Biochim. Biophys. Acta 345, 448.
Elgsaeter, A., and Branton, D. (1974), *J. Cell Biol. 63,* 1918.
Elgsaeter, A., Shotton, D. M., and Branton, D. (1976), *Biochim. Biophys. Acta 426,* 101.
Fairbanks, G., Avruch, J., Dino, J. E., and Bober, K. (1976), *J. Cell Biol. 70,* 243a.
Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.
Fuller, G. M., Boughter, J. M., and Morazzani, M. (1974), *Biochemistry 13,* 3036.
Gratzer, W. B., and Beaven, H. G. (1975), *Eur. J. Biochem. 54,* 403.
Gurthrow, C. E., Allen, J. E., and Rasmussen, H. (1972), *J. Biol. Chem. 247,* 8154.
Jacob, H. S., and Jandl, J. H. (1962), *J. Clin. Invest. 41,* 779.
Ji, T. H., and Nicolson, G. L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2212.
Kirkpatrick, F. H. (1976), *Life Sci. 19,* 1.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Liu, S. C., Fairbanks, G., and Palek, J. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 476.
Nicolson, G. L. (1973), *J. Cell Biol. 57,* 373.
Nicolson, G. L., and Painter, R. (1973), *J. Cell Biol. 59,* 395.
Palek, J., and Liu, S. C. (1976), *Blood 48,* 962 (Abstract).
Palek, J., Liu, S. C., Liu, A., Fortier, N., Synder, L. M., and Fairbanks, G. (1976), *Clin. Res. 24,* 316.
Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7,* 668.
Pinder, J. C., Bray, D., and Gratzer, W. B. (1975), *Nature (London) 258,* 765.
Pinto da Silva, P. (1972), *J. Cell Biol. 53,* 777.
Pinto da Silva, P., Moss, P. S., and Fudengerg, H. H. (1973), *Exp. Cell Res. 81,* 127.
Pinto da Silva, P., and Nicolson, G. L. (1974), *Biochim. Biophys. Acta 363,* 111.
Rothstein, A. (1971), *Exp. Eye Res. 11,* 329.
Rubin, C., and Rosen, O. (1973), *Biochem. Biophys. Res. Commun. 50,* 421.
Schechter, N. M., Sharp, M., Reynolds, J. A., and Tanford, C. (1976), *Biochemistry 15,* 1897.
Steck, T. L. (1972), *J. Mol. Biol. 66,* 295.
Strapazon, E., and Steck, T. L. (1976), *Biochemistry 15,* 1421.
Studier, F. W. (1973), *J. Mol. Biol. 79,* 237.
Tilney, L. G., and Detmers, P. (1975), *J. Cell Biol. 66,* 508.
Wang, K., and Richards, F. M. (1974), *J. Biol. Chem. 249,* 8005.
Wang, K., and Richards, F. M. (1975), *J. Biol. Chem. 250,* 6622.
Weed, R. I., and Chailley, B. (1973), in Red Cell Shape, Bessis, M., Week, R. I., and Leblond, P. F., Ed., Springer Verlag, New York, Heidelberg-Berlin, p 55.
Wolfe, L. C., and Lux, S. E. (1976), *Blood 48,* 963 (Abstract).
Yawata, Y., Howe, R., and Jacob, H. S. (1973), *Ann. Intern. Med. 79,* 362.
Yu, J., and Branton, D. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3891.
Yu, J., and Steck, T. L. (1975), *J. Biol. Chem. 250,* 9176.

Mouse Sperm Chromatin Proteins: Quantitative Isolation and Partial Characterization†

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ABSTRACT: Conditions are described that permit the quantitative extraction of chromatin proteins from the epididymal sperm of the mouse. These proteins have been isolated free of contaminating tail proteins following removal of the tails with cetyltrimethylammonium bromide (CTAB). Without this treatment, numerous acid-soluble tail proteins coextract with the nuclear proteins isolated from partially purified heads. The proteins isolated in this manner do not require prior modification with iodoacetamide and show no evidence of proteolytic degradation. In acid-urea polyacrylamide gels, 99% of the sperm protein migrates as one electrophoretic band. Evidence is presented that suggests that this single band contains two protamine-like proteins.

During spermatogenesis in most higher animals, differentiation of spermatogonia into mature sperm is accompanied by the replacement of the normal somatic histones and nonhistone proteins in chromatin by an extremely arginine- and cysteine-rich, protamine-like protein (Marushige and Dixon, 1969; Kumaroo et al., 1975). Extensive studies by Dixon (1972) show that the maturation and associated condensation of chromatin in trout sperm are directly correlated with the replacement of the histones on DNA by protamine. Although this process of repackaging may be more complex in mammals than fish, it appears to proceed in a similar fashion. Prior to the first meiotic division as the mammalian primary spermatocyte replicates its DNA, three new "meiotic histones" are synthesized (rat, mouse, rabbit, and monkey: Shires et al., 1975; Kistler and Geroch, 1975; Branson et al., 1975). These proteins are chemically and structurally similar to the H1, H3, and H2b

† From the Biomedical Research Division, Lawrence Livermore Laboratory, University of California, Livermore, California 94550. *Received March 29, 1977.* This work was sponsored by the U.S. Energy Research and Development Administration, Contract No. W-7405-ENG-48. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Energy Research and Development Administration to the exclusion of others that may be suitable.

histones and may replace, either partially or completely, their somatic counterparts. During the maturation of the spermatid following the second meiotic division, the histones, both somatic and meiotic, are acetylated and replaced on the DNA by a new lysine-rich protein TP (rat and mouse: Lam and Bruce, 1971; Kistler et al., 1973; Platz et al., 1975; Marushige and Marushige, 1975; Kumaroo et al., 1975; Kistler and Geroch, 1975). As a result of this repackaging of DNA, the chromatin within the nucleus takes on a more granular appearance (Dooher and Bennett, 1973). As the spermatid elongates, a small arginine- and cysteine-rich protein (protamine) is synthesized, deposited on the DNA replacing the TP protein, and is extensively phosphorylated (rat, mouse, rabbit, bull, and monkey: Monesi, 1965; Lam and Bruce, 1971; Dixon, 1972; Marushige and Marushige, 1975; Shires et al., 1975). Autoradiographic studies show that the synthesis and deposition of this protamine begin at the acrosomal end of the nucleus and move distally (Monesi, 1965). Coincident with the deposition of this new protein, both temporally and spatially, chromatin proceeds through an additional level of condensation (Dooher and Bennett, 1973). During elongation and continuing into the final steps of spermiogenesis, the newly deposited protamine is dephosphorylated (rat: Marushige and Marushige, 1975). As the sperm travel from the testes to the epididymi, the cysteine residues in the protamine are oxidized (rat: Marushige and Marushige, 1975), forming extensive intermolecular disulfide cross-links.

Previous analyses of chromatin proteins of mammalian epididymal sperm either by direct acid extraction of intact heads (Platz et al., 1975) or following solubilization of the heads in guanidine hydrochloride (Marushige and Marushige, 1975; Bellve et al., 1975) revealed the presence of one major, protamine-like and a number of minor histone-like proteins. The nature and exact number of proteins varied with the method of extraction. Both the isolation and characterization of these proteins have been hindered by the infrangibility of the sperm head, the highly condensed, insoluble structure of the chromatin complex, and the coextraction of proteins from contaminating tail fragments.

As a prelude to the analysis of chromatin structure in x-ray- and mutagen-induced abnormal sperm, we have devised a method for the isolation of the chromatin proteins of mouse sperm free of contaminating tail proteins. Under these conditions, the chromatin proteins are extracted quantitatively, and more than 99% of this protein migrates in polyacrylamide gels as one distinct electrophoretic species. Ethylenimine modification of this protein and N-terminal analysis indicate that this electrophoretic species is not homogeneous but contains two very similar proteins of identical electrophoretic mobility that differ in their cysteine content.

Materials and Methods

Isolation and Labeling of Sperm. Sperm were isolated from the caudal region of the epididymi of mice (either F_1 (C57BL/6J X SWR/J) bred in our colony or C57BL/6J from The Jackson Labs) by teasing the tissue in 0.01 M Tris[1]-saline, pH 8, and filtering the suspended sperm through silk gauze (80 μm). The sperm samples were washed three times in Tris-saline and either used immediately or stored as a frozen pellet at −20 °C.

Mice were injected intratesticularly with 1 μCi/g of body weight of [^{3}H]methylthymidine (18–25 Ci/mmol, Amersham/Searle) or [^{3}H]arginine monohydrochloride (8–25 Ci/mmol, Amersham/Searle). Maximal labeling of the DNA or sperm protein was obtained by sacrificing the [^{3}H]thymidine- and [^{3}H]arginine-labeled animals after 30 and 11 days, respectively.

Isolation of Sperm Chromatin Proteins. The sperm pellet (approximately 1.5×10^8 sperm) was suspended in 4 mL of 10 mM dithiothreitol (DTT), 0.05 M Tris, pH 8, with minimal sonication and incubated at 4 °C for 15 min. Cetyltrimethylammonium bromide (CTAB) was added to give a final concentration of 1% and the sample was incubated for an additional 30 min at 4 °C to allow dissolution of the sperm tails. The heads were subsequently pelleted by centrifugation at 3000g, washed twice in 1% CTAB, 0.01 M Tris, pH 8, and twice in Tris-saline. The purified heads were dissolved in 1 mL of 5 M guanidine hydrochloride (Gdn·HCl)–0.01 M Tris, pH 8, and the chromatin was allowed to expand at 4 °C for 30 min. After sonication the chromatin was diluted and urea, 2-mercaptoethanol (MSH), and sodium chloride (NaCl) were added to give a final concentration of 0.5 M Gdn·HCl, 3 M urea, 0.5 M MSH, and 2 M NaCl. Following dissociation of the proteins at 4 °C for 60 min, the DNA was precipitated by the addition of hydrochloric acid (HCl) to 0.5 M. After precipitation at 4 °C for 60 min, the DNA was pelleted by centrifugation at 14 500g for 10 min. The supernatant containing the chromatin proteins was desalted by chromatography on a 1 × 32 cm Sephadex G-10 column eluted with 0.01 N HCl. The proteins were precipitated from the appropriate pooled fractions with 20% trichloroacetic acid at 4 °C for 30 min, and the precipitate was pelleted by centrifugation at 14 500g, washed with acidified acetone, and dried in vacuo.

In specific experiments requiring acylation of the cysteine residues of protamine, iodoacetamide (final concentration, 0.2 M) was added to the chromatin following dissociation of the proteins in Gdn·HCl, MSH, NaCl, and urea and incubated in the dark at 37 °C for 90 min. The DNA was subsequently precipitated ,and the proteins were isolated as described above.

Polyacrylamide Gel Electrophoresis. The sperm protein was dissolved in 0.9 M acetic acid, 0.5 M MSH, 20% sucrose, and analyzed by electrophoresis on short (9 cm) polyacrylamide gels as described previously for histones (Panyim et al., 1971). After electrophoresis, the gels were stained in 0.1% Napthol Blue Black, 30% ethanol, 0.9 M acetic acid for 8 h. Microdensitometric traces of the destained gels were obtained using the Model SL504 Zeineh soft laser densitometer (Biomed Instruments, Inc.).

N-Terminal Analysis. N-terminal analysis of the isolated sperm protein (100 μg) was performed as described by Weiner et al. (1972). Two-dimensional, ascending chromatography of the dansylated amino acids was performed on 20 × 20 cm polyamide sheets (Brinkman Instruments, Inc.) in 1.5% aqueous formic acid (solvent 1) and 9:1 benzene–acetic acid (solvent 2).

N-terminal analyses of the ethylenimine-modified protamines (see below) were performed by dansylating the proteins eluted from polyacrylamide gels with [^{3}H]dansyl chloride (8.3 Ci/mmol, Amersham/Searle). The hydrolysate was chromatographed with a standard mixture of dansylated amino acids and the fluorescent spots were scraped into scintillation vials and counted.

Amino Acid Analysis. Following hydrolysis of the performic acid-oxidized-protamine samples in 6 M HCl at 110 °C for 20 h or 4 M methanesulfonic acid at 110 °C for 30 h, amino acid analyses were obtained using the Beckman 120C amino acid analyzer.

[1] Abbreviations used: CTAB, cetyltrimethylammonium bromide; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

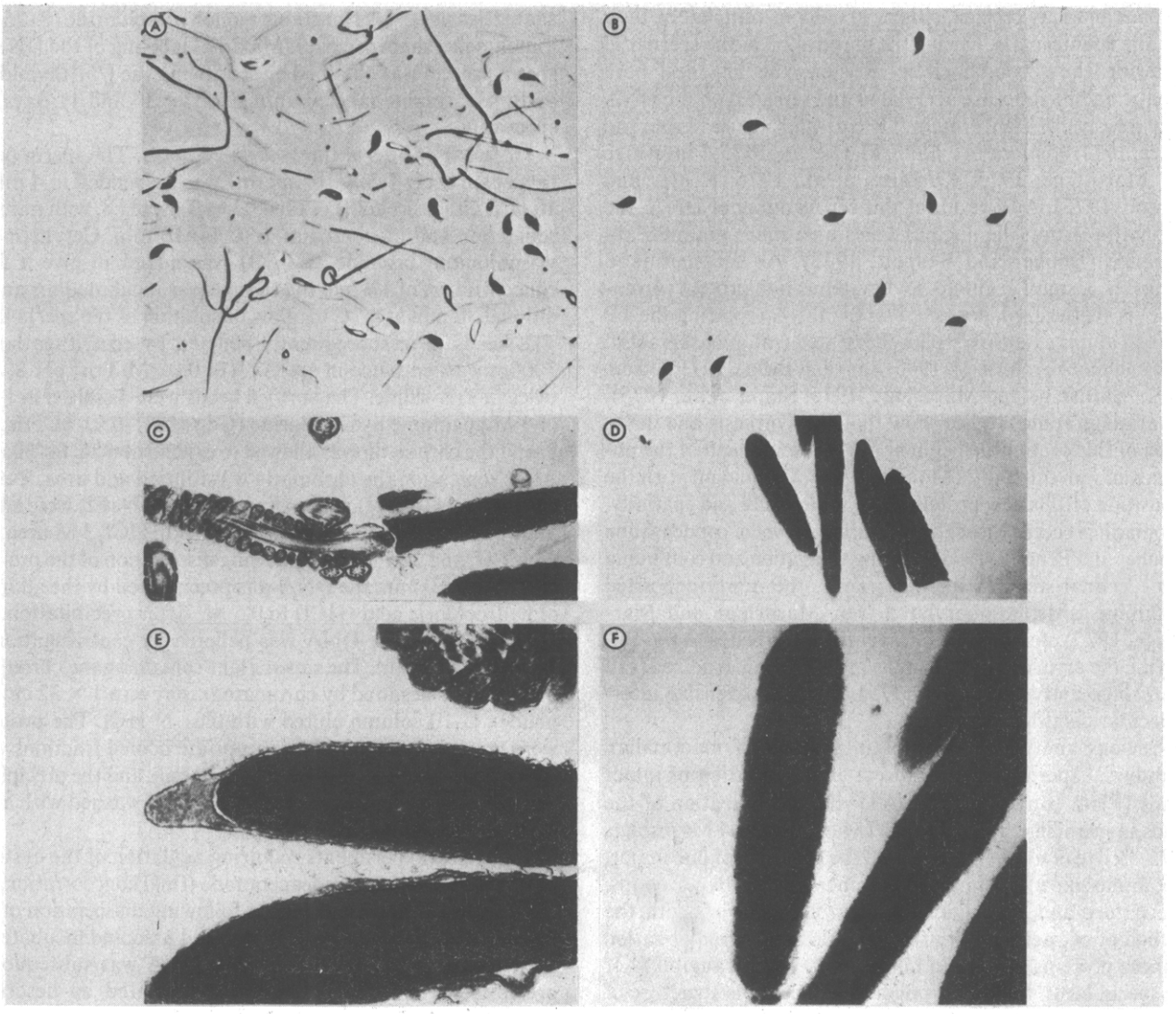

FIGURE 1: Light and electron micrographs of CTAB-treated mouse sperm. Suspension of sonicated mouse sperm in Tris–saline containing 10 mM DTT. Light photomicrographs: (A) untreated sample and (B) CTAB-treated sample after 30 min at 4 °C. Electron micrographs: untreated sperm are shown in C (×15 400) and E (×46 200). CTAB-treated sperm are shown in D (×15 400) and F (×46 200).

Ethylenimine Modification. Mouse protamine (100 μg) was dissolved in 3.2 mL of 0.5 M Tris, pH 8, 0.05 M dithiothreitol, and ethylenimine (Pierce Chemical Co.) was added to a final concentration of 0.25 M. After incubating at 37 °C for 1 h, the remaining ethylenimine was hydrolyzed upon the addition of HCl to 0.6 M. The aminoethylated protamine was precipitated with 20% Cl_3CCOOH at 4 °C and the protein pellet washed with acidified acetone and dried.

Following electrophoretic separation of the two modified protamine species in polyacrylamide gels, the bands were stained briefly in dilute stain ($^1/_{100}$ aqueous dilution) until just discernible and the regions containing the two proteins sliced out. Upon pooling samples from several gels, the proteins were eluted from the gel with 100 volumes of 0.01 M HCl–0.5 M MSH. The samples were filtered, concentrated by lyophilization, redissolved in 5 mL of 0.01 M HCl–0.5 M MSH, and precipitated with 20% Cl_3CCOOH. After washing the pellet three times with acidified acetone and drying in vacuo, the samples were taken up in 0.01 M HCl and chromatographed on a 1 × 32 cm Sephadex G-10 column to remove any residual acrylamide.

Electron Microscopy. Following prefixation of the whole sperm and sperm head pellets in 2% glutaraldehyde, 0.05 M calcium chloride, 6 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.4, for 10 min, the samples were fixed in 1% osmium tetroxide–veronal acetate buffer for 5 min at 23 °C. After staining with uranyl acetate, the samples were dehydrated with acetone and embedded with Epon. The samples were examined in a Siemens Elmiskop 1A electron microscope at an accelerating voltage of 80 keV.

Results

Sperm Tail Solubilization. Mechanical detachment of sperm tails by shearing, sonication, or freeze-thawing and their subsequent separation from the heads by centrifugation through sucrose, in our hands, proved to be a very inefficient means of obtaining purified sperm heads. Both free tails and heads with attached tail fragments invariably contaminated the purified head pellets. The sperm tails could, however, be completely removed by solubilization with DTT and the quaternary amine CTAB. Treatment of DTT-reduced mouse sperm for 30 min at 4 °C with 1% CTAB was sufficient to completely dissociate the tail structure (Figure 1). The nuclei remained intact, retaining their characteristic shape. While similar results have been obtained following CTAB treatment of human, guinea pig, bull, and ram sperm, this treatment was ineffective in dissociating the tails of Drosophila sperm. Electron-microscopic examination of the treated sperm con-

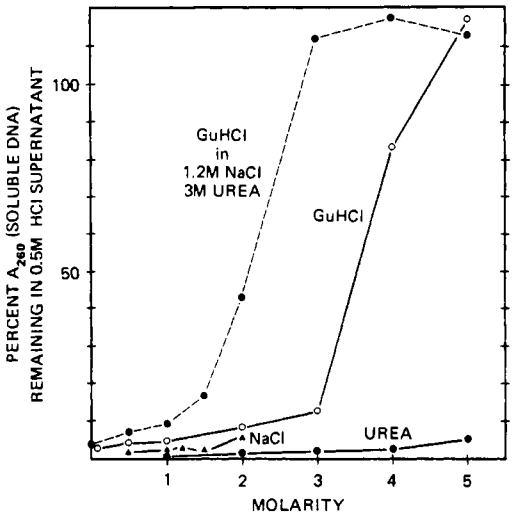

FIGURE 2: DNA solubility in acid. Effect of sodium chloride, urea, and guanidine hydrochloride concentration. Aliquots of salmon sperm DNA containing the appropriate concentrations of sodium chloride, urea, or guanidine hydrochloride were made 0.5 M in HCl and centrifuged to remove the insoluble DNA. Results are expressed as percent of the A_{260} (soluble DNA) remaining in the 0.5 M HCl supernatant after centrifugation. (●—●) Urea; (△—△) sodium chloride; (○—○) guanidine hydrochloride; (● - - - - ●) guanidine hydrochloride in 1.2 M sodium chloride-3 M urea.

TABLE I: Efficiency of Protamine Extraction and DNA Precipitation.

	cpm[a]	
	[^{3}H]Thymidine	[^{3}H]Arginine
Chromatin (Gdn·HCl solublized)	6120 (100%)	152 348 (100%)
Protamine extract[b]	ND[c] (0%)	158 674 (104%)
DNA pellet	6104 (99.7%)	852 (0.2%)

[a] Average of three experiments performed on equal aliquots of pooled, ^{3}H-labeled sperm. [b] 0.5 M Gdn·HCl, 1.2 M NaCl, 3 M urea, 0.5 M MSH, 0.2 M HCl. [c] None detectable.

TABLE II: Effect of Alkylation upon Extractibility of Protamine.

Expt	Extracted protamine[a]	
	Iodoacetamide blocked	Unblocked
1	128 ± 4	124 ± 3 (97%)
2	406 ± 8	404 ± 12 (99% +)

[a] Arbitrary units of integrated area from scans of the protamine peak analyzed in acid-urea gels.

TABLE III: Amino Acid Composition of Mouse Sperm Protein.[a]

Amino acid	mol/100 mol
Lys	4.9
His	14.0
Arg	56.9
Asp	0.3
Glu	0.5
Thr	0.5
Ser	6.0
Pro	0
Gly	2.6
Ala	0.5
Cys[b]	12.1
Val	0
Met	0
Ile	1.3
Leu	1.0
Tyr	1.4
Phe	0
Trp	0

[a] Hydrolysis in 4 M methanesulfonic acid, 30 h at 110 °C. Average of results from two separate protamine preparations. [b] Obtained as cysteic acid following performic acid oxidation and hydrolysis in methanesulfonic acid.

firmed the absence of tails and revealed that the acrosomes and essentially all of the membranes were removed (Figure 1).

Nuclear Protein Isolation. Direct acid extraction of purified sperm heads with 0.5 M HCl or 0.4 N sulfuric acid does not quantitatively solubilize the chromatin proteins. To quantitatively extract these proteins from sperm chromatin, the sperm head must first be solubilized. While sodium thioglycolate or low concentrations of Gdn·HCl (less than 2 M) were ineffective in solubilizing the heads at 4 °C, 5 M Gdn·HCl dissolves the heads upon contact.

Following solubilization of the purified heads in 5 M Gdn·HCl-0.05 M Tris, pH 8, the chromatin proteins are dissociated in high salt (1.2 M NaCl) and urea (3 M), and the DNA is precipitated by adding HCl. At Gdn·HCl concentrations above 3 M, however, DNA is quite soluble and will not precipitate when the pH is lowered (Figure 2). As is evident in Figure 2, the Gdn·HCl concentration in a 1.2 M NaCl-3 M urea solution of DNA must be reduced to less than 1 M before the DNA is quantitatively precipitated by 0.5 M HCl.

To confirm the efficiency of DNA precipitation and protamine extraction under these conditions, mice were injected with either [^{3}H]thymidine or [^{3}H]arginine and the epididymal sperm (caudal) isolated 30 or 11 days later, respectively. At these times maximal incorporation of [^{3}H]thymidine or [^{3}H]arginine into sperm DNA and protamine is observed (Meistrich et al., 1975; Monesi, 1965). The results in Table I clearly demonstrate that both the precipitation of DNA and the isolation of the chromatin proteins from these sperm are quantitative. Essentially all of the tritiated DNA is precipitated in the HCl-DNA pellet and less than 0.2% of the [^{3}H]arginine-labeled protein is found in the DNA pellet.

It should be noted that the isolation of the sperm proteins from mouse, hamster, bull, ram, and human sperm under these conditions does not require prior blockage of the sulfhydryl groups. As the data in Table II show, modification of the cysteine residues in mouse chromatin with iodoacetamide prior to extraction does not significantly alter the quantity of protein extracted.

Gel Electrophoretic Characterization of Sperm Protein. Using these methods, we have extracted and examined the basic proteins found in mouse sperm chromatin. Upon isolation of these proteins from CTAB-treated epididymal sperm heads, essentially all of the protein (99%) migrates in Panyim-Chalkley acid-urea gels as one electrophoretic band (Figure 3). None of the higher molecular weight, histone-like proteins observed by other workers is present. Also absent are the characteristic lower molecular weight proteins that are frequently observed as evidence of proteolytic degradation. The electrophoretic mobility of this protein, approximately twice that of histone H4, is characteristic of a protein molecule considerably smaller and more highly charged than histone. The amino acid composition of this protein (Table III) indicates that it is extremely rich in arginine (57%), cysteine (12%), and histidine (14%), and lacks aspartic and glutamic acids,

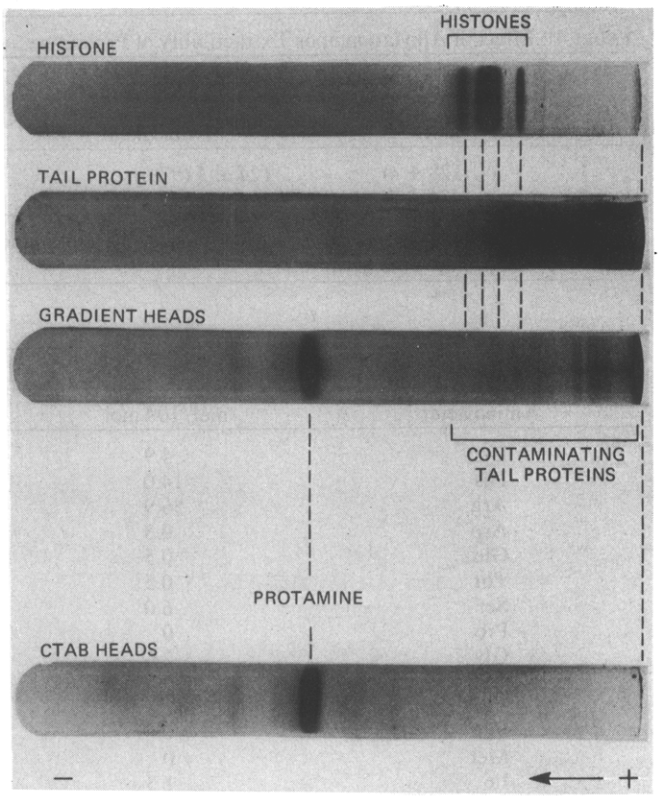

FIGURE 3: Gel electrophoretic patterns of mouse sperm protein. The sperm chromatin proteins were isolated from CTAB-treated or sucrose gradient purified heads as described in Materials and Methods. The acid-soluble sperm tail proteins were isolated from tails remaining after removal of the sperm heads by sedimentation through sucrose. The tails were solubilized in 1% CTAB and 10 mM DTT, brought to 0.5 M HCl, and the CTAB removed by chromatography on Sephadex G-10. Electrophoresis was performed at 130 V for 1.5 h.

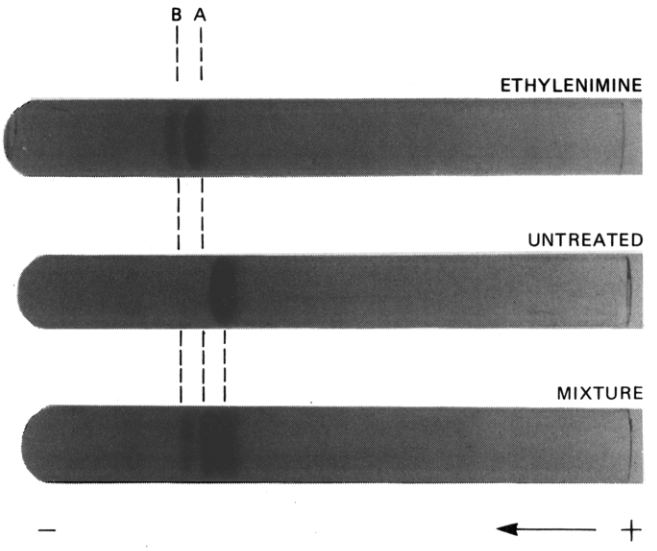

FIGURE 4: Ethylenimine modification of mouse protamine. Mouse protamine was modified as described in Materials and Methods. Electrophoresis was performed at 130 V for 1.5 h.

TABLE V: Ethylenimine Modification of Sperm Protein.[a]

Hours	% of total protein[b]		
	Unreacted	Band A	Band B
1	0.0	69	31
6	0.0	70	30
18	0.0	70	30

[a] Reaction in 50 mM DTT–0.5 M Tris (pH 8.0)–0.25 M ethylenimine at 37 °C. [b] As determined from microdensitometric scans of gel patterns from two separate experiments.

TABLE IV: N-Terminal Analysis of Protamine with [^{3}H]Dansyl Chloride.

Sample	cpm[a]	
	Dns-Ala	Dns-Gly
Whole protamine	606	178
	321	94
Protamine A (major)	7217	259
	5924	256

[a] cpm above background, with backgrounds at 50–100 cpm; corrected for loss during hydrolysis (Casola et al., 1974).

methionine, phenylalanine, proline, valine, and tryptophan. By comparison, proteins isolated from sucrose-purified heads frequently contained contaminating higher molecular weight proteins (Figure 3). Electrophoretic analysis of the acid-soluble proteins of the sperm tail (Figure 3) confirms that many of these larger proteins are tail proteins. Several of these proteins have electrophoretic mobilities quite similar to the somatic histones.

N-Terminal Analysis and Ethylenimine Modification. Since the electrophoretic homogeneity of a protein sample is rarely sufficient evidence that only one protein species is present, it was necessary to determine its complexity by some other means. N-terminal analysis of the dansylated protein (Table IV) showed two distinct N-terminal groups, alanine and glycine, in the approximate proportions of 3 to 1, respectively. Attempts to resolve these proteins by electrophoresis in sodium dodecyl sulfate gels were unsuccessful because of the insolubility of the protein in sodium dodecyl sulfate. Analysis of the protein in Triton gels under conditions in which the histones are known to migrate with mobilities very different than observed in acid–urea gels (Zweidler and Cohen, 1972) also failed to resolve the two proteins. Modification of the cysteine residues in the protein by ethylenimine, however, produced two electrophoretic species (Figure 4) of increased mobility upon electrophoresis in acid–urea gels. Coelectrophoresis of a mixture of the untreated and ethylenimine modified proteins (Figure 4) demonstrated that both of the ethylenimine treated proteins were modified forms of the slower migrating, untreated protamine. Conversion of the cysteine residues in the proteins to *S*-(2-aminoethyl)cysteine increased the cationic charge density on the two protein species, thus increasing their electrophoretic mobilities. As is shown in Figure 4, the increase in electrophoretic mobility of the minor protamine species upon modification is twice that of the major component, indicating that the cysteine content of this protamine must be approximately twice that of the major protamine species. The modification reaction was complete in less than 1 h (see Table V) producing the two modified species in the ratio of approximately 2.3 to 1.

To confirm the assignment of the N-terminal amino acids to these two proteins and rule out possible proteolytic effects, N-terminal analyses were performed on the modified proteins following elution from sliced gels. Since only a limited amount of the major protamine could be easily obtained by this method, the isolated sample was dansylated with [^{3}H]dansyl chloride. The results, shown in Table IV, confirm that the major, slower migrating protein (labeled A in Figure 4) has the alanine N

terminus. The minor protein must, therefore, have the glycine N terminus.

Discussion

Unlike the isolation of somatic histones from chromatin or nuclei, sperm chromatin proteins are not readily extracted from the intact mammalian sperm head. Acylation of the cysteine residues in these proteins prior to their dissociation from DNA in the reduced, Gdn·HCl-solublized sperm-head complex has been used to facilitate their extraction (Marushige and Marushige, 1975), but such procedures are clearly undesirable when unmodified proteins are needed for subsequent characterization. We have demonstrated that the sperm chromatin proteins may, under the appropriate conditions, be isolated quantitatively without modification. Following solubilization of the sperm head in 5 M Gdn·HCl, the proteins are dissociated in high salt and urea and the DNA is removed by acid precipitation. At Gdn·HCl concentrations above 3 M, DNA remains soluble in 0.5 M HCl. If, however, the Gdn·HCl concentration is lowered to 0.5 M, both the acid precipitation of DNA and the extraction of the chromatin proteins from mouse epididymal sperm are quantitative.

Previously the isolation and characterization of the sperm chromatin proteins have been complicated by the coisolation proteins from contaminating tail fragments and proteolytic effects, possibly arising as a result of the action of acrosomal proteases. The sperm tails may be solubilized, however, by treating reduced, whole sperm with the quaternary amine CTAB. Certain other quaternary amines, such as Hyamine 10X, act similarly but appear to solubilize the tails less efficiently. Examination of the CTAB-treated sperm by electron microscopy indicates that both the acrosome and various head membranes have been removed. Electrophoretic analyses of the unmodified sperm chromatin proteins isolated from CTAB-treated sperm heads have shown that 99% of the basic chromosomal proteins in mouse epididymal sperm migrate in acid–urea gels as one electrophoretic band. Proteins isolated from heads purified by centrifugation through sucrose were contaminated with numerous higher molecular weight proteins. Several of these proteins, as previously reported (Marushige and Marushige, 1975), have electrophoretic mobilities similar to the somatic histones. Electrophoretic analyses of the acid-soluble sperm-tail proteins have confirmed that these higher molecular weight proteins are contaminating tail proteins and not sperm chromatin proteins.

Although the use of CTAB in the removal of sperm tails has been applied successfully to a variety of mammalian sperm (mouse, hamster, bull, ram, and human), we must emphasize that caution should be used in the application of this technique to nonmammalian sperm. Specific quaternary amines will dissociate histones from isolated chromatin (Balhorn, manuscript in preparation) and CTAB treatment might extract considerable protein from nonmammalian sperm containing predominately histone. Histone or histone like proteins are not, however, extracted from mouse or human sperm by the CTAB treatment. Analyses of the CTAB-solubilized mouse sperm tail proteins in acid–urea gels demonstrated the absence of protamine and in sodium dodecyl sulfate gels confirmed the absence of somatic or meiotic histones. Similarly, CTAB treatment of human sperm, in which 12% of the chromatin proteins are histones (Puwaravutipanich and Panyim, 1975; Balhorn, unpublished observations), did not dissociate detectable amounts of histone from the human sperm head.

The amino acid composition of whole mouse protamine isolated from CTAB-purified heads proved to be quite similar to analyses reported previously by Calvin (1976) and Bellve et al. (1975). Significant differences were observed only in the serine and arginine contents. Protamine free of tail-protein contaminants contained less serine (6%) than reported by either Calvin (8.1%) or Bellve (12.2%), while the number of arginine residues (57%) was considerably higher than reported by Bellve (47%). The low levels of glutamic acid, threonine, alanine, isoleucine, leucine, and tyrosine do not appear to be contaminants but suggest, as observed in bull protamine (Coelingh et al., 1972), that only one or two of these residues may be present in either the major or minor protamine.

Subsequent characterization of the electrophoretically homogeneous mouse protamine by N-terminal analysis and ethylenimine modification has confirmed as first suggested by Bellve et al. (1975) that mouse sperm chromatin contains two protamine molecules of identical electrophoretic mobility. N-terminal analysis of the dansylated protein demonstrated two distinct N-terminal groups, alanine and glycine, in the approximate proportion of 3 to 1. Following modification of the cysteine residues with ethylenimine, two new electrophoretic species of increased mobility were obtained. Calculations based on the cysteine content of the protamine mixture and the relative increase in electrophoretic mobilities of the two protamine species indicate that the major protamine component may contain 9% cysteine and the minor protamine approximately 18% cysteine. N-terminal analyses of the major protein species isolated from gel slices have allowed assignment of the alanine N terminus to the major protamine and the glycine N terminus to the minor protamine species.

Acknowledgments

We thank Dr. Robert Smith, Jim Yoshiyama, and Dr. Judy Walton for providing their expertise in electron microscopy and Dr. Virgie Shore and Marie Laskaris for providing the amino acid analyses.

References

Bellve, A. R., Anderson, E., and Hanley-Bowdoin, L. (1975), *Dev. Biol. 47,* 349–365.

Branson, R. E., Grimes, S. R., Jr., Yonuschot, G., and Irvin, J. L. (1975), *Arch. Biochem. Biophys. 168,* 403–412.

Calvin, H. (1976), *Biochim. Biophys. Acta 434,* 377–389.

Candido, E. P. M., Honda, B. M., and Baillie, D. L. (1976), *FEBS Lett. 61,* 260–262.

Casola, L., Di Matteo, G., Di Prisco, G., and Cervone, F. (1974), *Anal. Biochem. 57,* 38–45.

Coelingh, J. P., Monfoort, C. H., Rozijn, T. H., Leuven, J. A. G., Schiphof, R., Steyn-Parve, E. P., Braunitzer, G., Schrank, B., and Ruhfus, A. (1972), *Biochim. Biophys. Acta 285,* 1–14.

Dixon, G. H. (1972), *Gene Transcription Reprod. Tissue, Trans. Karolinska Symp. Res. Methods Reprod. Endocrinol., 1972.*

Dooher, G. B., and Bennett, D. (1973), *Am. J. Anat. 136,* 339–362.

Kistler, W. S., and Geroch, M. E. (1975), *Biochem. Biophys. Res. Commun. 63,* 378–384.

Kistler, W. S., Geroch, M. E., and Williams-Ashman, H. G. (1973), *J. Biol. Chem. 248,* 4532–4543.

Kumaroo, K. K., Jahnke, G., and Irvin, J. L. (1975), *Arch. Biochem. Biophys. 168,* 413–424.

Lam, D. M. K., and Bruce, W. R. (1971), *J. Cell. Physiol. 78,* 13–24.

Marushige, K., and Dixon, G. H. (1969), *Dev. Biol. 19,* 397–414.

Marushige, Y., and Marushige, K. (1975), *J. Biol. Chem. 250,*

39-45.
Meistrich, M. L., Reid, B. O., and Barcellona, W. J. (1975), *J. Cell Biol. 64,* 211-222.
Monesi, V. (1965), *Exp. Cell Res. 39,* 197-224.
Panyim, S., Bilek, D., and Chalkley, R. (1971), *J. Biol. Chem. 246,* 4206-4215.
Platz, R. D., Grimes, S. R., Meistrich, M. L., and Hnilica, L. S. (1975), *J. Biol. Chem. 250,* 5791-5800.
Puwaravutipanich, T., and Panyim, S. (1975), *Exp. Cell Res. 90,* 153-158.
Shires, A., Carpenter, M. P., and Chalkley, R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2714-2718.
Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242-3251.
Zweidler, A., and Cohen, L. H. (1972), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31,* 4051.

Isolation and Characterization of a Unique Galactoside from Male *Drosophila melanogaster*†

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ABSTRACT: A ninhydrin-positive compound with presumptive hormonal activity, previously considered to be a peptide (Chen, P. S., and Bühler, R. (1970), *J. Insect Physiol. 16,* 615), has been isolated from adult male *Drosophila melanogaster.* Chromatographic analysis of the acid-hydrolyzed material revealed the presence of ethanolamine, phosphorus, galactose, and glycerol. Chemical analysis showed these to be present in equimolar amounts. Based on its phosphorus content, the nonreducing material took up 2 equiv of periodate, and released 1 equiv of formaldehyde. Characterization of the compound as 1-*O*-(4-*O*-(2-aminoethyl phosphate)-β-D-galactopyranosyl)-*x*-glycerol was achieved by gas chromatography–mass spectroscopy and ^1H and ^{31}P NMR using model compounds. In vivo synthesis from labeled precursors is in accord with the proposed structure.

Secretions from the male accessory glands (paragonial glands) of several insects have been examined with a view to identifying compounds having a physiological function in reproduction (cf. reviews by Chen, 1971; de Wilde and de Loof, 1973). The suggestions have been made that paragonial secretions have hormone-like activities, and are involved in the stimulation of egg laying and reduced receptivity of the female toward insemination by other males following an initial copulation. A dimeric protein termed "matrone" has been isolated from males of the yellow fever mosquito *Aedes aegypti* (Fuchs et al., 1969; Fuchs and Hiss, 1970; Hiss and Fuchs, 1972); both protein components were required to elicit monogamy while the α component alone stimulated egg laying. Proteins or protein-like materials having similar effects have been reported from the housefly *Musca domestica* (Nelson et al., 1969; Leopold et al., 1971), fruit fly *Drosophila funebris* (Baumann and Chen, 1973; Baumann et al., 1975), and the grasshopper (*Melanoplus sanquinipes*) (Pickford et al., 1969; Friedel and Gillott, 1976).

The present work is concerned with a major ninhydrin-positive compound from the paragonial glands of adult males of *D. melanogaster* which, from its location on paper chromatography (Chen and Diem, 1961), apparently corresponds to the "sex peptide" reported earlier by Fox (1956a,b) and Fox et al. (1959). This compound was subsequently examined on an amino acid analyzer (Chen and Bühler, 1970), where it eluted as an acidic material between phosphorylserine and glycerophosphorylethanolamine. At that time it was considered to be a peptide in view of the several free amino acids resulting from acid hydrolysis. We now report on the isolation and characterization from this so-called "sex peptide" of an ethanolamine-containing galactoside of unique structure, the earlier conclusions apparently being due to the presence of contaminating peptide(s) cochromatographing with the galactoside in crude preparations.

Materials and Methods

Materials. The wild stock (Sevelen) of *D. melanogaster* was raised on standard diet (corn-sugar-yeast-agar) at 25 °C. Male flies were separated from females about 2 h after adult emergence, and after aging for 8–10 days the males were frozen and stored at −20 °C until required.

Bis(trimethylsilyl)trifluoroacetamide (BSTFA), BSTFA-d_9, and gas chromatographic packings were purchased from Supelco, Inc., Bellefonte, Pa. D-Galactose, D-galactose 6-phosphate, and α-D-galactose 1-phosphate were obtained from Sigma Chemical Co. 1-*O*-(β-D-Galactopyranosyl)-D,L-glycerol was synthesized and kindly donated by Dr. Y. C. Lee (Johns Hopkins University); 1-*O*-(α-D-galactopyranosyl)-D-glycerol, isolated from *Porphyra perforata* (Su and Hassid, 1962), was a generous gift from the late Dr. W. Z. Hassid. Acid phosphatase, phosphodiesterase I, galactose oxidase, and β-galactosidase were purchased from Worthington Biochemical Corp., and the radioisotopes from Amersham-Searle Corp.

Analytical Procedures. Neutral sugar and phosphorus were determined according to Dubois et al. (1956) and Allen (1940), respectively. Ethanolamine was estimated on the amino acid analyzer or, less accurately, after separation by electrophoresis on cellulose TL plates at 25 V/cm at pH 1.9 or 3.7 and elution

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