ANALYSIS OF MOUSE SPERM PROTAMINE AND THE DEVELOPMENT OF A METHOD FOR THE ISOLATION OF ITS mRNA

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ABSTRACT

Tritium-labeled protamine was extracted from mouse caudal epidymides. Partial purification of the two protamine components was achieved using a series of chromatographic columns. It was determined that gel electrophoresis was not an adequate method for separating the two components for quantification. Mouse testes were used as a source of protamine mRNA. Procedures to isolate the protamine mRNA included utilization of a DEAE anion exchange column and an Oligo-Dt column. Results indicated that mRNA was not being recovered from the Oligo-Dt column.

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INTRODUCTION

Protamines are small basic proteins found in the mature sperm nuclei of most species including mammals. They have a high content of basic amino acids such as arginine, histine, and lysine. The protamines replace the somatic type histones associated with deoxyribonucleic acid (DNA). This occurs late in spermiogenesis when the spermatids are undergoing elongation and nuclear condensation (9). The replacement results in the compact packaging of the chromosomal DNA within the sperm nucleus.

This paper is concerned with the further characterization of mouse protamine and with the isolation of its messenger ribonucleic acid (mRNA). Sega and colleagues (23, 24, 25, 26) have found that mouse protamine is alkylated by mutagens such as ethyl methanesulfonate (EMS). Also in the case of EMS the germ cell stages most sensitive to genetic damage from this mutagen are near the stages when protamine replaces the histones. Because it is alkylated by mutagens, protamine could be useful in studying the mutagenic hazard posed by certain chemical agents. The isolation of protamine mRNA offers the possibility of learning more about the control of mRNA in the eukaryotic cell.

LITERATURE REVIEW

Analysis of Protamine

The protamines of fish, particularly salmon and trout, have been extensively studied and characterized (10, 11, 12, 13), since fish protamine is relatively easy to obtain using dilute mineral acids. In eutherian mammals however, protamine extraction is more difficult (1, 4, 17).

Gledhill et al. (18) have reported that there is an alteration in sperm histone to a more arginine-rich protein late in spermiogenesis in the bull. Additional work has shown that bull spermatozoan protamine is also rich in cysteine and the cysteine sulfhydryl groups form disulfide bonds (8).

Protamine has been isolated from rat spermatozoa (20). The protamine was found to be acid soluble and highly basic. A large number of cysteine residues was present in disulfide form.

Studies by Lam and Bruce (6) have revealed the presence of a protein rich in arginine in mouse testes. Balhorn et al. (3) described extraction of mouse sperm chromatin proteins. Their work confirms what was first suggested be Bellve (1) that mouse sperm chromatin contains two protamine molecules

of nearly identical electrophoretic mobility. They also found that the sulfhydryl groups of the cysteine residues contained in the protamine formed disulfide cross-links.

The evidence cited indicates the presence of cysteine in several mammalian protamines. The presence of the cysteine appears to result in a network of disulfide cross-links which seem to increase the structural stability of the condensed sperm nuclei (4). The replacement of somatic histones by protamine is a necessary event in the maturation of the spermatozoa.

Isolation of Protamine mRNA

Dixon et al. (15, 16) have done extensive experiments on isolating protamine mRNA from trout testes. They have shown that although translation of protamine mRNA occurs late in spermiogenesis, transcription takes place earlier. This means that the protamine mRNA is present in maturing germ cells 1-2 weeks before any protamine is synthesized. The protamine mRNA is therefore available for isolation and analysis.

Monesi (21) reported that during mouse spermiogenesis, RNA synthesis continues throughout spermiogenesis. Hence the mRNA must be preserved in the cell until utilized. It is reasonable to assume that protamine mRNA would be present in the developing germ cells.

MATERIALS AND METHODS

Animals Used for Protamine Analysis

Tritium ^{3}H-labeled arginine was obtained from New England Nuclear Corporation. Nine-month-old B1H male mice were injected intratesticularly with 9.0 uCi per testis of ^{3}H-arginine. Mice were sacrificed 5-12 days after injection. Three mice were killed at each of several time periods and the testes, caudal epididymides, caput epididymides, and vas deferens were removed and pooled in plastic vials, then stored in a liquid nitrogen freezer.

Recovery and Counting of Sperm Heads From Caudal Epididymides

Frozen caudal epididymides (pooled samples from three mice) were placed in 3 ml of a 10% sucrose + 0.2% sodium dodecyl sulfate (SDS) + 0.9% NaCl solution. Forceps were used to tease the tissue sample into small pieces. The samples were sonicated (Bronwill Biosonik III) after being transferred to corex tubes with an additional 3 ml of the 10% sucrose solution. The samples were then filtered through fine (200 mesh) wire cloth and centrifuged at 5000 rpm in a Beckman S-13 rotor with swinging buckets for 10 minutes at 10-15^{o}C. Pellets were washed in 5 ml of the 10% sucrose

solution and centrifuged as above. The resulting pellets were resuspended in 1 ml of a 0.9% NaCl + 0.2% SDS solution. The suspensions were then layered on top of 5 ml of a 20% sucrose + 0.9% NaCl + 0.2% SDS solution and centrifuged at 4000 rpm in a Beckman S-13 rotor for 10 min at 10-15^{o}C. This was done to remove sperm tails and debris. The remaining pellets were resuspended in 5 ml of a 0.9% NaCl + 0.2% SDS solution and centrifuged at 8000 rpm for 10 min at 10-15^{o}C. To the final pellets containing the recovered sperm heads 0.3 ml of a 0.9% NaCl + 0.2% SDS solution was added. These were the STOCK TUBES for the samples. For each sample an appropriate dilution tube for hemacytometer counting and a liquid scintillation vial were prepared. Liquid scintillation vials contained 50 ul of the sample (from the STOCK TUBE), 0.5 ml of NCS Tissue Solubilizer, and 17.5 ml of Omnifluor. Twenty-minute counts were made on a Beckman Liquid Scintillation Counter LS8100. A Pasteur pipette was used to apply a drop of the diluted sperm heads to a hemacytometer slide. A phase contrast microscope was used to count the number of sperm heads present in 10 different volumes of 0.5 mm^{3} each.

Extraction of Mouse Sperm Protamine

The samples of sperm heads remaining in the stock tubes were washed with 5 ml of saline to remove the SDS, then centrifuged at 5000 rpm for 10 min at 10-15^{o}C. The pellets were suspended in 1 ml of a 5M guanadine hydrochloride +

0.005M EDTA + 0.5M Tris (pH=8.5) solution. The samples were transferred to glass-teflon homogenizers and 50 ul of mercaptoethanol was added. The samples were then incubated in a 37^{o}C waterbath for 20 min with occasional homogenization. Homogenated samples were sonicated and 30 ul of ethylene imine (EI) was added to make the samples 0.56M EI. Again the samples were incubated and homogenized as above. The samples were brought to room temperature and 0.22 ml of a 5N HCL + 2.5M guanadine hydrochloride solution was added. The samples were then incubated for 20 min at room temperature. The samples were centrifuged for 30 min at 30K, 4^{o}C using an IEC SB405 rotor. The supernatants containing the first protamine extract were removed and stored in corex tubes in a refrigerator. The procedure was repeated to obtain second protamine extracts which were pooled with the first extracts for a final volume of approximately 1.8 ml.

Purification of Protamine Samples

Each protamine extract was applied to a Bio-gel P-6 chromatography column (27x1.5 cm) which had been equilibrated with a 0.2M acetic acid (HAc) buffer. The column was then washed with the same buffer and 20 fractions were collected, each containing approximately 2.0 ml. Appropriate fractions were pooled (usually #4-14) and applied to a Bio-Rad Cellex CM-30 Cation Exchange Cellulose Column (4.5x0.75 cm) which had been prewashed with the 0.2M HAc buffer. The column (now containing bound protamine) was washed with

50-75 ml of distilled water. Bound material was eluted with 5 ml of 0.05N HCl. The eluted samples of partially purified protamine were lyophilized in a Savant Speed Vac Concentrator (SVS-100) overnight. The lyophilized samples were redissolved in 100 ul of a 0.01N HCl + 0.025M NaCl buffer (pH=2) and applied to a Bio-gel P-10 column which had been equilibrated with the same buffer. Additional buffer was used to elute the column. Usually 40 fractions of approximately 0.25 ml each were collected. All fractions off the P-10 column were analyzed on a Beckman Du-8 Spectrophotometer for absorbance of UV light at 206 nm.

Polyacrylamide Gel Electrophoresis

Fractions off the P-10 column which showed strong absorbance and high levels of radioactivity were lyophilized. Lyophilized fractions were dissolved in a 10% sucrose + 0.188M glycine/0.188M HAc buffer. Polymerized gels (15x0.75 cm) were placed in an electrophoresis unit with a chamber buffer that was 0.188M glycine/0.188M HAc. Redissolved fractions were loaded onto the gels. Electrophoresis was performed at a constant potential of 100V for 1-2 hr. Removal of the gels was achieved by breaking the glass tube and rinsing the gels with water. Gels were stained for a minimum of 4 hr in a 0.5% solution of Coommasie Blue in 7% HAc/10% isopropanol. Destaining was done in a 7% HAc/10% isopropanol solution by diffusion. Destained gels were scanned on a Beckman Du-8 Spectrophotometer at 600 nm with visible light.

Animals for Protamine mRNA Isolation

Tritium-labeled uridine was obtained from New England Nuclear (specific activity = 37.6 Ci/mM). Eight-month-old BlH male mice were given intratesticular injections of 36 uCi per testis with 5,6 ^{3}H-uridine. Animals were sacrificed 3-9 days after injection and the testes were removed.

Extraction of mRNA

Testes (3 pairs pooled for each sample) were homogenized in a 7 ml glass-teflon homogenizer in 5 ml of pH=7.6 buffer (5mM Tris + 25mM EDTA = 75mM NaCl) on ice. Homogenized tissue samples were transferred to test tubes and centrifuged at 1400 rpm in a Beckman S-13 rotor for 20 min at 10-15^{o}C. The postnuclear supernatants were removed and centrifuged for 30 min in Beckman JA-21 rotor at 20,000 rpm (10-15^{o}C). Postmitochondrial supernatants were adjusted to 250mM NaCl + 10mM Tris + 0.5% SDS by adding an equal volume of a solution containing 425mM NaCl + 15 mM Tris + 1% SDS. In modifying the procedure some samples were adjusted to 200mM NaCl + 10mM Tris + 0.25% SDS using an equal volume of a solution that was 325mM NaCl + 15mM Tris +0.5% SDS (see Appendix for buffers).

DEAE Anion Exchange Column

Extracted samples were applied to a 9x1 cm DEAE column (see Appendix) which had been equilibrated with a 10mM Tris (pH=7.6) + 200mM NaCl buffer. The column was washed with 75-100 ml of additional equilibrating buffer. The column

was eluted with a 500mM NaCl + 10mM Tris + 0.5% SDS buffer. One-minute fractions were collected and analyzed on a Beckman Du-8 Spectrophotometer for absorbance of UV light at 260 nm against a blank containing 200mM NaCl + 10mM Tris buffer.

Oligo-Dt Column

Appropriate fractions from the DEAE column were pooled and applied to an Oligo-Dt column (2.5x0.75 cm) (see Appendix). The column had been equilibrated with a 500mM NaCl + 10mM Tris (pH=7.6) + 0.5% SDS buffer. Eluted material was collected and reapplied to the column. The column was then washed with 20 ml of equilibrating buffer minus the SDS. The column was eluted with a solution that was 0.1M NaCl + 10mM Tris. Fractions were collected and analyzed on a spectrophotometer for absorbance of UV light at 260 nm against a 0.1M NaCl + 10mM Tris buffer blank. The column was then eluted with distilled water. Fractions were collected and analyzed on a spectrophotometer for absorbance of UV light at 260 nm against a water blank.

RESULTS

Protamine Characterization

B1H mice were injected with ^{3}H-arginine. Tritium-labeled arginine was used to label the protamine. There was a suitable time interval to allow for incorporation of the label into synthesized protamine and to allow for migration of the sperm with labeled protamine to the caudal epididymus. The sperm heads were then recovered from mice sacrificed 5-12 days after injection following procedures outlined in Materials and Methods. Each sperm head sample was analyzed to determine the number of sperm heads in the sample and the amount of radioactivity present (see Tables 1 and 2). These two sets of data were then analyzed by a computer which was programmed to compute several values, most significantly, the DPM/MSP for each sample (see Table 3). When the DPM/MSP values are plotted against the number of days after injection as seen in Fig. 1, they increase sharply through day 7 and then they gradually decrease through day 12. This indicates that radioactively labeled material is present in the recovered sperm heads.

Protamine was extracted from each sperm head sample utilizing the techniques presented in Materials and Methods.

TABLE 1

Hemacytometer Counts for Sperm Head Samples Day 5-Day 12

Sperm Sample							
Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
175	125	204	144	65	128	77	101
187	143	199	153	90	109	83	93
187	143	322	141	88	129	78	92
183	139	204	144	84	136	73	112
180	124	223	153	93	130	72	84
188	125	204	149	89	128	69	102
192	143	193	141	87	136	85	93
162	133	209	140	85	119	87	90

Note: The 10 numbers are the number of sperm heads in 10 different volumes of 0.1 mm^3 each. Appropriate dilutions were made for each sample to obtain reasonable counts that were more accurate. This was taken into account in computer calculation.

TABLE 2

Liquid Scintillation Counts for Sperm Head Samples Day 5-Day 12

Sperm Sample	*CPM	**DPM
Day 5	969.50	2113.42
Day 6	3347.35	7149.43
Day 7	35273.70	73760.10
Day 8	90762.00	189791.00
Day 9	99809.05	208709.00
Day 10	29956.55	63982.70
Day 11	12623.35	26961.50
Day 12	73540.00	153780.00
Blank	0.08	0.00

*Counts per minute
**Disintegrations per minute

Note: Liquid scintillation vials contained: 0.5 ml NSC Tissue Solubilizer, 50 ul sperm sample, 17.5 ml Omnifluor. Counts were made on a Liquid Scintillation Counter LS8100.

TABLE 3

Values Calculated by a Computer Using Experimental Data

	Sperm Sample							
	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
AVE HEMO	184.250	134.375	207.250	145.625	85.125	125.875	75.500	95.875
STD DEV	5.339	8.700	10.498	5.344	8.610	9.280	6.414	8.709
MSP/LSC	9.213	8.398	20.725	21.844	25.538	7.867	4.719	28.763
STD DEV	0.267	8.544	1.050	0.802	2.583	0.580	0.401	2.613
DPM/MSP	229.408	851.281	3558.99	8688.55	8172.63	8132.85	5713.71	5346.53
STD DEV	6.869	55.246	184.57	333.04	831.53	599.85	485.71	489.24

NOTE: AVE HEMO = Average Hemacytometer Count
STD DEV = Standard Deviation
MSP = Million Sperm Heads
DPM = Disintegrations Per Minute
LSC = Liquid Scintillation Counts

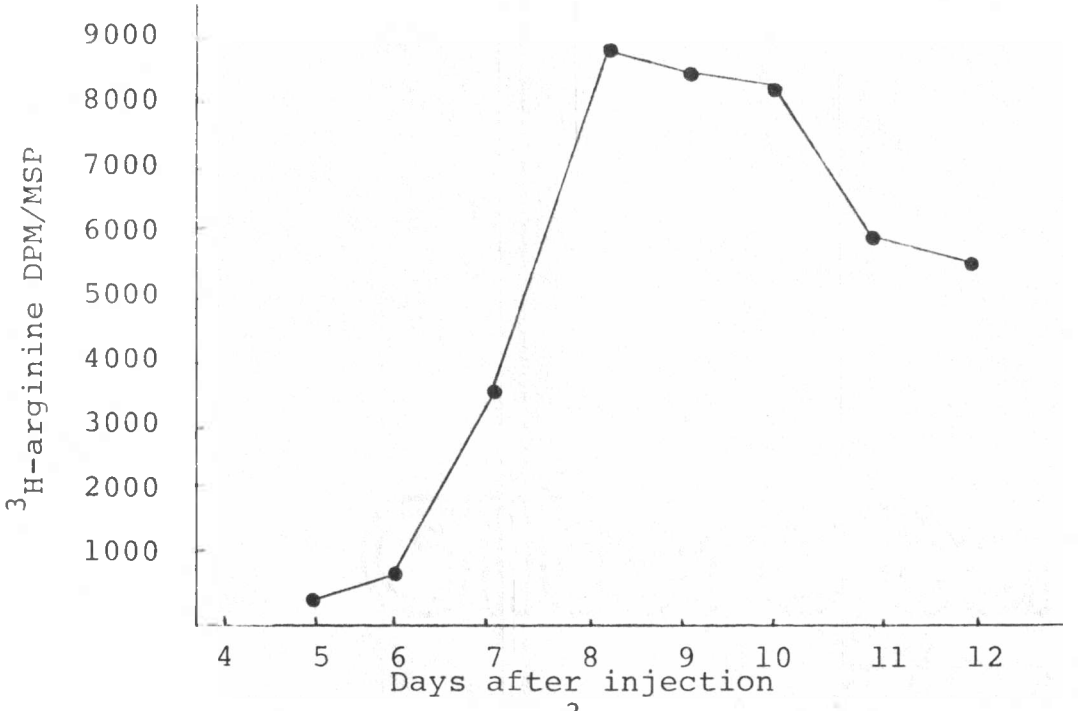


Fig. 1. Caudal sperm ^{3}H-arginine DPM/MSP plotted for each time point studied.

The final step in purification was to apply each sample to a Bio-gel P-10 column. Fractions were collected for each protamine sample. All fractions were analyzed on a spectrophotometer for absorbance of UV light at 206 nm. Plots were then made for each sample of the absorbance of each fraction (in Optical Density/OD units) against the fraction number. Characteristically the plots revealed a peak of absorbance beginning with fraction #10 and ending with fraction #20, and a lesser peak beginning with fraction #20 and ending with #35. The second peak corresponded to the eluted protamine.

Samples from fractions relating to the second peak were subjected to LSC. The DPM of each fraction was also plotted against the fraction number. Caudal protamine samples from days 7 and 8 showed the greatest activity. Therefore the plots from these two samples are shown in Figs. 2 and 3.

Selected fractions containing protamine were lyophilized then redissolved in 0.188M glycine/0.188M HAc buffer, and analyzed using gel electrophoresis. The procedures described in Materials and Methods were followed. After staining and destaining, two stained bands were visible in the gels. The faster migrating band was darker and slightly wider than the slower migrating band. Spectrophotometer scans of the gels at 600 nm with visible light recorded single peaks (Fig. 4). Apparently the bands were not separated enough for the spectrophotometer to record them as individual peaks.

Isolation of Protamine mRNA

BlH mice were injected intratesticularly with tritium-labeled uridine. The radioactive uridine was used to label the mRNA. After a suitable time interval to allow for incorporation of the label, the mice were sacrificed and their testes removed. Three pairs of testes were pooled for each sample day after injection. The pooled testes were homogenized and mRNA extracted according to procedures outlined in Materials and Methods. The post-mitochondrial supernatant of each sample was applied to a DEAE Cellulose Anion Exchange Column as described in Materials and Methods. The DEAE column binds

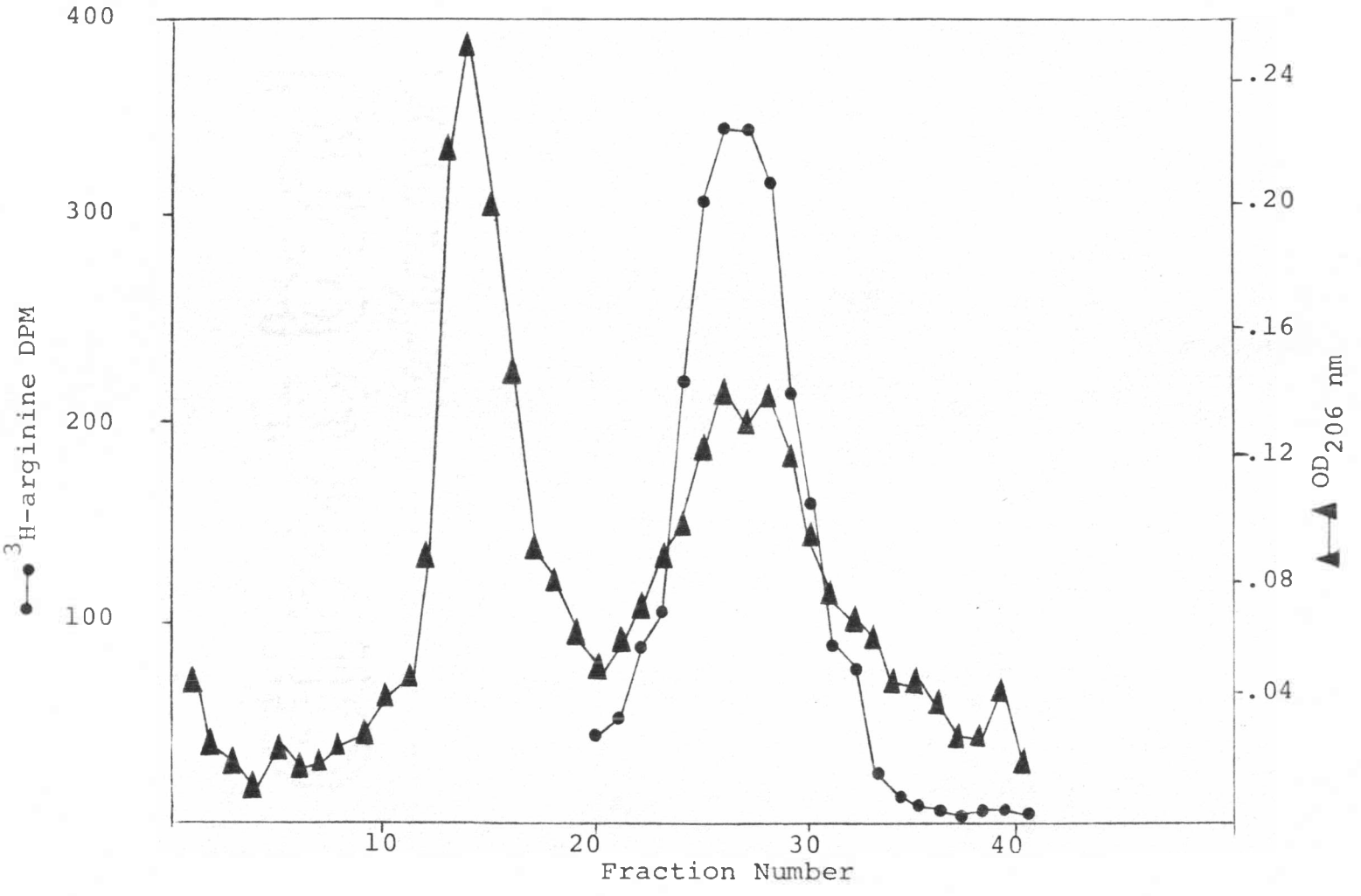


Fig. 2. Day 7 sample caudal protamine: analysis of fractions from the P-10 column.

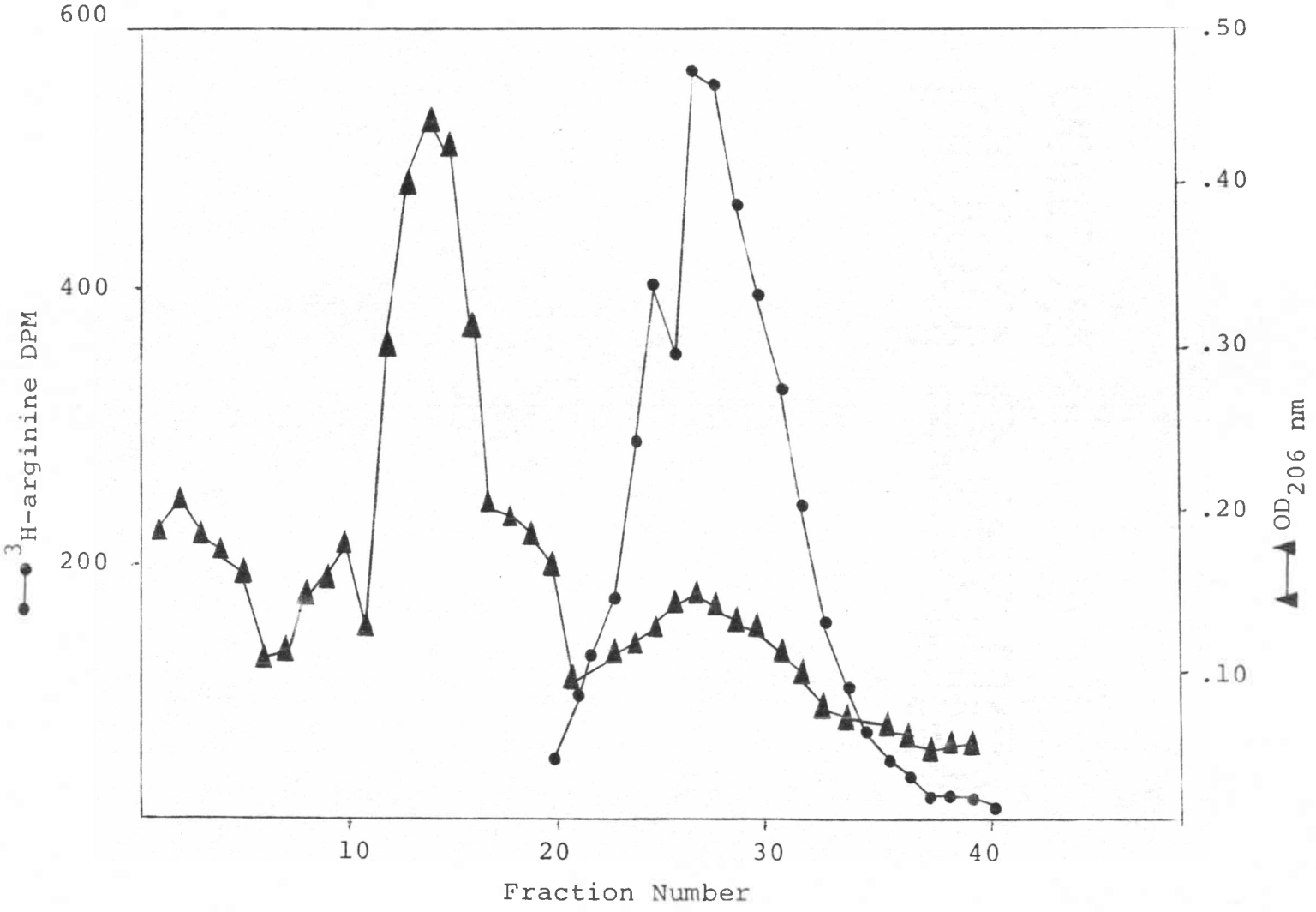
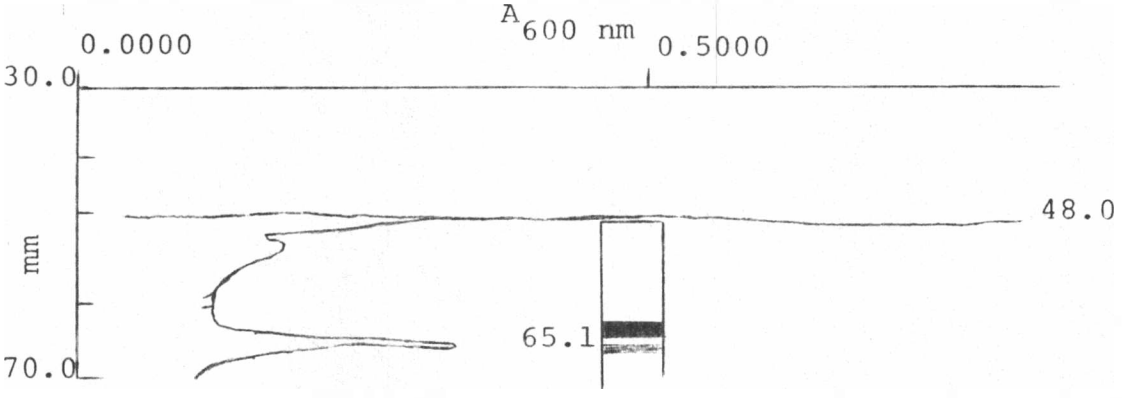
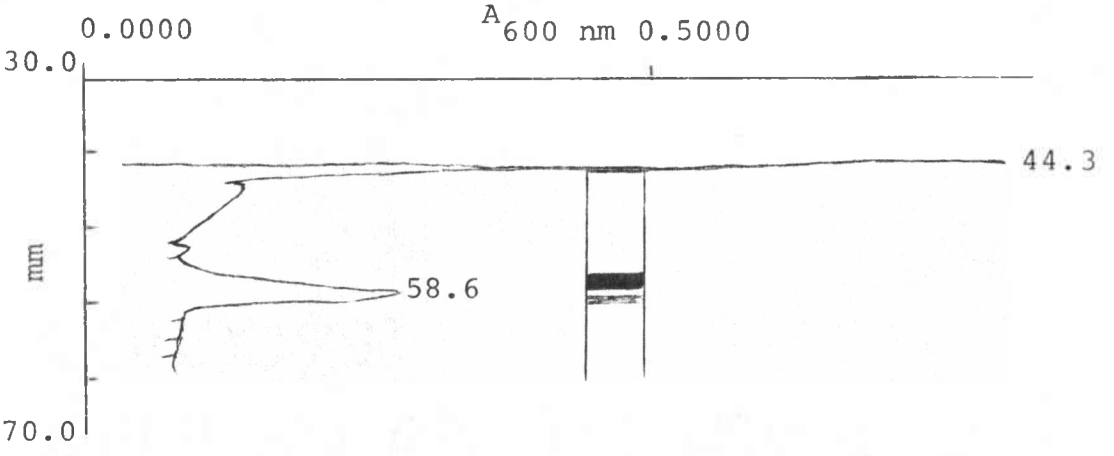


Fig. 3. Day 8 sample caudal protamine: analysis of fractions from the P-10 column.



NOTE: Gel contained fraction #29 from P-10 column. Stained band migrated 17.1 mm.



NOTE: Gel contained fraction #31 from Bio-gel P-10 column. Stained band containing protamine migrated 17.3 mm from the gel end.

Fig. 4. Spectrophotometer scans of gels with protamine bands from the analysis of selected fractions from the P-10 column (Day 7 sample). Gels were scanned at 600 nm with visible light. Only the top portion of the gels which contained the protamine bands was scanned. The first sharp peak indicates the beginning of the gel. The second peak corresponds to the stained band in the gel. Included are diagrammatic representations of the protamine extracted from the sperm heads and analyzed by gel electrophoresis.

negatively charged species, therefore RNA will bind to it. Eluted material from the column was collected in fractions which were analyzed for absorbance of UV light at 206 nm. The absorbance of each fraction was plotted against the fraction number to obtain an absorbance plot. Selected fractions were also sampled for liquid scintillation counting. The results indicated that radioactive material was not present in the eluted material, and hence, RNA was not binding to the DEAE column. A different brand of cellulose was prepared (see Appendix) and more fractions were collected for each sample. The resulting absorbance plots and data from LSC indicated improved recovery of RNA (Fig. 5).

The eluted material from the DEAE column contained other nucleic acids. An Oligo-Dt column was utilized to separate the mRNA from the other material. The Oligo-Dt column binds poly-adenylate (Poly-A) sequences. Most mRNA species have Poly-A sequences at the 3'-terminus and hence will selectively bind to the column. Fractions from the DEAE column having a high absorbance were pooled and applied to an Oligo-Dt column following the procedures outlined in Materials and Methods. Analysis of eluted material from the Oligo-DT column did not detect any RNA.

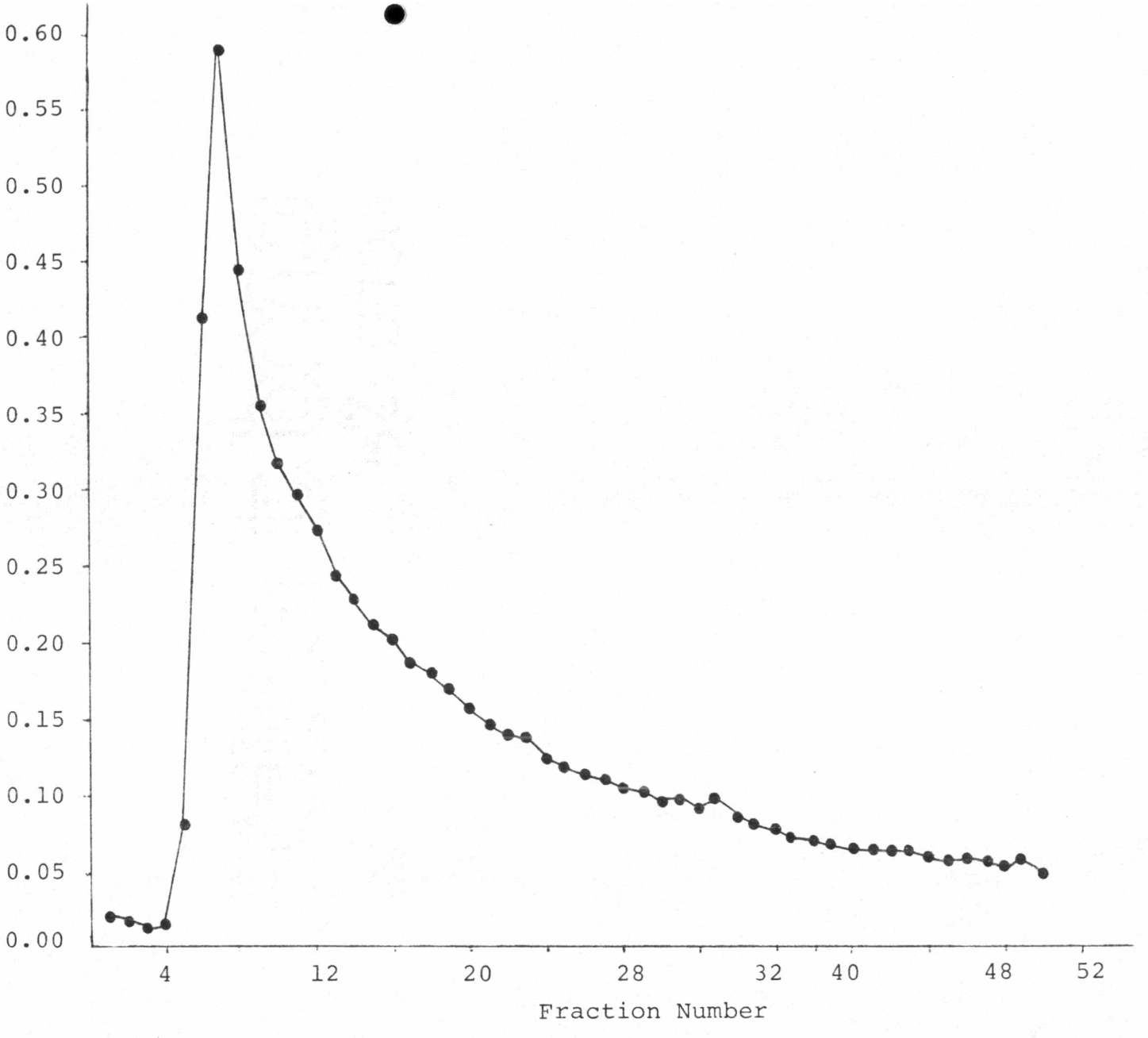


Fig. 5. Absorbance plot for fractions eluted from DEAE column (Day 9 Sample). The column was eluted with a 500mM NaCl + 10mM Tris + 5% SDS buffer. Fifty 1-min fractions were collected and were analyzed for absorbance of UV light at 260 nm against a blank that contained a 200mM NaCl + 10mM Tris buffer.

DISCUSSION

Each sperm sample was obtained from three pairs of caudal epididymides taken from male BlH mice. The purpose of pooling several pairs was twofold: 1) to obtain a greater number of sperm heads and hence more protamine, 2) to offset the chance that one mouse may have had an abnormally low number of sperm. The caudal epididymides were used as a source because they contain almost exclusively mature sperm. This would lessen the problem of recovering sperm in various stages of development.

Protamine is rich in arginine unlike most other proteins. Therefore ^{3}H-arginine was an appropriate label to use to indicate the presence of protamine in sperm samples that were analyzed. Appreciable amounts of radioactivity were detected in sperm samples taken 7-12 days after injection (Fig. 1). It was necessary to calculate the DPM/MSP for each sample for a more equal comparison of the amount of radioactivity observed in individual samples. This illustrates that there is a time element involved as the protamine is synthesized and incorporated into the chromosomal structure of the maturing sperm.

The methods used to extract the protamine were chosen because of the disulfide cross-links formed by the sulfhydryl

groups of the cysteine residues. Beta-mercaptoethanol causes the lysis of the sperm heads and the breakage of the disulfide bonds. Sonication aids this process. Ethylene imine is added to alkylate the exposed sulfhydryl groups and prevent the reformation of disulfide bonds. These procedures release the protamine from the sperm heads.

Once extracted the protamine sample is purified by using a series of columns. The Bio-gel P-6 chromatographic column separates molecules on the basis of size, hence salts are removed from the protamine sample. The eluent from the P-6 column was monitored at 206 nm to detect the presence of materials that show absorbance at this wavelength. A wavelength of 206 nm was used because protamine is poor in aromatic amino acids and does not absorb well at 280 nm. This characteristic absorbance was another means of detecting the presence of protamine throughout the purification procedures.

Appropriate fractions showing absorbance were pooled and applied to a Cellex CM-30 column which separates on the basis of charge. Basic species bind to the material in the column. Protamine is basic and will bind to the column while other acidic and neutral molecules are eluted from the column.

The protamine eluted from the CM-30 column is applied to a Bio-gel P-10 column that also separates molecules on the basis of size. Fractions collected from the P-10 column were analyzed for absorbance at 206 nm to detect the presence of protamine. Absorbance plots were then made for each

protamine sample (Figs. 2 and 3). The absorbance plots reveal two peaks. The first is not protamine, but some other absorbing species. The second peak corresponds to the fractions containing protamine. This is further verified when samples of fractions in this region are taken for liquid scintillation counting. As is seen in Figs. 2 and 3, the fractions showing absorbance (2nd peak) also contain higher amounts of radioactivity.

From the absorbance plot and the plot of disintegrations per min we determined which fractions contained protamine. It is our hypothesis that the beginning fractions of the second peak correspond to the heavier protamine component since it would be less retarded by the column whereas the lighter protamine component moves more slowly through the column and, therefore, corresponds to the later fractions of the peak. By analyzing individual fractions using gel electrophoresis, we hoped to further separate the lighter protamine component from the heavier one based on a difference in the migration rate of the two components. Fractions included in the second peak that were applied to acrylamide gels and allowed to migrate did leave definite bands in the gels which were visible after staining and destaining (Fig. 4). The slower migrating band containing the heavier protamine component stained darker than the faster migrating band. Since the degree of staining is proportional to the amount of substance present to be stained, this indicates that there is more of the heavier protamine present.

Although two bands could be detected visibly, spectrophotometer scans of the gels recorded only a single peak. Evidently the bands were not distinct enough to be distinguished by spectrophotometer gel scans which are needed to quantitate the amount of protamine.

It was possible to cut the darker, slower migrating band out of the gel without including the faster migrating one. We did preliminary work using 70% perchloric acid and 30% hydrogen peroxide to digest the gel slices which were then prepared for liquid scintillation counting. Counts from the slices were phenomenally high--apparently the perchloric acid was responsible for raising the background counts. Neutralization of the perchloric acid with 10N sodium hydroxide and small amounts of beta-mercaptoethanol brought the background counts down to a more reasonable level.

From the results obtained using gel electrophoresis to separate the two protamine components, we concluded that gel electrophoresis alone is not a satisfactory method for resolving the two components. In combination with the P-10 column gel electrophoresis does offer a method of further separating the protamine components for more accurate LSC. Further work is needed to improve procedures for digesting the gel slices. Another possible method for separating the two protamine components would be a High Power Liquid Chromatography System.

The work we did on isolating protamine mRNA was preliminary. We were developing a procedure for isolating protamine

mRNA based on methods employed by Dixon et al. (15). Because of this procedures were modified throughout the experiment. As was noted under Results, we had initial failures with the DEAE column; however, preparing a different brand of cellulose and collecting more fractions seemed to improve recovery of RNA.

Work with the Oligo-Dt column was less successful. We did not detect labeled material (i.e., RNA) in the eluent. Possible explanations for this are: 1) our procedures were incorrect; 2) not enough mRNA was present in the samples to be detected; 3) mouse protamine mRNA does not have a Poly-A sequence. Further steps in isolating protamine mRNA include testing the Oligo-Dt column with known Poly-A sequences, and using an alternative procedure to extract the RNA such as a phenol/chloroform extraction.

In conclusion the isolation of protamine and the recovery of its message offer a fruitful area of study. Much can be learned by studying the control mechanisms for translation of mRNA and by studying the interactions of chemical mutagens with protamine and its message.

APPENDIX

12% Acrylamide Gels

11.700 g acrylamide
 0.200 g N,N' methylenebis acrylamide
99.0 ml 0.188M glycine/0.188M acetic acid buffer
40.0 l tetramethylethylendiamine (TEMED)
 0.250 g ammonium peroxydisulfate in 10 ml glycine/HAc buffer
20 clean glass tubes sealed at one end with parafilm (3 layers)

Dissolve the acrylamide and bis-acrylamide in the buffer. Stir with a stirring bar for 15 min, then add the TEMED to make the solution 0.40% TEMED. Filter the solution and deaerate for 10 min. Add 1 ml ammonium peroxysulfate solution to make the solution 0.250% ammonium peroxysulfate. Fill clean tubes with approximately 2.6 ml acrylamide solution. Layer water saturated butanol on the surface using a Pasteur pipette to level the surface. Let stand exposed to light for 24 hr to polymerize. Run buffer through gels in electrophoresis unit before using.

DEAE Column

1. Suspend 10 g diethylaminoethyl (DEAE-Cellex D) in 500 ml of a solution that is 0.25M NaOH for 30 min. Filter the solution and rinse the filtrate with 2 l of deionized water.

2. Suspend the filtrate in 500 ml of a 0.25M HCl solution. Let stand, then decant the supernate. Add distilled water, stir, decant the water, repeat water wash.

3. Repeat Step 1.

4. Suspend filtrate in equilibrating buffer and load column.

Tris Buffers

Dissolve 1211.4 g Tris/HCl in 1000 ml of distilled water and adjust pH to 7.6 using dilute HCl. Add appropriate amounts of NaCl and/or SDS to make specific buffers.

LITERATURE CITED

1. Anderson, E., Bellve, A. R., and Hanley-Bowdoin, L. 1975. Synthesis and amino acid composition of basic proteins in mammalian sperm nuclei. Devel. Bio. 47: 349-365.

2. Ando, T., Suzuki, K., and Yamasaki, M. 1973. Protamines. Springer-Verlag, New York.

3. Balhorn, R., Gledhill, B. L., and Wyrobeck, A. J. 1979. Mouse sperm chromatin proteins: quantitative isolation and partial characterization. Biochem. 16: 4074-4079.

4. Bedford, J. M., and Calvin, H. I. 1971. Formation of disulfide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymus. J. Repro. Fert. Suppl. 13: 65-75.

5. Block, D. P. 1963. Genetic implications of histone behavior. J. Cell. Comp. Phys. 62 suppl. 1: 89-97.

6. Bruce, W. R., and Lam, D. M. K. 1971. The biosynthesis of protamines during spermiogenesis of the mouse: extraction, partial characterization, and site of synthesis. J. Cell. Phys. 78: 13-24.

7. Busch, H. 1965. Histones and other nuclear proteins. Academic Press, New York.

8. Coelingh, Jan P. et al. The complete amino acid sequence of the basic nuclear protein of bull spermatozoa. Biochim. et Biophys. Acta 285: 1-14.

9. Davis, J. R. 1970. The testis. Academic Press, New York.

10. Dixon, G. H., Ling, V., and Marushige, K. 1969. Phosphorylation of chromosomal basic proteins in maturing trout testis. J. Biol. Chem. 244: 5953-5958.

11. Dixon, G. H., and Ingles, C. J. 1967. Proc. Nat. Acad. USA 58: 1011-1018.

12. Dixon, G. H., and Marushige, K. 1969a. Develop. Biol. 397-414.

13. Dixon, G. H., Ling, V., and Marushige, K. 1969b. J. Biol. Chem. 244: 5953-5958.

14. Dixon, G. H., and Marushige, K. 1971. Transformation of trout testis chromatin. J. Biol. Chem. 246: 5799-6008.

15. Dixon, G. H., Gedamu, L., and Iastrou, K. 1978a. A simple procedure for the isolation and purification of protamine messenger ribonucleic acid from trout testis. J. of Biochem. 171: 589-599.

16. Dixon, G. H., Iatrou, K., and Spira, A. W. 1978. Protamine messenger ribonucleic acid: evidence for early synthesis and accumulation during spermatogenesis in rainbow trout. Devel. Biol. 64: 82-98.

17. Geroch, M. E. et al. Specific basic proteins from mammalian testis. J. Biol. Chem. 248: 4532-4543.

18. Gledhill, B. L., Gledhill, M. P., Ringertz, N. R., and Rigler, R., Jr. 1966. Changes in spermiogenesis in the bull. Exp. Cell. Res. 41: 652-665.

19. Gledhill, B. L. 1971. Changes in deoxyribonucleoprotein in relation to spermateliosis and the epididymal maturation of spermatozoa. J. Reprod. Fert. Suppl. 13: 77-78.

20. Marushige, K., and Marushige, Y. Transformation of sperm histone during formation and maturation of rat spermatozoa. J. Biol. Chem. 250: 39-45.

21. Monesi, V. 1965. Synthetic activities during spermiogenesis in the mouse. Expt. Cell. Res. 39: 197-222.

22. Reid, B. L. 1977. Protamines in the sperm heads of inbread and outbread mice. J. Reprod. Fert. 49: 169-171.

23. Sega, G. A. 1974. Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate. Proc. Natl. Acad. Sci. USA 71: 4955-4959.

24. Sega, G. A., Cumming, R. B., and Walton, M. F. 1974. Dosimetry studies on the ethylation of mouse sperm DNA in vivo exposure to ^{3}H-ethyl methanesulfonate. Mutation Res. 24: 317-333.

25. Sega, G. A., Owens, J. G., and Cumming, R. B. 1976. Studies on DNA repair in early spermatid stages of male mice after in vivo treatment with methyl-, ethyl-, propyl-, and isopropyl methanesulfonate. Mutation Res. 26: 193-212.

26. Sega, G. A., and Owens, J. G. 1978. Ethylation of DNA and protamine by ethyl methanesulfonate in the germ cells of male mice and the relevancy of these molecular targets to the induction of dominant lethals. Mutation Res. 52: 87-106.