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The Trypanocidal Cape Buffalo Serum Protein Is Xanthine Oxidase

MADHAVI MURANJAN,[1] QIN WANG,[2] YAN-LI LI,[2] ERIKA HAMILTON,[2] FREDERICK P. OTIENO-OMONDI,[3] JUN WANG,[2] ANDREW VAN PRAAGH,[2] JAN G. GROOTENHUIS,[4] AND SAMUEL J. BLACK[2]*

Department of Pathobiology, Michael Heidelburger Division of Immunology, NYU Medical Center, New York, New York 10016[1]; Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003[2]; Department of Microbiology, The Ohio State University, Columbus, Ohio 43216[3]; and International Laboratory for Research on Animal Diseases, Nairobi, Kenya[4]

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Plasma and serum from Cape buffalo (*Syncerus caffer*) kill bloodstream stages of all species of African trypanosomes in vitro. The trypanocidal serum component was isolated by sequential chromatography on hydroxylapatite, protein A-G, Mono Q, and Superose 12. The purified trypanocidal protein had a molecular mass of 150 kDa, and activity correlated with the presence of a 146-kDa polypeptide detected upon reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid sequences of three peptide fragments of the 146-kDa reduced polypeptide, ligand affinity and immunoaffinity chromatography of the native protein, and sensitivity to pharmacological inhibitors, identified the trypanocidal material as xanthine oxidase (EC 1.1.3.22). Trypanocidal activity resulted in the inhibition of trypanosome glycolysis and was due to H_2O_2 produced during catabolism of extracellular xanthine and hypoxanthine by the purine catabolic enzyme.

African trypanosomes are tsetse fly-transmitted protozoa that inhabit blood plasma and cause fatal sleeping sickness in humans and Nagana in cattle. Nagana excludes domestic livestock from much of the humid and semihumid zones of Africa (8), affecting an area of about 10 million square kilometers that stretches from the southern boundary of the Sahara to a few degrees north of the Tropic of Capricorn. The wide geographic distribution of African trypanosomes, the absence of conserved trypanosome antigens for use as vaccines, and the continuous requirement of susceptible hosts for chemotherapy while under challenge combine to make trypanosomiasis the major constraint to cattle-based agriculture in the humid and semihumid zones of Africa.

Cape buffalo coevolved with African trypanosomes, inhabit the humid and semihumid zones of Africa, and tolerate infection with African trypanosomes with few or no signs of disease (10, 15, 34, 38). Following experimental infection, Cape buffalo rapidly clear most trypanosomes from the bloodstream and subsequently maintain low-level parasitemia (10, 15, 34, 38). Effective control of trypanosome parasitemia by Cape buffalo is not associated with production by the buffalo of broadly protective antibodies or suppression of trypanosome antigenic variation (38). Hence, restrained trypanosome parasitemia in Cape buffalo may result from the presence of growth-inhibitory serum components that are not antibodies and/or from the limited availability of an essential growth factor or growth nutrient.

Serum from Cape buffalo kills *Trypanosoma brucei* subsp. *brucei*, *T. brucei* subsp. *rhodesiense*, *T. brucei* subsp. *gambiense*, *Trypanosoma vivax*, *Trypanosoma congolense*, and *Trypanosoma evansi* in vitro (38) or prevents their replication, depending on the assay buffer constituents. This activity is absent from the serum of a wide variety of domestic animals (38) and might be responsible for the superior capacity of Cape buffalo to restrain parasitemia. As a first step towards testing this hypothesis, it is essential to identify the trypanocidal or trypanostatic serum component(s). Our preliminary analyses (38) indicate that the Cape buffalo serum trypanocidal material is protein with a molecular mass of 150 kDa, lacks a lipid component, and is distinct in buoyant density and biological activity from previously described trypanocidal material (i.e., components of human serum that kill *T. brucei* subsp. *brucei* but not other species and subspecies of African trypanosomes) (16, 41). We now report the isolation of the trypanocidal Cape buffalo blood component and its identification as xanthine oxidase (EC 1.1.3.22).

MATERIALS AND METHODS

African buffalo serum. Serum samples were prepared from two Cape buffalo bulls at the Wildlife Disease Section of The Kenya Agricultural Research Institute and at the International Livestock Research Institute (both at Nairobi, Kenya), frozen, imported to the United States under U.S. Department of Agriculture permit no. 26091-A and 3984, and stored at −70°C until use.

Trypanosomes. *T. brucei* subsp. *brucei* IL3201 organisms of stock 427 were grown as described previously (7) in Baltz-modified minimal essential medium (4) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) and harvested by centrifugation at 1,000 × *g* for 10 min.

Assay of trypanocidal activity and inhibitors. Washed trypanosomes (2×10^4) were incubated in 100-μl aliquots of African buffalo serum or serum fractions diluted in 137 mM NaCl–20 mM phosphate buffer (pH 7.2) (PBS) containing 1% glucose and 10% FBS in wells of a 96-well tissue culture plate (Costar). Plates were incubated for 4 h at 37°C in a humid atmosphere of 9% CO_2 in air, and trypanosomes were examined for motility with an inverted tissue culture microscope; a complete loss of motility was associated with loss of infectivity (see text). Results were recorded either as the reciprocal end trypanocidal titer of serum or serum fraction or as units of trypanocidal activity, where 0.1 U of trypanocidal activity killed 2×10^4 trypanosomes in the assay mentioned above; units of activity in any sample are therefore calculated as (the reciprocal trypanocidal end titer) × (volume of the sample in milliliters). The concentrations and specificities of inhibitors (i.e., allopurinol, folic acid, mannitol, bovine catalase, bovine superoxide dismutase [Sigma], and staurosporine [Bethesda Research Laboratories]) are indicated in the text.

Sequential chromatography. Separations were carried out at 4°C with a fast protein liquid chromatograph (Pharmacia). For the hydroxylapatite step, Cape buffalo serum (40 ml) was dialyzed (25-kDa cutoff; Spectrum) into 200 mM potassium phosphate buffer (pH 6.8), loaded at 0.2 ml min^{-1} on an XK column (16-mm inside diameter) containing an 80-ml bed volume of hydroxylapatite (Bio-Rad), and eluted at the same flow rate by increasing molarity of potassium phosphate buffer (pH 6.8). For the protein A-G step, hydroxylapatite fractions with trypanocidal activity were pooled, adjusted to 400 mM potassium phosphate

* Corresponding author. Mailing address: Paige Laboratory, Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst MA 01003. Phone: (413) 545-2573. Fax: (413) 545-6326. E-mail: sblack@marlin.bio.umass.edu.

TABLE 1. Isolation of trypanocidal protein by sequential chromatography

Fractionation step	Sample volume (ml)	Amt of protein recovered (mg)	Activity (U)[a]	Sp act[b]	% Yield	Fold purification[c]
Buffalo serum	40	3,200	5,120	1.6	100	1
Hydroxylapatite	55	220	3,520	16	69	10
Protein A-G	55	137.5	3,520	25.6	69	16
Mono Q	4.5	0.9	513	570	10	356.3
Superose 12	0.5	0.05	80	1,600	1.6	1,000

[a] (Reciprocal end titer of trypanocidal activity) × (volume in milliliters).
[b] Units of trypanocidal activity per milligram of protein.
[c] (Specific activity of recovered protein)/(specific activity of Cape buffalo serum).

buffer (pH 6.8) by N_2 pressure dialysis over a YM 100 membrane (Amicon), and loaded at 1 ml min^{-1} on a C column (10-mm inside diameter) containing a 5-ml bed volume of protein A-G conjugated to agarose (Schleicher & Schuell). The column was washed with 0.1 M potassium phosphate buffer (pH 7.2) at 1 ml min^{-1} and eluted with 0.1 M glycine HCl (pH 2.5) at 1 ml min^{-1}. For the Mono Q step, the protein A-G run-through fraction was concentrated over a YM 100 membrane, dialyzed into 52.5 mM NaCl in 20 mM Tris buffer (pH 8.25), and loaded on an HR5/5 column containing a 1-ml bed volume of the anion-exchange resin Mono Q (Pharmacia) preequilibrated with the same buffer. The column was eluted at 0.12 ml min^{-1} with increasing molarity of NaCl in Tris buffer. For the Superose 12 step, Mono Q fractions with trypanocidal activity were pooled, concentrated to 100 μl over a Centricon 30 membrane (Amicon), loaded at 0.1 ml min^{-1} on an HR10/30 column packed with a 25-ml-bed-volume Superose 12 size exclusion matrix (Pharmacia), equilibrated with PBS, and eluted with PBS at 0.1 ml min^{-1}. The Superose 12 column was calibrated with blue dextran (2,000 kDa), serum amyloid protein polymer (230 kDa [a gift from R. Mortensen]), catalase (222 kDa), mouse immunoglobulin G (IgG) (150 kDa), bovine serum albumin (67 kDa), and cytochrome *c* (12.4 kDa).

Immunoaffinity chromatography. C-type columns (Pharmacia) packed with 5 ml of antibody-conjugated gels were equilibrated with PBS, loaded with serum in PBS at 0.1 ml min^{-1}, and washed with PBS, and bound material was eluted with 0.1 M triethylamine (pH 11.5) at 1 ml min^{-1}, neutralized with 1 M potassium phosphate buffer (pH 6.4), dialyzed into PBS at 4°C, and concentrated over a Centricon 30 or YM 100 membrane (Amicon). For antibodies, rabbits were primed by subcutaneous injection of antigen in Freund's complete adjuvant and given booster injections 4 and 8 weeks later with the same antigen in Freund's incomplete adjuvant. Serum was prepared from blood collected 10 days after the last booster injection. IgG was isolated by protein A-G chromatography (described above), and monospecific IgG was isolated by ligand affinity chromatography (described below). Anti-trypanocidal protein antibody (anti-TCP) was used as follows. Twenty-five micrograms of chromatographically pure trypanocidal protein (described in the text) was used for each immunization. IgG was conjugated to tresyl-activated agarose (Schleicher & Schuell [5 mg of IgG ml of gel^{-1}]). Anti-146 antibody was used as follows. The 146-kDa reduced polypeptide (≈2 μg), cut from a 7.5% homogeneous polyacrylamide gel after reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of anti-TCP-immunopurified trypanocidal protein was used for each immunization. IgG was conjugated to Affi-Prep HZ hydrazide support (Bio-Rad; 6.6 mg of IgG ml of gel^{-1}). Anti-cow's milk xanthine oxidase was used as follows. Two hundred micrograms of the 150-kDa fraction of cow's milk xanthine oxidase (isolated by fast protein liquid chromatography on Superose 12 from grade III chromatographically pure cow's milk xanthine oxidase [Sigma]) was used for each immunization. Xanthine oxidase-specific IgG was isolated from the immune serum by binding to (in 200 mM potassium phosphate buffer [pH 7.2]) and elution from (with 100 mM triethylamine [pH 11.5]) cow's milk xanthine oxidase (Sigma) conjugated to CNBr-activated Sepharose 4B (Pharmacia). Ten milligrams of xanthine oxidase affinity-purified rabbit IgG was conjugated to 3 ml of CNBr-activated Sepharose 4B (Pharmacia). Anti-buffalo Ig conjugated to Sepharose 4B (38), and anti-bovine lipoprotein conjugated to Affi-Prep HZ (29) have been described previously. Normal rabbit IgG conjugated to Affi-Prep HZ to give 5 mg of IgG ml of gel^{-1} was used as a control in some studies.

Folic acid affinity chromatography. Twenty-five milliliters of Cape buffalo serum diluted to 50 ml with loading buffer (20% 0.1 M pyrophosphate buffer [pH 8.5] containing 0.2 mM EDTA, 80% 0.05 M Tris-HCl buffer [pH 7.8] containing 0.2 mM EDTA) was loaded at 0.1 ml min^{-1} onto a C-type column packed with 5 ml of EAH Sepharose gel (Pharmacia) conjugated with 100 mg of folic acid (32), washed with 10 ml of wash buffer (30% 0.1 M pyrophosphate buffer [pH 8.5] containing 0.2 mM EDTA, 70% 0.05 M Tris-HCl buffer [pH 7.8] containing 0.2 mM EDTA) at 0.1 ml^{-1}, and eluted with wash buffer containing 0.5 mM hypoxanthine. Eluted protein was precipitated in 60% saturated ammonium sulfate and dialyzed into PBS.

SDS-PAGE, blotting, and peptide sequencing. SDS-PAGE on 7.5% homogeneous polyacrylamide gels was carried out with a Phast (Pharmacia) or minigel (Bio-Rad) system, and polypeptides were revealed by Phast silver stain (Pharmacia) or 0.05% Coomassie blue. The reducing sample buffer has been described previously (29), and broad-range molecular mass markers were from Bio-Rad. Protein bands were transferred from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore) with a transblot minigel wet transfer cell (Bio-Rad), polypeptides were stained with 0.1% amido black in methanol-acetic acid-water (4:1:5), and 146-kDa material was excised. The 146-kDa polypeptide was subjected to CNBr cleavage and peptide sequencing by Edman degradation at the Wistar Protein Sequencing Facility (Philadelphia, Pa.).

Effect of trypanocidal protein on trypanosomes. Trypanosomes at a concentration of 10^6 ml of PBS^{-1} supplemented with 10% FBS–1% glucose and with or without 5 U of trypanocidal activity Cape buffalo serum or anti-146-immunopurified trypanocidal protein were incubated at 37°C in a humid atmosphere of 9% CO_2 in air, and trypanosome plasma membrane integrity was monitored by addition of calcein AM and ethidium bromide homodimer (Molecular Probes Inc.) at the manufacturer's recommended concentrations (20). Trypanosome glucose metabolism was monitored by assaying changes in trypanosome and medium pyruvate content with time. To do this, trypanosomes were separated from medium by centrifugation at 1,000 × g, resuspended in PBS, centrifuged through glycerol (5), and lysed in water; the culture medium was filtered (0.22-μm pore diameter). Pyruvate in medium and trypanosome lysates was assayed by a standard procedure which measures lactate formation in the presence of lactate dehydrogenase (Sigma Pyruvate Procedure 726-UV, 1990). Trypanosome ATP content was assayed with the luciferin-luciferase assay (Sigma kit FL-ASC). Luminescence was measured on a Monolight luminometer, and ATP content was determined from a standard curve which was unaffected by addition of Cape buffalo serum or anti-146-immunopurified trypanocidal protein. Trypanosome replicative capacity was assayed after washing of the parasites in PBS to remove buffalo serum. Washed parasites were suspended at a concentration of 10^3 organisms ml^{-1} in Baltz-modified minimal essential medium (4) containing 10% FBS, triplicate cultures were incubated for various periods at 37°C in a humid atmosphere of 9% CO_2 in air, and trypanosomes were counted with a hemocytometer.

Xanthine catabolism. Catabolism of xanthine to uric acid was monitored by reverse-phase high-performance liquid chromatography (HPLC) by a modification of a standard technique (14). A 35-μl aliquot of either Cape buffalo serum, immunopurified trypanocidal protein, or cow's milk xanthine oxidase was mixed with an equal volume of 50 μM xanthine in 10 mM potassium phosphate buffer (pH 7.2) and incubated for up to 30 min at room temperature (≈20°C) in the case of Cape buffalo serum and 1 min for each of the other preparations. Each preparation was immediately cooled to 0°C and centrifuged at 4°C for 6 min at 10,000 × *g* over a Microcon 3 membrane (Amicon), and 15 μl of filtrate was loaded on a 3.9- by 150-mm NovaPac C_{18} column (Waters) with a Waters 600E HPLC with manual injection. The column was eluted with 20 mM KH_2PO_4 (pH 5.6) for 5 min at 1 ml min^{-1}; eluted material was scanned at A_{254} with a Waters Lamda Max model 481 LC spectrophotometer; results were recorded as *xy* plots; xanthine was eluted at 4 min, while uric acid eluted at 2 min; and peak areas were determined as one-half base × height. The molar concentration of each compound was determined by comparison with standard peak area curves.

Xanthine oxidase activity. Aliquots (25 μl) of immunopurified trypanocidal protein or chromatographically pure cow's milk xanthine oxidase (Sigma) in PBS (pH 7.2) were supplemented with 450 μl of 50 mM sodium carbonate buffer (pH 10.2) containing 2.4 mM Na_2EDTA, 1.2 mg of bovine serum albumin ml^{-1}, and 0.6 mM nitroblue tetrazolium (NBT), with or without 2.4 mM xanthine and with or without 500 μM NAD^+. Mixtures were incubated at 37°C for 30 min, and 25 μl of 6 mM $CuCl_2$ was added to each sample to stop the enzyme reaction. The optical density reading of each test sample was taken at A_{560} after zeroing in on a matched xanthine-free negative control sample.

RESULTS

Isolation of trypanocidal protein by sequential chromatography. The trypanocidal component of Cape buffalo serum was isolated by sequential chromatography (Table 1) on hydroxyla-

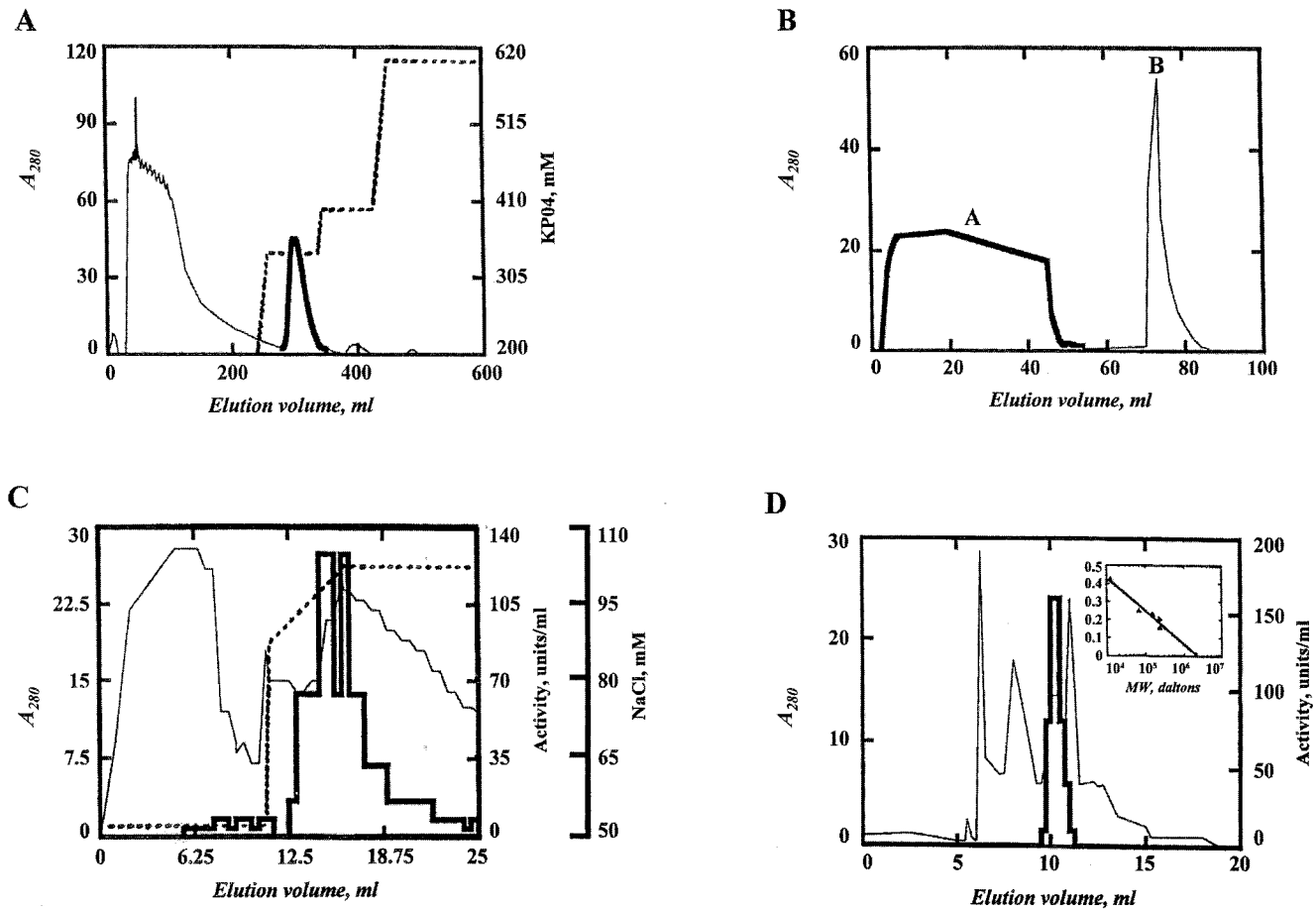

FIG. 1. Isolation of trypanocidal material from Cape buffalo serum by sequential chromatography on hydroxylapatite (A), protein A-G (B), Mono Q (C), and Superose 12 (D). In all panels, protein is recorded as relative A_{280} (thin lines). The presence of trypanocidal activity is indicated by thick lines and is overlaid on relevant regions of the A_{280} protein profiles in panels A and B because trypanocidal endpoints of individual fractions were not measured. Trypanocidal activity is presented as a separate curve in each of panels C and D where endpoint data were collected. MW, molecular mass. The molarities of elution buffers are recorded in panels A and C as dashed lines.

paptite (Fig. 1A), protein A-G (Fig. 1B), Mono Q anion-exchange resin (Fig. 1C), and a Superose 12 size exclusion matrix (Fig. 1D), which jointly achieved a 1,000-fold purification (Table 1). The trypanocidal protein had a molecular mass of 150 kDa (Fig. 1D), and trypanocidal activity correlated with the presence of a 146-kDa polypeptide detected by SDS-PAGE under reducing conditions (Fig. 2). Purified trypanocidal protein lost activity during 24 h of storage at 4°C, and during this period, the 148- and 28-kDa polypeptide components degraded to small peptides that ran with the buffer front in reducing SDS-PAGE. The inclusion of a cocktail of protease inhibitors (25 μg each of leupeptin, pepstatin, and aprotinin ml^{-1} and 1 mM phenylmethylsulfonyl fluoride) in the storage buffer did not preserve trypanocidal activity or polypeptide integrity. Attempts to blot and sequence the 146- and 28-kDa reduced polypeptides of sequentially purified protein were unsuccessful.

Isolation of trypanocidal protein by immunoaffinity chromatography. Trypanocidal activity of Cape buffalo serum was unaffected by chromatography on immobilized rabbit IgG or rabbit IgG raised against bovine lipoproteins or Ig (data not shown). Trypanocidal activity was completely removed from Cape buffalo serum by chromatography on immune IgG raised against purified trypanocidal protein (Fig. 2, fifth lane), and activity was recovered by elution with triethylamine buffer (pH 11.3) (anti-TCP [Table 2]). Anti-TCP-immunopurified trypanocidal protein resolved during reducing SDS-PAGE as several polypeptides spanning a molecular mass range of 166 to 55 kDa, of which the 146-kDa component was a minor species (not shown). Immunoaffinity chromatography with antibodies raised against the reduced 146-kDa component of anti-TCP-immunopurified protein also removed trypanocidal activity from Cape buffalo serum, and activity was recovered from the column by elution at pH 11.3 (anti-146 [Table 2]). Anti-146-immunopurified trypanocidal protein resolved as proteins with molecular masses of 300 and 150 kDa during chromatography on Superose 12 (Fig. 3A). Trypanocidal activity was solely associated with the 150-kDa material (Table 2), and this resolved as several polypeptides upon reducing SDS-PAGE, of which the 146-kDa reduced polypeptide was a minor species (Fig. 3B, lane 3).

Amino acid sequence analysis of the 146-kDa reduced polypeptide. The 146-kDa polypeptide blotted onto PVDF membrane after reducing SDS-PAGE of anti-146-immunopurified material was N blocked. Treatment of the PVDF-bound 146-kDa polypeptide with CNBr yielded numerous polypeptides. Three homogeneous polypeptide CNBr fragments of the 146-kDa reduced polypeptide showed (Fig. 4) 84 to 100% N-ter-

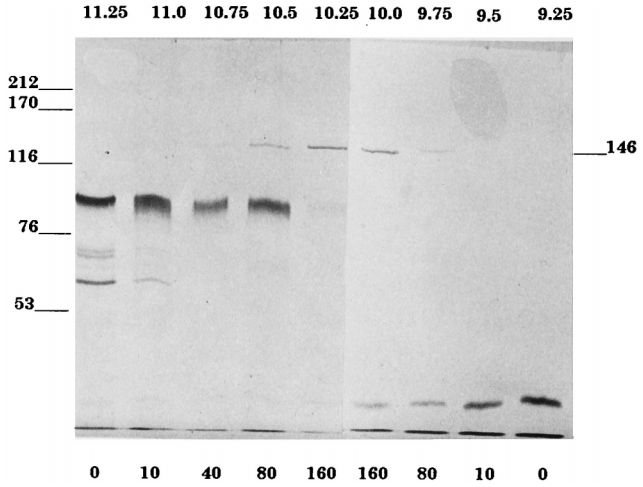

FIG. 2. Reducing SDS-PAGE of Fig. 1 Superose 12 fractions. Superose 12 fractions were subjected to reducing SDS-PAGE on a homogeneous 7.5% polyacrylamide gel and silver stained. The fraction elution volume in milliliters is shown at the top of each lane, and the reciprocal trypanocidal end titer is shown at the bottom, Molecular mass marker (Pharmacia) positions in kilodaltons are on the left, and the position of the 146-kDa material is shown on the right.

minal amino acid sequence homology, dependent on the fragment, with matched sequences of human, mouse, and rat xanthine:oxygen oxidoreductase, a dual-function enzyme that converts purines such as hypoxanthine and xanthine to uric acid (22, 23, 28, 31, 45).

Anti-146-immunopurified trypanocidal protein contains xanthine oxidase. The 150-kDa component of anti-146-immunopurified trypanocidal protein (Fig. 3A, peak 2) could convert xanthine to uric acid (1 nmol of xanthine catabolized min^{-1} 50 μg of immunopurified $protein^{-1}$), confirming the presence of xanthine:oxygen oxidoreductase. Chromatographically pure cow's milk xanthine oxidase (Sigma) had 50-fold higher activity (1 nmol of xanthine catabolized min^{-1} μg of xanthine $oxidase^{-1}$), and Cape buffalo serum had 600-fold lower activity (1 nmol of xanthine catabolized min^{-1} 3×10^4 μg of cape buffalo $serum^{-1}$). Xanthine:oxygen oxidoreductase was not detected in either Cape buffalo serum that was immunodepleted of anti-146-reactive protein, in the 300-kDa component of anti-146-kDa immunoaffinity-purified material (Fig. 3A, peak 1), or the fetal bovine serum used as a component of the bioassay buffer for trypanocidal protein.

Xanthine:oxygen oxidoreductase exists as xanthine oxidase or xanthine dehydrogenase, dependent on conformation (28, 45). Xanthine oxidase (EC 1.1.3.22) is reduced during catabolism of purine and in turn reduces O_2, yielding H_2O_2 plus O_2^-, which can be monitored by reduction of NBT read at A_{560}. Reduced xanthine dehydrogenase (EC 1.1.1.204) uses NAD^+ as an electron acceptor in preference to O_2, yielding NADH in the presence of NAD^+ but H_2O_2 and O_2^- in the absence of NAD^+ and presence of O_2 (17). Generation of reactive oxygen intermediates (ROIs) during catabolism of xanthine by anti-146-immunopurified protein (molecular mass of 300 kDa plus 150-kDa material) was inhibited to only a minor degree by addition of 0.5 mM NAD^+ to the incubation buffer (Fig. 5A), indicating that most of the xanthine:oxygen oxidoreductase in anti-146-immunopurified trypanocidal protein was present as xanthine oxidase. In contrast, production of ROIs during catabolism of xanthine by commercial cow's milk xanthine oxidase was inhibited by ≈75% by addition of NAD^+ to the reaction buffer (Fig. 5B), suggesting that most of the commercial enzyme could use NAD^+ as an electron acceptor and by that definition was present as xanthine dehydrogenase. In the absence of NAD^+, the commercial cow's milk xanthine oxidase had 100-fold-higher activity than anti-146-immunopurified Cape buffalo material (Fig. 5A and B).

Trypanocidal activity is due to xanthine oxidase. Although xanthine oxidase was a minor component of Cape buffalo serum and of the anti-146-immunopurified trypanocidal protein, the trypanocidal activity of both preparations was due to the purine catabolic enzyme, as shown by the following five findings.

(i) Chromatography of Cape buffalo serum on immobilized rabbit IgG specific for cow's milk xanthine oxidase completely removed trypanocidal activity, and activity was recovered upon elution of the bound protein (Table 2). The anti-cow's milk xanthine oxidase-immunopurified protein resolved as four proteins (Fig. 3C) with molecular masses of 300 (peak 1), 235 (peak 2), 150 (peak 3), and 50 (peak 4) kDa during chromatography on Superose 12. Trypanocidal (Table 2) and xanthine:oxygen oxidoreductase activities (data not shown) were solely associated with the 150-kDa material, and this material contained several polypeptides, of which the 146-kDa reduced polypeptide was a minor species (Fig. 3D, lane 4).

(ii) Allopurinol (at 0.1 mM) and folic acid (at 0.6 mM) abrogated the trypanocidal activity of the minimum effective concentration of anti-146 immunoaffinity-purified trypanocidal protein. Allopurinol and folic acid are inhibitors of xanthine:oxygen oxidoreductase (27, 33).

(iii) Folic acid conjugated to Sepharose 4B by its carboxy group binds in the purine binding site of xanthine:oxygen oxidoreductase (32) and captured trypanocidal protein from Cape buffalo serum (Table 2). Folic acid affinity-purified trypanocidal protein resolved as two proteins (Fig. 3E) with molecular masses of 300 (peak 1) and 150 (peak 2) kDa during chromatography on Superose 12. Trypanocidal (Table 2) and xanthine:oxygen oxidoreductase activities (data not shown) were solely associated with the 150-kDa material, and during reducing SDS-PAGE, this resolved as several polypeptides, of which the 146-kDa component was a minor species (Fig. 3F, lane 3). Recovery of trypanocidal activity and xanthine:oxygen oxidoreductase from the folic acid column was low (Table 2), and recovered material was very unstable, losing enzyme activity and polypeptide integrity within 24 h after isolation. Affinity chromatography on immobilized folic acid also removed all trypanocidal activity from anti-146- and anti-cow's milk xanthine oxidase-immunopurified preparations. In each case, less than 5% of the immunopurified protein bound to the immobilized folic acid, and the recovered protein was very unstable. Thus, the most enriched preparations of trypanocidal protein were the least stable, suggesting copurification of a protease activity. As was observed with trypanocidal protein that had been isolated by sequential chromatography (Fig. 1), the addition of 25 μg each of leupeptin, pepstatin, and aprotinin ml^{-1} and 1 mM phenylmethylsulfonyl fluoride to the storage buffer did not prevent loss of trypanocidal activity or preserve the polypeptide integrity of the folic acid affinity-isolated trypanocidal material.

(iv) Trypanocidal activity of anti-146-immunopurified trypanocidal protein required purine present in the FBS component of the reaction mixture; dialysis (25-kDa cutoff membrane) of the FBS abrogated expression of trypanocidal activity, and activity was restored upon addition of 1 μM hypoxanthine or xanthine to the buffer.

(v) Addition of 0.5 mM NAD^+ to the incubation buffer had no effect on the trypanocidal activity of Cape buffalo serum, consistent with the observation (Fig. 5A) that Cape buffalo

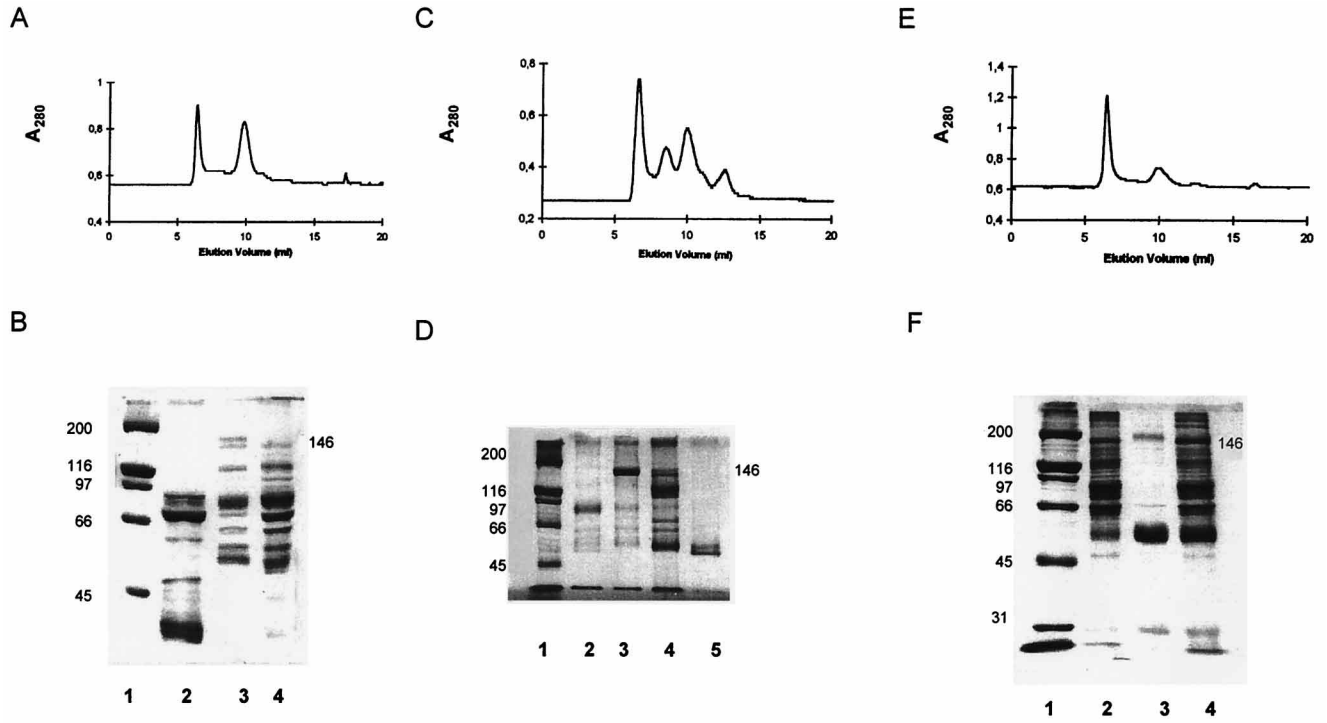

FIG. 3. Superose 12 fractionation and reducing SDS-PAGE of immunoaffinity-purified trypanocidal proteins. (A, C, and E) Superose 12 chromatographic profiles of, respectively, anti-146-, anti-cow's milk xanthine oxidase, and folic acid affinity-purified trypanocidal protein described in Table 2. (B, D, and F) Polypeptide content of the immunopurified proteins shown in panels A, B and C, respectively, separated by reducing SDS-PAGE on 7.5% homogeneous polyacrylamide gels and stained with Coomassie blue. Molecular mass markers (lane 1 in panels B, D, and F) in kilodaltons are displayed to the left of each panel; the position of the 146-kDa polypeptide is indicated to the right of each panel. Ten to 20 μg of protein was loaded in each lane. (B) Anti-146-purified protein. Lane 2 shows the 300-kDa peak 1 material, lane 3 shows the 150-kDa peak 2 material, and lane 4 shows the unfractionated anti-146-purified material. (D) Anti-cow's milk xanthine oxidase-purified protein. Lane 2 shows 300-kDa peak 1 material, lane 3 shows the 225-kDa peak 2 material, lane 4 shows the 150-kDa peak 3 material, and lane 5 shows the 50-kDa peak 4 material. (F) Folic acid affinity-purified protein. Lane 2 shows the 300-kDa peak 1 material, lane 3 shows the 150-kDa peak 2 material, and lane 4 shows the unfractionated folic acid affinity-isolated protein. Similar although not identical results were obtained in several separate studies of isolated proteins. In particular, the amounts of lower-molecular-mass polypeptides varied in different preparations, consistent with various degrees of degradation.

serum xanthine:oxygen oxidoreductase is predominantly present as xanthine oxidase.

Impact of trypanocidal protein on trypanosome replication. During incubation with anti-146-immunopurified material, trypanosomes first became sluggish, and by 1.5 h of incubation they were immobile. *T. brucei* organisms that had been incubated with anti-146-immunopurified protein for 1 h retained their capacity to replicate on transfer to fresh medium, and their growth characteristics were not different from those of control *T. brucei* organisms. In contrast, trypanosomes that had been incubated with immunopurified trypanocidal protein for 1.5 h and were completely immobile were unable to replicate when moved to fresh medium. Trypanosomes that had been incubated with anti-146-immunopurified trypanocidal protein for 1.5 h were able to exclude ethidium bromide and retain calcein preloaded as calcein AM (Molecular Probes, Inc.), indicating that their membranes were intact. However, the immobile organisms lost the capacity to retain calcein and exclude ethidium bromide after a further 6 to 8 h of incubation, and many broke into fragments. Similar results were obtained with trypanosomes that were incubated with intact Cape buffalo serum and with different preparations of anti-146-immunopurified trypanocidal protein, although the timing for complete loss of motility varied from 1.5 to 4 h in different experiments.

Trypanocidal activity is due to H_2O_2 and results in inhibition of trypanosome glycolysis. Catalase (EC 1.11.1.6; H_2O_2 plus H_2O_2 = O_2 plus $2H_2O$) at 30 μg of bioassay buffer per ml abrogated the trypanocidal activity of Cape buffalo serum and immunopurified trypanocidal protein, irrespective of the concentration of the trypanocidal material. Superoxide dismutase (EC 1.15.1.1; O_2^- plus O_2^- plus $2H^+$ = O_2 plus H_2O_2) at up to 2 mg ml of mannitol^{-1} (at 15 mM), which scavenges hydroxyl radicals, and thiourea or taurine (at up to 50 and 10 mM, respectively), which scavenge hypohalides, did not affect trypanocidal activity. Twenty-five micrograms of cycloheximide ml of bioassay buffer^{-1} and 10^{-7} M staurosporine, which respectively inhibit trypanosome protein synthesis (29) and protein phosphorylation (29), also did not inhibit trypanocidal activity of Cape buffalo serum or anti-146-immunopurified trypanocidal protein. Thus, trypanocidal activity may be due to a direct effect of H_2O_2 on the parasites. In support of this hypothesis, addition of 10 μM H_2O_2 to the bioassay buffer caused trypanosomes to become sluggish, lose motility, and die, which is the characteristic action of Cape bufalo serum (28) and of immunopurified trypanocidal material.

Catabolism of glucose to pyruvate is the sole energy-generating pathway in replicating *T. brucei* trypomastigotes (6), and loss of trypanosome motility during incubation with Cape buffalo serum or immunopurified trypanocidal protein may result from inhibition of energy metabolism. Incubation of *T. brucei* with anti-146-immunopurified protein caused a 90% reduction in ATP content within 1 min, and this was sustained thereafter (Fig. 6A). The treated organisms also had an impaired capacity

peptide A 1 TVPMDHTFFPSYR 13

rat XDH 381 TVRMDHTFFPGYR 393

human XDH 382 TVQMDHTFFPGYR 394

mouse XDH 384 TVWMDHTFFPGYR 396

peptide B 1 IPAFGSIPMEFR 12

rat XDH 1216 IPAFGSIPIEFR 1227

human XDH 1230 IPAFGSIPIEFR 1241

mouse XDH 1232 IPAFGSIPIEFR 1243

peptide C 1 VASIGGNIITASPISDLNPVFMASG 25

rat XDH 344 VASIGGNIITASPISDLNPVFMASG 368

human XDH 345 VASVGGNIITASPISDLNPVFMASG 369

mouse XDH 347 VASIGGNIITASPISDLNPVLMAS 370

FIG. 4. The NH_2-terminal sequences of three peptides, A, B, and C, from the CNBr digestion of the 146-kDa polypeptide of the trypanocidal protein aligned with those of peptides from rat, human, and mouse xanthine oxidase (XDH). Amino acid substitutions are underlined. Immunoaffinity chromatography on anti-146-kDa polypeptide IgG was used to obtain trypanocidal material from buffalo serum, which was subjected to reducing SDS-PAGE on a homogeneous 7.5% gel and transferred to an Immobilon-P membrane. The 146-kDa material was then cut out and cleaved by CNBr, and the N-terminal sequences of HPLC-purified peptides were obtained by Edman degradation at the Wistar Protein Sequencing Facility, Philadelphia, Pa. The homology search was conducted with the Swissprot protein sequencing database.

to catabolize glucose to pyruvate, as evidenced by reduced excretion of the glycolytic end product (Fig. 6B). Little or no pyruvate was detected within treated or control trypanosomes, indicating that reduction in pyruvate excretion was not due to inhibited transport. Incubation with Cape buffalo serum also caused a 90% reduction in trypanosome ATP content and pyruvate excretion, but the decline in metabolic activity was slower than that which occurred with trypanocidal protein, requiring an incubation period of 20 min before reaching a 90% reduction in trypanosome ATP content. Cape buffalo serum that was depleted of trypanocidal activity by anti-146 immunoaffinity chromatography had no effect on trypanosome ATP content or catabolism of glucose to pyruvate (data not shown).

DISCUSSION

Cape buffalo serum trypanocidal protein was purified by sequential chromatography on hydroxylapatite, protein A-G, Mono Q, and Superose 12. Chromatographically pure material had a molecular mass of 150 kDa and was composed of 146- and 28-kDa polypeptides revealed by reducing SDS-PAGE. The concentration of the 146-kDa reduced polypeptide correlated with trypanocidal activity, and this polypeptide was subsequently identified as the 146-kDa subunit of xanthine oxidase. The plasma xanthine oxidase appears to be present as a monomer, in contrast to tissue xanthine oxidase, which has a molecular mass of 300 kDa and is a noncovalent dimer of two identical subunits (23, 30, 31).

Trypanocidal protein was also isolated by immunoaffinity chromatography with immune rabbit IgG against either chromatographically pure Cape buffalo serum trypanocidal protein, the 146-kDa reduced polypeptide of Cape buffalo serum trypanocidal protein, or a commercial preparation of cow's milk xanthine oxidase. All of the immunopurified materials contained proteins with molecular masses of 300 and 150 kDa, of which the 150-kDa component was solely responsible for trypanocidal and xanthine oxidase activities. Irrespective of the immunoaffinity matrix used, the 150-kDa trypanocidal materials resolved as several polypeptides during reducing SDS-PAGE, and in each case, the 146-kDa reduced polypeptide was a minor species. The 150-kDa proteins were therefore for the most part assembled from lower-molecular-mass polypeptides. Xanthine oxidase has multiple cysteines (23), is highly susceptible to degradation (23, 31), and can accomodate extensive cleavage without any reduction in molecular mass (23, 31). However, it is unlikely that the immunopurified 150-kDa trypanocidal proteins were solely composed of intact and frayed xanthine oxidase; reducing SDS-PAGE of the different preparations revealed polypeptides that were unique to each preparation.

Although all of the immunopurified trypanocidal proteins contained material in addition to xanthine oxidase, there was compelling evidence that the xanthine catabolic enzyme was responsible for the trypanocidal activity. (i) Peptide fragments of a 146-kDa reduced polypeptide, the presence of which cor-

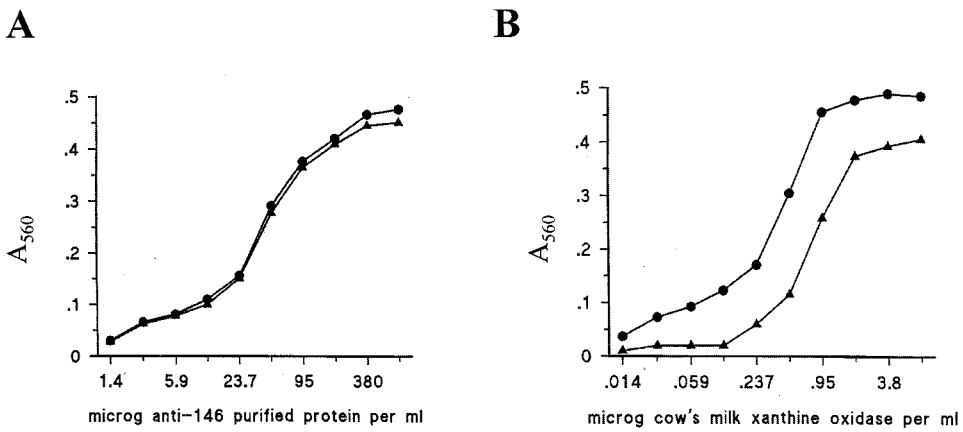

FIG. 5. NAD-resistant generation of ROIs during catabolism of xanthine by anti-146-immunopurified protein (A) and cow's milk xanthine oxidase (B). Mixtures of xanthine, anti-146-immunopurified protein, and tetrazolium salt (NBT) were incubated with (▲) and without (●) NAD^+, and reduction of NBT to formasan was measured as A_{560}. Reduction of NBT required xanthine in the buffer. microg, micrograms.

TABLE 2. Isolation of trypanocidal protein by affinity chromatography

Column(s) used	Protein loaded (mg)	Sp act of protein loaded[a]	Protein recovered (mg)	Sp act of protein recovered[a]	% Yield	Fold purification[b]
Anti-TCP	800	0.8	4	256	80	320
Anti-146	800	0.8	2	192	30	240
Anti-146–Superose 12[c]	2	192	0.5[d]	512[d]	66	640
Anti-cmxo[e]	2,000	0.8	6.8	75	40	94
Anti-cmxo–Superose 12	4	75	0.4[d]	320[d]	94	800
Folic acid	2,000	0.8	0.25	256	4	320
Folic acid[f] Superose 12[c]	0.72	256	0.14[d]	228[d]	35	285

[a] Units of trypanocidal activity per milligram of protein.
[b] (Specific activity of recovered protein)/(specific activity of Cape buffalo serum).
[c] One hundred-microliter load volume applied to Superose 12.
[d] One hundred-fifty-kilodalton peak (elution volume of 10 ml [Fig. 3]) only; other Superose 12 peaks had no activity.
[e] cmxo, cow's milk xanthine oxidase.
[f] Pool of three column runs concentrated over Centricon 3 membrane.

related directly with trypanocidal activity, were virtually identical in amino acid sequences to xanthine:oxygen oxidoreductase from humans, rats, and mice. (ii) Rabbit IgG raised against cow's milk xanthine oxidase captured all of the trypanocidal protein in Cape buffalo serum. (iii) Pharmacologic agents that inhibit xanthine:oxygen oxidoreductase (i.e., allopurinol [after catabolism to oxypurinol] and folic acid [27, 33]), inhibited the trypanocidal activity of Cape buffalo serum and immunopurified trypanocidal protein. (iv) Folic acid tethered by its carboxy group to Sepharose 4B, which is an affinity ligand for xanthine: oxygen oxidoreductase (32), bound Cape buffalo serum trypanocidal protein and removed the trypanocidal activity from anti-146 and anti-cow's milk xanthine oxidase-immunopurified proteins. (v) Immunopurified trypanocidal protein had xanthine oxidase activity evidenced by catabolism of xanthine to uric acid, with concomitant production of ROIs that was insensitive to inhibition by NAD^+. (vi) The requirements for expression of trypanocidal activity were consistent with the generation of H_2O_2 during catabolism of oxypurine by xanthine oxidase (i.e., activity required the presence of xanthine or hypoxanthine in the reaction buffer, was inhibited by addition of catalase, and was not inhibited by NAD^+). Identification of trypanocidal protein as xanthine:oxygen oxidoreductase clearly distinguishes it from human serum factors that selectively lyse *T. brucei* subsp. *brucei*. These factors are complexes of proteins that invariably include haptoglobin-related protein, are restricted to primates, and have peroxidase activity (16, 41).

The origin and secretory pathway of serum xanthine oxidase are not known. The enzyme is found in the cytosol but not the endoplasmic reticulum of hepatocytes; vascular endothelium; renal, intestinal, and skin epithelium; and muscle (3, 21, 24, 25, 30, 39). Cellular xanthine:oxygen oxidoreductase is mainly present as xanthine dehydrogenase, and expression of the xanthine:oxygen oxidoreductase gene is induced by inflammatory cytokines (35, 40), hypoxia followed by exposure to increased oxygen tension (19, 37), and exposure of the cell to activated neutrophils (36, 42). In all cases, increased gene expression is accompanied by increased conversion of cellular xanthine dehydrogenase to xanthine oxidase. This conversion results from limited proteolysis and/or thiol oxidation, the latter of which is reversible (28, 45). Conversion of xanthine reductase to xanthine oxidase in the cell cytosol results in the use of O_2 as the sole electron acceptor during purine oxidation and the generation of reactive oxygen intermediates. Perhaps the xanthine oxidase found in Cape buffalo serum leaked from cells that were dying as a result of oxidative stress, or there may be a more selective secretory process.

The trypanocidal activity of cape buffalo serum and of immunopurified trypanocidal protein required H_2O_2 that was generated during catabolism of xanthine and hypoxanthine in

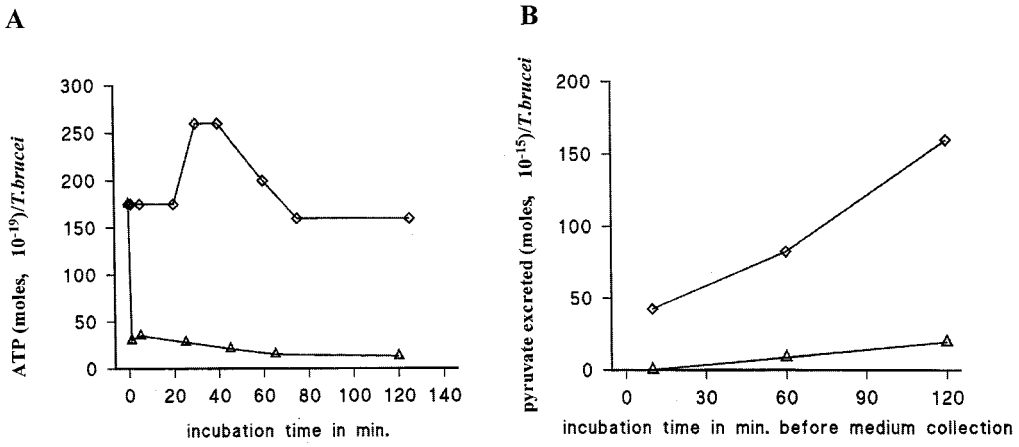

FIG. 6. Effect of immunoaffinity-isolated trypanocidal protein on trypanosome ATP content (A) and pyruvate excretion (B). (A) ATP content (10^{-19} mol/trypanosome) of a *T. brucei* isolate that was incubated for up to 2 h with (◇) or without (△) anti-146 immunoaffinity-isolated trypanocidal protein. (B) Pyruvate excreted (10^{-15} mol/trypanosome) by a *T. brucei* isolate during incubation for up to 2 h with (◇) or without (△) anti-146-immunopurified trypanocidal protein.

the incubation buffer. Trypanocidal activity did not involve O_2^-, OH^-, or hypohalides and was unaffected by inhibition of trypanosome protein synthesis or protein kinase activity, suggesting that it was likely due to a direct effect of H_2O_2 on the parasites. Trypanocidal activity resulted in a precipitous decline in trypanosome ATP content to about 10% of the normal value and maintenance at that level for some hours. Reduction in trypanosome ATP content was accompanied by and may have been due to a decline in the catabolism of glucose to pyruvate, which is the sole energy-generating pathway used by replicating bloodstream-stage trypanosomes (6). In this regard, H_2O_2 has been shown to inhibit glycolysis in higher eukaryotic cells (26). The ATP reserve of trypanosomes was 6×10^{-17} mol trypanosome^{-1} (mean of three experiments) in the present study, which is close to a previously reported value of 4.6×10^{-17} mol trypanosome^{-1} (6). The parasite ATP reserve is therefore only a minor portion of the 2×10^{-15} mol of ATP generated trypanosome^{-1} min^{-1} (6). The absence of catalase in *T. brucei* (13), its low energy reserve, and its profligate energy consumption render the parasite highly susceptible to H_2O_2-mediated inhibition of energy metabolism.

Trypanosomes that were exposed to trypanocidal protein for up to 1 h displayed sluggish movement but retained infectivity, indicating that the reduced ATP synthesis was adequate to support critical functions for a short period. Trypanosomes that were incubated with trypanocidal protein for longer periods irreversibly lost motility and infectivity but did not lyse until several hours later. Loss of motility and infectivity were not affected by inhibition of trypanosome protein synthesis or kinase activity, suggesting that an apoptotic program was not involved; the impact of these agents on eventual trypanosome fragmentation was not measured. Recent work in Larry Ruben's laboratory (38a) shows that extracellular xanthine oxidase and xanthine induce a death program in procyclic *T. brucei*, which is revealed as a gradual loss in motility accompanied by DNA cleavage and followed after several hours by cell lysis; Ca^{2+} and tyrosine protein kinase activity are required at some stages of the apoptotic program. Procyclic *T. brucei* organisms inhabit the gut of the tsetse vector of African trypanosomes, and unlike bloodstream-stage *T. brucei* organisms, do not rely solely on glycolysis for energy metabolism (6). It will be of interest to determine whether H_2O_2 or O_2^- is responsible for induction of the apoptotic program in procyclic *T. brucei* and whether the rapid depletion of ATP by H_2O_2 in the bloodstream-stage organisms prevents or delays activation of the program.

Paradoxically, the presence of xanthine:oxygen oxidoreductase in serum and plasma is not restricted to Cape buffalo. The enzyme has been reported in calf serum (9) and is also detected on the outer face of human vascular endothelium attached to heparin sulfate proteoglycans (1). Our ongoing investigations (42b) show that cow plasma or serum can catabolize xanthine to uric acid with between 8 and 100% (dependent on the donor cow) of the efficiency of Cape buffalo serum and that mouse (BALB/c, C57Bl/6, and C3H/He) serum and Cape buffalo serum have similar xanthine catabolic activities. The cow and mouse sera do not have trypanocidal activity (38). Lack of trypanocidal activity correlates with a failure to accumulate H_2O_2 in serum during catabolism of xanthine (42a). We have not resolved the mechanisms that prevent generation of trypanocidal H_2O_2 during catabolism of xanthine by xanthine:oxygen oxidoreductase in cow serum, but serum catalase is implicated.

Substrate availability will determine whether extracellular xanthine oxidase is involved in control of bloodstream *T. brucei* in Cape buffalo. In this regard, it is important to note that xanthine and hypoxanthine are not the only substrates that elicit trypanocidal H_2O_2 production in Cape buffalo serum. The serum also contains purine nucleoside phosphorylase (EC 2.4.2.1), adenosine deaminase (EC 3.5.4.4), and guanine deaminase (EC 3.5.4.3), which allow catabolism of adenosine, guanosine, and guanine to hypoxanthine and xanthine, which are then catabolized by xanthine oxidase, yielding H_2O_2 (42b). In addition, purines that cannot be catabolized directly to hypoxanthine and xanthine in serum may be converted to the oxypurines during processing by trypanosomes; the organisms are obligate purine auxotrophs (12, 18) and synthesize all of the cellular nucleotides after uptake of any of a variety of purines (12), and purine interconversion during salvage is accompanied by transport of xanthine and hypoxanthine out of the parasites (12). Interaction of released oxypurine with xanthine oxidase in Cape buffalo serum would lead to production of ROIs at the trypanosome surface, which, even if nonlethal, might affect survival of the parasite (e.g., by slowing trypanosome replication or by retarding antibody clearance from the parasite surface, either of which would enhance the efficiency of immune attack (44).

The data presented in this article show that a trypanocidal concentration of H_2O_2 can result from catabolism of xanthine and hypoxanthine by xanthine oxidase in Cape buffalo serum. Other oxidases can generate trypanocidal H_2O_2 (11), indicating that the xanthine oxidase-substrate axis may be one of several oxidase systems that could contribute to control of the parasites. Furthermore, H_2O_2 is highly diffusible. Hence, infection-induced intracellular oxidases might also contribute to the presence of H_2O_2 in plasma. In this context, it is of interest that expression of the xanthine:oxygen oxidoreductase gene and conversion of the enzyme to the oxidase mode are induced by inflammatory cytokines, including gamma interferon (35, 40), thus engaging this oxidase in both innate and acquired immune responses. The importance of xanthine oxidase (and any other oxidase) as a host resistance factor for trypanosomiasis will be determined by other host molecules that destroy H_2O_2. Based on this consideration, we hypothesize that accumulation of a trypanocidal concentration of H_2O_2 in serum during catabolism of xanthine, rather than xanthine oxidase concentration, is a phenotypic marker for innate resistance to African trypanosomiases and that any donor of serum that has this property will be resistant to trypanosomiasis.

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