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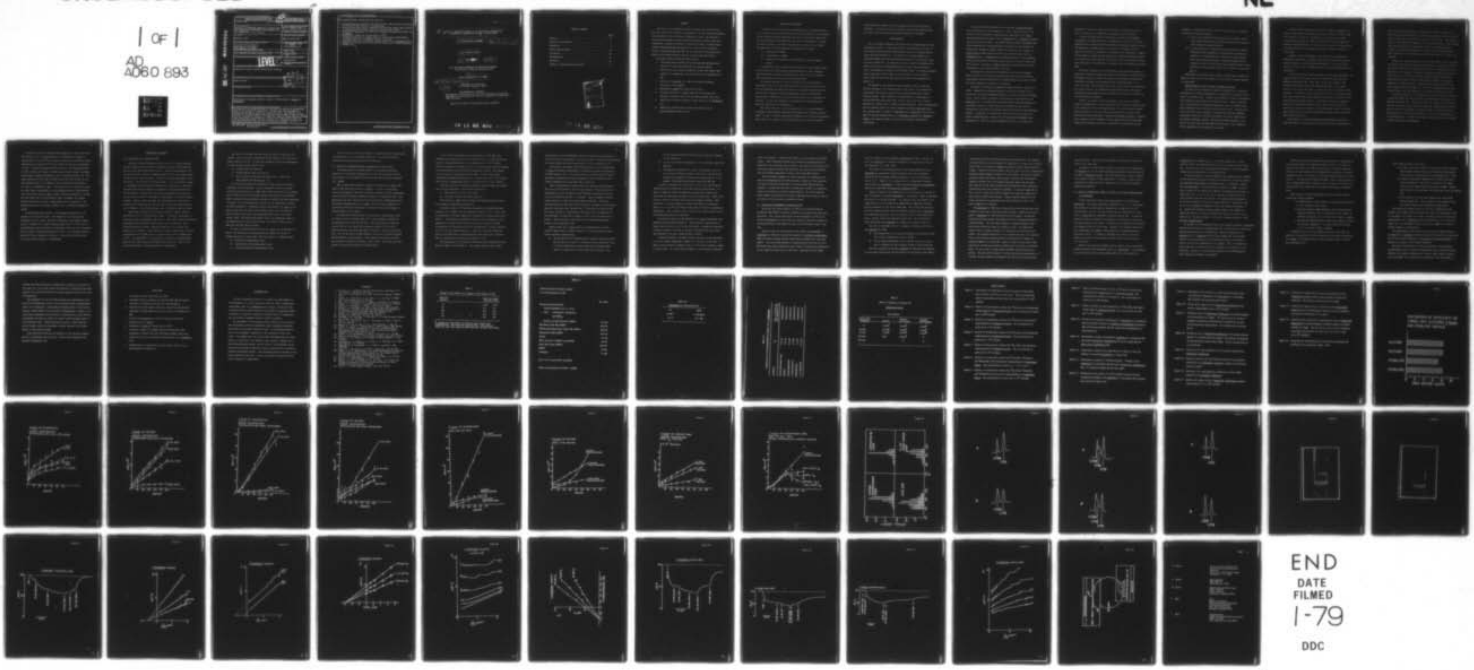

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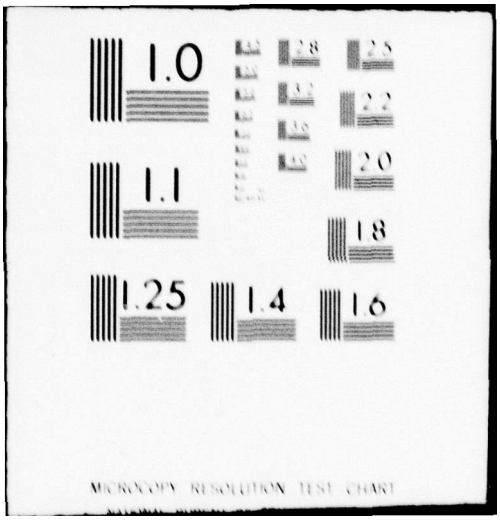
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The purpose of our studies has been to determine the effects of trypanocidal drugs on the function of trypanosomes. We have also been interested in determining the mode of action of trypanocidal drugs. Our approach to resolving this problem includes investigating various enzymes in host and trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

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a. Determined optimal conditions for measuring DNA, RNA and protein synthesis in bloodstream and procyclic trypomastigotes;
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c. Characterize Leptomonas sp. k-DNA and developed techniques appropriate for T. brucei;
d. Maintained T. brucei for several days at 25°C;
e. Initiated cultures of T. brucei infective bloodstream trypomastigotes on Buffalo lung cells and Chinese hamster lung cells;
f. Identified the electron transport systems present in T. rhodesiense (15);
g. Demonstrated suramin and five other drugs inhibit the L-α-glycerophosphate oxidase (15).

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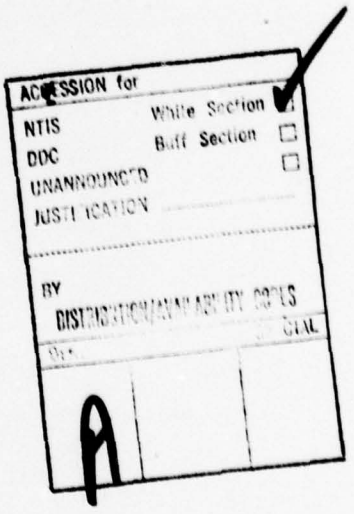

ABSTRACT

The purpose of our studies has been to determine the effects of trypanocidal drugs on the function of trypanosomes. We have also been interested in determining the mode of action of trypanocidal drugs. Our approach to resolving this problem includes investigating various enzymes in host and trypanosomes, studying the effects of trypanocidal drugs on enzyme systems isolated from trypanosomes and studying the structure and transcription ability of purified kinetoplast DNA. We are interested in determining the reason for the unique selective toxicity of known trypanocidal drugs.

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f. Identified the electron transport systems present in T. rhodesiense (14);

g. Demonstrated suramin and five other drugs inhibit the L-α-glycerophosphate oxidase (14,15).

APPROACH TO THE PROBLEM

Our approach to the problem of developing effective trypanocidal drugs is to study two specific and related areas of the biochemistry of trypanosomes. We are concerned with the regulation and control of the functioning of the electron transport system during the life cycle of trypanosomes. In order to identify targets for potential trypanocides, we must learn more about the properties of the mitochondrion in trypanosomes including:

a) replication of K-DNA;

b) transcription of K-DNA;

c) repression and synthesis of mitochondrial electron transport systems.

In addition we need to study the properties of the α-GP oxidase system. If we can alter the functioning of the mitochondrion or other essential electron transport systems in trypanosomes, we should be able to inhibit the continuation of the life cycle of the trypanosome.

Our working hypothesis is that the synthesis of the mitochondrial cytochrome system or the α-GP oxidase system is essential for the survival of the trypanosomes and under the control of mitochrondial and nuclear DNA. The electron transport systems in trypanosomes could be prevented from functioning by inhibition of the factors which control their synthesis and function. We feel this hypothesis is experimentally testable and pragmatically applicable for chemotherapy.

The overall objective of our research project is to gain biochemical knowledge on macromolecular synthesis and bioenergetics in African trypanosomes. We want to identify some unique properties in the role of mitochondrial DNA or RNA in the regulation of the functioning of the electron transport

system which will provide a tool for inhibiting the replication of the parasites. In this way, we hope to develop a more rational approach than now exists for the detection of potential trypanocidal compounds.

THE BACKGROUND

One of the major diseases in the world today is trypanosomiasis of man and animals in Africa. It has been placed by the WHO high on the list of the ten major health problems facing mankind, this list including malaria, cancer and heart diseases (1). The importance of trypanosomiasis as a veterinary disease has been well-documented recently in an excellent review by Losos and Ikede (2). The land mass of Africa, south of the Sahara, over which tsetse flies are distributed and that is thus virtually devoid of cattle is estimated to be about 4 million square miles. It has been estimated that if this area could be used, at least 125 million cattle could be raised, and this would more than double the present cattle population of Africa (3).

Our approach to the research on this problem has been to study the molecular biology of the causative agent of the disease - the trypanosome. Trypanosomes are parasitic protozoa of the order Kinetoplastida, Family Trypanosomatidae. These oganisms are characterized by a large amount of mitochondrial DNA (kinetoplast DNA), usually 5-20% of the total cellular DNA which is located within a single, long mitochondrion. These hemoflagellates are the causative agents for several diseases including Trypanosoma cruzi, the causative agent of Chagas' T. gambiense and T. rhodesiense, the causative agent of African sleeping sickness, and Leishmania donovani and Leishmania tropica, the causative agents of visceral and cutaneous leishmaniasis respectively.

We are particularly interested in one organism, Trypanosoma brucei, the causative agent of trypanosomiasis in cattle, sheep and horses. The division of the pleomorphic trypanosomes into three species, T. brucei infecting domesticated animals, and T. rhodesiense and T. gambiense infecting man, has no scientific basis. The human sleeping sickness trypanosomes and their morphologically indistinguishable counterparts in game animals are perhaps best regarded as genetic variants or subspecies of T. brucei. T. brucei brucei of game animals and cattle will not infect man, but T. b. rhodesiense and T. b. gambiense will, causing respectively, acute sleeping sickness in East Africa and a more chronic form of the disease in West and Central Africa. The three T. brucei subspecies are morphologically indistinguishable at all stages in their life cycle.

The question of the function of K-DNA has still not been answered. There are still no publications confirming the transcription of K-DNA in trypanosomes. Several laboratories are investigating the structure of K-DNA but little information is known on its function.

At present, the effects of trypanocidal drugs of K-DNA is difficult to assess. Drugs such as ethidium bromide or acriflavine are known to have many effects on the macromolecular synthesis in cells in addition to binding to DNA. We have shown the effects of acriflavine on the metabolism of C. fasciculata (4). The respiration of dyskinetoplastic organisms obtained by acriflavine treatment is lower than that of normal cells and could be attributed to a decrease in cytochrome content and activity of mitochrondrial enzymes. In addition, we also detected an increase in the activity of a number of dehydrogenases, particularly α-GP and glucose-6-phosphate dehydrogenases (5). However, it must be emphasized that it is certainly not

possible to conclusively state that the changes in enzyme activity are a direct consequence of an inhibition of kinetoplast DNA replication.

Simpson and Lasky (6) have reported the isolation from Leishmania tarentolae of two small RNA species, sedimenting at 9 and 12 S in sucrose gradients from a highly purified kinetoplast-mitochrondrion complex fraction. The labeling of this RNA in vivo was sensitive to ethidium bromide (2 µg/ml) but relatively insensitive to actinomycin D or camptothecin. Addition of these RNAs to a wheat germ in vitro protein synthesizing system resulted in a stimulation of incorporation of H-leucine into acid insoluble material. They proposed that these RNA species represent stable mitochondrial messenger RNAs.

The recent findings that the biosynthesis of mitochondrial enzymes is stimulated by agents blocking transcription and translation of mitochondrial DNA suggests that these enzymes are coded by nuclear genes, synthesized on cytoplasmic ribosomes, and transplanted into the mitochondrion. It is clear, therefore, that the functioning of the nuclear genes and mitochondrion are closely related. The cooperation of mitochondrial and nuclear genes specifying the mitochondrial genetic apparatus is essential to the function of this respiratory organelle.

Clearly, our interest in the functioning of the electron transport system in trypanosomes is closely associated not only with the identification of the properties of the α-GP oxidase system or the mitochondrial branched electron transport system, but also associated with the function of the mitochondrial and nuclear genes. Results with Neurospora crassa, an organism with a branched electron transport system, suggests that the nuclear genes coding for mitochondrial enzymes are controlled by mitochondrial protein

synthesis. The mechanism of this control is not yet known. Two general possibilities have been proposed:

(1) an indirect mechanism which would involve a metabolic control chain connecting respiration and ATP production in the mitochondrion with nuclear gene expression;

(2) a direct control by a repressor like protein which is coded by mitochondrial DNA, synthesized on mitochondrial ribosomes and exported to the nucleus where it controls the nuclear partner genes for mitochondrial proteins. Numerous examples are now available for role of both nuclear and mitochondrial genes in the synthesis of mitochondrial proteins such as ATPase or cytochrome $\underline{aa}_3$ (7).

Experiments to test these various ideas are needed as we consider the regulation and control of the synthesis of the electron transport systems in trypanosomes.

Identification of mode of action of trypanocidal drugs

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxia and treatment of early stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases, when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly, the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "bloodbrain barrier", this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review of the mode of action of trypanocidal drugs has been prepared by Williamson (8). More recent studies have suggested that berenil and ethidium bromide form complexes with DNA. In the case of ethidium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocylic chromophore of the drug molecules becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (9).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria of

many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA *in vivo* and give rise to dyskinetoplast trypanosomes (10) and "petite mutants" of yeast (11). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, an aromatic diamidine, has been shown under certain conditions to interact with DNA and selectively block kinetoplast replication (12,13). The earliest reported effect observed of berenil is the localization in the kinetoplast of *T. brucei*. This has been detected by ultraviolet microscopy within an hour of a curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an *in vitro* suspension of trypanosomes (13). Further work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (14).

A detailed examination of kinetoplast DNA isolated from berenil treated *T. cruzi* has shown that many of the small circular DNA molecules appear as branched structures (12). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of berenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. In vitro exposure of trypanosomes to suramin at concentrations as low as 10^{-5} M is known to reduce their infectivity whereas concentrations as high as 10^{-2} M do not affect the motility or respiration of cells. As would be expected from its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes, including hyaluronidase, fumarase, hexokinase, urease, and RNA polymerase (15). The ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as a prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that, when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

An outstanding characteristic of all pathogenic flagellates is the complexity of their life cycles. The changes that occur during their development must all result, directly or indirectly, from changes in gene activity. However, as in other differentiating systems, we know little of the mechanisms of gene repression and depression which give rise to parasites able to repond to changing environmental stimuli. Such knowledge should be the goal of future research in this area. It may well provide the basis for a more rational approach to chemotherapy.

RESULTS AND DISCUSSION

A. Cultivation of *T. brucei in vitro*

During our studies developing infective forms of T. brucei in vitro, we have studied the infectivity of organisms maintained in RPMI 1640 plus 5% fetal calf serum (FCS) at 25°C and 37°C. Organisms maintained at 37°C die after 24 hours. However, organisms grown at 25°C in this medium remain infective for at least four days. A result of a typical experiment is seen in figure 1. The cultures are initiated with $1 - 2 \times 10^6$ cells/ml. On day 4, trypanosomes from culture were injected into irradiated mice (800 rads) and infectivity results observed in comparison to inoculum obtained from frozen stabilates. Repeatedly, the cultured trypanosomes were infective. As can be seen in table I, the number of trypanosomes in cultures decreased slightly. However, after four days, the trypanosomes were still active. Incubation for periods greater than four days resulted in marked decreases in cell numbers, strange forms present, loss of infectivity and cell death.

These results indicated that infective forms of T. brucei could be maintained at 25°C for short periods of time. In addition, during days 1-3 numerous dividing trypanosomes could be seen. This system could be particularly advantageous since no "seed" tissue culture cells are required. Thus during incorporation studies, competition from the tissue culture cells would not be a problem. It has also been possible to remove these infective trypanosomes from the RPMI on day 3 and place them in the F-13 medium also at 25°C. The trypanosomes then transform to the procyclic trypomastigotes which are the forms found in the midgut of the tsetse fly. Thus at 25°C, it has been possible to obtain both forms of trypanosomes.

Our efforts to develop long-term infective forms in culture are continuing. These cells must be maintained in RPMI medium at 37°C over "seed" tissue culture cells (14). We have been attempting to grow the trypanosomes on three different tissue culture cells:

a) Buffalo lung cells (CCL 40)

b) Chinese hamster lung cells (CCL 16)

c) Steer fibroblast cells from peripheral blood. These cells were obtained from Hirumi et al. (1).

We now have the tissue cells required in large cultures and culture experiments are now beginning. Earlier experiments with tissue culture cells which grow rapidly (e.g., MDBK cells or CHO cells) were not successful as the tissue culture cells probably removed most nutrients from the RPMI media or perhaps produced products that made it difficult for the trypanosomes to develop. The slow growing lines that we are now using should be better for these experiments. Preliminary results show growth of T. brucei on the buffalo lung cells (CCL 40) and the Chinese hamster lung cells (CCL 16). With the buffalo lung cells, the trypanosomes grow close to the tissue culture cells. With the Chinese hamster cells, the trypanosomes remain in the medium above the tissue culture cells. The medium in both cases is RPMI 1640 plus 20% fetal calf serum.

We wish to establish all three forms of T. brucei in our laboratory so that we can distinguish them biochemically and observe the effects of trypanocidal drugs on these forms. The three forms of T. brucei include:

a) bloodstream trypomastigote forms;

b) cultured procyclic trypomastigote forms;

c) cultured bloodstream trypomastigote forms.

The first two are clearly established. Cultured procyclic trypomastigotes are growing well in the F-13 medium (Table II). The cultured infective bloodstream trypomastigotes must now be established.

B. Effects of trypanocidal drugs on synthesis of macromolecules

The identification of the RPMI medium as an excellent medium for incubation of bloodstream trypanosomes, as noted in the previous section, has provided us an environment for measuring DNA, RNA and protein synthesis in T. brucei.

Thus, we have made progress in looking at the effects of trypanocidal drugs on DNA, RNA and proteins in T. brucei. In order to study these activities in vitro, we have had to determine conditions for the trypanosomes under which they incorporate labeled precursors at a significant rate. The experiments have been done with both cultured procyclic trypomastigotes and bloodstream trypomastigotes of T. brucei. Once the proper conditions for incorporation have been determined, the effects of trypanocidal drugs on these processes can be studied.

The experiments involve growth of the procyclic trypomastigote forms in culture in the F-13 medium (Table II) or the obtaining of bloodstream trypomastigote forms from infected rats. The trypanosomes are collected and purified (2), and placed in an incubation medium at 25°C or 37°C. Labeled precursors are added and samples are taken at specified time intervals. We used ^{3}H-uridine for RNA labeling, ^{3}H-leucine for protein labeling and ^{3}H-thymidine for DNA labeling. These samples are placed in cold TCA and filtered to retain the precipitate on the filter. The filters are then placed in a liquid scintillation fluid and counted.

In the initial experiments low concentrations of cells were used. Bloodstream trypomastigotes were at a concentration of 1×10^8 cells/ml and procyclic trypomastigotes were at a concentration of 2×10^7 cells/ml. Then the techniques of growing the cells in culture improved and the technique of recovering the cells from the blood became more refined. This enabled us to increase the concentration of the cells. In later experiments, bloodstream trypomastigotes have been at a concentration of 2×10^8 cells/ml and procyclic trypomastigotes are at a concentration of 5×10^7 cells/ml.

Initially, we had to determine the proper incubation medium and temperature for the trypanosomes. The two media tested were:

a) RPMI 1640 plus 5% fetal calf serum;

b) F-13 plus 5% fetal calf serum (Table II).

These media are commercially available from GIBCO and have been used for growing the trypanosomes in vitro (2).

As shown in figure 2, ^{3}H-leucine incorporation by bloodstream trypomastigotes of T. brucei 110 occurs optimally in RPMI at 37°C. If the cells are incubated at the lower temperature, incorporation decreases after 10-20 minutes. Incorporation of bloodstream forms in the F-13 medium at either temperature is significantly less than the results obtained with RPMI. Incorporation of ^{3}H-leucine by procyclic trypomastigotes occurred optimally in 25°C in the F-13 medium (Figure 3). Many times when these forms were placed in the RPMI medium, the medium became acidic during the incubation and the trypanosomes were killed, particularly at 37°C.

The incorporation of ^{3}H-uridine by the bloodstream trypomastigotes was greater in RPMI at 37°C (Figure 4). The results indicated that uridine

incorporation is more significantly affected by lowering the temperature to 25°C than leucine incorporation. As noted in figure 5, ^{3}H-uridine incorporation by culture forms occurs more satisfactorily in the F-13 medium at 25°C.

From numerous experiments like those discussed, it was concluded that T. brucei bloodstream trypomastigotes incorporated precursors optimally in the RPMI medium at 37°C. On the other hand, the cultured procyclic trypomastigotes incorporated precursors optimally in the F-13 medium at 25°C. Further experiments were done under these conditions.

After running numerous incorporations on T. brucei using ^{3}H-uridine, ^{3}H-leucine and ^{3}H-thymidine, typical curves emerged. With bloodstream trypomastigotes, as seen in figure 6, with ^{3}H-uridine incorporation there is an initial lag period lasting 5 - 10 minutes. Then the uptake of uridine becomes linear at a high rate reaching about 25,000 cpm. ^{3}H-leucine incorporation has no lag period and remains linear throughout the 60 minutes, generally reaching no more than 4000 cpm. ^{3}H-thymidine incorporation has an initial lag period of approximately 50 minutes after the beginning of the experiment, at which time it begins to level off and incorporation is minimal. This may be due to the activity of a thymidine phosphorylase present in the trypanosomes (16).

Typical curves for T. brucei procyclic trypomastigotes were also obtained. These curves are shown in figure 7. As with bloodstream forms, certain patterns became apparent:

a) The incorporation of ^{3}H-uridine has an extended lag period, which has two parts, the first 0 to 20 minutes when incorporation is at a slow but steady rate, and the second 20 to 40 minutes where there is a transition to an increasing rate of incorporation.

The incorporation becomes linear and quite rapid with a maximum of about 6500 cpm;

b) As with bloodstream trypomastigotes, the incorporation approached 5000 cpm,

c) ^{3}H-thymidine incorporation in culture forms appears to be low. It is linear, and does not seem to level out at the end as does thymidine incorporation in bloodstream trypomastigotes. At its maximum, ^{3}H-thymidine incorporation approaches 1000 cpm.

In order to determine the optimal age of cells to be used ^{3}H-leucine incorporation was studied with cells from different growth periods. As shown in figure 8, greater incorporation occurred with younger cells. Cells that are three days old appear to be most satisfactory. These could be due to the fact that they are in the mid-log phase of growth. After three days, it is also possible to isolate from 300 mls of inocluated media, 1.5 - 2.0 x 10^{9} cells which is sufficient for several incorporation experiments. After 4 days in culture, the cells are quite inactive as far as incorporating leucine is concerned.

The effect of berenil on RNA synthesis in T. brucei bloodstream trypomastigotes was determined (figure 9). Three different concentrations of berenil were used (e.g., 2.5, 5.0 and 10.0 µg/ml). Although the experiment presented in figure 9 was done at 10 µg/ml, essentially the same results were obtained at the lower concentrations of berenil.

In these experiments, the basic procedure was that at plus 30 minutes, 5 µl of 1M uridine (unlabeled) is added as a cold chase to one flask. In order to see the effect of berenil alone, 5 µl of berenil solution is added to second flask. To a third flask is added 5 µl of 1M uridine (unlabeled)

and 5 µl of berenil. A fourth flask serves as a control and only receives buffer. These experiments indicate that bloodstream trypomastigotes are sensitive to low concentrations of berenil. The results of the berenil and cold chase together indicate that berenil may not only stop incorporation but also stimulate degradation of some of the stable RNAs present.

These experiments suggest that ideal conditions for bloodstream and procyclic trypomastigotes may differ markedly. However, we have been able to identify conditions under which we can measure DNA, RNA and protein synthesis. We have also been able to extend these studies to a characterization of the effects of berenil on RNA synthesis. We can now continue these studies on the effects of berenil on DNA synthesis as well as look at the effects of other potential trypanocidal drugs.

C. Structure of *Leptomonas* sp. kinetoplast DNA

During the last several months, our efforts in characterizing the kinetoplast DNA (k-DNA) by isopycnic gradient centrifugation has been quite successful. Also, during this period, we have completed our work on the thermal chromatography of this k-DNA, the results of which were reported at the Annual Meeting of the American Society for Microbiology in May of this year.

Figure 10 shows the final results of the thermal chromatography of Leptomonas sp. k-DNA and our purified standards of Crithidia acanthocephali kDNA, ϕ-29 (bacteriophage) DNA and lambda (bacteriophage) DNA on hydroxylapatite. Sonicated k-DNA networks or isolated subunit fractions in 0.12 M NAP buffer were eluted stepwise from the column by increasing the temperature of the column and buffer prior to each step. Using the ϕ-29 and lambda

DNA's as standards and the Crithidia acanthocephali k-DNA as control, the Tm of the Leptomonas sp. k-DNA was determined to be equivalent to that of the lambda DNA, i.e., 89°C - 90°C.

The results of our determinations of the bouyant densities of the Leptomonas sp. kinetoplast and nuclear DNA in CsCl using the Model E, Analytical Ultracentrifuge (Beckman Instruments, Inc.) are presented in figures 11-13. Figure 11 is a reproduction of the densitometer tracing with purified (A) Leptomonas sp. k-DNA and marker Micrococcus lysodeikticus DNA and (B) lambda DNA and Micrococcus lysodeikticus DNA.

Figure 12 shows the same components as are in (A) and (B) of Figure 11 with the addition of purified Leptomonas sp. nuclear DNA. Figure 13 indicates the results of a mixing experiment. An increase in the peak height of the k-DNA peak occurs on the addition of lambda DNA (B) suggesting a bouyant density equivalent to that of the lambda DNA. All of these determinations have been repeated several times with different preparations of Leptomonas sp. k-DNA using single-cell runs in the Model E with a counter-balance in place. Similar results were obtained in each case. The conclusions of these experiments with Leptomonas sp. k-DNA are presented in table III.

Two sets of experiments are now in progress to complete this work on the Leptomonas sp. kDNA.

a) Tm_i determination on the sonicated networks of the purified k-DNA will further establish the % G+C of the RNA.

b) Contour length measurements of the isolates minicircle will be used to determine the unit molecular weight of these structures.

The intact networks of purified Leptomonas sp. kDNA have been digested by restriction endonucleases and the products of the separate limit digests

visualized by agarose and polyacrylamide gel electrophoresis. The component circular kDNA molecules were shown to be comprised of at least three different types based on the number of fragments produced and their related molecular weights. Digestion by Eco RI, Hind II, Hind III, Bgl II and Alu I endonucleases generated single fragments of about 5.6×10^5 daltons. No pieces of greater molecular weight were made by these enzymes. However, Hae II and Bam HI digestion products were all of greater molecular weight than the above. Only about 15% of the intact networks were hydrolyzed by these restriction endonucleases even after exhaustive treatment. In contrast, digestion by Hpa I, Hpa II, and Hae III yielded fragments with molecular weights less than the unit length linear molecule. Almost complete digestion of the intact associations occurred when these three enzymes were used. Five major fragments were produced.

The Leptomonas sp. kDNA that we have used has been purified from cells harvested at late log or stationary phase of growth. Figure 14 is a photograph of Leptomonas sp. kDNA purified from late log phase. The DNA has attained equilibrium during centrifugation in CsCl containing 300 μg/ml of ethidium bromide. The lower band consists of complete networks of kDNA that contain minicircles that are covalently closed. The middle band consists also of complete networks, but the majority of minicircles are nicked. The uppermost band has yet to be characterized, but it is expected that it will be mostly linear molecules of minicircle unit length with possible fragments of nuclear DNA. Figure 15 shows the same type of kDNA preparation, but DNA was initially purified from cells grown to stationary phase. As can be seen, no upper band, presumably linear molecules, are present. The upper band in figure 14 could be replicating intermediates of the kDNA, as DNA synthesis has stopped by the time the cells have reached

stationary phase. Further work is being done to determine the source and nature of the upper band.

The purpose of these studies has been to characterize the kDNA present in Leptomonas sp. and to develop techniques appropriate for the analysis of the kDNA of Trypanosoma brucei. This has been desired in order to identify if the kDNA is template for RNA transcripts. At present, we plan on continuing these studies but with T. brucei since it can now be obtained in appropriate quantities.

D. Effect of trypanocidal drugs on function of electron transport system in trypanosomes

We have studied the steady state oxygen kinetics of bloodstream T. rhodesiense. Low oxygen concentration gradients (e.g., 0 - 0.5 μM O_2) have been employed for determining the apparent K_m for oxygen for the α-GP oxidase present (17,18). As seen in figure 16, the respiration of intact cells T. rhodesiense was stimulated for glucose and DL-α-GP. In this open electrode system, an increase in respiration is indicated by a decrease in the oxygen tension in the cuvette. The intact cell respiration is inhibited only slightly (0-10%) by CO or azide. However, it is markedly inhibited by salicylhydroxamic acid (SHAM), a known inhibitor of cyanide-insensitive respiration (19-21). In studies to determine the affinity of the α-GP oxidase for oxygen, we have observed that the intact cells have a K_m of 2.0 - 8.0 μM O_2.

However, more detailed information can be obtained using a particulate enzyme preparation from bloodstream forms of T. rhodesiense. In the presence of α-GP and defatted bovine serum albumin (BSA), the α-GP oxidase in T.

rhodesiense has an apparent K_m = 2.1 ± 0.5 μM O_2 (Figure 17). In the presence of BSA, the oxidase activity of K_m was increased 4-6 fold (Figure 18). The reciprocal plots in the presence and absence of BSA are parallel, demonstrating an activation of the oxidase by BSA.

As seen in figure 17, the α-GP oxidase is not inhibited by CO. However, we have identified the mode of inhibition of inhibitors of the α-GP oxidase including SHAM, diphenylamine, o-phenanthroline, α,α' dipyridyl, suramin and o-hydroxydiphenyl. The most effective inhibitor was SHAM. As can be seen in figure 19, the inhibition by SHAM is noncompetitive with respect to oxygen with a K_i = 5.4 ± 0.4 μM. The K_i and type of inhibition for the other inhibitors are given in table IV. The trypanocidal drug suramin is an effective inhibitor of the α-GP oxidase (Figure 20). As noted in table IV, it is an uncompetitive inhibitor of the α-GP oxidase with respect to O_2. Bowman and Fairlamb (19) have provided evidence that suramin is a competitive inhibitor of the α-GP oxidase with respect to L-α-GP, the K_i being 4.1 μM. As seen in figure 21, the K_i for o-phenanthroline is 1.0 mM.

Procyclic Trypomastigotes

In contrast to the bloodstream forms, early studies by several investigators demonstrated that procyclic trypomastigotes had cyanide-sensitive respiration, suggesting the presence of cytochrome aa_3. Spectral examination of T. gambiense (23), T. rhodesiense (24,25) and T. brucei (15) have provided evidence for cytochrome aa_3 in both CO-difference spectra and absolute spectra of procyclic trypomastigotes in T. brucei. Thus, the cyanide and azide sensitivity in these cells can be ascribed to the presence of cytochrome aa_3. However, no action spectral evidence for cytochrome aa_3 has been reported for pathogenic trypanosomes.

Studies with the open electrode system with procyclic trypomastigotes have provided evidence for additional terminal oxidases. In figure 22, several steady states are evident in the presence of various respiratory inhibitors. On the addition of 0.5 mM SHAM, 20-25% inhibition of cellular respiration occurs. In addition, 4.5 mM azide inhibits 50-60% of the respiration. The respiration inhibited by SHAM or azide is not sensitive to azide or SHAM respectively. However, as seen in Figure 22, 15-20% of the cell respiration is insensitive to both high concentrations of SHAM and azide.

These results provide strong evidence for three terminal oxidases functioning in these organisms:

a) A SHAM-sensitive, azide-insensitive oxidase supporting 15-20% of the respiration in the cells;

b) An azide-sensitive, SHAM-insensitive oxidase supporting the majority (50-60%) of the cell respiration. This oxidase is inhibited by CO with a K_i = 0.3 μM CO with respect to oxygen;

c) An azide and SHAM-insensitive oxidase which supports 15-20% of the cellular respiration. It is also inhibited by CO with K_i = 0.7 μM CO with respect to oxygen.

In figures 23 and 24, one can observe the steady states that are present on the addition of L-α-GP or succinate to particulate preparations of T. brucei. This organism also has three oxidases evident in steady state experiments (15).

Three important points can be noted:

a) All three steady state levels are evident with either substrate. This provides strong evidence that the SHAM-sensitive pathway is not specific in these procyclic trypomastigotes for L-α-GP;

b) A greater percentage of SHAM-sensitive activity occurs with L-α-GP as substrate. With L-α-GP as substrate, the steady state level was reduced 48% in the presence of SHAM. However, with succinate as the substrate, the steady state level was reduced 21%;

c) In the presence of L-α-GP, BSA stimulates the α-GP oxidase activity (Figure 23). This is similar to the response of the α-GP oxidase found in the bloodstream trypomastigotes.

The steady state oxygen kinetics of procyclic trypomastigotes have provided clear evidence that the affinity for oxygen for trypanosomes found in the midgut of the tsetse fly is high. Reciprocal plots of the procyclic trypomastigotes of T. rhodesiense reveal an apparent $K_m = 0.1 \pm 0.02$ μM O_2 (Figure 25). The multiphasic nature of the curves suggests that several terminal oxidases are functioning.

The significance of these results in the electron transport system in T. brucei and T. rhodesiense have been extensively discussed in the manuscript that we have recently submitted to The Journal of Biological Chemistry. A preprint is included in this renewal application.

During the transformation of African trypanosomes from bloodstream trypomastigotes forms to procyclic trypomastigote forms, oxidases with high affinities for oxygen are synthesized. With the oxygen tension extremely low in the midgut of the tsetse fly, the synthesis in the trypanosome of

oxidases with high affinities for oxygen may be essential for survival in the tsetse fly. Our present efforts are directed to studying factors which regulate the synthesis and function of these oxidases during the life cycle of trypanosomes.

The development of the α-GP oxidase during the transformation of the metacyclic trypomastigotes back to the bloodstream slender trypomastigotes needs to be investigated. No biochemical evidence exists on the electron transport system present in the metacyclic trypomastigotes. However, it is clear these trypanosomes are infectious to vertebrates and in most African trypanosomes have begun to produce the variant surface antigen present in the bloodstream forms. Further biochemical studies on these forms may provide evidence useful in developing a rational approach to the chemotherapy of this serious disease.

A more detailed description and discussion of the electron transport system in trypanosomes can be found in a recent review prepared by the principal investigator (26).

CONCLUSIONS

Our primary results during this last year:

a. Determined optimal conditions for measuring DNA, RNA and protein synthesis in bloodstream and procyclic trypomastigotes;

b. Observed that under optimal conditions, berenil does inhibit RNA synthesis in trypanosomes and increases the rate of degradation of RNA;

c. Characterized Leptomonas sp. K-DNA and developed techniques appropriate for T. brucei;

d. Maintained T. brucei for several days at 25°C;

e. Initiated cultures of T. brucei infective bloodstream trypomastigotes on Buffalo lung cells and Chinese hamster lung cells;

f. Identified the electron transport systems present in T. rhodesiense (15);

g. Demonstrated that suramin and five other drugs inhibit the L-α-glycerophosphate oxidase (15).

RECOMMENDATIONS

Our main recommendation would be to provide increased support to the development of a culture system for infective forms of bloodstream trypanosomes, such as T. rhodesiense and T. brucei. Progress in this area would provide a model system for the testing of trypanocidal drugs. In addition, a system for studying the differentiation of trypanosomes and the process of antigenic variation would be available.

The development of this in vitro culture system in order to study basic mechanisms of African trypanosomes under stringently controlled conditions is of paramount importance today. An outstanding characteristic of all pathogenic flagellates is the complexity of their life cycles. The changes that occur during their development must all result, directly or indirectly, from changes in gene activity. However, as in other differentiating systems, we know little of the mechanisms of gene repression and depression which give rise to parasites able to respond to changing environmental stimuli. Such knowledge should be the goal of the future research in this area. It may well provide the basis for a more rational approach to chemotherapy.

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Table I

Change in Cell Number of T. brucei in RPMI Medium at 25°C

Hours in Culture	Count in Flask (cells/ml x 10^6)	
0	1.0[a]	1.6[a]
24	1.1	1.7
48	0.9	1.9
72	1.1	2.0
96	0.8	1.8

[a] T. brucei was inoculated into flasks at this initial cell concentration. The cells were counted daily. The medium in which the cells were added was RPMI 1640 plus 5% fetal calf serum.

Table II

Modified Minimal Essential Medium

for Hemoflagellates (F-13)

	Per Liter
Minimum Essential Medium (Joklik-modified) cat. no. F-13 with: L-Glutamine, Antibiotics and $NaHCO_3$ without: Calcium Chloride (GIBCO)	13.4 gm
MEM Amino Acids 50X (GIBCO)	10.0 ml
MEM Non-Essential Amino Acids 100X (GIBCO)	10.0 ml
Na-pyruvate 100X (GIBCO)	11.0 ml
Biotin	0.1 mg
Hemin solution (1 mg/ml in pyridine)	6.0 ml
Fetal Calf Serum (GIBCO)	50.0 ml
HEPES*	6.0 gm
L-Proline	1.7 gm

pH to 7.2-7.4 and filter sterilize

*Final concentration of buffer = 0.025M

Table III

Leptomonas sp. Kinetoplast DNA

Tm	90°C
ρ CsCl	1.708 gm/ml
G + C	48 - 49%

Table IV

Inhibitors of L-α-Glycerophosphate Oxidase in *T. rhodesiense*

Inhibitor	K_i	Type of Inhibition with Respect to O_2
Salicylhydroxamic acid	5.4 μM	noncompetitive
Suramin	9.0 μM	uncompetitive
o-Hydroxydiphenyl	0.3 mM	uncompetitive
o-Phenanthroline	1.0 mM	noncompetitive
Diphenylamine	1.3 mM	uncompetitive
α, α'-dipyridyl	11.5 mM	uncompetitive

Table V

Effect of Suramin on Infection Of

Trypanosoma brucei

Cell Numbers

Hours after Treatment	Control	Suramin (5.0 mg/kg)	Suramin (10.0 mg/kg)
0	3.7×10^8	7.2×10^8	4.6×10^8
+2 hrs.	4.7×10^8	6.8×10^8	4.6×10^8
+5 hrs.	7.0×10^8	6.6×10^8	4.1×10^8
+19 hrs.	Dead	6.8×10^8	0
+26 hrs.		0	0

Figure Legends

Figure 1. Infectivity of trypanosomes cultured *in vitro* in RPMI medium plus 5% fetal calf serum for four days. Both cultured bloodstream trypanosomes and stabilates were inoculated at 1×10^6 cells/ml.

Figure 2. Results of incorporation studies with ^{3}H-leucine with bloodstream trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 1×10^8 cells/ml.

Figure 3. Results of incorporation studies with ^{3}H-leucine with procyclic trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 5×10^7 cells/ml.

Figure 4. Results of incorporation studies with ^{3}H-uridine with bloodstream trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 1×10^8 cells/ml.

Figure 5. Results of incorporation studies with ^{3}H-uridine with procyclic trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 2×10^7 cells/ml.

Figure 6. Results of incorporation studies with ^{3}H-uridine, ^{3}H-leucine and ^{3}H-thymidine with bloodstream trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 2×10^8 cells/ml.

Figure 7. Results of incorporation studies with ^{3}H-uridine, ^{3}H-leucine and ^{3}H-thymidine with procyclic trypomastigotes of *Trypanosoma brucei*. The concentration of cells was 5×10^7 cells/ml.

Figure 8. Effect of different ages of cells on ^{3}H-leucine incorporation with procyclic trypomastigotes of Trypanosoma brucei. The incubation was at 25°C in F-13 medium. The concentration of cells was 5×10^{7} cells/ml.

Figure 9. Effect of berenil on the incorporation of ^{3}H-uridine with blood-stream forms of Trypanosoma brucei. The concentration of cells was 1×10^{8} cells/ml.

Figure 10. Results of the thermal chromatography of Leptomonas sp. kinetoplast DNA and purified standards of Crithidia acanthocephali kinetoplast DNA, ϕ-29 (bacteriophage) DNA and lambda (bacteriophage) DNA on hydroxylapatite.

Figure 11. Densitometer tracing of purified A) Leptomonas sp. kinetoplast DNA and marker Micrococcus lysodeikticus DNA and B) lambda DNA and Micrococcus lysodeikticus DNA.

Figure 12. Densitometer tracing of the same DNAs as in Figure 12 with the addition of purified Leptomonas sp. nuclear DNA.

Figure 13. Densitometer tracing of a mixing experiment. Tracing A is of Leptomonas sp. kinetoplast DNA and marker Micrococcus lysodeckticus DNA. In tracing B, lambda DNA has been added.

Figure 14. Photograph of the results of a CsCl-ethidium bromide ultracentrifugation experiment with Leptomonas sp. kinetoplast DNA isolated from stationary phase cells.

Figure 15. Photograph of the results of a CsCl-ethidium bromide ultracentrifugation experiement with Leptomonas sp. kinetoplast DNA isolated from stationary phase cells.

Figure 16. Steady state oxygen trace of Trypanosoma rhodesiense bloodstream trypomastigotes (4.0×10^7 cells/ml).

Figure 17. Reciprocal plots of Trypanosoma rhodesiense α-GP oxidase against low concentrations of oxygen. The three curves (a, b and c) represent increasing concentrations of enzyme (4.0, 6.0 and 8.0 mg protein/ml respectively). The concentration of CO was 84 μM.

Figure 18. Reciprocal plot of Trypanosoma rhodesiense α-GP oxidase activity against low concentrations of oxygen in the absence and presence of defatted bovine serum albumin (BSA). Enzyme concentration is 4.0 protein/ml.

Figure 19. Dixon plot for SHAM inhibition of α-GP oxidase prepared from Trypanosoma rhodesiense.

Figure 20. Reciprocal plot of the α-GP oxidase activity in a particulate preparation from Trypanosoma rhodesiense against low concentrations of suramin.

Figure 21. Dixon plot for o-phenanthroline inhibition of α-GP oxidase prepared from Trypanosoma rhodesiense.

Figure 22. Steady state oxygen trace of Trypanosoma rhodesiense procyclic trypomastigotes (1.2×10^8 cells/ml).

Figure 23. Steady state oxygen trace of particulate preparation from Trypanosoma brucei procyclic trypomastigotes using DL-α-GP substrate. The protein concentration is 4.0 mg/ml.

Figure 24. Steady state oxygen trace of a particulate from Trypanosoma brucei procyclic trypomastigotes using succinate as substrate. The protein concentration is 4.0 mg/ml.

Figure 25. Reciprocal plot of procyclic trypomastigote forms of Trypanosoma rhodesiense DL-α-glycerophosphate oxidation against low concentrations of oxygen. The five curves (a,b,c,d and e) represent increasing concentrations of cells (3.0, 4.5, 6.0, 7.5 and 9.0×10^7 cells/ml).

Figure 26. Cooperation of mitochondrial and nuclear genes specifying the synthesis of an alternative oxidase (20).

FIGURE 1

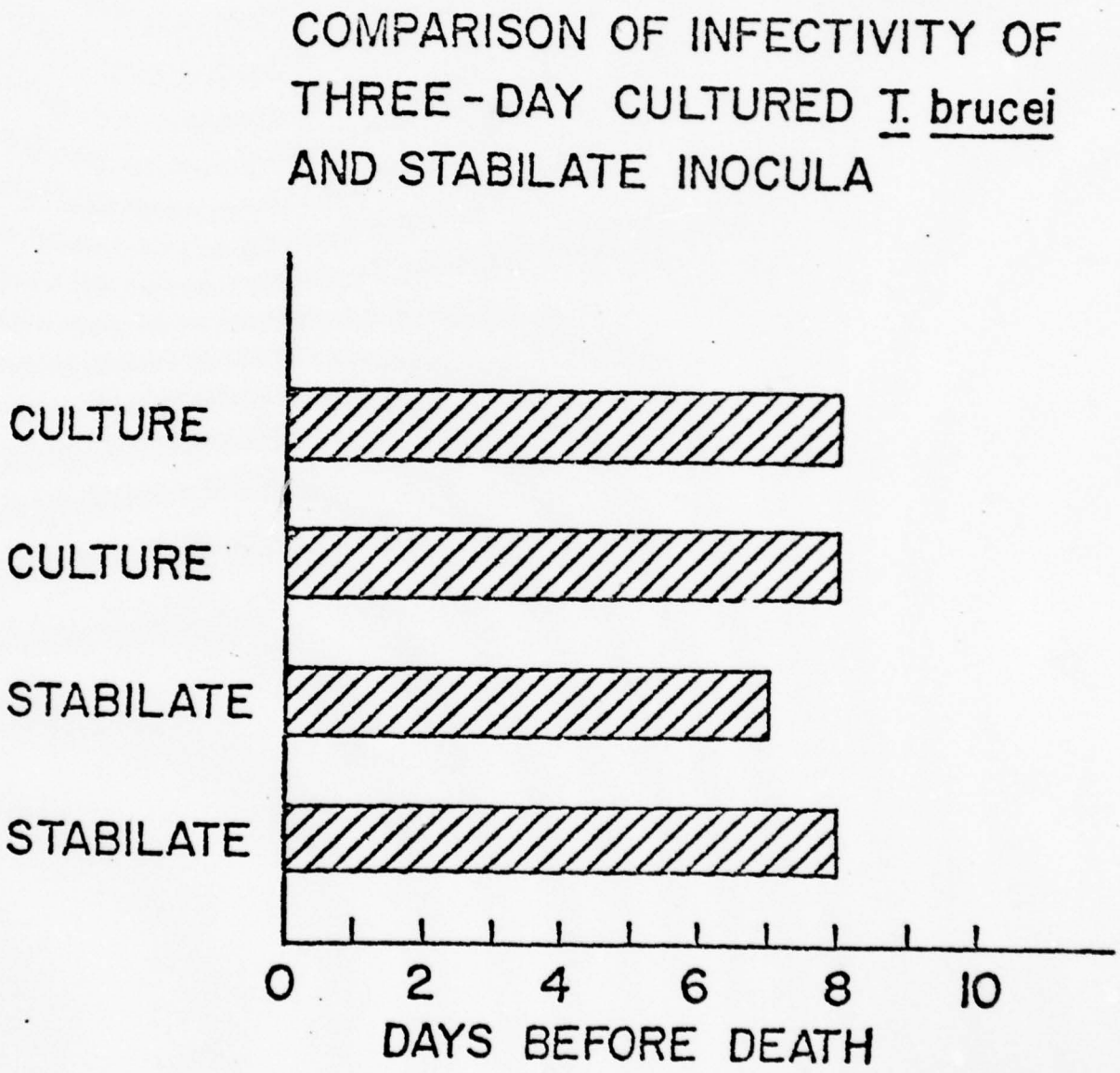

COMPARISON OF INFECTIVITY OF THREE-DAY CULTURED T. brucei AND STABILATE INOCULA
CULTURE
CULTURE
STABILATE
STABILATE
0
2
4
6
8
10
DAYS BEFORE DEATH

Figure 2

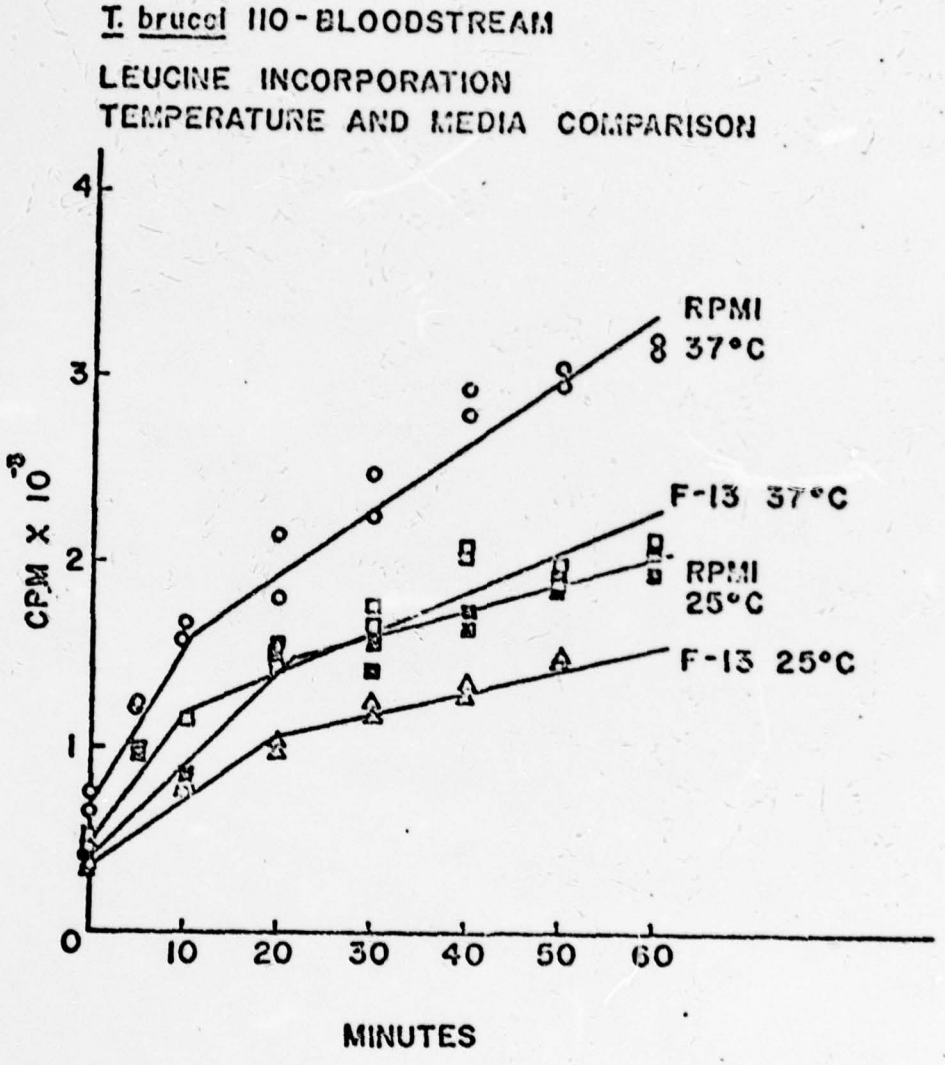

T. brucei 110-BLOODSTREAM
LEUCINE INCORPORATION
TEMPERATURE AND MEDIA COMPARISON
CPM X 10⁻³
0
1
2
3
4
10
20
30
40
50
60
MINUTES
RPMI 37°C
F-13 37°C
RPMI 25°C
F-13 25°C

Figure 3

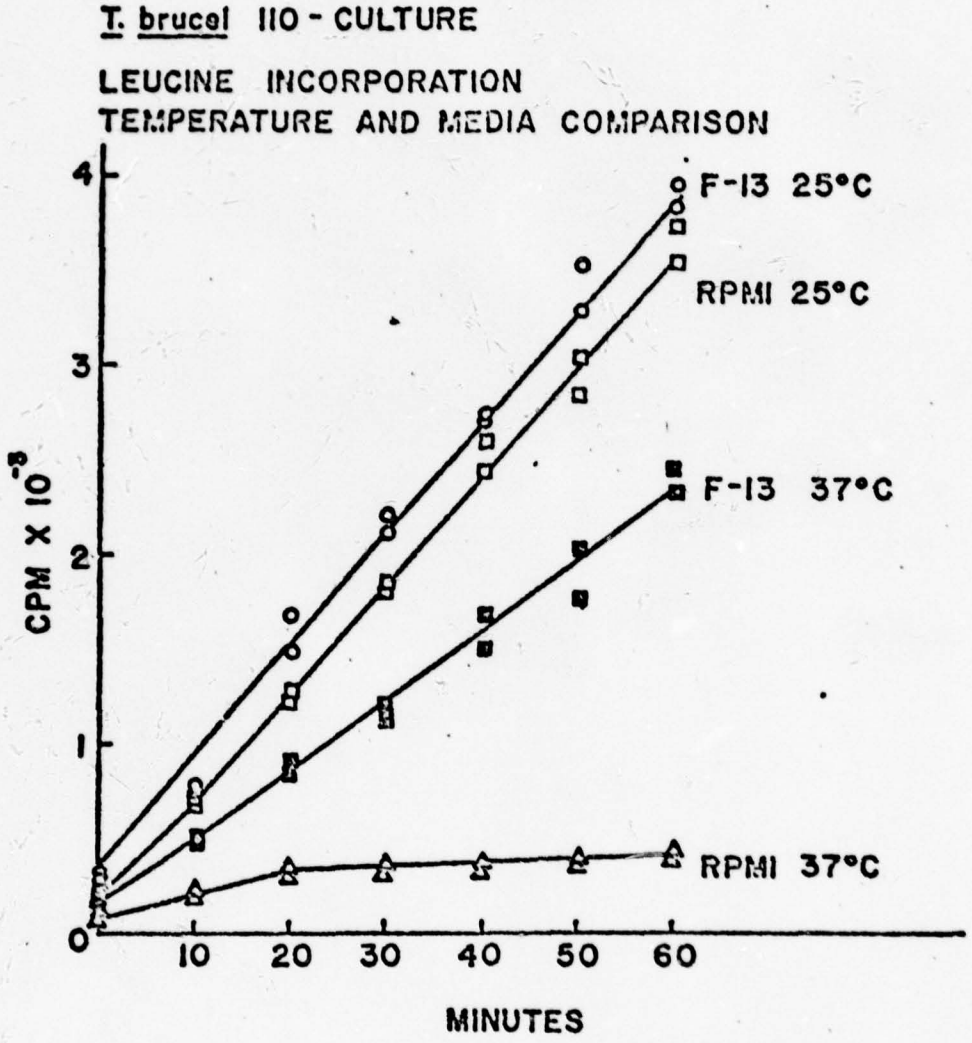

T. brucei 110 - CULTURE
LEUCINE INCORPORATION
TEMPERATURE AND MEDIA COMPARISON
F-13 25°C
RPMI 25°C
F-13 37°C
RPMI 37°C
CPM X 10⁻³
0
1
2
3
4
10
20
30
40
50
60
MINUTES

Figure 4

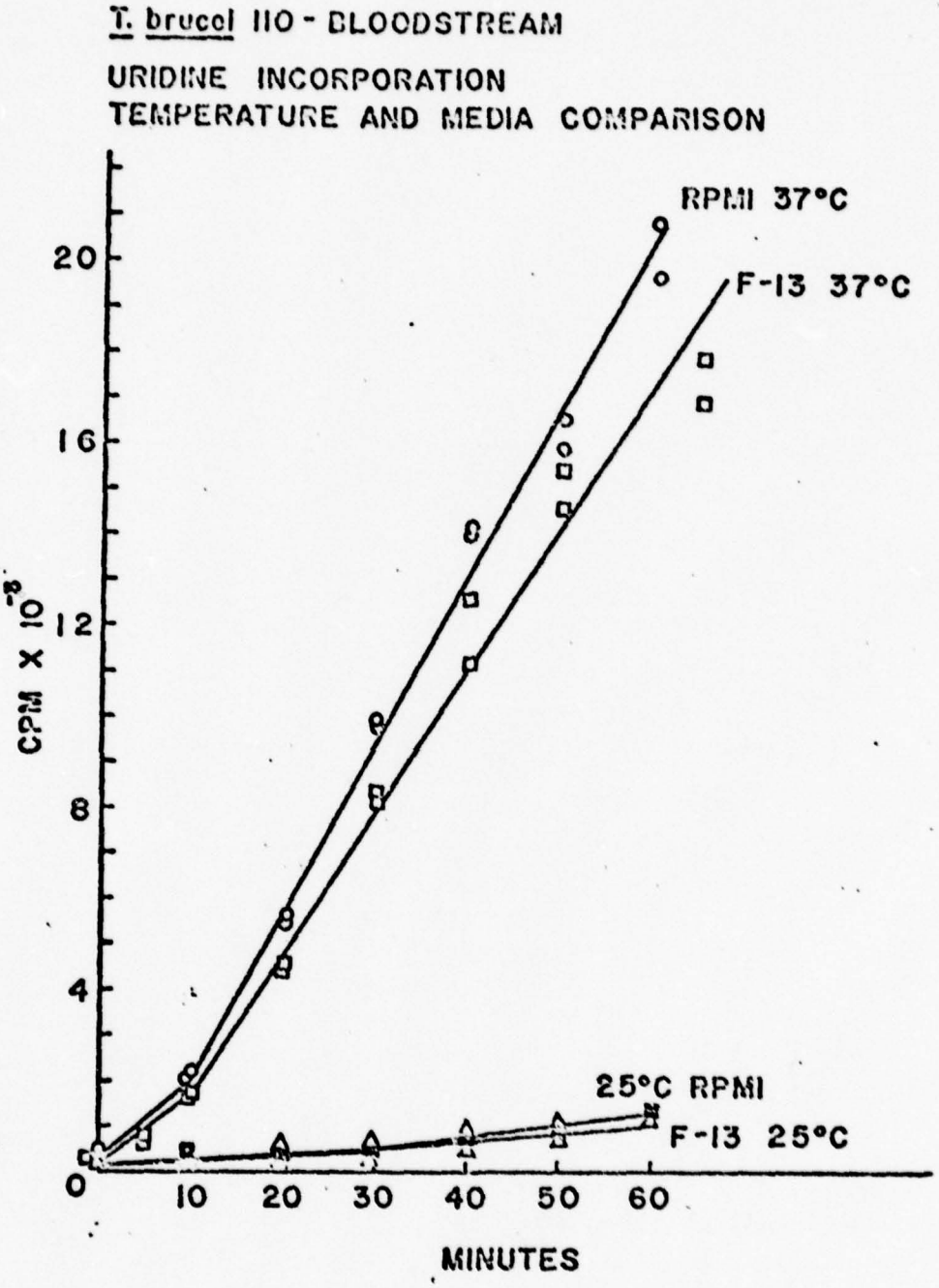

T. brucei 110 - BLOODSTREAM
URIDINE INCORPORATION
TEMPERATURE AND MEDIA COMPARISON
RPMI 37°C
F-13 37°C
25°C RPMI
F-13 25°C
CPM X 10⁻³
0
4
8
12
16
20
10
20
30
40
50
60
MINUTES

Figure 5

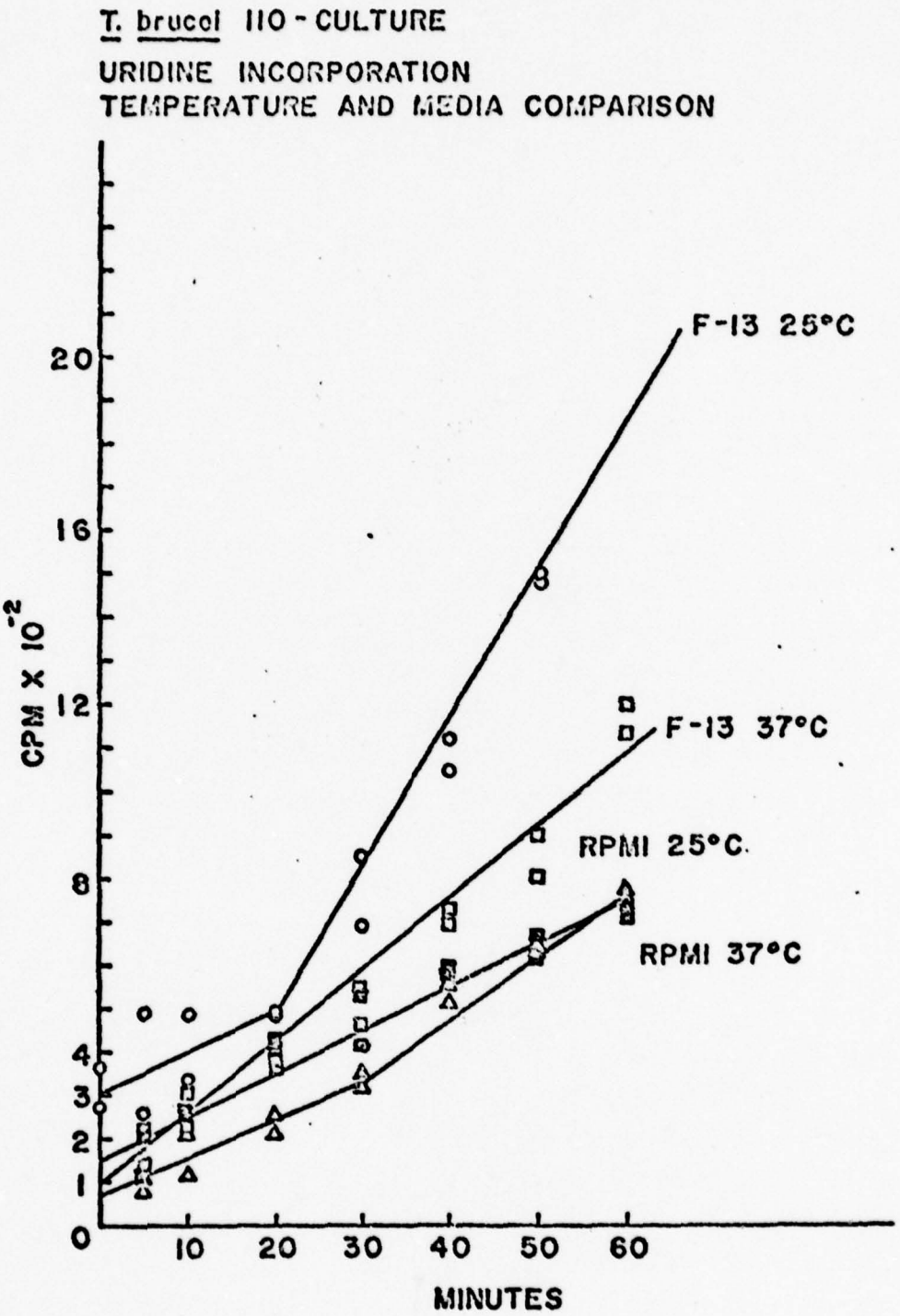

T. brucei 110 - CULTURE
URIDINE INCORPORATION
TEMPERATURE AND MEDIA COMPARISON
CPM X 10^{-2}
0
1
2
3
4
8
12
16
20
10
20
30
40
50
60
MINUTES
F-13 25°C
F-13 37°C
RPMI 25°C
RPMI 37°C

Figure 6

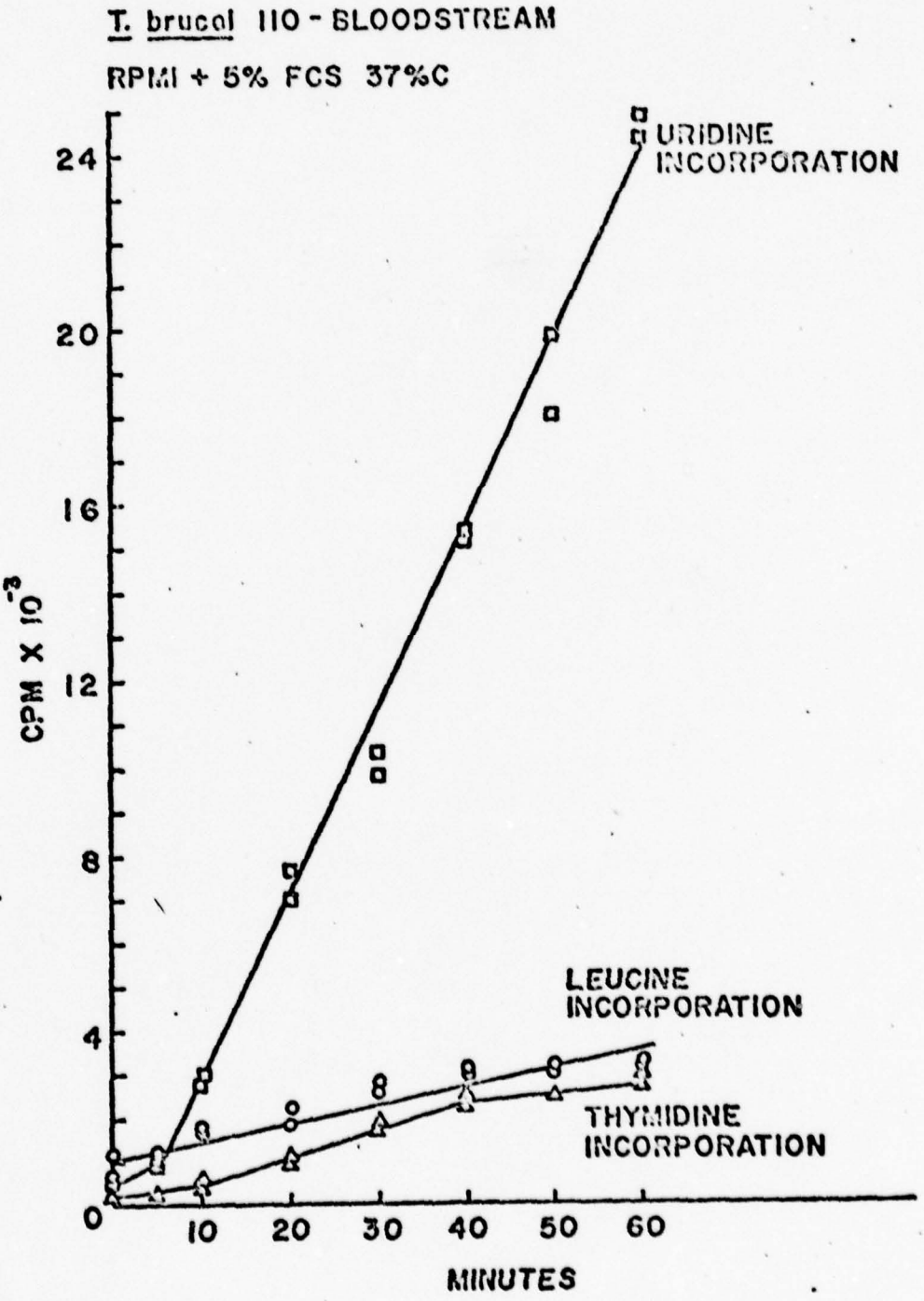

T. brucei 110 - BLOODSTREAM
RPMI + 5% FCS 37%C
URIDINE INCORPORATION
LEUCINE INCORPORATION
THYMIDINE INCORPORATION
CPM X 10⁻³
0
4
8
12
16
20
24
10
20
30
40
50
60
MINUTES

Figure 7

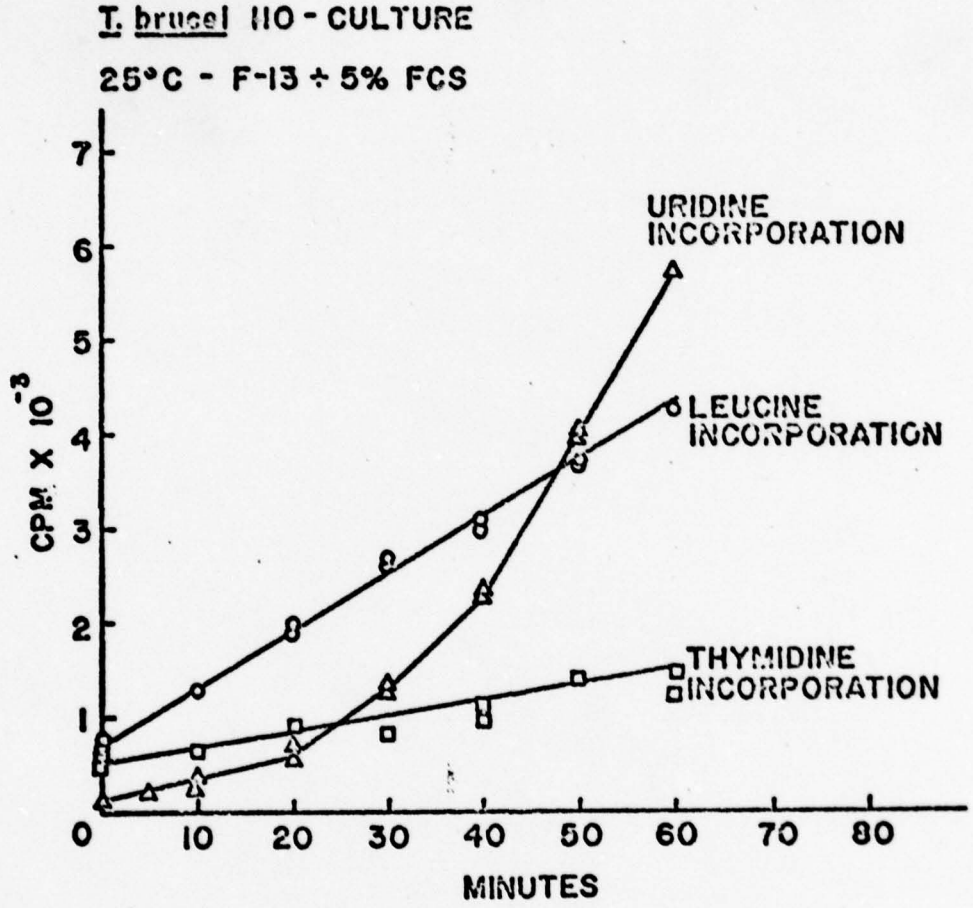

T. brucei 110 - CULTURE
25°C - F-13 + 5% FCS
CPM X 10^-3
0
1
2
3
4
5
6
7
10
20
30
40
50
60
70
80
MINUTES
URIDINE INCORPORATION
LEUCINE INCORPORATION
THYMIDINE INCORPORATION

Figure 8

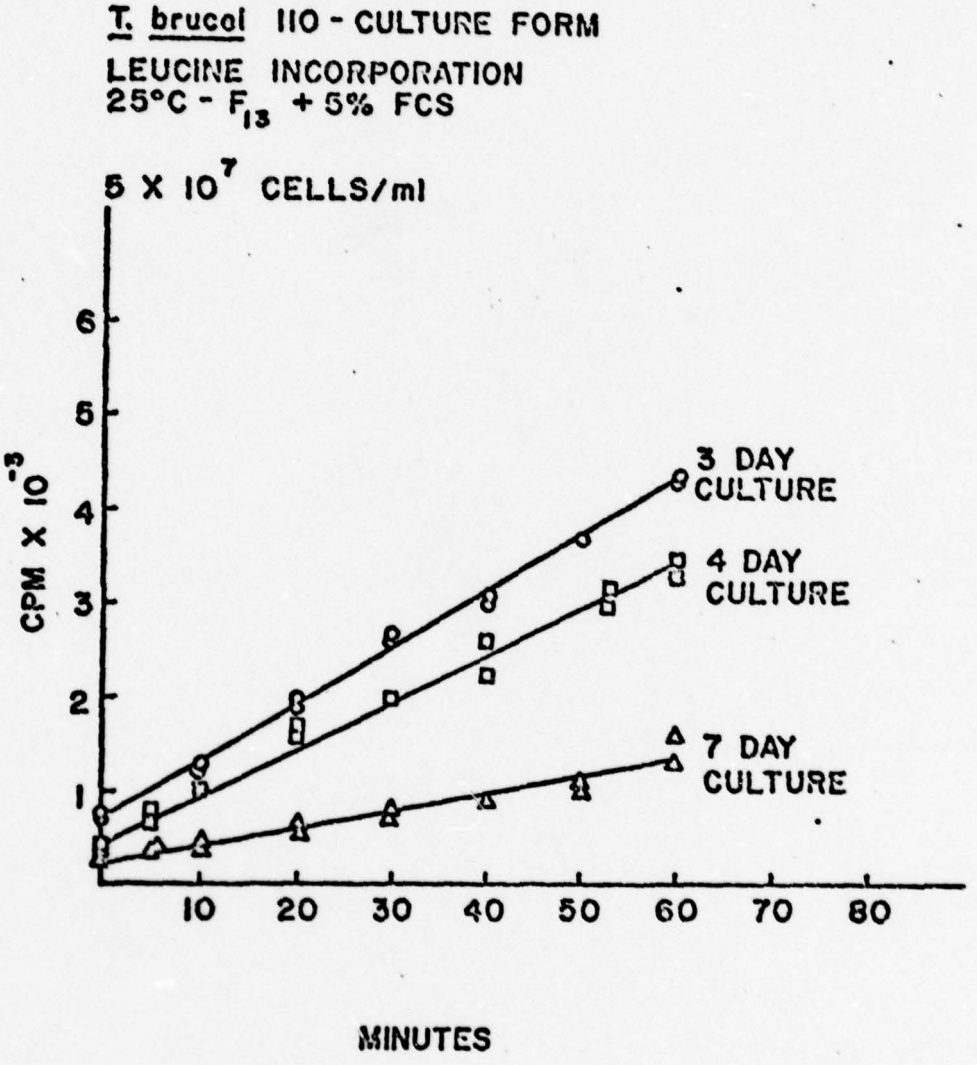
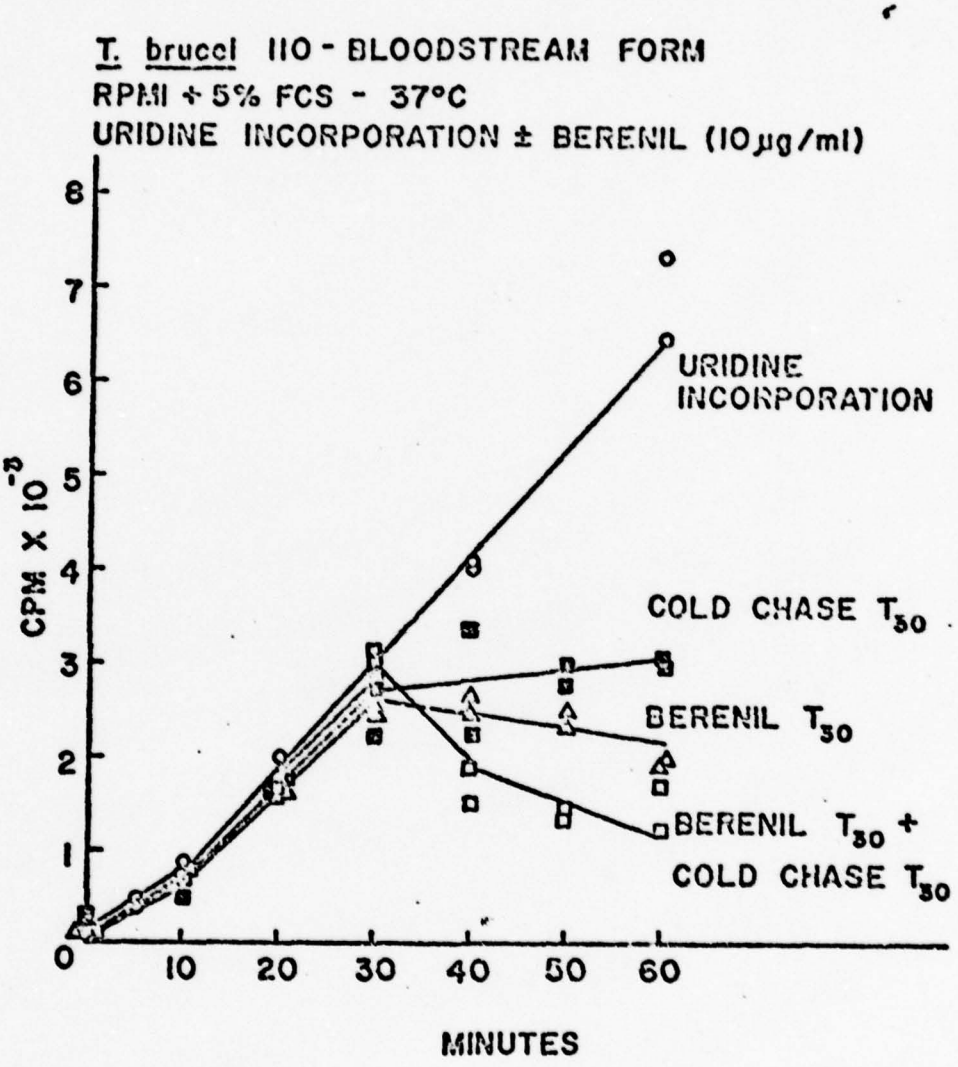

T. brucei 110 - CULTURE FORM
LEUCINE INCORPORATION
25°C - F13 + 5% FCS
5 X 10^7 CELLS/ml
CPM X 10^-3
6
5
4
3
2
1
3 DAY CULTURE
4 DAY CULTURE
7 DAY CULTURE
10
20
30
40
50
60
70
80
MINUTES

Figure 9


T. brucei 110 - BLOODSTREAM FORM
RPMI + 5% FCS - 37°C
URIDINE INCORPORATION ± BERENIL (10µg/ml)
CPM X 10⁻³
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
MINUTES
URIDINE INCORPORATION
COLD CHASE T50
BERENIL T50
BERENIL T50 +
COLD CHASE T50

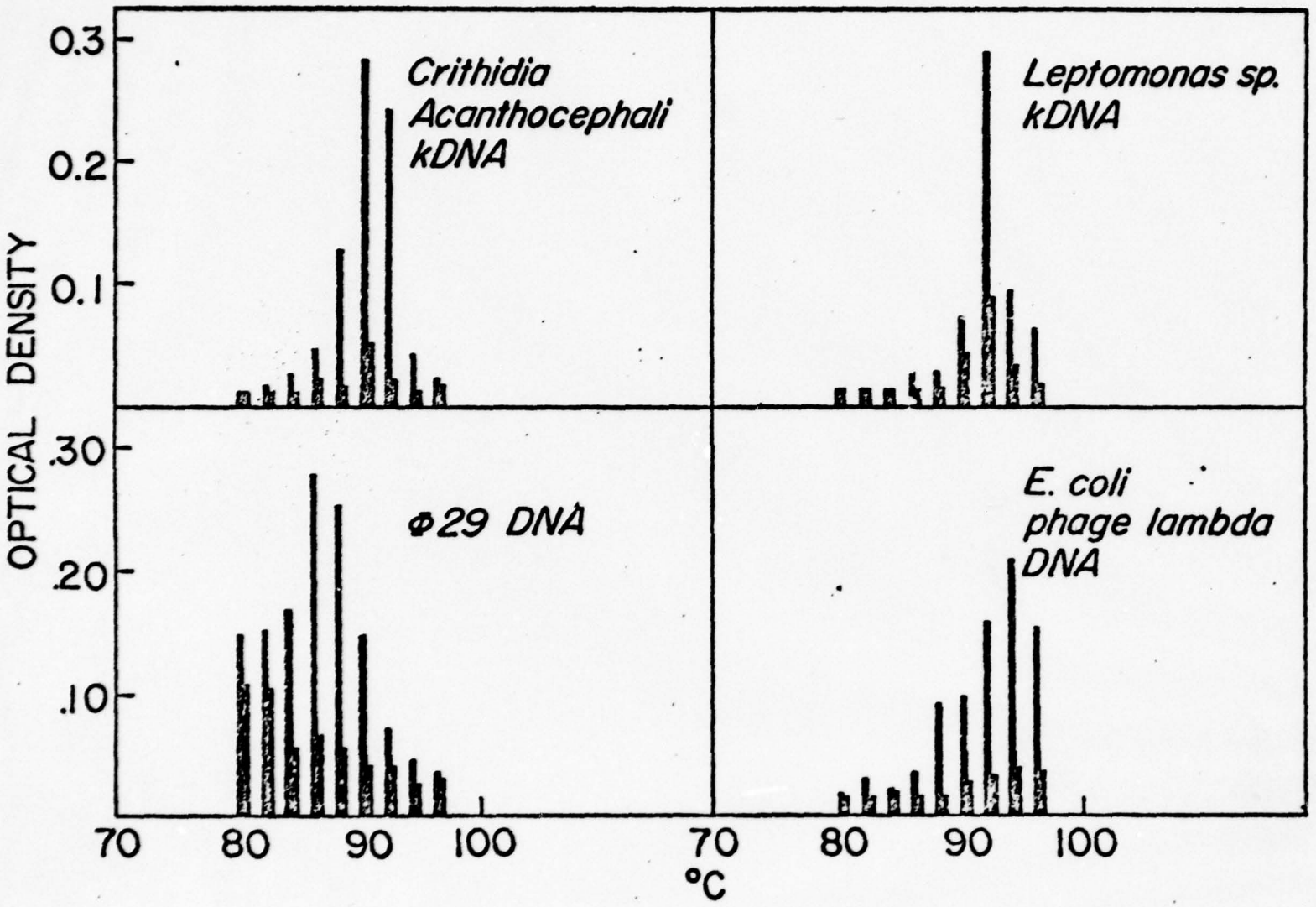

OPTICAL DENSITY
0.3
0.2
0.1
.30
.20
.10
Crithidia Acanthocephali kDNA
Leptomonas sp. kDNA
Φ29 DNA
E. coli phage lambda DNA
70
80
90
100
°C

Figure 10

A

B

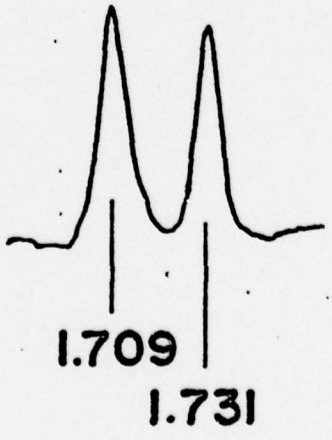
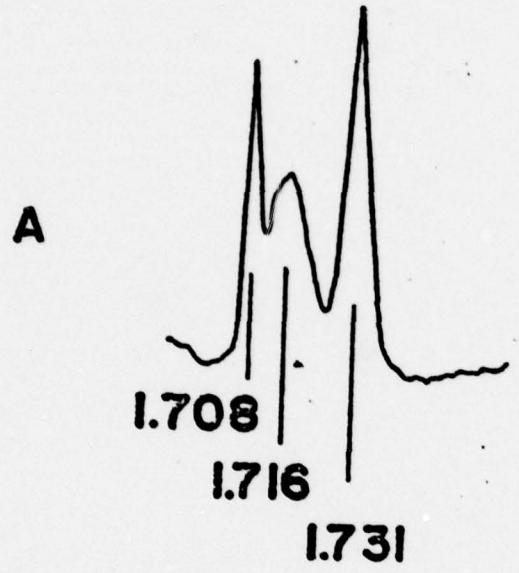
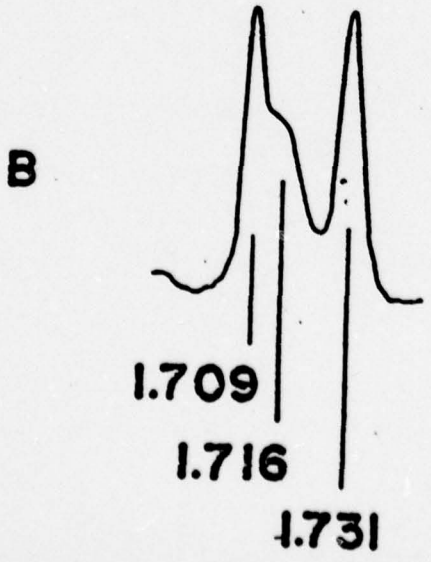
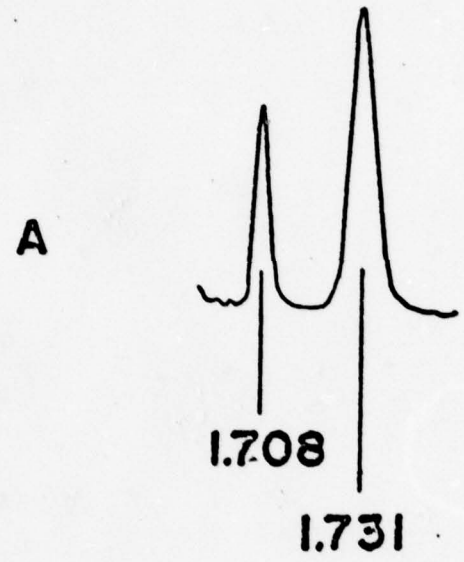


Figure 12






A
1.708
1.731

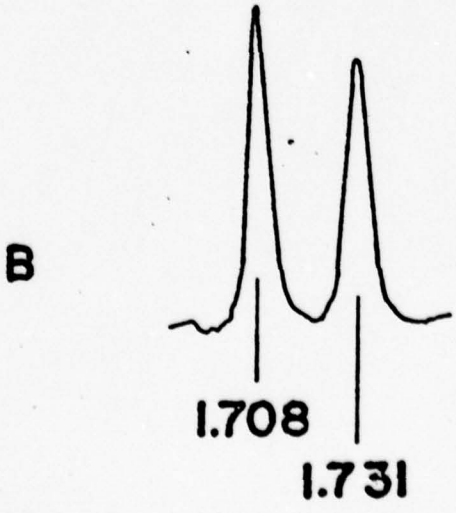

B
1.708
1.731

FIGURE 14

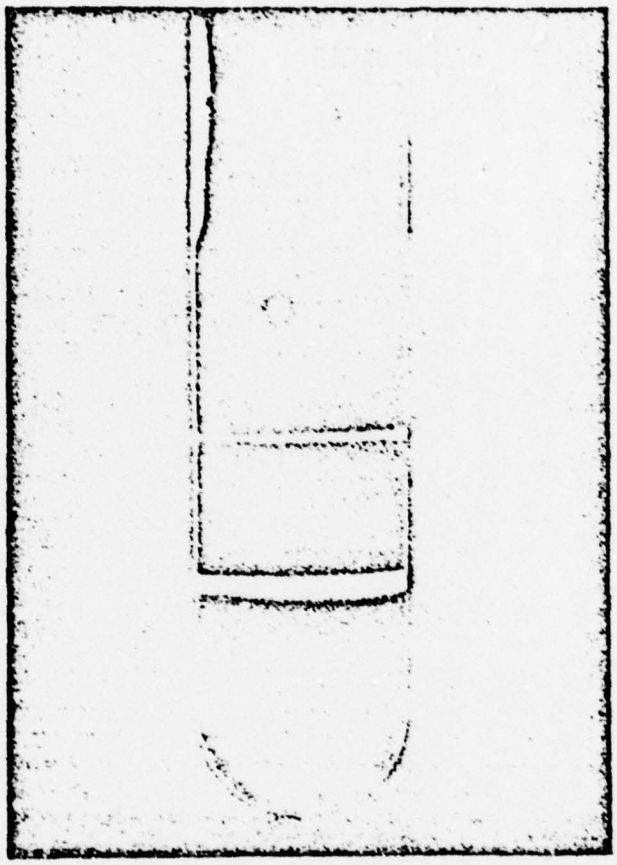


FIGURE 15

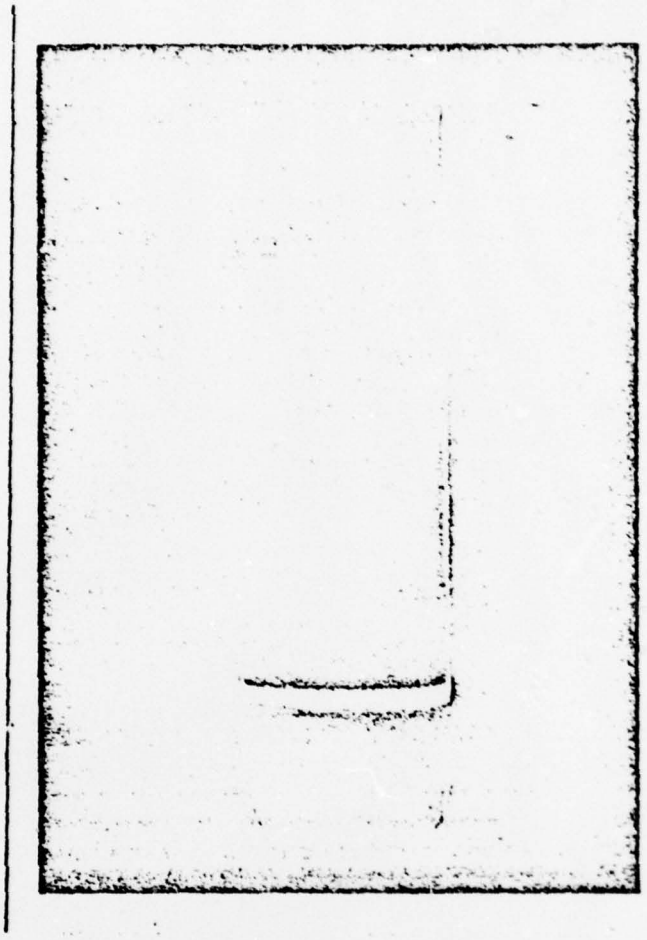


Figure 16

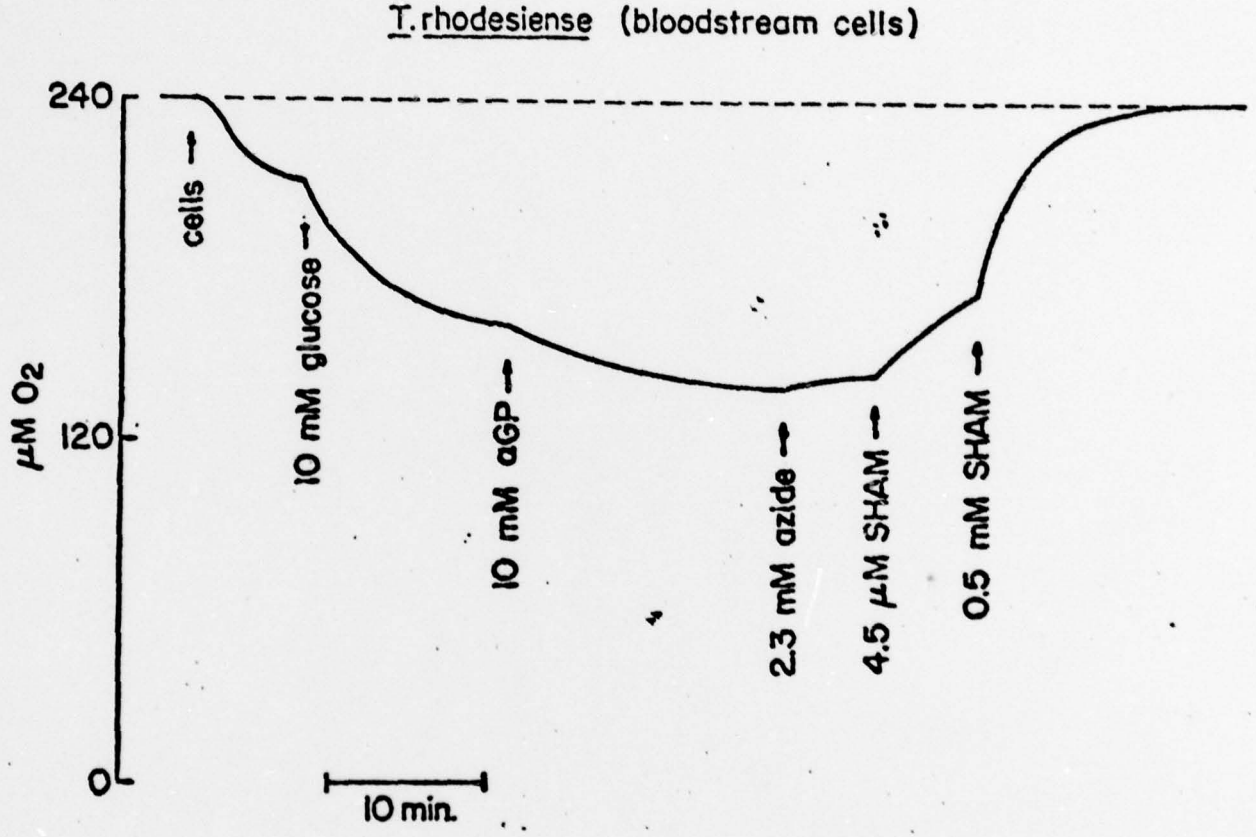

T. rhodesiense (bloodstream cells)
240
120
0
μM O2
cells
10 mM glucose
10 mM aGP
2.3 mM azide
4.5 μM SHAM
0.5 mM SHAM
10 min.

Figure 17

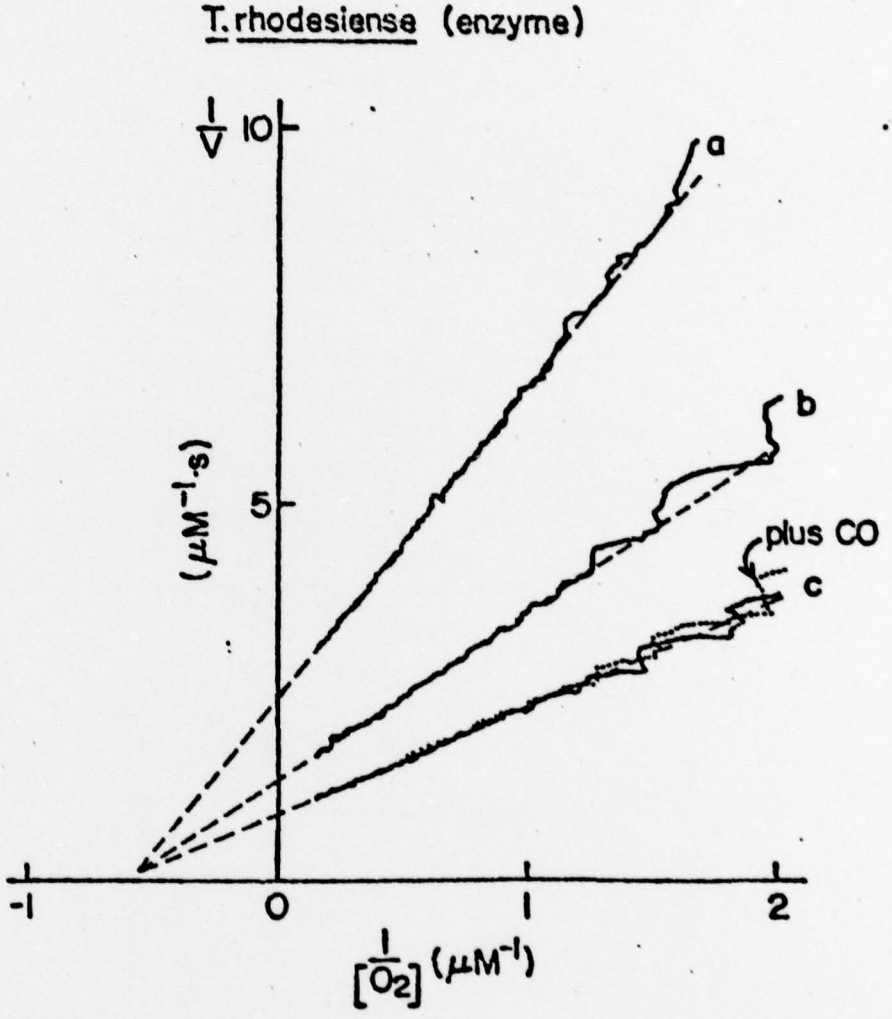


Figure 18

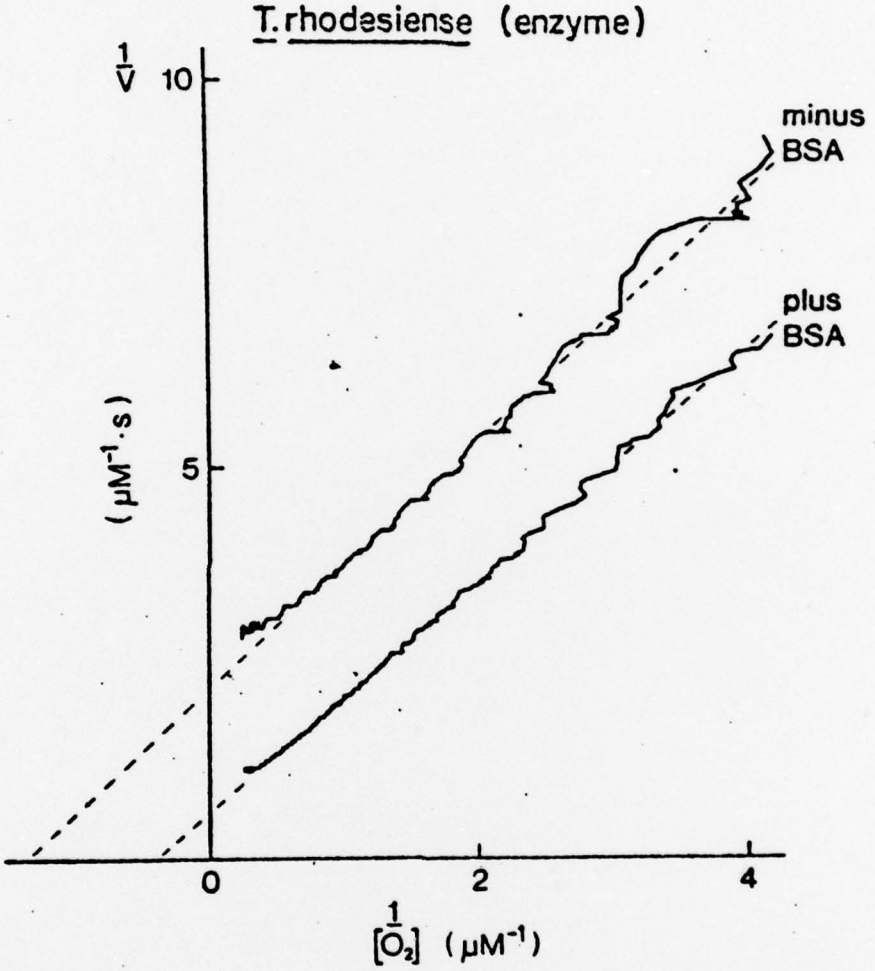

T. rhodesiense (enzyme)
1/V
10
5
(μM⁻¹·s)
minus BSA
plus BSA
0
2
4
1/[O₂] (μM⁻¹)

Figure 19

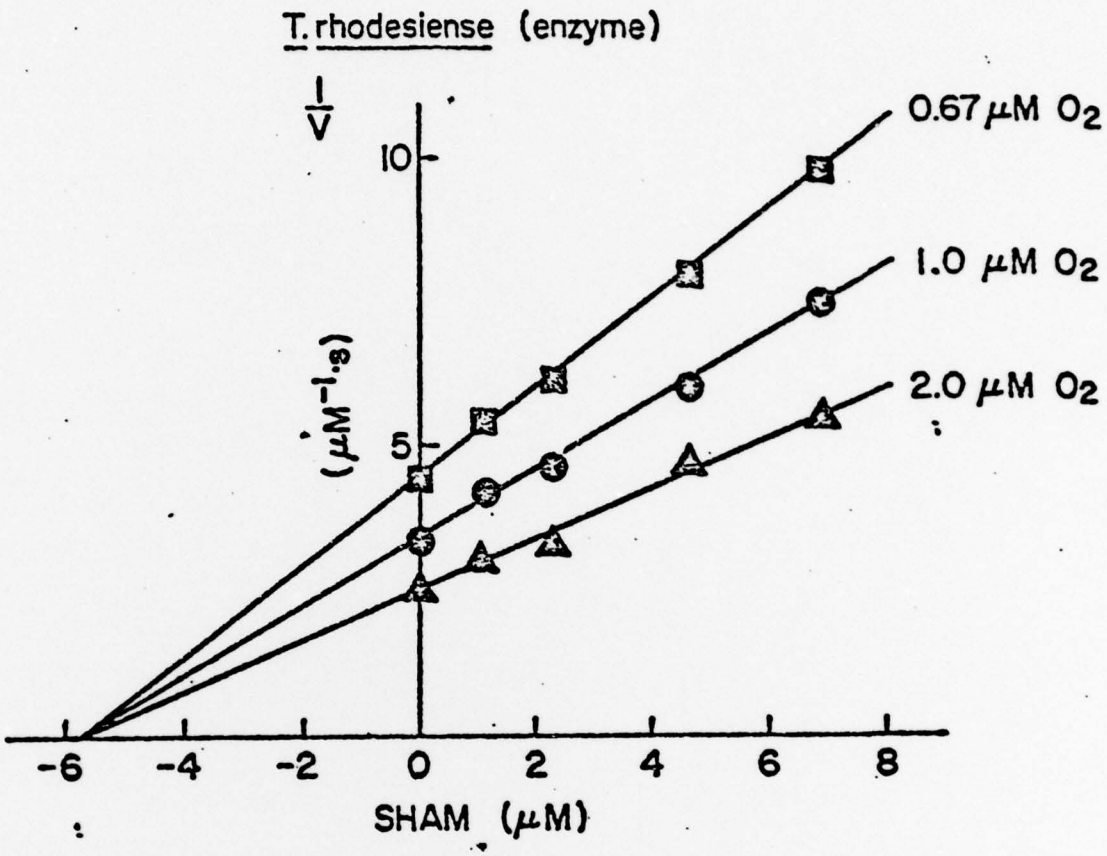

T. rhodesiense (enzyme)
1/V
(μM⁻¹.s)
10
5
0.67 μM O2
1.0 μM O2
2.0 μM O2
-6
-4
-2
0
2
4
6
8
SHAM (μM)

Figure 20

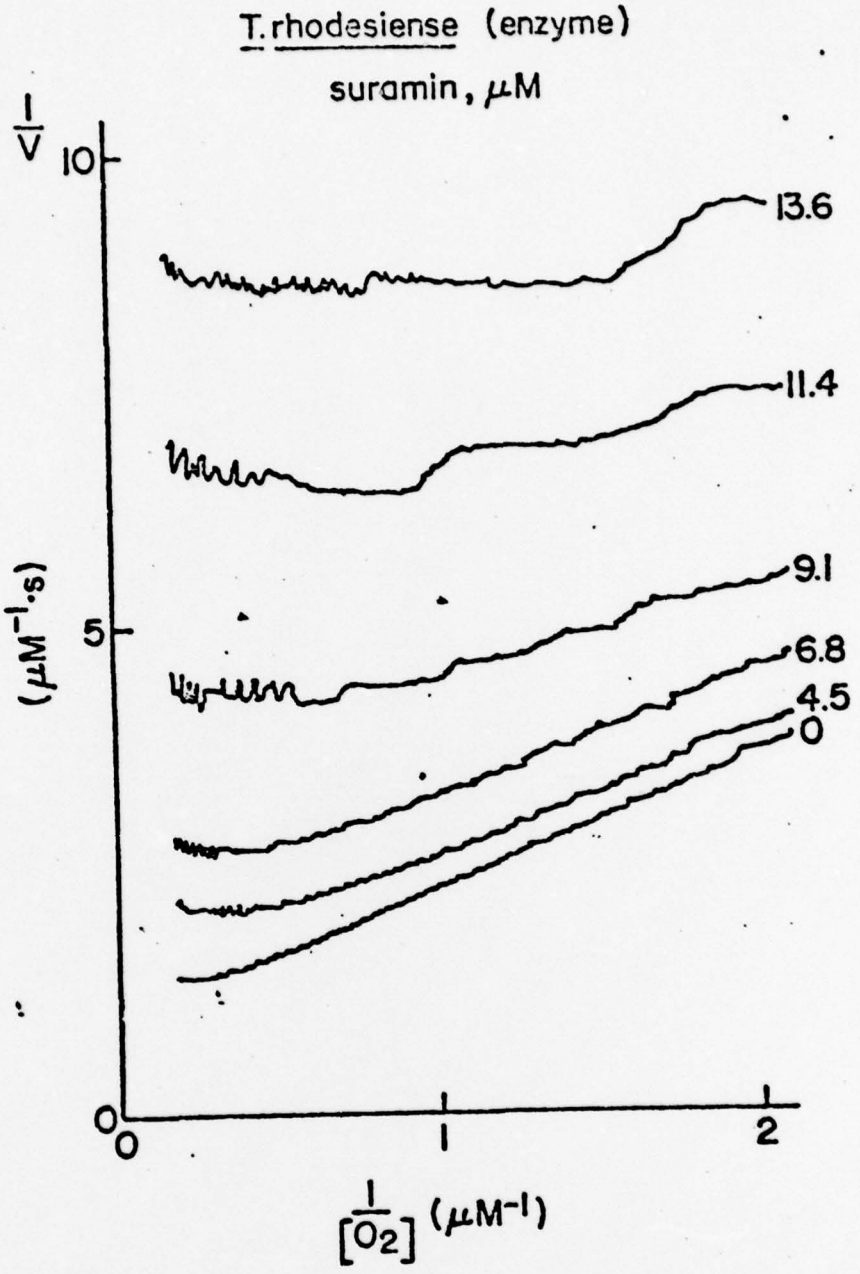

T. rhodesiense (enzyme)
suramin, μM
1/V
10
5
0
(μM⁻¹·s)
13.6
11.4
9.1
6.8
4.5
0
0
1
2
1/[O2] (μM⁻¹)

Figure 21

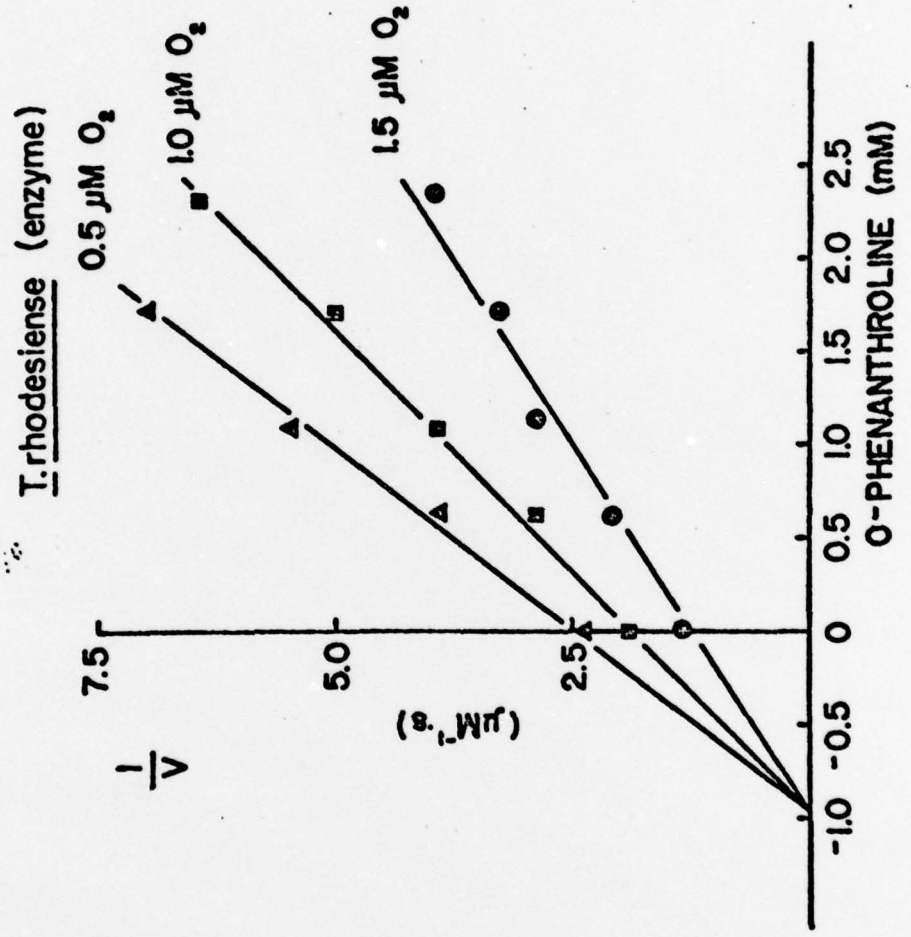

T. rhodesiense (enzyme)
0.5 μM O2
1.0 μM O2
1.5 μM O2
1/V
(μM⁻¹.s)
7.5
5.0
2.5
0
-1.0
-0.5
0.5
1.0
1.5
2.0
2.5
O-PHENANTHROLINE (mM)

Figure 22

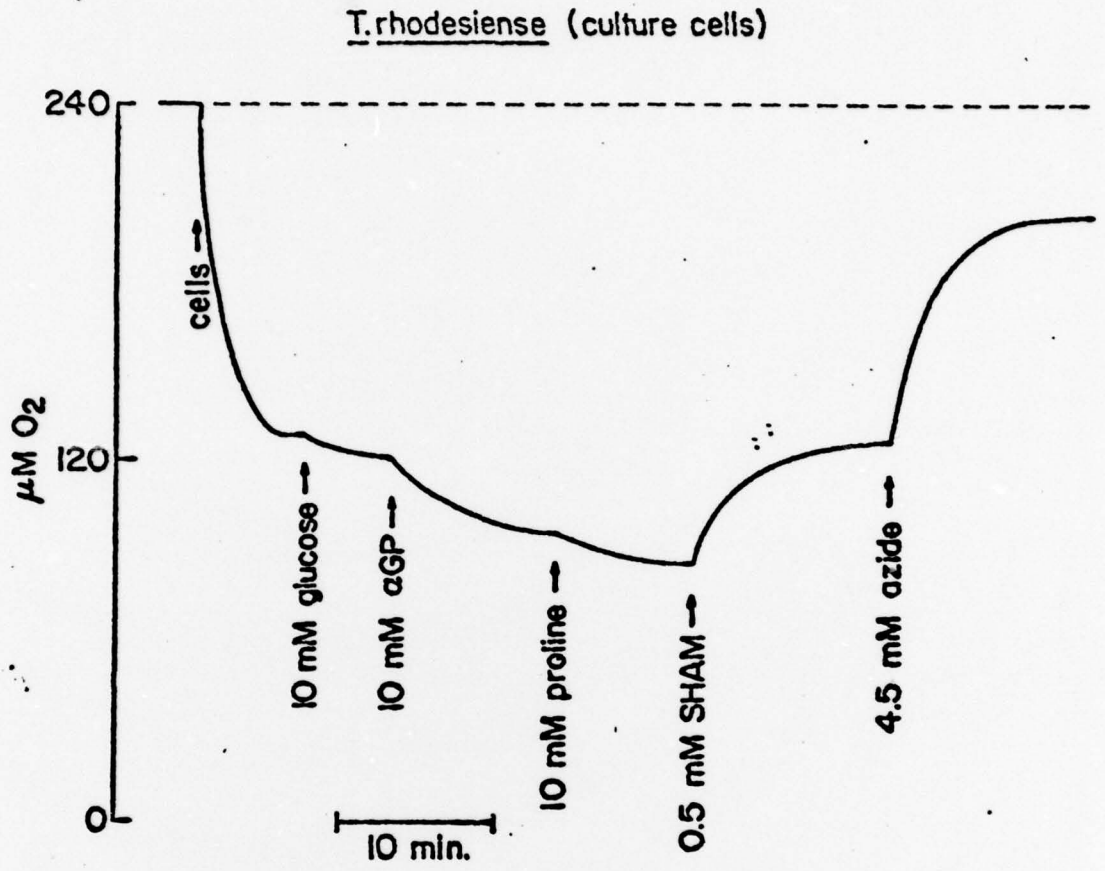

T. rhodesiense (culture cells)
240
120
0
μM O2
cells
10 mM glucose
10 mM aGP
10 mM proline
0.5 mM SHAM
4.5 mM azide
10 min.

Figure 23

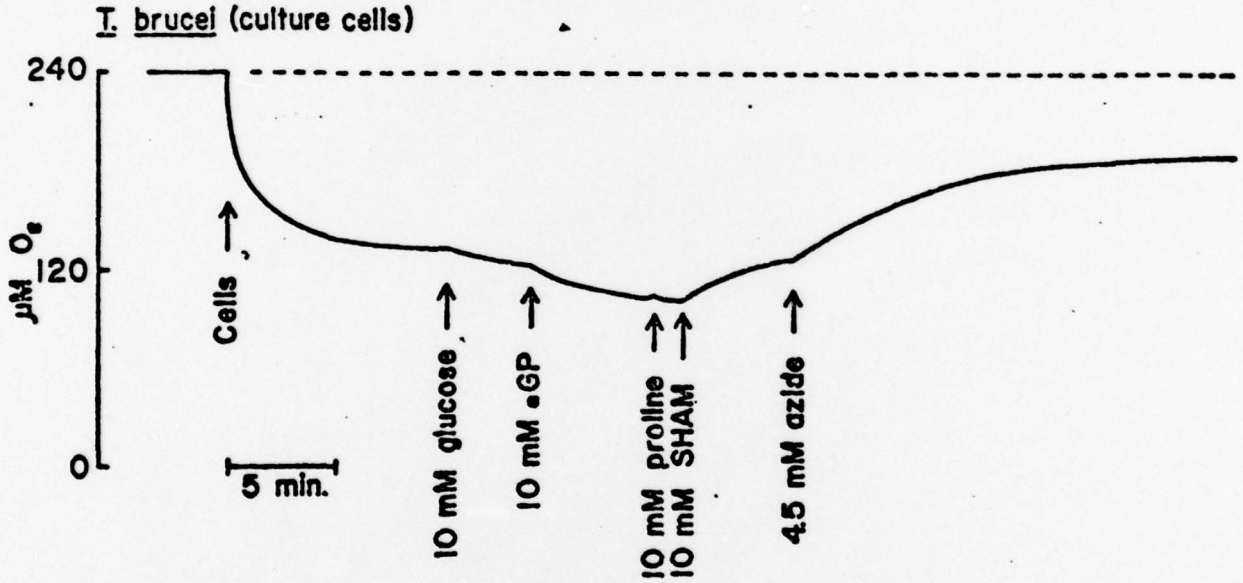

T. brucei (culture cells)
240
120
0
μM O_2
Cells
5 min.
10 mM glucose
10 mM α-GP
10 mM proline
10 mM SHAM
4.5 mM azide

Figure 24

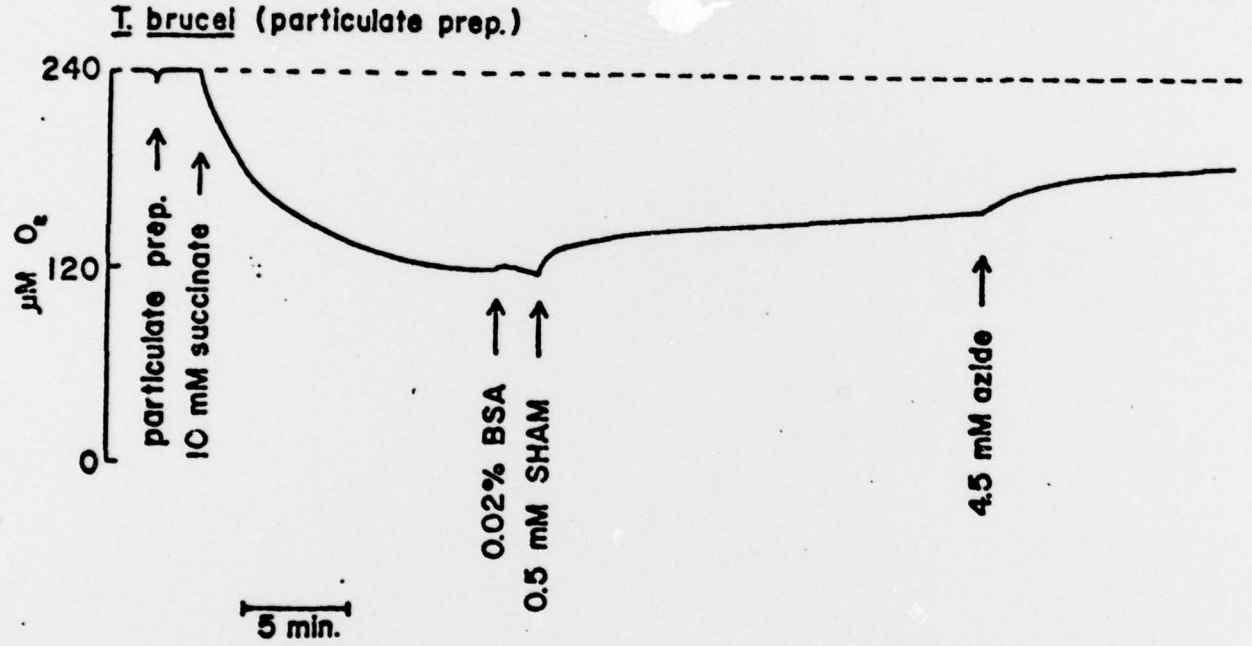

T. brucei (particulate prep.)
240
120
0
μM O_2
particulate prep.
10 mM succinate
0.02% BSA
0.5 mM SHAM
4.5 mM azide
5 min.

Figure 25

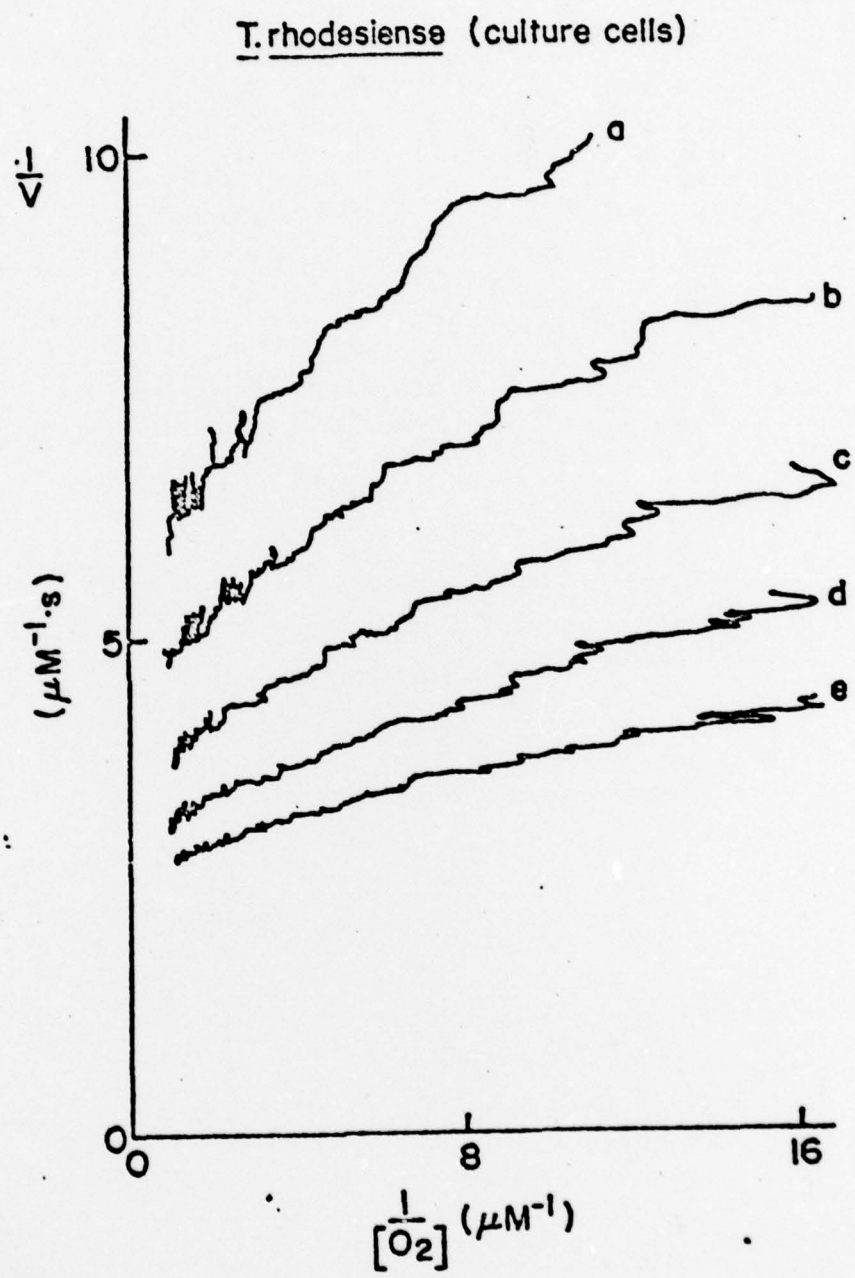
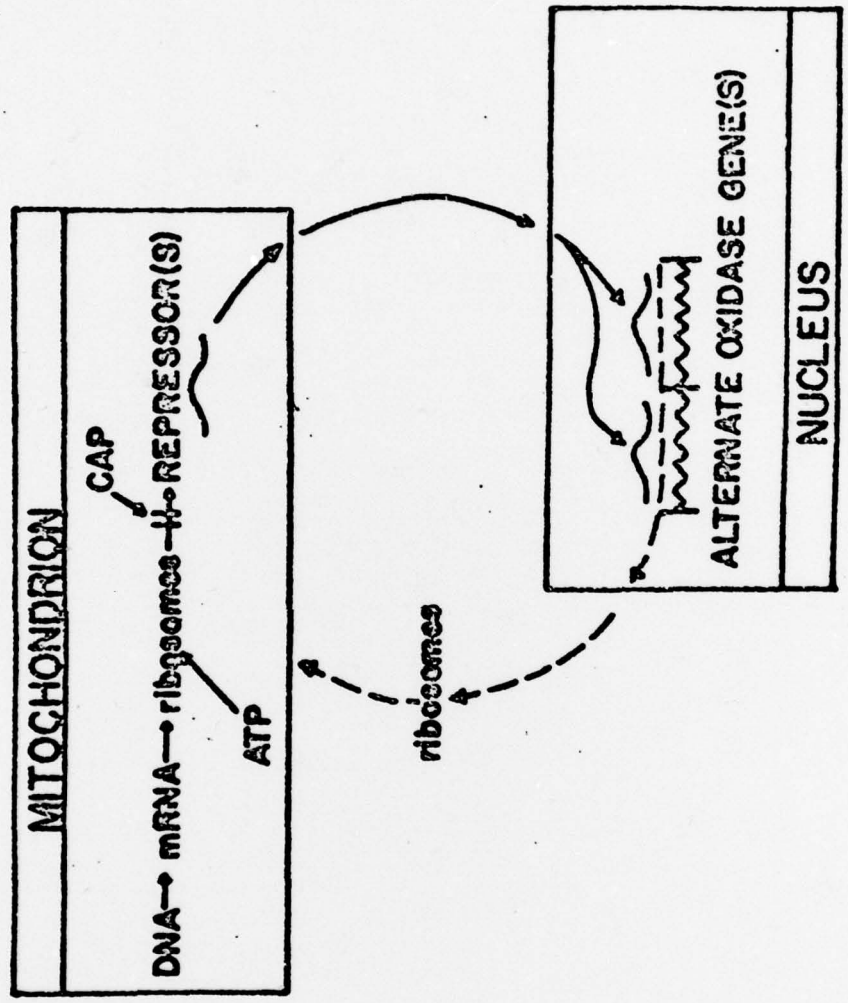

T. rhodesiense (culture cells)
$\overline{v}^{-1}$
10
5
0
$(\mu M^{-1} \cdot s)$
a
b
c
d
e
0
8
16
$\frac{1}{[O_2]}$ (μM^{-1})

Figure 26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ATP
ribosomes
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