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Steady state oxygen kinetics of Trypanosoma mega reveal the presence of three oxidases. These include an oxidase which is sensitive to salicylhydroxamic acid (SHAM) but insensitive to sodium azide. This oxidase could be the L-α-glycerophosphate oxidase present in bloodstream trypanoaomes. In addition, an oxidase is present which is azide-sensitive but SHAM-insensitive. This oxidase is inhibited by CO and is probably cytochrome aa_3. A 3rd oxidase is possibly cytochrome o. Reciprocal plots of T. mega reveal the presence of

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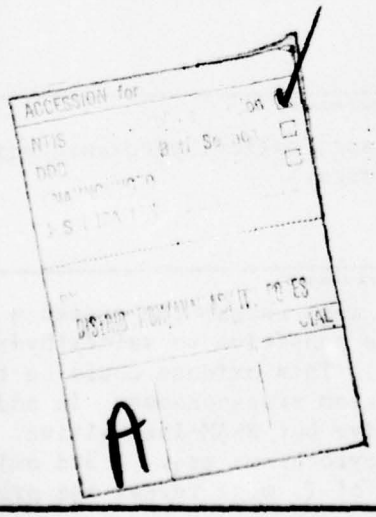
2 oxidases that are inhibited by CO. These results are discussed in the light of previous evidence suggesting the presence of several oxidases and a branched electron transport system in T. mega.



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EFFECTS OF TRYPANOCIDAL DRUGS ON THE REPLICATION AND FUNCTION OF KINETOPLAST (MITOCHONDRIAL) DNA IN TRYPANOSOMES

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FORWARD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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APPROACH TO THE PROBLEM

The numerous and necessarily speculate points raised in our discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell biochemistry. Given the necessary attention, this largely neglected but important field should yield results of considerable value, not only for an understanding both of trypanocidal drug action and of trypanosomal metabolism, but for cell biology in general. This is the purpose of this contract. So far as trypanocidal drug design is concerned, the era of intelligent emperic-ism is unlikely to be superseded until the balance of effort and expenditure on drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

Our approach to the problem of developing new trypanocidal agents includes investigating:

1. The effects of trypanocidal drugs on enzyme systems isolated from trypanosomes;
2. Detailed comparisons of homologous enzymes in host and trypanosomes;
3. Unique cell components or metabolic pathways in parasites;
4. The basis of the selective toxicity of known drugs.

BACKGROUND

During the last three months of this year (1975), we have been investigating the effects of various trypanocidal drugs on the function of the electron transport system in trypanosomes. Initially, our efforts have been directed towards the characterization of the steady state kinetics of terminal oxidases in Trypanosoma mega. Extensive studies with T. mega by Ray & Cross (12) and Kronick & Hill (8) have provided spectral evidence for several different terminal oxidases, including cytochrome aa_3 and cytochrome o. The results of these investigations support proposals for a branched electron transport system in this organism (6). Using the open-oxygen electrode system, we have identified 3 oxidases in this organism, each having a different response to respiratory inhibitors. In addition, we have studied the steady state oxygen kinetics of T. mega, particularly the affinity for O_2 of the oxidases in this organism.

ABSTRACT

Steady state oxygen kinetics of Trypanosoma mega reveal the presence of three oxidases. These include an oxidase which is sensitive to salicylhydroxamic acid (SHAM) but insensitive to sodium azide. This oxidase could be the L-α-glycerophosphate oxidase present in bloodstream trypanosomes. In addition, an oxidase is present which is azide-sensitive but SHAM-insensitive. This oxidase is inhibited by CO and is probably cytochrome aa_3. A 3rd oxidase is insensitive to both azide and SHAM but is inhibited by CO and is possibly cytochrome o. Reciprocal plots of T. mega reveal the presence of 2 oxidases that are inhibited by CO. These results are discussed in the light of previous evidence suggesting the presence of several oxidases and a branched electron transport system in T. mega.

MATERIALS AND METHODS

Trypanosoma mega was obtained from the Molteno Institute, Downing Street, Cambridge, courtesy of Dr. B. A. Newton. The organism was grown in a medium previously described by Ray & Cross (12). The technic for the measurement of respiration rate and O_2 concentration in systems open to O_2 has been previously described (3). The open system used in our experiments consists of a stirred liquid phase containing the respiring sample and a flowing gas phase with an adjustable O_2 content. The cells were suspended in a reaction mixture containing 40 mM KCl, 40 mM Tris HCl, 8 mM $MgCl_2$ and 1.6 mM EDTA adjusted to pH 7.8 with 1 M KOH.

The respirograph system consisted of a hexagonal cuvette containing 4.4 ml of sample (figure 1). The O_2 tensions in the liquid phase (T_L) and the gas phase (T_G) were measured with Clark electrodes. The liquid phase of the cuvette was in contact with a continuously flowing gas phase of known composition given by the gas-gradient mixer. Each experiment was started with pure N_2 in the gas phase. When the zero current of the oxygen electrodes had stabilized, progressively increasing amounts of O_2 were added to the gas phase as the steady state oxygen tension of the cells was measured.

An on-line computer system was adapted to the respirograph. The computer system has been previously described by Degn & Wohlrab (4) and Petersen et al. (11). The system was programmed in such a way that T_G and T_L were monitored every 10 seconds. The calculation and double-reciprocal plotting of the steady-state respiration rate (V_R) against T_L are performed automatically during the experiment from the recorded T_G and T_L values. Thus, the reciprocal plots of $1/T_L$ and $1/V_R$ were generated as the analog output and recorded on an x, y recorder.

RESULTS

Figure 2 presents a tracing of T. mega intact cells in the open oxygen electrode system. An increase in respiration is indicated by a decrease in O_2 tension in the liquid (T_L). The endogenous respiration in the cells was quite high. In this experiment, there is clear evidence for 3 terminal oxidases. One oxidase, which is inhibited by 0.5 mM salicylhydroxamic acid (SHAM), supports 15-20% of the respiration of the cells. Aromatic hydroxamic acids are inhibitors of cyanide-insensitive oxidases in several eukaryotic systems (9,13) and have been shown to inhibit the L-α-glycerophosphate oxidase system in trypanosomes (2,5-7,10). In experiments not shown, this SHAM-sensitive respiration is not sensitive to 4.5 mM azide.

A 2nd oxidase system is also present; it is inhibited by 4.5 mM azide and supports 55-60% of the cell respiration. In experiments not shown, the azide-sensitive respiration is not sensitive to 0.5 mM SHAM. Sodium azide is a known inhibitor of cytochrome aa_3. A 3rd oxidase is evident which is insensitive to both high concentrations of SHAM and azide and supports 20-25% of the cell respiration. Taken together, these results suggest the presence of 3 terminal oxidases in T. mega.

Figure 3 presents reciprocal plots of 3 different concentrations of intact cells of T. mega. It is obvious that these plots are not linear, suggesting the presence of more than one oxidase. Extrapolation of the 2 portions of the curves suggests that at least 2 oxidases are present, one with an apparent K_m of 0.14 μM O_2 and the other with an apparent K_m of 0.35 μM O_2. Figure 3 shows clear evidence that both of these terminal oxidases are inhibited competitively by 0.7 μM CO.

DISCUSSION

The electron transport system of T. mega has been studied by several investigators (8,12). The results reported here provide additional evidence for several terminal oxidases functioning in T. mega. This support comes from the following lines of experimental evidence:

Difference Spectral Studies

Spectral studies by Ray & Cross (12) and Kronick & Hill (8) have provided evidence for 2 CO-binding pigments in CO-reduced difference spectra. The CO difference spectra are dominated by evidence for cytochrome o with peaks (and relative intensities) at 418 (1), 538 (0.07) and 570 nm (0.08). Cytochrome a_3 with a trough at 443 nm is evident, but because of the preponderance of cytochrome o, the peaks of cytochrome a_3 CO and (430 nm, 550 nm and 595 nm) are difficult to detect (8,12). These spectral results suggest the presence of 2 CO-binding pigments in this organism.

Action Spectral Studies

Kronick & Hill (8) clearly demonstrated action spectral evidence for 2 functional terminal oxidases, cytochrome a_3 and cytochrome o in T. mega and several other trypanosomatids. In a very dilute cell suspension of T. mega where levels of oxygen greater than 1 torr could be maintained, the action spectrum revealed a small shoulder at 418 nm (cytochrome o) and a peak at 432 (cytochrome a_3) (8).

Cyanide-Insensitive Respiration

Ray & Cross (12) observed that a high percentage of cyanide-insensitive respiration was evident in T. mega, varying from 40 - 60% cyanide insensitivity.

0.1 mM KCN inhibited succinic oxidase activity only 60%. Na azide (5 mM) and antimycin (10 nmol/mg protein) provided similar inhibition (12). Dixon plots for the inhibition of succinate, proline and α-ketoglutarate oxidation by cyanide, azide or antimycin all gave biphasic curves (12). These results clearly suggest that at least 2 oxidases are present in T. mega, only one of which is sensitive to these inhibitors, but both of which are accessible to all 3 substrates.

Steady State Oxygen Kinetics

The results reported in this paper demonstrate that when different inhibitors are used, 3 different steady states are evident in T. mega (Fig. 1). The SHAM-sensitive oxidase is also azide-insensitive. This could be the L-α-glycerophosphate oxidase which is present in other Trypanosoma species. The azide-sensitive oxidase is also SHAM-insensitive. The proposal that this oxidase is cytochrome aa_3 is supported by action spectral evidence for cytochrome a_3 in T. mega. The azide and SHAM-insensitive oxidase is inhibited by CO. This could be cytochrome o, for which action spectral evidence has been demonstrated in T. mega.

Reciprocal Plot Curves

Reciprocal plots of T. mega succinate oxidation (Figs. 3,4) provide strong evidence for 2 oxidases. The curves are multiphasic, clearly suggesting the presence of 2 oxidases. In addition, the results presented in Fig. 3 provide strong evidence that these 2 oxidases in T. mega are inhibited by CO. The absence of evidence for the 3rd oxidase in these reciprocal plots is probably due to the fact that the L-α-glycerophosphate oxidase in trypanosomes

(e.g., T. evansi and T. brucei) has a high K_m for 0_2 in the range of 2-8 μM (6,7) and thus would not be evident with the 0_2 gradients employed in these studies.

The results presented in this paper strongly support the presence of several oxidases in T. mega. The presence of a branched electron transport system for T. mega has been proposed by several investigators (8,12) and recently reviewed (1,6). At present, there is no strong experimental evidence to support a branched electron transport system over several oxidases acting in parallel with one another. Further experiments on the fluxes of reducing equivalents and phosphorylation capacities for 2 or more pathways are needed in order to support proposals for a branched pathway in this and other trypanosomes, yet it is clear several oxidases are functioning in these organisms.

CONCLUSIONS

Steady state oxygen kinetics of *Trypanosoma mega* reveal the presence of three oxidases. These include an oxidase which is sensitive to salicylhydroxamic acid (SHAM) but insensitive to sodium azide. This oxidase could be the L-α-glycerophosphate oxidase present in bloodstream trypanosomes. In addition, an oxidase is present which is azide-sensitive but SHAM-insensitive. This oxidase is inhibited by CO and is probably cytochrome aa_3. A 3rd oxidase is insensitive to both azide and SHAM but is inhibited by CO and is possibly cytochrome *o*. Reciprocal plots of *T. mega* reveal the presence of 2 oxidases that are inhibited by CO. These results are discussed in the light of previous evidence suggesting the presence of several oxidases and a branched electron transport system in *T. mega*.

RECOMMENDATIONS

With the development of the proper techniques for the characterization of the steady state levels of terminal oxidases in T. mega, we recommend that studies now be undertaken to investigate the effects of trypanocidal drugs such as suramin on the terminal oxidases in T. brucei bloodstream trypomastigotes. Included in these studies could be investigations on the effect of suramin on the mode of inhibitor of the L-α-glycerophosphate oxidase, the terminal oxidase in bloodstream forms. These studies may provide a clue to the mode of action of this trypanocidal drug.

The mode of action of trypanocidal drugs is not known. We recommend a continued effort to determine the mode of action of some of the more effective drugs including berenil, antrycide and suramin. In these studies, we believe it is important and essential to couple the investigation of the mode of action of these drugs in vitro with their effects on pathogenic trypanosomes in mice or rats. These studies are essential if we are to develop a more rational drug development approach.

We would recommend that careful consideration be given to the systems to be used. It should be remembered that there is still no in vitro model for the in vivo infection in mice or rats of trypanosomiasis. In order to determine the mode of action of the drugs, one must consider the drug effects on the host-parasite system.

Support should be considered for studying the techniques required for maintaining the bloodstream forms in vitro. At the present time, this is not possible for any significant period of time. In addition, more research is needed on the pathobiology of trypanosomiasis. Little information is

available on the pathogenesis and pathology of this group of diseases which make up the tsetse-transmitted animal trypanosomiasis of Africa.

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FIGURE LEGENDS

Fig. 1. Scheme of respirograph and on-line computer system. The oxygen tension in the liquid and gas phases were measured with Clark electrodes.

Fig. 2. Steady state oxygen trace of intact cells of Trypanosoma mega. An increase in respiration is indicated by a decrease in oxygen tension in the cuvette. The concentrations of additions given are the final concentrations in the cuvette. Cells were added to a reaction mixture of 40 mM KCl, 40 mM Tris HCl 8 mM $MgCl_2$, and 1.6 mM EDTA, adjusted to pH 7.8 with 1 M KOH.

Fig. 3. Reciprocal plot of the succinate oxidation of Trypanosoma mega against low concentrations of oxygen. The three curves (a, b, and c) represent increasing concentrations of intact cells (3.0, 4.0 and 8.0 x 10^7 cells/ml) respectively.

Fig. 4. Reciprocal plot of the succinate oxidation of Trypanosoma mega against low concentrations of oxygen in the presence and absence of 0.7 μM CO. The concentration of intact cells was 6.0 x 10^7 cells/ml.

Figure 1

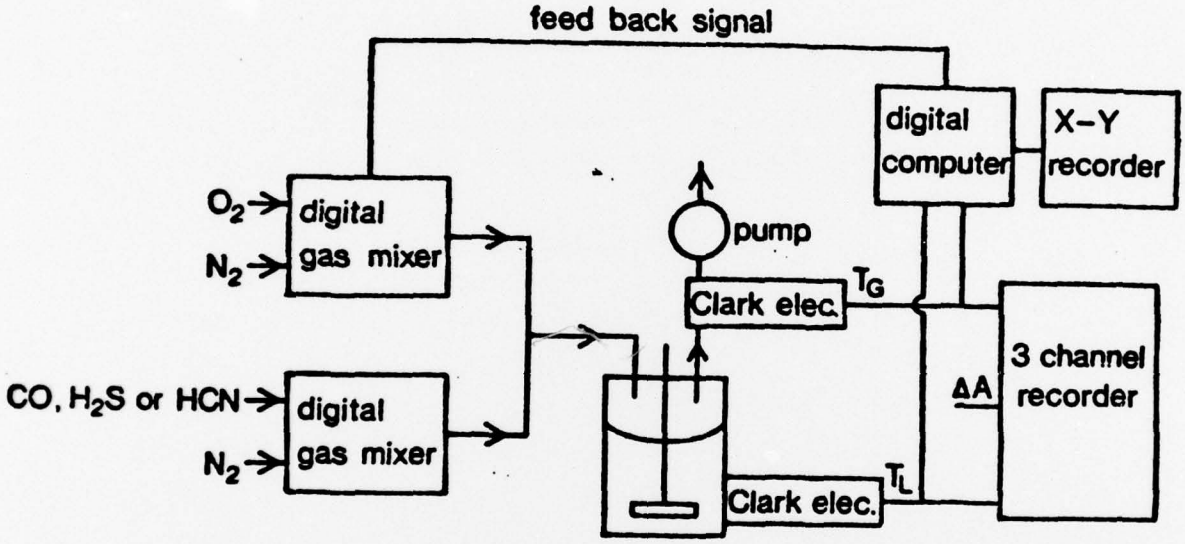
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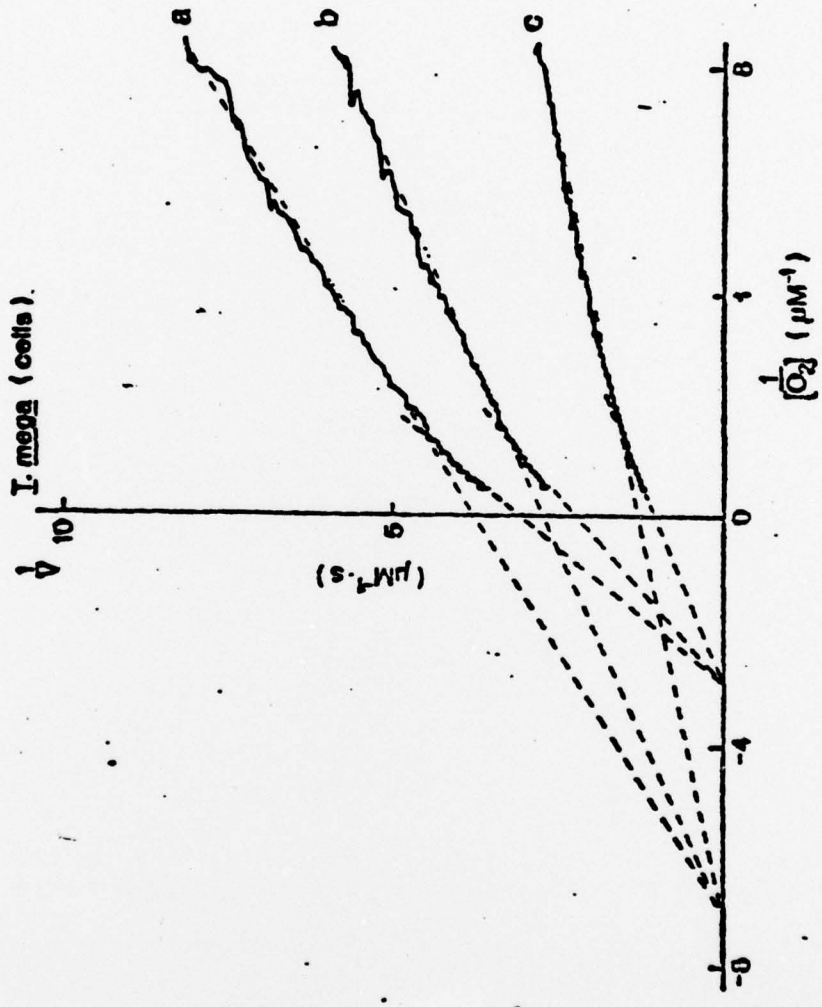
Figure 2



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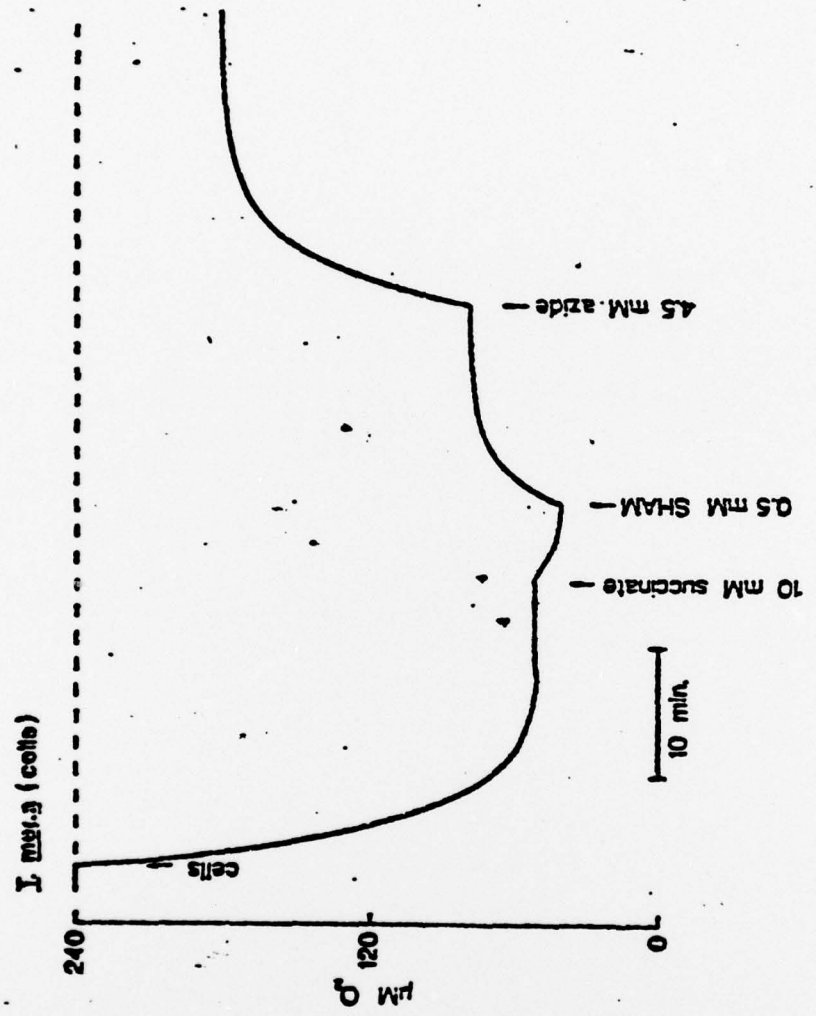
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10 mM succinate
10 min.
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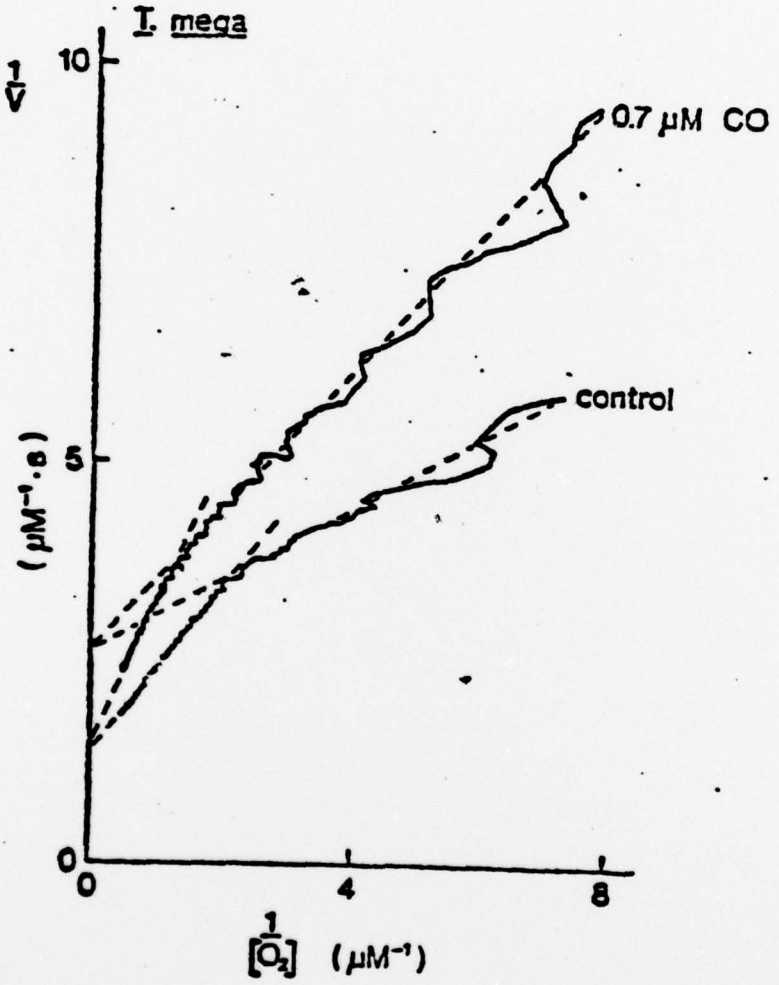
240
120
0
μM O_2

Figure 4



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