ly. We tested the fluidizing effect of ethanol on these preparations by increasing the concentration of ethanol in vitro from 8 to 130 m*M*. This caused a progressive decrease in the microviscosity (increased fluidity) of the membranes, which was similar in both groups. The decrement in the r_s value from 8 to 130 m*M* ethanol was highly significant ($\Delta r_s = 0.09$; $P < .01$). By contrast, the r_s values obtained in membranes from the Chow-fed group were initially lower than those of the other experimental groups ($r_s = 0.19 \pm 0.03$ versus 0.23 ± 0.05; $P < .05$), and increasing the concentrations of ethanol from 8 to 130 m*M* affected their microviscosity to a lesser degree (0.19 ± 0.030 to 0.17 ± 0.01). The data indicate that mitochondrial membranes from the Chow-fed rats fasted for 18 hours are more resistant to the fluiding effects of ethanol in vitro than membrane preparations from ethanol-fed rats. Thus, the addition of ethanol at concentrations comparable to those present in man and animals consuming ethanol altered the structural properties of the mitochondrial membranes from both the ethanol-fed and the pair-fed control rats to the same extent, decreasing their microviscosity (increasing the fluid state of the lipid portion of the membrane).

That the phospholipids are required for membrane integrity and for the optimal activity of the respiratory chain is not disputed here. However, the data do not support the hypothesis that functional changes in the activity of the respiratory chain are necessarily altered by changes in the properties of the membrane phospholipids. Our findings, and those of others (*1*), have demonstrated that long-term ethanol consumption reduces the state 3 respiration rate in mitochondria, especially for substrates linked to the reduced form of nicotinamide adenine dinucleotide (site I). We have detected no differences in the capacity of mitochondrial preparations from Chow-fed rats and those from rats fed the control liquid diet to oxidize substrates entering the respiratory chain at different levels. However, there were striking differences between these two groups not only in the proportions of the fatty acids of the phospholipids but also in their relative amounts. The microviscosity of these membranes also differed, as did their response upon the addition of physiological concentrations of ethanol. The initial r_s values for the preparations from the Chow-fed animals were lower than for the other experimental groups; preliminary data from our laboratory indicate a lower cholesterol content in these preparations, which is consistent with the observed results. Moreover, the membrane preparations from the Chow-fed animals were more resistant to the fluidizing effects of ethanol than the preparations from the other groups, a fact that may in part be due to the greater proportion of saturated fatty acids in the membrane phospholipids of these preparations. The relative proportions of saturated and unsaturated fatty acids were not significantly altered by the long-term consumption of ethanol; thus we lack evidence of a structural adaptation of the membrane under these circumstances.

The available data on changes in the lipid composition of mitochondrial membranes from ethanol-fed animals are conflicting (*7*). The fatty acid content of the diet, the duration of the ethanol treatment, or the fasting of the animals prior to the experiments could account for these discrepancies. Our findings are in agreement with those of some investigators (*8*) but in disagreement with those of others, who noted a decrease in polyunsaturated fatty acids, especially arachidonic (*7*). However, the differences described in these experiments are less than that observed between preparations from our two control groups, which exhibited similar functional activities. At this stage it would be premature to ascribe the severe functional derangements to relatively minor alterations in the lipid composition of the membranes. Moreover, the degree of saturation of the fatty acids is only one of many factors regulating the structural state of the membranes. Other factors, such as the cholesterol and calcium content and the protein composition of these preparations, remain to be defined. Thus, although our results confirm the effect of long-term ethanol intake on the mitochondrial function, a direct causal relationship could not be established between these changes and those in the structural properties of the membranes.

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Role of Calcium in Trypanocidal Drug Action

Abstract. *The synergistic effect of serum on the drug combination of salicylhydroxamic acid plus glycerol, which is active against* Trypanosoma brucei, *is due to diffusible calcium ions. The synergistic activity can be removed by dialysis of the serum or by addition of calcium chelating agents. A buffer containing calcium can mimic the synergistic activity of serum. This finding may have important implications in the clinical management of African trypanosomiasis in humans. Calcium also has a synergistic effect on melarsoprol, the only drug available for treating sleeping sickness patients with central nervous system involvement, and the concentration of calcium has been reported to be depressed in the serum of experimentally infected animals.*

The protozoan parasites *Trypanosoma brucei gambiense* and *T. b. rhodesiense* cause African sleeping sickness in humans, and the closely related *T. b. brucei* is one of several trypanosomes that cause a similar disease in animals. There has been little advance in the treatment of these diseases in the last 25 years, and the drugs in current use are inadequate (*1*). The therapeutic potential of a combination of salicylhydroxamic acid (SHAM) and glycerol, which blocks the carbohydrate catabolic pathways essential to these parasites, was previously reported by our laboratory (*2*). Subsequently, we found that this drug combination caused a more rapid and complete destruction of parasites when the infected rats were treated in vivo than when the parasites were treated in vitro suspended in a phosphate-saline-glucose (PSG) buffer (*3*). We attributed this dif-

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ference to a synergistic property of serum but were unable to identify the serum component responsible for the effect (*4*). We now report that this synergism is due to serum calcium. We have found that the synergistic action of serum is lost when the serum is subjected to dialysis or treated with calcium chelating agents. A Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] buffer with calcium added at a physiological concentration (1.2 m*M*) exhibits the same synergism as serum.

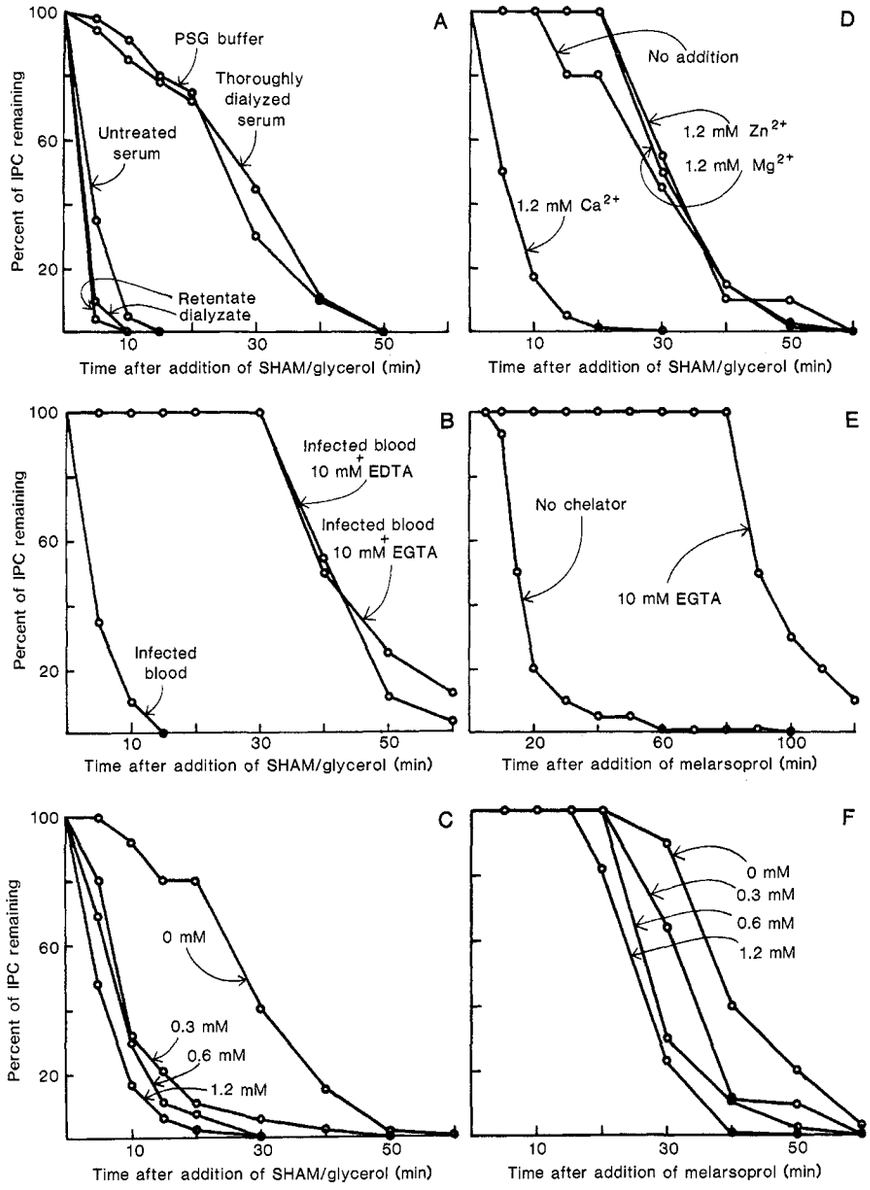

Fig. 1. The role of calcium in trypanocidal drug action. The concentrations of SHAM and glycerol were 2 m*M* and 10 m*M*, respectively, in all the experiments. *IPC*, initial parasite count. (A) The synergistic action of serum and SHAM/glycerol in the destruction of *Trypanosoma brucei brucei* (*5*). Dialysis of the serum abolished the synergistic effect of the serum on SHAM/glycerol. If a small volume of PSG buffer was used, activity could be found in both the dialyzate and the retentate. Control serum was held in a dialysis membrane that was not suspended in PSG dialysis buffer. (B) The effect of calcium chelators. Both EDTA and EGTA blocked the synergism of SHAM/glycerol and rat blood when SHAM/glycerol was added to whole, heparinized blood from *T. b. brucei*–infected rats. (C) The effect of added calcium to an incubation buffer. A buffer of 15 m*M* Bicine, 125 m*M* NaCl, and 1.5 percent glucose at *p*H 7.6 (Bicine buffer) was used. The calcium was added as a neutralized solution of calcium hydroxide. This calcium-containing solution mimicked the action of serum. (D) Comparison of the effect of zinc and magnesium with the effects of calcium. Neither zinc nor magnesium were able to substitute for calcium. Bicine buffer was used as in (C). (E) The effect of calcium chelation on the action of melarsoprol. Suspensions of partially purified parasites in rat serum were brought to a final concentration of 15 μg of melarsoprol per milliliter. The time required for 90 percent of the parasites to be lysed was delayed 90 minutes by chelation of calcium with EGTA. Note that the time scale in this figure is compressed relative to the other figures. (F) Effect of calcium on the action of melarsoprol. Bicine buffer was used as in (C). The melarsoprol concentration was 15 μg/ml; calcium concentrations were as indicated for the individual curves.

Figure 1A shows that *T. b. brucei* suspended in rat serum that has been dialyzed against a large volume of phosphate-saline (PS) buffer (0.1*M* sodium phosphate and 0.073*M* sodium chloride, *p*H 8.0) show the same rate of lysis when exposed to SHAM/glycerol as parasites suspended in PS buffer containing 1.5 percent glucose (PSG) (*5*). However, when rat serum is dialyzed against an equal volume of PSG buffer both the retenate and the dialyzate enhance the rate of lysis by SHAM/glycerol. Thus the active component is dialyzable and retains activity after passing through the membrane. Figure 1B shows that both the divalent cation chelator ethylenediaminetetraacetate (EDTA) and the calcium-specific chelator ethyleneglycoltetraacetate (EGTA) abolish the synergistic action of heparinized rat blood with SHAM/glycerol. When parasites are suspended in serum with EDTA or EGTA and exposed to SHAM/glycerol the rate of lysis is even slower than when they are suspended in PSG and similarly exposed. This is probably due to a stabilizing effect of certain serum components such as amino acids, vitamins, and proteins, since these have been shown to enhance and stabilize the metabolic activity of these parasites in vitro at 37°C (*6*). The enhancement of parasite destruction in the presence of calcium is therefore better demonstrated by the difference in the rate of destruction of the trypanosomes suspended in blood in the presence and absence of chelator rather than by the difference in the lysis rate of trypanosomes suspended in dialyzed and undialyzed serum.

Figure 1C shows that a physiological concentration of calcium [normal serum ionized calcium is 1.2 m*M* (*7*)] added to Bicine buffer mimics the effect of serum; essentially the same rate of lysis occurs at 0.3 and 0.6 m*M* Ca^{2+}. When the source of calcium is neutralized calcium hydroxide (Fig. 1C) the lytic activity is greater than when calcium lactate is added, especially at the lower concentrations of total calcium (data not shown). Since calcium lactate at physiological *p*H exists in solution as a complex, thus providing few free calcium ions (*7*), these data indicate that ionized calcium is the active agent. Figure 1D shows that neither zinc nor magnesium can substitute for calcium in this synergism.

We considered the possibility that the synergistic effect of calcium might not be limited to the SHAM/glycerol combination and indeed found that the rate of parasite dissolution induced by the gen-

eral metabolic inhibitor iodoacetamide was also enhanced by calcium (data not shown). These results led us to look for a similar interaction with malarsoprol, the only drug now available for treatment of cases of African trypanosomiasis with central nervous system involvement. Figure 1E shows that calcium is more critical for melarsoprol action than for SHAM/glycerol action. The time required for melarsoprol to cause lysis of 90 percent of the parasites was delayed 90 minutes by calcium chelation, whereas such chelation caused only a 40-minute delay in the time required for SHAM/glycerol to lyse 90 percent of the parasites.

Figure 1F shows that there is a dose-response curve with calcium and melarsoprol, although there is little difference between the rates of lysis at 1.2 and 0.6 m*M* Ca^{2+} for the first 30 minutes of incubation. Two repetitions of this experiment indicated that no intact parasites were present after 40 minutes in the presence of 1.2 m*M* Ca^{2+}, but that intact cells could be found in low numbers even after 60 minutes of incubation in the presence of 0.3 and 0.6 m*M* Ca^{2+}. These remaining parasites are important because one single cell is sufficient to maintain an infection.

That calcium influences the effects of several trypanocidal agents (SHAM/glycerol, melarsoprol, and iodoacetamide) indicates that its mechanism of action may be similar to that observed when calcium is added to hepatocytes in the presence of certain cytotoxic substances in vitro as reported by Schanne *et al.* (*8*) [despite recently reported, somewhat contradictory, results with hepatocytes (*9*)]. Because trypanosomes can be handled with relative ease and are lysed rapidly with the cytotoxic agents used, we were able to make a more detailed analysis than might be practical in the hepatocyte system. A possible mechanism for this calcium synergism is activation of phospholipases by an increase in intracellular calcium. The lysophospholipids resulting from this activation would cause lysis as a result of membrane damage. Such a role for calcium has been described in ischemic liver cell injury in vivo (*10*).

D'hondt *et al.* (*11*) reported that calcium is an essential cofactor for the trypanocidal action of human high-density lipoprotein (HDL) on *T. b. brucei* (*11*). Although these authors were unable to block the action of HDL in human serum with EDTA or EGTA, they could abolish the activity by dialysis of the serum. However, Rifkin (*12*), who originally described the action of HDL on *T. b. brucei*, did not find that dialysis of the serum abolished the action of HDL (*12*). Since the data of D'hondt *et al.* (*11*) clearly show that the action of HDL is enhanced by calcium, we suggest that calcium is not an essential cofactor but has a synergistic effect with HDL that is similar to its effect with SHAM/glycerol and melarsoprol as well as its effect with hepatotoxic agents as found by Schanne *et al.* (*8*).

Recently it was reported that a calcium influx provides the trigger for release of the trypanosome surface coat glycoprotein prior to its replacement with a new, antigenically variant, surface coat (*13*). The evidence provided was that external calcium ions (0.5 to 1.0 m*M*) together with the calcium ionophore A 23187 induced release of the surface coat glycoprotein. The authors were surprised that this process was enhanced by the inclusion of 2-deoxyglucose, a metabolic inhibitor, because they assumed that this putative differentiation process was one that required energy. The data we present in this report indicate than an enhanced calcium influx is generally toxic to *T. b. brucei*, whether the influx is induced by HDL, A 23187, or inhibition of production of the required energy to maintain low intracellular calcium. Loss of the trypanosome surface coat after cell damage is a well-known phenomenon, and the results obtained with calcium and A 23187 (*13*) therefore indicate that increased intracellular calcium is directly toxic to the parasite independent of other agents.

These data may have important implications in the clinical management of African trypanosomiasis. Infection of rabbits with *T. b. brucei* has been reported to cause a 34 percent reduction in total serum calcium and a 40 percent drop in total tissue fluid calcium (*14*). Although it is not possible from these data to determine the degree of reduction of physiologically active ionized calcium, it is unlikely that the reduction in total calcium simply reflects reduced blood albumin via loss of albumin-bound calcium (*14*). Since there is less albumin in tissue fluid than blood, the greater loss in tissue fluid calcium is inconsistent with hypoalbuminemia being the cause. The addition of SHAM/glycerol (2 m*M* and 10 m*M*, respectively) to infected whole mouse blood (incubated at 37°C in vitro for 1 hour) abolished the infectivity of the parasites; this was not possible in the absence of plasma or serum (*4*). Dilution of infected whole blood with 0.3*M* tris buffer, *p*H 7.8 (two parts of blood, one part of buffer), prevented loss of infectivity even when the blood was incubated for 1 hour at 37°C with 5 m*M* SHAM and 50 m*M* glycerol. We know of no studies of ionized calcium concentrations in the blood of patients with sleeping sickness, but if total calcium is reduced as much in these patients as it is in infected rabbits it is possible that supportive measures to restore calcium concentrations could improve the current therapy for this disease. In addition, screening for new trypanocidal agents in vitro may be more sensitive if a physiological concentration of calcium is included in the incubation medium.

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5. The strain of *Trypanosoma brucei brucei* used (EATRO 110) as well as the methods of infection of rats, harvesting of infected blood, and partial purification of parasites by differential centrifugation to remove noncellular host blood components were as described previously (*3*). The assay of parasite lysis (Fig. 1) depended on measuring the ratio of intact parasites to rat erythrocytes as seen in Giemsa-stained thin films made from the test parasite suspensions. As the integrity of erythrocytes is not affected by the trypanocidal compounds used, the erythrocytes provided a useful marker for the relative blood volume seen in the thin smears. Erythrocytes remaining in the partially purified trypanosome preparations similarly provided a volume marker for incubations in the buffer systems. All incubations were at 37°C. The parasite-to-erythrocyte ratio before adding a trypanocidal compound was defined as the initial parasite count. Ratios measured after the addition of trypanocidal compounds were thus expressed as a percentage of this initial ratio. The 0 time point refers to the time of addition of the trypanocidal agent. Smears were made from the incubated parasites at the indicated times and the ratio of intact parasites to erythrocytes was determined. Initial absolute parasite counts ranged from 0.5×10^7 to 1.0×10^7 parasites per milliliter of incubation medium as determined by hemocytometer counts.
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