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PARTIAL CHARACTERIZATION OF RAM SPERMATIDAL BASIC NUCLEAR PROTEINS

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SUMMARY : Several techniques were used to demonstrate that eight spermatidal proteins are present in the nucleus of ram non-round spermatids. Excepting one, they all contain cysteine and are partially intermolecularly cross-linked in the chromatin of non-round spermatids. Ion-exchange chromatography on carboxymethylcellulose shows that most of them have over-all basic charges similar to those of histones. The molecular weights determined by gel filtration in 6M guanidine hydrochloride, range between that of the smallest histone and that of the basic nuclear sperm protein.

INTRODUCTION

A preliminary study of the basic nuclear proteins in ram spermatids (1) showed that a dozen basic proteins (spermatidal proteins) are transiently present in non-round spermatid nuclei. In the present paper, we confirm their non-proteolytic origin and their monomeric nature and we point out that only eight are genuine nuclear proteins. To elucidate their biological function in the process of replacement of histones by the basic nuclear sperm protein (BNSP), we have begun to characterize them. Throughout spermiogenesis large amounts of DNA are packaged in compact form and become bound to a smaller protein than those present in diffuse chromatin (2). Because packaging of DNA by proteins involves possible -SS- cross-linking, charge and steric interactions, we have investigated the cysteine content and polymerized conformation by three different methods, the over-all basic charge by ion-exchange chromatography and the size of proteins by the sodium dodecyl sulfate (SDS) electrophoretic method and by gel filtration in 6M guanidine hydrochloride (Gu HCl).

MATERIAL AND METHODS

Adult Ile-de-France rams were used. All the work was performed at 1-3°C. Nuclei and cytoplasm preparations - A population of non-round spermatids was obtained by sedimentation at 1 x g and nuclei were prepared as described previously (1) with the following modification : the 6 % BSA solution used

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to wash the cells, contained an adequate quantity of Lima Bean Trypsin inhibitor (LBTI) and all solutions used to prepare the nuclei contained LBTI and 1 mM phenylmethyl sulfonyl fluoride (PMSF). After preparation with Triton X-100, the nuclei were collected by centrifugation while the supernatant, referred to as "Cytoplasm fraction" was centrifuged again (1500 x g ; 10 min.) to remove eventual contaminating nuclei.

Isolation of about 1.6 x 10^9 non-round nuclei was carried out as described elsewhere (3) by homogenizing 15 g of testis in 200 ml 3.5 mM EDTA in distilled water containing 50 mM sodium bisulfite. The nuclei were washed in 100 ml 0.06 % Triton X-100 in buffer A (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF) and purified by two centrifugations at 1000 x g for 30 min through one volume of 1.5 M sucrose in buffer A. About 1.2 x 10^9 elongated spermatid nuclei were prepared by sonication of crude EDTA-resistant nuclei according to Grimes et al. (4). The purity of the nuclear preparations was verified by electron microscopy (5).

Extraction and analysis of the basic proteins - Acid-soluble basic proteins were extracted from the nuclear preparations by 0.4 N H_2SO_4 and dialyzed against 0.01 N HCl then lyophilized. After 3 acid extractions no further acid-soluble proteins were extracted from the sonication-resistant nuclei. Cross-linked cysteine-containing basic proteins were then extracted by treatment with 0.28 M 2-mercaptoethanol (6), alkylation with 0.4 M iodoacetamide and extration with 0.2 N HCl (1). Proteins were dialyzed and lyophilized as above. The cytoplasm fractions were adjusted to 0.4 N H_2SO_4 and after centrifugation (17,000 x g ; 30 min), were dialyzed against 10 mM Lithium acetate pH 5.0. They were chromatographed on a CM52 column (1.6 x 7 cm) according to Sung & Dixon (7), the basic proteins being eluted with 0.2 N HCl then lyophilized after dialysis against 0.01 N HCl.

Prior to electrophoresis (8) on gels containing 6.25 M urea, the samples were treated at 37°C for 1 h with 0.5 M 2-mercapto-ethanol in 8 M urea - 0.9 N acetic acid. After staining with amido-black, the gels were scanned at 620 nm. Proteins were named as previously proposed (1). Band 13-15 corresponded to the BNSP which, in the testis, is partially acid-soluble (1, 3).

Determination of SH-group-containing proteins - Protein extracts were oxidized at 25°C with 0.3 % H_2O_2 in 0.05 M Tris HCl pH 7.3 for 15 min to 12 h, then dialyzed against the electrophoresis buffer. Acid-soluble proteins (2 mg) of EDTA-resistant nuclei and sonication-resistant nuclei were treated at 37°C with 0.28 M 2-mercaptoethanol in 1 ml 0.5 M Tris HCl pH 8.5 for 3 h. Then 250 µM (1- ^{14}C)-iodoacetamide (58 mCi/mM) were added, diluted 15 times with unlabelled iodoacetamide. The reaction was stopped 2 h later with HCl at 0.25 N final concentration and proteins were dialyzed and lyophilized as above.

Over-all charge and molecular weight determinations - Large amounts of spermatidal protein extracts were prepared from whole testis according to the method of Kistler et al. (9) with 0.4 N H_2SO_4, then fractionated with 20 or 100 % TCA, added to final concentrations ranging between 1 and 10 %. The relative basicity of most of the spermatidal proteins was evaluated by ion-exchange chromatography of 3 % TCA-soluble testicular extracts on carboxymethylcellulose (CM52 Whatman) eluted with a linear gradient of lithium chloride (Li Cl) as described by Wigle and Dixon (10). The molecular weight (M) of proteins was determined by two methods. The SDS electrophoretic method as modified for histones (11), was used at pH 7.6 and 10. The standard curve of mobility vs the logarithm of M was plotted with Calf Thymus histones. Gel filtration in 6 M Gu HCl of reduced alkylated proteins was performed as described by Fish et al. (12) on a column of Bio Gel A-1.5m, more convenient for small M than Bio Gel A-5m. The column was calibrated with Calf thymus histones. The samples used for molecular weight determinations usually contained 3 to 5 proteins. They were prepared from testicular basic proteins by fractionation with TCA concentrations ranging between 2 and 9 % and/or by ion-exchange chromatography.

moiety of adenylate cyclase (6,7,22,23).

Current evidence for the involvement of the cytoskeleton in locomotion of cell substances and organelles is based mainly on the pharmacological action of cytochalasins, which are believed to impair microfilament function (8,24,25), and of colchicine, which causes depolymerisation of microtubules (26). Cytochalasin B inhibits the stimulatory effect of TSH and ACTH on thyroidal and adrenal secretion (27-29). Colchicine was reported to inhibit the clustering of binding sites for concanavalin A on epithelial cells of toad bladder in response to vasopressin (30) and to inhibit insulin action on lipid and glycogen synthesis (31).

The follicular response to gonadotropins depends on functional coupling of receptors located on the outer cell surface to the membrane-bound enzyme adenylate cyclase (32). Microtubules and microfilaments are widely distributed in the Graafian follicle (33-36). In the present study, we examined whether the cytoskeletal system participates in gonadotropin- and prostaglandin-stimulated ovarian cyclic AMP production. To clarify this question, we used the drugs colchicine and cytochalasin B, as well as specific antibodies to actin and tubulin.

MATERIALS AND METHODS

Materials. LH (NIH-LH-S19) was kindly made available by the NIAMD, N.I.H., Bethesda, Maryland, FSH (G4-150C; 50 x NIH-FSH-S1) by Prof. H. Papkoff and PGE_2 by Dr. J. Pike of the Upjohn Co., Kalamazoo, Michigan. Purified cholera toxin was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by Dr. R.A. Finkelstein, The University of Texas, Southwestern Medical School, Dallas, Texas.

3-Isobutyl-1-methylxanthine (IBMX), cytochalasin A, B and D were purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. The cytochalasins were dissolved in dimethylsulfoxide (DMSO: Fluka AG, Buchs SG, Switzerland), as stock solutions of 5 mg/ml.

Highly purified human chorionic gonadotropin (hCG; 14,000 i.u./mg, Serono, Rome, Italy) was used for iodination and hCG (5000 i.u./mg, Organon, Holland) was used as unlabelled hormone in competition studies. Colchicine was purchased from Merck, Germany.

Rabbit antiserum to rabbit muscle actin was the gift of Drs. A. Schreiber and J. Hoebeke, Free University, Brussels and rabbit antiserum to calf-brain tubulin was the gift of Dr. U. Littauer and Mrs. I. Gozes, Department of Neurobiology, The Weizmann Institute of Science. Goat antiserum to rat uterine myosin was the gift of Drs. D. Wallach and I. Pastan, N.I.H. Rabbit anti-BSA was prepared by Dr. Y. Weinstein.

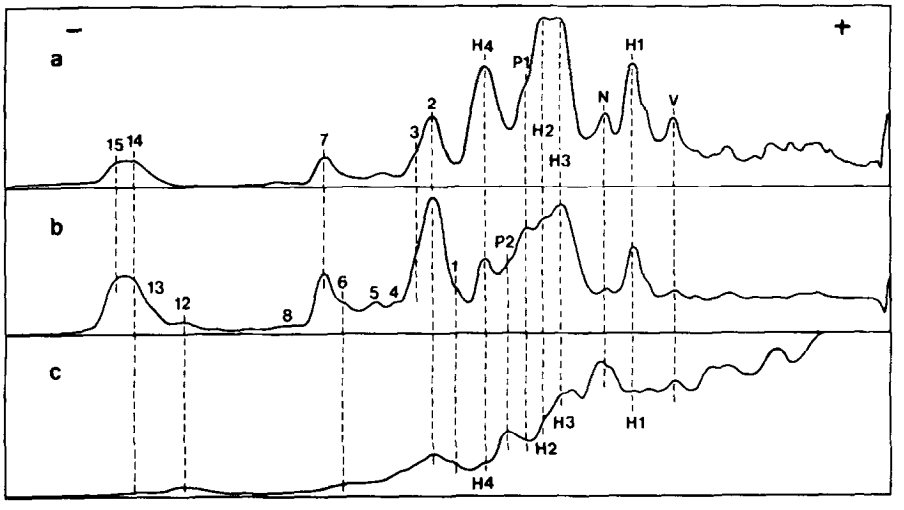

Figure 1 : Scans of the electrophoregrams of the basic proteins extracted by 0.4 N H_2SO_4 from (a) : EDTA-resistant nuclei purified through sucrose ; (b) : nuclei of a spermatid population obtained by sedimentation at 1 x g (3 % round spermatid nuclei, 29 % elongating spermatid nuclei, 66 % elongated spermatid nuclei) ; (c) : cytoplasm of the above spermatid population (see Methods).

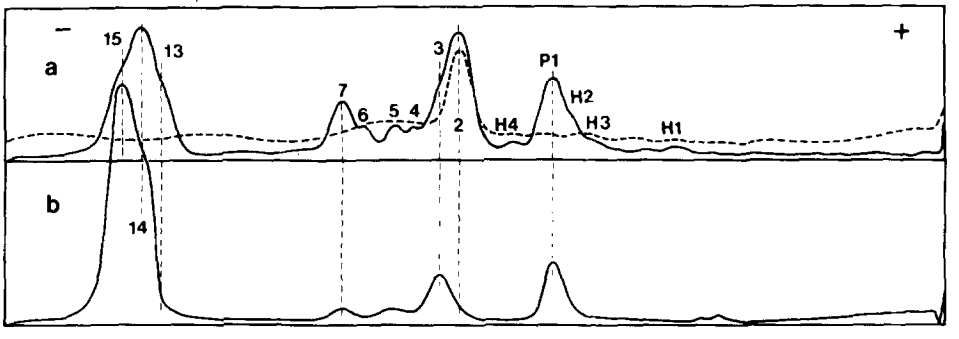

Figure 2 : Scans of the electrophoregrams of the basic proteins extracted from sonication-resistant nuclei (a), by 0.4 N H_2SO_4 ; (b), after additional reduction by 2-mercaptoethanol. ——: no oxidation ; – – – : after oxidation of the protein extract for 12 h.

linked into a polymerized form through formation of disulphide linkages.

Fractionation of acid-soluble testicular proteins at various TCA concentrations results in protein extracts enriched in specific spermatidal proteins as they become TCA-insoluble at concentrations ranging from 2 to 10 (Table 1). Fig. 4 shows the elution profile of 3 % TCA-soluble proteins

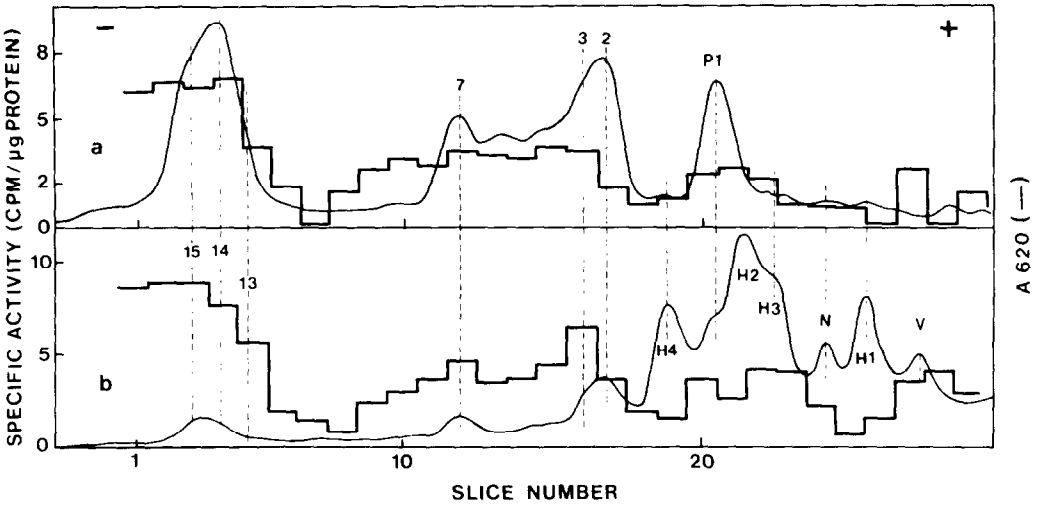

Figure 3 : Determination of the cysteine-containing proteins by alkylation with [^{14}C] iodoacetamide of the acid-soluble proteins from (A) sonication resistant nuclei and from (B) EDTA-resistant nuclei. After electrophoresis of the labelled proteins, the gels were scanned and sliced transversely into 2-mm slices. After solubilisation, the slices were counted in a liquid scintillation spectrometer. The specific activity (CPM/ug) was estimated for every slice by calculating the ratio of [^{14}C] activity to protein content as determined from the densitometric records by means of a standard curve made using calf thymus histones (3).

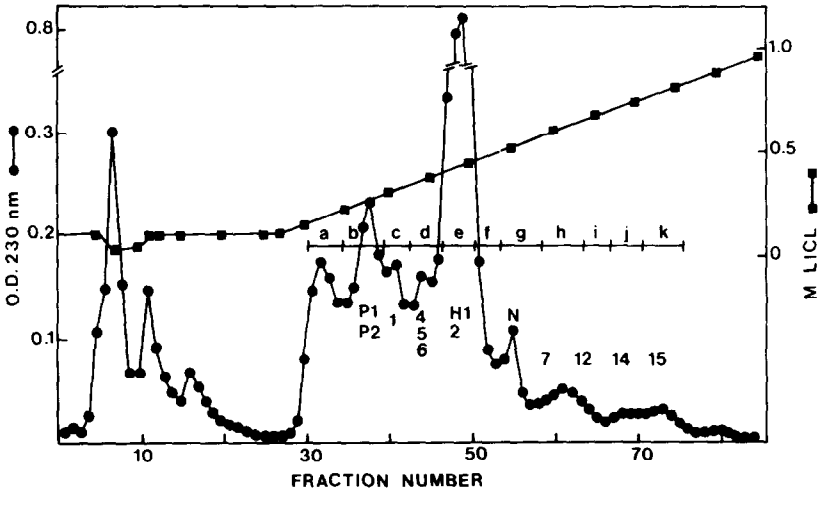

Figure 4 : Ion-exchange chromatography of 3 % TCA-soluble basic testicular proteins (25 mg) on a CM-cellulose column (1.6x28 cm) eluted with a linear gradient of Li Cl generated from 300 ml each of 0.10 M Li Cl and 2 M Li Cl in 0.01 M lithium acetate pH 5.0. 6 ml fractions were collected. Pooled eluant fractions (a to k) were subjected to electrophoresis after dialysis and lyophilisation.

TABLE 1

CHARACTERISTICS OF RAM SPERMATIDAL BASIC PROTEINS

Proteins		Insolubility % TCA	CM52 M LiCl	Molecular weight: Gel filtration 6M Gu HCl	Molecular weight: Electrophoresis SDS	Difference %
Nuclear	P1	4	0.25	9,600		
	2	10	0.40	12,000	13,400	+ 10
	3	2		10,300		
	4-6	4	0.32-0.37	10-11,000		
	7	4	0.55	8,700	12,000	+ 38
(BNSP)	14	7	0.70	6,600	10,400	+ 58
	15	7	0.75			
Cyto-plasmic	12	8	0.60	7,900	12,200	+ 55

chromatographed on CM-cellulose. Calf thymus histones were similarly chromatographed ; they were eluted with 0.35-0.55 M LiCl. As shown in Table 1, the spermatidal proteins have over-all charges similar to those of histones, with the exception of P1 which is less basic.

The molecular weight values obtained by the SDS electrophoretic method and by gel filtration in 6 M Gu HCl are shown in Fig. 5. They differ according to the method (Table 1). Particularly, the value obtained by gel filtration for the BNSP is in agreement with previous data (2) while that obtained by the SDS method is overestimated by 58 % (the BNSP extracted from sperm nuclei, with 2-mercaptoethanol and alkylated, is insoluble in the presence of SDS but that extracted from testis or spermatid nuclei with 0.4 N H_2SO_4 is soluble in SDS solutions). The overestimation of the molecular weight of proteins 2, 7, 12 and BNSP confirms, in agreement with previous reports (11, 13, 14) that the SDS method is unsatisfactory to measure the size of small and very basic proteins. The gel filtration data show that the spermatidal proteins have molecular weights ranging between 12,000 and 8,700 i.e. between the molecular weights of the smallest histone H4 and of the BNSP.

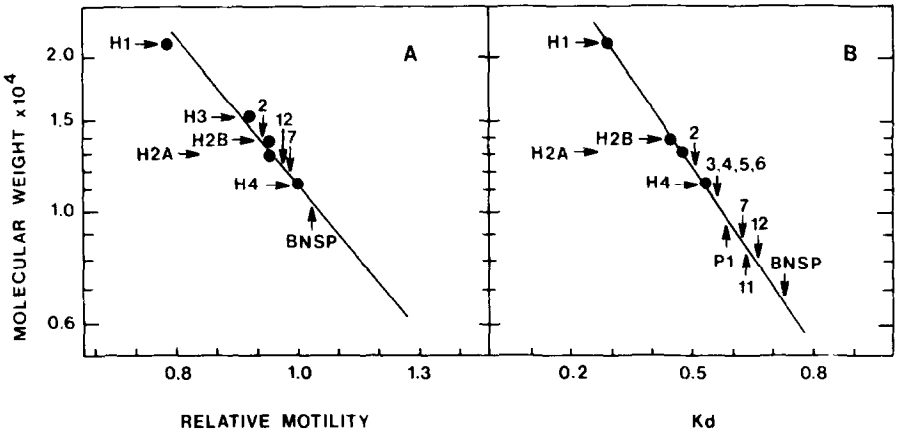

Figure 5 : Estimation of the molecular weight of ram spermatidal proteins (A), from electrophoretic mobility in SDS acrylamide gels at pH 10 and (B), from elution position after gel filtration on a Bio Gel A-1.5m column. Symbols : horizontal arrows : reference proteins (Calf thymus histones ; after 11) used to draw the calibration curves ; vertical arrows : ram proteins.

The nuclear spermatidal proteins are present only in the nuclei which are losing their histones and undergoing severe structural reorganization. They are de novo synthetized at the same time (3). The fact that all except one contain cysteine and are partially cross-linked in the chromatin, combined with the knowledge of their basicity and size, suggests that they play a crucial role in the structural rearrangement of the chromatin. Indeed, in the elongating nuclei, the expected expansion of the chromatin as a consequence of the removal of histones is not only counteracted, but the compaction of the chromatin increases progressively while chromatin fibers lose their beaded appearance to become smooth and coalesce to form thick bundles, as shown by ultrastructural observations (5). Thus, when the BNSP begins to cross-link, giving to the chromatin its final "keratinoïd" structure, the DNA is already tightly packaged by the cysteine-containing, intermediate-sized, basic spermatidal proteins.
The nuclear elongation phase is a critical one in the maturation process in ram germ cells (15) and is under hormonal control (16). It is fascinating to hypothesize that this control is mediated through the synthesis, binding,

then unbinding with DNA, of the nuclear spermatidal proteins.

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