DNA AND THE FINE STRUCTURE OF SYNAPTIC CHROMOSOMES IN THE DOMESTIC ROOSTER (*GALLUS DOMESTICUS*)

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ABSTRACT

The indium trichloride method of Watson and Aldridge (38) for staining nucleic acids for electron microscopy was employed to study the relationship of DNA to the structure of the synaptinemal complex in meiotic prophase chromosomes of the domestic rooster. The selectivity of the method was demonstrated in untreated and DNase-digested testis material by comparing the distribution of indium staining in the electron microscope to Feulgen staining and ultraviolet absorption in thicker sections seen with the light microscope. Following staining by indium, DNA was found mainly in the microfibril component of the synaptinemal complex. When DNA was known to have been removed from aldehyde-fixed material by digestion with DNase, indium stainability was also lost. However, staining of the digested material with non-selective heavy metal techniques demonstrated the presence of material other than DNA in the microfibrils and showed that little alteration in appearance of the chromosome resulted from DNA removal. The two dense lateral axial elements of the synaptinemal complex, but not the central one to any extent, also contained DNA, together with non-DNA material.

Meiotic prophase chromosomes have been shown with the electron microscope to embody a characteristic and unique substructure (24). The dense thickenings along the axes of such chromosomes adhere to a common structural pattern in most synaptic chromosomes so far studied, and have been termed "synaptinemal complexes" (20). Since these structures are found in such a wide variety of animals and plants (*cf.* 24), it is probable that they have been preserved through the evolutionary process as an advantageous mechanism for performing a specific functional role. It has been proposed that they are the morphological expression of the specific function of these chromosomes, *i.e.* of the point-by-point pairing of homologous chromosomes that is a prerequisite of genetic crossing-over and cytological chiasma formation. Other characteristic patterns of organization of chromosomes are known; for example, the "lampbrush" configuration of chromosomes in amphibian oocytes, and the polytene chromosomes of dipteran larvae. Although these are not distributed so widely as the synaptinemal complex, they, nevertheless, represent special organizations of the chromosome material concomitant with specific functional activities. Even mitotic (13) and interphase (8) chromosomes are considered to possess individual conformational states which facilitate their particular functions.

Common to all these chromosome types must be at least one chain or thread of DNA which contains sequentially ordered genetic information. In addition, there is associated with the DNA a protein moiety which is thought to be responsible

for the different states of chromosome organization related to various functions. Experimental evidence has suggested the simple nature of the DNA thread in lampbrush (5, 7) and polytene (35) chromosomes and has led to hypotheses of their organization. So far, the synaptic chromosomes have been mainly characterized by their morphology, and it is not known whether structurally distinct portions are also chemically distinct, or precisely where DNA is localized. An understanding of the structural or functional pattern underlying this chromosome depends on elucidating the relationship of DNA and protein to its organization.

Accordingly, the indium-trichloride ($InCl_3$) technique of Watson and Aldridge (38) was used to localize DNA with the electron microscope in the primary spermatocyte chromosomes of roosters (*Gallus domesticus*).[1] The latter provide a convenient and abundant source of non-seasonal material, with a high (*ca.* 70, see reference 29) chromosome number.

MATERIALS AND METHODS

Testes were removed rapidly from 6-month-old roosters, placed in cold (0–5°C) fixative, and cut into small pieces by drawing a razor blade across the tissue. Pieces about 1 mm^3 were then transferred to fresh, cold fixative on small strips of filter paper.

[1] Part of the results reported in this work has been presented at the 3rd Annual Meeting of the American Society for Cell Biology and appeared in abstract form in *The Journal of Cell Biology*, 1963, **19**, No. 2, 15A.

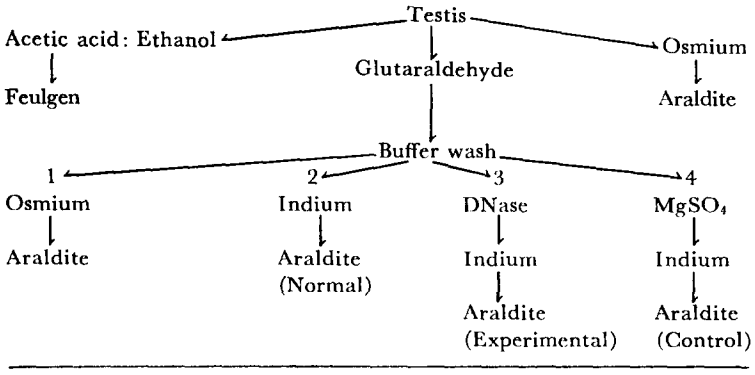
The following fixatives were used:

1. For light microscopy: 3 parts ethanol to 1 part glacial acetic acid (v/v).
2. For electron microscopic morphology: buffered 1 per cent osmium tetroxide, pH 7.2 (30), for 30 minutes to 1 hour in the cold, with rapid dehydration in alcohol.
3. For electron and light microscope cytochemistry:
   a. Glutaraldehyde, 6.5 per cent in 0.1 M phosphate buffer, pH 7.2, containing 0.1 M sucrose, for 4 hours in the cold (33).
   b. Formaldehyde, 4 per cent, in 0.067 M phosphate buffer, pH 7.2, containing 7.5 per cent sucrose (9), for 30 minutes to 12 hours in the cold.
   c. Acrolein (15) according to the procedure of Watson and Aldridge (38), for 30 minutes.

TABLE I

Fixation Plan for Tissues Fixed in Glutaraldehyde

A comparable plan was also followed with acrolein as the fixative.



The glutaraldehyde- and the acrolein-fixed tissue blocks were separated into four groups after washing for 12 hours in 0.1 M phosphate buffer containing 0.1 M sucrose in the cold and treated as follows (Table I):

1. Postfixed in osmium, as above, for 30 minutes, then dehydrated in ethanol and embedded as above in Araldite, using propylene oxide as a carrier (16).

2. (Normal) stained with indium trichloride according to the procedure outlined by Watson and Aldridge (38), except that the initial dehydration was carried out in a graded ethanol series, each step of which contained 10 per cent of the fixative. Some of the acrolein-fixed material was dehydrated and embedded in methacrylate according to the procedure of Watson and Aldridge.

3. (Experimental) digested at 37°C with deoxy-

ribonuclease (DNase I, Worthington Biochemical Corp., Freehold, New Jersey) at a concentration of 1 mg/ml dissolved in 0.003 M $MgSO_4$ and pH adjusted to 7.2 with 0.1 M NaOH. Digestion was continued until samples of tissues removed from the digestion mixture did not exhibit any Feulgen-positive staining in the nuclei of primary spermatocytes, when stained with the Feulgen squash procedure. The times required for digestion varied from 5 hours with formaldehyde fixation to 28 hours with glutaraldehyde and 48 hours with acrolein. For the longer periods of digestion, the DNase solution was changed every few hours in some cases. This did not appreciably shorten the time required for digestion, nor did reducing the size of the tissue block to the point at which it became, in effect, little more than a suspension of fixed cells. Blocks for electron microscopy were digested somewhat longer than necessary. They were not removed from the digestion mixture until about an hour after the end point had actually been reached. This additional length of time was required to remove and process a sample for Feulgen squash and light microscope monitoring. When it was determined that the end point had occurred, the blocks of tissue were stained with indium as in 2, dehydrated, and embedded in Araldite or methacrylate.

4. (Control) incubated in 0.003 M $MgSO_4$ at 37°C for the same length of time as the tissue in 3; then stained with indium as in 2, and embedded in Araldite or methacrylate.

Formaldehyde-fixed tissue was postfixed in osmium (as in 1), or digested with DNase (as in 3), or treated with $MgSO_4$ (as in 4), then embedded in Araldite.

Methacrylate was polymerized in a vacuum desiccator with long wavelength ultraviolet light, and Araldite was polymerized by heat in a 60°C oven.

Sections were cut on Porter-Blum (Servall) and Huxley (Cambridge) microtomes at various thicknesses, depending on the purpose. Sections varied in thickness between 50 and 100 mμ for examination in the electron microscope, and from ½ to 2 μ for examination in the light and ultraviolet microscopes. Thick sections were stained with either the Feulgen procedure or toluidine blue (18); or mounted in glycerol on quartz slides with a quartz coverslip and examined photographically and with an image converter at 262 mμ (absorption) and 350 mμ (scatter) in the ultraviolet microscope. In all cases, thick sections stained by the Feulgen procedure were compared with a control section which had received the same treatment but had not been hydrolyzed. This allowed assessment of the amount of stain bound by any of the aldehyde fixative remaining in the tissue. We detected no staining due to the fixative.

Thin sections were stained, in some cases, with a heavy metal according to one of the following procedures:

1. URANYL ACETATE: Grids were floated, section-side-down, on a drop of freshly filtered, saturated (*ca.* 0.2 M) aqueous uranyl acetate (36) for 20 minutes to 2 hours at room temperature, then rinsed vigorously in three changes of quartz-distilled water. The same procedure was used with 1, 2, and 4 per cent aqueous solutions as well as staining at elevated (60°C) temperatures. For the purposes of this study no advantages were found in any of these procedures, nor with alcoholic uranyl acetate, over saturated solutions at room temperature. Staining at elevated temperatures increased the density of stain bound in a given time, but also increased dirt deposits and tended to lift the sections or the carbon film from the grid.

2. LEAD HYDROXIDE: Grids were immersed in a drop of stain (prepared according to method A of Karnovsky (11) and diluted either 1:100 or 1:50 with quartz-distilled water) in a closed space containing NaOH pellets, for 5 to 30 minutes, then rinsed either with a thin stream of distilled water or vigorously shaken in three changes of quartz-distilled water.

3. POTASSIUM PERMANGANATE (14): Grids were immersed in aqueous 1 per cent $KMnO_4$ for 5 minutes, quickly rinsed in water, then rinsed in a 0.0125 M citric acid wash, and again in water.

In some cases, thin sections of indium-stained material which were picked up on carbon-coated grids were given an additional coat of evaporated carbon (38) either on the "under" or non-section side of the grid, or on the section side so that the sections were "sandwiched" between the carbon coats. This procedure decreased contrast and resolution, but prevented sublimation of indium from the section in the electron beam.

A drop of a dilute suspension of polystyrene latex particles was routinely placed on grids. These allowed an accurate assessment of focus in sections of low contrast, and with low beam intensities, and provided as well a reference for the estimation of astigmatism.

Electron micrographs were made on a Philips EM 100B, equipped with a Ladd anode and a 25-μ objective aperture, operating at 60 kv; Kodak Spectroscopic 649-0 film or Eastman Kodak Lantern Slide Plates in both medium and contrast grades were used. Original magnifications were from 1500 to 30,000, and micrographs were further enlarged photographically.

Tissues fixed in acetic acid–ethanol were stained by the Feulgen squash technique (3) to demonstrate DNA.

RESULTS

Because of unsatisfactory and inconsistent results with acrolein fixation (as mentioned by Watson and Aldridge, 38), as well as of the fact that the action of DNase was inhibited by this aldehyde, a more suitable substitute was sought. Glutaralde-

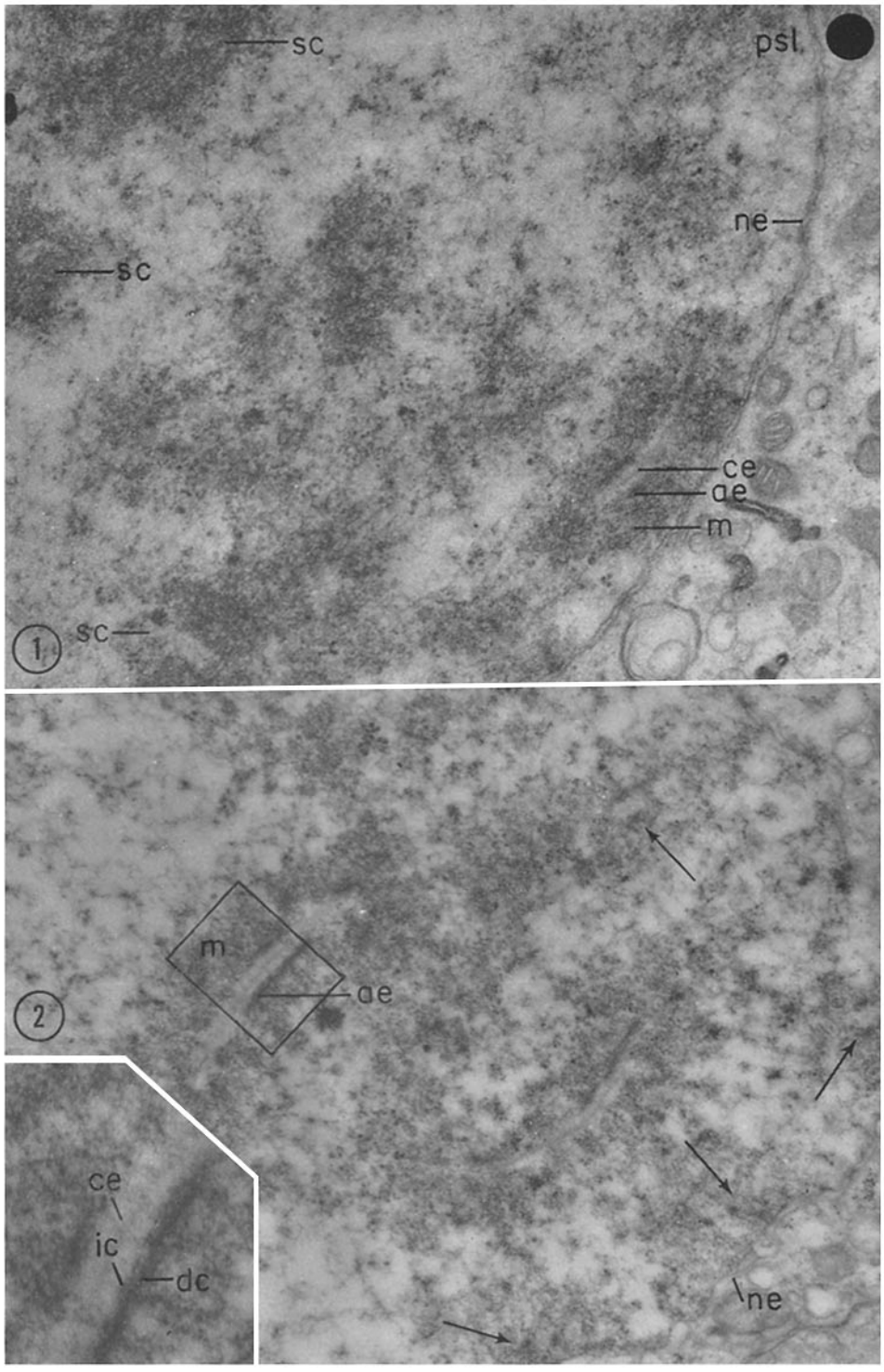
hyde afforded consistent fixation throughout the tissue blocks, and the time required for digestion with DNase to the point of Feulgen-negative primary spermatocyte nuclei was short enough so that little damage to the control tissue in $MgSO_4$ was noted. The intracellular morphology following fixation with OsO_4, and with glutaraldehyde and osmium postfixation, was taken as a standard for comparison. Since these preparations were dehydrated in ethanol and embedded in Araldite, the glutaraldehyde-fixed, indium-stained material was also dehydrated with ethanol (containing 10 per cent glutaraldehyde) and embedded in Araldite. Because of unpredictable results with both polymerization and tissue preservation in methacrylate, Araldite was used as the embedding material throughout most of the investigation. A disadvantage of Araldite is that the hardener used, dodecyl succinic anhydride, leaches indium, but this was overcome by cutting sections from freshly polymerized blocks before significant leaching could occur. Even when stain was leached, however, the selectivity of the stain was not altered, as could be seen by a comparison of sections cut from old and new blocks. Further, the density of Araldite to electrons more closely matched that of the tissue than methacrylate, thereby reducing the occurrence of density differences in the tissue unless caused by indium staining. Sections from tissue that had been handled according to Watson and Aldridge's original procedure were compared to those treated by our alternative procedure. The results were substantially the same, as was confirmed by Aldridge (personal communication). More particularly, with respect to the meiotic chromosomes of primary spermatocytes the same distribution of stain was observed with both methods, and the morphology, as far as size, number of elements, and general appearance, did not seem to be altered.

Since meiotic chromosomes of primary spermatocytes have been described mainly from osmium-fixed tissue, an attempt was made to correlate the results of the indium-staining technique with the familiar images of osmium-fixed material. Therefore, sections from tissue fixed in osmium alone were compared with those from glutaraldehyde-fixed, indium-stained material. Other portions of tissue were fixed in glutaraldehyde, then postfixed with osmium in an attempt to isolate the effects of glutaraldehyde fixation from those of osmium. The pictures presented by the osmium-fixed, the glutaraldehyde- and osmium-fixed, and the glutaraldehyde-fixed tissues stained with heavy metal were the same (Figs. 1 to 3). The prophase chromosomes of primary spermatocytes appeared as linear structures composed of microfibrils, a central element, and two double axial elements. The microfibrils, about 100 A in diameter, extend laterally into the nucleoplasm. The double axial element consists of an "inner" strand bordering on the light central zone of the complex and an "outer" strand nearer the microfibrillar part of the complex and away from the central light zone (Figs. 2 and 4). The central element, a linear structure midway between the two double axial elements, lies in a central pale zone. This pattern seen in tissue fixed in both ways is essentially the same as that originally described by Moses (20), and, except for the doubleness of the axial elements, that by Fawcett (4) and Moses (21).

In contrast to the above, the indium-stained tissue (Fig. 5) showed that only the microfibrils and the "inner" strand of the double axial element

FIGURE 1 Primary spermatocyte from glutaraldehyde-fixed, osmium-postfixed testis stained with saturated uranyl acetate, showing short segments of several meiotic chromosomes (*sc*), recognizable as light "cores" in dense chromatin, each composed of three parts: microfibrils (*m*), central element (*ce*), and axial elements (*ae*). One chromosome is tangent to the nuclear envelope (*ne*) and the other three are shown almost in cross-section. *psl*, polystyrene latex particle, 0.26 μ. × 25,000.

FIGURE 2 Primary spermatocyte from osmium-fixed material stained with saturated uranyl acetate and showing long segments of meiotic chromosome. The double nature of the axial elements is evident from the insert which is a higher magnification of the same chromosome. Four chromosomes are seen in near transverse sections (arrows) near the nuclear envelope (*ne*). The inset is a higher magnification of the area in the box. *m*, microfibrils. *ae*, axial elements. *dc*, dense outer component of axial element. *ic*, inner component of axial element. *ce*, central element. × 26,000; inset, × 60,000.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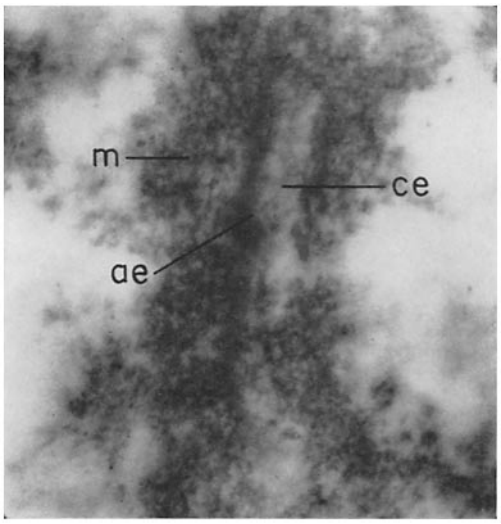


FIGURE 3 Longitudinal section through part of a synaptinemal complex from glutaraldehyde-fixed testis stained with saturated uranyl acetate. The microfibrils (*m*), axial elements (*ae*), and central element (*ce*) are all visible. × 57,000.

were stained (Table II). The area occupied by the "outer" strand in osmium-treated tissue is, in the indium-stained tissue, a zone of density intermediate between the densities of the microfibril area and the central light area. No central element is visible between the axial elements. This distribution of stain suggests that nucleic acid is concentrated in the microfibrils of the synaptinemal complex, and that the axial elements, though they contain nucleic acid, are probably not so rich in this material as the microfibrils. Further, the dense staining of the axial elements is not due pri-

TABLE II

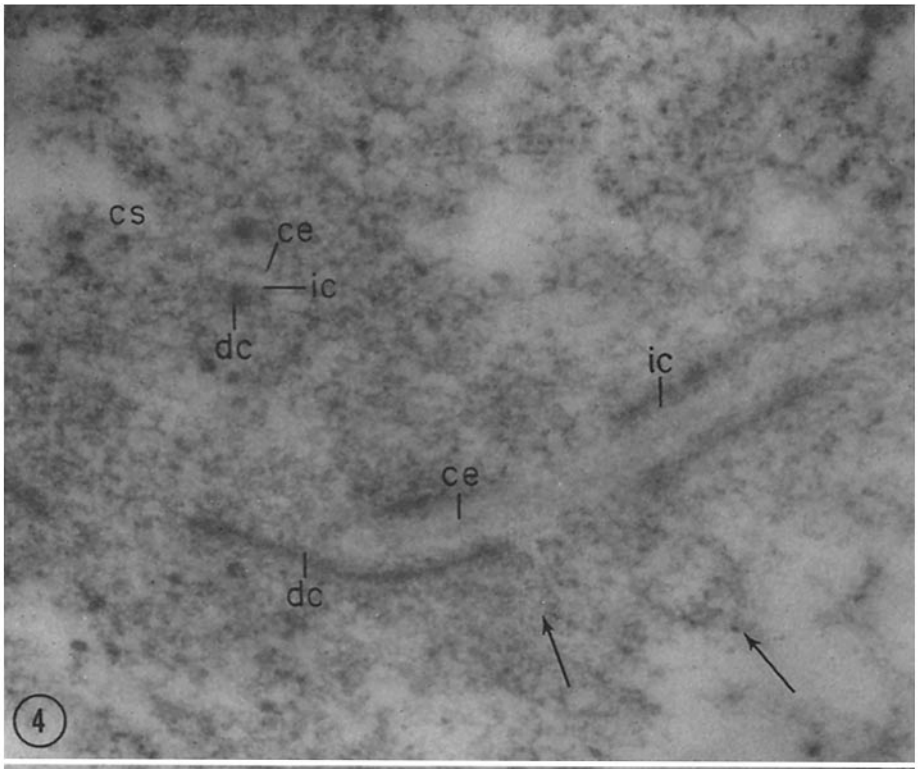
Visibility of Components of the Synaptinemal Complex in the Electron Microscope with Various Treatments

Treatment	Component: Microfibrils	Axial elements: Inner	Axial elements: Outer	Central element
Osmium	×	×	×	×
Glutaraldehyde+ Heavy metal (Os, Pb, U)	×	×	×	×
Indium	×	×	ø	0
Indium Heavy metal	×	×	×	×
DNase Indium	0	0	0	0
DNase Indium Heavy metal	×	×	×	×
$MgSO_4$ Indium	×	×	ø	0
$MgSO_4$ Indium Heavy metal	×	×	×	×

× = visible.
0 = invisible.
ø = stained, but different density than material treated with osmium.

FIGURE 4 Synaptinemal complex from osmium-fixed testis stained with saturated uranyl acetate. Both transverse (*cs*) and longitudinal sections are visible. In the longitudinal section the axial elements appear to be interrupted for part of their length, and in these areas (arrows) the microfibrils assume a slightly altered configuration. The microfibrils which can be traced in this area are about 25 A in diameter. The dense outer component (*dc*) and lighter inner component (*ic*) of the axial elements are clearly visible in several places. *ce*, central element. × 55,000.

FIGURE 5 Synaptinemal complex from glutaraldehyde-fixed, indium-stained testis. This grid was sandwiched with a layer of evaporated carbon, which somewhat reduces resolution. Both longitudinal and nearly transverse (*cs*) sections are visible, and the lack of the dense outer component of the axial elements is evident. Comparison with Fig. 3 shows that the area occupied by this component is filled by material continuous with the microfibrils (*m*), as the inner lighter component (*ic*) seems to be. There is no central element visible. × 55,000.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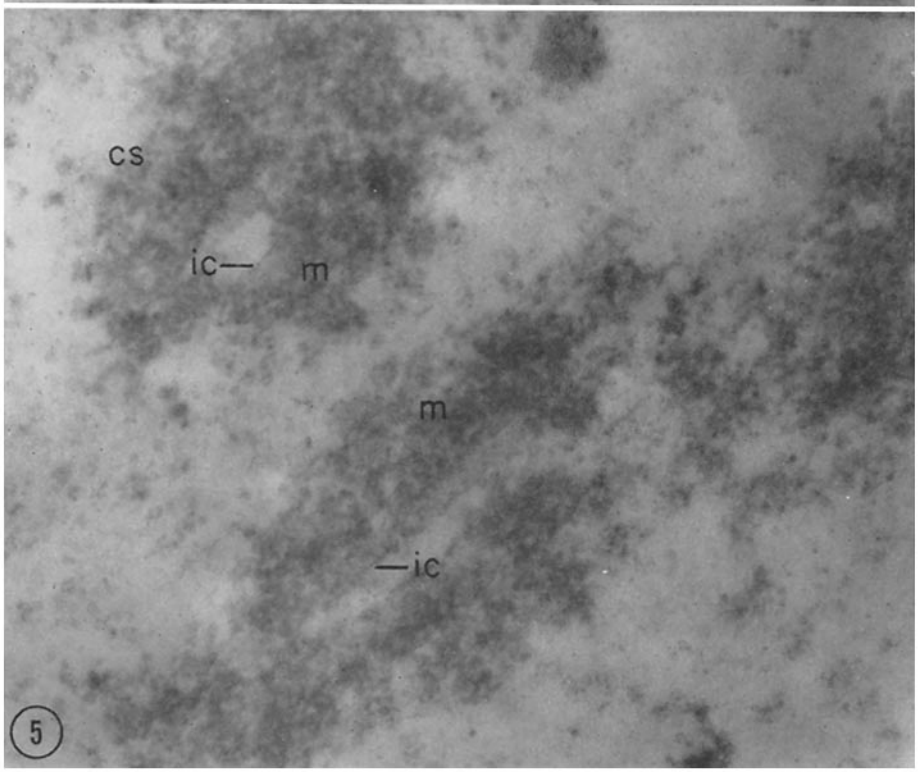
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marily to the presence of nucleic acids, as might be expected on the basis of osmium staining.

When glutaraldehyde-fixed, DNase-digested tissue is postfixed with osmium, no gross changes in structure, density, or dimension are found. But, glutaraldehyde-fixed, DNase-digested, indium-stained tissue shows no stain in the nuclei of primary spermatocytes (except for 100-A particles to be mentioned later) (Figs. 6 and 7). Yet, the nuclei of other cells (such as sustentacular cells and erythrocytes, Fig. 7), which are Feulgen positive in adjacent thick sections, show normal staining with indium. This is in contrast to the material treated with $MgSO_4$ for the same length of time and at the same temperature, which manifests no alterations in staining (Table III). Finally, when thin sections of DNase-digested, indium-stained tissues are further stained with other, less selective heavy metal techniques, all the elements of the meiotic chromosome are visualized (Figs. 8 to 11). Microfibrils, axial elements, and central elements are visible.

With digested material, the pattern of indium staining in the electron microscope follows that seen in thick sections stained with the Fuelgen procedure, which, in turn, corresponds to the distribution of absorbing and non-absorbing cells seen with the ultraviolet microscope (Table IV). That is, the same types of nuclei are seen to contain nucleic acid by all three methods. Furthermore, thick sections of the same material, when stained non-selectively with concentrated toluidine blue, revealed the presence of meiotic chromosomes in primary spermatocyte nuclei which had been unstained by the Feulgen procedure, and which had shown no appreciable absorption at 262 mμ. In a similar way, thin sections of the same material which had no chromosome staining with indium in primary spermatocyte nuclei were shown to contain intact meiotic chromosomes when stained with a less selective metal stain such as permanganate, lead, or uranium. Therefore, although DNA was removed from the meiotic chromosomes of primary spermatocytes, at least within the limits of detection by our methods the structural integrity of the chromosome was retained.

DISCUSSION

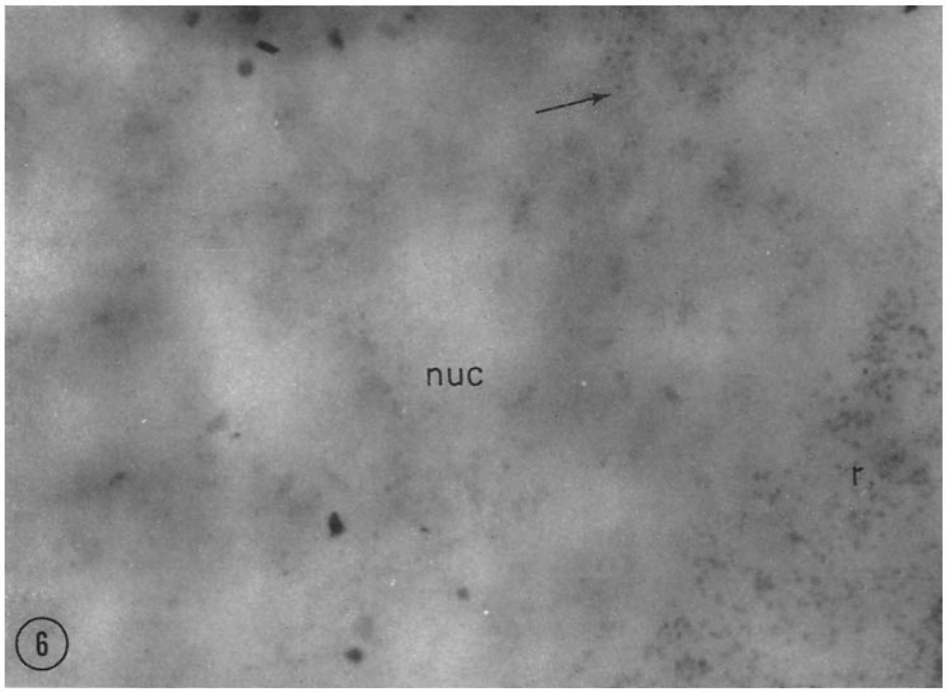
Techniques

Initial experiments in this study were aimed at describing changes in fine structure of the meiotic chromosome in general, and of the synaptinemal complex in particular, which result from the removal of DNA by DNase. The original approach consisted of primary fixation with an aldehyde (glutaraldehyde, acrolein, or formaldehyde), then digestion of the tissues with DNase until the primary spermatocytes were no longer Feulgen positive, whereupon they were postfixed in OsO_4. Unexpectedly, comparison with undigested controls, with both the light and electron microscopes, revealed no structural alterations that could be attributed to the removal of DNA. Both DNase-treated and $MgSO_4$-treated (control) blocks contained areas in which over-all deterioration was apparent. However, the position of the areas in the tissue blocks and their occurrence in both the digested (Feulgen negative) and undigested (Feulgen positive) material suggested that these were regions of poor fixation which had been adversely affected by the prolonged treatment.

One attempt to localize nucleoprotein in synaptic chromosomes by the use of enzymes has been reported by Nebel and Coulon (28). However, the resultant structural disorganization that is attributed to DNase treatment resembles the poorly preserved areas in our early preparations using formaldehyde. In our opinion, the results are more attributable to fixation effects than to the disrup-

FIGURE 6 Thin section of glutaraldehyde-fixed, DNase-digested, indium-stained material. The outline of the nucleus (*nuc*) of a primary spermatocyte is formed by indium-stained ribosomes (*r*). This was identified as a primary spermatocyte because a nearly adjacent thick section, when stained with toluidine blue, showed this area to be rich in primary spermatocytes at synapsis and because the size of the nucleus, as suggested by the ribosomes surrounding it, is the size expected of a primary spermatocyte. Groups of particles similar in size to ribosomes may be seen in the nucleus (arrow). × 55,000.

FIGURE 7 Nucleated erythrocyte present in a section of glutaraldehyde-fixed, DNase-digested, $InCl_3$-stained testis. This micrograph is presented for purposes of comparison with the nucleus of a primary spermatocyte directly above (Fig. 6). × 58,000.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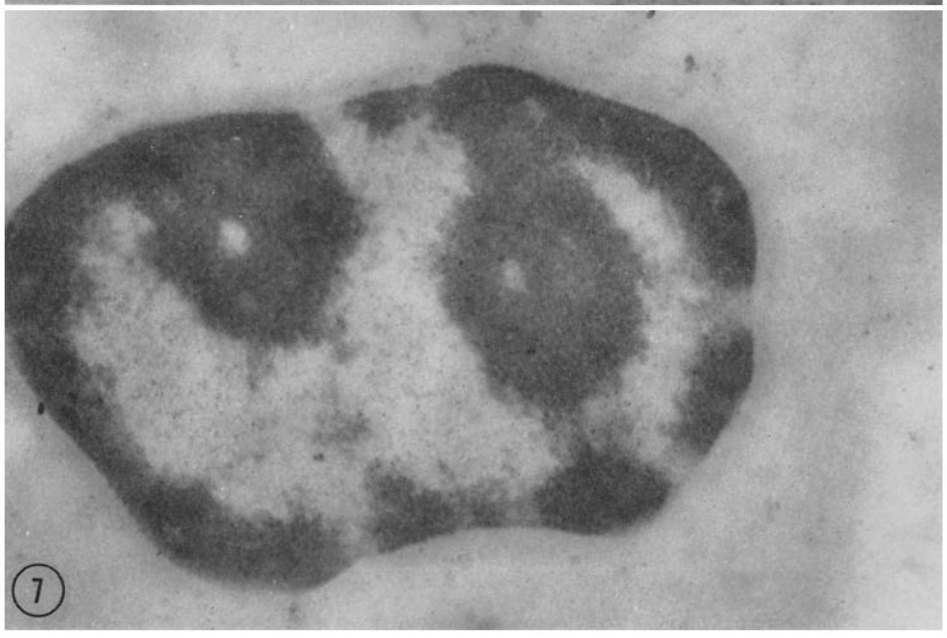
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tion of a fundamental structure dependent on DNA as postulated by these authors (28). Unfortunately, there is no way of knowing the extent to which any of the substrates were removed by the enzymes used in the study in question, since only structural modifications were reported.

Our failure to find structural differences as a consequence of DNA removal may rest on two factors: (1) In the aldehyde-fixed material, other components of the chromosome, particularly proteins, are probably stabilized through cross-linking and maintain the structural integrity of the chromosomes even though DNA, which in the living state may be essential to the maintenance of the chromosome, has been removed. (2) Since the osmium used in postfixation is mainly responsible for the contrast in the section, and since osmium is reduced primarily by non-DNA sites (1), removal of DNA would have little effect on the contrast contributed by osmium. The same argument holds for permanganate and lead which are also used to increase contrast. It was surprising, however, to find that the digested chromosome was stained almost as strongly as the intact one with uranyl acetate (*cf.* 28), which, according to other workers (10, 39, 35), attaches strongly and preferentially to nucleic acids, albeit under different fixing and staining conditions. Although it is probable that we could not detect subtle quantitative differences in uranium staining as a consequence of DNA digestion, the observation that the density of the stain was not appreciably altered leads us to conclude that certainly in the digested material, and probably in undigested material as well, the uranyl ion is binding to a significant

TABLE III

Results of Staining Normal, Digested, and Control Tissue with Indium

Tissue	Cell type: Sustentacular	Primary spermatocyte	Spermatid
Normal	×	×	×
Digested (DNase)	×	0	×
Control	×	×	×

× = visible.
0 = invisible.

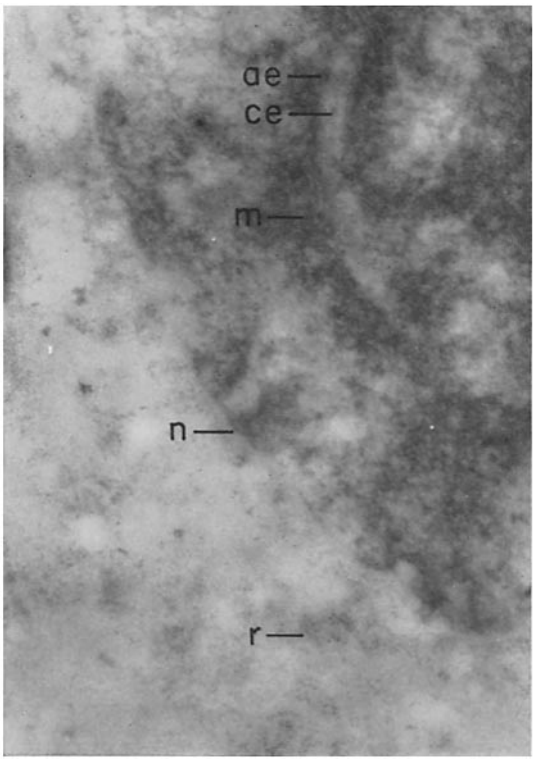

FIGURE 8 Acrolein-fixed, DNase-digested, indium-stained, methacrylate-embedded tissue; section stained with $KMnO_4$. Except for the digestion with DNase, this tissue was treated according to the procedure originally described by Watson and Aldridge. The greater difference between stained components and embedding material, as well as the non-homogeneous density to electrons of the methacrylate, is evident. Groups of ribosomes (*r*) are visible in the cytoplasm, and all three elements, microfibrils (*m*), axial elements (*ae*), and central element (*ce*), of the synaptinemal complex are seen to be present and apparently intact. *n*, limit of nucleus. × 30,000.

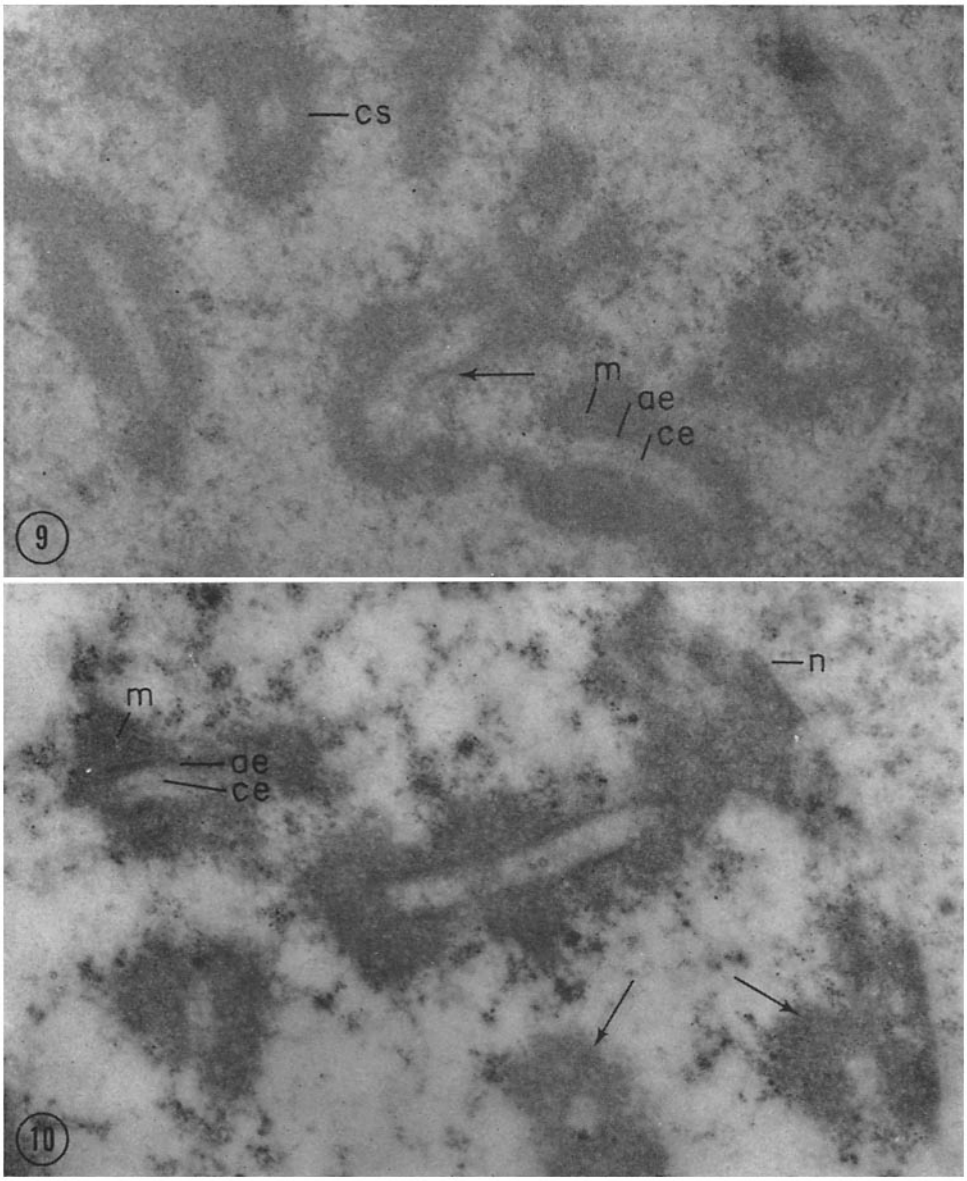

FIGURE 9 Synaptinemal complexes from glutaraldehyde-fixed, DNase-digested, $InCl_3$-treated tissue stained with lead hydroxide for 15 minutes at 60°C. The individual microfibrils (*m*) of the chromosomes are not resolved, yet the axial elements (*ae*) and central elements (*ce*) are clearly stained, and there are indications (arrow) of the doubleness of the axial elements. *cs*, section of complex in near transverse plane. × 26,500.

FIGURE 10 Synaptinemal complexes from glutaraldehyde-fixed, DNase-digested, $InCl_3$-treated tissue, stained with saturated uranyl acetate for 1 hour at room temperature. Most of the chromosomes are cut in the longitudinal plane, but two sections approach the transverse (arrows). The fibrillar nature of the microfibrils (*m*) is clear, the central element is visible (*ce*), and the doubleness of the axial elements (*ae*) can be seen at several places (arrows). *n*, limit of nucleus. × 30,000.

number of non-DNA sites. While some of this binding could conceivably be due to RNA, the ultraviolet-absorption picture following DNase suggests that this component is not present in very high concentrations. Rather, it seems likely that with our method the ion is binding non-selectively to groups such as the carboxyl of protein. But, although in the digested material virtually all of the binding of uranium must be by a non-DNA material, and although, under similar conditions, DNA-containing chromosomes stain intensely with uranium, it cannot be said how much of the staining in the latter is due to DNA and how much to protein. These observations raise a caution about any assumption that merely because a structure stains with uranyl acetate it contains nucleic acid.

TABLE IV

Results of Various Methods to Demonstrate Chromatin in Glutaraldehyde-Fixed, DNase-Digested, Indium-Stained Thick and Thin Sections in the Light and Electron Microscopes

Method	Cell type		
	Sustentacular	Primary spermatocyte	Spermatid
Feulgen	×	0	×
UV 262 mμ	×	0	×
Indium	×	0	×
UV 350 mμ	×	×	×
Toluidine blue	×	×	×
$KMnO_4$	×	×	×
Lead (Karnovsky)	×	×	×
Uranyl acetate	×	×	×
Osmium postfixation	×	×	×

× = stain.
0 = no stain.

The use of such demonstrably non-selective heavy metal staining was then abandoned in favor of the indium-trichloride method of Watson and Aldridge (38) for the staining of nucleic acids. Indium will combine with incompletely esterified groups such as phosphate, sulfate, and carboxyl. The basis of its selectivity resides largely in the blocking and reducing reactions that precede staining and that leave nucleic acid phosphate as the predominant binding site. Further, this method may also be of advantage in the preservation of chromosome fine structure, since indium is reported to have a less deleterious effect on the DNA-protein complex than the uranyl ion (37). It was hoped that this more rigorous method using a heavy metal and promising more consistent and demonstrable selectivity would allow the localization of DNA in the electron microscope.

Several observations adduce the selectivity of the indium methods employed in this study. All structures that are known to contain nucleic acids, *i.e.* chromosomes, nucleolar material, and cytoplasmic ribosomes, bind a significant amount of indium. The only other structures seen to stain with indium are the nuclear particles whose

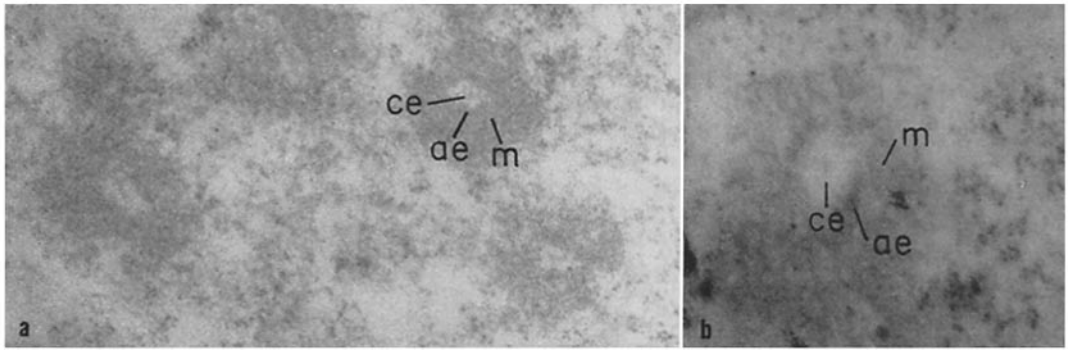

FIGURE 11 Nearly transverse sections of synaptinemal complexes from glutaraldehyde-fixed, DNase-digested, indium-treated tissue. *a*. Stained with uranyl acetate for 15 minutes at 60°C. *b*. Stained with uranyl acetate for 1 hour at room temperature. These sections show clearly the presence of all the elements, microfibrils (*m*), axial elements (*ae*), and central elements (*ce*), noted in the osmium-, the glutaraldehyde and osmium–, or the glutaraldehyde-fixed, heavy metal-stained material. *a*. × 26,500; *b*. × 56,000.

nucleic acid nature has not been demonstrated. Moreover, only specific structural components of the chromosome are stained with indium, in contrast to the general staining with uranium and other non-selective heavy metal techniques. Only the chromosomal affinity for indium is lost after digestion with DNase. Finally, two independent methods, Feulgen staining and ultraviolet absorption, show the same distribution of DNA among cells in the light microscope as is found in the electron microscope with indium (Table IV). This becomes of particular significance in view of the observation that DNase acts mainly on spermatocytes, affecting other cells little, if at all. All three methods reflect this pattern alike. We, therefore, place a high degree of confidence in our methods for demonstrating DNA in primary spermatocyte chromosomes.

The difference in susceptibility of the DNA of glutaraldehyde-fixed meiotic chromosomes to DNase compared to the resistance of DNA in other cell types (*e.g.* erythrocytes, Fig. 3) is curious, though it does not directly concern our study. It is possible that the association between DNA and protein varies among different cell types, and that this association could affect the accessibility of substrate to enzyme, thereby altering the rate of digestion. The nature of this association, whether or not it is a function of glutaraldehyde fixation, and how or why its effect on spermatocyte nuclei could be different from that on other nuclei is not known, nor is this concern germane to the present investigation.

Recently, Sabatini *et al.* (33) have contended that osmium fixation or post fixation could bring about distribution artifacts in the interphase nuclei of mitotic cells. This possibility is of some concern to our observations, since the morphologies of the chromosomes, particularly with respect to the synaptinemal complex, are similar in both osmium and glutaraldehyde-osmium preparations, but different in glutaraldehyde-indium preparations. The question is raised whether there has been an aggregation of axial material in the former preparations by osmium, but not in the latter. The answer is provided by both glutaraldehyde-fixed, lead- or uranyl-stained material (Fig. 3), and glutaraldehyde-fixed, DNase-digested, indium-stained material which, when further stained with heavy metals (Figs. 8 to 11), show the same structure and distribution of density as the osmium-fixed (Fig. 2) and the glutaraldehyde and osmium-fixed material (Fig. 1). In a similar manner, no readily noticeable deformations are present when thick sections of these preparations are stained for examination in the light microscope. Therefore, since in the chromosomes studied there was neither an alteration in their appearance nor a discernible redistribution of material within them, it is held that chromosomes fixed and treated by the various methods of this study are all morphologically comparable to one another. Of course, it is inescapable that fixation induces artifact at some level of organization, that fixed chromosomes are not identical to living ones, and that these differences will vary to some extent according to the different fixatives used. But our observation stands that however the variously fixed synaptic chromosomes may differ from those in the living cell they are morphologically similar to each other, especially with respect to their axial structures. We are thus not dealing with a redistribution or aggregation and disaggregation of structural components induced by different fixatives and subsequent treatments. In other words, any artifacts of structural distribution, if present, are subtle and obscure, and hence are negligible for the purposes of this study. It also follows that DNase digestion does not lead to any significant redistribution, since digested sections that have been stained with a non-specific heavy metal procedure also show the same chromosomal structures. The only structural anomaly noted in the undigested, indium-stained material was lack of distinct resolution of the chromosomal microfibrils associated with the axial complex, which could have been the result of indium polymer formation, scattering phenomena by the blocking molecules, reduced contrast due to the thickness of the section or to thick carbon coats, or even to small scale diffusion of indium into the methacrylate and Araldite. Since this lack of structural detail was noted in both normal and experimental materials, it is doubtful whether it would seriously affect the conclusions of this study.

Chromosomal Organization

The morphology of the synaptinemal complex immediately raises three questions concerning its component parts: (1) What is the composition of the 100 A microfibrils that make up the bulk of the chromatin and are associated radially with the two main elements of the complex? (2) Is the composition of the axial elements the same as that

of the microfibrils? (3) Is the composition of the central element, that appears only in paired chromosomes, related to either the microfibrils or axial elements? Our results provide partial answers in terms of DNA and non-DNA moieties.

From evidence to date, it appears that the basic structural unit of chromatin is a microfibril of indeterminate length and of the order of 100 A in diameter (*e.g.* 31), which may conceivably be composed of 1, 2, or possibly even more double helices of DNA (17; *cf.* 6). Although it is highly probable that in intact chromosomes these microfibrils do, indeed, contain DNA, and that most and possibly all of the chromosomal DNA is contained by them (*vis-à-vis* the amorphous material between them), the evidence that they actually contain DNA is circumstantial at best. Adjacent thick and thin sections have shown that Feulgen-positive areas are rich in microfibrils, while Feulgen-negative areas are not (21 and 22). In both light (25) and electron (8, 19, and 26) microscopic autoradiographs, labeling due to incorporated tritiated thymidine has been localized to clusters of microfibrils. But, the resolution obtained by these methods is inadequate to describe whether the DNA is actually in the fibrils. More to the point are selective staining methods with heavy metals (10, 35, 38) which show the substructures of chromatin to stain readily with indium and uranium. But, as Watson and Aldridge (38) have correctly pointed out, the specificity of heavy metal staining, even where precautions have been taken to remove competing sites, is capricious and must be proved; or at least a basis of confidence must be established by the use of specific extractants.

The abolition of indium staining in chromatin by DNase in our experiments constitutes strong evidence for the specificity of the stain for DNA. The substructures of chromatin to which the indium is bound must, therefore, contain DNA. While the most likely interpretation of the "dots" and "dashes" seen in thin sections of chromosomal material is that they represent various aspects of sectioned microfibrils, there is no evidence to say that at least some of the chromatin components may not be particulate, and this point must remain unsettled for the present. Not infrequently, however, lengths of twisted microfibrils can be identified (Fig. 4) usually near the axial complex, and these are clearly stained. There now seems no doubt that the microfibrils contain DNA.

But, the fact that the microfibrils (and the "dots" and "dashes") can be brought into view again by one of a variety of heavy metal stains after DNA removal means clearly that DNA is not the only constituent. Since uranyl is reported to complex readily with any available anionic group (38), it cannot be decided whether the complexing group is the carboxyl of protein or non-esterified phosphate of phospholipid, insoluble polyphosphate, phosphorylated polysaccharide, etc. However, it is most reasonable to consider protein as the component responsible for the "non-specific" staining because of its well known intimate association with DNA (*e.g.* discussion in 13).

In the material studied, the two dense axial elements of the complex, each of which is thought to be axial to a single homologous chromosome (21, 27; *cf.* 34), are not visible with indium (Fig. 4). The space occupied by them, however, is traversed by indium-stained fibrillar material, though the density of this demarcated space is lower than that of the contiguous chromosomal material, largely because the concentration of microfibrils is less. The inner border of this less dense area is more heavily stained; its position corresponds to that of the innermost compartment of the duplex axial element seen to have still greater density in non-selectively stained sections. Thus, while the main axial elements lack the concentration of DNA necessary to account for their density in osmium-fixed and comparable preparations, there is DNA present in the microfibrils which must be intimately associated with the axial elements. Some component other than DNA is present in these axial structures in sufficiently high concentration to complex with appreciable amounts of a variety of heavy metals. Again, the critical evidence is missing, but protein is a likely candidate.

The central element is co-axial with the bivalent and is usually, but not always, present in synaptic chromosomes (23). It has been considered to be a concomitant of pairing (21, 22, 13, and 32). In the present experiments it does not stain with indium (within the limits of detection of our methods), but the possibility that it may contain a few fine strands of DNA cannot be ruled out. Although it is stained by uranium, as well as by other non-selective heavy metal techniques, and thus resembles the axial elements flanking it, it must be considered a different structure by being practically devoid of associated DNA.

The question of localization of RNA in the

chromosomal components cannot be dealt with properly from the results of these experiments. From the negligible ultraviolet absorption of the nucleus after DNase, it would appear that the over-all concentration of RNA in the chromosomes (12) is not appreciable, but the resolution of these micrographs is inadequate to visualize a structure, such as the axial complex, the elements of which might contain higher concentrations of RNA. While the electron micrographs of digested, indium-stained chromosomes are devoid of visible structures (Figs. 6 and 7), the possibility cannot be completely ruled out that RNA is present in small amounts, and that the amount of indium bound is below the lower limit of detection with our methods, either because of its concentration or its association with protein which blocks it from complexing with indium. At least part of the staining by uranyl acetate might be due to the binding of such RNA with a more easily complexed uranyl ion. However, this possibility is less likely in view of the ready staining of other known RNA-containing structures (ribosomes and nucleolar fragments) with indium following DNase. Another possibility to be considered is that removal of DNA might also bring about the release of the RNA associated with it (12). Thus, our results do not permit us to say unequivocally that RNA is not a major component of some part of the synaptic chromosome.

Finally, the indium procedure brings out the presence of DNase-resistant, indium-binding particles in the nucleus which are similar to ribosomes in size, and which stain similarly with indium. These particles can be found near chromosomes in thick sections in DNase-digested material (Fig. 6). In non-selectively stained tissue these particles are not prominent because of their size and the presence of many other nuclear structures. As yet, nothing more can be said except to note their presence.

These results lead us to conclude that in fixed material, in contrast to living material, (5, 2, 7, and 13), DNA is not essential to the structural integrity of the chromosomes and that their DNA-containing components also contain other substance presumably responsible for maintaining the intactness of the chromosome when the DNA is removed. The axial elements are composed of a substantial amount of material other than DNA which accounts for their density when stained with osmium. This material may well be concerned with the unique appearance of meiotic prophase chromosomes, since the axial elements in which it occurs have been found only at this stage. Finally, the central element, which has been postulated to be associated with the occurrence of point-to-point pairing at synapsis, does not contain an appreciable amount of nucleic acid, and must be classed as one of the components of the chromosome low in nucleic acid content and whose exact nature and precise role has yet to be made clear.

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Note Added to Proof: Continued studies of indium-stained chromosomes employing contrast amplification reveal very fine filaments crossing the space between the axial elements. These correspond to the thicker, more obvious fibrils seen in this area following other procedures (Figs. 2 (inset), 3, and 4). The possible significance of these observations is discussed elsewhere (Moses, M. J., and Coleman, J. R., "Structural Patterns and the Functional Organization of Chromosomes," *in* Proceedings of the 23rd Symposium of the Society for the Study of Development and Growth, (Michael Locke, editor), New York, Academic Press, Inc., in press).

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