A STUDY OF NEWT MITOTIC CHROMOSOMES BY NEGATIVE STAINING

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ABSTRACT

A method is described for bursting single, selected mitotic cells on a fluid surface. Cells from cultures of newt heart tissue were burst on dilute solutions containing potassium and sodium with and without added calcium and also on dilute calcium chloride solution. The material was negatively stained with uranyl acetate or sometimes with ammonium molybdate or sodium phosphotungstate. The bodies of chromatids spread on NaCl/KCl solutions showed many parallel fibers about 150 A in diameter. Loops with a complex nodular structure were observed projecting from the sides and ends of chromatids. In calcium-containing solutions there was evidence of fiber coagulation; the chromatid body was more compact and laterally projecting fibers tended to be pulled out straight. Especially in the absence of calcium the chromosomal fibers had a nodular form and appeared to be composed of irregularly folded fibrillar elements. The question as to whether chromosomal fibers, which range in diameter from about 50 to 300 A, consist of single, folded threads or of two or more adjacent subunits is discussed.

INTRODUCTION

Although several models of chromosome structure have been suggested (Freese (14), Schwartz (34), Steffensen (36), Taylor (37), Cole (9), Du Praw (12)), most of them have been based more on genetical evidence and on what is known of the chemistry and replication of DNA than on direct observation. This is not surprising because, with the exception of certain unusual forms such as the very large chromosomes of Diptera and of Urodele oocytes, the finer structure of chromosomes has proved very difficult to elucidate by microscopy. Electron microscopy has so far provided very limited information. It is generally agreed (see Ris, 32, 33; Gall, 16; Moses and Coleman, 31; Hyde, 25) that fibers 100–300 A in diameter are the most conspicuous chromosomal components, but the interpretation of these fibers in terms of molecular structure is still uncertain. Nor have they been shown to have any definite arrangement to form regular structures of higher order, at least in more orthodox types of chromosomes or in interphase nuclei.

In thin sections, the fibers are presumably held in approximately their natural positions but their courses can only be followed for short distances, so that this method is not promising (Barnicot and Huxley, 2).

A different approach was initiated by Kleinschmidt and Lang (28) who spread the nuclear material of bacteria on a fluid surface by bursting the protoplasts osmotically. Gall (16) and Ris (33) have used a similar method to liberate and spread the nuclear contents of amphibian erythrocytes, and Wolfe (41, 42) and Du Praw (11, 12) have studied mitotic chromosomes by this procedure. Although the swelling and bursting of the cell and the subsequent exposure of the chromosome to surface tension forces are liable to disrupt and disarrange the chromosomal structure to some extent, the method seems better suited than sectioning to

the study of compact bodies composed of long, coiled threads.

The method of negative staining (Huxley, 22) has proved valuable in work on virus structure (see Horne and Wildy, 19) and also in the study of certain fibrous proteins (Huxley, 23; Hanson and Lowry, 18) and some of the larger globular ones (Horne and Greville, 20; Fernández-Morán, Van Bruggen, and Ohtsuki, 13). So far, it does not seem to have been used very much in the examination of spread chromosomes or interphase nuclear material though it should be ideally suited to such material provided the components are not destroyed by the reagents. However, Solari (35) used it effectively in a study of spread material from sea urchin sperm heads.

If a tissue or cell suspension is used for spreading, the chance of picking up chromosomes on an electron microscope grid is not high unless the material is exceptionally rich in mitoses. A technique was, therefore, devised by which single mitotic cells, selected under the light microscope, could be burst on a fluid surface and then negatively stained. Cultures of newt fibroblasts were chosen as material because the cells and chromosomes are relatively large and had already been studied by thin-sectioning (Barnicot and Huxley, 2; Bloom and Leider, 3; Bloom and Ozarslan, 4). Various floating-out fluids and several staining methods were investigated. 60-70 cells were examined but, since relatively few were prepared by each procedure, the results are to some extent preliminary.

METHODS

Selection and Bursting of Cells

The method of culturing newt heart tissue has already been described (Barnicot and Huxley, 2). The tissue was grown on narrow cover slips in sloped test tubes at room temperature. Plenty of mitoses were present in the monolayer outgrowth after 10 days or more in primary culture. The cover slip was then transferred to a shallow glass chamber (30 x 11 x 2 mm) so designed that it could be placed as a bridge over the floating-out trough (Fig. 1).

The chamber was made by cementing glass strips, cut from microscope slides, with Araldite. The trough, made of Araldite-cemented plate glass (internal dimensions 9 x 2.5 x 0.7 cm), was placed on the stage of a long working-distance binocular dissecting microscope (Zeiss stereomicroscope) and filled with the floating-out solution, the surface of which was swept clean with narrow glass strips coated with paraffin wax. The bridge-chamber, containing the culture immersed in the culture medium, was placed in position over the trough and inspected by transmitted light at × 100 magnification. Metaphase and anaphase mitotic cells are readily identifiable under these conditions but can be previously located by inspection under the phase contrast microscope, if desired. The chosen cell was then detached from the cover slip with needles. Small sewing needles, selected as having undamaged points, were found to be quite satisfactory. The magnification was changed to × 40 and the cell was picked up on a film suspended in a fine loop. The loop was about 1.0 mm diameter and was made of fine platinum wire (diameter 0.06 mm, 46 S.W.G.) twisted and sealed into a tapered glass tube. The film on this loop was at first made by dipping it into a collodion solution and allowing the drop to dry, but later Perspex cement (Tensol cement No. 6, G. H. Bloore, Ltd., London) diluted with a mixture of equal parts of chloroform and butyl acetate was found to give a stronger film. The cell was coaxed towards the surface by creating an upward current with the loop, and then lifted through the surface film so that it remained resting on the loop in a small drop of medium. The next stage of the procedure was performed rapidly so as to avoid drying of the drop. The bridge-chamber was removed; the loop was transferred to the left hand, the magnification changed to × 25, and a pair of fine forceps in which an electron microscope grid, bent at a suitable angle, had been clamped (by sliding a polythene ring up the jaws) was taken up in the right hand. The loop was gradually lowered towards the surface of the floating-out fluid, keeping it in focus until the fluid surface was seen. When the loop touched the surface, only a little fluid remained on it due to its hydrophobic nature. It was then lowered through the surface film and the cell was left floating. The cell was carefully watched until disintegration or bursting occurred, and the carboned side of the grid was then rapidly touched to the fluid surface over the area in which bursting had taken place.

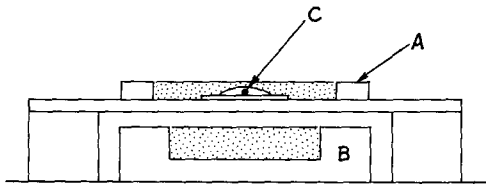


FIGURE 1 Floating-out chamber (*B*) and bridge-chamber (*A*) containing culture on coverslip (*C*) seen in transverse section. The diagram is not accurately to scale.

The carboned surface of the grid was immediately touched on the surface of the negative-staining reagent and held there for about 15 sec. The excess of reagent was removed with a fine glass capillary under the low power of the dissecting microscope so as to observe whether drying was even. The grid was then inspected for the presence of chromosomes under the phase contrast microscope and photographs were usually taken at this stage. Most of the cells examined were in metaphase. Anaphase cells tended to go into telophase during manipulation. The nuclei of interphase cells did not burst on the solutions used, but partial bursting of one prophase cell was observed.

Floating-Out Solutions

WATER: Many attempts were made to obtain chromosome preparations from cells burst on distilled water, but in only one instance was this successful, though the residue of the burst cells was often found on the grids.

M/500 AQUEOUS CALCIUM CHLORIDE: This solution was tried, partly with the aim of preserving spindle fibers (Barnicot, 1), and it was found that chromosomes were picked up on the grid in a high proportion of cases. They were often connected to the residue of the burst cell (Figs. 2 and 3), and it seems probable that using a calcium solution was more successful because bursting was less explosive than in water, so that the chromosomes were less likely to be flung away from the position of the burst cell and thus missed by the grid. It should be emphasized that the pH of the calcium solution was not adjusted and was about 5.2.

DILUTED CALLAN MEDIUM B: Medium B, made by mixing seven parts of a 5/1 mixture of 0.05 M KCl and 0.05 M NaCl with three parts of 0.001 M KH_2PO_4, was found by Callan and Lloyd (6) to be suitable for prolonged observation of fresh lampbrush chromosomes. For bursting cells, this medium was diluted 1/10, 1/20, or 1/30 with distilled water and the pH adjusted with dilute NaOH to 6.7–6.8.

In some experiments, $CaCl_2$ to a final concentration of M/2500 or M/4000 was added to the diluted Callan medium and the pH adjusted to 6.5. A few of these specimens were treated for 1 min with M/250 EDTA Na on the grid before negative staining. In other cases, 0.05 M trisodium citrate was used instead of 0.05 M sodium chloride in making up the stock medium, and this solution was then diluted 1/30 and adjusted to pH 6.7–7.0.

Fixing and Staining Reagents

For negative staining, 2% uranyl acetate in water adjusted to pH 4.7 with dilute NaOH and filtered was found to give the best results. 1% phosphotungstic acid in water adjusted to pH 7.0 with dilute NaOH or at pH 5.2, and 1% ammonium molybdate at the same pH values were also tried. All reagents

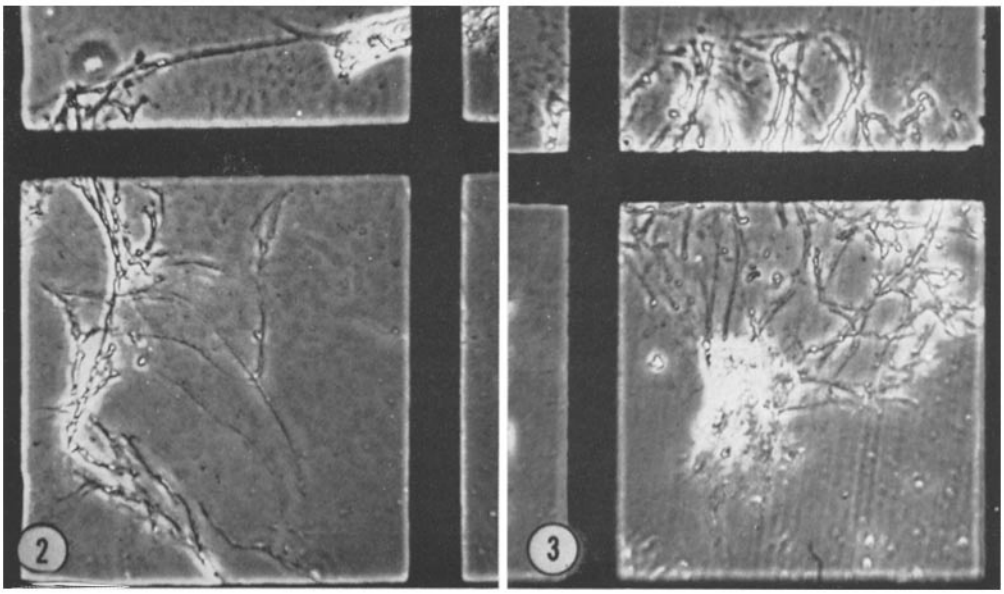


FIGURE 2 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. Note residue of cell body (top right) and chromosomes extending over lower grid square. Phase contrast. × 225.

FIGURE 3 Cell burst on Callan B–citrate 1/30; negatively stained with uranyl acetate. Cell body residue (low center) and extruded chromosomes. Phase contrast. × 225.

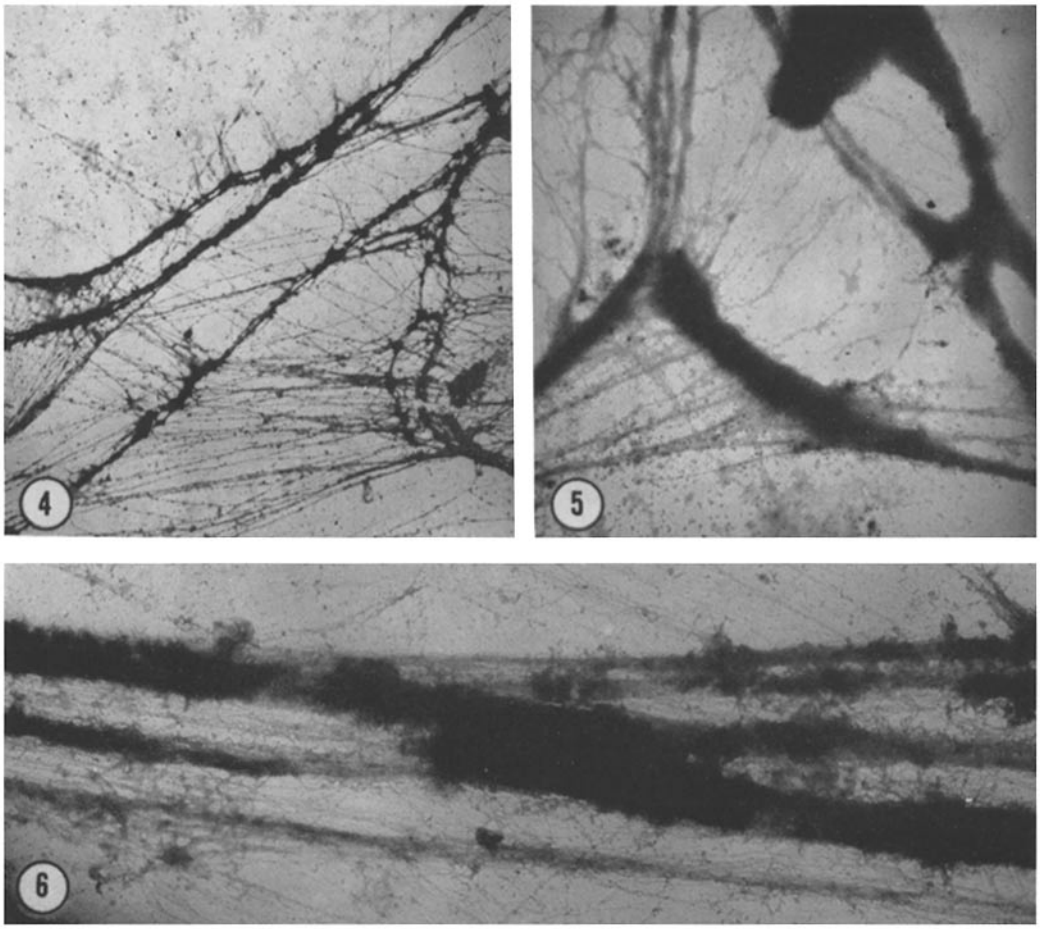


FIGURE 4 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. Chromosomes connected by stretched side branches. × 2350.

FIGURE 5 Cell burst on M/500 $CaCl_2$; negatively stained with ammonium molybdate. Chromosomes with side branches. Compare with uranyl acetate–stained specimen, Fig. 4. × 2350.

FIGURE 6 Cell burst on Callan B-citrate 1/30; negatively stained with uranyl acetate. Chromatids stretched to varying degrees. × 6000.

were freshly made before each experiment. A few preparations were negatively stained after floating the grid on either 5% glutaraldehyde or 5% formaldehyde in 0.1 M phosphate buffer, pH 7.2. The grid was washed in the buffer alone and then rinsed in water before staining. A few preparations were fixed in buffered 2% osmium tetroxide (pH 7.2) both with and without the addition of 10^{-3} M $CaCl_2$ (see Barnicot and Huxley, 2) or in 5% buffered glutaraldehyde, and were then positively stained with uranyl acetate (2% aqueous solution pH 4.7 for 6 hr) either alone or followed by lead hydroxide as described by Watson (39) and Huxley and Zubay (24) or by the method A of Karnovsky (26). Some preparations were positively stained by these methods after fixation in absolute alcohol and drying from butyl acetate. Chromosomal fibers were not strongly stained by any of these positive staining methods and yielded no useful information.

Electron Microscopy

Either single hole (L. K. B. Ltd., London, 800-μhole) or rectangular mesh grids (Smethurst Highlight Ltd., Type New 100, 3.0 mm in diameter) were used.

Chromosomes can often be picked up in the central area of a single-hole grid and viewed unobstructed, but it is difficult to avoid film breakage under the beam. A Formvar (0.3% in chloroform) or Pyroxylin film was laid on the grids which were then carboned and the film left in situ so as to obtain adequate strength in viewing thicker parts of the field. Formvar films proved to be the more satisfactory. Carbon films alone were tried, but were found to be too fragile.

The preparations were examined in an Siemens Elmiskop I B using double condenser illumination. The higher resolution photographs were taken at 60,000 or 76,000 magnification after careful correction for tilt and astigmatism.

After examination at higher magnifications, survey photographs at × 2000 were usually taken of the greater part of the field, and montages of these together with phase contrast photographs were useful in locating regions which had been studied at higher resolution.

RESULTS

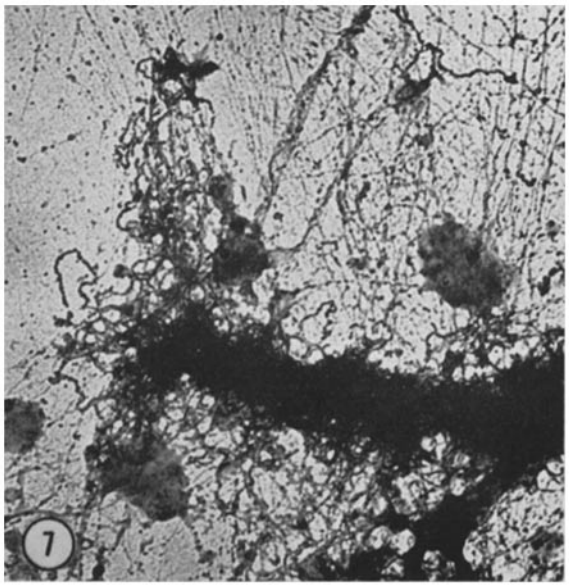
The results may be considered under two headings according to the type of medium used to burst the cells: 1. Cells burst on water or on diluted Callan medium B, with or without calcium or citrate. 2. Cells burst on M/500 calcium chloride.

1. *Cells Burst on Water or on Diluted Callan Medium B*

It so happened that in the one successful preparation from a cell burst on water the grid was unusually hydrophobic so that the little uranyl acetate remaining on it was restricted to the immediate vicinity of the chromosomal material. Certain features of spread chromosomes, which tend to be obscured by uranyl deposits when negative staining is better, were, therefore, particularly clear under low magnification.

Chromosomes which were not stretched much beyond their normal metaphase length showed numerous loops, composed of fibers about 300 A in diameter, projecting from their surfaces, and these were also present at the ends of chromatids (Fig. 7). The region of a partially spread chromosome shown in Fig. 8 suggests that at least some of these loops may arise by unraveling of the small surface projections which are a striking feature of newt metaphase chromosomes in thin sections (Barnicot and Huxley, 2).

Chromosomes which had been stretched to lengths of 50–100 μ, showed many fine, straight



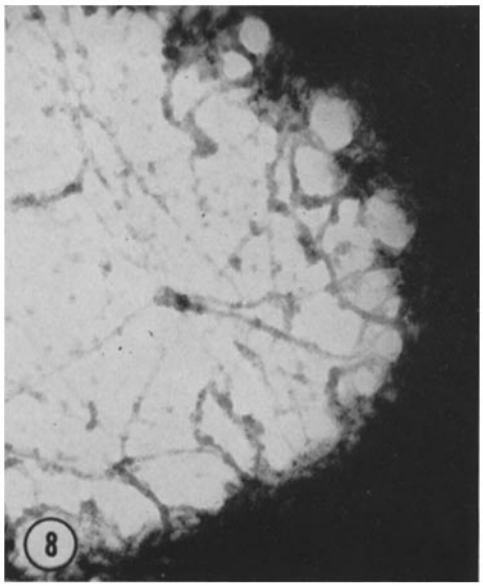


FIGURE 7 Cell burst on water; negativcly stained with uranyl acetate. Chromatid end showing loops. × 11,500.

FIGURE 8 Cell burst on water; negatively stained with uranyl acetate. Portion of a relatively unstretched chromosome showing surface projections and loops which appear to arise from them. × 40,000.

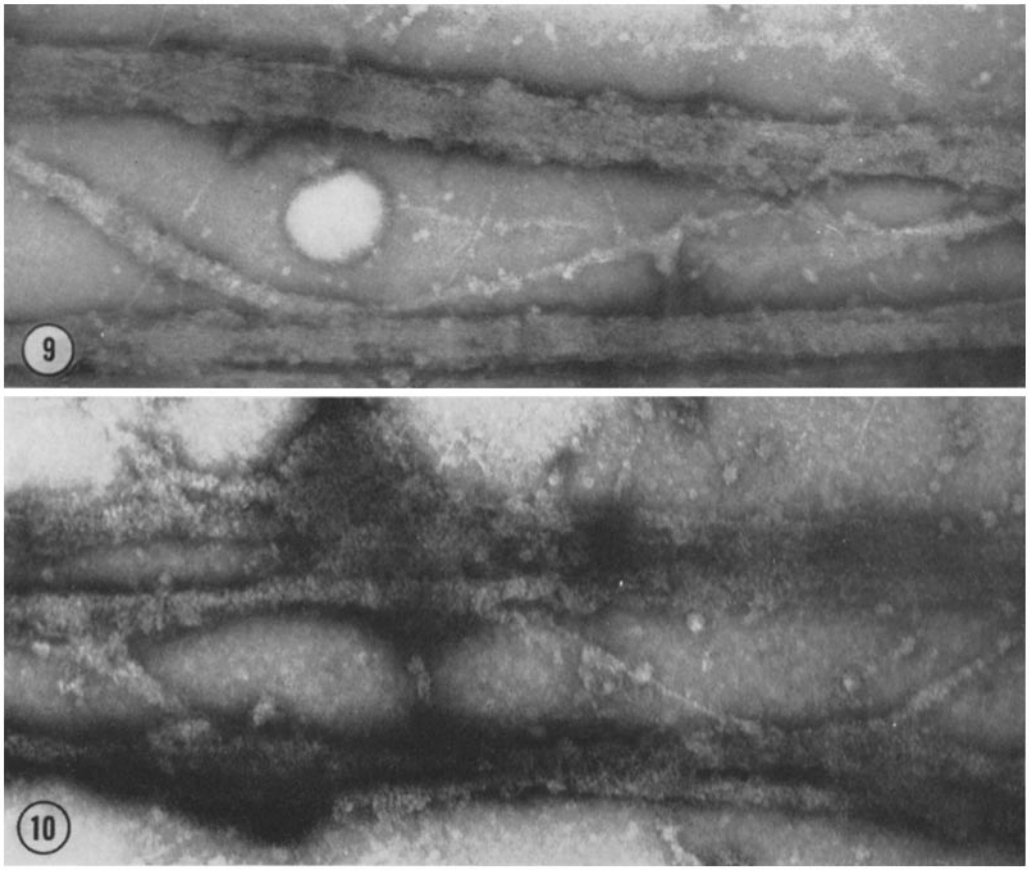


FIGURE 9 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. Two stretched chromatids, each composed of a single fiber about 1000 A in diameter. × 50,000.

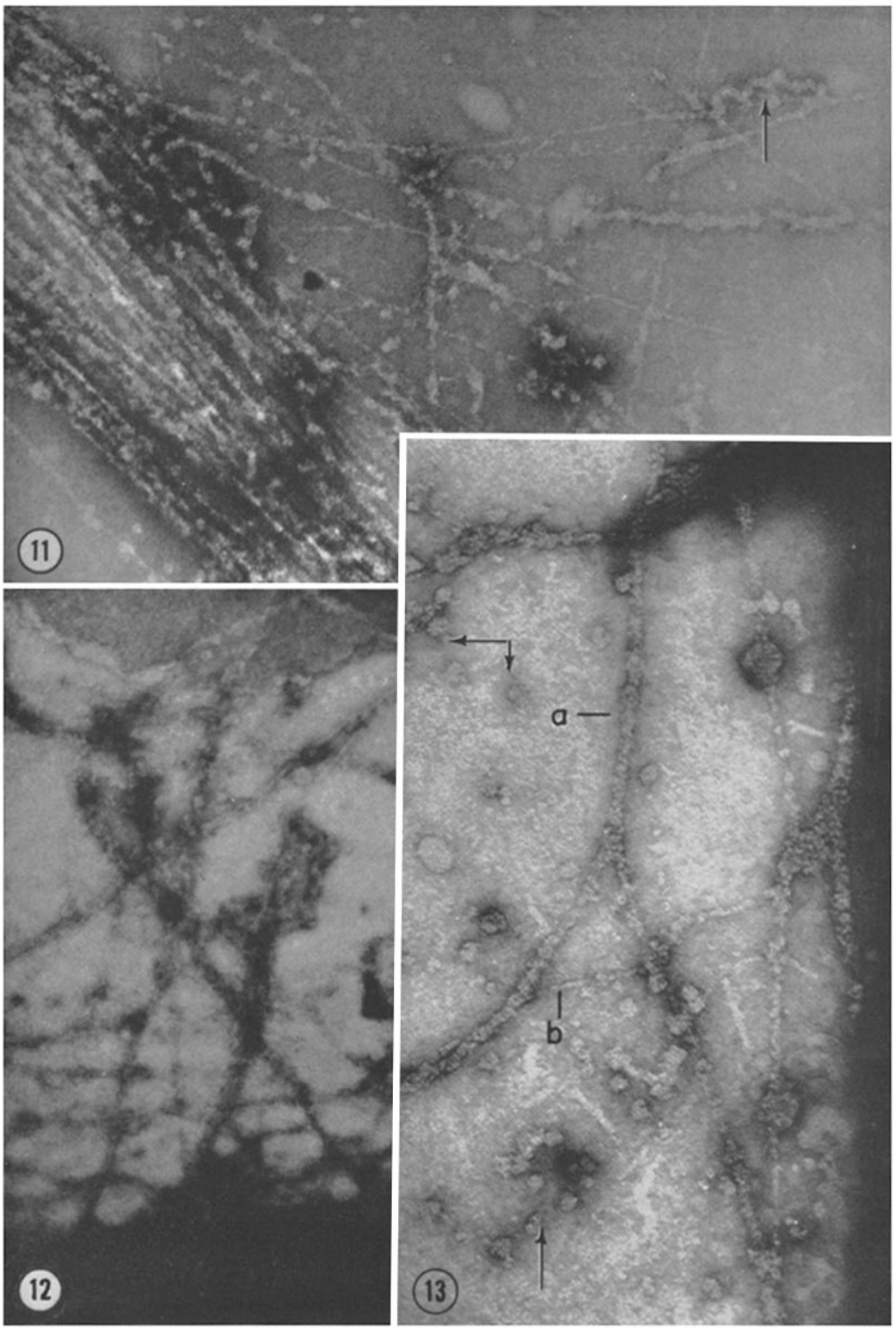
FIGURE 10 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. A stretched chromatid showing several thick interconnected fibers. × 90,000.

threads projecting more or less at right angles from their surfaces and often bifurcating at their junction with the chromatid body. Some of these threads, which could sometimes be traced for distances of 5–10 μ, probably represent loops greatly extended during spreading. In some places, adjacent chromosomes or chromatids were connected by fine straight threads as if these had been

FIGURE 11 Cell burst on Callan medium B 1/30 + M/4000 $CaCl_2$; negatively stained with uranyl acetate. Parallel fibers in 150 A range are seen in the body of a chromatid (left). On the right side of the chromatid some of these fibers are pulled out into the surrounding area. A detached loop is indicated by an arrow. × 95,000.

FIGURE 12 Cell burst on Callan medium B 1/20; negatively stained with uranyl acetate. Loops are seen projecting from a dense chromatid body (bottom). The loops appear to consist of aggregated nodules. × 65,000.

FIGURE 13 Cell burst on M/500 $CaCl_2$; negatively stained uranyl acetate. Bifurcations of fibers adjacent to a chromatid (right). The larger fiber (*a*) is 150–200 A diameter, and the thinnest (*b*) about 100 A. Note also ribosome-like particles (arrows). × 120,000.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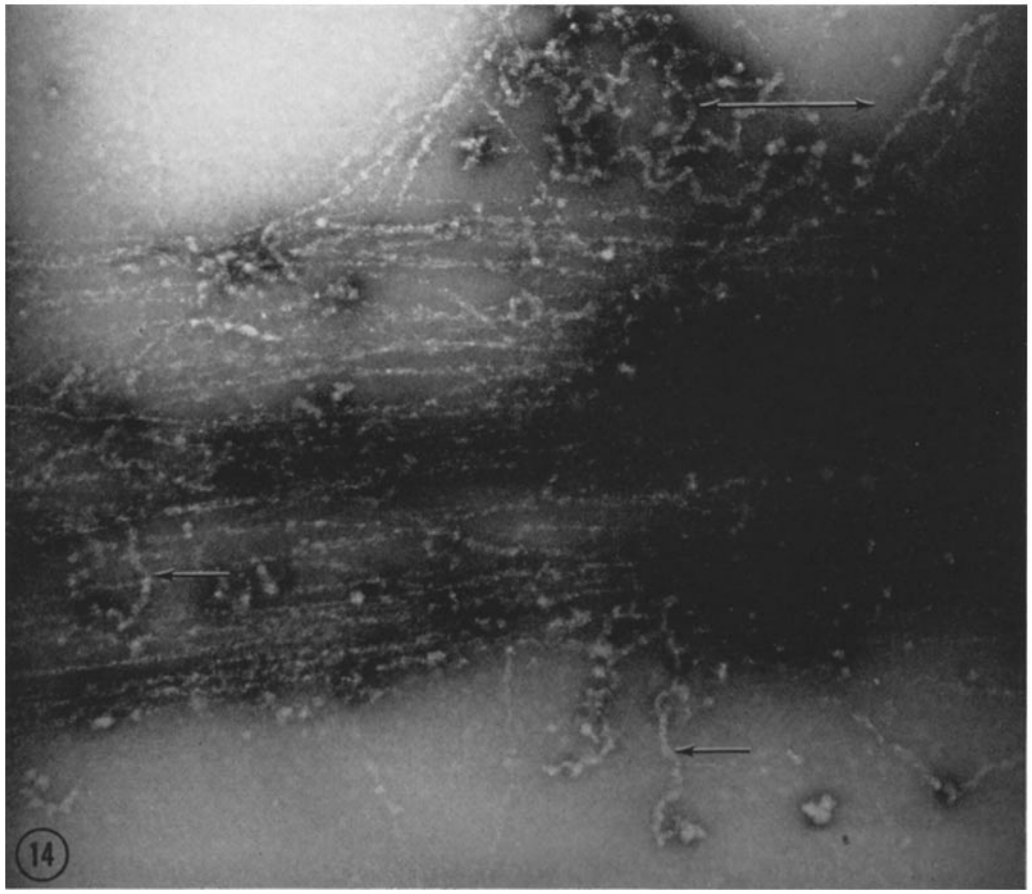


FIGURE 14 Cell burst on Callan medium B 1/30; negatively stained with uranyl acetate. Stretched region of a chromatid. The chromatid is here about 1.3 μ wide and is composed of many parallel 150 A fibers. Thicker, relaxed loops are seen at points marked with arrows. × 45,000.

stretched when adherent regions were pulled apart (Fig. 4).

The chromosomes of cells burst on diluted Callan medium showed essentially the same features at low magnifications as those described above. Cells floated on $\frac{1}{10}$ dilution burst slowly and the chromosomes were only slightly stretched and dispersed, but they were too thick for satisfactory electron microscopy. Cells floated on the $\frac{1}{20}$ or $\frac{1}{30}$ dilutions usually burst after about $\frac{1}{2}$ min, but bursting was not explosive. Considerable extension of some of the chromosomes was found to have occurred and, in some instances, they were at least 200 μ long. Chromatid pairs were often clearly identifiable. The chromatids of the more extended chromosomes were about 0.25–0.50 μ in diameter, but in some regions they were thinner and less dense and probably partially torn. A phase-contrast picture of a cell burst on the $\frac{1}{30}$ dilution with citrate is shown in Fig. 3, and a group of stretched chromatids from the same preparation in Fig. 6. The substitution of sodium citrate for sodium chloride made little difference to the low magnification appearance. If the pH of the diluted medium was not adjusted to 6.7–6.8 but was more acid, (pH 5.2) the chromosomes had a coagulated appearance.

In some areas of the preparations, where the chromatids were sufficiently thin, good negatively stained images of the chromatid body as a whole were obtained. Examples are shown in Figs. 14 and 15.

The body of a chromatid in such regions was composed of some 20 to 30 more or less straight

and parallel fibers which were generally about 150 A in diameter. A tendency for the fibers to be loosely associated as parallel pairs was sometimes apparent, but it was not a conspicuous feature and was noticeable mainly in regions in which the chromatid was fairly compact.

Although 150 A is a reasonable figure for the diameter of these chromatid fibers, there is certainly some variation in thickness. Also, individual fibers are not uniform in diameter, but have a nodular structure, with regions thinner than 150 A at somewhat irregular intervals along their length. The finer structure of these fibers was most clearly resolved in some of the material spread on diluted Callan medium with added calcium (see below).

In contrast to the relatively straight fibers seen in the main body of the stretched chromatid, thicker, irregularly folded fibers were often found projecting from the sides (Fig. 12) or lying straddled across its central area (Fig. 14). It could be clearly seen in some instances that these structures were loops. In a few places, apparent continuity between a loop and the straight fibers of the chromatid body was observed, but it was not possible to establish the existence of such connections beyond doubt.

The structure of less highly stretched regions of the chromatids was not easy to make out because they were usually too thick. However, no evidence of helical or other types of regular folding was observed. The material had a granular appearance which seemed to be due to irregular folding of fibers into nodules. Extension of the chromatid presumably leads to straightening out of these folds, but evidently some of the loop structures described above are not involved in this process and remain in a more relaxed and thicker condition.

Some improvement in the quality of the preparations for higher resolution microscopy was obtained by adding calcium chloride to a final concentration of either M/2500 or M/4000 to the diluted Callan medium. Especially in the higher of these two concentrations of calcium, the spread chromosomes showed distinct differences from those described above. These differences were presumably due largely to the presence of Ca^{++} since the solution was otherwise the same in composition and only slightly lower in pH. The fibers of the chromatid body tended to aggregate into thicker bundles, and laterally projecting fibers were more often pulled out straight. In both respects, the preparations resembled ones from cells

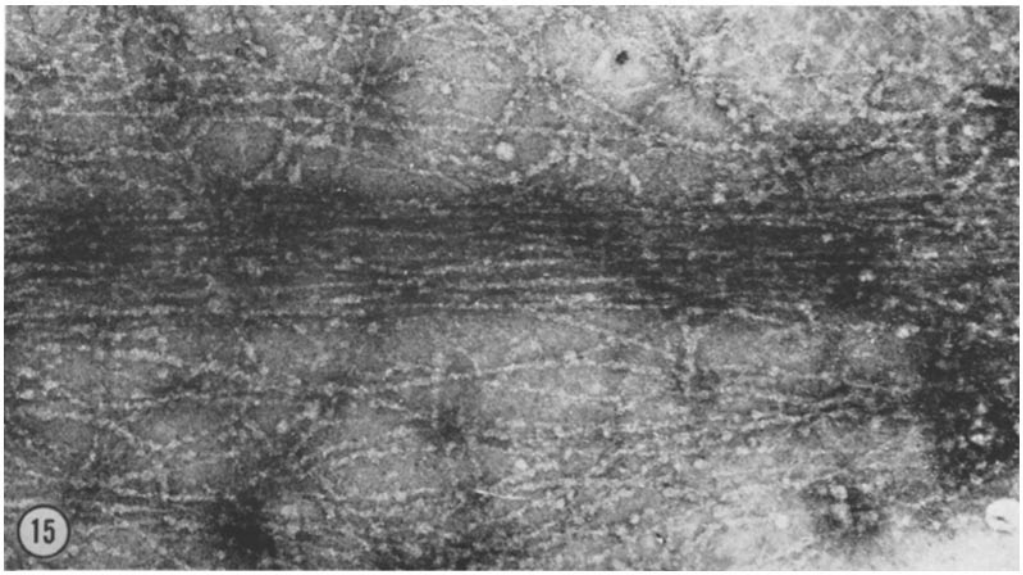


FIGURE 15 Cell burst on Callan medium B 1/20 + M/2500 $CaCl_2$; negatively stained with uranyl acetate. Portion of much stretched and spread chromatid. In the central region more closely packed parallel 150 A fibers are seen but these are more dissociated and form an interlacing network peripheral to this. × 50,000.

burst on M/500 calcium chloride (see below). Fig. 16 shows a dense tangle of fibers at the periphery of a stretched chromatid. It will be noted that the fibers vary in diameter from about 200 to 50 A and that the nodular structure mentioned above is much less pronounced. Bifurcations of fibers are to be seen in several places in this field.

The finer structure of loops was moderately well shown in some material prepared in 1/30 Callan medium containing M/4000 $CaCl_2$. A typical loop is seen in Fig. 18. The fiber is 200–300 A in diameter and is composed of a rather irregular linear aggregate of nodules. Fibers of similar dimensions and appearance but less tightly packed are shown in Figs. 19 and 20. In the latter, some fibers which appear to consist of similar material but are about 50 A in diameter are seen in the lower part of the field.

Further resolution of the structure of the chromosomal fibers was limited by the granularity of the background. The fibers appear to consist of a complex tangle of still finer elements about 20–30 A in diameter. Examples are shown in Figs. 23 and 24. The cell was burst on 1/30 Callan medium containing M/4000 $CaCl_2$ and briefly treated on the grid with M/250 EDTA Na 4 before staining. The appearance of the fibers is essentially the same as in preparations stained after bursting in M/500 $CaCl_2$ (see below) which are shown in Figs. 21 and 22. No definite evidence that the finer fibers (50 A) consist of two fibrillar subunits was found, though short regions occasionally suggest this. Ris (33) reported that citrate tended to dissociate chromosomal fibers in the 100–200 A range into finer elements. In the present experiments, replacement of the NaCl in the diluted Callan medium by sodium citrate yielded fibers with a diffuse appearance in which useful detail could not be seen.

2. *Cells Burst on M/500 Calcium Chloride Solution*

a. Chromosomes negatively stained with uranyl acetate after bursting cells on dilute calcium chloride showed certain characteristic differences from those prepared on dilute Callan medium containing potassium and sodium, but no calcium. It

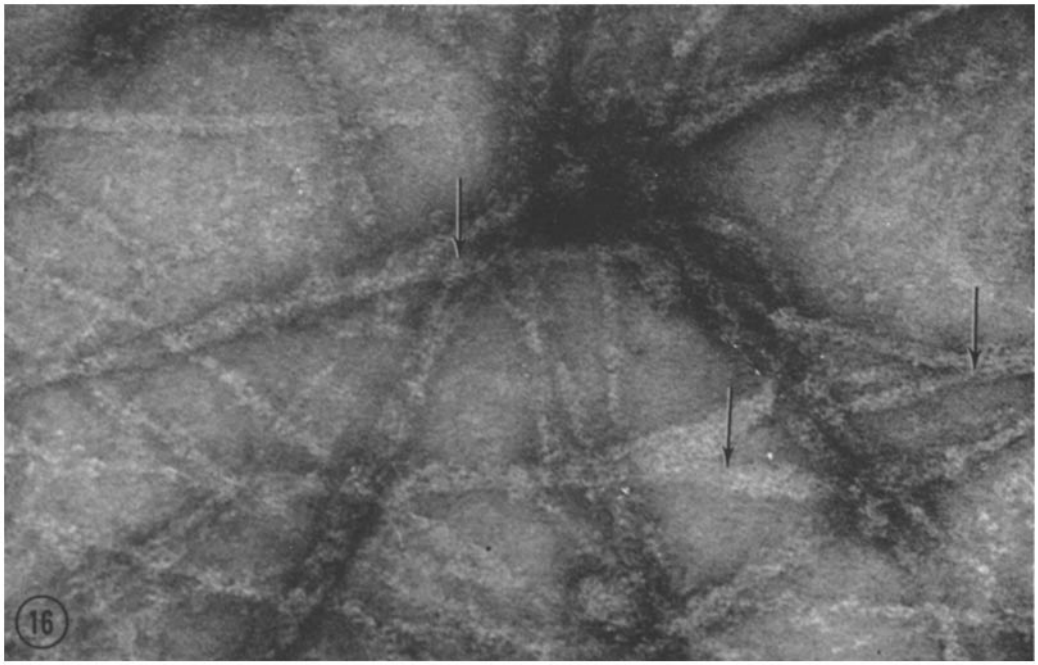


FIGURE 16 Cell burst on Callan medium B 1/20 + M/2500 $CaCl_2$; negatively stained with uranyl acetate. Tangle of straight fibers, 75–150 A in diameter, in the area adjacent to a chromatid. Note bifurcations of fibers (arrows). × 270,000.

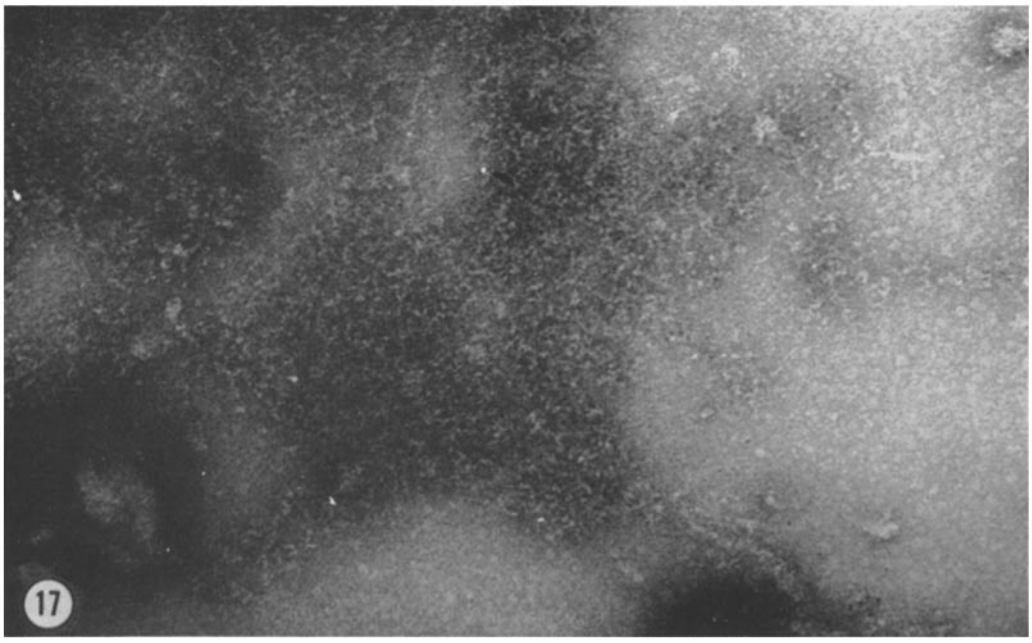


FIGURE 17 Cell burst on M/500 $CaCl_2$; negatively stained with ammonium molybdate. Material at peripheral region of a chromosome showing a disorganized conglomeration of fibers about 30 A in diameter. × 190,000.

should be noted, however, that the calcium solutions were unbuffered and the pH was about 5.2 whereas the Callan solutions were buffered and adjusted to pH 6.5–6.8. Stretching of the chromosomes was, in general, as great after bursting on calcium chloride (Fig. 2) as on Callan medium (Fig. 3), but after the former procedure the chromosomes showed a greater tendency to form complex networks in which it was often difficult to trace the limits of a single chromosome or to identify chromatid pairs with confidence. Adjacent chromosomes or chromatids were frequently interconnected by numerous fine, straight fibers (Fig. 4) and relaxed loops, as described above in material prepared on Callan medium, were not found. It seems probable that the loops were pulled out straight in the calcium preparations and, therefore, less easily recognisable as such.

The main body of a chromatid in the calcium material was often so dense that no fine structure could be seen. However, satisfactory negative staining of a chromatid body was found in some areas of a number of preparations, and, in these, it appeared either as a single strand 1000 A or more in diameter (Fig. 9) or as three or four roughly parallel strands (Fig. 10). Thinner fibers were often seen connecting two chromatids or branching off the main strands into the surrounding area. Under higher magnification, these thick fibers showed the same complex, tangled fine structure as the thinner strands described in the preceding section, but boundaries between constituent elements could not be detected.

Fibers of varying diameter from 200–300 down to 50 A were seen projecting from the chromosomes. The thicker ones were often seen to bifurcate where they joined the body of the chromatid. This appearance may be indicative of stretched loops, the two ends of which join the chromosomes at slightly different points, as already suggested in an earlier section, but it could also be due to adherence of independent strands. In some places, indeed, where the main body of the chromatid is is somewhat disrupted, fibers in the peripheral regions may undoubtedly be frayed out and project into the surrounding area, as is well shown in a specimen burst on Callan medium to which calcium was added (Fig. 11). Bifurcations of larger fibers to give rise to fibers 100 A or less in diameter were frequently to be seen in the complex network or strands adjacent to chromosomes and are probably artefacts of coagulation (Fig. 13). Where this

occurred, the strand from which the two branches arose showed no clear evidence of being double. Individual fibers in the range 100–300 A showed the same complex, irregular fine structure as those prepared in Callan medium with added calcium, as may be seen by comparing Figs. 21 and 22 with Figs. 23 and 24. Fibers as fine as 50 A in diameter (Fig. 21) could be found. Although the background was, in general, rather cleaner than in Callan medium material, there was still granularity which made it difficult to discern their structure clearly and it is uncertain whether they consist of single, slightly folded fibers or of two units intertwined.

The nodular character of the chromosomal fibers which was rather conspicuous in specimens prepared with Callan medium, especially in the absence of calcium, was less evident in this material though the fibers were certainly not uniform in diameter and showed thickenings at some points. Circular bodies 200–300 A in diameter, which closely resembled ribosomes, were often seen lying free among the chromosomal fibers (Fig. 13) and sometimes associated in groups of half a dozen or more. It was sometimes very difficult to be sure whether a nodule on a fine chromosomal fiber was really continuous with it, representing perhaps an uncoiled region, or whether it might not be a ribosome lying in close contact. Whether these ribosomes, if such they are, were intimately associated with the chromosomes prior to spreading or were fortuitously derived from the surrounding cytoplasm is uncertain. It may be noted in this connection that Cantor and Hearst (7) found that isolated chromosomes from mouse ascites tumor cells contained as much RNA as DNA, namely, 13.5%. However Chorazy, Bendich, Borenfreund and Hutchinson (8) found that in an acid medium which prevented chromosome disintegration, the chromosomes were liable to be contaminated by ribosomes and other cytoplasmic components.

b. In a few cases, the preparations were negatively stained with 1% ammonium molybdate at pH 6.5 or 5.2. The chromosomes at low magnification (Fig. 5) presented a striking contrast with those stained with uranyl acetate. The body of the chromosome was thick and homogeneous in appearance and lateral strands were rather infrequent, thick, and lacking in contrast. Examined at high magnifications, ammonium molybdate–stained material showed only a tangle of very fine fibers (Fig. 17) in which individual units about 30 A in diameter could be traced for short distances only, and formed a mass in which no regular arrangement could be discerned. The same result was obtained with the reagent at pH 5.2 as at pH 6.5.

c. Essentially similar results were obtained in material negatively stained with 1% sodium phosphotungstate (pH 6.7–7.0) or 1% phosphotungstic acid (pH 5.2), but, in some regions, the chromosomes were even more disrupted so that no intelligible structure remained. Although negative staining with uranyl acetate (pH 4.7) of material burst in M/500 $CaCl_2$ (pH 5.2) shows considerable coagulation of fibers, no trace of this remained when such preparations were stained with ammonium molybdate or with phosphotungstate either in acid or in nearly neutral solution.

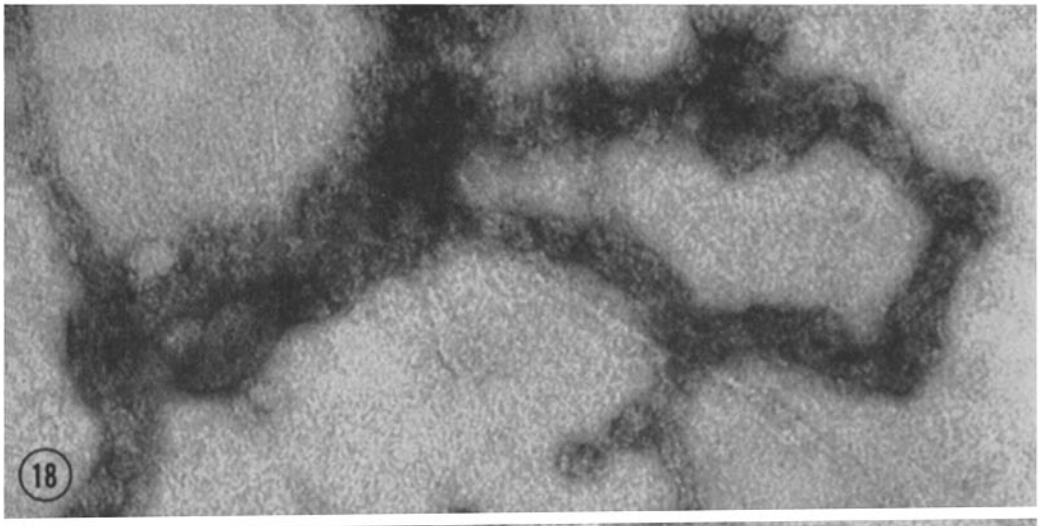
If the preparations were fixed in buffered glutaraldehyde or formalin prior to negative staining with either ammonium molybdate or phosphotungstate, the fibrous structure of the chromosomes was more like that seen in uranyl acetate preparations of unfixed material, but they were less satisfactory for high resolution work.

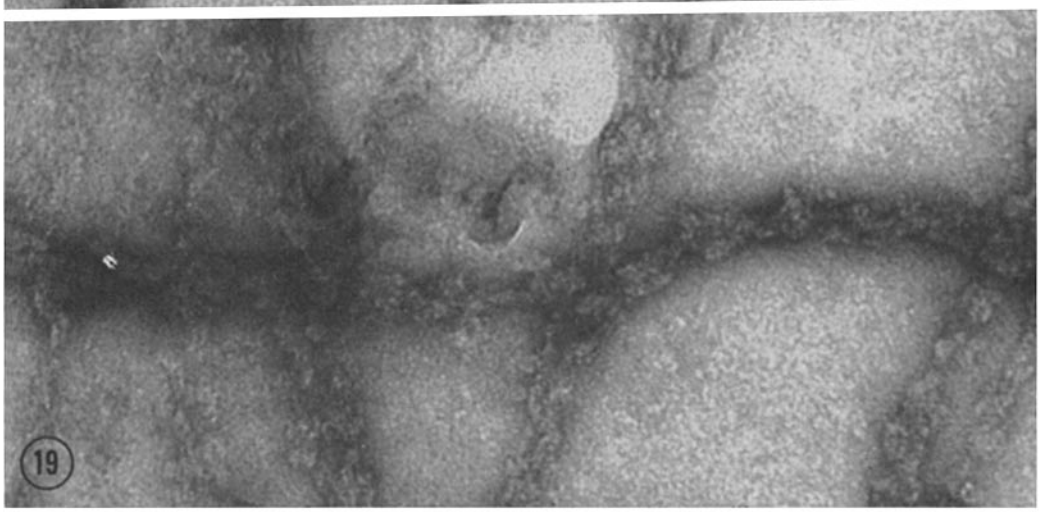
FIGURES 18–20 Cells burst on Callan medium B 1/30 + M/4000 $CaCl_2$; negatively stained with uranyl acetate. All the photographs are of chromosomal fibers adjacent to chromatids.

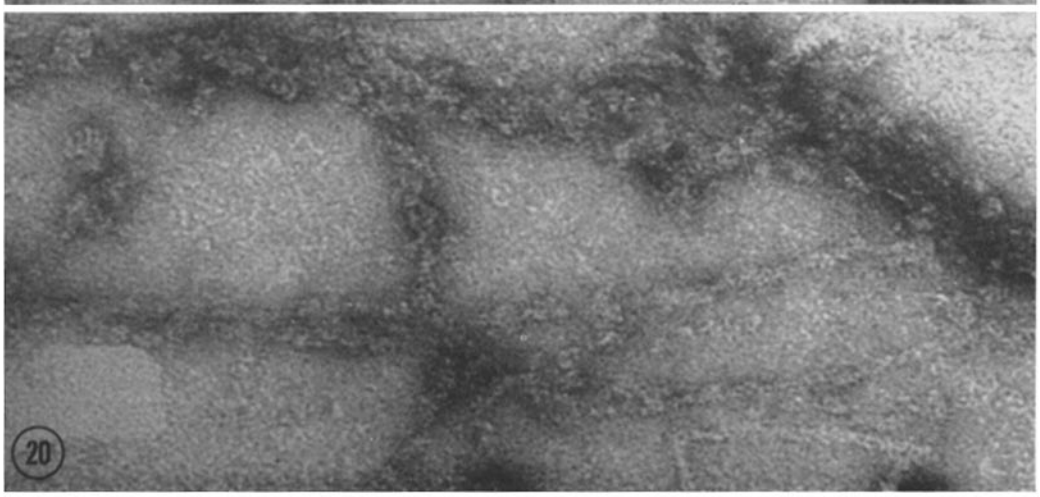
FIGURE 18 Part of a loop composed of a fiber about 200 A in diameter with a nodular structure. × 247,000.

FIGURE 19 Part of a loop running horizontally across field. Fibers which traverse the field vertically appear to consist of two or more associated nodular fibers. × 247,000.

FIGURE 20 Associations of nodular fibers. At the lower part of the field are seen two fibers about 50 A in diameter running horizontally (right). One of these at least becomes associated with another one (left) to form a thicker fiber. × 247,000.







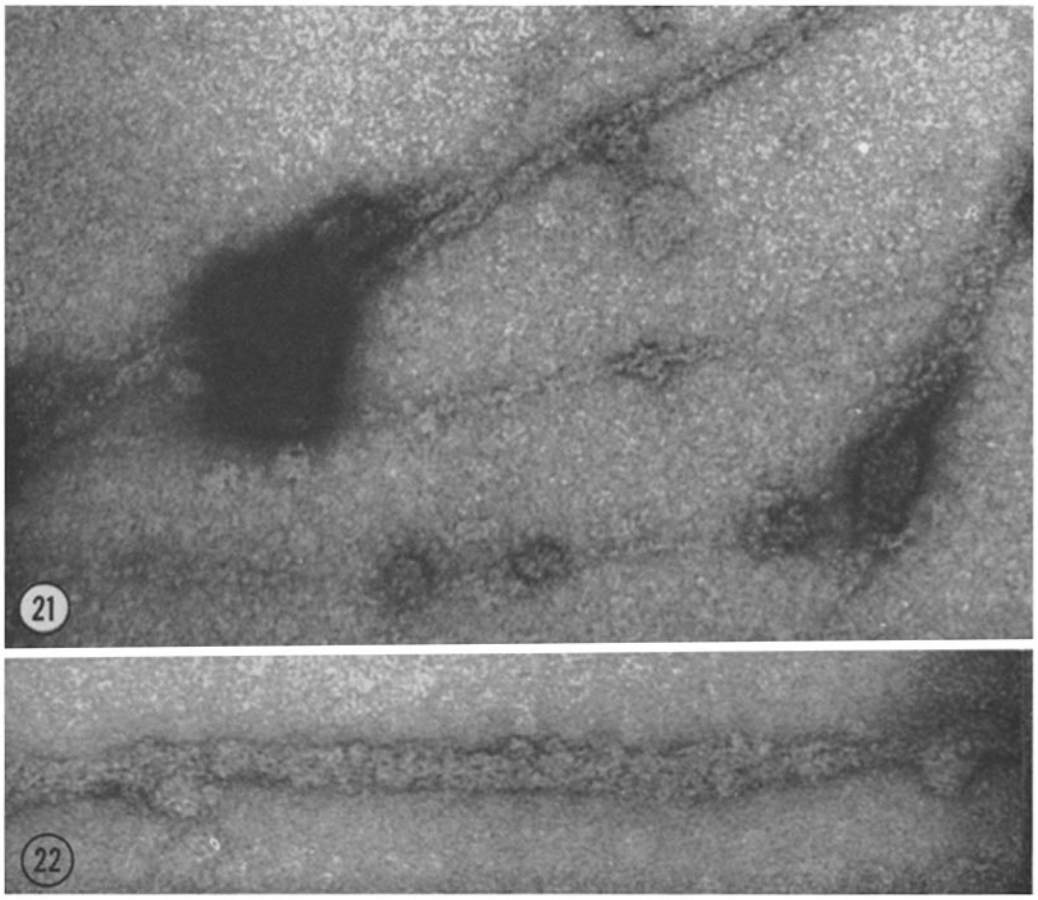


FIGURE 21 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. Chromosomal fibers ranging in diameter from 140 to 50 A. Note nodules on finer fibers. × 210,000.

FIGURE 22 Cell burst on M/500 $CaCl_2$; negatively stained with uranyl acetate. A chromosomal fiber about 200 A in diameter showing characteristic complex and irregular fine structure.

DISCUSSION

The appearance of spread newt chromosomes at low magnifications is, in some respects, similar to that of insect (Du Praw, 11) and mammalian ones (Wolfe, 41, 42; Du Praw, 12) treated in much the same way. Some of the chromosomes in the writer's preparations were, however, extended to greater lengths, amounting in some instances to 200 μ or more, and long fibers projecting at right angles to the chromatid axis were a striking feature. The lengths of the metaphase chromosomes in the intact cell range from about 10 to 20 μ, but the lampbrush chromosomes are between 200 and over 1000 μ long, depending on the particular chromosome of the set and on other factors (Callan and Lloyd, 6). Though the various members of the set cannot be identified in the spread material, it is doubtful whether any were extended as much as the lampbrush forms of the oocyte.

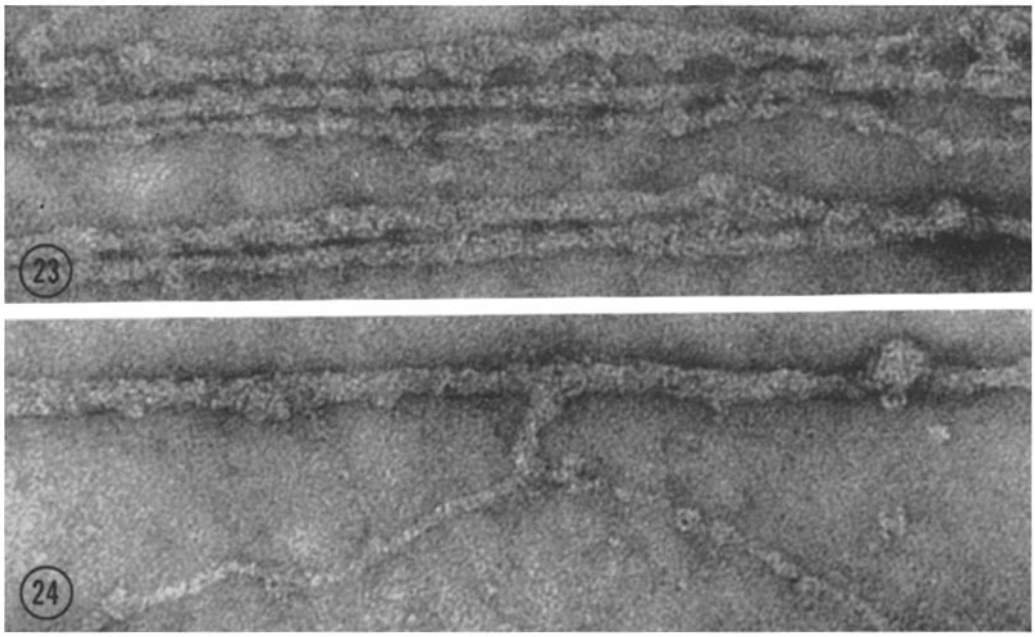
In some specimens, the surface layer, especially of relatively unstretched chromosomes, consisted of numerous loops, and similar loops are conspicuous in both Wolfe's (41, 42) and Du Praw's (12) photographs. They were also noted by Gall (16) in the spread contents of erythrocyte nuclei and are well shown in Ris's (33) pictures of this material. Solari (35) commented on the paucity of free fiber ends in the spread contents of sea urchin sperm heads and was able to trace continuous fibers for at least 50–90 μ. Using a radioautographic tech-

nique, Hubermann and Riggs (21) found continuous stretches of DNA up to 1.1 mm in length in hamster mitotic chromosomes liberated by lysis and treated with Pronase. They considered that such fibers might be single replication units. All these observations suggested that the fibrillar material of chromosomes consists of a few (and perhaps even only one) very long threads. The evidence on the structure of newt lampbrush chromosomes (Callan, 5; Gall, 17) also tends to the same conclusion. This view has been favored by geneticists (see Taylor, 37) because a mechanism for segregating replicates of DNA in a multistranded chromosome in a manner consistent with the radioautographic findings of Taylor, Woods, and Hughes (38) and others, is not easy to imagine and also because certain phenomena of mutation and crossing-over are more readily intelligible if each chromatid contains a single DNA double-helix.

On the other hand, light microscopists have long maintained (see Kaufmann, Gay, and McDonald, 27) that two or even four strands (chromonemata) are visible in anaphase chromosomes of some species, and earlier electron microscopic studies, mainly with whole mounts of selected material dried by the critical point method (see Ris, 32) have claimed to show from eight to 64 longitudinal strands in a chromatid.

In the present study, 20 to 30 parallel fibers about 150 A in diameter were clearly seen in stretched axial regions of chromatids spread on Callan medium. Very probably in the stretched regions which are most favourable for observation some fibers have been ruptured so that the number in unstretched regions may well be higher. These preparations certainly suggest that the chromatid is multistranded, but this appearance, as several workers have pointed out, is not incompatible with a structure involving a few strands (or even a single strand), since these might be folded back and forth as in a skein of wool or in some more complex pattern of this type (Du Praw (12). It should be noted that Mirsky and Ris (30) found that urodele nuclei had a relatively high DNA content and suggested that this might reflect a correspondingly high degree of polyteny.

The high incidence of loops at chromatid ends



FIGURES 23 and 24 Cell burst on Callan medium B 1/30 + M/4000 $CaCl_2$; treated for 1 min with M/250 EDTA Na; negatively stained with uranyl acetate. Fibers from adjacent areas of body of a stretched and spread chromatid. Fiber diameters range from about 160 down to 40 A. Some fibers show indications of a nodular structure. The fine structure of the fibers is similar to that shown in Fig. 21. Note in Fig. 24 that bifurcating fibers show no clear evidence of being double. × 247,000.

suggests that the latter are not simply bundles of free-ending fibers. If free ends are absent, this would provide a simple explanation of some properties of so called telomeres since the fibers would presumably have to be broken before joining to other free ends could occur. Fibers ranging in diameter from about 50–300 A were found in material spread on Callan medium without calcium. In much-stretched regions of chromatids they were about 150 A, but thicker in peripheral loops (200–300 A). Although these various fibers look similar in structure, it would be rash to assume on this basis alone that they are all made of the same material, in particular nucleohistone. Coleman and Moses (10) have emphasized the need to apply stringent tests in investigating fiber composition.

A further problem which has occupied other workers using different methods is whether the thicker fibers consist of single much-folded threads or whether they contain two or more filaments closely associated and perhaps intertwined. In the present work, no conclusive evidence was found that 50–100 A fibers contained fibrillar subunits, but appearances suggesting that this might be so in the case of thicker loops were not uncommon.

The fine structure of the fibers seemed to be an irregular tangle of fibrillar elements less than 50 A in diameter. Examination of fibers coagulated by calcium (see below) shows how difficult it may be to discern separate components where fibers of this kind lie in close proximity. Caution is certainly needed in interpreting the fine structure of the fibers, especially of the thinner ones, because of granularity of the background. This granularity may be due to dissolution of some of the fibers themselves as well as to contamination with cytoplasmic proteins. However, the fiber structure accords with that suggested by Wettstein and Sotelo (40) from very high resolution studies of sectioned chromosomes. They detected 20 A units within 100 A fibers, but here again it was unclear whether only one such unit was present. Rather similar fiber structure was reported by Huxley and Zubay (24) in sections of osmium tetroxide–fixed orientated nucleohistone stained with uranyl and lead. They described it as "usually in the form of a network or plaited appearance." These observations, gained by a quite different method, encourage the belief that the fine structure seen after negative staining with uranyl acetate may not be merely artefactual. If this is so and if the fine scale folding involves the DNA itself, it may be questioned whether the radius of curvature, which is less than 100 A in places, is compatible with the integrity of the double helix. Possibly the DNA runs a straighter course through a mesh of other material. If Cantor and Hearst's (7) analyses are a reliable guide, the relatively low DNA component (13%) might well be masked by protein (70%).

In material prepared on Callan medium without calcium, the fibers have a nodular structure and resemble those shown by Solari (35) in sperm-head chromatin negatively stained with uranyl acetate. The nodules appear to be regions of greater folding rather than adherent globular bodies. However, particles of similar size and structure occur in isolation or in small groups. Most of these are probably ribosomes, but some may be remnants of fibers in which dissolution of thin internodes has occurred. The fact that nodes are smaller and less numerous on the finer fibers (50 A) is consistent with the view that these represent a more extreme state of unfolding. If calcium-containing solutions are used in spreading, the nodular character of the fibers is less marked and this may be due to contraction closing up the thinner internodes.

It is tempting to equate the loops of spread newt mitotic chromosomes with those of the lampbrush form, especially since the latter withdraw their loops and come to look like mitotic chromosomes in the final stages of oocyte maturation. Evidence is presented in this paper that some of the loops arise by unraveling of the surface projections of metaphase chromosomes and that stretched loops may extend for several microns. Short loops are numerous at the periphery of chromosomes which are not greatly stretched, and it seems likely that stretching straightens some of these while some remain because the fibers are broken at some point. However, the relation of loops to axial fibers needs more clarification, and comparison with those of lampbrush forms should not be pressed until the latter have also been studied by spreading and negative staining.

A point which emerges clearly from the present work is the sensitivity of chromosome structure to certain technical conditions. The addition of calcium to the Callan solution, even if the pH is maintained substantially the same, leads to some fiber coagulation. This is not unexpected since Gall (15) has shown that calcium coagulates lampbrush chromosomes, and Zubay and Doty (43) found that divalent cations coagulated nucleohistone. Spreading on M/500 $CaCl_2$ at pH 5.2 leads to more

severe coagulation; the stretched chromatid body shows only a few thick fibers, and bifurcations, which are presumably due to adherence of fibers, are frequent in peripheral regions. Macgregor and Callan (29) reported that lampbrush chromosomes coagulated when the pH of their NaCl/KCl medium was 5.8 or less, and Barnicot and Huxley (2) found most marked evidence of coagulation when newt mitotic chromosomes were fixed in osmium tetroxide solutions which not only contained calcium but were buffered at low pH.

During preparation, the chromosomes were exposed to mechanical trauma, to the action of more or less unphysiological spreading solutions, and to drying in a negative-staining reagent. Callan medium has been found suitable for prolonged observation of lampbrush chromosomes, but this light microscope finding does not exclude more subtle changes of fine structure. Moreover, in order to burst cells, this medium had to be considerably diluted and the state of dispersion of nucleohistone is known to be sensitive to salt concentration (Zubay and Doty, 43).

The effect of the nature of the negative-staining reagent on chromosome structure is strikingly shown by comparing uranyl acetate preparations with those stained with phosphotungstate or ammonium molybdate. In the latter, only disordered fibers which approach nucleohistone in diameter (30 A; Zubay and Doty, 43) are seen, even when the pH of the reagent is near neutrality. Since uranyl acetate solutions cannot readily be prepared above pH 4.7–5.0, the possibility that this reagent may have induced some fiber coagulation must be admitted. In view of the high negative charge of phosphotungstate, it is not surprising that it should have a dispersive effect on chromosome fibers, but it is not clear why ammonium molybdate should do so. Fixation of the preparations in neutral glutaraldehyde or formalin prior to treatment with these reagents stabilizes the fibers to some extent so that the structure appears more like that in uranyl preparations.

Although there is a tradition of representing chromosomes as hierarchies of regular helices, simple helices have not been detected in the present material at any level of resolution, from Feulgen-stained light microscope preparations down to the finest fibers seen under the electron microscope. While it is true that helices might be pulled out and become difficult to detect in stretched regions, they were not seen in less stretched regions either. The bodies of relatively unstretched chromatids are usually too dense for effective electron microscopy, but a fair amount of detail can sometimes be seen; and if regular helices were a major feature of the folding pattern, they should have been detected. It does not follow that what is true of newt chromosomes is true of chromosomes in general, but it is noteworthy that Du Praw (12), studying both human and honeybee chromosomes spread on water, concluded that they were composed of irregularly folded fibers, only the whole chromatids being wound into the relatively large gyres which can be seen under the light microscope. Nothing observed in the present investigation gave any particular support for the ladder-like models of chromosome structure proposed by Freese (14) and Taylor (37).

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