931-1157

PD-AAQ-879

ISN 28791

ACTION MEMORANDUM

TO : AA/DS, Dr. E. J. Long

THRU : DS/PPU, Robert Simpson RS

FROM : DS/AGR, Leon F. Hesser

SUBJECT: Small Research Project - New York University Medical Center Proposal on an Approach to Chemotherapy for Trypanosomiasis Control

The purpose of this research is to learn whether trypanosomiasis (sleeping sickness) can be controlled by interferring with the carbohydrate metabolism system of the parasite. If this is successful, a new class of chemotherapeutic agents will be found. AID is being asked to fund half of the cost of the research, while the New York University Medical Center will fund the other half.

This research presents exciting possibilities to support AID's interests in controlling animal and human trypanosomiasis. The concept of eliminating or suppressing hemoparasites by administering chemotherapeutics is not unique, but the research that led to the submission of this proposal offers promise of new types of agents for the control of these diseases.

To date, the research results indicate: (1) low levels of toxicity to the host (a characteristic not generally noted with other trypanocidal or trypano-suppressant drugs), (2) effect on the morphology of the trypanosomes in vivo (It is anticipated that this latter characteristic may lead to the development of an immunogenic strain of pathogenic trypanosomes); and (3) the development of a new class of chemotherapeutic agents more effective against the disease. The preliminary work at the New York University Medical Center is a logical research approach in that it utilizes the most inexpensive screening system possible, i.e. laboratory animals, to determine applicability to the disease syndrome in natural hosts.

This proposal has been discussed with ILRAD and with the Washington State University personnel and both express interest in this approach. Discussion between researchers at New York University Medical Center and ILRAD indicate possible areas of complementarity. It has been suggested that the trypano-suppressant effect may be useful in development of a modified live vaccine, or if a vaccine is developed which might produce an adverse reaction that this material may ameliorate that effect. The matter has been discussed with AID/DS/H in relation to the human implications and their response is favorable to this research. The New York University Medical Center agrees to fund 50 percent of the costs and has its share of the funds already allocated.

Presently used chemotherapeutic material for trypanosomiasis control or treatment is expensive and relatively toxic. This material, if effective, will be much less costly and will probably be non-toxic.

DS/AGR considers that this project has good probability for a high payoff in the control of trypanosomiasis and at very low costs. Therefore, it is proposed that you approve the participation of A.I.D. in this research at a level of $25,000, which represents 50 percent of the total cost.

APPROVED: Caroline D McGraw

DISAPPROVED: _____

DATE: Jan 26, 1978

Clearances:

DS/AGR, NKonnerup	/S/ NK
DS/AGR, NSRaun	/S/ NK
DS/AGR, KBrundage	/S/ NK
DS/AGR, FWilliams	/S/ NK
DS/PPU, JEricson	[illegible]
DS/AGR, LHoward	/S/ NK

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Title: Carbohydrate Biochemistry of Trypanosomes and Inhibition of Energy Production by Iron Chelation and Glycerol as an Approach to Chemotherapy

Start Date: July 1, 1977

Co Principle Investigators: Allen B. Clarkson, Jr. PhD.

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Application Approvals:

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Head, Division of Parasitology

Dr. Milton Salton, PhD.
Chairman, Department of Microbiology

Dr. Ivan L. Bennett, Jr.,
Provost and Dean
New York University Medical Center

Carbohydrate Biochemistry of Trypanosomes and Inhibition of Energy Production by Iron Chelation and Glycerol as an Approach to Chemotherapy

Introduction

This proposal which is submitted to the AID for consideration for a Small Research Grant award is, in essence, the same proposal we submitted last fall from the Rockefeller University through Dr. William Trager. We resigned our temporary positions at Rockefeller on December 31, 1976 and accepted permanent positions on the faculty of the New York University School of Medicine on January 1, 1977. As we were the only investigators involved in this project at Rockefeller, Dr. Trager withdrew the previous application. We are, however, continuing our work together at NYU and following suggestions by Dr. Trager and by Dr. Konnerup of AID, we are resubmitting from NYU an application for support from AID.

The wording of this proposal is somewhat different than the previous application which reflects progress in our work and a better focus on our goals. A reprint of a paper which has appeared after our original proposal and a preprint of a recently finished manuscript are attached to this proposal.

Background

Veterinary trypanosome infections are presently most often treated by ethidium or berenil. A major reason is that infections resistant to one have never been found to be resistant to the other. Resistant strains do show cross resistance among the group of drugs represented by berenil and other strains to a group of drugs represented by ethidium. There has been as yet no report of an infection showing cross resistance between these groups, but this may be only a matter of time. If such a cross resistant strain arose, the diligent use of drugs would result in widespread dissemination of the resistant strain. Programs implemented to aid developing countries to control trypanosomiasis will increase the likelihood of the appearance and the spread of such highly resistant organisms. The consequences are obvious.

Human and veterinary trypanosomiases are inexorably linked and programs to control one without the other are short-sighted, especially in regard to real aid for endemic areas. A similar condition exists for medical drugs as for veterinary. To meet crises caused by human trypanosome strains resistant to drugs approved for human use, berenil is used to treat patients. These people would probably die without berenil, but berenil interacts strongly with DNA and may be mutagenic and carcinogenic. This property of berenil, combined with reports of prophylactic activity in cattle, (which implies the strong possibility of residual drug in meat and dairy products), underlines the precarious situation of the present state of chemotherapy for trypanosomiasis.

The standard phrase, that no new drugs for these diseases have been developed for twenty years, bears repetition. Most drug research has followed the procedure of screening thousands of chance compounds for activity. This is followed and combined with chemical modification of those compounds found to be active in order to improve their action. This empirical approach has produced what we have, but the well seems to be drying up. Another, sometimes called rational, approach is to study the disease-causing organism to understand its metabolism and develop selective inhibitors for pathways either unique to the pathogen or which are more sensitive or critical to the pathogen than the host. Recent improvements in techniques to work with trypanosomes and biochemical techniques in general should improve the probability of success.

We have enclosed a reprint of a recent Science paper and a preprint of a second paper. These describe our discovery of the therapeutic value of two agents, SHAM and glycerol, for trypanosomiasis. They themselves do not represent a practical drug combination, but clearly point the way for development of an entirely new class of trypanocidal agents. As SHAM and glycerol block the peculiar form of glycolysis unique to trypanosomes, this is an excellent example of such a rational approach.

Proposed Research

There are several lines of further research we plan to follow. These are briefly outlined below.

A. The action of SHAM is to block the aerobic glycolytic pathway. This forces the parasites to shift to an anaerobic system which can be blocked by glycerol. The combination stops energy production and leads to destruction of the parasites.

The anaerobic pathway is unknown. Glycerol is not a good chemotherapeutic agent as its activity is so short. We need to define the anaerobic pathway to be able to rationally design a substitute for glycerol. We are not even entirely sure at present how glycerol operates.

B. SHAM, while active in blocking the aerobic pathway, is not the best agent. We have already found an analogue, m-bromobenzhydroxamate, with three times the activity in an *in vitro* assay. Fortunately, we are able to measure the activity of these inhibitors both rapidly and with a high degree of precision. We plan to continue screening commercially available compounds for activity and collaborating with Dr. Robert Grady at Rockefeller, an organic chemist who has synthesized many chemical variants of hydroxamic acids.

C. We intend to determine if SHAM-glycerol is curative in a more suitable model system than rats infected with *Trypanosoma brucei*. The rat model is convenient and reproducable, but *T. brucei* kills in three days. This is unlike natural infections in which the host is able to show some resistance so the untreated disease lasts for several months before death. We plan to use a rabbit-*T. brucei* model which produces a more chronic infection.

D. Although SHAM alone has been shown by other investigators to be ineffective, the experiments are not conclusive. SHAM alone reduces the intracellular ATP level by 50%. If SHAM or a more effective analogue were present for several months instead of a few hours, a naturally infected animal might have all the advantage required to overcome the infection. Indeed, this would be analogous to an *in vivo* attenuation of the parasite which could lead to an acquired immunity.

Careful management of infection and treatment with existing drugs has been shown to produce immunity in field trials. The techniques required, however, make this approach not practical with existing drugs. The principle has, however, been demonstrated.

Any trypanocidal drug administered at sub-curative levels would theoretically produce an attenuation. This dose would have to be carefully monitored in the case of a potentially curative drug to keep the population suppressed and not eliminated. In the case of SHAM and its analogues, there could be no drug cure; the final cure would be by potentiation of the natural immune response.

Any drug given in subcurative doses produces conditions ideal for development of drug resistance. The best evidence for the mechanism of SHAM is that it chelates iron at the active site of an enzyme. SHAM does not seem to be actively transported into the cell. These two conditions make the development of drug resistance very unlikely. If iron is required by the parasite, SHAM will always be able to chelate it. If the parasite develops a means to degrade SHAM, new analogues could be developed.

To develop a long term effective SHAM analogue, the following steps will be required:

(a) Define the exact chemical groups required for activity. We are presently involved in this; (b) Modify the compound to increase retention time; and (c) Utilize slow release technology to extend the activity period.

A rabbit model with regular injections, or the use of commercially available implant pumps, will allow an evaluation of the probable outcome before full development from (a) through (c) above. We plan to begin this evaluation almost immediately.

In addition to our work on the final steps of aerobic and anaerobic glycolysis, we are collaborating with Dr. Cyrus Bacchi at Pace University. He is working on a different enzyme involved in glycolysis and attempting to exploit peculiarities in polyamine activation of this enzyme for a chemotherapeutic approach. We have clearly similar but separate interests and each group should strengthen the other.

BUDGET:

Present sources of support:

1) The NYU Medical Center is presently supporting the salaries of Drs. Clarkson and Brohn.

2) The Rockefeller Foundation has granted $33,000 for the period of 12/1/76 to 11/30/77 which has been used primarily to provide such basic equipment as a cold room, high speed centrifuge, water purification system and animal care technical help.

Proposed sources of support:

An application will be submitted for Institutional Basic Research Support (for a maximum of $10,000). If awarded this will cover a few small equipment items such as a top loading balance, microscope objectives and additional animal cages.

Request for AID support:

Personnel:

Technician, 100% time (104). This person will aid in the preparation of parasite material as well as biological and biochemical analyses.	$12,000
Fringe Benefits (19.5%)	2,340
Total	14,340

Animals:

Rats - 600 at 2.50	1,500
Rabbits - 50 at 16.00	800
Food and bedding	500
	2,800

Chemicals and small supply items:	960
Direct Costs	18,100
Indirect Costs (57.5% S and W)	6,900
GRAND TOTAL	$ 25,000

Justification of Personnel.

Salary increments included in future budget years are based on estimated percentages including merit and inflationary factors. Percentages applied vary depending on job descriptions, time and grade, etc. for each employee. Actual salaries to be paid in future years must be in agreement with the official wage and salary program consistently applied at NYU Medical Center.

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SCIENCE

Trypanosomiasis: An Approach to Chemotherapy by the Inhibition of Carbohydrate Catabolism

Allen B. Clarkson, Jr. and Frederick H. Brohn

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Trypanosomiasis: An Approach to Chemotherapy by the Inhibition of Carbohydrate Catabolism

Abstract. *When the infected mammalian host of* Trypanosoma brucei brucei *is injected with a solution of the iron chelator salicyl hydroxamic acid and glycerol, the aerobic and anaerobic glucose catabolism of the parasite is blocked and the parasite is rapidly destroyed.*

The protozoans *Trypanosoma brucei rhodesiense* and *T. brucei gambiense* are the causative agents of African human sleeping sickness, and the closely related *T. brucei brucei* is one of several tyrpanosomes that cause a similar disease in cattle. All three parasites are transmitted by the tsetse fly vector to a mammalian host (*1*). No new effective drugs against these diseases have been developed in recent years, and resistance of the parasites to most of the drugs now in use has been reported (*2*). We are able to block both aerobic and anaerobic pathways of adenosine triphosphate (ATP) production in these parasites by the simultaneous administration of salicyl hydroxamic acid (SHAM) and glycerol to the host. Because the production of energy in the form of ATP is entirely dependent on carbohydrate metabolism (*3*), the parasites are immediately immobilized and almost entirely cleared from the blood within 5 minutes.

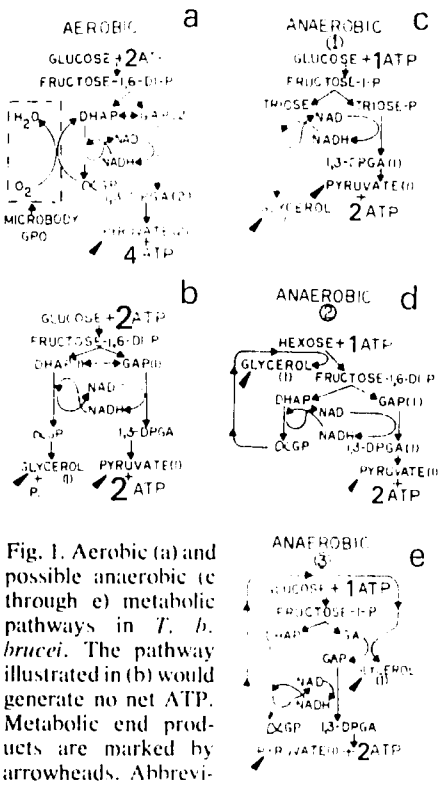

Fig. 1. Aerobic (a) and possible anaerobic (c through e) metabolic pathways in *T. b. brucei*. The pathway illustrated in (b) would generate no net ATP. Metabolic end products are marked by arrowheads. Abbreviations: ATP, adenosine triphosphate; fructose-1,6-di-P, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; αGP, L-α-glycerophosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; GPO, L-α-glycerophosphate oxidase; fructose-1,6-di-P, fructose-1,6-diphosphate; Pi, inorganic phosphate; fructose-1-P, fructose-1-phosphate; GA, glyceraldehyde.

Carbohydrate metabolism of the trypanosome in the insect vector is complete—that is, it includes glycolysis, a tricarboxylic acid (TCA) cycle, and reoxidation of coenzymes by cytochromes in the kinetoplast-mitochondrion (*4*). In contrast, only glycolysis occurs in the long slender (LS) form, the principal stage of the parasite in the bloodstream of the mammal. The other stage in mammals, the short stumpy (SS) form, develops in the bloodstream from the LS form. The SS forms are believed to be preadapted to the insect vector and have a partial TCA cycle but no functional cytochromes (*3*). It is the LS forms that divide in the mammal and probably account for the pathology of the disease. Evidence indicates that the SS forms are at an end point in the mammal and do not reproduce unless taken up by the vector (*5*). Parasitemias in mammals infected with these organisms differ in their ratios of LS forms to SS forms, the ratios varying with the strain of the trypanosome and the stage of infection, even in cloned populations of parasites. To diminish this variation and to concentrate on the LS form, we used a clone of *T. brucei brucei* that was isolated from EATRO (East African Trypanosomiasis Research Organization) strain 110 and made monomorphic (LS form only) by 30 rapid pas-

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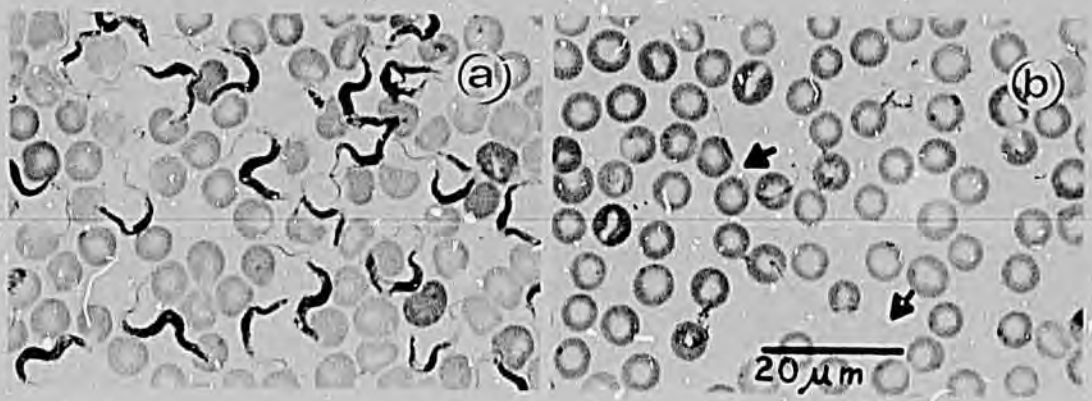

Fig. 2. Giemsa-stained blood film of a rat infected with *T. b. brucei*. (a) Before treatment. (b) Three minutes after treatment. Lysed trypanosome debris is evident and two flagella with attached kinetoplasts are marked with arrows.

sages in rats. In experiments with *T. brucei rhodesiense*, we used EATRO strain 1895 which produces both LS and SS forms.

No lactate is produced by the LS forms of either *T. b. brucei* or *T. b. rhodesiense*, and pyruvate is the chief end product of glucose catabolism. The highest reported yield of pyruvate generated from glucose by *T. b. brucei* is 92 percent (*6*). Although this is better than the previous high value of 83 percent, which was obtained from apparently rapidly deteriorating cells (*7*), we find that under the proper conditions the true conversion of glucose to pyruvate is 100 percent. By using a more suitable phosphate-saline buffer and better means of separating the protozoans from host blood cells (*8*), we are able to maintain the parasites in good morphological condition in vitro for at least 12 hours, and glucose consumption is linear over a 4-hour period at 25°C. Using Sigma glucostat reagent for glucose and the change in absorbance at 340 nm in the presence of excess oxidized nicotinamide adenine dinucleotide (NAD+) with lactate dehydrogenase added to measure pyruvate, we found that a suspension of the LS forms of *T. b. brucei* (2.5×10^8 cells per milliliter) consumed 4.06 ± 0.3 μmole ($\pm$ S.E.) of glucose and produced 8.15 ± 0.20 μmole of pyruvate per hour. These values were constant over a 3-hour period. Aeration was maintained by constantly bubbling air through the cell suspension. We conclude that in the LS forms, pyruvate is the only end product of glycolysis in the presence of adequate molecular oxygen.

Reoxidation of reduced nicotinamide adenine dinucleotide (NADH) produced by glycolysis is required for continuous energy production. However, this is not accomplished by either lactate dehydrogenase or cytochromes in the organisms but by a glycerophosphate oxidase system (*9*) which is localized in the microbody organelles (*10*) (Fig. 1a). The pathway shown in Fig. 1a requires the presence of molecular oxygen; however, both the LS and SS forms of these parasites can survive anaerobically for several hours. The end products of cell metabolism under anaerobic conditions are primarily pyruvate and glycerol in equimolar amounts (*6, 7*). To account for both the stoichiometry of these end products and the reoxidation of NADH formed under anaerobic conditions, it has been proposed (*6, 11*) that cells regenerate NAD+ from NADH by reducing dihydroxyacetone phosphate to L-α-glycerol phosphate (αGP), and subsequently degrade the αGP, which is not reoxidized anaerobically, to glycerol and orthophosphate (Fig. 1b). It has been pointed out most clearly by Opperdoes *et al.* (*6*) that because this pathway will generate zero net ATP it cannot possibly account for anaerobic glycolysis in these organisms.

We have reached somewhat the same conclusions reported by Opperdoes *et al.* (*6*). Hexose monophosphate aldolase activity could reduce ATP input and yield both a triose and a triose phosphate. The triose phosphate could yield two molecules of ATP per mole with the triose reduction to glycerol mediated by way of NADH (Fig. 1c). Aldolase activity with fructose-1-phosphate is present; however, we cannot detect triose reductase activity. It is also possible that αGP is used to transphosphorylate a hexose, thus reducing the ATP input requirement and yielding net positive ATP production (Fig. 1d). A third possibility—one not previously considered—is that a monophosphate aldolase produces a triose and a triose phosphate, the triose phosphate being reduced by NADH to αGP and the αGP used to transphosphorylate the triose. This would generate glycerol and a second triose phosphate which could yield two molecules of ATP (Fig. 1e).

Notwithstanding uncertainties concerning the pathway of anaerobic glycolysis in *T. b. brucei*, if aerobic and anaerobic glycolysis formed the only basis of energy production in these cells, simultaneous inhibition of these activities should result in cell death. All of the anaerobic schemes discussed here include glycerol as an end product of fermentation in a reaction that would involve a small negative change in Gibbs free energy (ΔG or ΔF) or a slightly positive ΔG, depending on the microenvironment. If this were the case, glycerol should block the alternate pathway by mass action. Glycerophosphate oxidase function is blocked by the lack of molecular oxygen and also by several hydroxamic acids, including SHAM (*6, 12*). We have found that 1 m*M* SHAM in the presence of 2.5 m*M* glycerol causes immediate loss of motility of *T. b. brucei* in vitro, and within 3 minutes extreme alteration and damage to the cells is visible by phase contrast microscopy.

The effect of SHAM and glycerol on the parasites in vivo was tested by using rats heavily infected with *T. b. brucei* (1.2×10^9 parasites per milliliter of blood). After injecting intravenously (5 ml per kilogram of body weight) a solution of SHAM (125 m*M*) and glycerol (600 m*M*) (96 and 276 mg/kg, respectively), all the parasites were immobilized within 1 minute, and no intact motile parasites and few ghosts were observed after 3 minutes. All of the uninfected control animals treated with six times the amount of SHAM and glycerol given the experimental animals survived.

A Giesma-stained blood smear of a rat infected with *T. b. brucei* (1.5×10^9 cells per milliliter) is shown in Fig. 2a. Blood taken from the same rat 3 minutes after we injected into the tail vein both SHAM and glycerol (182 mg/kg and 552 mg/kg, respectively) is shown in Fig. 2b. Only a few whole parasites are visible and these are clearly abnormal. Many flagella, some apparently with the kinetoplast portion of the kinetoplast-mitochondrion still attached, and what appeared to be parasite nuclei, were seen in the blood

from treated animals. No parasites or parasite debris were seen 24 hours after treatment.

Mice that were infected with *T. b. rhodesiense* and that showed both the LS forms and SS forms of the parasite in the blood were also injected with SHAM and glycerol. One hour after treatment only a monomorphic SS form was present in the blood, and after 24 hours no parasites were observed.

Six days after the treatment of rats and mice infected with *T. b. brucei*, the parasites reappeared in the blood and the animals died within a few days. Without treatment, the animals would have died within a few hours. Mice infected with *T. b. rhodesiense* also showed a recurrence of parasitemia after treatment. We have found no regimen of treatment that prevents the recurrence of parasitemia, although the response of the parasite population to successive treatments remains the same and the parasites do not appear to become resistant to the treatment. Two possible explanations for the recurrence are (i) the entire population of parasites contains a few resistant cells with a partial TCA cycle, and (ii) the effective trypanocidal levels of SHAM and glycerol are not reached in some tissues and parasites in those tissues survive. The second hypothesis seems more probable, because temporal separation of SHAM and glycerol administration by 5 minutes abolishes their therapeutic value, suggesting that they are cleared very rapidly from the blood. By finding a substitute for glycerol or a different method of administration, it might be possible to maintain therapeutic levels of the drugs long enough for all infected tissues to be reached. Similarly, other hydroxamic acids or iron chelators might be more effective in blocking the activity of glycerophosphate oxidase.

The results presented here promise a rational approach to trypanosome chemotherapy based on knowledge of the peculiar carbohydrate catabolic pathways of these parasites.

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13. We thank A. Balber and C. Patton for sharing their observation that in the presence of SHAM *T. b. brucei* glucose transport is inhibited by glycerol causing loss of motility and cell death in vitro. We thank M. R. Rifkin for her cloned isolate of *T. b. brucei* EATRO strain 110 from which we derived the monomorphic strain, and W. Trager, D. M. Dwyer, and P. A. D'Alesandro for their encouragement and criticism. This work was supported by training grant AI-00192 from the U.S. Public Health Service.

21 May 1976; revised 6 July 1976

QUANTITATIVE EFFECTS OF SALICYLHYDROXAMIC ACID AND GLYCEROL ON *TRYPANOSOMA BRUCEI* GLYCOLYSIS *IN VITRO* AND *IN VIVO*.

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and

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INTRODUCTION

The mammalian stage of the life cycle of African trypanosomes is primarily dependent on glycolysis for adenosine triphosphate (ATP) production. The brucei subgroup, which includes those forms that infect man and also several animals, is exclusively dependent on glycolysis. They produce pyruvate from glucose under aerobic conditions and pyruvate and glycerol in equimolar amounts under anaerobic conditions. There are at least two points in these pathways which are radically different from host metabolism. We have reported (1,2) that salicylhydroxamic acid (SHAM) and glycerol inhibit this glycolysis *in vitro* and that simultaneous administration of SHAM-glycerol rapidly clears these parasites from the blood of infected rats and mice.

Our demonstration of the chemotherapeutic potential of blocking the glycolysis of these parasites is the fifth link in a chain begun by Ryley in 1956 (3), when he showed that these parasites metabolize glucose both aerobically and anaerobically. The second was the discovery by Grant and Sargent in 1960 (4) of the peculiar L-α-glycerophosphate oxidase (GPO) present in the blood form of these cells. The third was the discovery of specific inhibitors of the GPO by Evans and Brown (5,6) which, disappointingly, had no therapeutic value even though the GPO could be completely blocked (7). The fourth link was the enlightened observation by Opperdoes et al that anaerobic glycolysis could not be accounted for by a simple modification of the aerobic pathway which utilizes the GPO (8). Building on Opperdoes' work, we outlined his proposed possibilities for the anaerobic pathway and all others we felt could explain the end products of glycolysis and the requirements for energy production. We noticed that all involved at least one

step yielding glycerol with little or no free energy and reasoned that exogenous glycerol should block this reaction (1,2).

What we report here are the results of experiments which show the effect of glycerol on anaerobic glycolysis *in vitro* and the effect of various amounts of SHAM and glycerol against an infection of *Trypanosoma brucei brucei* in rats.

MATERIALS AND METHODS

Chemicals and Equipment. Salicylhydroxamic acid (SHAM) was purchased from the Aldrich Chemical Co., Milwaukee, Wis. while adenosine triphosphate (ATP), oxidized and reduced nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from the Sigma Chemical Co., St. Louis, Mo. Enzymes used in the assay of glucose, pyruvate and glycerol were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. In addition, assays for glycerol were also done with a kit from Calbiochem, La Jolla, Ca. All other chemicals used were of reagent grade. Spectrophotometric assays were performed on a Gilford Instrument Laboratories modified Beckman DU spectrophotometer. Oxygen was measured using a Yellow Springs Instrument Co. Clarke type oxygen electrode. Cell counts were done on Neubauer hemacytometer.

Maintenance and Growth of the Parasites. Female rats from Charles River derived from Sprague-Dawley stock with a body weight from 125 to 250 g were used for all these studies. *Trypanosoma brucei brucei* (strain EATRO 110), made primarily monomorphic by 50 serial passages in rats at 3 day intervals, is maintained as a frozen stabilate. The stabilate is prepared from infected rat blood with a parasitemia of about 10^8 cells per ml of blood. The blood

is collected by cardiac puncture using diethyl ether anesthesia. One ml of heparin (30 mg heparin per 100 ml of 0.85% NaCl) is used as anticoagulant for each 10 ml of rat blood. The collected blood is immediately diluted with 3 volumes of ice cold phosphate buffered saline (6:4), pH 8.0, plus 1.5% glucose as described by Taylor et al (9) and hereafter referred to as PSG (and as PS with no glucose). With the diluted blood kept on ice and stirred, an equal volume of 20% (v/v) glycerol in PSG is slowly added. This is conveniently done by allowing the glycerol solution to drop from a syringe barrel fitted with a 22 gauge needle. Five ml aliquots of the diluted blood now containing 10% glycerol, are dispensed into 30 ml glass vials, capped and placed directly at -70°. Stabilates so prepared produce reliable infections for 6 months if held at -70° and for at least two years if removed from the -70° freezer after 24 hours to a liquid nitrogen refrigerator.

To initiate an infection, a vial is rapidly thawed and held at room temperature for 20-40 minutes. Injection of 0.02 ml of the stabilate (2×10^6 parasites) intraperitoneally produces a high parasitemia (from 10^8 - 10^9 cells/ml blood in 72 hours. It is worthwhile to note that the thawed stabilate should not be allowed to become anaerobic as the glycerol would then be toxic. The brightness of the rat hemoglobin is a useful guide to the oxygen level.

Parasite Isolation. For in vitro studies, blood is collected and diluted exactly as described for stabilates (no glycerol is added). All the following procedures are carried out on ice or at 4°. The diluted blood is spun at 2000 g for 15 minutes. The buffy coat, which contains the trypanosomes, is collected. This material, enriched in parasites, is passed through a DEAE cellulose (Whatman DE 52) column as described by Lanham (10), which removes all cells except the trypanosomes, which pass through. The eluted parasites are sedimented by centrifugation at 2000 g for 15 minutes, then resuspended in 40 volumes of PSG. This wash is repeated twice.

Measurements of glycolysis. All *in vitro* glycolysis studies were carried out in a 29 x 200 mm rimless pyrex test tube to which an 11 x 25 mm side arm, centered 30 mm from the base of the tube, had been fused. With the side arm plugged by a thin rubber stopper, 45 ml of PSG with 150 mg% bovine serum albumin (BSA, Armour Fraction V) and glucose at various concentrations was added to the tube. The top of the vessel was plugged with a one hole rubber stopper (No. 6) through which a 9 inch pasteur pipette had been inserted, with tip one cm. above the bottom of the test tube. A vent for this system was provided by inserting a 2 inch 18 gauge needle through the rubber stopper. Aeration was carried out by bubbling air through the pasteur pipette using an aquarium air pump. The air was humidified by bubbling it through the water prior to passage through the pasteur pipette. 20 ul of Dow Corning Antifoam was added to PSG to prevent foaming.

After 15 minutes of pre-aeration, 5 ml of a 10% cell suspension, prepared by suspending one volume of packed washed trypanosomes in 9 volumes of starting buffer, was added to the aerated buffer by injection through the test tube side arm too give a final volume of 50 ml with from 1.5 to 1.8 x 10^8 trypanosomes per ml.

Glycolysis was measured by removing aliquots at one hour intervals, centrifuging them immediately for one minute in a Beckman Microfuge B and removing the supernates for direct assay of glucose, pyruvate and glycerol. Glucose assays were carried out using the method of Bergmeyer *et al* which couples hexokinase, ATP and glucose - 6 - phosphate dehydrogenase and measures glucose indirectly as NADPH (11) while pyruvate was measured by the method of Czok and Lamprecht, which uses lactic dehydrogenase and NADH to reduce pyruvate to lactate (12). Glycerol levels were measured either by the method of Wieland, which uses glycerol kinase, ATP and glycerol phosphate dehydrogenase to measure gly-

cerol indirectly as NADH (13) or by the method of Eggstein and Kuhlmann, which also uses glycerol kinase, but follows ATP consumption by coupled enzyme system of pyruvate kinase and lactic dehydrogenase with phosphoenol pyruvate (PEP). Here, ADP formed during the phosphorylation of glycerol is converted back to ATP while PEP is converted to pyruvate. Pyruvate is then reduced to lactate with lactic dehydrogenase and NADH (14). Since pyruvate was present in these samples, the addition of glycerol kinase to the reaction mixture was delayed until a stable absorbance at 340 nm was observed, whereupon glycerol kinase was added and the resultant decrease in absorbance used to determine glycerol levels. An NADH and NADPH extinction coefficient of 6.22×10^{-6} ml per mole at 340 nm was used in all determinations.

Preparation and administration of SHAM-glycerol. For the dose response curve of SHAM-glycerol, stock solutions of 0.5 M SHAM and 2.4 glycerol were prepared. To prepare 50 ml of 0.5 M SHAM, 3.83 g SHAM (Aldrich Chem. Co.) is suspended by stirring in 25 ml distilled water. NaOH (1 N) is added dropwise to maintain the pH at 9 while the suspension is heated to 60° and stirred. After all the SHAM has dissolved, distilled water is added to bring the final volume to 50 ml. The glycerol stock was prepared directly in distilled water. A constant volume of 0.5 ml/100 g body weight was injected into the tail vein with the dose controlled by dilution of the stock solutions: SHAM stock was diluted with 0.85% NaCl and glycerol with distilled water. Injections were made while the animal was under diethyl ether anesthesia.

For all other experiments SHAM was prepared as a 0.25 M stock solution by suspending 1.92 g SHAM in 40 ml distilled water then adding 10 ml 1N NaOH. This suspension was heated to about 60°C and stirred until dissolved. The final pH is 8.3.

A stock SHAM-glycerol solution, 0.25 M in SHAM and 1.2 M in glycerol, is made as above except that 5.53 g glycerol is added to the SHAM and the final volume is adjusted to 50 ml. A dose of 0.5 ml/100 g body weight represents twice the minimum effective dose and is the standard dose for experiments designed to clear all peripheral blood parasites.

All injections are intravenous unless otherwise noted.

RESULTS

Aerobic Glycolysis. Under aerobic conditions, washed isolated trypanosome suspensions consumed glucose and generated pyruvate in linear fashion at room temperature for 4 hours (Fig. 1). Treating the data in figure one by the method of least squares, 1.07 umoles of glucose were consumed per 10^8 cells/hour at 22.5°C while 2.14 umoles of pyruvate were produced. By the average of 4 experiments, 98% $\pm$ 2% (Range 96-102%) of the glucose consumed under aerobic conditions at 25° could be accounted for as pyruvate. Using NAD, glycerol kinase, and glycerol dehydrogenase in a hydrazine buffer (13), 0.25 $\pm$ 0.13 umole of glycerol per ml of cell suspension were detected at all the time points shown in figure one. However, no increase or decrease of glycerol was observed. We could not maintain linear glycolysis with these cells at room temperature in the absence of BSA or 10% heat inactivated fetal calf serum (FCS); moreover, at 37°C we could not maintain a constant rate of glycolysis with these trypanosomes for more than 60 to 90 minutes, even with 1% BSA or 20% FCS present.

Anaerobic Glycolysis. When these trypanosomes were suspended and aerated as previously described - but now with 2 mM SHAM present, oxygen consumption was completely blocked and glycerol and pyruvate were formed as glycolytic end products. Under these conditions, however, glucose consumption and end product formation were no longer constant with time (Fig. 2).

Progressively less glucose was consumed and less end product pyruvate and glycerol formed until, after 4 hours, glycolysis had all but ceased. Cell counts made at every time point showed no decrease in cell number until 4 hours; however, the rate of of cell motility gradually decreased until, at 3 hours, the cells barely moved and, by 4 hours, the majority appeared as swollen ghosts by phase contrast microscopy. Although glycolysis was no longer linear, glucose consumption and end product formation remained stoichiometric throughout. Within the limits of experimental error, all the glucose consumed at every point in Figure 2 could be accounted for as pyruvate and glycerol.

When 5 mM pyruvate was included at zero time within cells suspended in phosphate buffered saline plus 20 mM glucose and 2 mM SHAM, no change was observed in the pattern of glucose consumption and end product formation; however, when 5 mM glycerol was present from the start, the net glycolytic activity was severely depressed (Fig. 3). As higher levels of glycerol were included with these cell suspensions at zero time, a progressively greater suspension of glycolysis was observed (Fig. 4). Again, within limits of experimental error, all of the glucose consumed in the data shown (Fig. 3, 4) could be accounted for as pyruvate and glycerol.

Dose Response in Rats. Three days after inoculation, rats with parasitemias ranging from 2×10^8 to 8×10^8 per ml of blood were treated with SHAM-glycerol injected intravenously. The parasitemias were counted just prior to treatment and 1 and 24 hours post treatment. The pattern of dose-response at 1 and 24 hours was very similar, with a slightly greater reduction in parasite numbers at 24 hrs. Figure 5 represents the 24 hour dose resposne. The points composing the curves showing the response to the 3 higher SHAM levels represent the average of at least three animals for each point. The course representing the lower two levels of SHAM have several

points representing two animals. The parasitemia data for each animal 24 hours post treatment has been normalized and expressed as the percentage of the parasitemia of that animal previous to treatment.

Treatment of infected animals causes complete loss of detectable parasitemia in the peripheral blood; however, 5 to 6 days post treatment, the parasitemia regularly returns. Several attempts were made to modify the regimen to produce a complete cure. A group of 10 rats were infected with T. b. brucei and three days later had parasitemias estimated about 5 x 10^8 per ml of blood. They were then treated with IV SHAM-glycerol at 96 and 276 mg/kg respectively. This abolished any detectable parasitemia. This treatment was repeated at 3 day intervals for total of 5 treatment cycles. All the animals developed parasitemias and died within 10 days after the last treatment. Without treatment they would have died within 24 hours of the time of the first treatment, thus this extended survival from an expected 3 days to 23 days and there was no reason to suspect that continued treatment would not have continued suppression of the disease.

DISCUSSION

With the exception of the preliminary report of our data (1,2), all published studies of glycolysis of T. b. brucei have depended on an initial and terminal assay of glucose, glycerol and pyruvate with the assumption that the rate was linear between these points. A steady rate of metabolism reflects a good maintenance system and allows more confidence that the results represent the normal functioning of the cell metabolism. Our measurements were made at 25° rather than the more desirable 37° for a blood stream parasite but were linear for 4 hours. This is a good indication that our data represents the real-stoichiometry of glucose catabolism. Under our rigorous

aerobic conditions we do not detect any increase in glycerol levels, thus our data confirms the 1956 prediction by Ryley that, under appropriate conditions of aeration, the conversion of glucose to pyruvate is complete.

Clearly, the suppression of glycolysis which we observe in the presence of 2 mM SHAM cannot be due to some innate deficiency of the PSG suspension medium, for constant rates of glycolysis are regularly seen in this suspension medium at room temperature in the absence of SHAM. It is equally unlikely that some unsuspected side-effect of SHAM could account for these results, for comparable patterns of glycolysis, as measured by glycerol and pyruvate production, can be observed when suspensions of these trypanosomes are bubbled with N_2 (100%) or N_2 (95%) and CO_2 (5%) atmospheres at room temperature (Brohn and Clarkson, unpublished results).

As early as 1962, Ryley reported that glycerol depressed glucose consumption in trypanosomes under anaerobic conditions (15). More recently, Balber and Patton, have reported that 1 mM glycerol in the presence of 1 mM SHAM blocks glucose uptake in suspensions of these cells at 37°C *in vitro* (Balber and Patton, personal communication), while Fairlamb *et al* have shown that, at similar concentrations of SHAM and glycerol *in vitro*, pyruvate production is blocked at 37°C (16). Our *in vitro* results confirm and extend these earlier observations. Pyruvate, the other end product of anaerobic glycolysis, does not inhibit anaerobic glycolysis in these monomorphic trypanosomes. Indeed, as Flynn and Bowman have shown, short stumpy forms of these cells do not appear to transport exogenous pyruvate, and LS forms may also lack this capacity (17). For glycolysis in the presence of 2 mM SHAM, despite the presence of atmospheric CO_2, no end products other than pyruvate and glycerol are necessary to account for the glucose consumption we have observed, confirming the 1957 report by Grant and Fulton, (18), that labeled pyruvate and glycerol could account for labeled glucose consumption under

anaerobic conditions in T. brucei during a two hour incubation in the presence of N_2 (95%) and CO_2 (5%). Whether derived as end product during anaerobic glycolysis, or added at the start of this glycolysis, glycerol clearly inhibits anaerobic glucose consumption in these cells and, at 25°C, in vitro levels of 3 - 5 mM glycerol rapidly depresses cell motility and eventually results in cell lysis and death.

The results of in vivo administration of SHAM-glycerol parallel and are similar to our in vitro experiments. One notable exception is the rapid clearance noted in vivo. With IV administration into the tailvein of SHAM-glycerol at 96 and 276 mg/kg, respectively, within 3 minutes parasites from blood of the snipped tail show signs of deterioration and there are no detectable parasites after 10 minutes. In vitro treated cells become abnormal in appearance but some retain a recognizable shape up to one hour in the presence of 2 mM SHAM and 10 mM glycerol.

We have as yet no explanation for the recurrence of parasitemia.

Our data confirm the existence of an as yet unknown portion of the glycolytic pathway of African trypanosomes as first proposed by Opperdoes et al. We do not as yet have any further information of the actual reactions involved.

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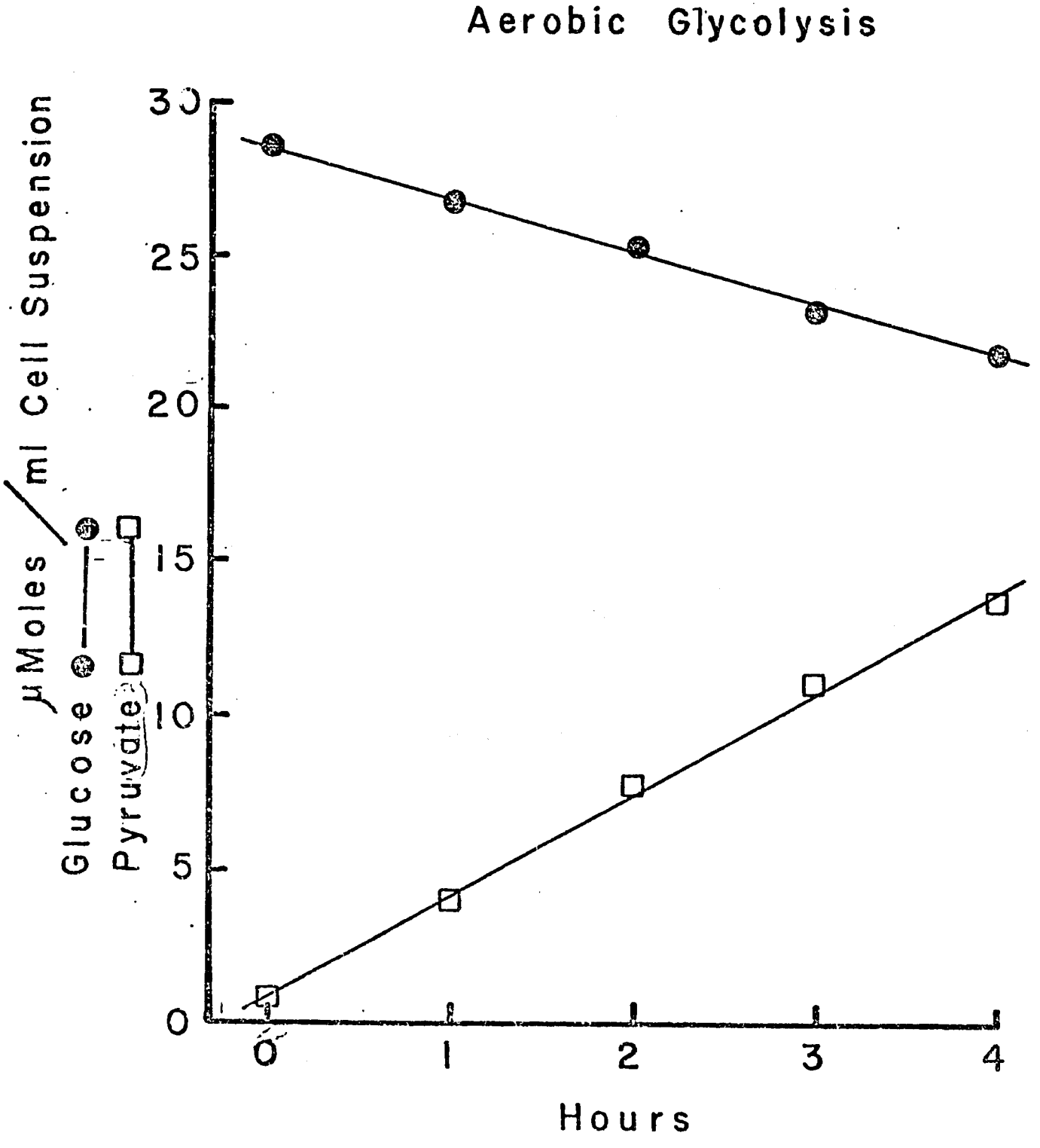
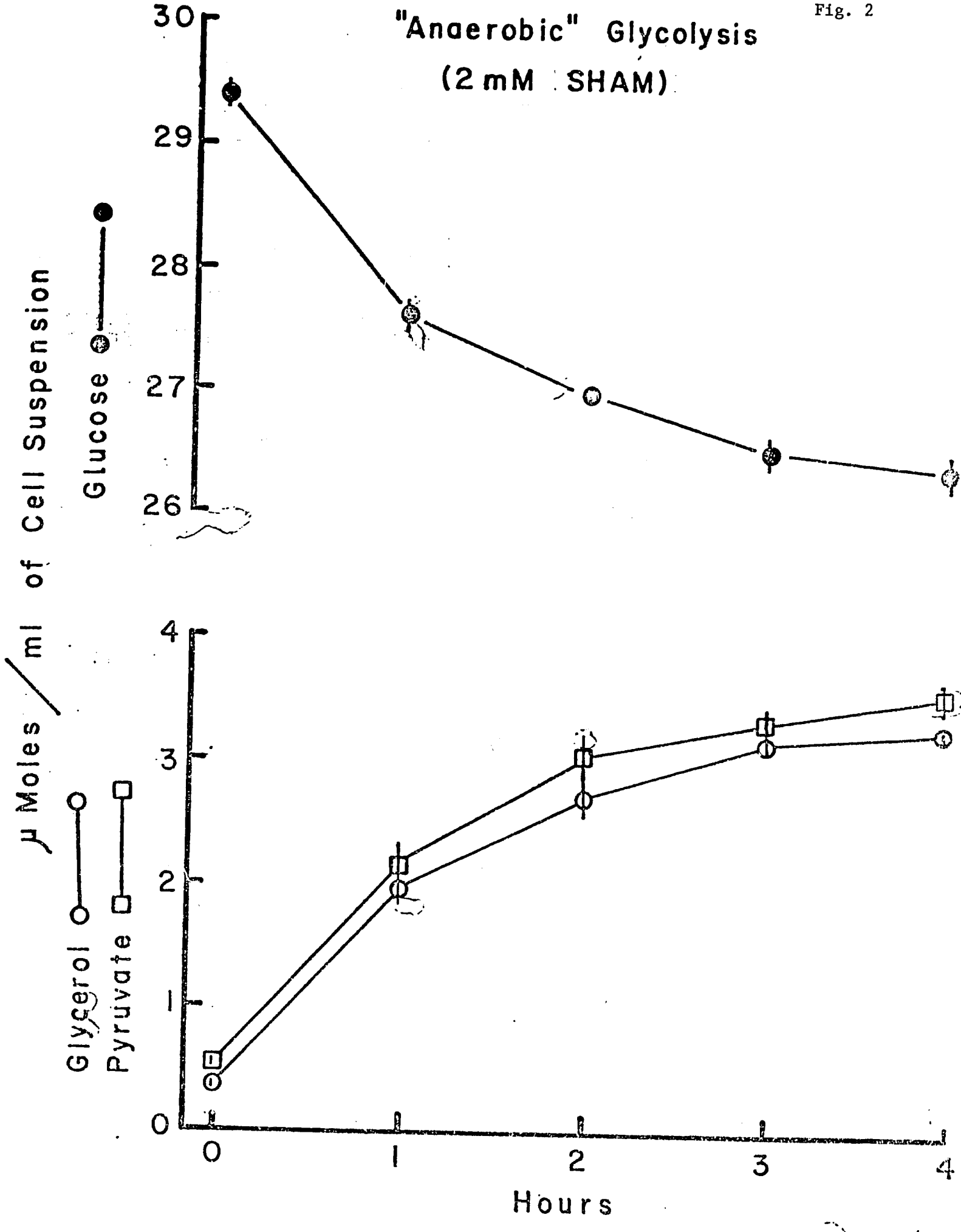

Aerobic Glycolysis
μMoles / ml Cell Suspension
Glucose
Pyruvate
30
25
20
15
10
5
0
0
1
2
3
4
Hours

Fig. 2


"Anaerobic" Glycolysis
(2 mM SHAM)
Glucose
μ Moles / ml of Cell Suspension
30
29
28
27
26
Glycerol
Pyruvate
4
3
2
1
0
0
1
2
3
4
Hours

Effect of Glycerol and Pyruvate under "Anaerobic" Conditions (2 mM SHAM)

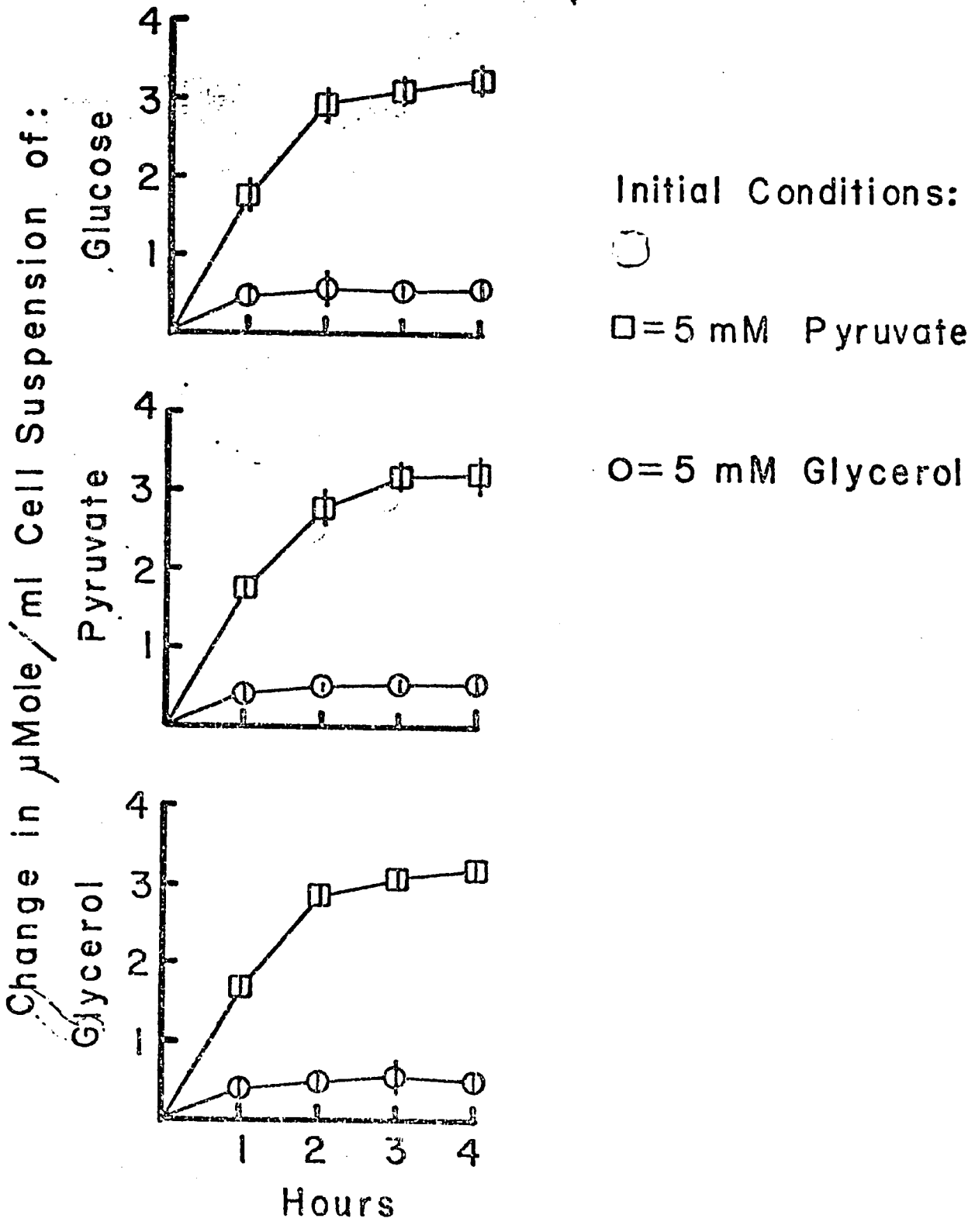


Fig. 4 .

on "Anaerobic" Glycolysis (2 mM SHAM)

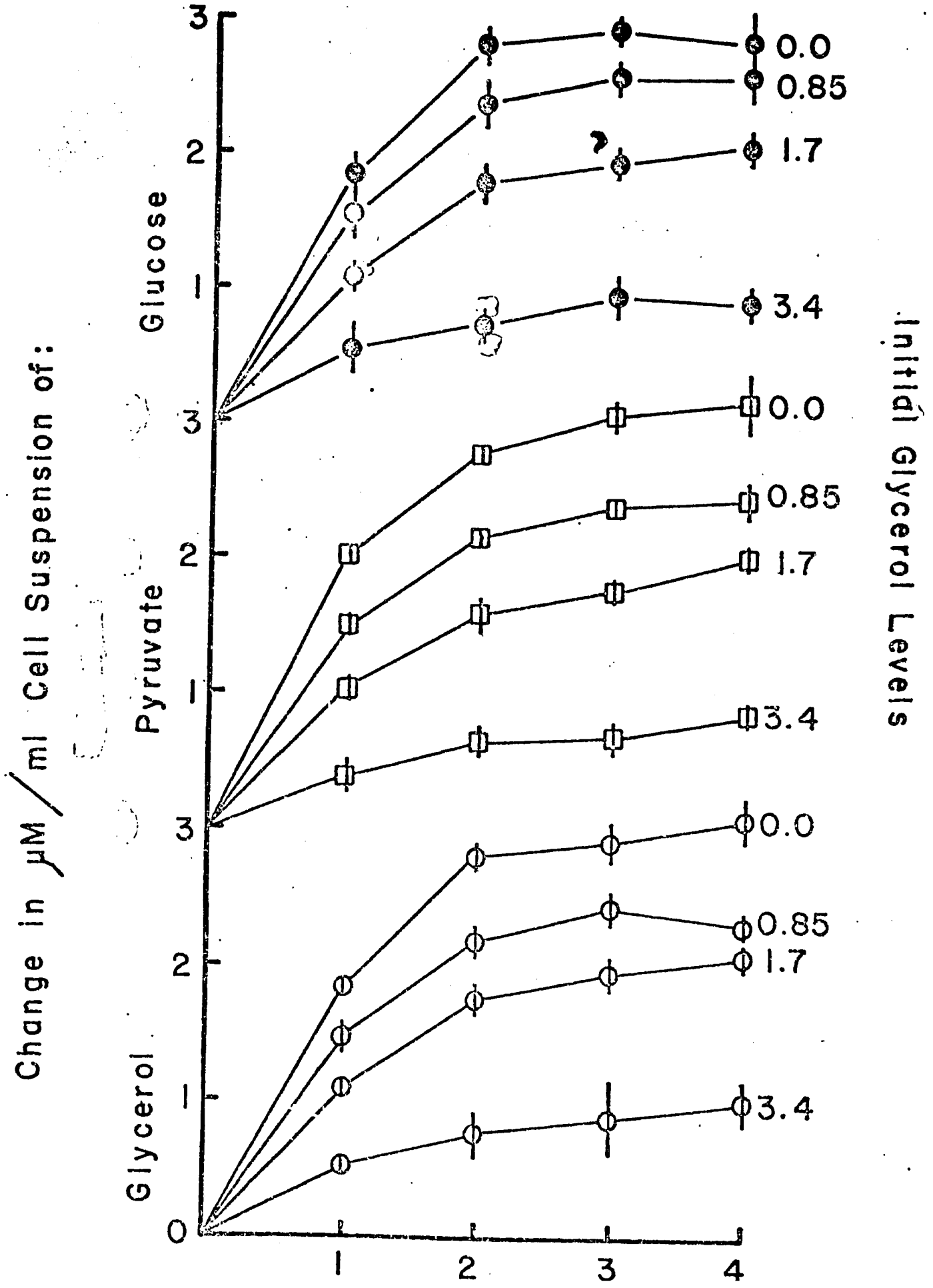
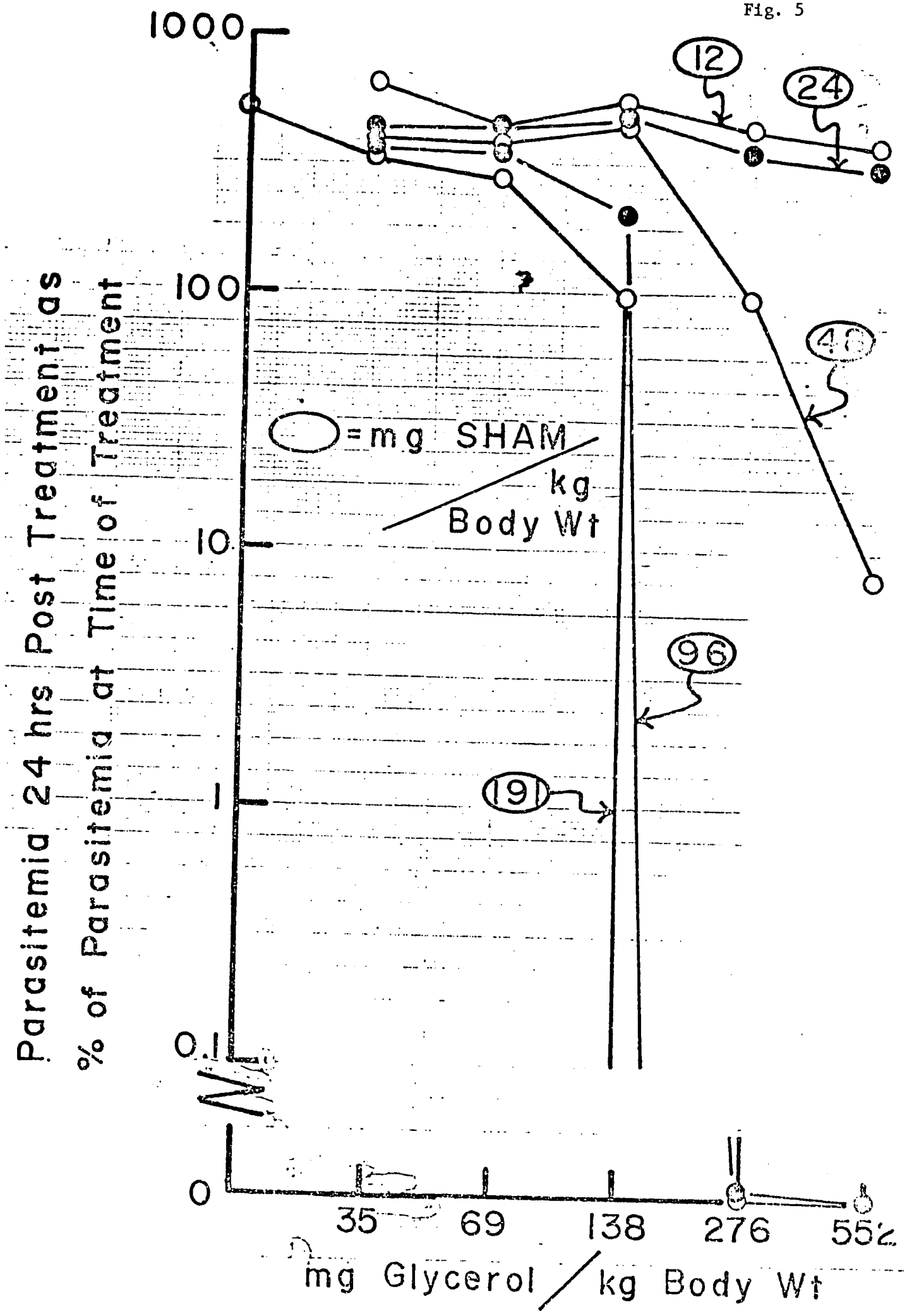


Fig. 5


1000
100
10
1
0.1
0
Parasitemia 24 hrs Post Treatment as
% of Parasitemia at Time of Treatment
12
24
48
96
19
= mg SHAM / kg Body Wt
35
69
138
276
552
mg Glycerol / kg Body Wt

ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE

SEVENTEENTH SEMINAR ON TRYPANOSOMIASIS

ORGANIZED BY THE TRYPANOSOMIASIS ADVISORY PANEL OF THE MINISTRY OF OVERSEAS DEVELOPMENT, HELD IN LONDON ON 23RD AND 24TH SEPTEMBER, 1976

Dr. L. G. Goodwin, F.R.C.P., F.R.S., *in the Chair*

On the first day, the Meeting was held at the Royal Society of Tropical Medicine and Hygiene, Manson House, London W1N 4EY, and was organized in four Sessions, each with its own Chairman. The introductory statement by the Chairman of each Session was followed by two papers read by invited speakers, and by discussion. Summaries of the papers presented are given below.

On the second day, Laboratory Demonstrations were presented at the London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT. A list of the exhibits is appended.

SESSION I

Trypanosome biochemistry and its relevance to control

Chairman's Introduction

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Metabolism and chemotherapy of African trypanosomes

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The work to be described was undertaken in collaboration with Drs. F. R. Opperdoes and A. H. Fairlamb.

The trypanosomes of the *brucei* group have a number of unique characteristics: the bloodstream forms are able to shut off completely the synthesis of the mitochondrial respiratory chain and live on glycolysis alone (Bowman & Flynn, 1976); the end product of glycolysis in bloodstream forms is pyruvate rather than lactate and the NADH produced in glycolysis is reoxidized by a glycerol-phosphate oxidase system (GPO) not present in the host (Bowman & Flynn, 1976), and the trypanosome shares with other Kinetoplastidae the presence of a bizarre form of mitochondrial DNA, called kinetoplast DNA. We are studying these aspects of trypanosome metabolism for their intrinsic biological interest, but we hope that this study will also produce spin-off for trypanosome chemotherapy.

The biochemical basis of trypanosome chemotherapy has been reviewed in detail (Williamson, 1976; Newton, 1976; Peters, 1976). Experiments in Amsterdam with (potential) trypanosomicidal drugs have followed two lines: the first line focuses on the GPO system, which co-operates with an NAD+-linked glycerol-phosphate dehydrogenase (NAD-GPD) to reoxidize NADH produced in glycolysis. Until recently, the GPO was thought to be in the microbodies (Bowman & Flynn, 1976) and the NAD-GPD in the cell sap. Experiments by Opperdoes with *T. brucei* have shown, however, that GPO is in fact a mitochondrial enzyme, whereas most of the NAD-GPD in trypanosomes is in microbodies (Opperdoes & Borst, 1976). Since microbodies are impermeable to NAD, there must be another enzyme in these bodies to permit a cyclic NAD oxidation and reduction. This enzyme may be glyceraldehyde-phosphate dehydrogenase because, in preliminary experiments, this was found to be largely particle-bound (Opperdoes, unpublished).

It was shown by Evans & Brown (1973) that the GPO system is inhibited by benzhydroxamates, like salicylhydroxamic acid (SHAM). Under anaerobic conditions bloodstream trypanosomes convert glucose into equimolar amounts of pyruvate and glycerol and we have verified (Opperdoes, Borst & Fonck, 1976) that the same result is obtained when GPO is completely inhibited with SHAM. One would expect from known metabolic pathways in bloodstream trypanosomes (Bowman & Flynn, 1976) that under these conditions net ATP synthesis would be zero, but this is not so (Opperdoes, Borst & Fonck, 1976). Cellular ATP concentrations are maintained at about half normal values and motility is not stopped. This is not due to a store of internal substrate because, in the absence of glucose, the trypanosomes stop wriggling within one minute and the ATP level drops to zero. We conclude, therefore, that trypanosome glycolysis is more complicated than originally thought and that there must be an alternative pathway allowing ATP synthesis when GPO is blocked. This also explains why high plasma SHAM concentrations have no effect on the course of a fulminant *T. brucei* infection in rats (Opperdoes *et al.*, 1976).

The nature of the alternative pathway has not been elucidated yet. It is clear, however, that an inhibitor of this pathway could be useful as a trypanosomicidal drug, if combined with a GPO inhibitor. We have tested this principle in a model experiment, which utilizes the old observation (Ryley, 1962) that glycerol inhibits glucose utilization by bloodstream trypanosomes under anaerobic conditions. We have found that 5 mM glycerol in the presence of SHAM blocks (>95%) the motility of *T. brucei* and inhibits the production of pyruvate from glucose, half-maximal inhibition being obtained at about 1 mM (Fairlamb & Opperdoes, unpublished). Glycerol does not inhibit in the absence of SHAM; it is even the preferred substrate in glucose-glycerol mixtures (Ryley, 1962).

Although we do not yet know exactly why trypanosomes are killed in the presence of SHAM+glycerol, the model experiments illustrate how insight into trypanosome metabolism could lead to the development of drug combinations that would not have been detected in a conventional screen for trypanosomicidal drugs, because neither of the drugs alone has measurable trypanosomicidal action.

Our second line of experiments on potential trypanosomicidal drugs has involved the testing of male antifertility drugs on *T. brucei*. Several of these drugs are known to inhibit glycolysis, and since their toxicity is known and usually low against the whole animal, we hoped to find a promising drug cheaply. As test systems we have used motility and O_2 uptake of *T. brucei* bloodstream forms. Entirely negative results were obtained with the glucose analogues 5-thio-D-glucose and glucosamine (though the toxic 2-deoxy-glucose inhibited trypanosome respiration half maximally at 0·8 mM), the glycerol analogues 3-chloro-1,2-propanediol and 1-amino-3-chloro-2-propanol, and two substituted bisdichloroacetamides (WIN 13,146 and 18,446) (Opperdoes & Fonck, unpublished). Via the latter compounds we came on tetraethylthiuramdisulphide (TETD, Antabuse), which had been shown already by Cross *et al.* (1975) to block the growth of *T. brucei* in culture. Opperdoes (unpublished) has now found that this compound inhibits O_2 uptake and motility of bloodstream *T. brucei*. Inhibition increases with time and is complete after seven minutes at 15 μM TETD. The effective killing of *T. brucei* by TETD was unexpected because in preliminary tests Cross *et al.* (1975) were unable to cure trypanosome infections in mice with TETD. This paradox is explained,

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however, by our finding that the inhibitory effects of low TETD concentrations on *T. brucei* are completely abolished by contact with whole rat blood, but not with plasma. Presumably, the hydrophobic drug is rapidly inactivated or sequestered by blood cells.

The fact that TETD does not reach the trypanosome *in vivo* does not necessarily make it useless for chemotherapy, because it may be possible to make analogues that preferentially enter trypanosomes. An interesting approach is to couple such drugs to macromolecules which are rapidly taken up and hydrolysed by trypanosomes, liberating the drug inside. Promising results with this "lysosomotropic" drug approach have been obtained already with ethidium-DNA complexes (TROUET, JADIN & VAN HOOF, 1976) and it may be worthwhile to extend this approach to other drugs that inhibit trypanosomes *in vitro*, but fail to cure trypanosome infections *in vivo*.

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