PN-AB6-262

Agency for International Development
Washington, D.C. 20523

APR 10

MEMORANDUM

TO: PPC/CDIE, Maury Brown

FROM: S&T/RUR, Curtis R. Jackson

SUBJECT: Final Report from Meharry Medical College

Attached is the final report from Dr. Jaswant S. Bhorjee on the research topic "Characterization of Trypanosome Non-histone Chromosomal Proteins" (DAN-5053-G-SS-8030-00). The research was supported by A.I.D.'s small research program for Historically Black Colleges and Universities.

Please make this information available through the A.I.D. Library to A.I.D. staff and others seeking information on this topic.

Attachment: a/s

FINAL REPORT

PROJECT TITLE: Characterization of Trypanosome Non-histone Chromosomal Proteins.

PRINCIPAL INVESTIGATOR: Jaswant S. Bhorjee, Ph.D.

GRANT NUMBER: DAN-5053-G-SS-8030-00

1. Research Objectives and Accomplishments.

A major research objective of the proposal was to develop reliable methodology for isolation of nuclei and chromatin from different life-cycle forms of Trypanosoma brucei. This has been accomplished. In the attached manuscript (Kochhar et al., submitted to Expl. Parasitol. 1/90), we have described the procedure which results in nuclear preparations whose biochemical and molecular integrity is retained. We have also characterized the nucleosomic structures of chromatins isolated from the procyclic (T.b. rhodesiense) and the bloodstream (T.b. brucei) forms of Trypanosoma brucei. And based on the nuclease sensitivity studies, we have shown that the chromatins from two different life cycle stages of T. brucei are different. We believe this observation may be of significance in our future research.

A significant, and a potentially important, finding in this project is the identification of a low molecular weight histone H1-like protein in T. brucei chromatin. Although indicated before, ours is the first report to show an immunologically cross reactive T. brucei histone H1-like protein to the chicken and Tetrahymena macronuclei H1 (Kochhar et al., manuscript submitted to Expl. Parasitol, 1/90, copy attached).

2. Objectives not Accomplished and Reasons.

One objective was the isolation and characterization of the High Mobility Group (HMG) chromosomal proteins from T. brucei. This has not be achieved. Several procedures, including use of 5% perchloric acid (PCA) and 0.35 M NaCl, failed to solubilize any proteins which had properties ascribed to the HMG proteins. The reasons for the absence of HMGs or HMG-like proteins in trypanosomes is not clear to us. It may be noted that several related protozoans and lower eukaryotes either do not have HMG proteins or have PCA soluble proteins which are somewhat different when compared to the classical mammalian or avian HMG proteins.

3. Papers Published/Submitted for Publication in Refereed Journals.

Kochhar, V.K., Mwasi, L.M. and Bhorjee, J.S. Trypanosoma brucei: Isolation of nuclei, characterization of chromatin subunit structure and evidence for the presence of histone H1-like protein. Expl. Parasitol. (submitted 1/90).

4. Contracts or Grants that resulted from the A.I.D. Grant.

Pending:

Agency: A.I.D.
Title: Characterization of Histone H1 Gene from Trypanosoma brucei
Period: 9/1/90 - 8/31/91

Total Cost: $100,000

5. Date this Report Submitted. 3/26/90.

Experimental Parasitology
(Submitted 1/25/90)

Trypanosoma brucei: Isolation of nuclei, characterization of chromatin subunit structure and evidence for the presence of histone H1-like protein

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Running title: *T. brucei:* Isolation of nuclei, chromatin structure and histone H1

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ABSTRACT

A rapid procedure for isolation of intact nuclei from *Trypanosoma brucei* subspecies *brucei* and *rhodesiense* is described. Cells were disrupted by Dounce homogenization followed by passage through a 26 guage needle in the absence of detergents, and the nuclei purified by centrifugation through sucrose-percoll density gradient. The yield of nuclei was greater than 50% of the starting material. Electron microscopy revealed that the purified nuclei retained their shape and ultrastructural integrity. Treatment of purified nuclei with micrococcal nuclease indicated that *T. brucei* chromatin is organized in a repeat subunit with mononucleosomes containing 180 bp of DNA in both *T. b. brucei* (bloodstream from) and *T. b. rhodesiense* (procyclic form); the kinetics of nuclease digestion, however, was different among the two trypanosome species and between the trypanosomes and the rat chromatin. Core-histones were found in both the life-cycle forms of the parasite, albeit with slightly different electrophoresis properties when compared to the mammalian histones. The data indicate molecular and biochemical integrity of the purified nuclei. Although a classical histone H1 was not found, fast migrating H1-like protein immunoreactive with chicken and protozoan *Tetrahymena* H1 antisera was present in *T. b. brucei* and *T. b. rhodesiense* species.

INDEX DESCRIPTIONS AND ABBREVIATIONS

Trypanosoma brucei; Nuclei; Chromatin subunit structure; Histone H1; High Mobility Group (HMG); Basal salts medium (BSM); Phosphate buffered saline (PBS); Phosphate buffered saline with 1% glucose (PBSGH); Polyethylene glycol (PEG); Perchloric acid (PCA); Phenylmethylsulfonyl fluoride (PMSF); Trichloroacetic acid (TCA); Ethylenediamine tetraacetic acid (EDTA).

INTRODUCTION

Protozoa of the family Trypanosomatidae are causative agents of a number of devastating diseases of man and animals; among these, African trypanosomiasis (sleeping sickness) caused by *Trypanosoma brucei* subspecies *brucei, rhodesiense,* and *gambiense,* is notable. The life cycle of these parasites is complex: a part of their life cycle is spent in insect vectors (procyclic forms) and the remainder in the mammalian host (bloodstream forms) (Englund et al. 1982). Interestingly, trypanosomatids present an intriguing and a complex system of eukaryotic cell differentiation.

Most studies of trypanosomes have concerned their life cycle patterns during pathogenesis, including studies of kinetoplast or mitochondrial metabolism, kDNA, and the surface antigens (reviewed in Donelson, 1988; Donelson and Rice-Ficht, 1985). Studies on the structural and molecular organization of the nucleus have been rather scanty (Donelson, 1988). The dramatic changes observed in cell morphology and expression of variant surface glycoproteins during the life cycle of the parasite must result from rearrangements and/or modifications in nuclear chromatin (genomic DNA). Although presence of a heterogeneous group of chromosomes is indicated by pulsed-field gradient gel electrophoresis (Gibson and Borst, 1986), strikingly, there is no chromatin condensation, and classical chromosomes have not been visualized in these protozoans. Amitotic division in trypanosomes occurs without nuclear disruption.

Analysis of chromatin structure of *T. cruzi* (Astolfi et al. 1980; Hecker and Gander, 1985; Rubio et al. 1980; Toro and Galanti, 1988) and *T. brucei* (Hecker and Gander, 1985; Shapiro and Doxsey, 1982) has revealed the presence of repeat subunit nucleosomic structure. Accordingly, core-histone like proteins have been shown to be present in these organisms (Hecker and Gander, 1985; Toro and Galanti, 1988). Unequivocal presence of histone H1, which has a critical role in chromatin condensation and supercoiling (McGhee and Felsenfeld, 1980), remains to be established.

Several procedures for isolating trypanosome nuclei, mostly from *T. cruzi,* have been published (Astolfi et al. 1980; Belnat et al. 1981; Hecker and Gander, 1985; Rubio et al. 1980; Toro and Galanti, 1988); most of these are unsatisfactory and involve use of detergents for disruption of cell membrane. Cellular fractionation of trypanosome organelles presents problems; in addition to the tough cell membrane, trypanosomes possess an extensive microtubular and microfilamentous cytoskeleton (Rubio et al, 1980; Vickerman and Preston, 1976). Moreover, in most procedures used, the integrity of isolated nuclei has been questionable and the yields low.

In the present paper, a simple and a rapid procedure for isolation of trypanosome nuclei in high yield is described. Using purified nuclei, we have examined chromatin structure and the

associated histone proteins of the bloodstream and procyclic forms of *T. brucei*, particularly, with reference to the presence of histone H1-like protein.

MATERIALS AND METHODS

Trypanosoma brucei strains and growth conditions. The studies reported here have been conducted with two subspecies representing the two life cycle forms of *Trypanosoma brucei: T. b. brucei* strain EATRC 110 (bloodstream form) and *T. b. rhodesiense* (procyclic form). The bloodstream form was obtained by injecting rats with 0.1 ml of the inoculum (10^8 parasites/ml) per 100 gm body weight. Blood was collected by heart puncture at a parasitemia of approximately 10^8 parasites/ml of blood, and the trypanosomes separated from blood cells by passage through a DEAE-cellulose column (Lanham and Godfrey, 1970) equilibrated in PBSG (phosphate buffered saline containing glucose: 3 mM sodium phosphate buffer, pH 8.0, 43.6 mM NaCl, 1% glucose) and 5 X 10^3 units heparin. The procyclic form was obtained by culturing cells in BSM (basal salts medium) as described previously (Njogu et al. 1980), harvested by low speed centrifugation and washed in PBSG. Both bloodstream and procyclic forms were processed similarly hereafter.

Isolation of nuclei. The bloodstream or the procyclic form trypanosomes were pelleted by centrifugation at 4,100 x g for 10 min in Sorval HB-4 rotor and washed once with PBSG. The parasites were lysed by several different means, including use of detergents, following which a number of appraoches for isolation of nuclei were tried (summarized in Table 1). The extent of cell lysis and nuclear integrity were monitored by phase-contrast microscopy. Based on reproducibility, integrity of isolated nuclei, yield and speed -- about two hours starting with the purified parasites, the following procedure was adopted. Briefly, the washed parasites were suspended in twenty volumes of cold lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM spermine, 1 mM spermidine, 0.5 mM PMSF, 7.0% polyethylene glycol) and kept on ice for 10 min. All procedures were performed at 4° C unless otherwise stated. The cells were lysed by homogenization with 50-100 strokes in a tight fitting Dounce homogenizer followed by two passages through a 26 guage needle under pressure. Cell lysis monitored by phase-contrast microscopy was greater than 90%. Unbroken cells, debris and aggregates were removed by centrifuging the lysate at 500 x g for 5 min. The supernatant containing most of the nuclei was placed on a discontinuous 0.75 M sucrose-35% percoll gradient (Shapiro and Doxsey, 1982) in lysis buffer in SW41 tubes and centrifuged at 60,000 x g for 30 min. The nuclei appeared as a loose pellet near the bottom of the tube and flagella and cellular membrane fragments formed two bands near the top (Fig. 1).

Preparation of nucleosomes. Purified nuclei were suspended in nuclease digestion buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$, 250 mM sucrose) and incubated with micrococcal nuclease (1.5 µg enzyme/500 µg DNA measured as A_{260} absorbance in 2 M NaCl-5 M urea) at 37° C as described previously (Bhorjee and Adler, 1982). Extent of nuclease digestion was

monitored as acid solubility of digested chromatin in 1 M NaCl-7% perchlorate solution. Nuclease reaction was terminated by chilling and addition of 1 mM EDTA. DNA was extracted from the enzyme treated nuclei by phenol-chloroform (Pederson and Bhorjee, 1979), and nucleosomes analyzed in 5% polyacrylamide gel electrophoresis in TBE buffer (50 mM Tris-HCl, pH 8.3, 50 mM boric acid, 1 mM EDTA) as described by Maniatis et al. (1982). The nucleosomes were stained with ethidium bromide and photographed with UV illumination using orange filter. Rat myoblast nuclei were processed similarly to serve as markers.

Extraction of acid soluble proteins. Purified nuclei or whole cells were extracted with 0.4 N H_2SO_4 for total histones, and with 5% PCA (perchloric acid) for PCA-soluble proteins which predominantly contain histone H1 and the high mobility group (HMG) proteins (Bhorjee, 1985; Nicolas and Goodwin, 1982). PCA extracts made acid by addition of 0.2 volumes of conc. HCl, and the H_2SO_4 extracts, were precipitated by addition of 5 volumes of cold acetone at -20° C overnight. The precipitates were collected by centrifugation at 20,000 x g for 30 min, and solubilized in Laemmli sample buffer for SDS-PAGE (Laemmli, 1970), and in acid-urea for acid-urea-PAGE (Panyim and Chalkley, 1969).

Polyacrylamide gel electrophoresis. Proteins were separated in 15% polyacrylamide-SDS (Laemmli, 1970) or acid-urea (Panyim and Chalkley, 1969) slab gels, stained with Coomassie Brilliant blue, destained and photographed as described previously (Bhorjee and Pederson, 1973; Bhorjee et al. 1983).

Immunoblot transfer of proteins to nitrocellulose. The SDS-PAGE and acid-urea PAGE separated proteins were transferred electrophoretically to nitrocellulose paper essentially by the procedure of Towbin et al. (1979). The efficiency of transfer was determined by staining parallel lanes with Amido Black. Alternatively, immunoblots were stained with Ponceau-S (0.4% solution in 8% TCA, 2% acetic acid) as previously described (Lin et al. 1989). Ponceau-S does not interfere with the immunoreaction; hence, the transferred proteins can be visualized directly on the nitrocellulose paper prior to reaction with the antibody. The antigens were visualized by the peroxidase-antiperoxiadse reaction. Briefly, blots were blocked with 3% bovine serum albumin in PBS (phosphate buffered saline), pH 7.2, containing 10% calf serum for 1 hr at 37° C. After 4 to 5 washes with PBS, blots were incubated with peroxidase-conjugated goat anti-rabbit IgG for 1 hr at room temperature and reacted with diaminobenzidine (3 mg/ml in 50 mM Tris-HCl, pH 7.5, 30% hydrogen peroxide) until brown color developed.

Antisera. The chicken antiserum containing histone H1 and the high mobility group (HMG) proteins was prepared as follows and was a gift from Judy Briggs. Briefly, chicken erythrocyte nuclei were extracted with 0.35 M NaCl and the salt extract treated with 2% TCA and centrifuged at 20,000 x g for 30 min in Sorvall SS-34 rotor. The supernatant which

contained histone H1 and the HMG proteins (Nicolas and Goodwin, 1982) was used as the antigen to inject rabbits. Histone H5, a major histone H1°-like chicken histone, is extracted sparingly, or not at all with 0.35 M NaCl (Isackson et al. 1980); hence, it was not detectable in the antigen used. The composition of the cognate antigens is evident from our results. The antiserum to *Tetrahymena* macronuclei histone H1 has been described previously (Chicoine et al. 1985; Schulman et al. 1987).

Chemical determinations. Protein content was determined by the Lowry procedure (Lowry et al. 1951) using bovine serum albumin as standard. DNA and RNA were determined by the diphenylamine and orcinol reactions, respectively, as described (Mellon and Bhorjee, 1982).

Materials. Reagents for *T. b. rhodesiense* culture, and other chemicals and reagents, including polyethylene glycol (mol. wt. 3,500), were from Sigma Chemical Co. Fetal bovine serum was from GIBCO (Grand Island, NY). Micrococcal nuclease was from Worthington. Organic solvents were from Baker Chemical Co. Ultrapure sucrose was from Schwartz Mann, and reagents for electrophoresis and hexylene glycol were from Eastman Kodak Co.

RESULTS

In this study we have used two closely related subspecies *T. b. brucei* and *T. b. rhodesiense*, bloodstream and procyclic forms, respectively, of *T. brucei*. Although these subspecies differ in their host range, biochemically and morphologically they are virtually identical (reviewed in Englund et al. 1982).

Purification of nuclei. A variety of hypotonic buffers were tried to lyse the parasites (see Table 1), and in most cases cell lysis was inefficient, being below 50%. Addition of non-ionic detergents (lubrol, NP40) although very effective in cell lysis, resulted, to varying degrees, in disruption of the nuclear envelope causing aggregation. For these and other obvious considerations, for example, enzyme and protein denaturation and selective loss of nuclear proteins (Smuckler et al. 1976), the use of detergents was avoided. Based on the observations listed in Table 1, including microscopic evaluations, the protocol selected (Table 1, protocol 5; Fig. 1) gave nuclei which retained their shape and molecular integrity as defined by maintenance of the chromatin subunit structure (shown later). The procedure outlined in Fig.1 avoids detergents, requires polyethylene glycol as a nuclei stabilizing agent, and uses a sucrose-percoll gradient for purification of nuclei. Phase-microscopy (not shown) of various fractions of the sucrose-percoll gradient revealed the presence of nuclei as a loose pellet near the bottom of the tube, while most flagellar and cellular membrane fragments were in the two bands near the top of the gradient (Fig. 1). Most other cell organelles including mitochondria were in the solution between the nuclear pellet and the two bands at the top. Fluorescense microscopy (not shown) of ethidium bromide-acridine orange stained preparations of purified nuclei indicated that nuclei were virtually free of cytoplasmic tags. Mitochondrial contamination judged by the small size of mitochondria relative to the size of nuclei in the fluorescense slides, was determined to be 5-10% of the nuclei present.

The electron micrograph (Fig. 2) of a typical preparation of purified nuclei from *T. b. brucei* (bloodstream form) revealed that the nuclei retained their shape and the ultrastructural integrity as indicated by preservation of the chromatin material. Contamination by cytoplasmic fragments and microtubular structures was minimal. However, in most nuclei the nuclear membranes were partially damaged. Similar observations were noted for the *T. b. rhodesiense* (procyclic form) purified nuclei (not shown).

The yield of nuclei was 50-60% of the starting parasites. The purity of nuclei was also determined by examining their protein/RNA/DNA contents. Protein : RNA : DNA ratios for the *T. brucei* bloodstream and procyclic forms were 5.90 : 0.25 : 1.00 and 5.54 : 0.22 : 1.00, respectively. The values indicated are averages of two separate experiments. These values of protein : DNA : RNA are in agreement with the values reported for several eukaryotic tissues and cells (Pederson, 1976), and indicate a high degree of chemical purity of trypanosome nuclei

preparations.

Chromatin structure: Nuclease digestion. Figure 3 shows the chromatin subunit structure of the bloodstream and the procyclic forms of *T. brucei*. Both procyclic (lanes 2 and 3) and the bloodstream (lanes 4 and 5) forms revealed typical nucleosomic subunit arrangement; the average mononucleosomic DNA size is approximately 180 bp in both forms. However, the nuclease sensitivities of the procyclic and bloodstream form chromatins were different. For example, under identical conditions of nuclease treatment, more of the oligonucleosomes in the bloodstream form (lane 5) were converted to the 180 bp mononucleosomes compared to their relative conversion to mononucleosomes in the procyclic form (lane 3). In turn, both the trypanosome chromatins were more nuclease sensitive than the rat chromatin (compare lanes 3, 5 and 7), indicating, perhaps the differences in compaction pattern of the chromatins in the trypanosome species and between the trypanosome and the mammalian chromatins (Hecker and Gander, 1985).

Analysis of histones. Figure 4 shows the electrophoresis distribution in acid-urea polyacrylamide gels of sulfuric acid extracted proteins from purified *T. b. brucei* and *T. b. rhodesiense* nuclei. Proteins comigrating in the region of mammalian core-histones can be seen in acid extracts from the bloodstream (lanes 2 and 3) and procyclic (lane 4) forms; however, their migration positions comapred to HeLa core-histones (lane 1) are different. Even though many more proteins were acid soluble in the bloodstream whole cells (lane 2) compared to the purified nuclei (lane 3), the electrophoretic distribution of the histones from whole cells was similar to the core-histones from purified nuclei. It is also apparent that in addition to the histones, several other major proteins are acid soluble in the trypanosomes, in particular, in the bloodstream form of the parasite. Histone H1-like protein migrating in the region of mammalian H1 seems to be absent in the trypanosome chromatins (lanes 2, 3 and 4). Although some protein bands in the bloodstream form (lane 2) seem to be comigrating with HeLa histone H1, as is shown later, these are not H1. Presence of the core-histone like proteins in both procyclic (Fig. 5, lane 2) and bloodstream forms (Fig. 5, lane 3) was also confirmed by electrophoresis in SDS-PAGE; again no histone H1-like protein comigrating with HeLa H1 (lane 4) was observed.

Immunoidentification of histone H1-like protein in T. brucei. Figure 6 shows an immunoblot of acid extracted proteins from procyclic and bloodstream forms of *T. brucei* separated in acid-urea-PAGE and reacted with chicken (panel A), and *Tetrahymena* H1 (panel B) antisera. For description of the antisera used see Materials and Methods. A fast migrating protein (unlabeled arrow, panel A) immunoreactive with chicken H1 is present in both procyclic (lane 1) and bloodstream (lanes 2 and 3) forms of *T. brucei*. HeLa 5% PCA extract (lane 5), containing H1 and HMG proteins (Bhorjee, 1985) run as marker, indicates

authenticity of the chicken antiserum; as was expected, it cross-reacted with histone H1 and the HMG proteins (bands above and below H1). Using both Coomassie staining and immunoreaction, we were not able to identify HMG-like proteins in the 5% PCA extracts of either the bloodstream or the procyclic forms of *T. brucei* (not shown). Similarly, *Tetrahymena* H1 antiserum reacted with a fast migrating protein (unlabeled arrow, panel B) in the trypanosome bloodstream (lane 6) and procyclic (lane 7) forms. *Tetrahymena* macronuclei (lane 1) and HeLa (lanes 3 and 4) acid soluble proteins run as markers indicate authenticity of the *Tetrahymena* H1 antiserum. Faster migration of *Tetrahymena* macronuclei H1 compared to mammalian H1 (see lanes 1 and 3 in panel B) in acid-urea-PAGE is in agreement with the electrophoresis migrating properties in acid gels of *Tetrahymena* macronuclei H1 reported previously (Gorovsky et al. 1974). The immunoblot in panel B electrophoresed under conditions similar to the immunoblot in panel A shows that the chicken H1 and *Tetrahymena* H1 cross-reactive H1-like proteins in trypanosomes migrate at the same position in the gel (see unlabeled arrows in panels A and B); hence, both chicken and *Tetrahymena* antisera recognize the same HI-like protein in trypanosomes. Preimmune sera from the rabbits used for eliciting antibodies to both chicken and *Tetrahymena* antigens gave no reaction with the proteins on the immunoblots (not shown). The presence of additional immunoreactive polypeptides seen in some of the preparations is possibly due to non-specific cross-reaction.

Figure 7 shows the immunoblots made from SDS-PAGE separated trypanosome acid soluble proteins. A low molecular weight protein (unlabeled arrow) migrating in the region of core-histones from bloodstream whole cells and nuclei (lanes 3 and 4, respectively, panel A) and procyclic nuclei (lanes 6 and 7, panel A) cross-reacted with the chicken antiserum. Similarly, a low molecular weight protein (unlabeled arrow) in the bloodstream form nuclei (lane 1, panel B) cross-reacted with the *Tetrahymena* H1 antiserum. HeLa H1 (lane 1, panel A; and lane 2, panel B) and *Tetrahymena* H1 (lane 3, panel B) run as markers, almost comigrate (panel B) in the SDS-PAGE system used and serve as controls for authenticity of the chicken and *Tetrahymena* H1 antisera. *Tetrahymena* macronuclei H1 migrates slightly slower than or with calf thymus H1 in pH 7.6 SDS-PAGE system; it has several characteristics, including size, similar to mammalian H1 (Gorovsky et al. 1974). The major fused bands cross-reacting with the chicken antiserum immediately below H1 in the HeLa sample (lane 1, panel A) are HMGs-1 and -2, while other immunoreactive bands are probably non-specific cross-reacting polypeptides. Preimmune sera from chicken and *Tetrahymena* antigen injected rabbits gave no reaction in the immunoblots (not shown). As was in the case of acid-urea-PAGE (Fig. 6), both chicken and *Tetrahymena* antisera reacted with a H1-like protein from the bloodstream and procyclic forms migrating in the same position in SDS-PAGE immunoblots (Fig. 7).

DISCUSSION

Fractionation and purification of intact nuclei and other cellular organelles from trypanosomatids have yet to be satisfactorily achieved. Some of the problems in obtaining clean intact nuclei including low yield and electrophoretic variability in the acid soluble proteins have been reviewed (Rizzo, 1985). The presence of extensive cytoskeleton of microtubules and microfilaments present around the surface coat of trypanosomes (Vickerman and Preston, 1976) makes it difficult to break the cells. Therefore, most published procedures (see Astolfi et al. 1980; Belnat et al. 1981; Rubio et al. 1980) for isolation of nuclei from trypanosomes have resorted to use of detergents for disruption of cell membrane. In almost all cases, including this study, where detergents have been used, the nuclear envelope is disrupted and the nuclei appear fragmented. For these reasons, we have developed a procedure for isolation of nuclei from *T. brucei* strains which avoids the use of detergents, is reproducible and rapid (approximately one and one-half to two hours starting with parasites), and which gives relatively intact and pure nuclei. The yield of purified nuclei was consistently 50% or better. Although there was partial disruption of nuclear membranes (Fig. 2), the nuclei retained their shape and their molecular and biochemical integrity as evidenced by maintenance of the chromatin subunit structure and the presence of similar total histones in nuclei and in whole cells. We note that inspite of trying several procedures for cell disruption and purification of "wholly" intact nuclei, based solely on intactness of the nuclear membranes, we have not fully succeeded. The inability to obtain trypanosome nuclei with "wholly" intact membranes has also been observed previously (see Figs. 1 in Hecker and Gander, 1985, and Shapiro and Doxsey, 1982). Important considerations and requirements in the procedure employed are: (1) use of EDTA in cell lysis buffer and passage through 26 guage needle for efficient lysis; (2) use of polyethylene glycol for stabilization of nuclei; and (3) use of the isopycnic banding properties of percoll in sucrose density gradients for efficient separation of nuclei from cell debris and other cell organelles.

Although our procedure of nuclei isolation is based, in principle, on the procedure described by Shapiro and Doxsey (1982) for isolation of nuclei from *T. brucei,* it differs in two important respects: One, we lyse cells by Dounce homogenization in hypotonic solutions followed by passage through a 26 guage needle as opposed to cell disruption in nitrogen cavitation bomb, which, in most laboratories, is not easily available, and, two, substitution of hexylene glycol with polyethylene glycol with no loss in nuclear stability; polyethylene glycol is more cost effective. The addition of glycol to nuclei isolating solutions influences hydrophobic interactions (Kane, 1965) imparting stability to nuclear membranes (Wray et al. 1977). In addition, our yield of 50% or more of purified nuclei is significantly better than ~35% yield obtained by the nitrogen cavitation bomb cell lysis procedure (Shapiro and Doxsey, 1982).

Recently, Toro and Galanti (1988) have indicated obtaining pure nuclei in good yield from *T. cruzi* by a procedure based on isolation of mammalian cell nuclei. However, it is difficult to compare the nuclear integrity of their preparations with ours since no microscopic evidence was presented to demonstrate purity and integrity of their purified nuclei. We note that published procedures for isolation of mammalian nuclei (Bhorjee and Pederson, 1973; Mellon and Bhorjee, 1982; Smuckler et al. 1976), including the one used by Toro and Galanti, were unsatisfactory for purifying nuclei from *T. brucei* (see Table 1). In agreement with the observations reported previously (Rubio et al. 1980), we find that trypanosome nuclei are very fragile; they disintegrate and form aggregates easily when centrifuged through sucrose (0.8 - 2.0 M), or even when just pelleted at forces around 1,000 x g for 10-15 min in hypotonic or isotonic buffers, steps routinely followed in mammalian nuclei isolation procedures. As is used in this study, and in agreement with Shapiro and Doxsey (1982), the presence of percoll in the sucrose-percoll gradients was found to be essential for circumventing the shearing effects of sucrose on the nuclear membranes.

Basic nucleosomic subunit is a fundamental repeat structure in all eukaryotic chromatins examined (reviewed in McGhee and Felsenfeld, 1980); the nucleosomic DNA repeat length, however, varies between 180 to 212 bp from lower to higher eukaryotes. The repeat subunit structure is formed by association of two molecules each of core histones, H2A, H2B, H3 and H4 with the nucleosomic DNA. Histone H1, which is not a component of the core particle, is associated with the internucleosomal linker-DNA and has a key role in compaction and condensation of chromatin (Thoma et al. 1979). The 180 bp DNA repeat length reported here for *T. brucei* bloodstream and procyclic form chromatins is in agreement with the DNA repeat length of 185-212 bp reported for *T. cruzi* chromatin subunits (Astolfi et al. 1980; Belnat et al. 1981; Rubio et al. 1980). The kinetics of nuclease digestion, however, is different in the chromatins from the two *T. brucei* life cycle forms, for example, the bloodstream form chromatin was more nuclease sensitive than the procyclic form chromatin, both of which in turn were more sensitive than the mammalian chromatin. It is noteworthy that *T. cruzi* (Rubio et al. 1980) and *Tetrahymena pyriformis* (Frado et al. 1977) chromatins have been reported to be more nuclease sensitive than vertebrate chromatins. Whether increased nuclease sensitivity of chromatin in one life cycle form as opposed to the other is due to relative differences between the extents of chromatin transcriptional activity, or their compaction states, cannot be answered from the data presented; it remains an interesting question. It is noteworthy that in mixing reconstitution experiments differences in compaction patterns of chromatin from *T. brucei* and *T. cruzi*, and between trypanosomes and rat liver chromatin, have been reported (Hecker and Gander, 1985).

Our SDS-PAGE and acid-urea-PAGE data indicate the presence of core-histone-like proteins in both bloodstream and procyclic forms of *T. brucei*. The electrophoresis patterns, however, are different in the two life cycle forms and between the trypanosome and the mammalian core-histones. These observed differences in electrophoretic mobilities of the core-histones are in agreement with similar differences reported between *T. cruzi* and vertebrate histones (Astolfi et al. 1980; Rubio et al. 1980), and between *T. cruzi* and *T. brucei* histones (Hecker and Gander, 1985).

The lack of a histone H1-like protein has been used as an argument for the decondensed state of chromatin during the amitotic division in trypanosomes. Using both the chicken and the protozoan *Tetrahymena* antisera, we have presented evidence of a fast migrating histone H1-like protein in both procyclic and bloodstream forms of *T. brucei*. H1-like protein is not visible in Coomassie stained gels (Figs. 4 and 5); it probably is masked by the core-histones since it migrates in that region of the gel. The possibility that the fast migrating H1-like protein may be a proteolytic degradation product seems unlikely. We have used PMSF, a serine-protease inhibitor, in our isolating solutions. It is noteworthy that in the presence or absence of PMSF and/or pepstatin A, two protease inhibitors, no differences were found in electrophoretic distribution of *T. brucei* and *T. cruzi* histones (Hecker and Gander, 1985). Atypical histone H1-like fast migrating protein(s) in the area of core-histones has also been reported from *Tetrahymena* micronucleus (Allis et al. 1979), *Oxytrichia* (Caplan, 1975), and *Crithidia* (Leaver and Ramponi, 1971). A recent report (Toro and Galanti, 1988) has described the presence of all the five histones in *T. cruzi*. Using sea-urchin H1 antiserum these authors have presented evidence for two H1-like proteins in *T. cruzi*, one comigrating with sea-urchin H1, and the other a fast migrating protein. Toro and Galanti's is the only report to show electrophoretic identity of *T. cruzi* histones with classical histones. We and others (Astolfi et al. 1980; Rubio et al. 1980), do not find a protein in trypanosomes which comigrates with classical H1 in SDS-PAGE or acid-urea-PAGE. The reason(s) why others and we do not find a classical histone H1 in trypanosomes is not clear. However, our results of a fast migrating antigenically similar H1-like protein in bloodstream and procyclic forms of *T. brucei* is in agreement with the observations of a fast migrating H1-like protein in *T. cruzi* reported by Toro and Galanti (1988) and suggested by others (Astolfi et al. 1980; Rubio et al. 1980). While no evidence is presented, the appearance of fused H1-like protein bands in the bloodstream form (Fig. 7, lanes 3 and 4) as opposed to a single band in the procyclic form (Fig. 7, lanes 6 and 7), could be due to the presence of H1 subtypes or the metabolically modified H1s in the bloodstream form of *T. brucei*. If true, this could effect the nature of chromatin compaction in bloodstream form of the parasite and, in part, explain the observed difference in the nuclease sensitivity between the bloodstream and the procyclic chromatins. The lysine rich

histone H1 which is implicated in higher order structural organization of chromatin is known to exhibit the most variability and specificity among the histone proteins (Isenberg, 1979).

In conclusion, we have described a rapid and a reproducible procedure for isolation of intact nuclei in good yield from two subspecies of *T. brucei.* The purified nuclei retain their shape and biochemical and molecular integrity. We have presented evidence for the existence of a fast migrating histone H1-like protein in both bloodstream and procyclic life cycle forms of *T. brucei,* albeit with different physical and chemical properties compared to the classical eukaryotic H1. It is this difference in the physical-chemical properties of *T. brucei* histone H1-like protein which may explain its inability to play a role in generating higher order organization of chromatin, or, alternatively, suggest a function different than in chromatin compaction. The presence of a H1-like histone in *T. brucei* suggests intriguing possibilities in life cycle related structure-function differences in chromatin of these parasites; these differences need characterization.

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FIGURE LEGENDS

FIGURE 1. Procedure for isolation of nuclei from *Trypanosoma brucei brucei* (bloodstream form) and *Trypanosoma brucei rhodesiense* (procyclic form).

FIGURE 2. Electron micrograph of *T. b. brucei* purified nuclei. Nuclear fractions were fixed in gluteraldehyde and osmium tetroxide and processed for electron microscopy as described previously (Mellon and Bhorjee, 1982). A representative field of nuclei. Note the nuclear double membrane is mostly intact. Magnification x 40,000.

FIGURE 3. Nucleosomic organization of *T. b. brucei* (bloodstream form) and *T. b. rhodesiense* (procyclic form) chromatins. Purified DNA samples from micrococcal nuclease treated nuclei were electrophoresed in 5% polyacrylamide gel slab and the nucleosomes visualized by ethidium bromide staining. Lane 1, 123 bp marker ladder; lanes 2 and 3, - 5 and 10 min nuclease treated procyclic nuclei, respectively; lanes 4 and 5, - 5 and 10 min nuclease treated bloodstream nuclei samples, respectively; lanes 6,7 and 8, - 5, 10 and 20 min nuclease treated rat L8 myoblast nuclei, respectively. Same amount of purified DNA as A_{260} units was loaded in lanes 2 - 8.

FIGURE 4. Acid-urea-polyacrylamide gel electrophoresis of histones. Acid extracted proteins from *T. b. brucei* (bloodstream form) and *T. b. rhodesiense* (procyclic form) were electrophoresed in 15% polyacrylamide gel slab in acid-urea and stained with Coomassie Brilliant blue. Lane 1, HeLa histones run as markers; lane 2, acid soluble proteins from bloodstream trypanosome whole cells; lane 3, acid soluble proteins form bloodstream trypanosome nuclei; lane 4, acid soluble proteins from procyclic trypanosome nuclei. 30 μg protein loaded in each lane.

FIGURE 5. SDS-polyacrylamide gel electrophoresis of histones. Acid extracted proteins from *T. b. brucei* (bloodstream form) and *T. b. rhodesiense* (procyclic form) were electrophoresed in 15% polyacrylamide-SDS gel slab and stained with Coomassie Brilliant blue. Lane 1, molecular weight markers (Bio-Rad); lane 2, acid soluble proteins from procyclic form trypanosome nuclei; lane 3, acid soluble proteins from bloodstream form trypanosome nuclei; lane 4, HeLa histones run as markers. 30 μg protein loaded in lanes 2 - 4.

FIGURE 6. Immunoblot analysis of histone H1-like protein in acid-urea-PAGE separated *T. b. brucei* (bloodstream form) and *T. b. rhodesiense* (procyclic form) acid soluble proteins. Acid

soluble proteins fom bloodstream and procyclic trypanosome forms were separated by electrophoresis in 15% polyacrylamide acid-urea gels as described in Fig. 4, electrophoretically transferred to nitrocellulose sheets and reacted with antisera described in Materials and Methods. *A.* Reaction with chicken antiserum: Lane 1, acid soluble proteins from procyclic form trypanosome nuclei; lane 2, acid soluble proteins from bloodstream trypanosome whole cells; lane 3, acid soluble proteins from bloodstream trypanosome nuclei; lane 4, empty; lane 5, HeLa 5% PCA extract containing histone H1 and the HMG proteins (Bhorjee, 1985) run as marker. *B.* Reaction with *Tetrahymena* macronuclei H1 antiserum: Lane 1, *Tetrahymena* macronuclei PCA-soluble extract containing histone H1 and HMG-like proteins (Schulman et al. 1987) run as marker; lane 2, empty; lanes 3 and 4, HeLa sulphuric acid and 5% PCA extracts, respectively, run as marker; lane 5, empty; lane 6, acid soluble proteins from bloodstream trypanosome nuclei; lane 7, acid soluble proteins form procyclic trypanosome nuclei. 30 µg protein loaded in each lane. Note: In both panels, H1 indicates migration position of HeLa histone H1. Unlabeled arrows indicate H1-like cross-reacting trypanosome protein.

FIGURE 7. Immunoblot analysis of histone H1-like protein in SDS-PAGE separated proteins from *T. b. brucei* (bloodstream form) and *T. b. rhodesiense* (procyclic form). Acid soluble trypanosome proteins were separated in 15% polyacrylamide-SDS gels as described in Fig. 5, electrophoretically transferred to nitrocellulose sheets, and reacted with chicken and *Tetrahymena* antisera described in Materials and Methods. *A.* Reaction with chicken antiserum: Lane 1, HeLa 5% PCA-soluble proteins containing H1 and the HMG proteins run as marker; lane 2, empty; lane 3, acid soluble proteins from bloodstream form trypanosome whole cells; lane 4, acid soluble proteins from bloodstream form trypanosome nuclei; lane 5, empty; lanes 6 and 7, two separate preparations of acid soluble proteins from procyclic trypanosome nuclei. *B.* Reaction with *Tetrahymena* macronuclei H1 antiserum: Lane 1, acid soluble proteins from bloodstream form trypanosome nuclei; lane 2, HeLa 5% PCA-soluble extract (see Fig. 6) run as marker; lane 3, *Tetrahymena* macronuclei PCA-soluble proteins (see Fig. 6) run as marker. 30 µg protein loaded in each lane. Note: In both panels, H1 indicates migration position of HeLa H1. Unlabeled arrows indicate trypanosome H1-like immunoreactive protein.

Table 1. METHODS TRIED FOR CELL LYSIS AND ISOLATION OF NUCLEI FROM THE BLOODSTREAM AND PROCYCLIC FORMS OF TRYPANOSOMA BRUCEI

Homogenization Buffer	Purification Procedure	Observations	Reference
1. 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 3 mM $CaCl_2$, 10 mM KCl, with or without 1.5 mM $MgCl_2$.	1. Lysed cells by Dounce homogenization (50 to 100 strokes) in homogenization buffer. 2. Nuclei pelleted at 1,000 x g, 10 minutes, followed by 3 washes with homogenization buffer. 3. Nuclear pellet centrifuged through 2.0 M sucrose using SW27•1 rotor, 130,000 x g, 90 minutes.	1. Lysis poor (<50%); nuclear pellet contaminated with flagella and cell debris causing extensive aggregation. 2. Centrifugation through 2.0 M sucrose lysed nuclei with extrusion of chromatin material; no pellet formation. 3. Procedure is ineffective for isolating trypanosome nuclei.	1. Procedure based on purification of mammalian nuclei (see Bhorjee and Pederson, 1973; Mellon and Bhorjee, 1982). In principle, also followed by Toro and Galanti (1988) for *T. cruzi* chromatin.
2. 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 5 mM KCl, 3 mM $CaCl_2$, 1 mM spermidine, 1.0% lubrol.	1. Lysed cells by Dounce homogenization (50 strokes) in homogenization buffer containing 250 mM sucrose. 2. Lysate centrifuged 600 x g to pellet unbroken cells and cell debris. 3. Lysate diluted with homogen zation buffer without lubrol. Centrifuged in SW41 rotor at 160,000 x g on a cushion of 2.0 M sucrose.	1. Lysis improved considerably in the presence of lubrol. 2. Electron microscopy (not shown) revealed extensive disruption of both outer and inner nuclear membranes with extrusion of chromatin. 3. Yield was 15% or less.	1. Procedure described by Astolfi et al. (1980) for isolation of *T. cruzi* nuclei. In principle, also followed by Belnat et al. (1981) for *T. cruzi*.

Homogenization Buffer	Purification Procedure	Observations	Reference
3. 1 mM PIPES (pH 7.4), 1 mM $CaCl_2$, 0.5 M hexylene glycol or polyethylene glycol (PEG), with or without lubrol.	1. Lysed cells by Dounce homogenization (50 strokes) in homogenization buffer. 2. Lysate centrifuged in SW41 rotor at 60,000 x g for 30 minutes in sucrose (0.75 M)-percoll (35%) gradient.	1. Dounce homogenization greatly aided cell lysis. Addition of lubrol (0.5%) further improved cell lysis. 2. Electron microscopy (not shown) revealed mostly broken nuclei with complete disruption of nuclear membrane and extrusion of chromatin as in (2).	1. Procedure described by Shapiro and Doxsey (1982) for purification of *T. brucei* nuclei. We, however, used Dounce homogenization instead of a nitrogen cavitation bomb for cell lysis.
4. 10 mM Tris-HCl (pH 7.6), 5 mM KCl, 3 mM $CaCl_2$, 1 mM EDTA, 3 mM $MgCl_2$, 7.0% PEG, 1 mM spermidine, 1 mM spermine, 0.5 mM PMSF, with or without detergents.	1. Lysed cells in homogenization buffer for 10 minutes on ice. 2. Dounce homogenization under following conditions: without detergent, 100 strokes; in the presence of 0.1% - 0.5% lubrol, NP40, or SDS, 50 strokes. 3. Centrifuged in SW41 rotor, 60,000 x g for 30 minutes on sucrose (0.75 M)-percoll (35%) gradient.	1. Cell lysis poor (<20%) in the absence of Dounce homogenization. 2. Addition of lubrol or NP40 greatly improved cell lysis; almost complete lysis obtained with 0.5% NP40. SDS disrupted nuclei with release of chromatin resulting in a viscous lysate. 3. Electron microscopy (not shown) of NP40 treated samples showed extensive nuclear membrane disruption with extrusion of chromatin as in (2).	1. Use of sucrose-percoll gradient based on observations by Shapiro and Doxsey (1982).

Homogenization Buffer	Purification Procedure	Observations	Reference
5. Homogenization buffer as in (4), no detergents.	1. Lysed cells by Dounce homogenization (80-100 strokes), followed by passing the lysate through 26G needle under pressure. 2. Centrifuged the lysate in sucrose (0.75 M)-percoll (35%) gradient at 60,000 x g for 30 minutes in SW41 rotor.	1. Passing the lysate through the needle resulted in complete lysis with intact nuclei as viewed by phase-contrast microscopy. 2. Presence of EDTA was important for good cell membrane disruption. 3. Electron microscopy (Fig. 2) revealed minimal contamination by flagella and cell debris. In approximately half the nuclei, nuclear membranes were partially disrupted. 4. Yield was 50-60%.	1. This report. Use of sucrose-percoll gradient based on observations by Shapiro and Doxsey (1982).

Fig. 1

PROTOCOL FOR NUCLEI PREPARATION FROM TRYPANOSOMES

BLOOD STREAM/PROCYCLIC FORMS

LYSE IN HYPOTONIC TRIS-HCL BUFFER pH 7.6
IN THE PRESENCE OF PEG, 10 MIN ON ICE

DISRUPT CELL MEMBRANE BY DOUNCE HOMOGENIZATION
(~100 STROKES)

PASS THROUGH 26G NEEDLE UNDER PRESSURE
(4-5 TIMES)

CENTRIFUGE AT 500 X g FOR 10 MIN. AT 4°C

PELLET
(MOSTLY CELL MEMBRANE FRAGMENTS & FLAGELLA)

SUPERNATANT
(MOSTLY NUCL & MIT)

SUSPEND IN LYSIS BUFFER

LAYER 2ML ALIQUOTS OVER 0.75M SUCROSE-35% PERCOLL GRADIENT IN SW 41 TUBES

CENTRIFUGE, 60,000 x g, 30 MIN

TWO BANDS
(MOSTLY FLAGELLA & CELL DEBRIS)

LOOSE PELLET
(ENRICHED IN NUCLEI)

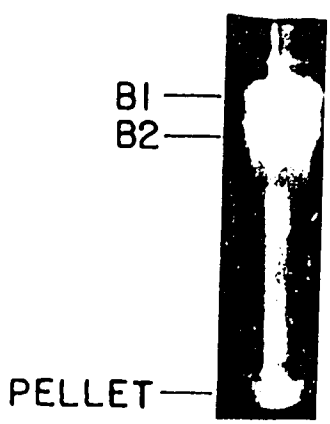

FIG. 2

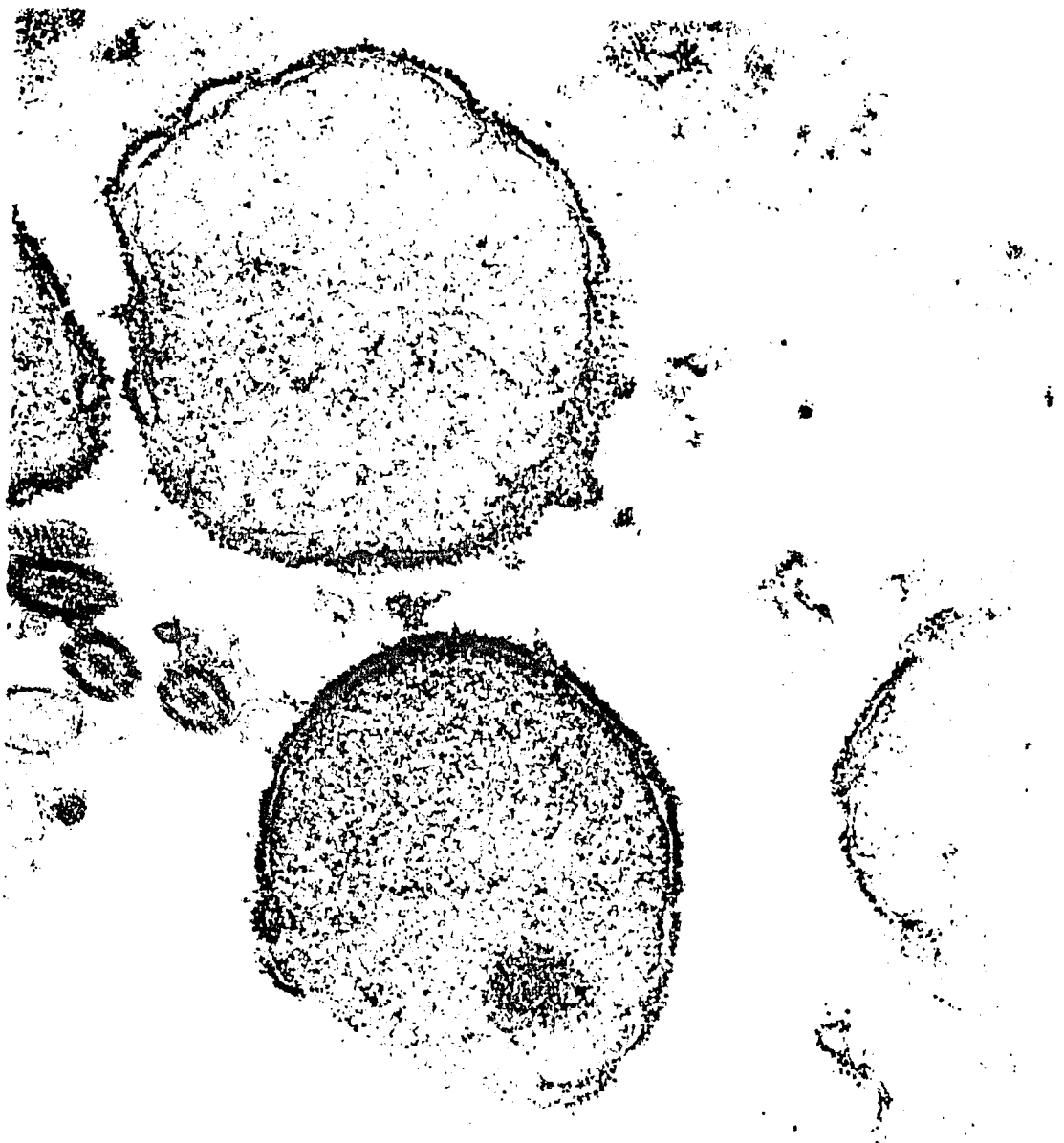

FIG. 3

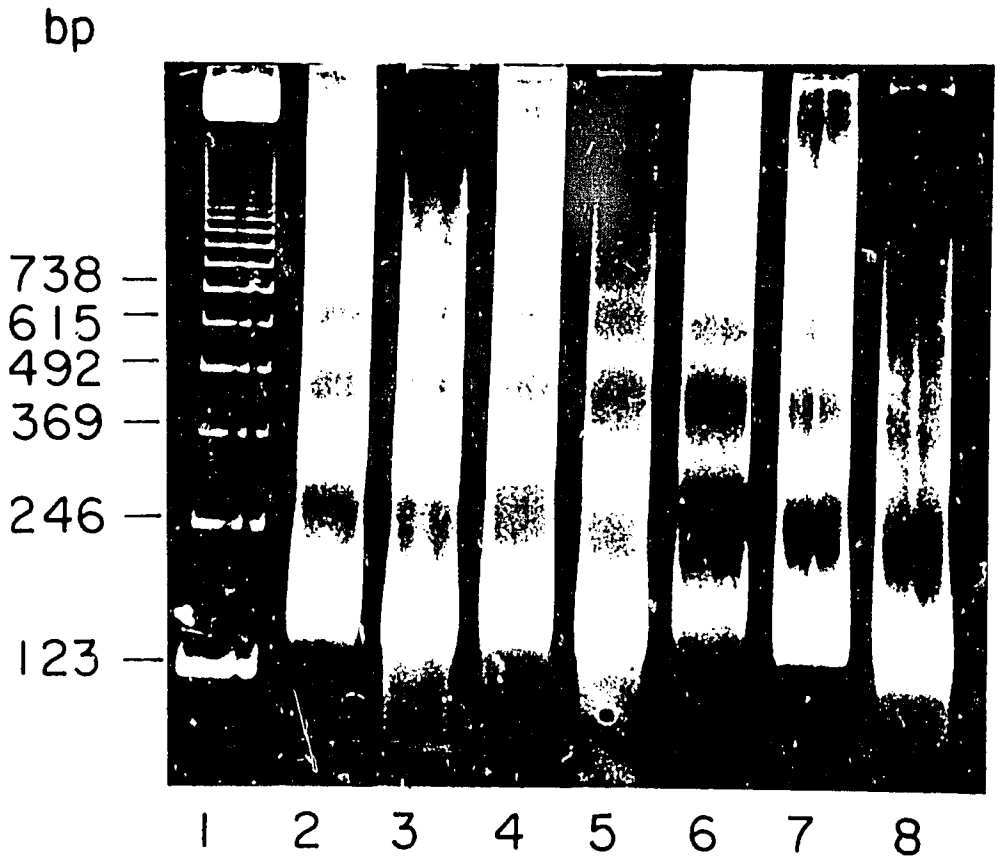

FIG. 4

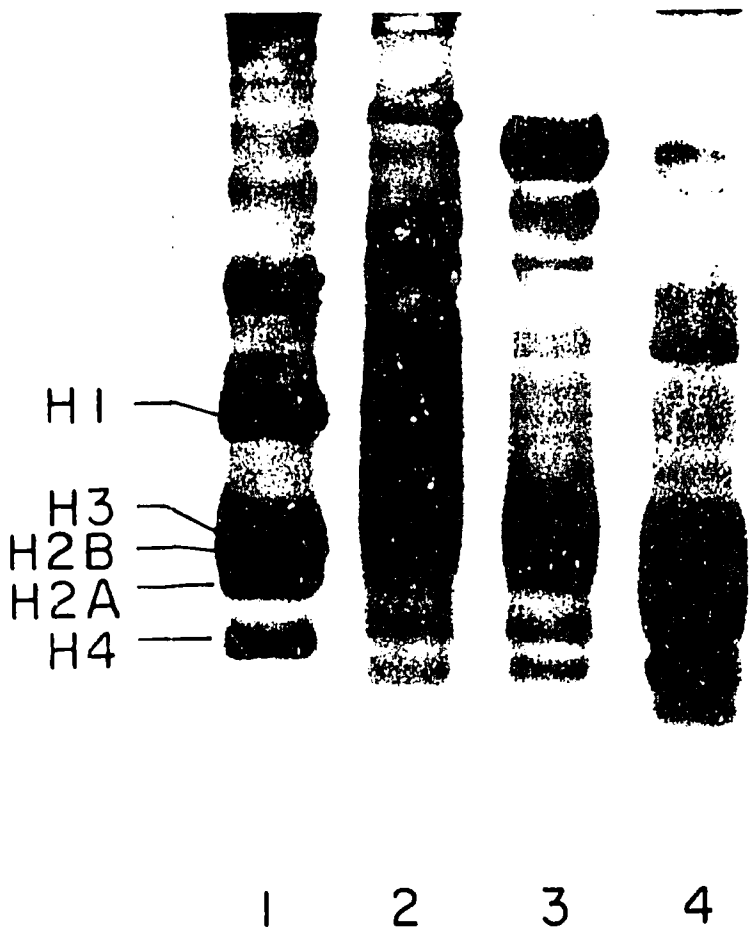

FIG. 5

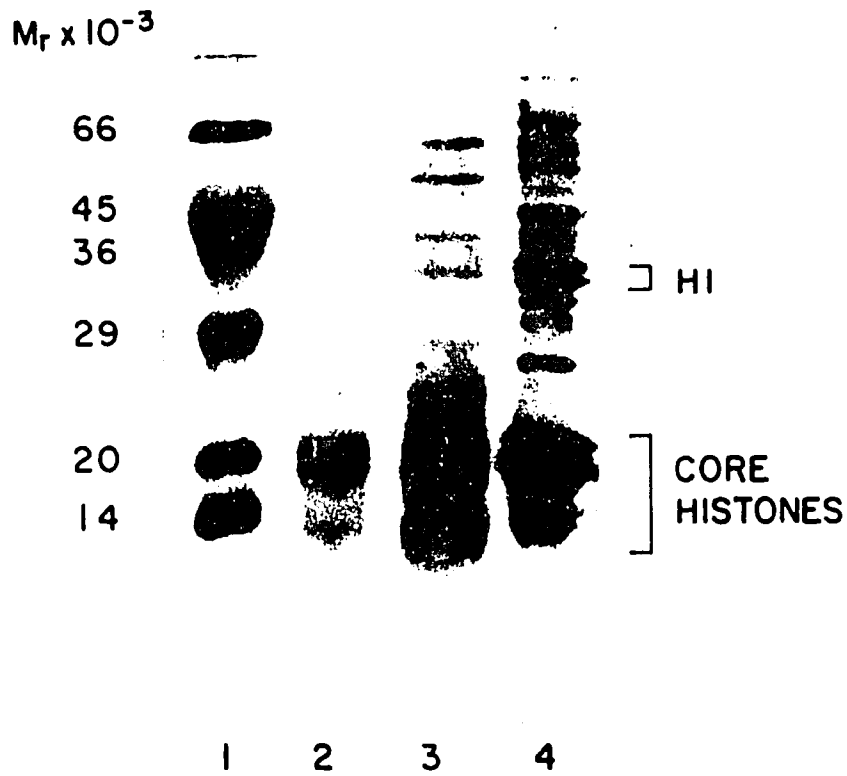

FIG. 6

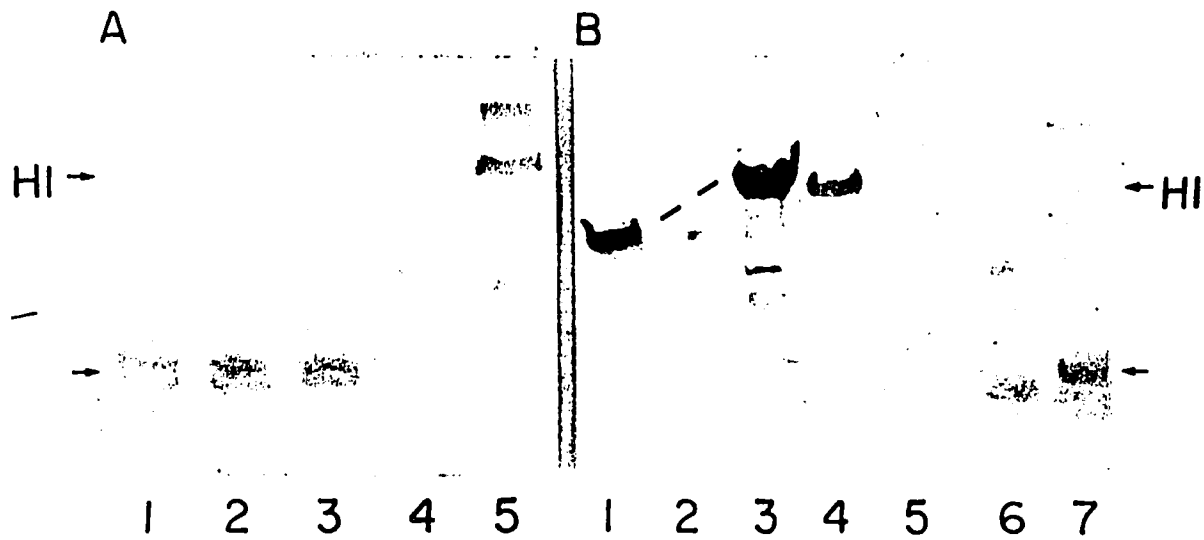
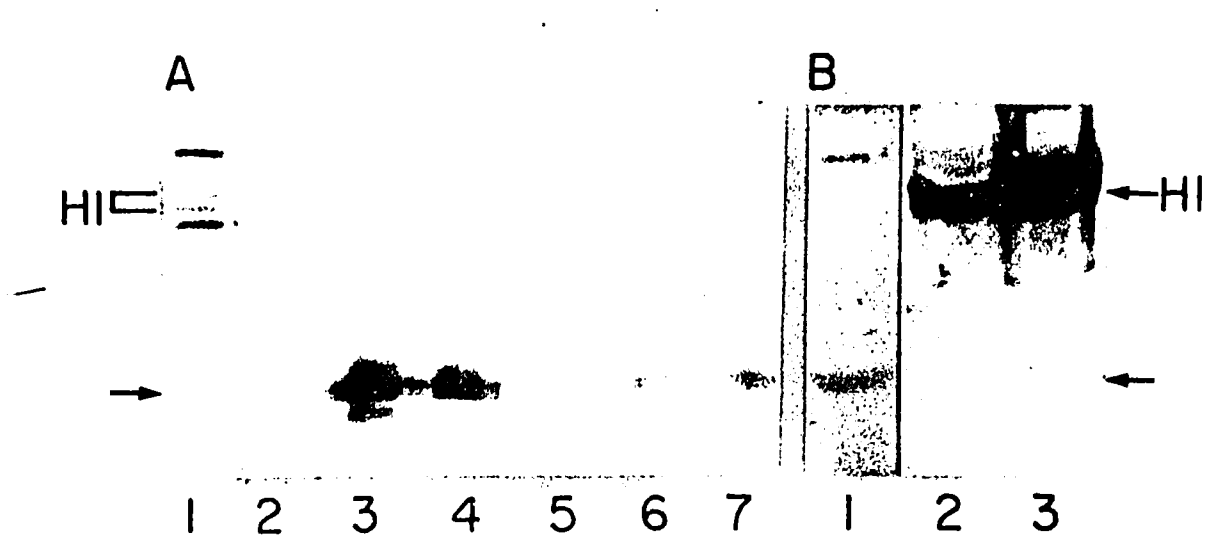
FIG. 7