Electron microscope observations on mitotic chromosomes

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With 17 plates

Summary

A method is described by which single mitotic cells growing in tissue culture can be selected under the light microscope and then sectioned for electron-microscopic study. The method has been applied to mitotic cells in newt heart cultures and to monolayer cultures of human fibroblasts. The structure of the chromosomes and of the mitotic apparatus at various phases of mitosis are described and discussed. The effects of pH and of divalent cations on the fixation of chromosomes by buffered osmic mixtures are also considered.

Introduction

Surprisingly few studies have been made of the characteristics of mitotic chromosomes as they appear in the electron microscope. In the normal course of events, cells undergoing mitosis are encountered only rather rarely in tissue sections, and when sectioned at random are often not cut in the optimal plane for study. Furthermore, the lack of readily characterized structure in interphase nuclei, as compared with that seen in other cell organelles, has tended to encourage the view that fixing and staining methods which were perfectly satisfactory for cytoplasmic structures might nevertheless fail to preserve or reveal structures containing large amounts of nucleic acids. However, the topic of chromosome structure is one of considerable interest, particularly in the light of recent developments in our knowledge of DNA replication. Moreover, the observation by Huxley and Zubay (1961) that oriented samples of purified nucleohistones could be fixed and stained perfectly satisfactorily for electron microscopy by appropriate choice of technique, and showed considerable organized fine structure when examined in thin sections, suggested that an attempt should be made to obtain satisfactory electron micrographs of the chromosomes in cells undergoing division. It was thought that such observations might be useful in providing a description of some aspects of the different stages of mitosis as they are observed in the electron microscope, as compared to the light microscope in which previous observations have usually been made; it was also thought worth while to find out whether or not any structural features exist within the chromosomes which might cast light on possible fine structure of the genetic material above the level of the molecular helix.

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In order to obtain sets of chromosomes at selected stages of mitosis, newt (Triturus triturus carnifex) tissue-culture cells growing on coverslips were employed as the principal experimental material. The chromosomes in these are readily seen in the phase-contrast microscope and the cells frequently show well-arranged mitotic figures. A few human fibroblasts growing in tissue culture were also examined, but the chromosomes seen during mitosis are too numerous and too short to be optimal material. Selected cells were appropriately fixed, and processed for electron microscopy in such a way that sections through the chosen cell could be obtained reliably and could be viewed without obstruction. These methods will be described below.

Methods

Preparation of carbon-coated coverslips

The cultures were grown on the surface of no. 1 coverslips (cut to 2 × 1 cm rectangles) which had previously been coated with a carbon film in a shadow-casting unit, so that the specimens could easily be detached after polymerization of the embedding medium. It is important to clean the coverslips thoroughly before coating, otherwise the carbon layer may become detached in the culture medium. This was done in the following way: the coverslips were boiled in Brylyanz detergent (G. Gurr) then left in 1% HCl for several hours, then washed in running tap-water followed by distilled water. They were finally dipped in absolute alcohol and dried with a clean cloth. Carbon was deposited in the shadow-caster at a distance of 12 cm from an arc struck between the pointed ends of two carbon rods. The rods were ground so as to have the form of a sharpened pencil, with a flat tip on one about 0.8 mm in diameter and on the other about 0.4 mm. A current of 20 to 40 amp passed between them when about 12 V a.c. was applied, and evaporation of carbon was allowed to take place for 25 sec.

Growth of newt tissue-culture cells

Various procedures for culturing amphibian tissues have been described, for example by Preston (1948-49) and Bloom and Leider (1962). Our own procedure was based on convenience in a laboratory devoted largely to culturing human cells.

The animal was killed with ether and the heart removed with due sterile precautions. The heart was freed as far as possible from blood and dissected in 0.6% NaCl. Pieces about 1.0 to 2.0 mm in diameter were cut off with sharp scissors. It was found that pieces of auricle or the roots of the great vessels gave more rapid growth than ventricle fragments. Each piece was placed in a drop of undiluted chicken plasma on a carbon-coated coverslip and 10-day chick embryo extract was mixed into the drop with a platinum loop which was also used to spread the drop. The drop was mixed by tilting the Petri dish containing the coverslip and when clotting occurred the coverslip was rapidly transferred to a tube containing the medium. These tubes were flattened on
one side at the closed end so as to provide a flat platform on which the cover-
slip rested with the explant side away from the wall of the tube. The tubes
were kept at room temperature at an angle of about 5° from the horizontal so
that 2.0 ml of medium spread as a shallow layer over the coverslip. The tubes
were closed with Esco silicone rubber bungs and a piece of sterilized alu-
minium foil was wrapped round the stoppered end to protect from dust. It
was found advantageous not to gas the tubes with a CO₂ in air mixture,
although the medium became rather alkaline. The medium was changed once
a week. Outgrowth of fibroblasts was generally moderate after one week,
and after two or three weeks large zones of outgrowth provided ample num-
bers of cells in various stages of mitosis. The cultures could be inspected
under a binocular at X 40 by rotating the tube so that the coverslip was left
adhering by capillarity to the flat surface.

The medium consisted of the mixture (A) employed for human cultures,
which was diluted by taking 2 parts of medium to 1 part of sterile distilled
water. The mixture A was 8 parts medium 199 (Glaxo), 1 part human serum,
1 part chick-embryo extract.

Observation, fixing, and marking of cells

When it was desired to fix cells for microscopy the coverslip was removed
and placed on a supporting chamber. These chambers were easily made by
cutting narrow slivers, the same length as the coverslips, from a microscope
slide and scaling them parallel to the edge of another slide so as to provide a
stepped support on which the coverslip could rest, supported on its two longer
sides. Medium was then run into the space between the coverslip and the
floor of the chamber. The upper surface of the coverslip was carefully cleaned
with tissue or filter paper. When a cell growing near the coverslip surface and
in the desired stage of mitosis had been found, a 0.1 mm ring was scribed
round it using a diamond-marker (Beck) which can be screwed into the
rotatable objective-mount of the microscope. The field was then photo-
graphed under low-power phase contrast so as to show the cell in relation to
the ring, and higher power photographs of the cell could also be taken at this
stage if required. For fixation, medium was drawn out by applying small
pieces of cleaning-tissue to one of the two open sides of the chamber and
fixative was simultaneously run in from the opposite side. The cell could be
photographed at intervals during fixation, which, in the case of osmic mix-
tures, was for 3 to 5 min. Other cells were fixed by placing the coverslip in
ice-cold fixative for the same period of time. The fixing fluid contained 1%
osmium tetroxide buffered with veronal acetate (Palade, 1952). A variety of
values for pH from pH 5.5 to pH 7.0, with and without added calcium or
magnesium ions (10⁻² and 10⁻³M CaCl₂, and 10⁻²M MgCl₂), were used in
various combinations; the most satisfactory was pH 6.0 or 6.5 with 10⁻²M
CaCl₂. Some cells were fixed in glutaraldehyde according to the method of
Sabatini, Bensch, and Barneett (1963), either with or without subsequent
osmium fixation. The coverslip was then transferred to 70% alcohol for at least one hour which gave adequate blackening of the tissue to distinguish the cells at later stages. At this stage the coverslip was remounted in the chamber and the surface away from the explant was carefully dried so that the scratched ring could be seen. A streak was then made over the ring with a black grease pencil so as to show its position at later stages when the coverslip was in Araldite.

**Embedding and locating the cell in the block**

The coverslip was passed through 85, 90, 95% alcohols and through three changes of absolute alcohol (about half an hour for each change). At this stage, some specimens were stained overnight in 1% phosphotungstic acid dissolved in absolute alcohol. After 1 h in a mixture of equal parts of Araldite plus hardener without accelerator, and absolute alcohol, the specimen was placed in Araldite plus hardener at 60° C for 1 h and then in a second change of the same mixture at 60° C overnight. It was then transferred to Araldite plus hardener plus accelerator at room temperature for a further period of 24 h. After this the coverslip was placed on a microscope slide with the explant surface upwards; the position of the marker ring was located under the low-power binocular and a gelatine capsule filled with the Araldite mixture was inverted over the area. The blocks were polymerized at 60° C, usually for about 3 days. Before removing the block from the microscope slide on which it had been polymerized the block face was inspected under the low-power binocular and when the marker ring was seen a sketch map was made of its position in relation to cells or tissue fragments in the vicinity. Photography could be used at this stage but was not in fact found necessary. The block was then removed from the slide by cutting away the surrounding excess of Araldite; during this process the block face usually separated from the coverslip carrying the carbon film and the culture with it. Inspection of the block face and reference to the sketch-map generally sufficed to locate the cell which had been selected and its position could then be marked on the block face by scratching a square around it with a fine needle.

After this stage the cylindrical Araldite block bearing the tissue was often cut across so as to yield a squat cylinder about 4 mm in height. This was still sufficiently long to grip in a standard microtome specimen-holder, and it was sufficiently short to view in phase contrast using a 20 X or even a 40 X objective. In this way, a good light-microscope image of the cell could be obtained, in which the chromosomes could be seen and compared with photographs taken at the fixing stage. Also, the depth of the cell below the surface could be measured, which facilitated subsequent sectioning. Additional mitoses could often be found in the same culture with a little searching and could be sectioned separately provided they were sufficiently far from the selected cell to allow the block to be cleaved with a razor between them. Since this work was completed, a rather similar procedure for selecting a mitotic cell for electron-microscope examination has been described by
Robbins and Gonatas (1964a), who have given an excellent description of mitosis in Hela cells (Robbins and Gonatas, 1964b).

Cutting and mounting of sections

The block was inserted in a Porter-Blum microtome and sectioned with a diamond knife. The use of a completely reliable cutting edge is essential as cells often lie very close to the surface of the block. The block face is set accurately parallel to the cutting edge, and parallel to the direction of movement of the specimen, before cutting is begun. This can be done with a little practice, using the reflection of the cutting edge in the block face, as seen through the viewing microscope, as a guide.

Sections were picked up on specially prepared Smethurst specimen grids in which a large central opening, 0.4 to 0.5 mm in diameter, had been punched using a sharpened hypodermic needle. The grids were filmed with collodion (initially deposited on a glass slide by the evaporation of a 0.33% solution in amyl acetate with the slide in a vertical position) and then coated with carbon in the usual way, but the collodion film was not then dissolved away. The resultant double film was then usually sufficiently strong to remain intact across the unusually large opening, and was not too thick to give satisfactory resolution. The sections were positioned over the open area, so that they could be viewed without obstruction, in the following way: they were first picked up using a tiny platinum loop about 1 mm in diameter so that they lay on the surface of a small drop of water in it. The loop was then held fixed with a dab of plasticine to some nearby object, examined by reflected light in the binocular viewer so that the section was readily seen, and a specimen grid applied to it from above so that the open area of the grid touched the section, which then adhered in the required position. Exploratory sections were examined immediately in the electron microscope, without staining; in these, the contrast was low, but could be improved by lowering the accelerating voltage from 80 kV to 40 kV. When the required cell was found, further sections were stained by immersing the grids bearing them in 2% unbuffered aqueous uranyl acetate for 4 to 8 hours and sometimes in lead hydroxide solutions as well (Watson, 1958 a, b; Huxley and Zubay, 1961). These were examined at 80 kV in a Siemens Elmiskop I using double condenser illumination and a 50 μ objective aperture.

Results

General observations on fixation and staining

In previous work on intact pieces of animal tissue, it had been found that the strongest staining of the embedded tissue with uranyl acetate was obtained when very short fixation times were employed (Huxley and Zubay, 1961). Accordingly, a fixation time of 3 min was tried out here and found to give perfectly satisfactory results, and it was confirmed that longer fixation (½ to 1 h) gave substantially weaker staining. In the previous work referred to it was also found that the presence of 10^{-2} M CaCl_2 in the fixing fluid, and a pH
around 6.0, was necessary for satisfactory results with extracted nucleohistone, and this was the medium we generally used here. Some cells were fixed at pH 7.0 in the absence of added calcium, and in these the chromosomes, as seen in the sectioned tissue, appeared to be perfectly well preserved, although they were stained somewhat less strongly by uranyl acetate. However, our attention was directed to changes in the appearance of the chromosomes during fixation itself by the observations of Davies and Spencer (1962) that chromosomes in a fluid culture of Hela cells became invisible in the phase-contrast microscope during fixation in osmium fixatives not containing added calcium, but reappeared again during subsequent dehydration. In the presence of $10^{-2}$ M CaCl$_2$, however, the chromosomes remained visible throughout. Somewhat similar results have also been reported by Robbins (1961).

We found that in our material too, the chromosomes became very faint or invisible during fixation in osmium tetroxide buffered at pH 7.0 without added calcium or magnesium, but reappeared apparently unchanged in form or in position during the subsequent dehydration (fig. 1 a, b, c). We found that at pH 6.0 in the presence of $10^{-2}$ M CaCl$_2$ the chromosomes remained visible throughout fixation, indeed becoming somewhat denser, and stayed visible and unchanged throughout the subsequent processing (fig. 2 a, b). The same was true, in the presence of $10^{-2}$ M CaCl$_2$, at pH 5.5 and 6.5, but if the fixation was carried out at pH 7.0, they again became faint even in the presence of these ions. In the presence of $10^{-3}$ M CaCl$_2$ at pH 6.0 the chromosomes became extremely faint during fixation. In the presence of $10^{-2}$ M MgCl$_2$ at pH 6.0 the chromosomes remained fully visible during fixation, and indeed underwent a substantial increase in density. At pH 6.5 with $10^{-2}$ M MgCl$_2$ the chromosomes remained more or less unchanged in appearance during fixation. However, we have not been able to relate this different behaviour during fixation to any striking changes in the appearance of the chromosomes as seen subsequently in the electron microscope. It seems likely that the changes are at least partly due to swelling and shrinkage of the

Figs. 1 and 2. Phase-contrast photographs of cultured newt cells to show effects of fixation on the appearance of the chromosomes.

Fig. 1. (a) a metaphase cell immediately prior to fixation. (b) same cell after 3 min in buffered 1% osmic pH 7.0 without added calcium or magnesium; note marked loss of contrast of chromosomes. (c) same cell after 30 min in 70% ethyl alcohol; contrast of chromosomes is partly restored ($\times$640).

Fig. 2. (a) prophase nucleus immediately before fixation. (b) same nucleus after 3 min fixation in buffered 1% osmic pH 6.5, $10^{-2}$ M CaCl$_2$; note that contrast of chromosomes is increased ($\times$1,350).

Fig. 3. Prophase nucleus of a cultured newt cell; fixed 1% buffered osmic pH 7.0 without added calcium or magnesium. Stained Feulgen; photographed without phase contrast. The chromosomes are strongly stained and can be seen to consist of two chromatids ($\times$1,120).

Fig. 4. (a) cultured newt cell before fixation. The cell appears to be in a transitional stage between prophase and metaphase but is unusual in that the chromosomes are arranged in two groups around two separate polar centriole centres. Phase contrast ($\times$680). (b) same cell after fixation in 6% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2. The contrast of chromosomes is slightly enhanced and the regions of the centrioles now appear as dense bodies. Phase contrast ($\times$680).
FIGS. 1 and 2

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FIGS. 3 and 4

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FIG. 5

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stage, but especially in Feulgen-stained preparations (fig. 3) their ends can
be seen to be flattened against the nuclear membrane (see also electron
micrograph, fig. 8) and the adjacent regions are sometimes thrown into two or
three gyres of a loose spiral. Centromere constrictions can also be seen in
Feulgen preparations and, in early prophase, paired chromatids are easily
detected especially when preparations are viewed without phase contrast
(fig. 3).

In sections stained with uranium for electron microscopy, especially if the
cell was fixed at acid pH in the presence of Ca++ or Mg++, the nuclear mem-
brane is clearly defined and the chromosomes are very dense structures which
stand out sharply from the surrounding nuclear material (fig. 5). In early
prophase their outline is irregular and their internal structure less uniform in
density than at later stages; in some places (e.g. figs. 9, 13) the chromosomes
appear to consist of two chromatids; denser regions, roughly 0.2 μ in diameter,
may perhaps be portions of convoluted threads which are themselves composed
of tangled finer filaments. Nucleoli are also seen at this stage as circular areas
of lower density than the chromosomes, and chromosomal material is often
closely attached to them. In some instances, as in interphase nuclei, some-
what denser material, which may be portions of a convoluted filament, may be
seen traversing their interior. The material between the prophase chromo-
somes consists of relatively dense particles, about 200 to 300 Å in diameter
associated with irregularly dispersed material of lower density; in more acid
fixatives containing Ca++ the latter material has a more fibrous character
(fig. 9). In sections close to the nuclear membrane the structure of the intra-
nuclear material is seen to differ from that of the adjoining cytoplasm which
contains mitochondria and also many small vacuoles often with associated
rows of dense particles which are smaller than those in the nucleus (fig. 12).
Stages in the disruption of the nuclear membrane can also be seen in prophase
nuclei (fig. 12); the inner and outer layers of the membrane become more
widely separated in places and local disruption into small vesicles finally
spreads throughout the structure. In one early prophase cell (fig. 10) sections
tangential to the surface of the nucleus revealed a centriole, composed of two

![Fig. 10. Newt cell; mid-prophase. Fixed buffered osmic pH 6.5, 10^{-6} M MgCl_2. Tangential
section showing centriole lying in cytoplasm adjacent to nuclear membrane (arrow) (x 4,650).
Inset. Higher-power view of centriole in this cell. Note faint fibrillar structures radiating
from region of centriole (x 30,000).](image1)

![Fig. 11. Cultured human fibroblast; fixed buffered osmic pH 7.0 without calcium or
magnesium. Stained uranyl acetate followed by lead hydroxide. Early metaphase. The
chromosomes are arranged around a relatively clear area in which a centriole is seen, cut
transversely (arrow) (x 9,300).](image2)

![Fig. 12. Newt cell; prophase. Fixed buffered osmic pH 7.0 without calcium or
magnesium. The nuclear membrane, which is beginning to break into vacuoles, is seen crossing the field
from top to bottom. On left cytoplasmic structures; fat globules, a mitochondrion, and a piece
of reticulum with adherent ribosomes. To right nuclear material; chromosome material and
also dense, relatively large interchromosomal particles (x 38,000).](image3)

![Fig. 13. Newt cell; late prophase; fixed buffered osmic pH 7.0 without calcium or
magnesium. The nuclear membrane has disappeared in some places but is still present in
others (top left). Chromosomes are thicker than in earlier prophase (x 4,650).](image4)
Fig. 10

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Figs. 11–13

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FIGS. 11–13

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Figs. 15 and 16

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dense tubular structures, lying in the adjacent cytoplasm. Even at this early stage, radially oriented vesicles and some fibres—presumably spindle fibres—can be seen. In glutaraldehyde-fixed material, fibres, having all the characteristics of the spindle fibres which we later find attached to the chromosomes, have been observed in considerable profusion in certain regions of the cytoplasm of cells in early prophase. No such fibres, however, are visible within the nucleus. Recently Behnke (1964) and Sandborn, Koen, McNabb, and Moore (1964) have reported the presence of 'microtubules' in the cytoplasm of various types of cell in interphase after glutaraldehyde fixation. These structures appear to be very similar to those which we have observed in prophase cells of the newt.

Transition to metaphase. In both human and newt fibroblasts growing on a glass surface, prophase is followed by a very characteristic stage in which the cell retains the extended form, and the chromosomes have a radial arrangement with their centromeres oriented towards a central area and their ends towards the periphery. This arrangement can sometimes be seen before breakdown of the nuclear membrane is complete. Sections of human (fig. 11) and newt cells at this stage show a centriole lying at the centre of the clear region, round which the chromosomes are disposed, but it is not yet clear whether two centrioles are in fact present at this time. If such a cell is kept under observation it is seen to round up, the cytoplasm being withdrawn from its processes, leaving the cell membrane in the form of fine anchoring filaments. As the cell becomes more spherical the plate of radially arranged chromosomes rotates so that its axis is now at right angles to the original direction. It appears that the spindle rapidly elongates at the same time to produce the characteristic metaphase form (figs. 14, 15). This sequence of events was described and filmed by Hughes (1948-9) in cultures of amphibian cells and our own observations fully confirm his. Apparently in cells which are flattened on a surface the metaphase arrangement of chromosomes precedes the final development of the spindle which occurs only when rounding of the cell provides room for its expansion.

**FIG. 14.** Cultured human fibroblast; metaphase; fixed buffered osmic pH 7.0 without calcium or magnesium. Stained uranyl acetate followed by lead hydroxide. The cell is cut through the longitudinal axis of the spindle and the section shows centrioles (indicated by arrows) at each end of the spindle. Some of the chromosomes, which are much contracted and irregular in shape, lie within the spindle. Note mitochondria and long strands of reticulum in surrounding cytoplasm (×9,300).

**FIG. 15.** Cultured newt cell; metaphase. Fixed in buffered osmic pH 7.0 without calcium or magnesium. Stained uranyl acetate. The spindle is seen near the centre of the field with its longitudinal axis lying transversely; centrioles are seen at the left-hand side. Strands of vacuole material, but not fibres, are seen within the spindle and there are a few mitochondria also. The attachments of six chromosomes to the spindle surface are seen. The chromosome is flattened against the spindle surface at its attachment and the two chromatids are slightly separated at this point. Note granular character of material surrounding the chromosomes in contrast to adjacent cytoplasm which contains mitochondria and strands of reticulum (×3,900).

**FIG. 16.** Adjacent section to fig. 15, showing separation of chromatids at point of junction with spindle (×3,900).
Metaphase. The chromosomes at this stage are more compact and uniform in structure than in earlier prophase. A characteristic feature is the presence of fine processes of chromosomal material which project at regular intervals from their surface (fig. 18). These projections are composed of a tangle of fibrils like the main body of the chromosome. They were not seen in sections of cultured human fibroblasts. As seen in sections parallel to the longitudinal axis of the spindle the centromere regions of the chromosomes are flattened against the periphery of the spindle (figs. 15, 16). In some sections chromosomes are seen to be attached to the spindle at various levels along its length (fig. 15) and not restricted to the equator; this arrangement and movements of the chromosomes bringing them to a more precise equatorial position are beautifully shown in the film by W. Bloom and R. E. Zirkle entitled 'Mitosis of newt cells in tissue culture'. The metaphase chromosomes are clearly composed of two chromatids (figs. 15, 16, 17) which are not twisted round each other but lie parallel with their adjacent surfaces slightly flattened. In some places the line of demarcation between the two members of a chromatid pair is impossible to detect but a neighbouring region may show a slight gap between them (fig. 18). The chromosomes are surrounded by granular material which is often rather sharply demarcated from the adjacent cytoplasm and is closely similar to the interstitial material of the prophase nucleus (e.g. figs. 15, 16).

The spindle. The main cytological constituents of the spindle are: (a) the spindle fibres, which are only seen clearly in cells fixed at pH 6·5, or less, in the presence of Ca++ or Mg++ (see figs. 19 to 23, 25, 26); (b) small vacuoles similar in size to those already mentioned as components of the cytoplasm in prophase; (c) small dense particles which are scattered in irregular groups and not noticeably associated with the vacuoles. The spindle fibres are about 150 Å in diameter and they have a denser surface layer giving them a tubular appearance (fig. 19 inset). Aggregation of particles on their surface increases the contrast in cells fixed at pH 6·0 or less in the presence of Ca++. The fibres are generally slightly wavy and show no evidence of any periodic structure. The chromosomes are connected to the centriolar regions by conspicuous bundles probably containing about 20 fibres which converge onto the centromeres (fig. 20). Other fibres, some of which are derived from the bundles as they approach the centromeres pass between the chromosomes.

Fig. 17. Newt cell; metaphase. Fixed buffered osmic pH 7·0 without calcium or magnesium. Stained uranyl acetate. Chromosome arms in transverse section showing chromatid pairs (×7,500).

Fig. 18. Newt cell; metaphase chromosome. Fixed buffered osmic pH 6·0 with 10^{-5}M CaCl_2. Stained uranyl acetate. The chromosomes are densely stained. Fine processes are seen to project from their surfaces. In some places the two chromatids of a chromosome area are slightly separated while in other places the line of contact between them is difficult to see (×8,000).

Fig. 19. Newt cell; metaphase. Fixed buffered osmic pH 5·5, 10^{-5}M CaCl_2 stained PTA before embedding. Spindle fibres and interstitial vacuoles (×38,600).

Inset. Spindle fibres at higher magnification showing relatively dense surface layer (×140,000).
FIGS. 17 and 18

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FIG. 19

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Figs. 20-23

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Figs. 24-26

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and probably traverse the spindle longitudinally from end to end. The fibres of the bundles interlace around the centrioles (figs. 25 and 26); although the diameter of the spindle fibres is similar to that of the fibrils which lie in the walls of the tubular centrioles, images suggesting any direct connexion between these structures are rare.

**The centromeres.** As noted above, each chromosome is flattened at its point of contact with the spindle and in this junctional region the two chromatids are clearly separated (fig. 16). In suitable sections bundles of spindle fibres are seen to converge to points on the polar aspect of each chromatid and where they meet it the fibres are surrounded by a distinct nodule of material which is less dense than the chromosome itself (figs. 21, 22). Most of the fibres seem to terminate at this point, though images suggesting some fibrous connexion between the centromeres of the two chromatids are occasionally seen. The structure of the fibre terminations at the centromere is difficult to interpret; the fibres seem to be slightly swollen at their ends and to be entangled in the dense fibrillar material of the chromosome.

**Anaphase.** In early and mid-anaphase the chromosomes still show the surface projections which occur in metaphase, but in later anaphase, as the whole chromosome becomes more compact, these are no longer seen; they appear to become fused together and incorporated into the chromosome (e.g. figs. 27, 28). Both from observation of living cells and from study of sections there seems little doubt that the fibre bundles connecting centromeres and centrioles must shorten during anaphase perhaps by as much as one-third or even half of their metaphase length, but there is no evidence that they increase in diameter. In late anaphase, when movement of the centromeres towards the centrioles has presumably ceased, chromosomal material extends a little way up the spindle-fibre bundle so that the centromere is sunk in a small depression in the chromosome (fig. 23).

The separation of the daughter sets of chromosomes is accompanied by elongation of the spindle in the intervening region but the fine structure

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**Fig. 20.** Newt cell. Fixed glutaraldehyde, post-fixed buffered osmic pH 7.0 without calcium or magnesium. Section stained with lead citrate. Spindle-fibre bundles converging to centromere (right, arrow) (×38,600).

**Fig. 21.** Newt cell. Fixed buffered osmic pH 5.5, 10-2M CaCl₂. Stained uranyl acetate. Centromere regions of a chromatid pair to which spindle-fibre bundles converge from above and below (×38,600).

**Fig. 22.** Newt cell. Fixed buffered osmic pH 6.0, 10-2M CaCl₂. Stained uranyl acetate followed by lead hydroxide. Spindle fibres converging to kinetochore (arrow) which is less densely stained than adjacent chromosomal material (×38,600).

**Fig. 23.** Newt cell. Fixed buffered osmic pH 6.0, 10-2M CaCl₂. Stained uranyl acetate. Late anaphase chromosome, showing spindle fibres attached in and around a concavity at centromere region (×38,600).

**Fig. 24.** Newt cell; early telophase. Fixed buffered osmic pH 7.0 without Ca²⁺ or Mg²⁺. Stained uranyl acetate and lead hydroxide. Surface of a chromosome with adherent vacuoles, an early stage in reconstitution of the nuclear membrane (×33,000).

**Fig. 25.** Newt cell; metaphase. Fixed buffered osmic pH 6.0, 10-2M CaCl₂. Section stained with PTA in absolute alcohol. Mesh of spindle fibres around centriole which has just been grazed by this section (×33,000).

**Fig. 26.** Centrioles and spindle fibres in another specimen (×33,000).
provides no obvious clue to the mechanism. In cells fixed at pH 7.0 to 7.3 without addition of Ca++ (figs. 27, 35) the chromosomes in the later part of anaphase show a denser axial core which is not apparent in material fixed at lower pH in the presence of this ion. It is possible that this appearance is an artefact due to swelling of the surface zone of the chromosome in the fixative. Although a duplex structure of anaphase chromosomes has been noted in various organisms by light microscopy and can sometimes be seen in newt cells stained in Feulgen or observed alive by phase contrast, electron-microscope sections show not a trace of bipartite structure.

**Telophase.** The contracted, dense chromosomes of late anaphase fuse at their points of contact to form an irregular ring of dense material surrounding the centriole (figs. 28, 31). At this stage aggregations of small vacuoles are seen to lie in contact with the surface of the chromosomal material (fig. 24) and the nuclear membrane is rapidly reconstituted by fusion of these vacuoles; the chromosomal material then begins to disperse and small aggregations, similar in structure to the nucleoli seen in prophase, are found in some of the remaining portions of undispersed chromosomal material (fig. 32).

In cultures of newt fibroblasts it is not infrequently found that the interphase nucleus is in the form of a ring surrounding a more or less central region of cytoplasm. It seems very likely that this region is the vestige of the central area containing the centriole around which the late anaphase chromosomes are aggregated; it may be conjectured that the nuclear membrane, which is re-formed in close association with the surface of the chromosome mass, sometimes fails to span this central area. It may be that the centriole remains in this region in the interphase cell and that at the onset of prophase the chromosomes, which are then elongated and tortuous, are in fact already arranged around it.

**Swelling in hypotonic KCl.** It was thought that swelling of mitotic cells in hypotonic solutions might lead to some extension of the chromosomes and loosening of their structure which would assist in interpretation of the normal condition. Metaphase cells were therefore irrigated with 0.27% KCl while under observation in phase contrast and this solution was left in contact with them for 20 min. The diameter of the cells increased by 50% or more and the image of the chromosomes became faint. Serial photographs show that the resting nuclei do not increase in diameter and may even shrink slightly;
Fig. 27

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Figs. 28-30

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FIGS. 31 and 32
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FIGS. 33 and 34

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aggregations of chromatin in their interior disappear so that they have a more homogeneous appearance and nucleoli become slightly smaller and increase in density. One swelled cell was fixed at pH 6.0 and another at pH 6.5 with addition of \(10^{-2}\)M MgCl\(_2\) to the fixative. Within a few minutes of fixation the elongated chromosomes became more clearly visible. In sections the two chromatids of each chromosome arm are clearly distinguishable (fig. 33). Each chromatid consists of a mass of fine, tangled threads which are more closely aggregated in the axial region than at the periphery (fig. 34). At the periphery of the chromatid the fibrils are more widely separated and often appear to be stretched in a direction at right angles to the long axis of the chromatid; this is more apparent after fixation at pH 6.0 and is probably due to slight shrinkage in the fixative. Under high power the stretched portions of these fibrils are not uniform in structure but show denser nodules at intervals (fig. 34). Spindle fibres of normal appearance are seen in the spindle area of swollen cells (fig. 33); the vacuoles in the spindle and cytoplasm are increased in diameter and are more widely separated from one another, but the mitochondria do not appear to be swollen.

The appearance of the chromosomal material in the electron microscope

Despite extensive examination of chromosomes of cells at all stages of mitosis, fixed by a variety of techniques, and stained by a number of different methods (see above), we have been unable to discern any organized fine structure within them whatsoever. In the sectioned material which we have studied (from newt and human cells) we can see virtually no evidence that the chromosomes have a helical internal structure, either near to or beyond the resolution of the light microscope, and there is no evidence for structural regularities at any level of organization visible in the electron microscope (i.e. down to 10 to 20 \(\AA\)). Moreover, the fine structure, such as it is (figs. 29, 30, 35, 36, 37), does not obviously correspond to the image one might anticipate if there were simply a single basic structural component—for instance, 30 \(\AA\) filaments, folded and tangled up into a compact but disorganized mass. Certainly filaments of this diameter, and of greater diameter, can be seen here and there, even in small groups sometimes, but a large part of the chromosomal

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**Fig. 31.** Newt cell. Telophase. Fixed buffered osmic pH 7.0 without Ca\(^{++}\) or Mg\(^{++}\). Stained uranyl acetate followed by lead hydroxide. Chromosomes forming a fused mass at each pole; equatorial cleavage furrow. Adjacent interphase nucleus (\(\times 2,080\)).

**Fig. 32.** Newt cells. Daughter cells at end of mitosis. Fixed buffered osmic pH 6.0, \(10^{-2}\)M CaCl\(_2\). Stained uranyl acetate. Nuclear membrane reconstituted; nucleoli and portions of undispersed chromosomes in nuclei. Note irregular shape of nuclei resembling that of chromosome mass of late telophase. Daughter cells remain connected by fine cytoplasmic strands (\(\times 2,350\)).

**Fig. 33.** Newt cell; metaphase. Swelled in hypotonic KCl. Fixed buffered osmic pH 6.5, \(10^{-2}\)M CaCl\(_2\). Stained uranyl acetate. Chromosome material partly dispersed; chromatid pair widely separated (left); swollen vacuoles; spindle-fibre bundles (right, arrow) (\(\times 10,000\)).

**Fig. 34.** Higher-power view of a portion of a chromatid pair in same cell as fig. 33. Stained uranyl acetate. Note chromatid on left shows axial patches of less dispersed material (\(\times 40,000\)).
material appears to be of a much more complex nature. This is illustrated in
the figures mentioned and as we are not able to put forward any positive
interpretation of this structure, there seems little point in describing its
appearance verbally in any further detail. The problem of the ultrastructure
of chromosomes therefore remains, in our opinion, unresolved.

Discussion

During the last five years electron-microscope studies on dividing animal
cells have done much to resolve certain doubtful points about the structure of
the mitotic apparatus. Thus, the centrioles are now known to be organized
bodies with a very distinctive fine structure, spindle fibres have been seen as
well-defined filaments of characteristic diameter and appearance, and material
which seems to correspond to the kinetochore has been observed at their
junction with the chromosomes (see Mazia, 1960; Harris and Mazia, 1962).

The electron microscope has also been used to examine chromosomes, inter-
phase nuclei, and extracted nucleohistone preparations. Various models of
chromosome structure have been proposed on genetical grounds (see Taylor,
1963) but so far none of them has received definite support from direct
observations on ultrastructure. It has been claimed by some electron micro-
scopists that the basic structural unit of chromosomes is a fibril of about 100 Å
diameter which is itself composed of two 40 Å fibrils (see Steffensen, 1959;
Ris, 1961). Davies and Spencer (1962), however, maintain that fibrils of this
size, as seen in sectioned nuclei of amphibian erythrocytes, are artefacts of fixa-
tion. The fibrils observed by Gall (1963) after floating intranuclear material
and chromosomes onto a water surface were 400 to 600 Å in diameter, but
had a denser core of about 150 Å diameter when stained in uranyl acetate.

The large cells of urodele amphibia with their long, easily visible chromo-
somes are classic material for the study of mitosis under the light microscope.
The advantages of this material have recently been exploited by Bloom and
Leider (1962) in their electron-microscope studies on the effect of highly
localized irradiation on the chromosomes of newt cells in culture. Studies on
the mitotic chromosomes of these animals have, perhaps, an added interest
in view of the remarkable extended lamppbrush structure of the pachytene
chromosomes of the oocyte which have been intensively investigated by
Callan and Lloyd (1960) and Gall (1954).

In this paper we have described a fairly simple method by which an indi-
vidual cell can be selected, its appearance during fixation and dehydration

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Fig. 35. Newt cell; late anaphase; chromosomes in transverse section. Fixed buffered
osmic pH 7.0 without Ca++ or Mg++. Stained uranyl acetate. Note slightly denser axial
core and absence of surface projections (X 44,000).

Fig. 36. Newt cell; metaphase; a chromatid in transverse section. Fixed buffered osmic
pH 6.0, 10-2M CaCl₂. Stained uranyl acetate (X 32,000).

Fig. 37. Newt cell; metaphase; chromosome in transverse section. Fixed buffered osmic
pH 6.0, 10-2M CaCl₂. Stained uranyl acetate. Line of contact between chromatids uncertain.
Note surface projections (X 32,000).
FIGS. 35-37

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observed, and its fine structure subsequently examined in thin sections under
the electron microscope.

It might perhaps be expected that the organization of the chromosomes
would be most easily seen in prophase when they are at their longest but this
did not prove to be the case in our material. In Feulgen preparations of
cultured newt cells the chromatids have a varicose appearance at this stage but
clear evidence of close spiralization was not obtained by light microscopy, and
electron microscopy of thin sections added no further information.

It appears that dissolution of the nuclear membrane at the end of prophase
occurs by disruption into small vesicles but in our material we did not see
evidence of stripping of the outer layer of the membrane as reported by
Barer, Joseph, and Meek (1960) in their work on locust spermatocytes; nor
did we find the close contacts between mitochondria and the nuclear membrane
or the formation of regular stacks of membrane material observed by these
workers. The inter-chromosomal material prior to breakdown of the nuclear
membrane appears granular after fixation at pH 7.2 but more fibrous if the
pH is 6.0 or lower. Ris (1962) believes that the dense particles of the 'nuclear
sap' may be coiled regions of fibres, since only fibrillar material was found in
tris extracts of isolated calf-thymus nuclei. We have drawn attention to the
fact that the dense intranuclear particles, if such they are, are noticeably
larger than the membrane-associated dense particles of the cytoplasm which
are presumably ribosomes.

At metaphase each chromosome is clearly seen in sections and in light-
microscope preparations to consist of two chromatids lying parallel in close
contact and not twisted around one another. The centromere regions at this
stage, as mentioned above, are flattened against the surface of the spindle,
and the two chromatid elements are slightly separated at this point. It is
interesting to note that Carlson (1953) in his microdissection studies on grass-
hopper neuroblasts found that the metaphase chromosomes were firmly
attached to the spindle. In sections which pass through the attachment-point
of a chromosome in a suitable plane it can be seen that a spindle-fibre bundle
converges from each pole to a knob-like structure on the polar face of each
chromatid. As far as we were able to tell there are few if any fibres passing
between these structures, which may be identified as kinetochores. The
centromere regions, it seems, do not become attached to a continuous spindle-
fibre bundle passing from pole to pole but rather each chromatid establishes
connexion by means of its kinetochore with the appropriate pole.

Nebel (1959) and Nebel and Coulon (1962) described kinetochore structures
in sections of dividing mouse cells and of pigeon spermatocytes, and Harris
(1962) observed them in sections of developing sea-urchin eggs. In the latter
material they took the form of a dense plate transverse to the direction of the
spindle fibres and their appearance was the same after various fixation
procedures. We have occasionally seen a somewhat denser line near the termina-
tion of the spindle fibres but more often the kinetochores were merely homo-
geneous bodies of lower density than the adjacent uranium or uranium-lead
stained chromosomes. Our best preparations of these structures were from glutaraldehyde-fixed cells. Schrader (1953), in reviewing the light-microscope evidence, noted that it was a matter of dispute whether the kinetochores are Feulgen-positive, but that they were coloured by mitochondrial stains. Their appearance in stained thin sections under the electron microscope is certainly consistent with their composition being different from that of the chromosomes.

The fact that spindle fibres converge sharply to the kinetochores, whereas, in our material at least, they are more widely separated and irregularly intermeshed around the centrioles, raises certain problems about their mode of formation. Evidence for the fibre-organizing functions of centromeres is reviewed by Schrader (1953) and Mazia (1960). However, the presence of spindle fibres around the centrioles prior to the breakdown of nuclear membrane has been described by Bernhard and de Harven (1960), and Harris and Mazia (1962) and we have made a similar observation in early prophase cells.

In cells growing on a flat surface in culture, as we have noted, there is a stage at which the chromosomes are arranged in a circle around and in the same plane as the centrioles and the distance between centrioles and centromeres is less than in the mature spindle. The formation of the mature spindle apparently happens rapidly after the breakdown of the nuclear membrane and it is not easy to catch informative intermediate stages. The possibility that some form of connexion between centromeres and centrioles is maintained even in the interphase nucleus is perhaps worth considering, though no evidence of such a connexion has been seen.

Spindle fibres have been observed by a number of electron microscopists (see Harris, 1962) working on a variety of materials and their findings have been remarkably consistent. Our own observations support the view that the distance between centrioles and centromeres is decreased in anaphase, but it remains problematical how this is brought about. Since, in thin sections, individual spindle-fibres can seldom if ever be followed throughout their full length the possibility that there may be inter-digitated fibres and that shortening occurs by a sliding-filament mechanism is perhaps worthy of consideration. Our findings with regard to the structural elements in the spindle are remarkably similar to those of Harris (1962) working on quite different material.

We have mentioned above that there is no evidence in thin sections that the chromosomes in anaphase are composed of two chromatids though appearances suggestive of this can sometimes be seen in Feulgen preparations or in the living cell under phase contrast. It is true that sectioned chromosomes as seen by electron microscopy consist of a tangle of fine fibrils and that where chromatids or even different chromosomes are in close contact it may sometimes be difficult or impossible to detect any line of division between them. This, indeed, may be one reason why it is so hard to discern any clear arrangement of the material within the confines of a single chromatid. Given that the finest visible fibrillar elements are not highly aligned, then any organization of the material on a larger scale may become very difficult to make out if
packing is close. Nevertheless, a bipartite structure consisting of two parallel and approximately straight chromatids, which could be recognized as such in the light microscope, should also be recognizable in some planes of sectioning in the electron microscope.

The reconstruction of the nuclear membrane in our material by fusion of vesicles which accumulate on the surface of the condensed mass of late anaphase chromosomes is essentially similar to the process described by Barer et al. (1960).

The coagulating effect of divalent cations on cytoplasmic material (see Chambers and Chambers, 1961) and also on chromosomes has long been known. Duryee (1937) pointed out that urodele lampbrush chromosomes are rendered inelastic by solutions of calcium salts, and Gall (1954) noted that calcium in excess of $10^{-3}$ M contracted them to a jumbled mass. Zubay and Doty (1959) remarked that solutions of purified nucleohistone were very sensitive to aggregation by divalent cations. The reduction in contrast of nuclear components produced by fixation in osmic solutions in the absence of divalent cations was described by Davies and Spencer (1962) working mainly on intact or isolated nuclei of amphibian red cells. In our material the chromosomes as observed in phase contrast virtually disappeared after treatment for a few minutes with such a fixative. It seems that in Davies and Spencer’s experiments the fixation times were longer and the loss of contrast of nuclear components occurred more slowly, especially in the case of intact cells, than in our material. Furthermore, we did not find that Ca++ or Mg++ prevented this change at neutral pH. There seems little doubt that in our material osmic fixation at pH 6·0 or lower in the presence of Ca++ or Mg++ causes shrinkage of the chromosomes so that their fine structure after this fixing procedure may not necessarily be better preserved than it is at pH 7·0 to 7·5 in the absence of these ions.

The absence of any interpretable fine structure in our electron micrographs of chromosomes is both disappointing and puzzling. It may, of course, be that structural regularities at various levels do exist in the chromosomes before fixation, but are lost during the preparative procedures. If this is the case, then such a loss of structure must occur without any significant change in the appearance of the mitotic figures as seen in the phase-contrast microscope. Moreover, the structure must be altered in the same way by a number of different fixation procedures. Finally, we must note that exactly similar preparative procedures were able to preserve a great deal of oriented fine structure in artificial fibres of nucleohistone (Huxley and Zubay, 1961). It may be that a three-dimensional reconstruction of the chromosomes would reveal characteristic structures which are not apparent in the thin sections one is obliged to used to obtain acceptable resolution, but the absence of an easily recognizable basic unit of structure suggests that the task of extracting useful information from micrographs of the present kind may not be a very rewarding one.
References


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References


