THE EFFECTS OF ENZYMES ON THE FINE STRUCTURE OF LAMPBRUSH CHROMOSOMES

by

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INTRODUCTION

"Lampbrush chromosomes", as Rückert (34) called them, have been found in oocytes of a number of vertebrates and invertebrates (4, 9, 15, 25). These chromosomes are the largest known and in some species of salamander attain lengths up to a millimeter (3, 15). For example, in the American newt, Triturus viridescens, the chromosomes which normally measure 10-30 μ undergo an elongation to 400-800 μ at meiotic prophase (3, 15). During this time, lateral loops develop. These loops extend 10-30 μ from the longitudinal axis of the chromosome (7, 9). Lampbrush chromosomes and their lateral loops reach their maximum extension in oocytes which are about half the size of mature oocytes (6, 15). Depending upon the species, the oocyte at this stage measures about 0.5-1 mm in diameter (15).

The main structural features of the chromosome as observed in the light microscope can be seen in Fig. 3. The axis appears to be a series of large and small granules (chromomeres) to which the lateral loops are attached.

As the oocytes continue development to maturity, the loops decrease in size while the chromomeres increase in size (6, 15). This inverse relationship between loop and chromomere size is not fully understood.

Important advances in technique made by Duryee (10, 11, 12) and by Gall (15) have influenced most of the modern studies on lampbrush chromosomes. Duryee demonstrated how easily the chromosomes may be dissected from the germinal vesicle and studied unfixed in saline solution. Gall, using a phase-contrast microscope with an inverted optical train, for the first time was able to observe intact, unfixed lampbrush chromosomes at the maximum magnification of the light microscope.

Lampbrush chromosomes have several characteristics which make them a favorable material for study: (1) The chromosomes are easily isolated manually from oocyte nuclei and provide good material for qualitative biochemical and morphological analyses of chromosomes. This property is peculiar to lampbrush chromosomes. Direct isolation of mitotic or meiotic chromosomes intact and free of contamination from other cellular components has yet to be accomplished with single cells from other sources. (2) The large size of lampbrush chromosomes facilitates observations in the light microscope. (3) There is evidence that the loops are involved in active synthesis of ribonucleic acid (RNA) and protein (18, 21, 23). The RNA has been reported to be "DNA-dependent" (23) for its synthesis and to have a base composition similar to the DNA (13). If these data are correct, then lampbrush chromosomes may prove useful in the understanding of how morphological changes in chromosome structure are related to synthesis of chromosomal RNA and protein.

The chemical composition of the various parts of lampbrush chromosomes has been studied by several investigators (8, 9, 12, 15, 21, 23). It is agreed that the Feulgen staining of the chromomeres indicates the presence of DNA (9, 15). Basic protein in the chromomeres is suggested by fast green staining (26). Although the loops are Feulgen negative, enzyme studies indicate the presence of DNA (8, 20, 26); but histone has not been demonstrated in the loops by any cytochemical method. Staining (9, 15, 26), autoradiography (18, 21, 23), and enzyme degradation (15, 26) suggest that the loops contain and synthesize RNA and protein.

The spatial arrangement of these chemical constituents in lampbrush chromosomes is not certain, and conflicting interpretations appear in the literature (2, 6, 9, 12, 14, 17, 22, 24, 28, 36). Until recently there were two completely different interpretations of lampbrush chromosome structure. One view originally suggested by Rückert (34) was supported by Ris (28). Basically, this model defined the loops as part of a long strand (or strands) continuous throughout the chromosome. Ris (28) regarded the chromomeres not as regions of linear differentiation of the chromosome but as points of overlap of the chromonemata. The other view, supported by Tomlin and Callan (36), Guyénot and Danon (22), Duryee (10, 11, 12), and Gall (14, 15) assumed the loops to be specific synthetic products of the chromomeres which were later shed. In this model, the basic unit of the chromosome was a submicroscopic thread connecting the chromomeres in some characteristic order.

Both models have been modified. Ris (24, 32) now holds that the chromomeres here, as in other chromosomes, are specific regions of tightly coiled chromonemata. The chromonemata are still regarded as continuing through both chromomeres and loops (Fig. 2). Gall (16, 17) and Callan (5, 6) revised their views and agree with Ris that the loops are part of the continuous chromonemata.

At the present time the main controversy, one which is also involved generally in the interpretation of chromosome structure, concerns the multiplicity of the chromatid (chromonema). Ris (30, 31, 32, 33), in line with his general ideas of multiplicity in chromosome structure, thinks that the loops are bundles of submicroscopic fibrils. Lafontaine and Ris (24, 25), in an electron microscope study, found the loops to contain 500 A thick fibrils which sometime appeared to consist of two units 200 A wide. The 500 A fibrils could be seen entering the chromomere regions, but the density of these regions in whole chromosome preparations did not allow the fibrils to be followed. However, they did report 500 A fibrils in cross sections of the chromosome axis. In the light microscope they also observed (their Fig. 1) two chromonemata traversing the space between chromomeres.

Gall and Callan (21) on the other hand believe that the chromomeres are connected by an interchromomeric fiber of submicroscopic dimensions. Each chromatid has a single axial fiber which is tightly coiled in the chromomeres and which continues into the loop (Fig. 2). They suggest that this fiber, continuous throughout the chromatid, is a single DNA molecule (5, 20). The predominant constituents of the loops are thought to be a matrix of RNA and protein arranged around the DNA axis (6, 17). Evidence for this model is taken from a series of investigations. Callan (3) observed that when lampbrush chromosomes are stretched, breaks occur not between chromomeres as might be expected, but in chromomere regions leaving the loops as bridges across the gap caused by the break. Several electron microscope studies have indicated that a single fiber, 200 A (36) or 200-400 A (17), connect the chromomeres. Other studies (22) have reported two 100-150 A fibrils. These size differences may be accounted for by various amounts of stretching during preparation (17).

Unfixed chromosomes have been subjected to ribonuclease, pepsin, trypsin, and deoxyribonuclease digestion (8, 20, 26). Deoxyribonuclease was the only enzyme reported to cause fragmentation of the loops and chromosome axis. This is the strongest direct evidence for DNA in the loops. Gall (20) has tried to obtain information on the number of strands from the kinetics of deoxyribonuclease action. The kinetics were reported to agree with a structure which has two subunits in the loop axis and four in the interchromomeric regions. He suggested that the two subunits in the loop axis were the two polynucleotide strands of a DNA molecule. Since each homologue has two chromatids, each having a continuous DNA molecule, there would be four subunits in the interchromomeric regions. Miller (27) has claimed that saturated potassium chloride dissolves RNA and protein from loops and leaves a single 40-50 A fibril. However, from his photographs the origin of this fibril is not clear. While these results seem to support the "Callan-Gall" model, they are often unconvincing or irreconcilable with other observations.

It seems that present techniques must be modified and new ones developed before we can interpretate the ultrastructure of these chromosomes.

It is the purpose of this study to investigate the following questions: (1) Is DNA continuous throughout the longitudinal axis and loops? (2) What is the degree of multiplicity of DNA in the axis and loops? (3) How is the RNA and protein present in the loops related to loop structure.

MATERIALS AND METHODS

Eggs from ovaries of the newt, Triturus viridescens, were used as the source of lampbrush chromosomes. After removal from anaesthetized animals, the ovaries were transferred to a glass container, covered and sealed to prevent evaporation, which was then kept in an ice bath until used. Portions of ovaries were then transferred to a 5:1 mixture of 0.1 M potassium chloride and sodium chloride (15) or Callan and Lloyd's medium C (7 parts, 5:1 KCl:NaCl and 3 parts 0.001 M potassium dihydrogen phosphate) (8). The nuclei from eggs 0.6-1.0 mm in diameter were isolated with the aid of fine forceps. The nuclei were cleaned rapidly with a braking pipette and transferred to a specially prepared slide originally designed by Gall (15) for use with an inverted microscope. A 1/4" hole was bored in the center of a slide 1.5" X 1" and a cover slip sealed over the hole with paraffin so as to produce a flat-bottomed well (Fig. 2). The nuclei were transferred to such a well containing saline and the nuclear membranes removed with the aid of fine forceps. With this procedure, chromosomes float free and sink to the bottom of the well. For light microscope studies, the well was sealed with a coverslip and the chromosomes observed unfixed with a Zeiss inverted phase microscope.

In order to attach the large chromosomes to a coverslip, the slide was centrifuged 5-10 minutes in a block designed for use in the International Clinical centrifuge (Fig. 1). The chromosomes can then be fixed in 10% neutral formalin for 15 minutes to an hour, dehydrated in an alcohol series and mounted. Heidenhain's hematoxylin was used for staining the chromosomes.

For electron microscopy, titanium grids were covered with a thin formvar membrane, carboned, and placed in the well. The chromosomes were isolated over the grids and the preparations centrifuged and fixed as described above. After fixation, the preparations were dehydrated in alcohol and transferred to amyl acetate, from which they were either dried in air or according to Anderson's (1) critical point method.

For enzyme studies, solutions of deoxyribonuclease (DNase), ribonuclease (RNase), trypsin, pepsin (Worthington Biochemical Corporation, Freehold, New Jersey) and Pronase (California Corporation for Biochemical Research, Los Angeles, California) were made up at concentrations of 1 mg/ml in Callan and Lloyd's medium C and adjusted to pH 6.2 (7). The effects of these enzymes on chromosome structure, before or after centrifugation, was studied by adding the enzyme solutions to wells to give a final concentration of 0.5 mg/ml. The action of the enzymes on the chromosomes was observed at room temperature with an inverted phase microscope. For treatment with more than one enzyme, the preparations were rinsed in saline before the second enzyme was added. After enzyme treatment, the preparations were rinsed in saline, fixed, and dehydrated as described above. After protease treatment, some preparations were transferred directly to absolute alcohol for fixation. The chromosomes are easily seen through the holes in the grids so that enzyme effects of selected regions can be studied at higher magnification in the electron microscope.

Centrifuged and uncentrifuged preparations were treated with saturated potassium chloride, rinsed with saturated ammonium sulfate, fixed in absolute alcohol and stained with uranyl acetate according to Miller (27). In some preparations, distilled water was substituted for ammonium sulfate. Staining was carried out in 1% aqueous uranyl acetate for an hour.

Chromosomes were spread on an air-liquid interface by pipetting isolated nuclei into 30% sucrose. The nuclei rise rapidly to the surface and burst. Carbon coated grids were touched to the surface, fixed in 10% neutral formalin, ethanol, or osmic acid vapors dehydrated in ethanol and air dried from amyl acetate.

All electron microscopic observations were made with a Siemens Elmiskop 2b electron microscope. The preparations were photographed on Kodak medium projector slide plates and developed in Kodak D-19 for four minutes.

RESULTS

GENERAL:

Lampbrush chromosomes isolated as described above can be kept in saline for several hours without any observable changes in structure. However, breaks and distortion are produced by the strong diffusion currents that occur on addition of solutions during fixation and dehydration. It was thought that these artefacts might be minimized by attaching the chromosomes to a coverslip or grid. This can be accomplished by centrifugation which does not seem to change the morphological appearance of the chromosomes.

For electron microscopy, these preparations must be dried. Air drying of uncentrifuged chromosomes from amyl acetate caused considerable breaking and clumping of chromosomes. Those dried by Anderson's critical point method are much less distorted. However, centrifuged chromosomes dried by either of these methods show essentially the same structure with a minimum of distortion.

1. Fixed Chromosomes:

(A) Loop Structure:

When studied with the light microscope, loops are seen to vary in size and thickness but otherwise appear morphologically similar (Fig. 3). Most of the loops in centrifuged and stained preparations appear to be coiled structures (Fig. 3,c). At the higher magnifications of the electron microscope, the loops appear as bundles of coiled and twisted microfibrils 200-250 A thick (Figs. 4-8). This is especially clear in stereoscopic micrographs (Figs. 4 and 5). The fuzzy appearance of loops at low magnifications in the electron microscope is due to fraying of loop microfibrils, as can be seen at higher magnifications (Figs. 4-10). Some of these 200-250 A fibrils appear individually coiled (Figs. 5 and 8) and in places two of these appear to be coiled together in a helical configuration to form a fibril approximately 500 A wide (Figs. 5 and 6, D). In formalin-fixed preparations, the smallest fibrils measure 200-250 A. Portions of the length of these fibrils are thinner, but these are obviously stretched regions as indicated by the gradual decrease in width along the length of a fibril from 200-250 A to 100 A or smaller. Such stretched fibrils are found in all preparations. Fibrils twice as thick are found after ethanol fixation.

(B) Structure of the Chromosome Axis:

In the light microscope, the chromosome axis appears as a row of irregular granules (chromomeres). At this level of resolution, there appear to be gaps between the chromomeres (Fig. 3). However, in uncentrifuged preparations, the distance between chromomeres on either side of these gaps does not change perceptibly when the chromosomes are being moved about by Brownian motion. This is taken as an indication of some submicroscopic connection. In some regions of fixed and stained preparations (Fig. 3, IC), suggestions of interchromomeric connections are barely visible with the light microscope. In the electron microscope, chromomeres are generally too dense to show any internal detail. However, in stereoscopic micrographs (Fig. 4), dense regions appear to result from a complex coiling and overlapping of strands continuous with the loops. A thin axial "fiber" connecting chromomeres at low magnification appears single (Fig. 9) but at higher magnification is seen to be a stretched region containing more than one strand (Fig. 10).

2. Enzyme Studies:

(A) The Effects of RNase, Trypsin, Pepsin and Pronase on Loop Structure:

Ten to thirty minutes after addition of any one of these enzymes (conc. 0.5 mg/ml) to uncentrifuged and unfixed preparation, loops seem to have disappeared and all that remains visible are "stumps" projecting from the chromosome axis. In the electron microscope, these stumps are too dense to reveal any detail. In contrast to this, the loops of centrifuged unfixed chromosomes remain clearly visible but get "thinner". Attachment of loops by centrifugation therefore appears to prevent the "contraction" on the axis suggested by Gall (15) and others (23).

In the electron microscope, many free 200-250 A fibrils (Figs. 11 and 12) are seen projecting from the frayed loops of RNase-treated chromosomes. Some regions of the loops are "loosened" or "uncoiled" (Figs. 11 and 12) and others are more tightly packed (Figs. 11 and 12). In unstretched regions, 200-250 A fibrils are seen to contain two subunits about 100 A wide (Figs. 11 and 12, D). Denser regions sometimes reveal 500 A fibrils (Fig. 11, C).

The disorganization in loops of trypsin-digested chromosomes is somewhat similar to that seen after RNase treatment. Fibrils 500 A wide (Fig. 13, C) are seen in dense regions, 200-250 A fibrils (Fig. 13, B) in frayed ends and "uncoiled" regions, and two 100 A subunits in the 200-250 A fibrils (Fig. 13, D). However, the loops generally contain less material and are more closely packed. Chromosomes incubated with RNase, rinsed in saline, and then treated with trypsin were found to look essentially the same (Fig. 14).

Loops of chromosome preparations treated with pepsin (Figs. 15 and 16) at the same concentration and for the same length of time (0.5 mg/ml for 30 min.) appear to contain fewer fibrils than those treated with trypsin or RNase. Electron micrographs (Figs. 15 and 16) show that the remaining loop structure consists of fibrils 200-250 A wide (Fig. 16, B) clumped in places to form 500 A fibrils (Fig. 16, C) and stretched into thinner regions elsewhere (Fig. 16, A).

In comparison to trypsin and pepsin, the effect of pronase on loop structure is quite different. As observed with the light microscope, loop digestion is much more rapid. Fifteen minutes after addition of pronase (0.5 mg/ml), the loops are barely visible. In some preparations the action of pronase was stopped after a shorter time (5-10 minutes) by rinsing and fixing while the loops could still be seen. Electron microscopic analyses of chromosomes prepared in this way (Figs. 18 and 19) show most of the usual 200-250 A fibrils split into 100 A fibrils. Some of these 100 A fibrils can be followed for considerable distances (Fig. 19).

It is interesting to note that at the light microscopic level neither RNase, trypsin, pepsin, nor pronase were observed to cause breaks across the chromosome or loop axis. However, even the most carefully prepared chromosomes, enzyme treated or untreated, invariably reveal some breaks visible in the electron microscope. Further careful attention will have to be given this specific problem since any breaks caused by enzymatic digestion would figure in the final elucidation of loop fine structure.

(B) The Effect of DNase on Loop Structure:

The action of DNase is dramatically different from the effects of the previously discussed enzymes. A few minutes after addition of the enzyme to uncentrifuged preparations, many loop and axial breaks are evident. The fragments produced are rapidly scattered by Brownian motion and after 30 minutes of digestion by enzyme at a concentration of 0.5 mg/ml all that remains of the chromosomes are many tiny fragments. In the electron microscope (Fig. 17), loops of partially digested chromosomes appear grossly frayed and broken. The complex coiling and packing of microfibrils is loosened, and the multiplicity of fibrils within the loops is clearly revealed. The broken fragments do not seem to have a characteristic size. They range from single pieces of 200-250 A fibrils to short loop fragments 50 mμ to 800 mμ in length. In many places partial breaking of the bundle of fibrils results in connection between loop fragments by one or several 200-250 A fibrils (Fig. 17, C).

(C) The Effect of Enzymes on the Chromosome Axis:

The chromomeres of preparations treated with pepsin (Fig. 15), trypsin (Fig. 14), or RNase (Fig. 11) are similar in appearance. Most of them are densely packed. However, in some regions they are loosened so that individual fibrils can be seen within these regions. Also, loop fibrils, in some cases, may be seen to continue into the interchromomeric region. In many places chromomeres are seen to be connected by several microfibrils (Figs. 15 and 18). The complex packing of microfibrils in chromomere regions is seen better in chromosomes treated with pronase (Fig. 18). In the light microscope the chromomeres are seen to swell visibly; and, after about 15 minutes, they are barely visible. In the electron microscope these swollen chromomeres resemble a tangle of 100 A fibrils (Fig. 18). The chromomeres of DNase-treated chromosomes are also loosened up to reveal a network of fibrils within them (Fig. 17). Also, however, DNase is the only enzyme observed to produce breaks (Fig. 17, BK) along the chromosome axis.

3. Spread Chromosomes:

Isolated nuclei pipetted into 30% sucrose solution rise rapidly to the surface and burst. Violent diffusion currents are seen as the nuclear contents are spread on the surface. Electron micrographs of such preparations (Fig. 20) show the same kinds of fibrils as found in whole chromosome preparations. Most of these fibrils, however, are uncoiled and stretched. Stretching of some fibrils produces regions that measure 40-50 A wide. Identification of loop and chromomere regions in most of these preparations is not possible. Enzyme studies to determine the chemical composition of these fibrils are in progress at the present time.

4. The Effect of Saturated KCl on Chromosomes:

When saturated KCl is added to uncentrifuged preparations, diffusion currents stretch and break the chromosomes. After periods of up to 30 minutes incubation, the chromosomes are somewhat clumped. Dissolution of any part of the chromosomes was not observed. In the electron microscope, the clumped chromosomes reveal little detail except in stretched regions where fibrils range from less than 100 A to 500 A in thickness.

DISCUSSION

1. The Effects of Methods of Preparation on Chromosome Structure.

Proponents for both models of lampbrush chromosome structure have presented observations with the electron microscope as support for their views. However, different methods of preparation of chromosomes were used in making these conflicting observations. It has been our experience that the method of preparation has a profound effect on the fine structure of lampbrush chromosomes.

There are three conditions that have to be satisfied before whole lampbrush chromosomes, reasonably preserved, can be studied in the electron microscope. These are attachment to a substrate, fixation, and dehydration.

Procedures that result in unattached or partially attached chromosomes are very frustrating since most of the chromosomes are washed away during subsequent treatments. More important, however, is that such preparations are grossly distorted. Gall (19) has used fixation in formaldehyde vapors as a method of attachment. In many cases, this procedure results in only partially attached chromosomes. Gall thinks that the attachment in formaldehyde vapor is due to small traces of formic acid in the commercial formaldehyde and is, therefore, a direct pH effect. He has used chromosomes prepared by this method for electron microscopy, but these preparations are unsuitable for detailed analyses. It is not surprising that his preparations reveal little detail since it is generally recognized that acid fixation contracts chromosomes (19, 26, 32). It should be emphasized that the loops in Gall's preparations are severely clumped.

Centrifugation, on the other hand, attaches the loops without shrinkage or clumping; and significant changes in their morphological appearance are not observed after fixation in neutral formalin. It has been recognized by many investigators (19, 26) that fixation near neutrality preserves chromosomes in an "extended state". To study chromosomes in the electron microscope in this extended state, gentle dehydration procedures must be used. Air drying from water or ethanol collapses, breaks or otherwise distorts the chromosomes. Those gradually dehydrated in ethanol and dried by the critical point method (1) or from amyl acetate are much less distorted. It seems likely that the different degrees of preservation of chromosomes by these methods are responsible for the conflicting views of lampbrush chromosome structure.

2. Structure of the Loops

We have found that the loops appear as bundles of fibrils when chromosomes are prepared with the modifications developed in this paper. This agrees with Lafontaine and Ris (25) who reported in the loops 500 A fibrils which sometimes appeared to have 200 A subunits. In addition, we have found evidence for subdivision of the 200-250 A fibrils into 100 A units. The demonstration of similar fibrils (Fig. 20) in chromosomes spread on water is further support for the multistranded nature of loops. However, since the chromosomes are almost completely "uncoiled" by spreading, distinction between loop and chromomere regions can rarely be made.

These observations are not in agreement with Gall's (16) studies utilizing thin sections. On the basis of these studies, he claims that the loops are "composed of loosely associated granules which generally do not exceed 300 to 400 A in diameter". Since in electron micrographs of intact chromosomes the loops appear as bundles of coiled and twisted microfibrils, we must conclude that Gall's "granules" are really sections through microfibrils.

It was mentioned in the introduction that the loops consist primarily of RNA and protein and that, although Feulgen negative, they are fragmented by DNase. It is impossible at present to identify the chemical constituents of the loop fibrils in the electron microscope. Gall (16, 17) attempted to do this by using specific enzymes. He described the loop structure remaining after pepsin treatment as a single fibril 200-400 A wide. We have not been able to confirm this. The remaining loop structure seen in Fig. 16 is clearly multifibrous. Similarly, RNase and trypsin remove considerable material from the loops, but the fibrils in the remaining structures resemble those in untreated preparations. So far, no definite conclusions can be drawn from these experiments as to the chemical nature of the fibrils.

Miller (27) has also claimed that a single fibril forms the axis of loops. However, the origin of his fibril is unclear in his photographs. We have prepared chromosomes according to Miller's method. These preparations do not reveal a single fibril as the loop axis except perhaps in regions where many of the fibrils are broken and a single remaining fibril spans the gap.

Indirect evidence for a single fibril is claimed in several studies (5, 20, 21), the most recent being Gall's observations on the kinetics of DNase action. It is important to recognize that his data were obtained with the light microscope. If a similar preparation is observed in the electron microscope, one sees that areas which appear as breaks in the light microscope are still connected by fibrils visible only in the electron microscope (Fig. 17,C). We, therefore, question that Gall's kinetic studies can be used to prove that continuity in loops is based on a single molecule of DNA. Such studies must be extended to the electron microscope level.

3. The Structure of the Chromomeres.

Lafontaine and Ris (25) have described the chromomeres as dense regions of tightly packed microfibrils which resemble those in the loops. Such a structure of chromomeres is confirmed by this study and is very evident in the chromosomes treated with DNase (Fig. 17) or Pronase (Fig. 18). In these preparations, the chromomeres are "loosened" and reveal the constituent microfibrils quite clearly. In view of the different chemical composition of loops and of chromomeres, it is surprising that the fibrils are similar both in appearance and dimensions. At present, the structure of the chromomeres can only be described as a complex network of microfibrils. The action of the crude protease, Pronase, is interesting in this respect. After several minutes of treatment with this enzyme, the chromomeres are swollen and "loosened" to reveal many 100 A fibrils in contrast to the usual 200-250 A fibrils (see section V for further discussion). These observations are an improvement on Gall's (16) report that the chromomeres are "relatively homogeneous bodies in which no internal detail has yet been seen".

4. Interchromomeric Connections.

Interchromomeric connections are claimed by Callan (7) and by Gall (17) to be submicroscopic. However, Lafontaine and Ris (25) reported seeing occasional connections approximating the size of loop bases (Fig. 1 (25)) with the light microscope. Connections barely visible in the light microscope were seen in the present study (Fig. 3).

In the light microscope, the numerous loops usually obscure any detailed analyses of axial structures. However, in many stretched regions chromomeres appear to be separated by a gap. In the electron microscope, some of these gaps are spanned by fibrils which would not be visible in the light microscope. Gall's Fig. 12 (17), which he uses as evidence for a single submicroscopic fiber connecting chromomeres, is similar to Fig. 9 in this study. However, in Fig. 10, we can see that the same apparently single fiber is resolved into more than one fibril at higher magnifications. Figure 15 clearly shows many fibrils connecting chromomeres after pepsin treatment. Whenever interchromomeric connections could be distinguished, they were multifibrous. The nature of interchromomeric connections is not yet clear, and further study will be needed before we can interpret their role in lampbrush chromosome structure.

5. Summary of Fine Structure as Indicated by Enzyme Treatments

Most of this study has dealt with the effects of enzymes on the fine structure of lampbrush chromosomes in an attempt to answer the questions stated in the introduction: (1) Is DNA continuous throughout the longitudinal axis and loops? (2) What is the degree of multiplicity of DNA in the axis and loops? (3) How is the RNA and protein present in the loops related to their structure?

The action of DNase indicates that DNA is present both in loops and axis of lampbrush chromosomes. However, whether DNA is continuous or joined by "linkers" such as have been suggested (35) remains an open question.

It is too early to make any predictions about DNA multiplicity in the loops. The action of Pronase on chromomeres has interesting implications. After treatments with this enzyme the usual 200-250 A fibrils are split into 100 A units. This may be compatible with Ris' view that 100 A nucleohistone fibrils are the main constituent of chromosomes. Ris (33) suggests that the 100 A fibrils contain two DNA molecules with associated histones. If this is the case, then the 200-250 A fibrils contain four DNA molecules. However, we must consider the experiments with Pronase as preliminary at this time.

The same is true for RNase, trypsin and pepsin treatments. These experiments have so far not produced any evidence which would identify RNA or protein in loop fibrils.

It is obvious that this study has not answered the original questions, nor has it added significantly to our knowledge of the stereochemistry of the fibrils in lampbrush chromosomes. However, we feel that it leaves little doubt that the loops and chromomeres consist of fibrils below the level of resolution of the light microscope.

Further studies with whole chromosomes as well as with thin sections might be helpful in determining the nature of these fibrils. Serial sections would have to be examined since profiles in random sections would be of little help in reconstructing three-dimensional patterns. The enzyme experiments reported here will have to be repeated with proper controls. It is hoped that a combination of enzyme studies on whole chromosomes and thin sections will shed some light on the fine structure of lampbrush chromosomes.

REFERENCES

1. Anderson, T. F. (1956). Physical Techniques in Biological Research, p. 177, A. W. Pollister and G. Oster, ed. New York: Academic Press.

2. Callan, H. G. (1952). A general account of experimental work on amphibian oocyte nuclei. Symp. Soc. Exp. Biol., 6, 243.

3. Callan, H. G. (1955). Recent work on the structure of cell nuclei. Symposium on Fine Structure of Cells. I.U.B.S. publ. Series B 21, 89.

4. Callan, H. G. (1957). The lampbrush chromosomes of *Sepia officinalis* L., *Anilocra phyrodes* L., and *Sycyllium Catulus* Cuv. and their structural relationship to the lampbrush chromosomes of amphibia. Pubb. Staz. Zool. Napoli, 29, 329.

5. Callan, H. G. (1963). Proc. 11th Internat. Cong. Genetics (In press).

6. Callan, H. G. and Lloyd, L. (1960). Lampbrush chromosomes. Internat. Congress of Zoology Symposium on New Approaches in Cell Biology, p. 23. London and New York: Academic Press.

7. Callan, H. G. and Lloyd, L. (1960). Lampbrush chromosomes of crested newts *Triturus Cristatus* (Laurenti). Phil. Trans. Roy. Soc. (London) B243, 135.

8. Callan, H. G. and Macgregor, H. C. (1958). Action of deoxyribonuclease on lampbrush chromosomes. Nature, 181, 1479.

9. Dodson, E. O. (1948). A morphological and biochemical study of lampbrush chromosomes of vertebrates. Univ. Calif. publ. Zool., 53, 281.

10. Duryee, W. R. (1937). Isolation of nuclei and non-mitotic chromosome pairs from frog eggs. Arch. Exp. Zellforsch., 19, 171.

11. Duryee, W. R. (1941). The chromosomes of the amphibian nucleus. University of Pennsylvania Bicentennial Conference on Cytology, Genetics and Evolution, p. 129. Philadelphia: Univ. Pennsylvania Press.

12. Duryee, W. R. (1950). Chromosome physiology in relation to nuclear structure. Ann. N. Y. Acad. Sci., 50, 920.

13. Edström, J. and Gall, J. G. (1963). The base composition of ribonucleic acid in lampbrush chromosomes, nucleoli, nuclear sap, and cytoplasm of Triturus oocytes. J. Cell Biology., 19, 279.

14. Gall, J. G. (1952). The lampbrush chromosomes of Triturus viridescens. Exp. Cell Res. (suppl.), 2, 95.

15. Gall, J. G. (1954). Lampbrush chromosomes from oocyte nuclei of the newt. J. Morph., 94, 283.

16. Gall, J. G. (1956). On the submicroscopic structure of chromosomes. Brookhaven Symp. Biol., 8, 17.

17. Gall, J. G. (1958). Chromosomal differentiation. McCollum-Pratt Symposium on The Chemical Basis of Development, p. 103. Baltimore: Johns Hopkins Press.

18. Gall, J. G. (1959). Uptake of nucleic acid precursors by oocytes of the newt (Triturus viridescens). Records of the Genetics Society of America., 28, 72.

19. Gall, J. G. (1962). Personal Communication.

20. Gall, J. G. (1963). Kinetics of deoxyribonuclease action on chromosomes. Nature., 198, 36.

21. Gall, J. G. and Callan, H. G. (1962). H^3-uridine incorporation in lampbrush chromosomes. Proc. Nat. Acad. Sci., 48, 562.

22. Guyénot, E. and Danon, M. (1953). Chromosomes et ovocytes de Batraciens. Revue Suisse de Zoologie, 60, 1.

23. Izawa, M., Allfrey, V. G. and Mirsky, A. E. (1963). The relationship between RNA synthesis and loop structure in lampbrush chromosomes. Proc. Nat. Acad. Sci., 48, 544.

24. Lafontaine, J. and Ris, H. (1955). A study of lampbrush chromosomes with the electron microscope. Genetics, 40, 579.

25. Lafontaine, J. and Ris, H. (1958). An electron microscope study of lampbrush chromosomes. J. Biophys. Biochem. Cytol., 4, 99.

26. Macgregor, H. C. and Callan, H. G. (1962). The action of enzymes on lampbrush chromosomes. Quart. J. Mich. Sci., 103, 173.

27. Miller, O. (1961). Annual Meeting of the American Society for Cell Biology. Abstracts , p. 142.

28. Ris, H. (1945). The structure of meiotic chromosomes in the grasshopper and its bearing on the nature of Chromomeres and Lampbrush Chromosomes. Biol. Bull. Wood's Hole, 189, 242.

29. Ris, H. (1952). The submicroscopic structure of chromosomes. Genetics, 37, 619.

30. Ris, H. (1955). The submicroscopic structure of chromosomes. Symposium on Fine Structure of Cells. I.U.B.S., Publ. Series B., 21, 121.

31. Ris, H. (1956). A study of chromosomes with the electron microscope. J. Biophys. Biochem. Cytol. 2 (Suppl.), p. 385.

32. Ris, H. (1957). Chromosome structure. McCollum-Pratt Symposium on The Chemical Basis of Heredity, p. 23. Baltimore: Johns Hopkins Press.

33. Ris, H. (1961). Ultrastructure and molecular organization of genetic systems. Can. J. Genet. Cytol., 3, 95.

34. Rückert, J. (1892). Zur Entwicklungsgeschichte des Ovarialeies bei Selachiern. Anat. Anz., 7, 107.

35. Taylor, H. (1963). The replication and organization of DNA in chromosomes. Molecular Genetics, p. 65. Academic Press: New York and London.

36. Tomlin, S. G. and Callan, H. G. (1951). Preliminary account of an electron microscope study of chromosomes from newt oocytes. Quart. J. Micr. Sci., 92, 221.

Fig. 1. (a) Sectional view of well slide.
(b) Centrifuge block with slide.
(c) Block in position for centrifugation

N	Nucleus	BC	Bottom coverslip
G	Grid	TC	Top coverslip
S	Slide	CA	Arm of centrifuge head

Arrow indicates direction of centrifugal force.

Fig. 2. Diagrams of postulated loop structure.

(A) Single stranded model (After Gall (17)).

(B) Multifibrous model

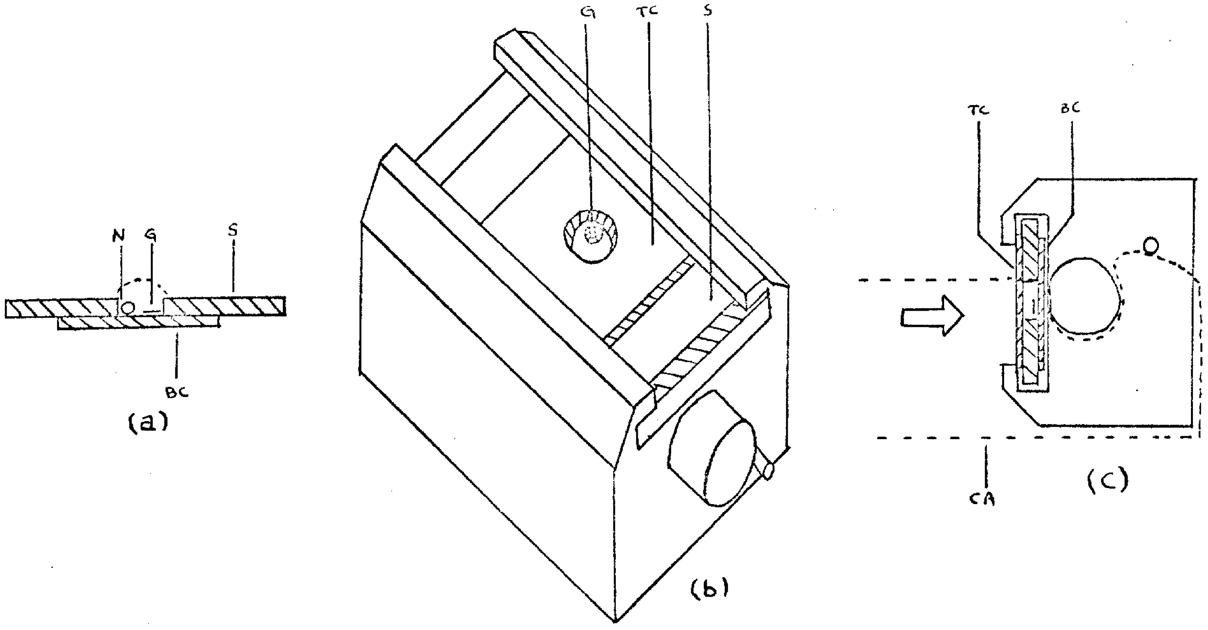

Fig 1.

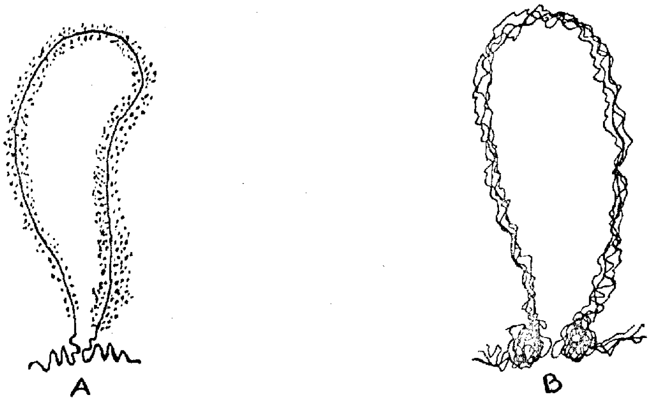

Fig. 2

Fig. 3. Light micrograph. A portion of a bivalent, centrifuged, fixed in 10% neutral formalin and stained with Heidenhain's hematoxylin. Gaps (g) can be seen along the chromosome axis between chromomeres. However, in some stretched regions the gap is spanned by an interchromomeric (IC) connection. Note coiled appearance of loops (C). X 2700

Fig. 4. Stereomicrograph* of centrifuged chromosome fixed in 10% neutral formalin and dried by Anderson's critical point method. Note that the loops are bundles of coiled and twisted microfibrils. The dense region (C) results from overlapping of several bundles of fibrils.

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*Figs. 4-20 are all electron micrographs.


IC
G
IC
C
3

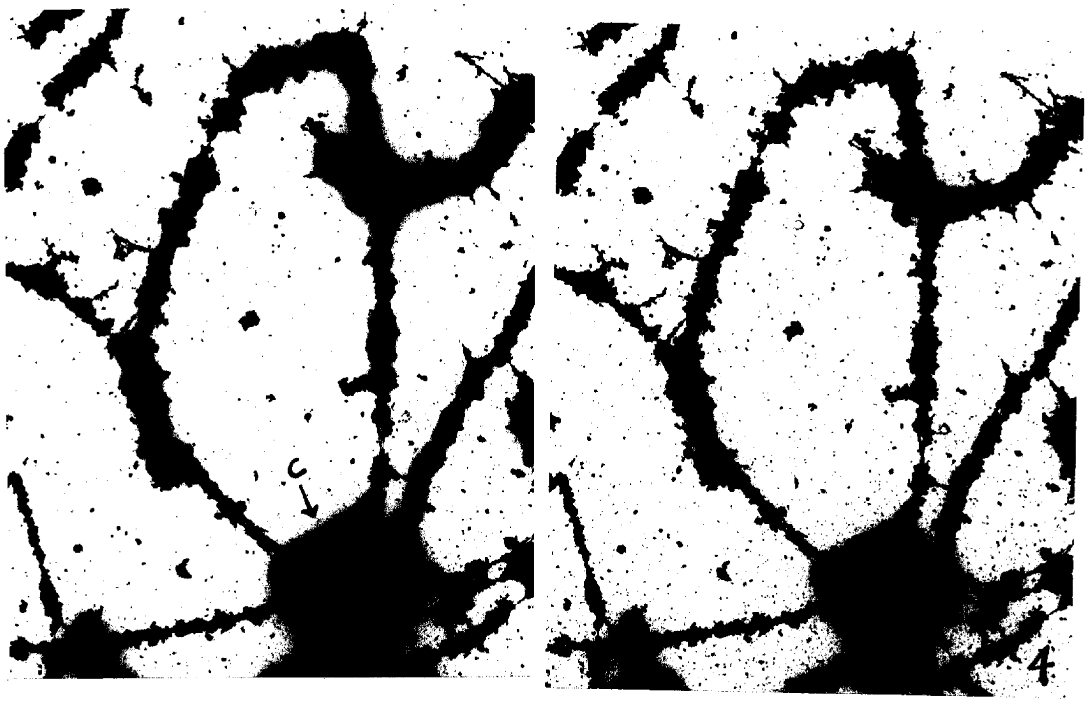
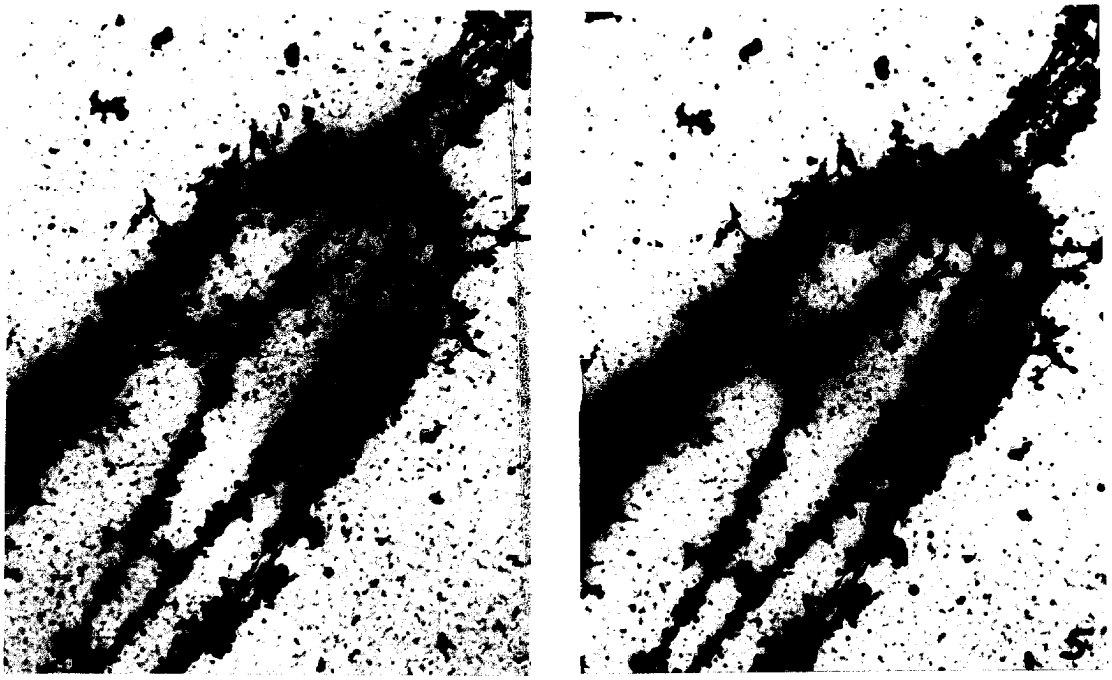

C
4

Fig. 5. Portion of a loop prepared as in Fig. 4. The multiplicity of 200-250 A fibrils (B) is clearly demonstrated by this stereomicrograph. Some of the fibrils show a single coiled structure (S). In other regions two 200-250 A fibrils can be seen coiled together in a helical configuration (D). X 20,000

Fig. 6. Higher magnification of upper region of Fig. 5. See Fig. 5 for explanation. X 59,000



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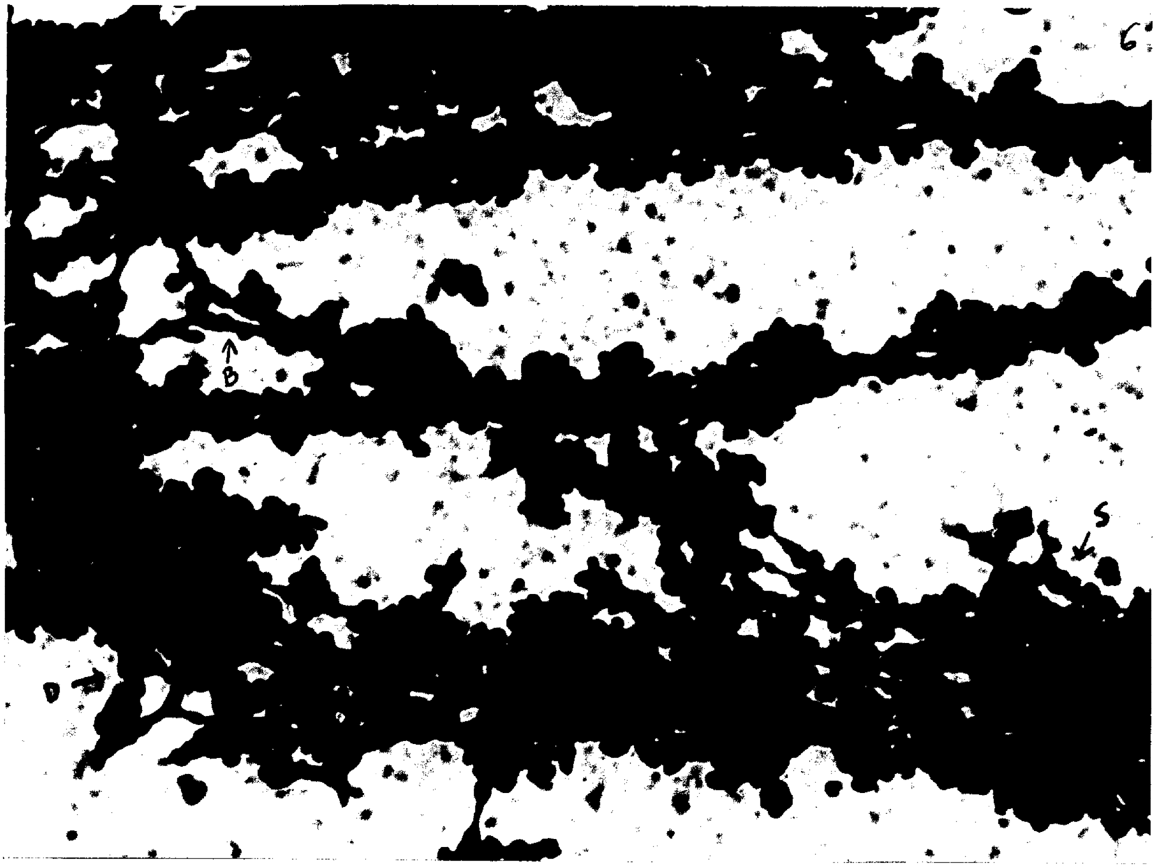

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Fig. 7. Loop of centrifuged chromosome, fixed in 10% neutral formalin and air dried from amyl acetate. Note that the preservation of loop structure is much the same as preparations dried by the critical point method. Coiling (S) of the 200-250 A fibrils (B) is also evident in this micrograph. X 14,000

Fig. 8. Higher magnification of lower middle portion of Fig. 7. See Fig. 7 for explanation. X 58,000

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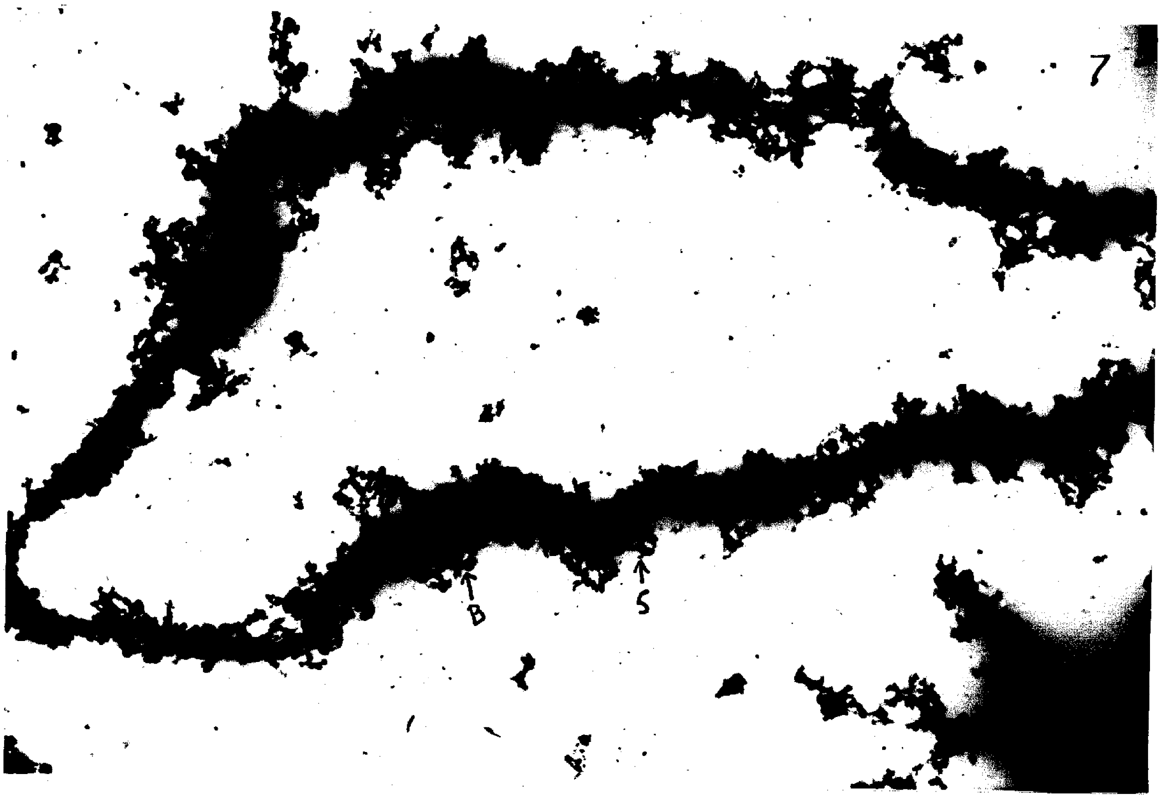
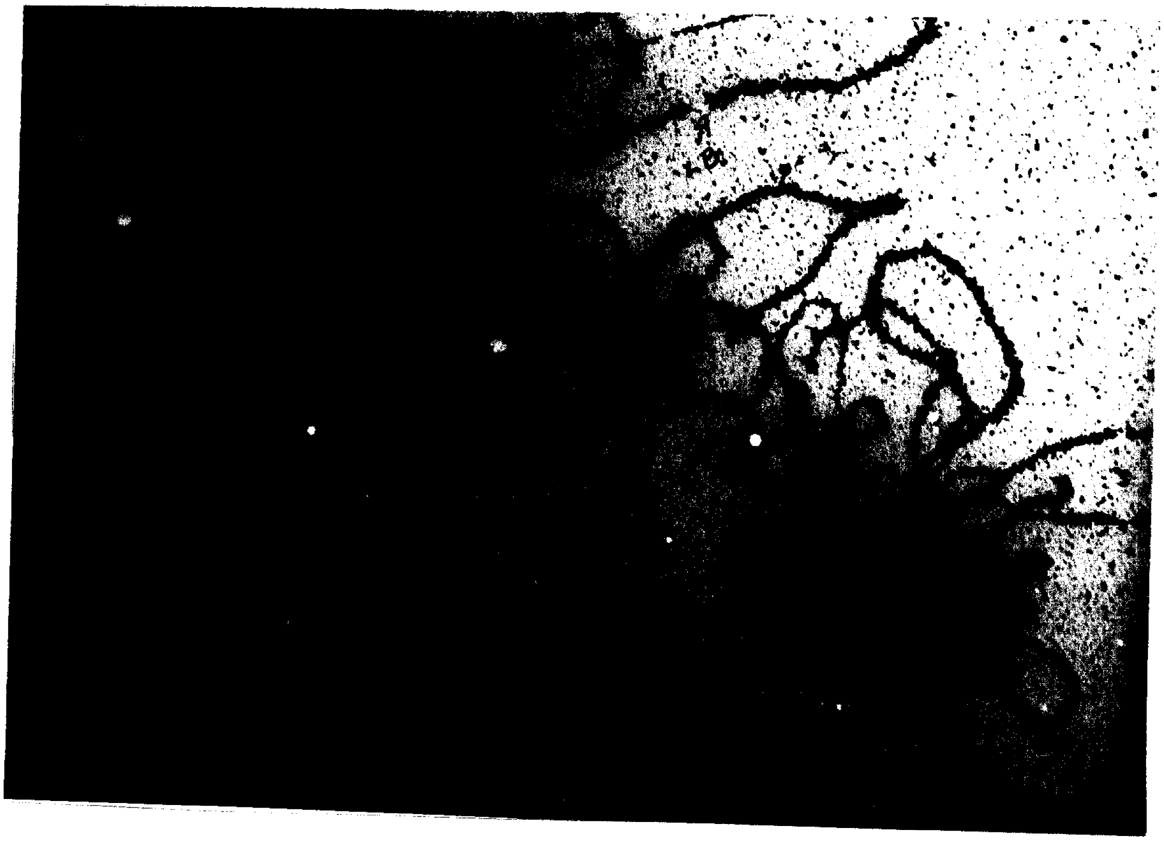
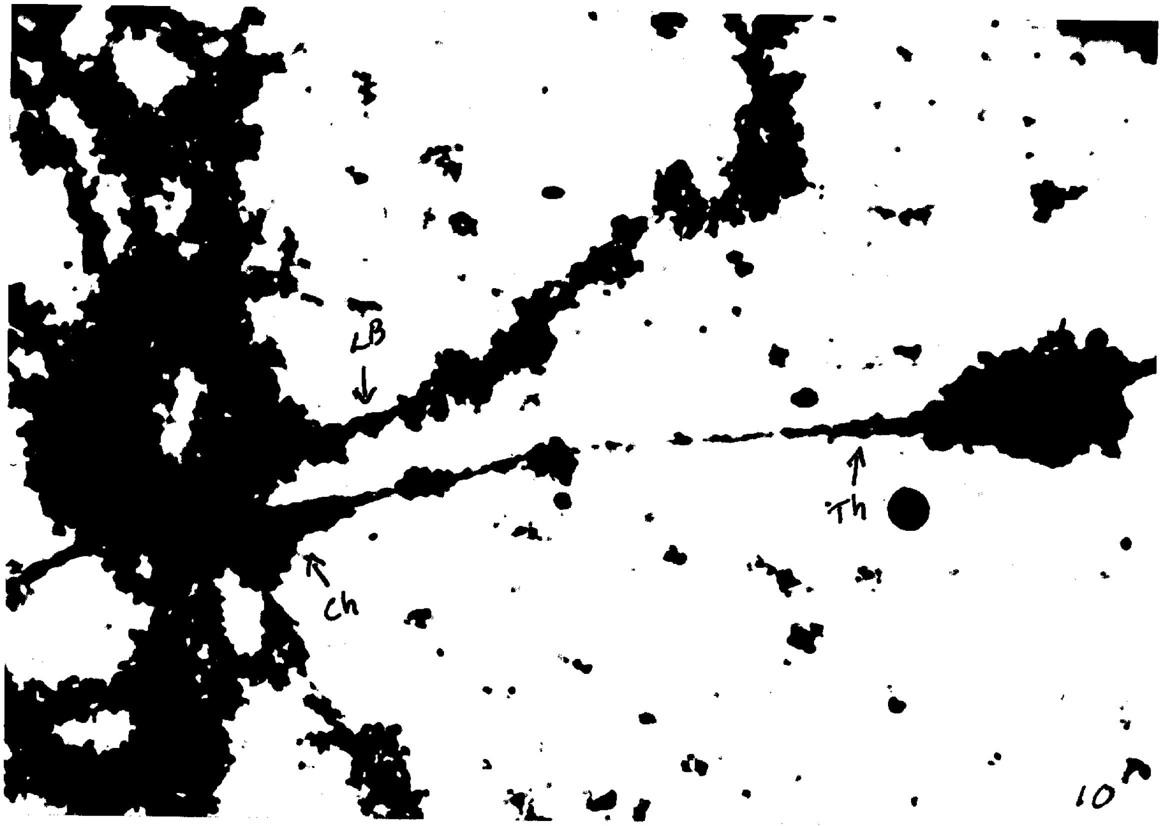


8

Fig. 9. Low power electron micrograph of chromosome centrifuged, fixed in 10% neutral formalin and air dried from amyl acetate. Fraying of the microfibrils causes the loops to look fuzzy at this magnification. Most preparations have a few loop breaks (LB) such as those seen in this micrograph. Note that fibril (S) which connects chromomeres in a stretched region appears single (however, see Fig. 10). X 4200

Fig. 10 Higher magnification of stretched region of Fig. 9. At this magnification the "single" fibril is seen to contain more than one unit in the thicker (Th) regions. Most of the fibrils have been broken and the remaining one stretched in the region where only one fibril can be seen. Loop bases (LB) can be seen entering the dense chromomere (Ch). X 29,000





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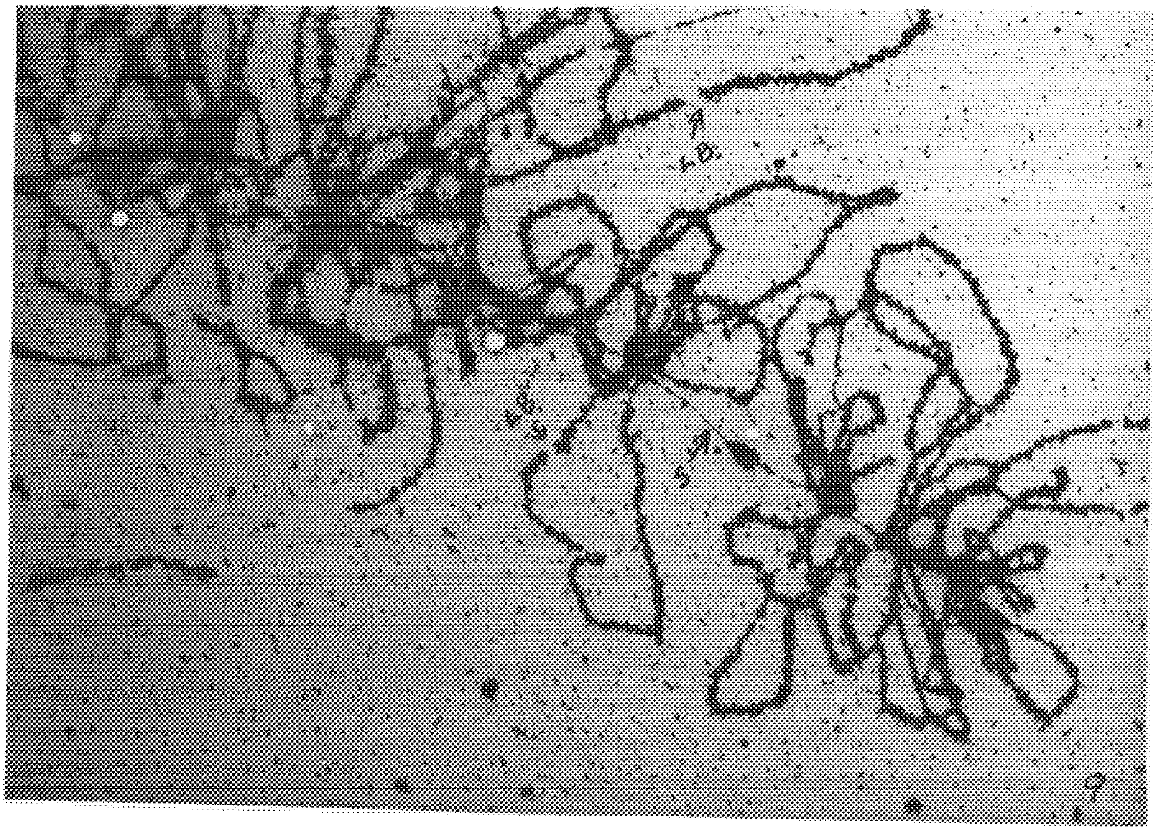

LB
LB
S
9

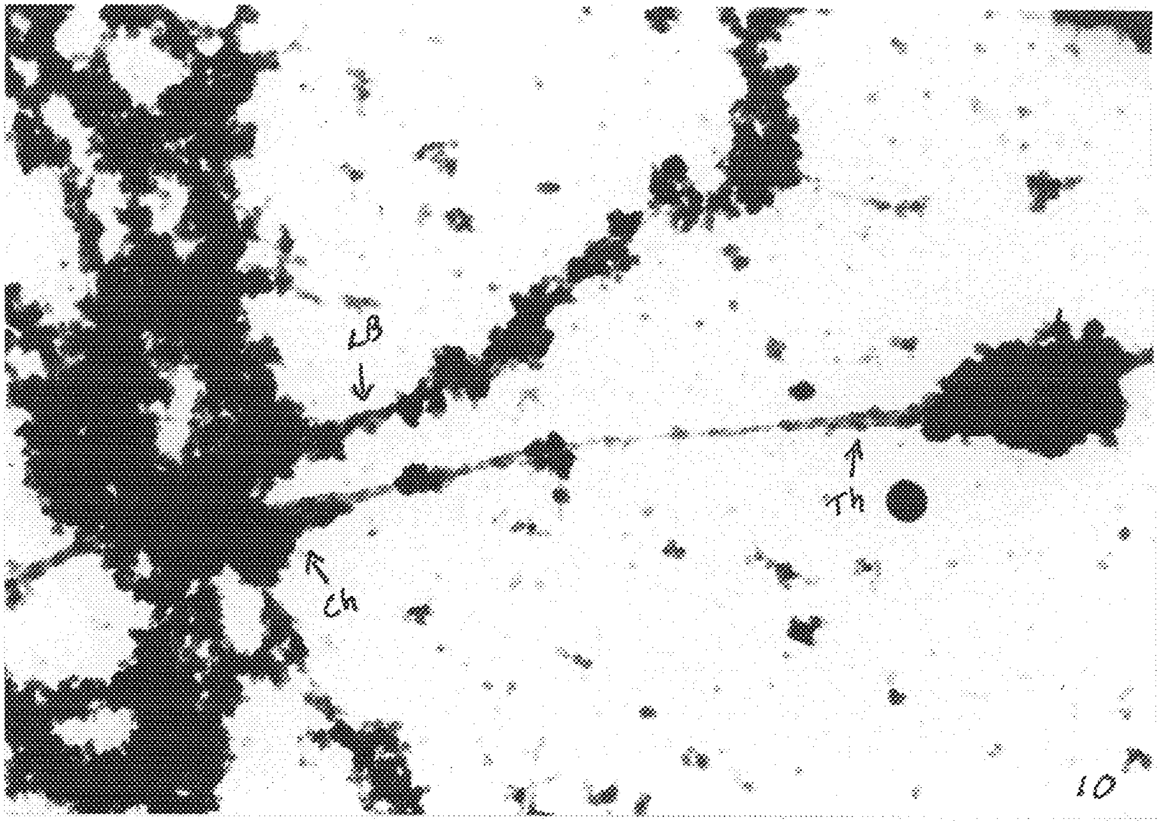
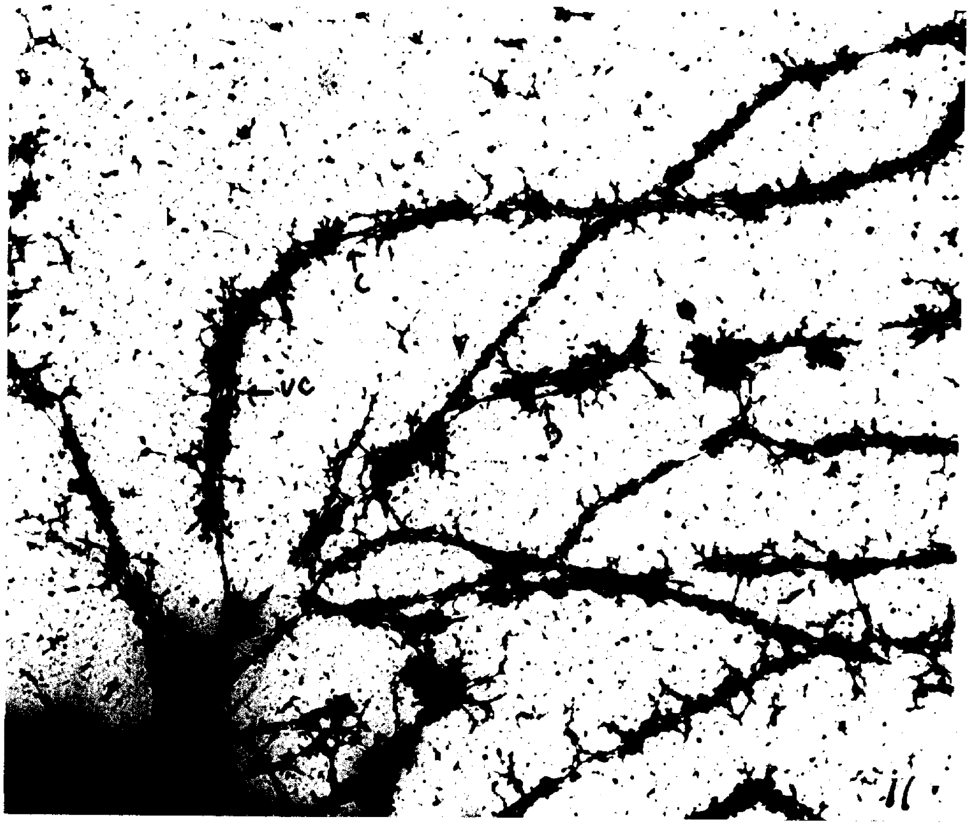
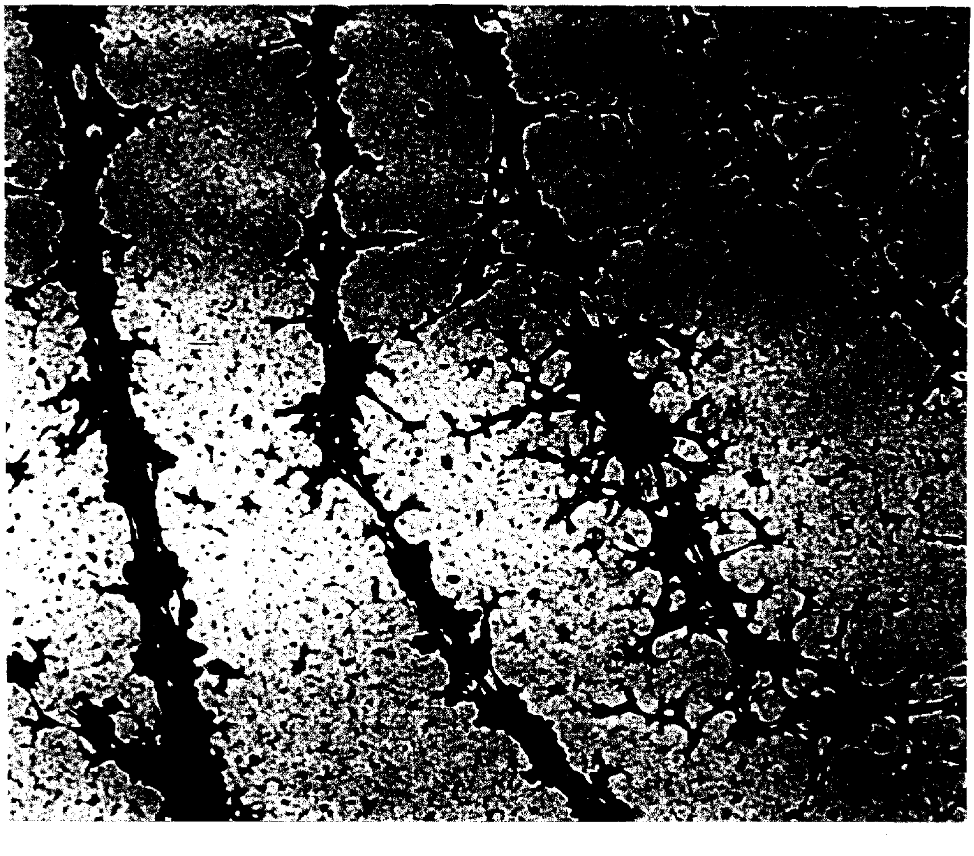
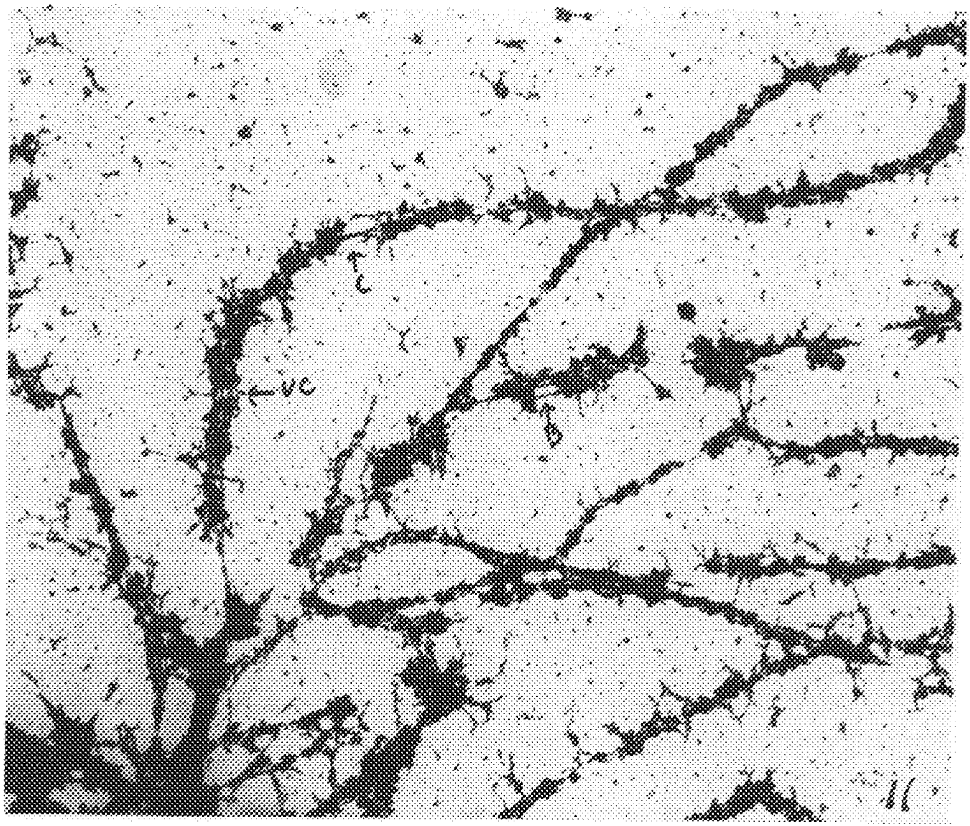

LB
Th
Ch
10

Fig. 11. Centrifuged preparation digested 30 minutes with RNase (0.5 mg/ml), fixed in 10% neutral formalin and dried by Anderson's critical point method. Fraying of the loop fibrils is more evident in these preparations. Many regions are "uncoiled" (UC) so that individual 200-250 A fibrils (B) can be followed for short distances. Some of these seem to associate to form 500 A fibrils (C). X 14,000

Fig. 12. Higher magnification of loop regions. Treatment was the same as Fig. 11. In some regions 200-250 A fibrils can be seen to contain two subunits about 100 A wide (D).
Note the multiplicity of fibrils. X 30,000


C
VC
B
11




C
VC
D

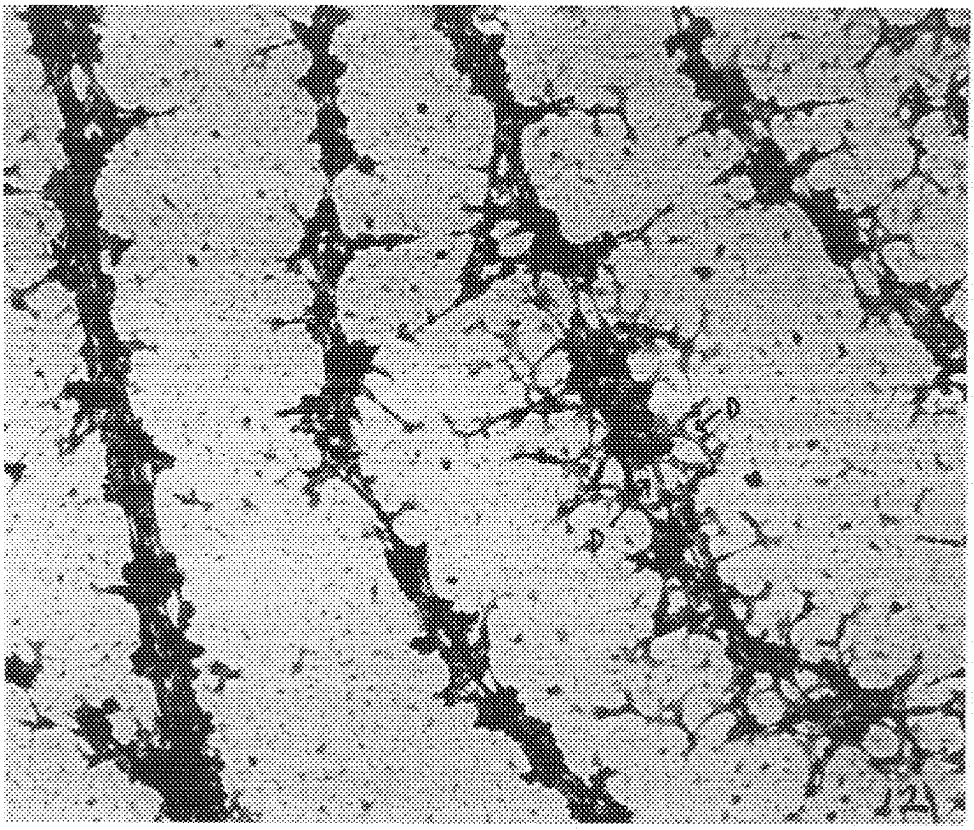
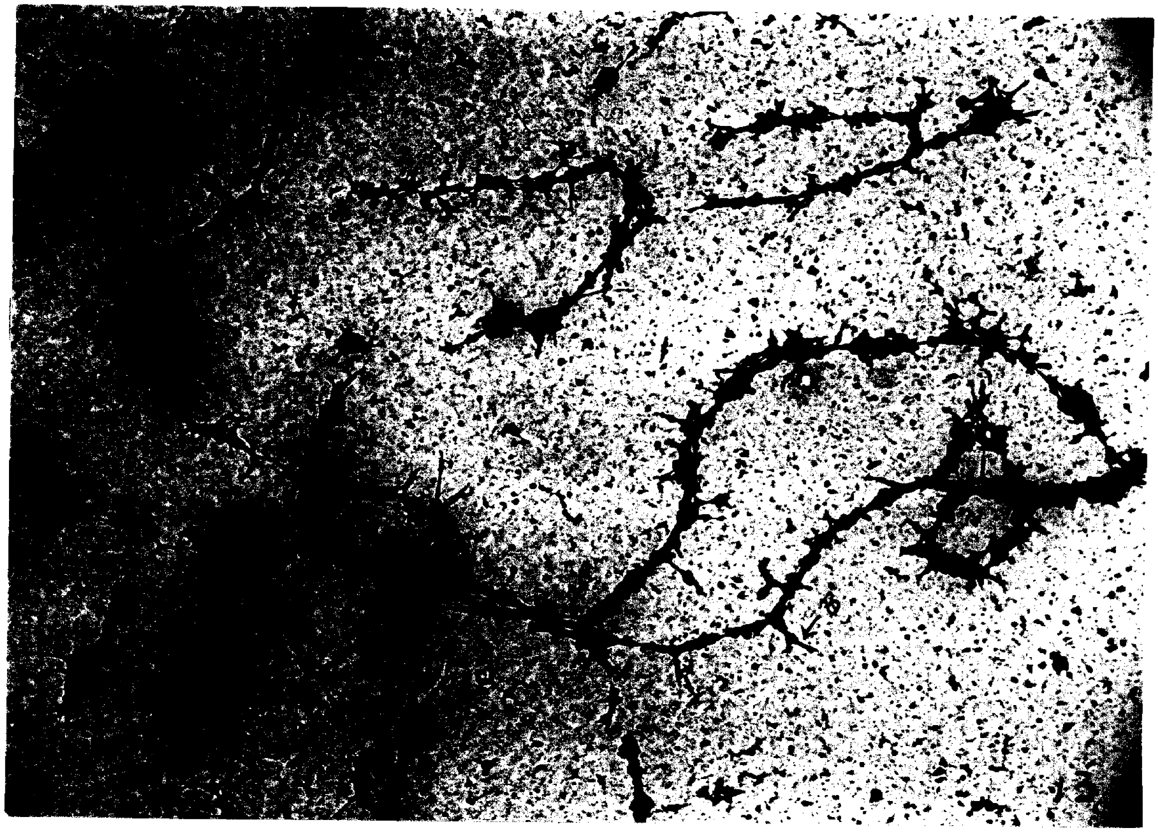
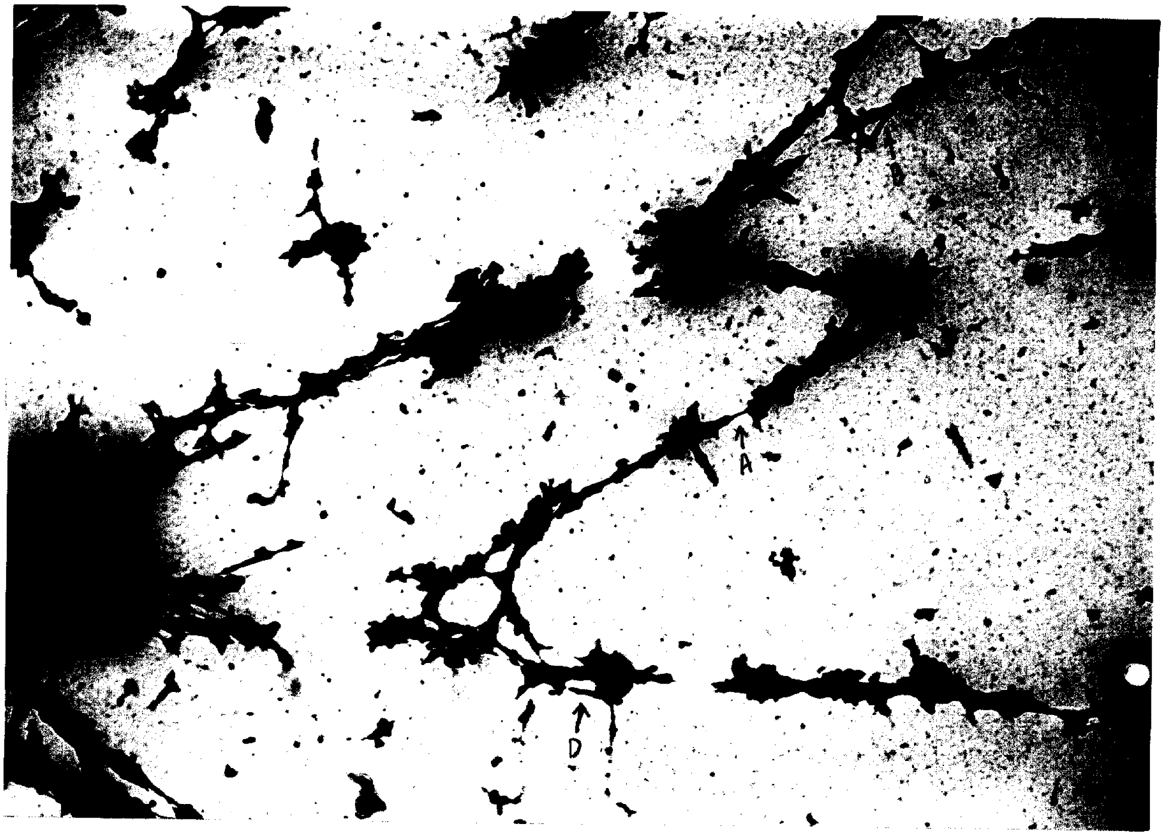
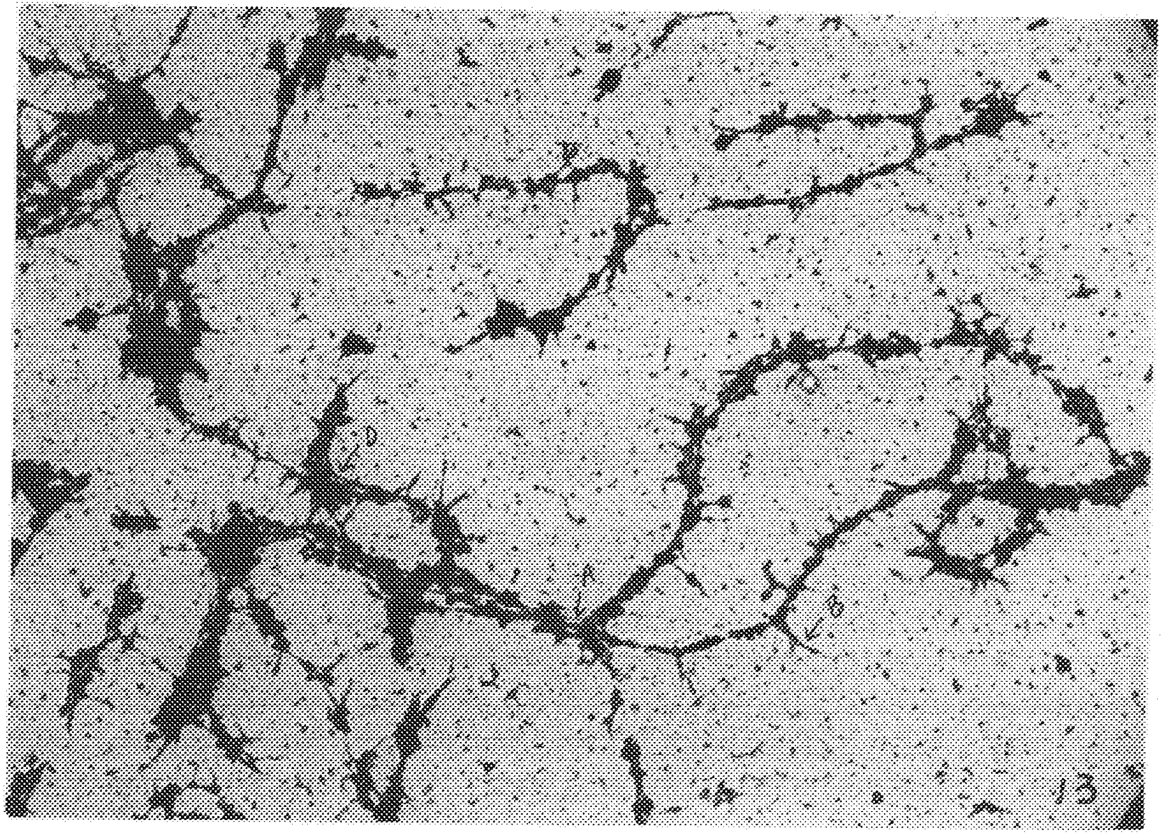

D
D

Fig. 13. Prepared as in Fig. 11 except digestion was with 0.5 mg/ml trypsin. Many of the loops of this preparation were broken during isolation. Fibrils 200-250 A (B) are seen in the frayed ends and "uncoiled" regions of the loops. Some of these fibrils show 100 A subunits (D), others are stretched to form 100 A regions (A). X 14,000

Fig. 14. This preparation was incubated with RNase, rinsed in saline, and then treated with trypsin. Fixation and dehydration were carried out as in Fig. 11. There does not appear to be any "characteristic" difference resulting from preincubation in RNase before trypsin treatment. See Fig. 13 for regions labeled A, B and D. X 30,000


B


A
D


D
A
B
13

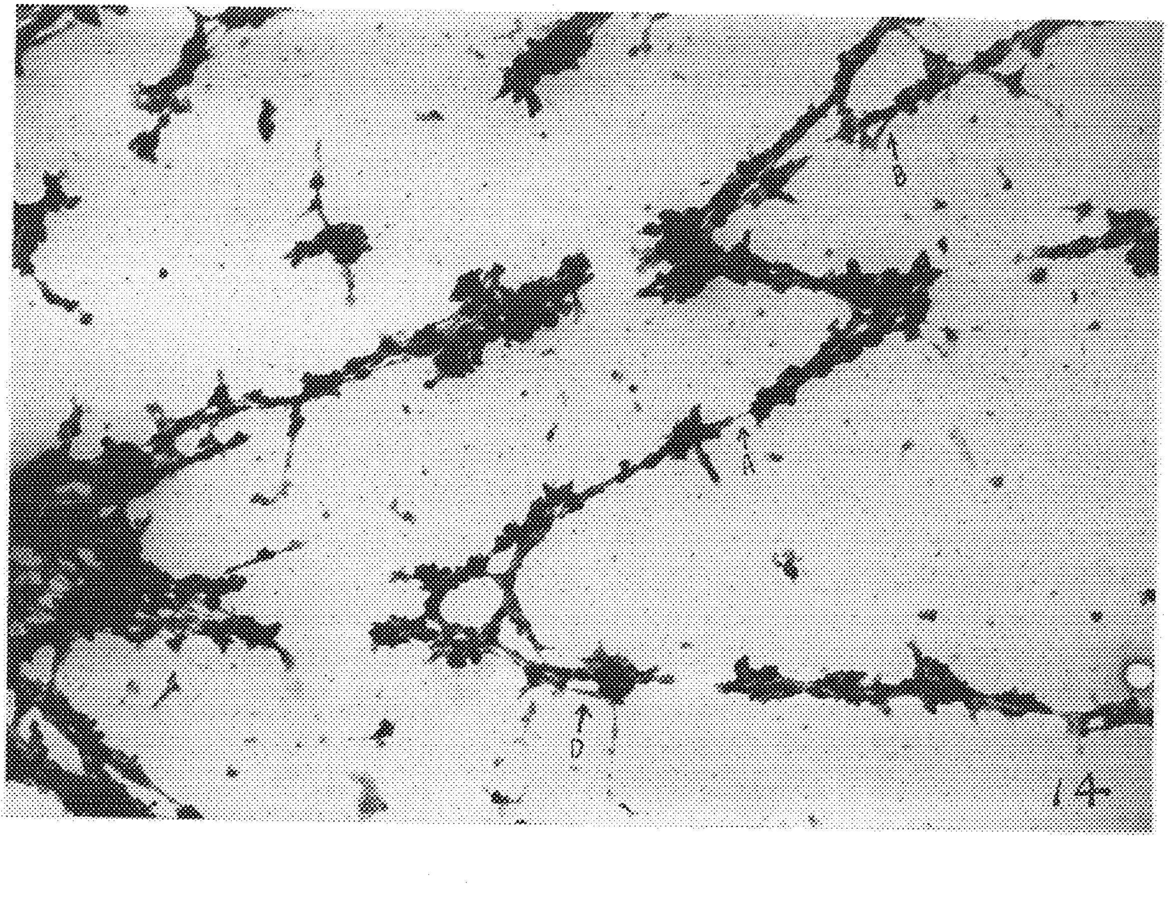
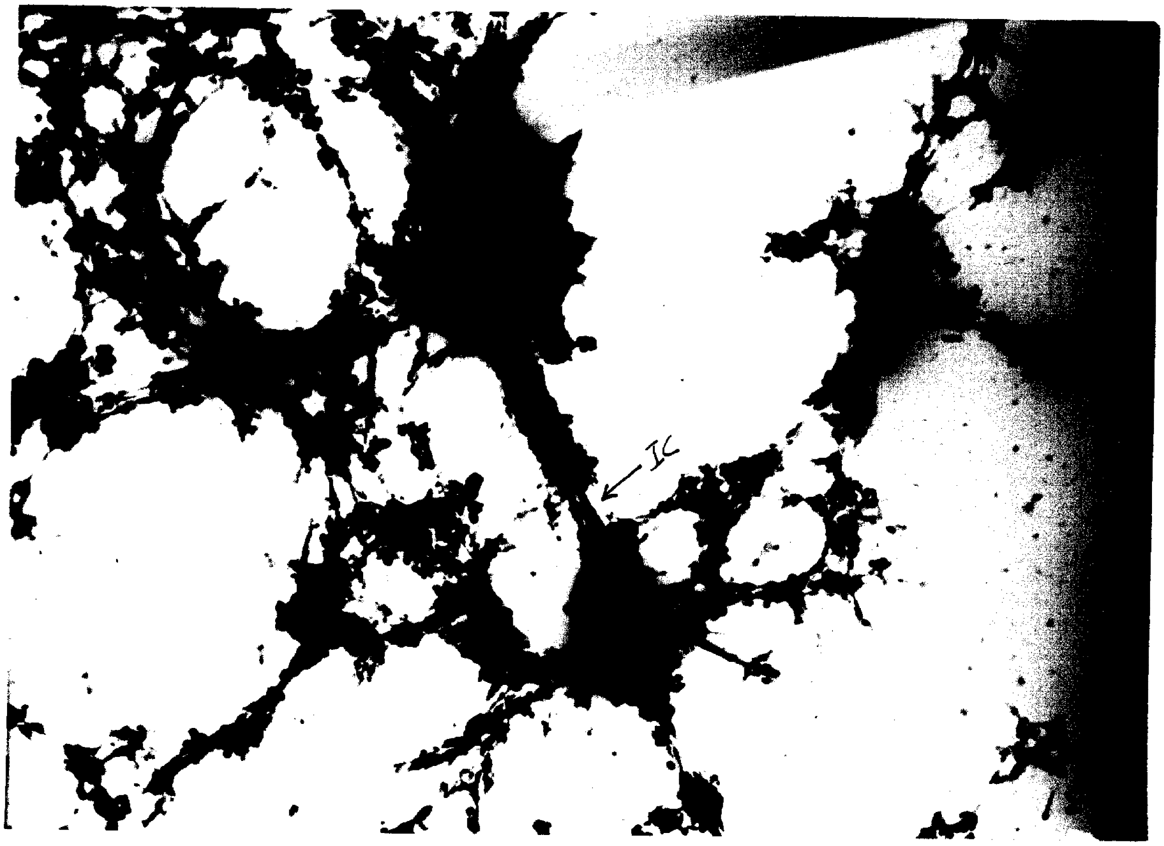
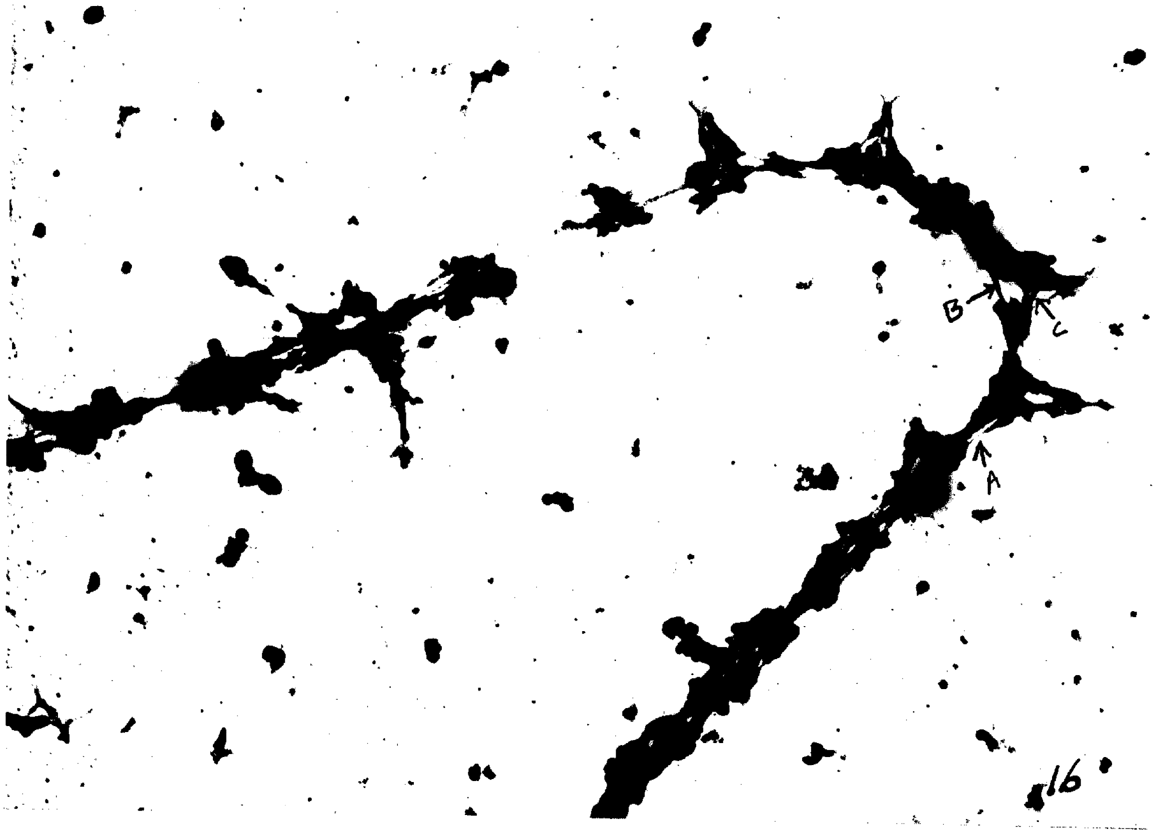
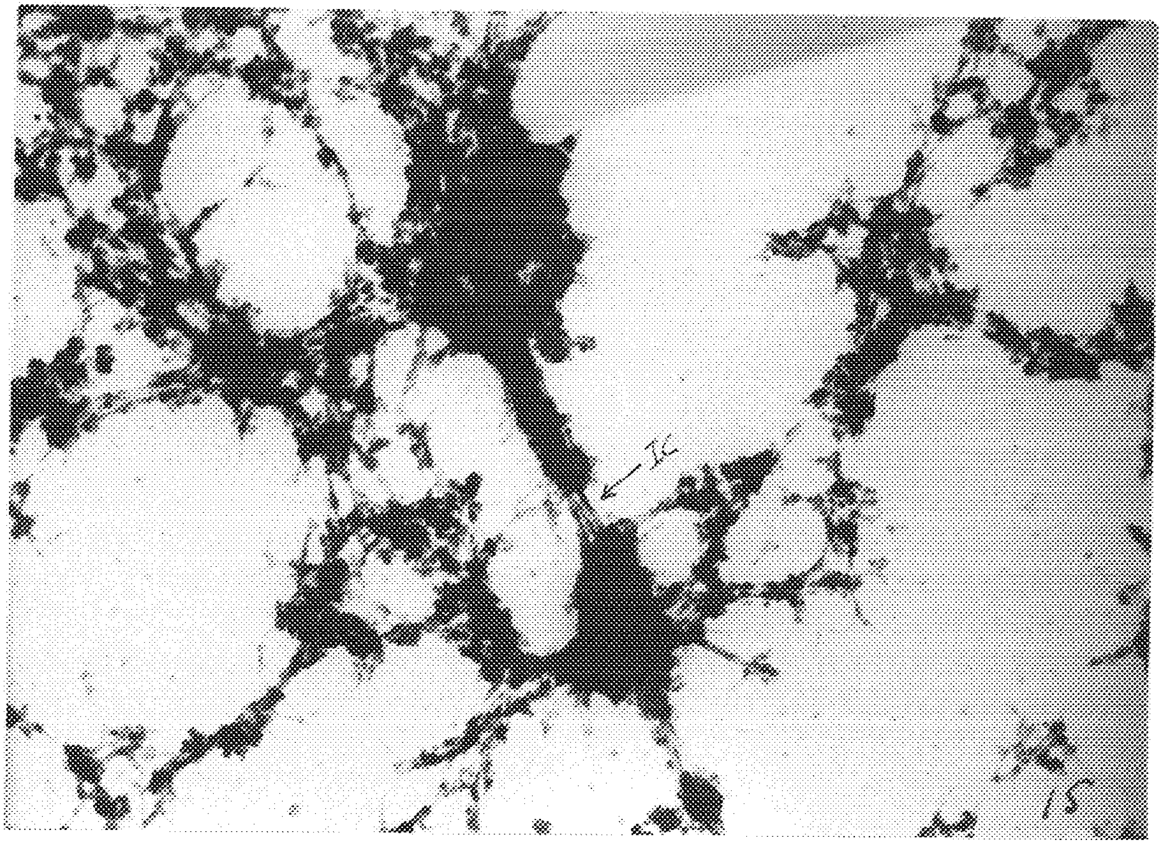

B
A
D
14

Fig. 15. Centrifuged preparation fixed first in 10% neutral formalin and then treated 30 minutes with 0.5 mg/ml pepsin. After enzyme treatment the preparation was rinsed in saline, dehydrated and air dried from amyl acetate. Note multiplicity of interchromomeric connections (IC). The remaining loop structures appear more severely damaged compared to RNase and trypsin treatments. X 14,000

Fig. 16 Portion of a loop prepared by treatment with 0.5 mg/ml pepsin after centrifugation, fixed in ethanol and dried as above. Note that the loop structure remaining after this treatment is also multifibrous. Some of the 200-250 A fibrils seem to be associated to form fibrils approximately 500 A wide (C). 200-250 A fibrils (B) and stretched regions of these fibrils (A) are also evident. X 30,000


IC


B
C
A
16


IC
15

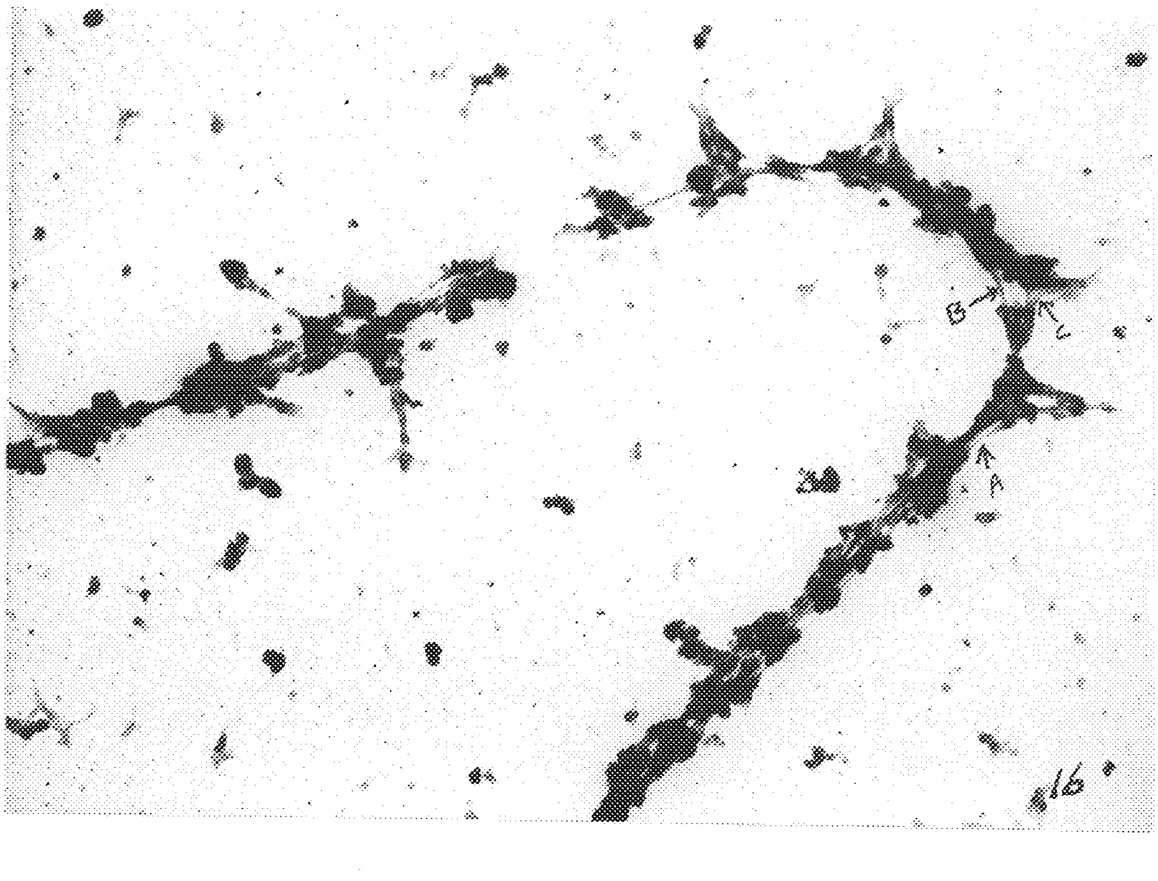

B
C
A
16

Fig. 17. Chromosome digested 5 minutes with 0.5 mg/ml DNase, centrifuged, fixed with 10% neutral formalin and air dried from amyl acetate. This micrograph shows the extensive fragmentation produced by DNase action. Many short fragments (SF) of various sizes as well as short pieces of 200-250 A fibrils are evident. Partial breaking of the bundles of fibrils results in connections between loop fragments by one or several 200-250 A fibrils (C). Note the tangled complex of fibrils in the chromomere regions (Ch). X 21,000

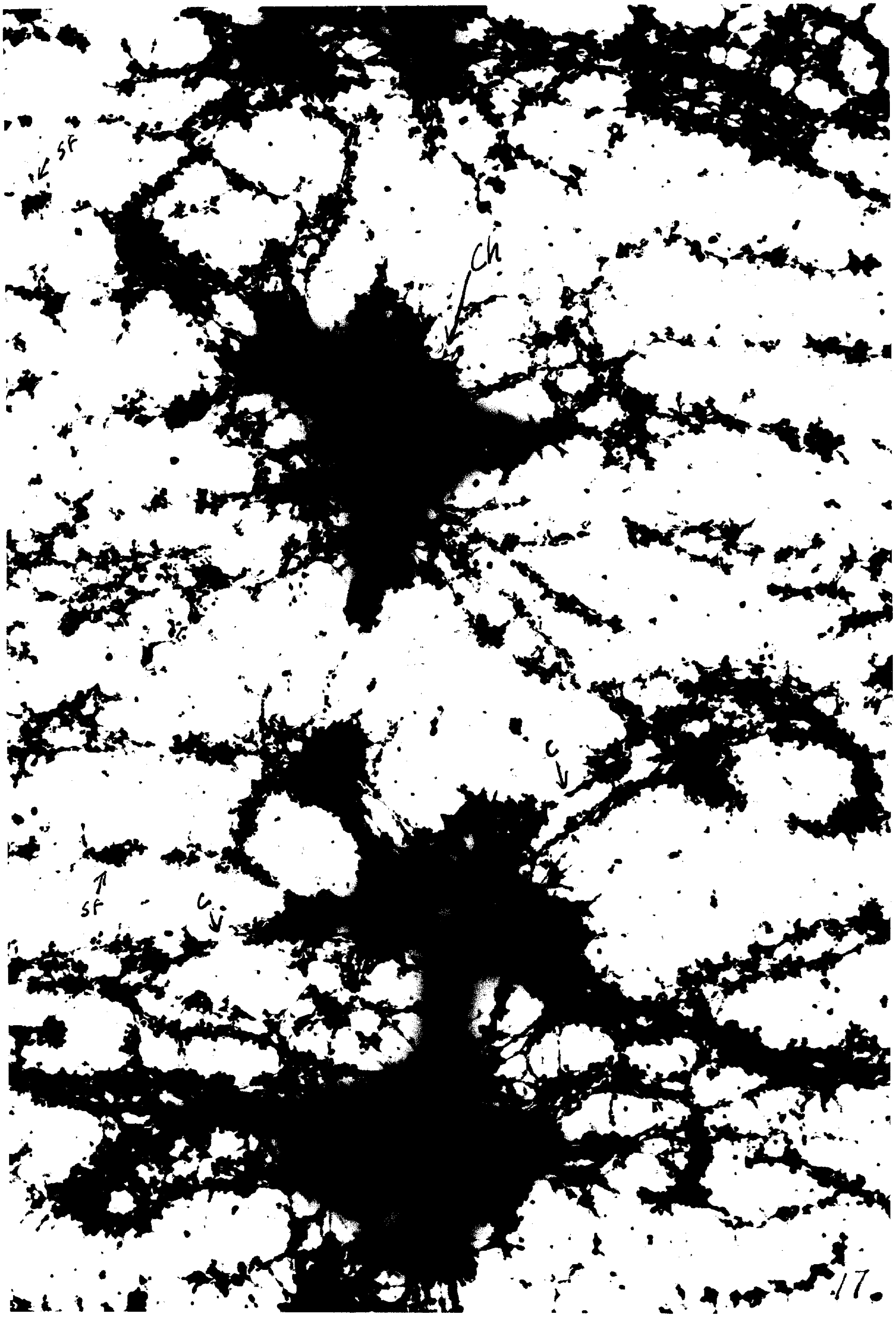
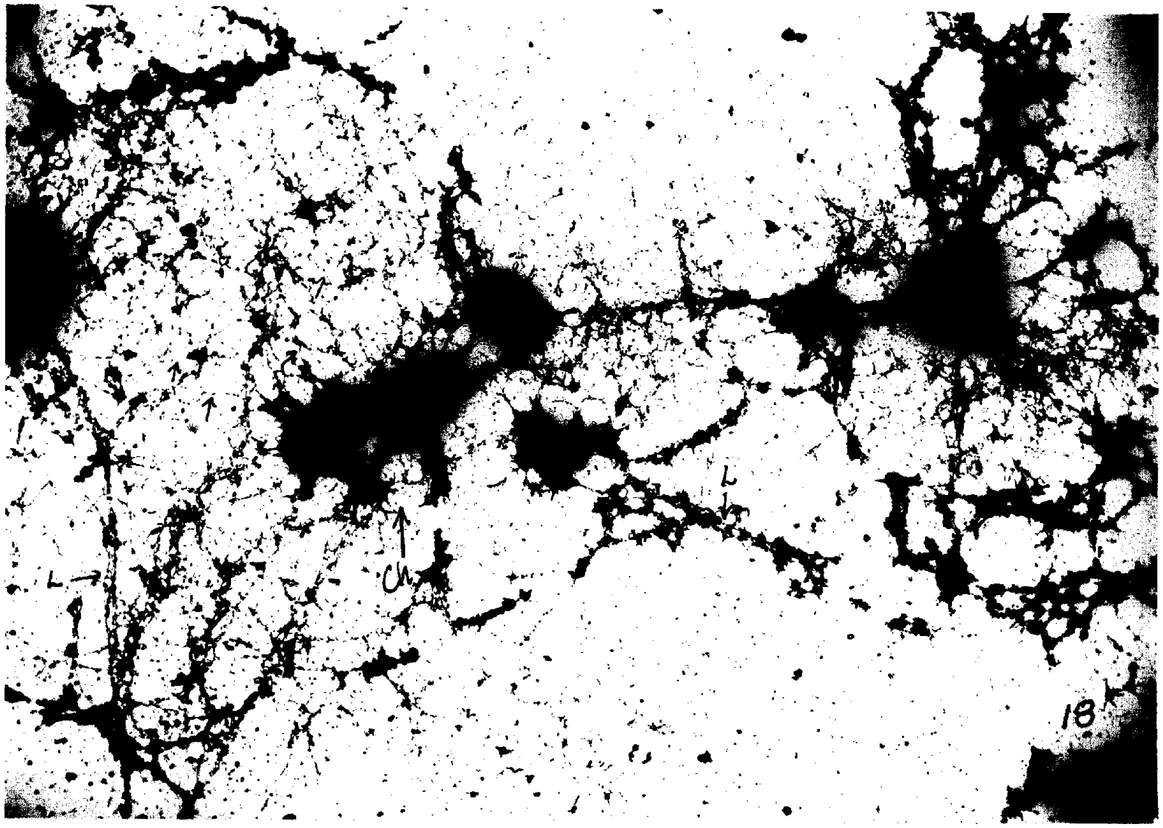
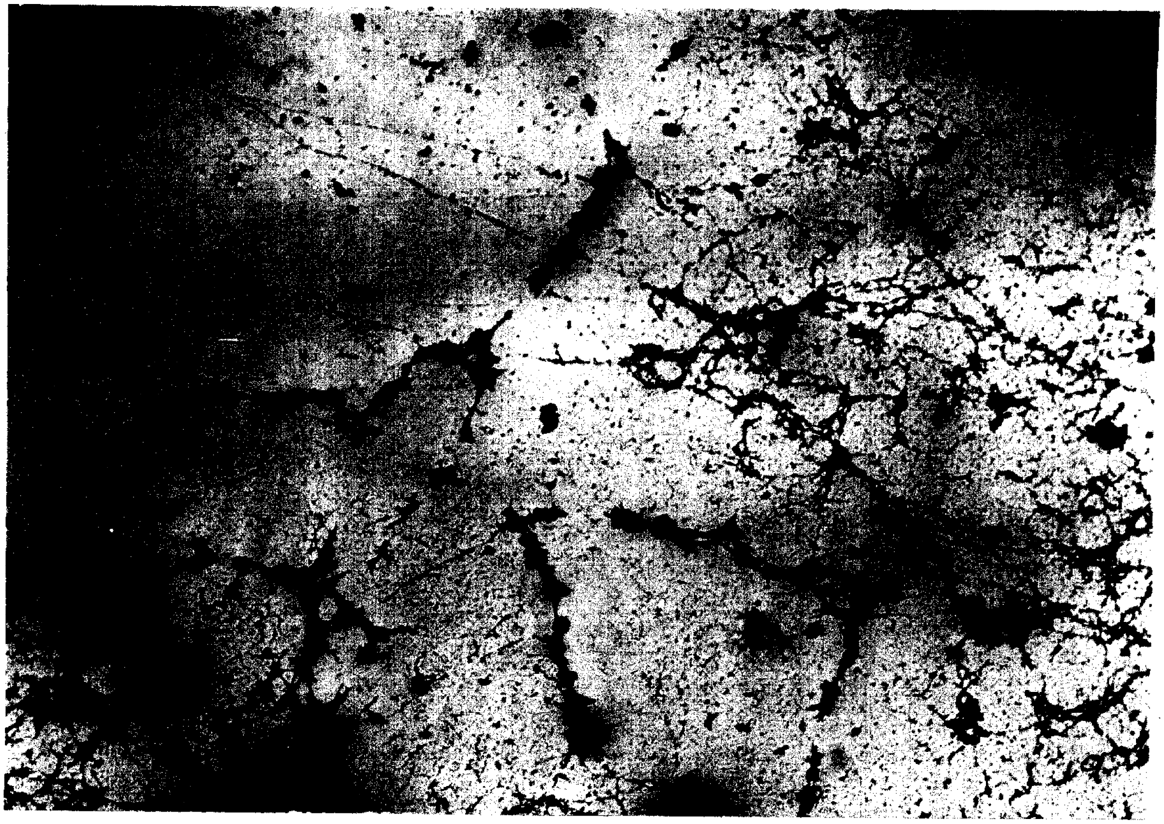
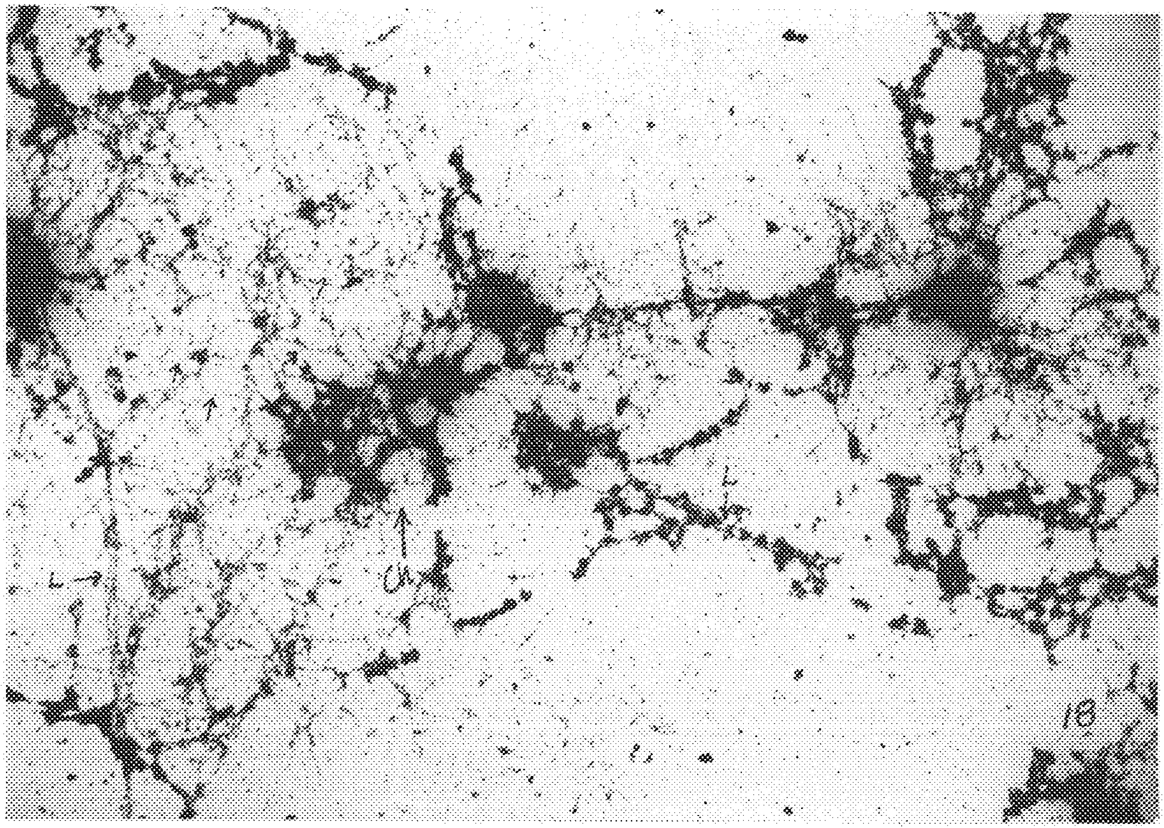

sf
ch
c
sf
c
17.

Fig. 18. Centrifuged chromosome treated five minutes with 0.5 mg/ml Pronase, fixed in ethanol, stained for one hour with 1% uranyl acetate and air dried from amyl acetate. The chromomeres (CH) are swollen by this treatment and reveal many 100 A fibrils (arrows). Several loop regions also show these 100 A fibrils (L). X 14,000

Fig. 19. Higher magnification of a different region of the above preparation shows 200-250 A fibrils which appear to be split into two fibrils measuring approximately 100 A. (arrows) X 29,000


L
Ch
L
18





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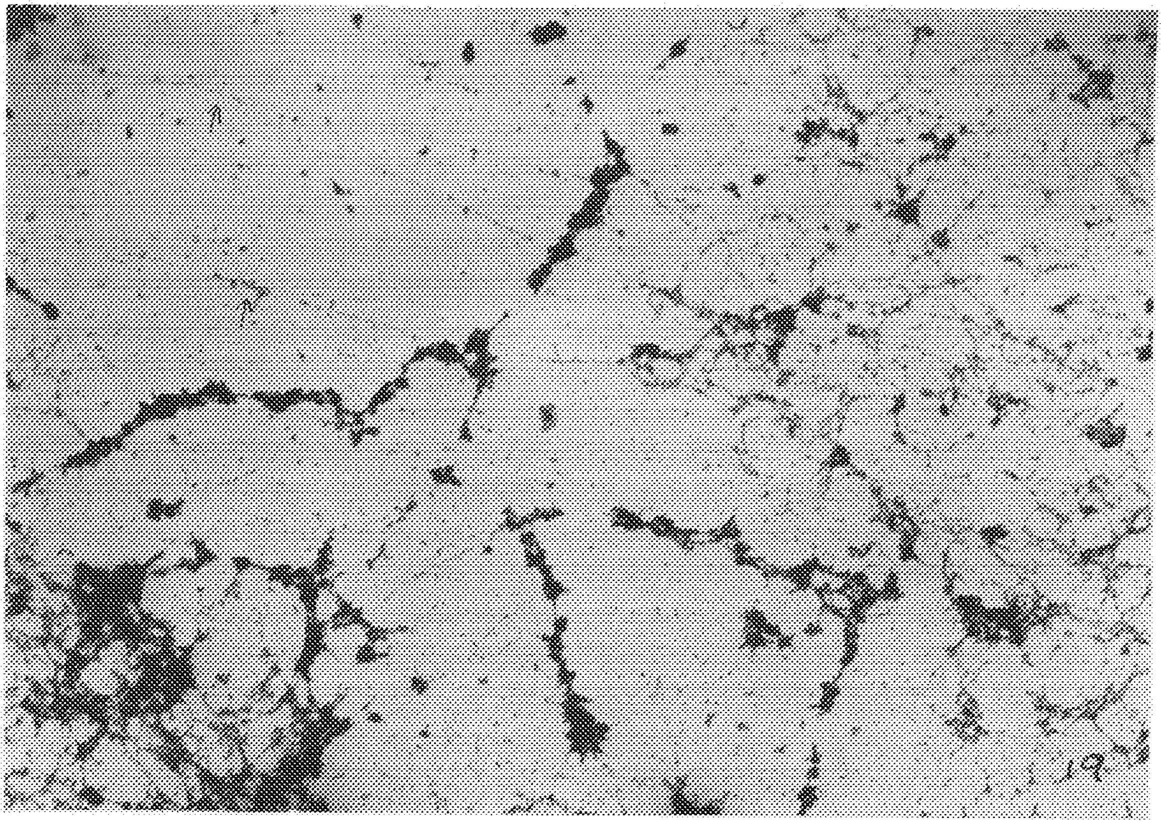
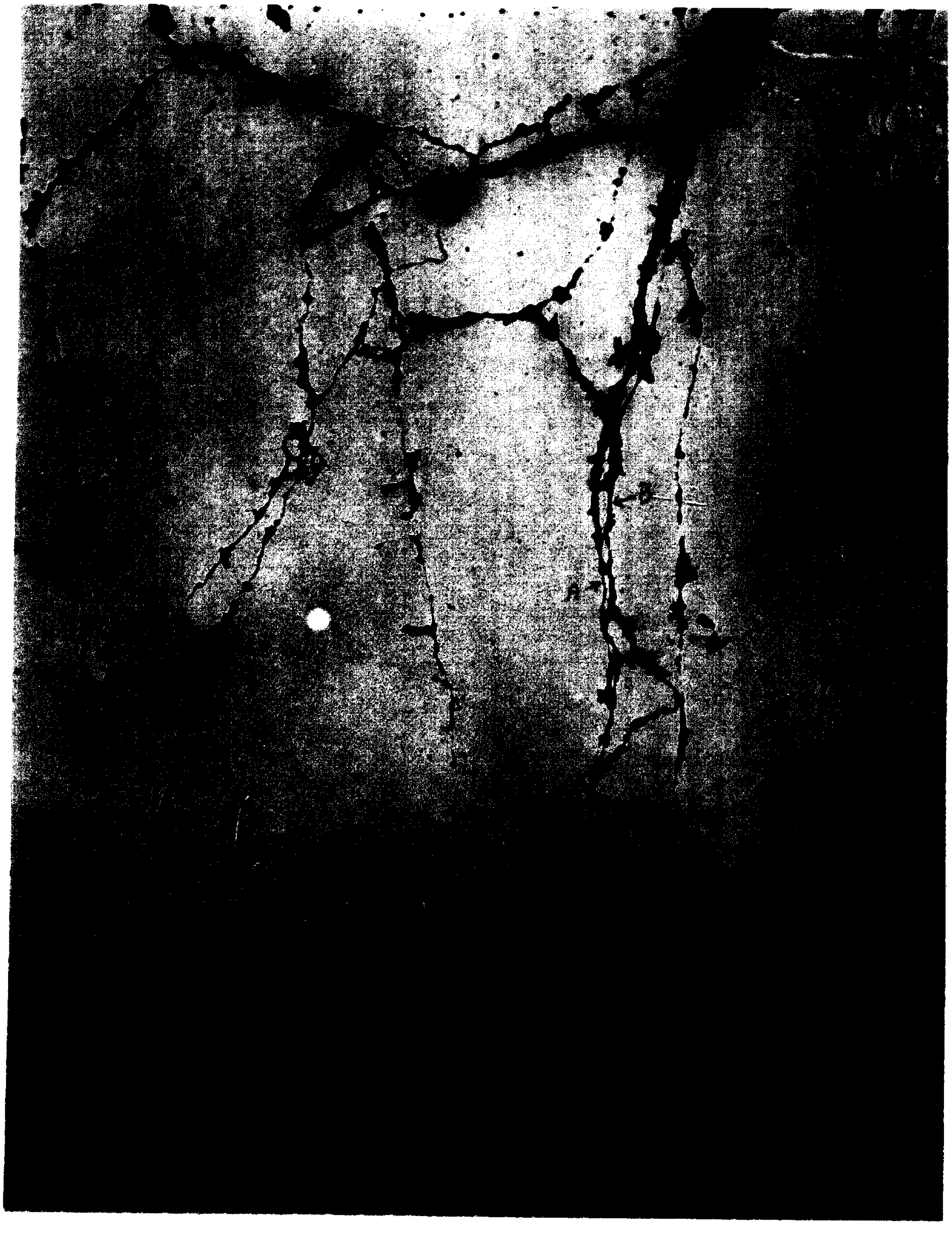
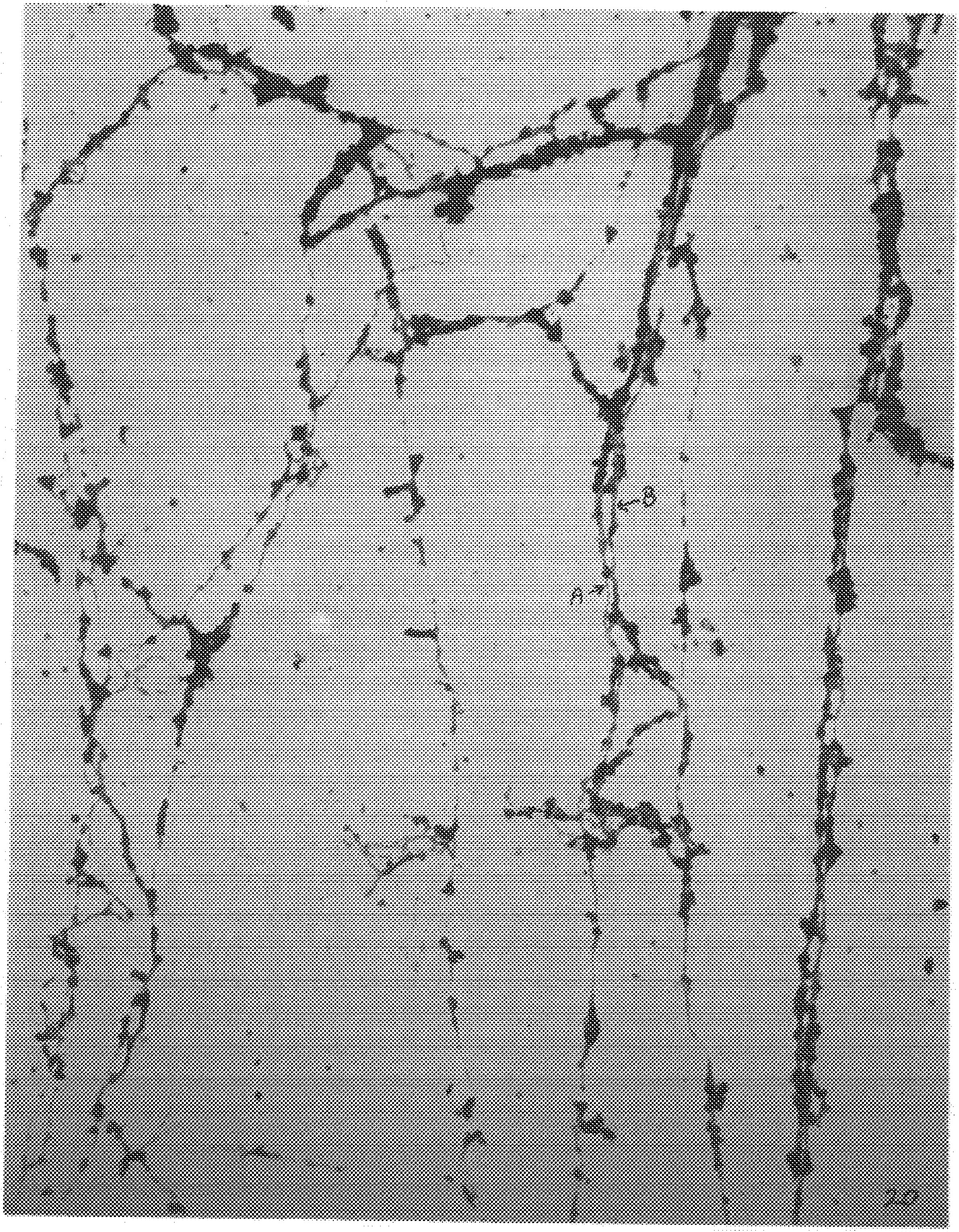


19

Fig. 20. Chromosome fibrils picked up from 30% sucrose-air interface, fixed for one minute in osmium vapors, and air dried from amyl acetate. Many 200-250 A fibrils (B) similar to those in loops and chromomeres of whole chromosomes can be seen. In some places these fibrils are stretched to regions 100 A in thickness (A) and smaller. X 21,000


B
A


B
A
22

APPROVED:

Hans Ris

Professor of Zoology

1/24/64

Date