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HEME LYSIS OF THE BLOODSTREAM FORMS OF *TRYPANOSOMA BRUCEI*

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Abstract—The bloodstream forms of *Trypanosoma brucei* were found to be lysed *in vitro* by the addition of heme. For each μg/ml of heme, approximately 2×10^5 organisms/ml were killed in 20 min at 37°. The mechanism for the lysis is believed to be mediated by heme-induced homolytic cleavage of intracellular H_2O_2 (70 μM) to form hydroxyl radicals. The highly reactive radicals particularly disrupt various membranes and eventually kill the organisms. Heme and several porphyrins which are active *in vitro* are inactive *in vivo* due to very tight binding to albumin and other body proteins. Hematoporphyrin, which binds albumin weakly, is curative *in vivo* (160 mg/kg). Hopefully, new and more effective agents will be found that will enter the trypanosomes and generate free radicals to kill the cells.

The development of new chemotherapeutic agents to treat infectious diseases can be aided by the identification of biochemical differences between the host and the invading organism. Although, traditionally, the mechanism of action has been elucidated after the drug has been found by screening, it is desirable to rationally design antimetabolites that exploit the biochemical differences. Consequently, we have been studying the biochemistry of the *Trypanosoma brucei* group of trypanosomes and their mammalian hosts in order to identify key points for which drugs can be designed.

In the current paper, we have explored one of the differences between the trypanosome and the mammalian host, namely the inability of these parasites to synthesize heme. As a result of this deficiency and the avid binding of heme by serum proteins of the host [1], the bloodstream stage of the life cycle has no detectable heme or hemoproteins such as cytochromes or catalase [2, 3]. We have found that this lack of catalase is associated with a substantial amount of intracellular hydrogen peroxide. This hydrogen peroxide leaves the organisms vulnerable to attack by compounds which can cause the homolytic cleavage of peroxide to form hydroxyl radicals. These radicals can react with vital cell components and kill the organism. We have found that heme and related compounds serve this function and lyse this group of organisms. Hopefully, this pharmacological mechanism can be exploited *in vivo* by the development of new drugs that will be useful in animals and man.

MATERIALS AND METHODS

T. brucei, EATRO laboratory strain 110, was obtained from Dr. William Trager and stored as stabilates at −80° in 10% glycerol. The strain had been passaged in rodents seven times, since it was last transmitted by tsetse flies. Intraperitoneal injections of 2×10^4 motile organisms into Swiss mice (20 g) yielded pleomorphic infections with slender forms predominating 5–7 days after infection.

A monomorphic strain of EATRO 110, EATRO 110M, was derived by over 100 serial passages in mice. These organisms were uniformly slender in morphology. They were routinely harvested 3–4 days after infection in mice or rats. Culture forms derived from EATRO 110 were kindly provided by Dr. Rolf Steiger [4]. The organisms were harvested in late log phase. The strain of *T. congolense* (TREU 1183) that was utilized had been passaged in rodents twelve times since it was last transmitted by tsetse flies and is described in more detail elsewhere [5]. These organisms were harvested 7 days after infection. Trypanosomes were isolated according to the procedure of Lanham and Godfrey [6]. Mice were bled retro-orbitally with heparinized pipettes. Whole blood (1 ml) was applied onto a column (15 × 0.8 cm) of DE-52 cellulose (Whatman) and eluted with phosphate buffered saline and glucose (PSG 6:4 [6]).

Heme (Grade 2), bilirubin, hematoporphyrin IX, microperoxidase and catalase were obtained from Sigma Chemical Co., St. Louis, MO. Protoporphyrin IX, cobalt protoporphyrin IX and uroporphyrin I were obtained from Porphyrin Products, Logan, UT. Riboflavin and leukoacetyldichlorofluorescein were purchased from Eastman Chemical Co., Rochester, NY. Bovine serum albumin was obtained from Miles Laboratories, Elkhardt, IN. Lipid-free diet, coconut and corn oil were purchased from ICN, Cleveland, OH. Diamide and horseradish peroxidase were obtained from CalBiochem, La Jolla, CA and Worthington Biochemicals, Freehold, NJ respectively. Hematoporphyrin was purified by the method of Lipson *et al.* [7]. Zinc hematoporphyrin was synthesized from purified hematoporphyrin by the procedure of Fuhrop and Smith [8].

The porphyrins were freshly dissolved either in a mixture of one part absolute methanol to one part 0.02 N KOH or in 0.25% Na_2CO_3 for studies *in vitro*. No more than 50 μl of these solutions was added to 1 ml of buffer. For intraperitoneal injections, the porphyrins were dissolved in 0.14 N NaOH. These solutions were brought to pH 7.4–8.0 by adding 0.14 N HCl. Subsequent dilutions were made in Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY).

Trypanocidal activity was assessed by monitoring the motility of the organisms. The motility was measured as follows. Plastic tubes containing the buffer PSG 6:4 and the test compound dissolved in the appropriate solvent were equilibrated at the desired temperature in a water bath. Minimum Essential Medium (MEM, Gibco, Grand Island, NY) containing the organism was added to this to a final ratio of 9 parts PSG 6:4 to one part MEM. The tubes were removed from the water bath at intervals and vortexed for 3 sec. An aliquot was removed and the organisms were counted on a Petroff–Hauser bacterial cell counter at 300x with phase contrast optics (Zeiss). The counting was completed within 1–2 min after the sample was removed. Non-motile ghosts were distinguished from debris by the presence of kinetoplast and flagella or nucleus and kinetoplast. Thirty to fifty organisms were counted at each time point. The effect on motility of the solvent used to dissolve the test compound was always observed simultaneously. The pH was measured and remained constant over the course of the incubation.

Anaerobic conditions were achieved in the following way: 2.5 ml PSG 6:4/MEM, 9/1, containing 6×10^6 *T. brucei*/ml, was incubated at 25° in a test tube with a sidearm containing 10 μl of a solution of 30 μg heme dissolved in methanolic potassium hydroxide. The system was deoxygenated under a stream of nitrogen gas for 5 min followed by the addition of 5 mg sodium dithionite. The nitrogen gassing was resumed and the heme was mixed with the solution containing the parasites.

Investigation of ultrastructural changes was performed in the following way. Column purified blood forms of *T. brucei* at 10^7 cells/ml were incubated at 37° in buffer with or without added heme (120 μg/ml). At intervals between 1 to 30 min, 0.5 ml aliquots were pipetted into 4.5 ml of 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were then centrifuged at 2000 *g* for 20–30 min. The pellets were fixed in freshly prepared glutaraldehyde followed by osmium tetroxide solution, stained with uranyl acetate and embedded in Epoxy resin [9]. Trypanocidal activity *in vivo* was assessed by the intraperitoneal injection of the test compound into five mice infected 24 hr previously with 5×10^4 organisms of *T. brucei* EATRO 110M.

The hydrogen peroxide content of trypanosomes was assayed by a modification of the procedure of Keston and Brandt [10], as described by Homan-Muller *et al.* [11]. A stock solution of 4 μM activated [10] leukodiacetyldichlorofluorescein (LDADCF) in 0.025 M sodium phosphate buffer at pH 7.2 was prepared containing 4% ethanol and 5 μg/ml of horseradish peroxidase. To 3 ml of the stock solution was added 0.2 ml of sample, and the solution was incubated for 20 min at room temperature. The fluorescence was measured in a Perkin–Elmer MPF2A spectrofluorimeter employing an excitation wavelength of 340 nm and observing the emission at 520 nm. A standard curve was determined by using freshly diluted hydrogen peroxide solutions utilizing an extinction coefficient of $E^{cm}_{230} = 81$. The enzymatic assay was linear from 10^{-7} to 10^{-4} M hydrogen peroxide. The hydrogen peroxide content of 10^{10} *T. brucei* EATRO 110M was determined. The purified

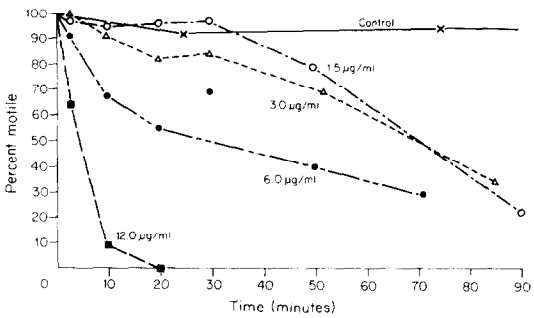


Fig. 1. Effect of heme on motility. *T. brucei* EATRO 110 at 4×10^6 organisms/ml was incubated at 37° with different concentrations of heme. At the times indicated, aliquots were removed and motility was assessed.

organisms were homogenized in 10 ml of phosphate buffer, pH 7.2, in a Virtis S45 for 25 sec. The background fluorescence of the homogenate was determined by adding 5 μg catalase to 2 ml of the homogenate 5 min prior to incubation with LDADCF. Neither catalase nor heme quenched the fluorescence of this assay. For the calculation of intracellular concentration of H_2O_2, 10^9 organisms were assumed to occupy 81 μl [12]. Protein concentration was determined by the method of Lowry *et al.* [13].

RESULTS

The addition of heme to buffered suspensions of *T. brucei* (EATRO 110) isolated from mouse blood was found with time to immobilize and disrupt the organisms. Injection of 10^3 organisms, treated with 6 μg/ml of heme for 10 min, into six mice did not result in an infection, confirming the trypanocidal effect of heme. The rate of immobilization was dependent on the concentration of heme and occurred at concentrations as low as 1.5 μg/ml (Fig. 1). At a fixed concentration of heme, the rate of killing of the organisms was dependent on the temperature of the incubation (Fig. 2). Calculation of the total number of organisms killed at various heme to trypanosome ratios revealed that 1 μg heme killed approximately 2×10^5 organisms after 20 min at 37°. This rate is for freshly isolated organisms. Organisms utilized several hours after isolation were found to be more

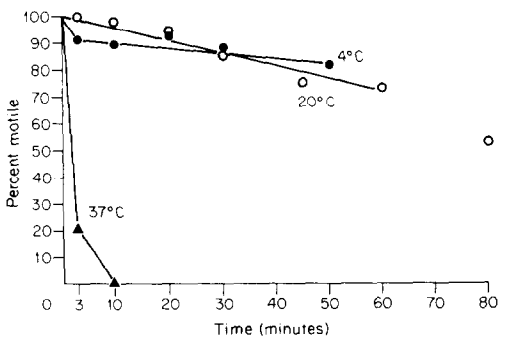


Fig. 2. Effect of temperature on the immobilization of organisms by heme. *T. brucei* EATRO 110 at 4×10^6 organisms/ml was incubated with 12 μg heme/ml at different temperatures.

Table 1. Trypanocidal activity of heme

	$T_{1/2}$ (min)*		
	(6 μg heme/ml at 37°)	(12 μg heme/ml at 37°)	(120 μg heme/ml at 24°)
Bloodstream forms			
T. brucei, dimorphic	30–40	3–6	
T. brucei, monomorphic	30–40	3–6	1.5
T. congolense	30–40	3–6	
Culture forms			
T. brucei			>80

* $T_{1/2}$ is the time required for lysis of one half of the organisms.

sensitive (~2-fold) to heme. The trypanocidal effect of heme was independent of the presence of oxygen. Organisms incubated under aerobic or anaerobic conditions were lysed at the same rate. In addition to the dimorphic strain, the monomorphic strain of *T. brucei* and the bloodstream form of *T. congolense*, were equally sensitive to heme lysis *in vitro*. The times necessary for immobilization of one half of the organisms (2×10^6 trypanosomes/ml) for the three types were 30–40 min at 6 μg heme/ml and 3–6 min at 12 μg heme/ml. In contrast to the bloodstream form of *T. brucei*, the culture forms were not affected by heme at concentrations as high as 120 μg heme/ml (Table 1). Ultrastructural studies revealed rapid cellular damage to the trypanosomes after incubation with heme. Trypanosomes incubated in medium alone remained intact throughout the 30 min (Fig. 3), whereas those treated with heme began to lyse within 1 min (Fig. 4), and were completely disrupted into membrane debris after 5 min (Fig. 5). Examination of a series of electron micrographs suggests that cell lysis is preceded by damage to various membranes of the cell. The membrane of the endoplasmic reticulum and the nuclear envelope are swollen and disrupted (Fig. 4). In addition, the plasma membrane is damaged as evidenced by the presence of denuded flagella and the apparent loss of cytoplasmic density (Fig. 4).

Albumin completely protected trypanosomes from the lytic effect of heme when added prior to the addition of heme and prevented further lysis when added during incubation with heme (Fig. 6). The protective effect depended on the binding of heme to albumin, since no protection occurred upon exposure of resuspended albumin-treated cells to heme in fresh albumin-free buffer. The strong binding of heme to albumin and other body proteins probably accounts for its lack of trypanocidal activity *in vivo* (Table 2).

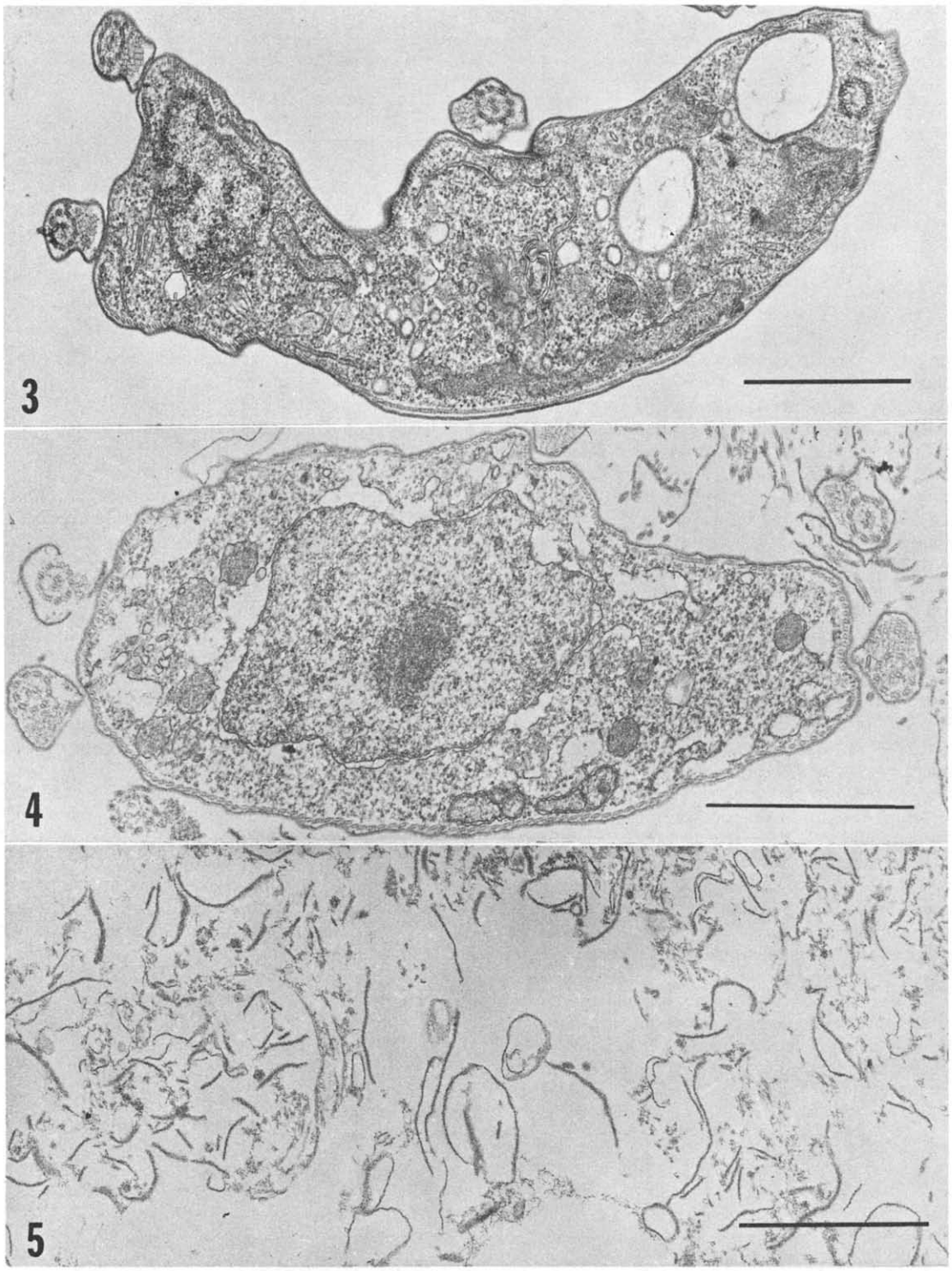
In attempting to explain the trypanocidal action of heme, we considered its role in catalyzing free radical formations. Heme is especially effective at catalyzing the homolytic cleavage of hydrogen peroxide [14]. Measurement of the hydrogen peroxide concentration in the bloodstream form of *T. brucei* revealed an intracellular concentration of 70 μM. This concentration (0.8 nmole/mg of protein) is approximately 30 times higher than that found in a liver homogenate (0.03 nmole/mg of protein). The presence of such a significant amount of hydrogen peroxide should make these organisms extremely vulnerable to compounds capable of generating free radicals.

A number of tetrapyrroles are known to produce activated chemical species when exposed to light [15]. Accordingly, the trypanocidal activity of a series of heme-related compounds was evaluated in the presence or absence of light, both *in vivo* and *in vitro* (Table 2). Of the compounds tested, only protoporphyrin IX had a light-dependent action. Cobalt protoporhyrin and zinc hematoporphyrin were approximately as effective as heme *in vitro*, while bilirubin and uroporphyrin were not effective *in vitro* or *in vivo*. Zinc hematoporphyrin was partially effective *in vivo*. Hematoporphyrin was not toxic *in vitro* but was active *in vivo* and was curative at 160 mg/kg.

Table 2. Lytic effect of porphyrins on *T. brucei*

Compound	Lytic concn *in vitro** (μg/ml)	*In vivo* Dose (mg/kg)	*In vivo* Mean survival of five mice (days)
Protoporphyrin IX	12 (0.24 in light)	40	4
Uroporphyrin I	> 80	200	4
Hematoporphyrin	> 600	160	Curative
Bilirubin	> 200	120	4
Heme	12	160	4
Cobalt protoporphyrin	<30†	ND‡	ND
Zinc hematoporphyrin	12	144	7§
Control			4

* Concentration which lyses 100 per cent of organisms in 20 min.
† Molar extinction coefficients of > 10^4 at both 530 nm and 562 nm were assumed.
‡ Not determined.
§ Three mice were used.



Figs. 3–5. (Fig. 3) Electron micrograph of *T. brucei* (EATRO 110M) incubated without heme for 30 min at 37°. Note the normal appearance of the nuclear envelope, endoplasmic reticulum and flagella. Bar represents 1 nm. (Fig. 4) Electron micrograph of *T. brucei* (EATRO 110M) incubated with 120 μg/ml of heme for 1 min at 37°. Note the swelling and disruption of the nuclear envelope and endoplasm reticulum and absence of membrane surrounding some sections of the flagella. Bar represents 1 nm. (Fig. 5) Electron micrograph of trypanosomal remnants after treatment with 120 μg/ml for 5 min at 37°.

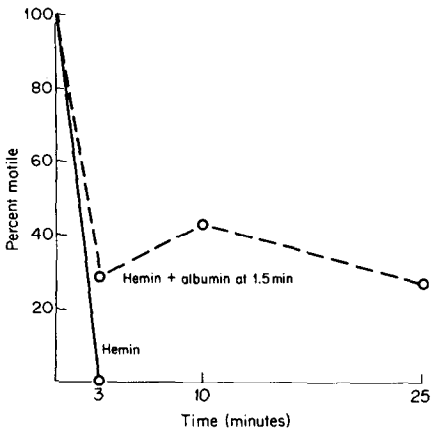


Fig. 6. Effect of albumin on the immobilization of organisms by heme. *T. brucei* EATRO 110 at 2×10^6 organisms/ml was incubated at 37° with 6 μg heme/ml. Bovine serum albumin was added at $t = 1.5$ min to a final concentration of 1 mg/ml.

Several experiments provide additional evidence indicating that the heme-mediated lysis of trypanosomes *in vitro* may be based on the generation of free radicals from hydrogen peroxide. First, trypanosomes grown in mice maintained on a corn oil diet, which is high in unsaturated fatty acids, were lysed four times more rapidly than organisms grown in mice maintained on a coconut oil diet, which is high in saturated fats (Fig. 7). Second, the free radical scavenger, riboflavin, partially protected the organisms from heme lysis. Half the cells were lysed in 5 min by 12 μg/ml of heme without riboflavin, while a period of 15 min was required in the presence of riboflavin. Third, pre-incubation of trypanosomes with diamide which has been shown to oxidize glutathione [16], potentiated the effect of heme presumably because the reduced glutathione is not available to act as a natural free radical scavenger. Prior incubation with 25 μM diamide reduced the time required for half-lysis from greater than 40 min with 3 μg/ml of heme to 1.5 min.

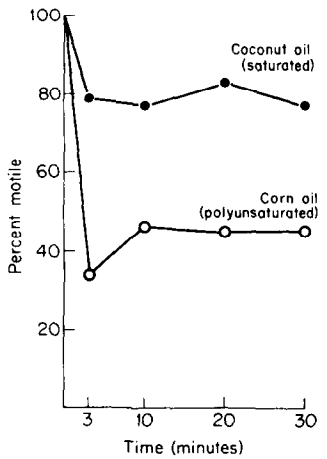


Fig. 7. Effect of host diet on the immobilization of organisms by heme. Mice were maintained on diets consisting of 17% coconut oil or corn oil in lipid-free diet (w/w) for 2 weeks prior to infection as well as during the infection. Mice were infected with 50,000 parasites (*T. brucei* EATRO 110M). Trypanosomes were harvested 5 days after infection; 4×10^6 organisms/ml were incubated at 37° with 6 μg of heme/ml.

DISCUSSION

During the course of evolution, trypanosomes seem to have lost the ability to synthesize heme; forms which contain heme are completely dependent on exogenous sources [17]. While many species of trypanosomes acquire heme from both their insect and vertebrate hosts, the *brucei* subgroup does not seem to obtain heme from its mammalian hosts, perhaps because of the latter's biochemical mechanisms for avid binding and disposal of heme [1]. Presumably the mammals have evolved such mechanisms to prevent auto-oxidation by free radicals, a process that heme so effectively catalyzes. Unsaturated fatty acids are particularly susceptible to these reactions [18]. The fact that animal fat becomes rancid much faster in the presence of trace amounts of heme accounts for its careful exclusion in the preparation of fat by food technologists [19]. The rapid lysis of bloodstream trypanosomes by heme which we report in the present paper might well be due to free radical damage to lipid components of membranes. The enhanced susceptibility of trypanosomes to damage by these reactions is due to the large intracellular concentration of hydrogen peroxide caused by a lack of catalase. Heme is taken up by the organism and catalyzes the homolytic scission of hydrogen peroxide to hydroxyl radicals. These radicals can participate in a myriad of chemical reactions with cell substituents. Since termination of this process occurs only when radicals combine with one another when they react to form stable products, it is apparent that the generation of even one radical can lead to considerable damage within the cell.

In recent years the importance of free radical reactions in biology has been recognized. Leukocyte killing of ingested bacteria [20], the pathological lesions associated with the accumulation of iron and copper [21, 22], and the damage caused by chemicals, such as carbon tetrachloride [23] are just a few examples where free radicals have been implicated.

One of the major difficulties in studying free radical reactions is the inability to identify the products of these ractions. This accounts for the fact that most of the data implicating free radical reactions are inferential. The evidence for the free radical reaction mechanism for the lysis of trypanosomes by heme is as follows. First, ultrastructural studies of heme-treated trypanosomes reveal a very rapid swelling and disruption of endoplasmic reticulum membranes (Fig. 4). This kind of cellular damage is similar to that seen in the liver after carbon tetrachloride intoxication [24], and might be related to the observation that the endoplasmic reticulum is especially rich in unsaturated fatty acids [25]. Second, trypanosomes from animals fed diets enriched with unsaturated fatty acids are more susceptible to heme lysis than organisms isolated from animals fed a diet enriched with saturated fatty acids. Third, the addition of riboflavin, which is a natural free radical trapping agent, retards the rate of heme lysis [26]. Fourth, the pretreatment of trypanosomes with diamide depletes the endogenous supply of reduced glutathione and poten-

tiates heme lysis. Reduced glutathione is one of the cell's mainstays in trapping free radicals and preventing free radical damage.

It appears unlikely that heme lysis is due to a detergent effect since bilirubin is not trypanocidal. Bilirubin is an open-chain tetrapyrrole with detergent activity but without the catalytic or photochemical properties of porphyrins and metalloporphyrins.

The uptake of porphyrins by trypanosomes must be fairly specific, since several porphyrins capable of generating radicals are ineffective at killing the organisms. Uroporphyrin I, with eight carboxylic acid groups, does not have the biological activity possessed by the dicarboxylic acid porphyrins, protoporphyrin IX and hematoporphyrin IX.

Hematoporphyrin, which does not lyse trypanosomes *in vitro*, is effective *in vivo* [27] and is thus presumably taken up by the organisms. A possible explanation for the ineffectiveness of hematoporphyrin *in vitro* is that its activity depends upon incorporation of a metal such as iron or zinc. Since zinc protoporphyrin is formed *in vivo* when protoporphyrin IX accumulates, as in lead poisoning [28], this could be the active metabolite. In contrast to hematoporphyrin, zinc hematoporphyrin is toxic to trypanosomes *in vitro* but is unfortunately too toxic *in vivo* to offer much hope as a drug. That protoporphyrin IX is effective at extremely low concentrations in the presence of light points to the use of free radical generators as potential trypanocidal drugs. In the future, we hope to find a molecule that will be selectively taken up by trypanosomes *in vivo*, catalyze the breakdown of intracellular hydrogen peroxide, and be therapeutically useful.

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