OBSERVATIONS ON THE RESPIRATION OF TRYPANOSOMA CRUZI IN CULTURE*

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Trypanosoma cruzi is the causative agent of Chagas' disease which is endemic over wide areas of South America, Central America, and Mexico. This species differs in many respects from *T. gambiense* and *T. rhodesiense*, the etiological agents of African sleeping sickness, and some other species of trypanosomes causing disease in animals. In particular, *T. cruzi* infections are resistant to drug treatment, whereas African sleeping sickness, at least in its early stages, can be combated successfully by chemotherapy. This difference may be due in part to the fact that *T. cruzi* exists as a trypanosome form in the blood stream of its final host for only a relatively brief period of time, after which it assumes a leishmania form in the tissues, in contradistinction to most trypanosomes, which continue to maintain themselves in the blood stream. Von Jancsó and von Jancsó (1935, 1936) showed that the carbohydrate metabolism of some of the blood stream forms may have some influence on the trypanocidal action of certain drugs. It is possible that resistance of *T. cruzi* to chemotherapy may be related to differences in its basic metabolism as compared with that of the blood stream trypanosomes, as well as to differences of localization within the final host. These considerations emphasize the need for investigations of the metabolism of *T. cruzi*.

T. cruzi is not host-specific since it occurs in a considerable number of vertebrate hosts and may be propagated in laboratory animals. It may also be maintained under laboratory conditions in species of the reduviid bug which acts as its invertebrate host. It is not known to what extent our experimental results might have differed had the organism been studied in either one of its vertebrate or invertebrate hosts rather than in cultures. Such information would be of value in comparing our data with those of other investigators on the metabolism of *T. rhodesiense*, *T. equiperdum*, and some other pathogenic species taken directly from the blood stream of the final host.

The work of Yorke, Adams, and Murgatroyd (1929), Geiger, Kligler, and Comaroff (1930), Regendanz (1930), von Brand (1933), and Chen and Geiling

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(1945) shows that *T. brucei*, *T. rhodesiense*, *T. congolense*, *T. evansi*, and *T. equiperdum* consume large amounts of sugar, and that *T. lewisi* and *T. cruzi* consume relatively small amounts of sugar. The underlying causes of these differences have not been ascertained. The course of the sugar breakdown by three trypanosome species has been studied, and it has been found to be different in each case (Reiner and Smythe, 1934; Reiner, Smythe, and Pedlow, 1936; Searle and Reiner, 1940, 1941; Fulton and Stevens, 1945). The main end-product of the aerobic sugar degradation by *T. equiperdum* is pyruvic acid; *T. lewisi* produces formic, acetic, and succinic acids, ethyl alcohol, and carbon dioxide; and *T. rhodesiense*, formic, acetic, pyruvic, succinic, and lactic acids, glycerol, ethyl alcohol, and carbon dioxide. *Leishmania tropica* consumes glucose, and protein substances in the absence of glucose (Salle and Schmidt, 1928). Glucose consumption results in the production of volatile acids and protein consumption in the production of ammonia. According to von Fenyvessy and Reiner (1924, 1928), and Christophers and Fulton (1938), the respiration of *T. equiperdum* and *T. rhodesiense* is not inhibited by cyanide. A. Lwoff (1934) described the aerobic respiration of *Leptomonas ctenocephali*, *Strigomonas oncopelti*, and *S. fasciculata* and reported inhibition of respiration by potassium cyanide and carbon monoxide. Using methods commonly employed for identification of bacteria, Senekjie (1943) reported that *T. cruzi* did not produce acid or gas in carbohydrate medium. It would be of interest to compare the results of these methods with the method used by Salle and Schmidt (1928) for detection of acid production by *Leishmania tropica*.

The respiratory metabolism of *T. cruzi* was chosen for the present investigation because of the importance of gaseous exchanges in the metabolism of most organisms. In particular, the investigation deals with the influence of the age of the cultures, the oxygen tension, and the temperature on oxygen consumption. In addition the growth rate and vitality of the organisms were considered in relation to the pH of the medium and the respiratory quotient. Finally, the effects of respiratory poisons were investigated for identification of some of the enzymes that are operative in the metabolism of *T. cruzi*.

Materials and Methods

A strain of *T. cruzi* isolated in 1942 from a case of Chagas' disease in Brazil was used in these studies. The strain was obtained by the Zoology Laboratory through the courtesy of Dr. H. A. Senekjie and Dr. Dorland J. Davis. Cultures isolated from a rat 2 months previously furnished the stock material. Stock cultures were grown in a diphasic medium prepared as follows: Senekjie's (1943) blood agar was overlaid with Locke's solution (NaCl, 8.0 gm.; $CaCl_2$, 0.2 gm.; KCl, 0.2 gm.; KH_2PO_4, 0.3 gm.; dextrose, 2.5 gm.; distilled water, 1000 cc.). Experimental cultures were grown in a liquid medium prepared from the overlay of the diphasic blood agar medium. The solid phase was dispensed in 25 cc. amounts in 250 ml. Erlenmeyer flasks and overlaid with approximately 15 cc. of Locke's solution. After 6 days' storage at 24–25°C.,

the overlay was pipetted in 10 cc. amounts into 50 ml. Erlenmeyer flasks forming a liquid medium with a pH of 7.0 ± 0.1 as determined with a Beckman pH meter. It was seeded from stock cultures of *T. cruzi*. Good growth occurred, with a predominance of crithidial forms.

The samples used for the experiments were withdrawn with a pipette at designated periods of incubation, the culture being agitated to insure uniform distribution of the organisms. In each set, pH determinations were made as previously described, and the concentration of the flagellates ascertained by hemocytometer counts. A Levy counting chamber was used with a 1–100 dilution prepared in a Hellige pipette, and all the flagellates were counted over the square used for erythrocyte counts.

The respiration of the flagellates was studied by means of the Warburg manometer equipped with flasks of about 14 cc. capacity; each vessel received 2 cc. of culture fluid. Although the vessels were sterilized by heat and the fluid was transferred by sterile pipettes, it was not possible to maintain sterile conditions during the tests. However, the regularity of the manometer changes indicated that bacterial contamination had no effect on the readings. Ten per cent KOH and strips of filter paper were placed in the center well to absorb carbon dioxide. The vessels were shaken at a rate of 120 cycles per minute with an amplitude of 3 cm. Except as otherwise indicated, the experiments were conducted with atmospheric air as the gas phase. All tests excepting those on the effects of temperature on respiration were conducted at 28.5°C. which was sufficiently above the temperature of the laboratory to permit control of the water bath. The readings on oxygen consumption were made at hourly, half-hourly, or quarter-hourly periods depending on whether the respective temperatures were below 20°C., at 28.5°C., or above 32°C. The duration of the tests was 4 hours but in tests on the influence of various oxygen tensions the respiratory rate was first followed for 2 hours under air; the experimental gas mixture was then passed from a steel cylinder through the vessels for 20 minutes, and the respiratory rate measured for another 2 hour period.

The respiratory quotient was ascertained by Warburg's direct method; all practical details recommended by Dixon (1943) were followed. Due consideration of the bound carbon dioxide was taken by acidifying the contents of a control flask at the beginning of the experiment and acidifying the experimental vessel at the end of the run.

In tests on non-volatile respiratory poisons (azide, pyrophosphate, carbamates, iodoacetate, and arsenite) the normal respiratory rate was determined during a preliminary 2 hour period; the active substance in the amount of 0.5 cc. was then tipped in from a side arm, and the respiration was followed for a second 2 hour period. Volatile poisons (KCN and H_2S) were added to the vessels at the beginning of the test, and normal respiration was followed in control vessels; the former contained an equal amount of fluid of the same culture as the latter. In the cyanide experiments balanced KOH/KCN mixtures were used as recommended by Krebs (1935) for the CO_2 absorption to prevent distillation of HCN into the solution of KOH. The poison solutions were adjusted to pH 7.0; it was impracticable to adjust them to the exact pH of the cultures since the pH of the latter was variable. In one sodium azide series the hydrogen ion concentration of both culture and experimental solution was adjusted to pH 5.5 since according to Keilin (1936) this poison exerts its maximum influence in an acid environment.

EXPERIMENTAL DATA

The data of Fig. 1 are presented to illustrate the manner of carrying out the tests and to give a general idea of normal respiration, respiration under the influence of a

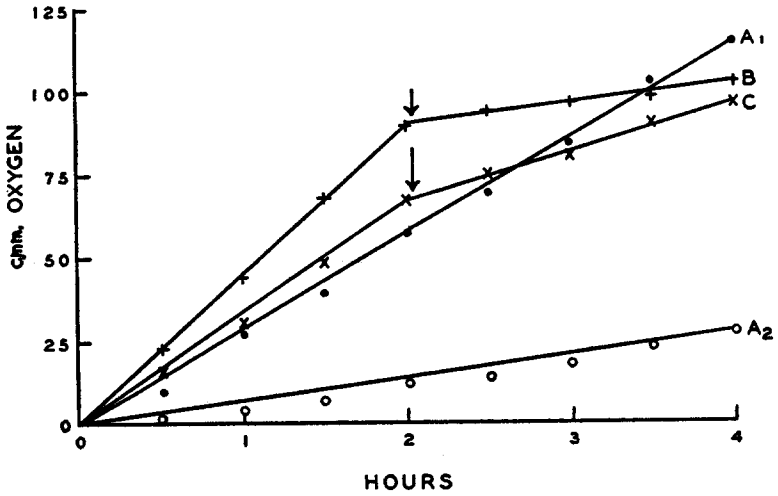


FIG. 1. Examples of single experiments on the oxygen consumption of cultural form of *Trypanosoma cruzi* under various conditions; in each case 2 cc. culture fluid were used and the temperature was 28.5°C.

A_1 and A_2 are parallel experiments with material from the same culture; culture 12 days old, 62 million organisms per cc., air in the gas phase. A_1, determination with the straight culture, A_2, with a M/100 KCN concentration in the culture fluid.

B, 8 day old culture, 84 million organisms per cc., air in the gas phase. First 2 hours show the respiration of the straight culture; at arrow addition of propyl carbamate through side arm of respiration vessel; final concentration in culture fluid, M/5.

C, 9 day old culture, 67 million organisms per cc. The gas phase consisted during the first 2 hours of air; at arrow the gas phase was changed to 0.8 per cent oxygen + 99.2 per cent nitrogen.

non-volatile and a volatile respiratory poison. Fig. 1 shows that under a given set of conditions the rate of oxygen consumption remained constant throughout the experiment, but that the rate of oxygen consumption was lowered under all of the indicated deviations from normal conditions.

The data presented in Figs. 2 to 4, and in Tables I to III represent average values based on a designated number of tests. Oxygen consumption was calculated as cubic millimeters of gas per 100 million organisms per hour. The customary method of calculating according to dry weight could not be followed because of the smallness of the sample and difficulties inherent in washing the

flagellates free from organic and inorganic constituents of the medium. Fig. 2 shows that the maximum concentration of organisms occurred at about the 10th day, and that the hydrogen ion concentration of the medium dropped from neutrality to the acid side during the early part of the incubation period, and rose to the alkaline side during the latter part of the period. Fig. 3 shows that

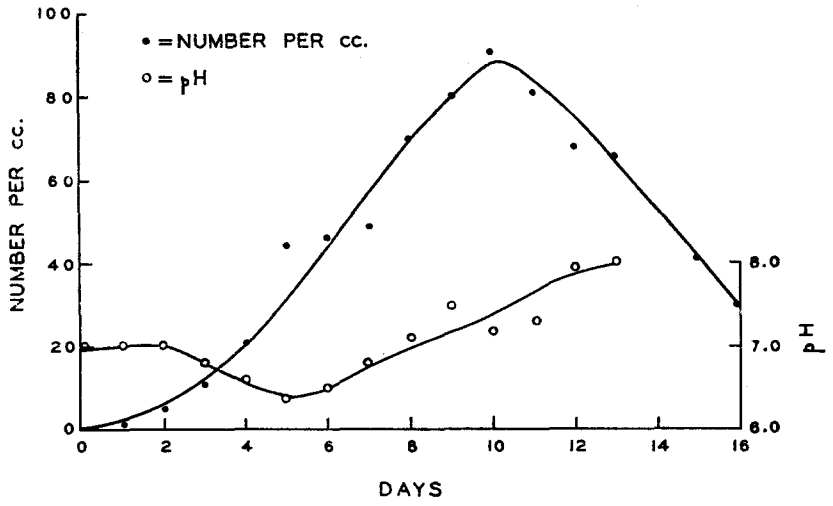


FIG. 2. Numbers of organisms and pH in the *Trypanosoma cruzi* cultures used for the respiration experiments shown in Fig. 3. The points on the numbers curve are mean values from 2 to 10 counts; the points on the pH curve are mean values from 2 to 9 determinations.

the rate of oxygen consumption fell steadily during the incubation period and was related to the age of the cultures.

Comparison of Figs. 2 and 3 shows a lack of correlation between oxygen consumption and the concentration of organisms, or their apparent vitality. The comparison fails to indicate any relationship between hydrogen ion concentration of the medium and the rate of oxygen consumption by *T. cruzi*. On the other hand, the data indicate relationships between the growth rate of the organisms and the respiratory quotient; the latter was at or slightly above 1.0 during the period of rapid growth and fell to a point approaching 0.7 during the period of declining growth. Further implications of these data are discussed subsequently.

Data concerning the effect of oxygen tension on the rate of oxygen consumption are summarized in Table I. An unaltered rate of consumption was maintained between O_2 tensions of 760 mm. Hg and 38 mm. Hg. At an undetermined point between 38 mm. Hg and 6 mm. Hg, the rate of oxygen consumption

began to decrease. At a tension of 6 mm. Hg, the rate was about half of normal, and at a tension of 1.1 mm. Hg, it was less than one-tenth of normal. The

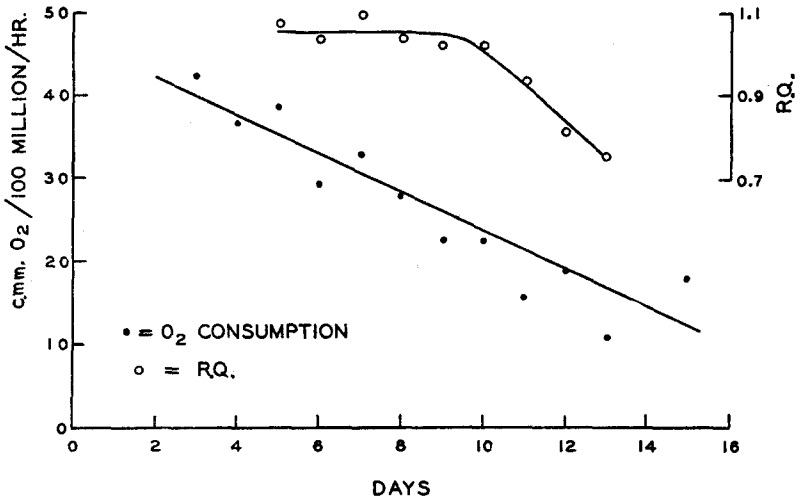


FIG. 3. Oxygen consumption and respiratory quotient of cultural forms of *Trypanosoma cruzi* in relation to the age of the cultures. All experiments were conducted at 28.5°C. and with air as gas phase. The points on the oxygen consumption curve are mean values from 4 to 20 determinations; those on the R.Q. curve from 2 to 3 determinations.

TABLE I

Dependency of Oxygen Consumption of Trypanosoma cruzi on Oxygen Tension
All experiments were conducted at 28.5°C.

No. of determinations	Initial oxygen consumption at oxygen tension of 160 mm. Hg (air)	Oxygen consumption at experimental oxygen tensions		
		Oxygen tension	Oxygen consumption	Per cent consumption of oxygen at 160 mm. Hg
	c.mm./100 million/hr.	*mm. Hg*	*c.mm./100 million/hr.*	
7	21.4	760.0	21.8	102
6	25.7	38.0	26.1	102
8	27.3	6.0	14.6	54
6	36.5	1.1	3.2	9

figures are valid for a temperature of 28.5°C., but the question whether different critical points might occur at lower or higher temperatures was not investigated. However, different critical points at different temperatures were noted in insects by Gaarder (1918) and von Buddenbrock and von Rohr (1922), and in crustaceans by Lindeman (1935).

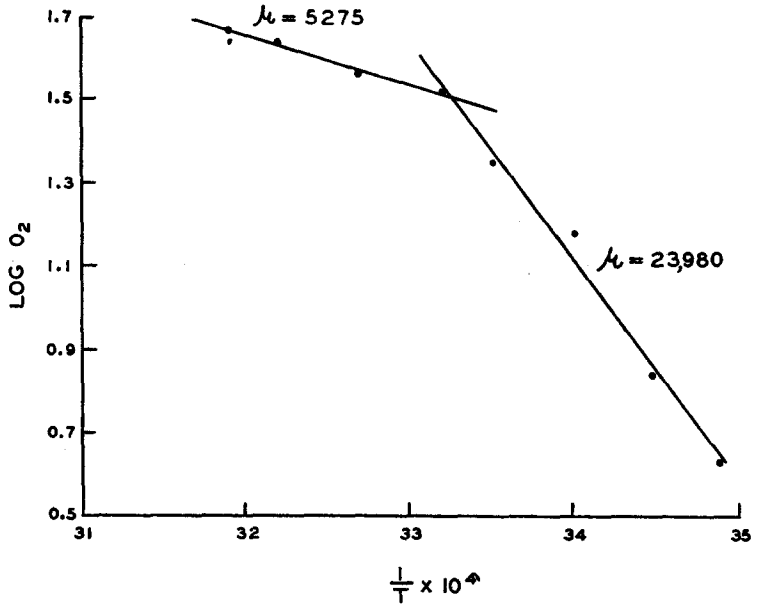


FIG. 4. Dependency of the oxygen consumption of cultural forms of *Trypanosoma cruzi* on the temperature, expressed according to Arrhenius' equation.

TABLE II

Dependency of Oxygen Consumption of Trypanosoma cruzi on Temperature

All cultures were 7 days old.

No. of determinations	Temperature	Oxygen consumption
	°C.	*c.mm./100 million/hr.*
6	13.4	4.3
6	16.9	7.0
12	21.5	15.2
6	25.5	22.4
20	28.5	33.1
6	32.5	37.2
6	37.5	43.5
6	40.4	46.6
4	44.3	(19.1)

The experiments concerning the effects of temperature on oxygen consumption are summarized in Table II and illustrated in Fig. 4. Table II shows that the rate of oxygen consumption increased between 13.4° and 40.4°C. and fell rapidly between 40.4° and 44.3°C. Exposure at 44.3°C. was lethal in less than 1 hour, but as determined by microscopic examination there was no damage to the parasites in the range 13.4° to 40.4°C. The graph of Arrhenius' equation,

Fig. 4, shows two straight lines intersecting at 28°C. The probable significance of this critical temperature is discussed later in the paper.

The results of the experiments on inhibition of oxygen consumption by respiratory poisons are summarized in Table III. In connection with these tests, the organisms were examined microscopically for evidence of marked damage at the end of the exposure period. In sodium azide at pH 5.5 there was considerable damage; some of the organisms were rounded up and appeared to be dying; others moved about sluggishly. Some reduction in activity was noted in hydrogen sulfide, M/2 ethyl carbamate, and M/5 propyl carbamate.

TABLE III

Influence of Respiratory Poisons on the Oxygen Consumption of Trypanosoma cruzi

No. of determinations	Age of culture	pH	Initial oxygen consumption	Active substance	Experimental oxygen consumption	Inhibition
	days		*c.mm. O_2/100 million/hr.*		*c.mm. O_2/100 million/hr.*	*per cent*
5	9 and 8	7.7; 7.2	27.8	M/1000 sodium azide	14.7	47
5	8	5.5	13.0	M/1000 sodium azide	0.9	93
4	9	7.3	26.1	M/1000 potassium cyanide	4.5	83
4	12	8.1	18.6	M/100 potassium cyanide	2.8	85
3	10	7.0	13.0	*Ca.* M/500 hydrogen sulfide	5.9	55
7	6 and 5	6.4; 6.6	33.4	M/200 sodium pyrophosphate	31.9	(4)
6	9 and 3	7.8; 6.7	33.1	M/5 methyl carbamate	26.2	21
6	13 and 6	7.6; 7.1	23.9	M/5 ethyl carbamate	17.2	28
6	13	8.2	9.2	M/2 ethyl carbamate	3.1	66
6	8	7.1	26.2	M/5 propyl carbamate	3.1	88
5	6	6.1	25.6	M/100 iodoacetate	10.1	60
6	7	7.4	27.2	M/3000 iodoacetate	23.8	13
6	7, 10, and 16	Not determined	33.2	M/1000 sodium arsenite	22.9	31

In all other solutions the parasites appeared normal. The extent to which the above mentioned injuries might have influenced the experiments could not be ascertained.

Table III shows that inhibition of oxygen consumption was produced by the oxidase inhibitors, sodium azide, potassium cyanide, and hydrogen sulfide, but not by sodium pyrophosphate. The figure reported for this last named poison is within the experimental error. Marked inhibition was produced by the dehydrogenase inhibitors, methyl, ethyl, propyl carbamate, and M/100 iodoacetate. Some inhibition was also produced by M/3000 iodoacetate and sodium arsenite. The last two substances probably affect sulfhydryl groups of various substances.

DISCUSSION

The data of the present paper do not indicate the causes of the observed correlation between the age of the cultures and the oxygen consumption of the

parasites, or of the observed lack of correlation between the rate of oxygen consumption and respiratory quotient, hydrogen ion concentration of the medium, and density of the parasite population. Reference to the literature has failed to furnish an explanation of these phenomena. Christophers and Fulton (1938) observed a lowering of the oxygen uptake of the blood form of *T. rhodesiense* when the sugar concentration of the medium dropped. A. Lwoff (1934) found that cultures of *Strigomonas* maintained without blood had a considerably lower oxygen consumption than when maintained with blood. He assumed, therefore, that certain Trypanosomidae cannot synthesize complete systems of catalytic respiratory enzymes. It is questionable whether these data are applicable in the case of *T. cruzi*.

The observed average respiratory quotient of 1.06 for *T. cruzi* during the early part of the incubation period is evidence of aerobic fermentation. Trypanosomidae studied by A. Lwoff (1934) showed an R.Q. of 1.0 and moderately developed aerobic fermentations. Von Brand (1946) has reviewed the pertinent literature on this subject. The decline in the R.Q. after the 10th day of incubation indicates that a change of metabolism occurred in *T. cruzi* after the population reached maximum levels. As pointed out by von Brand (1935), the respiratory quotients of protozoa are of limited application as guides to the kind of substances metabolized. However, the following hypothesis explains the observed results on *T. cruzi*: During the first 10 days the parasites metabolized predominantly carbohydrates with a resultant R.Q. of slightly above 1.0, and an acid pH; after 10 days, when the carbohydrate supply reached very low levels, the organisms metabolized protein with a resultant R.Q. lower than 1.0 and an alkaline pH. The derivation of acids from carbohydrates, as described by previous workers in pathogenic species of *Trypanosoma*, was noted earlier in the paper. The alkalinization of the medium in the later stages must be due to the elaboration of basic substances; these originate most commonly from a protein breakdown. The assumption that the protein metabolism becomes preponderant in the later stages finds a strong support in the lowering of the respiratory quotient. The previously reviewed experiments of Salle and Schmidt (1928) support the assumption of such a sequence for *L. tropica*.

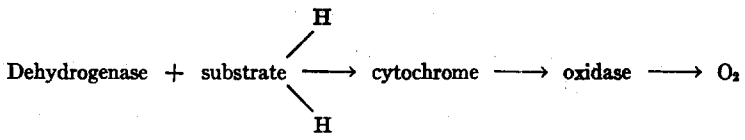
T. cruzi resembles many free living protozoa (Lund, 1918; Amberson, 1928; Adolph, 1928; and Baker and Baumberger, 1941) in its ability to maintain an unchanged rate of oxygen consumption over a wide range of tensions. This is not unexpected since the surface/volume ratio in such small organisms is favorable to the diffusion of oxygen even at low tensions. However, it differs in this respect from *Spirostomum*, which, according to Specht (1934), shows a clear dependency of its oxygen consumption on the tension over a wide range of tensions. No data on the behavior of any parasitic protozoan along this line have been published so far despite the fact that parasitic protozoa live in habitats with widely varying oxygen tensions. Furthermore, the oxygen tension in the intestine of the reduviid bug, the intermediate host of *T. cruzi*, is unfor-

tunately not known; it is consequently not possible at the present time to decide whether the organisms would be able to consume the maximum amount of oxygen in this habitat.

As observed by us, the response of *T. cruzi* to temperature changes differs from that of *Strigomonas fasciculata* (A. Lwoff, 1934) in the following respects: The rate of oxygen consumption of the former was continuous up to 40.4°C. and of the latter up to 34°C.; the respective thermal death points were 43.4° and 38°C. The plot of Arrhenius' equation gave two intersecting straight lines for *T. cruzi* and one straight line for *S. fasciculata* with an increment, μ, of 9380, which was between the two increments for *T. cruzi*. Since *S. fasciculata* is a one-host parasite of invertebrates and *T. cruzi* a two-host parasite of invertebrates and warm blooded vertebrates, the differences in temperature responses are probably related to these differences in the life cycle. In studies on *Eustrongylides ignotus*, von Brand (1943) found that a plot of Arrhenius' equation gave a picture similar to that found for *T. cruzi* in the present study. In the case of *E. ignotus* the temperature 27°C. where the two lines intersected was near that of the maximum encountered in the cold blooded intermediate host in nature. These data may indicate that within the cold blooded host a metabolic process predominates in the parasite (master reaction in the sense of Crozier, 1924) with a different temperature characteristic from that prevailing in the final host. The data indicate that a further investigation of this phenomenon in parasitic organisms may yield interesting results.

The oxidase mechanism of *T. cruzi*, as revealed by the influence of respiratory poisons, has greater similarity to that of bakers' yeast than to that of vertebrate muscle. Eighty per cent or more of the respiration of the latter is inhibited by pyrophosphate, while that of the former remains entirely unaffected (Dixon and Elliott, 1929), thus showing the same negative response as *T. cruzi*. The respiration of bakers' yeast depends almost exclusively on the cytochrome system (Haas, 1934). Cytochrome oxidase is strongly inhibited by cyanide, azide, and hydrogen sulfide, but not by pyrophosphate (Keilin, 1929). It is shown in Table III that the same response to these substances was given by *T. cruzi*. It appears, therefore, that this enzyme is the chief oxidase of *T. cruzi*. Definitive identification requires demonstration that the respiration is also inhibited by carbon monoxide and that this inhibition is reversed by light. In the present studies, inhibition by carbon monoxide was not investigated, but A. Lwoff (1934) reported a 90 per cent inhibition with carbon monoxide in the case of *Strigomonas*. He did not investigate the question of reversal.

It is of interest that oxidase inhibitors and dehydrogenase inhibitors both have a maximal effect of around 90 per cent on the respiration of *T. cruzi*. This may indicate that if one of the two enzyme systems is effectively blocked no alternate channel exists for the functioning of the non-inhibited system. In other words, both systems may be coupled together, perhaps according to the following well known scheme:



The system may be more complicated; we have, for example, not yet ascertained whether coenzymes are involved.

Roughly speaking, 10 per cent of the respiration of *T. cruzi* is not dependent on the foregoing system. It is possible that this fraction depends on substances, other than dehydrogenases, containing sulfhydryl groups. This may be indicated by the inhibition observed under the influence of M/3000 iodoacetate, and, though less convincing, under the influence of arsenite. However, these substances seem to be of much less importance in the present case than in the case of organisms that depend largely on sulfhydryl groups for their oxygen consumption. In *Glaucoma* for example, 75 to 80 per cent of the oxygen consumption was inhibited by analogous concentrations of iodoacetate and sodium arsenite (M. Lwoff, 1934).

SUMMARY

1. The oxygen consumption of cultural forms of *Trypanosoma cruzi* decreases in intensity with increasing age of the cultures; no correlation with any other factor studied could be established.

2. The respiratory quotient was high for the first 10 days, *i.e.* as long as the population increased; with the onset of a decline in numbers, the R.Q. began to drop. It is believed that the flagellates consume in the beginning predominantly sugar and later predominantly protein. Observations on the pH of the cultures bear out this view.

3. The oxygen consumption was independent of the oxygen tension over a wide range of tensions.

4. The oxygen consumption increased in the temperature range 13° to 40°C., while a temperature of 44°C. proved to be lethal. Upon application of Arrhenius' equation, two straight lines, intersecting at about 28°C., resulted. The μ values were 23,980 and 5275 for the lower and higher temperature range respectively.

5. Of the oxidase inhibitors tested, strong inhibition of the oxygen consumption was achieved with azide, cyanide, and hydrogen sulfide. Pyrophosphate had no influence at all. There is some probability that cytochrome oxidase is the chief oxidase present.

6. The strongest inhibitory influence due to dehydrogenase inhibitors was observed with propyl carbamate and high concentrations of ethyl carbamate.

7. A small fraction of the oxygen consumption, about 10 per cent, may be due to substances with sulfhydryl groups, as indicated by a slight but distinct inhibition due to dilute iodoacetate and to arsenite.

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