THE EFFECT OF PREFIXATION ON THE DIAMETER OF CHROMOSOME FIBERS ISOLATED BY THE LANGMUIR TROUGH–CRITICAL POINT METHOD

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ABSTRACT

The effect of prefixation on the diameter of chromosome fibers isolated by the Langmuir trough–critical point method has been investigated in several species of plants and animals. In barley, fibers isolated from endosperm without prefixation have an average diameter of between 240 and 250 A, and are similar in dimensions and structure to the chromosome fibers isolated from animals by this method. Chromosome fibers from other tissues of the same plant are smaller in diameter when isolated without prefixation, approximating 200 A. After prefixation in 2% buffered formalin, isolated fibers from the three barley tissues studied are reduced in diameter, to approximately 120–130 A for endosperm and leaflet and to 140 A for root tip. Chromosome fibers isolated from newt erythrocytes also show a significantly reduced diameter after formalin prefixation, to approximately 120 A.

INTRODUCTION

Chromosomes or nuclei isolated for electron microscopy by the Langmuir trough-critical point method (3) have been shown to contain a mass of irregularly coiled or folded fibers (2, 4, 9, 10). The average diameter of chromosome fibers isolated by this method has been reported by Ris (9) as 200 A, by DuPraw (2) as 230 A, by Wolfe as 250 ± 20 A (10), and by Gall (4) as 200–300 A. While it is safe to state, in view of these reports, that the diameter of these fibers lies between 200 and 300 A, some disagreement obviously exists concerning chromosome fiber diameter. The extreme limits of the diameter of isolated fibers actually extend from approximately 50 A to more than 500 A. These extremes are found, however, with a much lower frequency than dimensions between 200 and 300 A.

In contrast to the dimensions obtained in isolated chromosomes, the diameter of the fibers in thin-sectioned nuclei is much smaller (6). The most frequently reported diameters are near 100 A, although reports exist of fibers as small as the 20–30 A expected for a DNA helix up to a limit of about 500 A (for reviews see references 5 and 8). While the size ranges of the two classes of fibers, isolated and sectioned, thus overlap considerably, the average diameters are different by a factor of about 2 to 2.5. This discrepancy must be regarded as of fundamental importance in a consideration of the structure of isolated chromosomes, in view of the generally excellent preservation of cell structures obtained by osmium tetroxide or aldehyde–osmium tetroxide fixation and thin sectioning. For convenience, the two classes of fibers will be designated as "100 A" and "250 A" in this paper.

It is impossible to state with absolute assurance that the dimensions obtained in thin-sectioned chromosomes are closer to the true dimension for

the component DNA-protein fibers of living cells than are the dimensions of the larger fibers of isolated chromosomes. Recent results, however, have added weight to this interpretation. Wolfe and Grim (11) attempted to identify the stage in the isolation process at which a change in fiber diameter might occur. No change was found to result from surface-spreading or critical point drying; if these steps were circumvented, or if fibers were transferred at any stage in the technique after surface-spreading to an embedding medium and sectioned, the larger 250 A diameter was found to be present. However, when a surface film containing an excess number of cells was placed immediately in fixative, embedded, and sectioned, nuclear fibers showed transition areas from 100 to 250 A in cells which appeared to be in the process of breaking down. It was also possible in such preparations to find undamaged cells, with 100 A fibers present in the nucleus, surrounded by 250 A fibers from cells which were completely spread. These results were taken to mean that a change in fiber diameter from 100 to 250 A occurs at the time the cell membranes break down, possibly caused by the disturbance of the ionic environment of the nucleoprotein fibers of the nucleus.

Further work with isolated chromosomes has strengthened the interpretation that a change in diameter occurs during isolation to produce the 250-A fiber. Wolfe and Martin (12) undertook to study the chromosomes of plant cells, using a root tip press devised by McLeish (7) to isolate plant nuclei and metaphase-chromosomes for study. After surface spreading and critical point drying, the root tip chromosomes of the plants studied (*Vicia faba* and *V. sativa*) proved to be identical in fine structure to the chromosomes of animal cells, except that the basic fiber was of smaller diameter, approximately 150 A. In order to use the McLeish press to isolate nuclei for electron microscopy, it was found necessary to prefix plant tissue in formalin. Since the method used for plant tissue thus differed basically in that prefixation was a requirement, it was thought possible that the difference in fiber diameter resulted from this step in the method. In this case, the fixative would be expected to act by binding the sites involved in the transition to a larger diameter fiber. An alternate possibility, however, is that the smaller diameter found in the isolated plant chromosomes represents a basic difference in chromosome structure in plants and animals. Wolfe and Martin did not determine which of these possibilities is the case.

The immediate object of the research reported in this paper has been to investigate further the smaller diameter of fibers found in plant chromosomes. In order to carry out this research, it was necessary to develop a method for isolation of plant chromosomes without previous fixation. This problem has proved to be difficult with plants in the past, at least in respect to the surface-spreading technique, because the rigid cell walls prevent spreading of the cells. A source of plant nuclei which can be isolated readily without prefixation has been found, however, in the milky endosperm of grasses. At this stage, nuclei in the developing endosperm undergo rapid division without formation of cell membranes or walls. During a brief period after pollination, a suspension of nuclei can be obtained from this tissue which can be spread directly on the Langmuir trough without the requirement for formalin fixation or pressing. Another source of nuclei and chromosomes which can be isolated without prefixation has been found in the meristematic tissue of the roots and leaflets of recently germinated seeds, in which cell wall formation is less complete than in fully differentiated tissue.

These sources of plant nuclei were used in this study, and comparisons were made between the diameters of chromosome fibers from unfixed and prefixed nuclei of barley endosperm, leaflet, and root tip. Similar experiments were carried out with animal cells, for comparison of the effects of prefixation in animal tissue. The effect of trypsin digestion after prefixation was also tested. As a necessary part of this work, statistical analysis was made of dimensions obtained from measurement of fibers in the various groups and treatments studied.

METHODS AND MATERIALS

Unfixed Barley Nuclei

Developing endosperm was obtained from barley plants 2–3 wk after pollination. A single spikelet containing the embryo and endosperm was removed and pressed lightly between two glass slides. At the right stage, the drop of fluid obtained was viscous and milky, and contained a dense concentration of naked nuclei. The slides containing the endosperm nuclei were then passed through the surface of a Langmuir trough. The resulting film was picked up on carbon-formvar–covered grids by touching the grids lightly to the surface, and transferred immediately to the surface of a 2% uranyl acetate–staining solution for 8–10 min. After a brief rinse in distilled water,

the grids were placed in a plastic carrier (11) and dehydrated in an ethyl alcohol series: 1 min in each of 70 and 95% alcohols, and two 5-min changes and one 10-min change in 100% alcohol. The alcohol was replaced by immersing the grids in three changes of amyl acetate. The grids were then dried in a critical point apparatus (1). Identical methods were used for all of the preparations described below, once the material was placed on the surface of the trough.

Barley seedlings were germinated for 3–4 days in the dark at room temperature in damp vermiculite. Cells were obtained from leaflets by lightly macerating 1–2 mm of the proximal portion of a growing leaflet in a drop of distilled water between two glass slides. The slides were then dipped through the surface of a Langmuir trough containing distilled water. The resultant surface film was then picked up on grids. Subsequent techniques were the same as described for endosperm. Cells from root tips of barley seedlings were obtained similarly by the process of excising 1–2 mm from the tips of rootlets. Approximately 6–8 root tips were macerated between glass slides and spread on the trough.

Prefixed Barley Nuclei

Tissue excised from the tips of rootlets or from the proximal region of a leaflet was placed immediately in cold 2% formalin in 0.05 M phosphate buffer, pH 7.2, for 45 min. After fixation for 45 min, the root tip or stem tissue was rinsed and squashed in buffer solution without fixative between two glass slides. The slides were then passed through the surface of the Langmuir trough, and grids prepared as above. In some cases, seedlings were transferred after germination in the dark to aerated Hoagland's solution in the light. After 1–2 days in Hoagland's solution, approximately 2–3-mm pieces were cut from the root tips of the seedlings. These excised tips were then fixed in cold 2% formalin in 0.05 M phosphate buffer, pH 7.2, for 45 min. After fixation, the root tips were placed in the McLeish press (7) in 5 drops of fixative in the cold. The suspension of nuclei obtained after pressing was transferred directly to the surface of the Langmuir trough, stained, and dried by the critical point method. This variation in the preparation techniques appeared to have no effect on the diameter of chromosome fibers obtained after fixation.

Unfixed Chromosome Fibers from Animal Cells

For this study, nuclei were isolated from newt erythrocytes and from bovine kidney cells in tissue culture. Newt erythrocytes were spread directly by touching a small droplet of blood to the surface of the Langmuir trough. Bovine kidney cells in tissue culture were spread directly by passing the slides on which the cells were grown through the surface of the trough.

These preparations were subsequently dried by the critical point method.

Prefixed Fibers from Animal Cells

Testes from last instar nymphs of the milkweed bug were dissected out under insect Ringer's solution and placed immediately in cold 2% formalin in phosphate buffer at pH 7.2. After fixation for 45 min in the cold, the testes were rinsed in cold buffer and placed in a small drop of buffer solution on a glass slide, ground lightly, and spread and dried as before. To obtain trypsin-digested chromosomes, fixed testes were ground in a small drop of buffer solution to which trypsin was added to a concentration of 0.0025%. Digestion was carried out for 20 min in a moist chamber at room temperature.

Nuclei from newt erythrocytes were fixed by pipetting blood into cold formalin fixative in buffer. After fixation, the preparation was centrifuged, and the pellet washed several times in buffer solution. The pellet was then dispersed in a droplet of buffer solution, spread on the trough, and dried.

Measurement of Fibers

Grids were examined under a Hitachi HU 11A electron microscope. Photographs were made primarily at an instrumental magnification of slightly less than ×20,000, although a limited number were made at ×15,000. The instrumental magnification used was a compromise between a value high enough to produce good resolution in micrographs and a value low enough to permit a field of view sufficiently extensive to include a large sample of fibers. The microscope was calibrated frequently with a diffraction grating at the settings used for these magnifications. Actual variation in magnification during the period of this investigation was small, between 19,800 and 19,900 for the highest instrumental magnification used. Micrographs were then enlarged photographically to a final magnification of 100,000 on 11″ × 14″ prints.

Two methods of measurement were used. In one method, the diameter of chromosome fibers on the final prints was measured at 1.0-mm intervals along the length of unstretched (curved) fibers, by the use of a reticule marked at 0.1-mm units at ×7 magnification. At the ×100,000 magnification used in the final prints, this method is equivalent to measuring the fibers at points 100 A apart. In the second method, a transparent celluloid sheet, which was ruled at 0.5-cm intervals, was placed over the print. Fibers were measured at each point crossed by a line on the ruled sheet. After all fibers were measured at these points, the transparent sheet was rotated 90° and a second set of measurements was made. In the application of this method, the diameter of fibers was measured at all points crossed by a line, except in cases in which

TABLE I

Barley Chromosome Fibers

Source	Prefix-ation	Trypsin digestion	Method of measurement	No. of measurements	Mean diameter	Standard deviation
					A	A
I. Endosperm Preparation 1	—	—	100-A Intervals	1053	252	29.6
II. Endosperm Preparation 1	—	—	Grid	387	239	34.8
III. Endosperm Preparation 2	—	—	100-A Intervals	605	223	31.3
IV. Endosperm Preparation 3	—	—	100-A Intervals and Grid	230	270	36.3
V. Endosperm Combined data	—	—		2075	244	39.4
VI. Endosperm	+	—	Grid	367	122	29.5
VII. Leaflet	—	—	Grid	780	187	49.0
VIII. Leaflet	+	—	Grid	176	127	36.9
IX. Root tip	—	—	Grid	438	170	54.7
X. Root tip	+	—	100-A Intervals and Grid	433	142	35.3

superimposition of fibers prevented recognition of the outline of a single fiber. For the purpose of giving some idea of the differences in measurements produced by the two methods, one photograph (Preparation No. 1, Table I) was measured by both methods. As expected, slightly greater variability and a reduced average diameter was obtained by the second ("grid") method. The figures presented in this paper do not include the total area of the final prints used for measurements, since illustration limitations restrict the size and magnification possible for publication.

RESULTS

The results obtained from measurement of barley chromosome fibers are summarized in Table I and in the histograms in Figs. 1–6. Three different preparations were used for samples of unfixed barley endosperm (Figs. 1 and 7). Preparation 1, unfixed endosperm, was measured by both the 100 A interval and grid methods. As can be seen, measurement by the grid method gives a smaller average diameter for the chromosome fibers and somewhat greater variability in the data. In practice, selection of the measuring method used depended upon the quality of preservation of the fibers. If the preparation showed fibers of fairly uniform dimensions (see, for example, Fig. 7), the 100-A interval method was used. If some degree of distortion or obviously higher variability in diameter was present (Fig. 8, for example), the grid method was used, to eliminate bias.
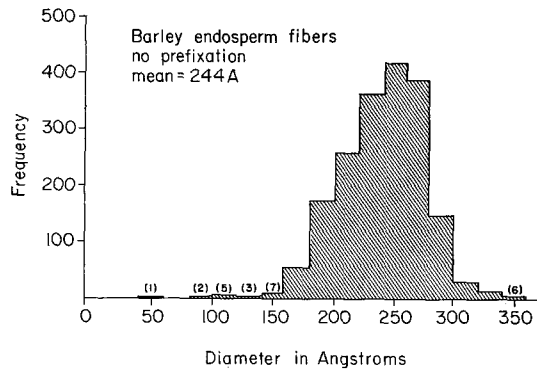

FIGURE 1 Histogram compiled from data from three preparations of barley endosperm chromosome fibers isolated without prefixation. "Frequency" refers to the number of times a given fiber diameter was encountered in the sample. Numbers in parentheses refer to the frequencies with which the extreme diameters were encountered.

Unfixed barley endosperm chromosomes show a mean fiber diameter of 244-A for the three preparations, which is very close to the 250-A fiber dimension reported previously for unfixed animal chromosome fibers. Unfixed fibers from barley leaflets are of smaller dimension, averaging 187 A in diameter (Fig. 3). The fibers from root tip (Fig. 5) are similarly reduced in diameter, to approximately 170 A. The 187- and 170-A diameters for
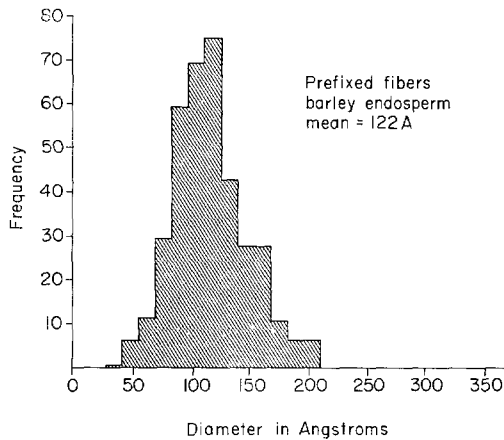
FIGURE 2 Histogram compiled from data from three preparations of barley endosperm chromosome fibers isolated after prefixation in 2% buffered formalin (see text).
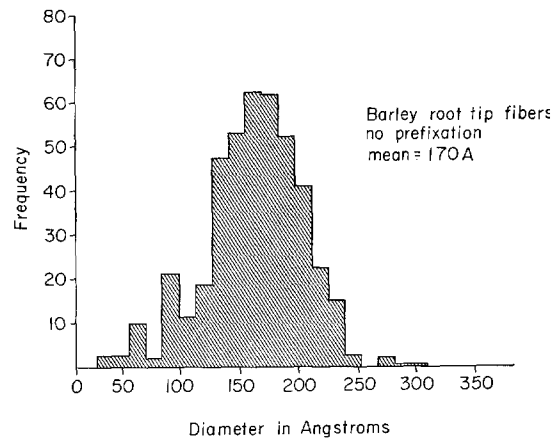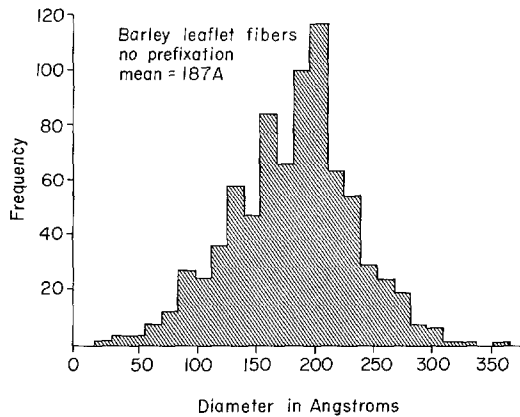

FIGURE 3 Histogram compiled from data from two preparations of barley leaflet chromosome fibers isolated without prefixation.

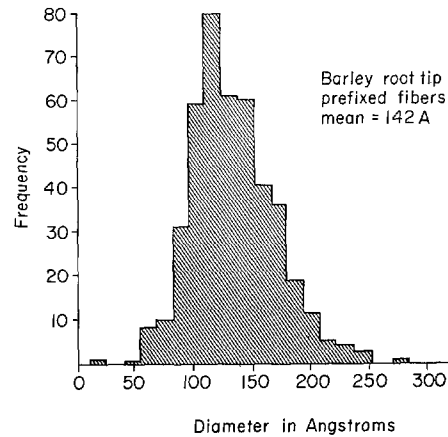FIGURE 4 Histogram compiled from data from two preparations of barley leaflet chromosome fibers isolated after prefixation in 2% buffered formalin.

FIGURE 5 Histogram compiled from data from two preparations of barley root tip chromosome fibers isolated without prefixation.

FIGURE 6 Histogram compiled from data from two preparations of barley root tip chromosome fibers isolated after prefixation in 2% buffered formalin.
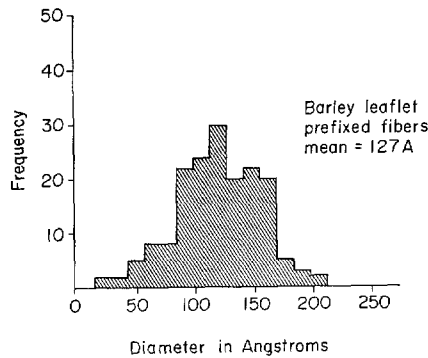
the fibers from leaflet and root tip, obtained by the grid method, represent values which are probably lower than the actual unstretched diameter for the various fibers isolated, since the inclusion of stretched fibers tends to produce a skewness in the data in the direction of reduced diameter, as has been noted. For this reason, the mean values for unstretched fibers in these two preparations would be expected to be near 200 A.

After prefixation in formalin, chromosome fibers from each tissue show a reduction in diameter, to 122 A for endosperm (Fig. 2), 127 A for leaflet (Fig. 4), and 142 A for root tip (Fig. 6). Fig. 8 shows chromosome fibers from barley root tip iso-
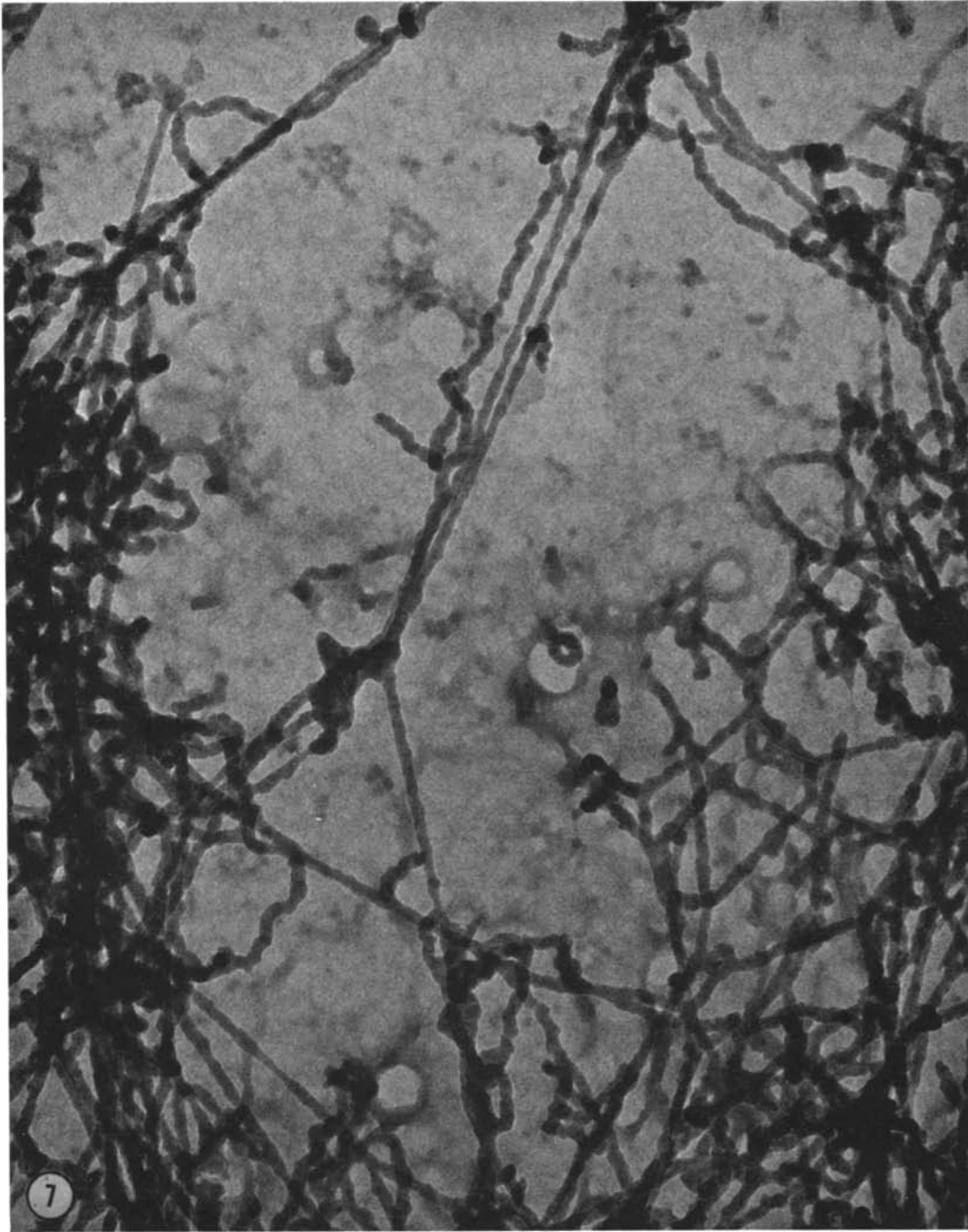
FIGURE 7 Chromosome fibers from a barley endosperm nucleus, isolated by the Langmuir trough–critical point method without prefixation. The structure and dimensions of the fibers in this nucleus are very close to those of animal fibers isolated by the same methods. The average diameter of the fibers of this nucleus is 252 A if measured by the "100-A interval" method, and 239 A if measured by the "grid" method (see text for a description of methods of measurement used). This figure includes approximately 25% of the area of this nucleus used for measurements. × 83,000.
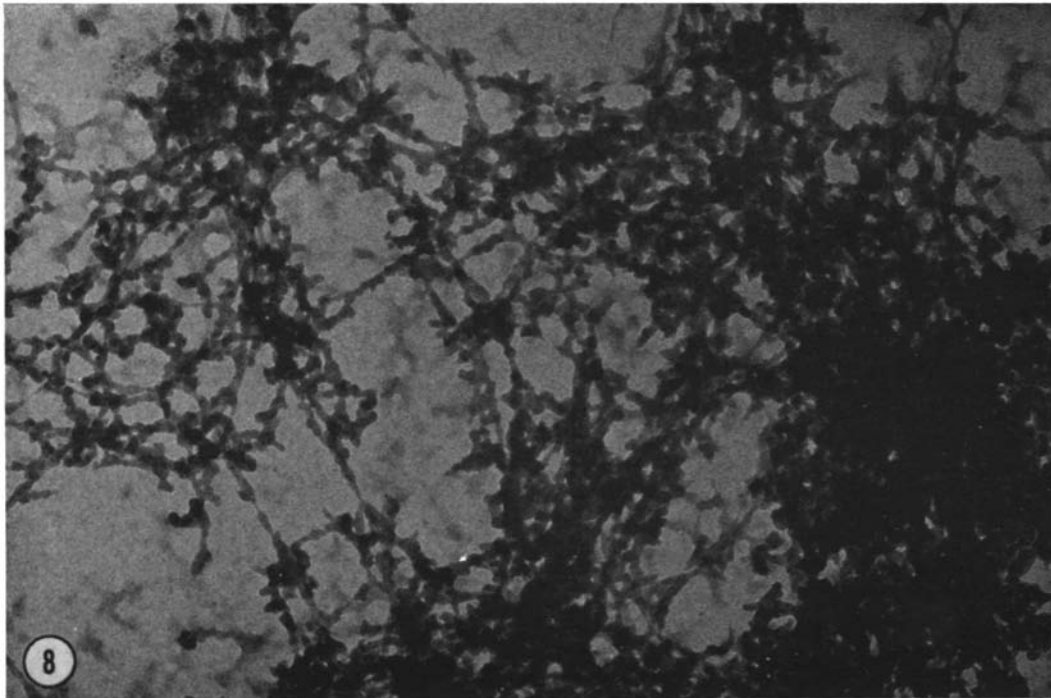
FIGURE 8 Chromosome fibers from a barley root tip nucleus, isolated after prefixation in 2% buffered formalin. Fibers in this preparation average 152 A in diameter. No obvious substructure is visible in the fibers in this type of preparation. This figure includes approximately 20% of the total area of this nucleus used for measurements; grid method. × 83,000.

lated after prefixation. Except for the reduced diameter and somewhat higher variability, the fibers in such preparations are essentially similar in structure to unfixed fibers.

Analysis of data from the unfixed animal chromosome fibers measured in this study (Table II) reveals that the fibers have a mean diameter close to the values reported by DuPraw (2), Gall (4), and Wolfe (10). A typical preparation is shown in Fig. 9. If the data for newt and bovine chromosome fibers are combined, the mean value of 240 A obtained for fiber diameter is very close to the combined mean for the diameter of unfixed chromosome fibers from barley endosperm. As in the case of prefixed barley chromosome fibers, prefixed chromosome fibers from the erythrocyte of the newt, *Triturus viridescens* (Fig. 10), are of smaller diameter, 117 A. Trypsin digestion of prefixed animal chromosome fibers (Fig. 11 and Table II) does not result in a significant change in fiber diameter or structure.

DISCUSSION

The fibers designated as "unfixed" in this study are actually subjected to a series of reagents, after isolation of the chromosomes, which may serve to fix the fibers to a certain degree. In particular, uranyl acetate and ethyl alcohol would be expected to act as fixatives. However, these possible fixatives act after the breakdown and spreading of cells on the trough. Since the point in the isolation procedure at which the fiber diameter appears to change is the time of disturbance of cell membranes (11), the application of fixatives after this time would not be expected to be significant in reducing or preventing the change in diameter.

A number of factors combine to reduce the significance of the mean diameters obtained for the various methods of isolation and the organisms investigated. Since the instrumental resolution under the best operating conditions approximates 10 A, values smaller than 10 A are not significant. The total magnification used for prints, 100,000,

TABLE II

Animal Chromosome Fibers

| Source	Prefix-ation	Trypsin digestion	Method of measurement	No. of measure-ments	Mean diameter	Standard deviation
					A	A
I. Newt erythrocyte	—	—	100-A Intervals	374	227	30.9
II. Bovine kidney cells	—	—	100-A Intervals	496	250	35.4
III. Newt erythrocyte and bo-vine kidney cells, combined data	—	—	100-A Intervals	774	250	37.0
IV. Newt erythrocyte	+	—	Grid	161	117	37.1
V. Milkweed bug (*Oncopeltus*) testis	+	+	Grid	352	129	35.1
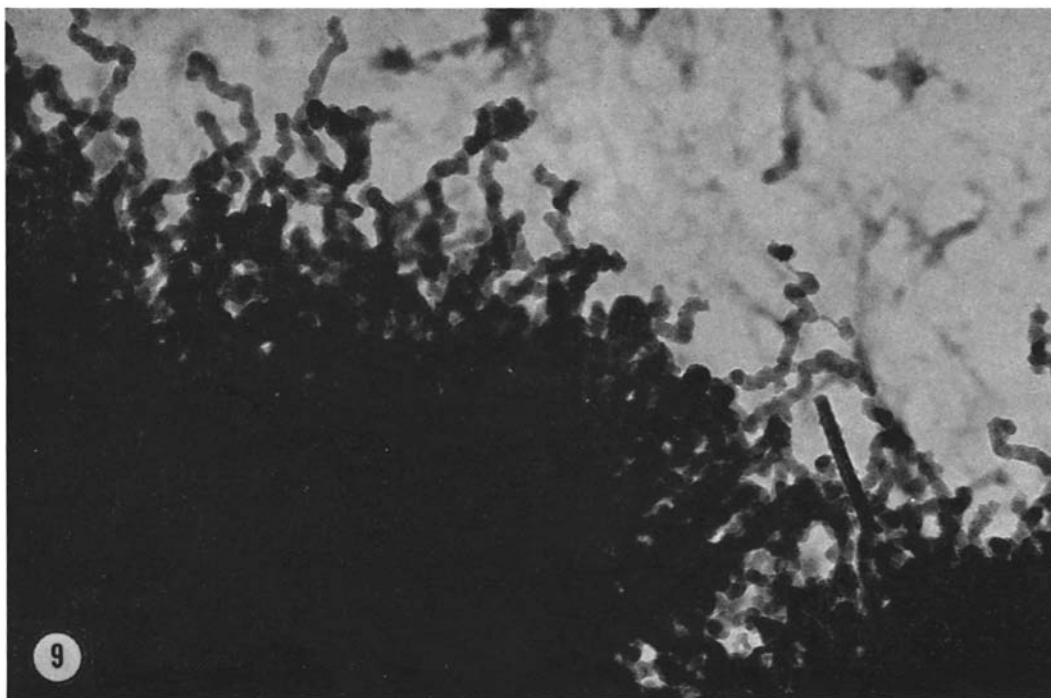
FIGURE 9 Fibers from a nucleus of bovine kidney cell in tissue culture, isolated without prefixation. Average fiber diameter, 250 A. This figure includes approximately 25% of the area used for measurements; 100-A interval method. × 83,000.

would permit recognition of size differences at, at least, the level of 20 A and possibly somewhat less. Additional errors are introduced by judgment in measurement, variation in instrumental magnification, and contamination of the fibers in the electron microscope. Further, as has been noted, the grid method of measurement produces a smaller mean value for fiber diameter than the 100-A interval method, because of the inclusion of stretched fibers among the measured fibers.

The results reported here for barley indicate that considerable differences exist in the diameter of unfixed chromosome fibers isolated from endosperm and leaflet and root tip. Whereas in endo-

FIGURE 10 Fibers from an erythrocyte of newt, *Triturus viridescens*, isolated after formalin prefixation. Average fiber diameter 117 A. This figure includes approximately 25% of the area used for measurement; grid method. × 83,000.

sperm the diameter of unfixed fibers approximates values previously reported for animal chromosomes, in leaflet and root tip the diameters of unfixed fibers are considerably smaller. The significance of this difference, approximately 50 A, is difficult to evaluate, but it would appear that the cumulative errors in the methods used are not so large that this difference can be ignored.

This variation in diameter is in contrast to the situation in animal chromosomes, in which fiber diameter has been described as constant not only between the tissues of an organism, but from species to species. It is possible, however, that a more detailed statistical study of animal chromosomes would reveal such differences. The small difference noted in this report between the mean value for unfixed fibers from newt erythrocyte and that for unfixed fibers from bovine kidney, approximately 20 A, must be regarded as probably not significant, in view of the degree of error. It is worth noting in this regard, however, that Gall (4) has reported that fibers isolated from grasshoper spermatocytes show a reduced diameter.

Prefixation of the three barley tissues and the

animal cells investigated causes a reduction in the diameter of isolated chromosome fibers in each case. This is most striking in endosperm, in which the fiber diameter is reduced from approximately 250 A to 120–130 A. Fibers from leaflet are reduced from approximately 200 A to 120–130 A. Because of the noted errors, it is difficult to determine whether the somewhat larger diameter obtained for fibers from prefixed root tip, 140–150 A, is significantly different from the diameter for fibers from prefixed leaflet and endosperm.

A general conclusion, which can be made from the results obtained with both the no-fixation and the prefixation isolation methods, is that the diameter of isolated chromosome fibers is variable, depending upon the method of isolation used. The dimensions obtained after isolation by a given method are, however, relatively constant. If this is indeed the case, the smaller diameters found for fibers in the leaflet and root tip chromosomes of barley can be regarded as resulting from special conditions of isolation rather than representing a basic difference between the chromosome fibers of plants and animals.
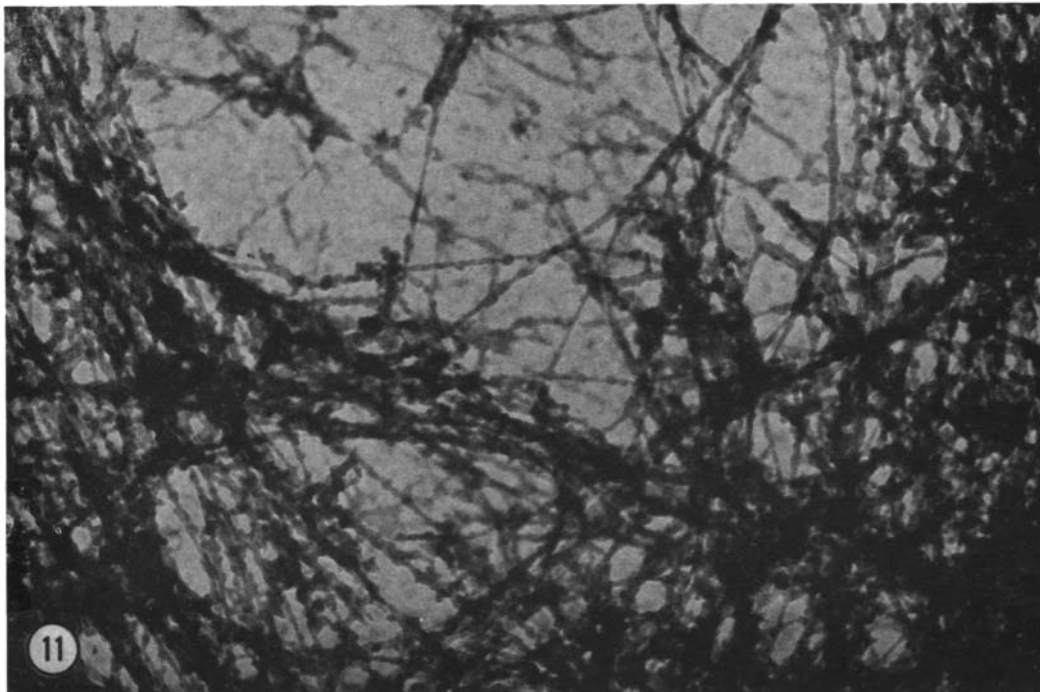
FIGURE 11 Fibers from newt erythrocyte (*T. viridescens*), digested with trypsin after prefixation in formalin. Average fiber diameter, 131 A. This figure includes approximately 20% of the area used for measurements; grid method. × 83,000.

Recent reports of fiber diameter in chromosomes isolated by the Langmuir trough–critical point method have been based on studies with animal chromosomes. The mean diameters found for the animal chromosomes included in the present work, 227 A and 250 A, are reasonably close to the value of 250 ± 20 A reported by Wolfe (10) for unfixed chromosome fibers from the Western salamander, *Taricha granulosa*, and the value of 230 A given by DuPraw (2) for unfixed chromosome fibers from the honeybee. Gall's estimate of 200–300 A (4) also includes the means obtained here. Ris's reported diameter of 200 A (9) for isolated, unfixed animal chromosome fibers would appear to be smaller than expected on the basis of the limited data included in this paper. While the chromosome fiber from barley endosperm is similar in diameter to the animal chromsome fibers studied, the diameters of the fibers from leaflet and root tip, which approximate 200 A, are much closer to the estimate for chromosome fiber diameter given by Ris.

The reduced diameter observed in prefixed chromosome fibers possibly reflects an interaction

of the fixative with the chromosome fibers so as to bind the sites involved in the transition to the larger diameter. Since the diameter of chromosome fibers isolated after formalin prefixation is still in excess of 100 A, it seems very likely that even with prefixation some degree of change occurs in fiber dimensions on isolation. The 80-A fiber diameter reported by Kaye (6) for thin-sectioned chromosomes lends support to this conclusion. The observed change in diameter could result from coiling or side-by-side aggregation of fibers or from interaction between the native fibers and surrounding molecules in the nucleus to produce a fiber of larger dimensions. It has not been possible in this study to determine which of these processes actually underlies the observed difference. Comparison of the gross morphologies of isolated metaphase chromosomes with and without prefixation is expected to provide some insight into which of these processes is involved. If, as seems to be the case in the limited work accomplished to date (12), the gross structures are found to be identical with and without prefixation, an interaction of the fibers

with surrounding molecules of the nucleus to produce a thicker fiber would seem the most likely process involved, since the alternatives would be expected to produce extensive rearrangement of the component fibers of the chromosome. Further work will be required to establish the validity of this conclusion.

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