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ANALYSIS OF A TRYPANODESTRUCTIVE BUFFALO SERUM PROTEIN

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Preliminary Studies

Plasma and serum prepared from any of 40 African buffalo tested, kills African trypanosomes *in vitro* (Appendix 1)*. The presence, and titer, of trypanocidal activity in plasma is not influenced by including EDTA, sodium azide, gentamycin, aprotinin, leupeptin, and PMSF, in blood collection buffer (Appendix 1). The combination of reagents prevent bacterial growth, the autoxidation of fatty acids and cholesterol, and general proteolysis. The absence of effect of these inhibitors on trypanocidal activity of buffalo serum, together with the absence of trypanocidal activity in serum prepared from uninfected cattle, horses, sheep, goats, rabbits, rats and mice, suggests that the trypanocidal activity is not likely to result from a collection artefact (Appendix 1).

To detect trypanocidal activity, bloodstream stage *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi*, *T. vivax*, or *T. congolense* organisms that are collected from the blood of infected mice, or are grown under axenic conditions *in vitro*, are washed 4 times in serum free medium, and 2 x 10^4 organisms are incubated at 37°C in wells of a 96 well microtiter plate, in 100 μl volumes of 150 mM NaCl in 20 mM PO_4 buffer pH 7.2 (PBS) containing 1% w/v glucose, 10% heat-inactivated (56°C, 30 m) fetal bovine serum (assay medium), and dilutions of buffalo serum. Fetal bovine serum is included in incubation buffer to maintain viability of control organisms and hence to improve end point measurements. The fetal bovine serum is not required for trypanocidal activity of the buffalo serum, or of fractions of the buffalo serum (Appendix 1).

Every 60 minutes, the wells are examined under an inverted tissue culture microscope. Between 1 h and 4h of incubation, all (100%) organisms that are incubated with a trypanocidal concentration of buffalo serum cease to move, whereas all other organisms remain highly motile. The immobile organisms do not infect mice. The kinetics of loss of motility and of infectivity for mice, is similar for the different species of African trypanosomes (Appendix 1).

With the exception of studies on the trypanosome metabolic pathway affected by African buffalo serum, all of the experiments described below were carried out using *T. b. brucei* S 427 cl 1. These were harvested during their exponential growth phase in axenic cultures maintained at 37°C.

Isolation of Trypanocidal molecules from buffalo Serum.

A volume of 12 liters of frozen serum from one male African buffalo was imported (USDA Permit numbers 22992, and 26091-A) from The Wildlife Disease Section of The Kenyan Agricultural Research Institute, Nairobi, to OSU, Columbus, via Plum Island. Further serum will be imported as required. Several procedures

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for fractionation of trypanocidal molecules from buffalo serum have been established (Table 1). The combination of procedures that we use to obtain an enriched preparation of trypanocidal molecules is shown in Table 2. We start with 50 ml buffalo serum, which contains 4 g protein and a total of 3200 units of trypanocidal activity. Units of activity are determined as: the reciprocal end titer that renders all trypanosomes immobile in the above assay. Total units of trypanocidal activity in a preparation is calculated as: [reciprocal end titer] x [volume in mls]. In a typical experiment we recover 100 μg of protein with an activity of 1600 units/mg protein (Table 2). This represents a 2000 fold enrichment of trypanocidal activity relative to activity in serum. The trypanocidal activity of this preparation, which elutes from Superose 12 as a tight peak (Fig 1A) with mean molecular mass of 133 kDa, is completely destroyed by incubation with immobilized proteinase K (Sigma, 2 units proteinase K/mg protein, 2 hr at 37°C).

The most enriched preparations of trypanocidal protein contain 3 polypeptides determined by SDS-PAGE (a representative results is shown in Fig 1B, track 2). These proteins are of 192 kDa, 149 kDa and 132 kDa relative molecular mass. Smearing of the 149 kDa protein in SDS-PAGE gels may reflect high glycosylation. Separation of the 132 kDa, 149 kDa, and 192 kDa proteins is not achieved by chromatography of enriched trypanocidal protein on phenyl Sepharose, or on Mono S, or on Con-A sepharose, or on AffiGel Blue Gel, and chromatofocussing or IEF destroys the trypanocidal activity.

Analysis of enriched trypanocidal proteins by SDS-PAGE in the presence of 2-mercapto-ethanol, indicates that the 149 kDa and 192 kDa molecules are composed of disulfide-bridged subunits. Two subunits, respectively of 50 kDa and 32 kDa are detected (Fig 1B, track 4). The 50 kDa subunit gives a broad smear similar to that observed with the 149 kDa unreduced molecule. The relationship of the subunits to the 149 kDa and 192 kDa molecules has not been established although it is tempting to consider that the 149 kDa protein is composed of 3 x 50 kDa subunits, and the 192 kDa protein is composed of 6 x 32 kDa subunits.

A SDS-PAGE comparison of highly active (1600 units/mg protein) trypanocidal protein (Fig 1B, track 2), and a less active flanking fraction (100 units/mg protein) (Fig 1B, track 1) indicates that trypanocidal activity correlates positively with the amounts of the 132 kDA and 192 kDa proteins in the preparation, and consequently with the 132 kDa and 32 kDa polypeptides after reduction (Fig 1B, tracks 3 and 4).

Cellular and Biochemical Analyses of *T. brucei* that have been incubated with African Buffalo Serum.

Trypanocidal activity of buffalo serum is not affected by inhibitors of parasite proteases, by chelation of Ca^{2+} in medium,

or by protein synthesis-, or protein kinase-inhibitors: Bloodstream stage *T. brucei* are killed during incubation with African buffalo serum in medium supplemented with 20 μM chloroquin, or with 20 mM ammonium chloride, a concentration of weak base that inhibits the processing, and hence action, of *T. b. brucei*-lytic human VHDL. They are also killed by trypanocidal protein in medium containing leupeptin, pepstatin, E64 and aprotinin each at a concentration of 25 μg per ml. These protease inhibitors prevent degradation by *T. brucei*, of endocytosed, VSG-specific, antibody.

Bloodstream stage *T. brucei* are killed by buffalo serum in medium supplemented with 15 mM EGTA, which is a 6.2 fold molar excess with respect to Ca^{2+}. EGTA chelates Ca^{2+} in the presence of other divalent ions and hence is expected to tie up all free Ca^{2+} in the medium. Addition of 20 μg cycloheximide/ml medium stops trypanosome protein synthesis, but does not affect parasite motility during 6 h incubation at 37°C, and does not influence killing of trypanosomes by African buffalo serum. " Staurosporin ", which inhibits protein kinase C and cAMP dependent kinases by interacting with the kinase catalytic domain, inhibits replication of *T. b. brucei* S 427 cl 1 organisms under axenic conditions at nanomolar concentration (Morgan and Black, unpub), but does not influence killing of trypanosomes by African buffalo serum even at 10^{-7} molar concentration. Similarly " Genestein ", which inhibits protein tyrosine kinase, and also prevents replication of bloodstream stage *T. brucei* when included at a concentration of 10^{-6} molar in incubation medium, does not inhibit killing of trypanosomes by buffalo serum.

Taken together, these studies indicate that the buffalo serum trypanocidal protein exerts a direct effect on African trypanosomes, and does not activate a death program involving Ca^{2+} influx, kinase activation, and/or synthesis of new proteins.

Incubation in African buffalo serum does not cause a change in *T. brucei* morphology or membrane integrity: Two lines of evidence show that incubation with African buffalo serum does not cause a change in trypanosome morphology;

[a] Motile, infective, bloodstream stage *T. brucei* cannot be distinguished by transmission electron microscopy (Fig 2, panel a), from organisms that had been incubated in African buffalo serum (Fig 2, panel b), and were neither motile, nor capable of replication (Fig 3) prior to fixation. Examination at higher magnification indicated that both the plasma membrane and VSG coat of organisms that had been incubated with trypanocidal protein were indistinguishable from those of control parasites.

[b] Non-motile, non-infective bloodstream stage *T. brucei*, prepared by incubating trypanosomes at 37°C in medium supplemented either with African buffalo serum, or isolated trypanocidal proteins, retain calcein (Molecular Probes), which

is a live cell stain and exclude ethidium bromide homodimer (Molecular Probes), which is a dead cell stain, indicating membrane integrity. Calcein, which fluoresces green under UV illumination, is taken up as a cell permeant derivative (Calcein AM), cleaved to calcein by cellular esterases and accumulates in the cytosol of intact cells, or rapidly leaks from cells with damaged membranes. Ethidium bromide only enters cells with damaged plasma membrane, and in these cells attaches to DNA and fluoresces red under UV illumination.

Incubation with African buffalo serum causes precipitous decline in trypanosome ATP content: Loss of motility and replicative capacity of bloodstream stage *T. brucei* during incubation in medium supplemented with African buffalo serum is preceeded by precipitous decline in cellular ATP content (Fig 4), assayed by the ATP dependent cleavage of luciferin by luciferase (Sigma Kit). Mathmatical analysis of these data indicate that decline in ATP content was exponential (Appendix 2), suggesting that a single process is affected. We are collecting more results during the first 30 minutes of incubation of trypanosomes with tyrypanocidal protein, in order to test the predicted decay rate of parasite ATP content. Mean parasite ATP content from 4 experiments was 6×10^{-17} moles per trypanosome, which is close to recently published values obtained by other investigators (4.6×10^{-17} moles/trypanosome; 41a), and less than the amount of ATP made each minute by the parasite. ATP synthesis by bloodstream stage *T. brucei* is predicted to be 2×10^{-15} moles per trypanosome per minute from analysis of glucose consumption rates, and pyruvate excretion rates. The organisms therefore consume almost all of the ATP they produce.

Buffalo serum-induced decline in *T. brucei* ATP content results from selective inhibition of glycolysis: Bloodstream stage *T. brucei* are pleomorphic. Rapidly dividing slender forms, which predominate during the exponential phase of growth in the bloodstream, transform to tsetse-infective, non-dividing ,short-lived, stumpy forms at peak parasitemia. This transformation is accompanied by a change in parasite metabolism. While slender *T. brucei* depend entirely on catabolism of glucose to pyruvate for ATP production, stumpy *T. brucei* have an additional capacity to generate ATP from alpha keto glutarate, if it is supplied to them (Fig 5). Thus only stumpy form *T. brucei* make ATP and remain motile when starved of glucose and provided with alpha keto glutarate at 37°C.

We obtained slender and stumpy *T. b. brucei* GUTat 3.1 by timed infection in irradiated mice. To indirectly examine the effect of trypanocidal protein on ATP production by the glycolytic, and alpha keto glutarate pathways, we incubated the slender and stumpy form *T. brucei* with buffalo serum in the presence and absence of alpha keto glutarate, and examined motility. The slender form *T. brucei* became immobile during 2 hours incubation at 37°C in medium supplemented with buffalo serum, and either

glucose, or alpha keto glutarate. The stumpy form *T. brucei* also became immobile during 2 hours incubation at 37°C in medium supplemented with buffalo serum and glucose, but retained full motility in medium supplemented with buffalo serum and 1.5 mM alpha keto glutarate. The trypanocidal protein is therefore unlikely to affect ATP generation via the alpha keto glutarate pathway, and consequently selectively inhibits ATP generation by the glycolytic pathway. We are assaying the ATP content of the various populations of organisms to confirm this interpretation.

Influence of buffalo serum on glucose-starved *T. brucei*. Every bloodstream stage *T. brucei* metabolizes about 10^{-15} moles of glucose/minute, with a nett gain of 2 ATP per glucose. The ATP store of a bloodstream stage *T. brucei* (Fig 4;) is therefore sufficient to meet the energy requirements of the organism for only a fraction of a minute. Bloodstream stage *T. brucei* do not have detectable glycolytic intermediates consistent with rapid metabolism of glucose to pyruvate. However, thoroughly washed slender *T. b. brucei* S 427 cl 1 organisms remained motile for 3 hours at 37°C in glucose-free medium supplemented with dialyzed fetal bovine serum, indicating the presence of substantial stores of glucose, or a usable polymer, and indicating that glucose uptake is not always a rate limiting step for glycolysis in bloodstream stage *T. brucei* at low exogenous glucose concentration. The glucose-starved organisms became immobile and lost their capacity to replicate during 1 hour of incubation at 37°C when dialysed buffalo serum was included in the medium. We conclude that buffalo serum trypanocidal protein affects trypanosome glycolysis at a step other than the uptake of glucose.

Inhibition of buffalo serum trypanocidal activity

The presence of a trypanocidal plasma protein seems to be inconsistent with the role of African buffalo as reservoir hosts of trypanosomes. However, erythrocytes, peripheral blood lymphocytes, endothelial cells, and fibroblasts, abrogate the trypanocidal activity of African buffalo serum *in vitro* revealing a trypanostatic effect. Bloodstream stage *T. brucei* S 427 cl 1 organisms could be incubated for a week at 37°C in Baltz-modified minimal essential medium supplemented with 50% v/v African buffalo serum and 5 x 10^8 buffalo, cow, or rat erythrocytes without loss of motility, without loss of replicative capacity determined by transfer to fresh medium lacking buffalo serum, **and without an increase in number.** Similar results were obtained when bloodstream stage *T. brucei* were incubated in medium supplemented with 50% v/v FBS, 10 μg enriched trypanocidal protein and 5 x 10^8 buffalo erythrocytes. In contrast, parasites that were incubated in the absence of erythrocytes were killed by the buffalo serum, and parasites that were incubated in medium supplemented with 50% v/v FBS and 5 x 10^8 erythrocytes/ml, replicated rapidly (doubling time 7.32 hours during exponential growth).

Trypanosomes that were separated from erythrocytes in a " Transwell " (Costar), were killed by buffalo serum, indicating a requirement for intimacy and perhaps contact. Erythrocyte membranes isolated after hypotonic lysis, were as effective as intact erythrocytes at preventing trypanocidal activity of buffalo serum in medium containing 1% v/v glucose. However, the erythrocyte membranes did not inhibit trypanocidal activity either in the absence of glucose, or after fixation with gluteraldehyde. Erythrocytes metabolize glucose to pyruvate and lactate using membrane-bound enzymes. These observations, suggest that protection against trypanocidal activity might be mediated by a product of glucose metabolism by erythrocytes. In fact, inclusion of pyruvate in incubation medium abrogated trypanocidal activity (Fig 6). The pyruvate, at a concentration of 1 mM to 2 mM, completely abolished the ability of trypanocidal protein to cause decline in parasite ATP content, completely prevented trypanocidal protein-induced loss of parasite motility, and completely prevented loss of parasite replicative capacity during incubation with trypanocidal protein. Inclusion of cycloheximide and staurosporin in incubation medium did not abrogate the protective effect of pyruvate, suggesting that the exogenous metabolite has a direct effect on the interaction between trypanocidal protein and trypanosomes.

Pyruvate is a 3 carbon alpha keto acid. Glyoxylate, oxaloacetate, and alpha keto butyrate, which are 2 to 4 carbon alpha keto acids, inhibited trypanocidal activity of buffalo serum at similar molar concentrations to pyruvate. Several related compounds including, beta keto butyrate, phenylpyruvate, methylpyruvate, alpha keto glutarate (with respect to slender *T. brucei*), succinate, acetate, citrate, malate, alanine, phosphoenolpyruvate, glucose, and glycerol had no effect on trypanocidal activity of buffalo serum. Several of these inactive compounds are intermediates in various metabolic pathways, further supporting the idea that the mechanism of inhibition of trypanocidal protein by pyruvate is not related to activation of alternative pathways of ATP generation. Structures of the compounds that inhibited trypanocidal activity, and of some of the compounds that had no effect on trypanocidal activity, are presented in Figure 7. Based on the above evidence we hypothesize that the 2 to 4 carbon alpha keto acids prevent interaction of the buffalo serum trypanocidal molecule with its target molecule in, or on, bloodstream stage trypanosomes. Pre-incubation of trypanosomes with the inhibitory compounds followed by washing did not alter their susceptibility to trypanocidal protein, and pre-incubation of buffalo serum with an inhibitory compound followed by its removal by dialysis, did not reduce trypanocidal activity. Therefore, the inhibitory molecules do not form a stable association with either the trypanosomes, or the buffalo serum trypanocidal protein.

Removal by *T. brucei* of trypanocidal activity from medium at 37°C and at 4°C - Trypanocidal activity was removed from incubation medium by trypanosomes at 37°C (Fig 8a), and at 4°C (Fig 8b)

indicating binding by trypanosome surface molecules.

Summary and working hypothesis.

Analyses of sera from naive African buffalo indicate the presence of trypanocidal material. This material kills bloodstream stage African trypanosomes of all species and serodemes and hence targets a conserved process. Trypanocidal material is enriched 2000 fold by sequential chromatography on hydroxylapatite, anion exchange and size exclusion gels. Enriched trypanocidal material contains 3 pseudoglobulins of 192 kDa, 150 kDa and 132 kDa, and all trypanocidal activity is destroyed by proteolysis of the preparation. Trypanocidal protein causes an exponential decline in parasite ATP content resulting in loss of motility, replicative capacity, and leading to death. Decline in cellular ATP results from trypanocidal protein-mediated inhibition of parasite glycolysis. The trypanocidal protein also inhibits glycolysis in bloodstream stage *T. brucei* that are glucose-starved and hence dependent on stored carbohydrate, in *T. brucei* that are unable to synthesize protein, and in *T. brucei* that are unable to phosphorylate proteins, indicating that it exerts a direct effect on a glycolytic step after glucose uptake. Trypanocidal activity is inhibited by 2 to 4 carbon alpha keto acids, but not by numerous related compounds indicating structural constraints on the inhibitory function. The ability of 2 mM pyruvate to inhibit trypanocidal protein, together with the demonstration that trypanocidal protein absorbs to trypanosomes at 37°C and at 4°C, suggests that trypanocidal protein may bind to a surface component of trypanosomes, that also associates with pyruvate at high concentration. The recent demonstration of a pyruvate transporter on *T. brucei* that mediates a small degree of pyruvate counter-flow at high exogenous pyruvate concentration, raises the possibility that trypanocidal protein binds to the pyruvate transporter. Because inhibition of pyruvate excretion would inevitably lead to shut down of glycolysis, this seems an attractive interpretation of our data. Based on the above arguments, we hypothesize;

[a] Trypanocidal protein binds to the apo form of pyruvate transporter on the surface of African trypanosomes (*ie.*, to pyruvate transporter after pyruvate has dissociated).
[b] Binding of trypanocidal protein to apo-pyruvate transporter inhibits pyruvate excretion.
[c] Excess accumulation of pyruvate in African trypanosomes during incubation with trypanocidal protein inhibits pyruvate kinase by mass action (feed-back inhibition), prevents gain of ATP during glycolysis, and hence profoundly affects the parasites, which have little or no store of ATP.
[e] Because trypanocidal protein is of a similar size to an IgG antibody, the pyruvate transporter is likely to be accessible to antibodies.

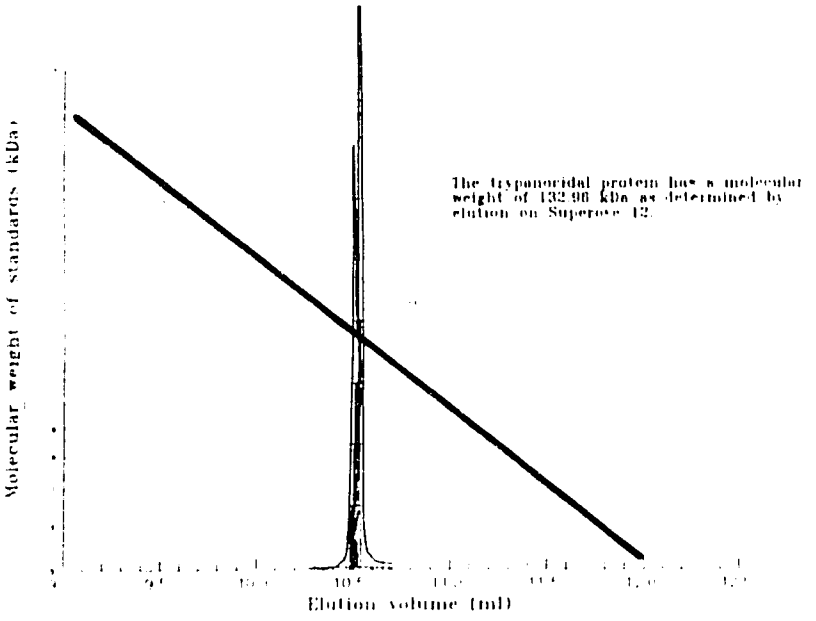
TABLE 1 **CONDITIONS FOR OBTAINING TRYPANOCIDAL PROTEIN**

Condition Tested	Recovery Condition	% Loss of Activity	Enrichment Factor
NH_4SO_4 ppt.	35% to 50% saturated NH_4SO_4	30 - 90	3
Dialysis vs H_2O	Water soluble	10 - 20	1.5
IEF	pH 6.2 - 6.6*	50 - 100*	0 - 8
Phenyl Sepharose	Does not adhere in 1.1 M NH_4SO_4	50 - 60	2
DEAE 52	Binds in 20 mM TRIS pH 8.25 Elutes between 80 and 120 mM NaCl	20 - 50	8
AffiGel Blue Gel	As above	20 - 50	6
Con A Sepharose	Does not Bind	20	2
Hydroxylapatite	Binds in 200 mM K-PO_4 pH 6.8 Elutes between 300 and 350 mM K-PO_4	10 - 30	20
Mono Q	Binds, 20 mM TRIS, 48 mM NaCl pH 8.25 Elutes between 60 - 90 mM NaCl	10 - 50	12
Superose 12	Elutes with molecules of 133 kDa	10 - 50	8

* Activity is completely destroyed when IEF is allowed to proceed to completion.

TABLE 2 **SEQUENTIAL ISOLATION OF TRYPANOCIDAL PROTEIN**

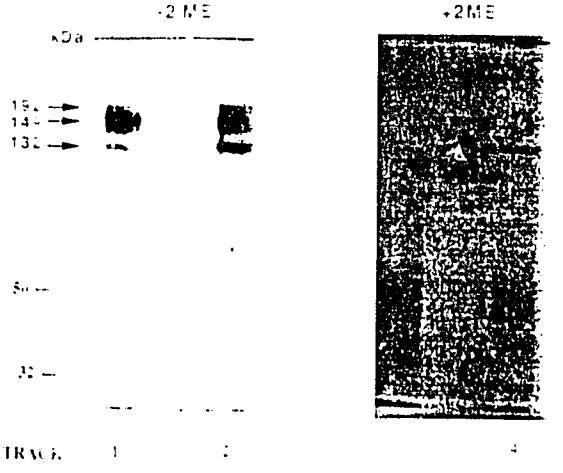
Condition Tested	Recovered Activity (relative to input)	Units Activity/mg protein
Dialysis, 200 mM K-PO_4, pH 6.8	90%	0.8
Hydroxylapatite fractionation	85%	16.0
Concentrate over XM 100	80%	19.0
Dialyze into 10 mM NaCl	60%	38.0
Adjust salt and fractionate, Mono Q	50%	380.0
Concentrate on Centricon 30	65%	380.0
Fractionate on Superose 12	50%	1600.0



Elution of Trypanocidal protein on Superose 12 sizing gel

FIGURE 1A : Superose 12 chromatography of buffalo serum that had been subjected to sequential chromatography on hydroxylapatite and Mono Q as outlined in Table 2. Results are presented as relative OD280 vs elution volume. The hatched zone between the 2 closely spaced protein peaks indicates trypanocidal activity. The molecular weight standard curve was obtained by best-fit analysis from data obtained with; Serum amyloid polymer, trimer bovine serum albumin, mouse IgG, and bovine serum albumin

SDS-PAGE ANALYSIS OF ENRICHED TRYPANOCIDAL PROTEIN



TRACK 1: 10 UNITS TRYPANOCIDAL ACTIVITY/ML
TRACK 2: 160 UNITS TRYPANOCIDAL ACTIVITY/ML
TRACK 3: SAME PREP AS TRACK 1
TRACK 4: SAME PREP AS TRACK 2

FIGURE 1B : Silver-stained SDS-PAGE under non-reducing (-2ME), and reducing (+2ME) conditions, of trypanocidal material obtained by fractionation of buffalo serum as described in Table 2, and Figure 1A. Samples (1μl) of a fraction with 1600 units activity/mg protein, and a flanking fraction with 16 times less trypanocidal activity were examined.

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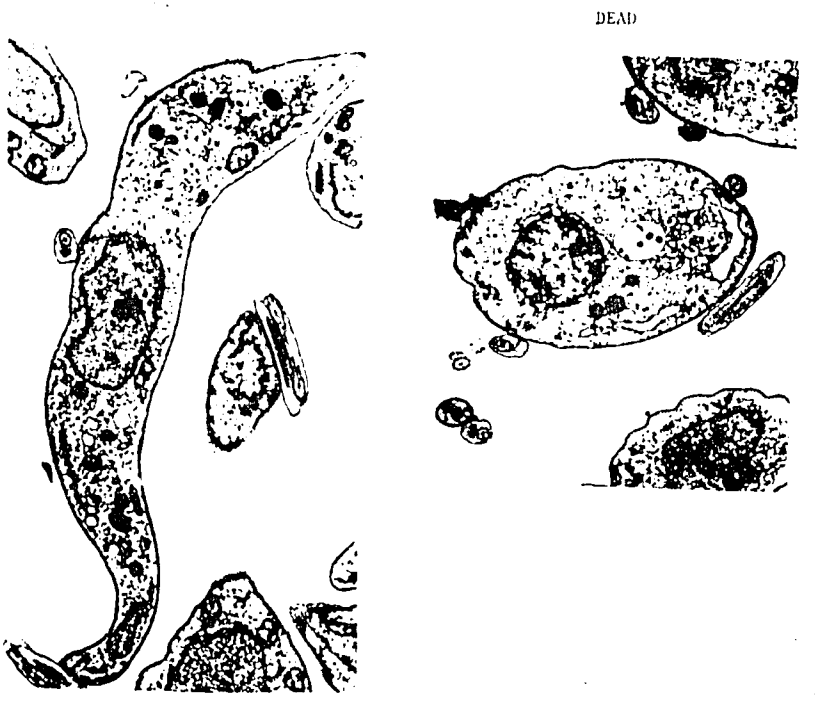

Figure 2 :
Trypanosomes were incubated for 2 h at 37°C in PBS supplemented with 1% v/v glucose, 10% v/v FBS, and with or without, 6.25% v/v heat- inactivated buffalo serum. The parasites were pelleted, fixed with 2% v/v gluteraldehyde, and stained en bloc with osmium tetroxide and uranyl acetate.

Similar results were obtained using enriched trypanocidal protein instead of whole buffalo serum.

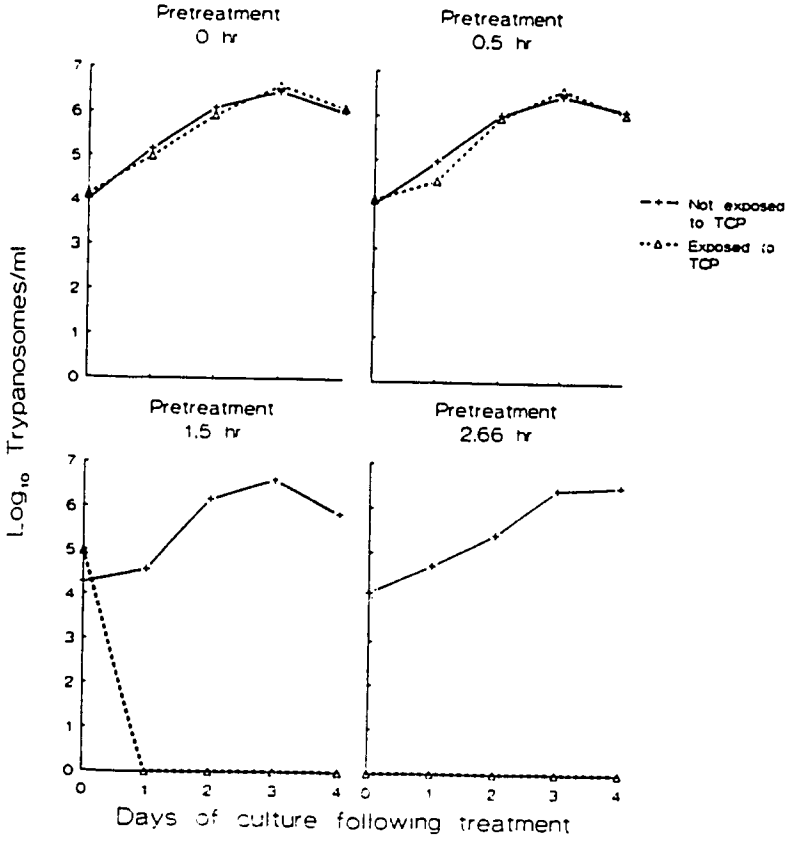

Figure 3 :
Trypanosomes were incubated at a density of 10^6 organisms per ml PBS supplemented with 1% v/v glucose, 10% v/v FBS, and with or without 6.25% heat inactivate buffalo serum. At the intervals shown, 10 μl samples containing 10^4 organisms were transferred to bloodstream stage *T. brucei* growth medium (27), incubated at 37°C and parasites were counted in a hemocytometer at daily intervals thereafter.
Similar results were obtained using enriched trypanocidal proptein instead of whole buffalo serum

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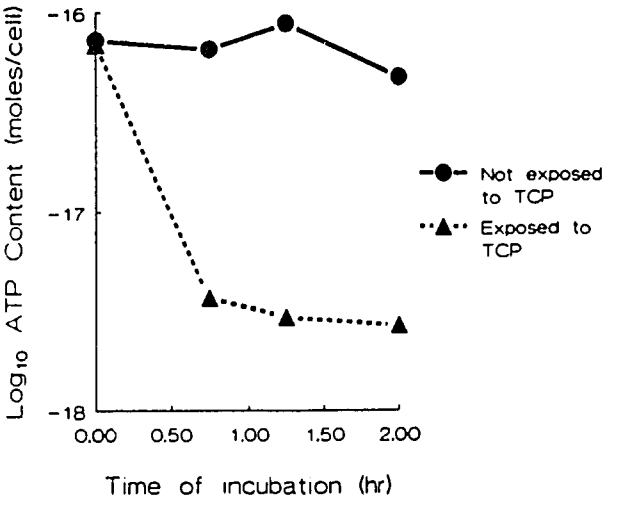

Figure 4 : Trypanosomes were incubated at 37°C at a density of 2 x 10^6/ml PBS supplemented with 1% v/v glucose, 10% v/v FBS, and with, or without 6.25% heat-inactivated buffalo serum. At the intervals noted, 100 μl aliquots were processed using a Sigma-kit to measure cellular ATP content by the ATP-dependent luciferin/luciferase assay. Results are presented as moles ATP/trypanosome, and are representative of 4 studies.

Similar results were obtained using enriched trypanocidal protein instead of whole buffalo serum.

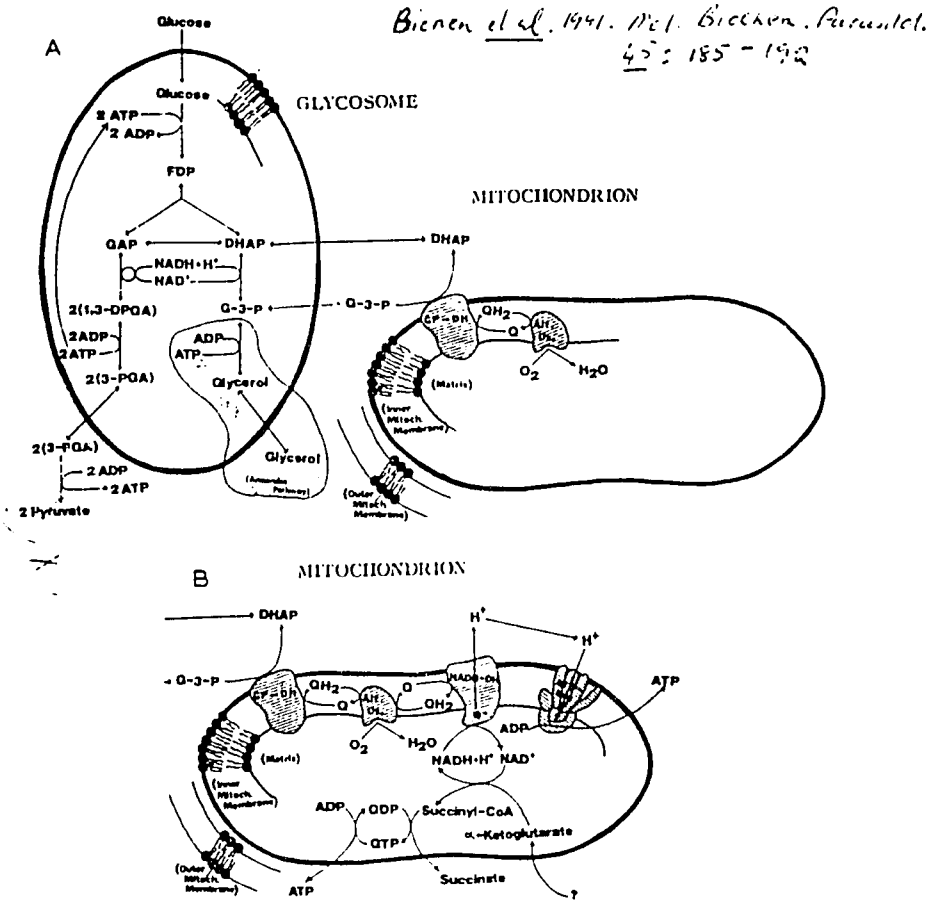

Fig. 1. (A) This schematic diagram presents the limited bioenergetic pathways present in the rapidly dividing long slender (LS) bloodstream forms of *T. b. brucei*. (B) This schematic diagram presents the probable mitochondrial bioenergetic pathways present in transitional intermediate and short stumpy bloodstream forms of *T. b. brucei*. The scheme of cytochrome-independent, trypanosome alternative oxidase (TAO)-dependent oxidative phosphorylation is illustrated. FDP, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; Q, oxidized coenzyme Q; QH_2, reduced coenzyme Q; GP-DH, glycerol-3-phosphate dehydrogenase.

Figure 5 : Bioenergetic pathways in bloodstream stage *T. brucei* as presented by Bienen *et al* (41).

A - Slender form *T. brucei* derive ATP exclusively from glycolysis under aerobic conditions

B - Stumpy form *T. brucei* also derive ATP from glycolysis but have an additional ability to generate ATP from alpha keto glutarate if it is provided.

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Pyruvate inhibits trypanocidal activity

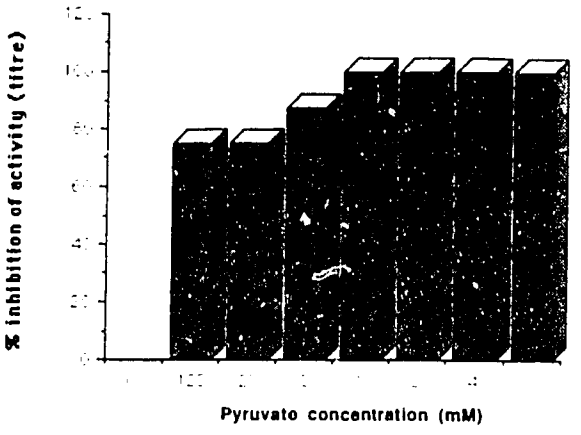

Figure 6 : Trypanosomes were incubated in PBS supplemented with 1% v/v glucose, 10% v/v FBS, with and without dilutions of buffalo serum, and with and without concentrations of pyruvate indicated in the figure. After 4 h at 37°C, the organisms were observed using an inverted tissue culture microscope and scored for motility. Pyruvate at a concentration of 62.5 μM reduced the trypanocidal titer of buffalo serum by 50% (not shown), and at higher concentrations had a greater effect.

Inhibitors of trypanocidal protein : 0.1mM – 1.6mM

pyruvate
CH3
C=O
COO-

α-ketobutyrate
CH3
CH2
C=O
COO-

glyoxylate
CHO
COO-

oxaloacetate
COO-
CH2
C=O
COO-

Structurally related non-inhibitors :

α-ketogluterate*
COO-
(CH2)2
C=O
COO-

acetate
CH3
COO-

acetylCoA
CH3
C=O
SCoA

phosphoenolpyruvate
CH2
C-O~P
COO-

methyl pyruvate
CH3
C=O
C-O-CH3
O

glycerol
CH2OH
CHOH
CH2OH

L-alanine
CH3
H-C-NH3+
COO-

phenylpyruvate
CH2
C=O
COO-

β-ketobutyrate
CH3
C=O
CH2
COO-

L-aspartate
COO-
CH2
H-C-NH3+
COO-

malate
COO-
HO-C-H
CH2
COO-

fumarate
COO-
H-C
C-H
COO-

succinate
COO-
(CH2)2
COO-

citrate
CH2-COO-
HO-C-COO-
CH2-COO-

*applies only to slender but not stumpy forms

Figure 7 : The compounds shown in the figure were examined for their capacity to inhibit trypanocidal activity of buffalo serum. Pyruvate, alpha keto butyrate, glyoxylate and oxaloacetate had similar inhibitory activity *ie.*, exerted the same effects at the same concentrations. The other compounds had no detectable inhibitory effect.

Accessory analyses of the ATP content of trypanosomes that were incubated at 37°C in medium with or without buffalo serum, and with or without 2 mM pyruvate showed that the organisms maintained normal levels of ATP in the presence of pyruvate, and in the presence of buffalo serum and pyruvate. The ability of trypanosomes to maintain glycolysis in the presence of high exogenous pyruvate concentration indicates that pyruvate counter-flow (25) is very inefficient, and hence that the pyruvate transporter is very asymmetric.

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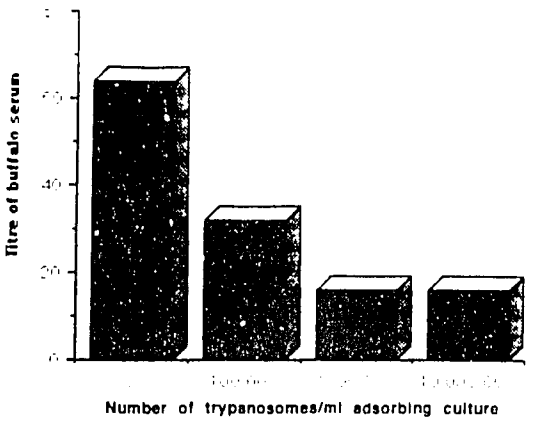
Adsorption of trypanocidal activity at 37°C



Adsorption of trypanocidal activity at 4°C

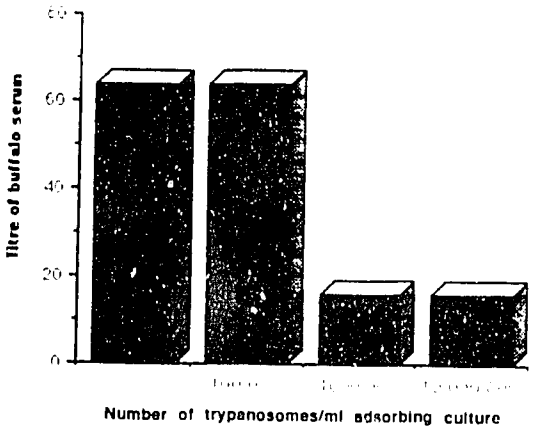


Figure 8a, 8b : Trypanosomes were washed, pelleted and incubated at the number and temperature stated in 1 ml volumes of PBS containing 1% glucose, 10% v/v FBS and with, or without 10% v/v heat-inactivated buffalo serum. After 1 h, the organisms were removed by centrifugation, and dilutions of the supernatant were screened for trypanocidal activity. Results are presented as the reciprocal end titer, and show that trypanosomes removed trypanocidal activity from the preparations at both 37°C and to a lesser degree, at 4°C. Incubation of heat-inactivated buffalo serum (10% v/v) with 5 x 10^8 erythrocytes/ml at 4°C, or with 10^8 mouse splenic lymphocytes/ml at 4°C did not affect trypanocidal activity indicating that removal by the trypanosomes was specific.

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