimulating antibody response of pantothenic acid-deficient rats. Proc. Soc. Exptl. Biol. Med. 77, 530-532.
- Lwoff, M. 1933. Recherches sur la nutrition des trypanosomides. Ann. Inst. Pasteur 51, 55-116.
- Lwoff, M. 1938. L'aneurine, facteur de croissance pour les strigomonas (Flagelles, Trypanosomides). Compt.-Rend. Soc. Biol. 128, 241-243.
- Lwoff, M. 1939. Le pouvoir de synthèse des Leishmanies. Compt.-Rend. Soc. Biol. 130, 406-408.
- Lwoff, M. 1951. The nutrition of parasitic flagellates (Trypanosomidae, Trichomonadinae). In Biochemistry and Physiology of Protozoa, A. Lwoff, ed., vol. 1. Acad. Press, Inc., New York.
- Lysenko, M. G. 1951. Concerning salicylate inhibition of ablasic activity in Trypanosoma levisi infection. J. Parasitol. 37, 535-544.
- MacNeal, W. J. 1904. The life history of Trypanosoma levisi and Trypanosoma brucei. J. Infect. Dis. 1, 517-543.
- Manteufel. 1909. Untersuchungen über spezifische Agglomeration und Komplettbildung bei Trypanosomen und Spirochaeten. Arb. a. d. kaiserl. Gesundh. 28, 172-197.

- Meyers, W. M., and Lysenko, M. G. 1953. Effect of sodium salicylate treatment on antibody titers in rats infected with Trypanosoma lewisi. Proc. Soc. Exptl. Biol. Med. 84, 97-98.
- Minchin, E. A., and Thomson, J. D. 1915. The rat-trypanosome, Trypanosoma lewisi, in its relation to the rat flea, Ceratophyllus fasciatus. Quart. J. Micr. Sci. 60, 463-492.
- Moulder, J. W. 1948 a. The oxidative metabolism of Trypanosoma lewisi in a phosphate saline medium. J. Infect. Dis. 83, 33-41.
- Moulder, J. W. 1948 b. Changes in the glucose metabolism of Trypanosoma lewisi during the course of infection in the rat. J. Infect. Dis. 83, 42-49.
- Noble, E. R. 1955. The morphology and life cycles of trypanosomes. Quart. Rev. Biol. 30, 1-28.
- Novy, F. G. 1907. Immunity against trypanosomes. Proc. Soc. Exptl. Biol. Med. 4, 42-44.
- Novy, F. G., and MacNeal, W. J. 1903. On the cultivation of Trypanosoma lewisi. In Contributions to Med. Res. Dedicated to V. C. Vaughn, p. 549.
- Novy, F. G., Perkins, W. A., and Chambers, R. 1912. Immunization by means of cultures of Trypanosoma lewisi. J. Infect. Dis. 11, 411-426.
- Oliver-Gonzalez, J. 1940. The in vitro action of immune serum on the larvae and adults of Trichinella spiralis. J. Infect. Dis. 67, 392-300.
- Oliver-Gonzalez, J. 1941. The dual antibody basis of acquired immunity in trichinosis. J. Infect. Dis. 69, 254-270.
- Otto, G. F. 1940. A serum antibody in dogs actively immunized against the hookworm, Ancylostoma caninum. Am. J. Hyg. 31, 23-27.
- Packchanian, A. 1934. On the cultivation of seven species of trypanosomes in vitro. Science 80, 407-408.
- Packchanian, A. 1943. On the viability of various species of Trypanosoma and Leishmania cultures. J. Parasitol. 29, 275-277.

- Papirmeister, B., and Bang, F. B. 1948. The in vitro action of immune sera on cercariae of Schistosoma mansoni. Am. J. Hyg. 48, 74-80.
- Rabinovitsch, L., and Kempner, W. 1899. Beitrag zur Kenntnis der Blutparasiten, speciell der Rattentrypanosomen. Ztschr. f. Hyg. Infektionskr. 30, 251-294.
- Regendanz, P., and Kikuth, W. 1927. Ueber die Bedeutung der Milz für die Bildung des vermehrungshindernden Reactions-produktes (Taliaferro) und dessen Wirkung auf den Infektionsverlauf der Ratten-Trypanosomiasis (Trypanosoma levisi). Versuche der Uebertragung des Trypanosoma levisi auf die weisse Maus. Centralbl. f. Bakt. Parasitenk. 1 Abt. (Orig.) 103, 271-279.
- Reiner, L., Smythe, C. V., and Pedlow, J. T. 1936. On the glucose metabolism of trypanosomes (Trypanosoma equiperdum and Trypanosoma levisi). J. Biol. Chem. 113, 75-88.
- Ryley, J. F. 1951. Studies on the metabolism of the Protozoa. 1. Metabolism of the parasitic flagellate, Trypanosoma levisi. Biochem. J. 49, 577-585.
- Ryley, J. F. 1956. Studies on the metabolism of the Protozoa. 7. Comparative carbohydrate metabolism of eleven species of trypanosomes. Biochem. J. 62, 215-222.
- Sadun, E. H. 1949. The antibody basis of immunity in chickens to the nematode, Ascaridia galli. Am. J. Hyg. 49, 101-116.
- Salvin-Moore, J. E., Breinl, A., and Hindle, E. 1908. The life history of Trypanosoma levisi. Ann. Trop. Med. Parasitol. 2, 197-220.
- Sarles, M. P. 1938. The in vitro action of immune rat serum on the nematode Nippostrongylus muris. J. Infect. Dis. 62, 337-348.
- Sarles, M. P., and Taliaferro, W. H. 1936. The local points of defense and the passive transfer of acquired immunity to Nippostrongylus muris in rats. J. Infect. Dis., 59, 207-220.
- Saul, L. A., and Becker, E. R. 1949. Course of Trypanosoma levisi infections in white rats treated with sodium salicylate. J. Parasitol. 35, 54-60.
- Scott, J. A., and MacDonald, E. M. 1951. Retarded growth

- of Litomosoides carinii after the introduction of non-living antigenic material into the host. J. Parasitol. 37 (Suppl.), 18.
- Seaman, G. R. 1953. The succinic dehydrogenase of Trypanosoma cruzi. Exptl. Parasitol. 2, 236-241.
- Seneca, H., and Henderson, E. 1951. Growth of hemoflagellates in dialyzing sacs and the production of growth inhibitory factor. Am. J. Hyg. 53, 17-21.
- Taliaferro, W. H. 1924. A reaction product in infections with Trypanosoma lewisi which inhibits the reproduction of the trypanosomes. J. Exptl. Med. 39, 171-190.
- Taliaferro, W. H. 1929. The Immunology of parasitic infections, The Century Co., New York and London.
- Taliaferro, W. H. 1932. Trypanocidal and reproduction-inhibiting antibodies to Trypanosoma lewisi in rats and rabbits. Am. J. Hyg. 16, 32-84.
- Taliaferro, W. H. 1938 a. The effects of splenectomy and blockade on the passive transfer of antibodies against Trypanosoma lewisi. J. Infect. Dis. 62, 98-111.
- Taliaferro, W. H. 1938 b. Ablastic and trypanocidal antibodies against Trypanosoma duttoni. J. Immunol. 35, 303-328.
- Taliaferro, W. H. 1948. The inhibition of reproduction of parasites by immune factors. Bact. Rev. 12, 1-17.
- Taliaferro, W. H., Cannon, P. R., and Goodloe, S. 1931. The resistance of rats to infections with Trypanosoma lewisi as affected by splenectomy. Am. J. Hyg. 14, 1-37.
- Taliaferro, W. H., and Pavlinova, Y. 1936. The course of infections of Trypanosoma duttoni in normal and in splenectomized and blockaded mice. J. Parasitol. 22, 29-41.
- Taliaferro, W. H., and Sarles, M. P. 1939. The cellular reactions in the skin, lungs and intestine of normal and immune rats after infection with Nippostrongylus muris. J. Infect. Dis. 64, 157-192.
- Taliaferro, W. H., and Taliaferro, L. G. 1922. The resistance of different hosts to experimental trypanosome infections with special reference to a new method of measuring this resistance. Am. J. Hyg. 2, 264-319.

- Taylor, J., and Becker, E. R. 1948. Liver changes in pantothenate-deficient rats infected with Trypanosoma lewisi. J. Infect. Dis. 82, 42-44.
- Thorson, R. E. 1951. The relation of the secretions and excretions of the larvae of Nippostrongylus muris to the production of protective antibodies. J. Parasitol. 37 (Suppl.), 18-19.
- Thorson, R. E. 1953. Studies on the mechanism of immunity in the rat to the nematode, Nippostrongylus muris. Am. J. Hyg. 58, 1-15.
- Thorson, R. E. 1956. Proteolytic activity in extracts of the esophagus of adults of Ancylostoma caninum and the effect of immune serum on this activity. J. Parasitol. 42, 21-25.
- Tobie, E. J., and Rees, C. W. 1948. The cultivation of Trypanosoma cruzi in dialysate medium. J. Parasitol. 34, 162-163.
- Tobie, E. J., von Brand, T., and Mehlman, B. 1950. Cultural and physiological observations on Trypanosoma rhodesiense and Trypanosoma gambiense. J. Parasitol. 36, 48-54.
- Zwisler, J. B., and Lysenko, M. G. 1954. The oxidative metabolism of Trypanosoma lewisi from salicylate treated infected white rats. J. Parasitol. 40, 531-535.