AN APPROACH TO THE DEVELOPMENT OF NEW DRUGS FOR AFRICAN TRYPANOSOMIASIS

By STEVEN R. MESHNICK, STEVEN H. BLOBSTEIN, ROBERT W. GRADY, AND ANTHONY CERAMI

(From the Laboratory of Medical Biochemistry, The Rockefeller University, New York)

African sleeping sickness is caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense.* This disease resulted in the deaths of several million people during the first half of the twentieth century (1) and continues to pose a threat of new epidemics (2). Of even more significance is the fact that animal trypanosomiasis or nagana (*T. congolense, T. b. brucei,* and *T. vivax*) makes four million square miles of the African continent unsuitable for the production of cattle and other livestock (3). The first trypanocidal agents were developed by Ehrlich and his collaborators in the early part of this century. Over the next 50 yr, drugs such as tryparsamide, suramin, pentamidine, berenil, ethidium, Antrycide, and melarsoprol became available for use in the treatment of both sleeping sickness and nagana (4). For the past 20 yr, however, there have been no new chemotherapeutic agents introduced. Moreover, the therapeutic usefulness of the older drugs is diminishing due to the increased incidence of resistant strains (5).

We have been attempting to develop new chemotherapeutic agents by elucidating biochemical differences between trypanosomes and their hosts and then designing drugs to take advantage of these differences. The present communication describes such an approach to drug development. The biochemical difference we have exploited is the inability of *T. b. brucei* to synthesize heme (6). As a result of this deficiency and the avid binding of heme to serum proteins in mammalian hosts, the bloodstream form of this organism has no detectable heme (S. R. Meshnick and S. Sassa, unpublished results) or hemoproteins such as cytochromes (7) or catalase (8). In a previous communication (9) we reported that the lack of catalase in *T. b. brucei* leads to an accumulation of intracellular hydrogen peroxide (H_2O_2) in these organisms, which should increase their susceptibility to killing by agents that promote the homolytic cleavage of H_2O_2 yielding hydroxy (HO·) or hydroperoxy (HOO·) radicals. Presumably, these radicals would react with unsaturated lipids and other cell constituents, thereby leading to cell destruction. Heme proved to be trypanocidal in vitro, whereas several other porphyrins showed in vivo activity (9). We ascribed this to their acting as initiators of homolytic cleavage. To enhance the efficacy of this therapeutic approach, we have attempted to determine the site of H_2O_2 production, the fate of the H_2O_2 generated, a means of increasing H_2O_2 production, and lastly, ways of rendering trypanosomes more susceptible to radical damage.

Materials and Methods

Chemicals. Hemin (type I, bovine), DL-alanine, DL-α-glycerol phosphate (DL-αGP),[1] glutathione (GSH), menadione, hematoporphyrin, D-amino acid oxidase (0.2 U/mg), and catalase (30,000 U/mg) were obtained from the Sigma Chemical Co., St. Louis, Mo. Salicylhydroxamic acid (SHAM) and 2-methoxy-naphthoquinone was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis. Both dichlorophenolindophenol (DCIP) and leucodiacetyldichlorofluorescin (LDADCF) were products of the Eastman Organic Chemicals Div. Eastman Kodak Co., Rochester, N.Y. Tryparsamide was purchased from K and K (Plainview, N. Y.), whereas melarsen and melarsenoxide were gifts from Dr. E. A. Friedheim of The Rockefeller University.

Horseradish peroxidase (880 U/mg) was obtained from Worthington Biochemical Corp., Freehold, N. J. Bovine serum albumin and minimal essential medium (MEM) were purchased from Miles Laboratories Inc., Elkhardt, Ind., and Grand Island Biological Co., Grand Island, N. Y., respectively. Hematoporphyrin D was prepared from hematoporphyrin by the method of Lipson et al. (11). Vitamin K-S(II) was a gift from Dr. C. C. Wang of Merck & Co., Inc., Rahway, N. J.

Animals. BALB/c and Swiss-Webster mice were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass., and The Rockefeller University, respectively. Sprague-Dawley rats were purchased from the Holtzman Co., Madison, Wis). Dutch-belted rabbits were from Dutchland, Denver, Pa.

Organisms. *T. b. brucei* (EATRO 110) were obtained from Dr. W. Trager (The Rockefeller University) and stored as stabilates in 10% glycerol at $-80°C$. This strain had been passaged seven times in rodents since it was last transmitted by tsetse flies. Intraperitoneal injection of 2×10^4 motile organisms into Swiss-Webster mice (20-g males) yielded pleomorphic infections with slender forms predominating 5–7 days after infection.

A monomorphic strain, EATRO 110 M, was derived from EATRO 110 via serial passages (>100) in mice. These organisms were uniformly slender in morphology. They were routinely harvested from Swiss-Webster mice or Sprague-Dawley rats (250–350-g females) 3–4 days after infection.

Isolation of Trypanosomes. Trypanosomes were isolated from blood according to the procedure of Lanham and Godfrey (11). Infected mice were bled retro-orbitally with heparinized pipettes, whereas infected rats were lightly etherized and then bled by cardiac puncture into heparinized syringes. Whole blood (10 ml) was applied to a column (2.5×7 cm) of Whatman DE-52 cellulose and eluted with phosphate-buffered saline and glucose (PSG 6:4). For preparation of mitochondrial fractions, whole blood was first centrifuged for 20 min at 2,000 *g* in a Sorvall RC 2B refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). The resulting buffy coat was gently removed and eluted from a column as described above. A column with a packed volume of 50 ml could then be used for the equivalent of 50 ml of blood.

Preparation of Mitochondria. Isolated bloodstream forms of *T. b. brucei* (EATRO 110 M) obtained from rats were washed three times in PSG 6:4. The organisms (1×10^{10}) were pelleted and resuspended in 40 ml of distilled water. After 1 min, lysis was stopped by the addition of 10 ml of 1 M mannitol and 1 ml of 1 M potassium phosphate buffer (pH 7.5). Approximately 90% of the organisms was found to be lysed. The lysate was centrifuged at 1,000 *g* for 10 min, and the supernate was stored on ice. The resulting pellet was resuspended in 10 ml of 20 mM potassium phosphate buffer containing 0.25 M mannitol (pH 7.5), and then homogenized (6–10 strokes) with an Eberbach homogenizer (Eberbach Corp., Ann Arbor, Mich.). The homogenate was centrifuged as above. The supernates were combined and centrifuged at 20,000 *g* for 20 min. The resulting pellet was resuspended in the above buffer and utilized for assays.

Measurement of Hydrogen Peroxide. Hydrogen peroxide was assayed as described by Homan-Muller and Roos (12). A stock solution of 4 μM of activated LDADCF was prepared in 0.025 M sodium phosphate buffer (pH 7.2) containing 4% ethanol and 5 μg/ml of horseradish peroxidase. To 3 ml of the stock solution was added 0.2 ml of sample, after which the solution was incubated at

[1] *Abbreviations used in this paper:* CoQ, coenzyme Q; DCIP, dichlorophenolindophenol; DL-αGP, DL-α-glycerol phosphate; DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; GSH, glutathione; GSSG, oxidized glutathione; LDADCF, leucodiacetyldichlorofluorescin; MEM, minimal essential medium; PSG, phosphate-buffered saline and glucose; SHAM, salicylhydroxamic acid.

room temperature for 20 min. Fluorescence was measured with a Perkin-Elmer spectrofluorometer (model 204; Perkin Elmer Corp., Norwalk, Conn.) employing an excitation wavelength of 340 nm, and observing the emission at 520 nm. A standard curve was determined for freshly diluted hydrogen peroxide solutions utilizing an extinction coefficient of $E^{cm}_{230} = 81$. The assay was found to be linear from 10^{-7} to 10^{-5} M hydrogen peroxide.

The hydrogen peroxide content of *T. b. brucei* (EATRO 110 M) was determined in the following manner. Isolated organisms (10^{10}) were washed three times in PSG 6:4 and then homogenized for 25 s in 10 ml of distilled water using a Virtis homogenizer (model S-45). Aliquots of the homogenate were assayed for their H_2O_2 content as described above. The background fluorescence of the homogenate was determined by adding 5 μg of catalase to 0.2 ml of the homogenate 5 min before incubation with LDADCF. Catalase did not quench the fluorescence in this assay system.

The αGP-dependent rate of production of hydrogen peroxide by mitochondrial preparations was determined at 23°C by the following procedure. The assay mixture contained enzyme substrate and activated LDADCF (2 μM) in 50 mM potassium phosphate buffer (pH 7.5) containing 2% ethanol and 5 μg/ml of horseradish peroxidase. The relative fluorescence of the assay mixture was recorded as a function of time. Calibration of the rate of change of fluorescence was achieved by adding varying amounts of D-amino acid oxidase to assay solutions containing 3.3 mM DL-alanine. The activity of the D-amino acid oxidase in these solutions was determined by measuring the oxygen consumption of an aliquot in the presence of 3.3 mM DL-alanine.

Assays for Glycerol Phosphate Oxidase and Dehydrogenase. Glycerol phosphate dehydrogenase activity was measured using DCIP as the electron acceptor (13). Glycerol phosphate oxidase activity was determined by following oxygen consumption in the presence of DL-αGP with a Clark-type, oxygen electrode (Rank Brothers, Cambridge, England).

Determination of Peroxidase Activity. Assays for endogenous peroxidase activity were performed as follows. Homogenates of *T. b. brucei* (EATRO 110 M) were prepared in 50 mM potassium phosphate buffer (pH 7.5). Aliquots of homogenate (1.5 mg) were incubated at 24°C in 3 ml of 50 mM potassium phosphate buffer (pH 7.5) containing activated LDADCF (2 μM) and H_2O_2 (0.3 μM), with either DL-αGP (0.4 mM) or GSH (0.04 mM). After 10 min, 15 μg of horseradish peroxidase was added. After an additional 20-min incubation, H_2O_2 was quantitated fluorometrically as described above. The fluorescence measured in the absence of H_2O_2 has been subtracted from all measurements.

Miscellaneous Biochemical Assays. Reduced and oxidized GSH was determined by the method of Tietze (14). Coenzyme Q (CoQ) was assayed by the method of Redfearn (15). Protein concentrations were determined by the method of Lowry et al. (16) using bovine serum albumin as a standard.

Assessment of Trypanocidal Activity in Vitro. The trypanocidal activity of various compounds was assessed in vitro by following their effect on the turbidity of suspensions of trypanosomes. *T. b. brucei* (EATRO 110 M) were suspended in PSG 6:4/MEM 9/1 at a concentration of 1–2 × 10^7 organisms/ml, and they were maintained at 37°C. The turbidity of such a suspension was measured at 750 nm in a 1-ml cuvette with a Beckman Acta III recording spectrophotometer (Beckman Instruments, Inc., Cedar Grove, N.J.). Heme in dimethyl formamide (DMF), naphthoquinones in dimethyl sulfoxide (DMSO) and/or arsenicals in DMSO were added to the final concentrations indicated. In no case was the volume of DMF or DMSO added greater than 10 μl/ml of suspension. These small amounts of organic solvents had no effect on the turbidity of the suspensions or the motility of the organisms. Where lysis was indicated by a decrease in turbidity, it was confirmed by inspection of the suspension by phase contrast microscopy.

Assessment of Trypanocidal Activity in Vivo. Groups of five BALB/c mice (20–25-g males) were infected with *T. b. brucei* (5 × 10^4 organisms). 24 h after infection, the animals were treated with single doses of tryparsamide, hematoporphyrin D, or a combination of both drugs at the concentrations indicated. The drugs were administered i.p. as suspensions in 0.25% methylcellulose. In calculating average survival times, we considered those mice alive at 28 days to be cured.

Results

Fig. 1 is a working model of the relevant biochemistry and shows the points at which we have attempted to intervene. As a consequence of its lacking cytochromes, the bloodstream form of *T. b. brucei* has an unusual metabolism;

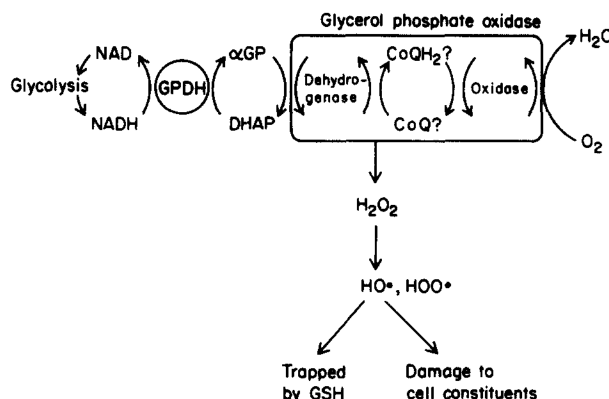

FIG. 1. A proposed scheme for electron transport in bloodstream forms of *T. b. brucei*. DHAP, dihydroxyacetone phosphate; GPDH, α-glycerol phosphate dehydrogenase.

namely, aerobic glycolysis. It is estimated that the organism consumes its dry weight in glucose every 90 min (17). Glucose is metabolized only as far as pyruvate (20); the NAD reduced during glycolysis is regenerated in microbodies by the reduction of dihydroxyacetone phosphate (19, 20). The αGP formed is in turn recycled by an oxidase complex which is associated with a rudimentary mitochondria (21).

Most of the electrons from the oxidation of αGP are incorporated into water. Mitochondrial preparations, in the presence of 7 mM DL-αGP at 23°C, consumed 21–29 nmoles of O_2/min/mg of protein, but produced only 0.2–0.7 nmoles of H_2O_2/min/mg of protein. Thus, ≅1–3% of the oxygen consumed forms H_2O_2. A similar partitioning of O_2 to form H_2O and H_2O_2 has been reported in mitochondrial preparations from rat liver (22) and *Crithidia fasciculata* (23).

The mitochondrial oxidase is a complex of at least two components, an αGP dehydrogenase and a terminal oxidase (21). The activity of the dehydrogenase can be measured using artificial electron acceptors such as DCIP, and it is not inhibited by either Triton X-100 or benzhydroxamic acids such as SHAM. The terminal oxidase, on the other hand, is inhibited by both Triton X and SHAM. The fact that the production of H_2O_2 by the mitochondrial preparation is not inhibited by Triton X (Table I) suggests that the dehydrogenase and not the oxidase is the source of hydrogen peroxide. However, since mitochondrial preparations contain microbodies (21), we cannot say with certainty which dehydrogenase is the source of H_2O_2.

The rate of H_2O_2 production described above for the trypanosome is similar to that measured in mammalian mitochondria. Thus, the increased concentration of H_2O_2 in trypanosomes must reflect an inability to degrade this compound. While the absence of heme in these organisms precluded the presence of catalase, other peroxidases might have been present. Accordingly, trypanosomal homogenates were assayed for peroxidase activity using a sensitive fluorometric technique (Table II). No decrease in H_2O_2 concentration could be observed after incubating for 10 min with an aliquot of trypanosomal homogenate (experiment 2). Similarly, no decrease in H_2O_2 concentration was observed when GSH was added to the homogenate (experiment 3). Thus, GSH peroxidase

TABLE I
The Effects of Triton X and SHAM on Various Activities of Glycerol Phosphate Oxidase Preparations

Exp.	Initial Rates* (nmoles/min/mg protein)			
	Triton X 0.05%		SHAM (1 mM)	
	−	+	−	+
1. O_2 Consumption‡	15.7	0.0	15.7	0.0
2. DCIP Reduction	28.8	69.0	26.9§	23.1
3. H_2O_2 Production	0.21	0.22	N.D.‖	N.D.¶
4. Menadione-supported O_2 consumption‡,**	268.0‡‡	393.0	258.0‡‡	225.0
5. Menadione-supported DCIP reduction§	159.0§§	266.0	N.D.	N.D.

* Initial rates were determined in the presence of 10 mM DL-αGP utilizing mitochondrial preparations.
‡ Oxygen consumption was measured as described in Fig. 2.
§ In this experiment a trypanosomal homogenate was utilized.
‖ N.D., not determined.
¶ SHAM inhibits horseradish peroxidase.
** Initial rates determined in the presence of 0.15 mM menadione.
‡‡ Initial rate in the absence of menadione 44.2 nmoles/min/mg protein.
§§ Initial rate in the absence of menadione 28.8 nmoles/min/mg protein.

is not likely to be present. Furthermore, no diminution in H_2O_2 concentration was observed when αGP was added to the homogenate (experiment 4). This latter experiment ruled out the possibility that H_2O_2 was an obligatory intermediate in the transfer of electrons from oxygen to water by the glycerol phosphate oxidase (24). Thus it appears that, lacking an enzymatic means of destroying H_2O_2, the organism must depend upon diffusion of this potentially lethal metabolite from the cell to its surroundings where it can be disposed of by mammalian enzymes.

It is apparent from Fig. 1 that increasing the rate of H_2O_2 production in *T. b. brucei* should increase the size of the intracellular H_2O_2 pool and thus make the organisms more susceptible to radical generators. We have been able to induce increased H_2O_2 production in vitro with a number of naphthoquinones. As shown in Fig. 2, the addition of menadione to a trypanosomal homogenate caused a threefold increase in the rate of oxygen consumption. The addition of catalase reduced the apparent rate of O_2 consumption by 30–40%, implying that 60–80% of the oxygen was being converted to H_2O_2. Thus, the naphthoquinone stimulated H_2O_2 production 200- to 300-fold over the normal state. Presumably, menadione was enzymatically reduced to the quinol which then reacted nonenzymatically with oxygen to form H_2O_2. Cycling of the naphthoquinone would explain its catalytic effect on the production of H_2O_2. Although we have not been able to definitively identify the site at which menadione interacts, we do not believe it is at the terminal oxidase since Triton X, which inhibits the oxidase, did not interfere with production of H_2O_2 in the presence of the naphthoquinone (Table I). The increased rate of reduction of DCIP in the presence of menadione, DL-αGP, and Triton X suggests that the naphthoquinone is reduced by the dehydrogenase. Since we find substantial amounts of CoQ (0.3

TABLE II
The Effect of Trypanosomal Homogenates on H_2O_2 in the Presence of DL αGP and GSH

Exp.	H_2O_2 preincubation	Trypanosomal homogenate	DL-αGP	Reduced GSH	H_2O_2 postincubation
	0.3 μM	*1.5 mg*	*0.4 mM*	*0.04 mM*	*μM*
1	+	−	−	−	0.30
2	+	+	−	−	0.29
3	+	+	−	+	0.26
4	+	+	+	−	0.25

nmoles/mg of protein) in the mitochondria of these organisms, we postulate that the naphthoquinone is substituting for CoQ as the electron acceptor of the dehydrogenase present in the glycerol phosphate oxidase complex.

The excess production of H_2O_2 caused by a naphthoquinone can be trypanocidal in vitro. The lowest concentrations of 2-methoxynaphthoquinone, menadione, and vitamin K-S(II) required to lyse trypanosomes are 0.3, 0.2, and 1.8 mM, respectively. As anticipated, naphthoquinones and heme act synergistically in vitro (Fig. 3). Although the addition of neither 60 μM menadione nor 3.5 μM heme had any lytic effect upon trypanosomes (Fig. 3, a and b), when administered together, the two caused a rapid lysis of the organisms (Fig. 3, a and c). Under these conditions, the rate of killing was equivalent to that observed in the presence of 20 μM heme alone (data not shown). The synergism was most evident when the naphthoquinone was added before heme, implying that an increased pool of intracellular H_2O_2 enhances the susceptibility of the organisms to heme lysis. Note that larger amounts of both menadione and heme were required to produce lysis when the naphthoquinone was added subsequent to heme (Fig. 3 b and c). Although naphthoquinones are trypanocidal in vitro both alone and in combination with heme, we have yet to find a naphthoquinone which is active in vivo either by itself or in combination with various porphyrins.

Another method of rendering trypanosomes more susceptible to radicals generated by heme-induced decomposition of H_2O_2 would be to decrease the level of intracellular radical scavengers. It has been suggested that reduced GSH plays such a protective role (25). Hence, if the GSH content of the trypanosomes were lowered, the organisms might be more susceptible to radical-mediated damage and killing. Accumulated evidence suggests that the trypanocidal arsenicals in their trivalent oxidation state react with thiols (26). Accordingly, the effect of arsenicals on intracellular GSH was studied by incubating isolated bloodstream forms of *T. b. brucei* (1.3×10^9 organisms/ml) at 23°C with 100-μM concentrations of either Melarsenoxide or Melarsen in PSG 6:4. After 20 min, the organisms treated with melarsenoxide, a trivalent arsenical, were immobilized but not lysed. Melarsen, a pentavalent arsenical, had no observable effect on the trypanosomes. The organisms were freeze-thawed three times, and the intracellular GSH content was determined (14). Melarsenoxide-treated trypanosomes were found to contain 67% less GSH (0.31 μg/10^9 cells) than either untreated trypanosomes (0.92 μg/10^9 cells), or Melar-

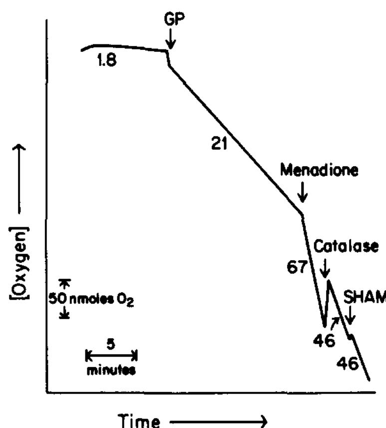

FIG. 2. The effect of menadione on the oxygen consumption of trypanosomal homogenates. An aliquot (100 μl) of homogenized bloodstream forms of *T. b. brucei* containing 1 mg of protein was added to 1.9 ml of sodium phosphate buffer (100 mM, pH 8.0) containing sodium chloride (73 mM). Oxygen consumption was measured in a Clark-type electrode at 37°C. At the indicated times, the following were added to obtain the final concentrations listed: DL-αGP (10 mM); menadione (0.15 mM); catalase (2,000 U/ml); SHAM (1 mM). The numbers shown next to the tracing are the slopes of each segment of the tracing in nmoles of oxygen consumed per min.

sen-treated trypanosomes (0.85 $\mu g/10^9$ cells). Melarsenoxide is not simply causing the accumulation of oxidized glutathione (GSSG), since only trace amounts of GSSG (0.015 $\mu g/10^9$ cells) were detectable (14). The fact that Melarsen does not cause a change in the concentration of GSH is consistent with the concept that in situ reduction of pentavalent arsenicals to the trivalent state is obligatory for trypanocidal activity (27).

The decreased GSH levels in Melarsenoxide-treated trypanosomes give the organisms an increased susceptibility to heme lysis. As shown in Fig. 3, combining sublytic concentrations of Melarsenoxide (0.5 μM) and heme (10 μM) caused lysis of the organisms at a faster rate than that observed with 60 μM heme alone (data not shown). A more pronounced synergism was observed when isolated trypanosomes were treated with sublytic concentrations of both menadione and Melarsenoxide before treatment with heme (Fig. 3f). This synergism points to the potential usefulness of such a combined therapeutic regimen.

An example of the synergism between arsenicals and porphyrins in vivo is presented in Table III. Both the rate of cure and the average survival time of mice infected with *T. b. brucei* are increased when the animals are treated with regimens involving both tryparsamide and hematoporphyrin D. The minimal curative doses of hematoporphyrin D and tryparsamide alone are 160 and 500 mg/kg, respectively. When administered separately, 25 mg/kg of hematoporphyrin D and 100 mg/kg of tryparsamide had no effect on survival, whereas the coadministration of both drugs resulted in a twofold increase in the average survival time of the mice. Unfortunately, the naphthoquinones tested to date have had no effect on survival when administered in combination with hematoporphyrin D, tryparsamide, or both. Nonetheless, we are continuing to screen

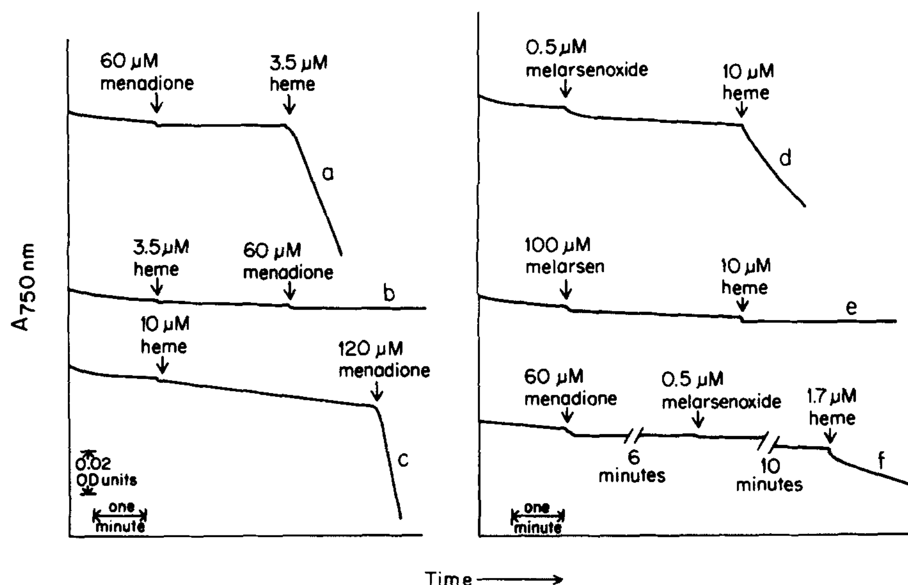

FIG. 3. The effect of menadione and arsenicals on the heme lysis of *T. b. brucei*. Purified bloodstream forms (1–2 × 10^7 organisms) were suspended in 1 ml of PSG 6:4/MEM 9/1 and maintained at 37°C. The turbidity of the suspensions was measured at 750 nm. Additions of Melarsenoxide, Melarsen, menadione, and heme were made as indicated by the arrows such that the final concentrations were those shown.

TABLE III
The Effects of a Combined Regimen of Tryparsamide and Hematoporphyrin D on the Survival of T. b. brucei-Infected Mice

Hematopor-phyrin D	Tryparsamide (mg/kg)			
	0	100	200	500
mg/kg				
0	3,3,3,4,4*	4,4,4,4,4	3,5,6,7,8	C,C,C,C,C
25	4,4,4,4,4	5,7,8,8,8	9,10,11,C,C	N.D.
50	8,8,8,9,9	9,9,9,C,C,	9,13,15,C,C,	N.D.
160	C,C,C,C,C,‡	N.D.§	N.D.	N.D.

* Days to death postinfection.
‡ C, cured (survived 28 days).
§ N.D., not determined.

naphthoquinones for in vitro effectiveness given the magnitude of their response in vitro.

Discussion

The presence of H_2O_2 in trypanosomes and the lack of enzymes to dispose of it represent an important biochemical difference between these parasites and their mammalian hosts. Fig. 1 shows that portion of the parasites biochemistry in which the production of H_2O_2 is thought to arise, and it depicts those sites where we have sought to intervene to increase the concentration of intracellular H_2O_2 or its homolytic cleavage products. We have previously shown that heme

in vitro and porphyrins in vivo are capable of killing the bloodstream form of *T. b. brucei*, presumably via their ability to initiate free radical reactions. This has prompted us to search for new agents with increased effectiveness in vivo. We have succeeded in substantially increasing the production of H_2O_2 by both isolated organisms and mitochondrial preparations via the addition of naphthoquinones. The increased intracellular H_2O_2 concentration confers upon the organisms an increased susceptibility to lysis by heme. Unfortunately, a naphthoquinone that is active in vivo has not been found, but we are still searching for such a compound. Another way in which we have increased the trypanocidal activity of heme in vitro and in vivo is by increasing the susceptibility of the organisms to radical damage. This has been accomplished by using arsenicals to diminish the concentration of reduced glutathione within the organisms. Thus, the main defense of the cells against radical damage is compromised. As in the case of the naphthoquinones, administering arsenicals in combination with heme and hematoporphyrin D resulted in a synergistic effect in vitro and in vivo, respectively. We hope that eventually it will be possible to develop a therapeutic regimen based on the scheme outlined here utilizing a combination of agents which will: (*a*) increase the production of H_2O_2, (*b*) lead to its homolytic cleavage, and (*c*) render trypanosomes more susceptible to damage by the free radicals produced.

Summary

The inability of the bloodstream form of *Trypanosoma brucei brucei* to decompose hydrogen peroxide forms the basis of our attempt to develop new pharmacological agents to kill these organisms. Approximately 1–3% of the oxygen consumed by these parasites appears in the form of hydrogen peroxide. Our previous observation that free radical initiators such as heme and hematoporphyrin D proved to be trypanocidal in vitro and in vivo, respectively, prompted this investigation into the mechanism of action of this class of compounds to enhance their therapeutic efficacy.

The locus of H_2O_2 production within the trypanosome was examined using cell-free homogenates. Experiments described herein suggest that H_2O_2 is formed by the α-glycerol phosphate dehydrogenase in an adventitious manner, and that no enzymatic means of disposing of this potentially toxic compound are present with the organisms. Naphthoquinones were found to substantially increase the rate of both oxygen consumption and H_2O_2 production by trypanosomal mitochondrial preparations. Presumably, the naphthoquinones are acting as coenzyme Q analogues. The addition of sublytic concentrations of both naphthoquinones and heme leads to a synergistic lysis of the organisms in vitro.

Another approach to increasing the susceptibility of *T. b. brucei* to free radical damage involved reduction of the intracellular concentration of glutathione. This was accomplished through the use of trypanocidal arsenicals. Melarsenoxide and heme acted synergistically in vitro, an effect which was further enhanced via addition of a naphthoquinone. Moreover, hematoporphyrin D and tryparsamide were shown to have a synergistic effect in *T. b. brucei*-infected mice.

Received for publication 10 April 1978.

References

1. Duggan, A. J. 1970. An historical perspective. *In* The African Trypanosomiases. H. W. Mulligan, editor. John Wiley & Sons, Inc., New York. xli.
2. DeRaadt, P. 1976. African sleeping sickness. *Trans. R. Soc. Trop. Med. Hyg.* **70**:114.
3. Wilson, S. G., K. R. S. Morris, I. J. Lewis, and E. Krog. 1963. The effects of trypanosomiases on rural economy. *Bull. W. H. O.* **28**:595.
4. Williamson, J. 1970. Review of chemotherapeutic and chemoprophylactic agents. *In* The African Trypanosomiases. H. W. Mulligan, editor. John Wiley & Sons, Inc., New York. 125.
5. Williamson, J. 1976. Chemotherapy of African trypanosomiasis. *Trans. R. Soc. Trop. Med. Hyg.* **70**:117.
6. Chang, K. P., C. S. Chang, and S. Sassa. 1975. Heme biosynthesis in bacterium protozoon symbioses: enzymatic defects in host hemoflagellates and complemental role of their intracellular symbiotes. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2979.
7. Baernstein, H. 1963. A review of electron transport mechanisms in parasitic protozoa. *J. Parasitol.* **49**:12.
8. Fulton, J., and J. Spooner. 1956. Inhibition of the respiration of *Trypanosoma rhodesiense* by thiols. *Biochem. J.* **63**:475.
9. Meshnick, S. R., K. P. Chang, and A. Cerami. 1977. Heme lysis of the bloodstream forms of *Trypanosoma brucei*. *Biochem. Pharmacol.* **26**:1923.
10. Lipson, R., E. Baldes, and A. Olsen. 1961. The use of a derivative of hematoporphyrin in tumor detection. *J. Natl. Cancer Inst.* **26**:1.
11. Lanham, S., and D. Godfrey. 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp. Parasitol.* **28**:521.
12. Homan-Muller, J., and D. Roos. 1975. Production of hydrogen peroxide by phagocytizing human granulocytes. *J. Lab. Clin. Med.* **85**:198.
13. Dawson, A. P., and C. J. R. Thorne. 1975. L-3-glycerophosphate dehydrogenase from pig brain mitochondrion. *Methods Enzymol.* **41**:254.
14. Tietze, F. 1969. Enzymatic methods for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27**:502.
15. Redfearn, E. R. 1967. Isolation and determination of ubiquinone. *Methods Enzymol.* **10**:381.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:2651.
17. Von Brand, T. 1973. Inorganic Substances. *In* Biochemistry of Parasites. Academic Press, Inc., New York. 20.
18. Grant, P. T., and J. Fulton. 1957. The catabolism of glucose by strains of *Trypanasoma rhodesiense*. *Biochem. J.* **66**:242.
19. Grant, P. T., and J. R. Sargent. 1960. Properties of L-α-glycerolphosphate and its role in the respiration of *Trypanosoma brucei*. *Biochem. J.* **76**:229.
20. Opperdoes, R. R., P. Borst, and H. Spits. 1977. Particle-bound enzymes in the bloodstream form *Trypanosoma brucei*. *Eur. J. Biochem.* **76**:21.
21. Opperdoes, F. R., P. Borst, S. Bakker, and W. Leene. 1977. Localization of glycerol-3-phosphate oxidase in the mitochondrion and particulate NAD-linked glycerol-3-phosphate dehydrogenase in the microbodies of the bloodstream form of *Trypanosoma brucei*. *Eur. J. Biochem.* **76**:29.
22. Boveris, A., N. Oshino, and B. Chance. 1972. The cellular production of hydrogen peroxide. *Biochem. J.* **128**:617.
23. Kusel, J. P., A. Boveris, and B. Storey. 1973. H_2O_2 production and cytochromic peroxidase activity in *Crithidia fasciculata*. *Arch. Biochem. Biophys.* **158**:799.
24. Grant, P. T., and I. B. R. Bowman. 1963. Respiration systems in protozoa with

special reference to the trypanosomidal. *Biochem. J.* **89**:89p.

25. Kosower, N. S., and E. M. Kosower. 1974. Effect of oxidized glutathione on protein synthesis. *In* Glutathione. H. Flohe, H. Benehr, H. Sieg, H. D. Waller, and A. Wendel, editors. Theime, Verlag KG, Stuggart, W. Germany 216.
26. Johnstone, R. M. 1963. Sulfhydryl agents: arsenicals. *In* Metabolic Inhibitors. R. M. Hochster and J. H. Quastel, editors. Academic Press, Inc., New York. 99.
27. Lourie, E. M., F. Murgatroyd, and W. Yorke. 1935. Studies in chemotherapy. *Ann. Trop. Med. Parasitol.* **29**:265.