UAB LIBRARIES
The University of Alabama at Birmingham

University of Alabama at Birmingham
UAB Digital Commons

All ETDs from UAB

UAB Theses & Dissertations

1984

Characteristics Of Sea Urchin Sperm Chromatin (Nucleus).

Naama Abdulgader Aboukarsh
University of Alabama at Birmingham

Follow this and additional works at: https://digitalcommons.library.uab.edu/etd-collection

Recommended Citation

Aboukarsh, Naama Abdulgader, "Characteristics Of Sea Urchin Sperm Chromatin (Nucleus)." (1984). *All ETDs from UAB*. 4218.
https://digitalcommons.library.uab.edu/etd-collection/4218

This content has been accepted for inclusion by an authorized administrator of the UAB Digital Commons, and is provided as a free open access item. All inquiries regarding this item or the UAB Digital Commons should be directed to the UAB Libraries Office of Scholarly Communication.

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106

8426737

Aboukarsh, Naama Abdulgader

CHARACTERISTICS OF SEA URCHIN SPERM CHROMATIN

The University of Alabama in Birmingham PH.D. 1984

University
Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1984
by
Aboukarsh, Naama Abdulgader
All Rights Reserved

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages __√__
2. Colored illustrations, paper or print _____
3. Photographs with dark background __√__
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages __√__
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) __________ lacking when material received, and not available from school or author.
12. Page(s) __________ seem to be missing in numbering only as text follows.
13. Two pages numbered __________. Text follows.
14. Curling and wrinkled pages _____
15. Other ____

University
Microfilms
International

Characteristics of
Sea Urchin Sperm Chromatin

by

Naama Abdulgader Aboukarsh

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Anatomy in The Graduate School,
University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1984

Copyright by

Naama Abdulgader Aboukarsh

1984

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Ph.D. Major Subject Anatomy

Name of Candidate Naama Abdulgader Aboukarsh

Title Characteristics of Sea Urchin Sperm Chromatin

The techniques of chromatin spreading, thin sectioning, and high resolution scanning electron microscopy were used on swollen and lysed sea urchin sperm nuclei to examine the morphological characteristics of chromatin. Isolated nuclei were lysed in low ionic strength buffer (1mM EDTA pH 7.5) and examined by electron microscopy to demonstrate dense aggregates of non-dispersed material and beaded filaments radiating from these aggregates. These beaded fibers are similar in size and appearance to the "beads-on-a-string" characteristic of chromatin of numerous interphase nuclei. The beads are nucleosomes which have an average diameter of 100Å. The interconnecting string is composed of DNA and chromosomal proteins ranging in size from 20Å to 25Å, and this corresponds to internucleosomal spacer DNA. In thin sections of swollen nuclei the sperm chromatin appears to be composed of 414Å spherical structures, (superbeads) which are closely apposed to form structures that appear to be 414Å-diameter rods. As the chromatin disperses, the superbeads are seen to be attached to one another by chromatin fibers of 100Å diameter. This interconnecting material is seen to be formed by the close association of nucleosomes (100Å diameter). In thin sections, the 414Å superbeads disperse directly into the 100Å fibers with no intervening structures. Experiments were carried out in an attempt to isolate superbeads: After

brief digestion of swollen nuclei with DNase I and centrifugation through 10%-30% linear sucrose gradients, fractions from the gradients were analyzed by chromatin spreading techniques to demonstrate that the superbeads are morphologically similar to those seen in thin sections. Polyacrylamide gel electrophoresis of the proteins extracted from both sperm nuclei and isolated superbeads indicated that the groups of proteins similar in both preparations were 58, 59, and 68 Kd. Using scanning electron microscopy, initial events of sperm nuclear lysis and chromatin dispersion were seen, demonstrating that even with this low resolution technique the organization of superbeads may be seen as a 414Å chromatin fiber.

Abstract Approved by: Committee Chairman ____________

Program Director ____________

Date 5/20/84 Dean of Graduate School ____________

ACKNOWLEDGEMENTS

I wish to express very special thanks to my graduate committee chairman, Dr. Mel Kunkle, for his support and assistance in all phases of my dissertation preparation.

I also wish to express my deepest appreciation to the members of my committee, Dr. Earl Hamel, Jr., Dr. George Hand, Dr. Wayne Finley, and Dr. Gary Poirer, for their continual support, the advice, and their valuable time they have given to me during the preparation of this work.

I wish to thank Roberta J. Meeks who helped me out in several technical problems, Sandy Silvers for scanning electron microscopy, Ralph Roseman and Larry Shory for their neat photography work.

I also thank the Anatomy Department faculty and graduate students for their encouragement and moral support.

The assistance of Carol McNeeley who provided the time and skill in typing this dissertation proved to be of great help.

Finally, I would like to offer a special word of gratitude to my husband, Senussi Sunni, M.D., for his encouragement and support and my children, Nadia, Nabeel, and Muna for their patience and understanding.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

INTRODUCTION 1

Chromatin Structure 2

Morphology of the Spermatozoon 6

The Sperm Nucleus 7

OBJECTIVES OF THE STUDY 10

MATERIALS AND METHODS 11

Isolation of Sperm Nuclei 11

Nuclear Swelling and Chromatin Spreading 13

Transmission Electron Microscopy (TEM) 13

Scanning Electron Microscopy (SEM) 14

Isolation of the 400Å units 14

Protein Extraction 15

Sodium Dodecylsulfate (SDS)-Gel Electrophoresis 16

Electron Microscopy of Different Fractions of Sucrose Gradients 17

RESULTS 18

Thin Sections of Sperm Nuclei 18

Chromatin Spreading 22

Scanning Electron Microscopy 24

Isolation of Superbeads from Digested Sperm Nuclei 25

DISCUSSION 30

Scanning Electron Microscopy 33

Transmission Electron Microscopy 35

Isolation of Superbeads 37

Relationship to the Events of Fertilization 38

REFERENCES 41

FIGURES 50

APPENDIX 111

LIST OF TABLES

1. Isolation of Sea Urchin Sperm Nuclei 12

2. Measurements of Sea Urchin Sperm Nuclear Chromatin by the Use of Different Techniques 112

3. Summary of Polypeptides in Gels 113

LIST OF FIGURES

1. Schematic diagram of mature spermatozoon of sea urchin *Strongylocentrotus purpuratus*. The head is almost entirely occupied by the sperm nucleus. The neck is a small constriction between the head and the tail 51

2. Photomicrograph of longitudinal section of mature sea urchin (*Strongylocentrotus purpuratus*) sperm which was not treated with either Triton X-100 or low ionic strength buffer (1mM EDTA). This figure demonstrates the different components of the mature spermatzaoon. The sperm head appears to be occupied almost entirely by the nucleus (SN). The latter is composed of tightly packed, darkly stained, electron dense chromatin (heterochromatin). The whole cell (spermatozoon) is surrounded by plasmalemma (P), while the nucleus is surrounded by the nuclear envelope (NE). The centriolar fossa (CF), the acrosome (A), the acrosomal fossa (AF), the sperm mitochrondria (SM) and axoneme (T) are apparent in this figure. This picture was taken by JEOL-GX100. X50,000 53

3. Photomicrograph of a longitudinal section of a sea urchin sperm nucleus in a control experiment which was treated with Triton X-100 in the process of nuclear isolation and purification. This nucleus was not allowed to swell since no EDTA was used. The nucleus (SN) is occupied entirely by the tightly packed, densely stained heterochromatin. Although heterochromatin appears uniformly granular, no definable substructures could be measured. No remnants of the nuclear envelope may be seen in this micrograph along the sides of the nucleus. X90,750 55

4. Electron micrograph showing a longitudinal section of sperm nucleus of sea urchin (*Strongylocentrotus purpuratus*). The nuclei were incubated only for 10 minutes in 1mM EDTA. The nuclei were fixed in 2.5% gluteraldehyde for 2 hours, post-fixed with 1% OSO_4 then cut into silver section (600-900Å) using MT-2B (Sorvall) microtomes. Sections were stained with 2% uranylacetate in 50% ethanol and Raynold's lead citrate.
The nuclear chromatin appears to be formed of discrete 400-500Å thick filaments (arrows). These

are loosely arranged in the areas around acrosomal fossa (AF) and around the poles of the centriolar fossa (CF). The 400Å thick filaments appear tightly packed in the middle region of the nucleus where it still has remnants of the nuclear envelope (NE-arrowheads). However, at this early stage of lysis, it is difficult to visualize the substructure of the 400Å filaments. X66,625 57

5. Electron micrograph of cross section of sea urchin sperm nucleus. The nuclei were incubated in 1mM EDTA for 30 minutes. The chromatin appears uniformly distributed throughout this section, and is formed of 400-500Å thick filaments. Most of the filaments have spiral courses (arrowheads) and are composed of discrete spherical structures (arrows) having diameters of 400-500Å which correspond to the diameters of the thick filaments. X77,000 59

6. Photomicrograph of sperm nucleus which was incubated in 1mM EDTA for 30 minutes, then processed for thin sectioning. In this section the 400-500Å filaments appear to be composed of 400-500Å spherical aggregates which are clearly seen (arrowheads) to be arranged as tandem. The filaments could be followed to variable distances. Several observations should be made in this section. First, there is better dispersion of the spherical units in the areas depleted of nuclear envelope (*). Second, there are areas at the periphery (arrows) which did not disperse to the corresponding stage of the rest of the chromatin. Third, this section was prepared from a sample which was less purified than is normally done, so several portions of axonemes (T) can be seen in the field. X71,000 61

7. Electron micrograph of sea urchin sperm nucleus at centriolar fossa level. The nucleus was incubated in 1mM EDTA for 60 minutes. The 400-500Å filaments are composed of discrete 400-500Å spherical units (arrowheads) which are well separated and interconnected by thin strands (arrows). In this plate there are some non-lysed sperm nuclei (NSN) which appear not to be affected by the process of lysis. Portions of the axoneme (T) are seen in the centriolar fossa (CF). X76,250 63

8. Photomicrograph of a survey view of sperm nuclei which have been incubated in 1mM EDTA for 90 minutes. Most of the chromatin appears in the form of well separated, discrete spherical granules (superbeads) of 414Å ± 60Å in diameter (arrowheads). These 400Å granules appear to be composed of very small circular subunits which are difficult to measure at

this stage. Thin strings connecting superbeads are seen at arrows. Scattered membranous vesicles (V) and occasional portions of axonemes (T) are seen in the lower left section of the photomicrograph. X62,000 65

9. Photomicrograph of sperm nuclei which were incubated in 1mM EDTA for 120 minutes. The chromatin appears to be composed of irregular spherical clusters (superbeads; *) of asterisks 414Å ± 60Å in diameter. These clusters appear closely packed in some areas (arrowheads) while loosely arranged in others and interconnected by 112Å fibers. These appear to be composed of closely packed smaller circular units of the same diameter chromatin filaments (arrows). The 400Å units appear not to have central holes, but they are composed of smaller circular subunits around 100Å in diameter. The 112Å units (nucleosomes) electron lucent central area of an average diameter of 30Å. Portion of the axoneme (T) is seen in the centriolar fossa (CF). X96,000 67

10. Photomicrograph of sea urchin sperm nuclei which were allowed to swell in 1mM EDTA for 120 minutes. The nuclear chromatin appears to be formed of superbeads (arrowheads) interconnected by 112Å chromatin fibers (arrows). The 112Å units are clearly formed of nucleosomes (112Å in diameter) and having a central electron lucent area of 30Å in diameter. X225,000 69

11. Stereomicrograph showing portions of sea urchin sperm nuclei lysed in 1mM EDTA for 90 minutes. Fixed in 2.5% gluteraldehyde for 2 hours, postfixed with 2% OSO_4, dehydrated, embedded in Epon 812, golden sectioned (900-1000Å) then stained with 2% alcoholic uranyl acetate and lead citrate. A - was taken at a tilt of +3°. B - was taken at a tilt of -3°. In both sections A and B, the chromatin appears to be formed of clusters (superbeads; arrowheads) roughly spherical variable in size and loosely arranged. The superbeads are composed of smaller circular subunits and interconnected by thin strings. At the right upper border of the lysed nuclei, part of the chromatin is still in the condensed state. Portions of the axonemes (T) and non-lysed sperm nuclei (NSN) are seen in this stereopair. By using the stereoscopic viewer, one can notice the three dimensional build up of the superbeads, their relation to each other and to the 100Å interconnecting fibers. X99,000 71

12. Photomicrograph illustrates stereopairs of sea urchin sperm nuclei which were allowed to swell in 1mM EDTA for 90 minutes, then were processed for EM golden

sections. The plate (A) was taken at 0 tilt, while plate (B) was taken at -5°. By the use of stereoscopic viewer one can see the three dimensional construction of superbeads (arrowheads) interconnected with thin chromatin strings (arrows). Portions of non-lysed sperm nuclei (NSN), and the nuclear envelope (NE) are seen in this plate. X79,000 73

13. Photomicrograph showing chromatin fibers "beads-on-a-string" configuration from sea urchin sperm nucleus which were lysed in 1mM EDTA pH 7.5 for 4 hours. The chromatin was then centrifuged onto formvar-carbon coated grids through paraformaldehyde-(3.7)-sucrose (3.4) cushion. The grids were washed with 1% phot-Flo, air dried and then were stained with 1% uranylacetate containing 2mM $MgCl_2$. The beads have diameters of 120Å (arrowheads) and spacer DNA (arrows) has a diameter of ~33Å. Note that the contrast of the beads is not very evident. X38,000 75

14. Chromatin spread of sea urchin sperm nuclei which were allowed to disperse for 4 hours in 1mM EDTA pH 7.5. The chromatin material was centrifuged onto formvar-carbon coated grids through paraformaldehyde-sucrose cushion (fixative). The grids were washed with 1% photoflo, dehydrated with 95% ethanol, then positively stained with 1% PTA. To increase the contrast of the beads, the grids were coated with platinum-palladium (80:20) at an angle of 6°. The beads (nucleosomes) appear very contrasting with a diameter of 132Å ± 22Å (arrowheads). The nucleosomes are separated by thin strings 38Å ± 9Å which correspond to spacer DNA. However the spacer DNA is of different length depending on the various forces imposed on the individual chromatin fiber. At the arrows, one can see the maximal stretch of spacer DNA around 200Å long. On the right upper corner of this figure, chromatin mass (Ch) which was not adequately dispersed. X28,600 77

15. Photomicrograph of chromatin spread of sea urchin sperm nuclei. The nuclear sample was allowed to lyse for 4 hours, then centrifuged onto the electron microscope grids through the fixative. The grids were coated with platinum-palladium (80:20) at an angle of 6 degrees. The nucleosomes (arrowheads) appear very contrasting with diameters of 132Å ± 22Å. The length of internucleosomal DNA is variable and appears maximal at arrow where the chromatin strings cross each other. The longest internucleosomal distance was around 200Å. X42,000 79

16. Electron microscopic plate showing chromatin spread of a 3-houred lysed sea urchin sperm nuclei. The chromatin spread was fixed and then negatively stained with 2% PTA for 2 minutes. The granules have ill defined borders and are of variable sizes ranging from 140Å (arrows) to 260Å (arrowheads). X230,000 81

17. A scanning electron micrograph showing a sperm nucleus which lysed in 1mM EDTA for 10 minutes, was centrifuged onto formvar-carbon coated grids through sucrose-paraformaldehyde cushion, critical-point dried and then sputter coated with gold. A loop of chromatin fiber appeared budding off at a certain spot (arrows) on the nucleus. Fibers of the extruding loop are of 500Å in diameter. The acrosomal fossa (AF) and the centriolar fossa (CF) are seen in this figure. X80,000 83

18. A high resolution scanning micrograph of sea urchin sperm nucleus which was lysed in 1mM EDTA, pH 7.5 for 20 minutes. This micrograph shows a chromatin fiber (arrowhead) spilled out of the nucleus. The general appearance of the cell has ill-defined nodules (arrow). The acrosome (A) and portion of the axoneme (T) projecting out of the centriolar fossa (CF) are also seen. X96,000 85

19. Scanning electron micrograph demonstrating a sea urchin sperm nucleus which was allowed to swell in low ionic strength buffer (1mM EDTA) for 30 minutes. The surface of the nucleus in general appears extensively convoluted with chromatin fibers having diameter of 400-500Å (arrows). X71,000 87

20. Scanning micrograph shows the posterior view of sea urchin sperm nucleus with portion of the axoneme (T) merging off the centriolar fossa. The nucleus was lysed for 60 minutes. In this figure numerous beaded chromatin fibers (arrowheads) of 400-500Å in diameter are very evident. X100,000 89

21. Photomicrograph of three sea urchin sperm nuclei as seen by high resolution SEM after 90 min lysis. The surface of nuclei appears highly convoluted. Chromatin fibers (arrows) of 400-500Å in diameter are seen coming out of the sperm nucleus labeled - II, while it is not ascertained the source of the rest of chromatin fibers seen in the field. X38,000 91

22. Scanning photomicrograph of sea urchin sperm nuclei after 120 minutes lysis in 1mM EDTA. The nuclear surface appears highly convoluted with deep

depressions and loosely arranged, interlacing chromatin fibers. These fibers appear uneven in thickness, some radiate from the nuclear mass in a way that makes it difficult to relate the fibers to one nucleus or another. The fibers are highly variable in thickness ranging from 200Å (arrowheads) to 700Å (arrows). The thicker fibers appear to be composed of merging of several smaller fibers. X35,000 93

23. The typical absorbance profile of sea urchin sperm nuclear chromatin. The nuclei were allowed to swell in 1mM EDTA for 2 hours, then were briefly digested with DNase I. The digest was layered on 10-30% linear sucrose gradient and centrifuged overnight (16 hours). The gradient was mechanically fractionated at an absorbance of 260 and yielded twenty six tubes. The superbeads were predominant at the area between tubes no. 6-10. The first arrow denotes 40S peak while the second denote the 60S peak. The gradient was divided into four regions denoted by the Latin numbers I, II, III and IV 95

24. Photomicrograph of chromatin material represented in region I of the sucrose gradient. The material was centrifuged onto the grids through sucrose paraformaldehyde cushion then positively stained with 1% PTA. The chromatin appear to be composed of fibers which are highly dispersed and the chromatin varied in packing from superbeads (*), and nucleosomes (arrowheads) to fibers of chromatin which contained no visible nucleosomes (arrows). X24,000 97

25. Photomicrograph of chromatin material which was liberated in region II and positively stained with 1% PTA. This region contained an enrichment of superbeads (arrowheads). X39,000 99

26. Electron micrograph of the chromatin material found in fourth region (IV) of sucrose gradient. The chromatin appears to be partially dispersed including dense aggregates. In this plate only few scattered superbeads (arrows) and small number of nucleosomes (arrowheads) could be seen. X39,000 101

27. SDS-polyacrylamide slab gel electrophoretic pattern with the acrylamide in a linear concentration ranging from 5% at the top to 12% at the bottom of the gel stained with silver stain. Lanes 1, 2, 3 and 4 represent the protein profiles of the material extracted from the chromatin found in the four regions of the sucrose gradient respectively. Lane (1) represent the chromatin extracted from region I

in which one can see the similarity of the polypeptides of this lane versus lane 2 which represents the protein extracted from region II. There are three polypeptides in both lanes 1 and 2: 68,000, 59,000 and 58,000. Note the heavier staining in lane 1 in the region of 70,000. Lane 4 represents the region IV of sucrose gradient and shows heavy staining in the area of molecular weight of 68,000 dalton 103

28. One-dimensional SDS-polyacrylamide slab gel (12%) showing electrophoretic banding patterns of acid soluble proteins of sea urchin sperm nuclei (Strongylocentrotus purpuratus, lane 2). The protein was extracted by using 0.25N HCl. The gel was stained with Coomassie brilliant blue. Lane 1 represents three major histones isolated from rat liver chromatin: H2A, H3 and H4. Lane 2 represents the sea urchin sperm nuclear histones. Histone H1 is formed of several subfractions. Histone H3 and H4 look very slightly different from those of rat liver chromatin in lane 1 105

29. Linear one-dimensional SDS-polyacrylamide gel gradient (5-12%) illustrating electrophoretic banding patterns of the acid soluble protein. This was extracted by the use of 0.25N HCl. Lane 1 is a molecular weight control. Lane 2 represents acid soluble proteins (histones) which were extracted from whole sperm nuclei without treatment with 1mM EDTA. Lane 2 illustrates the typical sperm nuclear histones H1, H2A, H2B, H3 and H4 as well as bands of molecular weights of 68Kd, 59Kd and 58Kd. Lane 3 shows the proteins released from sperm nuclei which were treated with 1mM EDTA in order to swell. In this lane the proteins of molecular weights 58Kd and 59Kd seem to be lacking. Lane 4 represents the proteins liberated from the supernatant as a result of swelling of nuclei by the use of 1mM EDTA used in lane 3. In lane 4 one can see two proteins of molecular weights of 59 and 58Kd 107

30. Schematic diagram illustrating the process of packaging and unpackaging of chromatin in sea urchin (Strongylocentrotus purpuratus) sperm nuclei.
a - 400Å-500Å thick chromatin fiber which is made of close association of 400Å chromatin globules.
b - 400Å-500Å fibers disrupt into superbeads which are connected by 112Å fibers.
c - superbeads disrupt to give 112Å fibers.
d - 112Å fibers unpack into beads-on-a-string configuration 109

INTRODUCTION

The interphase nucleus is morphologically defined as containing four major components - the nuclear envelope, chromatin, the nucleolus, and the nuclear sap (Fawcett, 1981; Bouteille, Laval, and Dupuy-Coin, 1974). The nuclear envelope is responsible for separation of cytoplasmic components from the genetic apparatus and is thought to be involved in regulation of transport of molecules and macromolecules from nucleus to cytoplasm (Franke, 1974). The nucleolus is the site of production of pre-ribosomal RNA synthesis and its initial packaging (Busch and Smetana, 1970). The nuclear sap (nucleoplasm) is composed of numerous factors which are either directly or indirectly involved in regulation of DNA replication, transcription, RNA processing, and migration of transcripts from the nucleus to the cytoplasm (Bouteille, Laval, and Dupuy-Coin, 1974). In addition, the interphase nucleus is composed of numerous different molecules and macromolecular complexes. Among the more prominent components are nucleoplasmic proteins, small molecular weight nuclear RNA's, lipids (associated mainly with the nuclear envelope), ribonucleoprotein particles, the nucleoside triphosphates, and enzymes.

Chromatin is an obvious morphological component within the nucleus; when viewed either with the light microscope or the electron microscope, it is seen to be composed of DNA (Adolph, Cheng, and Laemmli, 1977), a well characterized group of basic proteins (the histones [Hnilica, 1972]), a

much less well characterized group of proteins termed non-histone chromosomal proteins, and a small amount of RNA (Bonner, 1979).

Utilization of the light microscope has allowed identification of dispersed and condensed chromatin which was first referred to by the German cytologist, Heitz (1928), as euchromatin and heterochromatin, respectively. Euchromatin has been more recently described as a poorly staining dispersed material with high RNA synthetic activity (Milner, 1969; Hay and Revel, 1963), while heterochromatin has been identified by Heitz (1928; Ris and Kornberg, 1979) as the densely staining material which does not undergo cyclic behavior, and in many cases is preferentially located subjacent to the nuclear envelope. On the basis of these two major types of chromatin, a nucleus having more dispersed chromatin (euchromatin) is called vesicular (Wilson, 1925) and has been shown to be generally more active in RNA synthesis (Berkowitz and Doty, 1975; Fawcett, 1981). On the other hand, a nucleus is termed massive when there is relatively more condensed chromatin (heterochromatin) than dispersed, such as in the adult chicken erythrocyte nucleus (Wilson, 1925).

Chromatin Structure

Presently, attempts are being made to understand the higher ordered structure of chromatin, for this is one of the most important, yet outstanding, problems facing cellular and molecular biologists. Recent advances in the last decade, obtained through biochemical techniques, electron microscopic methodology, x-ray diffraction, and neutron-scattering (Ris and Kornberg, 1979), have indicated that the conformation in which chromatin resides reflects the availability of that chromatin for transcription.

In 1974, Kornberg proposed that chromatin is composed of repeating units (nucleosomes) consisting of approximately 200 base pairs (bp) of DNA, of which 140 base pairs are wound 1 3/4 times around an octamer of two each of the four histones - H2A, H2B, H3, and H4 (core histones, Kornberg, 1974, 1977; Axel, 1975; Felsenfeld, 1978). The core histones and 140 bp of DNA make up what is termed the core particle or core nucleosome. One molecule of the histone H1 per repeat unit appears to be present. This "linker histone" (H1) is believed to be responsible for the packaging of nucleosomes into higher ordered structures and stabilization of that configuration (Thoma and Koller, 1977). Oudet, Gross-Bellard, and Chambon (1975), working on H1 depleted, dissociated chicken erythrocyte chromatin, found that a slow decrease in salt (NaCl) concentration (2M NaCl to 10mM) resulted in reassociation of the chromatin into a beads-on-a-string configuration. Current models indicate that H1 is located in the entrance-exit region of DNA which wraps around the nucleosome (Thoma, Koller, and Klug, 1979). The additional 15-100bp of DNA (depending on cell line; Noll, 1974; Lohr and Courtens, 1979) connecting the nucleosomes is called the internucleosomal spacer DNA and is readily removed from the core particles by various nuclease treatments. Electron microscopic examination (EM) of nuclei lysed in low ionic strength buffer by Woodcock (1973), Olins and Olins (1974) and Oudet, Gross-Bellard, and Chambon (1975) has provided visual evidence that chromatin is packaged in a "beads-on-a-string" configuration. The beads, averaging 100Å in diameter, (initially termed nu-bodies by Olins) are now called nucleosomes, platysomes, or monosomes (Oudet, Gross-Bellard, and Chambon, 1975; Woodcock, 1976; Thoma, Koller, and Klug, 1979). The "string" which separates the nucleosomes (internucleosomal or linker DNA) ranges from 15Å-40Å in diameter (Oudet,

Gross-Bellard, and Chambon, 1975). By low resolution x-ray diffraction, it has been shown that the beads are disk-shaped, 110Å in diameter, 57Å high (Finch and Klug, 1978). Langmore and Wooley (1975) have also reported the apparent presence of a depression in the central region of the nucleosomes. Several studies have shown that the globular nucleosomal structure can be unfolded in the presence of urea to give a linear DNA-histone complex (Woodcock, Safer, and Stanchfield, 1976; Zoma et al., 1977), possibly analagous to "unfolded" active chromatin as visualized by Miller and Bakken (1972).

Further packaging of nucleosomes into higher ordered structures (superbeads, solenoids, fibers, filaments) has been the subject of numerous studies followed by much speculation. Various laboratories indicate that the higher ordered structure of chromatin is in the form of superbeads (Pruitt and Grainger, 1980) while other laboratories have demonstrated that the chromatin is packaged in the form of coiled or spiraling structures which they term solenoids (Thoma, Koller, and Klug, 1979). The demonstration of superbeads by electron microscopy and biochemical techniques comes from several different species with their nuclei in different states of transcriptional activity (compare Kiryanov et al., 1976 with Zentgraf, Muller, and Franke, 1980). Chromatin spreading techniques have shown superbeads in calf lymphocyte nuclei (Hozier, Renz, and Nehls, 1977), chick erythrocyte chromatin (Pruitt and Grainger, 1980; Olins and Olins, 1978) and in sea urchin sperm nuclei (Zentgraf, Muller, and Franke, 1980). Thin sections of embedded material have demonstrated superbeads in rat liver nuclei (Kiryanov et al., 1976) and also in sea urchin sperm chromatin (Aboukarsh and Kunkle, 1981). Using spreading techniques, the diameter of these superbeads has been seen to range from 200Å to 500Å

(Hozier, Renz, and Nehls, 1977; Renz, Nehls, and Hozier, 1977; Rattner and Hamkalo, 1978; Zentgraf, Muller, and Franke, 1980), while in thin sections the range in size is from 200Å to 400Å (Kiryanov et al., 1976; Aboukarsh and Kunkle, 1981). Using sucrose gradient centrifugation, superbeads were demonstrated to be composed of approximately eight nucleosomes (Hozier, Renz, and Nehls, 1977). A detailed description of possible means of packaging the 100Å fiber into these superbeads has not been attempted; neither has an analysis of the protein constituents of these superbeads been carried out. The question which arises is: what components other than the core histones (H2a, H2b, H3 and H4) plus histone H1 are present in these superbeads: are there non-histone proteins present which may be involved in the superstructure of chromatin?

Several laboratories suggested a helical or solenoidal form for the higher ordered packaging of chromatin (Finch and Klug, 1976; Carpenter et al., 1976; Ris and Kornberg, 1979; Worcel, 1978; Butler and Thomas, 1980). Finch and Klug (1976) have proposed a solenoid model for the compaction of the 100Å nucleosome filament into higher ordered structures where nucleosomes, when arranged into solenoids, have about 6.7 nucleosomes per turn with a pitch of 110Å. They have proved experimentally that the 100Å filaments wind into a helical or coiled structure to form 200-300Å fibers, then subsequently coil again to form supercoils of some 400-500Å diameter. However, Finch and Klug's proposal was based on their studies of the reconstituted chromatin in the presence of magnesium ions. This idea was strongly supported by Thoma, Koller, and Klug (1979) in an extensive study demonstrating the effect of varying the monovalent and divalent ion concentration on chromatin structure. They also demonstrated that H1 was the key to formation of the 250Å solenoidal structure (the nonhistone

proteins in their preparations were conveniently ignored). By the use of neutron scattering techniques on chicken erythrocyte chromatin, it was suggested that nucleosomes placed in buffers of increasing ionic strength pack into supercoils of 340Å diameter (Suau, Bradbury, and Baldwin, 1979; Butler and Thomas, 1980). The major drawback to these studies is that the DNA in the chromatin has been cut either with endogenous or added nucleases; this allows the DNA double helix to turn freely in space and may result in the solenoidal type compaction of the chromatin.

Morphology of the Spermatozoon

The structure of the spermatozoon has been well documented since 1897 by many studies using light and electron microscopic techniques (Meves, 1897; Retzius, 1910; Bowen, 1924; Gatenby and Woodger, 1921; Wilson, 1925). These early investigators reported a great deal of valuable information about its morphology using the light microscopy. In most eukaryotes the mature sperm is a flagellated motile cell (Fawcett, 1958); it consists of a head and tail joined by the neck as shown in Fig. 1. The tail consists of a middle piece, principal piece, and end piece. In the sea urchin, *Strongylocentrotus purpuratus*, the head is cone-shaped (conoid) with the base of the cone associated with the axoneme. The head is composed almost entirely of the nucleus, and it has compact intensely basophilic chromatin (heterochromatin) surrounded by a bilaminar nuclear envelope. The apex of the head has an indentation - the acrosomal fossa, a derivative of the Golgi complex which contains enzymes and polymerizable actin (Metz, 1932; Afzelius, 1955; Fawcett, 1975; 1981) which are important in sperm attachment and membrane fusion with the ovum during the early stages of fertilization. The base of the nucleus is indented to form the centriolar fossa which contains the proximal centriole and the proximal end of the

basal body; this area is termed the neck region. The middle piece has the typical structure of a flagellum (Fawcett and Porter, 1954; Fawcett, 1968) and prominent mitochondria. The principal piece is the longest part of the tail; it has an axial bundle of microtubules surrounded by a fibrous sheath. The end piece is relatively short and has the typical structure of a flagellum (Fawcett and Porter, 1954). The entire sperm is surrounded by a plasmalemma or cell membrane (Fawcett and Ito, 1965) which is characteristically highly granular in the region of the mid piece as viewed by freeze-fracture (Koehler, 1970).

The Sperm Nucleus

By light microscopic analysis (Retzius, 1910), the mature sperm nucleus is seen to be composed of a densely packed mass of chromatin (heterochromatin) of which the protein component is highly basic (Hamer, 1955; Bloch, 1969; Simpson and Bergman, 1980). In 1949 Wislocki examined the rat sperm head by histochemical and light microscopy methodology. He concluded that the sperm head (containing the nucleus) is highly basophilic and its chromatin is composed mainly of DNA, lacks RNA, and contains some lipids probably in the nuclear sheath.

The development of the electron microscope (EM) caused a great deal of excitement for biological scientists who thought they would be able to determine the structural organization and composition of cells, as well as their included nuclei. Initial EM studies began approximately three decades ago using whole mounts of nuclei either air dried followed by staining with osmium tetroxide or with heavy metal coating (Randall and Friedlaender, 1950; Schnall, 1952). The major difficulty with the above mentioned procedures was that the electron beam was unable to penetrate those nuclei with any degree of efficiency. Thin sectioning, dehydration,

and omission of washing after fixation (Watson, 1952) gave better results, yet the sperm nuclei still appeared as electron opaque structures, leading to a general feeling that chromatin structure would be unattackable by this type analysis (Friedlaender, 1952).

Freeze-fracturing techniques followed by analysis of the replicas resulted in reports that there was a granularity within the sperm head (Randall and Friedlaender, 1950; Koehler, 1970; Wilkins and Randall, 1953). Afzelius (1955) reported that granules (chromatin beads?) were present in sperm nuclei of the sea urchin (Stronglocentrotus drobachiensis) and that they were linearly arranged and had a mean diameter of 100Å. However, doubts surrounded this conclusion due to the fact that published micrographs lacked sufficient resolution and clarity, plus there was concern about possible artifacts due to a lack of understanding of fixation. In 1968, Lung studied whole mounts of human sperm chromatin by electron microscopy and reported 140-240Å diameter fibers with 200-400Å nodes along those fibers. Solari (1968) used spreading techniques, developed by Gall (1952) and Ris (1966), to study sperm nuclear chromatin and the effects of varying ionic strength on the morphology of chromatin fibers. Solari did not demonstrate nucleosomes, but he did report 250Å granules and 30Å fibrils.

In mammalian spermatozoa, chromatin analysis is complicated due to the presence of disulfide cross bridges; however, Tsanev and Avramova (1981) studying ram sperm nuclei demonstrated that chromatin fibers contained nucleosomal-sized beads and the sperm DNA may be organized into domains fixed by nuclear skeleton proteins. In 1979, Wagner and Yun reported that human sperm chromatin may be composed of spherical units of 400Å and 150Å diameter. Sperm nuclear chromatin of the sea urchin (Paracentrotus

lividus) is organized into nucleosomes (Spadafora et al., 1976a; Zentgraf, Muller, and Franke, 1980), and it is indicated that the nucleosomes are further packed into superbeads having a diameter of 400-500Å (Zentgraf, Muller, and Franke, 1980). Unlike the eutharian spermatozoa, these sea urchin sperm nuclei do not utilize disulfide bonds as a means of maintaining the compact configuration of their chromatin; hence decondensation *in vivo* or *in vitro* does not require agents which disrupt sulfur-sulfur crosslinks (eg. β-mercaptoethanol or dithiothreitol). Sea urchin sperm nuclear chromatin is composed of DNA, non-histone proteins (approximately 20%, Kunkle, Magun, and Longo, 1978b), and histones. The sea urchin sperm histones are classified as H1, H2A, H2B, H3, and H4; yet, H1, H2A, and H2B are different from embryonic and somatic cell histones (Strickland et al., 1974, 1977a, 1977b, 1982; Arceci and Gross, 1980), while histones H3 and H4 are essentially identical to somatic histones H3 and H4 (Simpson and Bergman, 1980). Histone H1 is both larger and more basic than that of embryonic (Carroll and Ozaki, 1979) and somatic cells (Keichline and Wasserman, 1977).

Despite these differences, Simpson and Bergman (1980) have shown that sea urchin sperm chromatin is organized at the most basic level in the form of nucleosomes. This indicates that the variability in the histones H2A and H2B may be less important than the similarity in the histones H3 and H4 as far as the basic conformation of the nucleosome is concerned. Further studies on the compaction of these nucleosomes have not been carried out, other than the description that there appear to be superbeads present in dispersed sea urchin sperm nuclei (Zentgraf, Muller, and Franke, 1980; Spadafora, Noviello, and Geraci, 1976b).

OBJECTIVES OF THE STUDY

The goal of this study is to determine the means of packaging the inactive chromatin within sperm nuclei of the sea urchin *Strongylocentrotus purpuratus* by electron microscopic techniques.

This goal will be met by obtaining information in the following areas:

1. What is the size of nucleosomes in sea urchin sperm chromatin?

2. How are the nucleosomes packaged in the sea urchin sperm nuclei? Are they packaged in the form of: filaments, coils, beads, or some other structures?

3. What is the mode of chromatin transformations from one structure to another?

4. What is the protein composition of the superstructures seen in *Strongylocentrotus purpuratus* sperm nuclei?

MATERIALS AND METHODS

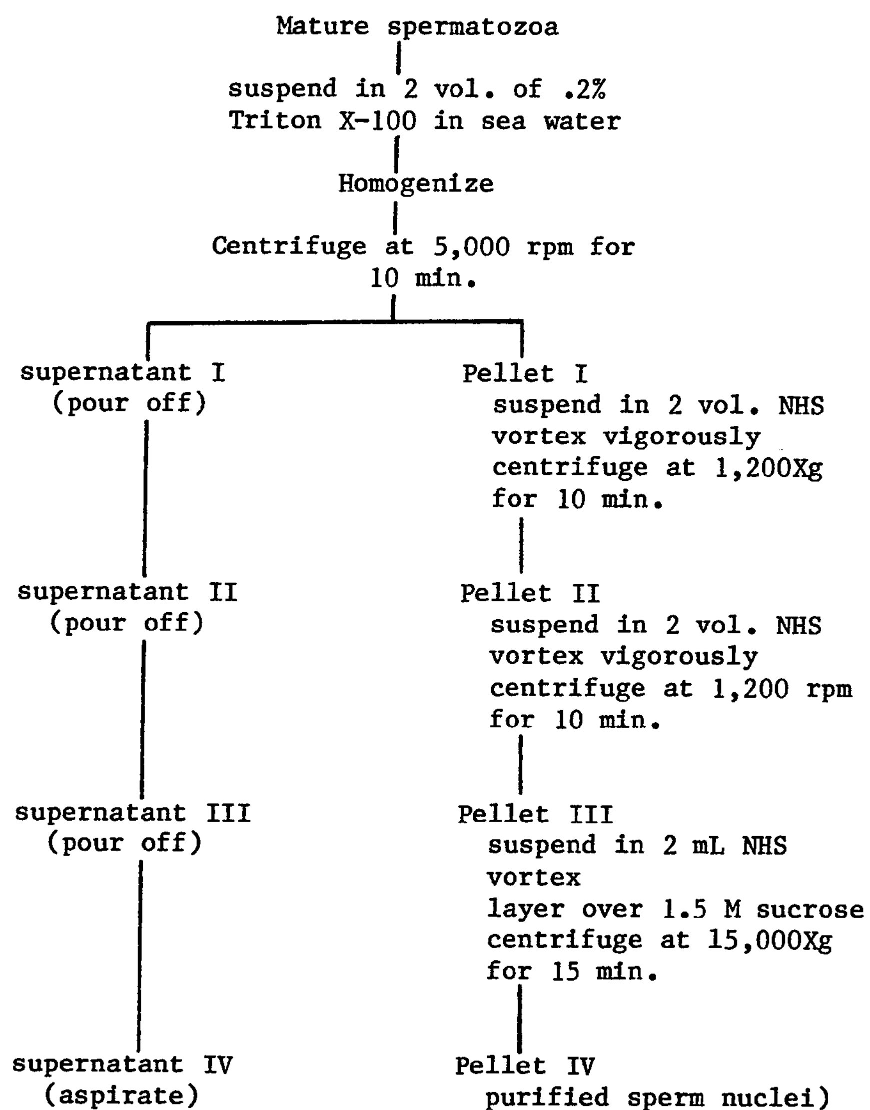
Isolation of Sperm Nuclei

The method of Kunkle et al. (1978a, 1978b) was used to isolate the sperm nuclei. All procedures were carried out at 4°C unless otherwise specified and all solutions contained 1mM phenylmethyl sulfonyl fluoride (PMSF) to inhibit serine-type proteases.

Sea urchins (Strongylocentrotus purpuratus) were injected with 0.5 M KCl to identify their sex. The testes were collected on ice, diced, and filtered through four layers of cheese cloth. The filtrate was cooled at 4°C for thirty minutes and then suspended in two volumes of 0.2% Triton X-100 in artificial sea water (Hawaiian Marine Imports or Instant Ocean), homogenized with a Teflon pestle and subsequently centrifuged at 1,000xg for ten minutes to pellet crude nuclei. The pellet was suspended in NHS (0.25M Sucrose - 10mM Tris - 10mM $CaCl_2$ - 0.1% Triton X-100 - 1mM PMSF - pH 7.5) and mixed by vortexing. Nuclei were pelleted by centrifugation at 1,200xg for ten minutes. The resulting crude nuclear pellet was resuspended in NHS and treated as in the previous step. The pellet was suspended by vortexing in a small volume of NHS and layered on 30ml of 1.5M sucrose - 10mM Tris pH 7.5 in a 50ml centrifuge tube and centrifuged at 1,500xg for fifteen minutes. The supernatant was removed by aspiration and the final pellet contained sperm nuclei lacking a nuclear envelope.

TABLE 1

Isolation of Sea Urchin Sperm Nuclei



Nuclear Swelling and Chromatin Spreading

Isolated nuclei were suspended in 10 ml of 1 mM ethylene diamine tetraacetic acid (EDTA) pH 7.5 and centrifuged at 1,250 xg for ten minutes. The pellet was resuspended in 20 to 25 volumes of 1 mM EDTA and aliquots were taken between ten minutes and four hours (every thirty minutes tubes were gently inverted to enhance lysis of nuclei). Some aliquots were centrifuged through a fixative containing 3.4% sucrose - 3.7% paraformaldehyde onto formvar-carbon-coated grids. They were washed in 0.5% Photoflo-200 (Eastman Kodak), stained with 1% phosphotungstic acid (PTA) or 1% uranyl acetate in 2 mM $MgCl_2$, dehydrated in 95% ethanol, and allowed to air dry (Miller and Bakken, 1972). Subsequently, some grids were coated with platinum-palladium (80:20) at an angle of 4°-6°. Some grids were negatively stained with 2% PTA prior to examination with a JEOL-100CX electron microscope.

Transmission Electron Microscopy (TEM)

Sperm nuclei, after various periods of time in 1mM EDTA, were fixed in 2% gluteraldehyde - 50mM sodium cacodylate pH 7.4 or 1mM EDTA for two hours or overnight. After fixation, specimens were washed three times in the appropriate buffer and postfixation was carried out for sixty minutes in 1% osmium tetroxide in cacodylate buffer or 1mM EDTA. Subsequently, nuclei were dehydrated in ethanol, infiltrated, and embedded in Epon 812. Gold to silver sections were cut with an MT-2B (Sorvall) and stained with 2% uranyl acetate in 50% alcohol followed by lead citrate (Reynolds, 1963). Specimens were examined by electron microscopy and photographs were taken. Using the goniometer on the JEOL-100CX, stereoscopic pictures were taken at angles between -5° and +5°. Measurements of chromatin superbeads,

filaments, and nucleosomes were done on photomicrographs with a magnifying ocular lens.

Scanning Electron Microscopy (SEM)

Sperm nuclei were swollen or lysed, fixed, and centrifuged onto formvar-carbon-coated grids as in the chromatin spreading technique. The grids with chromatin were divided into three groups. The first group was processed for SEM after simple dehydration with 95% ethanol. The second group was lyophilized overnight and the third group was critical point dried from acetonitrile in a Balzer critical point dryer. The samples were sputter coated with 100-200Å of gold-palladium in a Technics Hummer V Sputter Coater. Examination of the material was done using a JEOL-100CX TEM-SCAN high resolution scanning electron microscope with an accelerating voltage of 20 or 40KV.

Isolation of 400Å Units

Nuclei swollen or lysed in 1mM EDTA were digested with DNase I for short periods of time in order to obtain fragments of chromatin for morphological and biochemical analysis. After various intervals (one-four hours) of lysis in EDTA the suspension containing nuclei and chromatin was centrifuged at 5,000xg for ten minutes and the supernatant was discarded. The pelleted material was suspended in 0.25M Sucrose - 5mM magnesium acetate - 10mM Tris pH 7.5 at approximately 1mg DNA/ml and allowed to temperature equilibrate between 4° and 24°C. At that time DNase I was added to a final concentration of 100μg/ml and digestion was allowed to proceed for times ranging from ten minutes to overnight. Histones H2A and H2B decrease the susceptability of this chromatin to nuclease digestion (Simpson and Bergman, 1980), thus the longer incubation time. Digestion was stopped by addition of EDTA to a final concentration of 10 mM and

placing the aliquots on ice. For analysis of chromatin size, 400-1000μl aliquots of digested material were layered on linear sucrose gradients of 10% to 30% sucrose containing 50mM NaCl - 10mM Tris pH 7.5. Sucrose gradients were run at 28,000 rpm for one hour then 8,000 rpm overnight in a Beckman L5-75 centrifuge using a SW 28.1 rotor. The gradients were mechanically fractionated by using an ISCO model 185 fractionator. Absorbance of fractions was monitored at 254nm with an ISCO UA-5 monitor and recorder. Fractions (1/2ml) were collected and 10-100μl aliquots were processed for electron microscopy as previously described for chromatin spreading.

Protein Extraction

Fractions of 0.75ml were collected from the 10-30% linear sucrose gradients for this study. The gradients were divided into four groups according to the peaks recorded by the spectrophotometer. Tubes from the same portion of several gradients were pooled for the purpose of securing sufficient material for protein analysis.

Protein was extracted three times by using cold 0.25N hydrochloric acid (HCl). Chromatin was suspended in HCl, allowed to sit on ice with intermittent mixing for thirty minutes, and then centrifuged at 7,000Xg for ten minutes. The pellet was resuspended in HCl and treated as described above. Ten percent trichloroacetic acid (TCA, 50% stock solution) was used to precipitate protein from the three combined extractions. The unextracted material from the final pellet was analyzed for both protein (Bradford, 1973) and DNA (diphenylamine reaction).

In a control experiment to compare the protein constituents of sperm nuclei with the protein loss from nuclei during lysis, the following procedure was performed: frozen nuclei were divided in half. One half was

kept on ice while the other was placed in 1mM EDTA to lyse the nuclei. After four hours the lysed nuclei were centrifuged at 7,000Xg for 15 minutes. The resulting pellet of chromatin (and some nuclei) and the unlysed nuclei (conoid) were extracted for protein as described above. The supernatant, along with any released proteins, was made 10% in TCA, placed in the refrigerator (4°C) overnight, and the protein pelleted at 7,000Xg for fifteen minutes the next day. The three protein fractions (control nuclei, lysed nuclei, and supernatant from lysed nuclei) were analysed for protein composition as described below.

Sodium Dodecylsulfate (SDS)-Gel Electrophoresis

For the purpose of electrophoresis, 1-2 ml of 1% SDS (sodium dodecyl sulfate) - 1% β-mercaptoethanol was added to the TCA-precipitated material. The samples were heated to 100°C for two minutes, and then dialyzed against SDS-sample buffer (0.25M Sucrose - 10mM sodium phosphate - 0.1% SDS - 1% β-mercaptoethanol - pH 7.2) overnight. Dialyzed protein samples were either electrophoresed fresh or frozen until use. Protein samples were analyzed on one-dimensional SDS-polyacrylamide slab gels, by modifications of the procedures of Laemmli (1970) and LeStourgeon and Wray (1974). Polypeptides extracted were analyzed on 8.75%, 12%, 18%, and/or 5-12% linear gradient gels.

After migration of the bromophenol blue to the bottom of the gel, the resulting gels were placed in 0.1% Coomassie brilliant blue R-250 - 45% methanol - 10% acetic acid and allowed to stain overnight. Gels were then destained in 7.5% acetic acid until the background was clear. The silver stain procedure was carried out as per the "Bio-Rad" instructions for gels of greater than 1mm thickness.

EM of Different Fractions of Sucrose Gradients

Duplicate samples of chromatin material were subjected to both electron microscopy and electrophoresis in order to compare the morphological aspects of the different fractions of sucrose gradient with the biochemical composition of those fractions. Aliquots measuring 100-200μl of the four different fractions of sucrose gradient were centrifuged onto formvar-carbon-coated grids and processed for electron microscopy as was carried out in chromatin spreading.

RESULTS

Thin Sections of Sperm Nuclei

Thin sections of intact mature sea urchin sperm nuclei processed for transmission electron microscopy, without treatment with either Triton X-100 or low ionic strength buffer (1mM EDTA pH 7.4), show that the nucleus is completely occupied by chromatin. This material appeared tightly packed, darkly staining and electron dense, and is called condensed or heterochromatic chromatin (Figure 2). Figure 2 also reveals the different components normally seen in the mature sea urchin (*Strongylocentrotus purpuratus*) spermatozoon. The spermatozoon is surrounded by a cell membrane or plasmalemma (P). The centriolar fossa (CF), the sperm mitochondria (SM), and the axoneme (T) are also visible in this figure (2). While the intact mature sperm nucleus has a nuclear envelope (NE) which completely surrounds the nucleus, no remnants of the nuclear envelope appear in the micrograph of isolated sperm nuclei (Figure 3); this is a reflection of the use of Triton X-100 in the nuclear isolation procedure. The purified isolated sperm nuclei showed a morphological appearance of the chromatin identical to that of the mature intact (whole) spermatozoa (compare Figures 2 and 3).

It is impossible to measure the constituent chromatin units of intact sperm and isolated nuclei. Therefore, the procedure for attempting measurement of chromatin constituents was to remove the nuclear envelope with Triton X-100 and swell the nuclei by incubation in 1mM EDTA at pH 7.5

for intervals ranging from 10 to 240 minutes so that the chromatin organization could be visualized by electron microscopy. This procedure is relatively "mild" in that it seems to retain the morphological relationship between the chromatin components in the early stages of nuclear swelling; therefore, it allows the chromatin to disperse in a fairly controlled manner. The granulofibrillar appearance of the chromatin in these swollen nuclei becomes strikingly obvious by the use of this technique. Photomicrographs of final magnification between 25,000X and 120,000X were used to measure the chromatin subunit sizes by means of a scaled magnifying ocular lens 0.1mm/division, (Bausch and Lomb). The measurements were obtained from at least five different experiments, each comprised of a minimum of 25 subunits. The mean and the standard deviation (S.D.) were calculated for each; the final results represent the mean of these readings. In Figure 4 the sperm nuclei were incubated for only ten minutes in 1mM EDTA prior to processing for electron microscopy, and the chromatin is visualized as being in the form of 400Å-500Å thick filaments. At this stage of dispersion it is not possible to determine any further substructure of the chromatin due to the compact configuration. Allowing chromatin dispersion to proceed for a longer period of time (thirty minutes) permits one to see that the 400Å filament is composed of subunits (Figure 5). These structures appear to be spherical and have a diameter of 400Å-500Å which corresponds to the diameter of the filaments seen at the earlier stage of nuclear swelling.

Figure 6 is a section through a sperm nucleus at about the same stage of swelling as in the previous figure (5); however, several things should be noted. First, around the periphery there are areas which do not appear to have been dispersed (arrows). Second, this preparation was made with

nuclei which had not been as extensively purified as is normally done; here one can see portions of the axonemes in the field of view which indicates that the spreading conditions do not appear to be unduly harsh. In addition, after incubation of sperm nuclei in low ionic strength buffer for up to four hours, there was no noticable reduction in the presence of protein bands which might be due to proteolysis as seen on one dimensional SDS-polyacrylamide gels. This is most probably due to the fact that all isolation and incubation buffers contained phenylmethyl sulfonyl fluoride (PMSF) which inhibits serine-type proteases (James, 1978).

Incubation of isolated sperm nuclei for longer periods of time (sixty minutes) allowed the chromatin to disperse to an even greater extent (Figure 7) and it is possible to see that the spherical structures which were just barely visible in Figures 5 and 6 have become quite obvious at this point. In addition, at the arrows, one is able to see that there are thin strands of chromatin interconnecting the 400Å spherical granules. One other finding that is demonstrated on this Figure is that some of the isolated sperm nuclei are refractive to the swelling process and do not enlarge. In control experiments isolated sperm nuclei were dialyzed overnight against a large excess of 1mM EDTA with three changes of the buffer (100-fold excess per change). Analysis of the resulting chromatin by light and electron microscopy demonstrated that some nuclei remained in the compact conoid configuration. At present there is no reasonable explanation for this observation.

Figure 8 is a survey view of sperm nuclei which have been incubated in low ionic buffer for approximately ninety minutes. The majority of the chromatin appears in the form of superbeads (spherical granules) with some scattered membranous vesicles and an occasional portion of an axoneme.

Fields similar to this were used to determine the size of the spherical structures; the superbeads were seen to average 414Å in diameter with a standard deviation of ±60Å. If the sperm nuclei (Figure 9) were allowed to swell for two hours one could see more clearly the organization of the chromatin within the sperm nuclei. The superbeads are seen as irregular spherical structures, some of which have not separated to the same extent from one another as others (see arrowheads, Figure 9). Also, one is able to see that the interconnecting chromatin (arrows) is 112Å in width (S.D. of ±13Å). In none of the spread preparations has an intermediate structure between the superbead and the 112Å chromatin fiber been observed. Figure 10 demonstrates the interconnecting chromatin fibers (arrows) more clearly; one can see that the fiber has a series of central electron lucent areas which are circular in structure. Where these 112Å fibers are clearly visible, one sees electron lucent areas of about 32Å in diameter (S.D. of ±6Å). These clear areas in the 112Å fiber have been visualized only in material which has been thin sectioned prior to examination with the electron microscope. Material spread onto grids and visualized by high resolution transmission and scanning electron microscopy has failed to demonstrate this chromatin substructure. Also, in none of the preparations examined was there any evidence for a clear or electron lucent area in the central region of the superbead. This in itself argues against the idea that a solenoid or coiled form of the 112Å fiber is present in this chromatin.

Stereopairs were used to demonstrate the three-dimensional architecture of the superbeads. Sperm nuclei which had been incubated in low ionic buffer for ninety minutes were processed for electron microscopy and photographs were taken at varying tilt angles. Figure 11 is a

stereopair; plate A was taken at an angle of +3 degrees while plate B was taken at a tilt angle of -3 degrees. This pair demonstrates the three-dimensional organization of the superbeads and gives one an understanding of the organization in space. Figure 12 also demonstrates the organization of the superbeads and one is able to see the close packing of the beads to form filaments. The tilt angle in this figure (12) is only 5 degrees (0 to -5) whereas the previous plate was 6 degrees.

Chromatin Spreading

Isolated sperm nuclei were incubated in low ionic strength buffer until the chromatin was partially or completely lysed. Several different lysis procedures were tried (use of alkaline water - pH 9 or 2mM potassium chloride) but none were as effective as the use of 1mM EDTA as the lysis medium. After lysis, samples were centrifuged onto formvar-carbon-coated grids and stained. Electron microscopic examination of positively stained chromatin, using phosphotungstic acid (Figure 13), revealed an apparently intertwining mass of chromatin which demonstrated a typical beads-on-a-string appearance as seen in interphase nuclei. The beads are the nucleosomes which are 90Å-100Å in diameter. This is the typical size range for nucleosomes as seen in other systems. The internucleosomal spacer chromatin ranged in diameter from 20Å-25Å. The distance between nucleosomes was quite variable and probably reflected differing degrees of stretching forces on the individual chromatin fibers.

To improve the contrast of the chromatin and superbeads the lysed sperm nuclei when spread on grids were stained and subsequently coated with a thin layer of platinum:palladium (80:20) by vacuum evaporation at 1 x 10^{-5} torr. Figure 14 demonstrates the appearance of a typical spread preparation using this procedure. The average size of the nucleosomes seen

by this procedure was 132 (S.D. ± 22Å) and the width of the interconnecting chromatin fibers was 38Å (S.D. ± 9Å). The difference in size seen by this procedure as compared to the positively stained chromatin is a reflection of the thickness of the metal coating deposited on the chromatin. Thus the metal coating on the chromatin is approximately 21Å thick (42Å/2); the difference in thickness of the internucleosomal chromatin is only 9Å (18Å/2). The internucleosomal fiber is apparently hidden from the metal source by the adjacent nucleosomes as the platform is rotated; thus less metal is deposited here (the metal source is located at a very low angle of 4 to 6 degrees from horizontal). In this type preparation the spacing between nucleosomes is also quite variable; as one can see the range is from essentially no spacing to 200Å (arrows). This, again, is felt to be due to the various forces imposed on the individual fibers as they are centrifuged onto the electron microscope grids. Figure 15 is a higher magnification photomicrograph of chromatin from lysed nuclei which have been positively stained with phosphotungstic acid and shadowed with platinum:palladium (80:20) here one is able to see the nucleosomes (arrowheads) and the variable degree of spacing (arrows) between the nucleosomes. Lysed nuclei negatively stained with phosphotungstic acid (Figure 16) demonstrated spherical structures; however, the diameter was quite variable and did not fall into the size range expected for either nucleosomes or superbeads. The average size was 185Å (S.D. ± 25Å). This probably represents superbeads which have too much negative stain on them so that the stain encroaches on the periphery of the spherical structures and decreases their apparent size, since it does not allow electrons to pass through it. Another problem in measuring material visualized by

negative staining is that the borders of the spherical structures are not sharply delimited, making definition of the lateral limits tenuous.

Scanning Electron Microscopy

Sea urchin sperm nuclei were placed in low ionic buffer and allowed to swell. After ten minute time increments aliquots were taken out and centrifuged onto formvar-carbon-coated grids through 3.4% sucrose-3.7% paraformaldehyde. The swollen sperm nuclei were then critical point dried from acetonitrile. Finally, a coating of gold was deposited on the nuclei with a sputter-coater prior to examination with a JEOL-100CX STEMSCAN. Chromatin dispersion was generally seen to begin at one discrete point on the surface of the nucleus (Figure 17). This figure (17) demonstrates not only the initial decondensation of the chromatin (arrow) but also the visibility of the centriolar (CF) and acrosomal (AF) fossae. The diameter of the extruding chromatin fiber is 500Å. By scanning electron microscopy a later stage in swelling of the nuclei is seen in Figure 18, which shows a fiber of chromatin coming out of the nucleus and the convoluted surface has chromatin beginning to spill out of the confines of the nucleus (arrowheads). In addition, this figure (18) shows that a portion of the sperm axoneme (T) is left on the sperm head and also that the acrosome is visible (A) at the apex of the nucleus.

Figure 19 shows a sperm nucleus which in this case was centrifuged onto a filter prior to critical point drying. Here one sees that the surface is extensively convoluted with portions of chromatin fibers 400Å-500Å in diameter. In some areas (arrows) one can see profiles of slightly elongated appearing chromatin. Figure 20 is a view from the posterior aspect of the spermatozoa demonstrating the dispersion of the chromatin fibers; one can see numerous profiles of 450Å-500Å fibers on the

surface of this nucleus (arrowheads). Figure 21 demonstrates by scanning electron microscopy several nuclei which are in various stages of dispersion. At the arrows one sees a 400Å-500Å fiber coming out of the nucleus labeled II. The other two nuclei in the field have a convoluted surface, but it is difficult to determine whether chromatin fibers are eminating from them. Figure 21 demonstrates the latest stage in nuclear lysis where use of the scanning electron microscope is helpful in observation of the dispersing chromatin. Figure 22 shows several nuclei at a later stage in the lysis procedure and the chromatin fibers range widely in diameter and in many instances appear to be composed of several fibers which have collapsed on one another (arrows).

Isolation of Superbeads from Digested Sperm Nuclei

Sperm nuclei which had been swollen in low ionic strength buffer were digested with deoxyribonuclease I in an attempt to release superbeads from the swollen nuclei. Nuclei were digested for varying lengths of time (ten minutes to overnight with temperatures ranging between 4 and 24°C) in attempts to release the individual superbeads. It was found that incubation of swollen nuclei for thirty minutes at 20°C gave maximal digestion of chromatin to superbeads. To separate superbeads from the remainder of the chromatin and swollen nuclei, the digestion process was terminated by the addition of EDTA to a final concentration of 10mM. The digestion mixture was centrifuged at 2,000xg for ten minutes to pellet undigested nuclei and chromatin; the resulting supernatant was layered on a sucrose gradient to fractionate further the digested material.

Various sucrose concentrations were tried in attempts to obtain a useful separation of the various chromatin fragments. The gradients ranged from 5%-20% to 15%-60% in sucrose concentration. It was found that best

resolution of chromatin fragments was obtained on gradients of 10%-30% sucrose concentration. It was also necessary to vary the sodium chloride concentrations so that the chromatin did not unfold in the gradient as it was being centrifuged. The optimum NaCl concentration was found to be 50mM and this was utilized for gradients in which both the morphology and protein composition of the various fractions were determined. However, to preserve best the ultrastructural characteristics of the chromatin, gradients were formed with 1% formaldehyde in the gradient; use of this cross-linking agent did not allow further analysis of the proteins associated with these nuclei. Figure 23 demonstrates a typical absorbance profile from a preparation which began with 20 A_{260} units of chromatin in the digestion buffer, with approximately 8 A_{260} units of chromatin were loaded onto the gradient. The gradients were centrifuged overnight (sixteen hours) at 4°C centigrade in an SW-28 rotor spinning at 28,000 rpm for the first hour and then at 8,000 rpm for fifteen hours. After centrifugation gradients were fractionated on an ISCO 185 gradient fractionator and the absorbance profile is as seen in Figure 23. The majority of the chromatin was found in the first ten fractions of the gradient, while some of the material sedimented to the bottom region of the gradient (fractions 23-26). Each 1mL fraction was initially individually analyzed by electron microscopy in attempts to find the fraction or fractions enriched in superbeads. Previous investigators have found that the superbeads have been found on similar gradients in the region of the leading shoulder of chromatin at approximately fractions 7-10 in this figure (23, e.g., Hozier, Renz, and Nehls 1977). In this shoulder region it was found that there was an enrichment of superbeads in the fractions but in no fraction was there a relatively "pure" population of superbeads.

The fractions were pooled as indicated in Figure 23 and analyzed morphologically and biochemically (for protein composition only). Region I of the gradient was composed of fractions 1-5 and morphologically was seen to be composed of fibers of chromatin which were highly dispersed. The chromatin varied in packing from apparent superbeads to fibers of chromatin which contained no visible nucleosomes (Figure 24). The second region of the gradient (fractions 6-10) was the region which contained an enrichment of superbeads although it was by no means a fraction which was composed predominately of superbeads (Figure 25). Loading varying amounts of material on the gradients did not have an effect on the mixture of chromatin and superbeads in the various fractions, especially region II of the gradients. It was felt that the superbeads form aggregates as they are digested and do not separate into distinct entities until after they are centrifuged onto electron microscope grids and washed in Photo-flo 200 (Kodak). The third region of the gradient (III) including fractions 11-22 appeared to contain no A_{254} absorbing material. The final region of the gradient (region IV, Figure 23) included the fractions 23-26. By electron microscopic analysis, the material found in region IV can be seen in Figure 26. The chromatin was seen to be partially dispersed and included many dense aggregates of material which appeared to be only minimally acted upon by the endonuclease DNA'ase I. In this figure only a few scattered superbeads could be found (arrows) as well as only a minimal number of nucleosomes (arrowheads).

Electrophoretic analysis was carried out on the four regions of sucrose gradients as divided in Figure 23 to determine whether there were any differences or similarities in the proteins associated with the chromatin. This was done because it was felt that possibly the chromatin

was being fractionated or digested differently by the DNA'ase I due to a differing protein composition. Figure 27 is a photograph of an SDS-polyacrylamide gradient slab gel with the acrylamide in a linear concentration range from 5% at the top to 12% at the bottom of the gel. Lanes 1, 2, 3, and 4 of Figure 27 represent the protein profiles of material extracted from the chromatin found in each of the four corresponding regions of sucrose gradients as seen in Figure 23. One will note that lanes 1 and 2 are very similar in polypeptide profiles except that region I chromatin (Lane 1, Figure 27 vs. lane 2) has much more silver staining material in the region of the gradient above 70,000 apparent molecular weight than does the chromatin located in the second region of the gradient (II). In addition to the histones which run at the bottom of the gel in this figure, there are three polypeptides found in relatively high concentration in both lanes 1 and 2, and they have an apparent molecular weight of 68,000, 59,000, and 58,000. Lane 3 (Figure 27), even with the use of a silver stain which has an apparent sensitivity of 10 ng per band (10-50 fold more sensitive than Coomassie brilliant blue R-250 staining), fails to demonstrate a polypeptide profile which is different than the previous two regions of the gradients. Region IV of the sucrose gradients as seen in lane 4, Figure 27, is seen to have a preponderance of polypeptides which barely enter the gel (the smear at the top of the lane) with few proteins in the molecular weight region of less than 68,000.

Figure 28 is a micrograph of a 12% SDS-polyacrylamide gel which demonstrates the core proteins which are associated with sea urchin sperm nuclei when isolated by the procedure detailed in the Materials and Methods. In lane 1 (Figure 28) are three of the four core histones isolated from rat liver chromatin; in lane 2 are the total proteins which

are extracted from sperm nuclei with 0.25N HCl. The four histones are slightly different from those of rat liver in that histones H2a and H2b have different mobilities in this system and by sequence analysis (Strickland et al., 1974, 1977a, 1977b, 1982) are quite different in portions of their length. Also there is the presence of several electrophoretic forms of histone H1 as seen in this figure (28).

One of the questions of interest was, "During the swelling and lysis procedure, are there any proteins that come off of or are released from the chromatin and nuclei into the supernatant?" Frozen sperm nuclei were thawed on ice and divided into two aliquots. One was used for extraction of total protein while the other was placed in 1mM EDTA pH 7.5 to lyse the nuclei. After three hours in lysis buffer the swollen and lysed nuclei were centrifuged at 7,000xg for fifteen minutes to pellet the chromatin and nuclei. The resulting supernatant material was precipitated by addition of TCA to 10%. Three percent of the protein was released from the sperm nuclei during this lysis procedure and it appeared not to be a general release of all proteins from these nuclei (Figure 29). There are many polypeptides which are found in the supernatant yet two of the major proteins are seen as those which migrate in the molecular weight range of 59,000 and 58,000 daltons. Also, many of the higher molecular weight polypeptides are found in the supernatant after lysis yet they do not appear in high enough individual concentration to be visualized as discrete bands (Figure 29).

DISCUSSION

This study of sperm nuclear chromatin is meant to investigate and determine the means of packaging of chromatin at levels above that of the nucleosome. There is a great deal of controversy concerning the means by which chromatin is packed. Convincing evidence is presented by at least two laboratories which leads to different interpretations of how chromatin is packaged in the nucleus (Finch and Klug, 1976; Hozier, Renz, and Nehls, 1977). It was felt that by asking specific questions about a nucleus, which has a fairly well defined composition and contains only one type of chromatin, one might be able to make definite statements concerning the chromatin configuration. The questions which were asked to determine the organization of chromatin were: (1) Are nucleosomes arranged in larger units which can be visualized by electron microscopy? (2) Is it possible to isolate these units from nuclei for further investigation? (3) Are these units further packaged into larger structures within nuclei? (4) Is there another group of proteins that may share with histone H1 the responsibility in maintaining the higher order structure of these units? One problem which was felt to add confusion to the previously published reports is that there has essentially been no regard given to the fact that within a typical interphase nucleus one finds a nucleolus, inactive (hetero-) chromatin, and dispersed (active or eu-) chromatin (Fawcett, 1981). To circumvent this problem, nuclei from the sea urchin *Strongylocentrotus purpuratus* were used in this investigation. These nuclei have nucleosomes which are similar to those seen in interphase

somatic cell nuclei (Simpson and Bergman, 1980). In addition, the chromatin is packaged within the nuclei without the cross linking of cysteine residues in the histones. This allows the chromatin to be swollen in the presence of low ionic buffers without either β-mercaptoethanol or dithiothreitol. Yet most important is the fact that these nuclei are composed of condensed or heterochromatin without either euchromatin or nucleoli. Thus, when looking at chromatin spreads one has only to consider that all the chromatin is derived from one population that being inactive chromatin.

Recent investigations have suggested and there is fairly good agreement that the basic unit of chromatin packaging above that of the nucleosome is the 100Å fiber which is composed of nucleosomes (Mazia, 1954; Zirkin and Wolfe, 1972; Olins and Olins, 1974; Oudet, Gross-Bellard, and Chambon, 1975; Benyajati and Worcel, 1976; Woodcock, Safer, and Stanchfield, 1976; Adolph, Cheng, and Laemmli, 1977; Hozier, Renz, and Nehls, 1977; Becak and Fukuda, 1978; Carter, 1978; Thoma, Koller, and Klug, 1979; Adolph, 1980a; Labhart and Koller, 1981).

The question concerning the way in which 100Å fiber is further compacted in an organized manner within the nucleus has produced conflicting data (Gall, 1952, 1963; DuPraw, 1966; Pardon and Wilkins, 1972; Carpenter et al., 1976; Griffith, 1976; Evans, 1977, 1979; Evenson et al., 1978; Felsenfeld, 1978; Franke et al., 1978; Bonner, 1979; Butler and Thomas, 1980; Gusse and Chevaillier, 1980; Labhart and Koller, 1981, 1982). There are two methodologies which have been used to attack this problem. First, one can prepare chromatin from nuclei and digest it with either endogenous or exogenous nucleases to obtain fragments which one can manipulate either prior to placing them on an electron microscope grid or

while they are on the grid (Finch and Klug, 1976; Felsenfeld, 1978; Thoma, Koller, and Klug, 1979). This procedure has the advantage of allowing one to change the ionic environment and rapidly processing the material to be visualized by electron microscopy. However, there is a disadvantage which brings these data into question, that being, the DNA within the chromatin is free to rotate due to the cut ends. This allows any force acting on the chromatin through DNA supercoiling to be dissipated by rotation of the DNA. Thus, restraints on DNA which might be reflected in an altered chromatin configuration are not visualized in these studies.

The other methodology in widespread use is that of swelling nuclei in low ionic buffer and then processing the nuclei for electron microscopy by dehydration, embedment, and thin sectioning. This procedure is much more time consuming than the one previously described, yet it has the advantage of not requiring that the DNA be sliced with an endonuclease. (The difficulty with the Miller type spreading [Miller and Bakken, 1972] procedure is that until the nuclei are swollen almost to the point of lysis they cannot be centrifuged onto electron microscope grids and examined because the electron beam cannot penetrate the relatively thick specimen.) This inability to spread the nuclei on grids makes it much more difficult to obtain a three-dimensional image of the structure being examined because thin sections are used for examination and many times the structure being examined passes in and out of the plane of section. However, it is felt that the maintenance of the DNA in an uncut state so that it is not free to rotate about its longitudinal axis is more important than being able to rapidly process the material, so only at times when the chromatin was highly dispersed was the spreading technique utilized.

In Finch and Klug's initial experiments (1976) and in an extension of this work (Thoma, Koller, and Klug, 1979), chromatin from isolated rat liver nuclei was digested with either exogenous or endogenous nucleases to obtain chromatin which was up to 40 nucleosomes in length. The experiments demonstrated very elegantly that as the ionic strength of the medium in which the chromatin was placed was increased from 1mM to 150mM Na, the structure of the chromatin became more compact and finally resulted at 150mM Na in tightly compacted solenoidal structures. Based on this data Finch, Klug, Thoma, and Koller (Finch and Klug, 1976; Thoma, Koller, and Klug, 1979) have proposed that chromatin in interphase nuclei is compacted via a series of coils of increasing diameter. The 100Å fiber is wound to form a 200Å-300Å coil and this coiled chromatin is further compacted by winding to form a coil of 400Å-550Å diameter.

The idea of coiling of chromatin for its compaction has been questioned by numerous laboratories (Kiryanov et al., 1976; Hozier, Renz, and Nehls, 1977; Renz, Nehls, and Hozier, 1977; Olins and Olins, 1978; Rattner and Hamkalo, 1978; Pruitt and Grainger, 1980; Zentgraf, Muller, and Franke, 1980; Aboukarsh and Kunkle, 1981). However, in all these investigations only one or two points in the chromatin dispersion process was examined for chromatin superstructural organization and the data from each indicated that chromatin was organized in the form of superbeads. This present study was designed to investigate the morphological characteristics of chromatin superstructure.

Scanning Electron Microscopy

The scanning electron microscopy presented in this investigation was originally carried out in an attempt to visualize any remnants of the nuclear envelope that might be left on isolated sperm nuclei after

treatment with Triton X-100. However, it was found that by allowing sperm nuclei to swell in low ionic buffer and then processing them for scanning electron microscopy the initial stages of chromatin dispersion were observable. These results were quite informative in that they demonstrated that the sperm chromatin was indeed in the form of a 500Å fiber prior to its dispersion into superbeads. These results also demonstrated that as the sperm nuclei swell, generally there is one point from which the chromatin begins to be released from the nucleus. This observation is striking for most other investigations by scanning electron microscopy have dealt with chromatin which is dispersed to a much greater extent than in this investigation and there was no indication of the initial events which occur during chromatin lysis in these previously published scanning electron microscope investigations (Marsden and Laemmli, 1979; Daskal and Busch, 1978). The results obtained in this investigation agree quite well with those obtained by others; for example, by scanning-transmission electron microscopy the largest fiber observed in isolated metaphase chromosomes was 400Å-500Å (Adolph, Cheng, and Laemmli, 1977; Adolph, 1980a, 1980b; see also, Daskal and Busch, 1978 for review). This 400Å-500Å fiber was interpreted to be a coil of a smaller 200Å to 300Å solenoid and the evidence presented was convincing. This indicates that the packing of chromatin as seen in the metaphase chromosome may be different than that utilized in this sea urchin sperm nucleus. The major difference in the two systems is that the metaphase chromatin is, for the most part, only maximally compacted for a short period of time during mitosis (approximately sixty minutes maximum) while the chromatin within the sperm nucleus may be condensed for periods ranging up to six months (Harvey, 1956).

Analysis of chromatin at later stages of dispersion gave no clear indication of what was actually occuring because the chromatin was visualized as a tangled intertwining mass and the measurements of the chromatin diameters were quite variable (ranging from 100Å to over 500Å). In this regard these results are identical to many previous investigations of chromatin which has been prepared by numerous techniques and analyzed by scanning electron microscopy (Daskal and Busch, 1978).

Transmission Electron Microscopy

The results presented in this investigation were very informative for this is the first time that swelling and lysis of whole nuclei have been systematically studied using material which has been processed for thin sectioning-transmission electron microscopy. This study has demonstrated the events of chromatin dispersion from the most condensed form of the sea urchin sperm chromatin (500Å) to the nucleosome (112Å). It is easily seen that the superbeads are packed side by side with no intervening space in the most condensed configuration to form structures which appear as cylinders or rods of 500Å diameter. Generally, this large diameter structure is not seen in thin sections of interphase nuclei (Fawcett, 1981) but they are seen in scanning electron microscopic micrographs of metaphase chromosomes (Daskal and Busch, 1978). In analysis of scanning micrographs the question has been raised: Are these 400Å-500Å fibers really a natural form of the chromatin or are they an artifact of fixation? By demonstrating their presence in thin sections using a different set of buffers and dehydrating agents it is now possible to state that these 500Å fibers are probably not an artifact. In addition, 400Å superbeads have been seen in thin sectioned material using the Chironomus salivary gland nuclei (Anderson, Bjorkroth, and Daneholt, 1980). Using stereomicroscopy

it has been demonstrated that the condensed chromatin of these polytene chromosomes decondenses by breakdown of the superbeads into what appear to be 100Å fibers which are then transcribed after undergoing a further structural modulation.

The electron micrographs demonstrate that the superbeads are interconnected by 112Å fibers, which are equivalent to the diameter of nucleosomes placed side-by-side. It appears from the data obtained in this investigation that the superbeads are disrupted directly into these 112Å fibers without any intervening structures. Thus in this system there does not appear to be any structure of 200Å to 300Å in diameter. This is probably a reflection of the fact that in the sea urchin sperm nucleus there is no euchromatin (Paoletti and Huang, 1969) for in systems where there is chromatin actively engaged in transcription many areas may be visualized which have 200Å-300Å fibers (Puvion-Dutilleul and Puvion, 1981).

Sperm nuclei which had been partially lysed in low ionic buffer and then centrifuged onto electron microscope grids were also examined by transmission electron microscopy. This chromatin demonstrated the typical beads-on-a-string configuration as seen in many other cell types (Rill and Von Volde, 1973; Rill, Shaw, and Van Volde, 1978). One observation made was that at points in the nuclear swelling process when superbeads were seen in thin sections of sperm nuclei the chromatin was too densely packed for the electron beam to penetrate in spread preparations; thus it was impossible to see the superbeads in this type preparation and only with a greater degree of swelling was it possible to see the nucleosomes interconnected with the spacer DNA. Spadafora et al. (1976a, 1976b, 1979) and Zentgraf, Muller, and Franke (1980), in analysis of sperm chromatin in the sea urchin, _Sphaerechinus granulosus,_ demonstrated the presence of

superbeads with the same average diameter (450Å), yet they did not investigate the organization of the superbeads into higher ordered structures as was done in this investigation. It would be a logical extension of their results to say that the superbeads which they observed were ultimately packaged in the same type 500Å rod as seen in this investigation.

Isolation of Superbeads

In this investigation experiments were carried out in an attempt to isolate the superbeads which were seen in the swollen sea urchin sperm nuclei. Conditions were adjusted so that approximately 10% of the DNA was made acid soluble after digestion with DNA'ase I. After the digestion, chromatin was layered on 10-30% sucrose gradients and centrifuged overnight at varying speeds. These experiments resulted in an enrichment of the superbeads in one region of the gradient but in none of the fractions was there a relatively "pure" preparation of superbeads. In contrast to the findings reported here, Hozier, Renz, and Nehls (1977) were able to isolate superbeads from nuclei of calf lymphocytes by limited digestion with nuclease and subsequent centrifugation on sucrose gradients. The major difference in the two systems utilized is that the calf chromatin is readily susceptible to digestion by nucleases (Hozier, Renz, and Nehls, 1977) while the chromatin of the sea urchin sperm nucleus is relatively inaccessable (Simpson and Bergman, 1980). In fact, even after lysis of sperm nuclei, only approximately 20% of the DNA is digestable with DNA'ase I with prolonged digestion (overnight at 24°C); probably the superbeads are not being freed from the bulk of the chromatin and they only make a minor fraction of the total amount of chromatin in the digest. The results of the electron microscopy tend to confirm this idea because there are many

more large fragments of chromatin seen than superbeads and many of the superbeads seen have a chromatin tail on either one or both sides of the bead.

Relationship to the Events of Fertilization

The morphology of sea urchin sperm chromatin has been determined in order to increase our understanding of the events of fertilization. As the sperm nucleus enters the egg cytoplasm during the early aspects of fertilization the morphology of the paternal chromatin is rapidly altered from that of a condensed mass of inactive material to one which is active in both DNA replication (Longo and Plunkett, 1973) and RNA transcription (Longo and Kunkle, 1977). Prior to this activation of the paternal genome there occurs a massive reorganization of its heterochromatin (Kunkle, 1982; see also Longo and Kunkle, 1978) into dispersed chromatin. It has been shown that concomitant with this change in chromatin structure there is a change in the proteins associated with the paternal genome (Kunkle, Longo, and Magun, 1978a; Carroll and Ozaki, 1979; Poccia, Salik, and Krystal, 1981). These changes involve the removal of the sperm specific basic proteins and their replacement with cleavage stage specific basic proteins (Johnson and Hnilica, 1970; Carroll and Ozaki, 1979; Poccia, Salik, and Krystal, 1981). In addition, the nonhistone proteins of the sperm nucleus are removed and replaced with other acidic proteins apparently of maternal origin (Kunkle, Longo, and Magun, 1978a). These results demonstrate that there is a massive reorganization of the paternal genome at fertilization, yet they do not indicate which events are necessary for the dispersion of the sperm chromatin. It is possible that most, if not all, of the events described above are secondary to initiation of chromatin dispersion and are only a reflection of the primary intracellular events. In order to

circumvent this problem of not knowing the order of events, sperm nuclei were incubated in cytoplasm from sea urchin eggs and it was determined that nuclei did expand and form structures similar to male pronuclei (Kunkle, Magun, and Longo, 1978b). However, this did not greatly reduce the complexity in attempting to understand the events of sperm nuclear enlargement. The experiments described in this investigation are an attempt to learn what is involved in dispersion of the paternal chromatin. Placing sperm nuclei in a low ionic strength buffer allows one to determine what structures are present and are also capable of being disrupted by reducing the divalent ion concentration. Of course, at the time the sperm nucleus enters the egg at fertilization the calcium concentration is elevated rather than being depressed (Epel, 1978). However, the local concentration around and within the sperm nucleus has not yet been determined experimentally.

The determination of the higher ordered structure of this paternal chromatin will enable further investigations to be carried out in attempts to identify the factors within the egg cytoplasm which are involved in the breakdown of the superbeads into 100Å fibers and the mechanisms necessary to reorganize the sperm histones and remove them so that a different set of proteins might come onto the DNA to initiate or allow its involvement in DNA and RNA synthesis. As the fractionation procedures for the egg cytoplasm become further refined (Kunkle, Magun, and Longo, 1978b; Longo and Kunkle, 1978) one should be able to determine what molecule(s) is involved in removing or adding proteins (or other components) to the superbeads to disrupt their organization. In addition, one will be able to investigate the ordering of the superbeads so that the questions about the inactivation of large segments of a genome might be approached through this

system, by examination of the factors responsible for the lateral apposition and dispersion of superbeads.

One other off-shoot from these investigations is that by understanding the mechanisms of chromatin dispersion and activation, one might be able to inhibit the activation process by altering the environment in which the spermatozoa are maintained (epididymis or ductus deferens), leading to the inability of the paternal genome to participate in chromatin dispersion after it enters the egg at the time of fertilization.

REFERENCES

Aboukarsh, N., and Kunkle, M. (1981). Ultrastructural characteristics of sea urchin sperm chromatin. J. Cell Biol. 91:189.

Adolph, K. W. (1980a). Organization of chromosomes in mitotic HeLa cells. Exp. Cell Res. 125:95-103.

Adolph, K. W. (1980b). Isolation and structural organization of human mitotic chromosomes. Chromosoma (Berl.) 76:23-33.

Adolph, K. W., Cheng, S. M. and Laemmli, U. K. (1977). Role of non-histone proteins in metaphase chromosome structure. Cell 12:805-816.

Afzelius, B. A. (1955). The fine structure of the sea urchin spermatozoa as revealed by the electron microscopy. Zeitschrift fur Zellforschung Bd. 42, S. 134-148.

Anderson, K., Bjorkroth, B., and Daneholt, B. (1980). The in situ structure of the active 75S RNA genes in Balbiani rings of Chironomus tentans. Exp. Cell Res. 130:313-326.

Arceci, R. J., and Gross, P. R. (1980). Sea urchin sperm chromatin structure as probed by Pancreatic DNase 1: Evidence for a novel cutting periodicity. Develop. Biol. 80:210-224.

Axel, R. (1975). Cleavage of DNA in nuclei and chromatin with Staphylococcal nuclease. Biochemistry 14:2921-2925.

Becak, M. L., and Fukuda, K. (1978). Arrangement of nucleosomes in condensed chromatin fibres. Experientia 35:24-26.

Benyajati, C., and Worcel, A. (1976). Isolation, characterization and structure of the folded interphase genome of Dorsophilia melanogaster. Cell 9:393-407.

Berkowitz, E. M., and Doty, P. (1975). Chemical and physical properties of fractionated chromatin. Proc. Natl. Acad. Sci. USA 72:3328-3332.

Bloch, D. P. (1969). A catalog of sperm histones. Genetics 1:93-111.

Bloch, D. P., and Hew, H. Y. C. (1960). Changes in nuclear histone during fertilization and early embryonic development in the pulmonate snail, Helix aspersa. J. Biophys. Biochem. Cytol. 8:69-81.

Bonner, J. (1979). Properties and composition of isolated chromatin. In Nicolini, C. A. (ed.), Chromatin structure and function, Plenum Press, New York, pp. 3-13.

Bouteille, M., Laval, M., and Dupuy-Coin, A. M. (1974). Localization of nuclear functions as revealed by ultrastructural autoradiography and cytochemistry. In Busch, H. (ed.), The Cell Nucleus, Vol. I, Academic Press, New York, pp. 3-71.

Bowen, R. H. (1924). On the acrosome of the animal sperm. Anat. Rec. 28:1-13.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.

Busch, H., and Smetana, K. (1970). The cytochemistry and ultrastructure of the nucleus. In Busch, H., and Smetana, K. (eds.), The Nucleolus, Academic Press, New York, pp. 59-114.

Butler, P. J. G., and Thomas, J. O. (1980). Changes in chromatin folding in solution. J. Mol. Biol. 140:505-529.

Carpenter, B. G., Baldwin, J. P., Bradbury, E. M., and Ibel, K. (1976). Organization of subunits in chromatin. Nuc. Acid Res. 3:1739-1746.

Carroll, A. G., and Ozaki, H. (1979). Changes in the histones of the sea urchin Strongylocentrotus purpuratus at fertilization. Exp. Cell Res. 119:307-315.

Carter, C. W., Jr. (1978). Histone packing in the nucleosome core particle of chromatin. Proc. Natl. Acad. Sci. USA 75:3649-3653.

Daskal, Y., and Busch, H. (1978). Ultrastructure of chromatin and chromosomes as visualized by scanning electron microscopy. In Busch, H. (ed.), The Cell Nucleus, Vol. 4, Academic Press, New York, pp. 3-45.

DuPraw, E. J. (1966). Evidence for a "folded-fiber" organization in human chromosomes. Nature 209:577-581.

Epel, D. (1978). Mechanisms of activation of sperm and egg during fertilization of sea urchin gametes. In Moscona, A. A., and Monroy, A. (eds.), Current Topics in Developmental Biology, Vol. 12, pp. 186-246.

Evans, H. J. (1977). Some facts and fancies relating to chromosome structure. In Harris, H. and Hirschhorn, K. (eds.), Advances in Human Genetics, Vol. 8, Plenum Press, New York, pp 347-438.

Evans, H. J. (1979). Chromosome structure in man and related organisms. In Dion, A. S. (ed.), Concepts of the Structure and Function of DNA, Chromatin and Chromosomes, Symposia Specialists Inc., Chicago, pp. 81-121.

Evenson, D. P., Witkin, S. S., Harven, E. D. E., and Bendich, A. (1978). Ultrastructure of partially decondensed human spermatozoal chromatin. J. Ultrastruct. Res. 63:178-187.

Fawcett, D. W. (1958). The structure of the mammalian spermatozoon. International Review of Cytology 7:195-234.

Fawcett, D. W. (1968). The topographical relationship between the plane of the central pair of flagellar fibers and transverse axis of the head in guinea pig spermatozoa. J. Cell Sci. 3:187-198.

Fawcett, D. W. (1975). Review Article, The Mammalian Spermatozoon. Develop. Biology 44:394-436.

Fawcett, D. W. (1981). Nucleus. In The Cell, Saunders, Philadelphia, pp. 195-302.

Fawcett, D. W., and Ito, S. (1965). The fine structure of bat spermatozoa. Am. J. Anat. 116:567-610.

Fawcett, D. W., and Porter, K. R. (1954). A study of the fine structure of ciliated epithelium. J. Morphology 94:221-281.

Felsenfeld, G. (1978). Chromatin. Nature 271:115-122.

Finch, J. T., and Klug, A. (1976). Solenoidal model for superstructure in chromatin. Proc. Natl. Acad. Sci. USA 73:1897-1901.

Finch, J. T., and Klug, A. (1978). X-ray and electron microscope analysis of crystals of nucleosome cores. Cold Spring Harb. Symp. Quant. Biol. 42:1-9.

Franke, W. W. (1974). Nuclear envelopes: Structure, biochemistry and functions of the nuclear envelope. Phil. Trans. Roy. Soc. London (B) 268:67-93.

Franke, W. W., Scheer, U., Trendelenburg, M., Zentgraf, H., and Spring, H. (1978). Morphology of transcriptionally active chromatin. Cold Spring Harbor Symp. Quant. Biol. 42:755-772.

Friedlaender, M. H. G. (1952). Observation on the structure of human spermatozoa, an electron microscope inquiry. Proc. Roy. Soc. B 140:60-69.

Gall, J. G. (1952). Chromosome fibers from an interphase nucleus. Science 139:120-121.

Gall, J. G. (1963). Chromosomes and cytodifferentiation. In Locke, M. (ed.), A cytodifferentiation and macromolecular synthesis, Academic Press, New York, pp. 119-143.

Gatenby, J. B., and Woodger, J. H. (1921). The cytoplasmic inclusions of the germ cells. Part IX. On the origin of the Golgi apparatus on the middle piece of the ripe sperm of Cavia, and the development of the acrosome. Qurt. Jour. Micr. Sci., N.S., 65, 258:265-291.

Griffith, J. D. (1976). Visualization of prokaryotic DNA in a regularly condensed chromatin-like fiber. Proc. Natl. Acad. Sci. USA 73:563-567.

Gusse, M., and Chevaillier, P. H. (1980). Molecular structure of chromatin during sperm differentiation of the Dogfish Scaliorhynus caniculus (L). Chromosoma 77:57-68.

Hamer, D. (1955). The composition of the basic proteins of Echinoderm sperm. Biol. Bull. 108:35-39.

Harvey, E. B. (1956). Sperm. The American arbacia and other sea urchins, Princeton University Press, New Jersey, pp. 87-94.

Hay, E. D., and Revel, J. P. (1963). The fine structure of DNA component of the nucleus. J. Cell Biol. 16:29-51.

Heitz, E. (1928). Das heterochromatin der Mooe I. Jahrb. Wiss. Bot. 69:762-818.

Hnilica, L. S. (1972). Histone interaction with DNA. In The structure and biological function of histones, vol. 1, CRC Press, Florida, pp. 93-123.

Hozier, J., Renz, M., and Nehls, P. (1977). The chromosome fiber: evidence for an ordered superstructure of nucleosomes. Chromosoma 62:301-317.

James, G. T. (1978). Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Anal. Biochem. 86:574-579.

Johnson, A. W., and Hnilica, L. S. (1970). In vitro RNA synthesis and nuclear proteins of isolated sea urchin embryo nuclei. Biochim. Biophys. Acta 224:518-530.

Keichline, L. D., and Wasserman, P. M. (1977). Developmental study of the structure of sea urchin embryo and sperm chromatin using micrococcal nuclease. Biochem. Biophys. Acta. 475:139-151.

Kiryanov, G. I., Manamshjan, T. A., Polyakov, V. Y., Fais, D., and Chentsov, Y. S. (1976). Levels of granular organization of chromatin fibers. FEBS Lett. 67:323-327.

Koehler, J. K. (1970). A freeze-etching study of rabbit spermatozoa with particular reference to head structure. J. Ultrastruct. Res. 33:598-614.

Kornberg, R. D. (1974). Chromatin Structure: A repeating unit of histones and DNA. Science 184:868-870.

Kornberg, R. D. (1977). Structure of Chromatin. Ann. Rev. Biochem. 46:931-954.

Kunkle, M. (1982). Chromatin transition in the fertilizing sperm nucleus of the sea urchin, Strongylocentrotus purpuratus. Gamete Research 5:181-190.

Kunkle, M., Longo, F. J., and Magun, B. E. (1978a). Nuclear protein changes in the maternally and paternally derived chromatin at fertilization. J. Expt. Zool. 203:371-380.

Kunkle, M., Magun, B. E., and Longo, F. J. (1978b). Analysis of isolated sea urchin nuclei incubated in egg cytosol. J. Exp. Zool. 203:381-390.

Labhart, P., and Koller, T. (1981). Electron microscope specimen preparation of rat liver chromatin by a modified Miller spreading technique. Eur. J. Cell Biol. 24:309-316.

Labhart, P., and Koller, T. (1982). Structure of the active nucleolar chromatin of Xenopus laevis oocytes. Cell 28:279-292.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227:680-685.

Langmore, J. P., and Wooley, J. C. (1975). Chromatin architecture: Investigation of a subunit of chromatin by dark field electron microscopy. Proc. Nat. Acad. Sci. USA 72:2691-2695.

LeStourgeon, W. M., and Wray, W. (1974). Extraction and characterization of the phenol-soluble acidic nuclear chromatin. In Cameron, I. L. and Jeter, J. (eds.), Acidic proteins of the nucleus, Academic Press, New York, pp. 59-102.

Lohr, M., and Courtens, J.-L. (1979). Nuclear reorganization in ram spermatids. J. Ultrastruct. Res. 67:309-324.

Longo, F. J., and Kunkle, M. (1977). Activation of paternally derived chromatin in fertilized sea urchin eggs. J. Exp. Zool. 201:431-438.

Longo, F. J., and Kunkle, M. (1978). Transformation of sperm nuclei upon insemination. Cur. Top. Develop. Biol. 12:149-184.

Longo, F. J., and Plunkett, W. (1973). The onset of DNA synthesis and its relation to morphogenetic events of the pronuclei. Dev. Biol. 30:56-67.

Lung, B. (1968). Whole-mount electron microscopy of chromatin and membranes of bull and human sperm heads. J. Ultrastruct. Res. 22:485-493.

Marsden, M. P. F., and Laemmli, U. K. (1979). Metaphase chromosome structure: Evidence for a radial loop model. Cell 17:849-858.

Mazia, D. (1954). The particulate organization of the chromosome. Proc. Natl. Acad. Sci. USA 40:521-527.

Metz, C. W. (1932). The male germ cells. In Cowdry, E. V. (ed.), Special Cytology, Vol. 3, Hoeber, New York, pp. 1727-1770.

Meves, F. (1897). Zur Entstehung der Achsenfaden menschlicher spermatozoen. Anat. Anz., Bd. 14, S. 168-170.

Miller, O. L., and Bakken, A. H. (1972). Morphological studies of transcription. Acta Endocrinol. Suppl. 168:155-177.

Milner, G. R. (1969). Nuclear morphology and ultrastructural localization of DNA synthesis during interphase. J. Cell Sci. 4:569-582.

Noll, M. (1974). Subunit structure of chromatin. Nature 251:249-251.

Olins, A. L., and Olins, D. E. (1974). Spheroid chromatin units (μ bodies). Science 183:330-332.

Olins, D. E., and Olins, A. L. (1978). Nucleosomes: The structural quantum in chromosomes. Am. Scientist 66:704-711.

Oudet, P., Gross-Bellard, M., and Chambon, P. (1975). Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. Cell 4:281-300.

Paoletti, R. A., and Huang, R. C. C. (1969). Characterization of sea urchin sperm chromatin and its basic proteins. Biochemistry 8:1615-1625.

Pardon, J. F., and Wilkins, M. H. F. (1972). A supercoil model for nucleohistone. J. Molec. Biol. 68:115-124.

Poccia, D., Salik, J., and Krystal, G. 1981. Transitions in histone variants of the male pronucleus following fertilization and evidence for a maternal store of cleavage-stage histones in the sea urchin egg. Dev. Biol. 82:282-296.

Pruitt, S. C., and Grainger, R. M. (1980). A repeating unit of higher order chromatin structure in chick red blood cell nuclei. Chromosoma 78:257-274.

Puvion-Dutilleul, F., and Puvion, E. (1981). New aspects of intranuclear decondensation of chromatin: a cytochemical and high-resolution autoradiographical study. J. Cell Sci. 42:305-321.

Randall, J. T., and Friedlaender, M. H. G. (1950). The microstructure of ram spermatozoa. Exp. Cell Res. 1:1-32.

Rattner, J. B., and Hamkalo, B. A. (1978). Higher order structure in metaphase chromosome. II. The relationship between the 250Å fiber, superbeads and beads-on-a-string. Chromosoma (Berl.) 69:373-379.

Renz, M., Nehls, P., and Hozier, J. (1977). Involvement of histone H1 in the organization of the chromosome fiber. Proc. Nat. Acad. Sci. USA 74:1879-1883.

Retzius, G. (1910). Zur Kenntnis der Spermien der Echinodermin. Biol. Untersuch. 15:55-62.

Reynolds, E. S. (1963). The use of lead citrate at high pH as electron-opaque stain in electron microscopy. J. Cell Biol. 17:208-212.

Rill, R., and Von Volde, K. E. (1973). Properties of nuclease-resistant fragments of calf thymus chromatin. J. Biol. Chem. 248:1080-1083.

Rill, R., Shaw, B. R., and Von Volde, K. E. (1978). Isolation and characterization of chromatin subunits. In Stein, G., Stein, J., and Kleinsmith, L. J. (eds.), Methods in Cell Biology, Vol. 18, Academic Press, New York, pp. 69-103.

Ris, H. (1966). B-chromosomes and genes. Fine structure of chromosomes. Proc. Roy. Soc. B. 164:246-257.

Ris, H., and Kornberg, J. (1979). Chromosome structure and levels of chromosome organization. In Prescott, D. M. and Goldstein, L. (eds.), Cell biology. A comprehensive treatise, the structure and replication of genetic material, Vol. 2, Academic Press, New York, pp. 267-361.

Schnall, M. (1952). Electron microscopic study of human spermatozoa. Fertility and Sterility 3:62-82.

Simpson, R. T., and Bergman, L. W. (1980). Structure of sea urchin chromatin core particle. J. Biol. Chem. 255:10702-10709.

Smetana, K., and Busch, H. (1974). The nucleolus and nucleolar DNA. In Busch, H. (ed.), The Cell Nucleus, Vol. 1, Academic Press, New York, pp. 73-147.

Solari, A. J. (1968). The ultrastructure of chromatin fibers. 1. The effect of spreading condition. Exp. Cell Res. 53:553-566.

Spadafora, C., Bellard, M., Compton, J. L., and Chambon, P. (1976a). The DNA repeat lengths in chromatins from sea urchin sperm and gastrula cells are markedly different. FEBS Letters 69:281-285.

Spadafora, C., Noviello, L., and Geraci, G. (1976b). Chromatin organization in nuclei of sea urchin embryos. Comparison with the chromatin organization of the sperm. Cell Differentiation 5:225-231.

Spadafora, C., Oudet, P., and Chambon, P. (1979). Rearrangement of chromatin structure induced by increasing ionic strength and temperature. Eur. J. Biochem. 100:225-235.

Strickland, M., Strickland, W. N., Morgan, M., and von Holt, C. (1974). Sequences of the cysteine-containing protein of histone F2a1 from the sea urchin Parachinus angulosus. FEBS Lett. 40:346-348.

Strickland, M., Strickland, W. N., Brandt, W. F., and von Holt, C. (1977a). The complete amino-acid sequence of histone H2b1 from sperm of the sea urchin Parachinus angulosus. Eur. J. Biochem. 77:263-275.

Strickland, W. N., Strickland, M., Brandt, W. F., and von Holt, C. (1977b). The complete amino-acid sequence of histone H2b2 from sperm of the sea urchin Parachinus angulosus. Eur. J. Biochem. 77:277-286.

Strickland, W. N., Strickland, M., von Holt, C., and Gianotti, V. (1982). A partial structure of histone H1 from sperm of the sea urchin Sphaerechinus granulosus. Biochim. et Biophys. Acta 703:95-100.

Suau, P., Bradbury, M., and Baldwin, J. P. (1979). Higher-order structures of chromatin in solution. Eur. J. Biochem. 97:593-602.

Thoma, F., and Koller, T. (1977). Influence of histone H1 on chromatin structure. Cell 12:101-107.

Thoma, F., Koller, T. H., and Klug, A. (1979). Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. J. Cell Biol. 83:403-427.

Tsanev, R., and Avramova, Z. (1981). Non-protamine nucleoprotein ultrastructures in mature ram sperm nuclei. Eur. J. Cell Biol. 24:139-145.

Wagner, T. E., and Yun, J. S. (1979). Fine structure of human sperm chromatin. Arch. Androl. 2:291-294.

Watson, M. L. (1952). Spermatogenesis in the albino rat as revealed by electron microscopy. A preliminary report. Biochem. et Biophys. Acta 8:369-374.

Wilkins, M. H. F., and Randall, J. T. (1953). Crystallinity in sperm heads: Molecular structure of nucleoprotein in vivo. Biochem. et Biophys. Acta 10:192-193.

Wilson, E. B. (1925). The nucleus. In The cell in development and heredity. 3rd ed. Macmillan, New York, pp. 78-113.

Wislocki, G. B. (1949). Seasonal changes in the testes, epididymes and seminal vesicles of deer investigated by histochemical methods. Endocrinology 44:167-189.

Woodcock, C. L. F. (1973). Ultrastructure of inactive chromatin. J. Cell Biol. 59:368a.

Woodcock, C. L. F., Safer, J. P., and Stanchfield, J. E. (1976). Structural repeating units in chromatin. I. Evidence for their general occurrence. Exp. Cell Res. 97:101-110.

Worcel, A. (1978). Molecular architecture of the chromatin fiber. Cold Spring Harbor Symp. Quant. Biol. 42:313-324.

Zentgraf, H., Muller, U., and Franke, W. W. (1980). Supranucleosomal organization of sea urchin sperm chromatin in regularly arranged 40 to 50 nm large granular subunits. Eur. J. Cell Biol. 20:254-264.

Zirkin, B. R., and Wolfe, S. L. (1972). Fiber ultrastructure and dimensions in thin sectioned chromatin. J. Ultrastruct. Res. 39:496-508.

Zoma, M., Bryan, P. N., Harrington, R. E., Olins, A. L., and Olins, D. E. (1977). Conformational states of chromatin. Cold Spring Harbor Symp. Quant. Biol. 42:31-41.

FIGURES

Figure 1. Schematic diagram of mature spermatozoon of sea urchin *Strongylocentrotus purpuratus*. The head is almost entirely occupied by the sperm nucleus. The neck is a small constriction between the head and the tail.

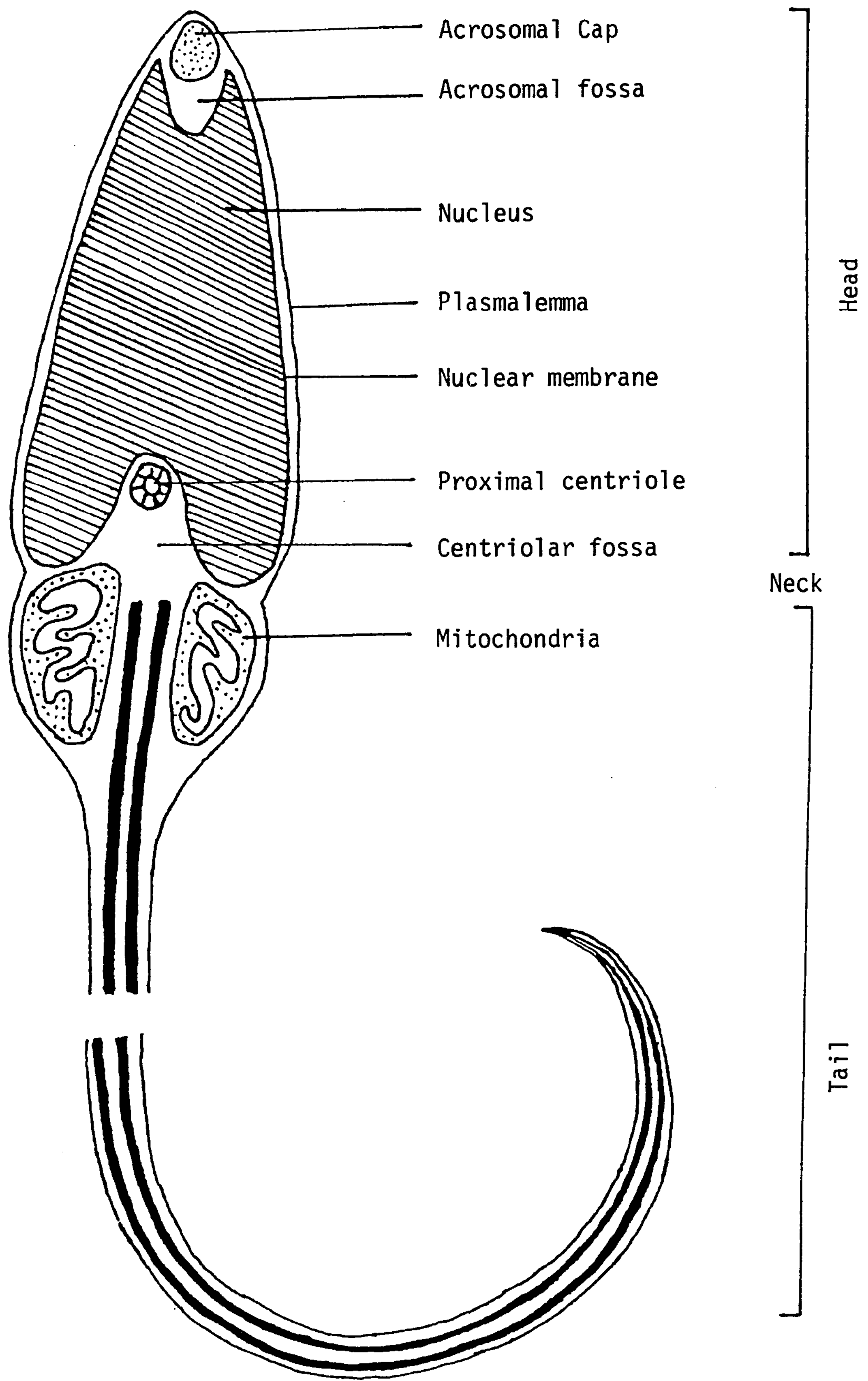

Acrosomal Cap
Acrosomal fossa
Nucleus
Plasmalemma
Nuclear membrane
Proximal centriole
Centriolar fossa
Mitochondria
Head
Neck
Tail

Figure 2. Photomicrograph of longitudinal section of mature sea urchin (Strongylocentrotus purpuratus) sperm which was not treated with either Triton X-100 or low ionic strength buffer (1mM EDTA). This figure demonstrates the different components of the mature spermatzaoon. The sperm head appears to be occupied almost entirely by the nucleus (SN). The latter is composed of tightly packed, darkly stained, electron dense chromatin (heterochromatin). The whole cell (spermatozoon) is surrounded by plasmalemma (P), while the nucleus is surrounded by the nuclear envelope (NE). The centriolar fossa (CF), the acrosome (A), the acrosomal fossa (AF), the sperm mitochrondria (SM) and axoneme (T) are apparent in this figure. This picture was taken by JEOL-GX100. X50,000.

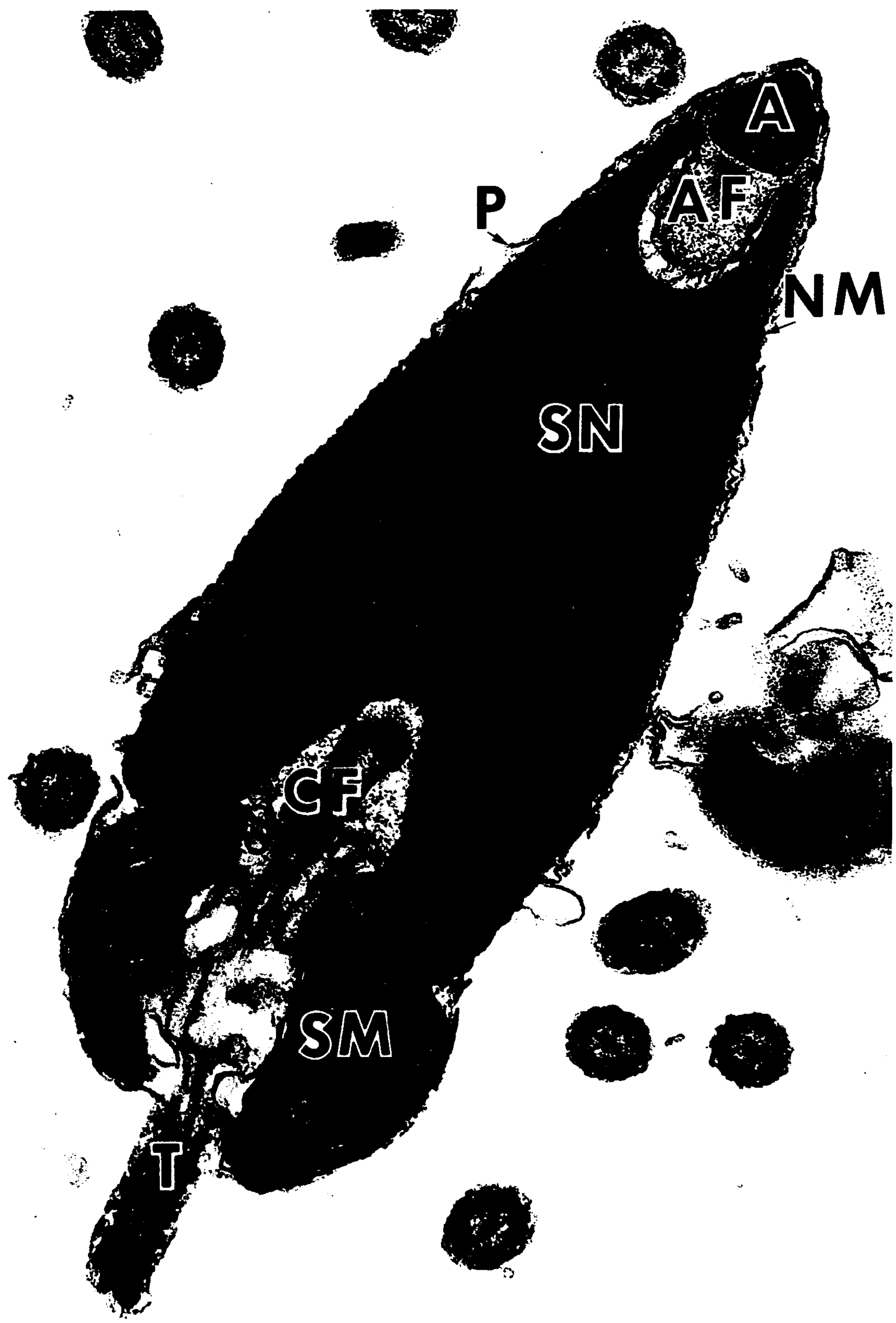

A
AF
P
NM
SN
CF
SM
T

Figure 3. Photomicrograph of a longitudinal section of a sea urchin sperm nucleus in a control experiment which was treated with Triton X-100 in the process of nuclear isolation and purification. This nucleus was not allowed to swell since no EDTA was used. The nucleus (SN) is occupied entirely by the tightly packed, densely stained heterochromatin. Although heterochromatin appears uniformly granular, no definable substructures could be measured. No remnants of the nuclear envelope may be seen in this micrograph along the sides of the nucleus. X90,750.

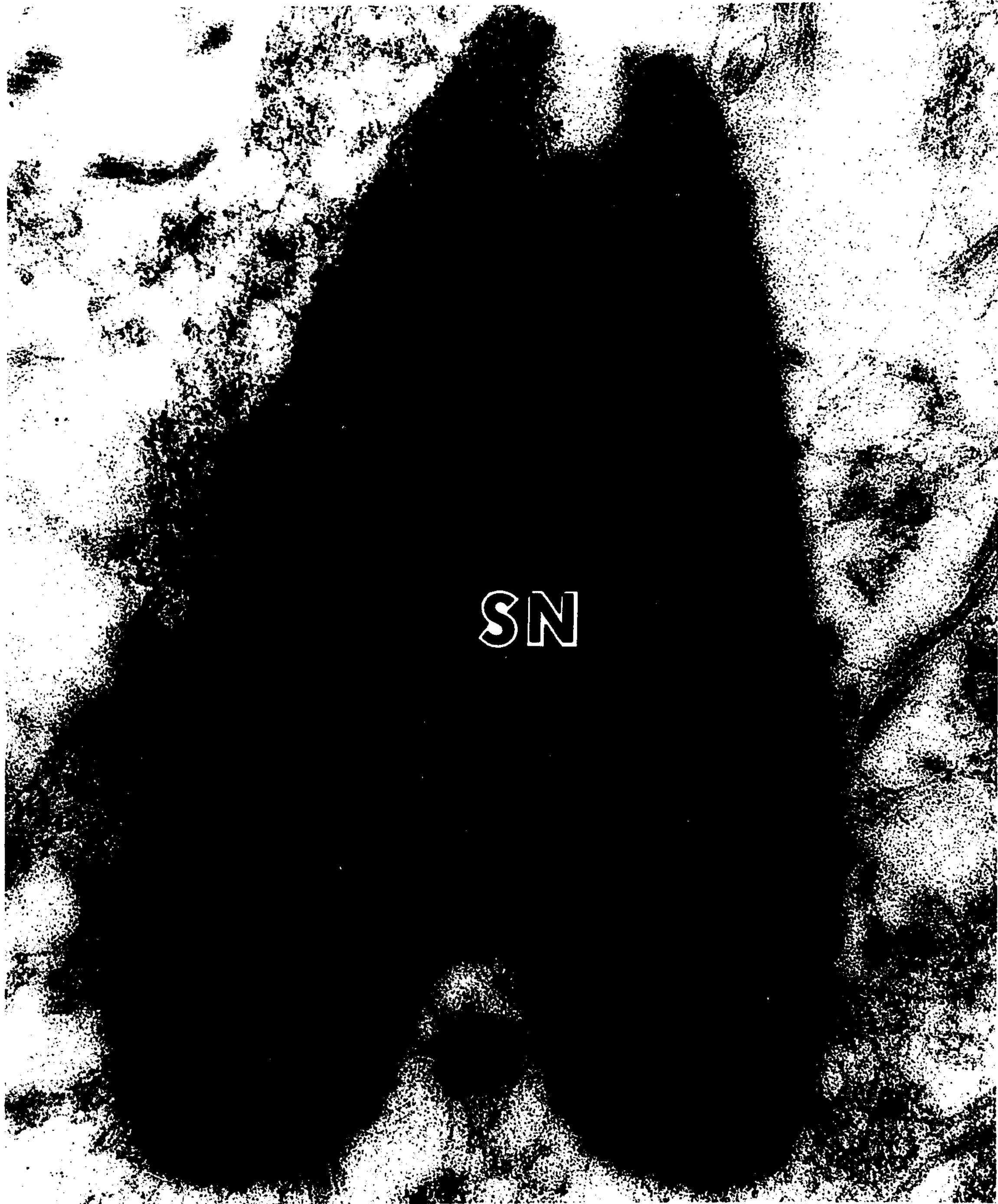

SN

Figure 4. Electron micrograph showing a longitudinal section of sperm nucleus of sea urchin (Strongylocentrotus purpuratus). The nuclei were incubated only for ten minutes in lmM EDTA. The nuclei were fixed in 2.5% gluteraldehyde for two hours, post-fixed with 1% OSO_4 then cut into silver section (600-900Å) using MT-2B (Sorvall) microtomes. Sections were stained with 2% uranylacetate in 50% ethanol and Raynold's lead citrate.

The nuclear chromatin appears to be formed of discrete 400-500Å thick filaments (arrows). These are loosely arranged in the areas around acrosomal fossa (AF) and around the poles of the centriolar fossa (CF). The 400Å thick filaments appear tightly packed in the middle region of the nucleus where it still has remnants of the nuclear envelope (NE-arrowheads). However, at this early stage of lysis, it is difficult to visualize the substructure of the 400Å filaments. X66,625.

AF
CF
NE

Figure 5. Electron micrograph of cross section of sea urchin sperm nucleus. The nuclei were incubated in 1mM EDTA for thirty minutes. The chromatin appears uniformly distributed throughout this section, and is formed of 400-500Å thick filaments. Most of the filaments have spiral courses (arrowheads) and are composed of discrete spherical structures (arrows) having diameters of 400-500Å which correspond to the diameters of the thick filaments. X77,000.

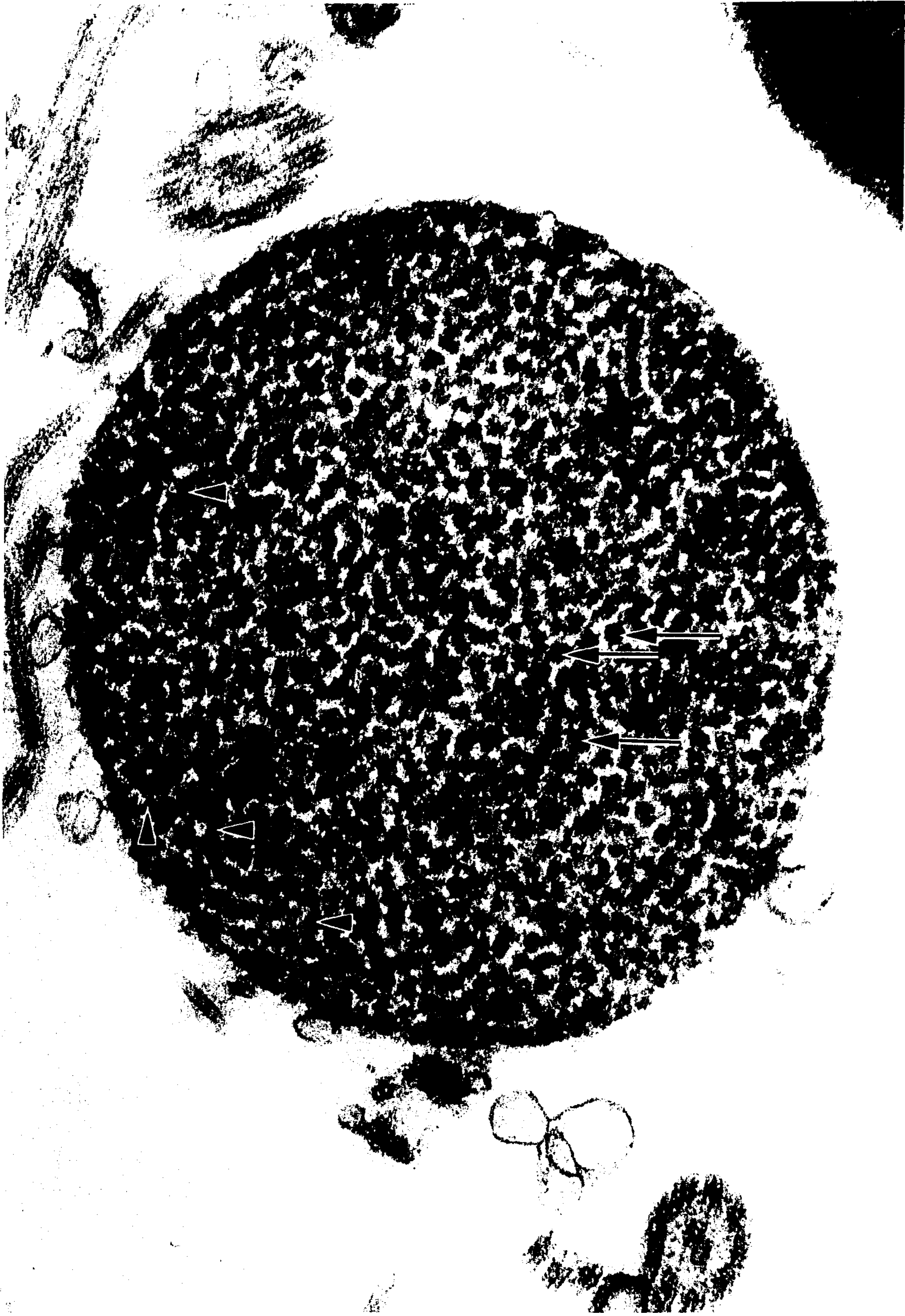


Figure 6. Photomicrograph of sperm nucleus which was incubated in 1mM EDTA for thirty minutes, then processed for thin sectioning. In this section the 400-500Å filaments appear to be composed of 400-500Å spherical aggregates which are clearly seen (arrowheads) to be arranged as tandem. The filaments could be followed to variable distances. Several observations should be made in this section. First, there is better dispersion of the spherical units in the areas depleted of nuclear envelope (*). Second, there are areas at the periphery (arrows) which did not disperse to the corresponding stage of the rest of the chromatin. Third, this section was prepared from a sample which was less purified than is normally done, so several portions of axonemes (T) can be seen in the field. X71,000.

T

Figure 7. Electron micrograph of sea urchin sperm nucleus at centriolar fossa level. The nucleus was incubated in 1mM EDTA for sixty minutes. The 400-500Å filaments are composed of discrete 400-500Å spherical units (arrowheads) which are well separated and interconnected by thin strands (arrows). In this plate there are some non-lysed sperm nuclei (NSN) which appear not to be affected by the process of lysis. Portions of the axoneme (T) are seen in the centriolar fossa (CF). X76,250.

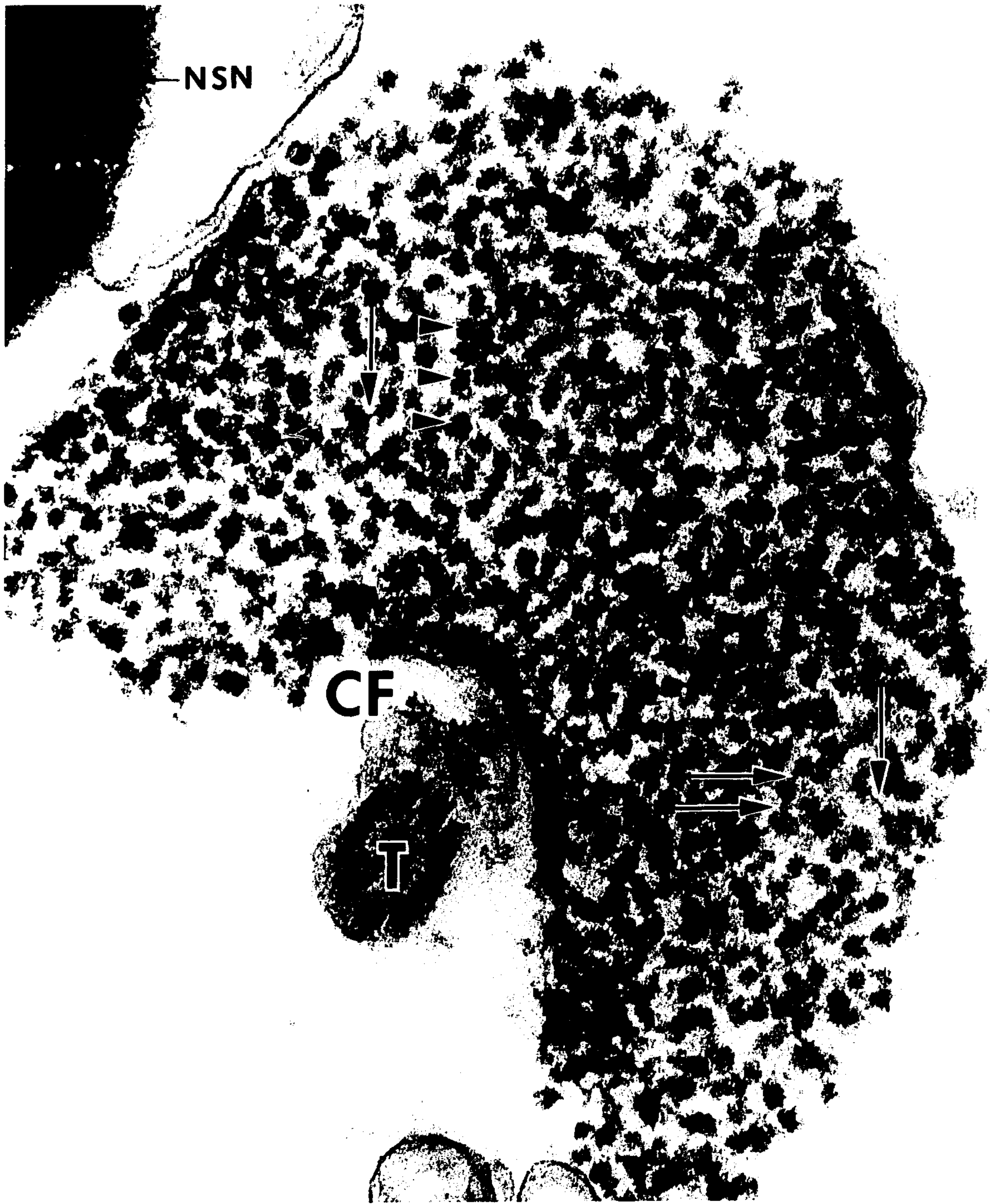

NSN
CF
T

Figure 8. Photomicrograph of a survey view of sperm nuclei which have been incubated in 1mM EDTA for ninety minutes. Most of the chromatin appears in the form of well separated, discrete spherical granules (superbeads) of 414Å ± 60Å in diameter (arrowheads). These 400Å granules appear to be composed of very small circular subunits which are difficult to measure at this stage. Thin strings connecting superbeads are seen at arrows. Scattered membranous vesicles (V) and occasional portions of axonemes (T) are seen in the lower left section of the photomicrograph. X62,000.

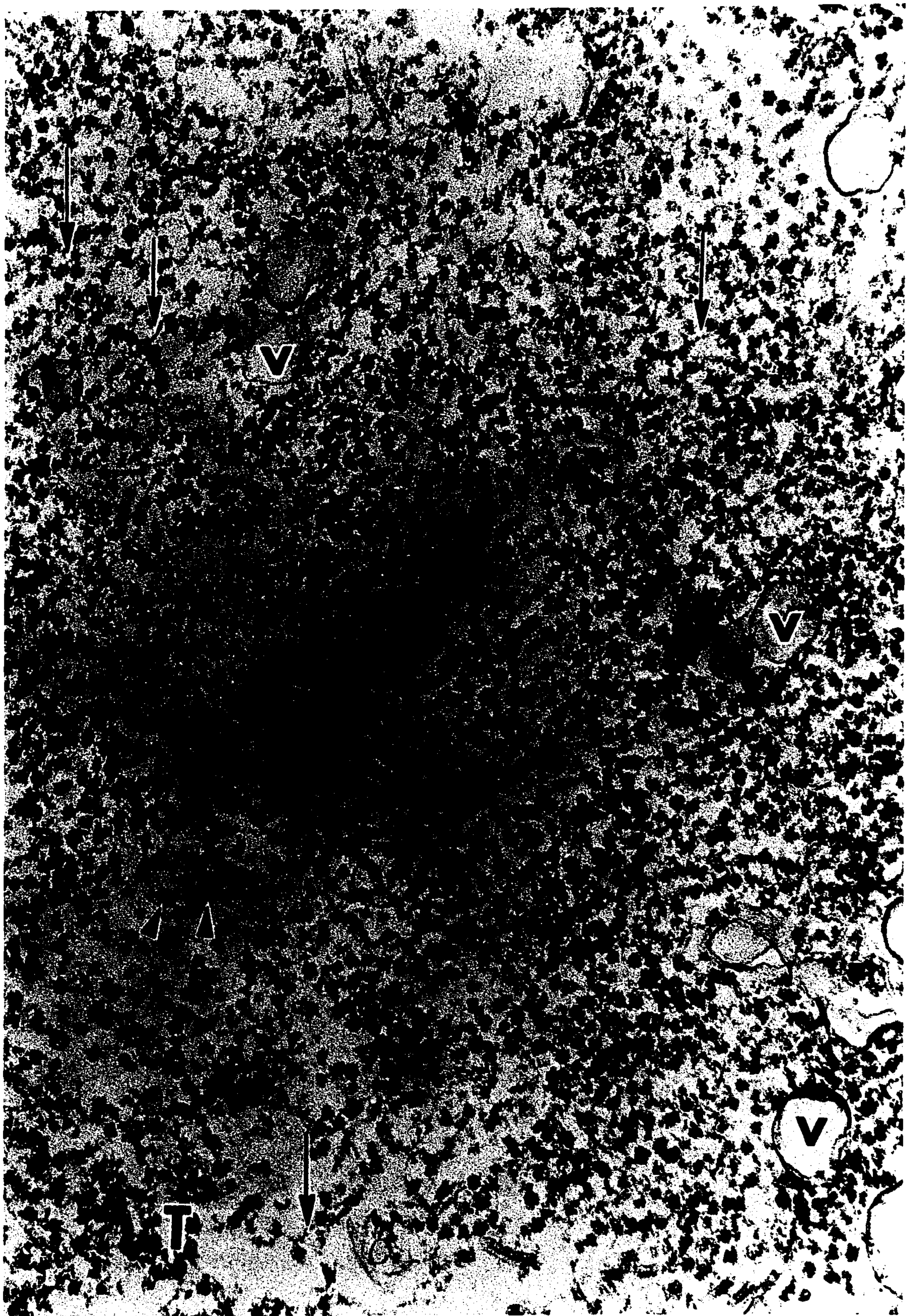

V
V
V
T

Figure 9. Photomicrograph of sperm nuclei which were incubated in 1mM EDTA for 120 minutes. The chromatin appears to be composed of irregular spherical clusters (superbeads; *) of asterisks 414Å ± 60Å in diameter. These clusters appear closely packed in some areas (arrowheads) while loosely arranged in others and interconnected by 112Å fibers. These appear to be composed of closely packed smaller circular units of the same diameter chromatin filaments (arrows). The 400Å units appear not to have central holes, but they are composed of smaller circular subunits around 100Å in diameter. The 112Å units (nucleosomes) electron lucent central area of an average diameter of 30Å. Portion of the axoneme (T) is seen in the centriolar fossa (CF). X96,000.

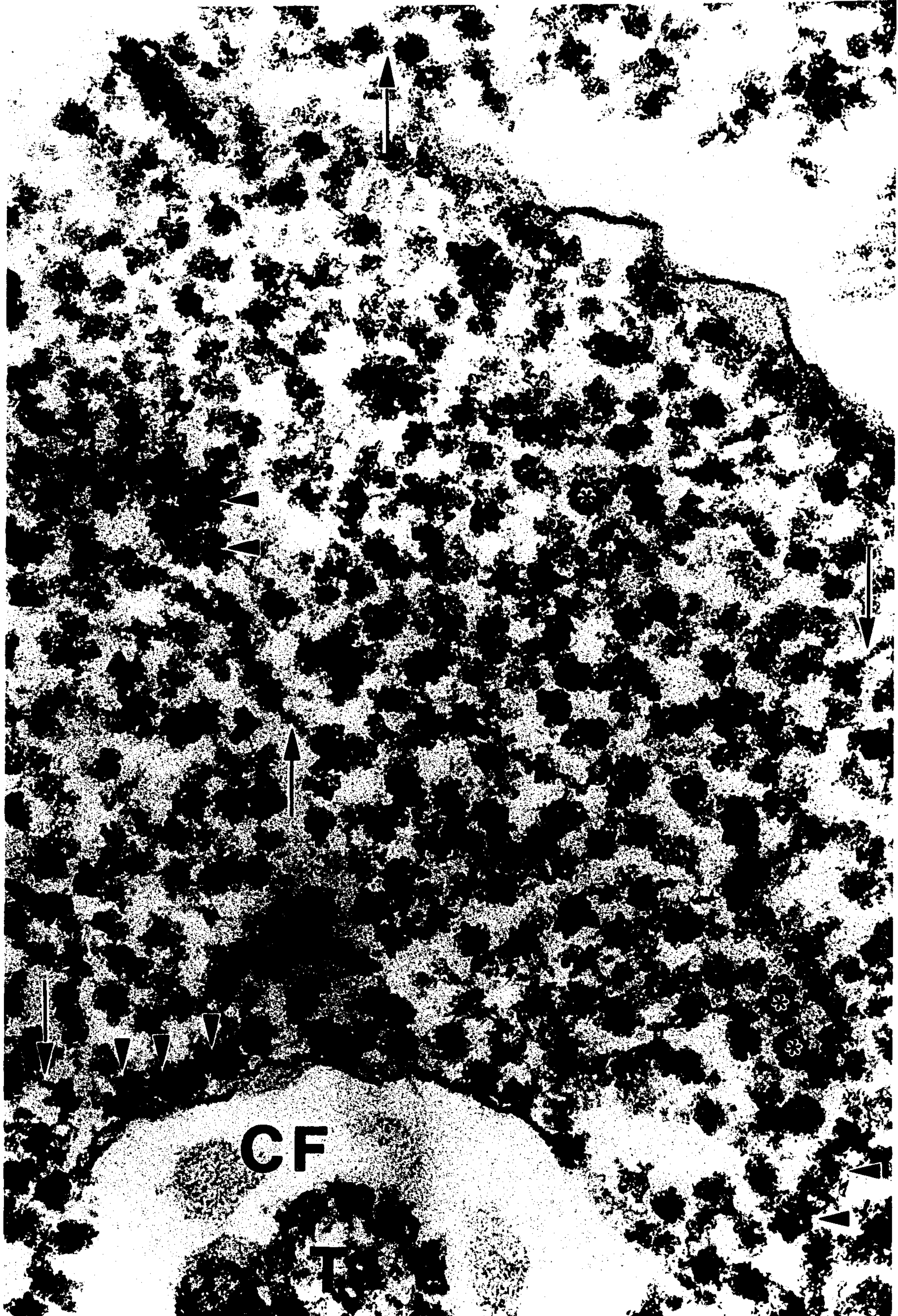

CF
T

Figure 10. Photomicrograph of sea urchin sperm nuclei which were allowed to swell in 1mM EDTA for 120 minutes. The nuclear chromatin appears to be formed of superbeads (arrowheads) interconnected by 112Å chromatin fibers (arrows). The 112Å units are clearly formed of nucleosomes (112Å in diameter) and having a central electron lucent area of 30Å in diameter. X225,000.

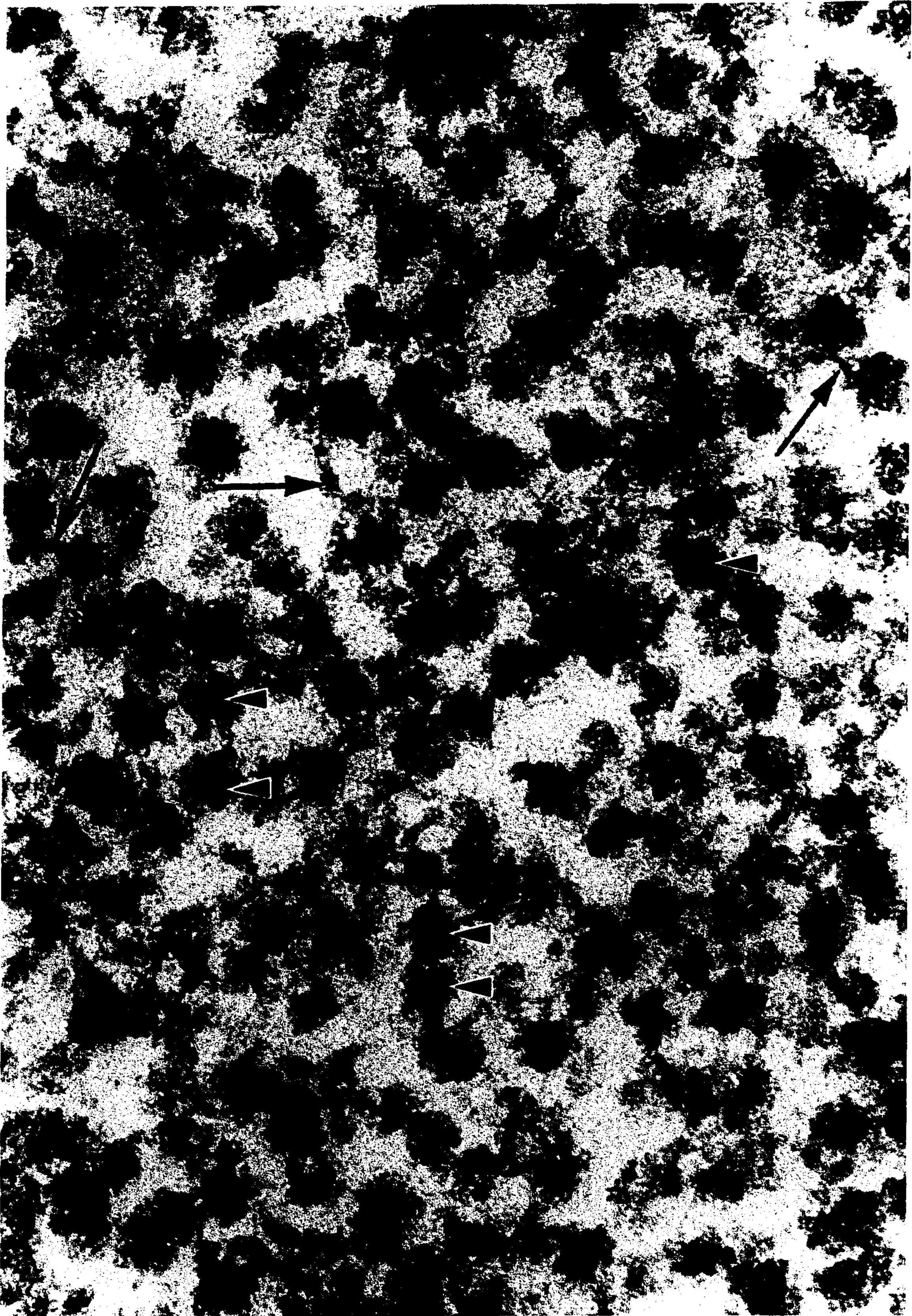


Figure 11. Stereomicrograph showing portions of sea urchin sperm nuclei lysed in 1mM EDTA for ninety minutes. Fixed in 2.5% gluteraldehyde for two hours, postfixed with 2% OSO_4, dehydrated, embedded in Epon 812, golden sectioned (900-1000Å) then stained with 2% alcoholic uranyl acetate and lead citrate. A - was taken at a tilt of +3°. B - was taken at a tilt of -3°. In both sections A and B, the chromatin appears to be formed of clusters (superbeads; arrowheads) roughly spherical variable in size and loosely arranged. The superbeads are composed of smaller circular subunits and interconnected by thin strings. At the right upper border of the lysed nuclei, part of the chromatin is still in the condensed state. Portions of the axonemes (T) and non-lysed sperm nuclei (NSN) are seen in this stereopair. By using the stereoscopic viewer, one can notice the three dimensional build up of the superbeads, their relation to each other and to the 100Å interconnecting fibers. X99,000.

NSN
A

NSN
B

Figure 12. Photomicrograph illustrates stereopairs of sea urchin sperm nuclei which were allowed to swell in 1mM EDTA for ninety minutes, then were processed for EM golden sections. The plate (A) was taken at 0 tilt, while plate (B) was taken at -5°. By the use of stereoscopic viewer one can see the three dimensional construction of superbeads (arrowheads) interconnected with thin chromatin strings (arrows). Portions of non-lysed sperm nuclei (NSN), and the nuclear envelope (NE) are seen in this plate. X79,000.

NSN
NE
A

NSN
NE
B

Figure 13. Photomicrograph showing chromatin fibers "beads-on-a-string" configuration from sea urchin sperm nucleus which were lysed in 1mM EDTA pH 7.5 for four hours. The chromatin was then centrifuged onto formvar-carbon-coated grids through paraformaldehyde-(3.7)-sucrose (3.4) cushion. The grids were washed with 1% phot-Flo, air dried and then were stained with 1% uranylacetate containing 2mM $MgCl_2$. The beads have diameters of 120Å (arrowheads) and spacer DNA (arrows) has a diameter of ~33Å. Note that the contrast of the beads is not very evident. X38,000.

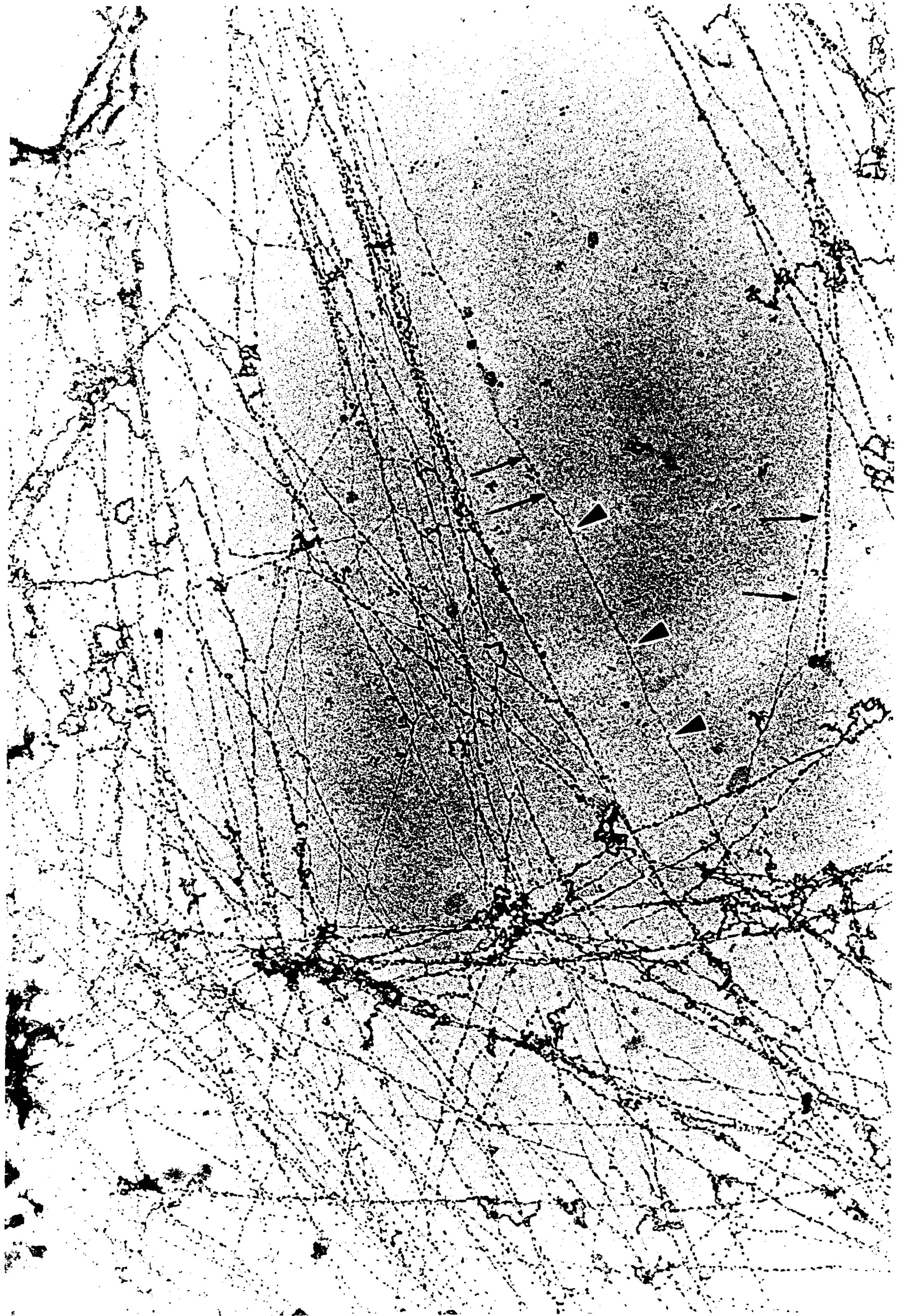


Figure 14. Chromatin spread of sea urchin sperm nuclei which were allowed to disperse for four hours in 1mM EDTA pH 7.5. The chromatin material was centrifuged onto formvar-carbon-coated grids through paraformaldehyde-sucrose cushion (fixative). The grids were washed with 1% photoflo, dehydrated with 95% ethanol, then positively stained with 1% PTA. To increase the contrast of the beads, the grids were coated with platinum-palladium (80:20) at an angle of 6°. The beads (nucleosomes) appear very contrasting with a diameter of 132Å ± 22Å (arrowheads). The nucleosomes are separated by thin strings 38Å ± 9Å which correspond to spacer DNA. However the spacer DNA is of different length depending on the various forces imposed on the individual chromatin fiber. At the arrows, one can see the maximal stretch of spacer DNA around 200Å long. On the right upper corner of this figure, chromatin mass (Ch) which was not adequately dispersed. X28,600.

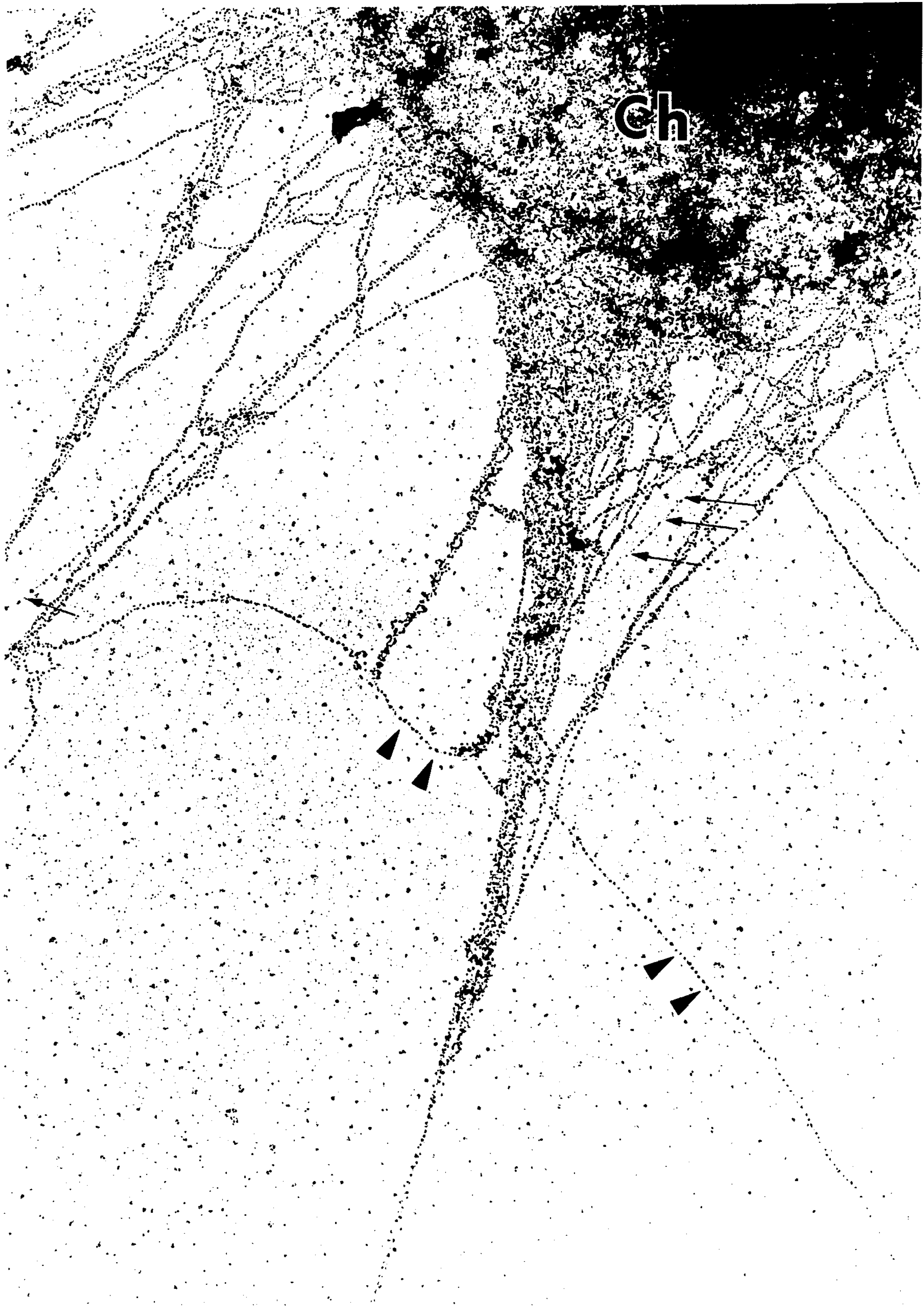

Ch

Figure 15. Photomicrograph of chromatin spread of sea urchin sperm nuclei. The nuclear sample was allowed to lyse for four hours, then centrifuged onto the electron microscope grids through the fixative. The grids were coated with platinum-palladium (80:20) at an angle of 6 degrees. The nucleosomes (arrowheads) appear very contrasting with diameters of 132Å ± 22Å. The length of internucleosomal DNA is variable and appears maximal at arrow where the chromatin strings cross each other. The longest internucleosomal distance was around 200Å. X42,000.

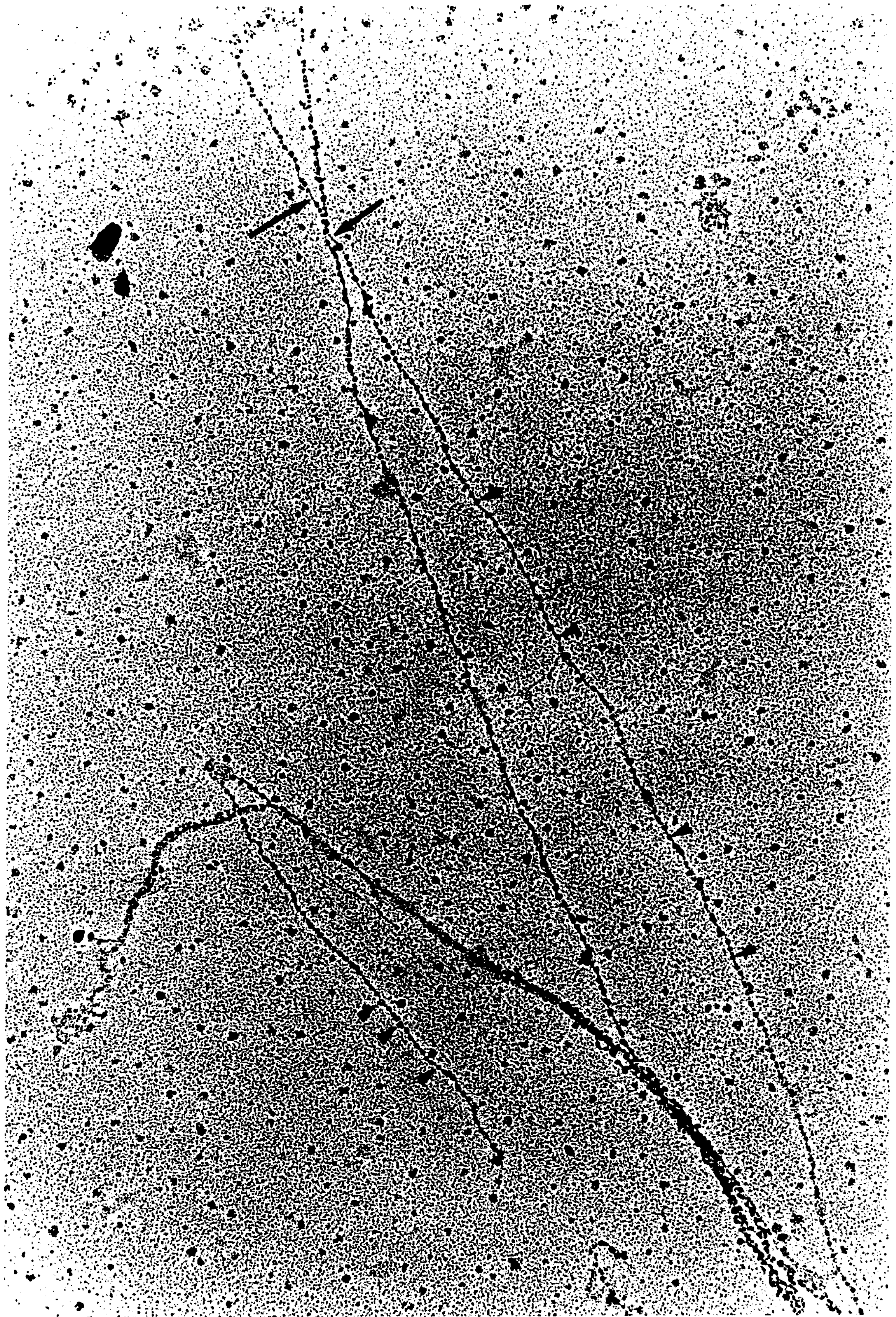


Figure 16. Electron microscopic plate showing chromatin spread of a 3-houred lysed sea urchin sperm nuclei. The chromatin spread was fixed and then negatively stained with 2% PTA for two minutes. The granules have ill defined borders and are of variable sizes ranging from 140Å (arrows) to 260Å (arrowheads). X230,000.

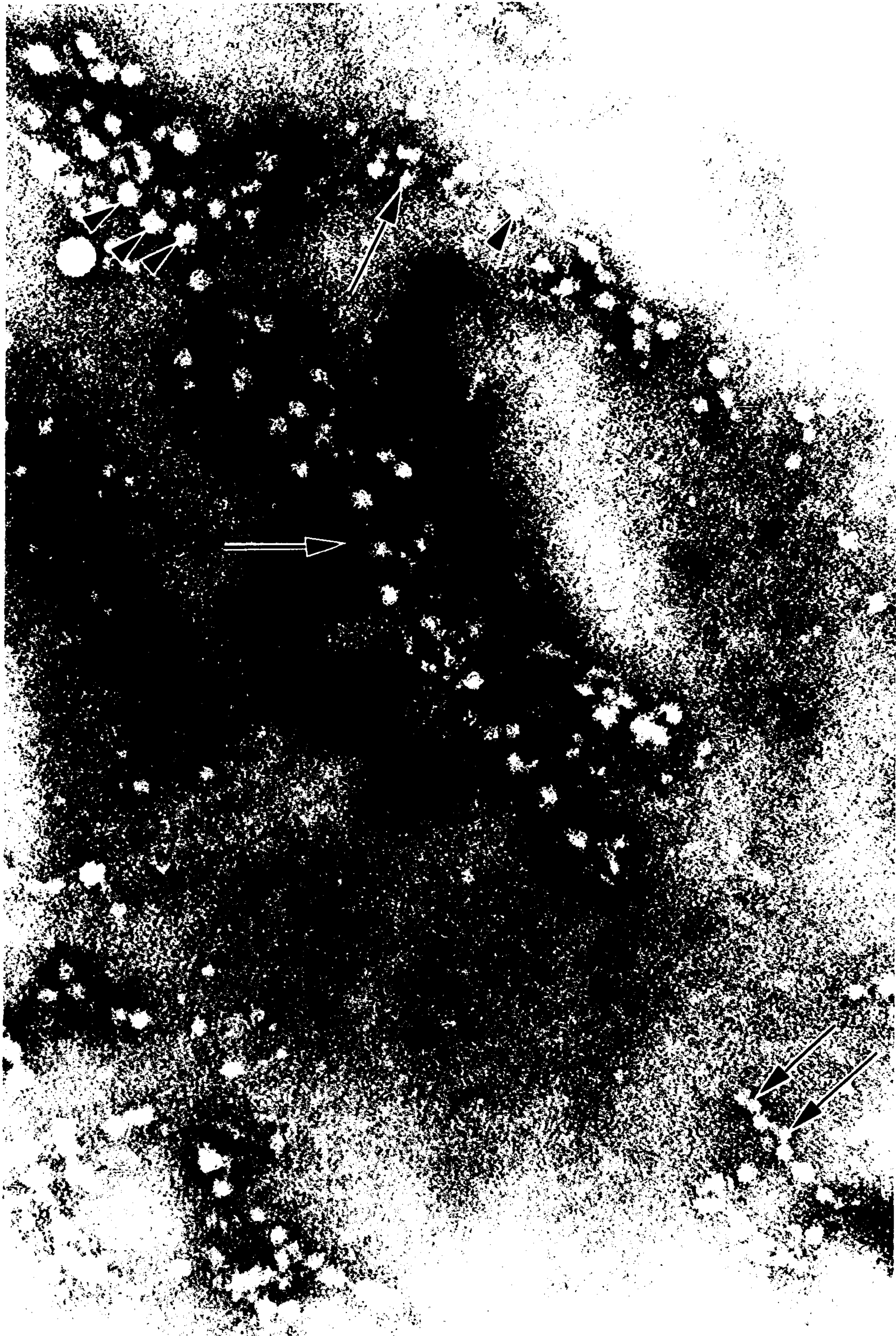


Figure 17. A scanning electron micrograph showing a sperm nucleus which lysed in 1mM EDTA for ten minutes, was centrifuged onto formvar-carbon-coated grids through sucrose-paraformaldehyde cushion, critical-point dried and then sputter coated with gold. A loop of chromatin fiber appeared budding off at a certain spot (arrows) on the nucleus. Fibers of the extruding loop are of 500Å in diameter. The acrosomal fossa (AF) and the centriolar fossa (CF) are seen in this figure. X80,000.

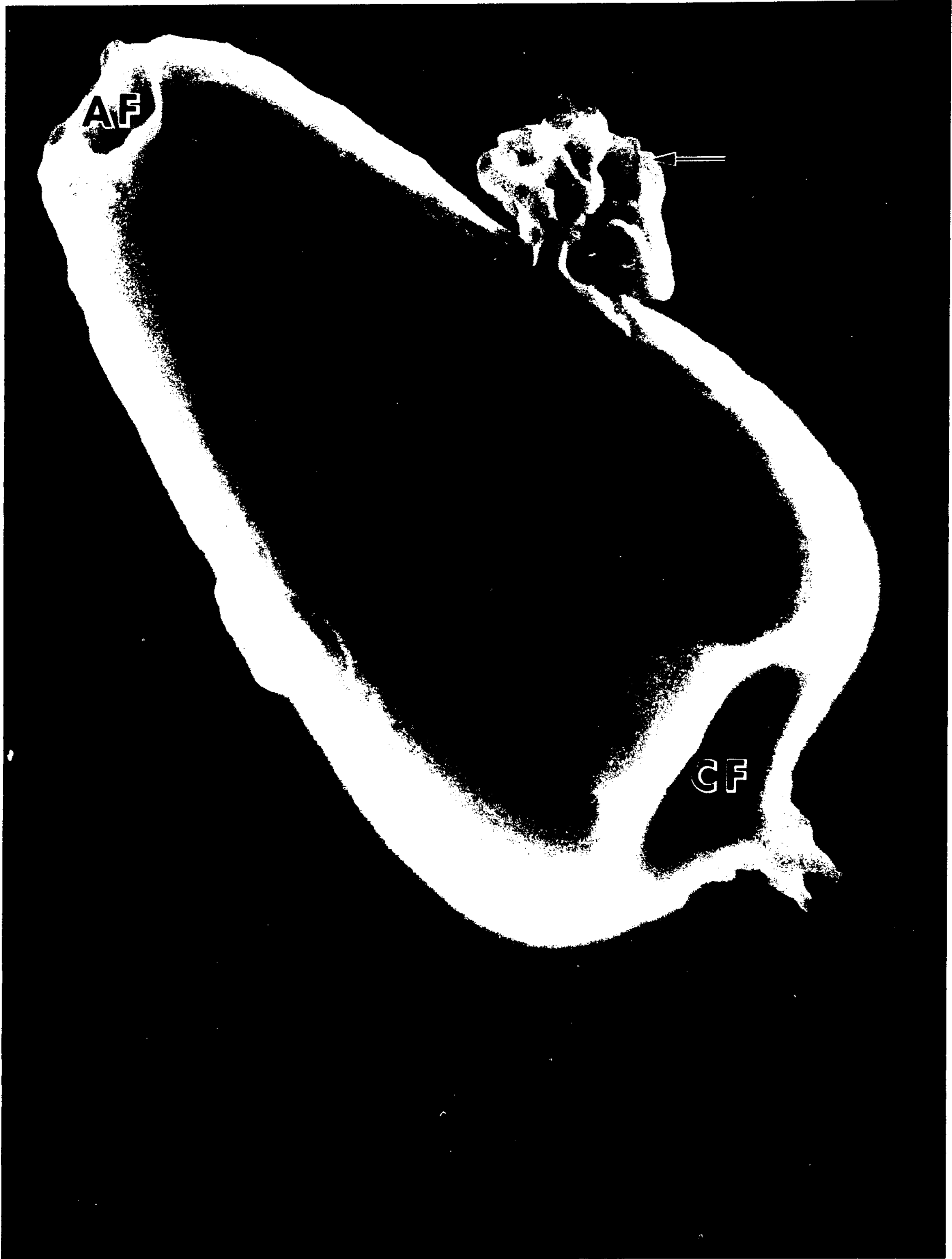

AF
CF

Figure 18. A high resolution scanning micrograph of sea urchin sperm nucleus which was lysed in 1mM EDTA, pH 7.5 for twenty minutes. This micrograph shows a chromatin fiber (arrowhead) spilled out of the nucleus. The general appearance of the cell has ill-defined nodules (arrow). The acrosome (A) and portion of the axoneme (T) projecting out of the centriolar fossa (CF) are also seen. X96,000.

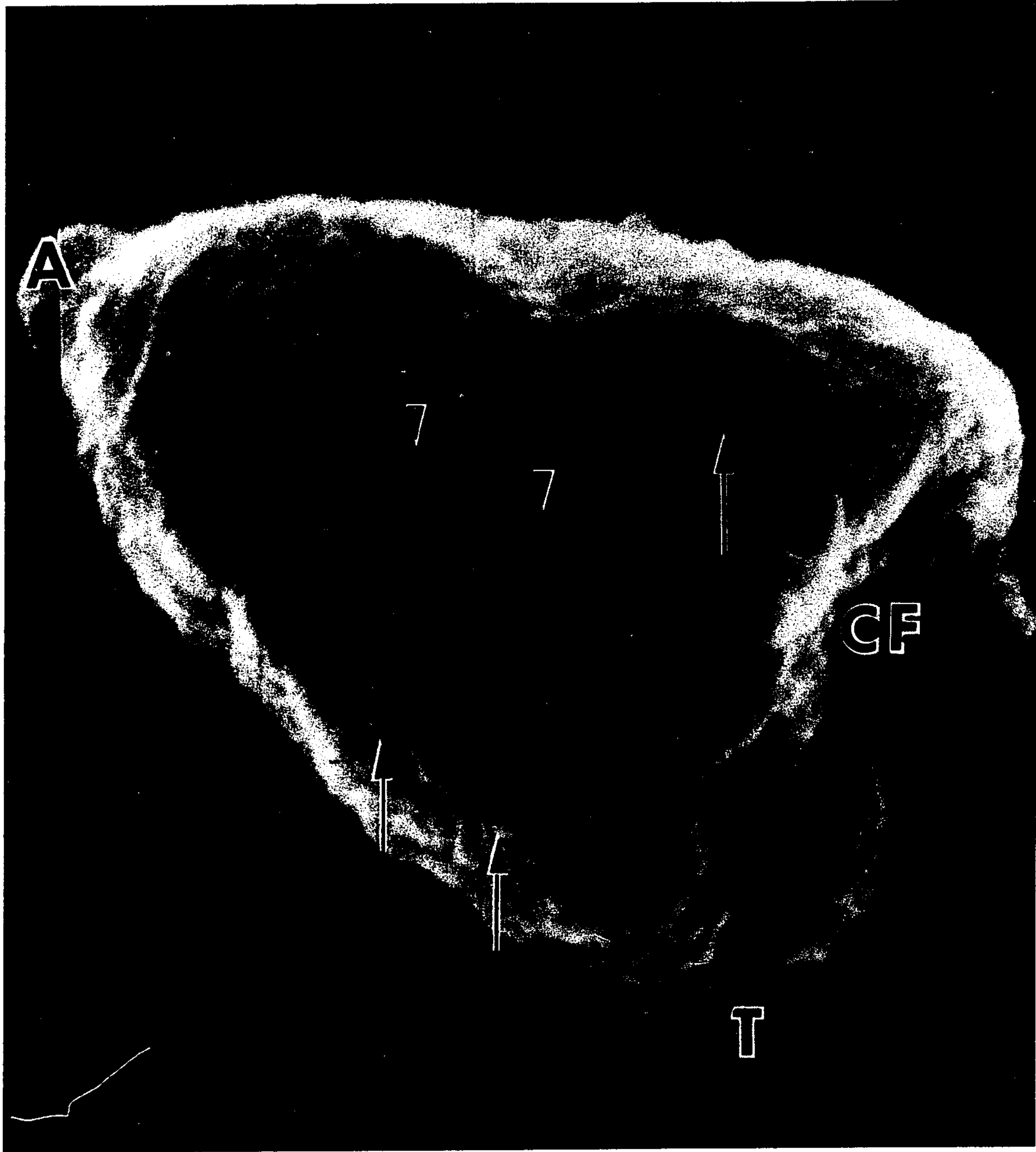

A
CF
T

Figure 19. Scanning electron micrograph demonstrating a sea urchin sperm nucleus which was allowed to swell in low ionic strength buffer (1mM EDTA) for thirty minutes. The surface of the nucleus in general appears extensively convoluted with chromatin fibers having a diameter of 400-500Å (arrows). X71,000.

T

Figure 20. Scanning micrograph shows the posterior view of sea urchin sperm nucleus with portion of the axoneme (T) merging off the centriolar fossa. The nucleus was lysed for sixty minutes. In this figure numerous beaded chromatin fibers (arrowheads) of 400-500Å in diameter are very evident. X100,000.

T

Figure 21. Photomicrograph of three sea urchin sperm nuclei as seen by high resolution SEM after ninety minutes lysis. The surface of nuclei appears highly convoluted. Chromatin fibers (arrows) of 400-500Å in diameter are seen coming out of the sperm nucleus labeled - II, while it is not ascertained the source of the rest of chromatin fibers seen in the field. X38,000.

II

Figure 22. Scanning photomicrograph of sea urchin sperm nuclei after 120 minutes lysis in 1mM EDTA. The nuclear surface appears highly convoluted with deep depressions and loosely arranged, interlacing chromatin fibers. These fibers appear uneven in thickness, some radiate from the nuclear mass in a way that makes it difficult to relate the fibers to one nucleus or another. The fibers are highly variable in thickness ranging from 200Å (arrowheads) to 700Å (arrows). The thicker fibers appear to be composed of merging of several smaller fibers. X35,000.

Figure 23. The typical absorbance profile of sea urchin sperm nuclear chromatin. The nuclei were allowed to swell in 1mM EDTA for two hours, then were briefly digested with DNase I. The digest was layered on 10-30% linear sucrose gradient and centrifuged overnight (sixteen hours). The gradient was mechanically fractionated at an absorbance of 260 and yielded twenty six tubes. The superbeads were predominant at the area between tubes no. 6-10. The first arrow denotes 40S peak while the second denote the 60S peak. The gradient was divided into four regions denoted by the Latin numbers I, II, III and IV.

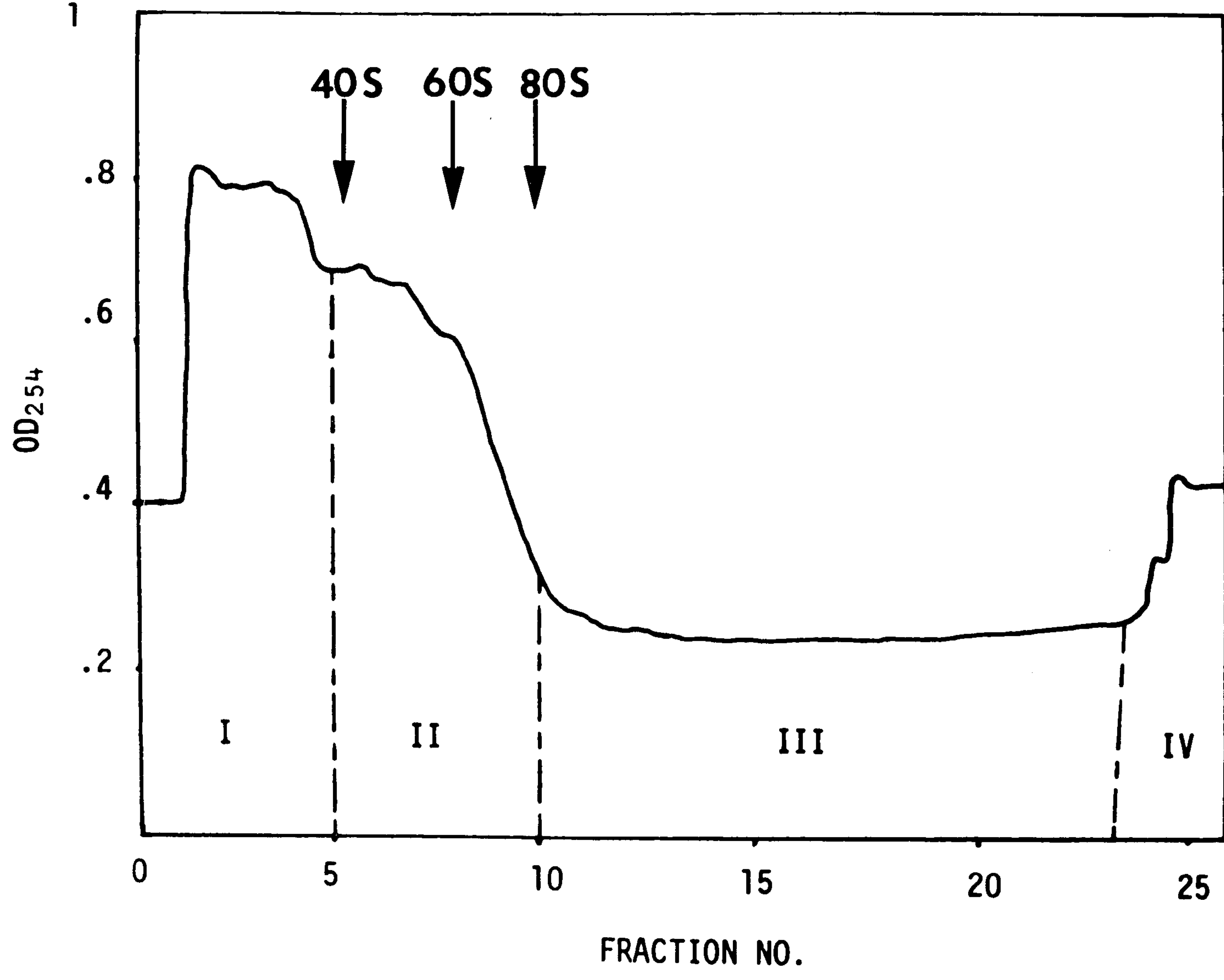

40S
60S
80S
1
.8
.6
.4
.2
OD_{254}
I
II
III
IV
0
5
10
15
20
25
FRACTION NO.

Figure 24. Photomicrograph of chromatin material represented in region I of the sucrose gradient. The material was centrifuged onto the grids through sucrose paraformaldehyde cushion then positively stained with 1% PTA. The chromatin appear to be composed of fibers which are highly dispersed and the chromatin varied in packing from superbeads (*), and nucleosomes (arrowheads) to fibers of chromatin which contained no visible nucleosomes (arrows). X24,000.

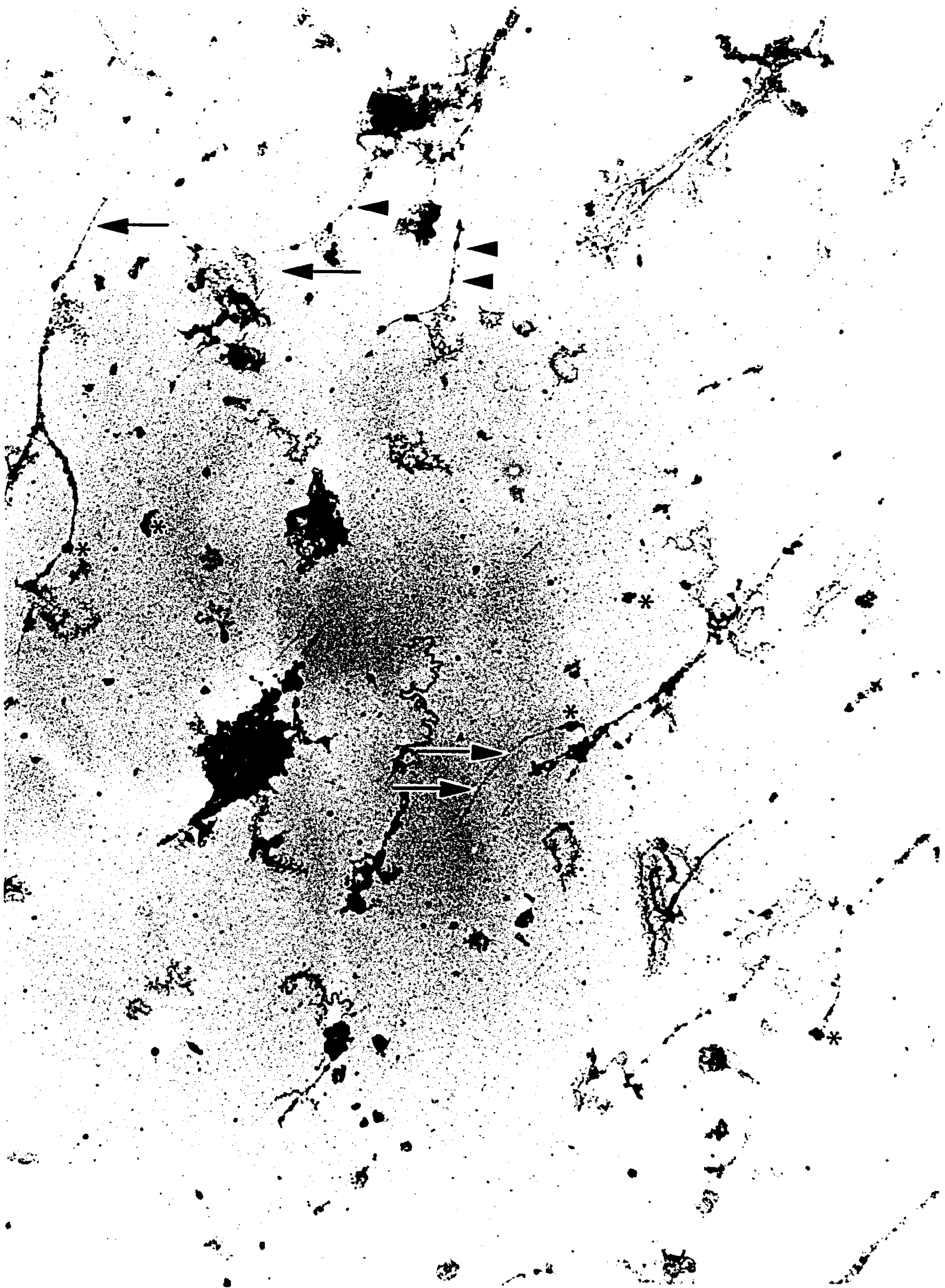


Figure 25. Photomicrograph of chromatin material which was liberated in region II and positively stained with 1% PTA. This region contained an enrichment of superbeads (arrowheads). X39,000.

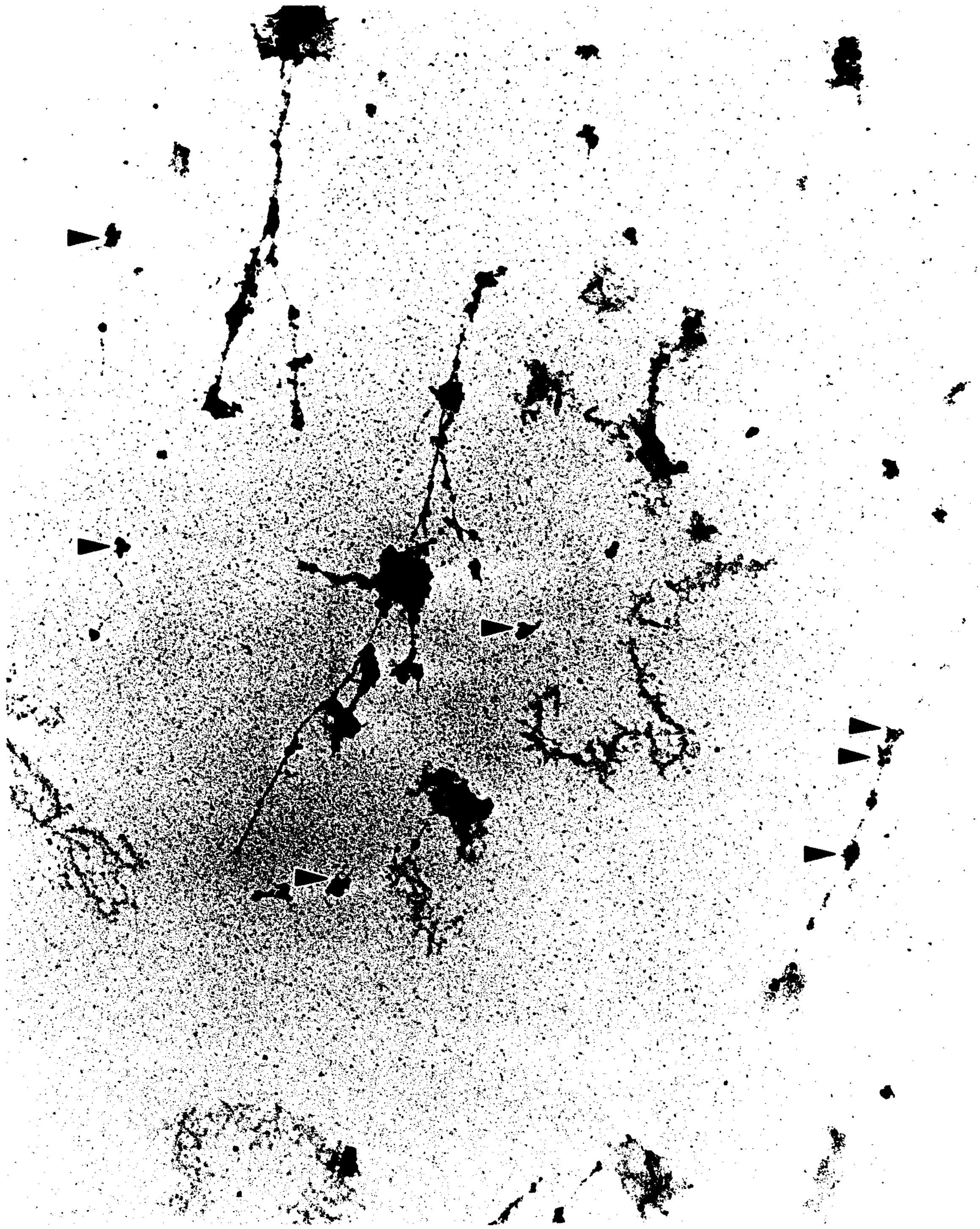


Figure 26. Electron micrograph of the chromatin material found in fourth region (IV) of sucrose gradient. The chromatin appears to be partially dispersed including dense aggregates. In this plate only few scattered superbeads (arrows) and small number of nucleosomes (arrowheads) could be seen. X39,000.

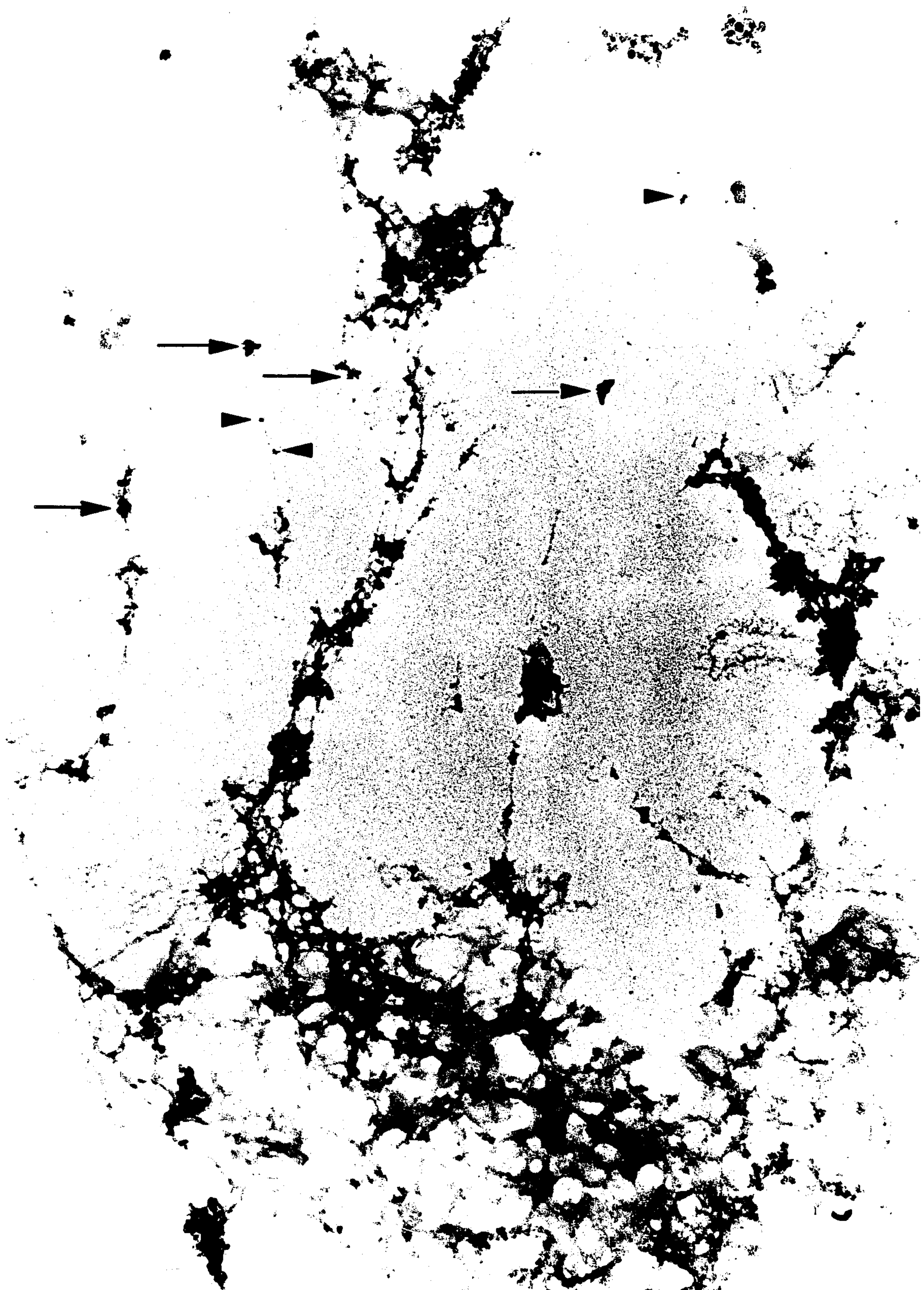


Figure 27. SDS-polyacrylamide slab gel electrophoretic pattern with the acrylamide in a linear concentration ranging from 5% at the top to 12% at the bottom of the gel stained with silver stain. Lanes 1, 2, 3 and 4 represent the protein profiles of the material extracted from the chromatin found in the four regions of the sucrose gradient respectively. Lane (1) represent the chromatin extracted from region I in which one can see the similarity of the polypeptides of this lane versus lane 2 which represents the protein extracted from region II. There are three polypeptides in both lanes 1 and 2: 68,000, 59,000 and 58,000. Note the heavier staining in lane 1 in the region of 70,000. Lane 4 represents the region IV of sucrose gradient and shows heavy staining in the area of molecular weight of 68,000 dalton.

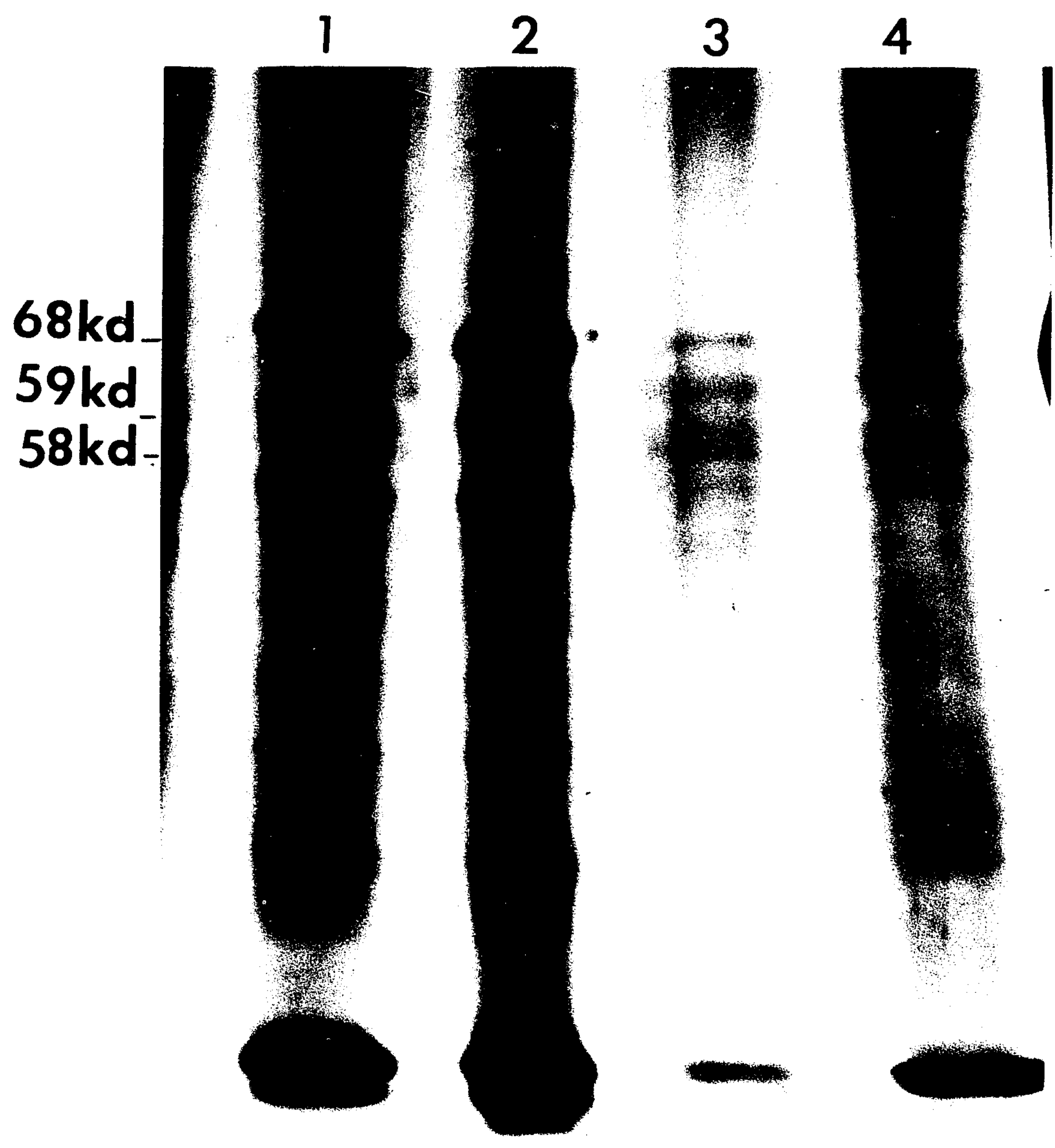

1
2
3
4
68kd
59kd
58kd

Figure 28. One-dimensional SDS-polyacrylamide slab gel (12%) showing electrophoretic banding patterns of acid soluble proteins of sea urchin sperm nuclei (Strongylocentrotus purpuratus, lane 2). The protein was extracted by using 0.25N HCl. The gel was stained with Coomassie brilliant blue. Lane 1 represents three major histones isolated from rat liver chromatin: H2A, H3 and H4. Lane 2 represents the sea urchin sperm nuclear histones. Histone H1 is formed of several subfractions. Histone H3 and H4 look very slightly different from those of rat liver chromatin in lane 1.

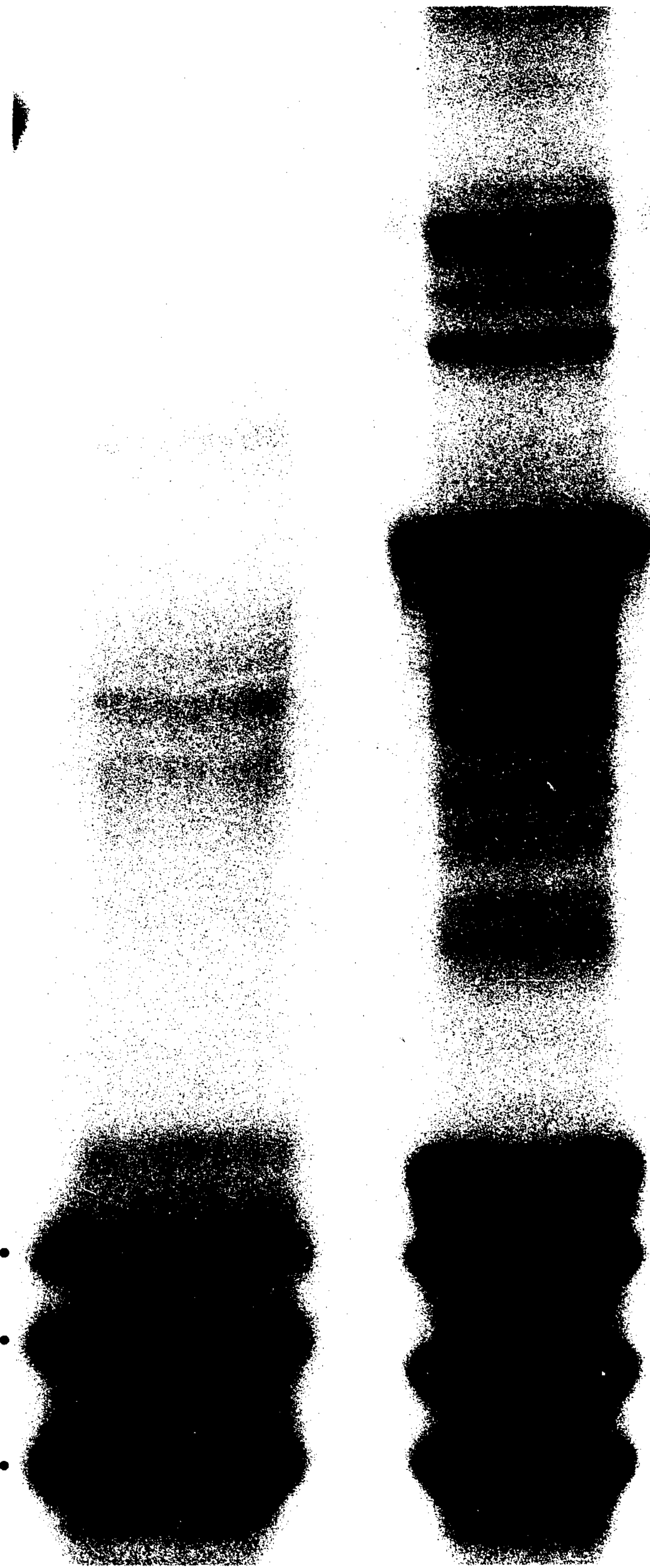

1
2
H1
H2B
H3
H2A
H4
H3
H2A
H4

Figure 29. Linear one-dimensional SDS-polyacrylamide gel gradient (5-12%) illustrating electrophoretic banding patterns of the acid soluble protein. This was extracted by the use of 0.25N HCl. Lane 1 is a molecular weight control. Lane 2 represents acid soluble proteins (histones) which were extracted from whole sperm nuclei without treatment with 1mM EDTA. Lane 2 illustrates the typical sperm nuclear histones H1, H2A, H2B, H3 and H4 as well as bands of molecular weights of 68Kd, 59Kd and 58Kd. Lane 3 shows the proteins released from sperm nuclei which were treated with 1mM EDTA in order to swell. In this lane the proteins of molecular weights 58Kd and 59Kd seem to be lacking. Lane 4 represents the proteins liberated from the supernatant as a result of swelling of nuclei by the use of 1mM EDTA used in lane 3. In lane 4 one can see two proteins of molecular weights of 59 and 58Kd.

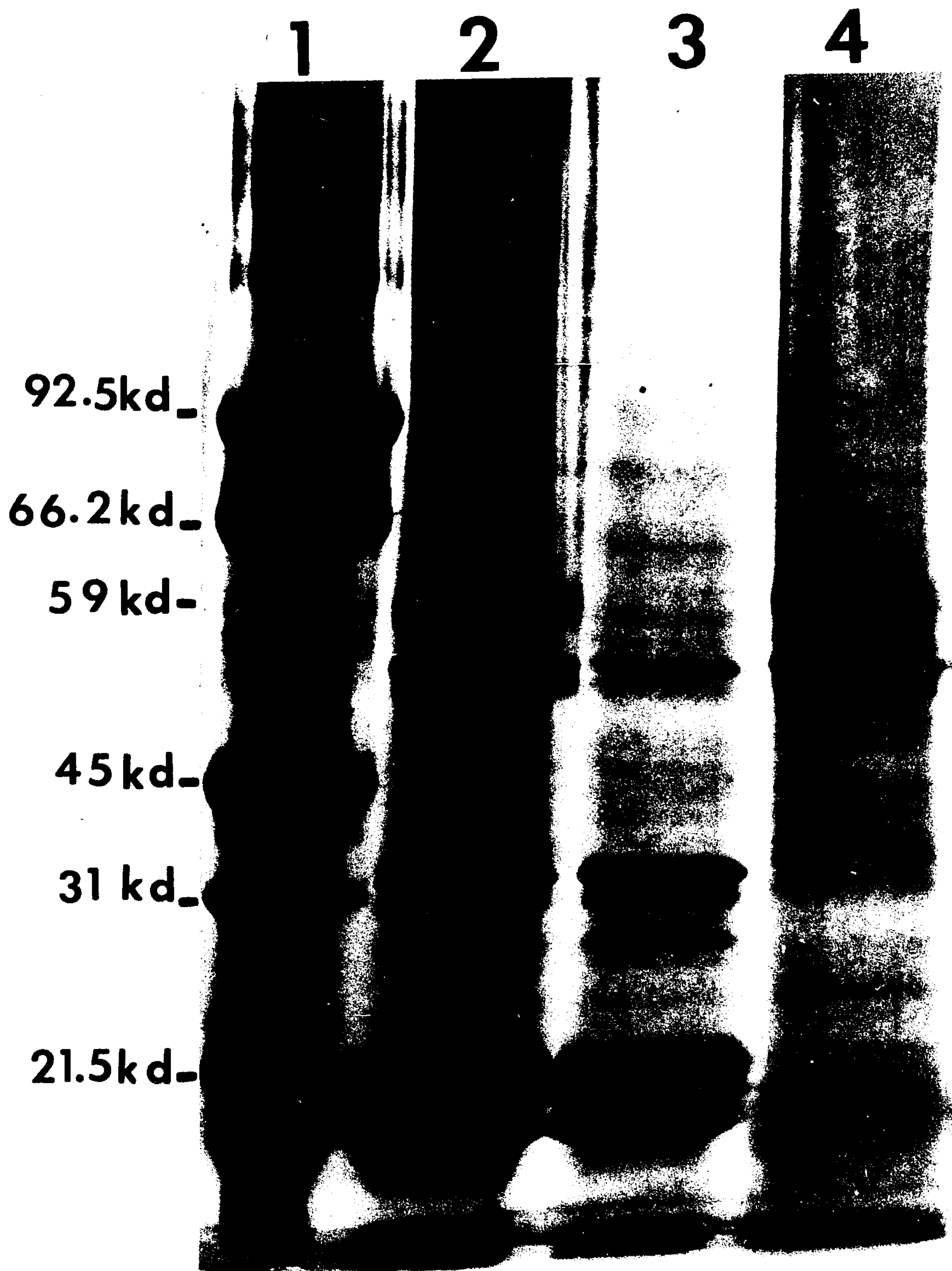
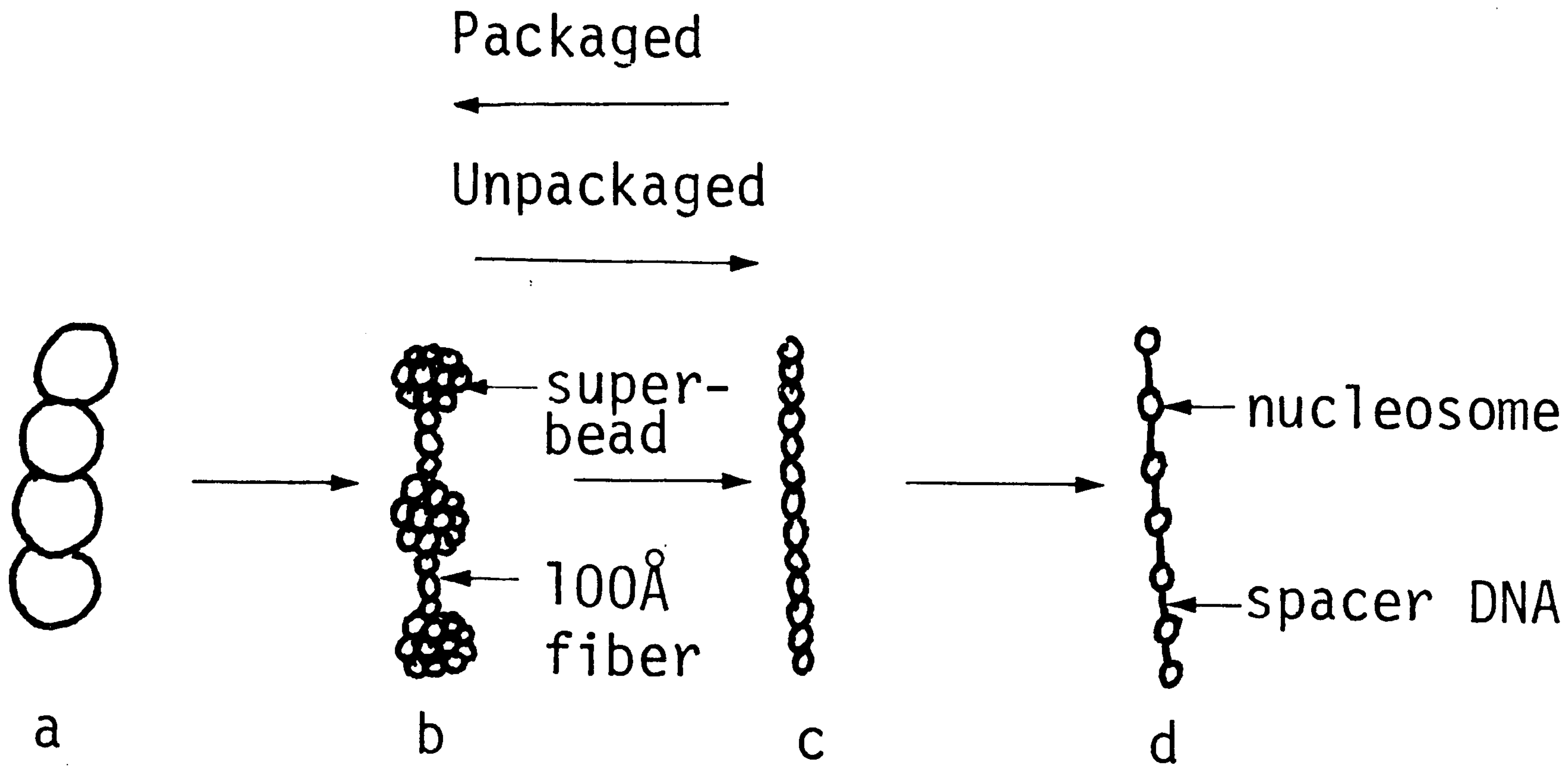

1
2
3
4
92.5kd
66.2kd
59kd
45kd
31 kd
21.5kd

Figure 30. Schematic diagram illustrating the process of packaging and unpackaging of chromatin in sea urchin (*Strongylocentrotus purpuratus*) sperm nuclei.

a - 400Å-500Å thick chromatin fiber which is made of close association of 400Å chromatin globules.
b - 400Å-500Å fibers disrupt into superbeads which are connected by 112Å fibers.
c - superbeads disrupt to give 112Å fibers.
d - 112Å fibers unpack into beads-on-a-string configuration.


Packaged
Unpackaged
super-
bead
100Å
fiber
nucleosome
spacer DNA
a
b
c
d

APPENDIX

TABLE 2

Measurements of Sea Urchin Sperm Nuclear Chromatin by the Use of Different Techniques

Structure	Size	Technique
Nucleosome	95Å ± 10Å	Chromatin spreading - no coating
Nucleosome	132Å ± 22Å	Chromatin spreading - platinum-palladium coating
Nucleosome	112Å ± 13Å	Thin sectioning
Spacer DNA (diameter)	22Å ± 3Å	Chromatin spreading - no coating
Spacer DNA (diameter)	38Å ± 9Å	Chromatin spreading - platinum-palladium coating
Spacer DNA (length)	0Å - 200Å	Chromatin spreading - platinum-palladium coating
Electron lucent area in the center of nucleosome	32Å ± 6Å	Thin sectioning
Superbeads?	185Å ± 25Å	Chromatin spreading - negative stain
Superbeads	414Å ± 60Å	Thin sectioning
Thick chromatin fiber	414Å ± 60Å	Thin sectioning
Thick chromatin fiber	400Å - 700Å	SEM - gold coating

TABLE 3

Summary of Polypeptides in Gels

Protein MW Kd	Sucrose Gradient Fractions I	II	III	IV	Non-lysed nuclei	Lysed nuclei	Supernatant of lysed nuclei
58	++++	++++	+	+	++++	++	++
59	++++	++++	+	+	++++	-	++++
68	++++	++++	+	++	++++	-,+	+
Above 70	++++	++	+	++	++++	+	++++
Histones	++++	++++	+++	+++	++++	++++	+

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Naama Abdul-Gader Aboukarsh

Major Subject Anatomy

Title of Dissertation Characteristics of Sea Urchin Sperm Chromatin

Dissertation Committee:

Mel Kunkle, **Chairman**

Gary P. Poirier

Wayne H. Finley

George S. Hand

Director of Graduate Program Earl M. Hammer

Dean, UAB Graduate School Blaine A. Brownell

Date 4/19/83