University of the Pacific
Scholarly Commons

University of the Pacific Theses and Dissertations

Graduate School

1970

Immunodiffusion and immunoelectrophoretic studies on Trypanosoma lewisi (Kent) during the course of an infection in the albino rat, Rattus rattus

Carol Louise Perkins Drew
University of the Pacific

Follow this and additional works at: https://scholarlycommons.pacific.edu/uop_etds

Part of the Life Sciences Commons

Recommended Citation

Drew, Carol Louise Perkins. (1970). *Immunodiffusion and immunoelectrophoretic studies on Trypanosoma lewisi (Kent) during the course of an infection in the albino rat, Rattus rattus*. University of the Pacific, Thesis. https://scholarlycommons.pacific.edu/uop_etds/1711

This Thesis is brought to you for free and open access by the Graduate School at Scholarly Commons. It has been accepted for inclusion in University of the Pacific Theses and Dissertations by an authorized administrator of Scholarly Commons. For more information, please contact mgibney@pacific.edu.

IMMUNODIFFUSION AND IMMUNOELECTROPHORETIC STUDIES ON *TRYPANOSOMA LEWISI* (KENT) DURING THE COURSE OF AN INFECTION IN THE ALBINO RAT, *RATTUS RATTUS*

A Thesis

Presented to

the Faculty of the Department of Biological Sciences

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Carol Louise Perkins Drew

June 1970

This thesis, written and submitted by

Carol Louise Perkins Drew,

is approved for recommendation to the

Graduate Council, University of the Pacific.

Department Chairman or Dean:

Thesis Committee:

, Chairman

Dated May 7, 1970

ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. Fuad M. Nahhas under whose guidance, helpful criticism, and encouragement this study was made. Thanks are also due to Dr. James R. Thompson of the Department of Physiology and Pharmacology, School of Pharmacy, University of the Pacific, for making the facilities of his Department available; to Dr. John D. McCrone for the use of his LKB Immunodiffusion apparatus; to Mr. Paul C. Edmiston for his assistance in the inoculation of rats, and to my husband, C. Irvin Drew, for his invaluable help and patience.

TABLE OF CONTENTS

	PAGE
BIOLOGY OF TRYPANOSOMA LEWISI	1
FIGURE 1. TRYPANOSOMA LEWISI	2
FIGURE 2. LIFE CYCLE OF T. LEWISI	4
REVIEW OF LITERATURE	7
MATERIALS AND METHODS	19
TABLE 1. TIME TABLE FOR SAMPLING	22
TABLE 2. FLOW SHEET	23
RESULTS	28
TABLE 3. RESULTS	31
PLATE 1. PHOTOGRAPHS OF IMMUNOELECTRO - PHORETIC SLIDES	32
DISCUSSION	33
SUMMARY	39
LITERATURE CITED	40

THE BIOLOGY OF TRYPANOSOMA LEWISI (Kent)

Trypanosoma lewisi (Kent), a member of the Family Trypanosomatidae, Order Protomonadida, Class Mastigophora in the Phylum Protozoa, is an extracellular haemoflagellate of the Lewisi group which, also, includes the human pathogen, *T. cruzi*. It is a widely distributed, non-pathogenic parasite of wild rats of the genus *Rattus*. The parasite is generally considered refractive to all other laboratory animals, though Laveran and von Mesnil (1901) claimed success with the guinea pig.

Morphology: *Trypanosoma lewisi* (Figure One) has a slender, flattened body approximately 30 microns long and pointed at both ends. A long flagellum, arising from the blepharoplast, extends along the body forming the outer boundary of the undulating membrane and continues anteriorly as a free flagellum. The nucleus tends to be central with a large karyosome.

Life Cycle: Transmission to the rat is through a wound contaminated with feces of the infected flea *Nosopsyllus fasciatus*. The life cycle of *T. lewisi* (Figure Two) in

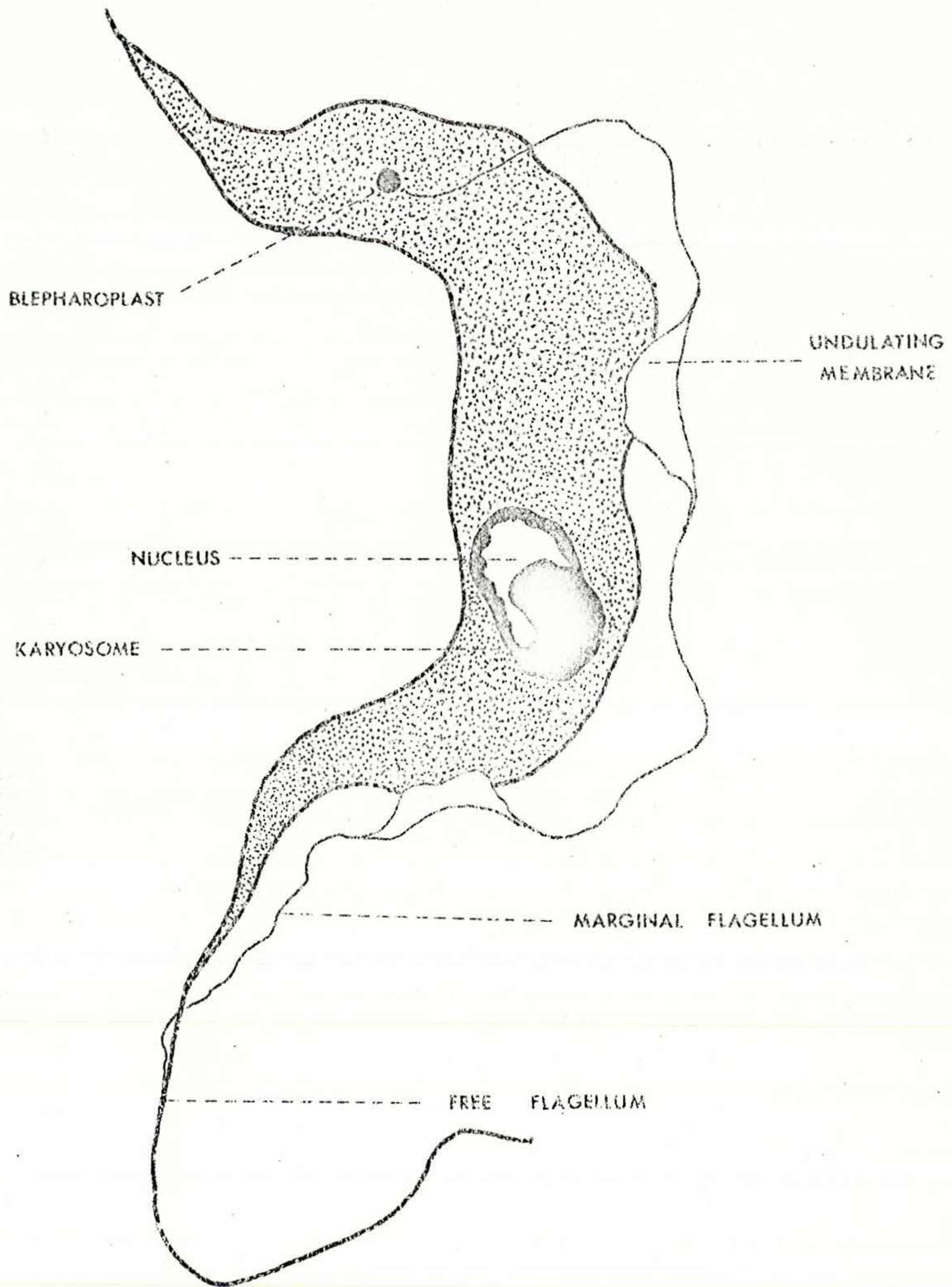


Fig I. Trypanosoma lewisi

the rat host involves three phases: first, a period of reproduction lasting four to six days followed by a second phase characterized by non-reproducing, stumpy adult forms. About the ninth or tenth day the parasites agglutinate in rosettes and a crisis occurs in which the majority of the parasites are destroyed. A third period of gradually declining population follows, interrupted after several weeks by a second crisis and the infection is terminated.

Trypanosoma lewisi can be maintained in the laboratory by weekly serial passage through the rat host. Though this species is readily cultured *in vitro* on blood agar, it can not be propagated beyond three sub-inoculations (Sanchez and Dusanic, 1968c).

Trypanosoma lewisi is a metacyclic parasite requiring transmission through the invertebrate host for completion of its life cycle. In the stomach of the flea, the trypanosomes undergo multiplication and morphological changes. As the trypanosomes pass into the hindgut and rectum, they assume the crithidial forms, a stage followed by the infective, metacyclic form which is discharged in feces.

Immunity: Immunity is quickly established in the rat and has been attributed to several factors which will be discussed later. Though *T. lewisi* is considered non-

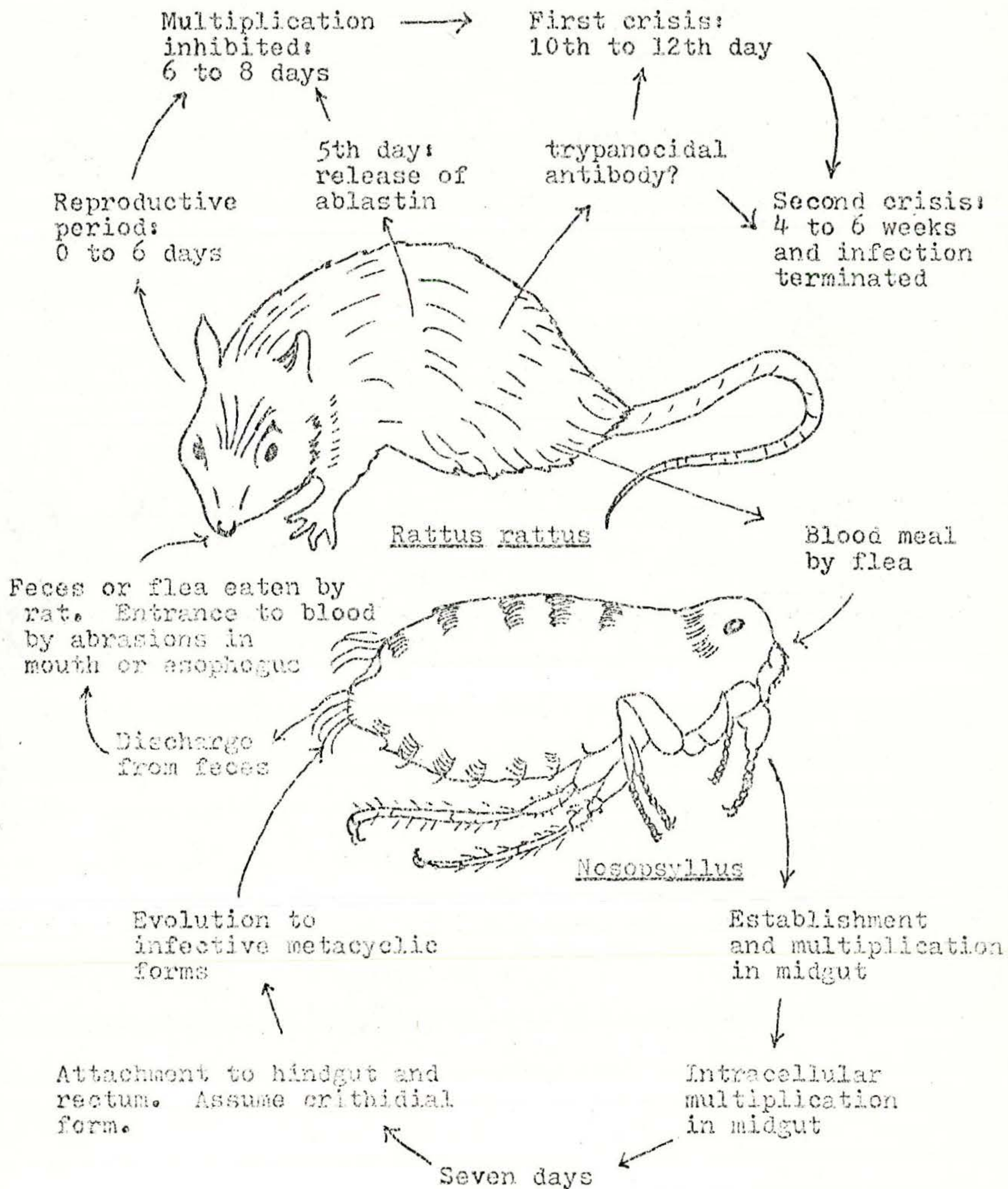


Fig 2. Life Cycle of Trypanosoma lewisi

pathogenic in rats, Lysenko (1951) noted that drug treatment with sodium salicyclate depresses the anti-reproductive activity of the antibody ablastin, reproduction continues, and the infection becomes pathogenic.

Biochemistry: In the blood stream, glucose is readily available to meet the energy requirements of the flagellate. As in most protozoan parasites, glucose is not completely oxidized to carbon dioxide and water. Von Brand (1966) noted that in aerobic respiration, acetic acid is the primary end product with some lactic and succinic acids. However, in anaerobic metabolism, lactic and succinic acids are primarily accumulated.

The exact metabolic pathways have not been established for any of the trypanosomes, and as von Brand (1966) pointed out, the trypanosomes can readily shift to alternate metabolic pathways. Ryley (1951) reported a cytochrome system for respiration of adult forms that is sensitive to cyanide. He noted that anaerobic respiration required extracellular carbohydrate and an unco-ordinated heavy metal. No direct evidence has been given for a phosphorylative mechanism for carbohydrate metabolism.

Von Brand (1966) noted that the mechanisms by which trypanosomes affect the host have not been established.

Production of toxins, changes in the host cell permiability, changes in enzyme levels, utilization of host nutrients, and mechanical blockage of vital capillaries have all been suggested.

REVIEW OF LITERATURE

A variety of antibodies have been assumed to be the cause of cessation of trypanosome infections in rats. Weitz (1962) stated that Francis (1903), who was among the first investigators, considered only agglutination as responsible for the crisis and subsequent elimination of the parasites from the host.

The classic studies on antibody production to *T. lewisi* were done by Taliaferro between 1924-1938. *Trypanosoma lewisi* is known to undergo rapid reproduction up to the sixth day. Either before or shortly after the first numbers crisis, reproduction entirely ceases and the stumpy adult forms are the most evident. Taliaferro (1924) attributed the inhibition of reproduction to a specific antibody which he termed ablastin. He suggested that actually three antibodies are involved in the complete elimination of trypanosomes from the rat host: ablastin and two trypanocidal antibodies. First to appear on about the fifth day is the reproduction-inhibiting antibody (ablastin) which maintains itself until the thirty-fifth day. Next, a trypanocidal antibody is produced and assumed to be responsible for the agglutination

of the parasite and the subsequent crisis on approximately the tenth day. A second type of trypanocidal antibody accounts for the final elimination of the parasites several weeks later.

Taliaferro (1932) characterized ablastin as a globulin, suggested that ablastic immunity is not abolished in immune sera by absorption with living parasites, and that ablastin does not kill the parasites or affect their motility, vitality, or infectivity. Taliaferro (1938) further suggested that the terminal antibody may be a lysin.

Coventry (1930) supported Taliaferro's report of two trypanocidal antibodies and demonstrated by titration methods that there were differences in antibody concentrations during the course of an infection.

Culbertson (1941) stated that only two antibodies were involved: the typical ablastin and a lysin.

According to Weitz (1962), Augustine (1943) hypothesized that antibodies of the agglutinin and opsonin types rendered the dividing trypanosomes sensitive to phagocytosis and agglutinated the adult forms, leaving their removal to the mechanics of the liver and spleen.

Moulder (1948) correlated the appearance of ablastin and the resulting cessation of reproduction of the trypanosomes with the qualitative and quantitative changes in glucose metabolism of the trypanosomes. He found that the ablastin-inhibited parasites utilized less glucose than those that were reproducing freely, and showed that not only were adult forms of T. lewisi capable of oxidizing glucose more efficiently, but that the trypanosomes were able to oxidize substrates other than glucose such as glycerol, lactate, and glutamate. Moulder also noted that while the rate of glucose utilization decreased, the rate of oxygen consumption increased with the age of the infection. On the basis of these studies he concluded that the trypanocidal antibody does not interfere with or affect the glucose metabolism of surviving trypanosomes and that ablastin interferes with the oxidative metabolism of glucose. As a result, cell division and growth of T. lewisi are stopped.

Barnes (1951), while studying the effect of benzene and selected salicylates on the level of immunity to trypanosomes, reported three antibodies: ablastin, a trypanocidal, and an agglutinin.

Thillet and Chandler (1957) noted that ablastin, unlike the trypanocidal antibodies, does not sensitize

trypanosomes *in vitro* and can not be removed from immune serum by absorption with living parasites. They demonstrated that rats could be rendered completely refractory to *T. lewisi* infections even when given challenge doses of twenty million trypanosomes. Immunity was established by repeated injections of metabolic products which had been collected from trypanosomes suspended in a mixture of rat serum and saline for twenty-four hours. It was also demonstrated that the serum of rats immunized with metabolic products like that of rats recovered from a natural infection, caused the rapid agglutination of trypanosomes *in vitro*, whereas serum from rats in which immunization had been attempted by injection of dead, titrated trypanosomes had practically no agglutinating potency. This antigen, unlike the living parasite, was capable of absorption of ablastin from the serum. They identified the antigen as metabolic products which they termed ablastinogen.

Based on this work, Chandler (1958) stated that ablastin was solely responsible for the development of immunity in rats to *T. lewisi*. The inhibition of reproduction, the agglutination, and the final elimination of the trypanosomes can be explained entirely by ablastin working with phagocytosis and a possible lysin at the end. He stated that the low titers of ablastin occurring on

about the fifth day impede reproduction. This results from neutralization "by antibodies of excreted metabolic products, probably enzymes, which enable the parasites to avail themselves of some constituent or constituents of the environment that is required for growth and reproduction" (Chandler, 1958:134). As the titer of ablastin increases, a viscous antibody-antigen complex is formed about the trypanosomes causing them to agglutinate. Agglutination of the parasites leaves them susceptable to phagocytosis and hence, the first numbers crisis. He discredits a trypanocidal antibody basis for the first crisis by arguing that "there is no evidence of any appreciable number of trypanosomes having been destroyed to provide a trypanocidal antibody" (Chandler, 1958:131). With the reduced numbers of trypanosomes, final elimination is accomplished by normal phagocytosis and possibly an immobilizing antibody stimulated by the destruction of numerous trypanosomes at the first numbers crisis. He substantiated the presence of such an antibody by the work of Laveran and von Mesnil (1901) who obtained an immobilizing antibody from disintegrated trypanosome bodies.

Ormerod (1963) gave indirect evidence for the influence of antibody on T. lewisi. During the course of an infection with T. lewisi, morphological changes in shape and the appearance in large numbers of volutin granules coincide with the production of antibodies by

the host. Ormerod noted that antibodies are stimulated more by trypanosomes containing multiple granules than by those with a single granule.

D'Alesandro (1959) utilizing electrophoretic and ultracentrifuge techniques disagreed with the conclusions of Chandler (1958) and supported the concept that antibodies are distinguishable by their serological specificities and not by their physiochemical properties. D'Alesandro demonstrated the presence of at least two antibodies and quite possibly three. Ablastin and the early trypanocidal antibody appear together and migrate between the beta and gamma globulins. This would indicate a small molecular weight with a sedimentation coefficient of 6 Svedbergs. However, in the hyperimmune serum the large molecular weight 16S globulins increase. This was correlated with a terminal trypanocidal antibody. He noted that electrophoretic and ultracentrifuge analysis revealed no qualitative differences between normal and immune sera as to the number of components, but did show a definite rise in the gamma globulin levels and a decline in the albumin levels of immune sera.

Based on these findings, D'Alesandro hypothesized the following: the destruction of trypanosomes at the first crisis is incomplete. However, the titer of ablastin

continues to rise. If ablastin is the sole cause of destruction, then destruction of trypanosomes should continue as the titer rises. He considered the incomplete destruction to be due to the resistance of the survivors to the early trypanocidal antibody. Therefore, even though ablastin and the early trypanocidal antibody arise together, they should not be considered identical.

Pizzi and Taliaferro (1960) absorbed trypanocidal antibodies from serum known to contain ablastin and then passively transferred the ablastic serum to infected rats. Not only did reproduction cease, but the high rate of synthesis of proteins and nucleic acids was markedly reduced. By using the rate of incorporation of S^{35} amino acids into protein, they determined that the rate of protein synthesis is much higher in reproducing trypanosomes than in adult forms.

In a later study of ablastin, D'Alesandro (1962) brought out several points concerning its action. Ablastin is not complement-dependent since ablastic sera inactivated at 56°C for 20 minutes do not lose their inhibitory activity. He noted that in addition to physiological, morphological, and enzymological differences between blood stream and culture forms, an immunological one can

also demonstrated. Culture forms grown at room temperature and on media are not affected by ablastin. This would indicated a basic antigenic difference between them. He also attempted to duplicate the results of Thillet and Chandler (1957), but was unable to confirm their findings and suggested that due to the diverse nature of ablastin, more than one antigen may be involved, i.e., several related antibodies each directed toward a different but vital metabolic product.

Gray (1960:1059) noted that trypanosomes isolated from animals in which they had been exposed "to antibodies or to subcurative doses of drugs may be serologically different from the organisms used to infect the animals in the first place." In his study of antigenic variation in _T. brucei_, Gray (1962:11) noted that a series of antibody peaks coincide with the appearance of new antigenic variants. Samples of the variants were injected into other rabbits and they, too, produced their own "variant specific" antibodies. Gray was even able to passively immunize the rabbit to unwanted antigenic types and elicit a predetermined antigenic type. Also, Gray suggested that each variant has a major "antigenic component and smaller amounts of other antigens." Serum taken at the same time as a sample of trypanosomes would not agglutinate them, but serum taken from an older infection would.

Weitz (1962:183) concluded on the basis of Gray's work and his own research that "there appears to be no end to the number of antigenic variants and the consequent antibodies which can be produced from a single strain of trypanosomes. The rise in antibody coincides with the disappearance of the homologous variant and the production of a new variant immediately following the elimination of a given antigenic type." He concluded that this left no doubt "that the production of antigenically variable trypanosomes depends on the ability of an animal to produce antibody."

D'Alessandro and Sherman (1964) demonstrated that the level of lactic dehydrogenase (LDH) is reduced with the appearance of ablastin. They were unable to neutralize the enzyme directly with ablastin or show qualitative changes between dividing and inhibited trypanosomes. They concluded that ablastin has an indirect effect on enzyme levels of the parasite. However, they were not able to determine whether ablastin acts at many points along the synthetic pathways or only at a single key point and whether this activity occurs on the cell membrane or internally as a result of free passage of ablastin across the membrane. They also noted that the LDH levels of the plasma of the rat host were lower in the reproductive phase of the parasites but returned to normal upon

inhibition of the parasites.

Sanchez and Dusanic (1968b:361,369) noted alterations in the physiology and biochemistry of the parasite following the course of an infection of *T. lewisi*. They also tested the effect of metabolic inhibitors and 4, 8, 12, and 16 day post-inoculum plasma against 4, 8, 12, and 16 day infections with respect to respiration and glucose utilization. They found that "reproducing trypanosomes have primarily a glycolytic pathway, shifting to dependence on the tricarboxylic cycle at eight day post-inoculation and possibly back to glycolysis at 12 days." Since the levels of inorganic phophate and ATPase "correspond to the changes in respiration" while oxygen uptake and glucose utilization were differentially affected by the post-inoculation plasma, they concluded that the host's biochemistry may be influencing the parasites metabolism. However, they added, "that an alternate explanation can be proposed by taking into consideration possible antigenic alterations in the trypanosomes. The observation that glucose utilization and oxygen consumption were inhibited greatest by 16 day plasma may support this. Antibody is gradually being formed in higher amounts to this antigenic type of trypanosome. More complete antigenic analysis is necessary."

In another study, Sanchez and Dusanic (1968a:377)

noted that the metabolic changes in the parasite occur concurrently with physiological and biochemical alternations in the host during the course of an infection. Metabolic shifts might be the result of substrate and coenzyme levels in the host triggering regulatory mechanisms in the parasite. High inorganic phosphate and ATP-hydrolyzing enzyme levels during the peak of the parasitemia, the Pasteur effect, and "respiration control" would make it energetically more feasible for the parasite to assume oxidative metabolism at the height of the infection, while rapid reproduction favors anaerobic fermentation. Following the crisis, with much lower titers of parasites, the host returns to normal physiological balance of enzymes. Therefore, "metabolic changes that occur in _T. lewisi_ during infection may be manifestations of a changing environment."

If antibodies are produced in response to metabolic products, it should be possible to directly demonstrate this specificity through immunodiffusion and immunoelectrophoresis. In turn, they would provide information concerning changes in antigenicity of the trypanosomes in response to changes in the host and the trypanosomes.

The course of the infection will be viewed as having four stages: a 4 day infection where ablastin has

not appeared in the blood, the trypanosomes are respiring anaerobically, and multiplying rapidly; an 8 day infection just prior to the first numbers crisis where respiration is aerobic and reproduction has ceased; a 12 day infection in which the trypanosomes have returned to anaerobic respiration following the crisis; and a 16 day infection in which the titer of ablastin is high and the trypanosomes numbers are very small.

Blood taken at these four stages will be used for sera containing ablastin while the trypanosomes separated from the blood will be used for the production of metabolic products. Sera from 4-, 8-, 12-, and 16-day infections will be reacted with metabolic products from trypanosomes removed from a rat at day 4, 8, 12, and 16. If immunological specificity exists between serum and metabolic products or trypanosomal extracts, then precipitin lines should be demonstrated by Immunodiffusion and Immunoelectrophoresis.

MATERIALS AND METHODS

The strain of *Trypanosoma lewisi* used in this study was obtained from Dr. Gilbert Sanchez of the New Mexico Institute of Mining and Technology and was originally isolated by Dr. W. H. Taliaferro in 1951. The strain was maintained by weekly sub-inoculations into female, randombred Sprague-Dawley albino rats (Bio-Science Animal Laboratory, Oakland, California) provided by Dr. James Thompson of the School of Pharmacy, University of the Pacific. Rats weighing 150 to 200 grams were used for all work. All injections were made intraperitoneally from tail-vein blood diluted in 0.85% saline.

Three complete sets of 16 rats each were run, resulting in the utilization of 48 rats. Each set consisted of a 4 day sample, 8 day sample, 12 day sample, and a 16 day sample (Table One). On day 0, 16 rats were injected with 0.5cc of trypanosome-saline suspension. On the appropriate post-inoculation day, 5-6cc of blood were drawn by cardiac puncture from an etherized rat. For each sample, no less than three rats were used and the blood was pooled. One-half of the blood drawn was placed

in a sterile tube (A) and set aside as a source of trypanosomal antibodies and one-half of the blood was added to a tube containing heparinized saline (Tube B) and used as a source of trypanosomes. (Table Two).

Collection of Sera (Tube A)

Blood was drawn by cardiac puncture from rats sacrificed on the appropriate post-inoculation day, pooled, and allowed to clot for one hour at room temperature. The sample was then placed in the refrigerator overnight.

Serum was separated from the clot the following day by centrifugation at 2250G for 15 minutes in the Sorvall Refrigerated Centrifuge, Model RC2 and kept in the frozen state until needed.

The same process was followed for obtaining normal sera.

Collection of Trypanosomes (Tube B)

The sample containing the trypanosomes was added to 2.5cc of 0.85% saline containing .5cc heparin. Trypanosomes were separated from infected blood by centrifugation at 75G for 15 minutes. The trypanosomes remained in the cloudy supernatant or in the white layer over the sedimented red blood cells. The supernatant was pipetted into a centrifuge tube, resuspended in saline, and sedimented

at 2250G for 15 minutes. The parasites were washed twice in physiological saline and resuspended in equal parts of normal rat serum and saline.

Preparation of Metabolic Products

All suspensions were incubated for 24 hours, one set (to be known as Set I) at 27°C and two sets (to be known as Set II and Set III) at 33-37°C. After incubation the trypanosomes were sedimented by centrifugation for 15 minutes at 2250G and the supernatant containing the metabolic products was filtered and stored in the frozen state until needed.

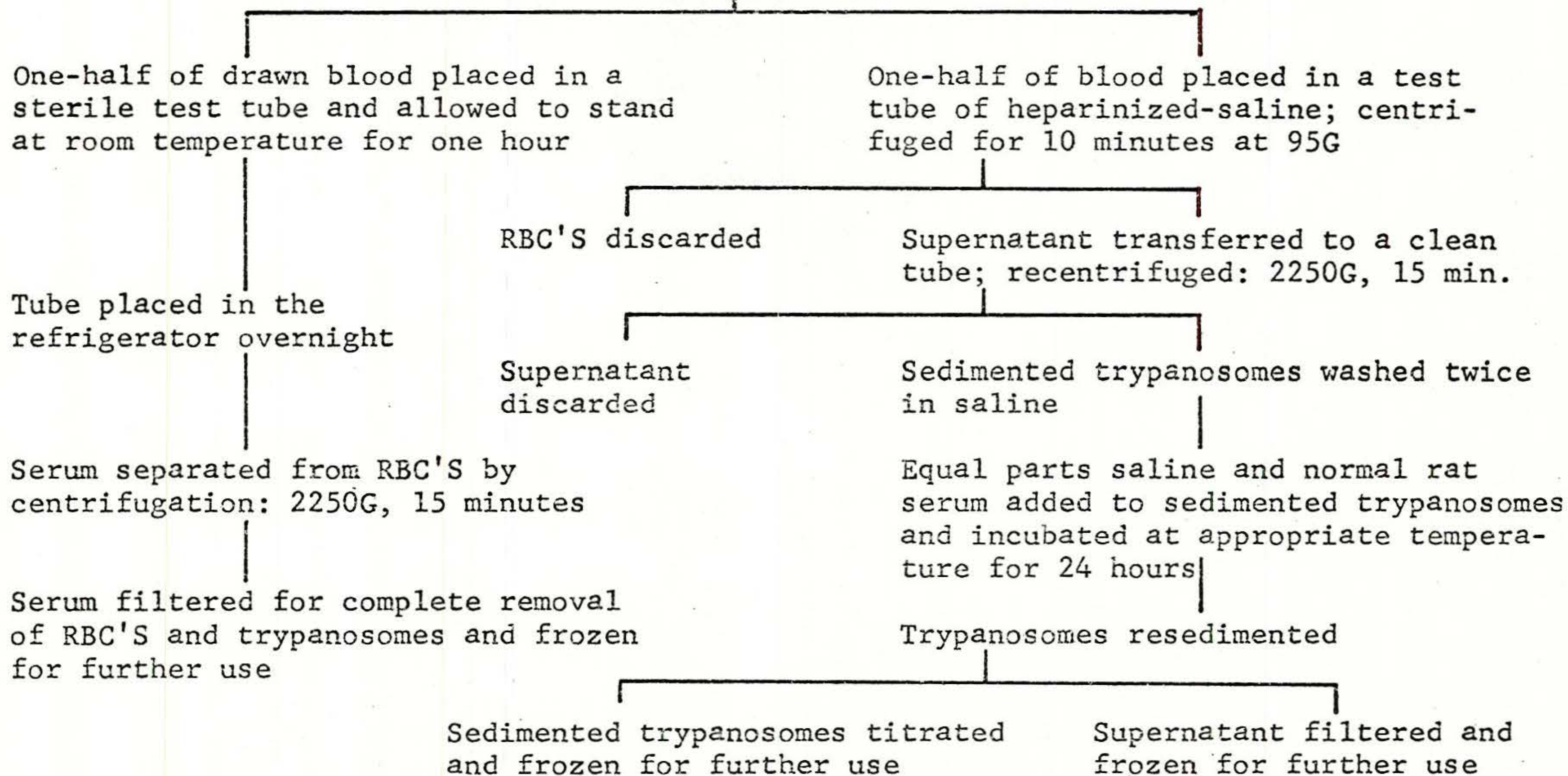
Preparation of Trypanosome Extract

The sedimented trypanosomes were washed twice and resuspended in saline. Following the method of D'Alesandro and Sherman (1964), the suspensions were alternately frozen and thawed three times, centrifuged in the cold for 20 minutes at 12,800G, and stored in the frozen state until needed. Only one set of trypanosomal extracts was collected. This was prepared from set III.

Table 1. Time Table for Sampling

Day	Number of Rats Inoculated per Set				
0	Rat	1 2 3 4	5 6 7 8	9 10 11 12	13 14 15 16
1					
2					
3					
4					
5		Rats sacrificed;			
6		blood drawn			
7		and pooled			
8					
9			Rats sacrificed;		
10			blood drawn		
11			and pooled		
12					
13				Rats sacrificed;	
14				blood drawn	
15				and pooled	
16					
					Rats sacrificed;
					blood drawn
					and pooled

Table 2. Flow Sheet



* This same process was repeated for blood drawn from rats in their 8th, 12th, and 16th day of infection.

Immunoelctrophoresis

A Millipore Immuno-phoroslide system was used. 0.8 microliter of serum was deposited in the center well and electrophoretically separated for 18 minutes at 100 volts. Thirty microliters of antigen were then added to the side trough and allowed to diffuse for one hour in a diffusion chamber at room temperature, followed by the addition of an equal amount of the same antigen to each trough and diffusion for 48 additional hours.

After 48 hours, slides were removed from the diffusion chamber and washed for 90 minutes at 40 ^{o}C in physiological saline to remove unreacted proteins. Saline solutions were changed every 30 minutes. Washed slides were transferred to staining trays containing a solution of dilute Nigrosine and allowed to stain for 4 to 12 hours.

Unreacted stain was removed by three one-minute washes in 5% acetic acid. Following the final acetic acid rinse, the strips were rendered transparant by rewetting with isopropanol and immediately immersed in clearing solution (30ml ethyl acetate and 70ml acetic acid) for one minute.

Each antibody sample (4, 8, 12, and 16 day serum) from Set I was cross reacted with 4, 8, 12, and 16 day metabolic products from SetI. The same was done for

set II. The sera samples from Set III were reacted against both 4, 8, 12, and 16 day metabolic products and 4, 8, 12, and 16 day trypanosomal extracts. Immunoelectrophoretic tests of 4 day metabolic products against sera were repeated in both Set II and Set III.

Controls using clinical human sera and anti-sera were run with each set to insure accurate technique and proper functioning of equipment.

Immunodiffusion

The standard Immunodiffusion set, Model 6800A-7 manufactured by LKB, Sweden, was used.

Solutions:

Agar Solution - Difco Purified Agar, Sodium Chloride and distilled water in the proportions 1:1:98 and merthiolate (1:10,000) as a preservative.

Impregnation Agar - 0.1% Purified Noble Agar and 0.05% glycerine in distilled water.

Sodium Chloride Solution - 1% NaCl dissolved in distilled water.

Rinsing Solution - methyl alcohol, acetic acid, and water in the proportions 45:10:45.

Staining Solution - concentrated Nigrosine in 2% acetic acid.

A hot film of impregnation agar was applied to glass slides, placed in a frame, and allowed to air dry. Frames were then placed on a levelling table and 10cc of hot agar evenly distributed across each frame. After allowing the frames to set in a humid chamber for thirty minutes, holes were punched in the gell layer in the appropriate pattern. Excess agar was removed from the wells with a pipette. Sera and antigen were added to the wells with a micropipette and the frames were incubated in the humid chamber for up to 48 hours. All frames were periodically checked for signs of a reaction.

After 48 hours, excess proteins were washed off by immersion in 1% NaCl for six hours, followed by re-immersion in a fresh bath of 1% NaCl for 16 hours and a final rinse for one hour in distilled water. Frames were air dried, stained for 12 hours in dilute Nigrosine, and rinsed in four baths of rinsing solution for ten minutes each. Slides were then removed from the frames, allowed to air dry, and examined. As with the immuno-electrophoretic tests, controls were run with human sera and anti-sera to check equipment and technique.

<u>Alternate Methods for Immunodiffusion</u>

1. One test of 4 day metabolic products against 4, 8, 12, and 16 day sera was carried out in agar prepared without the addition of NaCl. incubated at

room temperature for 48 hours, and examined periodically.

2. For one test of 4 day metabolic products against 4, 8, 12, and 16 day sera, frames were incubated for 48 hours at 30°C while a second frame was incubated for 48 hours at 37°C.

3. The constant-feed double diffusion technique of Crowle (1961) was applied to one frame of 4 day metabolic products reacted with sera. Serum was continually added for 24 hours to the serum well as soon as it had diffused into the agar medium. If no reaction occurred after the last addition of serum, the frame was re-incubated for an additional 24 hours with periodic checks for a reaction.

4. If no reaction had occurred in 48 hours, one frame was soaked in a weak cationic solution (0.0125% cadmium acetate in physiological barbital buffer) for ten minutes in the event that the solution might enhance the visibility of the precipitation bands should there be any.

RESULTS

All rats inoculated with 0.5cc of trypanosome-saline suspension readily took the infection. Sub-inoculations made between day 4 and day 12 yielded viable infections. Rats so infected proved refractive to further attempts at a later date to reintroduce the parasite.

Immunodiffusion

No antibody-antigen reaction bands were demonstrated by any of the methods or variations attempted (Table Three).

Treatment with cadmium ions which will on occasion reveal hidden precipitin lines yielded large amounts of non-specific precipitation with the diffused sera but no distinct precipitation lines. Staining also showed the presence of non-specific fixing of stain with proteins that for some reason were not removed with washing even when the washing time was increased.

All controls with human blood, however, showed clear and definitive precipitation bands.

Immunoelectrophoresis

Electrophoresis of antigen in saline shows one homologous band moving slightly behind the albumin fraction.

In all immunoelectrophoretic tests of all combinations of 8, 12, and 16 day serum, no definitive precipitation bands were shown (Table 3).

Several unusual results were noted, however, when 4 day metabolic products were reacted with 8, 12, and 16 day serum. One small, faint, and short band occurred in the region of the gamma globulins and was strongest with the combination of 4 day metabolic products and 16 day serum (Plate 1). These bands occurred in only two of the six tests run on these combinations.

In all of the tests run with 4 day metabolic products and 8, 12, and 16 day serum, non-specific precipitation bands appeared at the positive end of the trough in the region of the albumin bands. Normally one heavy band was noted, but on two occasions with 4 day metabolic products and 16 day serum, two bands could clearly be seen. These bands occurred on a few of the tests run with 8 day metabolic products, but on none of the samples of 12 and 16 day metabolic products.

Immunoelctrophoresis of trypanosomal extract with serum revealed a single faint band in approximately the same position as those found with metabolic products. The reaction occurred only between 4 day trypanosome extract and 16 day serum (Plate One).

Table 3. Results

Immunodiffusion

Set I:

sera	metabolic products 4	8	12	16
4	-	-	-	-
8	-	-	-	-
12	-	-	-	-
16	-	-	-	-

Set II:

sera	metabolic products 4	8	12	16
4	-	-	-	-
8	-	-	-	-
12	-	-	-	-
16	-	-	-	-

Set III:

sera	metabolic products 4	8	12	16
4	-	-	-	-
8	-	-	-	-
12	-	-	-	-
16	-	-	-	-

sera	trypanosomal extracts 4	8	12	16
4	-	-	-	-
8	-	-	-	-
12	-	-	-	-
16	-	-	-	-

Immunoelectrophoresis

Set I:

sera	metabolic products 4*	8	12	16
4	-/-	-	-	-
8	-/-	-	-	-
12	-/-	-	-	-
16	-/-	-	-	-

Set II:

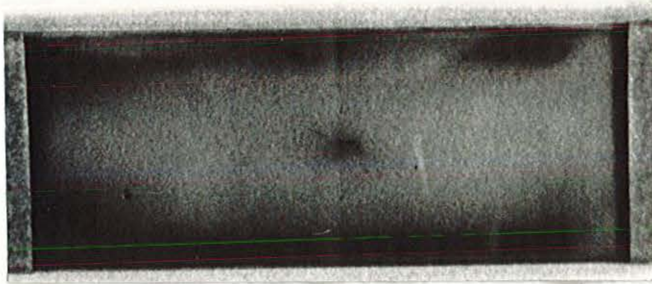
sera	metabolic products 4*	8	12	16
4	-/-	-	-	-
8	+/-	-	-	-
12	+/-	-	-	-
16	+/-	-	-	-

Set III:

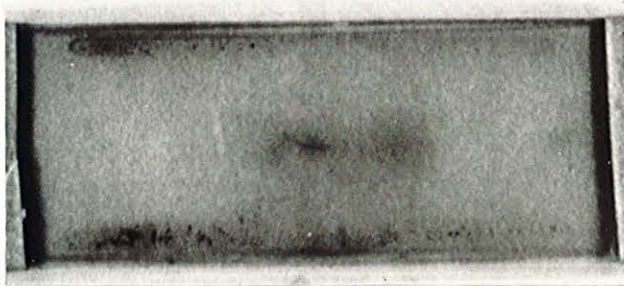
sera	metabolic products 4*	8	12	16
4	-/-	-	-	-
8	+/-	-	-	-
12	+/-	-	-	-
16	+/-	-	-	-

sera	trypanosomal extract 4*	8	12	16
4	-/-	-	-	-
8	-/-	-	-	-
12	-/-	-	-	-
16	+/-	-	-	-

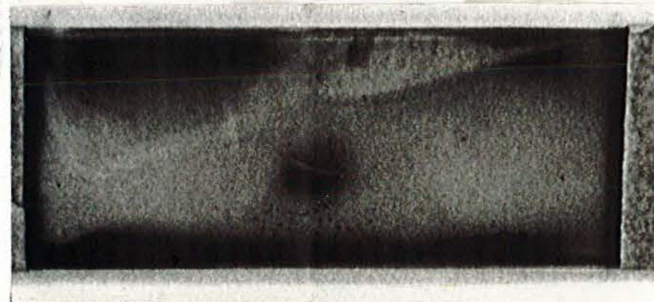
* These sets were run twice, results from both tests indicated by the slashed line.



4 Day Metabolic Products Against 8 Day Sera



4 Day Metabolic Products Against 12 Day Sera



4 Day Metabolic Products Against 16 Day Sera

4 Day Trypanosomal Extract Against 16 Day Sera

Plate 1. Photographs of Immunoelectrophoretic Slides

DISCUSSION

The role of ablastin has been eluding researchers for over fifty years. It has been called the "mediator" of metabolic alterations by some authors (Sanchez and Dusanic, (1968b:377) and its effects on both glucose metabolism (Moulder, 1948) and protein and nucleic acid synthesis (Taliaferro et al, 1958) have been noted. However the mechanism of action and the specific metabolites to which it is directed remain unknown.

Though the results of this research have not elucidated the role of ablastin or the metabolites to which it is directed, they do conform to our present knowledge about the antibody and its possible antigenic instigator. Except for the immunoelectrophoretic reaction in two tests of 4 day antigen with 8, 12, and 16 day serum and in two tests of 4 day trypanosomal extract with 16 day serum, there were no other reactions.

Based on physiochemical and immunological properties, immunoelectrophoresis and immunodiffusion have become two of the most important contributions to the

characterization of antibodies. Immunoelectrophoresis goes one step farther than immunodiffusion to allow the simultaneous analysis and separation of serum components. There are, however, limits to both techniques. Normally, as Crowle (1960:162) pointed out, specific precipitation occurs in two stages: first, combination of specific antibody and antigen, and secondly, aggregation of the complex into visible precipitate. However, "the first stage of the antigen-antibody complexing seems to occur under broader experimental conditions than does the second." Certain types of antibody form complexes within a very narrow range of antibody-antigen ratios and are readily soluble in an excess of either the antigen or the antibody. Also, it has been noted by Crowle that the reaction is more governed by the quantity of antibody available for reaction than the antigen concentration, salt concentration, or non-antibody serum constituents. However, even when varying the amount of antibody used, it was not possible in this investigation to determine the proper ratio for complexing.

Crowle (1961:227) has further noted that "when antigen-antibody aggregates grow to a certain size in agar, they are fixed *in situ* by their very size, being unable to diffuse through the spaces in the network of the supporting medium." However, these aggregates remain invisible.

Though these invisible aggragates may be revealed by specific staining, the enhancing effect of cations, interferometric scopes, or radioactive labelling, they often will elude the most sensitive techniques.

There is the possibility that these limitations apply to the reaction of ablastin and metabolic products. As previously mentioned, D'Alesandro (1959) found that ablastin migrated between the beta and gamma fractions of immune serum. The lack of a visible precipitate may be due to this migration of ablastin in the beta range which in many cases is known for not giving a visible precipitate (Crowle, 1960).

The fact that bands formed in only two of the tests run and only with 4 day antigen against 8, 12, and 16 day serum is not entirely surprising. In two of the 8 tests run, metabolic products were obtained from trypanosomes incubated at 27.5 °C as in the original work of Thillet and Chandler (1957). However, D'Alesandro (1962) has shown that ablastin is not effective against trypanosomes incubated at a temperature below 30 °C since trypanosomes at these temperatures are immunologically and metabolically different from blood stream forms. Therefore, the two tests run on room-temperature-metabolic products were negative as expected.

Why were four of the remaining tests negative? Crowle (1960:173) has elucidated this point in stating that antibody to a given antigen is "discouragingly heterogeneous, as is responsiveness itself to antigens. These antigens may provoke formation of no antibody, antibody of one kind, or antibody of several kinds." Therefore, not only can antibody produced by a single animal vary in its specificities, but antibody produced from different animals of the same species can be of a precipitating type in one case and a non-precipitating type in another.

The fact that a reaction occurred only with 4 day antigen and 8, 12, and 16 day sera does not mean that a reaction is not possible with metabolic products from later samples. However, as Sanchez and Dusanic (1968a) pointed out, the respiration of 8, 12, and 16 day trypanosomes indicates that they are metabolically different from the rapidly multiplying, young infection. It might be expected, then, that the antigen to which ablastin is directed is no longer present, in very small amounts, or in a state of neutralization. It was noted that the reaction appeared greater with the 16 day serum and not at all with the 4 day serum. Sixteen day serum represents hyperimmune serum, the antibodies of which are capable of detecting more determinant groups on an antigen and are, therefore, more sensitive to similar constituents in different

antigenic mixtures (Grabar, 1959).

Present research would indicate that if metabolic products are antigenic, they are not strongly antigenic. Since faint bands appeared for trypanosomal extract and serum, it would seem more likely that they work in conjunction with other metabolites and it is highly unlikely that the metabolic products reported by Thillet and Chandler (1957) are the exclusive instigators of ablastic production. Their cumulative effect is an antigenic response on the part of the animal. Therefore, it will be difficult to isolate any one portion without greater knowledge of the specific metabolites involved.

The successful neutralization of ablastin with an antigen produced by the _in vitro_ incubation of living trypanosomes in normal rat sera may be more a result of the methods used than any real antigenic powers on the part of metabolic products. As already suggested by Grabar (1959), the injection of antigen causes the formation of antibodies able to react more avidly and against more determinant groups on an antigen than antibodies provoked by normal immunization. Thillet and Chandler (1957) made six intraperitoneal injections at three day intervals from 4 billion trypanosomes. The striking results could be a result of such hyperimmunization and quite

possibly not in proportion to the normal role played by metabolic products in the stimulation of ablastin in the rat. Crowle (1960:162) observed in his own research that "antibody capable of reacting with antigen but not precipitating it can be formed by animals in response to the injection of antigen." It has, also, been found by Atsumi et al (1969) while doing research on the combining sites of gamma globulin and macroglobulin that antibodies produced in response to a single determinant on an antigen will in time not only require less time to respond but will be able to respond to larger portions and more determinant groups of the same antigen.

As D'Alesandro pointed out, ablastin works through diverse inhibitory actions suggesting the activity of more than one antigen. Ablastin, then, would be the composite of several related antibodies directed to common or similar determinants on different metabolites. Therefore, ablastin can be effective against protein synthesis, nucleic acid synthesis, and glucose metabolism by attacking common determinant sites on diverse metabolites necessary in some way to all three processes. From this, it might be deduced that ablastin does not have a direct effect on enzyme systems, but intervenes at several points along the synthetic pathways. Quite probably, it does not discriminate as to the synthetic pathway, but merely to the metabolite or determinant site on the metabolite involved.

SUMMARY

Immunoelectrophoresis revealed an antigen-antibody response between 4 day metabolic products and 8, 12 and 16 day sera and between 4 day trypanosomal extract and 16 day serum.

Metabolic products from trypanosomes incubated at room temperature do not appear to be antigenic.

The limitations of immundiffusion are discussed in reference to the results. It is suggested that some of the antibodies to metabolic products may be of the precipitating type while others are not.

Since a faint reaction also occurred between 4 day trypanosomal extract and 16 day serum, it may be concluded that metabolic products contribute to only a portion of the antibody response of the rat and are by no means the exclusive angents. They possibly work in conjunction with other metabolites within or on the surface of the trypanosome.

LITERATURE CITED

Atsumi, T., Adachi, M., Kinoshita, Y., Kawasaki, M., and Horiuchi, Y. 1969. The heterogeneity of combining sites of anti-benzylpenicilloly antibodies obtained from individual rabbits: changes in combining sites of yG and yM antibodies during the immune response. J. Immun. 101:1016-1022.

Augustine, D. L. 1943. Some factors in the defense mechanism against reinfection with Trypanosoma lewisi. Proc. Amer. Acad. Sci. 75:85-93.

Barnes, E. A. 1951. Effect of benzene and six selected salicylates on the development of immunity in Trypanosoma lewisi infection and on various aspects of the blood picture. Iowa State Coll. J. 26:1-17.

Chandler, A. C. 1958. Some considerations relative to the nature of immunity in Trypanosoma lewisi infections. J. Parasit. 44:129-135.

Coventry, R. A. 1930. The trypanocidal action of specific antiserums on Trypanosoma lewisi in vivo. Am. J. Hyg. 12:366-380.

Crowle, A. J. 1960. Immunodiffusion Tests. Ann. Rev. Microbiol. 14:161-176.

__________. 1961. Immunodiffusion. Academic Press, Inc. New York. 333 pages.

Culbertson, J. T. 1941. Immunity Against Animal Parasites. Columbia University Press, New York. 274 pages.

D'Alesandro, P. A. 1959. Electrophoretic and ultracentrifuge studies of Trypanosoma lewisi. J. Inf. Dis. 105:76-95.

__________. 1962. In vitro studies of ablastin, the reproduction-inhibiting antibody to Trypanosoma lewisi. J. Protoz. 9:351-358.

__________ and Sherman, I. W. 1964. Changes in lactic dehydrogenase levels of *Trypanosoma lewisi* associated with appearance of ablastic immunity. Exp. Parasit. 15:430-438.

Francis, E. 1903. An experimental investigation of *Trypanosoma lewisi*. Hyg. Lab. Bull. 11:26pp.

Grabar, P. 1959. In *Methods of Biochemical Analysis*, Edited by D. Glick, Interscience Publications, New York.

Gray, A. R. 1960. Precipitating antibodies in trypanosomiasis of cattle and other animals. Nature (London) 186:1058-1059.

__________. 1962. The influence of antibody on serological variation in *T. brucei*. Ann. Trop. Med. Parasit. 56:4-13.

Laveran, A. and von Mesnil, F. 1901. Recherches morphologique et experimentales sur le trypanosome des rats. Ann. Inst. Pasteur. 15: 673-714.

Lysenko, M. G. 1951. Concerning salicylate inhibition of ablastic activity in *Trypanosoma lewisi* infection. J. Parasit. 37:535-544.

Moulder, J. M. 1948. Changes in the glucose metabolism of *Trypanosoma lewisi* during the course of infection in the rat. J. Inf. Dis. 83:42-49.

Ormerod, W. E. 1963. In *Immunity to Protozoa*, Edited by P. C. Garnham, A. E. Pierce, and I. M. Roitt, Blackwell, Oxford.

Pizzi, T. and Taliaferro, W. H. 1960. A comparative study of protein and nucleic acid synthesis in different species of trypanosomes. J. Inf. Dis. 107:100-107.

Ryley, J. F. 1951. Studies on the metabolism of Protozoa. Biochem. J. 49:577-586.

Sanchez, G. and Dusanic, D. 1968a. Respriatory activity of *Trypanosoma lewisi* during several phases of infection in the rat. Exp. Parasit. 23:361-370.

____________. 1968b. Trypanosoma lewisi: creatine phosphokinase, ornithine carbamyl transferase, ATPase, Pi, and glucose levels in the rat host. Exp. Parasit. 23:371-377.

____________. 1968c. Growth of the bloodstream form of Trypanosoma lewisi in vitro. J. Parasit. 54:601-605.

Taliaferro, W. H. 1924. A reaction product in infections with Trypanosoma lewisi which inhibits the reproduction of the trypanosomes. J. Exp. Med. 39:171-190.

____________. 1932. Trypanocidal and reproduction-inhibiting antibodies to Trypanosoma lewisi in rats and rabbits. Am. J. Hyg. 16:32-64.

____________. 1938. The effects of splenectomy and blockade on the passive transfer of antibodies against Trypanosoma lewisi. J. Inf. Dis. 63:98-111.

____________, Pizzi, T. and D'Alesandro, P. A. 1958. Lytic and reproduction-inhibiting antibodies against T. lewisi. Science 127:1063.

Thillet, C. and Chandler, A. C. 1957. Immunization against Trypanosoma lewisi in rats by injection of metabolic products. Science 125:346-347.

Von Brand, T. 1966. Biochemistry of Parasites. Academic Press, Inc., New York. 429 pages.

Weitz, B. 1962. In Drugs, Parasites, and Hosts, Edited by L. G. Goodwin, R. H. Nimmo-Smith, Churchill, London.