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Trypanosoma cruzi: Fatty Acid Metabolism *in Vitro*[1]

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WOOD, DUELL E. 1975. *Trypanosoma cruzi:* Fatty acid metabolism *in vitro*. *Experimental Parasitology* 37, 60–66. *Trypanosoma cruzi* populations, composed primarily of trypomastigote forms, readily converted palmitic acid, linoleic acid, oleic acid, and stearic acid to CO_2. Appreciable amounts of carbon from these four fatty acids were also incorporated into neutral and phospholipid lipids by these parasites. Palmitic acid, a 16 carbon saturated fatty acid, was converted at rates greater than those of the other three fatty acids.

INDEX DESCRIPTORS: *Trypanosoma cruzi;* Metabolism; Fatty acids; Trypomastigotes; Palmitic acid; Linoleic acid; Oleic acid; Stearic acid; Carbon dioxide; Carbon isotopes.

INTRODUCTION

In comprehensive reviews of the studies on the metabolism of parasites von Brand (1966) and Honigberg (1967) point out that carbohydrates have been found to be the most important source of energy for the culture forms of the hemoflagellates. Additionally, Ryley (1959) reported that the bloodstream forms (trypomastigotes) of many species of trypanosomes utilized glucose when it was added to media in which these parasites were incubated. In his studies, the glucose consumption of *Trypanosoma cruzi* isolated from the blood of infected mice was almost twice that of culture forms (epimastigotes) harvested from a diphasic medium.

A review of the literature indicates that fatty acids have a minor, if any, role in the energy metabolism of the hemoflagellates. Until recently, the only reported utilization of fatty acids in the energy processes of trypanosomes was in the studies of Manozzi-Torini (1940). In these studies, Manozzi-Torini reported that slight increases, measured manometrically, in respiration rates of culture forms of *T. evansi* occurred when short chain fatty acids were added to the incubation medium.

In recent years, fatty acid metabolism has been found to be an important component of the total metabolism of certain microorganisms and vertebrates tissues (Neptune *et al.* 1964; Meyer and Holz 1966). Indeed, the finding that fatty acids are of importance in the energy metabolism of various tissues has often necessitated the revision of earlier concepts. Much of this new knowledge can be attributed to improvements in the techniques used to study the metabolic processes. Especially useful has been the development of radioisotopic techniques to trace the fate of fatty

[1] The opinions or assertions contained herein are those of the author and are not to be construed as reflecting the views of the Navy Department or the Naval Service at large.

acid components in the metabolism of microorganisms and vertebrate tissues.

Utilizing labeled palmitic acid, Dixon (1967) found that trypomastigotes of *T. lewisi* from the blood of infected rats were able to convert this long chain fatty acid to CO_2. This was the first definitive evidence that a long chain fatty acid was utilized in the energy metabolism of trypanosomes.

In view of the results obtained by Dixon (1967) and with the availability of a culture system from which large numbers of trypomastigotes of *T. cruzi* could be harvested (Wood and Pipkin 1969) the present studies were initiated.

Materials and Methods

The Goble strain of *T. cruzi* maintained in the laboratory in C_3H mice was used in this study.

In vitro cultures of *T. cruzi* were established and maintained according to Wood and Pipkin (1969). Primary isolates and subsequent transfers were in Grace's (1962) medium for cells from the moth, *Antheraea eucalypti*, (GMA) + 10% heat-inactivated fetal calf serum (FCS) + 0.5% hemolymph from the moth, *Philosamia cynthia*. Only parasite population harvested from second subcultures, *in vitro*, were used in the studies described herein.

To determine the percentage of each morphological form present in a given culture, two drops of culture medium containing parasites were placed on an alcohol cleaned slide and allowed to dry. These preparations were fixed in 5% gluteraldehyde for 30 min. After fixation slides were rinsed thoroughly in distilled water, stained with Giemsa's stain, and examined by light microscopy. Differentiation between epimastigote and trypomastigote forms was based on the position of the kinetoplast within the parasite. Forms with the kinetoplast in a pre- or juxtanuclear position were designated epimastigote and those with the kinetoplast posterior to the nucleus, trypomastigote. "Differential" counts were performed as follows. The number of each morphological form present in a microscopic field was recorded until a total of 100 parasites had been counted; this procedure was repeated five times and averaged for each sample.

To harvest parasites from culture for use in metabolic studies, cultures were first mixed and then placed in sterile centrifuge tubes, capped, and centrifuged at 500 *g* for 30 min. The supernatant solutions were discarded and the parasite pellets resuspended in sterile GMA. The samples were recentrifuged as described and then resuspended in one half their original volume in GMA. The densities of the *T. cruzi* populations were then determined using a Coulter Counter (Coulter Electronics, Hialeah, Florida).

Once the number of parasites per ml was determined, parasite suspension were diluted with sterile GMA in accordance with each experimental protocol and dispensed in 5-ml aliquots into 25-ml reaction flasks (Kontes Glass, Vineland, New Jersey).

All experiments on the metabolism of ^{14}C-labeled substrates by *T. cruzi* were carried out in reaction flasks fitted with airtight rubber caps. Each rubber cap held a plastic cup for collection of CO_2. A $1\frac{1}{2} \times 3$ cm wick of Whatman No. 50 filter paper was folded and placed into the plastic cup to provide additional surface area for the adsorption of free CO_2. The reaction flasks were modified by the addition of a glass side arm fitted with a serum stopper. These side arms facilitated the addition of labeled substrates to the flasks and reduced the chance of contaminating the CO_2 collection cup with labeled material.

Labeled long chain fatty acids, [1-^{14}C]-palmitic acid, [1-^{14}C]-linoleic acid, [1-^{14}C]-oleic acid, and [1-^{14}C]-stearic acid, were obtained from commercial sources. In order to get each of these fatty acids into solution, their sodium salts were prepared and

TABLE I[a]

Conversion product[b]	Fatty acid			
	Palmitic	Linoleic	Oleic	Stearic
CO_2	0.129 ± 0.0040	0.048 ± 0.0007	0.036 ± 0.0015	0.014 ± 0.0003
Neutral lipids	0.586 ± 0.0016	0.148 ± 0.0028	0.186 ± 0.0044	0.105 ± 0.0024
Phospholipids	0.348 ± 0.0258	0.118 ± 0.0056	0.100 ± 0.0174	0.094 ± 0.0028

[a] Conversion of four, long-chain fatty acids into CO_2, neutral lipids and phospholipids by a population of *Trypanosoma cruzi*, consisting of 68% trypomastigotes and 32% epimastigotes, from second subcultures. Each value is the mean of one duplicate determination ± the standard error.

[b] The quantities of each conversion product collected are expressed as the nmoles of fatty acids converted/10^8 parasites/hr.

complexed to crystalline bovine plasma albumin (BPA) which was dissolved in glass distilled water.

In the preparation of stock labeled material for use in these experiments an attempt was made to get 10 μCi of activity in a final volume of 1 ml. This would allow addition of 1 μCi of activity to each reaction flask by the injection of one-tenth milliliter of the stock solution. However, because of the conditions required for complexing long chain fatty acids to BPA, it was not always possible to attain precise uniformity of concentration. Therefore, initial added activity was normalized to 10^6 desintegrations per minute (dpm) to facilitate analyses of all experimental results.

In the experimental protocols 12 flasks containing *T. cruzi* in 5 ml of GMA and four flasks with GMA alone (blank) were used. All flasks were placed in a 37 C shaking water bath and allowed to equilibrate for 5 min. After equilibration, one tenth ml of [1-^{14}C]-palmitic acid + BPA complex was injected into each of three flasks containing parasites and into one blank flask. This procedure was repeated using each of the other three fatty acid–BPA complexes. One flask, containing parasites, from each group was immediately brought to 5% with trichloroacetic acid (TCA). The addition of TCA stopped all metabolic activity in these flasks and they served as zero-time controls for each subtrate. After one hour incubation the remaining flasks were brought to 5% TCA and the labeled CO_2 was collected. The contents of each flask were then harvested and processed for extraction of lipids.

After incubation and the termination of the metabolism by the addition of TCA, 0.2 ml of Hyamine (Registered Trademark of Rohm and Haas available from Packard Instrument Company, Downers Grove, Illinois) hydroxide was injected into the CO_2 collection cup in each reaction flask. The Hyamine quickly absorbed onto the paper wick. All flasks were shaken in the 37 C water bath for an additional 30 min to allow adsorption of the liberated $^{14}CO_2$. After this, the plastic cup with paper wick from each reaction flask was carefully removed and placed in individual scintillation vials. Two and one-half milliliters of absolute methanol were then added to each vial, followed by 15-ml of Liquiflor (New England Nuclear, Boston, Massachusetts) in toluene. The vials were capped, mixed, and then counted in a liquid scintillation counter.

After collection of CO_2, the contents of each reaction flask were harvested and washed twice in saline by centrifuging at 600 *g* for 15 min and discarding the supernatant solutions. Each pellet was resuspended in 10 volumes of absolute methanol and shaken for 20 min. Twenty volumes of chloroform were then added to the methanol suspensions and the mixtures

TABLE II[a]

Conversion product[b]	Fatty acid			
	Palmitic	Linoleic	Oleic	Stearic
CO_2	0.239 ± 0.0003	0.108 ± 0.0013	0.068 ± 0.0290	0.048 ± 0.0005
Neutral lipids	0.144 ± 0.0070	0.070 ± 0.0140	0.082 ± 0.0231	0.028 ± 0.0011
Phospholipids	0.349 ± 0.1332	0.170 ± 0.0420	0.144 ± 0.0566	0.107 ± 0.0600

[a] Conversion of four, long chain fatty acids into CO_2, neutral lipids, and phospholipids by a population of *Trypanosoma cruzi*, consisting of 84% trypomastigotes and 16% epimastigotes, from second subcultures. Each value is the mean of one duplicate determination ± the standard error.

[b] The quantities of each conversion product collected are expressed as the nmoles of fatty acid converted/10^8 parasites/hr.

shaken for an additional 20 min. These mixtures were centrifuged at 600 g for 15 min, the supernates decanted, and the precipitates discarded. Equal volumes of 0.4% $CaCl_2$ solutions were then added to each of the $CHCl_3$:MeOH solutions. These mixtures were shaken thoroughly and allowed to settle into two phases. The upper phases were aspirated and discarded. This process was repeated twice. Each of the lower phases was then dried at 40 C under a stream of nitrogen (N_2). The resultant lipid extracts were each redissolved in 1 ml of chloroform and the neutral lipids and phospholipid lipids partitioned according to the procedure described by Neptune *et al.* (1964).

Each neutral lipid fraction was dried under a stream of N_2 in a 40 C water bath and redissolved in 4 ml of spectrograde hexane. The hexane, containing the neutral lipids was mixed thoroughly with 2 ml of alkaline aqueous alcohol and allowed to settle in two phases. The lower phase, containing the nonesterified fatty acids, was removed and discarded. This procedure was repeated twice. The upper phase containing the neutral lipids was dried at 40 C under a stream of N_2 and then redissolved in 1 ml $CHCl_3$. One-tenth milliliter of this solution was placed in a scintillation vial and diluted with 2.5 ml absolute methanol and 15 ml of scintillation fluid. The sample was then counted in a liquid scintillation counter.

Each phospholipid fraction was dried in the same manner as the neutral lipids and then redissolved in 1 ml of $CHCl_3$:MeOH (2:1). One-tenth milliliter of the resulting solution was placed in a scintillation fluid, and then counted.

In order to determine the rate of substrate utilization and facilitate analyses of the experimental data, the results of the metabolic studies were converted from dpm's to nmoles of substrate utilized. The incubation time of each experiment was one hour. Therefore, the data were standardized to nmoles of substrate utilized per 10^8 parasites per hour and are expressed as nmoles/hr.

Unless otherwise stated, the results on substrate utilization are expressed as means ± the standard error of two or more replicate determinations. Student's t test was used for testing the differences between means.

Results

In these experiments two separate populations of *T. cruzi*, differing only in the percentage of morphological forms present in each were utilized. The first population consisted of 68% trypomastigotes (T = 68%) and 32% epimastigotes. The second population consisted of 84% trypomastigotes (T = 84%) and 16% epimastigotes. The results from each of these populations will be presented separately and compared.

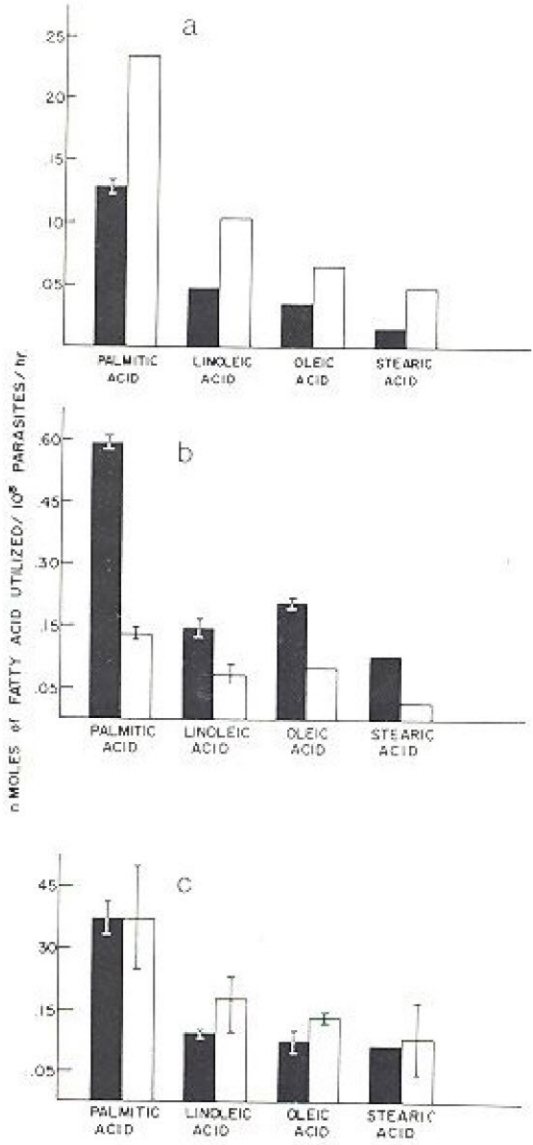

FIG. 1. Comparison of the mean rates of conversion of four long chain fatty acids to CO_2 (a), neutral lipids (b), and phospholipids (c) by two different populations of *Trypanosoma cruzi* from second subcultures *in vitro*. Vertical lines = The range of values of the duplicate determination. Shaded bars = *Trypanosoma cruzi* 68% trypomastigote population; unshaded bars = *Trypanosoma cruzi* 84% trypomastigote population.

The rates of conversion of each of the fatty acids to CO_2 by the $T = 68\%$ population of *T. cruzi*, as outlined in the Table I, showed the following relationship.

Palmitic acid >> linoleic acid > oleic acid > stearic acid.

Palmitic acid was incorporated into neutral and phospholipid lipids at a greater rate than the other three fatty acids studied (Table I). The comparative rates of incorporation of fatty acids into neutral lipids by the first population were as follows.

Palmitic acid >> oleic acid > linoleic acid >> stearic acid.

For incorporation into phospholipids, the same type of comparison showed.

Palmitic acid > linoleic acid ≥ oleic acid ≥ stearic acid.

The $T = 84\%$ population of *T. cruzi* also utilized palmitic acid at a greater rate than the other three fatty acids (Table II).

The conversion rates of the four fatty acids into CO_2, neutral lipids and phospholipids showed the same relationship as illustrated for the $T = 68\%$ population.

A comparison of the data from the $T = 68\%$ population with that of the $T = 84\%$ population shows a number of differences (Fig. 1). The conversion rates of each of the fatty acids to CO_2 were greater in the $T = 84\%$ population than in the $T = 68\%$ population. When these differences were analyzed by the Student's t test, they were all found to be significant ($P < 0.01$).

The rates of conversion of fatty acids into neutral lipids were the inverse of the above comparison. That is, the rates of conversion of each fatty acid into neutral lipids were greater by the $T = 68\%$ population. The differences in the conversion rates for palmitic acid, oleic acid and stearic acid were found to be statistically significant ($P < 0.01$). The differences in the conversion rates of linoleic acid also were significant ($P < 0.05$).

The data indicate that conversion rates of fatty acids into phospholipids were similar in both experimental populations. Analyses of the differences between each of the fatty acids showed them not to be statistically significant.

Discussion

The results obtained herein revealed that *T. cruzi* populations composed primarily of trypomastigote forms readily converted long chain fatty acids to CO_2. Thus, it was shown that these fatty acids could serve as sources of energy for these trypanosomes. In addition, appreciable amounts of labeled carbon were found in the neutral and phospholipid lipids extracted from these populations, indicating that these hemoflagellates were also capable of utilizing long chain fatty acids in their biosynthetic processes.

It is of interest to note that the rates of conversion of palmitic acid to CO_2 were more than twice those of linoleic acid. The mean rates of conversion of stearic acid to CO_2 were lower than those of the other fatty acids tested, being from five to nine times less than the rates of palmitic acid.

Palmitic acid was incorporated into neutral and phospholipid lipids at rates in excess of the rates of incorporation of the other three fatty acids. Differences in the rates of incorporation of linoleic, oleic, and stearic acid were relatively small.

Boné and Parent (1963) reported that stearic acid is an essential growth factor for the culture forms of *T. cruzi*. In their studies, palmitic acid and linoleic acid were said to be inhibitory to survival of the parasites *in vitro*. Their results appear to be contradictory to the findings in this study. However, direct comparisons are impractical because their experiments were based on long term survival of parasites in media containing added fatty acids, whereas the present study was designed to measure utilization of fatty acids over a short term. As another explanation, these contradictory results might be attributable to the differences in the strains of *T. cruzi* under investigation.

Perhaps the most interesting observations of the experiments on utilization of the four long chain fatty acids were revealed in the comparisons of the data from the parasite population composed of 68% trypomastigotes with those from the parasite population composed of 84% trypomastigotes. Analyses of these data suggest that differences in the rates of conversion of the fatty acids to CO_2 and neutral lipids might be related to the relative proportions of the different morphological forms present in each of the populations. These data infer that: (1) the rate of conversion of fatty acids to CO_2 is attributable to the number of trypomastigotes in the experimental population; and (2) the rate of fatty acid incorporation into neutral lipid is due to the number of epimastigotes in the experimental population.

The above inferences are supported by the results obtained from experiments in which rates of conversion of palmitic acid to CO_2, neutral lipids, and phospholipid lipids were measured in populations of *T. cruzi* composed of different ratios of epimastigote to trypomastigote forms. Studies designed to investigate further these inferences have been completed and their results will be presented in a subsequent paper.

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