EFFECTS OF TRYpanocidal DRUGS ON THE FUNCTION OF TRYpanosomes

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George C. Hill

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Effects of Trypanocidal Drugs on the Function of Trypanosomes

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During the past year, we have placed an emphasis on several aspects of our research concerned with the effects of trypanocidal drugs on the function of African trypanosomes. One of our goals this past year was to grow and maintain Trypanosoma gambiense TREU 1309 in vitro and this has been accomplished. These organisms grow as cultured-infective trypomastigotes as we have previously accomplished for several strains T. brucei and T. rhodesiense EATRO 1895 (1,2). In addition, another strain of T. brucei (IUMP 1026) has been initiated in culture.
Using our in vitro culturing system, we have observed the trypanocidal effects of suramin and SHAM on the *T. brucei* cultured infective trypomastigotes. This provides strong evidence that this system will be beneficial in detecting potential trypanocidal drugs. The effects of suramin and berenil on the fine structure and biochemistry of *T. brucei* have been continued and a manuscript submitted on our results.

We have continued to study the L-α-glycerophosphate oxidase and identified an alternate electron donor, ubiquinol-1, which will be extremely important in the further characterization of this important enzyme. Finally, we have made significant progress in identifying in the mitochondria of *T. brucei* a translation system showing sensitivity to a similar spectrum of antibiotics as that determined for eukaryotes.
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ABSTRACT

During the past year, we have placed an emphasis on several aspects of our research concerned with the effects of trypanocidal drugs on the function of African trypanosomes. One of our goals this past year was to grow and maintain *Trypanosoma gambiense* TREU 1309 *in vitro* and this has been accomplished. These organisms grow as cultured-infective trypomastigotes as we have previously accomplished for several strains *T. brucei* and *T. rhodesiense* EATRO 1895 (1,2). In addition, another strain of *T. brucei* (LUMP 1026) has been initiated in culture.

Using our *in vitro* culturing system, we have observed the trypanocidal effects of suramin and SHAM on the *T. brucei* cultured infective trypomastigotes. This provides strong evidence that this system will be beneficial in detecting potential trypanocidal drugs. The effects of suramin and berenil on the fine structure and biochemistry of *T. brucei* have been continued and a manuscript submitted on our results.

We have continued to study the L-α-glycerophosphate oxidase and identified an alternate electron donor, ubiquinol-1, which will be extremely important in the further characterization of this important enzyme. Finally, we have made significant progress in identifying in the mitochondria of *T. brucei* a translation system showing sensitivity to a similar spectrum of antibiotics as that determined for eukaryotes.
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, nuclear and mitochondrial gene expression. The interrelationships of these two systems can be seen in Figure 1. In addition, we are concerned with the regulation and control of the functioning of the terminal oxidases in bloodstream trypomastigotes. In order to identify targets for potential trypanocides, we must learn more about the properties of the mitochondrion in trypanosomes including the replication and transcription of KDNA and the repression and synthesis of mitochondrial electron transport systems. We also 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.

We are also interested in the inhibition of novel enzymes on processes which are under control of the nuclear genome. These include processes such as antigenic variation or synthesis of L-α-GP oxidase on cytoplasmic ribosomes. Our goal in the development of novel trypanocidal drugs is to gain additional knowledge on the control mechanisms involved in the transcription of nuclear and mitochondrial genes in these organisms. In this way, we hope to identify potential targets for trypanocides that so far have not been investigated. The proper establishment of the primary mode of action of a drug requires a systematic study of its effect on the various metabolic processes of the cell at the lowest concentrations that inhibit growth. Only when such a survey is complete can it be concluded confidently that a particular pathway or reaction is most sensitive to inhibition by a drug and is therefore the primary target of that drug. We are carrying
out this type of systematic study with several trypanocidal drugs including berenil, suramin, antrycide and ethidium bromide. These types of studies have only rarely been performed with trypanocidal drugs.

The numerous and necessarily speculative points raised in any discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell biochemistry. Given the necessary attention, this largely neglected but important field should yield results of considerable value, not only for an understanding both of trypanocidal drug action and of trypanosomal metabolism, but for cell biology in general. This is the purpose of this contract. So far as trypanocidal drug design is concerned, the era of intelligent empiricism is unlikely to be superseded until the balance of effort and expenditure on drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

In developing new trypanocidal agents, we are investigating:

1. Effects of trypanocidal drugs affecting enzyme systems in trypanosomes in hosts;
2. Comparisons of homologous enzymes in host and trypanosomes;
3. Unique cell components or metabolic pathways in trypanosomes;
4. The basis of the selective toxicity of known drugs.
THE BACKGROUND

African trypanosomiasis is confined to Africa by the distribution of its vectors. *T. gambiense* infection occurs over a broad belt from Senegal in the west to the great lakes Victoria, Albert, Benguelo, and Tanzania in the east, extending as far south as north Angola. *T. rhodesiense* is located in east and central Africa and is scattered over a longitudinal band from the south Sudan to Mozambique. The importance of trypanosomiasis of man and animals in Africa has been summarized recently by Kershaw (3) in the statement:

"The World Health Organization in determining the ten major health problems facing mankind places trypanosomiasis of man and his domestic animals high on the list along with malaria, cancer and heart diseases."

Identification of New 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 prophylaxis 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 (e.g., antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. As pointed out recently by Newton (4), 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 "blood-brain barrier," this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review on the mode of action of trypanocidal drugs has been prepared by Williamson (5). 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 heterocyclic 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 (4).

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 dyskinetoplastic trypanosomes (6) and "petite mutants" of yeast (7). 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 to interact with DNA and can selectively block kinetoplast replication (8, 9). The earliest reported effect observed of berenil is the localization in the kinetoplast of T. brucei. This has been detected by ultraviolet microscopy with 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 (9). Recent 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 (10).

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 (8). 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 to 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 (5). The most sensitive enzymes examined appear to be hyaluronidase, inhibited at $10^{-5}$-$10^{-6}$ M, fumarase, inhibited at ca. $10^{-7}$ M, urease at pH 5 (ca. $10^{-4}$ M), hexokinase ($10^{-4}$-$10^{-5}$ M), and RNA polymerase ($10^{-5}$ M) (11). Recent studies by our laboratory supported by this contract (12) and other investigators (13, 14) have demonstrated that suramin also inhibits the L-a-GP oxidase in bloodstream trypanosome *in vitro*. Whether this is its mode of action *in vivo* remains to be determined.

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.

**The Importance of African Trypanosomiasis as a Public Health Problem**

Human trypanosomiasis, causing sleeping sickness, and animal trypanosomiasis, affecting cattle and other domesticated animals, are the two classical notorious plagues of Africa rooted in the continent since time immemorial.
Sleeping sickness constitutes a permanent and serious risk to the health and well-being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the vast potential for livestock production in the continent. Any involvement of our military troops in areas endemic with African trypanosomiasis would be disastrous.

Ten thousand new cases of human trypanosomiasis are known to occur yearly, but this figure does not truly reflect the importance of the disease as a public health problem. As with many tropical diseases, prevalence figures are underestimates due to failure to recognize the disease and to under-reporting. The relatively low prevalence is due to the major control efforts which have been made over the past 50 years. Without these, sleeping sickness would still be a major cause of death, as it was at the turn of the century, with great epidemics raging along the Congo river and the northern shores of Lake Victoria costing the lives of some 750,000 people.

At the present time, some 10 million people at risk are examined annually by mobile teams at an estimated cost of 5 million dollars. Expenditure for control of tsetse flies is at least ten times higher, but the total is difficult to evaluate since most efforts are directed towards control related to animal disease.

In view of the potential serious danger of sleeping sickness, national health services accord high priority to control services but efforts are frequently inadequate since sufficient resources in terms of finance, manpower and administrative facilities may not be available.
An outbreak of sleeping sickness is a dramatic event in a community since the disease causes severe symptoms due to lesions of the central nervous system and is fatal if not treated. Outbreaks may cause populations to abandon villages and fertile farmlands, and the effect is such that even after two generations, fear of re-exposure may prevent the people from returning.

Current control measures do not usually eliminate the disease; moreover, they are costly and cumbersome. With the available tools, control is a continuing effort, producing suppression rather than eradication. The experience of the past 50 years has been that whenever control efforts are interrupted, for example due to political or economic circumstances, or out of complacency, a flare-up of the disease will sooner or later occur.

A recent example was the resurgence of trypanosomiasis in Zaire in the early 1960's, when after six years of interruption of surveillance, prevalence figures rose from 0.017 to 127, and even to 18% in some areas. Outbreaks of unknown severity are now reported from Angola, Cameroon and the Sudan. It is to be expected that more outbreaks will occur in coming years unless improved control measures can be found. Development of new tools is therefore a matter of urgency, not only as a means to eradicate the disease but to provide measures which are more effective and can be more widely applied than those presently available.
RESULTS AND DISCUSSION

A. Cultivation of Trypanosomes in Vitro

We have continued our efforts to cultivate African trypanosomes and in the past year extending the success to Trypanosoma brucei LUMP 1026 and Trypanosoma gambiense TREU 1309. Other organisms that we have grown in vitro as cultured infective trypanastigotes include:

a. T. brucei FATRO 427
b. T. brucei FATRO 110
c. T. rhodesiense FATRO 1895
d. T. equiperdum ATCC 30023

The results with T. rhodesiense have been published in Science (2). The results with T. brucei are discussed in a paper published in Acta Tropica (1). Reprints are available in the appendix.

1. Cultivation of T. brucei LUMP 1026

The growth of T. brucei LUMP 1026 has been extremely important. This strain of T. brucei is one of the few strains now available which undergoes transformation of bloodstream trypanastigotes to procyclic trypanastigotes and also can be grown in vitro as cultured-infective trypanastigotes. Thus, it is possible to do numerous comparative studies with these organisms. The T. brucei LUMP 1026 were initiated into culture by procedures similar to those methods used for T. rhodesiense FATRO 1895 (2). A 150-200 gm albino rat was
inoculated at $2 \times 10^6$ trypanosomes. This strain undergoes antigenic variation for 20-35 days. The rat was sacrificed on day 25 at $3.0 \times 10^8$ trypanosomes/ml. The cultures were inoculated at $5 \times 10^5$ cells with trypanosomes from the buffy coat. In addition some cultures were started using whole blood adjusted to $2 \times 10^6$ trypanosomes/ml. In both cases, T-25 flasks were used. Established *T. brucei LUMP 1026* cultured infective trypomastigotes grow very well in vitro as can be seen in Table I. However, there is often a delay of 5-7 days after a fresh culture is started before the numbers begin to increase. The organisms reach a level of $2-4 \times 10^6$ trypanosomes/ml and maintain this high growth until changed to fresh tissue culture cells. As with other trypanosomes cultured in vitro, the organisms are slender trypomastigotes and grow in the tissues and the supernatant (figures 2 and 3). As can be seen in figure 4, the *T. brucei LUMP 1026* cultured infective trypomastigotes have a surface coat which is quite distinct. It is absent on procyclic trypomastigotes (figure 5).

The tissue culture cell line we have used recently has been bovine embryonic tracheal tissue culture cells (ATCC CCL 44 EBTtr) which are available from the American Type Culture Collection. A description of these cells is given in Table II. We have determined that irradiated tissue culture cells can serve as an excellent feeder layer for the trypanosomes. Irradiation at 5000 rads slows the growth of the tissue culture cells to 1-2 generations per week. The tissue culture cells require transfer only once every 4-5 weeks. We have observed no
difference in the trypanosomes with irradiated or non-irradiated tissue culture cells.

The *T. brucei* LUMP 1026 cultured-infective trypomastigotes are infective to mice. The organisms have been maintained in vitro for 50 days and still produce a parasitemia in mice with an inoculum of $5 \times 10^5$ trypanosomes. As we have noted with other organisms cultured in this system, after four weeks, the cultured trypomastigotes develop an initial parasitemia at the same time bloodstream stabilates but the mice infected with cultured trypomastigotes die 2-3 weeks later than the bloodstream stabilates.

The respiration of the *T. brucei* LUMP 1026 cultured infective trypomastigotes is similar to bloodstream trypomastigotes. The respiration was measured in the closed system and was 100% sensitive to 0.5 mM salicylhydroxamic acid and insensitive to 0.1 mM KCN. These results are comparable to those obtained with bloodstream trypomastigotes (see manuscript in appendix by Njogu, M., Whittaker, C., and Hill, G.C.) and suggest that cultured infective trypomastigotes have the a GP oxidase found in the bloodstream trypomastigotes.

2. Cultivation of *T. gambiense*

During the past few months, we have been able to grow and maintain cultured infective trypomastigotes of *Trypanosoma brucei gambiense* (TREU 1309). This strain was isolated from a patient at Kwara Tera, a sleeping sickness focus in Nigeria in 1967 by inoculation of lymph gland material into a rat. Subsequently, the trypanosomes were serially subpassaged in 23 adult rats, 23 nursling rats, and 2 nursla
mice before being adapted and stabilatcd in mouse blood in 1970. Stabilates were received from Dr. Bron M. Honigberg, Department of Zoology, University of Massachusetts, Amherst, MA.

The *T. gambiense* TREU 1309 cultured infective trypomastigotes were grown and maintained following procedures similar to those used for *T. rhodesiense* EATRO 1895 (2) except that bovine embryonic trachea tissue culture cells (ATCC CCL 44 EBTr) were used. The trypanosomes grow similar to *T. rhodesiense*, reaching 3-5 x 10^6 trypanosomes/ml 4-5 days after subcultures are made from established cultures. The trypanosomes grow in the medium as well as close to the tissue culture cells (figure 6). The surface coat is quite pronounced (figure 7). Infectivity studies demonstrate the *T. gambiense* (TREU 1309) cultured infective trypomastigotes are infective to mice 25 days after growth in vitro. *T. gambiense* procyclic trypomastigotes have also been obtained following procedures outlined by Cunningham (15) as we have modified in our laboratories (see Rienen, E.J. and Hill, G.C., submitted, manuscript in appendix).
B. Transformation Studies of *T. brucei* LUMP 1026 Cultured Infective Trypomastigotes

In order to evaluate the similarity of the *T. brucei* LUMP 1026 cultured infective trypomastigotes to the bloodstream trypomastigotes, we have evaluated their ability to undergo transformation. We have studied extensively the ability of *T. brucei* bloodstream trypomastigotes to undergo transformation to procyclic trypomastigotes. This transformation that occurs is highly reproducible. The medium that has been used is a semidefined medium developed by Cunningham (15). We recently submitted a manuscript for publication detailing the methodology for this procedure (Bienen, E. J. and Hill, G. C., submitted, see manuscript in appendix). As seen in figure 8, *T. brucei* LUMP 1026 undergoes morphological transformation within 72-96 hours and then multiplies. However, experiments in the same medium using *T. brucei* LUMP 1026 cultured infective trypomastigotes have not been successful. As seen in Table III, the cultured infective trypomastigotes undergo morphological transformation, but they do not grow after undergoing transformation. In addition, the trypanosomes present often do not look healthy. Clearly, the proper conditions under which cultured infective trypomastigotes will undergo transformation remain to be determined and experiments designed to pursue this question are presented in the continuation proposal.
C. Effectiveness of In Vitro System for Drug Evaluation

We have had significant success in using the in vitro culturing system as a potential system for compounds which may have trypanocidal activity. Our initial experiments were with SHAM. This compound has been identified by several laboratories as a potent inhibitor of the α GP oxidase system. However, its effectiveness as a trypanocidal drug in vitro has been limited, except in combination with glycerol (16). Table IV presents results from a typical experiment with SHAM which provides evidence that SHAM added alone does have trypanocidal activity. Addition of 0.05 mM SHAM had marked effect on cell growth. However, it is not clear whether the results are due to SHAM alone or the presence of SHAM and glycerol, the glycerol, possibly produced by the tissue culture cells present. Experiments are now in progress in collaboration with Fred Brohn and Allan Clarkson at NYU in order to measure the concentration of glycerol present during SHAM inhibition.

Suramin has also been tested in the system at 5, 10, and 20 μg/ml (Table V). For comparison with SHAM, the concentrations in Table V are given in molar concentrations. Clearly, after two days of growth, suramin has had an effect. This is significant since suramin has no effect on procyclic trypomastigotes. Thus, this system has potential as an assay system for compounds being evaluated for their trypanocidal activity.
D. Identification of Alternate Electron Donor for α-GP Oxidase

Bloodstream forms of salivarian African trypanosomes have been shown to oxidize glucose via the glycolytic pathway with no significant functioning of the citric acid cycle or the pentose phosphate pathway for glucose metabolism. The NADH formed in the glyceraldehyde-3-phosphate reaction during glycolysis is reoxidized to NAD⁺ by linkage to the oxidation of dihydroxyacetone phosphate (DHAP) to α-glycerophosphate (α GP) by an α GP dehydrogenase, an enzyme located in the glycosome. L-α-GP in turn is reduced back to DHAP in the mitochondrion by the L-α-GP oxidase system. This terminal oxidase system (figure 9) consists of two components, the L-α-GP dehydrogenase (reaction 4) and the L-α-GP oxidase (reaction 5). L-α-GP is reduced back to DHAP in the mitochondrion by an L-α-GP dehydrogenase coupled to a SHAM sensitive, KCN insensitive L-α-CP oxidase. This enzyme is the only terminal oxidase in bloodstream trypomastigotes and a key enzyme in their aerobic metabolism. Thus, it could be an effective target for trypanocidal drugs. The absence of an identical system in mammals makes this terminal oxidase a prime target for rational chemotherapy. An intensive investigation of this system may prove to be productive.

In order to further characterize the L-α-GP oxidase, we have been trying to identify alternate electron donors which can donate electrons directly to the terminal oxidase. In this way, we could begin to purify the terminal oxidase as it is removed from other membrane proteins. The uncoupling of the α GP dehydrogenase from the α GP oxidase has been accomplished recently by the use of various quinols as alternate donors to the L-α-GP oxidase. As seen in figure 10, we have been successful in identifying ubiquinol-1 as a potential electron donor.
donor. The activity with ubiquinol-1 alone is 4-5 fold greater than with α-GP. This activity with ubiquinol-1 is completely inhibited by 1 mM SHAM and insensitive to 1 mM KCN, both properties of the α GP oxidase. Table VI presents results with other electron donors. However, none have been as active as ubiquinol-1. We now are testing coenzyme Q9, identified in C. fasciculata (17), in order to determine if it will have any significant activity.
E. Biochemical and Ultrastructural Effects of Trypanocidal Drugs on T. brucei Treated In Vivo

During the past year, the results of the experiments that we have performed to evaluate the effects of trypanocidal drugs (e.g., suramin, berenil, ethidium bromide and antrycide) have been submitted for publication (Hill, G. C., and Shimer, S., submitted to The Journal of Protozoology). A detailed presentation of our results are included in the manuscript in the appendix. A system has been developed for the study of the biochemistry and fine structure of Trypanosoma brucei treated in vivo with four trypanocidal drugs including suramin, berenil, ethidium bromide, and antrycide. Marked inhibition of RNA synthesis (e.g., 80-90%) was noted from bloodstream trypomastigotes treated in vivo with suramin, berenil, and antrycide. In antrycide-treated bloodstream trypomastigotes, protein synthesis was inhibited 60-65%. Suramin-treated cells had a marked increase in respiration. Effects on the fine structure of the berenil, ethidium bromide, and antrycide-treated bloodstream trypomastigotes were observed.

These studies have provided some additional information on the effects of trypanocidal drugs on African trypanosomes. In our studies, we have concentrated on looking at the effects of the drugs on trypanosomes exposed in vivo. The model system we have used here is highly reproducible and allows for obtaining large quantities of drug-treated trypanosomes for biochemical investigations. From one rat, it is possible to easily obtain $7-8 \times 10^8$ trypanosomes which can be separated cleanly from blood elements and used further in other experiments. The bloodstream trypomastigotes have been removed from the laboratory animals after treatment of the infected rats has reduced the parasitemia significantly.
The effects of berenil on the trypanosomes is especially significant. This drug has been observed to produce in vitro dyskinetoplastic trypanosomes and inhibit the replication of kinetoplast DNA (18, 19). However, within 15-30 minutes after treatment of infected animals with berenil, the trypanosomes show gross morphological effects. Within two hours, no trypanosomes are observed in the blood. These results clearly suggest that the primary mode of action of this drug is not specifically on the replication of the kinetoplast DNA. In contrast, the rapid effect on the motility and morphology of the trypanosomes could indicate an effect on the surface membranes or microtubules present. It is interesting to note that similar effects have been observed with cultured-infected trypomastigotes (2, 20) treated at 5-10 μg/ml but not with procyclic trypomastigotes (Hill and Shimer, unpublished). During these studies, we have purposely avoided examining bloodstream trypanosomes treated with drugs in vitro, as we feel these experiments may not actually reflect what occurs when the trypanosomes are treated in a host. The ultrastructural and biochemical effects which occur in the in vitro system may have little relationship to the mode of action of the trypanocides in vivo. For example, in a large number of in vitro experiments, dyskinetoplasty is produced after trypanosomes are exposed to ethidium bromide or berenil (4). However, our results in vivo do not suggest that the kinetoplast is a primary or specific site of action for these trypanocides.

The drugs that we have examined have required varying times for inhibition of growth to occur. Studies with suramin and antroline often were done overnight. However, ethidium bromide and berenil were effective within several hours. Suramin has been investigated extensively in our
From previous experiments in several laboratories, it is clear that suramin inhibits the L-α-glycerophosphate oxidase system (α-GP oxidase) in bloodstream trypomastigotes. With respect to L-α-GP, the $K_i$ is 4.1 μM (21). With respect to $O_2$, the $K_i$ is 8.0 μM (22,23). However, since the complete inhibition of the α-GP oxidase by salicylhydroxamic acid is not sufficient to kill the bloodstream trypomastigotes (16), suramin must be inhibiting some other site or function within the trypanosome. Previously published studies have demonstrated that suramin is an active inhibitor of numerous enzyme systems including hyaluronidase, fumarase, hexokinase, urease, and RNA polymerase (24). In our current studies, it is clear that suramin is an extremely effective inhibitor in vivo of RNA synthesis in trypanosomes. It certainly is possible that a combined effect of this drug on the functioning of the α-GP oxidase and RNA synthesis could result in the death of suramin-treated trypanosomes. The marked stimulation of respiration after the trypanosomes had been exposed to suramin was surprising. Since suramin inhibits the α-GP oxidase, a decrease in the respiration of the cells was expected. Clearly, the stimulation observed reflects some modification in the normal aerobic metabolism in these drug-treated cells which needs to be investigated.

Antrycide also had a marked effect on the RNA and protein synthesis of bloodstream trypomastigotes. Previous reports on the effects of this drug provide clear evidence that antrycide reduces the growth rate of Crithidia oncopelti in vitro by inactivating cytoplasmic ribosomes (25). In addition, the aggregation of cytoplasmic ribosomes results in the formation of basophilic granules and bound antrycide appears in the cytoplasm of organisms after a period of growth in the presence of antrycide (25). In studies with T. brucei isolated from infected animals,
treated with anticycide, Omerod (26, 27) observed similar basophilic granules. As noted in our experiments, it has been observed previously that anticycide does not immediately inhibit the growth of trypanosomes in rats or mice (28, 26). Other studies by Sen et al. (29) have presented information that anticycide had reduced effect in splenectomized mice, suggesting that host defense mechanisms could play an important role in the action of anticycide in vivo. As a result, Newton (4) has suggested that anticycide is not a trypanocidal drug but reduces the growth rate of the trypanosomes to a level where host defense mechanisms can control the infection. The marked inhibition observed in our studies on the RNA and protein synthesis in trypanosomes in vivo could certainly result in decreased growth of the trypanosomes.

With the development of the in vitro system for the growth of T. brucei and T. rhodesiense cultured-infective trypomastigotes (1, 2, 20), it is now possible to study the biochemical effects of the drugs on the trypanosomes and the feeder layer cells in order to determine whether any differences can be observed between the tissue culture cells and the trypanosomes. The culturing system for bloodstream trypomastigotes may serve as a model system for determining the effects of the trypanocides on the trypanosomes and also on mammalian tissue culture cells. In addition, the study of the action of trypanocidal drugs in vivo should certainly be encouraged. It would be important to continue these in vivo studies with recently isolated strains of T. brucei brucei and T. brucei rhodesiense which are now available in several laboratories. In this way, the effects of the drugs on the slow-killing strains which undergo continual antigenic variation could be explored.
Future examination of drug-treated trypanosomes in kinetic studies is important in order to determine what the initial biochemical effects of the trypanocides are and how the effects increase as the decrease in parasitemia and increase time exposure of the trypanosomes to the drugs occurs. If progress in the development of trypanocides is to occur at an accelerated pace in the future, research on the biochemistry and chemotherapy control of trypanosomes treated in vivo with different trypanocides must be encouraged and pursued. Newton (4) has correctly emphasized that the knowledge of the biochemistry of bloodstream trypanosomes is essential if significant progress on the mechanisms of action of trypanocides is to be made. In this area, new innovative approaches are essential.
F. Identification of the Mitochondrial Protein Synthesis System and the Effects of Antibiotics on Cellular Functions in T. brucei

The effects of the metabolic inhibitors cycloheximide (CHX), D-chloramphenicol (CAP), erythromycin (ERY), tetracycline (TET), oleandomycin (OLE), lincomycin (LIN), paromomycin (PAR), neomycin (NEO), and antimycin A (ANA) on cellular functions in procyclic trypanosomes of Trypanosoma brucei LUMP 1026 have been investigated. The parameters investigated include total protein synthesis and respiration in vivo, as well as cytoplasmic and mitochondrial protein synthesis in vitro.

Total protein synthesis in vivo was examined by determining the incorporation of L-(\(^3\)H)-leucine into protein (figure 1). CHX, a specific inhibitor of cytoplasmic ribosome function in eukaryotes, inhibited total protein synthesis 50% at 0.3 μg/ml and, at 100-1000 μg CHX/ml, incorporation was inhibited 95-97% (figure 11). The insensitive incorporation (3-5% of total) presumptively, but not necessarily, represents the activity of the mitochondrial translation system. Surprisingly, CAP and ERY (each at 1500-2000 μg/ml) and TET (at 400-3000 μg/ml), all inhibitors of bacterial and mitochondrial ribosome function, also inhibit total protein synthesis in vivo by 50-95% (figure 12). The other bacterial antibiotics, OLE, LIN, PAR, and NEO have no significant inhibitory effect at similar concentrations (data not shown).

The effects of the drugs on the incorporation of L-(\(^3\)H)-phenylalanine into protein in vitro by a cytoplasmic ribosome S30 extract from T. brucei LUMP 1026 was tested. This S30 incorporation system represents the ATP-dependent, puromycin-sensitive translation of natural endogenous mRNAs since the incorporation is independent of
added poly U (Table VII). It was found that at concentrations inhibitory to total protein synthesis in vivo, CHX and TET strongly inhibited the function of cytoplasmic ribosomes in vitro whereas CAP and ERY had no inhibitory effect (figure 13). Thus, the inhibitory effect of CAP and ERY in vivo was not due to a direct effect on the cytoplasmic ribosome.

In order to elucidate the precise inhibitory site of action of CAP and ERY, the effects of the drugs on respiratory systems in *T. brucei* LUMP 1026 were studied. The total respiration of procyclic trypanosomes of *T. brucei* LUMP 1026 is inhibited 70-80% by 0.1 mM KCN and represents predominantly the activity of the cytochrome-mediated mitochondrial electron transport chain. It was found that CAP, ERY, and TET are potent inhibitors of mitochondrial respiration (figure 14). Moreover, this inhibition is elicited over the same concentration range as that at which total protein synthesis is affected (cf. figure 12). Note that a concentration of drug sufficient to inhibit total respiration 60% (3000 µg CAP or ERY/ml; 1500 µg TET/ml) (figure 14) produces a 90% inhibition of total protein synthesis (figure 12). In contrast, CHX has no effect on total respiration in the concentration range (100-1000 µg/ml) where protein synthesis is affected.

These results suggest that the inhibitory effect of CAP and ERY on total protein synthesis is a secondary consequence of a primary inhibition of respiration. This conclusion is supported by the observation that antimycin A, a specific inhibitor of mitochondrial respiration at complex III, also produces a 98% inhibition of total protein synthesis.
synthesis following a 60% inhibition of total respiration (figure 15).

Interestingly, these results indicate that, in the absence of an ATP supply from mitochondrial respiration, procyclic trypomastigotes of T. brucei LIMP 1026 cannot produce sufficient ATP from the fermentation of glucose to maintain total protein synthesis.

In an attempt to identify the activity of the presumptive mitochondrial protein synthesis system in vivo, the effects of the bacterial antibiotics on the CHX-insensitive incorporation of L-(3H)-leucine into protein in vivo was examined. It is well documented that the mitochondrial ribosome in many eukaryote systems is sensitive to bacterial ribosome inhibitors including CAP, ERY, TET, OLE, LIN, NFO, and PAR but insensitive to CHX (30). The effects of CAP, ERY, and TET, at concentrations which do not affect respiration, on the CHX-insensitive incorporation of L-(3H)-leucine into protein by procyclic forms of T. brucei LIMP 1026 is shown in figure 16. At the low concentrations tested (<100 μg/ml), only TET was found to significantly inhibit incorporation. OLE, LIN, PAR, and NFO also had no inhibitory effect in the concentration range 100-1000 μg/ml (data not shown). The relative lack of effect of all these drugs may be due to in vivo permeability barriers at the level of the whole cell and mitochondrial membrane.

In order to eliminate the possible complicating effects of in vivo permeability barriers, an in vitro mitochondrial translation system was developed employing enriched mitochondrial fractions from T. brucei LIMP 1026. The properties of this in vitro system and the effects of the drugs on the incorporation of L-(3H)-phenylalanine into protein by mitochondria are given in Table VIII. The mitochondrial fraction
is probably contaminated with cytoplasmic ribosomes since the total incorporation is partially sensitive to CHX. The CHX-insensitive incorporation is dependent on the exogenous ATP supply, sensitive to puromycin and relatively independent of added poly U. It is evident that mitochondrial fractions of *T. brucei* LUMP 1026 exhibit a translation activity, the function of which is insensitive to CHX but sensitive to the range of bacterial antibiotics tested. TET, CAP, and PAR appear to be the most potent inhibitors. These inhibitions are not a secondary consequence of a primary inhibition of mitochondrial respiration since the activity is not inhibited by antimycin A and is dependent upon the exogenous ATP supply. The activity does not represent incorporation by contaminating bacteria since the sterile cultures are free of bacteria, the mitochondrial preparations are performed under sterile conditions and the isolated fractions contain <100 bacteria/ml, a level insufficient to produce significant incorporation under our conditions. These preliminary results suggest that mitochondria of *T. brucei* LUMP 1026 possess a translation system showing sensitivity to a similar spectrum of antibiotics as that described for other eukaryotes.
CONCLUSIONS

Our primary accomplishments during this past year are:

a. Established *Trypanosoma brucei* LUMP 1026 *in vitro* as cultured infective trypomastigotes;

b. Established *T. gambiense* TREU 1309 *in vitro* as cultured infective trypomastigotes;

c. Observed the inhibition by SHAM and suramin of the growth of *T. brucei* LUMP 1026 cultured infective trypomastigotes;

d. Identified ubiquinol-1 as an alternate electron donor to the a-CP oxidase;

e. Identified in *T. brucei* LUMP 1026 a translation system showing sensitivity to a similar spectrum of antibiotics as that described for other eucaryotes.

f. Prepared a manuscript on our results describing the biochemical and ultrastructural effects of trypanocidal drugs on *T. brucei* treated *in vivo*. 
References


TABLE I

GROWTH OF *T. brucei* LUMP 1026 ON BOVINE EMBRYONIC TRACHEA CELLS

<table>
<thead>
<tr>
<th>Day</th>
<th>Cell Numbers(^a) ((\text{trypanosomes/ml}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(2.0 \times 10^5)</td>
</tr>
<tr>
<td>1</td>
<td>(5.0 \times 10^5)</td>
</tr>
<tr>
<td>2</td>
<td>(1.1 \times 10^6)</td>
</tr>
<tr>
<td>3</td>
<td>(3.0 \times 10^6)</td>
</tr>
<tr>
<td>4</td>
<td>(2.5 \times 10^6)</td>
</tr>
<tr>
<td>5</td>
<td>(3.0 \times 10^6)</td>
</tr>
</tbody>
</table>

\(^a\) = One-third of the RPMI medium was changed daily.
ATCC CCL 44 EBTr (NBL-4) *(Embryonic trachea, Bovine, *Bos taurus*)

**HISTORY**

The EBTr (NBL-4) cell line was initiated by A.J. Kniazeff, W.A. Nelson-Rees and N.B. Darby, Jr., from the minced, whole trachea of a male fetus (9" long), in April, 1964. The cells have been grown, since origin, in Eagle's MEM with non-essential amino acids, 90%; newborn calf serum, 10%; antibiotic-free.

The cell line is excellent for isolation, replication and quantitation of bovine viral diarrhea (mucosal disease) virus, infectious bovine rhinotracheitis virus, and parainfluenza type 3 virus. When used in passage range 30-55, optimal results were obtained. (Abstr. paper XVI Ann. Meeting T.C A. 1965; Excerpta Medica, Section 1, Vol. 19, Oct. 1965). Although the cell line has been cultivated through the 80th passage, the growth and morphological characteristics of the cells declined beyond the 55th passage. The cell line, therefore should not be considered to be established. The reference seed stock was frozen in December, 1965, at the 25th serial passage.

**DESCRIPTION OF REPOSITORY REFERENCE SEED STOCK**

- **Number of Serial Subcultures from Tissue of Origin:** 25
- **Freeze Medium:** Culture medium, 90%; dimethyl sulfoxide (DMSO), 10%; antibiotic-free.
- **Viability:** Approximately 92% (dye exclusion).
- **Culture Medium:** Minimum essential medium (Eagle), with non-essential amino acids, and Earle's BSS with reduced bicarbonate (0.85 gm per l), 90%; newborn calf serum, 10%, antibiotic-free.
- **Growth Characteristics of Thawed Cells:** An inoculum of 4 x 10^5 viable cells/3 oz. prescription bottle will yield 3.2 x 10^9 cells in 6-7 days when the medium is changed on day 3 or 4 and the pH is maintained at 7.3 - 7.6 in a closed system.
- **Plating Efficiency:** Approximately 9% in the above culture medium.
- **Morphology:** Fibroblast-like.
- **Karyology:** Chromosome Frequency Distribution 70 Cells: 2n = 60

<table>
<thead>
<tr>
<th>Cells</th>
<th>1</th>
<th>1</th>
<th>2</th>
<th>2</th>
<th>2</th>
<th>1</th>
<th>2</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>3</th>
<th>5</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes:</td>
<td>35-41-44</td>
<td>45-48</td>
<td>49</td>
<td>50</td>
<td>51-53</td>
<td>54</td>
<td>55</td>
<td>56</td>
<td>57</td>
<td>58</td>
<td>59</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparations of cells from various passages of this line since its initiation have always revealed a considerable number of hypodiploid cells not believed to be the result of manipulative cellular fragmentation. In about 4% of the cells there now appear neometacentric chromosomes.

- **Sterility Tests:** Free of mycoplasmas, bacteria, and fungi.
- **Species:** Confirmed as bovine by fluorescent antibody (indirect) technique using bovine specific antiserum.
- **Virus Susceptibility:** Susceptible to bovine viral diarrhea, infectious bovine rhinotracheitis, parainfluenza type 3, herpes simplex, reovirus type 3, vaccinia, vesicular stomatitis (Ogden Strain) viruses. Not susceptible to human adenovirus type 5, poliovirus type 2, and Coxsackie A-3 and B-5.

Submitted by: A.J. Kniazeff, W.A. Nelson-Rees and N.B. Darby, Jr., University of California, School of Public Health Naval Biological Laboratory, Berkeley, California.

Prepared and characterized by: University of California Naval Biological Laboratory, Berkeley, California.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Trypanosomes x $10^6$/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>Bloodstream Trypomastigotes in whole blood</td>
<td>1.5</td>
</tr>
<tr>
<td>Bloodstream Trypomastigotes separated from red blood cells</td>
<td>1.8</td>
</tr>
<tr>
<td>Bloodstream Trypomastigotes separated from red blood cells and whole blood added</td>
<td>2.1</td>
</tr>
<tr>
<td>Cultured Infective Trypomastigotes</td>
<td>1.7</td>
</tr>
<tr>
<td>Cultured Infective Trypomastigotes with whole blood added</td>
<td>2.1</td>
</tr>
</tbody>
</table>
TABLE IV

Effect of SHAM on Growth of *T. brucei*

Cultured-Infected Trypomastigotes

<table>
<thead>
<tr>
<th>Concentration of SHAM</th>
<th>(trypomastigotes/ml) Group 1</th>
<th>(trypomastigotes/ml) Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$8.9 \times 10^6$</td>
<td>$8.9 \times 10^6$</td>
</tr>
<tr>
<td>0.01 mM</td>
<td>$7.6 \times 10^6$</td>
<td>$7.5 \times 10^6$</td>
</tr>
<tr>
<td>0.05 mM</td>
<td>$8.1 \times 10^5$</td>
<td>$9.2 \times 10^5$</td>
</tr>
<tr>
<td>0.10 mM</td>
<td>$6.5 \times 10^5$</td>
<td>$2.0 \times 10^5$</td>
</tr>
</tbody>
</table>

All flasks were inoculated at $0.5 \times 10^6$ trypanosomes/ml. The counts given are after 5 days of growth. One third of the medium was changed daily, and fresh media was added containing the appropriate concentration of drug.
### TABLE V

Effect of Suramin on Growth of *T. rhodesiense* (EATRO 1895) Cultured-Infective Trypomastigotes

<table>
<thead>
<tr>
<th>Concentration of Suramin (M x 10^{-9})</th>
<th>Growth (trypanosomes/ml)</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.0 x 10^6</td>
<td>2.4 x 10^6</td>
</tr>
<tr>
<td>3.5</td>
<td></td>
<td>1.2 x 10^6</td>
<td>1.4 x 10^6</td>
</tr>
<tr>
<td>7.1</td>
<td></td>
<td>0.7 x 10^6</td>
<td>0.5 x 10^6</td>
</tr>
<tr>
<td>14.2</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

All flasks were inoculated at 0.5 x 10^6 trypanosomes/ml. The counts given are after 48 hours of growth. One third of the medium was changed daily, and fresh media was added containing the appropriate concentration of drug.
TABLE VI

Effect of Various Substrates as Electron Donors to
L-α-Glycerophosphate Oxidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GP</td>
<td>1.00</td>
</tr>
<tr>
<td>Ubiquinol-1</td>
<td>4.00</td>
</tr>
<tr>
<td>Menadiol</td>
<td>0.33</td>
</tr>
<tr>
<td>Coenzyme Q₁₀</td>
<td>0.28</td>
</tr>
<tr>
<td>Plastoquinol-1</td>
<td>0.00</td>
</tr>
</tbody>
</table>
TABLE VII

Properties of the Cytoplasmic S30 Incorporation System from *T. brucei*

<table>
<thead>
<tr>
<th>System</th>
<th>% of Control Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>- ATP</td>
<td>7</td>
</tr>
<tr>
<td>- Poly U</td>
<td>92</td>
</tr>
<tr>
<td>+ Puromycin (1mM)</td>
<td>25</td>
</tr>
<tr>
<td>+ Antimycin A (10 μg/ml)</td>
<td>103</td>
</tr>
</tbody>
</table>

*Control Incorporation: 6544 DPM $^3$H-Phenylalanine/mg protein/10'

Washed procyclic trypomastigotes were lyed by grinding with glass beads, the lysate was extracted with SIVMK buffer (0.25 M Sucrose, 25 mM Hepes, pH 7.7 at 4°C, 5 mM Mg Acetate, 150 mM KCl, 6 mM 2-mercaptoethanol, 3 mg Heparin/ml) and centrifuged at 30,000g for 35 min. This supernatant (S30) was dialysed for 5 hrs. at 4°C against 1000 volumes of SIVMK buffer and stored at -70°C. The incorporation system was modified from that described by Lamb et al. (31), employing 0.1 ml reaction volumes containing L-($^3$H)-phenylalanine (20mCi/ml; 3.72 Ci/m mole), 100 mg polyuridylic acid/ml, and 0.02 ml of S30 extract. Incorporated radioactivity was determined as described in the legend to Fig.11A.
### TABLE VIII

**Properties of In Vitro Amino Acid Incorporation System with Isolated Mitochondria of T. brucei**

<table>
<thead>
<tr>
<th>Complete System</th>
<th>% Inhibition of CHX-Insensitive Incorporation *</th>
</tr>
</thead>
<tbody>
<tr>
<td>- ATP</td>
<td>79</td>
</tr>
<tr>
<td>- Poly U</td>
<td>16</td>
</tr>
<tr>
<td>+ Puromycin (1mM)</td>
<td>84</td>
</tr>
<tr>
<td>+ Antimycin A (10 μg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>+ CAP (0.01 - 1mM)</td>
<td>28 - 55</td>
</tr>
<tr>
<td>+ ERY</td>
<td>20 - 43</td>
</tr>
<tr>
<td>+ TET</td>
<td>24 - 77</td>
</tr>
<tr>
<td>+ PAR</td>
<td>6 - 48</td>
</tr>
<tr>
<td>+ NEO</td>
<td>13 - 42</td>
</tr>
<tr>
<td>+ OLE</td>
<td>13 - 45</td>
</tr>
<tr>
<td>+ LIN</td>
<td>15 - 32</td>
</tr>
</tbody>
</table>

*CHX-Insensitive Incorporation: 5424 DPM $^3$H-Phenylalanine/mg protein/15', which is 84% of the control rate.
Legend for Table VIII. Properties of the in vitro mitochondrial amino acid incorporation system isolated from T. brucei LUMP 1026. Washed procyclic trypomastigotes were lysed by grinding with glass beads, the lysate was extracted with MHEM buffer (0.6M Mannitol, 20mM Hepes, pH 7.7 at 4°C, 1mM Na EDTA, 2.5 mM Mg Acetate containing 0.15% w/v BSA) and an enriched mitochondrial fraction was prepared by differential centrifugation. The incorporation system was modified from that described by Groot Obbink et al. (32) employing L-(3H)-phenylalanine (10 mCi/ml; 1.93 Ci/mmmole), 134 mg polyuridylic acid/ml and 0.6-2 mg mitochondrial protein/ml. Certain antibiotics (ANA, CAP, ERY, TET, OLE, LIX), were added as DMSO solutions. The final DMSO concentration (1% v/v) had no effect on the control incorporation. The incorporation insensitive to cycloheximide (1000 mg/ml) (5424 dpm L-(3H)-phenylalanine/mg protein/35 min) was determined as described in the legend to Fig.11A and represents 84% of the control incorporation in the absence of antibiotics.
FIGURE LEGENDS

Fig. 1. The site of action of various inhibitors on the nucleo-cell sap system and the mitochondrial system.

Fig. 2. *T. brucei* LUMP 1026 cultured infective trypomastigotes growing on bovine embryonic trachea tissue culture cells. The trypanosomes were free-swimming in the medium and had been growing in culture for 50 days. X200.

Fig. 3. *T. brucei* LUMP 1026 cultured infective trypomastigotes growing in the spaces between the bovine embryonic trachea tissue culture cells. The trypanosomes had been growing in culture for 50 days. X200.

Fig. 4. Electron micrograph of *T. brucei* LUMP 1026 cultured infective trypomastigotes which have been grown on bovine embryonic trachea tissue culture cells for 50 days. Note the presence of the surface coat. X45,000.

Fig. 5. Electron micrograph of *T. brucei* LUMP 1026 procyclic trypomastigotes which have been transformed from bloodstream trypomastigotes in Cunningham's (SM) media. Note the absence of the surface coat. X45,000.

Fig. 6. *T. gambiense* TREU 1309 cultured infective trypomastigotes growing in the spaces between the bovine embryonic trachea tissue culture cells. The organisms had been growing in culture for 25 days. X200.

Fig. 7. Electron micrograph of *T. gambiense* TREU 1309 cultured infective trypomastigotes which had been grown on bovine embryonic trachea tissue culture cells for 25 days. Note the presence of the surface coat. X45,000.
Fig. 8. Transformation experiment with *T. brucei* LUMP 1026. The initial inoculum was from a rat at $2.1 \times 10^8$ trypanosomes/ml and a day 8 parasitemia. By morphological criteria, transformation from bloodstream trypomastigotes to procyclic trypomastigotes was complete within 72 hours.

Fig. 9. Relationship of various $\alpha$-glycerophosphate enzymes present in African trypanosomes. The various enzymes identified are: (1) glyceraldehyde-3-phosphate dehydrogenase; (2) triosephosphate isomerase; (3) NAD$^+$-linked (+) $\alpha$-glycerophosphate dehydrogenase; (4) L-$\alpha$-glycerophosphate dehydrogenase; (5) L-$\alpha$-glycerophosphate oxidase.

Fig. 10. Comparison of L-$\alpha$-GP and ubiquinol-1 as substrates for the aGP oxidase.

Fig. 11A. Time course of incorporation of L-($^3$H) leucine into protein by procyclic trypomastigotes of *T. brucei* LUMP 1026. Washed cells were pre-incubated for 10 min. (with or without antibiotics) at 25°C in 0.1 ml of Locke's solution (0.9% w/v NaCl, 0.02% w/v CaCl$_2$, 0.03% w/v KCl, 0.02% w/v NaHCO$_3$ containing 0.5% v/v glycerol, 0.5% w/v glucose, 5% v/v fetal bovine serum) at a cell density of $1 \times 10^8$ ml and then chilled on ice. L-($^3$H)-leucine (30 µCi/ml, 65 Ci/m mole) was added and the cells were incubated at 25°C for the indicated times. The incubation was terminated by addition of 3 ml of ice-cold TCA-Leu (or TCA-Phe, for incubations incorporating L-($^3$H)-phenylalanine) solution (5% w/v TCA containing 1 mg leucine or phenylalanine/ml). The precipitated protein was heated at 95°C for 20 min., chilled, collected onto glass fiber filters, washed with three, 1 ml portions of cold TCA-Leu or TCA-Phe, dried and placed in glass scintillation vials. Radioactivity was extracted from the filter by a 3 hour incubation at 50°C with Protosolv (0.5 ml), dissolved in 5 ml of Econofluor and determined in a Beckman Liquid Scintillation Spectrometer employing channel ratio quench correction. The efficiency for tritium was 30%. 
Fig. 11B. Effect of cycloheximide (CHX) on the incorporation of L-(\(^3\)H)-leucine into protein by procyclic trypomastigotes of \(T.\) \(brucei\) LUMP 1026. Incorporation was determined in 0.1 ml reaction volumes as described above (Part A). The control incorporation was 373,760 dpm/10\(^8\) cells/10 min.

Fig. 12. Effect of antibiotics on the incorporation of L-(\(^3\)H)-leucine into protein by procyclic trypomastigotes of \(T.\) \(brucei\) LUMP 1026. Incorporation was determined over a 10 min. time period as described in the legend to Fig. 11A. Antibiotics were added as dimethyl sulfoxide (DMSO) solutions such that the final DMSO concentration was 2% v/v. This concentration of DMSO has no inhibitory effect on the control incorporation. Control incorporation in several experiments were in the range 85–530,000 dpm/10\(^8\) cells/10 min. CAP, D-chloramphenicol; ERY, erythromycin; TET, tetracycline; CHX, cycloheximide.

Fig. 13. Effect of antibiotics on the incorporation of L-(\(^3\)H)-phenylalanine into protein by the cytoplasmic S30 incorporation system from \(T.\) \(brucei\) LUMP 1026. Incorporation was determined as described in the legend to Table VII. Antibiotics were added as DMSO solutions such that the final DMSO concentration was 1% v/v. This DMSO concentration had no inhibitory effect on the control incorporation (6544 dpm/mg protein/10 min).

Fig. 14. Effect of antibiotics on the rate of total respiration by procyclic trypomastigotes of \(T.\) \(brucei\) LUMP 1026. Washed cells (0.3–2 x 10\(^8\)/ml) were incubated at 25\(^\circ\)C in the Locke's glycerol-glucose-fetal bovine serum solution employed for the protein synthesis studies (legend Fig. 11A). The rate of oxygen consumption was determined polarographically in a 3 ml incubation cuvette using a Clark-type oxygen electrode.
Following addition of cells to the curvette, the initial rate of oxygen consumption was determined over a 4 min. time period. Antibiotics were then added, as DMSO solutions, to give the final concentrations indicated in the figure and the rate of oxygen consumption was determined after a further 20 min. incubation. Thus, the effects of antibiotics on protein synthesis (Fig. 12) and respiration are both determined after a 20 min. exposure of the cells to the drugs. The effect of an antibiotic is expressed as: (the test respiration rate at 20 min./control (+DMSO) respiration rate at 20 min.) x 100. The final DMSO concentrations (1 or 2% v/v) had a slight inhibitory effect on the rate of respiration. The control respiration rates in several experiments were in the range 12.3 - 15.0 nmoles O$_2$ consumed/min/10$^8$ cells.

**Fig. 15.** Effect of antimycin A on the rate of total respiration and the incorporation of L-(3H)-leucine into protein by procyclic trypomastigotes of *T. brucei* LUMP 1026. Determinations were carried out as described in the legends to Fig. 11A (protein synthesis) and Fig. 14 (respiration). The control activities in the absence of antimycin A were: for protein synthesis, 202,654 dpm incorporated/10$^8$ cells/10 min.; for respiration 17.1 nmoles O$_2$ consumed/min/10$^8$ cells.

**Fig. 16.** Effect of antibiotics on the cycloheximide-insensitive incorporation of L-(3H)-leucine into protein by procyclic trypomastigotes of *T. brucei* LUMP 1026. Washed cells were preincubated for 10 min. at 25°C in the presence of cycloheximide (1000 μg/ml) and the indicated concentration of antibiotic. Following addition of L-(3H)-leucine (30 μCi/ml), incubation was continued for a further 20 min. at 25°C, terminated and the incorporated radioactivity was determined as...
Fig. 16. described in the legend to Fig. 11A. The incorporations in the presence of cycloheximide alone in several experiments were 22,688 - 24,846 dpm L-($^3$H)-leucine incorporated/10^8 cells/20 min. which represents 3-5% of the control incorporation in the absence of drugs.
Figure 1
Figure 8

T. brucei Lump 1026 TRANSFORMATION

TRYPANOSOMES x 10^6/ml OF CULTURE

PERCENT TRANSFORMATION

HOURS

0 24 48 72 96 120

0 10 20 30 40 50 60 70 80 90 100
Figure 10

nmol O₂/min/mg protein

10mM α-GP

Enzyme

1mM SHAM

0.5mM UQ, H₂

512

12

20

This figure shows a diagram with various conditions and measurements related to oxygen production.
Time Course of $^3$H-Leucine Incorporation and Effect of Cycloheximide on in Vivo Protein Synthesis by T. brucel

**Figure 11**
Effect of Antibiotics on In Vivo Respiration by T. brucel

Figure 14
In Vivo Respiration and Protein Synthesis by T. brucel

Effect of Antimycin A on

- Respiration
- Protein Synthesis

Figure 15
Effect of Antibiotics on Cycloheximide-Insensitive
In Vivo Protein Synthesis by *T. brucella*

**Figure 16**
ATE
ME