AN ELECTRON MICROSCOPE STUDY OF SALIVARY GLAND CHROMOSOMES BY THE REPLICA METHOD

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Plates 18 to 20

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Information concerning the structure of the giant chromosomes of dipteran salivary glands has derived mainly from the application of cytological procedures, histochemical techniques, and micromanipulation (1), methods which have utilized the limited resolving power of the light microscope. No studies have been recorded in which the increased resolution afforded by the electron microscope has been used to elucidate the organization of the giant chromosomes, although a number of attempts have been made to study the chromosomes of plants (2, 3), mammals (4), and birds (5) by means of this instrument. Such attempts have been impeded by the thickness of the chromosomes and their opacity to the electron beam; hence little or no fine structure has been revealed. In the present investigation this technical difficulty was obviated by resorting to a modification of the simple replica method (6) used previously in the study of metal surfaces. Replicas of salivary gland chromosomes obtained by this method reveal considerable detail not apparent in electron micrographs of the original chromosomes.

Material and Method

Preparation of Chromosomes.—Full grown larvae of *Drosophila pseudoobscura* and *Drosophila melanogaster* $Sc^{8}w^{a}$ and Ore R, cultured on corn meal–molasses–agar, were used for this study.[1] The larvae were placed in a drop of 45 per cent acetic acid and the salivary glands were isolated under the dissecting microscope by means of stainless steel needles. Adherent fat body was discarded. Only those glands filled with secretion and composed of large, flat cells were selected for study. The large-cell tip of the gland was then transferred to a fresh drop of 45 per cent acetic acid on a glass slide usually coated beforehand with a thin film of Mayer's albumen. A coverslip was laid over the preparation, the slide was inverted onto a paper towel, and the cells were crushed by the application of pressure through the slide. By this maneuver the tissue was spread out very thin and flat so that Liesegang's rings appeared. Examination under the light microscope showed that in successful preparations practically all the cells had been disrupted, with the chromosomes dispersed, individually extended, or stretched out, many of them isolated and apparently free from nuclear and cytoplasmic debris. For further fixation and progressive dehydration these preparations were exposed to

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[1] The original cultures of these larvae were kindly provided by Mr. Bruce Wallace of the Carnegie Institution of Washington, Cold Spring Harbor, New York, for whose continued interest and aid we are grateful.

alcohol vapors for 12 to 24 hours by keeping them in a closed Coplin jar containing just enough 95 per cent alcohol to reach the lower edges of the coverslips. The preparations were finally immersed in 95 per cent ethyl alcohol for at least 24 hours.

Preparation of Replicas.—The coverslip was carefully pried loose from the slide while still immersed in 95 per cent alcohol. The slide and its separated coverslip were each passed through three changes of fresh absolute ethyl alcohol and placed in a desiccator over phosphorus pentoxide until all visible moisture had vanished.

In many preparations the chromosomes remained adherent to the coverslip rather than to the slide after they had been separated. When the coverslip was used in making replicas it was first cemented with clarite to a glass slide, tissue side up.

Replicas were made by dipping the slide vertically into a 0.75 per cent solution of formvar[2] in ethylene dichloride just long enough to immerse and withdraw. Upon removal the slide was kept in the vertical position while the excess formvar was drained off, the back of the slide was wiped with a gauze pad, and the film was allowed to dry in air. When the humidity was high it was necessary to dry the film in a phosphorus pentoxide desiccator, because the cooling effect of the rapidly evaporating ethylene dichloride would cause water to condense on the film, which would then become opaque and brittle.

Stripping of the film from the slide was carried out in water under the dissecting microscope by means of sharpened watchmaker's forceps. In regions where the chromosomes appeared properly spread out and numerous, circular areas were outlined and discs of the film, just large enough to cover the specimen screen of the electron microscope, were peeled off and brought to the surface of the water where they immediately flattened out, and picked up on 160 mesh stainless steel screens. The screens with their films were drained by touching them to a lintless towel and were allowed to dry in air. Replicas thus obtained were then ready for examination in the electron microscope. The whole procedure of coating the slide with formvar and mounting the film on the specimen screen took about 15 minutes.

Chromosome preparations from which replicas have been obtained can be used repeatedly for the same purpose if the slide is freed of remaining formvar by stripping and then dried after passage through several changes of absolute alcohol.

All micrographs were made with the RCA (type E.M.U.) electron microscope.[3]

Comments on the Method.—Dehydration and complete drying of the spreads were found necessary for successful preparation of replicas. If the material dried from an aqueous medium, the chromosomes shrank considerably and were distorted. If the spread was not allowed to dry in the air, but was passed from absolute alcohol through ethylene dichloride into the formvar solution, the chromosomes became embedded in the film and came off the slide with it. Even when the slide had been dried in air, the chromosomes would come off with the film if the slide was allowed to stay in the formvar solution too long. It is necessary to withdraw the slide as soon as it has been immersed.

The thickness of the chromosomes is another important factor in making replicas. Good replicas could be made only when the dried chromosomes were sufficiently thin, as indicated by their transparency and lack of distortion when examined with the high dry power of the light microscope (× 600). Such chromosomes were almost invisible unless examined by diffused or diminished light. The thickness of these preparations depended upon the pressure applied in crushing the salivary glands and the length of time they were held between slide and coverslip in the alcohol vapor. If the preparation was too thick the chromosomes shrank

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[2] Formvar E (grade No. 15-95), obtained from the Shawinigan Products Corporation, New York.

[3] Made available through the courtesy of Dr. R. M. Taylor, Director of the laboratories of the International Health Division of The Rockefeller Foundation.

in drying and appeared opaque and distorted. Thus the appearance of the dried chromosomes under the light microscope indicated whether they were suitable for the preparation of replicas.

A certain thickness of the formvar film was also found to be essential for the success of the method. Films that were too thin were not only difficult to strip but also gave no detail. Films that were too thick could be detached readily but gave poor contrast in the electron microscope and exhibited many defects, such as tears and holes in the replicas. Whether the film was of optimal thickness could be readily ascertained while it was still on the slide immersed in water. Under favorable conditions numerous tiny water droplets penetrate under the film, causing minute elevations which have a golden color when viewed under the dissecting microscope. Such a film can be easily detached from the slide and when placed on a screen and dried has a golden color in daylight (angle of incidence approximately 80°). According to Schaefer and Harker (6), this color is given by a film 70 mμ in thickness, but measurements of our films with polarized light[4] revealed an average thickness in the area of the replicas of about 130 mμ.

Care had to be taken that the slides and coverslips were free from all traces of grease, for this prevents stripping of the plastic film. Only new slides and coverslips were used, cleaned in sulfuric acid–bichromate solution and dried from alcohol.

Numerous attempts were made to change the fixation of the chromosomes in order to improve the definition of the replicas. Osmium tetroxide, trichloracetic acid, phosphotungstic acid, and Flemming's mixture were all tried in various combinations, but without success. Either the chromosomes were not preserved or replicas could not be made from them.

Experimental.—In an attempt to localize desoxyribonucleic acid in the chromosomes, dried squash preparations of salivary glands were subjected to digestion by desoxyribonuclease (7) in a mixture containing, as final concentrations, 0.01 mg. enzyme[5] per ml., 0.025 M phosphate buffer of pH 7.3, and 0.005 M magnesium sulfate. Control mixtures were identical except that no desoxyribonuclease was added.

The salt mixtures were prepared on the day before the experiment and were kept at 37°C. overnight. The dry enzyme was dissolved in the warm salt mixture just before use. Both the digesting and control mixtures had a final pH of 7.45. The preparations were incubated at 37° for 60 minutes, washed in five changes of distilled water, passed through three changes of fresh absolute alcohol, and dried in air for 3 days. Formvar replicas of the chromosomes were then made in the usual manner. After the replicas had been taken, both controls and the digested preparations were treated according to the Feulgen nucleal technique.

OBSERVATIONS

As the replicas described in this report are, in effect, casts of the original chromosomes, the electron micrographs obtained from them are negative images. Therefore, in the final prints the thinner portions of the chromosomes are represented by dark grey or black, and the thicker portions by light grey or white.

Because the replicas were surface impressions it was essential that the chromosomes be separated from one another and be free of overlying debris which would obscure structural details. For this reason the replicas studied

[4] We are indebted to Dr. A. Rothen of The Rockefeller Institute for Medical Research, who made the measurements.

[5] The sample of partially purified desoxyribonuclease was kindly supplied by Dr. M. McCarty of the Hospital of The Rockefeller Institute for Medical Research.

were selected from those preparations in which the chromosomes appeared untangled and cleanly separated from cellular debris. Even so, it was not possible to obtain a complete, satisfactory record of a whole chromosome, because twists and turns sometimes distorted considerable portions of it and because the thicker bands often revealed little or no detail. Since moderately stretched chromosomes of *Drosophila*, exclusive of the short fourth chromosome, are 220 to 485 μ in length (8), an entirely uncoiled chromosome would extend across several squares of the specimen screen, and appreciable portions would be hidden by the intervening wires of the screen. Hence a complete picture of an uncoiled chromosome could not be made.

As can be seen from an examination of Figs. 1, 2, and 3, the giant salivary gland chromosome in the unstretched condition appears to consist of a series of closely apposed rows or bands of small round bodies extending across its width. In the clearest band in Fig. 1, a row of bodies can be discerned measuring from 250 to 330 mμ in diameter. Between granules and between rows there are no obvious connections. The discreteness of the bodies can be seen in Fig. 2 where the chromosome is slightly stretched. In Fig. 3 the striated and granular structure is also evident, with no indication of strands or threads between granules or bands. In all the micrographs these granules have been measured wherever their contours were clearly discernible. They were found to vary from 210 to 330 mμ in diameter, the majority being in the range of 250 to 290 mμ. Since these granules are therefore just at the limit of resolution of the light microscope, they are considered to be identical with the small basophilic granules and vesicles that have been designated as chromomeres (1).

The micrographs of the replicas reveal no evidence of a limiting membrane surrounding the chromosomes, either in lax or stretched specimens. The chromomeres extend to the edge of the chromosome, and no sheath or pellicle appears to intervene between the outermost chromomeres in a band and the material around the chromosome (Figs. 1, 3, and 4). A definite matrix of intrachromosomal substance between chromomeres also appears to be absent.

When chromosomes are moderately or greatly stretched, the bands of chromomeres separate, and more or less coarse longitudinal strands appear between them (Figs. 4, 5, and 6). In some places these filaments stretch between corresponding granules of neighboring bands as at *a* in Fig. 4; in other places (*b* in Figs. 4 and 5) they form an interweaving longitudinal meshwork. Nowhere could the same strand be clearly traced across more than one row of granules. The replica method has not revealed any fine periodic structure in the strands such as has been found in myofibrils or collagen threads (9). They seem, on the contrary, to be homogeneous fibers which divide and anastomose between bands, their configuration depending upon the amount and direction of the stresses developed in stretching the chromosomes (Fig. 6).

The digestion experiments with desoxyribonuclease provided further infor-

mation about the strands. Figs. 7 and 8 show portions of moderately and greatly stretched chromosomes after they had been subjected to the action of desoxyribonuclease for 60 minutes at 37°C. These chromosomes give a negative Feulgen reaction when tested after the replicas had been made, thus indicating that the desoxyribonucleic acid had been removed by the digestion. The continuity of the chromosomes was not affected by the procedure but the chromomeres appeared to be more discrete than those in untreated preparations. In the heavier bands small granules can be discerned that are not obvious in such bands of undigested chromosomes (compare Fig. 7 with Fig. 3). The distinct chromomeres, measuring from 210 to 250 mμ in diameter, were also a little smaller than those of the control chromosomes. Between the bands no fibers or filaments remained, but in their place was an amorphous material that had no apparent orientation in relation to the chromomeres. The control preparations resembled in all respects the untreated chromosomes (compare Fig. 8 with Figs. 4 and 5).

DISCUSSION

The replica method has already been applied to blood cells and bacteria with some success (10), and it seems probable that with certain modifications it can be used to study tissues which have thus far proved inaccessible to electron microscopy because of their opacity to the electron beam. In the present study on giant salivary gland chromosomes by means of the replica technique, no detail has been revealed that had not been suggested by examination of stained preparations with the light microscope. However, the increased resolution and magnification provided by the electron microscope have made it possible to characterize the structure of these chromosomes more fully.

According to the most widely accepted view, the giant chromosomes consist of a number of parallel and closely approximated threads or chromonemata bearing homologous chromomeres at regular intervals marked by Feulgen-positive, basophilic bands. This polytene structure was postulated by Koltzoff (11) and Bridges (8) on the basis of fixed and stained preparations and has been supported by the work of Bauer (12), Painter and Griffen (13), and d'Angelo (14). Metz and Lawrence (15) have presented contrary evidence to the effect that the chromosomes are alveolar in structure, made up of achromatic vesicles or droplets in a chromatic matrix, and that the strands visible in stretched chromosomes are not true chromonemata but artifacts, stress lines in the matrix produced by the stretching of chromatic material from the band regions. Buck's micromanipulation studies (16) on fixed salivary gland chromosomes have tended to confirm this interpretation. The polytene theory has also been challenged by Ris and Crouse (17), who state that the bands are actually caused by the complex coiling of a bundle of chromonemata which weave back

and forth across the width of the chromosome. According to this view the chromonema is uniformly Feulgen-positive and the so called chromomeres are optical sections of gyres in the chromonema. The electron micrographs presented in this paper do not substantia te any one of these theories.

Although there is general agreement (1) that a sheath envelops the chromosomes, we could find no evidence for it in the replicas. Chromomeres occupy the entire width of the chromosomes. It is possible that the sheath may be too delicate to withstand squashing of the chromosomes, but the fact that the nuclear membranes of blood cells can be demonstrated in replicas (10) makes it seem improbable that the replica technique would not disclose a sheath in the chromosomes if it were present. However, in view of the apparently conclusive micrurgical demonstration by d'Angelo (14) of the existence of a membrane in the living giant chromosomes of *Chironomus*, it may be inferred that in our material the sheath was destroyed or dispersed by the process of making squash preparations.

The existence and character of a chromosomal matrix in which the chromonemata and chromomeres are embedded have been controversial subjects for many years (1). The observations reported here provide no evidence for the presence of any intrachromosomal substance distinct from the chromomeres. Neighboring rows of chromomeres lie in very close apposition in the unstretched chromosomes. The appearance of achromatic interband regions in stained lax chromosomes may be merely the result of the relatively poor nucleic acid content of certain bands.

The nature of the strands seen in electron micrographs of the interband regions of stretched chromosomes is more difficult to determine. The facts that these filaments are not apparent in the lax chromosomes, that they are evident only in stretched preparations, that they divide and anastomose between bands, and that they disappear when digested by desoxyribonuclease suggest that they are artifacts, as Metz and his coworkers maintain. Some indication of their nature can be obtained by comparing Fig. 5 in the present paper with the electron micrographs of thymonucleohistone in the paper of Mazia, Hayashi, and Yudowitch (18). These workers compressed films of thymonucleoprotein into fibers which under the electron microscope appear as narrow, thin, anastomosing sheets, resembling the strands that form between bands of stretched chromosomes. This similarity is further borne out by the results of our digestion experiments with desoxyribonuclease. These results suggest that when the chromosome is stretched desoxyribonucleoprotein is pulled out from the surface of adjoining chromomeres into sheets extending between them and that these sheets tear or shred into strands according to the irregular stresses produced by pressure on the coverslip and the varying resistances of the bands. When nucleic acid is removed by enzyme action, the strand-like appearance is destroyed and only the protein remains as amor-

phous material. This interpretation of the strands, furthermore, is in accord with the fact that the interband regions of salivary gland chromosomes are positively birefringent to polarized light only when in the stretched condition (19).

The electron micrographs show that the transverse bands are indeed composed of small round granules arranged in rows across the width of the chromosomes. Since the replicas are surface impressions there can be no possibility of confusion arising from misinterpretation of optical sections. It is difficult to visualize how the small round bodies seen in these preparations could be caused by gyres of a continuous uniform thread. Moreover, the persistence of these bodies after the chromosome has been subjected to digestion by desoxyribonuclease indicates that they have a complex constitution. They contain at least two types of substances, desoxyribonucleic acid and other constituents, probably protein in nature. Since the granules are smaller after digestion and since strands that are destroyed by desoxyribonuclease appear between them when the chromosome is stretched, at least part of the desoxyribonucleic acid lies on the surface. The fact that after digestion granules are more readily discerned in the heavy bands also supports this inference. Evidently the nucleic acid component does not determine the shape of the chromomeres.

In general the observations reported here lead to the concept that the giant salivary gland chromosome is essentially chromomeric in structure. The chromomeres lie in close apposition to one another to form continuous, parallel, adherent chains without interpolated threads. Each member of a chromosome pair is, then, a single giant chromonema composed of chromomeres that are more numerous, or perhaps larger, or both, than those in the chromosomes of ordinary cells.

SUMMARY

A method for preparing replicas of salivary gland chromosomes for electron microscopy is described.

Electron micrographs of these replicas show that the giant chromosomes are composed of a series of small granules of approximately equal size arranged transversely across the chromosome.

In stretched preparations a linear network of filaments appears between the rows of granules. These fibers cannot be traced between corresponding granules of more than two consecutive rows. When the chromosomes are digested by desoxyribonuclease, these fibers disappear and only amorphous material remains between the bands. The characteristics of the strands suggest that they are artifacts produced when the chromosomes are stretched.

The small granules are composed of desoxyribonucleic acid and at least one other component, probably a protein. The nucleic acid seems to lie at least in part on the surface of each granule.

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EXPLANATION OF PLATES

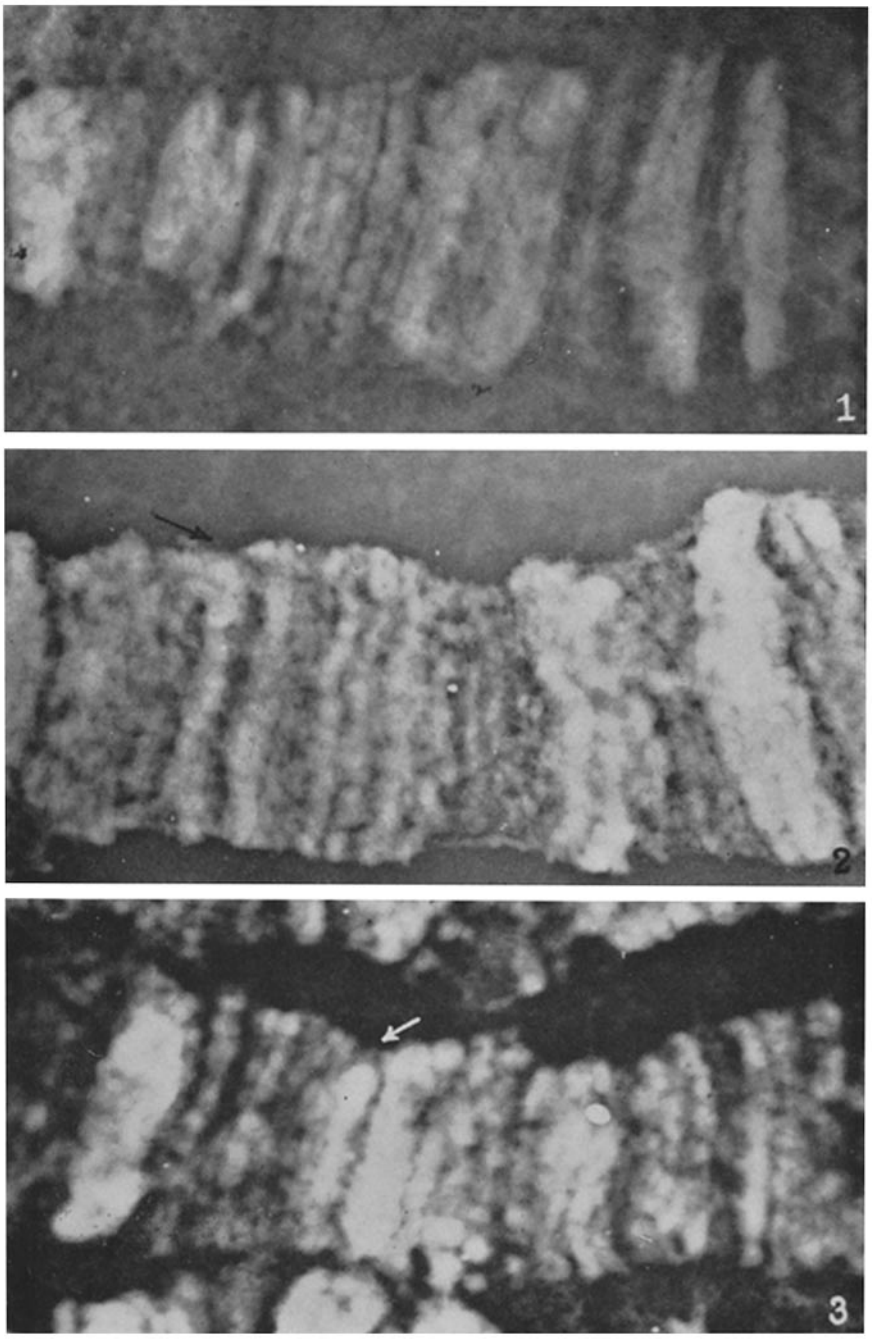
All figures are electron micrographs of formvar replicas made from giant salivary gland chromosomes fixed in 45 per cent acetic acid and dehydrated and dried from absolute ethyl alcohol. The thicker portions of the chromosomes are represented by light grey or white, the thinner portions by dark grey or black.

PLATE 18

FIG. 1. Portion of an unstretched chromosome of *D. melanogaster* Sc^8w^a. The rows of granules (chromomeres) are close together, with no apparent interconnecting threads. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 2. Part of a slightly stretched chromosome of *D. melanogaster* Sc^8w^a, showing rows of chromomeres separated by short distances. Fiber formation is restricted to the lower edge of the chromosome. The thin, diagonal, dark line (arrow) extending along the length of the chromosome marks the groove between the two component chromatids coiled loosely about each other. Micrograph taken at a magnification of 2200 enlarged to 8140.

FIG. 3. Portion of a chromosome of *D. melanogaster* Sc^8w^a, showing chromomeric structure and coiling of the chromatids. The arrow points to the groove between chromatids. Micrograph taken at a magnification of 2600, enlarged to 9620.



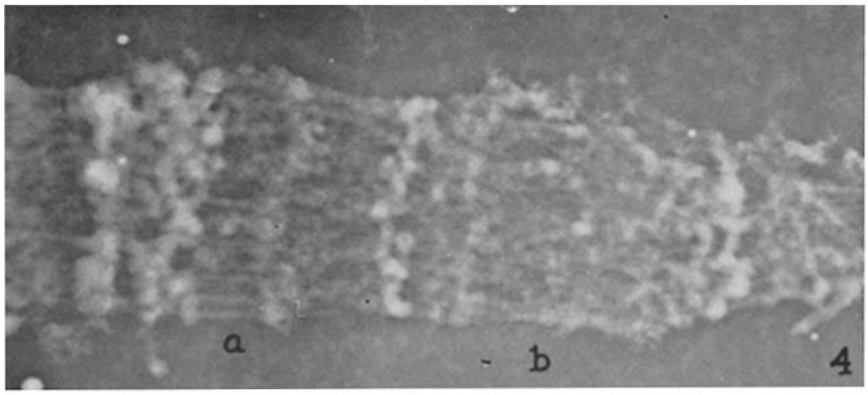
(Palay and Claude: Electron microscopy of salivary gland chromosomes)

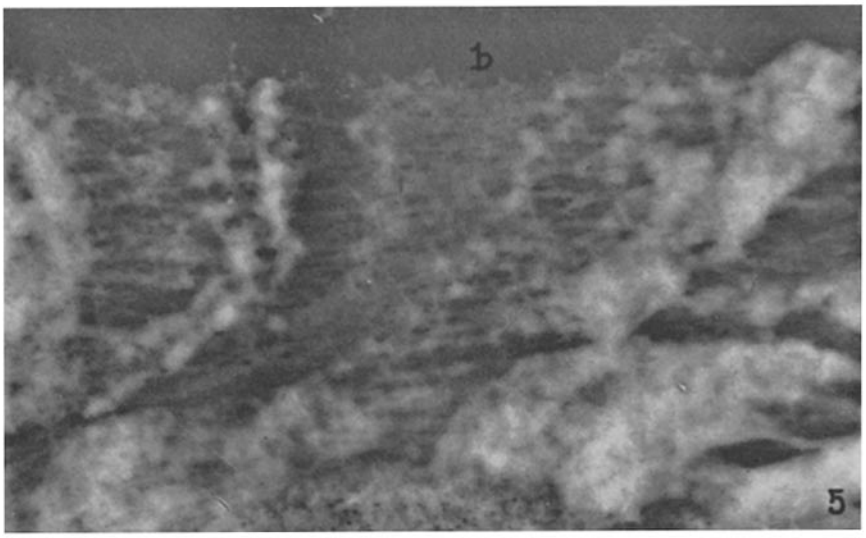
PLATE 19

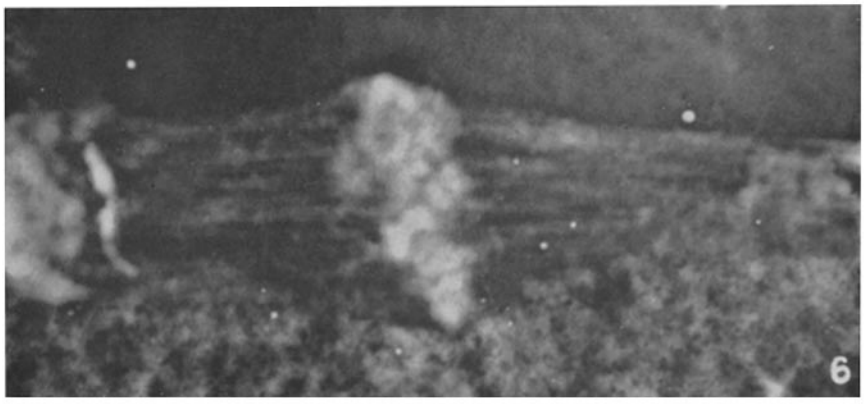
FIG. 4. Portion of a stretched chromosome of *D. melanogaster* Sc^8w^a, showing at *a* the filaments extending between corresponding chromomeres of neighboring rows. At *b* the filaments are interconnected to form a meshwork. Stretching of the chromosome has somewhat separated the round bodies or granules from one another. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 5. Portion of a greatly stretched chromosome of *D. melanogaster* Ore R, showing meshwork of filaments between bands of chromomeres (*b*). Micrograph taken at a magnification of 2600, enlarged to 9620.

FIG. 6. Portion of a greatly stretched chromosome of *D. melanogaster* Sc^8w^a, showing two bands composed of granules approximately 240 mμ in diameter, connected by longitudinal strands. Micrograph taken at a magnification of 3100, enlarged to 9455.



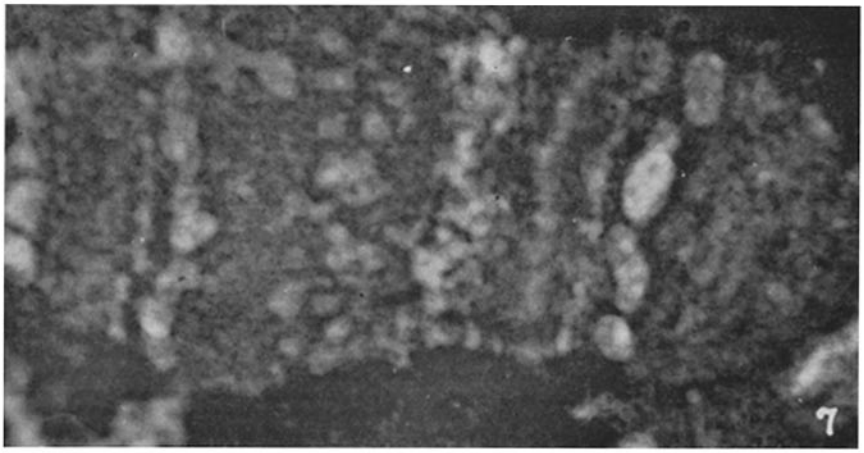


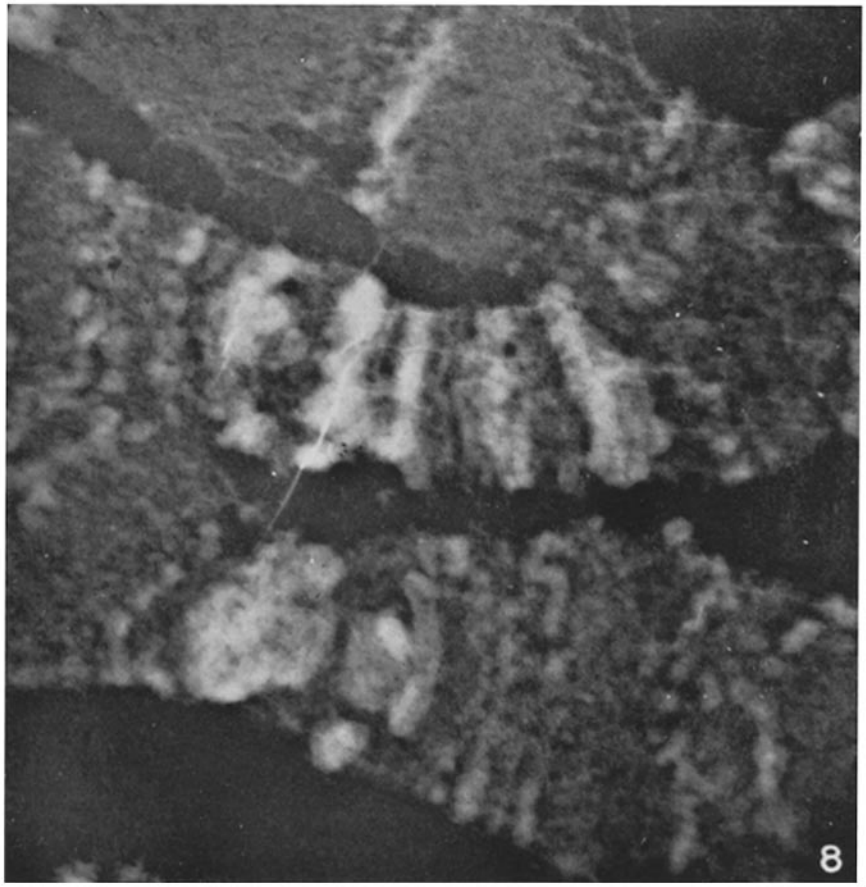


(Palay and Claude: Electron microscopy of salivary gland chromosomes)

PLATE 20

FIGS. 7 and 8. Portions of moderately and greatly stretched chromosomes of *D. melanogaster* Ore R after digestion with desoxyribonuclease for 60 minutes at 37°C. The chromosomes from which the replicas were made were Feulgen-negative. The chromomeres appear to be smaller and more distinct than those of undigested preparations. No filaments remain after digestion, but in their place an amorphous coagulum appears between the bands. Electron micrograph taken at a magnification of 3100, enlarged to 9455.





(Palay and Claude: Electron microscopy of salivary gland chromosomes)