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ANTIGENIC RELATIONSHIPS IN AMERICAN TRYPANOSOMES

CHAPTER I

INTRODUCTION

Trypanosoma (Schizotrypanum) cruzi*, a hemoflagellate of man was discovered by Chagas (1909a, b, c). Following the original communication a series of papers was published describing the clinical symptoms (Chagas, 1910), the life cycle (Chagas, 1911a), and the pathology of the disease (Vianna, 1911).

T.(S.) cruzi is the agent of American trypanosomiasis or Chagas' disease. Its geographical distribution is confined to the American continent where it is endemic in a wide area. In lower animals the parasite has been found from Washington, D.C. to southern Argentina, and in man from the southern U.S. to Argentina. Where the infection is of human importance, the vector, a reduviid bug, lives in close contact with man. Reduviid bugs acquire the parasite while feeding on infected vertebrate hosts, and harbour it in their digestive tracts with no apparent harmful

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*The scientific name of the trypanosomes here and in the following pages is that proposed by Hoare (1966).

effects. Cyclical development of the flagellate takes place in the stomach and intestine of the invertebrate host giving rise to the infective stages which are passed in the insect's feces. A susceptible host will normally acquire the infection through contamination of the mucosa or the damaged skin with feces excreted by the bugs during the feeding process. Inside the new host the parasite invades the cells, transforms into the amastigote* form, and begins multiplying. Any cell type of the vertebrate provides a good environment for the intracellular life cycle of the flagellate. Reticulotropic (Dias, 1932; Badinez, 1945; Pizzi, 1945), neurotropic (Villela and Villela, 1932), and myotropic strains (Badinez, loc. cit.; Pizzi, 1945) have been reported. In these instances the organisms may occur more frequently in certain tissues, but other cell types can also be invaded (Pizzi, 1957).

Following intracellular multiplication the organisms pass into the blood stream as trypomastigotes. In the blood the parasite occurs as slender and stumpy forms. There is much controversy concerning the significance of this dimorphism, as pointed out by Silva (1959). Explanations include sexual differences of the parasite (Chagas, 1909a), age of the parasite (Brumpt, 1912), different sites of development (Wood, 1953), and host body temperatures (Trejos et al. 1963). No experimental evidence is

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* Life cycle stages of the family Trypanosomatidae are named here as proposed by Hoare and Wallace (1966). The former leishmania, leptomonad, crithidia, and trypanosome stages are called amastigote, promastigote, epimastigote, and trypomastigote, respectively.

available concerning these two blood forms of T.(S.) cruzi. African trypanosomes of the Trypanosoma (Trypanozoon) brucei group show a similar dimorphism, and each type occurs at a different time in the life cycle. The stumpy ones, considered to be more "mature" develop in the insect while the slender ones do not (Vickerman, 1965). Also, observed physiological differences in the aerobic metabolism of the two forms correlate with variations in ultrastructure of the kinetoplast-chondriome system (Vickerman, loc. cit.; Vickerman and Cox, 1967).

T.(S.) cruzi infection in man can be either asymptomatic or symptomatic. The symptomatic phase has an acute period manifested by fever, adenitis, edema, a lesion at the point of entry, mild liver and spleen enlargement, acute myocarditis, and occasionally death (Amato-Neto, 1958). The portal of entry varies, and the best known one is through the conjunctiva, producing edema of the soft parts of the eye. This is known as the Romana sign. In most cases the patient survives and the chronic stage of the disease follows with manifestations of deterioration of the autonomous nervous system, of which one of the best known clinically is chronic cardiac damage, or Chagas' cardiopathy.

The physiopathology of the chronic damage in Chagas' disease has been explained in several ways. The classical theories refer to mechanical cellular damage (Vianna, 1911), and to toxins liberated by the parasite (Chagas, 1911b), though no experimental evidence for these theories, especially that concerning toxins, is available today (Alencar

and Elejalde, 1959; Musacchio and Meyer, 1959; Jorg, 1964). An allergic factor has also been suggested (Chagas, 1934; Okumura et al. 1960, 1962, 1963), but it is not accepted by all workers (Koberle, 1968). Autoantibodies against heart muscle in chagasic patients have been demonstrated (Kozma, 1962), but convincing evidence for an autoimmune mechanism of cellular damage requires further documentation. The diversity of ideas explaining the physiopathology of the infection indicates that the problem is a difficult one, and yet to be solved. In essence, the sequelae of T. (S.) cruzi infection in man and animals are due to a diminution in the number of ganglion cells in the central or peripheral nervous system or both (Koberle and Nador, 1956; Koberle, 1957, 1958, 1960, 1968; Koberle and Penha, 1959; Koberle and Gomez-de-Alcantara, 1960). These authors advocate Chagas' idea that a toxin liberated by the amastigotes during the acute phase of the infection is responsible for the physiopathology of the disease. Damage with the actual reduction in number of ganglionic cells affects the tone of the smooth muscle and results in enlargement of the hollow viscera. A high incidence of enlargement of the esophagus, stomach, colon, and other organs as well as disturbances in the motility of the hollow muscular organs is common in T. (S.) cruzi endemic areas (Koberle, loc. cit.). Heart pathology also results from damage to the ganglionic cells, and it is manifested as a chronic cardiopathy, with heart enlargement and A-V or intraventricular blockage (Laranja, 1956).

After the discovery of T. (S.) cruzi, the significance of the para-

site as a cause of human illness was overestimated. Several pathological conditions, poorly understood at the time, such as mental retardation, paralysis, hypothyroidism and asthenia, were erroneously attributed to the parasite (Clark-Bacellar, 1963). This fact led to much skepticism about the actual pathogenicity of the newly discovered protozoan, however certain important contributions to our knowledge of diagnosis and pathology of this disease were made by the early workers. Guerrero and Machado (1913) adapted the Bordet-Gengou test (CF) for serological diagnosis of the infection, using extracts from spleen and liver of parasitized animals as antigen. Brumpt (1914) described xenodiagnosis as a method for detecting the parasite. Vianna (1911) described the pathology of the disease in man, discovered the curious intracellular life cycle of the parasite, and pointed out that reproduction occurs in the amastigote form. Also, the cardiac involvement both in acute and chronic phases was recognized by Chagas and Villela (1922).

The public health problems presented by the parasite were overlooked by early workers. Today this is a well recognized point, but the economic impact of the infection (both in loss of productivity and patient care costs) on the Latin-American countries is still unknown (World Health Organization, 1960, 1969a). In acute cases a mortality of 10% has been reported (Laranja et al. 1956; WHO, 1960), but no accurate estimates of morbidity and mortality of the chronic illness are available today. It has been mentioned that there are at least 7 million people infected in the

endemic areas (WHO, 1960) but public health authorities are studying the feasibility of carrying out extensive surveys to determine accurately this figure, and the geographic distribution of the parasite.

In 1920 Tejera described another flagellate from Venezuela from the intestinal contents of Rhodnius prolixus, a vector of T. (S.) cruzi. This parasite, named T. (H.) rangeli, was later found to infect man (Leon, 1942, 1949; Pifano et al. 1948; Hernandez-de-Paredes et al. 1949) and to have a life cycle different from that of T. (S.) cruzi. Tejera's description of the organism (loc. cit.) was incomplete since he reported only on the intermediate forms found in the invertebrate. It is not surprising that several authors later described the same parasite under different names, such as T. guatemalense (Leon, 1946) and T. ariarii (Groot et al. 1951a). It has also been mentioned (Yorke, 1920) that a trypanosome from Perú identified as T. (S.) cruzi (Escomel, 1919) has morphological characteristics which enable the establishment of a new species, T. escomeli (Yorke, loc. cit.). This flagellate also corresponds to T. (H.) rangeli. In animals, especially American monkeys, rangeli-like flagellates have also been described under different specific names (for review, see Aeledon, 1954; Groot, 1954).

The life cycle of this protozoan was studied in its main vector, R. prolixus (Groot, Renjifo-Salcedo, and Uribe-Piedrahita, 1951b; Groot, 1952a,b,c,d; Groot, Osorno, and Escobar, 1952; Groot and Sanmartin, 1952). After the invertebrate acquires the parasite, development takes place in the

stomach and intestine, with subsequent invasion of the hemocoele and salivary glands. The parasite is a potential pathogen for the invertebrate host causing a high percentage of deformities during the molting process, and may cause death (Grewal, 1957; Tobie, 1968). Metacyclical trypomastigotes develop in the salivary glands and are inoculated at the time of feeding into the vertebrate host where they live and reproduce in the blood in the trypomastigote form.

The exact geographic distribution of T. (H.) rangeli is not known today. It exists in South America, especially Colombia, Venezuela, and the Guianas, where R. prolixus is its main vector (Zeledon, 1954). It also occurs in Central America where it is transmitted by R. pallences, especially in Panama. Reports of the parasite in Chile and Argentina have been questioned (Zeledon, loc. cit., 1954).

At present, it is not well established whether all isolates now called T. (H.) rangeli correspond to a single species (Tobie, 1968). Under experimental conditions there are quantitative differences in the abilities of various strains to migrate to the salivary glands in the various species of invertebrate hosts (Tobie, 1961; Zeledon, Monge, and Perez, 1963; Zeledon and Blanco, 1965). In spite of these strain differences in the life cycle, the parasite seems to be uniform in the fact that no symptomatology ensues when man acquires the infection, either naturally (Pifano et al. 1948; Hernandez-de-Paredes et al. 1949) or experimentally (Groot et al. 1950, 1951a). The parasitemia is low (Groot et al. 1950, 1951a), difficult to

detect, and perhaps a transient one, but nothing is known about the actual duration of the patent period. These considerations may account for the fact that only in recent times has attention been brought to the possibility of a wider occurrence of T. (H.) rangeli in man. Studies have been made of trypanosome isolates from humans living under endemic conditions for both T. (S.) cruzi and T. (H.) rangeli and it has been found that mixed infections are almost the rule (D'Alessandro and Gutierrez, unpublished).

The relationship between T. (S.) cruzi and T. (H.) rangeli in mixed infections in man is not known, either in nature or under experimental conditions. The same observation is true in the invertebrate host. However, at present the importance of T. (H.) rangeli infections in man, animals, or reduviid bugs lies in the possibility of mistaking it for T. (S.) cruzi (WHO, 1960). This is especially true during surveys carried out to assess the incidence of the flagellates in the reduviid, or in the interpretation of xenodiagnosis (WHO, loc. cit.).

Studies on immunological phenomena in American trypanosomiasis started soon after the discovery of T. (S.) cruzi. The first observations were on animals inoculated with non-virulent strains of the parasite and subsequently challenged with a virulent strain (Brumpt, 1913), but the importance of these observations was overlooked for many years. In 1925, Nino reported experimental infections with T. (S.) cruzi in the frog, Bufo marinus, but his work has never been confirmed by other investigators (Bruni, 1926; Dios, Wergren, and Perez, 1929a, b; Dias, 1933, 1934; Dios

and Bonacci, 1943; Rubio, 1956), suggesting that the parasites detected by Nino were probably frog trypanosomes. In fact the study of the figures presented by Nino strongly suggests that this is the case. The importance of these studies lies in the fact that they brought attenion to the natural resistance of some animal species to the parasite.

In the late 1920's and early 1930's several authors showed interest in the role of the spleen in natural resistance to T. (S.) cruzi infections. Kritschewiski and Schwarzmenn (1928), Regendanz (1930), Nielschulz and Wawo-Rontoe (1930), Galliard (1930), and more recently Galliard, Lapierre, and Coste (1962a) demonstrated that splenectomized mice, rats, guinea pigs, and dogs do not have a different course of infection from that of control groups. Also,differences in natural resistance between younger and old rats were observed by Regendanz (loc. cit.) and later confirmed by Kolodny (1939a, b). However, attempts to demonstrate the possible role of natural antibodies in this type of resistance failed to yield positive results (Regendanz, loc. cit.).

At present there is no explanation at the mechanistic level for the immunological process in T. (S.) cruzi infections in man. As a rule, human infections in nature are asymptomatic. Whether or not superinfection with virulent strains in individuals already infected with non-virulent strains plays a role in the development of chronic Chagas' disease is not known. Several authors suggest that circulating antibodies may be important in resistance. Culbertson and Kolodny (1938) protected rats from severe

infection by administering hyperimmune serum at the time of inoculation. Kagan and Norman (1961, 1962a, b) also showed that protection occurred in mice when the serum was administered in amounts as low as 0.2 ml. In contrast, Cultberson and Kolodny (loc. cit.) failed to alter significantly the course of infection when serum was given 5 days after inoculation with flagellates. These results support those of Mayer and Rocha-Lima (1914), Dias (1934), and Muniz (1962).

A cellular basis for the immunological process in experimental Chagas' disease has also been explored in some detail. The first attempt to relate the reticulo-endothelial system (RES) to the defense mechanism in T. (S.) cruzi infections was made by Denison (1943). Rats infected with a non-virulent strain of the parasite, after RES blockade with trypan blue, did not have a significantly different course of infection. However, there were detectable differences in the levels of circulating antibodies between blocked and control animals as shown by lysin titers with culture forms. These experiments have been considered inconclusive by some authors (Goble and Singer, 1960). With the use of different RES blocking agents these authors found that, "by the use of intravenous colloidal thorium dioxide prior to inoculation, we have been able to obtain early and high parasitemia in mice with avirulent strains that ordinarily produce only low infections". They also reported results similar to those obtained by Pizzi using X-irradiated animals (Goble and Boyd, 1962).

Indirect evidence of RES involvement in T. (S.) cruzi infections

has been provided by the use of cortisone in infected animals (Agosin et al. 1951a,b, 1952; Neghme et al. 1951, 1952; Jarpa et al. 1951; Wolf et al. 1951, 1953; Pizzi, 1952; Pizzi et al. 1952, 1954a,b, 1955; Thierman and Christen, 1952; Seneca and Rockenbach, 1952; Rubio, 1954, 1955; Galliard, Lapierre, and Coste, 1962a,b,c). In general these studies have demonstrated that with the administration of cortisone latent T. (S.) cruzi infections in animals are brought to patency, or parasitemia is higher, or mortality rate increases, or all of these. Though these results seem to be quite conclusive, other findings are more difficult to interpret. Robles, Perrin, and Balcazar (1951) found that ACTH, which stimulates cortisone secretion, improved the clinical picture of Chagas' disease in dogs.

Other experiments designed to test the effect of sex hormones on the course of Chagas' disease in mice yielded negative results (Goble, 1952). In spite of this, it has been observed that male and female mice exhibit marked quantitative differences in the course of T. (S.) cruzi infections (Hauschka, 1947), however they are perhaps due to weight differences rather than sex differences (Kagan and Norman, 1960).

More direct evidence that a cellular type of immunity is important in American trypanosomiasis has been provided by Pizzi and co-workers. In a series of papers which report experiments conducted over a period of 10 years, the authors showed that cellular immunity of both natural and acquired types, is the main mechanism of resistance in animals (Pizzi and Prager, 1952; Pizzi, Rubio, and Knierim, 1953, 1954a,b; Pizzi and Rubio,

1955; Pizzi and Knierim, 1955; Pizzi and Chemke, 1955; Taliaferro and Pizzi, 1955; Pizzi, 1957, 1961). It was shown in these studies that when either blood or culture forms of an avirulent strain were inoculated into a susceptible host, the parasites were quickly ingested and destroyed by the macrophages. The site of inoculation always showed a marked inflammatory reaction, and only a few parasites developed and produced a transient infection. A similar picture was seen when virulent strains and suppressive treatment were given concurrently. Both cases suggest the existence of strong natural cellular type of immunity in some forms of experimental Chagas' disease of animals.

An acquired type of cellular immunity was more evident in animals inoculated with non-virulent strains and subsequently challenged with virulent strains. These immunized animals always showed a more intense inflammatory reaction at the site of inoculation, with cellular infiltration consisting mainly of lymphoid and polymorphonuclear cells. Phagocytosis and destruction of the parasites was completed within a shorter period of time and their destruction at a cellular level obviously reduced the parasitemia. These observations suggest a partial explanation for T. (S.) cruzi immunity in experimental animals, but unfortunately no data are available suggesting that the same situation prevails in man.

An interesting aspect of T. (S.) cruzi immunity is that of vaccination with antigenic material other than living organisms. Muniz et al. (1946) inoculated a suspension of killed culture forms (Merthiolate 1:10,000)

into Macaca mullata. Vaccination via cutaneous, subcutaneous, intraperitoneal, and intravenous routes failed to achieve protection to a challenge with virulent organisms. Hauschka et al. (1950) used formalin-killed blood forms subcutaneously in mice, and Kagan and Norman (1961) used mouse and culture forms in merthiolate (route not given). Both groups of workers obtained negative results, however, Johnson and Neal (1963) observed protection in mice using blood forms of the flagellate killed by freezing and thawing and administering them subcutaneously in adjuvant. Other authors also observed protection in mice using culture forms killed by other physical means, such as the French pressure cell, sonic oscillator, or ballotini in the Shockman apparatus (Goble et al. 1964) and pressure (Gonzales-Cappa et al. 1968) which mechanically disrupted the organism. The use of adjuvants was not necessary and protection lasted up to 4 months, the longest time interval tested (Goble et al. loc. cit.)

These results seem contradictory, and a clear explanation is lacking at present. It is possible that labile functional antigen(s) present in both the culture and the blood forms of the flagellate are destroyed by chemical treatment (formalin, merthiolate). On the contrary when physical methods such as those reported by Johnson and Neal (1963) and Goble et al. (1964) are used, these antigens are not destroyed and they elicit an antibody response which protects the animals. More difficult to interpret are reports of protection in mice inoculated with a chemical extract of culture forms of T. (S.) cruzi (Seneca, Peer, and Hampar, 1966; Seneca and Peer,

1961a, b). This extract possessed all the characteristics of a toxin (Chagastoxin), but the results need confirmation.

As previously mentioned non-virulent living culture forms of the parasite produce protection against virulent strains in experimental animals. Emmett (1950) used X-irradiated culture forms of a highly virulent strain of T. (S.) cruzi to vaccinate mice. Results showed that the flagellate could be attenuated with this treatment and that protection ensued after the initial dose. Interestingly, the original level of virulence was regained in the culture tube 1 month after irradiation.

Serological tests have been used by many workers to aid in the diagnosis of Chagas' disease. Early workers used antigens prepared from tissues of infected animals and the tests invariably demonstrated cross-reactions with other diseases (Guerrero and Machado, 1913; Villela and Bicalho, 1923; Lacorte, 1927). No significant progress was made until Kelser (1936) described a method of antigen preparation in glycerine using culture forms of the organism. However, the activity of this antigen lasted only 1 month. This prompted Liem and Thiel (1941) to use a phenol extract; Romana and Dias (1942) an alcoholic extract; and Davis (1943) a saline-merthiolate one. These antigens all possessed the disadvantage of producing cross-reactions with sera from syphilitic patients (Liem and Thiel, loc. cit.; Pellegrino and Mesquita, 1947), and in most cases had a low complement fixing power (Freitas and Almeida, 1949; Pellegrino and Brener, 1952a).

A quantitative complement fixation (CF) test for Chagas' disease, using 50% hemolysis, was described by Freitas and Almeida (1949) and Freitas (1951) following the technique of Wadsworth (1947). The antigen used by Freitas and Almeida (loc. cit.) was a benzene extract of the culture forms of the parasite. The antigen was free from the cross-reactivity observed with the previous antigens in sera from syphilitic patients. Further purification of the antigen by ether extraction of the culture forms (Chaffee, Fife, and Kent, 1956) did not result in any improvement. These authors, however, speculated that the cross-reactivity was due to some factor(s) in the lipidic fraction of the T. (S.) cruzi antigen. Recently this has been demonstrated to be the case (Almeida et al. 1969). A further improvement of the antigen preparation was introduced by Maekelt (1960, 1964) who adapted the dialysis sac culture technique (Tobie and Rees, 1948) for mass culture of the parasite. A definite advantage of growing the flagellates in cellulose sacs is the elimination of extraneous medium contaminants from the antigen preparation.

Cross-reactivity has also been reported with Leishmania organisms (Pessoa and Cardozo, 1942; Pellegrino and Brener, 1952b; Brener and Pellegrino, 1958; Fife and Kent, 1960) but this is still controversial since the geographic distribution of both Leishmania donovani and L. brasiliensis overlaps that of T. (S.) cruzi in many areas of Latin America.

At present, the CF test in Chagas' disease is widely used both in serological surveys and for diagnostic purposes, but it is not sufficiently

standardized to allow comparisons of results from different laboratories. Efforts directed toward standardization are being made (WHO, 1969b) that should provide basic guide lines for antigen preparation and testing.

Many other serological methods have been described for the diagnosis of Chagas' disease. One of the most interesting is the precipitin reaction developed by Muniz and Freitas (1944) and Muniz (1947a,b, 1959). The antigen used in this test is a polysaccharide fraction of the culture form and it gives a positive reaction in the acute phase of the disease when the CF test is still negative. Together with the CF test, these two serological reactions are the most widely used in routine work for diagnosis of both the acute and the chronic stages of the disease. Other serological reactions have been used more as research tools than for diagnostic purposes, for example agglutination (Packchanian, 1935, 1940; Senekjie, 1943; Chang, 1947), dye test (Scorza et al. 1959), flourescent antibody techniques (Fife and Mueschel, 1959; Sadun et al. 1963; Biagi, Tay, and Martinez, 1964; Toussaint, Tarrant, and Anderson, 1965) and hemagglutination (Bouisset, Ducos, and Ruffie, 1960; Cerisola, Fatala, and Lazzari, 1962).

In spite of the wide use of serological reactions in the diagnosis of Chagas' disease, little is known about the nature of the antigens used in these tests. The first attempts to fractionate T. (S.) cruzi antigen were made by Muniz and Freitas (1944) who extracted the polysaccharide fraction of the parasite for use in precipitin tests. The polysaccharide

extraction was carried out as recommended by Fuller (1938) for the extraction of polysaccharides from hemolytic streptococci. Packchanian (1952) tried to further purify the antigenic components of T. (S.) cruzi, using the method described by Sevag (1938) for polysaccharide extraction. Packchanian (loc. cit.) obtained three fractions from the culture forms referred to as A, B, and C. Fraction A was a protein complex with contaminants such as nucleic acids and carbohydrates. Fraction B was a pure protein and fraction C was a polysaccharide complex with little protein contamination. The three fractions were reported to be active serologically, but unfortunately no information was given on the type of serological tests employed.

Using the same methods as those described by Packchanian (1952), Fife and Kent (1958, 1960) separated a carbohydrate and a protein fraction. The two fractions did not react non-specifically with normal or syphilitic sera whereas the whole extract did. On the other hand, both the protein and whole fractions showed a higher capacity for binding complement than the carbohydrate. Serum from cases of L. braziliensis and L. tropica gave positive complement fixation tests with the two fractions. No cross-reactions were observed using serum from patients infected with other parasites, especially helminths. Goncalves and Yamaha (1956, 1969) studied the polysaccharide fraction of T. (S.) cruzi culture forms in more detail and found that 11 amino acids constituted the protein "contaminants" of this fraction. They also found that immunological activity, as detected

by the precipitating ring test and Oudin's serum agar technique, was present in the polysaccharide fraction. Since trypsin and chymotrypsin treatment of the fraction did not alter the results, the authors concluded that the serological activity resided in the polysaccharide fraction.

Maekelt (1962) carried out further fractionations of the culture forms of T. (S.) cruzi and studied them in CF tests. Twelve fractions were prepared, of which the first four corresponded to whole extracts of the flagellate in different media, another three to different supernatant fluids after homogenization and centrifugation of the antigen, and the remainder to precipitates obtained by treatment with increasing concentrations of various salt solutions. Results reported by the author are of doubtful significance since the methods of fractionation were very crude, and the complement fixing activity was present in lesser or greater amounts in each one of the 12 fractions.

The fractionation of culture forms of T. (S.) cruzi for the study of their antigenic composition, as outlined above, has practical application for example in the case of the precipitin test. An interesting aspect of this area is the study of the antigenic relationships of strains of the same species. Several authors have demonstrated that all of the T. (S.) cruzi strains and isolates are antigenically related. Hauschka and co-workers (1950), Norman and Kagan (1960), and Nussenzweig, Kloetzel, and Deane (1963) have given some attention to this problem. These authors concluded that, since infection with any T. (S.) cruzi-like isolate protected mice

against a pathogenic strain they were antigenically related. Also, the same conclusion has been reached with agglutination reactions between different strains (Hauschka, loc. cit.; Nussenzweig, Deane, and Kloetzel, 1963a, b). Moreover, the latter group of workers also showed that agglutination reactions after absorption of experimentally prepared immune horse serum with flagellates enabled them to separate the strains into two distinct groups. These groups, referred to as A and B, correspond to strains isolated from humans, reduviid bugs, and bats (A) and various wild animals from endemic areas (B). These two distinct groups, however, are closely related, since protection studies in mice did not reveal any differences between them (Nussenzweig et al. 1963).

Nussenzweig et al. (1963a,b) used agar diffusion techniques and immunoelectrophoresis to study further the antigenic relationships of various T. (S.) cruzi strains and isolates. Results of agar diffusion studies supported the idea of the existence of the two distinct serological types A and B. However, immunoelectrophoretic results were inconclusive, since only two precipitin lines were obtained. Later, Nussenzweig and Goble (1966) studied other strains and isolates in the same fashion and expanded the previous classification to include a third group, C. This group was composed of a strain from a carnivore (Tayra barbosa) with a specific antigen different from the other two, A and B. Other workers (Gonzales-Cappa and Kagan, 1969) also used agar diffusion and immunoelectrophoretic techniques to study several T. (S.) cruzi human isolates from different

geographical areas in Latin America. They confirmed the existence of the A, B, and C types proposed by Nussenzweig and co-workers.

Thillet and Chandler (1957) found that Trypanosoma (Herpetosoma) lewisi produced (released?) some substances in vitro which protected animals from infection. This material was called exoantigen and they speculated that it was a metabolic product of the parasite. Weitz (1960a,b, 1963a,b) demonstrated that another exoantigen is present in the serum of animals experimentally infected with the African T. (Trypanozoon) brucei group. Tarrant, Fife, and Anderson (1964a,b,1965) found exoantigen in supernatant fluids of dialysis sac cultures of T. (S.) cruzi, as detected by CF tests. Kagan (personal communication) was unable to confirm these results, nor was the present writer with the use of immunoelectrophoresis as the detection tool (unpublished). Siqueira et al. (1966), however, reported detecting exoantigen of T. (S.) cruzi in the sera of experimentally infected animals.

To date no study has been undertaken to compare T. (S.) cruzi with a related species, T. (H.) rangeli, which infects man in Latin America. More important, cross-reactivity in Chagas' disease antigen-antibody reactions could occur with T. (H.) rangeli infections. As mentioned previously this flagellate infects man under natural conditions in apparently a higher incidence than was originally thought (D'Alessandro and Gutierrez, unpublished).

A report on the role of T. (H.) rangeli infections in CF tests in

Chagas' disease (Maekelt and Diaz-Vazquez, 1962) is not a conclusive one, because serological (CF) data were obtained from people living in endemic areas of both T. (S.) cruzi and T. (H.) rangeli. At present, further study of the problem is considered necessary. The present work was undertaken in order to study especially the following aspects:

1. The antigenic make-up of T. (S.) cruzi and T. (H.) rangeli and their antigenic relationships.

2. A physical and chemical characterization of the soluble antigens of each species.

3. The possible occurrence of cross-reactivity in agglutination, and hemagglutination (HA) reactions using antigens from both species.

CHAPTER II

MATERIALS AND METHODS

Source of Trypanosome Strains

Six strains of trypanosomes belonging to two species were used in these experiments. Three strains of T. (S.) cruzi and three strains of T. (H.) rangeli were used, as described below:

1. T. (S.) cruzi Brazil was obtained from Dr. E.J. Tobie, National Institutes of Health (Bethesda, Maryland). This strain was isolated from a human patient in 1942 (Hauschka et al. 1950) in Brazil.

2. T. (S.) cruzi Patuxent was obtained from Dr. I.G. Kagan, Center for Disease Control (Atlanta, Georgia). It was isolated from a raccoon in Laurel, Maryland in 1955 (Walton et al. 1956, 1958).

3. T. (S.) cruzi Raccoon was isolated in Georgia from a raccoon (Norman et al. 1959) and was obtained from Dr. I.G. Kagan.

All of the T. (S.) cruzi strains were maintained by serial passage in Tobie's medium.

4. T. (H.) rangeli 321

5. T. (H.) rangeli 347

6. T. (H.) rangeli 546

The T. (H.) rangeli strains were isolated from various locations in Colombia (South America) from R. prolixus by Dr. A. D'Alessandro in 1962, and were identified as T. (H.) rangeli by biological criteria related to transmission (D'Alessandro 1963a; D'Alessandro and Mandel, 1969). Isolates were recovered from mice that had been bitten by infected wild reduviid bugs in such a manner that it was impossible for the bug feces to come in contact with the mouse (D'Alessandro, 1963b). Thus, transmission occurred via the salivary glands, a route that excludes T. (S.) cruzi as the infecting organism. Hemoculture of mice infected in this fashion yielded the T. (H.) rangeli strains. Later experimental work showed that the behavior of the different isolates was constant with respect to hemolymph and salivary gland migration (D'Alessandro, personal communication). All of the stock cultures were maintained in Tobie's medium.

Batch Cultures

The cellulose sac culture principle as outlined by Tobie and Rees (1948) for trypanosomes and then adapted for batch culture of T. (S.) cruzi by Maekelt (1960, 1964) was used with some modifications. The culture medium used was a modification of Chang (1947) liquid medium made by R.G. Yaeger (personal communication; Fernandez and Castellani, 1966) and designated as LIT medium (Appendix I).

A hole, 14mm in diameter, was made at the bottom of a No. 10 rubber stopper, approximately 1 cm deep, and a glass tube (6.8 cm x 13mm,

id) was place in the hole. A 90 cm length of dialysis tubing (Curtin, #625-D, 1-1/7 inch) was sealed at one end by means of a strong knot. The other end was fitted on the glass tube and securely tied with a cord. Fifty ml of dialysis bag medium (Appendix I) was added to the bag. The stopper with the cellulose sac containing the medium was suspended in a 2 liter Erlenmeyer flask containing 450 ml of flask medium (Appendix I). A 15 gauge, 3-1/2 in hypodermic needle was inserted through the top of the rubber stopper into the glass tube. The needle permitted access to the inside of the dialysis sac and allowed addition of nonautoclavable medium components.

After 24-48 hr incubation for sterility test, flagellates from one week old LIT cultures were washed with sterile saline solution and transferred aseptically into the flask medium. Flasks were then placed in a G.E. incubator, model 805, and kept at 28 C. Depending upon the size of the initial inoculum, full growth was attained in 6-10 days.

Initial attempts to mass culture T. (H.) rangeli in LIT medium as described above were unsuccessful. Bulk cultivation was possible when the organism was grown in medium containing all of the LIT components minus the dialysis bag arrangement. A liquid culture medium was later developed and adopted for batch culture of T. (H.) rangeli using dialysis tubing as described above (Appendix II).

Antigen Preparation

Flasks containing one week old cultures were opened and the dialysis sacs were carefully removed and discarded. Cultures were studied microscopically for contaminants, and Giemsa-stained smears were prepared in order to study the morphological characteristics of the flagellates. This technique permitted the identification of the species with every batch of antigen (WHO,1960).

The contents of several flasks were pooled, centrifuged at 10,000 G for 15 min at 5 C, and washed three times in saline solution. The flagellates were resuspended in a small amount of saline solution and stored at - 20 C. The organisms were thawed as needed and subjected to sonic oscillation for 5 min at 10 KC, under 250 Watts power in a Raytheon model DF 101 sonic oscillator. After centrifugation at 10,000 G for 20 min the supernatant liquid was collected, dialyzed against distilled water (3 changes in 24 hrs), and lyophilized on a Virtis freeze-dry unit. The white powder, considered the crude soluble antigen, was stored at 4 C. Culture medium controls were prepared from non-inoculated flasks. These antigens and controls were used throughout the present study.

Antigen for hemagglutination (HA) tests was similar to that described above, with the exception that it was suspended in buffer pH 6.4, centrifuged in a clinical centrifuge for 5 min, and the supernatant was saved for use in the test.

Antiserum Preparation

Three different types of immune sera were used in these studies.

1. Twelve adult male and female albino rabbits weighing from 1.5 to 2.5 kg were used to prepare immune sera reactive with soluble antigen. Two animals were used for each of the six trypanosome strains studied. Preimmune sera were collected from all animals prior to immunization and served as control sera in all experiments. The antigen was dissolved in 1 ml of saline, added to 1 ml of Freund's incomplete adjuvant (Difco # 0639-60) and emulsified in a Sorval Omnimixer equipped with a micro-attachment. The initial immunizing dose consisted of a total of 2 mg of antigen injected subcutaneously at four to five different sites on both flanks of each animals. Booster doses were given at one week (2mg), three weeks (4 mg), five weeks (4 mg), and eight weeks (4 mg) after the initial dose. Bleeding of the animals was begun the fourth week after the initial dose and sera were tested for precipitating antibody with immunoelectrophoresis. Complete bleeding was done when the maximum number of precipitin lines were obtained. In a few instances another booster dose of 4 mg was given to animals which responded slowly. The maximum time interval one animal was kept before being exsanguinated was 150 days.

2. Albino, Hartley strain guinea pigs were used for the production of antisera. Ten animals were infected with culture forms of T. (S.) cruzi Brazil strain and 10 were infected with T. (H.) rangeli 321. One week old cultures of the flagellates were washed in saline solution, counted,

and diluted to 5 million per ml. After bleeding for preimmune sera, each animal was inoculated intraperitoneally with 1×10^6 living flagellates. A second dose of a similar number of organisms was given 2 weeks later and the animals were bled 5 weeks after the initial dose.

3. Human sera, collected in Argentina, and positive by the CF test were provided by Dr. I.G. Kagan from CDC, Atlanta, Georgia. Some of the samples came from the laboratory of Dr. J.A. Cerisola. The remainder were of undetermined origin.

Disc Electrophoresis

The alternate procedure described by Davis (1964) was used for disc electrophoresis. The power supply consisted of a Heathkit model IP-32. All six different preparations of crude soluble antigen were electrophoresed and stained for various groups of compounds. The amount of antigen used for each gel contained 40 ug of N as determined by the micro-Kjeldahl technique, modified by Lang (1958). Proteins were stained according to techniques of Davis (loc. cit.); lipids by the methods of McDonald and Ribeiro (1959); carbohydrates by the periodic acid Schiff method; and nucleic acids by the Feulgen reaction.

Immunoelectrophoretic Analysis (IEA)

Immunoelectrophoresis (Grabar and William, 1953) was carried out with the microtechnique modification of Scheidegger (1955), as described in the LKB (6800 A) immunoelectrophoresis operational manual.

All of the analyses were performed in precooled (4 C) 0.1 M Veronal buffer, pH 8.6. The power supply was set at 50 MA, 250 volts for 1 hr. The optimal antigen concentrations and well/trough distances were determined for each antigen. In general 10 mg dry weight of antigen per 0.1 ml of saline solution, and 2 mm well/trough distances were found to be suitable. Analyses of both homologous and heterologous antigen-antibody systems were carried out. Identities of precipitin lines were determined by absorption studies (see below) or by modifying the well/trough distance until precipitin lines joined. After application of serum the system was allowed to react for 24 hr. Occassionally, longer periods of incubation were used in order to enhance the chances for demonstration of identity of certain components. Both preimmune sera and lyophilized culture media were used as controls. All IEA slides were dried and stained for proteins as recommended by the LKB manual. Both rabbits in each group were tested and the precipitin lines observed were recorded in a composite drawing.

Absorption of Antisera

To absorb antibody from antisera 1 mg of lyophilized antigen was placed in a test tube and 1.0 ml of the serum to be absorbed was then added. The mixture was incubated at 37 C for 1 hr, placed in the refrigerator (4 C) overnight, centrifuged at 5000 G for 10 min and the sediment was discarded. This procedure was repeated at least two times on two consec-

utive days. On a few occasions it was repeated three times.

Physical and Chemical Characterization

The crude soluble antigens from T. (S.) cruzi Brazil and T. (H.) rangeli 321 were chosen for the characterization studies. The effect of different treatments was studied by IEA. Partial or total disappearance of precipitin lines after physical treatments was studied using immune sera from two rabbits. For study of chemical treatments, immune serum from only one rabbit was used. Physical and chemical treatments of the crude antigens were performed as described below:

Physical Treatment

Heat. Twelve 6 x 50 mm test tubes containing crude antigen in the optimal concentration for IEA (10 mg/0.1 ml) were prepared. Four of the tubes were placed in a 37 C water bath, and one tube was removed after 15, 30, 45, and 60 min time intervals. Another group of four similar tubes was incubated at 45 C and individual tubes were removed at 5, 10, 15, and 30 min. A third group was incubated at 60 C and tubes were removed at 5, 10, 15, and 20 min. After removal from the water bath, the tubes were stored at -20 C until the contents were tested by IEA.

Freezing and thawing. For this experiment six test tubes were used containing amounts of antigen similar to those described above. The crude antigen was frozen at - 70 C and thawed at 37 C for 3, 6, 10, 15, or 25 times, and stored at - 20 C until ready for use in IEA.

Chemical Treatment

The chemical characterization of the crude soluble antigens was carried out according to the methods used by Williamson and Brown (1964) with the African trypanosomes. These methods are those used for investigating the essential groups for enzyme activity, and are described in detail by Fraenkel-Conrat (1957). All reactions were carried out in 6x50mm test tubes. Amounts of 2.5mg dry weight of crude antigen of T. (S.) cruzi Brazil and T. (H.) rangeli 321 were placed in each tube. All reagents containing the estimated working concentrations in 0.2 ml of the solvent were added to each tube. Proper controls were set up for all reactions. Incubations were carried out in accordance with each protocol (see below) after which the tubes were stored at - 20 C until used. After incubation, a piece of dialyzing membrane was stretched over the open end of all test tubes and securely tied. They were allowed to dialyze in an inverted position against five changes of distilled water over a 48 hr period at 4 C. The tubes were agitated at frequent intervals. When dialysis was completed the dialyzed fluid was frozen at - 70 C and freeze-dried. After lyophilization the contents of each tube was resuspended in 0.25 ml of saline solution and studied by IEA. The antigens were reacted with the chemicals or enzymes as described below:

Reactions affecting proteins. (1) Urea 4 M (aq), incubated for 18 hr at 37 C. (2) Formalin (20% neutral formalin) in phosphate-buffer-saline (PBS), pH 7.2, incubated at 22-24 C for 3 days. (3) Formalin-

alanine (50 mg of alanine per ml in 0.5% neutral formalin) in PBS, pH 7.2, reacted for 3 days at 22-24 C. (4) Formalin-acetamide (50 mg of acetamide in 0.5% neutral formalin) in PBS, pH 7.2, reacted for 3 days at 22-24 C. (5) Methanol-HCl (0.1 N HCl in methanol) reacted for 3 days at 4 C. (6) Propylene oxide (1:2-epoxypropane). The pH of diluted (0.1 ml) crude antigen was adjusted to 3.5-4.0 with acetic acid. Then 0.1 ml of propylene oxide was added and the reactants were incubated 6 days at 22-24 C. (7) Sodium p-chloromercuribenzoate (7 mM in 0.03 M acetate buffer, pH 5) incubated at 22-24 C for 6 days. (8) Diazobenzene-sulfonic acid was prepared as described by Fraenkel-Conrat (1957). Five mg was dissolved in 0.2 ml PBS, pH 7.2, before use and the mixture was neutralized with sodium bicarbonate, then incubated for 24 hr at 22-24 C. (9) Trypsin (2X crystallized, bovine origin, Sigma Chemical Co.), 0.2 mg per ml in PBS, pH 7.2, incubated for 18 hr at 37 C. (10) Pepsin (2X crystallized, Sigma Chemical Co), 1.0 mg per ml at pH 2.0 incubated for 30 min at 37 C. (11) Papain (Nutritional Biochemical Co) 0.1 mg per ml in 33 mM phosphate buffer, pH 7.0, containing 0.5 mg per ml cysteine and 0.25% (v/v) saturated aqueous versene reacted for 18 hr at 37 C. (12) Carboxypeptidase-A (crystalline suspension, Sigma Chemical Co, 50 mg per ml in aqueous toluol), 5 mg per ml in 20 mM veronal-acetate buffer, pH 7.5, containing 50 mM NaCl, incubated for 18 hr at 37 C.

Reactions affecting carbohydrates. (1) Acetylating mixture (10%, v/v, acetic anhydride in pyridine) reacted for 24 hr at 22-24 C. (2) Sodium

metaperiodate (50 mM) incubated for 15 min in the dark at 22-24 C. (3) Lysozyme (3X crystallized, Nutritional Biochemical Co) 0.5 mg per ml placed in 25 mM ammonium acetate buffer, pH 6.5, incubated for 18 hr at 37C.

Reactions affecting lipids. (1) Lipase (from pancreas, Nutritional Biochemical Co) 5 mg per ml in 0.5 M NH_4OH-NH_4Cl buffer, pH 9.2, containing 5 mM $CaCl_2$ reacted for 18 hr at 37 C.

Reactions affecting nucleic acids. (1) Deoxyribonuclease (crystallized, Sigma Chemical Co) 50 ug per ml of PBS, pH 7.2, containing 4 mM Mg^{++} incubated for 30 min at 37 C. (2) Ribonuclease (crystallized, Sigma Chemical Co) 10 ug per ml of PBS, pH 7.2, incubated for 15 min at 37C.

Reactions affecting phosphate esters. (1) Alkaline phosphatase (crude, Sigma Chemical Co), 5 mg per ml in M veronal-acetate buffer, pH 9.5, reacted for 18 hr at 37 C.

Serological Reactions

The serological reactions were carried out with all of the sera described above. With each experimentally produced serum the preimmune sample was used as a control. Other proper controls were also used.

Agglutination reaction: These reactions were performed according to the technique of Cunningham and Vickerman (1961, 1962), using Microtiter equipment (Cooke Engineering Co., Alexandria, Virginia). Calibrated

pipette droppers, 0.05 ml, were used to deliver the sera to the U-shaped wells in lucite plates. Two-fold dilutions of sera up to 1:4096 were made in saline solution. Living organisms from fully grown cultures (1 week old) were used after they had been washed in saline solution. The optimal concentration of organisms for the test was found to be 1 million per ml. Organisms were placed in each well with the calibrated pipette dropper (0.05 ml). Plates were shaken to allow good mixing of reactants, and incubated for 30 min at 37 C. Plates were not stacked at any time during the incubation period. Reactions were read with a microscope with a 3X objective. The results were recorded as described by Cunningham and Vickerman (1961, 1962) as follows: 4+, spherical clumps up to 0.2 mm in diameter; 3+, spherical clumps up to 0.08 mm in diameter; 2+, many clumps up to 0.05 mm; and +, very few clumps up to 0.05 mm. Readings above 2+ were considered to be positive, and titers are expressed as the highest dilution giving positive results.

Hemagglutination reaction: HA was carried out as described by CDC (personal communication) and patterned after the technique of Boyden (1951). Several modifications were introduced, the main one being the microtechnique (Sever, 1962) using the Microtiter equipment (Cooke Engineering Co., Alexandria, Virginia). Other modifications consisted of utilization of tannic acid at a 1:40,000 dilution incubated at 4 C for 15 min and sensitization of tanned cells at 37 C for 15 min (Bouisset et al. 1960; Cuadrado and Kagan, 1967).

The optimal antigen concentration was determined for each batch used and was found to be 60 to 80 ug of N per ml. Since repeated tests of a few initial samples showed consistently reproducible results, tests were repeated only twice with all remaining sera. Readings were made at 3 and 24 hrs. If differences in titer were found, the lowest titer was recorded.

CHAPTER III

RESULTS

Batch Cultures

No attempt was made to determine the exact number of flagellates in the batch cultures. Turbidity of the media was used as an indication of the general yield and efforts were directed towards obtaining as high yields as possible.

The LIT medium-dialysis bag technique provided excellent yields of T. (S.) cruzi but not T. (H.) rangeli. Better yields of T. (H.) rangeli occurred when the organism was cultured in LIT medium minus the dialysis bag. However, the antigen obtained from these cultures showed some degree of "contamination" with medium proteins, especially human serum proteins. This occurred despite repeated washing of the organisms before sonication. Subsequently, the development of a modified liquid medium allowed utilization of the cellulose sac technique (Appendix II) and overcame the contamination problem.

Antigen Preparation

Microscopic study of the flagellates before harvesting did not

reveal any detectable bacterial contamination. Study of the Giemsa stained smears revealed that 100% of all flagellates seen were epimastigotes. No other intermediate forms were seen at any time.

Differentiation between T. (S.) cruzi and T. (H.) rangeli was readily made in the stained smears, since T. (H.) rangeli consistently showed a predominance of the long, slender epimastigote forms characteristic of this species. Such forms were not seen in any of the T. (S.) cruzi smears.

The antigens collected had a nitrogen content which varied from 60 to 100 ug per mg and a carbohydrate content of from 20 to 25 ug per mg of antigen (dry weight).

Antiserum Preparation

In general the rabbits used in this study gave good antibody titers, as detected by IEA using sera collected after the end of the 4th week of immunization. High titers were obtained in agglutination and HA tests using the same sera. In contrast, the guinea pigs inoculated with live culture forms failed to give antibody responses detectable with the agglutination and HA tests.

Disc Electrophoresis

Results of disc electrophoresis are summarized in figures 1 to 4. The number of bands shown represents the maximum number detected by the method used and are represented according to their migration in relation to

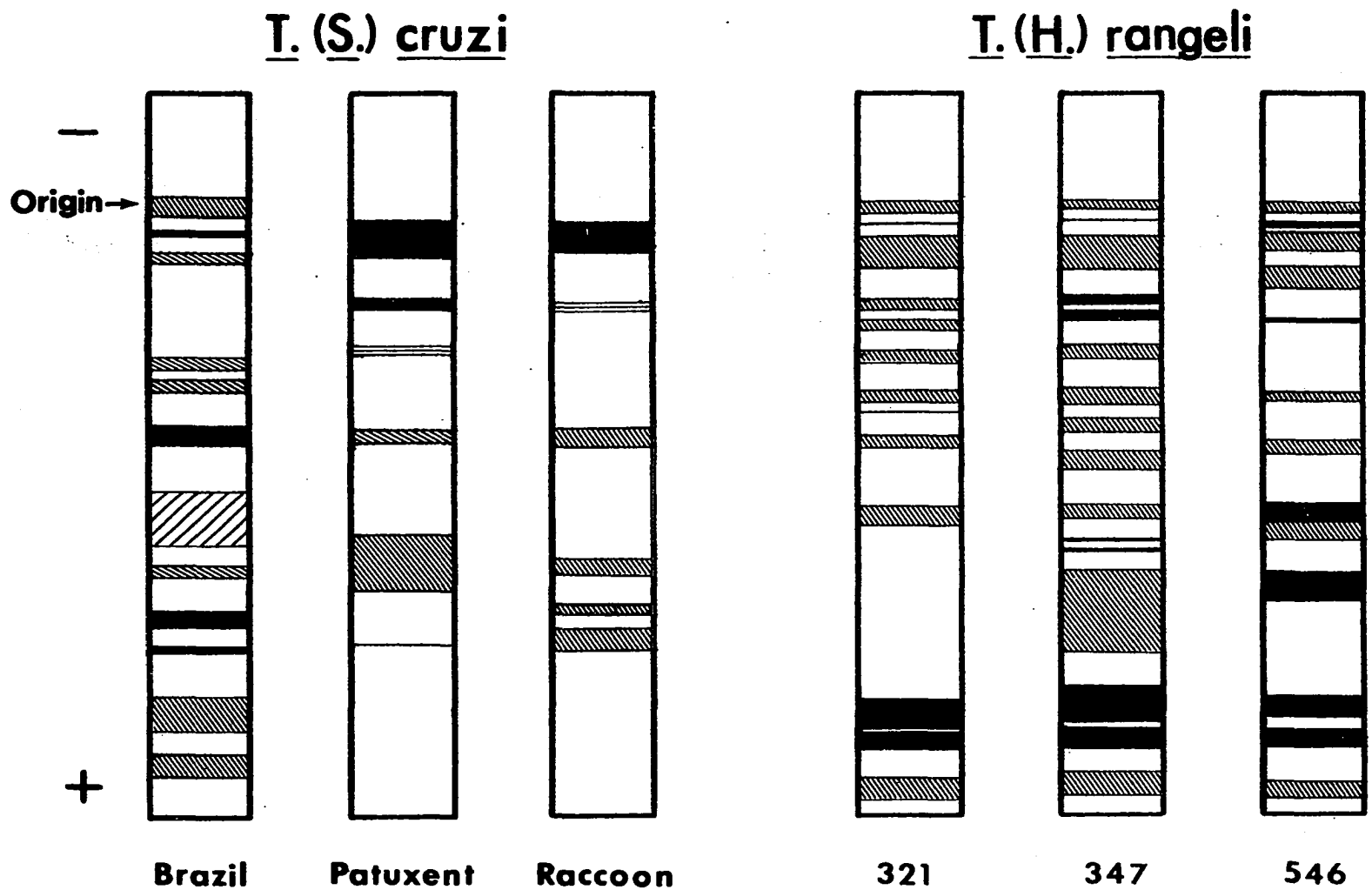

Fig. 1 - Disc electrophoresis of soluble antigen extracts from six trypanosome strains, stained to reveal proteins. Drawings approximate relative intensity of bands.

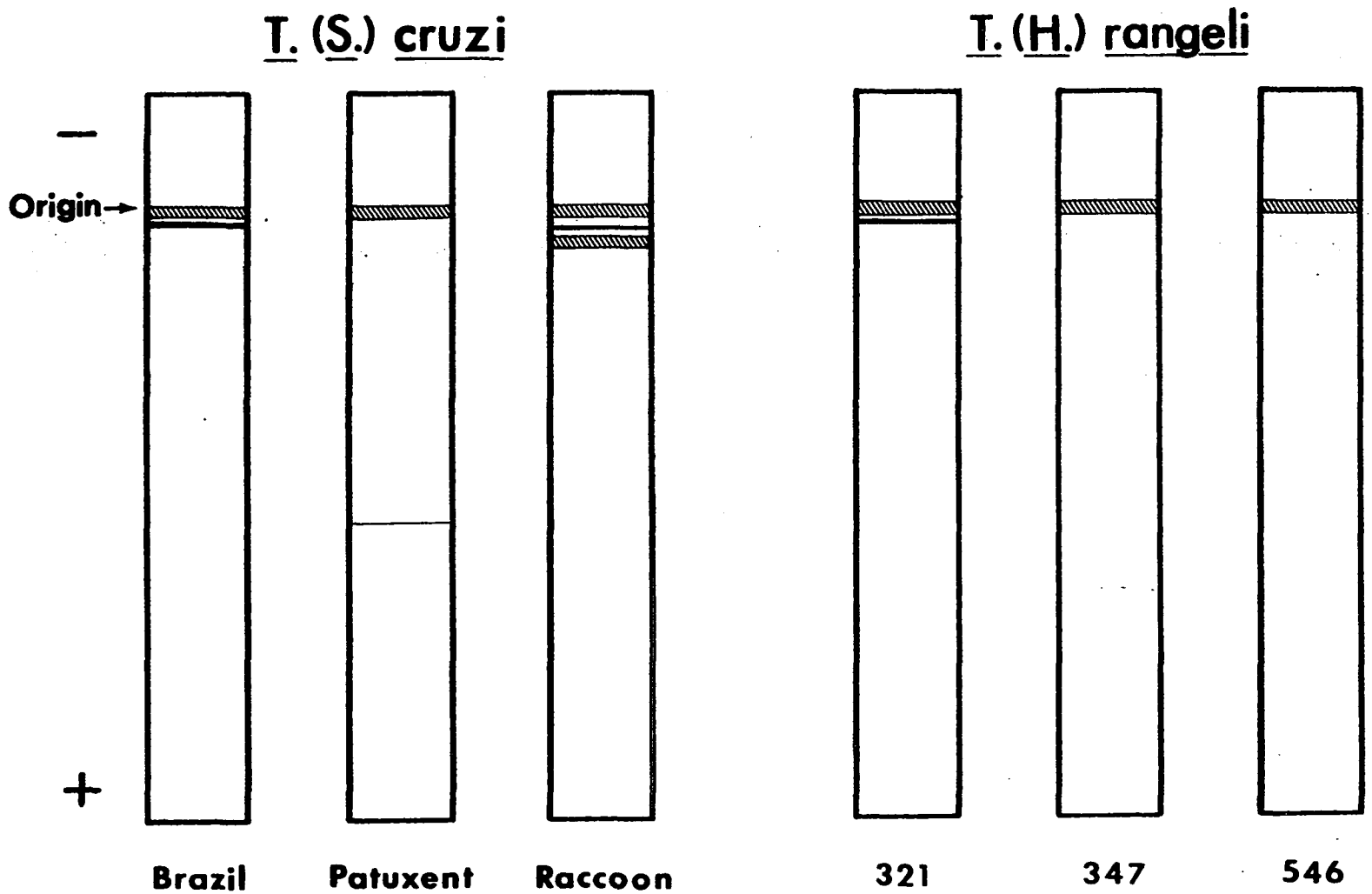

Fig. 2 - Disc electrophoresis of soluble antigen extracts from six trypanosome strains, stained to reveal carbohydrates. Drawings approximate relative intensity of bands.

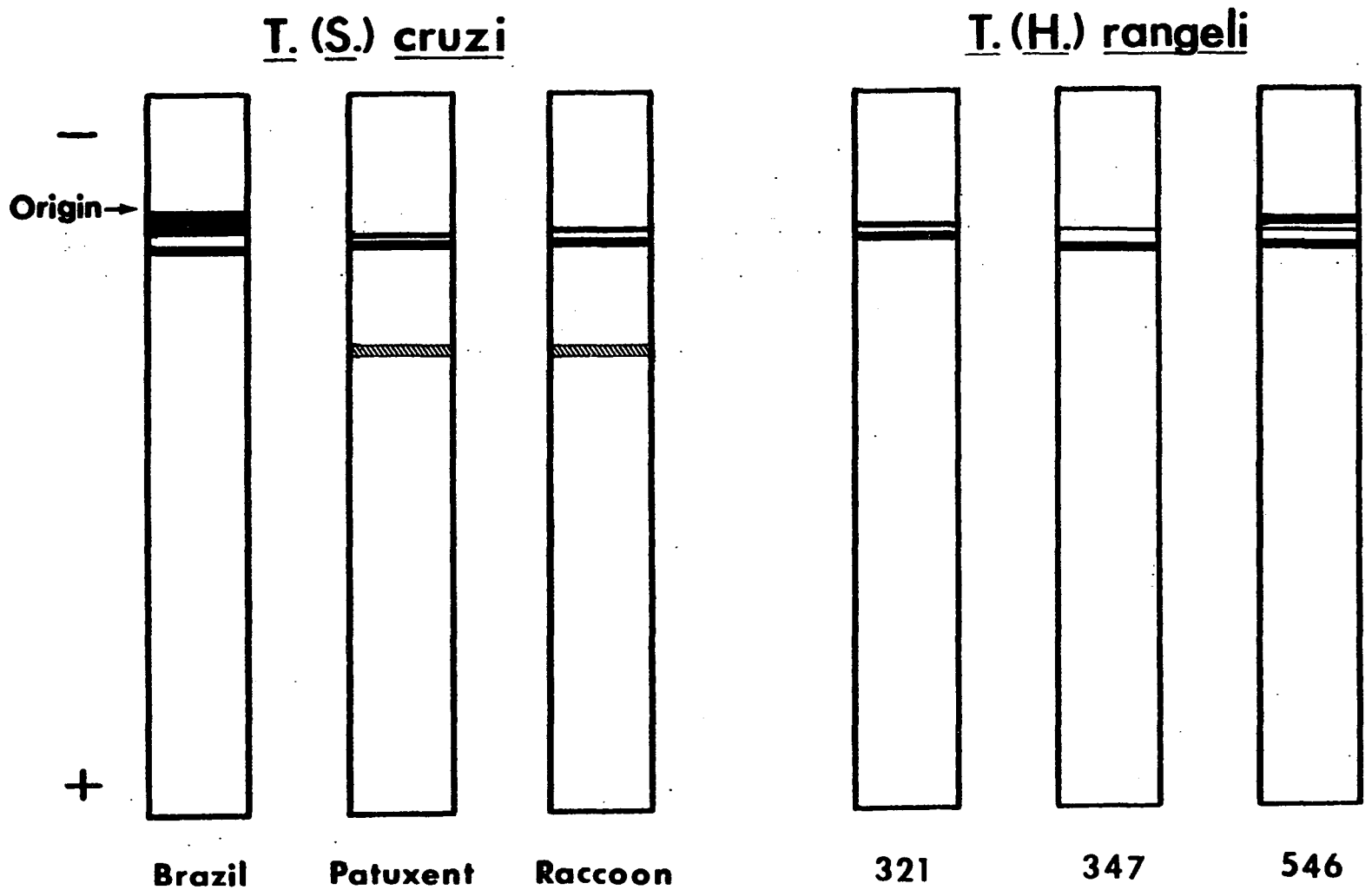

Fig. 3 - Disc electrophoresis of soluble antigen extracts from six trypanosome strains, stained to reveal lipids. Drawings approximate relative intensity of bands

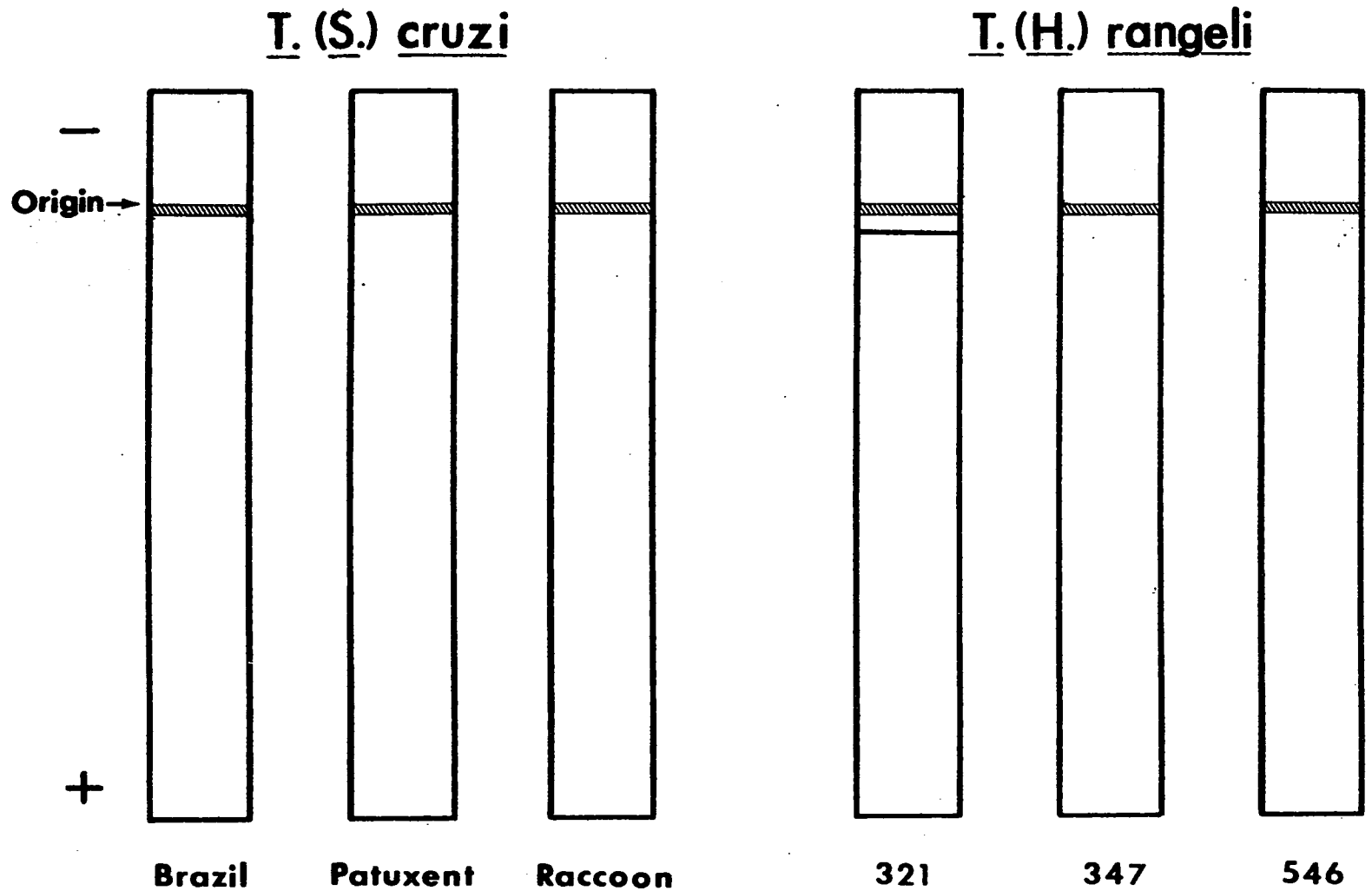

Fig. 4 - Disc electrophoresis of soluble antigen extracts from six trypanosome strains, stained to reveal DNA-RNA. Drawings approximate relative intensity of bands.

the tracking dye front. The relative concentrations of the components are indicated by appropriate shading.

The disc electrophoresis revealed that most, if not all, of the fractions found in both T. (S.) cruzi and T. (H.) rangeli soluble antigens were proteins (Fig. 1). In general, the nucleic acids, lipids, and carbohydrates were each represented by only one to three bands. These bands were detected either at the bottom of the spacer gel or at the beginning of the separation gel.

The lipid stain of certain strains revealed one band at the end of the spacer gel and one at the beginning of the separation gel (Fig. 3). Two exceptions to this are the Patuxent and Raccoon strains which showed one band in the separation gel migrating at the same rate. The results of carbohydrate analyses (Fig. 2) were somewhat similar to those of the lipids. The nucleic acids were represented by only one band, in most cases at the end of the spacer gel (Fig. 4).

IEA and Absorption Studies

Results of the IEA are represented in Figure 5. In general, animals immunized with the same antigen produced sera that gave similar precipitin line patterns. In some cases one or more precipitin lines detected with the serum of one animal were not detected with the serum of a second one. The composite drawings were prepared by incorporating all precipitin lines given separately by sera from the two rabbits immunized

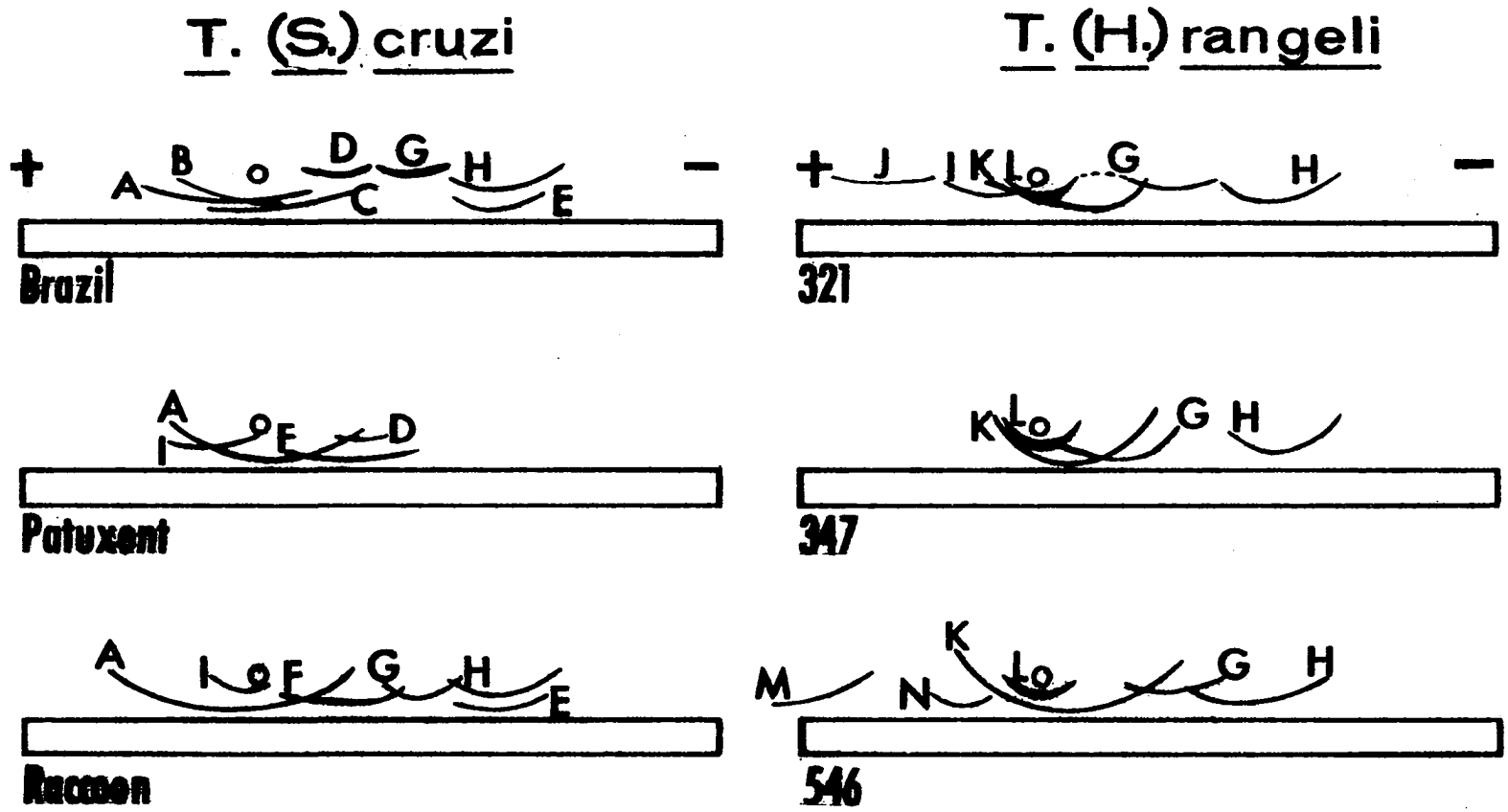

Fig. 5 - Immunoelectrophoretic patterns of various strains of T. (S.) cruzi and T. (H.) rangeli. Composite drawn from results obtained with sera from two rabbits.

with a particular strain. This resulted in the characteristic IEA pattern for each strain as shown in Figure 5.

Precipitin lines are designated with letters A through N. To denote antigenic identity the same letter is applied to identical precipitin lines appearing in different strains throughout the entire study.

Identity of the different components was said to exist when precipitin lines joined across the slide to form continuous round or oval-shaped bands. This method, though very effective in determining identity of antigenic components in agar systems, has certain limitations. The main one is that identical components do not always join. Therefore, absorption of the immune sera with a given antigen was also used to determine identity.

Results of all absorption studies are presented in tables 1 through 12. One set of experiments involved the use of homologous antigen-antisera systems after the antisera had been absorbed with antigens from each trypanosome strain used in the study (Tables 1,2,3,4,5, and 6). In general the results showed that antigens can absorb more components when reacted with immune sera prepared against the homologous than against a heterologous strain. For example, it will be seen in Table 1 that Raccoon antigen absorbed antibody responsible for all precipitin lines of the Brazil antiserum, and Patuxent antigen absorbed 3 components (C,G,H). On the contrary, absorption of the same Brazil serum with strains belonging to T. (H.) rangeli did not result in the deletion of as many components. A

TABLE 1

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (BRAZIL) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected[1]					
	A	B	C	D	G	H
Non-absorbed	+	+	+	+	+	+
Brazil	-	-	-	-	-	-
Patuxent	+	+	-	+	-	-
Raccoon	-	-	-	-	-	-
Tr-321	+	+	+	+	-	-
Tr-347	+	+	+	+	-	-
Tr-546	+	+	+	+	-	-

[1] + = Presence of line; - = Absence of line.

TABLE 2

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (PATUXENT) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected [1]			
	A	I	D	F
Non-absorbed	+	+	+	+
Brazil	-	-	-	-
Patuxent	-	-	-	-
Raccoon	-	-	-	-
Tr-321	+	-	±	+
Tr-347	+	-	+	+
Tr-546	+	-	+	+

[1] + = Presence of line; - = Absence of line; ± = Very faint line.

TABLE 3

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (RACCOON) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected [1]				
	A	I	F	G	H
Non-absorbed	+	+	+	+	+
Brazil	-	-	-	-	-
Patuxent	-	-	-	-	-
Raccoon	-	-	-	-	-
Tr-321	+	-	+	-	-
Tr-347	+	+	+	-	-
Tr-546	+	+	+	-	-

[1] + = Presence of line; - = Absence of line.

TABLE 4

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-321) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected[1]				
	J	I	K	L	G
Non-absorbed	+	+	+	+	+
Brazil	+	-	+	+	-
Patuxent	+	-	+	+	-
Raccoon	+	-	+	+	-
Tr-321	-	-	-	-	-
Tr-347	-	-	-	-	-
Tr-546	-	-	-	-	-

[1] + = Presence of line; - = Absence of line.

TABLE 5

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-347) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected[1]			
	K	L	G	H
Non- absorbed	+	+	+	+
Brazil	+	+	-	-
Patuxent	+	+	-	-
Raccoon	+	+	-	-
Tr-321	-	-	-	-
Tr-347	-	-	-	-
Tr-546	-	-	-	-

[1] + = Presence of line; - = Absence of line.

TABLE 6

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-546) ANTIGEN WITH ABSORBED HOMOLOGOUS ANTISERUM

Antiserum absorbed with following antigens	Precipitin lines detected[1]					
	K	L	G	H	M	N
Non-absorbed	+	+	+	+	+	+
Brazil	+	+	-	-	+	+
Patuxent	+	+	-	-	+	+
Raccoon	+	+	-	-	+	+
Tr-321	-	-	-	-	-	-
Tr-347	-	-	-	-	-	-
Tr-546	-	-	-	-	-	-

[1] + = Presence of line; - = Absence of line.

similar situation is seen in Tables 2,3,4,5, and 6 for the other five strains studied.

In another set of experiments each of the six antisera was absorbed, as described in Materials and Methods, with each of the six antigens under study. Each serum sample was then used in the IEA against the absorbing antigen (Tables 7,8,9,10,11, and 12). It can be seen that absorption of a serum sample with its homologous antigen alwasy resulted in the disappearance of all components.

As expected, the identity of antigenic components within species was more marked than that between species (Fig. 5). Absorption studies tended to confirm this observation (Tables 1 through 12).

Antibody to certain antigenic components of one strain or species was frequently absorbed by heterologous antigens. This was interpreted as evidence for the existence of that component in the absorbing antigen. An example of this is component I which appeared in the Patuxent (Tables 2 and 8), Raccoon (Tables 3 and 9) and Tr-321 strains (Tables 4 and 10) and was consistently absorbed by the Brazil strain. This component was therefore considered to be present in the Brazil antigen, although it was not demonstrated when Brazil antigen was reacted with homologous antibody (Fig. 5). It is included instead in Table 13 where a general resume of the antigenic make-up of all strains in study is presented. Table 13 is based on both the actual pattern obtained with the agar electrophoresis and the theoretical components implied by absorption studies.

TABLE 7

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (BRAZIL) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (S.) cruzi Brazil antigen precipitin lines[2]					
		A	B	C	D	G	H
Brazil	Non-absorbed	+	+	+	+	+	+
	Absorbed	-	-	-	-	-	-
Patuxent	Non-absorbed	-	-	±	±	-	-
	Absorbed	-	-	-	-	-	-
Raccoon	Non-absorbed	±	±	-	-	+	+
	Absorbed	-	-	-	-	-	-
Tr-321	Non-absorbed	-	-	-	-	+	+
	Absorbed	-	-	-	-	-	-
Tr-347	Non-absorbed	-	-	-	-	+	+
	Absorbed	-	-	-	-	-	-
Tr-546	Non-absorbed	-	-	-	-	+	+
	Absorbed	-	-	-	-	-	-

[1] Absorbed with T. (S.) cruzi Brazil.
[2] + = Presence of line; - = Absence of line; ± = Faint line.

TABLE 8

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (PATUXENT) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (S.) cruzi Patuxent antigen precipitin lines[2]				
		A	I	D	F	H
Brazil	Non-absorbed	+	-	+	-	-
	Absorbed	-	-	-	-	-
Patuxent	Non-absorbed	+	+	+	+	-
	Absorbed	-	-	-	-	-
Raccoon	Non-absorbed	+	+	-	+	+
	Absorbed	-	-	-	-	-
Tr-321	Non-absorbed	-	-	-	-	-
	Absorbed	-	-	-	-	-
Tr-347	Non-absorbed	-	-	-	-	-
	Absorbed	-	-	-	-	-
Tr-546	Non-absorbed	-	-	-	-	-
	Absorbed	-	-	-	-	-

[1] Absorbed with T. (S.) cruzi Patuxent.
[2] + = Presence of line; - = Absence of line.

TABLE 9

IMMUNOELECTROPHORESIS OF T. (S.) CRUZI (RACCOON) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (S.) cruzi Raccoon antigen precipitin lines[2]				
		A	I	F	G	H
Brazil	Non-absorbed	+	+	+	+	+
	Absorbed	-	-	-	-	-
Patuxent	Non-absorbed	+	-	+	-	-
	Absorbed	-	-	-	-	-
Raccoon	Non-absorbed	+	+	+	+	+
	Absorbed	-	-	-	-	-
Tr-321	Non-absorbed	-	-	-	+	+
	Absorbed	-	-	-	-	-
Tr-347	Non-absorbed	-	-	-	+	+
	Absorbed	-	-	-	-	-
Tr-546	Non-absorbed	-	-	-	+	+
	Absorbed	-	-	-	-	-

[1] Absorbed with T. (S.) cruzi Raccoon.
[2] + = Presence of line; - = Absence of line.

TABLE 10

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-321) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (H.) rangeli Tr-321 antigen precipitin lines[2]				
		J	I	K	L	G
Brazil	Non-absorbed	-	-	-	-	+
	Absorbed	-	-	-	-	-
Patuxent	Non-absorbed	-	+	-	-	-
	Absorbed	-	-	-	-	-
Raccoon	Non-absorbed	-	+	-	-	+
	Absorbed	-	-	-	-	-
Tr-321	Non-absorbed	+	+	+	+	+
	Absorbed	-	-	-	-	-
Tr-347	Non-absorbed	-	-	+	+	+
	Absorbed	-	-	-	-	-
Tr-546	Non-absorbed	-	-	+	+	+
	Absorbed	-	-	-	-	-

[1] Absorbed with T. (H.) rangeli 321.
[2] + = Presence of line; - = Absence of line.

TABLE 11

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-347) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (H.) rangeli Tr-347 antigen precipitin lines[2]			
		K	L	G	H
Brazil	Non-absorbed	-	-	+	-
	Absorbed	-	-	-	-
Patuxent	Non-absorbed	-	-	-	-
	Absorbed	-	-	-	-
Raccoon	Non-absorbed	-	-	-	+
	Absorbed	-	-	-	-
Tr-321	Non-absorbed	+	+	+	+
	Absorbed	-	-	-	-
Tr-347	Non-absorbed	+	+	+	+
	Absorbed	-	-	-	-
Tr-546	Non-absorbed	+	+	+	+
	Absorbed	-	-	-	-

[1] Absorbed with T. (H.) rangeli 347.
[2] + = Presence of line; - = Absence of line.

TABLE 12

IMMUNOELECTROPHORESIS OF T. (H.) RANGELI (Tr-546) ANTIGEN WITH NON-ABSORBED AND ABSORBED[1] SERA

Antiserum		T. (H.) rangeli Tr-546 antigen precipitin lines[2]					
		K	L	G	H	M	N
Brazil	Non-absorbed	-	-	+	+	-	-
	Absorbed	-	-	-	-	-	-
Patuxent	Non-absorbed	-	-	-	-	-	-
	Absorbed	-	-	-	-	-	-
Raccoon	Non-absorbed	-	-	+	+	-	-
	Absorbed	-	-	-	-	-	-
Tr-321	Non-absorbed	+	+	+	+	-	-
	Absorbed	-	-	-	-	-	-
Tr-347	Non-absorbed	+	+	+	+	+	-
	Absorbed	-	-	-	-	-	-
Tr-546	Non-absorbed	+	+	+	+	+	+
	Absorbed	-	-	-	-	-	-

[1] Absorbed with T. (H.) rangeli 546.
[2] + = Presence of line; - = Absence of line.

TABLE 13

SUMMARY OF ANTIGENIC COMPONENTS[1] OF T. (S.) CRUZI AND T. (H.) RANGELI AS DETECTED BY IMMUNOELECTROPHORESIS

Trypanosome strain	Antigens (precipitin lines) detected[2]													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Brazil	+	+	+	+	+	(+)	+	+	(+)	(+)				
Patuxent	+	(+)	(+)	+	-	+	(+)	(+)	+	-				
Raccoon	+	(+)	(+)	(+)	+	+	+	+	+	-				
Tr-321							+	+	+	+	+	+	(+)	(+)
Tr-347							+	+	(+)	(+)	+	+	(+)	(+)
Tr-546							+	+	(+)	(+)	+	+	+	+

[1] Composite of results of two different rabbit sera for each antigen.
[2] + = Presence of line; - = Absence of line; (+) = Precipitin line was present and/or demonstrable indirectly by ability of antigen to remove component in heterologous system.

The representation of the antigenic make-up of these trypanosomes shows, in essence, that antigens A through J are found in the cruzi species, while G through N antigens are present in the rangeli species. An intermediate set of components, G through J, are therefore common antigens of both species. The relative positions of all of the species-specific components for each species, as well as the shared ones, are shown in Figure 6.

No reactions were obtained in the IEA with the preimmune sera or with lyophilized culture medium used as antigen. One exception was the sera from animals immunized with T. (H.) rangeli grown in LIT total medium. In this case two distinct components, one migrating to the positive pole and the other to the negative, were consistently detected in all three strains. These two components were absorbed with lyophilized LIT culture medium. IEA precipitin lines due to the medium were not present with antigen from T. (H.) rangeli grown in the modified liquid medium (Appendix II).

Physical and Chemical Characterization

Results of studies on the effect of heat on the different antigenic components of T. (S.) cruzi, Brazil strain and T. (H.) rangeli, Tr-321 strain, are shown in Tables 14 and 15.

In general, the T. (S.) cruzi components were more stable than the T. (H.) rangeli components at all temperatures tested. The T. (S.) cruzi

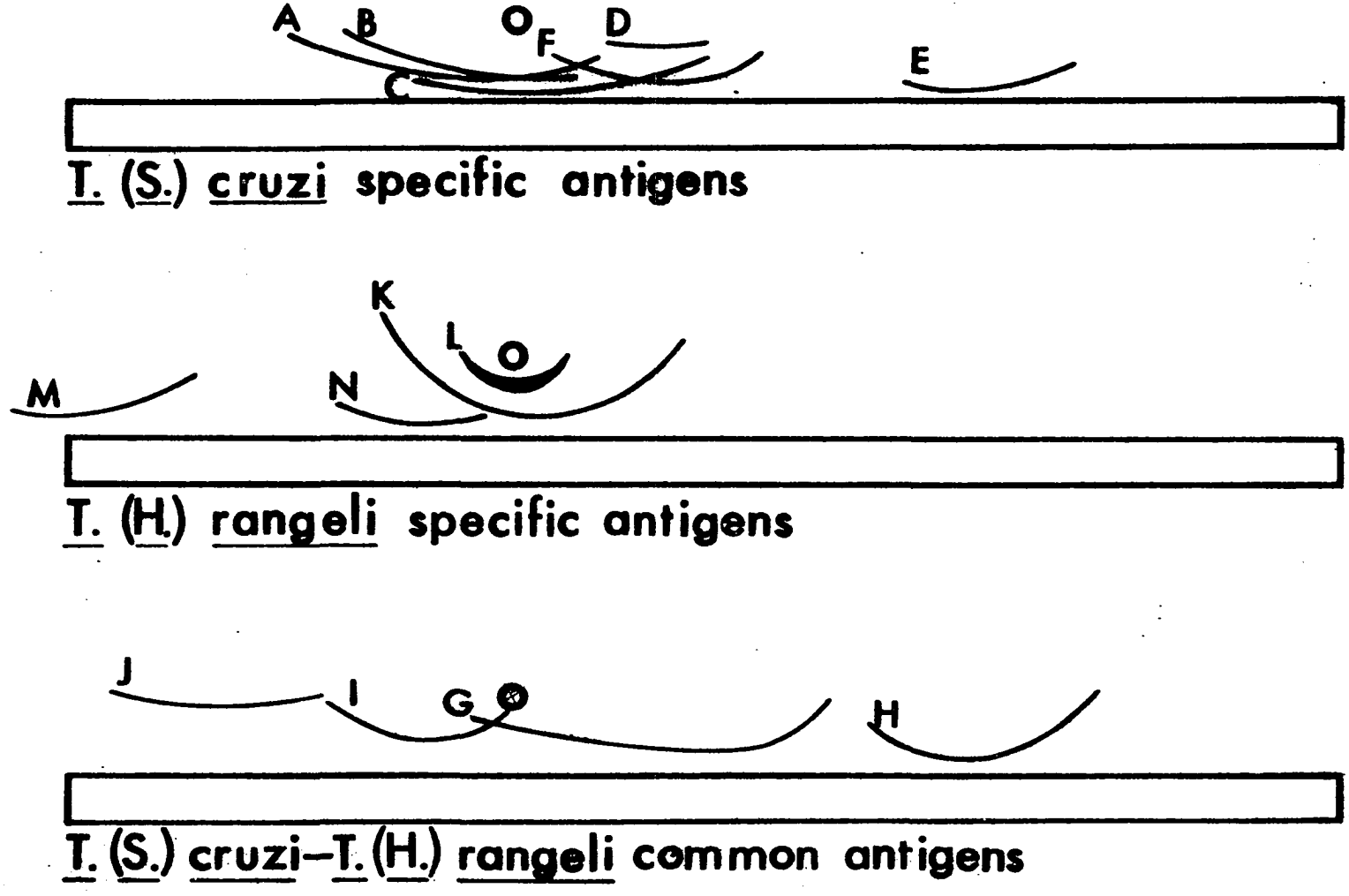

Fig. 6 - Precipitin lines corresponding to specific and common antigens of T. (S.) cruzi and T. (H.) rangeli. The figure is a composite from experiments with six strains in homologous and heterologous systems. The precipitin lines are demonstrated by the immunoelectrophoretic analysis.

TABLE 14

EFFECT OF HEAT ON THE SOLUBLE ANTIGENIC COMPONENTS OF T. (S.) CRUZI (BRAZIL) AS DETECTED BY IEA [1]

Temperature (C)	Incubation time (min)	T. (S.) cruzi Brazil antigen precipitin lines[2]						
		A	B	C	D	E	G	H
37	15	+	+	+	+	+	+	+
	30	+	+	+	+	+	+	+
	45	+	+	+	+	+	+	+
	60	+	+	+	+	+	+	+
45	5	+	+	+	±	+	±	+
	10	±	±	+	±	+	±	+
	15	±	±	+	-	+	±	+
	30	±	±	+	-	+	±	+
60	5	±	±	±	-	+	±	+
	10	-	-	-	-	+	-	+
	15	-	-	-	-	+	-	+
	20	-	-	-	-	+	-	+

[1] Composite of sera from two rabbits.
[2] + = Presence of line; ± = Very faint or distorted appearence.
- = Absence of line.

TABLE 15

EFFECT OF HEAT ON THE SOLUBLE ANTIGENIC COMPONENTS OF T. (H.) RANGELI (Tr-321) AS DETECTED BY IEA[1]

Temperature (C)	Incubation time (min)	T. (H.) rangeli Tr-321 antigen precipitin lines[2]					
		G	H	I	J	K	L
37	15	±	+	+	-	+	+
	30	±	+	+	-	+	+
	45	±	+	±	-	-	+
	60	-	+	-	-	-	+
45	5	+	+	+	+	±	+
	10	+	+	+	-	±	+
	15	±	+	-	-	-	±
	30	±	+	-	-	-	±
60	5	±	+	±	-	±	+
	10	±	+	-	-	±	±
	15	±	+	-	-	-	±
	20	±	+	-	-	-	±

1 Composite of sera from two rabbits.

2 + = Presence of line; ± = Very faint or distorted appearence.
- = Absence of line.

antigen was quite stable at 37 C for up to 60 min. At 45 C the antigenic components were more noticeably affected. A temperature of 60 C for 10 min inactivated all components except E and H (Table 14).

T. (H.) rangeli components H, G, and L exhibited the greatest resistance to the heat treatment. I and K showed only a partial resistance and component J was extremely labile (Table 15).

The freezing and thawing experiment with T. (S.) cruzi antigen revealed no effect on any of the antigenic components thawed and frozen up to 25 times. However, T. (H.) rangeli antigen showed slight deterioration, as evidence by faint precipitin lines, of components G and I after being frozen and thawed 20 times. Component J was similarly affected after being frozen and thawed 25 times.

Results of the chemical treatment of antigens of both species are shown in Tables 16 and 17. Neither the T. (S.) cruzi antigens (Table 16) nor the T. (H.) rangeli antigens (Table 17) were affected by chemicals acting on nucleic acids. The same was true for the carbohydrates since lysozyme did not affect any of the antigenic components of either species. However, sodium metaperiodate and the acetylating mixture which can act on both carbohydrates and proteins totally destroyed the antigenic activity of all components of both species. This is apparently due to their action on the proteins rather than on the carbohydrates.

Reactions affecting lipids of the T. (S.) cruzi antigen (Table 16) gave mostly inconclusive results. Antigenic components A and C were

TABLE 16

EFFECTS OF VARIOUS CHEMICAL AND ENZYMATIC TREATMENTS ON THE SOLUBLE ANTIGENS OF T. (S.) CRUZI (BRAZIL) AS DETECTED BY IMMUNOELECTROPHORETIC ANALYSIS[1]

Treatment	Locus of action		Precipitin lines[2]				
			A	B	C	D	H
No treatment			+	+	+	+	+
4 M Urea		Tertiary structure	-	-	-	-	-
Methanol-HCl		Carboxyl	-	-	-	-	-
Formalin-acetamide		Amino , guanidyl, amide	+	+	-	-	-
Formalin-alanine		Guanidyl, amide, amino	+	+	-	-	-
Formalin 20%		Amino	-	-	-	-	-
Propylene oxide		Carboxyl, phenol, thiol	-	-	-	-	-
p-Chloromercuribenzoate	Proteins	Thiol	+	?	?	?	?
Diazobenzenesulfonic acid		Phenol, imidazole	+	+	?	?	?
Sodium metaperiodate		Hydroxyl, thiol, phenol, indole	-	-	-	-	-
Acetylating mixture		Amino, thiol, phenol	-	-	-	-	?
Pepsin		Whole molecule	?	?	?	?	?
Trypsin		" "	+	+	+	+	?
Papain		" "	+	+	+	+	?
Carboxypeptidase-A		" "	+	+	?	?	?
Lysozyme			+	+	+	+	?
Sodium metaperiodate	Carbohydrates		-	-	-	-	-
Acetylating mixture			-	-	-	-	-

TABLE 16-Continued

EFFECTS OF VARIOUS CHEMICAL AND ENZYMATIC TREATMENTS ON THE SOLUBLE ANTIGENS OF T. (S.) CRUZI (BRAZIL) AS DETECTED BY IMMUNOELECTROPHORETIC ANALYSIS[1]

Treatment	Locus of action	Precipitin lines[2]				
		A	B	C	D	H
Lipase	Lipids	+	?	+	?	?
Ribonuclease	Nucleic acids	+	+	+	+	?
Deoxyribonuclease		+	+	+	+	?
Alkaline phosphatase	Phosphoric esters	-	-	?	?	?

[1] Results from one rabbit serum. Component G was not present in this animal's serum.
[2] + = Presence of line; - = Absence of line; ? = Inconclusive results.

TABLE 17

EFFECTS OF VARIOUS CHEMICAL AND ENZYMATIC TREATMENTS ON THE SOLUBLE ANTIGENS OF T. (H.) RANGELI (Tr-321) AS DETECTED BY IMMUNOELECTROPHORETIC ANALYSIS [1]

Treatment	Locus of action		Precipitin lines [2]				
			I	K	L	G	H
No treatment			+	+	+	+	+
4 M Urea		Tertiary structure	-	-	+	-	?
Methanol-HCl		Carboxyl	-	-	-	-	-
Formalin-acetamide		Amino , guanidyl, amide	+	-	-	-	-
Formalin-alanine		Guanidyl, amide, amino	-	+	-	-	-
Formalin 20%		Amino	-	-	-	-	-
Propylene oxide		Carboxyl, phenol, thiol	-	-	-	-	-
p-Chloromercuribenzoate	Proteins	Thiol	?	?	?	?	?
Diazobenzenesulfonic acid		Phenol, imidazole	-	+	+	+	+
Sodium metaperiodate		Hydroxyl, thiol, phenol, indole	-	-	-	-	-
Acetylating mixture		Amino, thiol, phenol	-	-	-	-	-
Pepsin		Whole molecule	?	?	+	?	?
Trypsin		" "	-	+	+	+	-
Papain		" "	-	-	+	+	+
Carboxypeptidase-A		" "	-	+	+	+	+
Lysozyme			?	+	+	+	+
Sodium metaperiodate	Carbohydrates		-	-	-	-	-
Acetylating mixture			-	-	-	-	-

TABLE 17 - Continued

EFFECTS OF VARIOUS CHEMICAL AND ENZYMATIC TREATMENTS ON THE SOLUBLE ANTIGENS OF T. (H.) RANGELI (Tr-321) AS DETECTED BY IMMUNOELECTROPHORETIC ANALYSIS [1]

Treatment	Locus of action	Precipitin lines [2]				
		I	K	L	G	H
Lipase	Lipids	?	+	±	+	+
Ribonuclease	Nucleic acids	+	+	+	+	+
Deoxyribonuclease		+	+	+	+	+
Alkaline phosphatase	Phosphoric esters	?	+	?	?	+

[1] Results from one rabbit serum. Component J was not present in this animal's serum.
[2] + = Presence of line; ± = Partially present; - = absence of line; ? = Inconclusive results.

not affected by lipase. In the case of T. (H.) rangeli, however, the enzyme did not destroy the antigenic characteristics of components K, G, and H (Table 17).

The most significant results were those concerning reactions affecting proteins. In general, these reactions altered antigenic components of both species of trypanosomes. Essentially every protein chemical group was affected by the treatments and the antigenic activity as detected by the IEA was destroyed (Tables 16 and 17).

Serological Reactions

Results of agglutination of culture flagellates by various sera are shown in Table 18. Neither T. (S.) cruzi nor T. (H.) rangeli reacted with rabbit or guinea pig sera from animals inoculated with live culture forms or injected with soluble antigen. On the other hand, human sera positive to T. (S.) cruzi by the CF test gave extremely low titers with both species of organisms. The homologous system however, produced higher titers than did the heterologous one.

Results of the hemagglutination reactions are shown in Tables 19 and 20. None of the sera from guinea pigs inoculated with either species reacted with the antigens tested. However, the immune rabbit and human sera reacted with both antigens. Interestingly, the T. (H.) rangeli antigen gave consistently higher titers in both homologous and heterologous systems.

TABLE 18

AGGLUTINATION REACTIONS OF T. (S.) CRUZI AND T. (H.) RANGELI CULTURE FORMS IN RABBIT[1], HUMAN[2], OR GUINEA PIG[3] SERA

T. (S.) cruzi serum source	Reciprocal of agglutination titer	
	T. (S.) cruzi Brazil	T. (H.) rangeli Tr-321
Brazil rabbit A	0	0
Brazil rabbit B	0	0
Patuxent rabbit A	0	0
Patuxent rabbit B	0	0
Raccoon rabbit A	0	0
Raccoon rabbit B	0	0
Human Q 2395	16	4
Human Q 2409	4	4
Human Q 2391	64	8
Human Aq 13	8	2
Human Q 2408	64	2
Guinea pig 1	0	0
Guinea pig 2	0	0
Guinea pig 3	0	0
Guinea pig 17	0	0
Guinea pig 18	0	0
Guinea pig 19	0	0
Guinea pig 20	0	0
Guinea pig 21	0	0
Guinea pig 22	0	0

1 Immunized with crude soluble antigen from culture forms of three different T. (S.) cruzi strains. Antigens suspended in Freund's adjuvant.

2 Human sera giving positive CF reactions came from CDC and originally from Argentina.

3 Guinea pigs inoculated with living culture forms of T. (S.) cruzi Brazil.

TABLE 19

HEMAGGLUTINATION REACTIONS OF T. (S.) CRUZI SERA (RABBIT [1], HUMAN [2] , AND GUINEA PIG [3]) WITH T. (S.) CRUZI AND T. (H.) RANGELI ANTIGENS

Serum source	Reciprocal of HA titer	
	T. (S.) cruzi (Brazil) antigen	T. (H.) rangeli (Tr-321) antigen
Brazil rabbit A	320	320
Brazil rabbit B	320	1,280
Patuxent rabbit A	320	160
Patuxent rabbit B	320	1,280
Raccoon rabbit A	640	5,120
Raccoon rabbit B	640	640
Human Q 2395	320	5,120
Human Q 2409	80	320
Human Q 2391	80	640
Human Aq 13	40	320
Human Q 2408	320	640
Human Aq 1	80	1,280
Human Q 2925	160	2,560
Human Aq 3	160	1,280
Human 16 m	160	40
Human 10 m	80	1,280
Human 20 m	320	1,280
Human 11 m	160	320
Guinea pig 1	0	0
Guinea pig 2	0	0
Guinea pig 3	0	0
Guinea pig 17	0	0
Guinea pig 18	0	0
Guinea pig 19	0	0
Guinea pig 20	0	0
Guinea pig 21	0	0
Guinea pig 23	0	0

1 Immunized with crude soluble antigen from culture forms of three different T. (S.) cruzi strains. Antigens suspended in Freund's adjuvant.

2 Human sera giving positive CF reactions came from CDC and originally from Argentina.

3 Guinea pigs inoculated with living culture forms of T. (S.) cruzi Brazil.

TABLE 20

HEMAGGLUTINATION REACTIONS OF T. (H.) RANGELI SERA (RABBIT[1], AND GUINEA PIG [2]) WITH T. (S.) CRUZI AND T. (H.) RANGELI ANTIGENS

Serum source	Reciprocal of HA titer	
	T. (S.) cruzi (Brazil) antigen	T. (H.) rangeli (Tr-321) antigen
Tr-321 rabbit A	40	1,280
Tr-321 rabbit B	40	320
Tr-546 rabbit A	80	640
Tr-546 rabbit B	80	1,280
Tr-347 rabbit A	80	1,280
Tr-347 rabbit B	40	2,560
Guinea pig 5	0	0
Guinea pig 6	0	0
Guinea pig 7	0	0
Guinea pig 8	0	0
Guinea pig 10	0	0
Guinea pig 11	0	0
Guinea pig 13	0	0
Guinea pig 14	0	0
Guinea pig 16	0	0

1 Immunized with crude soluble antigen from culture forms of three different T. (H.) rangeli strains. Antigens suspended in Freund's adjuvant.

2 Guinea pigs inoculated with living culture forms of T. (H.) rangeli Tr-321.

CHAPTER IV

DISCUSSION

The disc electrophoresis data permit only certain broad generalizations since the relatively similar position of bands from different samples does not necessarily imply identity. It only suggests that identity is a possibility, and therefore interpretations of similarity of fractions on the basis of the migration rates is purely speculative.

Disc electrophoresis patterns revealed the complexity of the soluble antigens of each strain. Though the number of bands detected indicates only the very minimum number of fractions which could be present, it was invariably greater than the number detected by IEA. This is the result of two factors: 1) a greater concentration of antigen was used in disc electrophoresis, and 2) the fractions were directly stained after completion of the run. In contrast IEA relies on an antigen-antibody reaction to produce precipitin lines indicative of the various components.

The lipid, carbohydrate, nucleic acid, and disc electrophoresis studies permit certain generalizations. Almost all of these components were found at the end of the spacer gel and at the beginning of the separation gel, indicating that materials constituting the band at the end of

the separation gel were in all probability greater than 1 million in molecular weight (Davies, 1964; Koshawa et al. 1964).

In almost all strains the nucleic acids were located as a single band at the bottom of the spacer gel. Obviously, their carbohydrate moiety would stain and show a band in the same position. It is not known whether the carbohydrate band shown in all samples at this point was composed of carbohydrates other than the ones which are constituents of nucleic acids. T. (S.) cruzi Raccoon antigen gave one band penetrating a short distance into the separation gel and T. (S.) cruzi Patuxent gave a small component in the middle of the gel.

Lipids were detected as a band at the end of the spacer gel or as two bands penetrating slightly into the separation gel. This suggests that they had a molecular weight of around 1 million, or that they had a very slight negative charge. In addition, the Patuxent and the Raccoon strains showed a heavy lipidic component migrating into the separation gel. The significance of these components is not known but their presence suggests that the two strains are related in some manner.

All three strains of T. (H.) rangeli were isolated from naturally infected reduviids captured in a relatively small geographical area and showed striking similarities in their disc electrophoresis patterns. On the contrary, T. (S.) cruzi Brazil was isolated from a human in South America and was somewhat different from the other two strains (Raccoon and Patuxent), both of which were isolated from Procyon lotor in the U.S.

The IEA of two species of American trypanosomes revealed the existence of both shared and species-specific antigens (Figs. 5 and 6). There are reports which indicate that this is usually the case when related species of organisms are studied with the IEA. For example, differences and similarities in IEA patterns have been found in two species of Plasmodium (Zuckerman, 1964). At the strain level the situation has also been explored with other protozoa. Different strains of Entamoeba histolytica have been reported to have different IE patterns as well as shared and specific antigens (Krupp, 1966; Ali Khan and Meerovitch, 1968). On the other hand when sub-strains have been studied antigenic differences have not been found. Goldman and Siddiqui (1965) reported no differences in IE patterns between two sub-strains of E. histolytica derived from the same stock culture. Differentiation of both sub-strains was based on their relative size.

In the present study IEA revealed seven major antigenic components in T. (S.) cruzi Brazil soluble extracts. Six components were detected in the Raccoon and only four in the Patuxent strain (Fig. 5). Goldman and Siddiqui (1965) found eight to nine components in the Brazil strain, and Nussensweig et al. (1963b) found only two to three for T. (S.) cruzi isolates. On the contrary Afchain and Capron (1969) reported 19 components. These differences in the number of major antigenic fractions emphasize the variability of the method, differences in the composition of the strains, and perhaps differences in method-

ology. Afchain and Capron (loc.cit.) used soluble extracts of the parasite in complete Freund's adjuvant for the production of immune serum, while Nussensweig et al. (loc. cit.) used culture forms without adjuvant.

IEA revealed four to six antigenic components in T.(H.) rangeli strains (Fig. 6). To the writer's knowledge this is the first such report dealing with this parasite. The precipitin lines found by IEA in the soluble extracts of these trypanosomes represent the minimum number possible for each strain, and represent the major antigenic components to which the animals responded. The minor components were detected by the absorption studies with heterologous sera both at the species and strain level (Table 13).

Discrepancies in the IEA patterns of homologous systems were encountered often. For example, component E in T.(S.) cruzi Brazil and T.(S.) cruzi Raccoon did not occur consistently (Fig. 5). The same situation was true for component J of strain Tr-321 (Fig. 5). There is no clear explanation at present for these inconsistencies.

The present study has shown that all six strains of flagellates are related antigenically. Both cruzi-specific and rangeli-specific antigens were detected. One major component (for example G in Fig. 5) was a consituent of all three strains of T.(H.) rangeli and two of the T.(S.) cruzi strains. The importance of these findings lies in the possibility of T.(H.) rangeli infections giving false positive results in

serological tests used for the diagnosis of T. (S.) cruzi infections.

Reports of other work using IEA for a comparison of T. (S.) cruzi strains are more difficult to relate to the present study. Nussensweig et al. (1963) and Gonzales-Cappa and Kagan (1969) have worked with isolates and strains of T. (S.) cruzi using IEA and Ouchterlony plates. They found that all strains studied were antigenically related, and could be classified into three different serotypes. The present work showed antigenic relationships among T. (S.) cruzi strains, but not enough strains were analyzed to permit a confirmation of the groups found by the previous authors nor was this an objective of this study.

Antigenic relationships among T. (S.) cruzi strains and isolates have also been shown in other serological systems. Walton et al. (1958), Packchanian (1935), Hauschka et al. (1950), and Chang and Negherbon (1947) using agglutination tests found that several isolates or strains of T. (S.) cruzi were related antigenically. Antigenic relationships among different isolates of T. (S.) cruzi which produce low parasitemias and scanty histopathological lesions have also been shown by the protection test in mice (Norman and Kagan, 1960; Kagan and Norman, 1961). This test is a well accepted criterion today for classifying the species as cruzi in undetermined isolates. On the contrary, no antigenic relationships have been demonstrated previously in serological studies comparing T. (S.) cruzi and T. (H.) rangeli.

D'Alessandro (1963a) found no protection in animals infected with T. (H.) rangeli isolates and subsequently challenged with T. (S.) cruzi. This suggests indirectly that none of the common antigens detected in the present study with the IEA, was important in eliciting "protection" in animals challenged with virulent T. (S.) cruzi strains.

The IEA of the six trypanosome strains revealed major antigenic components which were different from one another. Differences exist between the species as well as among the three strains within each species. The differences in the characteristic IEA patterns of the three strains of each species are valid with respect to the major antigenic components (Fig. 5). When the minor or secondary components found with the absorption studies are added to the antigenic composite of each strain, all the three strains within each species have essentially the same set of antigens (Table 13). On the contrary, there were clear differences between the species (Table 13).

In spite of the fact that characteristic antigens exist for each species of flagellates, the small number of strains studied here do not allow generalizations regarding separation of unknown isolates into species on the bases of IEA patterns.

All of the information obtained via physical and chemical characterization is expressed in terms of changes in the antigenic components as revealed by IEA. The same method has been used for studies of Trichinella spiralis antigens (Tanner, 1963a, 1963b), and for the African trypano-

somes (Williamson and Brown, 1964).

The freeze-thaw experiments gave essentially similar results with both species. Generally, freezing and thawing up to 20 times did not have a deleterious effect on the antigens. Similar results were reported by Thompson et al. (1968) for antigens derived from E. histolytica.

The results of heat stability experiments showed that T.(H.) rangeli antigens were more labile than T.(S.) cruzi antigens. Some differences in response of the shared antigen G to temperature treatment are shown in Tables 14 and 15. This component was resistant to treatment at 37 C in the T.(S.) cruzi system but showed a partial degree of lability in the T.(H.) rangeli system. There is no good explanation for this discrepancy at present.

T.(S.) cruzi antigenic components were not affected by exposure to 37 C for one hour, whereas some T.(H.) rangeli components were altered at this temperature. Higher temperatures, 45 C and 60 C destroyed most of the activity of both antigens. Interestingly, Thompson et al. (1968) found that 37 C and 56 C temperatures for 8 hours did not affect E. histolytica antigens but a 100 C temperature destroyed the antigen in 10 - 20 min. However, their experiments differed from those reported here in that they used lyophilized material instead of a saline suspension of antigen. Similarly, Williamson and Brown (1964) found that 37 C for 18 hours did not destroy antigens of the

brucei group of trypanosomes.

The chemical characterization studies showed that carbohydrates, lipids, or nucleic acids were either not present, or if present were not affected in any of the components detected by the IEA. These results correlated well with results obtained by disc electrophoresis.

The principle moiety of all soluble antigens detected with IEA was therefore protein. The results suggest that components A and B of T. (S.) cruzi Brazil are related in their antigenic properties since they were both affected by urea, methanol-HCl, formalin, propylene oxide, metaperiodate, and acetylating mixture treatments. Similarly, components C,D, and H were affected by the same treatments plus the formalin-acetamide and formalin-alanine (Table 16). It is suggested that the difference between antigens A and B and antigens C, D, and H is the guanidyl, or the amide, or both the guanidyl and amide chemical groups. These groups seem to be an essential part of the epitope in antigenic fractions C, D, and H but not in fractions A and B (Table 16).

The results of the chemical characterization of T. (H.) rangeli (Tr-321 were similar to those obtained with T. (S.) cruzi. Nucleic acids, carbohydrates, and lipids were not detected in any of the fractions (Table 17). However, the chemicals affecting proteins produced similar reactions with all antigenic components. The I component was especially sensitive and was the only one affected by trypsin, papain, and carboxypeptidase-A.

Pepsin rapidly hydrolyzes non-denatured and denatured proteins

at pH 2.0 (White, Handler, and Smith, 1968). However, in the present study the results obtained were inconclusive since the control buffer, pH 2.0, also destroyed the antigenic fractions of both species of trypanosomes. Trypsin acts upon carboxyl groups of arginine and lysine (White et al. loc. cit.). Papain affects the thiol group of cystein (Mahler and Cordes, 1966) and carboxypeptidase-A the free alpha carboxyl group (White et al. loc. cit.). It is apparent from the above description that sites of action of these enzymes, if present in the different antigen fractions do not enter into the constitution of the antigenically active site of the molecule. In fact, with few exceptions, enzyme treatment did not affect the ability of antigenic fractions to react with antibodies in the IEA system.

The data obtained with the serological studies, and especially with the HA also indicate a close antigenic relationship between the two human trypanosomes. Agglutination of living culture forms of both species was detected only when T. (S.) cruzi human sera were used (Table 18). Titers were low in the five sera tested but this is not unusual and has been found both in chronic Chagas disease patients and in experimental animals (Muniz et al. 1946). The possibility exists that titers obtained here non-specific. This is not considered to be the case because 1) washed and non-washed culture forms gave the same titers, 2) the normal human serum control was consistently negative, and 3) reactivity was absorbed by the culture forms.

The immune sera obtained from rabbits inoculated with either T. (S.) cruzi or T. (H.) rangeli strains gave negative results in agglutination tests. This is an apparent contradiction to earlier reports, especially with T. (S.) cruzi. Sera from animals experimentally inoculated with dead or living culture forms have usually shown high agglutinin titers (Packchanian, 1935; Chang et al. 1947). However, until now there have been no reports of experiments using soluble antigen extracts from culture forms to produce immune sera for agglutination studies.

It is well established that agglutinating antibodies attach to the specific binding sites on the cell membrane (Wallach and Kamat, 1964; Wallach, 1967; Benedetti and Emmelot, 1968). It has also been shown that sites identical to the cell membrane binding sites for agglutinating antibodies are present on membranes of intraplasmatic cell organelles (Wallach and Kamat, loc. cit.). The results of T. (S.) cruzi and T. (H.) rangeli agglutination studies described here suggest that the soluble antigens used contained very few, or none of the surface antigens of the parasite. Cell membrane components being insoluble were probably discarded with the pellet after centrifugation, or if soluble, they were not present in high concentrations and thus the immunized animals did not develop detectable agglutinating antibodies. The finding of Chang et al. (1947) that ghost T. (S.) cruzi cells (cell membranes) agglutinated in the presence of antibody in the same fashion as living organisms, supports this interpretation.

Sera from guinea pigs inoculated with living culture forms was also negative in all serological tests. It is possible that the number of organisms used for immunization was insufficient. For example, animals inoculated with very large numbers of living culture forms produced agglutinating antibodies (Packchanian, 1935; Change et al. 1947). The specific purpose of the small dose of flagellates used in the present report was to obtain an infection which would resemble that seen in nature. Large numbers of culture forms are not desirable for immunizing animals whose sera are going to be tested with antigens derived from the same forms. Results obtained under such conditions are of little or no significance since they cannot be compared with what is occurring in nature.

Certain findings of Simpson (1968) point to the importance of obtaining infections with the life cycle stages occurring in natural infections. In a related organism, Leishmania donovani, he found antigenic differences between the amastigote and the promastigote forms.

The HA reactions gave positive results with rabbit and human sera, using either cruzi or rangeli antigens. This cross-reactivity is in all probability based on the fact that the shared antigens both provoked production of antibodies and reacted in the HA test. This dual function is seen in the sera of animals inoculated with soluble antigens. More important, the evidence presented here indicates that this cross-reactivity is also present in sera from humans infected with T. (S.) cruzi (Table 19). However, the possibility of double (cruzi-rangeli) infections in humans

must always be considered (D'Alessandro and Gutierrez, unpublished). All samples used here originated in Argentina where T. (H.) rangeli infection has been reported in man only once (Borzone et al. 1949, 1950) and the validity of this finding has been questioned (Zeledon, 1954). Thus the possibility of double infections in the donors of these sera appears unlikely. Quite possibly the cross-reactions were the result of the shared antigens which were demonstrated to exist by IEA. In the case of T. (S.) cruzi natural infections in man these antigens probably give rise to antibodies which can react with either T. (S.) cruzi or T. (H.) rangeli antigens in HA tests. This cross-reactivity was demonstrated here only when T. (H.) rangeli antigen was used in HA reactions with T. (S.) cruzi human antisera.

The lack of T. (H.) rangeli antisera (from naturally acquired infections) has been a limiting factor in evaluating whether the same situation is true for this system. Experimental animals inoculated with insect infective forms should be used in order to find an answer to this problem because with human T. (H.) rangeli sera it is not possible to ascertain that a mixed cruzi-rangeli infection is not present. No geographical area is known today where only T. (H.) rangeli infections are present in man.

Interestingly, titers were almost invariably higher with T. (H.) rangeli than with T. (S.) cruzi antigen when tested against human or rabbit T. (S.) cruzi sera. If such findings can be confirmed, together with the fact that T. (H.) rangeli mass culture is now possible for production of

antigen, an important contribution to T. (S.) cruzi serology could result. The routine use of T. (H.) rangeli as a source of antigen for HA and perhaps other serological reactions could be feasible, with the advantage that there would not be the danger of laboratory infections with T. (S.) cruzi.

CHAPTER V

SUMMARY

The soluble extracts of six strains, belonging to two species of American trypanosomes, were compared by disc electrophoresis and immunoelectrophoretic analysis. Certain physical and chemical characteristics of the major antigenic components of a strain representative of each species were determined. Also, both species were compared serologically using agglutination and hemagglutination tests.

Disc electrophoresis of soluble extracts revealed that the major antigenic components were proteins. Carbohydrates, lipids, and nucleic acids were present in small amounts, and almost invariably were molecules one million or greater in molecular weight.

Immunoelectrophoretic analysis revealed a characteristic pattern of major antigenic components for each of the six strains of trypanosomes. These components were arbitrarily labeled with letters from A to N. Absorption studies of homologous and heterologous sera of all six strains showed that there were antigenic components characteristic of both the cruzi (A to J), and the rangeli species (G to N). The set of antigens, G to J, were shared by both species. The actual number of major components varied for

each strain. T. (S.) cruzi strains were found to have from four to seven components, while T. (H.) rangeli had from four to six.

The physical characterization studies showed that 37 C temperatures for up to one hour did not affect T. (S.) cruzi components, 45 C for 30 minutes only slightly altered antigenic activity, whereas 60 C for 5 minutes usually destroyed it. T. (H.) rangeli antigens were somewhat more labile with temperature treatments. Freezing and thawing up to 25 times had no deleterious effects on T. (S.) cruzi components but T. (H.) rangeli components showed deterioration after freezing and thawing 20 times.

The chemical characterization studies showed that all of the major antigenic components of both species were proteins. No carbohydrates, lipids, or DNA-RNA were detected in the antigens by chemical and enzyme treatments of the crude soluble extracts.

Serum from humans serologically positive for T. (S.) cruzi gave very low agglutinin titers with living culture forms of both strains, but lower titers with T. (H.) rangeli. Hemagglutination reactions were positive with antigens from both species. In both sera from animals immunized with soluble extracts of T. (S.) cruzi and human sera positive for T. (S.) cruzi, antibody titers were higher when T. (H.) rangeli antigen was used.

APPENDIX I

LIT CULTURE MEDIUM FOR T. (S.) CRUZI

1. Dialysis bag components

Bacto liver	10.0 gm
Bacto Tryptose	2.5 "
Dextrose	1.0 "
H_2O	50.0 ml

Dissolve and adjust pH to 7.2. with 1.0 N NaOH. Place the above solution in the dialysis bag.

2. Flask components (outside the dialysis bag).

KCl	0,2 gm
Na_2HPO_4	2.5 "
NaCl	2.0 "
H_2O	450.0 ml

Dissolve, adjust pH to 7.2 and place in a 2000 ml Erlenmeyer flask. Sterilize at 20 lb for 15 min. Cool and add 1 ml of frozen and thawed blood (rabbit or human) to the bag through the needle. Then add 100 ml of sterile serum in the same fashion (Pooled normal human sera was used here because it was in abundant supply at practically no cost). Antibiotics were added to each bag to get approximately 100 ug of streptomycin and 100 units penicillin G per ml of the dialysis bag medium.

APPENDIX II

T. (H.) RANGELI CULTURE MEDIUM

1. Dialysis bag components

Bacto Beef	1.50 gm
Bacto Peptone	2.50 "
$CaCl_2$	0.07 "
H_2O	50.00 ml

Adjust the pH to 7.2 and place inside the dialysis sac.

2. Flask components (outside the dialysis bag)

KCl	0.07 gm
NaCl	4.00 "
KH_2PO_4	0.10 "
Dextrose	0.80 "
H_2O	450.00 ml

Adjust the pH to 7.2 and add to a 2000 ml Erlenmeyer flask. Sterilize at 20 lb for 15 min, cool, and add 150 ml of inactivated human blood (56 C for 30 min) to the dialysis sac. Antibiotics in the proportions used for LIT medium are also added.

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