OPTICAL AND ELECTRON MICROSCOPIC CHANGES IN ULTRAVIOLET-IRRADIATED CHROMOSOME SEGMENTS

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ABSTRACT

Chromosome segments of urodele cells lose some substance after irradiation with about 10^{-1} ergs/μ^2 of heterochromatic ultraviolet light. These segments stain faintly or negatively with the Feulgen and pyronine- methyl-green methods and weakly with the Alfert-Geschwind stain for basic protein. In the living cells, Perry found in these chromosome segments a decrease of 50 to 60 per cent in absorption at 2400, 2600, and 2800 A, *i.e.*, in the region of intense chromosomal absorption that is maximal at 2600 A. Apparently the material lost contains DNA (?DNP) and we call the process *DNA-steresis*. In such cells, fixed in neutral formalin in Tyrode's solution and stained with phosphotungstic acid, electron microscopy shows that the unirradiated parts of the chromosomes consist of (*a*) a homogeneous or finely fibrillar material (component-A) filling the meshes of (*b*) an irregular network with bars 40 to 300 A in diameter, some of which continue into a similar interchromosomal network. DNA-steretic portions of the chromosomes consist mainly of this network and only small amounts of component-A, which presumably contains the DNA. We have not been able to demonstrate DNA-steresis with the electron microscope after primary fixation with OsO_4 or $KMnO_4$. Structural changes due to DNA-steresis are compared with certain nuclear changes in the mitotic cycle.

A small portion of a chromosome or of an interkinetic nucleus of a salamander cell in culture, after exposure to ultraviolet light in the Uretz microbeam (Uretz, Bloom, and Zirkle, 1, Uretz and Perry, 2), undergoes a prompt change in refractive index at the irradiated site and appears as a pale area in the living cell by dark phase-contrast microscopy (Bloom, Zirkle, and Uretz, 3). This happens with a flux of ultraviolet within certain limits and with certain lengths of exposure. The affected area appears optically empty in contrast to the sharp black and gray details of the adjacent chromosomal or other nuclear structures (Fig. 1). This change extends gradually beyond the original site of bombardment. The larger the volume of chromosomes irradiated, the less intense is the local reaction; none is visible after ultraviolet irradiation of all of them.

When phase contrast is replaced by ordinary transillumination there is little or no indication of a difference and the continuity of the chromosome through the irradiated area appears uninterrupted. With such optics, the effect of irradiation may be indicated by a change in brilliance in the irradiated area. Izutsu (4), using an ultraviolet microbeam of the Uretz type, found that the irradiated portions of chromosomes of grasshopper spermatocytes "became pale with or without phase-contrast microscopy immediately after exposure of fifteen seconds or more." He did not report what the UV flux was.

The change in refractive index suggests a

profound upset in structure and a probable loss of substance. One of our first tests of the nature of this localized alteration in the chromosomes was the application of a variety of the fixing and staining methods of light microscopy. Some of our early results were reported briefly by Bloom and Leider (5) who found that the irradiated areas stained only faintly with the Feulgen method or Delafield's hematoxylin and did not stain blue as chromatin does with Mallory's phosphotungstic acid hematoxylin, or green with the pyronine-methyl-green stain. The weak or negative reactions with the Feulgen and pyronine-methyl-green stains suggest that DNA may have been greatly changed or lost at the site of irradiation. This conclusion is bolstered by Perry's observation (6) of a decrease of about 50 per cent in absorption at 2400, 2600, and 2800 A in the irradiated portions of chromosomes. In some preliminary measurements by interference microscopy, Haynes and Stodolsky (7) have observed a decrease in optical path length ($\sim$50 mμ) between the unirradiated and irradiated segments that would suggest a substantial loss of dry mass from the latter.

The chromosomes in the living cells in our cultures are not birefringent and we have been unable to discern any type of birefringence in the irradiated parts of the chromosomes. Some of the changes found with the electron microscope in the irradiated parts of the chromosomes have been reported briefly by Bloom (8, 9).

In several previous communications we have spoken of the visible effect of local ultraviolet irradiation as "paling," for with dark phase-contrast microscopy the irradiated area appeared as a pale segment in an otherwise dark chromosome. Had we used bright phase-contrast microscopy we probably would have called the change in these areas "darkening." Although we are reluctant to introduce another term, "steresis," indicating loss of a specific part, expresses our meaning more accurately. Since the accumulating evidence suggests strongly that DNA is part of the substance lost, the process might be called "DNA-steresis" and we will use this term for brevity.

During our study of partial cell irradiation, we found that exposure of parts of chromosomes to very large amounts of ultraviolet light inhibited completion of DNA-steresis and made the irradiated parts very dark, instead of light, in the dark phase-contrast microscope. We also found with the electron microscope that such parts did not show all the changes in structure characteristic of DNA-steresis. A low power micrograph of such overirradiated chromosomes has been published by Bloom (10). Out of this finding grew our experiments using ultraviolet irradiation of large parts of cells as a method of fixation. In the present paper we will include some mention of its usefulness as a primary fixative either alone or followed by formalin or OsO_4.

For the purposes of this report, the term "chromosome" covers not only the stages in development of typical metaphase chromosomes, but also the less characteristic structures forming the fused chromosome masses of telephase and their changes in early reconstruction. We are unable to visualize and define "chromosomes" in late reconstruction, interkinesis, and exceedingly early prophase.

In the following pages we present illustrations and details of our findings on the changes in the locally irradiated parts of chromosomes, including those seen with the electron microscope. It will also be shown how the changes we found in the irradiated parts have offered some clues helpful in interpreting chromosome structure.

MATERIAL AND METHODS

Practically all of the cells used in this work were in the epithelial sheet growing out of the cardiac mesothelium of *Triturus viridescens* and *Amblystoma tigrinum* in tissue culture. This tissue was chosen for several reasons: (*a*) this epithelium forms an optically favorable material, since the cells usually remain in contact with their neighbors throughout the mitotic cycle and do not round up enough in metaphase to produce the disturbing halos seen with phase contrast in isolated cells in culture; (*b*) the relatively large cells have 22 long, conspicuous chromosomes; (*c*) the mesothelium, like other epithelial tissues in culture, liquefies the fibrin of the medium, with the result that the cells become closely attached to the coverslip as the sheet grows out from the explant; and (*d*) a room maintained at 70 to 72°F (a favorable temperature for the urodele cells) obviates the need for incubators around the observing and microbeam microscopes.

The simple culture medium consists of 1 part of heparinized or reconstituted lyophilized chicken plasma, 6 parts of Tyrode's solution and 3 parts of distilled water (to approximate the lower tonicity of urodele plasma).

In experiments with ultraviolet light the cultures must be made on quartz coverslips which are then mounted on hollow chambers. These are made by boring a ½-inch hole in a 2 by 3 inch slide 1 mm thick; a glass coverslip is then placed under the hole

and sealed to the slide with an acetone solution of polyvinyl acetate (Gelva); the acetone is allowed to evaporate before sterilization of the slide by dry heat. For studies of ultraviolet absorption, quartz coverslips must be used instead of glass to cover the base of the hole. Several drops of the dilute plasma are placed in the well and most of it aspirated before the coverslip with culture is applied. The residual thin layer of plasma in the well aids in maintaining the tonicity of the culture and in preventing the deposition of small droplets of condensed water vapor which would disturb the path of light for microscopic study.

The peak of mitoses in the cultures is usually reached between the 5th and 7th days after explantation. The advancing mesothelial sheet of cells liquefies the fibrin, leaving the cells closely applied to the coverslip. Mitoses frequently occur in waves, often in a particular part of a culture at a given time, although scattered dividing cells are found at almost any time of the day or night. Four to 8 hours usually elapse from the earliest indication of the onset of mitosis until reconstruction is well advanced (at 72°F).

With the Uretz ultraviolet microbeam apparatus one aims at the desired spot (demarcated by crosshairs in the ocular) and the morphological changes which result can be observed directly. This flexible instrument permits irradiation of targets with circular spots as small as 1 μ, or with long narrow beams, or squares, triangles, or any desired shape. In the observations reported here most of the irradiations lasted 5 to 10 seconds; in some instances, especially those involving late metaphase chromosomes, the irradiations were from 12 to 15 seconds. They were made with an 8 μ spot or with a long band about 3 μ wide; the energy flux was about 10^{-2} ergs/μ^2/second with heterochromatic ultraviolet from an AH6 mercury arc lamp. The energy delivered was about the same as in the earlier experiments in which the irradiations were made with heterochromatic ultraviolet light from a Hanovia germicidal lamp for exposures of 3 to 7 minutes. Although the spectra from these two sources are not identical, in the present studies there were only minor differences in their effects, and these were apparently due to the much longer exposure required with the weaker Hanovia source.

The relative effectiveness of the various wave lengths of ultraviolet light in producing the localized change in the chromosome has been studied by Zirkle and Uretz, whose findings are being published separately. Contrary to expectations they found that 2600 A was not the most effective wave length.

If the irradiation is carried out in metaphase and one chromosome lies above another, the upper one can be made DNA-steretic without much visible change in the lower one. This result is in part due to the precise focal plane of the Uretz microbeam and, probably to a lesser extent, to absorption by the upper chromosome. This makes it difficult to determine, in the living cell, how extensive the change in the chromosome is. By varying the focus during irradiation so as to include portions of both the upper and lower chromosomes, both will be DNA-steretic. Owing to the hollow conical shape of the ultraviolet beam above the focal point, one result of varying the position of the focal point is to broaden the lightly irradiated, narrow zone of chromosome adjacent to the main irradiated portion of the upper chromosome. After we found that unavoidable irradiation of the "marginal zone" was of great help in analyzing the changes seen with the electron microscope, we often broadened this zone intentionally by focussing up and down during irradiation of the target.

Histological Technics

After a cell is selected for study its location is marked by drawing a ring about 250 μ in diameter around it on the coverslip with a diamond marker. This serves to identify the cell as it passes through the various experimental and histological procedures.

Most of the cells for cytological study were fixed in Zenker-formol (9 parts of Zenker stock solution and 1 part full strength formalin neutralized with an excess of $MgCO_3$) for 12 to 15 minutes, washed in several changes of water until the yellow stain with bichromate was removed, passed through 50 per cent alcohol with iodine, then into 75 per cent alcohol, after which they were stained in various ways. Among the many stains applied after this fixation, the most useful were found to be dilute Delafield's hematoxylin, Mallory's phosphotungstic acid hematoxylin, Heidenhain's iron-hematoxylin, azure II-eosin, azan, the Feulgen method. The cultures for the Alfert-Geschwind method for basic proteins were fixed in aqueous 10 per cent neutral formalin; those to be stained with the pyronine-methyl-green method were fixed in Carnoy's mixture. In addition, many preparations for the Feulgen and other stains were made after fixation in 1 per cent OsO_4 or after 10 per cent neutral formalin. In all cases where the Feulgen stain was used, the period of hydrolysis in HCl was carefully limited to 12 minutes at 60°C.

Technics for Electron Microscopy

The method of identifying, fixing, embedding, and mounting the experimental cell for electron microtomy has been described by Bloom (10). Here it is necessary to repeat only briefly the principle of the special technic developed for the study of a particular cell in a culture or smear. As mentioned above, the position of the selected cell is marked by drawing a small ring around it on the coverslip with a diamond

marker. This helps to locate the cell at any stage of subsequent operations such as the partial cell irradiation, fixation, dehydration, embedding in the plastics, and above all in mounting it for microtomy. Careful diagrams and, during the last 2 years, Polaroid Land photomicrographs were made of the experimental cell and its neighbors. These are indispensable aids in the identification of the cell and the portion of it which was irradiated.

Fixation by Liquids

The problem of fixation of cultures is different in some aspects from that of a small block of tissue containing perhaps 10,000 to 20,000 cells or even more. For study of the living cell, we selected the flattened epithelium (mesothelium) adhering to the coverslip in the migration zone of the cultures. The periphery of these cells is perhaps 1 or 2 μ thick and the greatest thickness of the nuclear portion, even at the height of metaphase, is probably not more than 7 or 8 μ, usually less than 6 μ. It is obvious that fixing, dehydrating, and embedding agents come into immediate contact with the cell without being diluted with materials extracted from intervening cells, as occurs in the penetration of a fixative through a block. In addition to 1 per cent OsO_4 in distilled water or in Veronal buffer at pH 7.4 or pH 7.8 (Palade) a number of other fixatives were used. For the study of nuclear changes in the mitotic cycle and the effects of irradiating segments of urodele chromosomes we found the following fixing solution to give the most informative preparations: 1 volume of 40 per cent formaldehyde saturated with $MgCO_3$, 6 volumes of Tyrode's solution and 3 of distilled H_2O. We shall refer to this fixative as "neutral formalin–Tyrode."

Zenker-formol-osmic, Zenker stock with or without formalin, Low's solution, Dalton's solution, $KMnO_4$ as advised by Luft, Carnoy's, and many other fixatives were tried and found to be of little value for our purposes because of vacuolization, or extreme shrinkage, or failure to demonstrate the irradiated area.

For fixation in osmium tetroxide the coverslips, with culture side down, were floated on 1 per cent solutions of this reagent for 10 to 20 minutes and after a rapid rinse in distilled water were passed (culture side up) through a succession of alcohols (5 minutes each in 25, 50, 75, 95, and 100 per cent). Those fixed in neutral formalin–Tyrode were fixed for 30 minutes, rinsed in water, and then transferred to 25 per cent alcohol. Early in this work we passed such cultures rapidly through the alcohols as indicated for the OsO_4-fixed cultures. However, for reasons given in the third paragraph of the Discussion it is better to leave them in 75 per cent alcohol for several hours. For convenience we have standardized on 5 minutes each in 25 per cent and 50 per cent alcohol, overnight in 75 per cent alcohol, 5 minutes each in 95 per cent and absolute alcohol, and then through the embedding procedures.

Fixation by Ultraviolet Light

The largest area we can irradiate in the microscopic field with our present apparatus, using a × 50 objective with the intense AH6 high pressure mercury arc, is a circle 80 μ in diameter. This is large enough to irradiate much more than the nuclear area but not the entire cytoplasm of the greatly extended urodele mesothelial cells.

With a flux of heterochromatic ultraviolet light of about 10^{-2} ergs/μ^2/second we have irradiated the whole nuclear zone of cells for periods of 10 to 70 seconds. No visible changes occur in the first 10 to 20 seconds, but after 30 to 40 seconds small blebs sometimes appear at the edge of the irradiated area. After the irradiation we exposed the cells to the usual liquid fixatives or directly to dilute alcohols and have obtained results which convince us that the irradiation acts as a good fixing agent for some structures.

Staining

The most informative stains have been 0.5 per cent phosphotungstic acid (PTA) or uranyl acetate in the absolute alcohol used for dehydrating the cultures. The cultures stayed in the stain for 5 minutes. In a few instances they were left in for much longer times, but the resulting sections were too dense to show much structure in the electron beam. We found that this procedure gave us more regular results than staining the sectioned cells on the grids.

Embedding

Practically all of our work was done by dissolving polymerized mixtures of varying percentages of butyl and methyl methacrylates in ethylene dichloride. Benzol could also be used as the solvent except for osmium-fixed material. The procedure is simple: the coverslip with the culture passes from absolute alcohol into a mixture of equal parts of absolute alcohol and ethylene dichloride, then into two changes of ethylene dichloride. The excess is removed by touching the edge of the coverslip with a bit of filter paper (care being taken to prevent any drying of the cells) and then a drop of a viscous solution of the polymers in ethylene dichloride is placed on the culture. After perhaps ½ hour it will have lost much of its solvent and another drop is added. We usually allow this to dry overnight although this is not necessary. The cell under observation is identified by the diagrams and Polaroid Land photographs made at the time of fixation. The surrounding unwanted parts of the culture are then cut away with a clean

razor blade. The embedded culture has now to be mounted onto the tip of a Lucite rod which fits the chuck of the microtome. This is accomplished by covering the tip of the rod with a small drop of the viscous solution of polymerized methacrylates in ethylene dichloride and placing it in position over the selected cell. This had previously been centered by placing the circle circumscribing it on the coverslip over a cross inscribed in the glass (or quartz) plate forming the base of the centering ring and holder devised for this purpose (10).

After a couple of days, or longer if desired, the Lucite rod, now firmly attached to the culture and with its long axis perpendicular to the plane of the coverslip, is removed from the positioning device. Before removing the coverslip it is again necessary to compare the topography of the cell or cells in the inscribed circle with the diagrams and Polaroid Land photographs previously made so as to be certain of identifying the selected cell under study. If the quartz coverslip was carbon coated before the culture was made, it is easily removed by cooling with solid CO_2. If it has not been carbon coated the cells often stick to the quartz. We have had no difficulty removing the cells from uncoated glass coverslips.

The culture then is trimmed for cutting. If the plastic is a bit tacky, the free surface is exposed in a closed Stender dish overnight to let the last traces of the ethylene dichloride evaporate. The trimming can be done free hand, with the help of a dissecting microscope, using clean razor blades. For the past year we have been using a device with which it is possible to control the descent of the razor blade in a guillotine-like holder and to rotate the block at successive 90° turns. This permits rapid and more accurate trimming of the block to include only the selected cell or cells (and incidentally removes some of the tension on the person trimming the block), but is dispensable.

The universal joint on the chuck in the Porter-Blum microtome was replaced by a rigid chuck which holds the Lucite rod with the embedded cell on its tip. This ensures sectioning the cell in the plane of the original surface of contact of the cell with the coverslip. Most of the cells studied were in gray or silver gray sections. Slightly thicker sections did not show much structure in the formalin-fixed, phosphotungstic acid–stained cells. The sections, usually mounted on carbon-coated Formvar films on the grids, were studied with an EMU-3C (RCA) microscope.

We have obtained a few good preparations after embedding in Vestopal. We had little success in embedding with heat polymerization of mixtures of butyl and methyl methacrylate or with the water-soluble epoxy resin (11) which Dr. W. Stäubli sent us. The greatly increased viscosity of the latter in the higher concentrations caused difficulty in infiltrating the cultures thoroughly; in addition, the tough polymer of the embedded cultures adhered to the quartz coverslips. A few cultures were embedded by using the methacrylate monomer mixture instead of ethylene dichloride as solvent for the polymerized methacrylates. We found no advantage in using the monomers.

OBSERVATIONS

Living Cells

Unless otherwise noted, all of these observations were made with medium dark phase-contrast microscopy; after an appropriate dose of ultraviolet light the change in refractive index of the irradiated chromosome segment starts promptly and reaches a maximum intensity within 5 minutes, although longer periods are occasionally required (Fig. 1). With time the steretic change may extend for some microns beyond the irradiated zone. Indeed, we have seen it extend from an original 8 μ segment to over 20 μ in the course of a couple of hours.

The clearest observations are those made when the chromosomes are in a single layer, as in the mesothelial cells in late prophase or in prometaphase. In such cells, a lightening of the dark gray of the chromosomes appears a minute or less after irradiation with an AH6 source for 6 to 10 seconds, (or 1 or 2 minutes later during the course of a 5 minute irradiation with a Hanovia lamp). Within the next few minutes this change progresses so that the outlines of the irradiated portions of chromosomes disappear and this area becomes an optically empty zone demarcated by adjacent unirradiated structures. A micron or so of the chromosome adjoining the irradiated area may appear darker than the rest of the chromosome, particularly if the irradiation is carried out in prophase.

If too little ultraviolet is used, the effects may not be appreciable or they may be incomplete in which case the irradiated portion appears light gray in contrast to the dark gray of the unirradiated portions.

With too much ultraviolet, brightening may start but will not progress and the center of such an overirradiated area may become very dark or show a slight brownish tinge in the dark phase-contrast microscope. Depending upon the amount of ultraviolet used there may or may not be a narrow, bright zone at the periphery of the overirradiated area.

There are other factors which modify the onset

of the change as seen in the living cells. It appears more rapidly and after less irradiation, the earlier the stage in the mitotic cycle. In late anaphase it is elicited only by large doses and in telophase it may be impossible to produce it without causing other severe and obscuring effects of the irradiation. Indeed, amounts of energy insufficient to produce visible change in these stages may retard reconstruction of an irradiated daughter nucleus as compared with its unirradiated mate which serves as an excellent control. The changed chromosome may be incorporated within the daughter cell, especially if the kinetochore region has not been irradiated. Such areas may be observed for days in the daughter cells; they do not resume the appearance of normal nuclear structure.

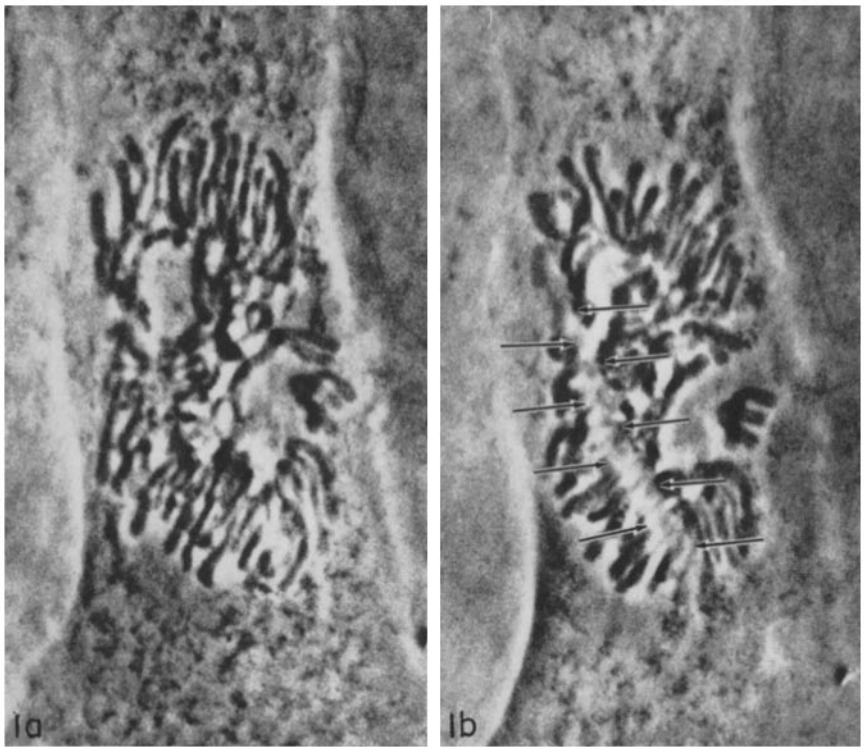


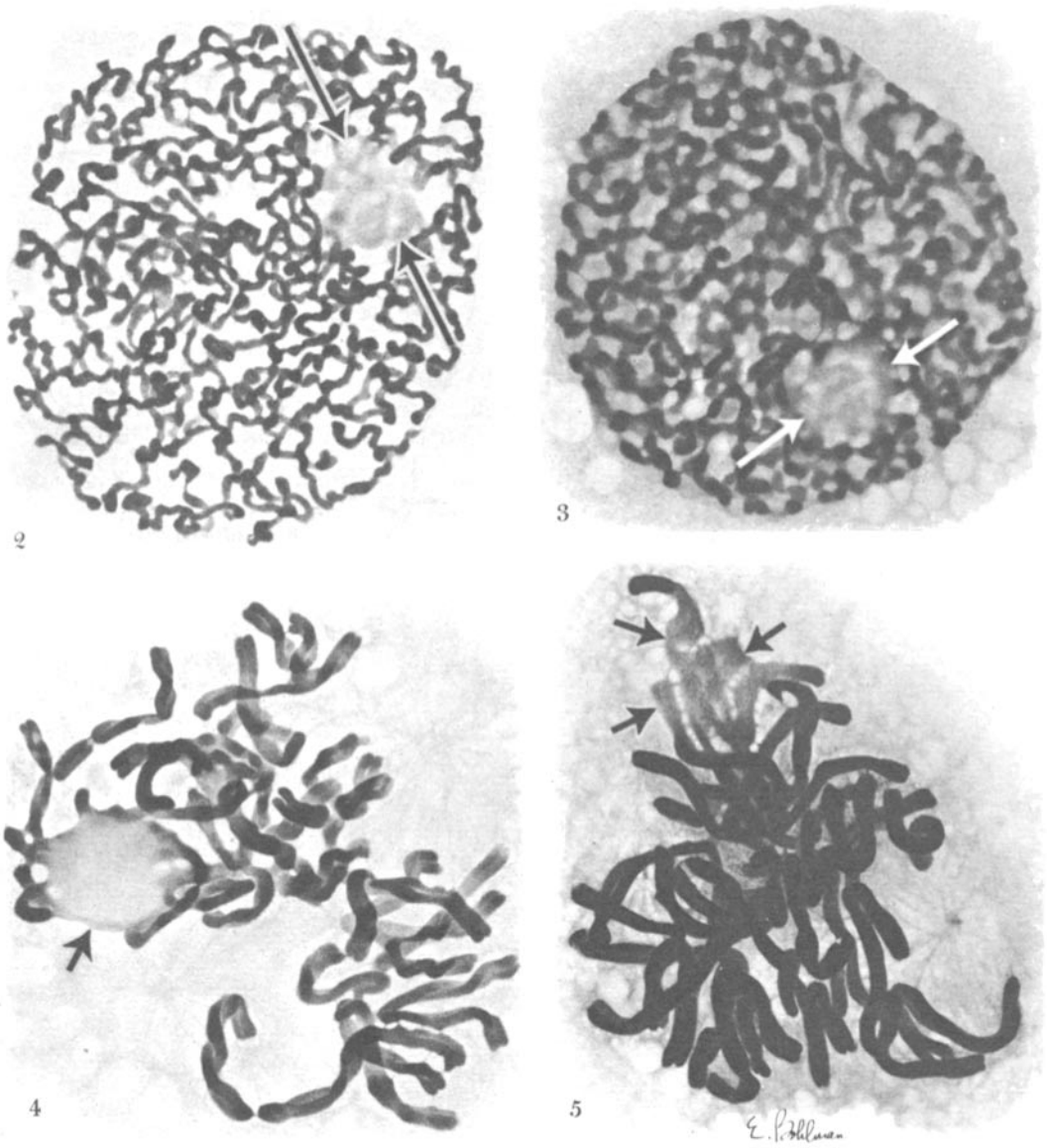
FIGURE 1

Metaphase of living mesothelial cell before (*1 a*) and after (*1 b*) irradiation with a narrow stripe of ultraviolet light. In the almost complete DNA-steretic area (arrows) pale gray traces of chromosomes can still be seen. Medium dark phase contrast, × 1100.

Fixed and Stained Preparations

Living cells which showed the typical DNA-steretic change of the irradiated portions of chromosomes with the phase microscope were fixed and stained in a variety of ways. Some of the technics did not demonstrate these areas as, for example, those for phosphatase tried by Amenta (12). However, a number of the routine histological methods showed the steretic places nicely, as did several of the more specific methods for chromosomes. For brevity's sake, the following description is limited to the results we obtained with the more informative of these procedures applied to cells irradiated with heterochromatic ultraviolet light over a circle 8 μ in diameter or a long narrow stripe.

In the whole mount preparations, the DNA-steretic areas are clearly demonstrated after fixation with Zenker-formol and staining with routine hematoxylin methods. The irradiated cells pictured in Figs. 2 to 6 and 8 were fixed with Zenker-formol. After staining with hematoxylin and eosin (Fig. 2), the portions of chromosomes in the DNA-steretic area have lost their



Each of the cells in Figs. 2 and 3, in prophase, and Figs. 4 and 5, in metaphase, was irradiated for 5 minutes with 8 μ spots of heterochromatic ultraviolet light from a Hanovia lamp to produce DNA-steresis. These areas are indicated by arrows. Fixation in Zenker-formol. × 1640.

FIGURE 2

The steretic chromosome segments have lost their basophilia and appear somewhat swollen or fused. Hematoxylin-eosin stain.

FIGURE 3

The steretic portions do not stain so deeply with Heidenhain's iron-hematoxylin as do the unirradiated.

FIGURE 4

Chromosomes are not seen in the DNA-steretic area which stains a diffuse pale blue with dilute Delafield's hematoxylin.

FIGURE 5

Steretic chromosome segments stain paler than the unirradiated parts. Heidenhain's iron-hematoxylin stain.

DNA steresis, indicated by arrows, was produced in the cells of Figs. 6 to 8, and 10 to 12 by irradiation with 8 μ spots, and in cells of Figs. 9 and 13 with narrow stripes of ultraviolet light. Cells of Figs. 6 to 8 were irradiated for 5 minutes with the Hanovia lamp; cells of Figs. 9 to 13 were irradiated for 10, 15, or 20 seconds with the AH6 arc. The cells were fixed and stained in several ways. × 1640.

FIGURE 6

Prophase. Fixed in Zenker-formol and stained with the Feulgen method for DNA. There is a faint diffuse staining in the steretic area.

FIGURE 7

Metaphase. The DNA steretic chromosome segments do not stain with the methyl-green of the pyronine-methyl-green mixture. Carnoy's fixation.

FIGURE 8

Prophase. The continuity of chromosomes in the DNA-steretic area is obvious but the characteristic staining for chromatin is negative. Zenker-formol fixation; stained with Mallory's phosphotungstic acid hematoxylin.

FIGURE 9

The steretic area stains faintly with the Alfert-Geschwind method for basic proteins. Formalin fixation.

FIGURE 10

Prophase. The Feulgen stain is negative in the DNA-steretic area. Fixed 24 minutes after irradiation in 1 per cent osmium tetroxide.

FIGURE 11

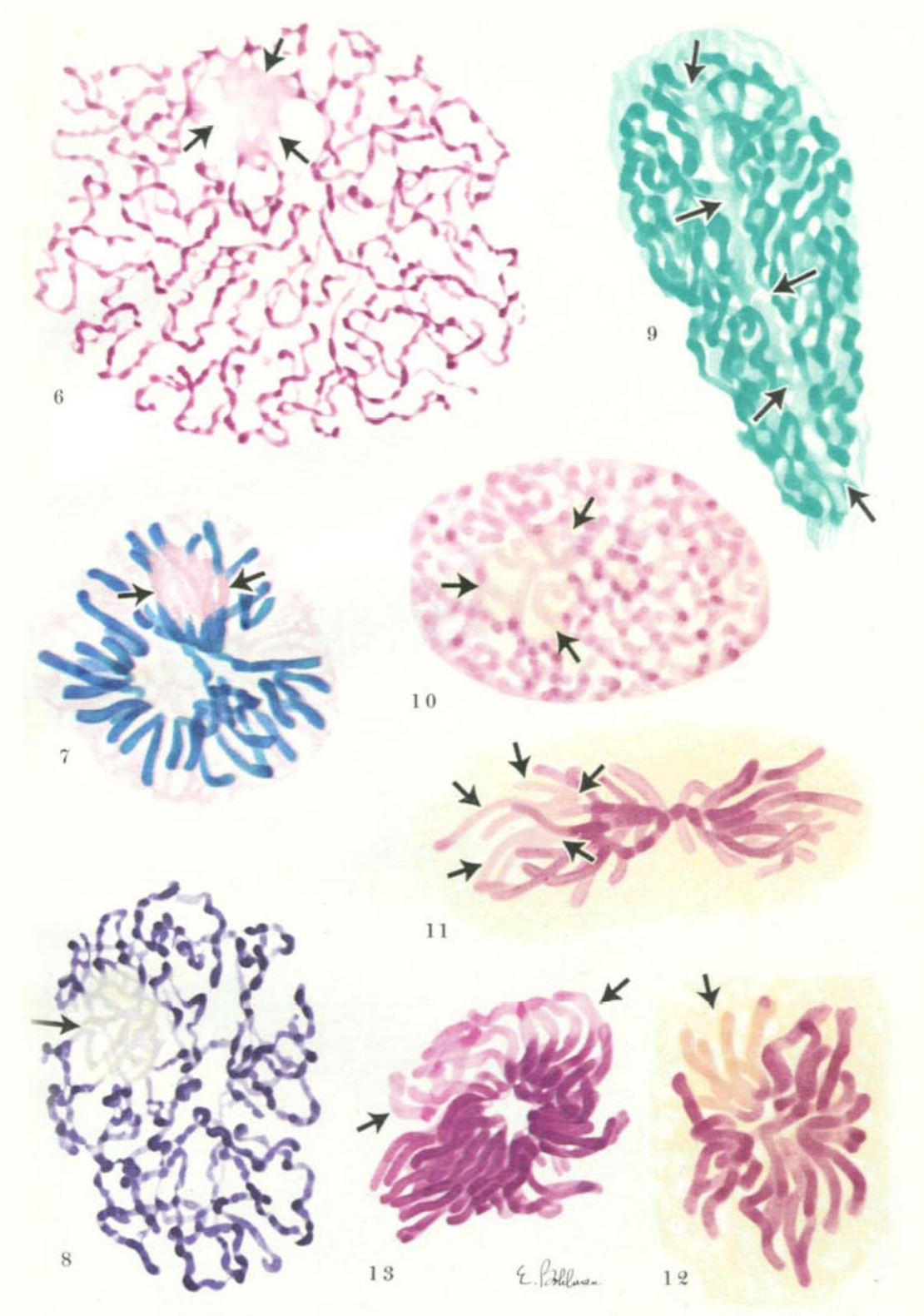
Metaphase. Irradiated in prometaphase with 8 μ spot for 10 seconds. The Feulgen reaction is almost negative in the DNA-steretic area. During the 53 minutes between irradiation and fixation, portions of two chromosomes moved over part of the irradiated area. 1 per cent osmium tetroxide fixation.

FIGURE 12

Metaphase. Irradiated in prometaphase with 8 μ spot for 15 seconds. The Feulgen reaction is negative in the DNA-steretic area. Fixed in 1 per cent osmium tetroxide, 54 minutes after irradiation.

FIGURE 13

Metaphase. Partial cell ultraviolet irradiation of chromosome segments for 20 seconds produced DNA-steresis in zone indicated by arrows. Fixation was by total nuclear irradiation followed by 10 per cent neutral formalin. Feulgen staining of the steretic area, although greatly diminished, is still definitely positive.



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basophilia and stain with eosin; they appear slightly swollen. Actually, this may be an earlier stage of movement of material resulting from the irradiation than that shown in Fig. 4. The latter was stained only with dilute Delafield's hematoxylin. The irradiated area appears as a pale staining oval in which the continuity of the chromosomes is not visible; presumably, it represents material liberated from them by the irradiation and precipitated either by the long continued irradiation or the fixing agent. We have seen a similar precipitation of chromosome material after severe overirradiation with the AH6 lamp.

Typical appearances of steretic areas after staining with Heidenhain's iron hematoxylin are shown in Figs. 3 and 5. The relatively dark staining of the chromosomes in this portion of the metaphase cell (Fig. 5) may be due to insufficient differentiation of the stain in the iron alum.

After similar irradiation and fixation, staining with the Feulgen method shows a diffuse and faintly positive staining of the DNA-steretic area (Fig. 6), suggestively like the diffuse staining of a comparable area in the cell of Fig. 4 stained with Delafield's hematoxylin.

An equally striking demonstration of DNA-steresis follows staining with the phosphotungstic acid hematoxylin of Mallory as shown, typically, in a cell in prophase (Fig. 8). The chromosomes stain deep blue except in the irradiated area where the residual chromosomal material stains light brown. In such preparations the chromosomes are seen to maintain their continuity as they pass into and through the irradiated area and the two zones are sharply separated by their contrasting staining reactions.

Staining with pyronine-methyl-green, after DNA-steresis and fixation in Carnoy's solution, reveals that the irradiated parts of the chromosomes do not stain with the methyl green and are sharply demarcated from the unirradiated contiguous parts (Fig. 7).

The cell shown in Fig. 9 was fixed in 10 per cent neutral formalin and stained with the Alfert-Geschwind method for basic proteins. The chromosome segments in the DNA-steretic area stain a faint green in contrast to the brilliant deep green of the unirradiated parts of the chromosomes.

Because of our use of OsO_4 solutions in fixing cells for electron microscopic study of the irradiated parts of their chromosomes, we also fixed many cells in these solutions for Feulgen staining. Three cells shown in Figs. 10 to 12 were from a culture fixed in 1 per cent aqueous OsO_4 stained with the Feulgen method after localized irradiation with an AH6 lamp. The cell in Fig. 10, in the granular stage of early prophase, showed the steretic change in the irradiated area and some darkening of the chromosomes at its margin within 4 minutes after irradiation. The change occurred more promptly in this cell than in those in early metaphase in the same culture. The DNA-steretic area is prominent and it is questionable whether even a trace of Feulgen-positive material is present. The interval from irradiation to fixation was longer for the other two cells. The chromosome segments in the irradiated area of the cell shown in Fig. 12 stain tan from the OsO_4. If a trace of Feulgen-positive material is present in this area, it is definitely less than the faint pink staining in the comparable area of the cell shown in Fig. 11. It is noteworthy that this cell received two-thirds as much irradiation as that of Fig. 12. In Fig. 11 the ends of several unirradiated chromosomes moved over part of the DNA-steretic area during the interval before fixation.

In addition to the cells which show the reactions of DNA-steretic areas to fixation and staining, such as those described, other cells were exposed similarly to partial nuclear irradiation to produce steresis and were then fixed by irradiating the entire nucleus with ultraviolet light. After this some of these were placed in formalin and subsequently stained with the Alfert-Geschwind method for basic proteins or the Feulgen method while others were processed for electron microscopy. Those stained with the Alfert-Geschwind technic show the same change as those fixed only in formalin, as in Fig. 9. None of those stained with Feulgen shows a completely negative reaction in the steretic area. An example of this is seen in Fig. 13, of a cell in metaphase which was irradiated for 20 seconds with a narrow band of ultraviolet light. DNA-steresis occurred promptly and completely. Forty minutes after the partial nuclear irradiation, the entire nuclear zone was irradiated with the same flux of ultraviolet light for 10 seconds; the cell was fixed in 10 per cent neutral formalin and stained with the Feulgen method. In the steretic area the Feulgen reaction is much weaker than in the other parts of the chromosomes.

Electron Microscopic Observations

Neutral Formalin–Tyrode Fixation: When this study was begun, it was anticipated that there would be great difficulties in following the experimental cell in the tissue culture through all the fixing, embedding, and sectioning procedures preliminary to electron microscopy, but, as mentioned above under Material and Methods, this proved to be relatively easy. We did encounter an unexpected and disconcerting obstacle of another type: despite the striking changes seen with light microscopy, we could not find the irradiated areas with the electron microscope in cells fixed with solutions of osmium tetroxide (see Fig. 23) or potassium permanganate. However, after fixation in neutral formalin-Tyrode without heavy atom staining, the irradiated parts of the chromosomes stand out as strikingly in the electron microscope (Figs. 14 and 15) as in the living cell (Fig. 1) or after fixation and staining for the light microscope (Figs. 2 to 13).

In such neutral formalin-Tyrode-fixed cells, these urodele chromosomes have a homogeneous appearance (*Ch* in Figs. 14 and 15). This resembles that produced by several other fixatives, such as absolute alcohol, and the appearance of chromosomes of Earle's L strain of mouse cells after freeze-drying as found by Dales (13).

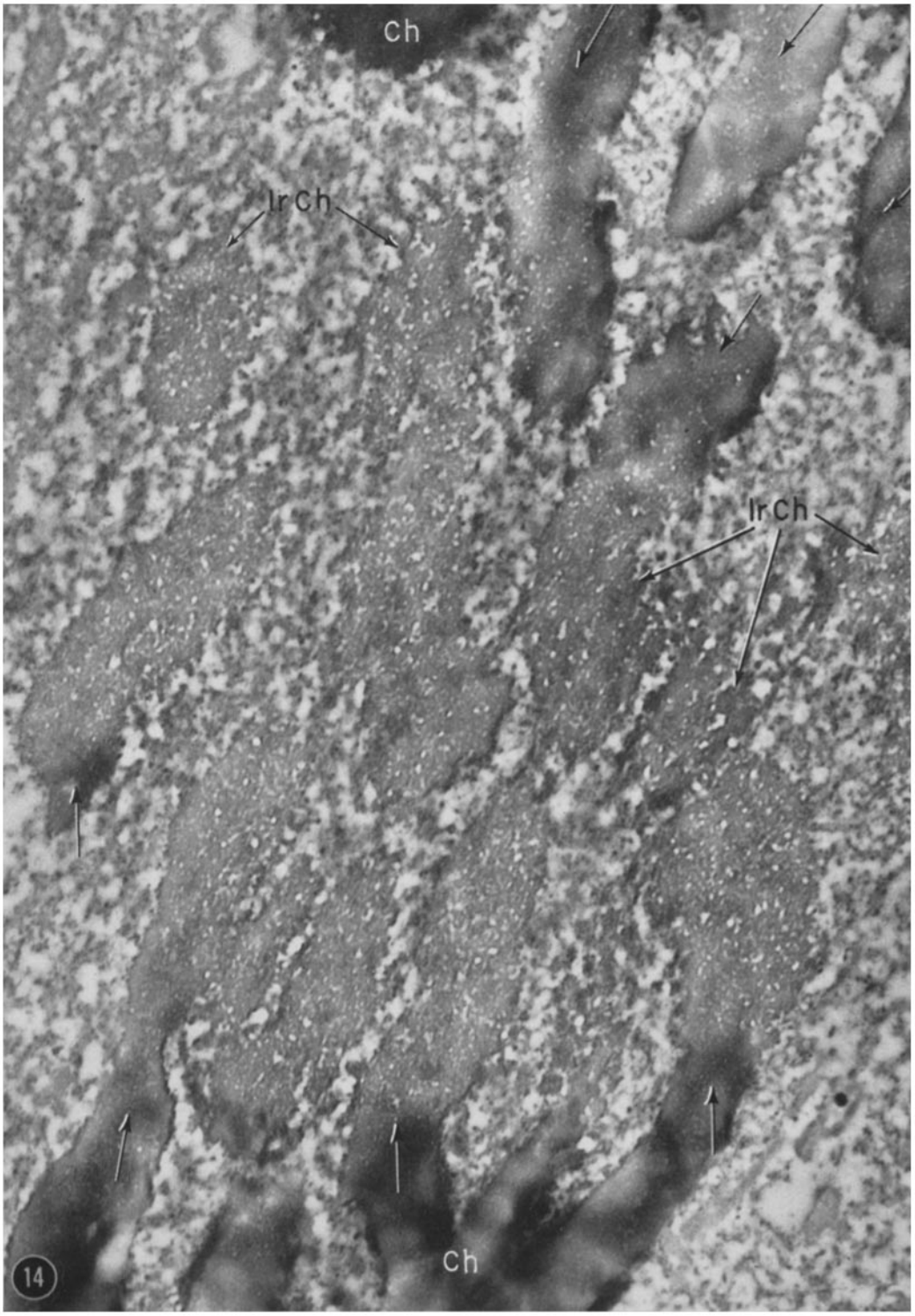
Although these chromosomes do not have a distinct surface membrane they are sharply demarcated in late prophase, metaphase, and anaphase from interchromosomal structures. The homogeneous, sharply contoured chromosomes are quite different in appearance from the indistinctly outlined masses of rodlets and dots seen with the electron microscope after OsO_4 fixation (Fig. 23). Between the chromosomes are scattered fine, dark granules and somewhat paler, irregular, ill-defined bands or cords. Sometimes there are small granules in these cords but more frequently they occur at their margins. Some of the interchromosomal cords and the less well defined strands meet and fuse with the edges of the chromosomes, giving them an irregular outline. Scattered among these structures are apparent open spaces of varying size which probably represent shrinkage or places from which substances have been dissolved.

Unlike the non-irradiated portion where the edge of the chromosome is definite, the edges of the chromosomes in the heavily irradiated segments are ragged and blurred (Fig. 14, *IrCh*). Indeed it is impossible in many places in such areas to determine exactly where the chromosome ends. This is suggestively like the indefinite delimitation of non-irradiated chromosomes fixed in OsO_4 (Fig. 23). In the transition zone between the irradiated and non-irradiated parts of the chromosomes there are closely packed small vacuoles indicated by arrows in Fig. 14. The smallest of them are fairly regular in contrast to the larger ones which may vary considerably. At higher magnifications the vacuoles measure 75 to 250 A and may appear to be oval or ellipsoidal—perhaps as a result of compression during sectioning (Fig. 15, arrows). They are smaller than the irregular vacuoles in the more intensely irradiated central areas. The large interchromosomal vacuoles of Fig. 15 are not a result of the irradiation since they occurred throughout the nuclear part of the cell; they probably arose from damage during preparation for microscopy.

Neutral Formalin–Tyrode Fixation and Phosphotungstic Acid Staining: Since the DNA-steretic portions were distinctly different from the rest of the chromosomes in neutral formalin–Tyrode-fixed cells, the next step was to stain some of them with heavy elements. Most informative have been staining with phosphotungstic acid or uranyl acetate dissolved in the absolute alcohol prior to embedding. This method gave us uniformly better results than staining the sections on grids. In comparing our findings with those of others, it must be borne in mind that most investigators apply these compounds to cells fixed in OsO_4

FIGURE 14

Metaphase chromosomes with DNA-steresis resulting from irradiation with a narrow band of ultraviolet. Homogeneous parts of the chromosomes (*Ch*) merge into the vacuolated and paler irradiated segments (*IrCh*). In places, it is difficult to see limits between DNA-steretic chromosomes and interchromosomal constituents. Arrows on the chromosomes indicate fine vacuoles in the zone of marginal irradiation. 10 per cent neutral formalin–Tyrode fixation. No stain. Methacrylate embedding. × 24,300.


Ch
Ir Ch
Ir Ch
Ch
14

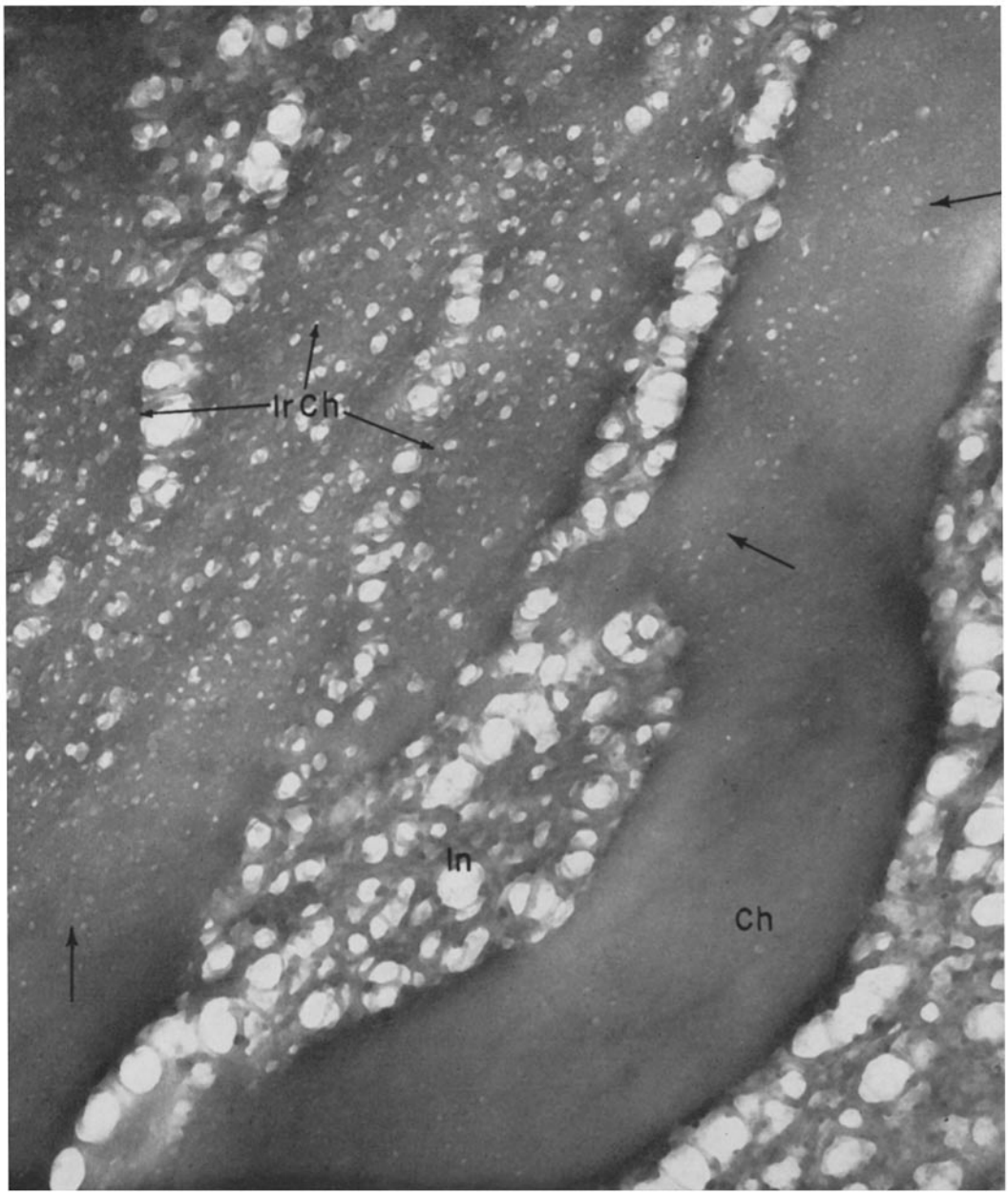


FIGURE 15

Metaphase chromosomes showing transition from non-irradiated chromosome (*Ch*) through a marginal zone with small vacuoles (arrows) to DNA-steretic chromosome segments (*IrCh*). Interchromosomal structures (*In*) are unusually heavily vacuolated, possibly owing to damage during preparation. Irradiated with 8 μ spot of ultraviolet light. Fixation in 10 per cent neutral formalin–Tyrode; lightly stained with Delafield's hematoxylin. Methacrylate embedding. × 35,700.

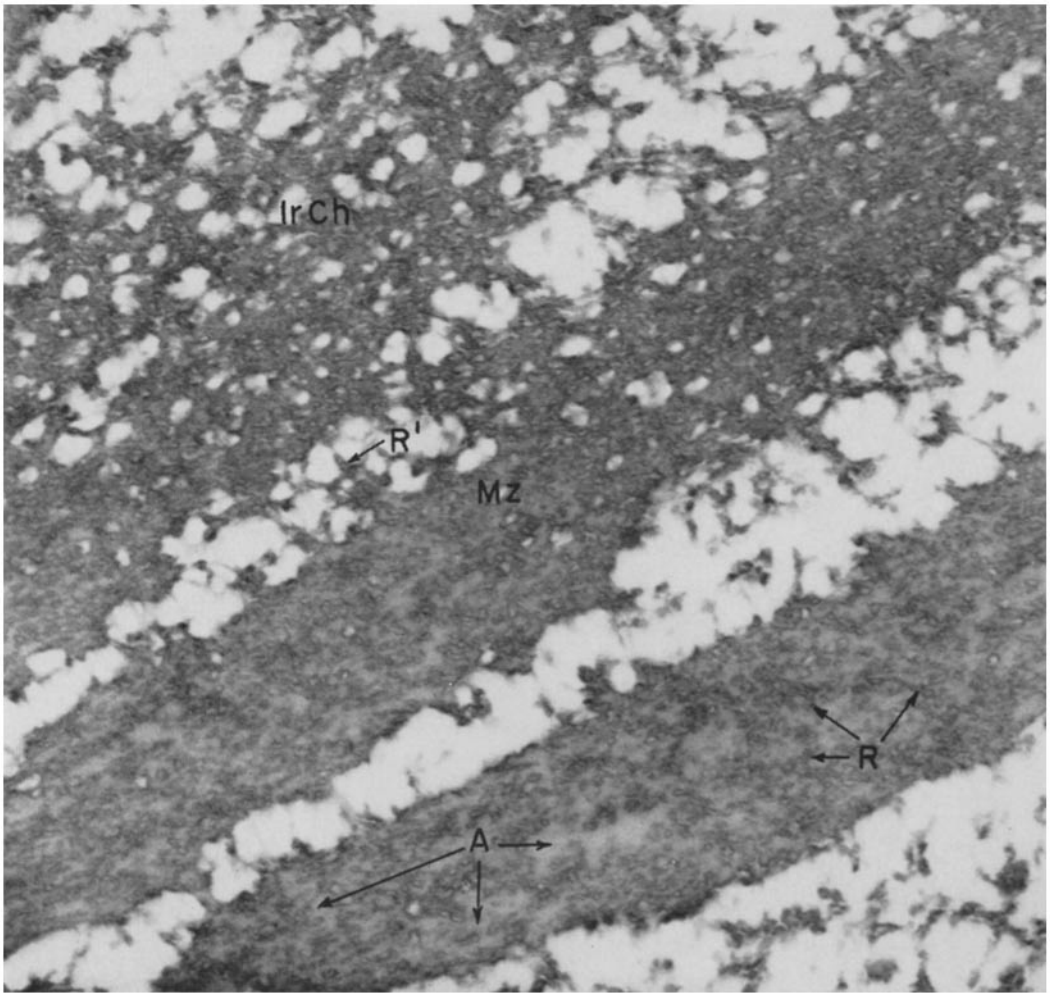


FIGURE 16

This micrograph is of metaphase chromosomes at edge of DNA-steretic zone (*IrCh*) produced by irradiation with a narrow stripe of ultraviolet light. In the lowest, unirradiated chromosome, component-A (*A*) and strands of reticulum (*R*) are indicated. *Mz*, marginal zone; *R'* interchromosomal reticulum. Fixed in 10 per cent neutral formalin–Tyrode; stained with alcoholic phosphotungstic acid. Methacrylate embedding. × 64,000.

solutions. This accounts for some of the differences between our observations on the neutral formalin–Tyrode-fixed chromosomes and nuclei and those obtained on preparations fixed in OsO_4 by others and by ourselves.

In the neutral formalin–Tyrode-fixed cells stained with alcoholic phosphotungstic acid the interchromosomal material is found to consist of an irregularly meshed reticulum (*R'*) of elongate, branching strands usually 75 to 150 A in diameter, although occasional ones may be as small as 40 A and others up to 300 A in their short axis (see *IR* in Figs. 17 and 18). These strands are often darker at their margins than at their centers and are attached to and continue, singly or in small bundles, into the chromosomes (*R'* in Figs. 18, 21).

Similar structures are much more numerous within the chromosomes (*R* in Figs. 16 to 19, 21). In rare places they may be arranged in the long axis of the chromosome, but their usual disposition, as seen in the thin sections, is as short segments of wavy, or bent, branching filaments which are parts of a network with irregular meshes cut at random. Although they are frequently close to

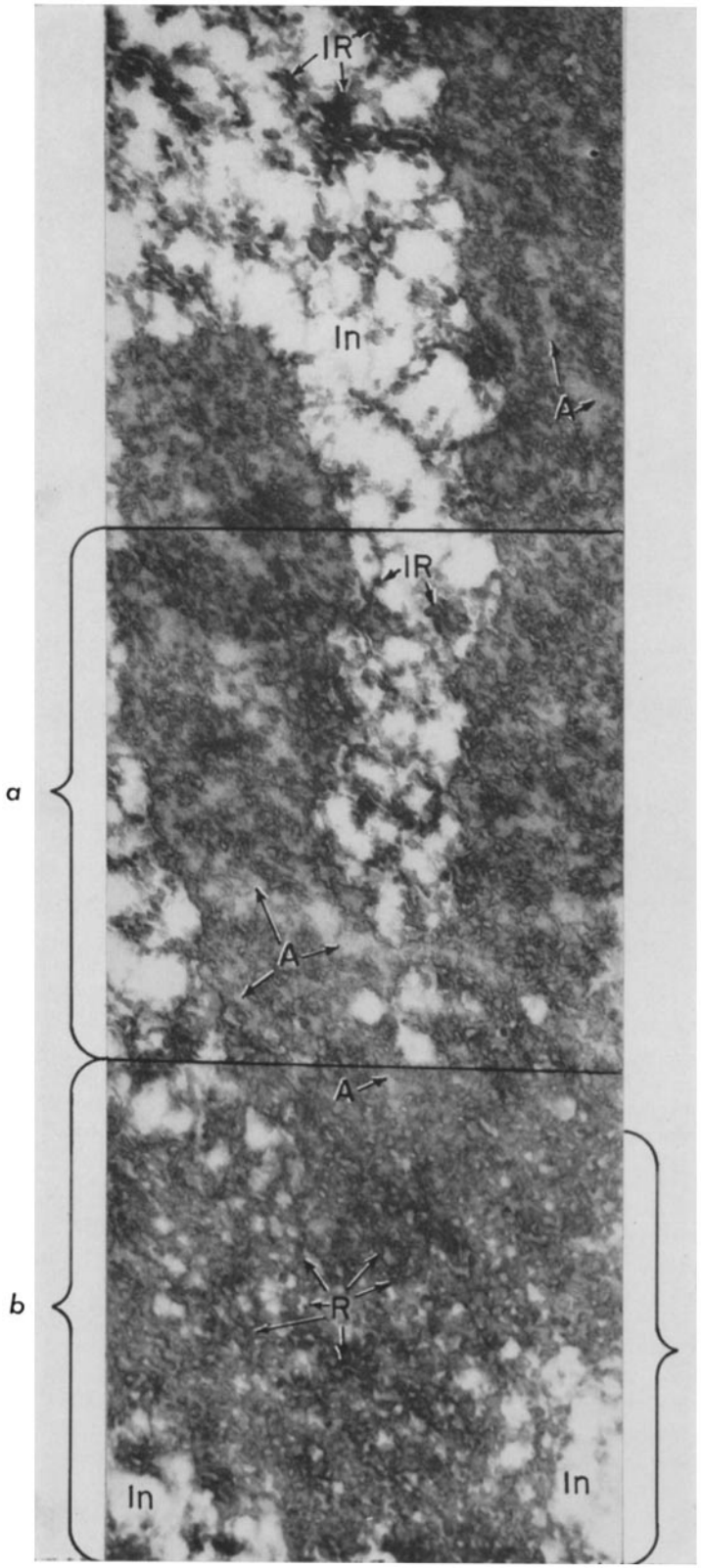


FIGURE 17

A montage of three adjacent micrographs of metaphase chromosome, including the ultraviolet-irradiated, DNA-steretic part (*Irrad. zone*). Component-A (*A*) seems to be slightly diminished in the lower margin of micrograph *a* and seems largely gone in most of *b*. Strands of interchromosomal reticulum (*IR*) continue into the chromosome; (seen better at higher magnification at *R'* in Fig. 18). In the DNA-steretic portion the strands (*R*) are continuous with those in the interchromosomal regions (*In*). Fixed in neutral formalin-Tyrode. Stained with alcoholic phosphotungstic acid before embedding and with uranyl acetate on the grids. Methacrylate embedding. × 63,000.

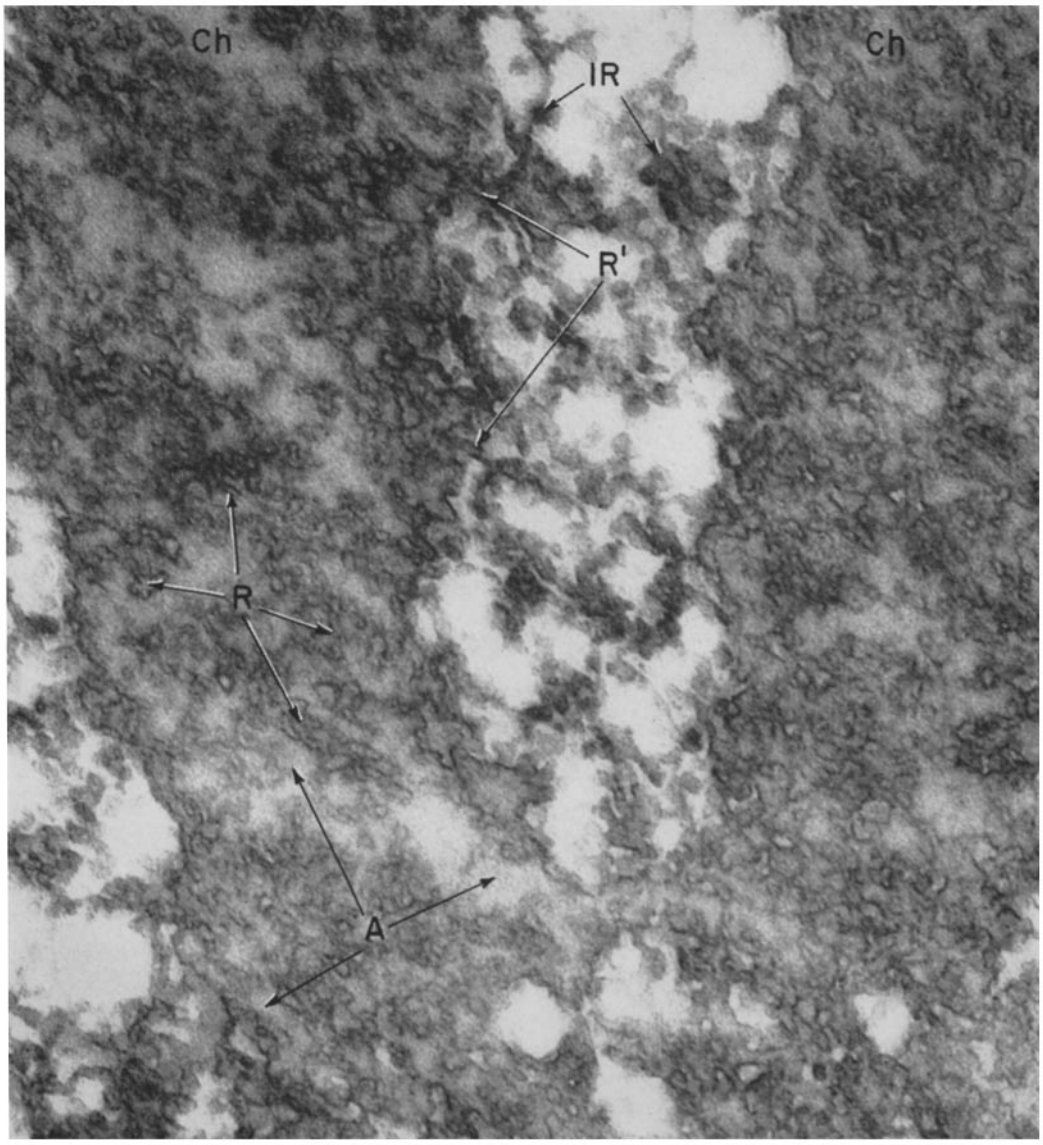


FIGURE 18

Higher magnification of Fig. 17*a*. Component-A is prominent (*A*) in contrast to its paucity in the irradiated part of the chromosome (Fig. 17*b* and Fig. 19). The chromosomal reticulum (*R*) is continuous (*R'*) with the interchromosomal reticulum (*IR*). *Ch*, chromosomes. × 129,000.

one another, they are usually separated, singly or in groups, by a homogeneous or finely granular or fibrillated material. Because it varies in appearance with the phases of mitosis and with different fixatives and stains, we will designate it for the present as "component-A" (*A* in Figs. 16, 18). When it is homogeneous it looks much like the chromosomal substance in unstained neutral formalin–Tyrode-fixed preparations. Component-A tends to be suggestively fibrillar in early prophase, finely granular or homogeneous in metaphase, and homogeneous in late anaphase and in telophase. It would seem that the more condensed the developing chromosomes become, the more

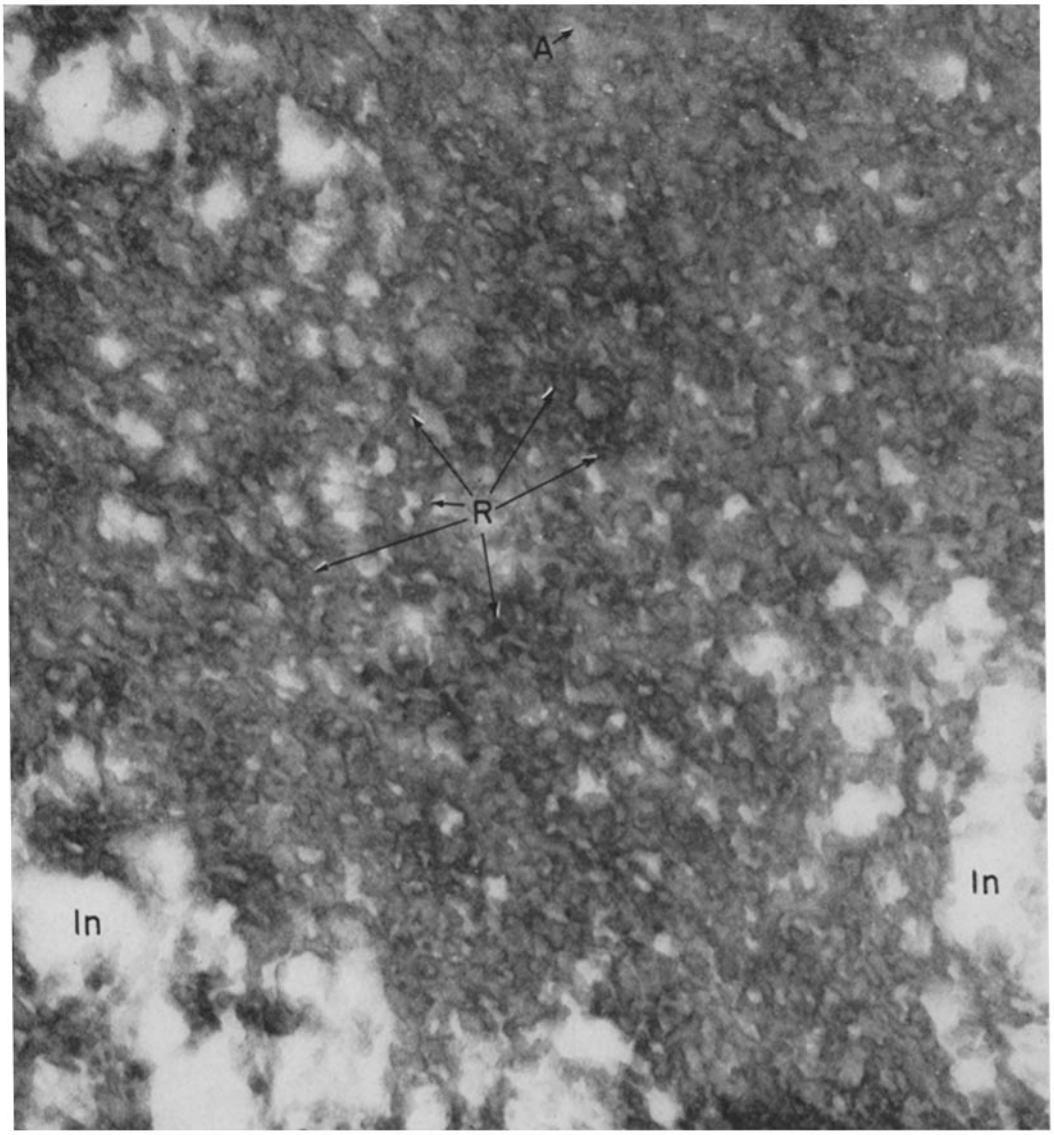


FIGURE 19

Higher magnification of Fig. 17*b*, the irradiated, DNA-steretic zone. Owing to the absence of component-A (except at *A*), the close-meshed chromosomal reticulum (*R*) is now obvious. × 129,000.

homogeneous is the appearance of this component. Very often, bars of the reticulum (R) within the chromosomes stain paler after they become surrounded by component-A.

With low magnification (5000) in the formalin–phosphotungstic acid preparations, the irradiated areas stand out rather prominently because of some vacuolization of the chromosomes and the much greater irregularity of their margins. With higher magnification it is obvious that in the steretic areas the chromosomes have undergone a great change. In Fig. 16 the lowest chromosome was not irradiated, while the upper parts of the other two were irradiated and show DNA-steresis. In addition to the vacuolization, which probably varies with the amount of irradiation and may be quite prominent (Fig. 16, *IrCh*), there is a striking change due to the disappearance of component-A,

with the result that the chromosomes here consist largely of a branching network (*R*). In the transition zone (*Mz*) between the obviously unirradiated and the irradiated portions, one can follow the progressive decrease in component-A.

In a montage of three adjacent micrographs in Fig. 17, and in Figs. 18 and 19, which are enlargements of two of them, we can follow the transition between the unirradiated and DNA-steretic portions of the chromosomes. These micrographs are from a cell in metaphase fixed in neutral formalin–Tyrode, stained *in toto* with alcoholic phosphotungstic acid and then stained on the grid with uranyl acetate. This combined stain gives more contrast to some of the structures, especially the edges of the chromosomes and the outlines of the reticulum within them (*R*). It also darkens the inner parts of the interchromosomal reticulum (*IR*). The DNA-steretic area fills most of Fig. 19. Here the reticulum (*R*) forms a clearly anastomosing network which extends throughout this portion of the chromosome and also is continuous with the interchromosomal reticulum (at *In*), so that it is difficult to see just where the chromosome ends (Fig. 19). There is a bit of component-A at and near (*A*) in this figure.

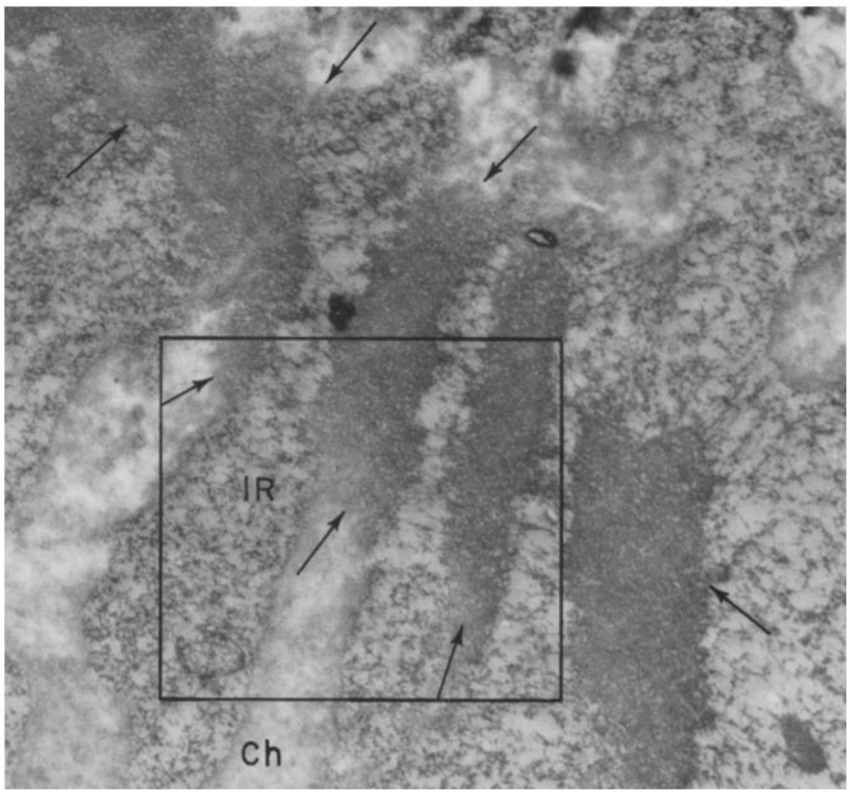


FIGURE 20

Metaphase chromosomes irradiated with a narrow stripe of ultraviolet light. Arrows point to the DNA-steretic area which appears as a dark band cutting across the mottled grey chromosomes (*Ch*). *IR*, interchromosomal reticulum. The outlined area is shown in Fig. 21 at higher magnification. Fixed in 10 per cent neutral formalin–Tyrode and stained with alcoholic phosphotungstic acid before embedding in Vestopal. × 12,900.

The results were no different when we substituted a mixture of the methacrylate monomers for ethylene dichloride as the solvent for the polymerized methacrylates in our embedding process, or when we used Vestopal instead of the methacrylates for embedding. Figs. 20 and 21 show that after neutral formalin–Tyrode fixation, phosphotungstic acid staining and Vestopal embedding, we obtained essentially the same results as with methacrylate embedding. That is,

the steretic area appears as a dark mass at low magnification and at higher magnifications this part of the chromosome is seen to consist of a close meshed network (*R*) continuous (*R'*) with the looser interchromosomal one (*IR*). There is a narrow zone of transition (*Mz* in Fig. 21) at the sharply delineated in this preparation as in metaphase chromosomes of most of our methacrylate-embedded cultures. Some portions of the interchromosomal reticulum appear to be stretched between adjacent chromosomes, perhaps because of shrinkage.

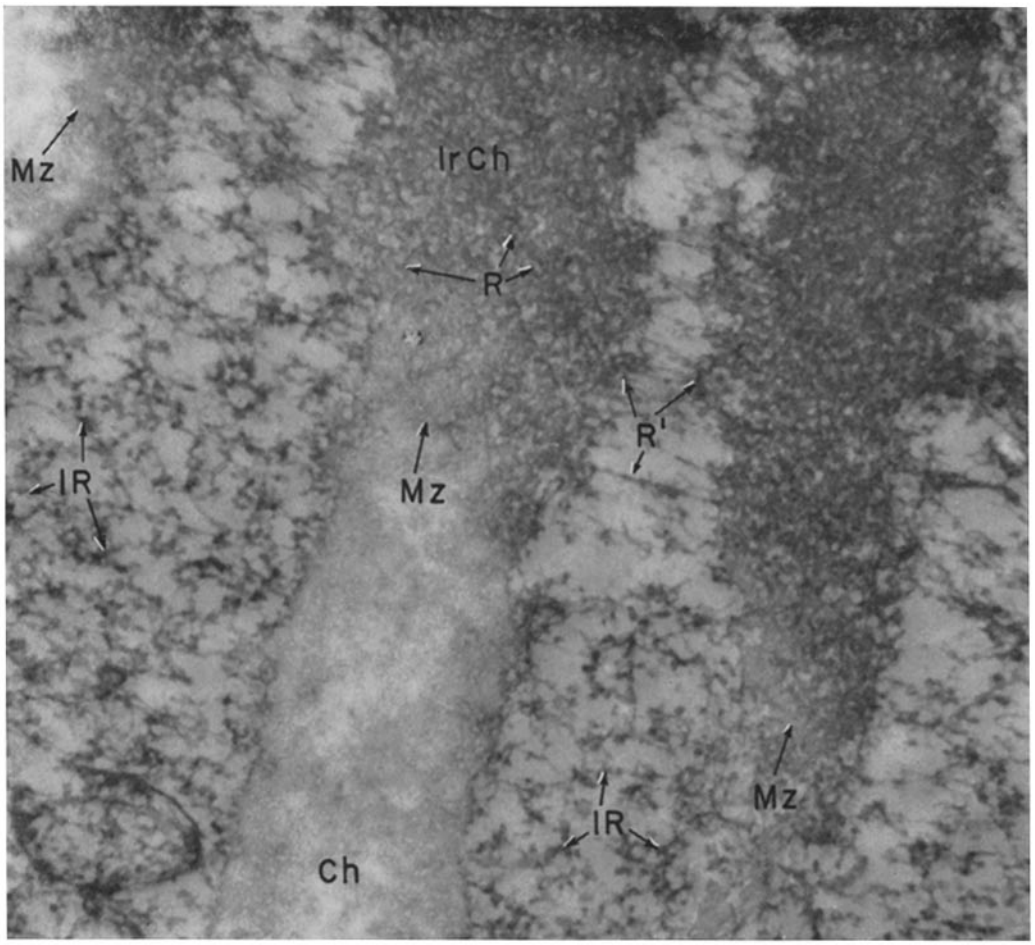


FIGURE 21

This is higher magnification of the rectangle outlined in Fig. 20. The DNA-steretic area (*IrCh*) consists almost entirely of the intrachromosomal reticulum (*R*) which is connected (*R'*) with that between the chromosomes (*IR*). *Mz*, margin of irradiated zone. *Ch*, chromosome. Vestopal embedding. × 44,000.

margin of the irradiated area. The unirradiated chromosomes are sharply demarcated from the interchromosomal areas and component-A is a mottled gray, just as in many of the similarly fixed and stained cultures embedded in methacrylate. The strands of intrachromosomal reticulum surrounded by component-A are not quite so

Neutral Formalin–Tyrode Fixation and Uranyl Acetate Staining: Somewhat different pictures are obtained with this fixation and subsequent staining with uranyl acetate in absolute alcohol before embedding. With this stain, the chromosomes in late prophase are fairly sharply delimited and are composed of a finely granular or suggestively

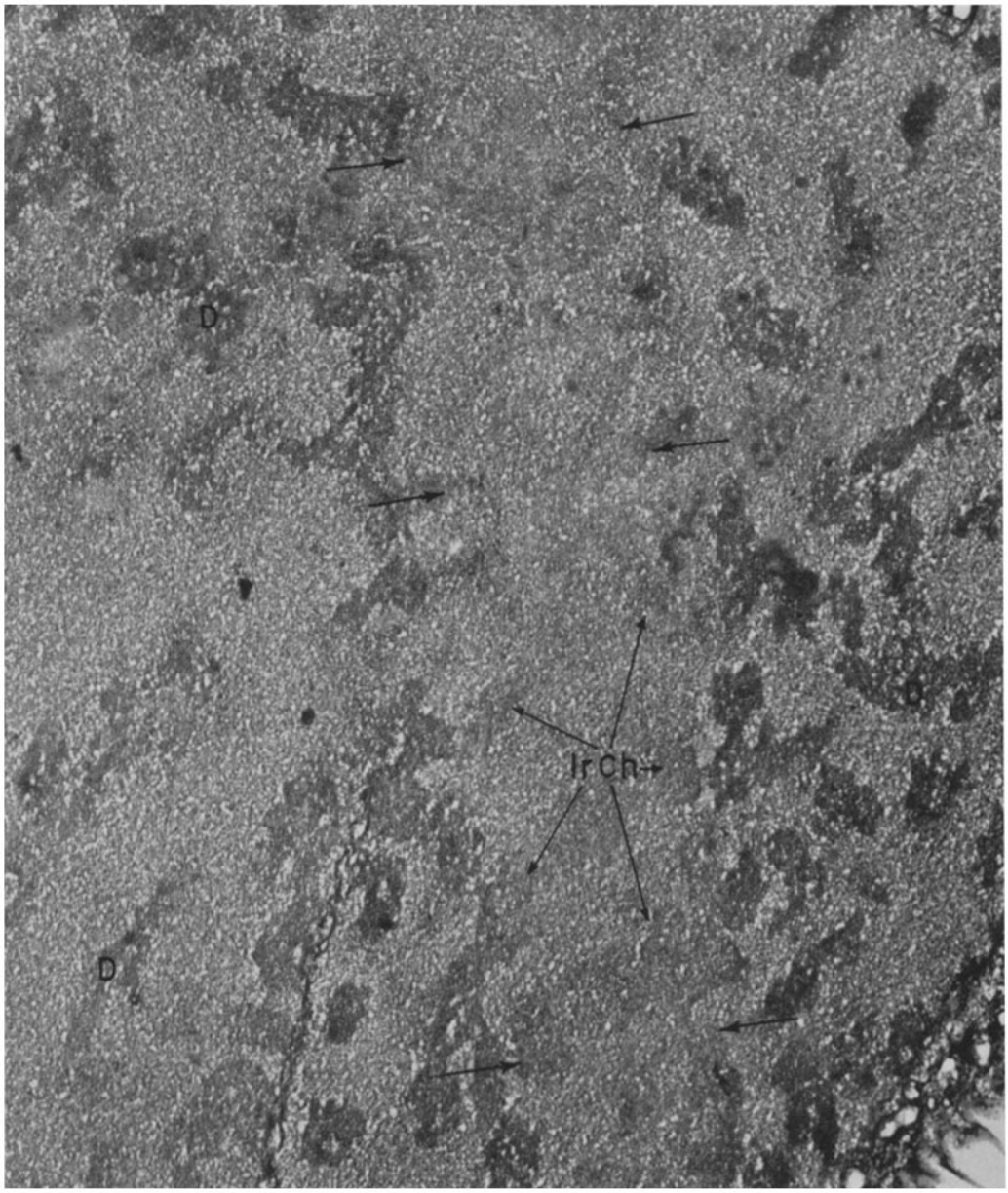


FIGURE 22

Prophase irradiated with a narrow stripe of ultraviolet light. In the obvious DNA-steretic area (*IrCh*), demarcated by arrows, residues of the chromosomes are barely detectable. The non-irradiated developing chromosomes (*D*) are distinct. Fixed in neutral formalin–Tyrode. Stained with 1.2 per cent $KMnO_4$ in refrigerator for 22 hours. Methacrylate embedding. × 7,700.

fibrillar material and an irregular network of open spaces communicating with interchromosomal areas. Component-A fills the meshes between these spaces and is more positively stained by uranyl acetate. It appears more granular or fibrillar than after phosphotungstic acid staining. In prophase the interchromosomal structures consist of a dark granular network and an ill formed, fuzzy or finely granular, moderately dark gray material much like that of component-A and often connected with it. Such parts of the interchromosomal substance will presumably become an integral part of the chromosomes by the time they reach maximum size in metaphase.

As the developing chromosomes condense into the metaphase state they take on a paler, more homogeneous appearance. In the DNA-steretic area they lose their distinctive margins and most if not all of component-A. This part of the chromosomes now appears as a dark reticulum obviously continuous with the looser interchromosomal reticulum.

Fixation in Potassium Permanganate: We have not seen the steretic area in preparations fixed in potassium permanganate. However, if the cells showing DNA-steresis in the living are fixed in neutral formalin–Tyrode and then stained with potassium permanganate at 0°C for 24 hours or longer, the change is clearly visible in the electron microscope. Fig. 22, from a cell in early prophase, shows the differences between irradiated (*IrCh*) and unirradiated portions of the developing chromosomes (*D*). The darkly staining chromosomes merge into the ill defined structures in the DNA-steretic area.

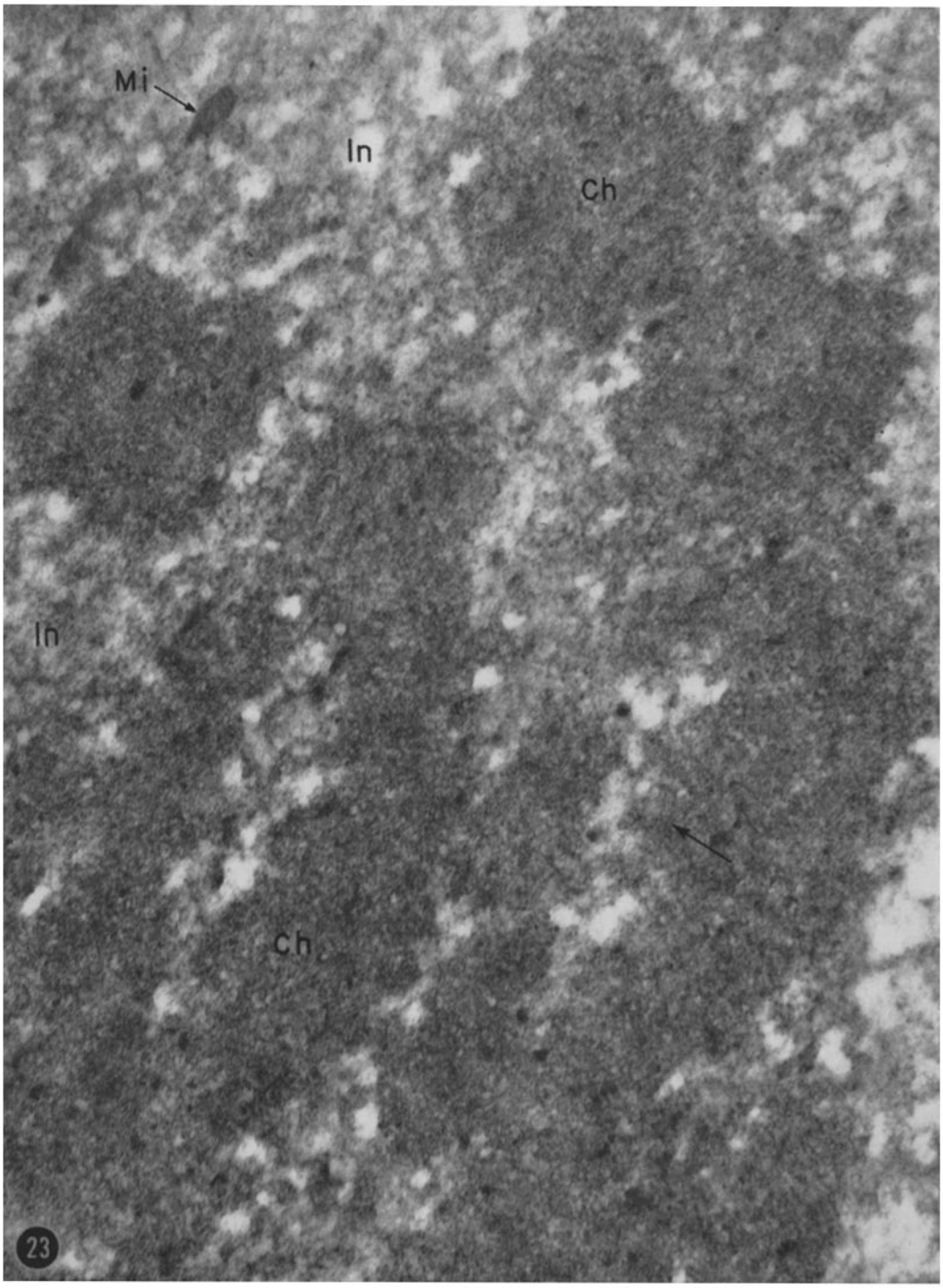
OsO_4 Fixation Compared with Exposure to OsO_4 after Other Types of Fixation: Chromosomes with DNA-steresis show no change by which this area can be recognized after OsO_4 fixation when examined with the electron microscope. In such experiments all the chromosomes look alike (Fig. 23, *Ch*). In many places it is not possible to tell exactly where they end and interchromosomal material (*In*) begins. In thin sections the chromosomes consist of small dots and rods and some longer, double-contoured structures (arrow) 75 to 300 A in diameter. The latter probably correspond to the reticular structures seen in the phosphotungstic acid-stained preparations after neutral formalin–Tyrode fixation. In some OSO_4-fixed preparations, larger black bodies are scattered in the chromosomes; we do not know what they represent.

Although the steretic area cannot be found with the electron microscope after primary OsO_4 fixation, it is readily demonstrable if exposure of the cell to OsO_4 is preceded by fixation with total nuclear UV irradiation, as in Fig. 24. DNA-steresis was produced by a band of ultraviolet light running through the nuclear zone; the margin of the area exposed to partial nuclear irradiation and then to total nuclear irradiation (*P.N.I.* + *T.N.I.*) is indicated by arrows. In the part exposed only to total nuclear irradiation (*T.N.I.*) the chromosomes (*Ch*) have sharply demarcated contours and are formed of densely packed granules and rods, while the chromosome segments in the steretic part (*IrCh*) consist of fewer granules and have vague outlines which gradually merge into interchromosomal areas (*In*).

Fixation by Total Nuclear Irradiation Followed by Neutral Formalin–Tyrode and Phosphotungstic Acid: Cells so treated (Figs. 25 and 26, *P.N.I* + *T.N.I.*) show somewhat more of component-A in the steretic parts of the chromosomes than do cells treated in similar fashion except for the omission of total nuclear irradiation (*T.N I.*). In Fig. 26 the right-hand portion of the micrograph shows dense homogeneous component-A in the parts of the chromosomes exposed only to total nuclear irradiation (*T.N.I.*). At the margin of this zone (arrows) component-A becomes darker and faint

FIGURE 23

This micrograph of a cell after fixation in OsO_4 is an instance of the failure of this reagent to demonstrate differences between unirradiated and irradiated chromosomes. Despite the obvious DNA-steresis seen in the living cell, after fixation all of the metaphase chromosomes (*Ch*) have the same appearance. They consist of short rods and granules and their margins often merge with interchromosomal structures (*In*). Occasional double-contoured structures (arrow) are probably the same as the strands of chromosomal reticulum seen in micrographs of cells fixed in neutral formalin–Tyrode and stained with phosphotungstic acid. *Mi*, mitochondrion. OsO_4 fixation. Methacrylate embedding. × 30,400.


Mi
In
Ch
In
Ch
23

outlines of elements of the chromosomal reticulum begin to be visible. Toward the left side of the micrograph more of the reticular structure (*R*) is revealed, but here less of the dark component-A is seen. In this region the continuity of the interchromosomal reticulum (*IR*) and that within the chromosome (*R*) can be followed.

Unirradiated Chromosomes in Early and Late Stages of Mitosis: In the course of our experiments, we also studied the nuclear changes in unirradiated cells throughout the mitotic cycle. Here we shall make brief mention of certain changes in prophase and telophase which have helped us to analyze DNA-steresis.

Our practice has been to observe a particular living cell and photograph it before it was fixed for electron microscopic study. In very early stages of mitosis the nuclear substance, except for the nucleoli and chromatin particles, appears as a dark network after neutral formalin–Tyrode fixation and phosphotungstic acid staining (Fig. 27, *IR*). When portions of this reticulum begin to aggregate (*R*), component-A becomes visible (*A*). The developing chromosomes continue to enlarge by accretion of more and more of the condensing portions of the reticulum with attached component-A and by fusion of the masses already formed (Fig. 27, *D1* to *D4;* Fig. 28, *D*). From the start of formation of small chromosomal masses until the fully formed chromosomes of metaphase are reached (Figs. 16 to 21), the dark strands of interchromosomal reticulum become paler as they become surrounded by obvious component-A. In late anaphase and especially in early telophase, as the chromosomes fuse, component-A becomes exceedingly dense and appears unstained or nearly so (Figs. 29, 30). Although the continuity of some strands of the reticulum (*R'* in Figs. 29 to 31) into the chromosomes is maintained, they cannot be traced far into the dense component-A. However, persistent traces of the reticulum remain along lines of fusion of the chromosomes (Fig. 29, *F*), often demarcated by irregular rows and groups of vacuoles (Fig. 30, *R''*). In such places the few strands of reticulum are darkly stained.

As the fused chromosome mass begins to enlarge and reconstruct into a daughter nucleus, component-A begins to disperse and the reticulum of the nucleus gradually becomes apparent, at first as ghost-like images (near *A*, Fig. 31) and later as sharply demarcated dark structures (*R*). At this stage the strands attain their greatest width, some of them measuring 400 A across. Occasional ones seem to be larger, but these may represent branchings.

While the nucleus enlarges progressively, more and more of component-A disappears from view, with the result that most of the nucleus appears to consist of reticulum. The number of strands of the reticulum passing through the nuclear membrane (Figs. 30 and 31, *R'*) seems small as compared with the number which aggregated to form the chromosomes in the preceding prophase. Component-A disappears last from a thin zone beneath the nuclear membrane (Fig. 31).

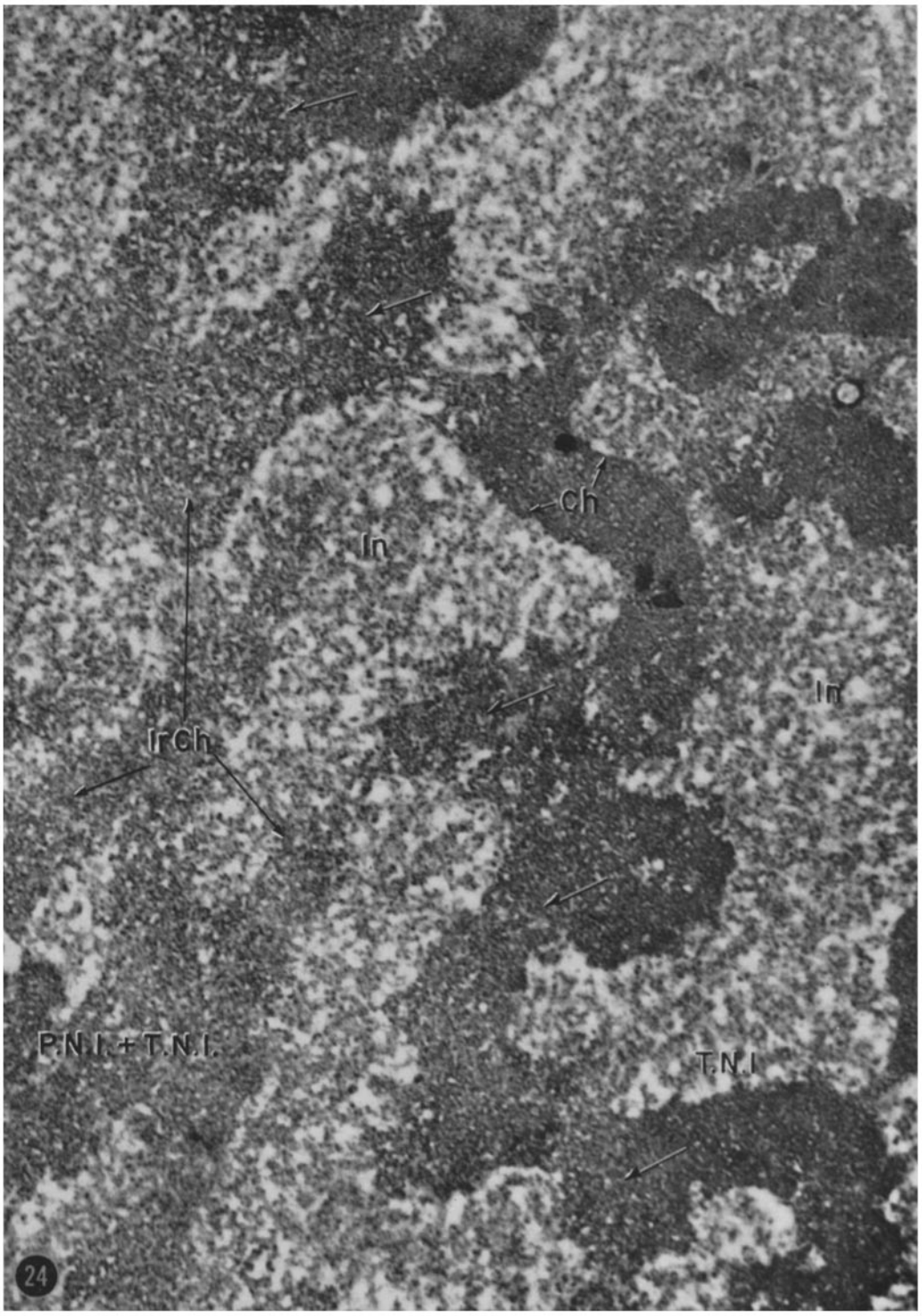
Thus, in the course of the mitotic cycle there are dramatic changes in the relative visibility of component-A and the nuclear reticulum, varying from the picture at the height of telophase, when component-A completely obscures the reticulum, to that during interkinesis, when the latter is the main structure visible. However, the unirradiated interkinetic nuclear reticulum has not lost any of its DNA, whereas irradiated, steretic chromosome segments have.

DISCUSSION

All of our observations on developing and reconstructing chromosomes and on DNA-steresis indicate that DNA is contained in the submicroscopic material (component-A) attached to the chromosomal reticulum. In the early stages of

FIGURE 24

DNA-steretic changes in chromosomes, after partial nuclear irradiation (*P.N.I.*), are demonstrated by OsO_4 after fixation of the whole nuclear zone with ultraviolet light (*T.N.I.*). The label *P.N.I.* + *T.N.I.* indicates that this zone was irradiated with ultraviolet light to produce DNA-steresis and again as a part of total nuclear irradiation for fixation. The margin of the area exposed to partial nuclear irradiation and then to total nuclear irradiation is indicated by arrows. Localized postirradiation steresis (*IrCh*) is apparent. The non-steretic parts of chromosomes (*Ch*) have sharp outlines in contrast to the steretic ones. *In*, interchromosomal structure. Compare this figure with Fig. 23. Methacrylate embedding. × 24,000.


Ch
In
In
IrCh
P.N.I. + T.N.I.
T.N.I
24

chromosome formation there are occasional places which at first glance suggest that component-A might be within the strands of the reticulum. However, after thorough study we are convinced that these sites arise by condensation of the strands with their attached wisps of component-A. This is apparently the same process responsible for the paler staining of the portions of reticulum within chromosomes. The condensation reaches its greatest extent in late anaphase and early telophase when nearly all of the chromosomal reticulum is completely obscured.

It is difficult to say how much DNA remains in the steretic segments. The weak or negative Feulgen staining of these portions indicated that much of the DNA was gone (Figs. 11 and 12), a conclusion supported by the decreased absorption at 2400, 2600 and 2800 A as measured by Perry. When nuclei were fixed by ultraviolet irradiation followed by neutral formalin–Tyrode, the Feulgen reaction in the steretic parts became weak but was still positive (Fig. 13) and component-A was present in small amounts (Figs. 25 and 26). Our conclusion is that a large part of the DNA leaves promptly after irradiation and that some of the residue may be removed from the irradiated parts by the dehydrating or embedding or staining reagents. The complete removal of DNA may possibly be obtained by the delivery of an optimal amount of ultraviolet irradiation delicately balanced between over- and underirradiation.

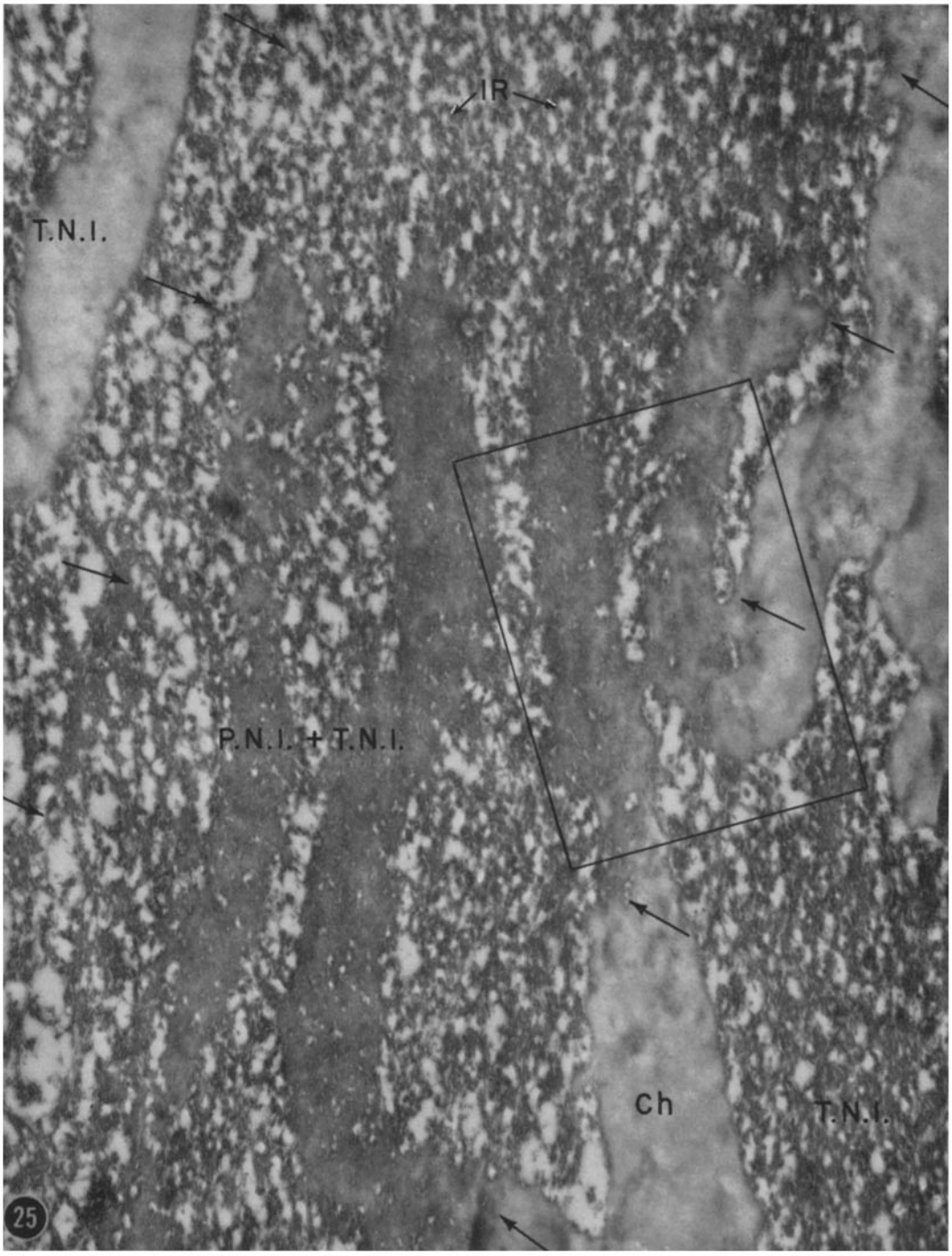
Our attempt to localize the position of component-A, and implicitly of DNA, in the chromosomes has been hindered by the complex solubilities of chromosomal constituents in the fixing agents and in the various concentrations of alcohol and other solvents used for dehydration and embedding. For instance, when cultures, after neutral formalin–Tyrode fixation, were passed quickly through the alcohols (5 minutes in each concentration) and stained with phosphotungstic acid, the metaphase chromosomes showed component-A in the meshes of the reticulum, but the small aggregations of reticular strands in beginning prophase did not show any component-A. However, when such cultures were left in 75 per cent alcohol for several hours, component-A was just as obvious in early prophase as in metaphase. It thus appears that in chromosomes, after fixation in neutral formalin–Tyrode, finely divided DNA (?DNP) becomes less soluble in 95 per cent and absolute alcohol after a prolonged stay in 75 per cent alcohol.

Since the sum of our findings is that much of the DNA (?DNP) has left the irradiated portions of the chromosomes, we sought an explanation of the fact that the DNA-steretic regions are not distinguishable from the unirradiated chromosomes in electron micrographs of cells fixed with OsO_4 or $KMnO_4$. This failure may mean either that these agents do not precipitate the DNA (?DNP) or do not react with it to produce a material opaque to the electron beam. We incline to the latter interpretation since unirradiated interkinetic nuclei and chromosomes fixed in OsO_4 give positive Feulgen reactions and the DNA-steretic areas do not (Figs. 10 to 12). This also agrees with Bahr's (14) observation that solutions of DNA and of DNP do not react visibly with OsO_4 *in vitro*.

We have failed to explain, or prevent, some variations in appearance of component-A from preparation to preparation. Sometimes it is light gray or medium gray or almost white; at other times it is irregularly mottled in shades of gray. In the neutral formalin–Tyrode-fixed cultures, especially when stained with phosphotungstic acid, the non-irradiated parts of chromosomes show a greater susceptibility to compression during sectioning than the DNA-steretic portions of the same chromosomes or the other cellular structures. We have not noticed this reaction after other types of fixation. We have also encountered differences in staining of the reticular strands, especially in metaphase chromosomes; they may be a dark

FIGURE 25

The nuclear zone of this cell, in prometaphase, received a narrow ribbon of partial nuclear UV irradiation (*P.N.I.*) to produce DNA-steresis and 12 minutes later was fixed with total nuclear UV irradiation (*T.N.I.*). The culture was then placed in neutral formalin. The arrows indicate the margins of DNA-steresis. Note the mottled gray of the chromosomes (*Ch*) due to sectioning. The steretic area (*P.N.I.* + *T.N.I.*) is darker and has lost most but not all of its component-A. *IR*, strands of interchromosomal reticulum. Stained with alcoholic phosphotungstic acid and embedded in methacrylate. The outlined area is enlarged in Fig. 26. × 33,000.


IR
T.N.I.
P.N.I. + T.N.I.
Ch
T.N.I.
25

or medium gray, or a pale gray difficult to discriminate in some instances from that of component-A.

The neutral formalin–Tyrode-fixed cells when stained with alcoholic phosphotungstic acid seem to show much greater vacuolization of the DNA-steretic parts of chromosomes and of the interchromosomal structures, and more shrinkage of the cytological structures in general than do similarly fixed cells, with or without subsequent staining with alcoholic uranyl acetate.

Neutral formalin–Tyrode fixation, although of crucial help in this investigation, is defective in some respects, a fallibility shared with the other fixatives used for electron microscopy. For example, neither it nor OsO_4 solutions permit the visualization, in the electron microscope, of the kinetochores which we see in the living cells. We must emphasize, however, that the use of this formalin solution has permitted us to study the portions of chromosomes which we irradiated and in which we saw striking changes in the living cells and with histochemical procedures, and this we were not able to do in our OsO_4-fixed cells.

SUMMARY AND CONCLUSIONS

Parts of chromosomes of urodele mesothelial cells, in culture, were locally irradiated with approximately 10^{-1} ergs/μ^2 of heterochromatic ultraviolet light. At the irradiated places the chromosomes showed a marked change in refractive index which reflects a loss of substance.

Preliminary measurements of the living cells with the interference microscope (by Haynes and Stodolsky) suggest that there is a substantial loss of material from the irradiated portions of the chromosomes.

We have concluded that the material lost contains DNA (and possibly some basic protein) since we found that the irradiated segments stain faintly or not at all with the Feulgen method or with the methyl green of the pyronine-methyl-green mixture, and that they stain weakly with the Alfert-Geschwind procedure for basic protein. This conclusion is also supported by Perry's observation that, in living cells, the irradiated parts show a 50 to 60 per cent decrease in absorption at 2400, 2600, and 2800 A, that is, in the region of intense chromosomal absorption that has a peak at or near 2600 A.

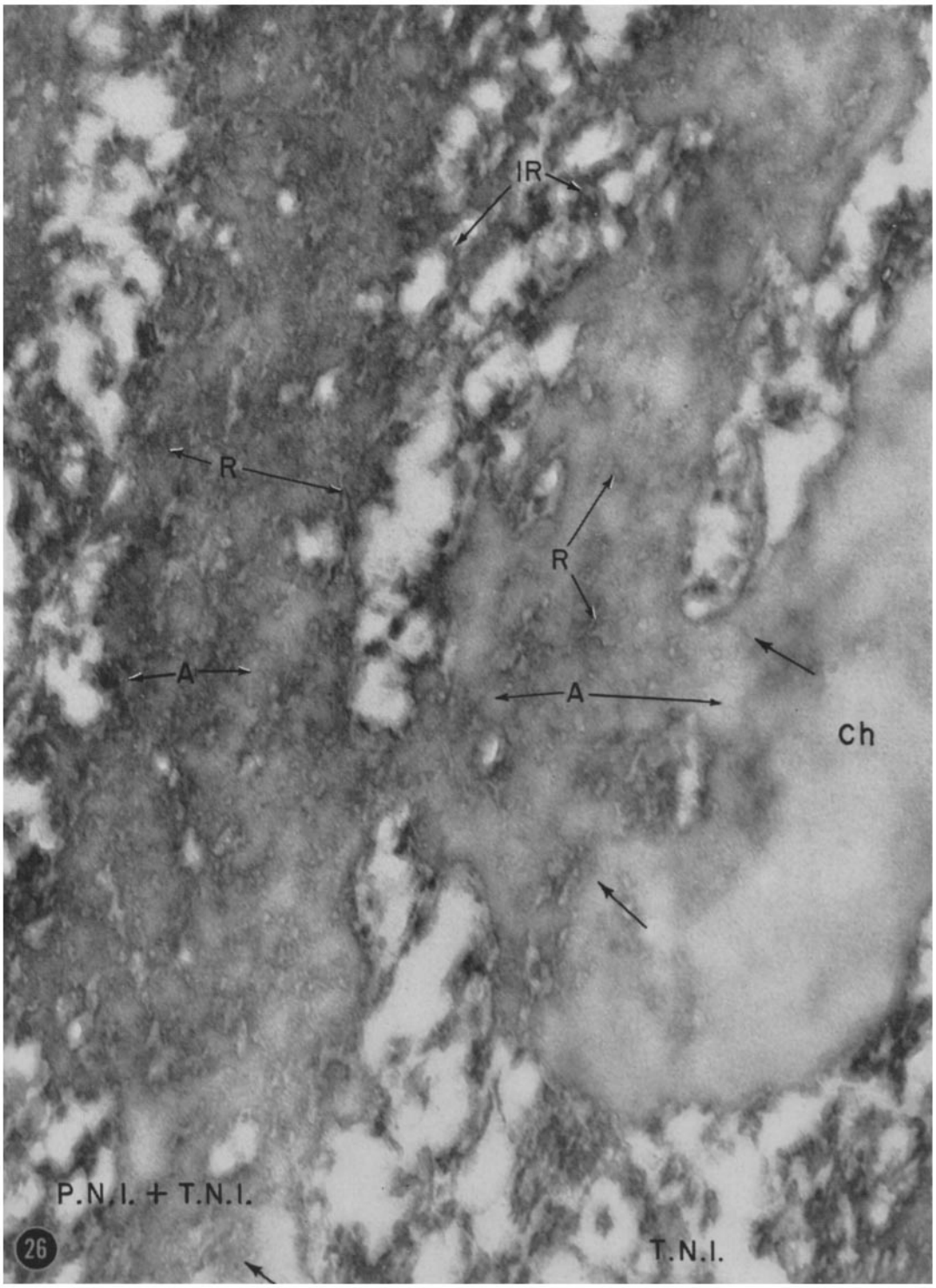
Our electron microscopic studies, based largely on cells fixed in 10 per cent neutral formalin–Tyrode and stained with phosphotungstic acid or uranyl acetate, indicate that these urodele somatic chromosomes consist of at least two main constituents: (*a*) a dense reticulum and (*b*) a material (component-A) which fills its meshes. The strands of the reticulum (40 to more than 300 A in diameter) are continuous in places with a similar network between the chromosomes. The ultraviolet-irradiated portions of the chromosomes appear to consist mainly of the reticulum, with little component-A visible. Since stages of progressive decrease of component-A are obvious in the marginal zone between the unirradiated and irradiated areas, it would seem that this component contains the DNA (or perhaps DNP) which disappears after irradiation. We have used the term "DNA-steresis" for this process.

The irregular network, visible throughout the nucleus of the interkinetic cell (in preparations fixed in neutral formalin–Tyrode and stained with phosphotungstic acid), is interpreted as forming a framework—presumably of protein—to parts of which DNA (?DNP) is attached; these two components condense to form the visible chromosomes of prophase and metaphase. They become especially dense in telophase and undergo equally striking reverse changes in the reconstructing nuclei of the daughter cells.

As the dispersed chromosomal constituents of the interkinetic nucleus aggregate and condense in foci during prophase, dispersed component-A becomes readily visible in the electron microscope as part of the chromosomes. It almost completely obscures the reticular strands in the condensed chromosome mass of late anaphase and telophase.

FIGURE 26

Enlargement of area in rectangle of Fig. 25. The steretic area is to the left (*P.N.I.* + *T.N.I.*) and its margin is indicated by arrows. Chromosome (*Ch*) shows a little mottling, a result of sectioning. *IR*, strands of interchromosomal reticulum continue into chromosomes. The chromosomal reticular strands (*R*) appear more numerous as component-A (*A*) decreases progressively from the chromosome at the right (*Ch*) to the one at the left. Irradiated, fixed and stained as described in Fig. 25. × 99,000.


IR
R
R
A
A
Ch
P.N.I. + T.N.I.
26
T.N.I.

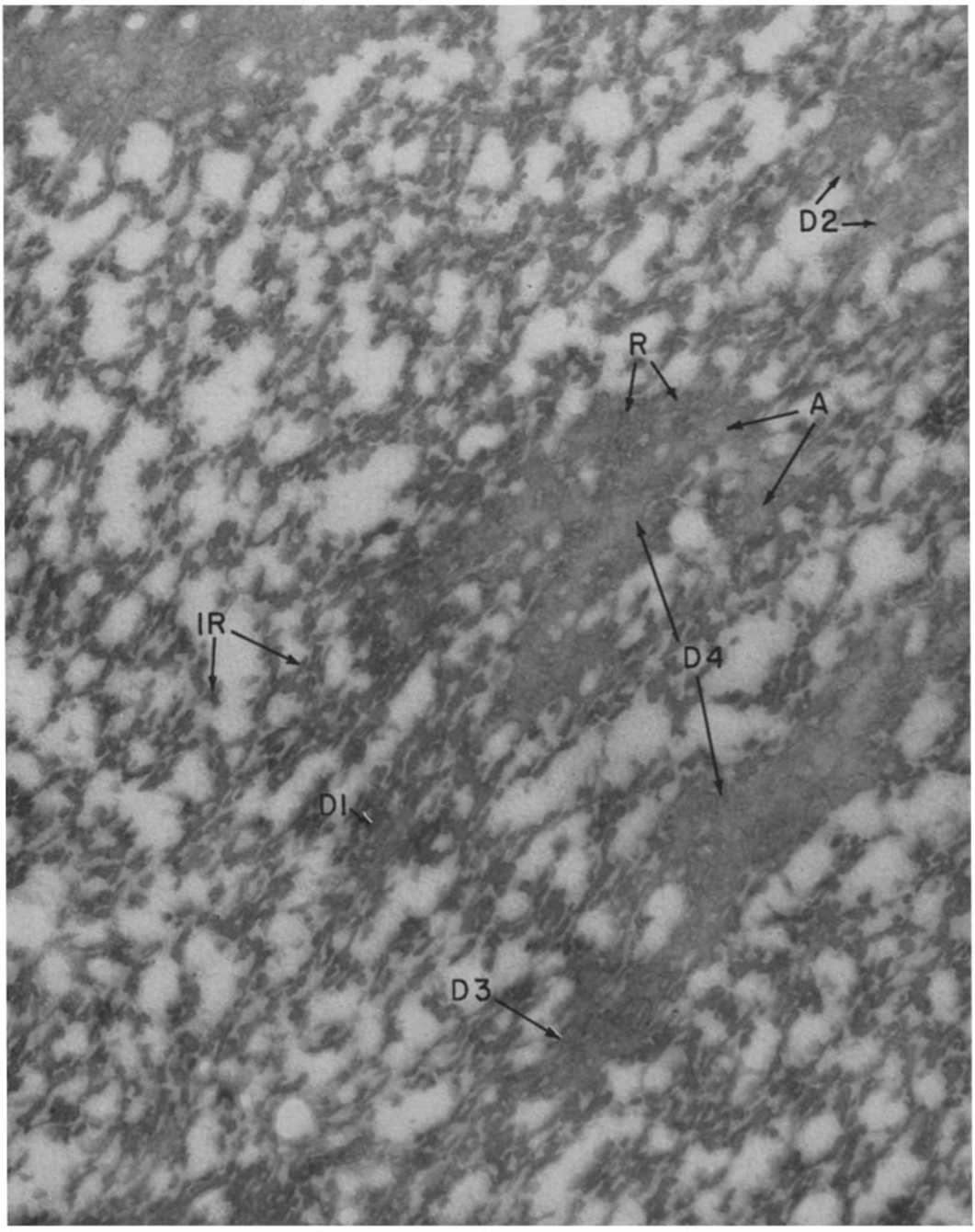


FIGURE 27

Early prophase, not irradiated, showing aggregation of strands of interchromosomal reticulum (*IR*) into progressively larger masses (*D1* to *D4*) and accumulation of component-A between the strands (*A*). *R*, chromosomal reticulum. Fixed in neutral formalin–Tyrode. Stained in alcoholic phosphotungstic acid and embedded in methacrylate. × 85,000.

When it disperses in the reconstructing nucleus the strands become visible again.

A part of the nuclear reticulum persists in metaphase as interchromosomal material connected with the chromosomal reticulum; some of the reticulum extends through the membrane of the

The failure to differentiate the irradiated from the unirradiated parts of the chromosomes with primary OsO_4 or $KMnO_4$ fixation is probably due to a failure of these substances to form an electron-opaque combination with component-A which presumably contains DNA and most of which has

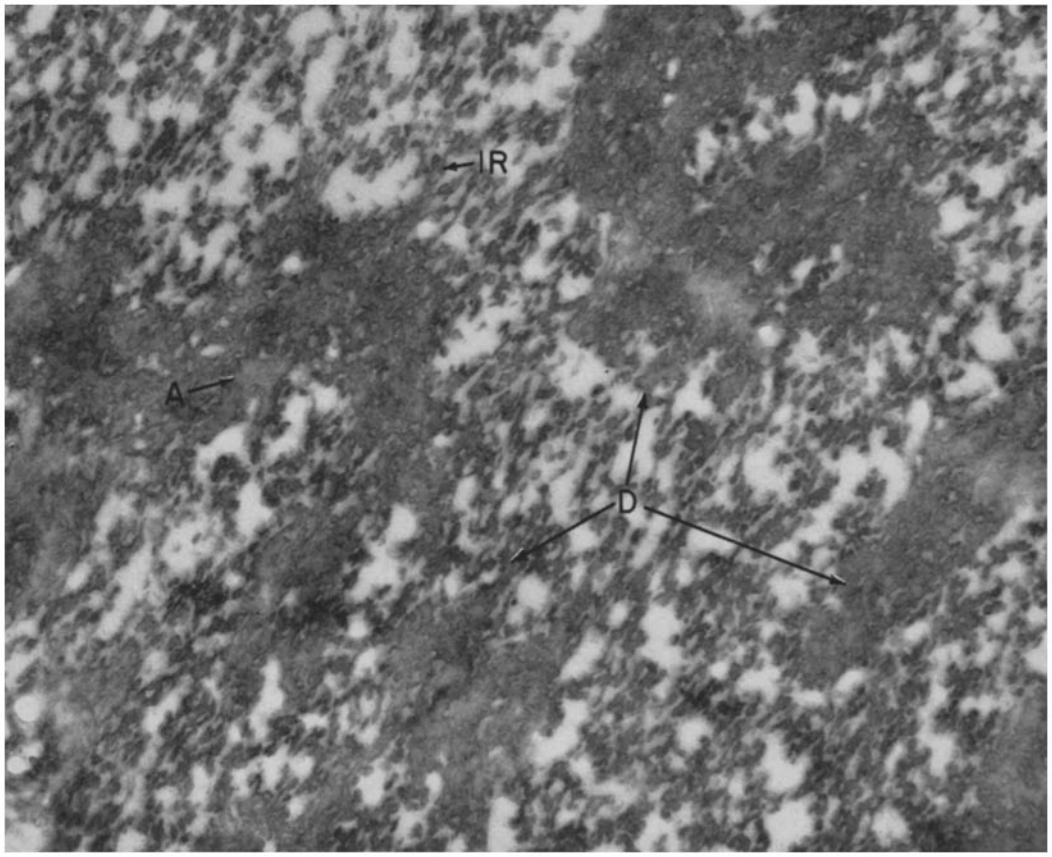


FIGURE 28

Early prophase, not irradiated, with further stages in aggregation of interchrosomal reticulum (*IR*) and component-A (*A*) into larger masses (*D*) than those shown in Fig. 27. Fixed in neutral formalin–Tyrode and stained in alcoholic phosphotungstic acid; methacrylate embedding. × 52,000.

fused telophase chromosome mass into the cytoplasm. We have not determined exactly when this connection disappears.

After primary fixation of the nuclear part of the cells with ultraviolet light, followed by treatment with formaldehyde or dilute alcohols, in the DNA-steretic segments definite but small amounts of Feulgen-positive material—and of component-A—resist the solvent action of the reagents used to prepare the cells for optical and electron microscopy.

left the irradiated portion. However, if the cell with irradiated chromosome segments is first fixed in neutral formalin–Tyrode solution or is subjected to total cell ultraviolet irradiation and then treated with 1 per cent OsO_4, this reagent will demonstrate differences between the irradiated and unirradiated parts.

Primary fixation of the whole nuclear portion of the cell by short but intense ultraviolet irradiation has been a great aid in our analysis of chromosome structure.

We are indebted to Mr. John Costa for designing and making much of the delicate apparatus used in this work and to Mrs. Esther Bohlman Patterson for her meticulous drawings of the cells.

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FIGURE 29

End of anaphase with fusion of the tails of chromosomes. (The kinetochore regions, the first parts to fuse, are far beyond the right-hand side of the micrograph.) *Ch*, chromosomes with mottled component-A. Small collections of chromosomal strands are visible at (*F*) along the lines of chromosome fusion. Strands of interchromosomal reticulum (*R'*) are attached to and penetrate the chromosomes but are visible only for short distances before being masked by component-A. Fixed in neutral formalin–Tyrode and stained with alcoholic phosphotungstic acid. Methacrylate embedding, × 42,000.

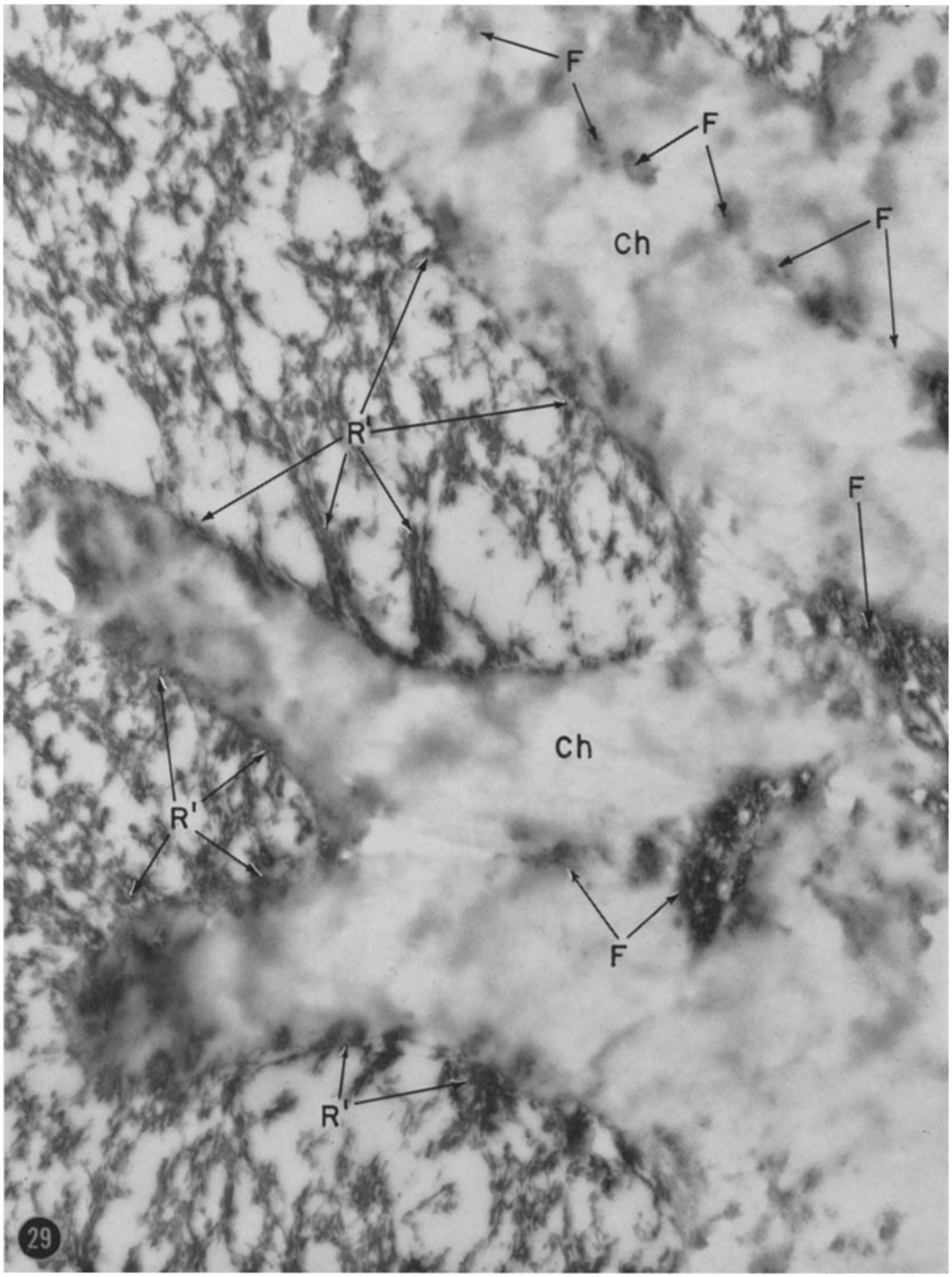

F
F
F
Ch
R'
F
Ch
R'
F
R'
29

FIGURE 30

The telophase nucleus, slightly later in the cycle than that of Fig. 29, shows scattered, faintly outlined reticular strands in the masses of homogeneous component-A (*A*) The mottling is apparently a result of compression in sectioning. A dark edge (*N*) delimits the nuclear mass from cytoplasm. Attached to this limiting structure are dark strands (*R'*) like strands (*R'*) of Fig. 29. Irregular lines and groups of chromosomal strands (*R''*) probably represent surfaces of fusion of anaphase chromosomes and, perhaps, the earliest stages of reconstruction. Fixed in neutral formalin–Tyrode and stained with phosphotungstic acid. Methacrylate embedding. × 49,500.

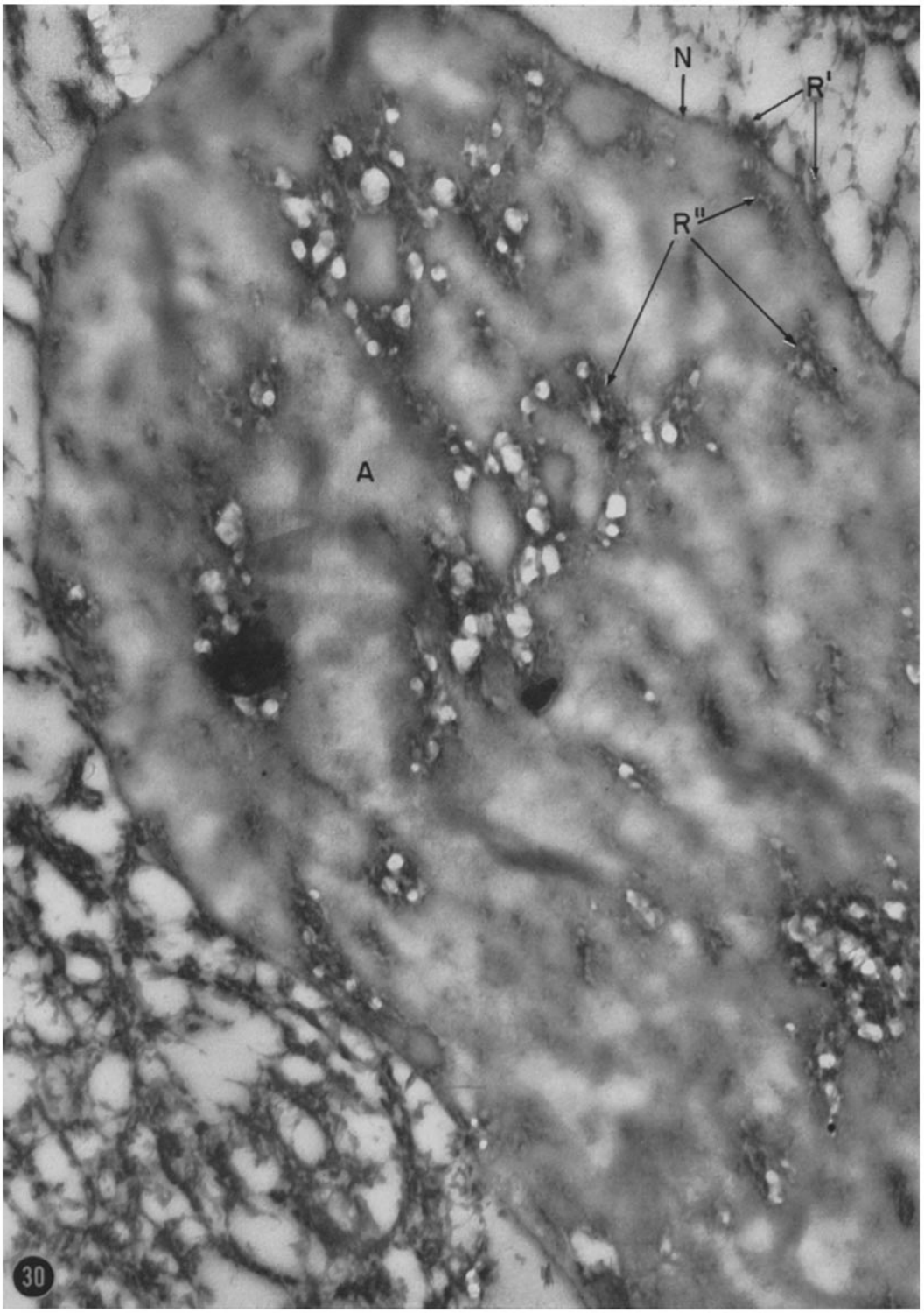
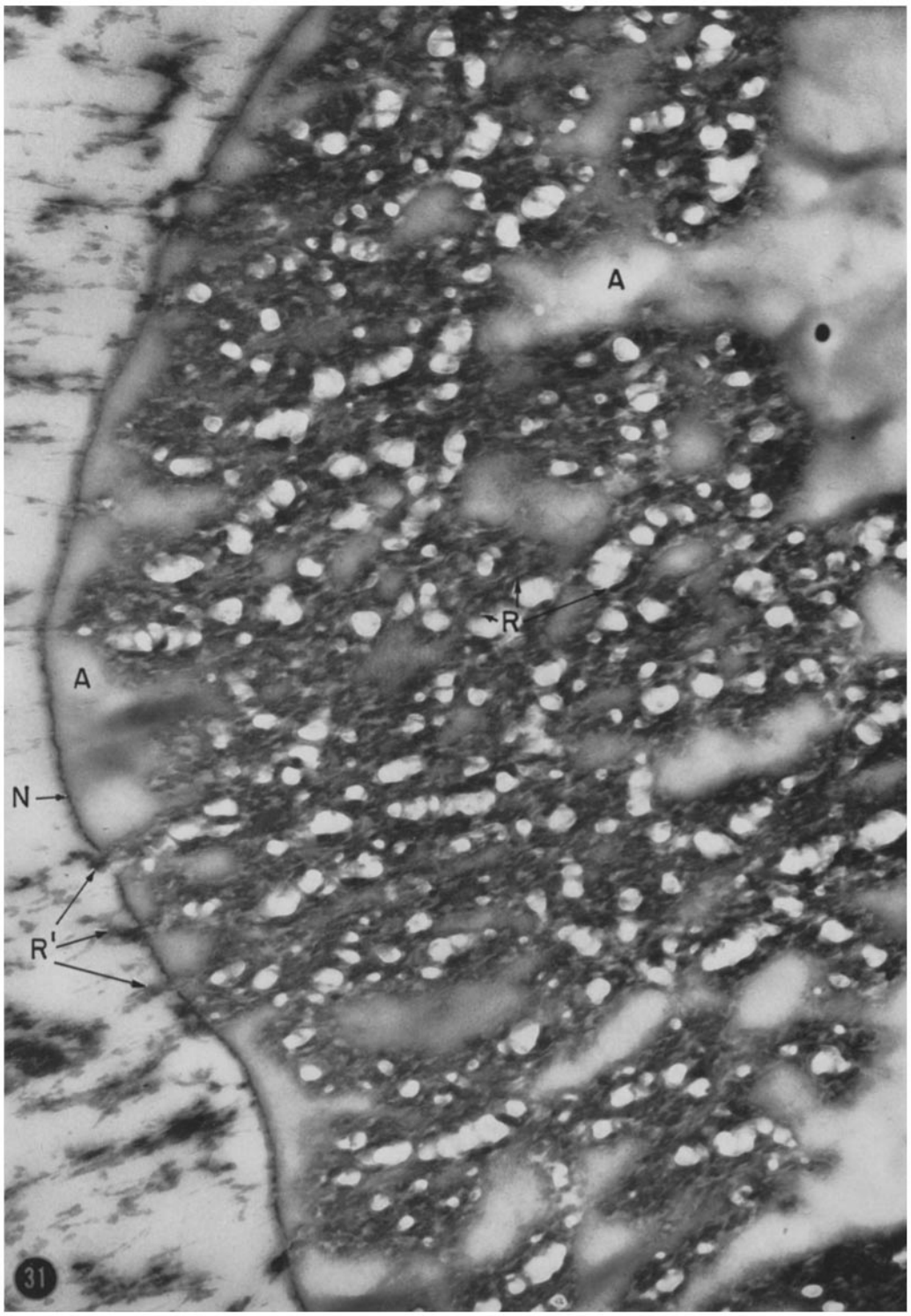

N
R'
R''
A
30

FIGURE 31

Stage in nuclear reconstruction shortly after that shown in Fig. 30. Much less component-A is visible (*A*) and much more of the chromosomal reticulum (*R*) is seen. Reticular strands (*R'*) passing through the edge of the nucleus (*N*) can still be seen. Fixed in neutral formalin–Tyrode and stained with alcoholic phosphotungstic acid. Methacrylate embedding. × 49,500.


A
R
A
N
R'
31