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Histone-DNA interactions in the chromatin of procyclic *Trypanosoma brucei brucei*

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Abstract. The dissociation of histone proteins a–d from the chromatin of *Trypanosoma brucei brucei* procyclic culture forms was investigated by removing the proteins from the DNA by centrifugation of soluble chromatin through isokinetic sucrose gradients in the presence of NaCl. The dissociation of the *T. b. brucei* histones was compared with that of their higher-eukaryote counterparts H3, H2A, H2B and H4. All four histones of *T. b. brucei* remained bound to the DNA at 500 mM NaCl, were partially released at 750 mM NaCl and were completely dissociated from the DNA at 1 M NaCl. These interactions of histones a–d with the DNA were comparable with those of the H2 histones in the chromatin of higher eukaryotes, and histones a and d interacted with the DNA more weakly than did their higher-eukaryote counterparts H3 and H4. Substoichiometric amounts of an additional protein were recovered in the top fractions of the gradients under all dissociation conditions. This protein migrated in the H1 region of rat-liver chromatin in various gel systems. Its early release from the DNA also indicated a resemblance to histone H1. The presence of only small amounts of this protein and the relatively weak interactions of histones a and d with the DNA suggest that the mechanisms involved in chromatin compaction in *T. b. brucei* are different from those in higher eukaryotes.

It is generally accepted that gene expression is accompanied by modifications of the chromatin structure (Eissenberg et al. 1985; Pederson et al. 1986; Yanif and Cereghini 1986; van Holde 1989; Gross and Garrard 1987; Yager et al. 1989). The stability of the chromatin structure partially depends on the nature and strength of the DNA-protein interactions (Yager et al. 1989). In the physiological pH range, the histones are firmly bound to the DNA by a combination of electrostatic and hydrophobic forces (Bradbury et al. 1967; Bartley and Chalkley 1972; Nelson et al. 1982).

The stability of the DNA-protein interactions can be investigated by modifying the binding forces via salt, urea, pH or temperature changes (Bartley and Chalkley 1972, 1973; Burton et al. 1978; Wilhelm et al. 1978; Jorcano and Ruiz-Carrillo 1979; Thoma and Koller 1981; Kawashima and Imahori 1982; Oohara and Wada 1987; Yager et al. 1989). It has been shown for the chromatin of higher eukaryotes that the lysine-rich histone H1 dissociates first, at an ionic strength of about 500 mM NaCl (Thoma and Koller 1981). The moderately lysine-rich histones H2A and H2B (Burton et al. 1978; Wilhelm et al. 1978; Jorcano and Ruiz-Carrillo 1979; Oohara and Wada 1987; Yager et al. 1989) and, finally, the arginine-rich histones H3 and H4 (Burton et al. 1978) dissociate at salt concentrations of between 0.8 and 2 M.

The nuclear chromatin of procyclic culture forms of *Trypanosoma brucei brucei* is organized in nucleosome filaments; however, the nucleosomes are arranged less regularly than in higher eukaryotes (Hecker and Gander 1985). Four DNA-bound proteins, a–d, were recently isolated and characterised (Bender et al. 1991), and similarities were found between proteins a, b, c and d and eukaryote histones H3, H2A, H2B and H4, respectively. *T. b. brucei* histones could be discriminated from their higher eukaryote counterparts on the basis of their primary structure (Bender et al. 1992) as well as by their behaviour in different gel systems.

Histones a and d showed remarkably low hydrophobicity as compared with H3 and H4 (Bender et al. 1991). No H1-like protein was detected, and no salt-dependent condensation of the extended nucleosomal filament into a 30-nm fiber took place (Hecker and Gander 1985). The chromatin structure of procyclic *T. b. brucei* could more easily be destabilised by changes in the experimental conditions than could the structure of rat-liver chromatin, which indicated that the DNA-protein interactions are less stable in the chromatin of the protozoan parasite (Hecker et al. 1989).

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To analyse the basis for the low stability of the chromatin of procyclic *T. b. brucei* as compared with that of higher eukaryotes, we investigated the process of dissociation of the four histones a–d from the DNA at various ionic strengths.

Materials and methods

Procyclic culture forms of *Trypanosoma brucei brucei* (STIB 247) cultivated in SDM 79 medium (Brun and Schoenenberger 1979) were used in the present study. All experiments were performed at 0–4° C and pH 7–7.4.

Preparation of nuclei and soluble chromatin

Nuclei were isolated from $1.5–3.5 \times 10^{10}$ exponentially growing procyclic *T. b. brucei* as previously described by Shapiro and Doxsey (1982) and Hecker et al. (1989). Rat-liver nuclei were purified according to the method of Thoma et al. (1979). Nuclear chromatin of *T. b. brucei* was digested with micrococcal nuclease (Sigma, N-3755, 0.2 IU/20 A260) for 50 s at 30° C. Rat-liver chromatin was digested for 50 s at 37° C (0.4 IU/20 A260). Chromatin was solubilised by nuclear lysis in a low-salt buffer [1 mM tetraethylammonium chloride (TEACl) and 0.2 mM ethylenediaminetetraacetic acid (EDTA), pH 7; Thoma et al. 1979].

DNA-agarose gel electrophoresis

DNA of soluble chromatin was purified with phenol and chloroform/isoamylalcohol, precipitated with ethanol, vacuum-dried and resuspended in sample buffer. The DNA was electrophoretically fractionated in a horizontal 1.5% agarose gel, BRL 5510 UA (Maniatis et al. 1982).

Dissociation of chromatin-bound components

Aliquots of soluble chromatin (8–22 A260) were adjusted to the dissociation conditions by the addition of equal volumes of double-concentrated dissociation buffer (10 mM TEACl, 0.4 mM Na_2-EDTA and either 0–2 M NaCl or 1 M NaCl and 1 M urea; Thoma and Koller 1981). Samples were layered onto isokinetic sucrose gradients in 38-ml tubes (McCarty et al. 1974; 5.5%–28.8% sucrose, 5 mM TEACl, 0.2 mM Na_2-EDTA in the absence of salt or with in the presence of either 500, 750 or 1,000 mM NaCl or 500 mM NaCl and 500 mM urea) and the chromatin was fractionated by centrifugation at 130,000 *g* for 24 h. The centrifugation time for the gradients containing either 750 mM NaCl, or 500 mM NaCl and 500 mM urea was reduced to 11 and 15 h, respectively. The density of the DNA was recorded at 260 nm during the collection of the fractions, after which 3-ml fractions were dialysed against 1 mM phenylmethylsulfonylfluoride (PMSF) in water and then lyophilised and the proteins were analysed (Bender et al. 1991) by acid-urea and Triton-acid-urea gel electrophoresis.

Acid-urea gel electrophoresis

The lyophilised chromatin samples were dissolved in sample buffer [0.5% cetyltrimethylammonium bromide (CTAB), 2.5 M urea, 3% 2-mercaptoethanol, 0.01% Pyronin G and 0.9 M acetic acid] and incubated at 37° C for 30 min prior to loading. Gels containing 0.9 M acetic acid and 2.5 M urea in 15% acrylamide were pre-run in 0.9 M acetic acid at 20 mA for 1–2 h, and electrophoresis was carried out overnight at 8 mA (Panyim and Chalkley 1969; Shmatchenko and Varshavsky 1978). The upper electrode buffer contained 0.01% CTAB in 0.9 M acetic acid.

Triton-acid-urea gel electrophoresis

The lyophilised chromatin samples were dissolved and incubated as described above and were then centrifuged at 12,000 *g* for 4 min. The proteins in the supernates were precipitated with 5 vol. acetone and pelleted by centrifugation (12,000 *g* for 4 min). Prior to loading, these pellets were suspended in sample buffer in the absence of CTAB. The gels contained 6 mM Triton X-100, 0.9 M acetic acid and 2.5 M urea in 15% acrylamide (Alfageme et al. 1974). Following the first pre-run (20 mA, 1–2 h), each lane was loaded with 80 µl 1 M cysteamine in 0.9 *M* acetic acid and the second pre-run was performed at 20 mA for 1–2 h to prevent oxidation of methionine residues during the subsequent separation of proteins. Electrophoresis was carried out overnight at 15 mA.

The gels were stained with 0.25% Coomassie blue R250 in methanol:water:glacial acetic acid (5:5:1, by vol.) and were then scanned using an LKB Ultroscan recorder.

Results

The size distribution of soluble chromatin fragments was assessed by agarose gel electrophoresis (Fig. 1). A relatively small amount of DNA pieces corresponded to mono- and oligonucleosomes; the bulk of the DNA fragments were longer than 1,400 bp. For the investigation of the electrostatic interactions of histones a–d with the DNA, soluble chromatin was fractionated by sucrose gradient centrifugation at various ionic strengths (500, 750 and 1,000 mM NaCl).

The distribution of the DNA (Figs. 3, 4) and proteins (Figs. 2–4) in the gradients were monitored. In the absence of salt, DNA and proteins were recovered together in the bottom part of the gradients (Figs. 2a, 3, 4). In all, 50%–60% of both the proteins and the DNA were found in fractions 1–4, and 30% of the proteins and 20% of the DNA were concentrated in fractions 5–8 (Figs. 3, 4).

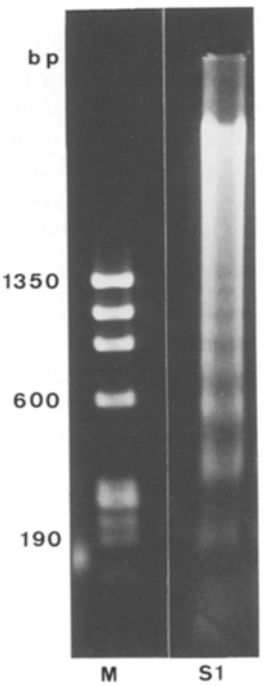

Fig. 1. Electrophoretic separation of DNA fragments of soluble chromatin of *Trypanosoma brucei brucei* procyclic culture forms in a 1.5% agarose gel. Digestion of nuclear chromatin was carried out using 0.2 IU micrococcal nuclease/20 A260 for 50 s at 30° C. bp, Base pair numbers; *M*, Hae III fragments of phiX174 RF DNA; *S1*, DNA fragments of soluble chromatin

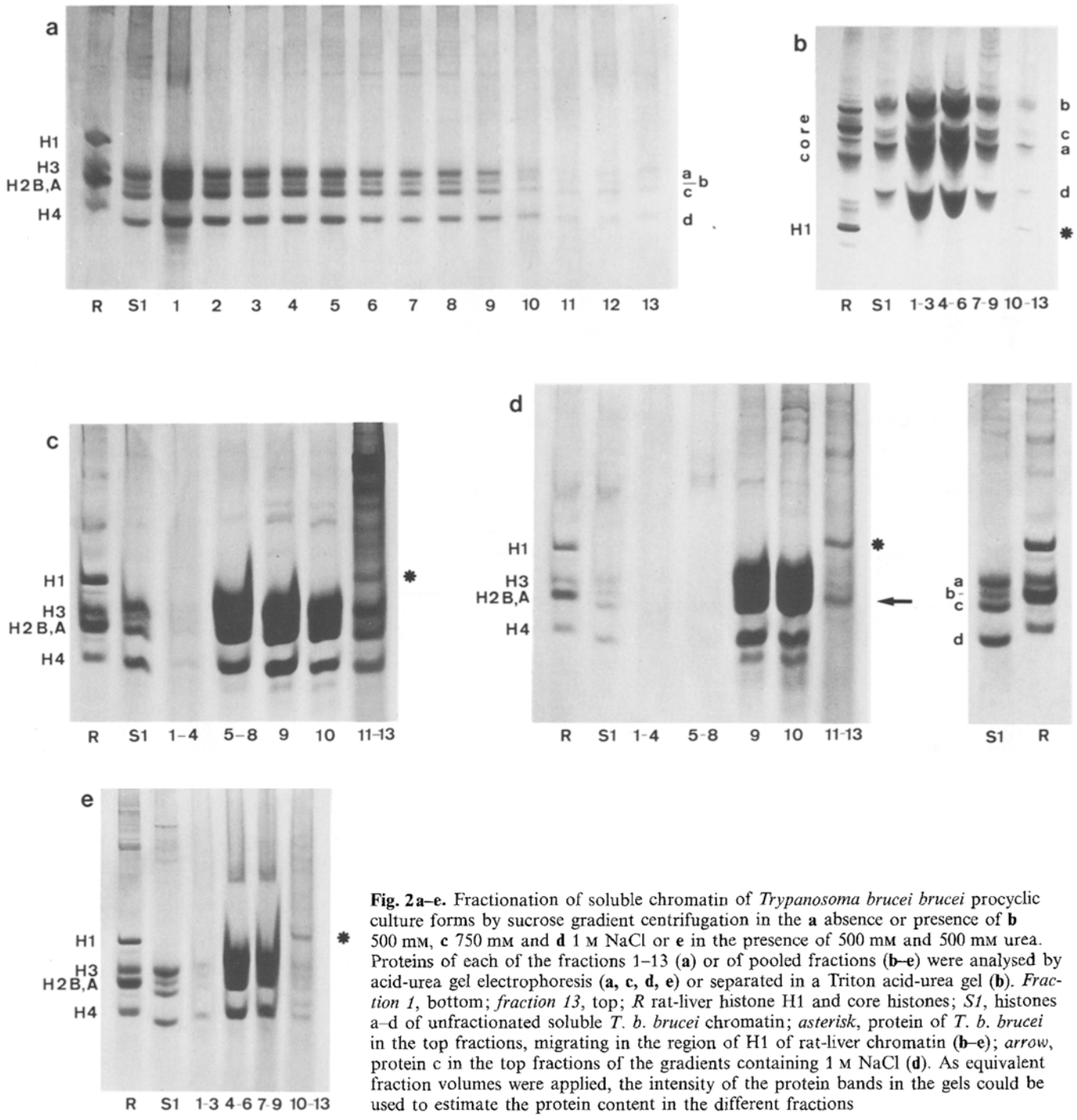

Fig. 2a–e. Fractionation of soluble chromatin of *Trypanosoma brucei brucei* procyclic culture forms by sucrose gradient centrifugation in the **a** absence or presence of **b** 500 mM, **c** 750 mM and **d** 1 M NaCl or **e** in the presence of 500 mM and 500 mM urea. Proteins of each of the fractions 1–13 (**a**) or of pooled fractions (**b–e**) were analysed by acid-urea gel electrophoresis (**a, c, d, e**) or separated in a Triton acid-urea gel (**b**). *Fraction 1*, bottom; *fraction 13*, top; *R* rat-liver histone H1 and core histones; *S1*, histones a–d of unfractionated soluble *T. b. brucei* chromatin; *asterisk*, protein of *T. b. brucei* in the top fractions, migrating in the region of H1 of rat-liver chromatin (**b–e**); *arrow*, protein c in the top fractions of the gradients containing 1 M NaCl (**d**). As equivalent fraction volumes were applied, the intensity of the protein bands in the gels could be used to estimate the protein content in the different fractions

At 500 m*M* NaCl, the distribution of the proteins and DNA was comparable to that in the low-salt gradients; the bulk of the material was recovered from the bottom fractions (Figs. 2b, 4). About 76% of the protein was concentrated in fractions 1–6 and 21%, in fractions 7–9. The distribution of the proteins corresponded to that of the DNA, 60% of which was recovered from fractions 1–6 and 20%, from fractions 7–9 (Fig. 4). At 750 mM NaCl (Figs. 2c, 3), about 50% of the DNA was found in fractions 1–8 together with only 30% of the protein, whereas 30% of the DNA was recovered from fractions 9 and 10 together with 50% of the protein and 24% of the DNA was concentrated in fractions 11–13 along with 18% of the protein. At 1 MNaCl (Figs. 2d, 3), about 70% of the DNA was found in fractions 1–8 and only 28% was recovered from fractions 9–13. Almost all of the protein was concentrated in fractions 9 and 10. A small amount of histone c was detected in the top fractions 11–13 (Fig. 2d, arrow).

Soluble chromatin was also fractionated in the pres-

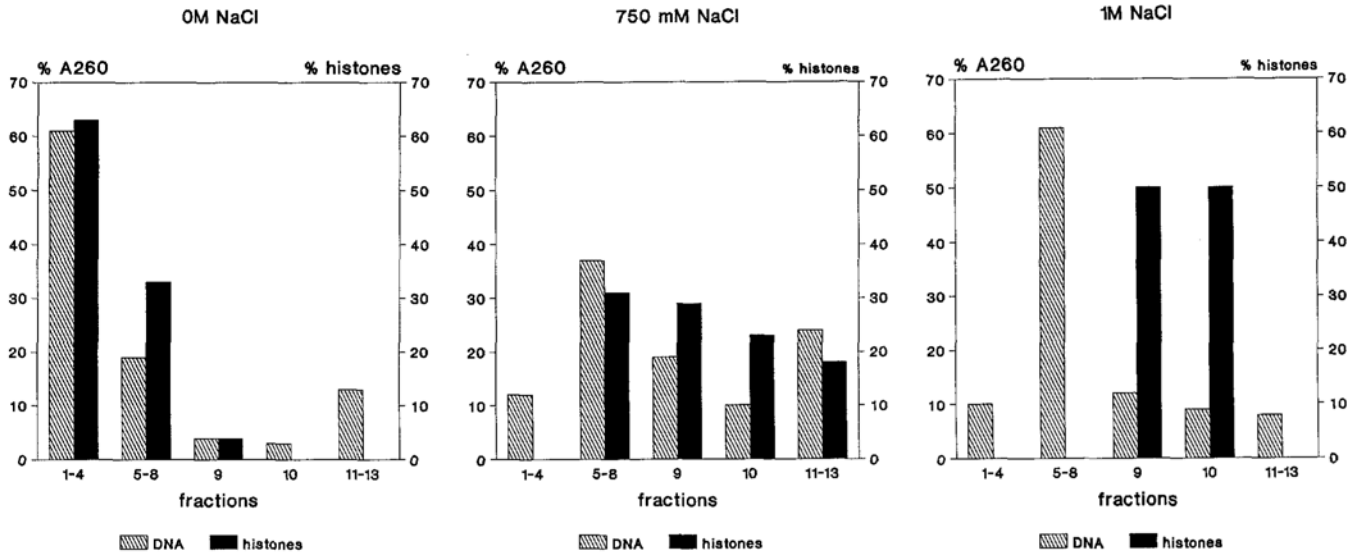

Fig. 3. Distribution of DNA and histones (percentage of recovered material, respectively) after fractionation of soluble chromatin of *Trypanosoma brucei brucei* procyclic culture forms in isokinetic sucrose gradients in the absence or presence of 750 mM or 1 M NaCl. Absorbance was measured at 260 nm. Pooled fractions *1–4, 5–8, 9, 10 and 11–13* were analyzed (*1*, bottom; *13*, top)

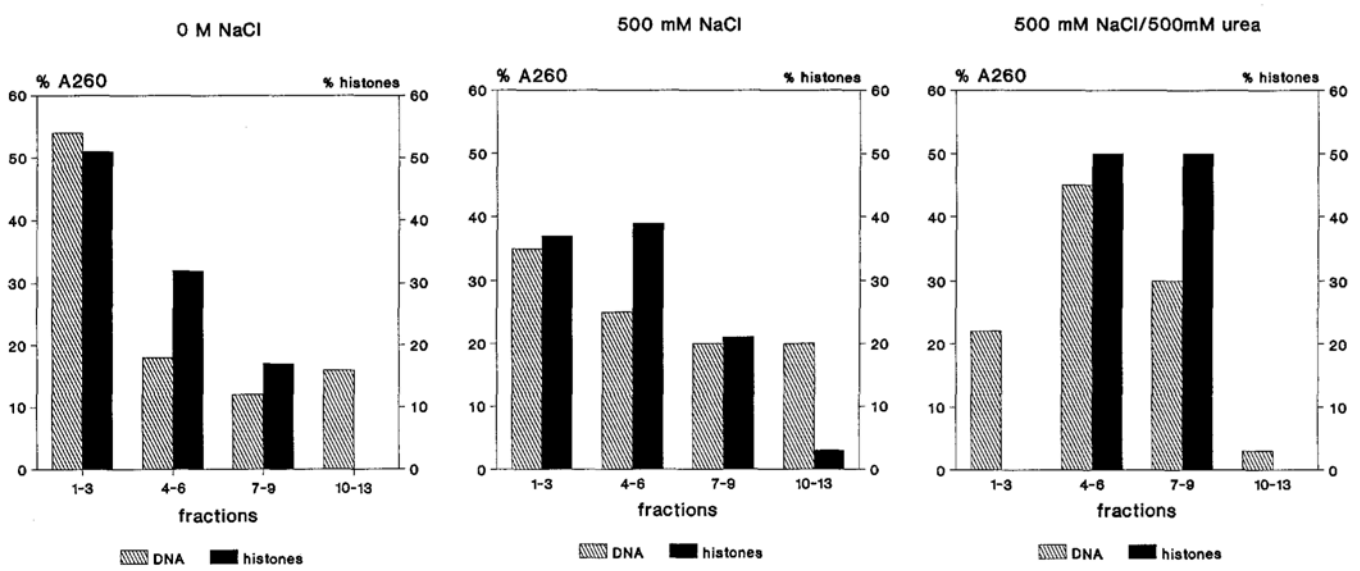

Fig. 4. Distribution of DNA and histones after fractionation of soluble chromatin of *Trypanosoma brucei brucei* procyclic culture forms in isokinetic sucrose gradients in the absence or presence of either 500 mM NaCl or 500 mM NaCl and 500 mM urea. Absorbance was measured at 260 nm. Pooled fractions *1–3, 4–6, 7–9 and 10–13* were analyzed (*1*, bottom; *13*, top)

ence of 500 mM NaCl and 500 mM urea. Almost all of the protein was recovered from fractions 4–9 together with about 75% of the DNA. More than 20% of the DNA, free of protein, was found in fractions 1–3 (Figs. 2e, 4).

At 500, 750 and 1,000 mM NaCl and in the presence of 500 mM NaCl and 500 mM urea, a small amount of protein was found in the top fractions 10(11)–13. This protein ran as a single band in the same region occupied by histone H1 of rat-liver chromatin in acid-urea and Triton-acid-urea gels (asterisk, Fig. 2). When the top fractions of the gradients containing 1 M NaCl were electrophoresed in the same two gel systems, a protein that migrated in the same region as did histone H1 of rat-liver chromatin was also found in both systems (Fig. 2d). When the protein of fractions 10–13 of the gradient containing 500 mM NaCl (Fig. 2b) was mixed with the core histones and histone H1 of rat-liver chromatin, only one band was detected in the H1 region of the gels (data not shown).

Discussion

Proteins a–d of procyclic *Trypanosoma brucei brucei* have been characterised according to their behaviour in different gels systems, the strength of their binding to a reversed-phase column, and their amino acid composi-

tion, as well as by partial sequence analysis (Hecker and Gander 1985; Bender et al. 1991, 1992). They could be classified as core histones, and strong similarities were evident between proteins a, b, c and d and eukaryote histones H3, H2A, H2B and H4, respectively.

The bulk of the chromatin-bound histones a–d were centrifuged into the bottom part of the gradients together with the DNA in the absence of salt or in the presence of 500 mM NaCl. These results indicated that under these conditions, the histones remained bound to the DNA. The distribution of proteins between the fractions of these gradients may reflect the amounts of different size classes of chromatin fragments that are present in soluble chromatin preparations. At 1 M NaCl, the rate of sedimention of the bulk of the DNA and proteins was significantly reduced. DNA and proteins were separated extensively as compared with both the controls and the gradients containing 500 mM NaCl. A relatively small amount of DNA was detected in fractions 9 and 10 together with most of the protein, which strongly indicated that all of the histones (a–d) had been released from the DNA. The dissociation of histones a and d from the DNA at 1 M Nacl contrasts with the behaviour of their higher-eukaryote counterparts H3 and H4, which are released from the DNA only at higher ionic strengths of between 1.2 and 2 M NaCl (Burton et al. 1978).

The somewhat lower ratio of basic to acidic amino acid residues of histones a (1.2) and d (2.0) as compared with H3 (1.6) and H4 (2.2), respectively (Bender et al. 1991), indicated that there are charge differences between the *T. b. brucei* histones and those of higher eukaryotes at physiological pH values. Histones a and d could also be distinguished from H3 and H4 on the basis of their low hydrophobicity and their higher electrophoretic mobility in Triton-acid-urea gels (Bender et al. 1991). Hydrophobic interactions play an important role in the binding of histones to DNA (Bartley and Chalkley 1972). If the ionic strength is increased, apolar amino acids in the arginine-rich histones become involved in α-helical segments of the histone secondary structure (Bradbury et al. 1967; Boublik et al. 1970). The arginine-rich histones might bind DNA not only via electrostatic interactions but also through hydrophobic interactions between apolar residues in the α-helical segments of histones and the inner regions of the DNA double helix (Bradbury et al. 1967; Bartley and Chalkley 1972). Given that NaCl concentrations above 0.6 M abolish the electrostatic interactions (Bartley and Chalkley 1972, 1973) and that the secondary structure of *T. b. brucei* histones resembles that of the histones of higher eukaryotes (Bender et al. 1991), it can be assumed that the low hydrophobicity of histones a and d was partially responsible for their early release at 1 M NaCl.

For the investigation as to whether hydrophobic forces may indeed have been involved in DNA-protein interactions in *T. b. brucei* chromatin, soluble chromatin was fractionated in the presence of 500 mM NaCl and 500 mM urea. The appearance of traces of proteins together with about 25% of the DNA in the bottom fractions 1–3 and the different distribution of proteins and DNA as compared with that observed in the gradients containing 500 mM or 1 M NaCl in the absence of urea strongly indicated a partial release of all histones under the influence of urea. The nature of urea-sensitive forces may be hydrophobic, and in the chromatin of higher eukaryotes the urea sensitivity was most marked for the arginine-rich histones (Bartley and Chalkley 1972). Since at 500 mM NaCl and 500 mM urea the release of *T. b. brucei* histones a–d from the DNA was not selective, it must be assumed that the differences between histones a and d and histones b and c are less pronounced than those between the arginine-rich histones H3 and H4 and the H2 histones of higher eukaryotes. The weak interactions of histones a and d with the DNA may partially explain the low stability of the chromatin of procyclic *T. b. brucei* as compared with that of rat liver (Conconi et al. 1984; Widmer et al. 1988; Hecker et al. 1989) under any experimental condition.

No clearly selective release of any of the histones a–d from the DNA was observed at 1 M NaCl. However, the appearance of some histone c in the top fractions suggested that it began to dissociate first. To monitor the release of histones a–d more precisely, fractions 9 and 10 were subdivided during their collection and the smaller fractions were analysed separately (data not shown). Since the amount of histone d was reduced in the top of fraction 10, it appeared that histone d was released from the DNA somewhat later than histones a–c in the presence of 1 M NaCl.

To elucidate whether histones a–d could be distinguished from each other by a sequence of dissociation at an ionic strength lower than 1 mM NaCl, soluble chromatin was fractionated at 750 mM NaCl. The presence of naked DNA (about 12% of the total DNA) in the bottom fractions 1–4 and the recovery of DNA together with proteins from fractions 5–8, which contained DNA only after fractionation at 1 M NaCl, strongly indicated both a partial release of histones and the existence of an equilibrium between chromatin, free DNA and free histones at 750 mM NaCl. As in the experiments using 1 M NaCl, no selective dissociation of proteins was observed. The interactions of histones a–d of procyclic *T. b. brucei* with the DNA thus appear to be similar to those of H2-histones in the chromatin of higher eukaryotes (Burton et al. 1978; Wilhelm et al. 1978; Jorcano and Ruiz-Carrillo 1979; Oohora and Wada 1987).

At all salt concentrations investigated as well as in the presence of salt together with urea, small amounts of protein were detected in the top fractions. This protein migrated in the same position as did histone H1 of rat-liver chromatin in acid-urea and Triton-acid-urea gels. Its early release from the DNA at 500 mM NaCl also indicated that this protein somewhat resembled histone H1 (Burton et al. 1978; Thoma and Koller 1981). These findings contradict the results of previous studies (Hecker and Gander 1985; Hecker et al. 1989; Bender et al. 1991) in which no H1-like protein could be detected in *T. b. brucei* chromatin. However, it should be borne in mind that in the present experiments the amount of soluble chromatin that was loaded onto the gradients was 20- to 40-fold that used for gel electrophoresis and

that the protein was subsequently enriched tremendously in the top fractions. It cannot be ruled out that this protein represents a non-histone component. If it does resemble histone H1, it occurs in smaller quantities than does H1, which is usally found at a ratio of one molecule per eight molecules of core histones (Kornberg 1977). The possible existence of a histone H1-like protein in a substoichiometric ratio to the core histones as well as its functional and evolutionary significance for the chromatin structure of *T. b. brucei* needs further experimental elucidation.

The presence of only small amounts of an H1-like protein, the less significant role of histones a and d in the stabilisation of the core particle as compared with that of H3 and H4 in the chromatin of higher eukaryotes, and the finding that the nucleosomal filament does not condense into a 30-nm fiber (Hecker and Gander 1985) indicate that chromatin compaction in this protozoan parasite is different from that in higher eukaryotes. The precise role played by the core histones in conformational changes in the chromatin of procyclic *T. b. brucei* needs further elucidation.

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