STUDIES ON THE *IN VIVO* AND *IN VITRO* GROWTH OF *TRYPANOSOMA CRUZI* (CHAGAS, 1909)

By

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INTRODUCTION

Trypanosoma cruzi was first described and identified as the causative agent of American trypanosomiasis or Chagas' disease by Chagas in 1909. The disease, once believed limited to the state of Minas Gerais, Brazil, is now known to affect millions of persons from Mexico to Argentina (Dias and Laranja, 1948). Naturally infected triatomids and mammals have frequently been found in Texas, California, and Arizona (Hall, 1953). The first indigenous case of human Chagas' disease in this country was reported in 1955 from Corpus Christi, Texas (Woody and Woody, 1955).

While information concerning its distribution continues to increase, knowledge of other aspects of the disease remains scanty. There is still no treatment for the infection (Goble, 1956; Hawking, 1953). Nor is the manner in which the parasite harms the host known. The idea that the trypanosome injures its host through the consumption of large amounts of blood glucose has been discarded (von Brand *et al.*, 1949). Evidence concerning the production of endotoxins by *T. cruzi* is highly contradictory (von Brand, 1951). Cameron (1956) believes that the effects of this parasite are due chiefly to mechanical injury to host tissues. It is unlikely that solutions to these problems of treatment and causation will be found until much more basic information on the metabolism and nutritional requirements of *T. cruzi* has been gained. The present study was undertaken in an effort to gain some further insight into the physiology of this organism.

Several excellent reviews of our present day knowledge of the physiology of T. cruzi have recently appeared (von Brand, 1951, 1952; Lwoff, 1951; Hutner and Provasoli, 1955). It is well established that culture forms of T. cruzi, which correspond to the form found in the invertebrate host, utilize glucose (von Brand et al., 1949). Ryley (1956) demonstrated glucose consumption by blood stream forms. Baernstein and Rees (1952) found aldolase in culture forms of the parasite, and Baernstein (1953b) demonstrated the presence of isomerase and triosephosphate dehydrogenase. From this, he concluded that T. cruzi probably has the classical Embden-Myerhof-Parnas glycolytic scheme.

Considerable evidence for the presence in the parasite of a functional tricarboxylic acid cycle has been accumulated. Succinic dehydrogenase activity has been shown by Seaman (1953), Agosin and von Brand (1955), and Ryley (1956). Malic dehydrogenase and fumarase were demonstrated by Baernstein (1953a), and isocitric dehydrogenase by Agosin and Weinbach (1956). Von Brand and Agosin (1955) found that the respiration of T. cruzi was stimulated by the addition of various Krebs cycle intermediates to the medium. Ryley (1956) detected small amounts of citric acid in media containing T. cruzi. He suggested that the tricarboxylic acid cycle was operating in these organisms and that traces of this key metabolite leaked from the cells. Ryley found no significant differences in glucose metabolism between blood stream and culture forms. Therefore, the above findings on culture types of T. cruzi are probably applicable to the blood stream forms also, and suggest that aerobic metabolism of the organism is carried out via the tricarboxylic acid cycle.

In view of these data, the report that injection of adenosine triphosphate reduced the mortality of cortisone-injected mice infected with T. cruzi (Adams, 1954) appeared to be highly significant.

Different strains of T. cruzi vary widely in their pathogenicity for mice, ranging from a complete avirulence to an extreme virulence (Hauschka, 1947). Since 1951 when Jarpa et al. found that the administration of cortisone acetate to mice infected with T. cruzi increased the parasitemia and the mortality rate, this compound has been used to enhance the effect of the less virulent strains on host organisms. Cortisone has this same effect on various other spontaneous and experimentally induced infections, including tuberculosis, poliomyelitis, and certain kinds of malaria. The mechanism of this action is not definitely known, but there seems to be rather general agreement that cortisone depresses host resistance to infection rather than having any direct effect on the infectious agent. It has been found that the hormone affects metabolism through its action on various enzymes and that it acts on lymphoid tissue, reducing capillary permeability and inhibiting phagocytosis, antibody formation, and reticulo-endothelial activity. Any or all of these effects may be related to its infection-enhancing ability. This subject has been reviewed in a series of papers edited by Shwartzman (1953).

The nutritional requirements of T. cruzi are still unknown. The chief obstacle to the determination of these growth needs and to the study of their metabolism has been the need for a defined synthetic medium in which to grow the organism. Until quite recently it was be-

lieved that T. cruzi could not be grown in the absence of erythrocytes (Little and Oleson, 1951) or serum (Lwoff, 1951). Citri and Grossowicz (1954) then devised a liquid medium in which crystalline serum albumin, hematin, and tomato juice completely replaced blood. They refined the medium further by substituting known growth factors for the tomato juice (1955a). This medium supported satisfactory growth of the Culbertson strain of T. cruzi. Seaman (1957) suggested that their medium might be rather selective for this strain, since several other strains did not grow well in it.

The aims of the present study were: (1) to repeat Adams' (1954) experiments with adenosine triphosphate, (2) to extend his work to include the study of the effects of other compounds involved in carbohydrate metabolism on trypanosome-infected mice, (3) to test the ability of Citri and Grossowicz's medium (1955a) to support growth of T. cruzi strains other than the Culbertson strain, (4) to define further the partially defined medium of Citri and Grossowicz.

MATERIALS AND METHODS

Organisms and Their Maintenance

The "Brazil" and "Costa Rica" strains of Trypanosoma cruzi were used for in vivo studies. In addition to these two strains the Culbertson strain was utilized in the culture work for comparative purposes, since it was for this strain that Citri and Grossowicz (1954, 1955a) devised their media. Axenic cultures were used throughout this study.

The Brazilian strain was obtained from Dr. R. G. Yeager (Tulane Medical Center) who had maintained it in a diphasic medium consisting of Difco brain-heart infusion agar plus 25 per cent defibrinated rabbit blood overlaid with 2 to 3 ml. of sterile Locke's solution. This strain was originally isolated from a Brazilian patient in 1942 (Goble, 1951) and has been maintained in several laboratories in this country since that time.

The Costa Rican strain was secured in Costa Rica by Dr. Herbert Johnstone of the University of California Medical Center (Noble et al., 1953) and was given to me by Dr. E. R. Noble (University of California, Santa Barbara College). The latter grew the organisms in a medium composed of glucose, NaCl, and peptone to which was added red cell coagulum or hemoglobin coagulum (McRary et al., 1953).

Dr. N. Grossowicz supplied a culture of the Culbertson strain which had been maintained at Hebrew University, Jerusalem, on Adler's medium (Adler and Theodor, 1926) since 1949. The early history of this strain is incomplete (Hauschka, 1947).

All three strains grow well in a modification of the medium used by Yeager. This modification is prepared as follows:

Difco brain-heart infusion broth	3.7 gm.
Bacto-agar	1.5 gm.
Pyrex-distilled water	100 ml.

This was autoclaved for 15 minutes at 15 p.s.i., cooled to approximately 46° C., and to it was added:

Whole blood	10 ml.

Citrated (ACD) blood discarded by the J. H. Thomas Memorial Blood Bank, Gainesville, Florida, was used and found to produce highly satisfactory growth. Each slant of this medium was overlaid with 5 ml. of sterile Locke's solution (NaCl, 8.0 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; KH_2PO_4, 0.3 gm.; dextrose, 2.5 gm.; Pyrex-distilled water, 1000 ml.) to which were added penicillin (60 μg./ml.) and streptomycin (100 μg./ml.) to decrease the risk of bacterial contamination. Stock cultures of all strains were grown in this medium at 27° C., and subcultures were made every 2 to 3 weeks.

All of the mice used in this study were bred in the laboratory from a small initial stock of white mice, CF No. 1 strain, obtained from Carworth Farms, Inc., New City, New York. Although this procedure limited the number of mice available for experimentation, it did assure that the mice used were in good health and that they were of the desired age. The mice were permitted to feed ad libitum on Purina Laboratory Chow, and a constant supply of water was provided. Young mice were weaned at 28 days of age.

In vivo Experiments

1. Injection of mice. Mice to be infected were injected with both cortisone acetate and trypanosomes, since the Brazilian and Costa Rican strains of T. cruzi are only moderately virulent. Five mg. of cortisone acetate (saline suspension, Cortone - Merck) were injected subcutaneously. One-half ml. of trypanosome-containing overlay from two- to three-week-old stock cultures was injected subcutaneously or intraperitoneally. The split litter technique, with consideration for sex, was used in all experiments.

At the beginning of this study, it was felt that it might be desirable to inject the mice after they had been weaned. The earliest age suggested for weaning is 21 days, and 28 days is the recommended time (Snell, 1941). Culbertson and Kessler (1942) found that when mice under 25 days of age were inoculated with T. cruzi (strain not indicated) they acquired an infection of greater intensity than that exhibited by older mice and generally died, while mice above this age usually survived. Therefore, three litters of weaned 21-day-old mice were injected to determine whether infections could be produced at that age. Each litter was divided into the following treatment groups: (1) no injection (no inj); (2) cortisone (cort); (3) trypanosomes, Brazilian (Br) or Costa Rican (CR) strains; (4) trypanosomes and cortisone. At the end of 60 days only two mice (and one of these not infected) had died (see Table 1). With such a low mortality rate, it would be difficult to determine whether injections of adenosine triphosphate or other compounds

TABLE 1

MORTALITY OF 21-DAY-OLD MICE INJECTED WITH TRYPANOSOMES AND/OR CORTISONE

Treatment	No. mice injected	Deaths No.	Deaths %	No. days between injection and death
No inj	2	0	0	
Cort	5	1	20.0	9
Br	4	0	0	
Br + cort	5	2	40.0	155, 321
CR	4	1	25.0	78
CR + cort	5	2	40.0	43, 312

reduced the number of deaths resulting from the disease. For this reason, 17-day-old mice, which are more susceptible to infection with T. cruzi, were used for all of the experiments in this study, following the practice of Adams (1954).

Haemocytometer counts of trypanosomes used for injection were made by diluting the culture with a 1:10 dilution of neutral formalin (36% formaldehyde) in distilled water. The organisms were counted in 80 squares (as recommended for erythrocyte counts) with a magnification of 430X. Duplicate counts were made in each case and the results of the two averaged to give the number of organisms per ml. of sample.

Other injections were administered as indicated in the description of the individual experiments.

Experimental mice were weighed on the day of injection and every other day thereafter for 1 month. Weights were taken every fourth day for a second month.

2. Histological techniques. All dead mice were autopsied, and macroscopically visible changes in internal organs noted, as soon after death as the animals were discovered. Tissues for histological study were immediately fixed in Bouin's fluid, and 8 μ paraffin sections were stained in Harris' haematoxylin and counterstained in a saturated solution of orange G in 95 per cent ethanol. Spinal and brain smears were fixed in methanol and stained with Wright's blood stain. Fresh tissues to be examined were teased or macerated in a drop of Neff's (1955) basic salt solution (NaCl, 120 mg.; $MgCl_2.6H_2O$, 3 mg.; $CaCl_2$, 3 mg.; $FeSO_4.7H_2O$,

3 mg.; 0.1M KH_2PO_4 and 0.1M Na_2HPO_4, 20 ml. to adjust pH to 6.5; Pyrex-distilled water to 1000 ml.) and were examined under variable phase contrast at a magnification of 400X with an AO-Baker Interference Microscope.

3. Trypanosomes and/or cortisone. A series of experiments was performed to determine what percentage of injected mice died, the number of days elapsing between time of injection and death, and the growth pattern (based on weight) of injected mice. These data provide a basis against which to compare the results of later series of experiments in which the mice were, in addition, injected with nucleotides or thiamine, and also serve for comparing the effects of the Brazilian and Costa Rican strains of T. cruzi.

Ten litters of 17-day-old mice were used in these experiments. Each litter was divided into groups, usually four, each of which was treated in one of the following ways: (1) no injection, (2) cortisone, (3) trypanosomes, (4) trypanosomes and cortisone. Occasionally, large litters were divided into more groups in order to test the two strains of trypanosomes on members of the same litter.

4. Adenosine triphosphate (ATP). Each litter was divided into groups which were injected as follows: (1) trypanosomes and cortisone, (2) trypanosomes, cortisone, and ATP, (3) cortisone and ATP. Later, some of the litters were further subdivided to test the effects of different amounts of ATP on members of the same litter. The trypanosomes and cortisone were injected as previously described, and 8 hours later the ATP solution was injected subcutaneously. Nine litters of mice were

used in these experiments.

The disodium salt of ATP was dissolved in sterile Locke's solution to give the desired concentration. The solution was prepared immediately before it was to be injected, for ATP breaks down rapidly in solution.

To repeat Adams' (1954) work, 5.0 mg. of ATP were given to each mouse. When it was found that more of the infected mice treated in this way were dying than those untreated, the amount was reduced to 2.5 mg. of ATP.

5. Thiamine. Several mice in the preceding experimental series which had been injected with Brazilian strain and cortisone displayed symptoms similar to those of thiamine deficiency (Morris, 1947; Woolley and White, 1943). They were unable to retain their balance and rolled over and over; they went into convulsions when held by the tail; and spasticity of the legs and feet was apparent. These observations, coupled with the reports that culture forms of T. cruzi may produce appreciable amounts of pyruvic acid (Chang, 1948) and that blood stream forms produce at least small amounts of it (Ryley, 1956), suggested that a thiamine deficiency might be involved in the course of the infection, since thiamine is required for the metabolism of pyruvate via the Krebs tricarboxylic acid cycle. It seemed possible that: (1) the trypanosomes were using thiamine in amounts that caused the host to become deficient, (2) the trypanosomes were producing pyruvic acid to such an extent that the mouse was unable to metabolize it, and so the accumulated pyruvate

proved toxic (Robinson, 1951; Grant and Fulton, 1957), or (3) an independently occurring thiamine deficiency might exist in some mice, perhaps making them more susceptible to infection. Mice were, therefore, treated with thiamine to see what effect it might have on the infection.

Each litter was divided into three treatment groups: (1) trypanosomes and cortisone, (2) trypanosomes, cortisone, and thiamine, (3) thiamine. Mice from two litters were given 60 µg. of thiamine hydrochloride (amount adopted from Woolley and White, 1943) orally on the day following injection and every other day thereafter for 4 weeks, giving a total of fourteen doses. Mice from five litters were injected subcutaneously with 25 µg. or 50 µg. of thiamine hydrochloride on the day following the trypanosome injection and every other day thereafter for 4 weeks. The thiamine solution was prepared by dissolving thiamine hydrochloride in Locke's solution and was sterilized by filtration.

6. Thiamine, magnesium, and adenosine triphosphate. In the conversion of pyruvate to acetyl coenzyme A, thiamine is in the form of thiamine pyrophosphate or cocarboxylase. It has been known for some time that ATP is necessary for the phosphorylation of thiamine *in vitro* (Glass, 1951), and it has recently been demonstrated (Rossi-Fanelli *et al.*, 1954) that this reaction also occurs in the living animal. Within 1 hour of injecting thiamine and ATP into intact rats, phosphoric groups are transferred to thiamine, producing di- and triphosphothiamine. Magnesium ions are probably necessary for the phosphorylation of thiamine (Glass, 1951; Neilands and Stumpf, 1955).

Therefore, in conjunction with the thiamine experiments, four litters of mice were treated with thiamine, Mg^{++}, and ATP. The injection solution was prepared to give a 1:1:1 molar ratio of the components. The desired amounts of thiamine hydrochloride and $MgCl_2.6H_2O$ were dissolved in Pyrex-distilled water and sterilized by filtration. The ATP was dissolved in this solution just before the mice were injected. Each litter was divided into these treatment groups: (1) trypanosomes and cortisone, (2) trypanosomes, cortisone, thiamine, Mg^{++}, and ATP, (3) thiamine, Mg^{++}, and ATP. On the day following injection of the trypanosomes and every other day thereafter for 4 weeks, the mice were given subcutaneous injections of thiamine hydrochloride, 200 µg.; $MgCl_2.6H_2O$, 120.6 µg.; and ATP (disodium salt), 370 µg.

7. Adenosine diphosphate and inorganic phosphate. Under physiological conditions the coupling of oxidation and phosphorylation appears to be obligatory, and both reactions are dependent upon the supply of adenosine diphosphate (ADP) and inorganic phosphate (iP) (Krebs, 1957). In other words, phosphate and/or phosphate acceptors are the rate-limiting factors in oxidation under different conditions which have been studied (Lardy, 1956), and Krebs (1957) has suggested that both are probably important in vivo. Since, in most tissues the available concentrations of ADP and iP are below the critical level, the rate of oxygen consumption depends upon the rate at which ATP splits to form ADP and iP (Krebs, 1956).

In view of these facts, it seemed possible that whatever effect

ATP had upon the trypanosome-infected mice might be hastened and/or enhanced by supplying ADP and iP rather than ATP. Five litters of mice were divided into these treatment groups: (1) trypanosomes and cortisone, (2) trypanosomes, cortisone, ADP, and iP, (3) cortisone, ADP, and iP. The injection solution was prepared so that an amount of ADP equivalent in moles to 2.5 mg. of ATP would be administered and with the molar ratio of ADP to iP 1:1. The desired amount of $Na_2HPO_4.12H_2O$ was dissolved in Pyrex-distilled water and the solution sterilized at 15 p.s.i. for 20 minutes. The ADP (sodium salt) was dissolved in the phosphate solution immediately before injection. Eight hours after receiving trypanosome injections, the mice received 2.4 mg. of ADP and 1.8 mg. of $Na_2HPO_4.12\ H_2O$ subcutaneously.

In vitro Experiments

1. Inoculation of cultures. To establish initial cultures of trypanosomes in the partially defined medium of Citri and Grossowicz (1955a), several stock cultures of each strain were pooled and centrifuged at 1400 rpm (r = 15.0 cm.) for 10 minutes. The organisms were washed twice in 0.85 per cent NaCl solution and finally suspended in 5 ml. of this. This suspension was used to inoculate tubes of Citri's medium, and samples were taken for haemocytometer counts.

Other experimental media were inoculated with washed organisms grown in Citri's medium.

Cultures were grown in 15x125 mm. Pyrex culture tubes, each containing 5 ml. of medium. These were incubated at 27° C. in a slanted

position. Both experimental and stock cultures were routinely checked for bacteria, using nutrient agar pour-plates.

Population densities were determined by making direct haemocytometer counts as previously described. The counts from duplicate tubes were averaged to give a measure of growth for each experiment. All experiments were run at least in triplicate.

2. Preparation of media. Water redistilled in a Pyrex glass still was used in compounding all media. Vitamins, amino acids, and hemin were obtained from Nutritional Biochemicals Corporation. Concentrated stock solutions of vitamins and metal mixes were prepared and stored in the refrigerator. Because the activity of folic acid deteriorates during storage (Trager, 1957), solutions of it were used within 2 months of preparation. The solution was prepared by dissolving the folic acid in a few drops of 0.01N NaOH, then adding water and heating gently. Hemin was dissolved in a small amount of 5N NH_4OH, then made up to volume with Pyrex-distilled water. Solutions of glucose, hemin, serum albumin fraction V, thiamine, and riboflavin were sterilized by filtration through a Pyrex brand fritted glass filter disc of ultrafine porosity and added aseptically to the autoclaved medium.

After experimenting with various modifications on Citri's medium, it was found that a known mixture of amino acids, the composition of which is based on an analysis of β-lactoglobulin (Tristram, 1953), could be substituted for the casein hydrolysate. Serum albumin fraction V was omitted from the medium, various metals were added, and slight changes

were made in the concentrations of some other ingredients. The growth factors and their concentrations were not altered. The composition of the medium is shown in Table 2.

The amino acids were dissolved in three-fifths of the final volume of boiling water, then the salts were added, and the mixture cooled. Tween 80 (polyoxyethylene sorbitan monooleate), cytidylic acid, creatine, creatinine, and ribonucleic acid were added to this solution, and 1.0N NaOH was used to increase the pH before the vitamins were added. The pH was finally adjusted to 8.0, the volume made up with redistilled water, and the medium autoclaved at 15 p.s.i. for 20 minutes. The remaining ingredients, as indicated in the table, were added aseptically to the cooled, sterile medium.

In Medium 1 the amount of hemin was increased to 2.5 mg./100 ml. of medium. Medium 2 was identical to Medium 1 except that the hemin was added before autoclaving.

Medium 3 differed from the basal medium in several ways. Hemin polymerizes in alkaline solution so that part of it may not be available for use by the organisms; therefore, the hemin for this medium was dissolved in triethanolamine (5 mg. hemin/ml. 50 per cent (w/v) aqueous triethanolamine) as suggested by Cowperthwaite et al. (1953). The amount of triethanolamine added to the medium with the hemin was only half the total amount to be included; the remainder was added as the 50 per cent (w/v) aqueous solution. The triethanolamine also served as a buffer in the medium. The concentrations of $CaCl_2$, $MgSO_4.7H_2O$, and

TABLE 2

BASAL MEDIUM

	mg./100 ml.		mg./100 ml.
L-Alanine	70.0	Para-aminobenzoic acid	0.01
L-Arginine	30.0	Biotin	0.02
L-Aspartic acid	115.0	Choline.Cl	0.3
L-Cystine	20.0	Folic acid	0.25
L-Glutamic acid	190.0	Inositol	15.0
Glycine	15.0	Nicotinamide	1.5
L-Histidine	15.0	Pyridoxine.HCl	0.2
DL-Isoleucine	60.0	Pyridoxal.HCl	0.2
L-Leucine	155.0	Pyridoxamine.2HCl	0.2
L-Lysine.HCl	110.0	Riboflavin*	0.1
DL-Methionine	30.0	Thiamine.HCl*	0.1
DL-Phenylalanine	40.0	Cobalamin	0.00001
L-Proline	50.0		
DL-Serine	40.0	Ribonucleic acid	8.0
DL-Threonine	50.0	Cytidylic acid	2.0
L-Tryptophane	20.0		
L-Tyrosine	35.0	Creatine	2.0
DL-Valine	55.0	Creatinine	2.0
NaCl	200.0	Hemin*	1.0
$Na_2HPO_4.12H_2O$	300.0	Glucose*	200.0
KH_2PO_4	50.0	Tween 80	1.0
$CaCl_2$**	1.3		
$MgSO_4.7H_2O$**	50.0		
Metal mix B** +	0.2 ml.		

*Stock solution sterilized by filtering through ultra-fine sintered glass filter and added aseptically to the autoclaved medium.

**Stock solution autoclaved at 15 p.s.i. for 15 min. and added aseptically to the autoclaved medium.

+1 ml. contains: H_3BO_3, 0.057 mg.; $CoSO_4.7H_2O$, 0.238 mg.; $CuSO_4.5H_2O$, 0.394 mg.; Fe $SO_4.7H_2O$; 5.0 mg.; $MnSO_4.H_2O$, 1.54 mg.; $ZnSO_4.7H_2O$, 11.0 mg. (Nathan and Cowperthwaite, 1955).

glucose were changed, and ethylenediamine-tetra-acetic acid (EDTA) and $FeSO_4.7H_2O$ were added to the medium. Metal mix A was used instead of metal mix B. These changes are summarized in Table 3.

TABLE 3

MEDIUM 3

Basal Medium with Following Changes and Additions

	mg./100 ml.
Before autoclaving add:	
Triethanolamine	500.0
Hemin	2.5
EDTA	60.0
$FeSO_4.7H_2O$	0.5
Metal mix A+	2.5 ml.
$CaCl_2$	0.55
$MgSO_4.7H_2O$	40.0
After autoclaving add:	
Glucose*	500.0

*Stock solution sterilized by filtering through ultra-fine sintered glass filter and added aseptically to the autoclaved medium.

+1 ml. contains: H_3BO_3, 0.023 mg.; $CoSO_4.7H_2O$, 0.095 mg.; $CuSO_4.5H_2O$, 0.039 mg.; $MnSO_4.H_2O$, 3.076 mg.; $ZnSO_4.7H_2O$, 4.398 mg.; NaI, 0.001 mg.; EDTA, 1.0 mg. (Nathan and Cowperthwaite, 1954).

RESULTS

In vivo Experiments

Before the results of these experiments could be summarized, it was necessary to determine whether there were statistically significant differences among litters which would have an effect upon experimental results. For this purpose, an analysis of variance was carried out on all the litters (14) which contained at least three mice injected with Brazilian strain and cortisone. The per cent weight change from the day of injection to the twelfth day after injection in Br + cort-injected mice was the character selected for analysis.

Analysis of Variance

Source of variance	D.f.	Sum of squares	Mean square	F ratio
Between litters	13	60,460.89	4,650.84	$F = 4650.84/2780.616 = 1.6725$
Within litters	28	77,857.25	2,780.616	$F_{.95}(13, 28)$ is between 2.12 and 2.06
Total	41	138,318.14		

Therefore, we accept the null hypothesis: the litter means are not significantly different. Litter differences, with respect to a basic physiological characteristic, growth rate, are not such as to affect experimental results significantly, and data from similar tests on different litters may be pooled.

For each series of experiments a mortality table covering the 60-day period following injection is presented. A graph portraying additive growth rate curves of each experimental group for this period is also included. Each point on a graph represents the mean per cent change in weight on a given day from that 4 days earlier, with the weight at the time of injection taken as 100 per cent. Only the weights of mice which survived for at least 60 days were used in computing these curves. The mortality tables may then be considered to show the effect of acute trypanosomiasis, and the growth curves, that of the chronic disease.

Statistical analyses of results presented in the mortality tables were performed using Fisher's exact treatment of the 2x2 table. Selected points (those which appeared most likely to show differences) on growth curves were analyzed using the t-test for the comparison of 2 means (with pooled variances). The 0.05 level of significance was selected.

1. Trypanosomes and/or cortisone. The results of these experiments are presented in Table 4.

There was no statistically significant difference in mortalities between the following treatment groups: No inj vs. cort (P = 0.22); cort vs. Br + cort (0.25); cort vs. CR + cort (0.15); Br vs. Br + cort (0.13); CR vs. CR + cort (0.63). The probability indicated is that of obtaining values at least this widely separated by chance alone. The differencesin mortalities between the following groups were statistically significant: no inj vs. Br. + cort (0.0478) and no inj vs. CR + cort (0.028); but that between Br + cort vs. CR + cort (0.82) was

TABLE 4

MORTALITY OF 17-DAY-OLD MICE INJECTED WITH TRYPANOSOMES AND/OR CORTISONE

Treatment	No. mice injected	Deaths No.	Deaths %	No. days between injection and death Mean	No. days between injection and death Range
No inj	15	0	0		
Cort	24	3	12.5	16.0	5 - 33
Br	9	0	0		
Br + cort	20	5	25.0	11.4	8 - 17
CR	4	1	25.0	41.0	
CR + cort	12	4	33.3	13.25	7 - 22

not. We therefore reject the null hypothesis of no effect, for injection of the mice with trypanosomes and cortisone does affect their survival. It may also be observed in Table 4 that the mean numbers of days elapsing between injection and death for the latter two groups are very close.

The additive growth rates for each experimental group are shown in Figure 1. The similarities of the growth patterns shown are evident. These are especially interesting since some of the mice represented in the data of Graphs b, e, and f of Figure 1 had chronic trypanosomiasis as evidenced by their subsequent deaths and the appearance of heavily parasitized organs upon autopsy.

On day 4 only, statistically significant differences exist between the mean per cent weight changes of the following groups: no inj vs. Br + cort ($0.05 > P > 0.02$); Br vs. Br + cort ($0.01 > P > 0.001$); no inj vs. CR + cort ($0.01 > P > 0.001$); CR vs. CR + cort ($0.01 > P > 0.001$). The probability given is that of the differences between the means being this great by chance alone. By day 8 this difference is made up and is no longer statistically significant. Differences on subsequent days are not statistically significant. CR + cort and Br + cort groups differ significantly only on day 12 ($0.02 > P > 0.01$). The mean values for other groups showed such slight differences upon inspection that analyses were not carried out on them. Actual weights of some of the above groups were also compared (t-test for comparison of two means) for the days on which they were most different; no statistically significant differences were found.

Figure 1. Additive growth rates of 17-day-old mice injected with trypanosomes and/or cortisone.

a. No injection

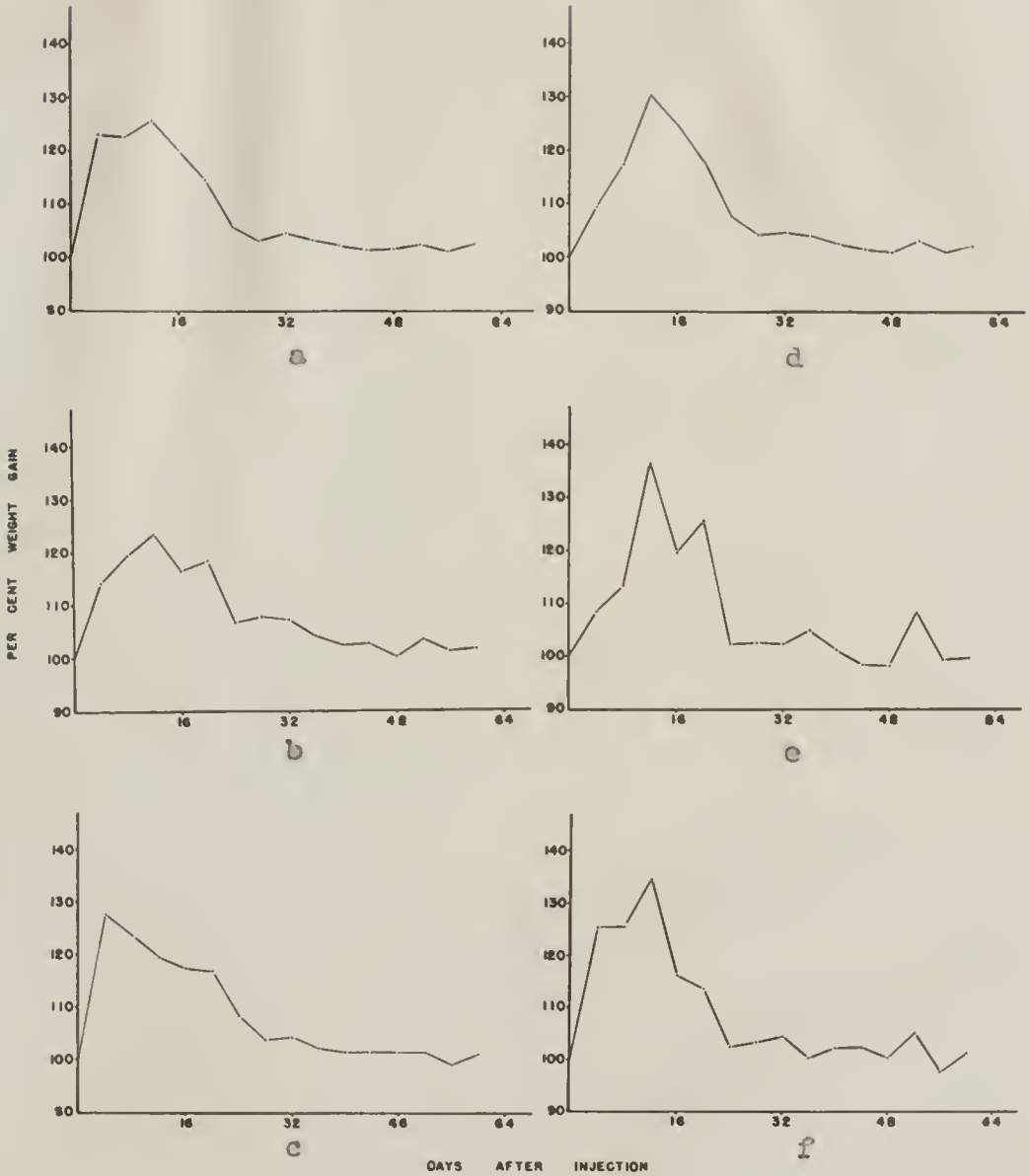
b. Brazilian strain + cortisone

c. Brazilian strain

d. Cortisone

e. Costa Rican strain + cortisone

f. Costa Rican strain


PER CENT WEIGHT GAIN
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DAYS AFTER INJECTION

Figure 1.

2. Adenosine triphosphate. The results of these experiments are summarized in Table 5.

The difference in mortalities between groups of mice injected with cort + 5.0 mg. ATP and cort + 2.5 mg. ATP was not statistically significant (P = 0.49). The mortality difference between Br + cort- and Br + cort + 2.5 mg. ATP-treated mice is significant (0.016), as is that for Br + cort + 2.5 mg. ATP- and Br + cort + 5.0 mg. ATP-treated ones (0.000); while that between Br + cort- and Br + cort + 5.0 mg. ATP-treated ones is probably biologically significant, although it is not significant statistically (0.052). No statistically significant difference exists between mortality rates of CR + cort- and CR + cort + 2.5 mg. ATP-treated groups (0.78).

Additive growth rate curves for these groups are shown in Figure 2. Again, statistically significant differences in mean per cent weight gains occur only on single isolated days. The differences between the means for the cort + 2.5 mg. ATP- and cort + 5.0 mg. ATP-treated groups are significantly different on day 24 ($0.01 > P > 0.001$), as are those for Br + cort- and Br + cort + 2.5 mg. ATP-injected ones on day 32 ($0.05 > P > 0.02$).

3. Thiamine. Results of these experiments are shown in Table 6.

Because the numbers of mice in some of the experimental groups were small, the mortality values for all mice treated with thiamine were combined, as were all those for mice treated with BR + cort + thiamine (differences in mortality rates among the pooled groups were not

TABLE 5

MORTALITY OF 17-DAY-OLD MICE INJECTED
WITH TRYPANOSOMES, CORTISONE, AND ADENOSINE TRIPHOSPHATE

Treatment	No. mice injected	Deaths		No. days between injection and death	
		No.	%	Mean	Range
Cort + 5.0 mg. ATP	11	2	18.2	9.0	7 - 11
Cort + 2.5 mg. ATP	18	2	11.1	12.0	3 - 21
Br + cort	20	8	40.0	11.5	6 - 30
Br + cort + 5.0 mg. ATP	15	11	73.3	10.8	5 - 39
Br + cort + 2.5 mg. ATP	11	0	0		
CR + cort	9	2	22.2	9.0	4-14
CR + cort + 2.5 mg. ATP	11	2	18.2	17.0	

Figure 2. Additive growth rates of 17-day-old mice injected with trypanosomes, cortisone, and adenosine triphosphate.

a. Brazilian strain + cortisone + 5.0 mg. ATP

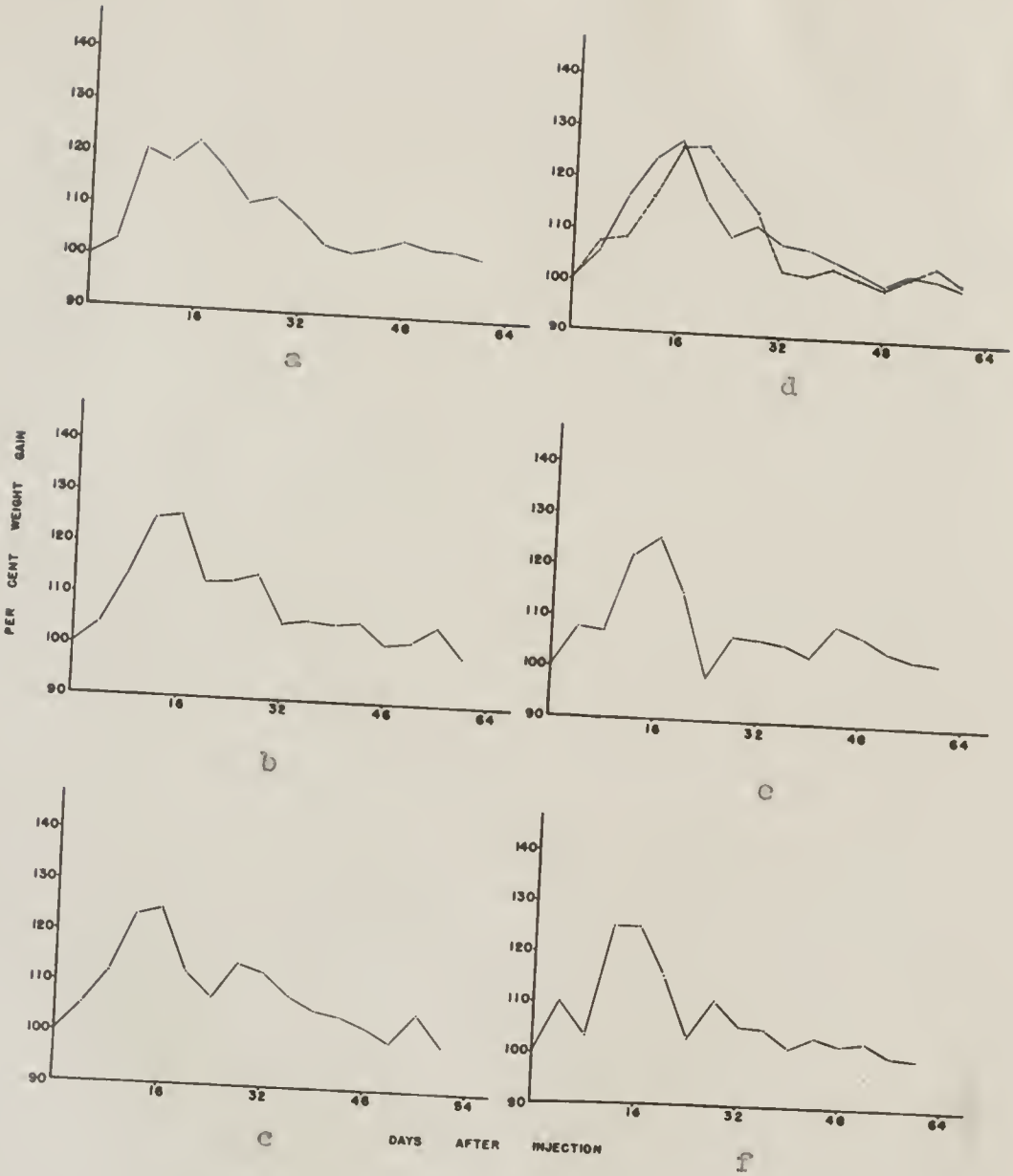
b. Brazilian strain + cortisone

c. Brazilian strain + cortisone + 2.5 mg. ATP

d. Cortisone + ATP
. _____ . = 2.5 mg. ATP
x-------x = 5.0 mg. ATP

e. Costa Rican strain + cortisone

f. Costa Rican strain + cortisone + 2.5 mg. ATP


PER CENT WEIGHT GAIN
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DAYS AFTER INJECTION

Figure 2.

TABLE 6

MORTALITY OF 17-DAY-OLD MICE INJECTED WITH TRYPANOSOMES AND CORTISONE, TREATED WITH THIAMINE

Treatment	No. mice injected	Deaths No.	Deaths %	No. days between injection and death Mean	No. days between injection and death Range
No inj	6	0	0		
Thiamine, 60 µg., oral	6	0	0		
Thiamine, 25 µg., inj	4	0	0		
Thiamine, 50 µg., inj	7	0	0		
Total	17	0	0		
Br + cort	14	3	21.4	16.3	13 - 21
Br + cort + thiamine, 60 µg., oral	6	3	50.0	16.7	13 - 22
Br + cort + thiamine, 25 µg., inj	5	0	0		
Br + cort + thiamine, 50 µg., inj	3	2	66.7	19.5	11 - 28
Total	14	5	35.7		
CR + cort	5	1	20.0	51.0	
CR + cort + thiamine, 50 µg., inj	5	0	0		

statistically significant) for statistical analyses. No statistically significant differences in mortality rates were found between the experimental groups in this series: no inj vs. Br + cort ($P = 0.32$); Br + cort vs. Br + cort + thiamine (0.34); no inj vs. CR + cort (0.45); CR + cort vs. CR + cort + thiamine (0.50). There is an indication that cortisone-injected mice infected with the Costa Rican strain may react differently to thiamine treatment than do those infected with the Brazilian strain.

Growth curves for this series of experiments are presented in Figure 3. Values for all mice treated with thiamine and for those given Br + cort + thiamine were also pooled here. Mean per cent weight change values for Br + cort + thiamine-treated mice are never significantly different from those for Br + cort-injected ones, nor are those for the CR + cort + thiamine and CR + cort groups. The difference in means on day 12 of the no inj and thiamine-treated groups is statistically significant ($0.02 > P > 0.01$).

4. Thiamine, magnesium, and adenosine triphosphate. The results of these experiments are shown in Table 7, and the growth curves in Figure 4. The number of litters used in this set of experiments and in the next series is so small that it was not felt that extensive statistical analyses would be meaningful. The differences between mortalities in Br + cort- and Br + cort + thiamine, Mg^{++}, ATP-treated mice ($P = 0.45$), and in CR + cort- and CR + cort + thiamine, Mg^{++}, ATP-treated ones (0.27) are not statistically significant. Growth rate curves for treated and untreated animals in these two groups also appear to be very similar.

Figure 3. Additive growth rates of 17-day-old mice injected with trypanosomes and cortisone, treated with thiamine.

a. No injection

b. Brazilian strain + cortisone

c. Brazilian strain + cortisone + thiamine

d. Thiamine

e. Costa Rican strain + cortisone

f. Costa Rican strain + cortisone + thiamine

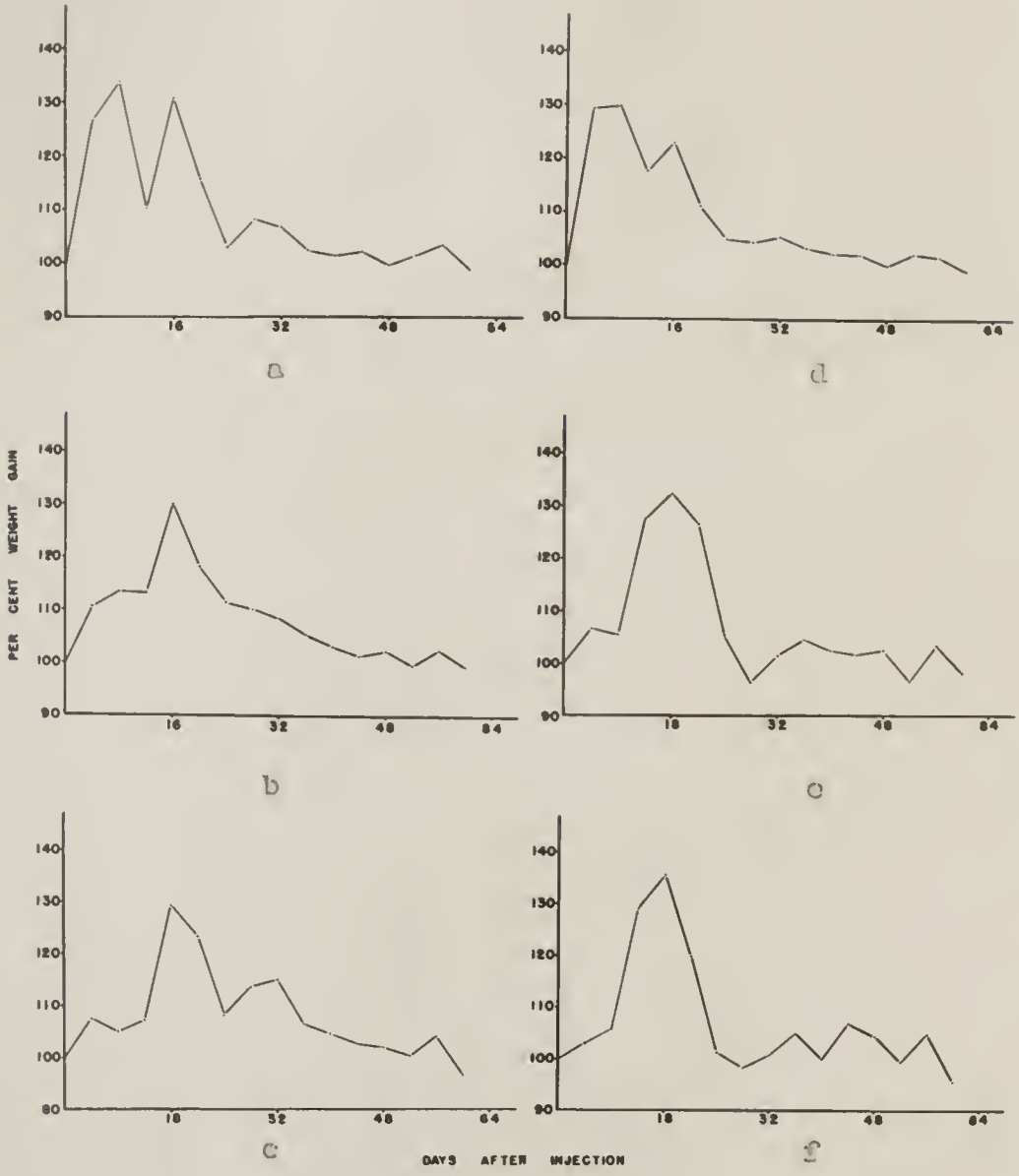
PER CENT WEIGHT GAIN
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Figure 3.

TABLE 7

MORTALITY OF 17-DAY-OLD MICE INJECTED WITH TRYPANOSOMES, CORTISONE, THIAMINE, MAGNESIUM, AND ADENOSINE TRIPHOSPHATE

Treatment	No. mice injected	Deaths No.	Deaths %	No. days between injection and death Mean	No. days between injection and death Range
Thiamine, Mg^{++}, ATP	10	0	0		
Br + cort	5	1	20.0	3.0	
Br + cort + thiamine, Mg^{++}, ATP	5	0	0		
CR + cort	6	1	16.7	24.0	
CR + cort + thiamine, Mg^{++}, ATP	6	3	50.0	38.3	11 - 56

Figure 4. Additive growth rates of 17-day-old mice injected with trypanosomes, cortisone, thiamine, magnesium, and adenosine triphosphate.

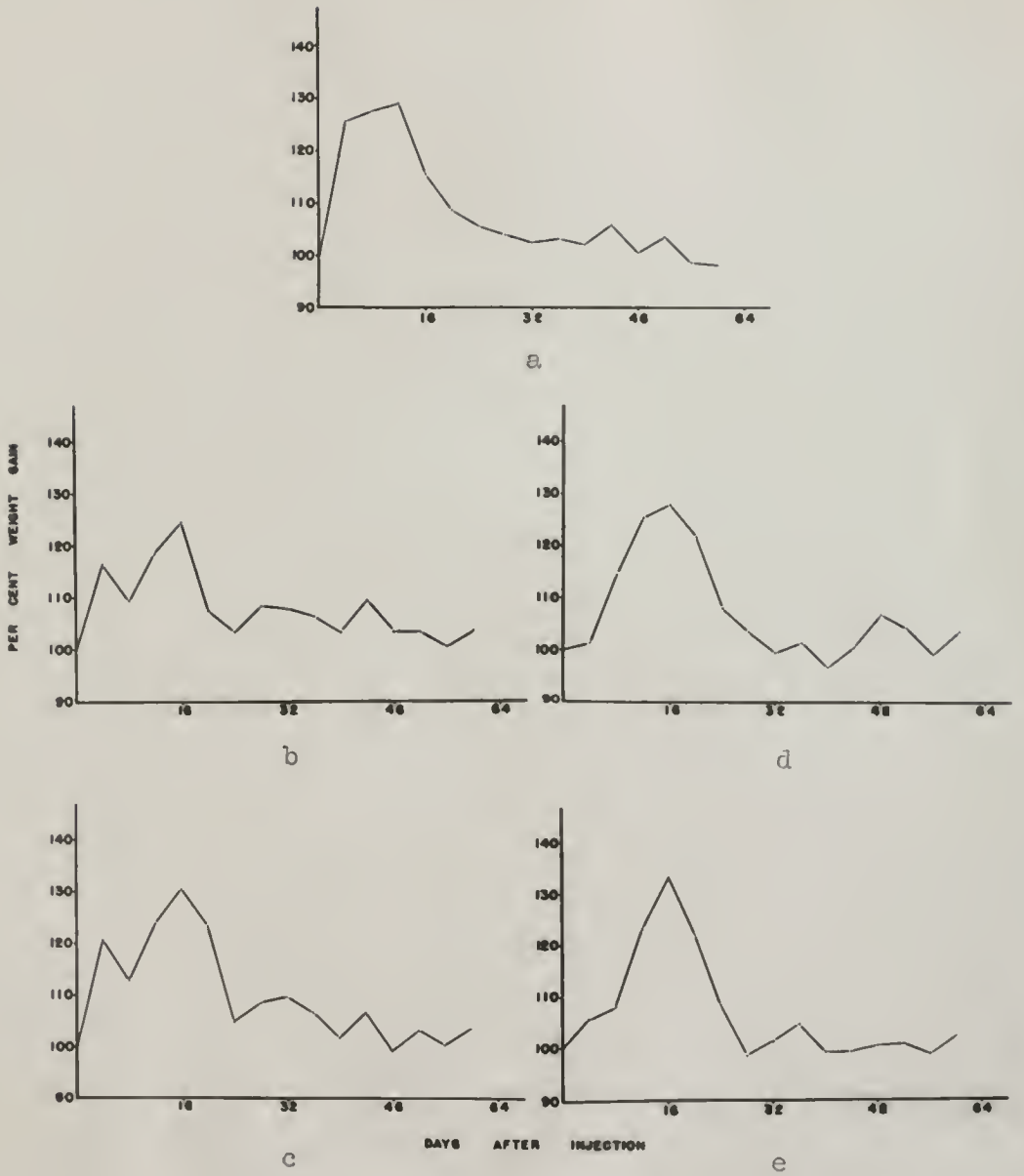
a. Thiamine, magnesium, and ATP

b. Brazilian strain + cortisone

c. Brazilian strain + cortisone + thiamine, magnesium, and ATP

d. Costa Rican strain + cortisone

e. Costa Rican strain + cortisone + thiamine, magnesium, and ATP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DAYS AFTER INJECTION

Figure 4.

5. Adenosine diphosphate and inorganic phosphate. The results of this experimental treatment are presented in Table 8 and Figure 5. There was no statistically significant difference in mortalities of treated and untreated CR + cort-injected mice, but the difference between the two Br + cort-injected ones was close to the significance level ($P = 0.077$). Here again there appears to be a difference in reaction to treatment between mice infected with the Costa Rican and Brazilian strains. Growth curves for the treated and untreated groups are quite similar.

If the mortality rates of all the mice injected with Br + cort and those injected with CR + cort from all experiments are compared, the difference between them is not statistically significant ($P = 0.24$). Twenty-nine per cent of the mice injected with Br + cort died within 60 days after injection, as compared to 21 per cent of the CR + cort-injected ones.

When the numbers of days elapsing between injection and death for these same two groups are compared, it is found that the two variances are significantly different ($P < 0.02$ of obtaining an F-value as large as that found by chance alone). The means cannot, therefore, be analyzed using the usual t-test with pooled variances. However, when the means are compared by the approximate method of Cochran and Cox (Snedecor, 1946) with no hypothesis about the variances, it is found that the means probably are not significantly different ($P > 0.05$ of obtaining the t-value calculated). The mean number of days elapsing between injection and death of Br + cort-injected mice is 10.7 (3 - 30);

TABLE 8

MORTALITY OF 17-DAY-OLD MICE INJECTED WITH TRYPANOSOMES, CORTISONE, ADENOSINE DIPHOSPHATE, AND INORGANIC PHOSPHATE

Treatment	No. mice injected	Deaths No.	Deaths %	No. days between injection and death Mean	No. days between injection and death Range
Cort + ADP, iP	12	1	8.3	3.0	
Br + cort	9	3	33.3	4.3	3 - 6
Br + cort + ADP, iP	9	7	77.8	19.0	6 - 31
CR + cort	6	0	0		
CR + cort + ADP, iP	7	0	0		

Figure 5. Additive growth rates of 17-day-old mice injected with trypanosomes, cortisone, adenosine diphosphate, and inorganic phosphate.

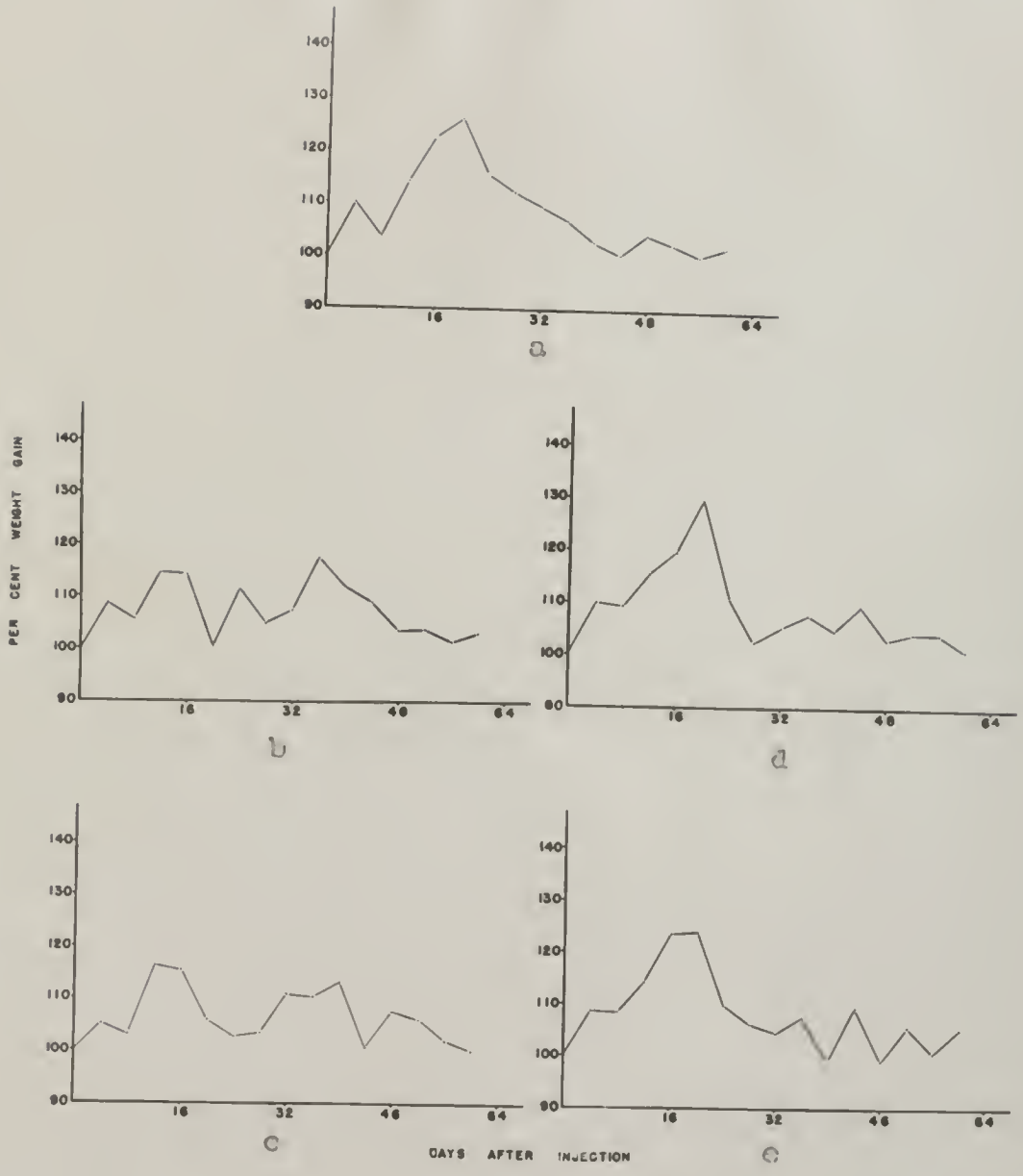
a. Cortisone + ADP and iP

b. Brazilian strain + cortisone

c. Brazilian strain + cortisone + ADP and iP

d. Costa Rican strain + cortisone

e. Costa Rican strain + cortisone + ADP and iP


PER CENT WEIGHT GAIN
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DAYS AFTER INJECTION

Figure 5.

for CR + cort-injected ones, 18.25 (4 - 51).

6. Histology. Histological examination of tissues from mice infected with both Brazilian and Costa Rican strains showed the following organs to be parasitized: esophagus, stomach, small intestine, heart, liver, lung, spleen, urinary bladder, brain, and spinal cord. Leishman bodies were not observed in the kidney or pancreas. These findings compare with those of other workers (Hauschka, 1947; von Brand et al., 1949) using the Brazilian and different strains of T. cruzi.

In about half of the mice dying of the acute disease the stomachs were enormously distended. They were filled with partially digested food, and the material at the pyloric end of the stomach sometimes contained blood. Gray to black spots were frequently visible macroscopically on the external stomach wall. Microscopic examination of sections through these regions and of the fresh tissue macerated in a drop of saline solution showed these spots to be dense concentrations of leishman bodies. In several cases the stomachs were actually perforated at these points.

This observation of stomach infections confirms the findings of Lesser and Lukeman (1957) who have presented the only previous report of stomach involvement. Their studies were done with the Pilcher strain of T. cruzi.

7. Priapism. Two cases of priapism were observed, one in a mouse injected with CR + cort + thiamine, Mg^{++}, and ATP, the other in one injected with Br + cort + thiamine. The condition in each lasted

for five days and was followed by paraplegia in one of the animals.

This phenomenon has been reported in young rats suffering from a cortisone-aggravated thiamine deficiency (Wilwerth and Meites, 1953). When these authors treated the animals with suboptimal amounts of thiamine, the priapism disappeared although cortisone injections were continued.

8. Paraplegia. Ten mice were afflicted with paraplegia. Information concerning these cases is summarized in Table 9. The mice seemed to fall rather definitely into two groups: those which died very soon after the onset of paralysis, and those which lived for a long time afterward. In the latter group the condition of two of the mice alternately improved and regressed, but in no case did a mouse completely recover from the paralysis. Even when some improvement was shown here, the mice remained thin. The female infected with Brazilian strain also developed visual troubles several weeks before its death, one eye finally becoming completely opaque.

Spinal smears of all the paraplegic mice which died showed leishman bodies, but never in great numbers. The parasites were also observed in fresh material from the spinal cord which was macerated in a drop of saline solution. Brain smears were prepared for two mice, and the brains of four others were sectioned. One brain smear was positive for leishman bodies, and three of the sectioned brains showed them.

9. Chronic infections. During the course of this study 15 deaths resulted from chronic trypanosomiasis. These mice died from

TABLE 9

PARAPLEGIA IN INFECTED MICE

Sex	Treatment	No. days after injection First appearance of paralysis	Died	Leishman bodies present: Spinal cord	Brain
F	Br	153	334	+	+
M	CR + cort	56	326	+	-
M	CR + cort	61	82	+	+
M	CR + cort	63	83	+	-
M	CR + cort	48	*		
F	CR + cort	48	51	+	+
M	CR + cort	79	**		
M	CR + cort + thiamine	75	81	+	
M	CR + cort + thiamine, Mg^{++}, ATP	39	48	+	+
M	CR + cort + thiamine, Mg^{++}, ATP	52	*		

*Still living 124 days after injection.

**Still living 114 days after injection.

78 - 454 days (mean = 216) after injection. During this period no uninjected mice of the same ages died. Chronic infections were found in mice with the following injections: (1) Br, (2) Br + cort, (3) Br + cort + ATP, (4) Br + cort + thiamine, (5) CR, (6) CR + cort, (7) CR + cort + thiamine, with the largest number of cases (five) occurring in the CR + cort-injected group. Since not all of the mice used in experiments could be kept indefinitely, no statement can be made concerning the total number of deaths which might have occurred.

Upon histological examination the same organs mentioned previously were found to be parasitized. Hearts, spleens, and livers were frequently enlarged. Stomachs were not distended with food as in the acute cases although leishman bodies were frequently present in the tissues. Liver, lung, and urinary bladder, in about half of these cases, contained unusually large numbers of leishman bodies.

In vitro Experiments

1. Growth in Citri's partially defined medium. It has been found that the Brazilian and Costa Rican strains of T. cruzi grow as well as, or better than, the Culbertson (C) strain in Citri and Grossowicz's partially defined medium and that this growth is steadily maintained through at least eight subcultures. Counts from a representative series of samples are presented in Table 10.

These population densities are just slightly lower than those of the same strains in the diphasic medium. For the Brazilian strain the mean of counts from 26 4-week-old cultures in diphasic medium was

TABLE 10

GROWTH IN THE PARTIALLY DEFINED MEDIUM
OF CITRI AND GROSSOWICZ (1955a)

Strain	Organisms per ml. (x 10^6)			
	At time of inoculation	After 2 weeks	After 3 weeks	After 4 weeks
C	0.28	1.7 (6x)	11.0 (39x)	19.4 (69x)
CR	0.06	5.7 (95x)	20.9 (348x)	29.6 (493x)
Br	0.03	7.9 (263x)	19.2 (640x)	28.2 (940x)

31.6 x 10^6 organisms per ml.; for the Costa Rican strain, the mean from 19 cultures of the same age was 42.7 x 10^6.

2. Growth in experimental media. A synthetic medium completely defined except for ribonucleic acid, has been devised. The Costa Rican strain grew as well in each of the modifications of this medium as it did in control tubes of Citri's medium. The results of these experiments are summarized in Table 11.

TABLE 11

GROWTH OF COSTA RICAN STRAIN IN EXPERIMENTAL MEDIA

Medium	Organisms per ml. ($\times 10^6$) At time of inoculation	After 5 weeks
Basal medium	0.9	1.2 (1.33x)
Citri's medium	0.9	0.9 (1.00x)
Medium 1	0.75	1.4 (1.87x)
Citri's medium	0.75	0.9 (1.20x)
Medium 2	0.75	1.3 (1.73x)
Citri's medium	0.75	1.4 (1.87x)
Medium 3	0.9	1.5 (1.67x)
Citri's medium	0.9	1.75 (1.94x)

DISCUSSION

Incomplete oxidation of carbohydrates is characteristic of the metabolism of all the trypanosomes studied to date. For example, the pathogenic African trypanosomes break down glucose only as far as pyruvate. Both blood stream and culture forms of T. cruzi are able to oxidize glucose more completely, and about half of the glucose molecule is converted to carbon dioxide, the remainder being degraded to succinic, acetic, and lactic acids (Ryley, 1956). Chang (1948) and von Brand et al. (1949) both demonstrated glucose utilization by culture forms of T. cruzi, but glucose consumption was not shown for blood stream forms (von Brand et al., 1949). Ryley (1956) reported that both forms use glucose and that they do not differ markedly in their metabolism of it.

Ryley's analyses for organic acids were carried out on two to two and one-half hour incubates in Ringer-bicarbonate-glucose medium. Chang (1948), in analyzing 1- to 2-week-old diphasic cultures of T. cruzi, found appreciable amounts of succinic, pyruvic, and lactic acids and small amounts of carbon dioxide and formic acid, but no acetic acid. Unfortunately, no metabolic studies have been carried out on tissue forms of T. cruzi.

Pyruvate is the only one of these metabolic end-products for which host tissues have been analyzed. Coleman and von Brand (1957) studied blood pyruvate levels in rats infected with various trypanosomes. They found that in all cases, whether the host was infected with T. cruzi or with one of the trypanosomes producing much larger amounts of pyruvic acid, the host animals were able to maintain normal blood pyruvate

levels. However, they made no mention in their report of how long the host animals had been infected. In T. cruzi infections there is frequently extensive damage to liver and muscle tissues. It is well known that with severe liver disorders in man there is a decreased utilization, and consequent accumulation in the blood, of pyruvate (Parida and Kark, 1956). Therefore, maintenance of normal pyruvate levels may very well depend upon the extent of tissue, especially liver, damage in the host. As tissue damage progressed it is quite likely that pyruvate levels would rise, eventually to concentrations toxic to the host.

Under the conditions of the present experiments the treatment of T. cruzi-infected mice with thiamine or with thiamine, magnesium, and ATP apparently had no effect. If, as suggested above, the mice are able to metabolize pyruvate satisfactorily until tissue damage becomes too extensive, it can be seen why thiamine did not affect the course of the disease. However, if an animal were suffering from a slight pre-existent thiamine deficiency, the increased amount of thiamine necessary to metabolize the pyruvate produced during a trypanosome infection might be enough to make the deficiency pronounced. In such a case, thiamine therapy might be effective, at least temporarily. It should be borne in mind also that even if a thiamine deficiency did occur during a trypanosome infection and were treated, it is very likely that the disease has other effects which might completely mask any results of the thiamine therapy.

Grant and Fulton (1957) found abnormally high levels of pyruvate in the blood of rats infected with T. rhodesiense and showed a positive

correlation to exist between the pyruvate level and the degree of parasitemia. Upon treating the rats with a trypanocidal drug, they noted that parasites had disappeared from the blood 90 minutes after treatment, and at 120 minutes the keto-acid levels in these rats were almost identical to those of control animals. Here there was apparently no extensive tissue damage. The failure to maintain normal blood pyruvate levels in this case may have been due to a limiting amount of some cofactor required for pyruvate metabolism. The possibility that the parasite is in some way able to inhibit this metabolic reaction should not be ruled out.

These ideas presented concerning pyruvate metabolism are probably applicable also to the utilization by the host of the other organic acids produced by T. cruzi. The host should be able to maintain normal levels of succinate, lactate, and acetate until tissue damage becomes extensive. Perhaps in early infections slight pre-existing deficiencies of vitamins or other compounds required for the oxidation of these organic acids may be aggravated. In this case, treatment of the host with the needed factor would prevent the accumulation of these acids to toxic levels, staving off death from this cause and giving the animal time to fight off, or adjust to, the infection. Such deficiencies might also occur in chronic cases of trypanosomiasis. Deficiencies of thiamine, α-lipoic acid, pantothenic acid, riboflavin, and nicotinamide are among those which might be expected to occur.

The one factor common to the utilization of all these metabolic end-products of the trypanosome is ATP or ADP and iP. As pointed out

earlier, the rate of substrate degradation is dependent upon the rate of oxidative phosphorylation, which is in turn controlled by the rate at which ATP is split to ADP and iP.

The results of the present experiments confirm Adams' (1954) finding that ATP treatment reduces the mortality of mice infected with the Brazilian strain of T. cruzi. Failure of the treatment to have the same effect on mice infected with the Costa Rican strain may be attributed to: (1) the smaller number of experimental animals involved, (2) the greater variability of the Costa Rican strain, or (3) a real difference in reaction to the treatment between the two strains.

The finding that 2.5 mg. of ATP reduce the mortality of infected mice while 5.0 mg. increase it was unexpected. It was the latter amount which Adams used in his experiments. The larger amount of ATP was not in itself toxic, for when given to cortisone-injected mice it was not fatal. Only when the mice had previously been injected with trypanosomes did the 5.0 mg. dosage have an adverse effect. I have no explanation for the reaction to this dosage difference, if indeed it is a real thing. It is interesting, however, to imagine that the host may be able to claim for its own use certain amounts of ATP, but that above a certain level the ATP is not immediately utilized and becomes available to the trypanosomes.

The manner in which ATP acts to reduce the mortality of trypanosome-infected mice remains hypothetical. Adams (1954) suggested that it was used in combating a hypoglycemia produced in the host by the trypanosomes. Since it has been shown that animals infected with T. cruzi usually maintain normal blood sugar levels (von Brand et al., 1949),

this would not seem to be the explanation. The fact that the ATP was administered only once, very early in the infection, precludes the possibility that its energy was available for repair of damaged tissues. It is possible however that it could be used for synthesis in the production of antibodies or phagocytic cells. The ATP injections were administered at about the time that the metacyclic trypanosomes injected are beginning to change to leishman forms (Perez-Reyes, 1953), the forms which reproduce within the vertebrate host. It seems more likely, however, that the ATP becomes involved directly in the metabolism of the mouse or the trypanosome or both. It may very well be used to provide phosphate and/or phosphate acceptors necessary for the oxidation of the metabolic end-products of the trypanosomes, giving the host time to adjust its metabolic pattern to accommodate this new situation.

Surprisingly, hardly any studies have been made of the effect of ATP administration on intact animals. From the few experiments performed it has been learned that there is a direct transfer of phosphate groups from ATP to thiamine and incorporation of the resultant di- and triphosphothiamines into the liver within 1 hour of injecting rats with ATP^{32} and thiamine (Rossi-Fanelli et al., 1954). Mascitelli-Coriandoli and Boldrini (1957) found that the injection of ATP raises the levels of ATP-phosphorus, creatine phosphate-phosphorus, and inorganic phosphorus to almost normal levels in the heart muscle of rats treated with thyroxine. Until more is known concerning the fate of injected ATP in animals, and until the nutritional requirements of T. cruzi and the effect of this parasite upon its host are better understood, no final explanation

for the effect of ATP on trypanosomiasis can be given.

The treatment of trypanosome-infected mice with ADP and iP did not reduce the mortality rate. In view of the small number of mice used in this set of experiments, these results cannot be considered to be conclusive. With a large enough sample, the results of this treatment should be similar to those obtained with ATP injection.

The high degree of adaptation between T. cruzi and its host is well exemplified in the very close similarity of growth rates of infected and uninfected mice. Further evidence of this adaptation is found in the relatively large number of infected mice which survived the acute infection and continued to live with chronic infections. At least a partial explanation for this host-parasite relationship lies in the very nice metabolic exchange between the two. The parasite takes glucose from the host, but does not completely metabolize it, returning at least half of the molecule to the host in the form of organic acids which the host is then able to utilize. In this way both organisms obtain energy from the substrate.

The occurrence of chronic trypanosomiasis in laboratory animals is rarely mentioned in the literature. Johnson (1938) reported that of 19 dogs experimentally infected with T. cruzi, 9 of them developed acute infections and 11, chronic infections. Goble (1951) and Hauschka (1947) have both noted the occurrence of the chronic disease in mice. Since chronic trypanosomiasis is the most frequent form encountered in humans, it is suggested that mice with chronic infections should lend themselves

well to experimental work concerned with the effect of the chronic disease on the host and with therapy of the disease.

Paraplegia in mice infected with T. cruzi has been reported, to my knowledge, only once before. De Souza Campos (1925) stated that 4 of 12 infected mice showed paraplegia. The condition is fairly common in infected dogs (Goble, 1952; Villela, 1924) and rabbits (de Souza Campos, 1924). De Souza Campos (1925) reported that lesions of the central nervous system were not observed in all cases of paraplegia and suggested that this might depend on the duration of the infection. The aflagellated forms were found in the central nervous system of all the other paraplegic cases reported, although they were never numerous. Inflammatory centers formed by infiltration of macrophages were encountered more frequently during microscopic examination of brain and spinal cord sections than were leishman bodies. Goble (1952) found that dogs may die within a few days of becoming paraplegic or may continue to live for some time with varying degrees of weakness and paralysis. The neurotropic form of trypanosomiasis appears to be very similar in dogs and in the mice observed in the present experiments. Goble, however, also reported the recovery of one paraplegic dog. In view of these reports where leishman bodies could not be demonstrated in the central nervous system and where there was a complete recovery from paraplegia, paralysis in trypanosome-infected mice should not be haphazardly attributed to the direct effect of the trypanosome. It might be pointed out that a phosphorus deficiency will produce paralysis of the hind legs (Foster et al., 1949) in mice. A thiamine deficiency also affects the legs of mice,

making them so weak that they are unable to support the body (Woolley and White, 1943). Therefore, it is quite possible that paraplegia in trypanosome-infected mice should not be wholly attributed to a direct effect of the parasites in the central nervous system.

It is now known that the partially defined synthetic medium of Citri and Grossowicz (1955a) will support growth of three strains of T. cruzi, as shown by the results presented earlier in this paper, and 31 different strains of Leishmania tropica (Citri and Grossowicz, 1955b). The latter authors found that the medium did not support growth of L. infantum, L. donovani, L. brasiliensis, and L. agamae, however. This suggests that the growth requirements of L. tropica and T. cruzi are more similar than are those of L. tropica and the other leishmanias of man.

During the time that growth experiments in media containing known amino acids were being conducted, the cultures in this laboratory were undergoing one of the unexplained growth depressions which occasionally affect trypanosome cultures (Balamuth, 1957). It is for this reason that the populations attained in the experimental media and in the Citri's medium controls were much lower than would be expected. The relative amount of growth obtained in the experimental media as compared to that in the control medium appears to be significant, and there is no reason to believe that this will change under improved growth conditions. No differences in growth supporting ability were observed among the different experimental media. Future experiments are planned in which

purines and pyrimidines or nucleotides will be substituted for the ribonucleic acid in the media now being used. If this substitution is accomplished, the medium for T. cruzi will be completely defined.

SUMMARY AND CONCLUSIONS

Injection with adenosine triphosphate reduced the mortality of mice infected with Trypanosoma cruzi. Treatment of infected mice with thiamine or with thiamine, magnesium, and adenosine triphosphate apparently had no effect on the course of the disease. With a small number of mice, adenosine diphosphate and inorganic phosphate appeared to produce no effect on the infection.

Chronic trypanosomiasis resulted frequently when mice were injected with trypanosomes and cortisone. Growth rates of normal, uninfected mice were not significantly different from those of mice with the chronic disease.

Leishman bodies were observed in heart, spleen, liver, lung, and other organs commonly parasitized by T. cruzi. In addition, the aflagellated forms were found in stomach tissues of about one-half of all mice dying of acute trypanosomiasis, confirming a single previous report of stomach infections.

Ten infected mice developed paraplegia. Leishman bodies were present in the central nervous systems of these animals.

The partially defined medium of Citri and Grossowicz (1955a) was found to support growth of the Brazilian and Costa Rican strains of T. cruzi as well as it did that of the Culbertson strain for which it was devised. Modifications of the medium were made, and ribonucleic acid is now the only undefined component. A known mixture of amino acids completely replaced the protein source. Growth of T. cruzi (Costa Rican strain) was as good in amino acid-substituted media as in the original medium.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 9, 1958

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