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Quantitative effects of salycylhydroxamic acid and glycerol on *Trypanosoma brucei* glycolysis in vitro and in vivo

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Summary

During anaerobic glycolysis in vitro in the presence of salicylhydroxamic acid, *Trypanosoma brucei brucei* converts glucose to equimolar amounts of glycerol and pyruvate as end products. Glycerol, whether generated endogenously or added exogenously, can inhibit anaerobic glycolysis sufficiently in vitro to result in cell death. The concomitant administration of salicylhydroxamic acid and glycerol to rats infected with *T. brucei brucei* results in a rapid clearance of parasitemia. Our results clearly demonstrate a new and approachable chemotherapeutic target for African trypanosomes.

Key words: T. brucei brucei; aerobic glycolysis; anaerobic glycolysis; salicylhydroxamic acid; glycerol; chemotherapy.

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 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 (Clarkson and Brohn, 1976a, b) 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.

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Our demonstration of the chemotherapeutic potential of blocking the glycolysis of these parasites is the fifth link in a chain begun by Ryley (1956), when he showed that these parasites metabolize glucose both aerobically and anaerobically. The second was the discovery by Grant and Sargent (1960) of the peculiar L-α-glycerophosphate oxidase (GPO) present in the blood form of these cells. The third was the discovery by Evans & Brown (1973a, b) of specific inhibitors of the GPO, including SHAM which, disappointingly, had no therapeutic value under the conditions used even though the GPO could be completely blocked (Opperdoes et al., 1976a). The fourth link was the enlightened observation by Opperdoes et al. (1976b) that anaerobic glycolysis could not be accounted for by a simple modification of the aerobic pathway which utilizes the GPO. 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 (Clarkson and Brohn, 1976a, b).

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), oxidised 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, was maintained as a frozen stabilate. The stabilate was prepared from infected rat blood with a parasitemia of about 10^8 cells per ml of blood. The blood was collected by cardiac puncture using diethyl ether anesthesia. One ml of heparin (30 mg heparin per 100 ml of 0.85% NaCl) was used as anticoagulant for each 10 ml of rat blood. The collected blood was 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. (1974) 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% (w/v) glycerol in PSG was 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, were 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 h to a liquid nitrogen refrigerator.

To initiate an infection, a vial was rapidly thawed and held at room temperature for 20–40 min. Injection of 0.2 ml of the stabilate (2×10^6 parasites) intraperitoneally produced a high parasitemia (from 10^8–10^9 cells/ml blood) in 72 h. 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 was collected and diluted exactly as described for stabilates (no glycerol is added). All the following procedures were carried out on ice or at 4°. The diluted blood was spun at 2000 *g* for 15 min. The buffy coat, which contains the trypanosomes, was collected. This material, enriched in parasites, was passed through a DEAE cellulose (Whatman DE 52) column as described by Lanham (1968), which removes all cells except the trypanosomes, which pass through. The eluted parasites were sedimented by centrifugation at 2000 *g* for 15 min, then resuspended in 40 volumes of PSG. This wash was repeated twice.

Measurements of glycolysis. All in vitro glycolysis studies were carried out in a 29×200 mm rimless pyrex test tube to which an 11×25 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 PS with 150 mg% bovine serum albumin (BSA, Armour Fraction V) and glucose at various concentrations was added to the tube. SHAM, when present in this mixture, was at a concentration of 2 m*M*. 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 1 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 water prior to passage through the pasteur pipette. 20 μl of Dow Corning Antifoam was added to PSG to prevent foaming.

After 15 min 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 to give a final volume of 50 ml with from 1.5 to 1.8×10^8 trypanosomes per ml.

Glycolysis was measured by removing aliquots at 1 h intervals, centrifuging them immediately for 1 min 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. (1974) which couples hexokinase, ATP and glucose – 6 – phosphate dehydrogenase and measures glucose indirectly as NADPH while pyruvate was measured by the method of Czok and Lamprecht (1974), which uses lactic dehydrogenase and NADH to reduce pyruvate to lactate. Glycerol levels in the absence of SHAM were measured by the method of Wieland (1974), which uses glycerol kinase, ATP and glycerol phosphate dehydrogenase to measure glycerol indirectly as NADH. In the presence of SHAM, the method of Eggstein and Kuhlmann (1974), which also uses glycerol kinase, but follows ATP consumption by a coupled enzyme system of pyruvate kinase and lactic dehydrogenase with phosphoenal pyruvate (PEP) was used to measure glycerol. 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. Since pyruvate was present in these samples, the addition of glycerol kinase to the reaction mixture was delayed until a stable absorbance at 340 n*m* 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 n*m* was used in all determinations.

Preparation and administration of SHAM-glycerol. For in vitro glycolysis studies 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 1*N* NaOH. This suspension was heated to about 60° C and stirred until dissolved. The final pH was 8.3 and 0.8 ml of this solution was added to 60 ml of stock PS. Glucose and BSA were added to yield the final concentration described under *Measurements of glycolysis*, and the mixture diluted to 100 ml with H_2O.

For the dose response curve of SHAM-glycerol, stock solutions of 0.5 *M* SHAM and 2.4 *M*

glycerol were prepared. To prepare 50 ml of 0.5 *M* SHAM, 3.83 g SHAM (Aldrich Chem. Co.) was suspended by stirring in 25 ml distilled water. NaOH (1 *N*) was added dropwise to maintain the pH at 9 while the suspension was heated to 60° and stirred. After all the SHAM dissolved, distilled water was 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 a 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.

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

All injections were 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 h (Fig. 1). Treating the data in Fig. 1 by the method of least squares, 1.07 μmoles of glucose were consumed per 10^8 cells/h at 22.5° C while 2.14 μmoles of pyruvate were produced. By the average of 4 experiments, 98% ± 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 (Wieland, 1974), 0.25 ± 0.13 μmole of glycerol per ml of cell suspension were detected at all the time points shown in Fig. 1. 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 min, even with 1% BSA or 20% FCS present.

Anaerobic glycolysis. When these trypanosomes were suspended and aerated as previously described – but now with 2 m*M* 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 h, glycolysis had all but ceased. Cell counts made at every time point showed no decrease in cell number until 4 h; however, the rate of cell motility gradually decreased until, at 3 h, the cells barely moved and, by 4 h, 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 Fig. 2 could be accounted for as pyruvate and glycerol.

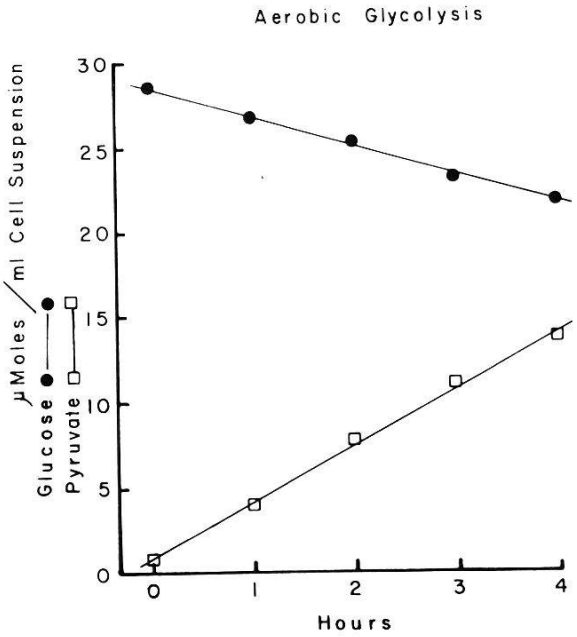

Fig. 1. Glucose consumption and pyruvate production in suspensions of *T. brucei* under aerobic conditions. The rate of metabolism is constant over four hours. 1.5×10^8 cells per ml were suspended in PS with 30 m*M* glucose and 150 mg% BSA. T = 22.5° C.

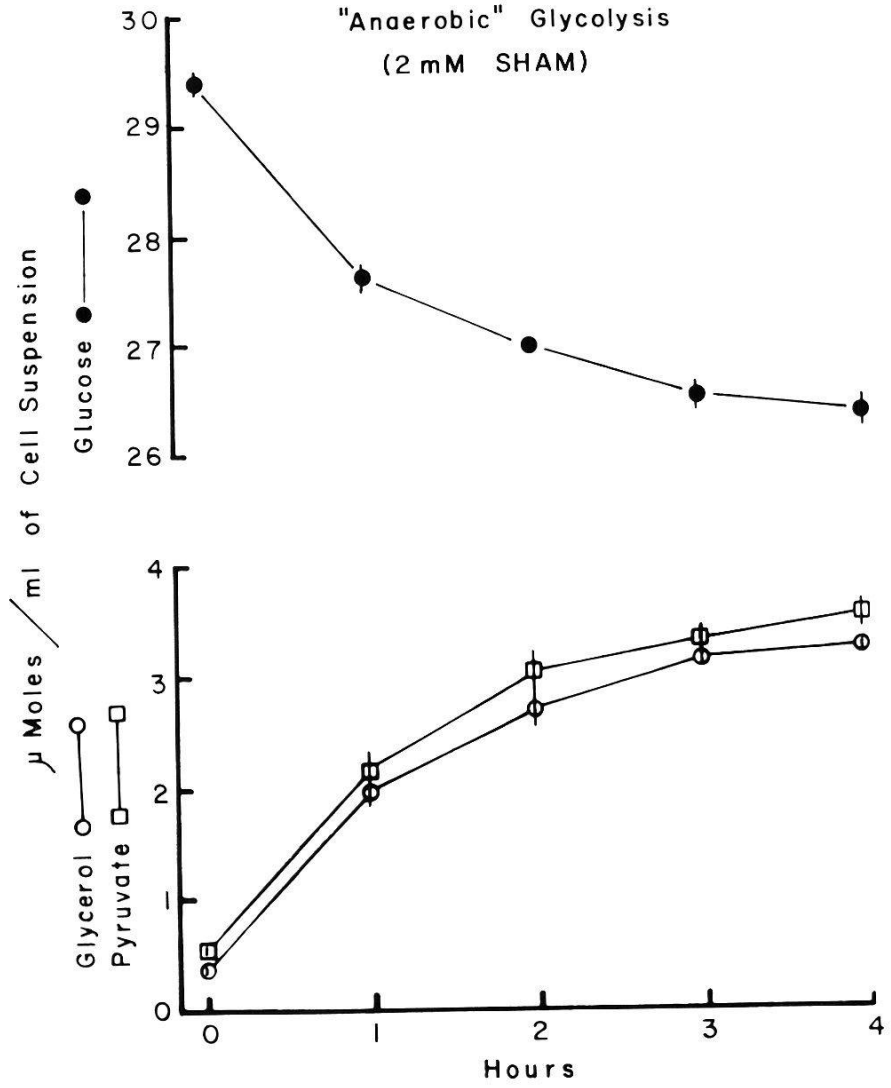

Fig. 2. Glucose consumption and pyruvate and glycerol production in suspensions of *T. brucei* with 2 m*M* SHAM present. Glycolytic rate decreases with time. 1.8×10^8 cells per ml were suspended in PS with 30 m*M* glucose and 150 mg% BSA. T = 25° C. The vertical bars at each sample point represent the Standard Error in a mean of 3 samples.

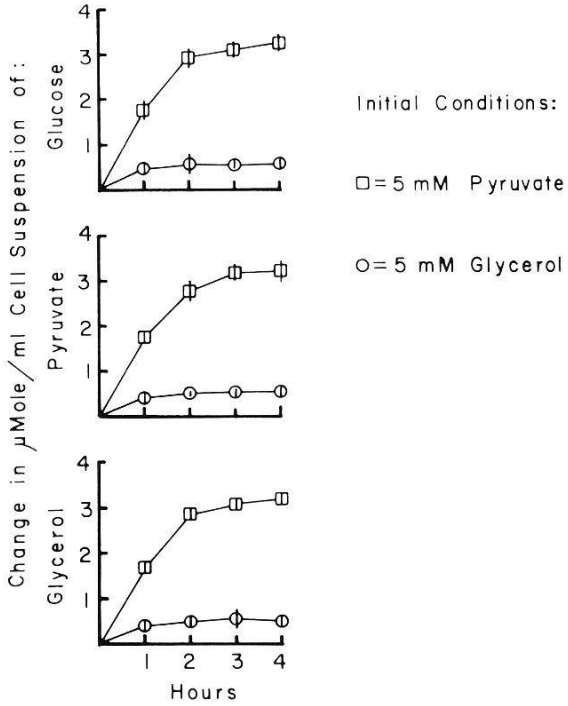

Fig. 3. Effect of glycerol and pyruvate under "anaerobic" conditions (2 m*M* SHAM). Glucose consumption and pyruvate and glycerol production in suspensions of *T. brucei* made 2 m*M* in SHAM and 5 m*M* in either pyruvate or glycerol before cells were added. Approximately 1.8×10^8 cells per ml were suspended in PS with 20 m*M* glucose and 150 mg% BSA. T = 25° C. The vertical bars at each sample point are as described in Fig. 2. To illustrate the constant stoichiometry of catabolism, the glucose, glycerol and pyruvate levels are shown as net change to emphasize the carbon balance even though glucose declines with time while pyruvate and glycerol increase. Glycerol suppresses glycolysis while pyruvate does not.

When 5 m*M* pyruvate was included at zero time with cells suspended in phosphate buffered saline plus 20 m*M* glucose and 2 m*M* SHAM, no change was observed in the pattern of glucose consumption and end product formation; however, when 5 m*M* 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 suppression 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 h post treatment. The pattern of dose response at 1 and 24 h was very similar, with a slightly greater reduction in parasite numbers at 24 h. Fig. 5 represents the 24 h dose response. 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

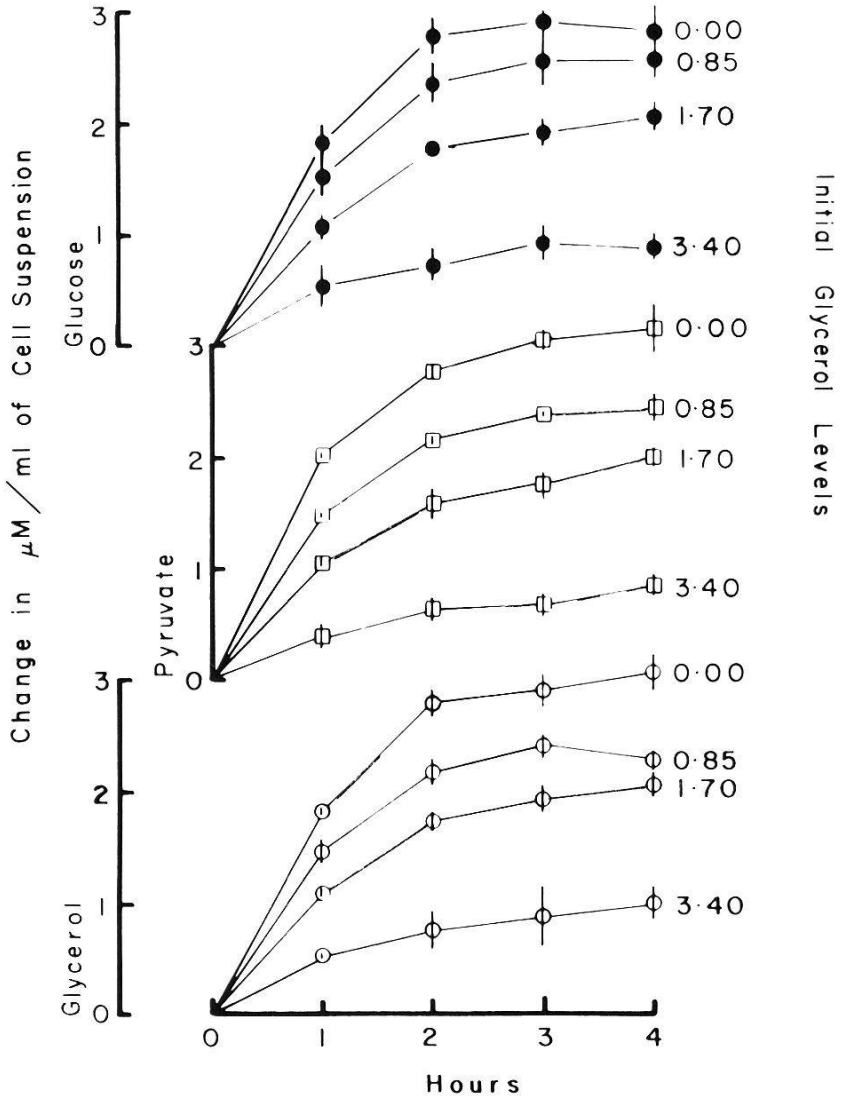

Fig. 4. Effect of various levels of glycerol on "anaerobic" glycolysis (2 m*M* SHAM). Glucose consumption and pyruvate and glycerol production in suspensions of *T. brucei* made 2 m*M* SHAM and with increasing levels of glycerol present prior to the addition of cells. Approximately 1.8×10^8 cells per ml suspended in PS with 10 m*M* glucose and 150 mg% BSA. T = 25° C. The vertical bars at each sample point are as described in Fig. 2. The glucose, glycerol and pyruvate levels are shown here as described in Fig. 3. As higher levels of glycerol are included before adding cells, a greater overall suppression of glycolysis is seen.

each animal 24 h 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 5×10^8 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 parasite-

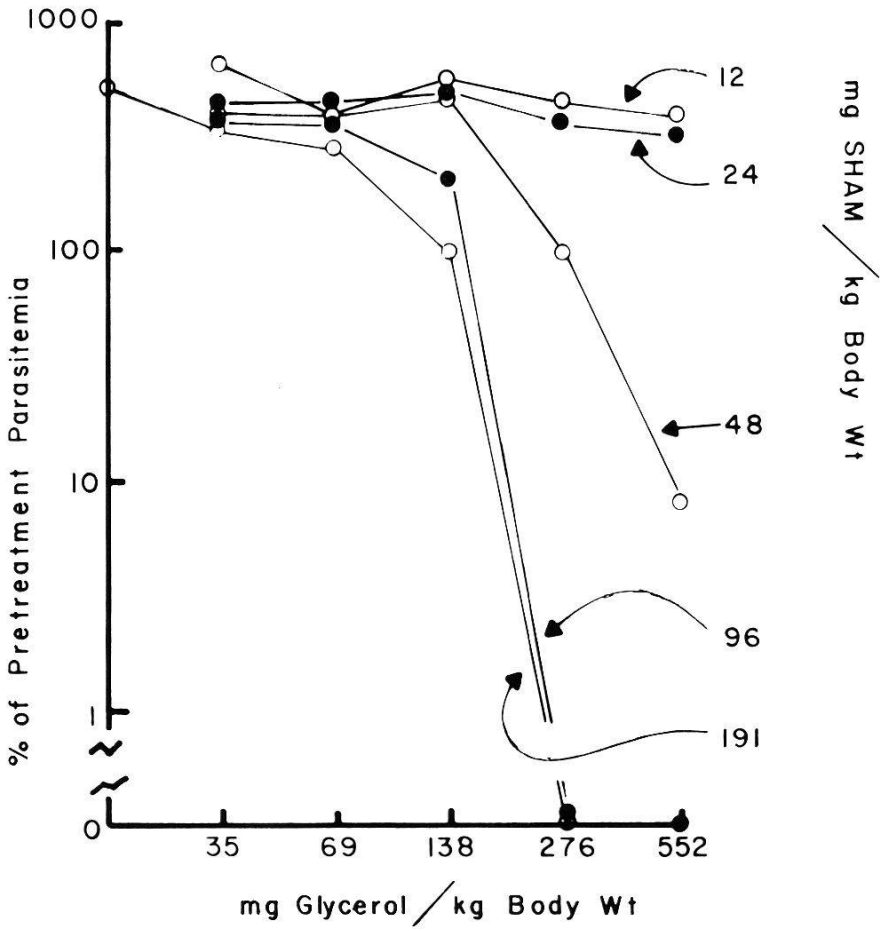

Fig. 5. Dose response. Parasitemia 24 h post treatment expressed as % of parasitemia just prior to treatment. The clearance of trypanosomes from the peripheral blood of rats infected with *T. brucei* is shown as a family of curves. In order to normalize the data from many rats, the parasitemia before treatment was defined as 100% (the actual value ranged from 10^8 to 10^9 parasites per ml of blood). Consequently, with no treatment or ineffective treatment the parasitemia will be greater than 100% 24 h later. Each curve represents a constant level of SHAM and shows that the clearance is dependant on the level of glycerol administered with that level of SHAM. However, both SHAM and glycerol must be at a minimum level for these to be of any effect.

mias and died within 10 days after the last treatment. Without treatment they would have died within 24 h 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, 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 bloodstream parasite but were linear for 4 h.

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 prediction by Ryley (1956) 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 m*M* 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. More recently, Balber and Patton, have reported that 1 m*M* glycerol in the presence of 1 m*M* SHAM blocks glucose uptake in suspensions of these cells at 37° C in vitro (Balber and Patton, personal communication), while Fairlamb et al. (1977) have shown that, at 1 m*M* SHAM and 5 m*M* glycerol in vitro, 99% of pyruvate production is blocked at 37° C. 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 (1973), short stumpy forms of these cells do not appear to transport exogenous pyruvate, and LS forms may also lack this capacity. For glycolysis in the presence of 2 m*M* 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, that labeled pyruvate and glycerol could account for labeled glucose consumption under anaerobic conditions in *T. brucei* during a 2 h 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 m*M* 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 a tail vein of SHAM-glycerol at 96 and 276 mg/kg, respectively, within 3 min parasites from blood of the snipped tail show signs of deterioration and there are no detectable parasites after 10 min. In vitro treated cells become abnormal in appearance but some retain a recognizable shape up to 1 h in the presence of 2 m*M* SHAM and 10 m*M* glycerol.

There are at least three possible reasons for the recurrence of parasitemia which we observed in our in vivo experiments: First, effective cidal levels of SHAM and glycerol may not have been maintained long enough in our experimental animals to kill all of the parasites. The observations by Opperdoes et al. (1976a, b) that SHAM is rapidly eliminated from the blood plasma of rats, lend support to this possibility. Second, some parasites may be sequestered in sites where they cannot be reached by SHAM and glycerol even with prolonged maintenance of high blood stream levels of these compounds. Evans et al. (1977) recently showed that, when SHAM and glycerol are administered to mice infected with *T. brucei brucei* or *T. brucei rhodesiense*, no recurrent parasitemia is observed if the animals are treated prior to the appearance of parasites in the peripheral blood. Moreover, the treatment with SHAM and glycerol of animals infected with *T. vivax* always resulted in a cure, whether the parasitemia was pre-patent or patent. Evans et al. have related this difference in curative effect to the differences in host distribution of *T. vivax* and the *T. brucei* subgroup. The former is restricted to host blood vessels, while the latter is distributed in both blood and tissue fluids. Finally, at high parasitemias there may exist a sub-population of parasites refractory to treatment with SHAM and glycerol but capable of reinfecting a treated animal. Preliminary data collected by the authors suggest that this may be the case. Blood from infected animals was diluted four fold in Lanham's PSG buffer with heparin added. This diluted blood was then made 2.5 m*M* with respect to SHAM and 12 m*M* with respect to glycerol. The blood was incubated at 37° for various times up to 45 min. At the end of these incubations, a volume of this suspension, which would have contained about 5×10^8 trypanosomes and which contained no recognizable moving trypanosomes by phase contrast microscopy, was injected into each of five rats. All subsequently developed parasitemias. Clearly more data is needed before a solid explanation can be made of the recurrence of *T. brucei* and cure of *T. vivax*.

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. (1976). Recently, Opperdoes and Borst proposed a very interesting model to explain the glycolytic production of glycerol. Their hypothesis is that levels of α-glycerophosphate become high enough in the glycosome (microbody containing most of the glycolytic enzymes) to reverse the action of glycerol kinase thus producing ATP and glycerol from ADP and α-glycerophosphate. This would also explain the action of glycerol as a mass action inhibition as we proposed (Clarkson and Brohn, 1976b). Fortunately, their hypothesis is readily testable by examining the levels of α-glycerophosphate in the cytosol and the glycosome when glycolysis is blocked by SHAM-glycerol, by SHAM and with no block. This data combined with ADP/ATP ratios should confirm or deny their model.

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