Moreover, if when $\alpha = 0$ *and* A *is singular,* $\int_0^\sigma \phi(\tau)d\tau$ *tends to* $+\infty$ *with* $|\sigma|$, *then* V *tends to* $+\infty$ *with* $\|x\|$. *Thus, under the above conditions, the solution* $x = 0$ *of (1) is asymptotically stable in the large.*

Lemmas 1 and 2 can equally well be used to consider the system (1) when $\phi(\sigma)$ is restricted to $0 < \sigma\phi(\sigma) < k\sigma^2$ and a sharper result can be obtained. Another example of the consequences of Lemma 1 is the following:

THEOREM 2. *Let* A *be any real* $n \times n$ *matrix and* b, c *as before. Let* λ *be any real number that is strictly greater than the real part of all the characteristic roots of* A. *If*

$$\operatorname{Re} c'(zI - A)^{-1}b \geqq 0$$

for all $z = i\omega + \lambda$, ω *real, then there exists a nonnegative function* K *defined on* $[0, \infty)$ *such that*

$$\|x(t)\| \leqq K(\|x_0\|)e^{\lambda t}$$

for all $t \geqq 0$ *where* $x(t)$ *is the solution of (1) such that* $x(0) = x_0$.

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[4] *Note added in proof: Assume also that if* $i\omega_0$, ω_0 *real, is a characteristic root of* A *of multiplicity* ν, *then* $\lim_{z \to i\omega_0} (z - i\omega_0)^\nu c'(zI - A)b \neq 0$.

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*STRUCTURE OF THE CHROMATIN IN SEA URCHIN SPERM**

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The nuclei of the sea urchin sperm, the subject of the present work, have been studied both chemically and by electron microscopy, and information is available about their DNA,[1, 2] histones,[3] nucleoprotein,[4] and the ultrastructure of the sperm head.[5] X-ray diffraction data on the sperm heads of sea urchins have been reported.[6]

In the present study, some earlier observations of the effects of divalent cations and ionic strength on the behavior of the nucleoproteins[4, 7] have been adapted to techniques of spreading and staining molecules for high-resolution electron microscopy. The chromatin has also been studied after several enzymatic and extraction procedures.

Materials and Methods.—Fresh sperm from the sea urchin *Strongylocentrotus purpuratus* was used in this work. Several techniques for the preparation of the electron microscopical specimens were used.

Spreading on liquid surfaces:[8] The sperm, washed in sea water, was transferred either to 0.125 *M* sodium citrate or 10^{-3} *M* EDTA at pH 6.6, in 0.14 *M* NaCl, for 10–20 min in the cold. After this treatment the sperm were immobile but retained their normal appearance under the phase contrast microscope. After centrifugation concentrated sperm was taken up in minute quantities on a needle and gently put in contact with a clean surface of distilled water.[9] The material spread instantly to form a thin film. Grids coated with formvar and stabilized with carbon were touched to the surface and were processed by various techniques *without drying.* Usually, the grids were floated on a saturated solution of uranyl acetate at pH 4.5 for 2 min to 2 hr. Then they were drained by touching their edges to filter paper and finally allowed to dry rapidly without washing, or were floated on water for 30 sec and dried.

Negative staining and staining with PTA (phosphotungstic acid): Several procedures were tried for the negative staining of the chromatin, but only uranyl acetate gave adequate results. The grids with the spread chromatin were floated on a saturated solution of uranyl acetate in water at pH 4.5 for 30 sec and immediately drained with filter paper leaving a thin film that dried very rapidly.

Positive staining with PTA was performed by floating the grids on a 1% solution of PTA brought to pH 4.5 by 1 *N* NaOH, for 10–30 min and then washing in water for 1 min.

Enzyme digestions: The grids with the chromatin were floated on the following solutions: (*a*) trypsin (Nutritional Biochem. Corp., 2 × cryst.) 0.3–0.1% in water brought to pH 8 with 0.01 *N* NaOH, for 5 min to 2 hr at 37°C; the same solution of the enzyme, inactivated by boiling, was used as control; (*b*) deoxyribonuclease (Worthington Biochem. Corp., 1 × cryst.) 0.2% in a 6×10^{-3} *M* solution of $MgCl_2$, adjusted to pH 6.5 with 0.01 *N* NaOH, for 5 min to 2 hr at 37°C; controls were treated with either the enzyme in presence of 1×10^{-2} *M* $ZnSO_4$ as inhibitor[10] or the enzyme inactivated by boiling. In addition to enzyme digestions, extraction with 0.01 *N* HCl was performed by floating the grids on the solution for 2 hr at room temperature.

DNA-protein monomolecular films:[11] During this work it was found that the successive action of EDTA and chymotrypsin made it possible to obtain films of the sperm DNA and chymotrypsin, without any mechanical procedure for the isolation of the DNA that might involve shearing forces and consequent degradation of the DNA.

Living sperm was washed in sea water and suspended in a 0.14 *M* NaCl solution containing 1×10^{-3} *M* EDTA adjusted to pH 6.6 as above. The sperm was centrifuged at low speed, and a small quantity of the loose pellet was mixed with 50–100 vol of the incubation medium. This medium contained chymotrypsin (Worthington Biochem. Corp., 3 × cryst.), 0.1 mg per ml; 0.2 *M* sucrose; and EDTA (disodium salt), 1×10^{-3} *M*, adjusted to pH 7 with NaOH. It has been found that an additional buffer is not necessary; furthermore, the complex DNA-chymotrypsin will dissociate when the ionic concentration is beyond 10^{-3} *M* as Hofstee has reported.[12] The sperm was incubated for 20 min to 1 hr at 37° C. The resulting solution was spread on the surface of distilled water[11] by a glass rod. The film was picked in carbon-coated grids and stained by floating on uranyl acetate or dried and shadowed. The shadowing was done with platinum-carbon pellets, at an angle of 8° while rotating the specimen with a small electric motor.

Sectioning and microscopy: Sea urchin testes and normal sperm were fixed in Caulfield's fixative[13] which contained, in addition, $CaCl_2$ and $MgCl_2$ in concentrations 5×10^{-3} *M* and 2×10^{-3} *M*, respectively, for 2 hr at 4°C. The specimen was then transferred to a saturated solution of uranyl acetate for 1 hr and then dehydrated and embedded in Epon. Swollen sperm was obtained by putting a small volume of living sperm in distilled water. After 5 min in the water at 4°C, it was mixed with equal volume of a 2% unbuffered solution of OsO_4, or with the same OsO_4 solution in 1×10^{-3} *M* $CaCl_2$, then fixed and embedded as before. Sections were stained with lead hydroxide or, better still, with a saturated solution of uranyl acetate in absolute methanol. Micrographs were taken with a RCA EMU 3F electron microscope and a Siemens-Elmiskop I. The instruments were calibrated with a carbon grating replica (28,000 lines/inch; Ernest Fullan, Inc., Schenectady).

Results.—(a) *Chromatin fibrils spread on water:* The effects of pretreating the sperm with 0.125 *M* sodium citrate and with 1×10^{-3} *M* EDTA were similar. In the latter the spreading of the chromatin was more complete, and we will refer generally to EDTA-treated sperm.

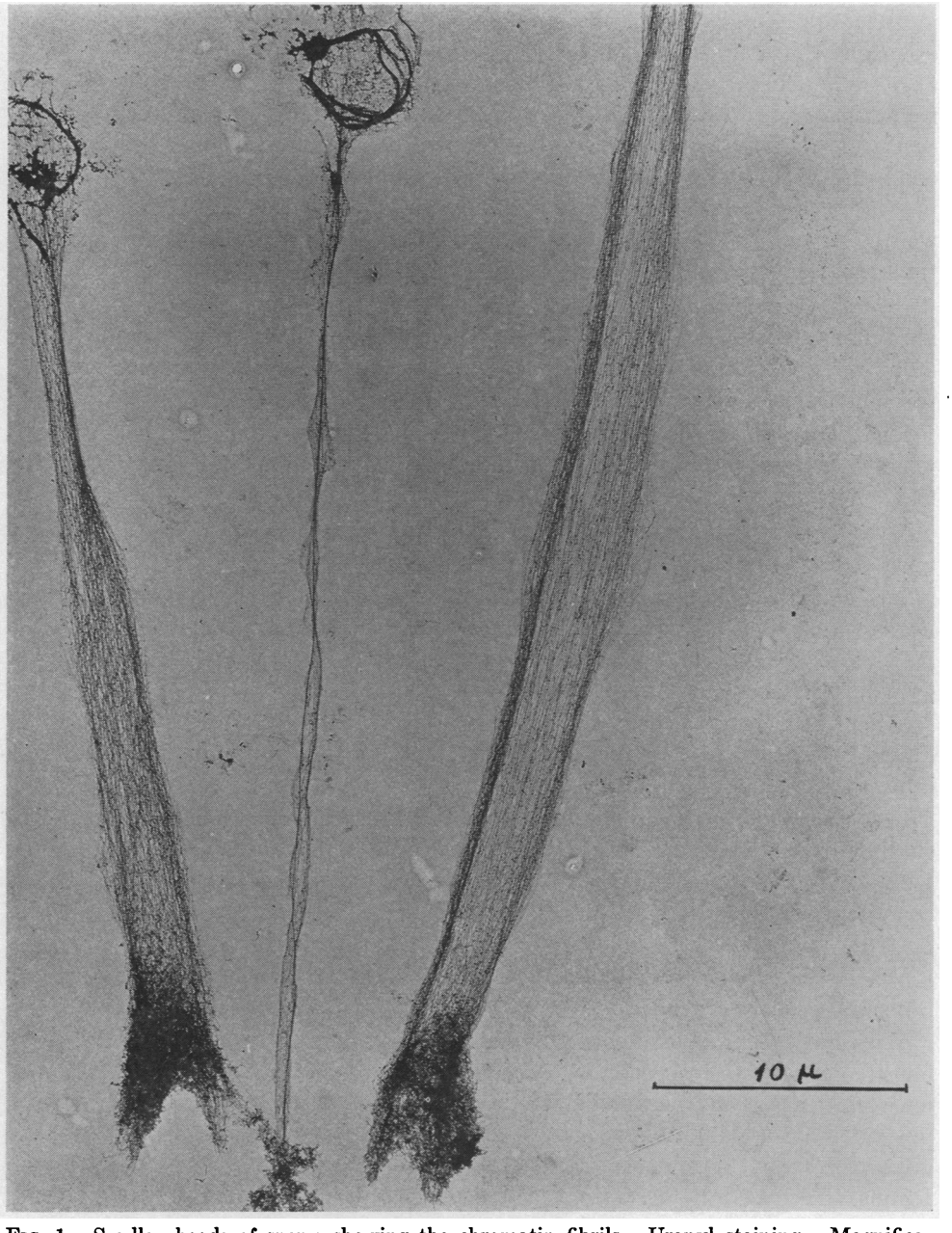

FIG. 1.—Swollen heads of sperm showing the chromatin fibrils. Uranyl staining. Magnification: 3,700 ×.

The spreading of the treated sperm was similar when done on distilled water and on 0.01 *M* NaCl. The heads did not spread at all when put directly on 1 per cent uranyl acetate or in 0.01 *M* $CaCl_2$, but appeared under the electron microscope as swollen masses retaining a spherical shape, with a dense, structureless content.

The sperm spread on water presented a consistent structure when stained with

uranyl acetate. The heads, very swollen, were devoid of any membrane and formed exclusively by very thin and long fibrils that stained intensely (Fig. 1). These fibrils very seldom were seen with free ends and they appear rather as loops emerging from a bundle. The length of these fibrils could be followed for 10 μ, and in some cases considerably more. The width was irregular along each fibril, the minimal one being 30 $\pm$ 10 Å. Frequently the fibrils were associated in pairs as irregularly interlaced coils. At some places along the fibrils pieces of tangled fibrillar material were observed. It was difficult to decide whether the fibrils were continuous in such tight tangles.

(b) *Enzymatic digestion:* The action of DNAase on these fibrils was very rapid and intense. After 5 min of treatment most of the fibrils had disappeared; after 40 min of digestion, only pieces of dense material at the base of the head and the tail constituents are seen.

Digestion with trypsin did not result in loss of the fibrils, nor did it cause breaks in their continuity over long (5 μ or more) distances. They did seem to become thinner and smoother.

Hydrochloric acid extraction resulted in a gross coagulation of the fibrils, but most of the material seemed not to be extracted.

(c) *Positive stain with PTA:* PTA at pH 4.5 stained the fibrillar material of the head as well as the tail constituents. As seen in medium magnification pictures, the PTA-stained material was homogeneously distributed along the fibrils. At high magnification, however, the stain was discontinuous.

When the grids with the spread chromatin were floated on 2 *M* NaCl for 15 min, most of the material stainable with PTA at pH 4.5 was lost; only the tail fibrils retained the same stainability.

(d) *Molecular morphology of sperm DNA:* The sperm DNA in the chymotrypsin films appeared as very long and homogeneously thin strands. The width of the DNA-chymotrypsin complex when seen after the rotary shadowing with Pt-C was consistently 130 $\pm$ 25 Å. This is in good correspondence with the magnitude reported by Hofstee.[12]

When stained with uranyl acetate, the width of the strands was also homogeneous but smaller, of the order of 25 Å.

With both techniques the strands appeared as indefinitely long (Fig. 2). Very few free ends were recorded in good preparations. Minimal lengths (lengths followed until a tangling mass forbids the assessment of continuity) were measured both in shadowed and stained preparations, in seriated photographs finally composed to give large areas. It was possible to trace lengths from 50 to 93 μ by this method. It must be stressed that these figures do *not* represent lengths from an end to an end, but only the stretches of much longer fibrils that can be followed by this tedious method. It is certain that the true lengths of chromatin fibrils are much longer; in fact, the paucity of free ends is one of the striking features of these preparations. Some "clustering" in centers was recorded. Along the length of the fibrils there were no obvious heterogeneous regions with respect to uranyl affinity or width of the protein coat as seen in the shadowed preparations.

(e) *Sections:* Sections of mature sperm rendered no information on the structure of the head; the chromatin seemed almost homogeneous.

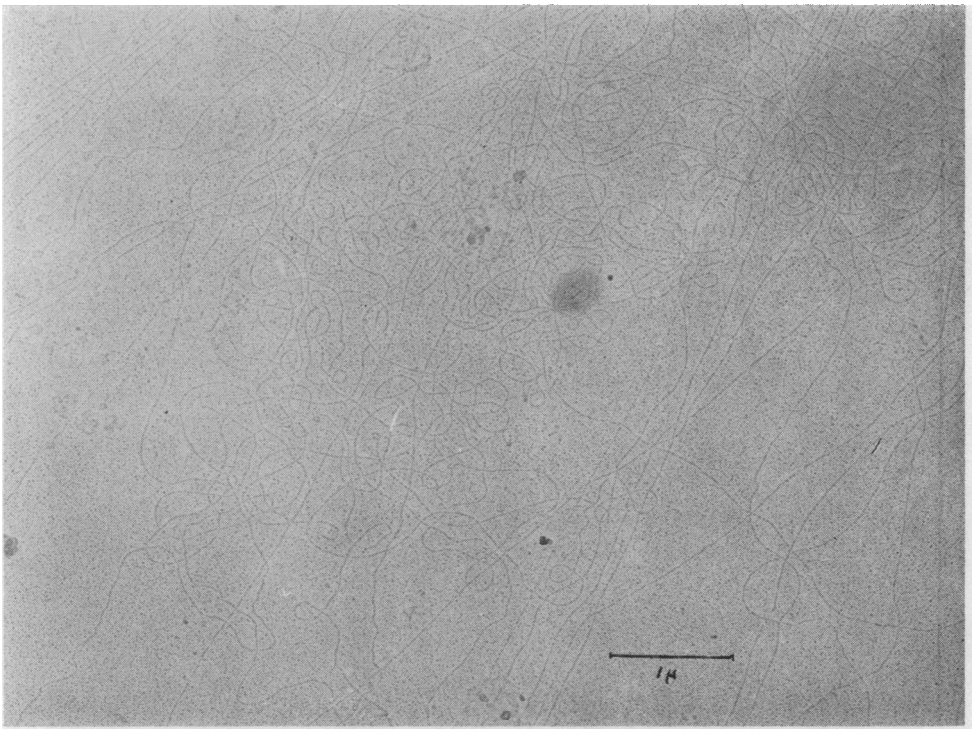

FIG. 2.—Sperm DNA in a film of chymotrypsin. Very few free ends are seen. Shadowed with Pt-C. Magnification: 22,000 ×.

In the immature sperm the chromatin appears as gross threads of irregular and possibly coiled path, whose diameter is about 250 Å. The chromatin at the base of the head presents a condensed layer of 200-Å thickness that covers the invagination formed by the centriole.

In the water-treated sperm, regardless of the type of fixation, the chromatin was present as a relaxed mesh formed by beaded-like threads. The condensations on the threads were approximately 200 Å in diameter, but no periodicity or finer fibrillar structure could be seen.

(f) *Negative stain and high salt concentration effect:* Negative staining with uranyl acetate coupled by extraction of the spread chromatin with 2 *M* NaCl gave additional evidence of the presence of another material besides DNA in the chromatin fibrils. While the original chromatin fibrils appeared as fibrils of irregular outline (Fig. 3) 40 ± 10 Å in width, after floating the grids on 2 *M* NaCl for 20 min very thin and smoothly outlined fibrils were seen (Fig. 4).

Discussion.—Chromatin structure has been extensively studied with the sectioning techniques of electron microscopy (for reviews see refs. 14, 15, and 16). Although that literature will not be discussed here, it can be pointed out that our observation of chromatin fibrils, about 30 Å thick (40 ± 10 Å with negative stain) in the sea urchin sperm, seem to correspond to the microfibrils described by de Robertis[17] in the meiotic nucleus of locusts and in the chromosomes of the onion[16] and the "lines" described by Davies and Spencer in erythrocyte nuclei.[18]

Using a spreading technique, Gall[8] has observed much thicker chromatin fibrils in

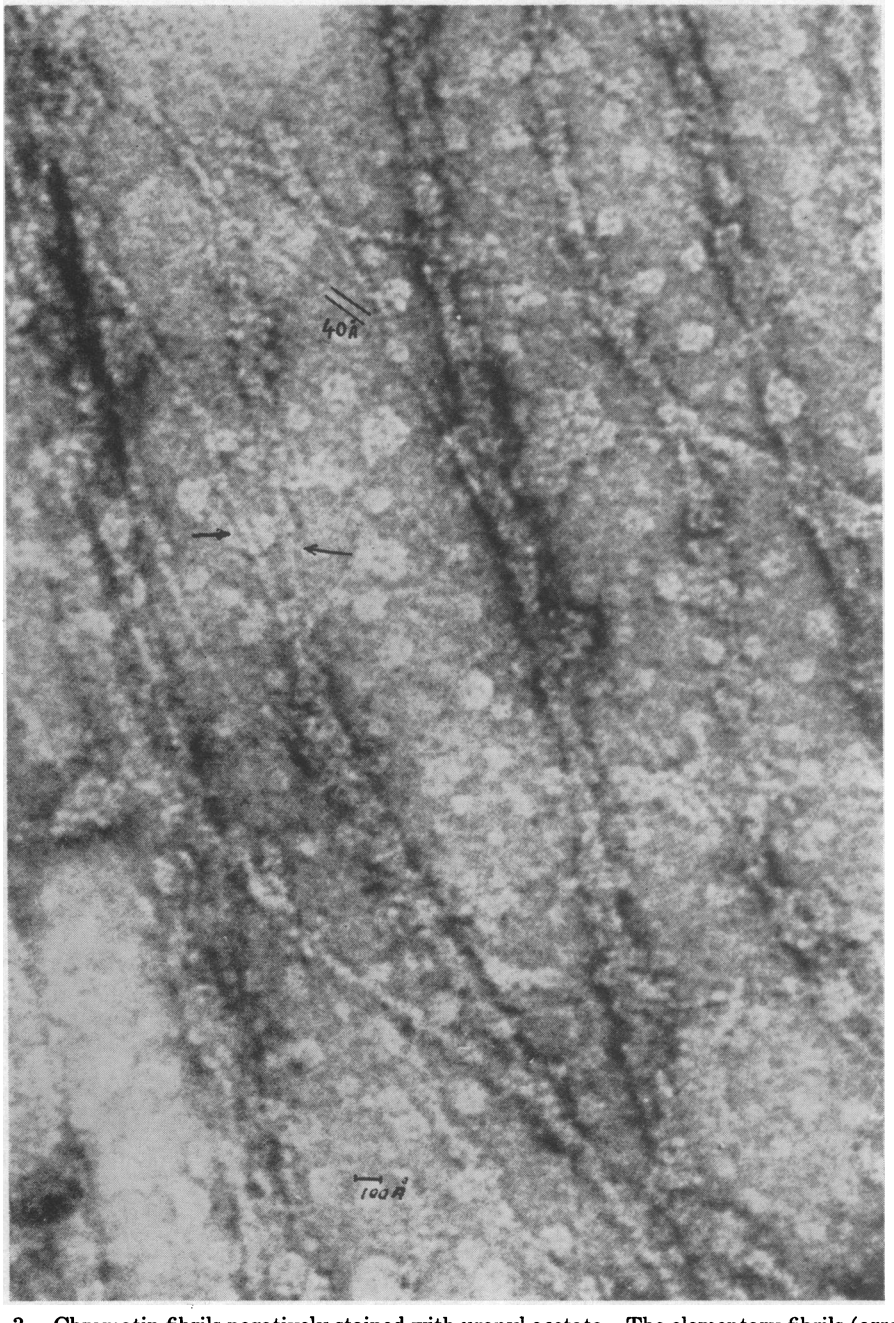

FIG. 3.—Chromatin fibrils negatively stained with uranyl acetate. The elementary fibrils (arrow) are forming pairs or bundles. Magnification: 330,000 ×.

Triturus erythrocyte nuclei, where the hemoglobin may have formed a thicker coat during the procedure.

Ris[19] has described chromatin fibrils after a similar spreading procedure in *Triturus* erythrocytes, although again the fibrils are thicker, about 100 Å, and more

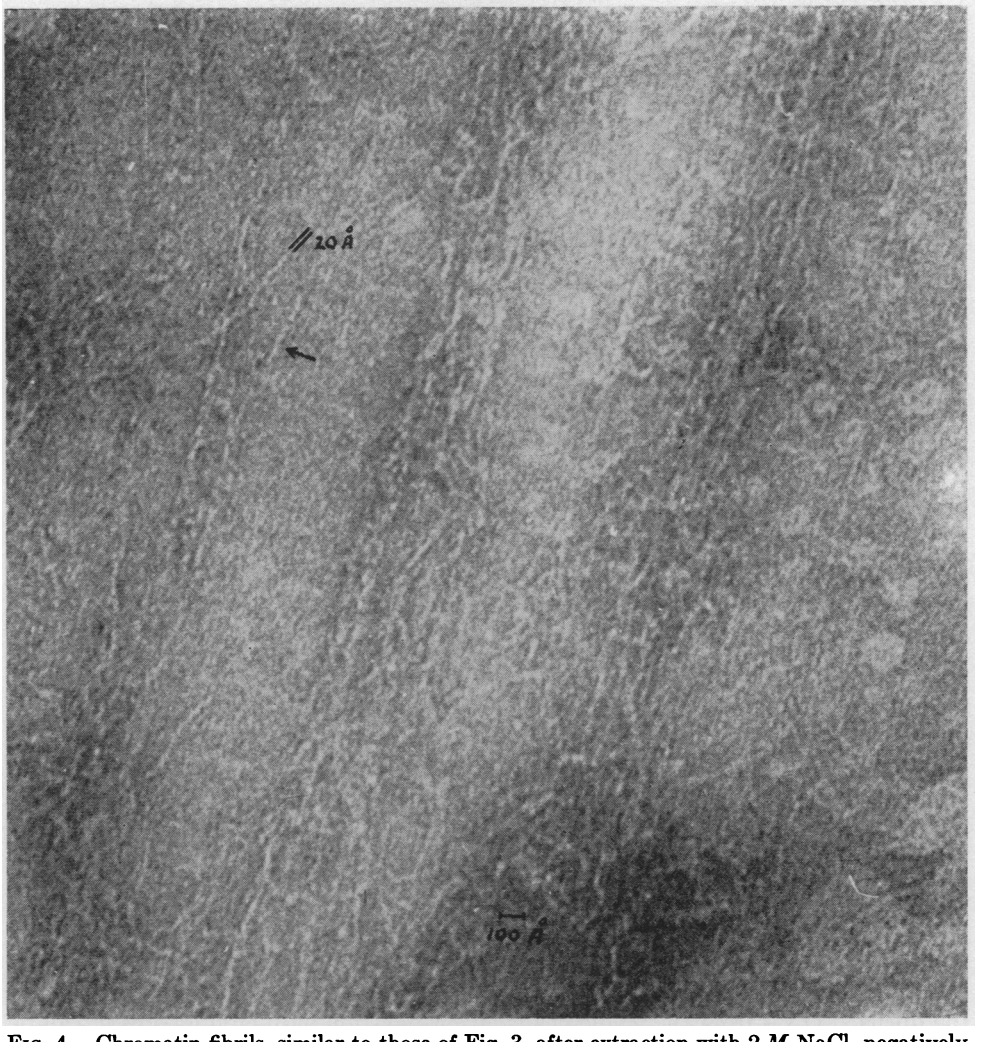

FIG. 4.—Chromatin fibrils, similar to those of Fig. 3, after extraction with 2 *M* NaCl, negatively stained with uranyl acetate. Magnification: 330,000 ×.

irregular. After hydrochloric acid extraction these fibrils show two 40 Å components. Du Praw[20] has recently used the water-spreading technique coupled with the critical point method in honeybee embryo cells where the chromosomes appear to be formed by fibrils wider than those reported here, but coinciding with the present observations in the lack of free ends and indefinite lengths.

None of these observations, including the present ones, seem to agree with the existence of deoxyribonucleoprotein "particles" of molecular weight 18.5×10^6 or other deoxyribonucleoprotein preparations[21, 22] that may be degraded during isolation.

Our observations on the DNA of the sea urchin sperm rule out the possibility of DNA *units* shorter than 90 μ. Assuming that the state of the DNA in solution was equivalent to the B crystallographic form, that length would correspond[23] to a molecular weight of 172×10^6. "Linkers" between DNA molecules, if present, must

be insensitive to chymotrypsin and small enough (less than 100 Å) not to be observed after uranyl staining.

From our data we suggest that the fibrils are the fundamental unit in the sperm chromatin. The fibrils have a continuous backbone of DNA, as shown by digestion experiments and the staining affinities. The morphology of the fibrils is not that of pure DNA.[24-26] The staining at low pH with PTA shows some material constituting the fibrils that behaves like a base, presumably the histone. This assumption is strengthened by the disappearance of the stainable material upon extraction with 2 *M* NaCl. This extraction also shows a definite loss of material from the chromatin fibrils when seen with the negative stain. It is assumed that the DNA backbone, in the form of a very much longer polymer than histone, remains adsorbed to the film. Thus, after the extraction, thinner strands corresponding to DNA width are seen. Preliminary observations of sperm deoxyribonucleoprotein isolated by an earlier method[7] gave a good agreement with the *in situ* fibrils.

The structure of this nucleohistone complex does not correspond fully to models proposed mainly on the basis of X-ray diffraction data.[6, 27]

The packing of the chromatin seems dependent on the presence of divalent cations. Although the importance of these cations in chromatin structure has been repeatedly stated,[28, 18] the mechanism of its action is not fully understood. Osgood *et al.*[29] have observed extensive uncoiling and dissociation into subunits in human chromosomes treated with hypotonic solution. The authors interpret this effect as caused by a reduction in the concentration of divalent cations that are necessary to maintain the integrity of the chromatin.

Finally, it may be reported that undegraded chromatin has recently been isolated from sea urchin embryos, and that the DNA-chymotrypsin complexes obtained from somatic chromosomes are similar to those shown in Figure 2.

Summary.—An electron microscopical study was performed on the morphology and composition of sea urchin sperm chromatin. The spreading technique renders visible the fibrillar units composing the chromatin. The fibrils are 40 ± 10 Å wide, indefinitely long and frequently paired. Digestion with DNAase destroys the fibrils completely. Digestion with trypsin changes the width but does not destroy the fibrils. DNA from the whole heads, after digestion with chymotrypsin, can be spread in a film using the chymotrypsin as support. The DNA appears as "molecules" longer than 90 μ. There is no aggregation of the DNA in these conditions, nor evidence of discontinuities or "linkers." PTA at pH 4.5 stains the chromatin fibrils. Extraction with 2 *M* NaCl produces morphological changes in the fibrils. Sections of immature sperm and hydrated sperm gave evidence of some higher degree of organization of the fibrils as threads of 200-250 Å in diameter.

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*SALIVARY GLAND SECRETION AND ITS RELATION TO CHROMOSOMAL PUFFING IN THE DIPTERAN, CHIRONOMUS THUMMI**

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Puffs of the dipteran salivary gland chromosome probably reflect gene activity. Support for this belief is derived from the tissue- and stage-specificity of the puffing patterns (cf. Beermann).[1] Further supporting evidence is obtained from the observations of heightened RNA synthesis in puffed loci (cf. Pelling).[2] Tissue-specific puffs, particularly the Balbiani ring loci, have been correlated with secretion.[3–11]

In the present study we have examined the manner in which puffs are correlated with salivary secretion. Either elements of the secretion must be synthesized directly in the gland, or else their synthesis occurs elsewhere in the body and the