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PROBING MITOTIC CHROMOSOME STRUCTURE AND ARRANGEMENT BY MICROMANIPULATION

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Bruce R. Korf, A.B.

27 March 1979
The Rockefeller University
New York

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Some of this work has been published (Korf and Diacumakos, 1977, 1978). Some text passages and figures 4, 5, 9, 10, 11, and 13 are used with the permission of the publishers.

Finally, I thank my wife, Michele, first for preparing the drawings for this thesis, but most importantly for sharing the pleasure and excitement of these years

PREFACE

This thesis deals with the long standing question of the arrangement of chromosomes in the eukaryotic cell and the possibility that chromosomes are interconnected. These problems have been difficult to study in the past because they concern three-dimensional spatial relations which are readily disrupted. Micromanipulation provides a means of obtaining chromosome preparations in which these relations are preserved and the production of artifacts is avoided. The Indian deer, Muntiacus muntjak, the mammal with the lowest known diploid chromosome number, is an ideal system for such studies. The entire diploid chromosome complement can be microsurgically removed from cultured cells along with the mitotic apparatus. The micromanipulation studies reported in this thesis show that mitotic chromosomes are not connected to one another in the cell and that they are positioned randomly on the mitotic spindle. In addition, the usefulness of microsurgically extracted chromosomes for studies of chromosome structure is demonstrated.

SUMMARY

The Indian deer, (Muntiacus muntjak), has the lowest known diploid chromosome number (seven in the male) for a mammal. Mitotic chromosomes can be removed from cells either by extraction with microneedles or by inducing chromosome expulsion with a drop of silicone oil applied to the cell. The entire diploid complement is removed by virtue of interconnecting fibers among the chromosomes. The chromosomes are brought to the surface of the cover slip for analysis, where each can be identified morphologically with phase contrast optics. The chromosomes are sticky and extensible, and parts of chromosomes can be dissected by cutting with microneedles.

Within muntjac metaphase cells, chromosomes are arranged radially on the mitotic spindle, with centromeres facing the center and arms pointing outwards. The position of individual chromosomes on the spindle is random however. There is no homologous pairing or tendency towards association of particular chromosomes. The radial array is preserved when chromosomes are microsurgically removed from cells. Immunofluorescent staining with tubulin antiserum reveals that the spindle is at the center of the extracted array and holds it together. When cells are treated with spindle poisons, the mitotic chromosomes are not radially arranged and chromosomes can be removed from the cells individually. The spindle is therefore responsible for maintaining the configuration of mitoitic chromosomes.

Interconnecting fibers among microsurgically extracted chromosomes are of several types. Some are adhesions of sticky chromosome fibers accidentally brought into contact with one another during micromanipulation. Cognizance of this possibility allows such artifacts to be avoided. Centromeres are connected to the spindle, which appears as a fibrous network at the center of the radial array with phase contrast and scanning electron microscopy. When chromosomes are displaced from the spindle during micromanipulation, their connections to the spindle stain for DNA and histone and are sensitive to DNase. These connections may represent stretched chromosome regions which are still attached to the

spindle. Further studies may elucidate the mode of connection of chromosomes to the spindle. Examination of intact mitotic cells with fluorescent stains for DNA confirm that chromosomes are not connected to one another by DNA during mitosis.

Scanning electron microscopy, immunofluorescent staining for histone, and the effects of enzyme treatments on microsurgically-isolated chromosomes provide insights into chromosome structure. Chromosome arms are labile to DNase but not protease. This suggests that chromosomes do not contain protein cores which run from one end to the other. Centromere regions are resistant to nuclease and protease treatments, and consist of tightly packed fibers. The remaining surface of the chromosome consists of looping fibers, except for the nucleolus organizers, where fibers are loosely packed and longitudinally oriented. The latter regions are especially prone to breakage by DNase. Staining for histones H1 and H2B reveals that these are present throughout the lengths of the chromosomes and are distributed more or less uniformly.

These studies exploit the ability of micromanipulation to obtain chromosome preparations from cells without chemical treatments or destruction of three-dimensional relations. Production of artifacts can be monitored and often avoided. Micromanipulation can therefore be a valuable adjunct to other forms of analysis in studying chromosome structure and function.

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I. GENERAL INTRODUCTION

In 1903 - 1904, Sutton and Boveri formulated the chromosome theory of heredity. Now one of the major canons of biology, it postulates that the chromosomes are the structures into which the eukaryotic nuclear genome is organized. Chromosomes consist chiefly of deoxyribonucleic acid (DNA), the molecule in which genetic information is encoded, and protein. Most of the time, the chromosomes are extended in the nucleus as long, thin threads and are accessible for transcription of RNA, whereby the genetic information is utilized. When cells are preparing to divide, the chromosomes replicate. During cell division, the replicated products condense and separate to provide a complete copy of the genome for each daughter cell. Most chromosome regions then revert to the decondensed state and again become active in RNA synthesis.

The relative ease of morphological study of the condensed mitotic chromosomes and the importance of mitosis render the chromosomes of particular interest at this stage. Their distinct and characteristic morphologies are useful in gene mapping, diagnosis of certain human disorders, and in studies of evolution and the response of the genome to environmental agents. Yet, while the mitotic chromosome is in some ways the most accessible portion of the eukaryotic genome, we have relatively little precise information regarding its structure and function. Thus, although the interactions of DNA and proteins are becoming better understood, the mode of packaging of chromatin at mitosis remains a mystery. Also, the precise nature of certain chromosomal components, such as heterochromatic regions and centromeres, is unknown. Finally, we are ignorant about many aspects of mitosis, including the mode of interaction of chromosomes with the mitotic apparatus.

This thesis is concerned with studies of the structure and arrangement of chromosomes on the mitotic spindle. In the past, many different methods have been used to investigate these matters, with varied and equivocal results to be reviewed in a later chapter. This introduction will survey the different means of studying chromosomes. It will be shown that micromanipulation is a valuable complement to these ap-

proaches, with distinctive advantages and limitations. Also to be introduced is the particular mammalian karyotype used as a model system in the present work.

Methods of Studying Chromosomes

Mitotic chromosomes may be studied either *in situ* within fixed or living cells or after isolation from mitotic cells. *In situ* preparations are examined with the light or electron microscope, and chemical analyses may be done with specific staining or enzyme reactions. Isolated chromosomes also may be studied microscopically but lend themselves particularly well to biochemical analysis.

Fixed Preparations The microscopical examination of fixed mitotic cells is the oldest and simplest means of studying chromosomes. The first preparations were obtained by the usual histological techniques of sectioning and staining fixed tissues (*e.g.*, Flemming, 1880). Belling (1926) introduced the use of squash preparations for obtaining clear spreads of chromosomes, facilitating the accumulation of data on chromosome number and morphology in many plant and animal species. The treatment of tissues prior to fixation with the spindle poison colchicine was found (Levan, 1938) to increase the yield of mitoses and to improve the definition of chromosome structure by leading to contraction and separation of chromatids at all points but the centromeres. However, mammalian chromosomes remained, for the most part, difficult to study due to overlap within the cell. The advent of hypotonic treatment to swell cells (Hsu, 1952; Hughes, 1952; Hsu and Pomerat, 1953), and the application of simple cell culture techniques (Moorhead *et al.*, 1960) permitted the precise examination of chromosomes in mammals. Examination of cytological preparations has provided the bulk of our knowledge of chromosome morphology.

Fixed preparations have been employed to analyze chromosome instability, both experimentally-induced (Kihlman, 1966; Evans, 1962) and naturally-occurring (German, 1973), and have been instrumental in the delineation of certain human chromosome abnormalities (*e.g.*, Lé́jeune *et*

al., 1959). Taylor (1958) introduced the use of labeled precursors to DNA synthesis for the study of chromosome replication in cytological preparations. Special staining techniques have long been used to probe the chemical constitution of chromosomes (see Sharma and Sharma, 1972) and, most recently, have revealed something of their structural organization (Caspersson et al., 1968; Hsu, 1973). Finally, the localization of specific DNA sequences on fixed chromosomes has been made possible by the technique of cytological hybridization devised by Pardue and Gall (1970), Jones (1970), and Buongiorno-Nardelli and Amaldi (1970).

The electron microscope was first applied to the study of chromosomes in dividing cells by Ris (1956). He fixed and thin-sectioned germ cells from a number of plant species and noted pairs of 200 Å fibers within the chromosomes. Due to the thickness of the mitotic chromosome, transmission electron microscopy used to examine thin sections has contributed little additional information about mitotic chromosome fibers. However, several investigators have reported on the structure of kinetochores (e.g., Brinkley and Stubblefield, 1966) and the mitotic apparatus (see Bajer and Molé-Bajer, 1971).

About a century of cytological examination has yielded much information on chromosome morphology and behavior at mitosis; yet the induction of artifacts renders fixed preparations of limited value in structural studies. While the primary goal of fixation is to preserve in vivo architecture, it can not be assumed that such preservation is complete. It probably rarely is. Fixatives extract proteins from structures (e.g., methanol:acetic acid -- Dick and Johns, 1967), induce swelling or contraction (see Sharma and Sharma, 1972), or render them brittle. Processes of dehydration, mounting, and staining also detract from the fidelity of preservation. Another drawback to cytological preparation is that materials are usually rendered nearly two-dimensional for examination. This is achieved by sectioning, squashing, or subjecting the specimens to surface tension; in any case, the effect is to portray three-dimensional relations as two-dimensional. Finally, chromosomes prepared for microscopic examination are often obtained from cells treated with spindle poisons which lead to contraction and separation of

chromatids as well as disruption of the arrangement of the chromosomes on the spindle.

Observation of mitosis in live cells The study of chromosomes in live cells usually requires special optical systems. Most commonly used is phase contrast microscopy, with which structures are visualized by virtue of differences in refractive index. Numerous investigators have used phase contrast optics to observe mitosis in isolated tissues or cultured cells. Bajer and Molé-Bajer (1963) have made impressive cinemicrographic records of mitosis in endosperm cells of the plant, Haemanthus katherinae, which are nearly flat and lack cellulose walls. Among mammals, rat kangaroo (Potorus tridactylus) fibroblasts are flat enough during mitosis to permit similar observations (Roos, 1976). Differential interference phase contrast microscopy, which reveals three-dimensional contours, has been used to follow structural changes during mitosis (Bajer and Molé-Bajer, 1971). Inoué (1952) refined the technique of polarized light microscopy to allow precise analysis of mitotic spindle birefringence. The occurrence of birefringence constitutes strong evidence that spindle fibers exist in living cells and are not merely fixation artifacts. Polarized light microscopy has been used to follow changes in the spindle during mitosis, and to monitor responses to agents such as colchicine (Inoué, 1953), low temperature (Inoué, 1964), and ultra-violet irradiation (Forer, 1965). Live cells have thus been helpful for studying the dynamics of mitosis, but have provided little information about the chromosomes, which are usually not seen clearly within the cell.

Isolated Chromosomes To isolate chromosomes from the cell, modifications of the technique of Claude and Potter (1943) are used. Cells are disrupted, usually in the presence of detergent, and the released chromosomes are collected by centrifugation. Frenster et al. (1963) separated sonicated interphase chromatin into condensed and extended fractions by centrifugation in cation-free sucrose. While these fractions have been extensively studied, their correlation with the cytological entities, heterochromatin and euchromatin, is uncertain. Other investigators have isolated mitotic chromosomes, using a variety

of techniques. The stability of chromosomes is enhanced at low pH or in the presence of high concentrations of divalent cations (Chorazy et al., 1963; Somers et al., 1963) or hexylene glycol (Wray and Stubblefield, 1970). Isolated chromosomes have been subjected to chemical analyses, but such analyses are plagued by the contamination of the chromosomes with other cellular components (Hearst and Botchan, 1969). Several groups have attempted to fractionate chromosomes on the basis of charge (Landel et al., 1972), size (Mendelsohn et al., 1968), and DNA content (Gray et al., 1975). Isolated chromosomes and, in some cases, fractionated chromosomes have been used to transfer genetic traits between cells (McBride and Athawal, 1976). Chromosomes added to the culture medium are taken up by the cells, and portions become associated with or even integrated into the recipient genome.

Isolated mitotic chromosomes have been prepared for transmission and scanning electron microscopy by the whole mount method. Originally used for studying eukaryote interphase chromatin by Gall (1963), the technique was adapted for the mitotic chromosome by DuPraw (1966). The first step is the release of chromosomes from cells. DuPraw accomplished this by bursting colchicine-treated cells with hypotonic solution and spreading the chromosomes at an air-water interface. However, other methods of chromosome isolation (see above) have also been employed. The released chromosomes are then picked up on electron microscope grids and subjected to critical point drying. In this process, devised by Anderson (1951), the fluid bathing the specimen is brought to its critical point so that dehydration is accomplished without the distorting effects of a fluid interface. The specimens are then stained or coated with metal and viewed with the transmission or scanning electron microscope, respectively. Mitotic chromosomes consist of looping fibers of diameter 200 - 500 Å (e.g., Golomb and Bahr, 1974a,b; Daskal et al., 1976), and are organized into nucleosomes (Rattner et al., 1975). Some investigators have noted that nuclear membrane fragments are attached to chromosomes (Comings and Okada, 1970b), suggesting that chromosomes may be associated with the nuclear envelope in the cell. The internal structure of chromosomes has been probed with enzymes (Golomb and Bahr, 1974a) and

chemicals (Adolph et al., 1977b), and the distribution of dry mass along the chromosome has been analyzed by quantitative electron microscopy (DuPraw and Bahr, 1969).

Most methods for isolating chromosomes involve the treatment of cells with spindle poisons to accumulate mitoses. If this is avoided, and special efforts are made to insure its stability, the entire mitotic apparatus can be isolated. Mazia and Dan (1952) removed the spindles from sea urchin eggs by fixing the cells and lysing them with detergent. A sulfhydryl oxidizing agent was added to preserve the mitotic apparatus. Kane (1962) experimented with the use of six carbon alcohols in the place of sulfhydryl agents. The method of Wray and Stubblefield (1970) employs hexylene glycol in the presence of a pH 6.5 buffer and small amounts of calcium. Isolated spindles have been a useful source of information concerning the chemical nature and structure of the mitotic apparatus and have provided proof of its existence as a discrete set of structures.

Studies of isolated chromosomes are limited chiefly by three drawbacks. First, the chromosomes are released from cells in the presence of various chemicals, which may lead to distortion of physical and chemical properties. The second problem is that three-dimensional or spatial relations are damaged or destroyed. The third drawback is that when preparations containing millions of isolated chromosomes are subjected to biochemical analysis, the properties of different chromosome regions are averaged together. Many of the most interesting questions about chromosomes concern specific regions seen with the microscope, and it would be preferable to isolate and purify these for analysis.

Other Modes of Analysis The means of studying mitotic chromosomes discussed so far in no way exhaust the armamentarium of the chromosome researcher. Before discussing micromanipulation, a few additional approaches should be mentioned. Somatic cell genetic analysis using interspecific hybrid cells has led to great strides in mapping the mammalian genome (see Ruddle, 1975). The ability to transfer genes between cells via isolated chromosomes has already been mentioned. Chromosome-

mediated gene transfer has also been accomplished with chromosomes sequestered in liposomes (Mukherjee et al., 1978) and with microcells obtained from metaphase-arrested cells treated with cytochalasin B (Fournier and Ruddle, 1977). The brilliant cytogenetic investigations in maize by McClintock (1965) also deserve consideration. McClintock examined a series of genetic elements which move from place to place in the genome, causing mutations and chromosome breakage. It has recently been suggested (Nevers and Saedler, 1977) that these are similar to prokaryote insertion elements. The findings may thus have presaged a molecular phenomenon which may be of profound importance in chromosome evolution, and perhaps in gene regulation. Finally, the characterization of DNA associated with specific chromosome regions should be mentioned. DNA specific to the human Y chromosome has been isolated by hybridization (Kunkel et al., 1976) and identified in restriction enzyme digests (Cooke, 1976). Studies in males with structurally rearranged Y's (Kunkel et al., 1977) have permitted the mapping of the sequences on the chromosomes. The application of a similar approach to other chromosome markers may provide a DNA sequence map to complement the genetic map of the chromosome.

Micromanipulation of Chromosomes

The earliest published work in which a micromanipulator was employed is that of Schmidt (1859), who studied the structure of liver tissue by microdissection. Microtools, including needles, forceps, and scissors, were moved by means of a screw-thread mechanism. The tissue was bathed in dilute alcohol and viewed at a magnification of 400. However, the modern era of micromanipulation may be traced to the development in 1901 - 1904 by Barber and Schouten of methods for the isolation of single bacteria. Barber's apparatus (Barber, 1914) held a glass capillary and controlled its position by adjusting screws. Pressure for injecting or aspirating fluids with the capillary was controlled through a tube to the operator's mouth. The use of oil immersion optics was possible since the specimen was suspended in a hanging drop on a cover slip. McClendon (1906) used a similar apparatus to suck the chromosomes

out of Chaetopterus and Asterias eggs. However, George L. Kite was mainly responsible for adapting the Barber-Schouten method for eukaryotic cells. Kite joined Barber in Kansas to learn the method which he then brought to Wood's Hole. His work from 1911 - 1918 comprises the birth of modern microsurgery. After Kite's untimely death in 1918, his work was continued and extended by Robert Chambers.

Chromosome Isolation Kite and Chambers (1912) first reported the removal of chromosomes from cells by micromanipulation. Working with insect spermatogonia and spermatocytes, they described chromosomes as consisting of a "concentrated, refractive gel". In 1914 Chambers reported that the distinctness of chromosomes in the nucleus could be increased by damaging the cell with a microneedle. When freed from the cell into Ringer's solution, the chromosomes became swollen and eventually dissolved. In 1923, Chambers and Sands applied a newly designed micromanipulator to the extraction of chromosomes from pollen mother cells of Trandescantia. The spindle area was noted to be of jelly-like consistency, and the chromosomes were found to be elastic and clumped on removal from the cell. Similar properties were seen in prophase and metaphase chromosomes extracted from grasshopper spermatocytes. From these observations, Chambers (1924) formulated a model of mitotic chromosome structure, in which granules were coalesced around a hyaline core. Wada (1935) also reported the extraction of mitotic chromosomes from Trandescantia. He found that the chromosome fibers were coiled but could be straightened by stretching.

Beginning in the 1930's, micromanipulation was applied to the study of insect polytene chromosomes. These are actually interphase chromosomes greatly thickened by the precise lateral association of several thousand identical replicated elements. They lend themselves well to micromanipulation given their size, with a favorite chromosome source being the large salivary gland cells of Chironomous larvae. Several investigators have microsurgically isolated these chromosomes and noted that they are sticky and elastic, with the regions between dark bands the more flexible (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; Pfeiffer, 1940; Glancy, 1940; Buck, 1942;

Kroeger, 1966). Extensive studies were reported by Glancy d'Angelo (1946, 1950). She found that the chromosomes are sticky only after removal from the cell. Touching two chromosomes together within the cell did not result in their adhering, and oil drops or carbon particles injected into nuclei did not stick to the chromosomes. The consistency of extracted chromosomes was jelly-like, with dark bands more rigid than interbands. The fibrillar structure of polytene chromosomes was revealed by transverse stretching and also by lifting a strand of membrane-like material from the surface of the chromosomes, under which carbon particles could be injected. She also studied the effects of ionic media on isolated chromosomes. Thus, microsurgical isolation has been a useful means of obtaining polytene chromosomes for physical and chemical study. Edström and Beerman (1962) have recently refined the chemical analysis of microsurgically isolated chromosomes, using microelectrophoresis to determine the base composition of RNA associated with polytene chromosomes.

The lampbrush chromosome is another type which is found in cells whose large size render them convenient for micromanipulative studies. Lampbrush chromosomes occur in primary oocytes late in prophase I, a period of very active RNA synthesis. They consist of a central core, along which are alternating regions of greater and lesser density, with loops extending laterally. Duryee (1937, 1950) isolated lampbrush chromosomes from amphibian oocytes by micromanipulation and examined their physical and chemical properties. These chromosomes were also noted to be highly extensible and elastic. Stretching the chromosomes did not straighten the lateral loops, however, but merely increased their distance from one another. The effects of various chemical treatments, some of which selectively dissolved lateral loops, were studied. Gall (1966) has reviewed the more recent use of micromanipulation in obtaining specimens of lampbrush chromosomes for chemical and electron microscopical analyses. Edström and Gall (1963), for example, applied microelectrophoresis to the characterization of RNA associated with isolated lampbrush chromosomes in a study similar to the one with polytene chromosomes.

The work discussed so far has involved the study of the properties of microsurgically isolated chromosomes or the probing of chromosomes inside the cell. Such handling minimizes the induction of artifacts and allows the precise control of the chemical environment of isolated chromosomes. These studies also provided the first opportunity to directly manipulate chromosomes, contributing to our awareness of their fibrillar nature.

Studies of Mitosis Another application of micromanipulation is in the study of mitosis. Several investigators have challenged the cell to complete division after removing some component of the mitotic apparatus. The work of McClendon (1906) has already been mentioned. Lorch (1952) removed organelles from sea urchin blastomeres by suction with a micropipette. Enucleation was followed by a period of aster multiplication without formation of cleavage furrows. Ultimately, partition of the cytoplasm into a large number of anucleate compartments occurred. Removal of both the nucleus and the asters resulted again in the formation of a mass of anucleate compartments. However, if only the aster was removed, division was delayed until the asters had regenerated. Hiramoto (1956) examined the effects of removal of the mitotic apparatus on the division of sea urchin zygotes. Cleavage was unaffected by extraction of the mitotic figure at or after anaphase. In some cases, a cleavage furrow even formed after removal at metaphase, showing that the cleavage plane may already have been determined by this stage.

Carlson (1952) used a Chambers-type micromanipulator to probe various aspects of mitosis in the grasshopper neuroblast. This cell is unusual in that division is unequal: the products are a small ganglion cell and a larger neuroblast. By inserting a microneedle into the spindle region, Carlson found that the area was of semi-solid consistency, in contrast to the fluidity of other parts of the cell. A needle which was inserted into the equatorial region of the spindle was observed to slowly move towards one pole. Assuming that the needle followed the path of least resistance and did not intersect spindle fibers, it was inferred that the spindle consists of longitudinally-oriented fibers running from equator to pole. It was fairly easy to move the spindle and

chromosomes around in the metaphase cell as a unit but not possible to remove chromosomes from the spindle or to separate sister chromatids. The region between separating sister chromatids at anaphase was comparatively fluid. However, movement of the microneedle across the spindle axis did produce displacement of sister chromatids, indicating that some connections persist between the latter. By moving the spindle through the cell at different periods of mitosis, it was found that the cleavage furrow is formed independently of the presence of the spindle, but its position is to some extent dependent on the location of the spindle.

The mechanism of unequal division of grasshopper neuroblasts was further studied by Kawamura (1960, 1977), who microsurgically removed the mitotic apparatus at different times in division. The position of the cleavage furrow appears to be determined by the position of the spindle until late anaphase. The furrow may occur anywhere in the cell, and is always located across the spindle equator, regardless of where the latter has been moved. Thus, unequal cleavage of these cells may be the result of unequal growth of the asters, which results in displacement of the equator of the spindle closer to one side of the cell than the other.

Carlson's characterization of the spindle as a rigid gel was at variance with Chambers' finding that the spindle region is as fluid as the rest of the cell. It is true that Chambers probed the spindle by moving a microneedle through the region, which might itself have disrupted spindle rigidity. Moreover, considerable evidence had been amassed through the years in favor of the fibrillar nature of the spindle. Yet, to confirm and extend Carlson's studies, Nicklas (Nicklas and Staehly, 1967; Nicklas, 1967) initiated micromanipulative investigations of meiotically-dividing spermatocytes of the grasshopper Melanoplus differentialis.

By probing prometaphase and metaphase primary spermatocytes, chromosomes could be stretched from the equator of the spindle towards one of the poles. The kinetochore region moved very little when force was applied in this direction, but could be moved in the equatorial plane.

Thus, spindle fibers are less elastic than the chromosomes, and each chromosome is attached independently to spindle fibers. Displacement of chromosomes at anaphase I produced remarkably little disturbance in chromosome motion. By continually prodding a chromosome, however, it could be detached from the spindle. When released, these chromosomes inevitably moved back to the spindle and reformed connections with the poles. In some cases, detached bivalents reoriented on the spindle in the reverse of their original orientation, showing that separation from the spindle had been functionally complete. Ultrastructural confirmation of the reattachment of spindle fibers to the kinetochores has been obtained (Nicklas, 1971).

In further studies on the mechanism of reorientation of chromosomes detached from the spindle, Nicklas and Koch (1969) showed that microsurgically induced unipolar orientations could be maintained by applying tension to one end of the bivalent, without reestablishment of the bipolar configuration. Similar results were achieved when the unipolar orientations of two bivalents were maintained by manipulating them so that the U-shaped chromosomes became interlocked (Henderson and Koch, 1970). These results are consistent with the hypothesis that the bipolar orientation is favored because it is the most stable.

Nicklas and Koch (1972) also applied their micromanipulative system to the study of chromosome motion. Insertion of granules into the metaphase spindle resulted in their poleward movement. However, little motion was seen if the granules were placed in the interzonal region at anaphase. Displacement of chromosomes from the spindle at anaphase produced the surprising result that the chromosomes moved to the nearer pole, regardless of which pole the kinetochores faced. These results imply that forces other than those generated by kinetochore to pole attachments may be responsible for motion within the spindle.

The elegant studies of Carlson (1952) and Nicklas *et al.* (1967, 1971, 1972) exploited the ability of micromanipulation to nondestructively interfere with a dynamic process so that its response can be observed. It should be noted, however, that both investigators ex-

pressed the belief that microneedles rarely, if ever, entered the cells in their studies. The manipulations were thought to have occurred by indentation of the cell membrane by the microneedle. These experiments complement studies of the effects on mitosis of stresses such as cold, pressure, or ultraviolet irradiation, as well as investigations of biochemical and ultrastructural features of mitosis. They have been instrumental in characterizing the properties of the functioning mitotic apparatus and in testing hypotheses about chromosome orientation and motion at anaphase.

Studies in Mammalian Cells The last micromanipulative studies of chromosomes to be discussed are those employing mammalian cells. Cells in culture are the usual source of material for these studies. Diacumakos et al. (1971, 1972) developed methods for and applied microsurgery to the analysis of human cell disjunction. By mobilizing matrix from dividing cells, it was shown that equal distribution of the more fluid portion of the cell is not required for disjunction. Also, displacement of chromosomes at various stages of mitosis did not impede cell division. When chromosomes were pulled away from the spindle with a microneedle and then released, they moved back. At prometaphase, a chromosome could be extracted from the cell by touching the chromosome with a microneedle which was then withdrawn. Pulling the chromosome further from the cell led to the removal of the other chromosomes, which were attached to one another. Extrusion of metaphase chromosomes from fibroblastic cells could be induced by applying a fresh drop of silicone oil onto the cell membrane. Chromosomes were removed from non-fibroblastic cells (HeLa) by injecting aqueous material between the cytoplasm and spindle region and then tearing the cell apart. However, regardless of the means of obtaining chromosomes, they were found to be interconnected by fibers.

The presence of interconnecting fibers among microsurgically extracted human, mouse, and Chinese hamster chromosomes was also noted by Hoskins (1968, 1969). The fibers were sensitive to breakage by deoxyribonuclease (Hoskins, 1968) and were assumed to represent spindle fibers which connected adjacent chromosomes. Hoskins (1965, 1969) examined mi-

crosurgically isolated chromosomes with the electron microscope, and proposed a model for centromere and spindle structure which provided for the association of DNA with spindle fibers. While these interpretations are not widely credited, the observation of chromosome interconnections is provocative.

These pioneering methods of isolating mammalian chromosomes by microsurgery make possible a great variety of studies. The absence of chemical treatments avoids the formation of many of the artifacts that plague other types of preparations. Microsurgically isolated chromosomes are therefore useful for chemical and ultrastructural studies. Also, the careful extraction of chromosomes from cells offers the possibility of studying the three-dimensional relations among mitotic chromosomes and the spindle. The finding of interconnecting fibers among the chromosomes is especially intriguing. Many investigators have seen interconnections among chromosomes prepared in a variety of ways (see Chapter II), but it has been uncertain whether or not they are artifacts. The presence of interconnections among microsurgically extracted chromomosomes has been regarded as the strongest evidence for their existence within the cell. Another application of extracted chromosomes, anticipated by Diacumakos et al. (1970), is in the transplantation of chromosomes between cells. It is clear that studies of microsurgically extracted chromosomes can be of great value in conjunction with other approaches.

However, a problem in working with the cells of many mammalian species is that the relatively large number of chromosomes and difficulty in identifying each on the basis of morphology impedes the precise study of the entire extracted genome. Usually only a few chromosomes can be viewed clearly and identified; the remainder often become entangled with each other. The work to be described in this thesis represents an effort to exploit the advantages of micromanipulation for the study of chromosomes using a mammalian species with a far simpler karyotype, the Indian deer, Muntiacus muntjak. This system will be described in the next section.

Indian Muntjac Chromosomes

Karyotype The Indian muntjac, Muntiacus muntjak vaginalis, has the lowest diploid chromosome number known for a mammal (Wurster and Benirschke, 1970). The male karyotype, consisting of seven chromosomes, is shown in figure 1. There are two autosomal pairs, a metacentric and an acrocentric. The X chromosome is acrocentric, with a thin "neck" connecting the long and short arms. The long arms of the X are homologous with the long arms of the acrocentric Y_2, and the Y_1 is a small metacentric. In primary spermatocytes, the long arms of the X and Y_2 pair, and the short arms of the X and the Y_1 are terminally associated (Sasaki and Makino, 1971; Sharma, 1972). The female Indian muntjac has six chromosomes, with two pairs of autosomes, and two X's, but no Y_1 or Y_2 (Wurster and Benirschke, 1970). The X is derived from a translocation of the primordial X onto an autosome. Such an XY_1Y_2 sex chromosome system has also been found in other mammals (Fredga, 1970).

Some landmark features of the chromosomes should also be noted. The neck region of the X chromosome is heterochromatic (Comings, 1971), and the prominent secondary constrictions on the long arms of the X and Y_2 are nucleolus organizer sites (Goodpasture and Bloom, 1975). Small nucleolus organizers are located on chromosome number 1, and may lead to a constriction at this site in some cells. These features are not only helpful in identifying the chromosomes, but are also interesting subjects for study.

Evolution The Indian muntjac is a small deer (figure 2) found only in parts of Asia and represents an extreme and unexplained case of chromosome evolution (Wurster and Benirschke, 1970; Wurster and Atkin, 1972). The subfamily Muntiacinae of the deer family Cervidae contains six species. Among these, Muntiacus reevsi (Reeve's muntjac) has 46 chromosomes, Muntiacus muntjak muntjak (Javan muntjac) has 8 or 9 (in the female or male, respectively), and Muntiacus muntjak vaginalis (Indian muntjac) has 6 or 7. These animals resemble one another physically, but are differently distributed geographically. The evolution of M. reevsi to M. muntjak appears to have involved both changes in DNA con-

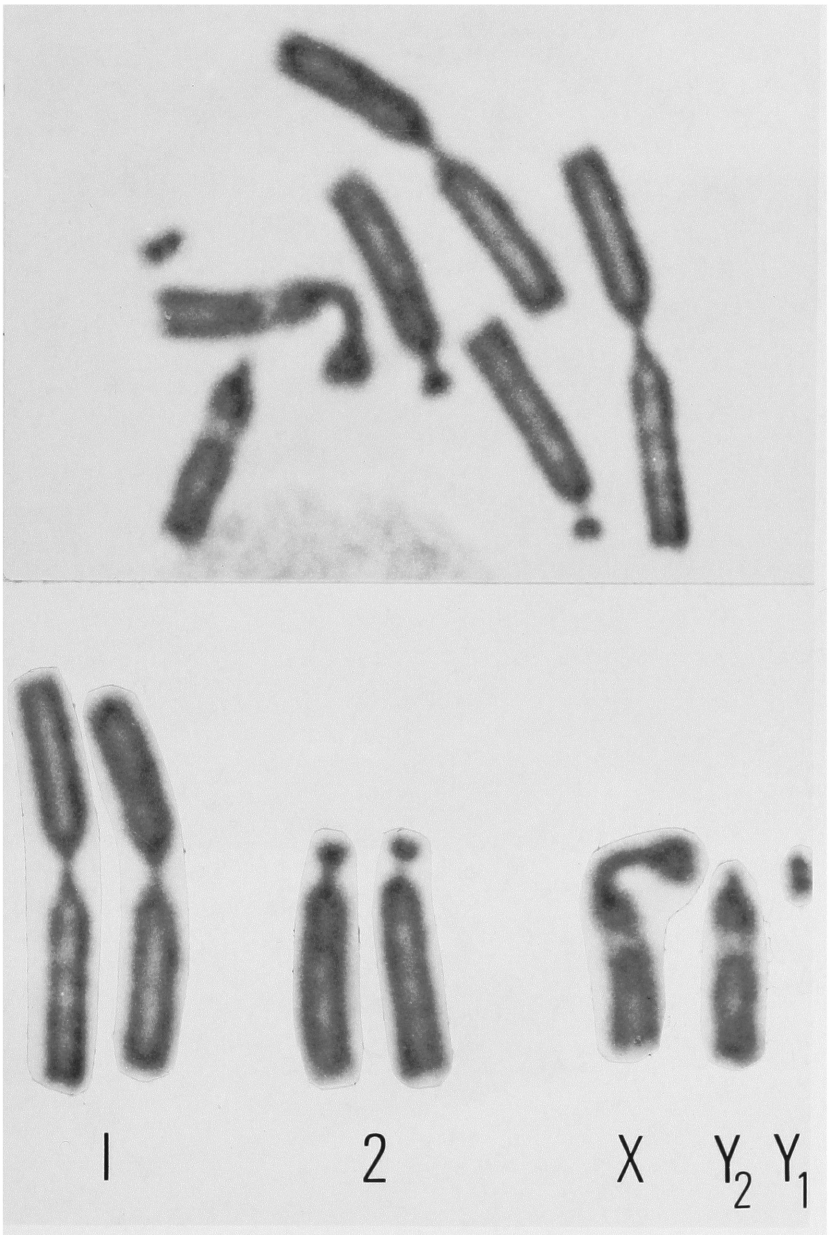


Figure 1. Indian muntjac male metaphase chromosomes and karyotype. Cells were treated with colcemid and hypotonic solution, spread on slides, air dried, and Giemsa stained. Note secondary constrictions on the X and Y_2 and the neck connecting the long and short arms of the X.

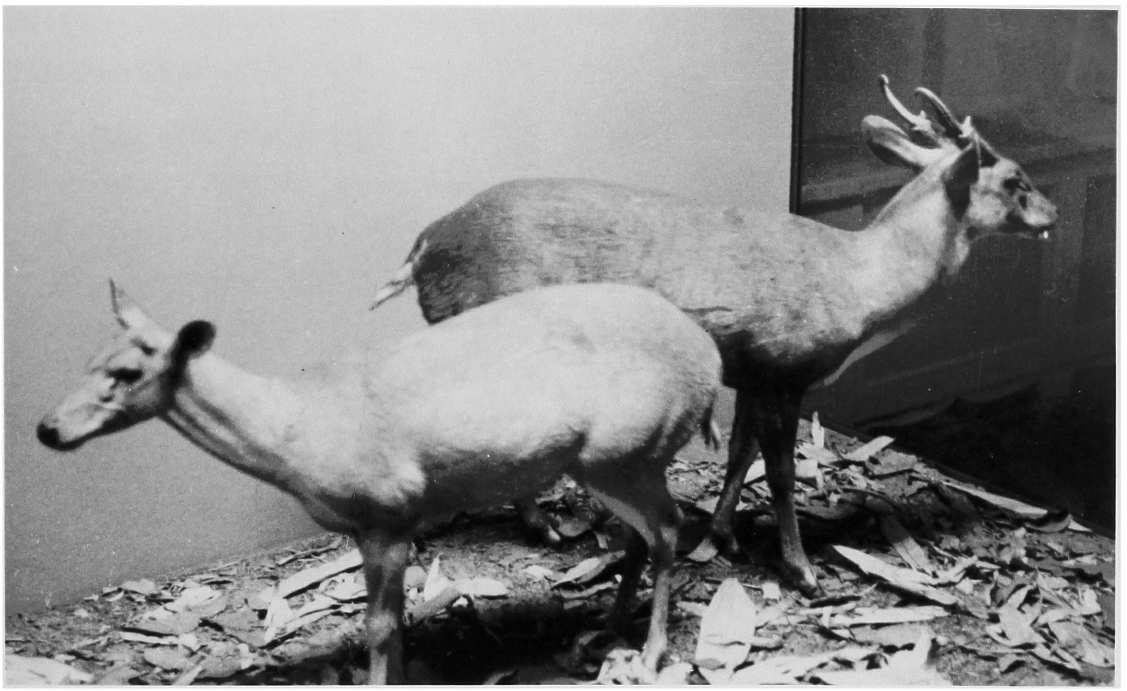


Figure 2. Indian muntjac male (background, right) and female (foreground, left). (Stuffed animals displayed at the American Museum of Natural History, New York.)

tent and chromosome structure. It is remarkable that while the Indian muntjac has 88% of the DNA content per nucleus of the Reeve's muntjac, the DNA content in Javan muntjac cells is slightly greater than in the Reeve's. Also, the autosome onto which the X is translocated is different in the Javan and Indian muntjacs. Thus, it is not clear if the Javan represents an intermediate step between the Reeve's and Indian muntjacs or whether the Javan and Indian muntjacs arose independently from the Reeve's.

Use as a Model System The utility of the muntjac karyotype in cytogenetic studies is obvious: the chromosomes are few, large, and can be distinguished from one another on the basis of morphology. However, to use the Indian muntjac as a model system it must be established that, in spite of their remarkable evolution, the chromosomes are not unusual in terms of structure or function. The evolution of this karyotype is believed to have proceeded by chromosome fusion and inversion, with loss of extra centromeric material (Wurster and Benirschke, 1970; Matthey, 1973), and perhaps other DNA. The percent DNA by weight in muntjac chromosomes was determined by Green and Bahr (1975). Dividing total mass of DNA per cell, as determined by Feulgen cytophotometry, into total dry mass of the chromosomes, as determined by quantitative electron microscopy, results in a figure of 10.2% DNA. The corresponding value for the human genome is 13.6%. Thus, the muntjac chromosomes are not packed with an unusually large amount of DNA per unit length. Comings (1971) suggested that the folding of chromatin in muntjac chromosomes is unusually tight, since he failed to see a segmental pattern of chromosomal DNA replication in autoradiographic studies. However, Sharma and Dhaliwal (1974) did observe segmental patterns in chromosomes labeled near the end of DNA synthesis. Thus, DNA replication occurs uniformly along the chromosome until almost complete, at which time a segmental pattern occurs which is not obscured by any peculiarity of chromosome structure. Muntjac chromosomes respond in the usual way to special staining methods to produce heterochromatin (Hsu and Arrighi, 1971), Giemsa- (Sharma and Gary, 1973), and quinacrine-bands (Fredga, 1971). Finally, studies of meiosis (Sasaki and Makino, 1971; Sharma, 1972) and

the extensive observations of mitosis to be reported in this thesis have not revealed any peculiarities. Indian muntjac cells have been used in studies of heterochromatin (Comings, 1971), the ultrastructure of chromosome banding (Green and Bahr, 1975), effects of chemicals on chromosomes (Huttner and Ruddle, 1976; Carrano and Johnston, 1977), sister chromatid exchange (Carrano and Wolff, 1975), host cell-virus interaction (d'Alisa and Gershey, 1978), and in efforts to fractionate isolated chromosomes (Carrano et al., 1976).

In this thesis, micromanipulative studies of the structure and arrangement of Indian muntjac chromosomes on the mitotic spindle will be described. The muntjac karyotype is ideal for studying chromosome arrangement, since there are enough chromosomes to permit different arrangements to be distinguished, yet the analysis is relatively simple. Micromanipulation provides a means of orienting live mitotic cells so that the arrangement of chromosomes can be noted. Moreover, the large and morphologically distinctive chromosomes are easy to handle by micromanipulation, allowing study of the interactions of chromosomes with one another and with the mitotic spindle.

II. REVIEW OF LITERATURE
CHROMOSOME ARRANGEMENTS AND INTERCONNECTIONS

The spatial arrangement of chromosomes in eukaryotic cells has long been a provocative subject for study. When chromosomes are assembled on the mitotic spindle at metaphase, they assume a characteristic configuration which may be important to the normal function of the mitotic apparatus. Specific positions may also obtain for chromosomes at interphase, and might play a role in regulating genetic activity. Moreover, if homologous chromosome regions are paired together in somatic cells, genetic recombination may occur. The arrangement of chromosomes at interphase has been studied both by directly analyzing the interphase nucleus and by noting the positions of mitotic chromosomes in cells in which spindle formation has been prevented.

A separate but related question concerns chromosome interconnections. While mitotic chromosomes appear to be discrete structures, there have been reports that the chromosomes are actually connected to one another. The significance of chromosome interconnections has been much debated; suggestions have ranged from suspicion that the fibers are artifacts, to the intriguing idea (Dupraw, 1970) that the chromosomes are parts of a single DNA molecule. In this chapter, studies of the spatial arrangement of chromosomes in the cell and evidence that chromosomes are interconnected, will be reviewed.

Spatial Arrangements of Chromosomes in Cells

Arrangement of Chromosomes on the Mitotic Spindle At metaphase in most eukaryotic cells, the chromosomes are arranged radially about the longitudinal axis of the spindle. The larger chromosomes occupy the most peripheral positions, and the smaller lie near the center. In cells which lack very small chromosomes the central region of the spindle is free of chromosomes. Such a configuration is referred to as a hollow spindle (Wilson, 1925), and is found in a great diversity of organisms, including the amoeba, Amoeba glabaen and Amoeba fluvialis

(Dobell, 1914), the marine flatworm, Polychoerus carmelensis (Costello, 1961), many species of insects (Gaulden and Carlson, 1951; Hess, 1965; Nur, 1973; Fox et al. 1975), mammals such as the rat kangaroo (Hsu et al., 1967), Chinese hamster (Juricek, 1975), Indian muntjac (Heneen and Nichols, 1972), the human (e.g., Darlington and LaCour, 1972), and also plants (Darlington and Shaw, 1959). Studies of the mitotic figures of diatoms are particularly illuminating (Pickett-Heaps and Tippet, 1978). As in most eukaryotes, the spindles of these organisms consist of two classes of fibers: continuous fibers that run from pole to pole and chromosome fibers that run from the poles to the chromosomes. The continuous fibers do not actually connect the two poles; rather, fibers from the poles interdigitate in the equatorial region. The continuous fibers are gathered together in a dense bundle. At metaphase, the chromosomes girdle this bundle at the equator of the cell. A different arrangement from the hollow spindle occurs in species having microchromosomes. Microchromosomes (chromosomes less then one micrometer in length) occupy the central region of the spindle. Such an arrangement is found in some reptiles (Painter, 1921) and birds (Boring, 1923; Shiwago, 1924; Werner, 1927; Bammi et al., 1966).

What accounts for the exclusion of larger chromosomes from the central spindle region? Oestergren (1949) suggested that the spindle is a liquid crystal which pushes out intruding chromatin. Another possibility is that the hollow spindle results from the manner in which chromosomes interact with microtubules. Birefringence studies of Haemanthus (Inoué and Bajer, 1961) indicate that the central spindle is at least partly formed before the nuclear membrane breaks down at prophase. From electron microscopic studies, it seems that microtubules are closely associated with one another, and may be in contact via crossbridges (McIntosh et al., 1969; Bajer, 1977). Thus, when chromosomes attach to the spindle, the larger chromosome arms might be unable to penetrate the tightly packed continuous fibers.

Homologous Pairing and Crossing Over In 1908, Stevens reported studies of spermatogonial and oogonial mitotic figures in 8 species of Dipteran insects. The chromosomes were arranged radially on the spindle

with homologues adjacent to one another. Metz (1914, 1916) examined 80 species of Diptera, and noted that homologues were paired intimately at prophase, less tightly at metaphase, and again tightly at anaphase. In most Diptera, homologous pairing occurs in both somatic and germ cells, as well as in cultured cells (Nichols et al., 1972; Halfer and Barigozzi, 1972). In other insects, homologous pairing is found only in certain tissues, or not at all. In Sciarids, homologous chromosomes are paired in oogonia and somatic cells, but not in spermatogonia (Metz et al., 1926; Metz, 1931). This may be due to the presence of supernumerary chromosomes in the male germ line of these species. In certain Orthoptera, somatic pairing occurs only in germ cells in the last premeiotic division (McClung, 1927). Neuroblasts of the Orthopteran Locusta migratoria have hollow spindles, but the chromosomes are positioned at random in the radial array at metaphase (Fox et al., 1975).

In Diptera, the association of homologous chromosomes is very intimate. Pairing of chromosomes that differ by an inversion results in the formation of a loop (van Heemert, 1977). That homologous pairing in somatic cells may have genetic consequences was demonstrated in a classic paper by Stern (1936). Flies were obtained which were doubly heterozygous for mutations affecting the body surface (yellow color and singed bristles). When the mutant alleles were present in repulsion, adjacent patches of tissue expressing one or the other mutation (a twin-spot) were found. Rarely, unpaired yellow or singed spots occurred. These somatic segregations could be most easily explained by single and double cross-overs, respectively. When the mutant alleles were in coupling, no twin spots occurred, only single yellow or singed spots. From this it was inferred that crossing over occurs at the four strand stage. Although somatic recombination resembles meiotic crossing over in this respect, the two processes are different in that somatic crossing over is not suppressed in flies carrying the C3G allele (LeClerc, 1946).

In fungi, somatic recombination is a regular occurrence, and provides an important means for genetic analysis. Roper and Pritchard (1955) documented the process in Aspergillus by isolating the complemen-

tary genetic products of the exchange. Evidence for spontaneous mitotic recombination has also been obtained for Penicillium (Sermonti, 1957), Neurospora (Pittenger and Coyle, 1963), and Verticillium (Hastie, 1967). The presence of homologous pairing has not been confirmed cytologically in these species, since the chromosomes are difficult to analyze. The frequency of recombination is increased by irradiation (James and Lee-Whiting, 1955; Kafer, 1960) or chemical treatment of cells (Morpurgo, 1963; Holliday, 1964; Shanfield and Kafer, 1971).

In a review of the literature, Metz (1916) found reports of homologous chromosome pairing in somatic cells of 17 species of plants. Staining of heterochromatin in interphase nuclei has provided evidence that homologues are paired at interphase in some plants not having clear homologous pairing at mitosis (Stack and Clarke, 1973; Singh et al., 1976; Fussell, 1977). Statistical analysis of chromosome positions in squash preparations has also uncovered a tendency towards homologous pairing in cases where it is not obvious at metaphase. Feldman et al. (1966) noted that a homologous pair of telocentrics in wheat, Triticum aestivum, lay nearer one another than expected by chance in squashes of cold-treated root tips. Darvey and Driscoll (1972) disputed the validity of this claim, finding that certain telocentric non-homologues also tend to be near one another. These studies in wheat require the use of telocentric markers due to the difficulty in identifying other homologous pairs. Horn and Walden (1978) performed a similar type of analysis in maize, where each chromosome could be identified. They found that homologues tend to be associated, and, in addition, certain sets of non-homologues are nearer one another than expected by chance. Thus both homologues and certain non-homologues may group together, perhaps explaining the discrepant findings in wheat. Nearness of homologues, but not of non-homologues, has been reported in diploid and hexaploid oat, Avena (Sadasivaiah et al., 1969; Dubuc and McGinnis, 1970; Thomas, 1973), and in barley, Hordeum (Fedak and Helgason, 1970). Ferrer and Lacadena (1977) noted the frequency of adjacent homologous chromosomes in radial metaphases of three species of Crepis. A strong tendency towards homologous pairing was seen in each. Finally, the occurrence of

homologous pairing during interphase has been inferred in some species from the fact that chromosome interchanges induced by radiation or chemicals preferentially involve homologues (Revell, 1953; Evans, 1961).

In accordance with the occurrence of somatic pairing, there is ample evidence for mitotic recombination in plants. Jones (1936) found evidence for a number of somatic segregation events, including crossing over and non-disjunction, by examining variegation patterns of aleurone in maize kernels. Harrison and Carpenter (1977) estimated that the frequency of spontaneous mitotic crossing over in Antirrhinum majus is approximately 10^{-5} per division. Treatment of plants with caffeine increased this frequency.

Mitotic and meiotic chromosome pairing in plants seem to have at least some properties in common. In wheat, the chromosomal locus $5B^L$ affects meiotic and mitotic pairing: in allopolyploids nullisomic for $5B^L$, both homologous and homeologous (i.e., involving genetically equivalent chromosomes from the different parent strains) pairing occurs (Feldman, 1966; Feldman et al., 1972). In barley nullisomic for chromosome IV, asynapsis occurs at meiosis and the tendency for proximity of homologues at mitosis is decreased (Thomas, 1973). Stack and Brown (1969) proposed that meiotic pairing and recombination evolved from the somatic process. Homologous pairing has been reported to occur in the last premeiotic gonial interphase (Maguire, 1967; Chauhan and Abel, 1968; Bowman and Rajhathy, 1977), although John (1976) pointed out that this is not always seen. Although the matter of evolution is unresolved, it seems likely that some processes, such as identification and pairing of homologues, are similar in meiosis and mitosis (Comings and Riggs, 1971). Other aspects, however are probably different; for example intimate synapsis may be unique to meiosis.

In vertebrates, the search for somatic pairing and recombination of homologous chromosomes has been long, and the goal elusive. Although Montgomery (1903) reported that homologues are loosely paired in spermatogonial cells of an amphibian, there have been very few other observations of somatic pairing in higher animals. Boss (1954, 1955) noted

that homologues were paired at anaphase in newt cells in culture. Gibson (1970) published two remarkable photographs of metaphases from cultured rat kangaroo cells. In one cell, the homologues were intimately paired in a manner reminiscent of an endoreduplication figure, except that each homologue was present in a single pair rather than two. The second picture showed a pair of homologous chromosomes that were in contact at the ends of their long arms. These were exceedingly rare findings, however, and their significance is unknown. Juricek (1975) analyzed the positions of chromosomes in radial mitoses of the Chinese hamster. In corneal epithelial cells (five examined), no homologous pairing was seen. The smallest chromosomes were located at the center of the array, but did not engage in homologous pairing. A diagram of the average position of chromosomes in metaphase II spermatocytes revealed that the chromosomes were distributed according to size, so that mass was balanced in the metaphase plate. Hens (1976) measured a number of parameters of chromosome position in metaphase spreads from colchicine and hypotonic treated cells: centromere to centromere distance, chromosome to center of metaphase plate distance, and the angle between pairs of chromosomes and the center of the metaphase plate. In contrast to Juricek's (1975) findings, smaller chromosomes were distributed at the periphery of the metaphase plate, presumably due to absence of a spindle to maintain the radial array. Only one pair of homologues tended to be nearer one another than expected by chance.

Cohen et al. (1972) found random positioning of chromosomes in colchicine and hypotonic treated Indian muntjac cells. Heneen and Nichols (1972) looked at radial metaphases in Indian muntjac cells. There are 11 possible radial configurations if the small Y_1 chromosome is neglected and the X and Y_2 are regarded as homologues. Although these authors interpreted their data as favoring the occurrence of homologous pairing, Nur (1976) pointed out an error in their statistical analysis. Re-analysis of the data indicates a tendency only for the large metacentric autosome to associate with its homologue. However, the number of cells observed was relatively small; results with a larger number of cells to be reported in this thesis (Korf and Diacumakos, 1977) show

that the arrangement of Indian muntjac chromosomes on the spindle is random.

In human cells, homologous pairing has not been directly demonstrated, although some evidence has been suggestive. Many investigators have examined chromosome positions in fixed mitotic cells treated with colchicine and hypotonic solution, fixed, and spread on slides. The most frequent associations are between the short arms of acrocentric chromosomes (e.g., Ferguson-Smith and Handmaker, 1961), which are the sites of nucleolus organizers. Schneiderman and Smith (1962) found that certain non-homologous chromosomes tend to lie near one another in spreads. Barton and David (1962, 1963) and Barton et al. (1963) compared the distance between homologous chromosomes with the distance between each member of a homologous pair and every other chromosome. A tendency for homologous pairing was found in mitoses from normal female subjects, but not from normal males or patients with trisomy 21. With the advent of chromosome banding to identify each chromosome, more precise studies are possible. Warburton and Naylor (1973), Naylor and Warburton (1973), and Rodman et al. (1978) have examined banded preparations and noted that homologues do not tend to associate. On the other hand, Hens et al. (1975) and Galperin-Lemâitre et al. (1977) have reported that human chromosomes are not randomly arranged in metaphase spreads. Homologous associations were seen mainly among acrocentrics, although some other pairs are proximate to one another more than expected at random.

Although not regularly paired at mitosis, homologous chromosome regions may be paired at least some of the time in human cells. In peripheral blood cultures from patients with Bloom's syndrome, occasional cells contain a pair of chromosomes which are associated with one another through part of their lengths (German, 1964). Such associations are referred to as quadriradials and represent geneticexchanges between chromosomes. In Bloom's syndrome, quadriradials tend to involve homologues (Schroeder and German, 1974). Bloom's syndrome is recessively inherited and is characterized by dwarfism, sun-sensitive facial rash, spontaneous chromosome breakage, and increased sister chromatid exchange (German, 1973; Chaganti et al., 1974). The frequency of quadriradials

is increased in normal cells by treatment with mitomycin C (Nowell, 1964; Shaw and Cohen, 1965; German and LaRock, 1969; Huttner and Ruddle, 1976). Mitomycin C also increases the frequency of somatic recombination in fungi (Holliday, 1964) and the frequency of sister chromatid exchange (Latt, 1974). Vogel and Schroeder (1974) concluded that human chromosomes must be homologously paired at interphase to explain the formation of homologous quadriradials. However, in Fanconi's anemia, another chromosome breakage syndrome, quadriradials involve nonhomologues (Schroeder and German, 1974). Comings (1975) suggested that the nature of the DNA damage occurring spontaneously in Bloom's syndrome or induced by mitomycin C might only be repairable by the annealing of homologous sequences. It is thus not clear whether the formation of quadriradials between homologues implies that homologous pairing is a regular event; homologous pairing may either be a rare, but necessary prerequisite for this type of aberration, or may not occur at all.

Genetic evidence for somatic recombination in animals is not convincing. Serebovsky (1925) interpreted feather color patterns in chickens as evidence of "somatic segregation", and others (Carter, 1952; Grunberg, 1966; Bateman, 1967) have cited examples of alleged twin spotting in mice. These cases are rare and poorly documented, however. The only known example of spontaneous somatic recombination occurring at high frequency in mammals involves the loci for immunoglobulin V and C sequences (Brack et al., 1978) in lymphoid cells. However in this case, recombination is not between homologous loci.

In Chinese hamster cell cultures, spontaneous mitotic recombination occurs rarely, if ever. Rosenstraus and Chasin (1978) and Tarrant and Holliday (1977) each examined more than 10^7 cells for spontaneous intraallelic recombination at the X-linked hypoxanthine-guanine phosphoribosyl transferase (hgprt) locus. Intraspecific hybrids, containing two active X chromosomes and heteroallelic for hgprt mutations, were screened for wild type segregants. No spontaneous recombination occurred, while mitomycin C treatment produced a total of four potential recombinants (only one was confirmed) of approximately 10^8 colonies screened. In contrast, the frequency of spontaneous intraallelic somat-

ic recombination in fungi is approximately 10^{-6} per cell; treatment with mitomycin C increases this rate 100 fold. Rosenstraus and Chasin (1978) also searched for interallelic recombination on the X chromosome, using hybrid cells marked at both the hgprt and glucose-6-phosphate dehydrogenase loci. All mutant segregants from parental heterozygous clones were the result of chromosome breakage events, not recombination.

Arrangement of Chromosomes in the Interphase Nucleus Comings (1968) reviewed the evidence that the arrangement of chromosomes in the interphase nucleus is ordered. He proposed that chromosomes are attached to the nuclear membrane, that these attachments might be at specific chromosomal sites, and that the spatial arrangement might be involved in the regulation of DNA replication and genetic activity. Current evidence supports an orderly arrangement of interphase chromatin at three levels: 1) attachment to the nuclear membrane; 2) association of certain chromosome regions with one another; 3) polarization of chromosomes in the nucleus so that the centromeres are clustered at one end, telomeres at the other.

Evidence that chromosomes are attached to the nuclear membrane dates back to observations that densely staining chromatin is often found at the periphery of the nucleus (see Vanderlyn, 1948). Brenner (1953) centrifuged rat liver nuclei and noticed that the chromatin mass was connected by strands to the centripetal border of the nucleus. DuPraw (1965) examined honeybee interphase chromatin by whole mount transmission electron microscopy and noted that some chromatin fibers were attached to nuclear pore complexes. Association of interphase and prophase chromosomes with nuclear pore complexes has also been reported by Maul (1970) and Comings and Okada (1970a). Moreover, isolated nuclear membranes are associated with DNA (see Franke, 1974). At leptotene in meiosis, chromosome ends are attached to the nuclear envelope (Moens, 1969). Sved (1966) suggested that movement of chromosome ends along the nuclear membrane might lead to homologous pairing at meiosis. Such movements were inferred by Church (1977), who compared centromere positions in the grasshopper Brachystola magna at premeiotic interphase and meiotic prophase. The notion that DNA replication is initiated at

the nuclear membrane was originated by Jacob et al. (1963) for bacteria. Comings and Kakefuda (1968) showed by autoradiography in human amnion cells that DNA synthesis for the first 10 minutes following release of amethopterin block is confined to the periphery of the nucleus, while at later times synthesis could be detected elsewhere.

The second level of organization involves the association of chromosome regions with one another. Heterochromatic regions, which remain condensed throughout the cell cycle (see Heitz, 1935), are particularly prone to such associations. Vanderlyn (1948) reviewed evidence that heterochromatic chromocenters in plants tend to fuse at interphase. In Drosophila melanogaster, Heitz (see Swanson, 1957) found that the centromeres of autosomes and the X chromosome, as well as the entire Y, are heterochromatic. In polytene cells, the heterochromatic regions are clustered in a tight chromocenter. The tendency of homologous heterochromatic regions to fuse at interphase in plants has already been noted (Stack and Clarke, 1973; Singh et al., 1976; Fussell, 1977). In mammals, heterochromatic associations have been reported by Hsu et al. (1971) and Schmid et al. (1975). The association of nucleolus-associated heterochromatin in human cells (Lima-de-Faria and Reitalu, 1963) may be responsible for acrocentric associations and nucleolus organization. The basis for heterochromatic association is not known. Maguire (1972) pointed out that it may be indicative of homologous pairing in some species, the paired heterochromatic regions merely being the most conspicuous. Heterochromatin is usually rich in highly repetitive DNA sequences (Yunis and Yasmineh, 1971). Mayfield and Ellison (1975) proposed that repetitive DNA's of similar base compositions might aggregate, with sequences of different families forming separate chromocenters. Otner chromosome associations may or may not involve heterochromatin. Employing cytological hybridization, Steffensen (1977) nas located the position of genes for 5s RNA, tRNA, and histones in interphase nuclei of Drosophila melanogaster. Tissue-specific locations for these loci were seen, suggesting that the three-dimensional arrangement of DNA in the nucleus may be involved in the regulation of genetic activity. Finally, Comings (1977) has proposed that at intervals along

each chromosome, the chromatin is condensed and bound to the protein matrix of the nucleus.

The third level of chromosome arrangement is the polarized array. The polarized array occurs in many, but not all, plant and animal cells (Hsu et al., 1971; Maguire, 1972). In the onion, Allium, heterochromatin staining reveals that the centromeres are located near one another, often in a ring, on one side of the nucleus, and the telomeres are located at the other side (Fussell, 1977). Moens and Church (1977) examined serial sections of Allium microspores (haploid cells) with the transmission electron microscope. While the chromosomes were polarized in cells at different developmental stages, their positions in the nuclei were different. Early in interphase, centromeres were clustered near the former pole of the spindle. However, at the next prophase, prior to breakdown of the nuclear envelope, the centromeres were no longer aggregated into groups, but were arranged in a ring, each centromere separated from the other. Thus, the simple interpretation that the polarized array is a passive result of chromosome arrangement at the previous telophase may be incorrect; some movements of chromosomes may occur independently of the spindle during interphase.

Chromosome Interconnections

Evidence for the Existence of Chromosome Interconnections In the early cytological literature, there was occasional mention of chromosomes appearing to be interconnected by fibers. Werner (1927) noted filaments running between microchromosomes at the center of radial metaphase arrays of the Indian runner duck. Janaki-Ammal (1931) found that in interphase nuclei of the plant Nicandra physaloides, chromocenters of homologous chromosomes were paired, and the ten pairs of chromocenters were connected together by a fine thread. Dearing (1934) reported that the telophase chromosomes of the axolotl, Ambystoma tigrinum, were enmeshed in fibers. These reports were all based on examinations of sectioned and stained fixed cells. Since there is a great hazard of inducing artifacts with such preparations, the significance of the findings

is uncertain. Boss (1972) and Klástrská et al. (1977) have demonstrated that hypotonic treatment may lead to the artifactual appearance of chromosome interconnections, since the fibrous structure of the chromosomes is loosened. However, Klástrská (1978) has pointed out that in some species, interconnections appear among meiotic chromosomes without hypotonic treatment of the tissue and with the use of various fixatives. She concluded that the structure of chromosomes may differ in various species, and in some, interconnections may exist.

Wagenaar (1969) and Ashley and Wagenaar (1974) have studied chromosome interconnections in several plants. These studies employed squash preparations of various cell types, without hypotonic treatment or chemical disruption of the mitotic spindle. In prophase and telophase cells of Allium cepa, the chromosomes, all metacentric, are polarized, and the telomeres are attached to one another by thin strands. When the nuclear membrane was broken during squashing, most of the chromosomes were found to be connected end to end in a chain. In Crepis capillaris (2n=6), four chromosomes are connected in a ring, and the two which carry the nucleolus organizers are each attached to the ring at one point. Similar findings were made in several other plants: chromosomes are attached end to end, in specific orders, at prophase and telophase. Homologous pairing is usually present; in haploid pollen cells, a single unpaired chain was seen. The connections between chromosome ends break down with the nuclear membrane. The chromosomes are thus independent of one another during metaphase and anaphase, although at these times they are held in position by the spindle. It was suggested that the interconnections are important for the maintenance of homologous pairing. However, it is not clear if interconnections actually maintain chromosome arrangements or whether they result from the artifactual adhesion of chromosome ends held in proximity to one another by other means (such as attachment to the nuclear membrane). Godin and Stack (1975, 1976) showed that interchromosomal connectives in Ornithogalum virens and Secale cereale occur at heterochromatic regions, and suggested that heterochromatin might be involved in forming the interconnections.

In mammalian cells, chromosome interconnections have been seen both with light and electron microscopy. Some cases are very likely the result of preparative artifacts. Lácá (1972), Chen (1973), and de Grouchy and Chavin-Colin (1973) noted fibers running between chromosomes after fixation and staining with Giemsa banding methods. These are fairly harsh procedures, involving treatment of chromosomes with alkali, proteolytic enzymes, or sodium dodecyl sulfate. Schwartzacher et al. (1976) have shown that such treatments lead to a swelling and partial dispersal of chromosome fibers; overlap of dispersed fibers from two adjacent chromosomes probably explains the interconnections.

On the assumption that the brittleness of fixed preparations leads to breakage of fragile interconnections, several investigators have examined mammalian chromosomes in unfixed cells. Burdick et al. (1974) and Burdick (1976) squashed pieces of unfixed testis from mouse and Chinese hamster, suspending the tissue in an isotonic solution containing trypan blue dye at the time of squashing. Both somatic and pachytene chromosomes were found to be enmeshed in a network of fibers. There was no specific arrangement of the chromosomes. A similar network was reported by Takayama (1975) in cultured mouse fibroblastic cells (L-cells) which were treated with hypotonic solution, mixed with Giemsa stain, and squashed on a slide. Some cells were incubated with colchicine for 2 - 6 hours prior to hypotonic treatment; interconnecting fibers were seen after this treatment as well. Myhra and Brøgger (1975) observed end to end attachments between human chromosomes in cells treated with hypotonic solution, spread onto the surface of distilled water, picked up onto slides, and air dried. The interconnecting fibers were resistant to treatment with pronase and incorporated tritiated thymidine as demonstrated by autoradiography.

Unfixed chromosomes which have been whole-mounted and viewed with the electron microscope are also interconnected by fibers. Lampert et al. (1969) noted fibers of the same dimensions as chromatin fibers interconnecting human chromosomes. Their specimen was obtained from a Burkitt's lymphoma, and included a structurally rearranged chromosome. The chromosomes shown to be interconnected were acrocentric, and may

have been associated with the nucleolus. A network of interconnections, reminiscent of findings with the light microscope, has been seen by scanning and transmission electron microscopy of whole mounted human chromosomes from cells with or without colchicine treatment (Golomb and Bahr, 1973, 1974a; Jaffray and Geneix, 1974). The interconnections are of the same dimensions as chromosome fibers, but may extend for several micrometers across the metaphase spread. They are sensitive to DNase (Golomb and Bahr, 1973).

There are two reports in which fixed mammalian chromosomes were viewed with the electron microscope and interconnections were seen. Emmerich (1973) mounted colchicine and hypotonic treated, methanol- acetic acid fixed cells on a synthetic polymer to obtain whole metaphase spreads for electron microscopy. Interchromosomal connecting fibers, 1 - 20 μm long and 500 - 1700 Å wide, were seen, and interpreted as being responsible for fixing chromosomes on the nuclear membrane. Hsu et al. (1967) published an electron micrograph of a radial metaphase from the rat kangaroo, in which the chromosomes appeared to be attached to one another at their centromeres. In this picture, however, it is not clear that the attachments are discrete connecting fibers; they may actually be regions where adjacent chromosome fibers intermingled.

A drawback to working with unfixed chromosomes is that they might adhere to one another during spreading or squashing. Also, squashes and spreads offer opportunities for overlaps and adhesions to occur. A more controlled manner of releasing chromosomes from cells is microsurgery. The findings by Diacumakos et al. (1971) and Hoskins (1965, 1968, 1969) of interconnecting fibers among microsurgically extracted chromosomes have already been discussed in Chapter I. It may be recalled that Hoskins had shown that the fibers were sensitive to DNase and resistant to colchicine.

There is one study which indirectly supports the existence of chromosome interconnections at some stage of the cell cycle, although fibers were not visualized. Costello (1970) reported a remarkable chromosome arrangement in a first cleavage division zygote of the flatworm

Polychoerus carmelensis. The 17 paternal and 17 maternal chromosomes were in separate groups, yet were arranged in the same linear order in each group. This would be very unlikely to occur by chance, athough only one cell was examined. It suggests that in some cells at least, chromosomes are arranged in a chain-like configuration, which might involve interconnections among the chromosomes.

Are Different Chromosomes Part of the Same DNA Molecule? DuPraw (1970) reviewed the data on chromosome interconnections and proposed that single DNA molecules might be packaged into more than one chromosome. Folding of the DNA and protein would determine the number of mitotic chromosomes and their morphologies. In the extreme, it was proposed that the entire genome in a eukaryote nucleus might consist of a single circular DNA molecule which condenses at mitosis into an interconnected complement of chromosomes having characteristic morphologies. Alternatively the chromosomes comprising a haploid genome might be part of a single DNA molecule, there being two such molecules in a diploid cell.

The consequences of such models for genetic linkage and chromosome rearrangement are intriguing. Connected chromosomes would tend to act as a single linkage group, but if the connections between them could engage in recombination, this tendency would be slight. A very simple mechanism for certain types of chromosome rearragements can be envisioned: for example, alternative packing modes of DNA and protein could produce a metacentric or an interconnected pair of telocentrics. This mechanism was invoked by Kraemer et al. (1972) to explain the fact that in cultures of heteroploid cells, chromosome number and morphology may be highly variable, but DNA content per cell is constant.

DuPraw's hypothesis is especially important because it stimulated a number of investigations into chromosome structure and arrangement. However, several findings fail to lend support to the model. Nur (1976) and Fox et al. (1975) analyzed the positions of chromosomes around radial metaphase arrays in two insects, Locusta migratoria and Melanoplus rubrum. In both species, chromosomes were positioned at random. Yet

these results can be accomodated by the model: the arrangement of chromosomes along the DNA might be readily and frequently changed by recombination so that, in a large population of cells, the arrangement is random.

Another difficulty with the model is in explaining the disjunction of chromatids at anaphase. Lark et al. (1966) and Lark (1967) suggested that sister chromatids do not disjoin randomly at anaphase. They grew cells in tritiated thymidine, followed by one division in unlabeled thymidine. This resulted in the differential labeling of sister chromatids visualized by autoradiography. Grain counts in the daughter cells after anaphase revealed that labeled chromatids were unevenly distributed, more being present in one cell than the other. However Heddle et al. (1967) challenged this result, pointing out that grain counts in anaphase cells could be unequal due to sister chromatid exchange. Since chromatids were not visualized and it was not known exactly how many divisions each cell had completed in unlabeled thymidine, it was not possible to know the extent of sister chromatid exchange. These investigators analyzed the distribution of labeled centromeres at metaphase in cells grown for three cycles after pulsing with tritiated thymidine. They found that the disjunction of sister chromatids in the plant Vicia faba and the animal Potorus tridactylus was random. More recently the same conclusion has been reached from an analysis of disjunction in cells of the Indian muntjac (Mayron and Wise, 1976), human (Brøgger, 1976), and chicken (Morris, 1977). Sister centromeric regions were distinguished by differential staining following two rounds of replication in the thymidine analog 5-bromodeoxyuridine. Random disjunction of sister chromatids, however, also does not refute DuPraw's hypothesis. Bahr (1977) suggested that crossing over would relieve the entanglements of interconnecting fibers caused by random alignment of chromatids on the spindle at metaphase.

The observations most damaging to the model are those of chromosome-sized DNA in eukaryotic cells. The yeast Saccharomyces cerevisae contains 8.4×10^9 daltons of DNA packed into 17 chromosomes. Therefore, the average DNA content per chromosome should be $4.9 - 7.1 \times$

10^8 daltons. Petes and Fangman (1972) layered yeast spheroplasts on a sucrose gradient and lysed the cells. Sedimentation velocity measurements revealed an average DNA molecular weight of 6.2×10^8 daltons, suggesting that the entire genome is not contained in a single molecule of DNA. Kavenoff and Zimm (1973) determined the size of the largest DNA isolated from Drosophila melanogaster cells by measuring viscoelastic retardation time. The largest piece of DNA was 4.1×10^{10} daltons, which compares favorably with an estimated DNA content of $4.0 - 4.3 \times 10^{10}$ daltons for the largest chromosome. A Drosophila strain carrying a translocation chromosome longer than the longest normal chromosome, has a correspondingly sized largest DNA. It may be that chromosome interconnecting fibers are more labile to shear-breakage than chromosome fibers, thus leading to fragmentation of DNA into chromosome-sized lengths. Yet the methods employed avoided shear forces until all the protein had been removed from the DNA, after which there is no reason to expect interconnecting DNA to be especially labile. It is further possible that the species examined are different from those in which chromosomes are interconnected.

There is indirect evidence that chromosomes are not interconnected during cell division. Nicklas (1967) was able to detach grasshopper chromosomes from the meiotic spindle without disturbing other chromosomes. Interspecific hybrid cells often lose some of the chromosomes of one species and retain others (Weiss and Green, 1967). Individual chromosomes have been obtained in micronuclei from cells treated with colchicine and cytochalasin B (Sekiguchi et al., 1978). Thus the evidence against DuPraw's model is considerable. Yet as long as there is convincing cytological evidence for chromosome interconnections, the model is viable.

Other Interpretations of Chromosome Interconnections The possibility that chromosome interconnections are due to heterochromatic associations has already been mentioned. Godin and Stack (1975) invoked this explanation for interconnections in some plants. As previously noted, heterochromatic regions tend to associate at interphase. In Drosophila polytene chromosomes, intercalary (i.e., non-centromeric)

heterochromatic sites are often in contact with one another (Hinton, 1945). This is referred to as ectopic pairing. Occasionally the paired regions are separated during squashing but remain connected by a thin strand. Interconnecting fibers may thus reflect the juxtaposition of chromosome regions prior to cytological preparation; the fibers are the effects, not causes, of this association.

Dancis and Holmquist (1977) have set forth a model for chromosome replication which involves the transient telomeric fusion of chromosomes. The problem is how RNA primers for DNA synthesis at 5' telomeric ends are replaced with DNA, since removal of the RNA at these sites leaves no 5' primer for DNA synthesis. According to the hypothesis, chromosomes fuse during interphase so that there are no free 5' ends. After DNA synthesis, fission of the chromosomes from the chain takes place. Occasional failure of fission might result in translocations, and may explain the chains of chromosomes seen rarely in cells treated with 5-bromodeoxyuridine or in cells of patients with the Thiberge-Wissenbach syndrome (Dutrillaux et al., 1977). This model is also consistent with the behavior of plant chromosome interconnecting fibers, which break down after prophase. Whether some of the observations in mammalian chromosomes represent a similar phenomenon is not known; cases in which chromosomes are enmeshed in a fibrous network clearly do not.

Among other interpretations of chromosome interconnections, Hoskins's proposal should be recalled. Hoskins (1968) assumed that the fibers are part of the mitoitc spindle and suggested that DNA-containing spindle fibers connect adjacent chromosomes. Schneider (1973) found that in oppossum (Didelphis virginiana) cells incubated with tritiated uridine for 15 - 20 minutes prior to hypotonic treatment, label was found over fibers that run between the chromosomes. He suggested that the interconnections represent chromosome regions that are active in DNA synthesis after the chromosomes condense for mitosis. Henderson and Atwood (1973) have shown by cytological hybridization that fibers running between acrocentric chromosomes in human metaphase spreads contain rRNA coding sequences. These fibers are probably remnants of the association of acrocentrics involved in organizing the nucleolus.

Finally, none of the reports of chromosome interconnections is free from the possibility that cytological preparation or manipulation has produced these structures, and that, therefore, they may be artifacts. This possibility must be born in mind when evaluating any such observation. Moreover, the above hypotheses may each be valid for certain cases; interconnecting fibers may exist in some cell types, while in others they may be artifactual.

III. MATERIALS AND METHODS

Muntjac Cell Culture

Source of Cells A Muntiacus muntjak vaginalis fibroblastic cell culture was obtained from the American Type Culture Collection (CCL 157). It had been established from a skin biopsy of a male Indian muntjac by Wurster and Benirschke. The number of population doublings prior to receipt by the American Type Culture Collection is unknown. The culture obtained by our laboratory was passed six times, and thirty cultures were frozen and stored with 10% glycerol in liquid nitrogen. Experiments were performed on cells obtained by thawing an ampoule and subculturing until "sensecence" occurred (see below), whereupon the culture was replaced by thawing another ampoule.

Growth Conditions Cultures were maintained in Dulbecco's modified Eagle's medium (GIBCO) with heat-inactivated (56°C, 30 minutes) fetal calf serum (10%, v/v, Flow Laboratories) and l-glutamine (2 mM). For about the first eighteen months, potassium penicillin G (500 U/ml), streptomycin sulfate (0.10 mg/ml), and mycostatin (25 u/ml) were added to the medium. When a laminar-flow hood was acquired, use of these antibiotics was discontinued. No muntjac culture ever developed a bacterial or fungal contamination. Cells were seeded into 75 cm^2 plastic flasks (Falcon, no. 3024) at a density of 0.5 - 1.5 x 10^4 cells per cm^2 and split either 1:5 every week or 1:3 twice a week. For subculturing, cells were washed twice in calcium- and magnesium-free phosphate buffered saline (PBS-deficient) and suspended by treatment with 0.25% trypsin in PBS-deficient with 0.5% Versene. Cultures were gassed with a mixture of 95% air - 5% CO_2 for 20 seconds and incubated at 37°C.

Mycoplasma Testing Cultures were periodically checked for mycoplasma contamination by scanning electron microscopy (Brown et al., 1974) or fluorescent staining with Hoechst 33258 (Chen et al., 1977). Flagrant contamination with mycoplasma was found only on one occasion. The infected culture was discarded and replaced from frozen stocks.

Karyotype Stability The karyotype of the cultures was monitored

at frequent intervals to insure that all work was done on cells having a normal diploid chromosome complement. For this analysis, cultures were treated with colcemid (GIBCO) at 0.05 - 0.25 μg/ml for 4 - 6 hours. Mitotic cells were shaken from the surface of flasks or treated *in situ* when grown in petri dishes on glass cover slips. In either case, they were incubated for 10 - 12 minutes in 0.075 M KCl at 25 - 37°C., and fixed three times in 3:1 methanol:acetic acid. Cover slips were dried over a flame, while cell suspensions were dropped onto cold, wet slides and air-dried. Preparations were stained in 4% Giemsa in 0.1 M PO_4 buffer (pH 6.8).

Table I displays karyological data obtained from analyses of subsequent passages of a muntjac culture. At early passages, cells were euploid, and about 75 - 85% were diploid, the remainder being tetraploid. Higher ploidy cells began to appear after the 20th passage. By the 30th passage, chromosome rearrangements, primarily dicentrics and rings, appeared. The frequency of polyploidy went up slightly. At about this time, the rate of growth of the cells decreased, making subculturing very difficult. This point probably represents the onset of senescence of the culture (Hayflick and Moorhead, 1961). Cultures showing evidence of senescence (altered karyotype or growth rate) were discarded and replaced from frozen stocks.

Equipment for Micromanipulation

Equipment and procedures for micromanipulation have been described by Diacumakos (1973).

Microsurgery Chamber Cells to be used for microsurgery were seeded into 60 mm plastic petri dishes (Falcon, no. 3002) on number 0, 18 mm square, acid-washed sterile glass cover slips (Gold Seal). Inoculation density was approximately 5 x 10^3 cells/cm^2. The microsurgery chamber is shown in figure 3a. It consists of a 50 x 75 mm glass slide onto which four glass supports have been cemented. The cover slip, with cells growing on one side, is placed cell-side down on the supports, and fastened at the corners with a dab of high-vacuum grease. Culture medi-

TABLE I

Karyotype at Subsequent Passages of Muntjac Culture

Passage*	2n No.	2n %	4n No.	4n %	8n No.	No.** Mitoses with Abnormal Chromosomes	Total
12	27	79	7	21	0	0	34
14	41	84	8	16	0	0	49
16	30	79	8	21	0	0	38
21	32	70	13	28	1	0	46
24	11	65	5	29	1	0	17
25	35	61	21	37	1	0	57
31	48	72	12	18	0	6 ring; 1 dicentric	67
32	25	73	7	21	0	1 ring; 1 dicentric	34
33	15	65	6	26	0	1 ring; 1 dicentric	23

*Each passage = approximately 2.5 population doublings

**Each abnormal chromosome found in separate mitosis (i.e., 6 ring means 6 mitoses found with a ring chromosome)

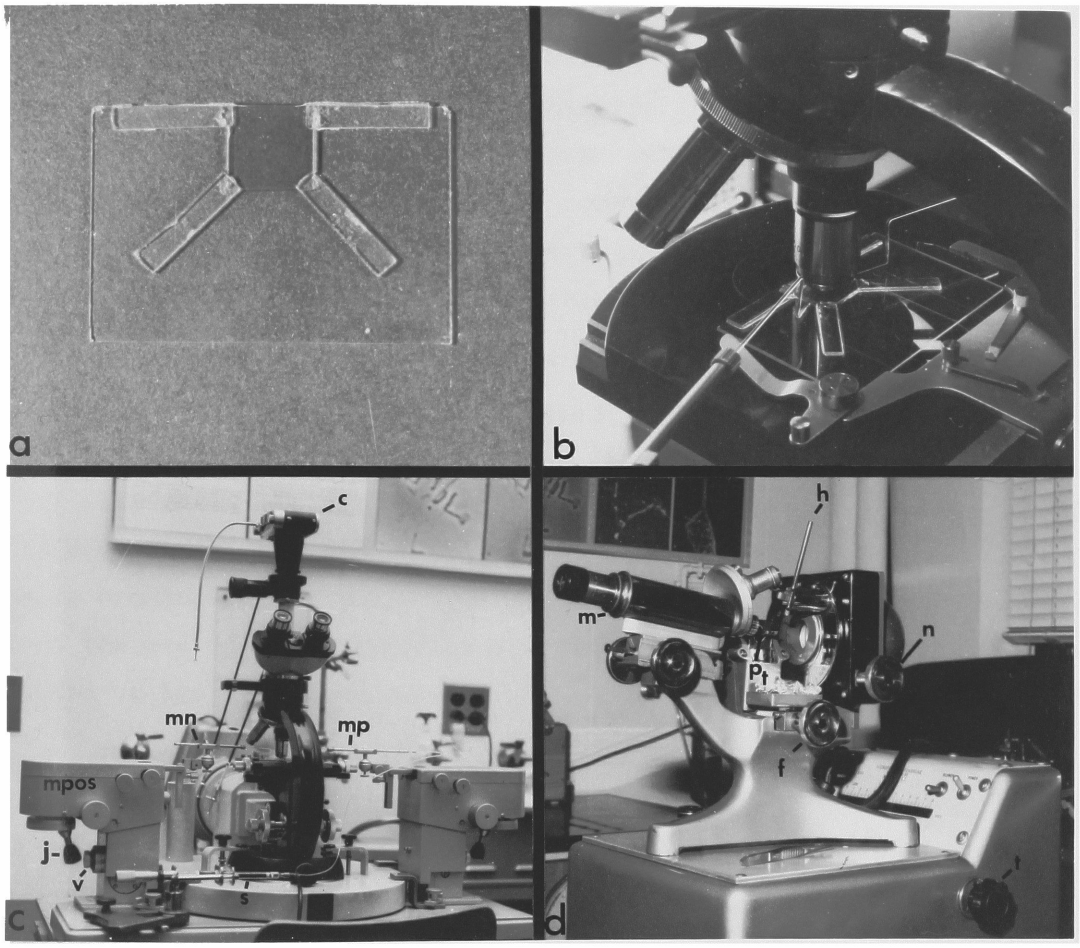


Figure 3. Equipment for micromanipulation. (a) Microsurgery chamber with cover slip in place; (b) microsurgery chamber in place on the microscope stage, with microtools entering from either side; (c) complete setup for microsurgery. The microsurgery chamber is on the stage; a micropipette (mp) enters on the right, a microneedle (mn) on the left. The micropipette is connected by a plastic tube to the syringe (s) which is fitted with a micrometer head. The microscope is flanked by a pair of micropositioners (mpos); horizontal movements are controlled with a joystick (j), vertical with a knob (v) connected to a rack and pinion. A Leica camera (c) is on top of the microscope trinocular head. (d) de Fonbrune microforge. The microtool is placed in a brass holder (h) and brought next to a heated platinum filament (pt) to the temperatures controlled by knob (t). Movement of the filament and the microtool are controlled by adjusting screws (n and f, respectively). The procedure is monitored through the microscope (m).

um, or any other desired solution (see below) is added to the space between the cover slip and the base of the chamber (approximately 0.3 ml). The edges of the cover slip are sealed with silicone oil (Dow Corning 200 fluid), which prevents contamination and retards evaporation of the medium. The top of the cover slip is then dried with a cotton swab and polished by wiping with ethanol.

Micromanipulators The microsurgery chamber is placed on the microscope stage as shown in figure 3b. In these studies a Zeiss phase contrast microscope, equipped with a 100x neofluor objective and Galileo 15x wide field oculars, was used. A drop of immersion oil is placed on the cover slip, the microscope objective carefully lowered, and the cells brought into focus. The thinness of the microsurgical chamber allows the use of high magnification without overly compromising the resolution of the optical system.

The microscope is flanked by a pair of Leitz micropositioners (figure 3c). These scale down hand movements by an adjustable factor of up to 800 times. Movements in the horizontal plane are controlled by a joystick, while fine and coarse vertical movements are made with a rack and pinion. Glass microtools are clamped in place on the micropositioners and enter the microsurgery chamber from either side. The micropipette is connected by a polyethylene tube to a water-filled, spring-loaded Hamilton gas-tight syringe (No. 1002) fitted with a Starrett micrometer head.

Microtools Microneedles and silicone oil-filled pipettes are made by hand as described by Diacumakos (1973). Microneedles are fashioned from 1 mm Pyrex rod (Corning), while 1 mm outer diameter thin-walled capillary tubing (Kimble), acid-washed and coated with Desicote (Beckman), is used for micropipettes. The glass is drawn out in a small flame, and a set of bends are made. The final shaping and formation of the tip is accomplished on a deFonbrune microforge (figure 3d). The microtool is held in place adjacent to an electrically heated platinum wire or glass bead and moved by adjusting screws. Movements are monitored through a microscope. This setup allows fine control of

the dimensions and shape of the tip of the needle or pipette. For the work to be described in this thesis, microneedles with a fine tip (less than 0.1 μm), and pipette tips of inner diameter less than 0.5 μm, were used.

Phase Contrast Micrography Phase contrast micrographs were taken with a Leitz camera attachment using Panatomic-X 35 mm film (Kodak), exposed for 15 - 20 seconds with 6 volt, blue-filtered (Tiffen Photar 80B), tungsten illumination.

Analysis of Chromosome Position at Metaphase

Live Cells Cells were grown on cover slips and prepared for microsurgery. The chamber was filled with culture medium from the petri dish in which the cells had been growing. Metaphase cells were rotated with microneedles so that the radial metaphase plate was visible with phase contrast optics at 1500 magnification.

Fixed Cells Cells were plated in plastic roller bottles and grown for 48 hours. Mitotic cells were then collected by shaking in 30 ml of culture medium by a Telandic cell cycle analysis machine. Cells were harvested by centrifugation, treated with 0.075 M KCl for 10 minutes at room temperature, fixed in two changes of 3:1 methanol:acetic acid, spread onto clean glass slides, and air dried. Approximately 2 x 10^6 cells, obtained from five identical cultures, were pooled and spread onto four slides. The mitotic index was approximately 98%, and about 60% of the mitoses were in metaphase. Slides were stained by the Feulgen reaction and examined with the light microscope at 600 magnification.

Extraction of Chromosomes

Microsurgery Medium A special medium, to be referred to as microsurgery medium (MSM), was formulated for chromosome extraction. Most microsurgery done in this laboratory employs the same culture medium used for growing cells, i.e., Dulbecco's modified Eagle's medium with

fetal calf serum. This is a bicarbonate buffered solution, the pH being maintained at 7.3 in an atmosphere of 5% CO_2. In early experiments with extracted chromosomes it was found that if the Dulbecco's medium was allowed to equilibrate with room air (to pH approximately 8) the chromosomes were less condensed and easier to manipulate. It was thus desirable to work with a medium buffered at this pH and lacking fetal calf serum, which is not well defined chemically and interferes with certain enzyme reactions. MSM (Table II) contains a balanced salt solution similar to Dulbecco's medium, a Tris buffer (pH 8.1) and glucose. This medium is not immediately toxic to cells, but after 2 - 3 hours some cells do begin to show toxic effects. However, microsurgery generally did not last longer than 1 hour, after which cultures were discarded or fixed, and during which time no perceptible changes occurred in the cells. Also, cells which are in prophase at the time the microsurgery chamber is set up continue through mitosis in MSM. Thus, the medium does not disrupt normal chromosome movements. Finally, the morphology, arrangement, and properties of chromosomes extracted in MSM are the same as those extracted in pH 8 Dulbecco's medium.

Extraction with Microneedles Mitotic cells are easily identified in fibroblastic cultures since they are nearly spherical. The chromosomes on the metaphase spindle are seen on edge as a dark band (figure 4a). To extract chromosomes, the cell is braced with one microneedle, while another is inserted a short distance within the cell to contact one of the chromosomes (figure 4b). The inserted microneedle is then carefully withdrawn, pulling the chromosomes along with it (figure 4c). Gentle pressure exerted on the cell by the bracing needle facilitates the removal of the chromosomes. The entire complement of chromosomes is extracted in this way by virtue of interconnections among the chromosomes. The chromosomes are brought to the surface of the cover slip for analysis.

Extraction with Silicone Oil The second method of chromosome extraction employs silicone oil to cause chromosomes to be expelled from the cell (Diacumakos et al., 1971). A droplet of silicone oil (Dow Corning 200 fluid) is expelled from a micropipette directly onto the

TABLE II

Microsurgery Medium

NaCl	134.0 mM	
KCL	3.3 mM	
$CaCl_2$	0.5 mM	
$MgSO_4$	0.5 mM	
glucose	15.0 mM	
Tris	10.0 mM	pH 8.1

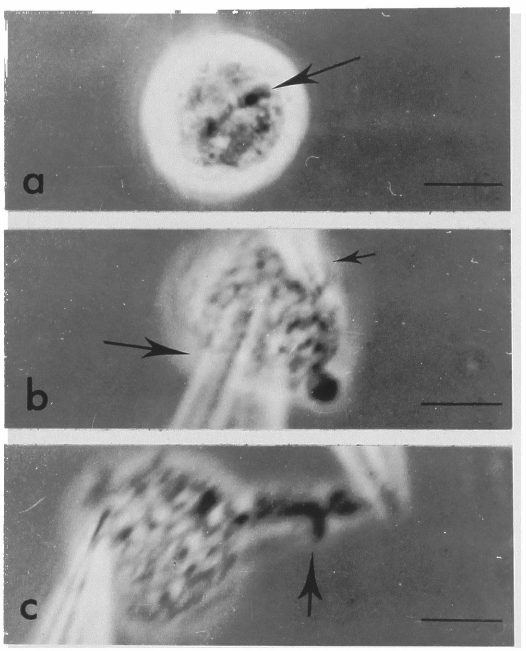


Figure 4. Extraction of chromosomes using microneedles. (a) Metaphase Indian muntjac cell. Chromosomes are indicated by arrow; (b) the cell is braced with one microneedle (thick arrow) and another needle is inserted within the cell and in contact with one of the chromosomes (thin arrow); (c) having withdrawn the inserted microneedle, the chromosomes (arrow) are seen being pulled away from the cell. Bars, 10 μm.

surface of a metaphase cell between the equator and one of the poles (figure 5a,b). The diameter of the oil droplet is approximately equal to that of the cell for best results. Within seconds of the application of the silicone oil, some or all of the chromosomes are expelled from the cell and appear adjacent to the oil (figure 5c). Using a microneedle, the entire chromosome complement is then pulled away from the cell and brought to the surface of the cover slip for analysis. Silicone oil is dimethylpolysiloxane (figure 6). Other oils, including paraffin oil and Bayol F, also have this effect (Diacumakos, personal communication). The mechanism of chromosome extrusion is not known. For the purposes of obtaining chromosomes from mitotic cells, the silicone oil method is preferred. Apparently, a larger portion of the cell surface is weakened by silicone oil than is the case using a microneedle; hence the entire metaphase array is removed more easily. Thus, most of the chromosome extractions to be described in this thesis were done with the silicone oil method, although no differences could be discerned in the properties of chromosomes extracted by either means.

Treatment of Cells with Mitotic Poisons

For some experiments, mitotic poisons were used to inhibit spindle formation. Since the spindle, once formed, is not readily disrupted (Gaulden and Carlson, 1951), cells were treated for 7 - 8 hours before mitosis to insure that spindles never formed. Either colchicine (50 μM) or vinblastine sulfate (0.5 μM) was added to the culture medium of cells growing in petri dishes on glass cover slips. Microsurgery was done in MSM also containing either colchicine or vinblastine sulfate at the same concentrations as above.

Staining Extracted Chromosomes

Marking the Location of Extracted Chromosomes To facilitate relocation of extracted chromosomes after the cover slip was removed from the microsurgical chamber, a mark was made on the top of the cover glass with a diamond scriber. Mitotic cells were brought to this region with

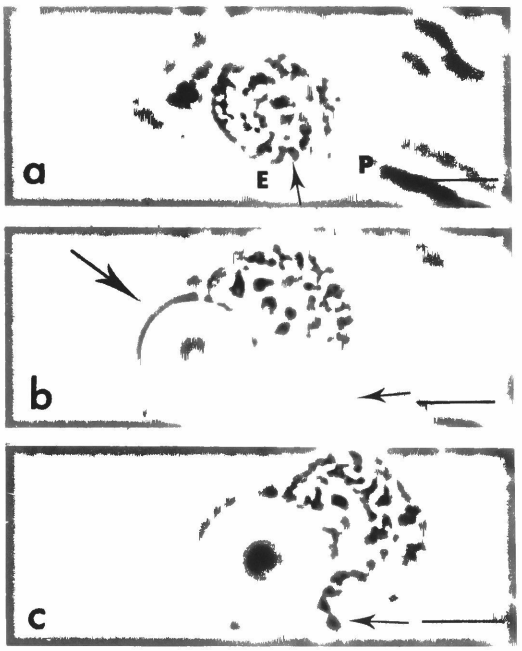


Figure 5. Extraction of chromosomes using silicone oil. (a) Metaphase cell with poles (P) and equator (E) indicated. The chromosomes are visible at the cell equator (arrow); (b) micropipette (thin arrow) is shown just after applying oil droplet (thick arrow) onto cell surface; (c) chromosomes (arrow) partially expelled from the cell. The oil droplet is still in place, and the micropipette has been removed from the field. Bars, 10 μm.

Dimethylpolysiloxane
Silicone Oil
Dow Corning 200 Fluid

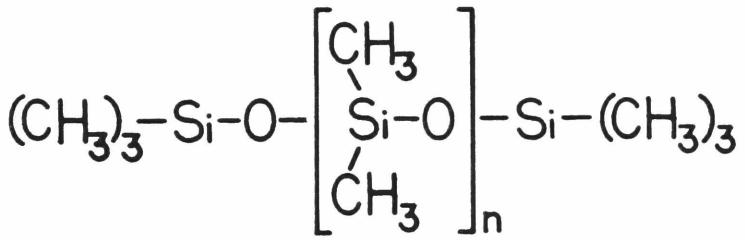


Figure 6. Chemical structure of silicone oil.

a microneedle, and extracted chromosomes placed near the mark.

Glutaraldehyde Fixation of Extracted Chromosomes After the extracted chromosomes were arranged on the cover slip, a solution of 1% glutaraldehyde in a 0.1 M borate buffer (pH 8.1) with 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, and 50 mM glucose was added to the microsurgery chamber, while MSM in the chamber was drawn off. Cover slips were left on the microsurgery chambers in fixative for 1 - 5 minutes and then removed and immersed for 1 minute in a Columbia dish with fixative. They were then rinsed for 2 minutes in a Columbia dish with borate buffer.

Staining with Hoechst 33258 Cover slips were stained for 5 minutes in Hoechst 33258, 0.50 µg/ml in H_2O. They were then immersed in distilled H_2O for 5 minutes, and mounted in pH 7.0 citrate-phosphate buffer. The preparations were sealed with nail polish and examined with the fluorescence microscope.

The fluorescence of Hoechst 33258 is quenched by DNA containing the base analog 5-bromodeoxyuridine (BrdU) in place of thymidine (Latt, 1973). This effect was used to confirm that Hoechst-stained structures in isolated chromosomes were in fact DNA. Cells growing on glass cover slips in petri dishes were incubated overnight in the presence of 100 µM BrdU, 0.4 µM fluorodeoxyuridine, and 6 µM uridine added to the culture medium. Control cultures were incubated in medium without these nucleosides. Chromosomes were extracted from both control and treated cells and fixed in 1% glutaraldehyde. The Hoechst 33258 staining procedure was as above except that a lower concentration of dye, 0.025 µg/ml, provided better distinction of BrdU-containing DNA from normal DNA.

Staining with Acridine Orange Glutaraldehyde-fixed preparations were immersed in an aqueous solution of acridine orange (0.125 mg/ml) for 5 minutes. They were then rinsed in H_2O for 2 minutes, mounted in pH 7.0 citrate-phosphate buffer, sealed with nail polish, and viewed with the fluorescence microscope.

Immunofluorescent Staining with Histone Antisera Antisera to histones H1 and H2B were obtained from Dr. H. Peter Hoffmann. Histones were purified by molecular sieve chromatography and purity was confirmed

by electrophoresis (Panyim and Chalkley, 1969). Histones H1 and H2B were then complexed with yeast RNA and injected into rabbits with Freund's adjuvant (Bustin, 1973). Sera were cleared by centrifugation and used at a dilution of 1:20 in phosphate-buffered saline with 0.2% bovine serum albumin (PBS-BSA). Cross reactivity between H1 and H2B antisera was not detected on radioimmune assay.

In preliminary experiments it was found that glutaraldehyde-fixed chromosomes could not not be stained for histone. However, other fixatives which did not interfere with staining also did not preserve the structure of the chromosomes. Hence, histone staining was carried out on unfixed chromosomes while they were still in the microsurgery chamber. Staining reagents were added to the chamber in the same manner as described previously: a drop of solution was placed at one edge of the cover slip, while a cotton swab was used to draw fluid out of the chamber from other side. After repeating this 5 times, the exchange was deemed complete. The immunofluorescent staining procedure consisted of the following sequence of 10 minute treatments at room temperature: 1) histone antiserum, 1:20 in PBS-BSA; 2) PBS-BSA; 3) fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum (Miles Biochemicals), 1:20 in PBS-BSA; 3) PBS-BSA. After staining, 1% glutaraldehyde in borate buffer was added, and cover slips were fixed as was previously described. They were then mounted on slides in a drop of borate buffer, sealed with nail polish, and viewed with the fluorescence microscope.

As a control, this procedure was also carried out using pre-immune rabbit serum, 1:20 in PBS-BSA, in the place of histone antiserum. In addition, the specificities of the antisera were tested by staining cells with histone antisera pre-absorbed with purified histone H1 or H2B. Cells were grown in multiwell plastic plates (Falcon, 3008), and fixed with methanol at 4°C for 30 minutes. Antisera to H1 and H2B were incubated for 30 minutes at 37°C with electrophoretically purified histone H1 and H2B (obtained from Dr. H. Peter Hoffmann) at a concentration of 5 µg/ml in PBS-BSA. Each antiserum was incubated separately with each histone at each concentration, and with a control containing only PBS-BSA. The antisera were then used to stain the fixed cells by

indirect immunofluorescence using the sequence of reagents described above.

Immunofluorescent Staining with Tubulin Antiserum Anti-tubulin serum was obtained from the laboratory of Prof. Gerald M. Edelman. It had been prepared by immunizing rabbits with sodium dodecyl sulfate-treated, electrophoretically-purified bovine tubulin along with Freund's adjuvant. The antiserum forms a single band by immunoprecipitation with mouse tubulin.

Tubulin staining could be carried out on glutaraldehyde-fixed chromosomes. Extracted chromosomes on cover slips were fixed for 5 minutes in 1% glutaraldehyde in borate buffer and rinsed twice for 2 minutes in borate buffer. To render cells permeable for staining (presence of normal microtubule staining patterns in cells provided a control for successful staining), cover slips were immersed in the following reagents at 4°C: 1) 1:1 acetone:H_2O, 3 minutes; 2) acetone, 5 minutes; and 3) 1:1 acetone:H_2O, 3 minutes. To reduce background fluorescence due to glutaraldehyde, the cover slips were treated with $NaBH_4$ (0.5 mg/ml in PBS) three times at room temperature and rinsed three times with PBS (Weber et al., 1978). They were then rinsed in PBS-BSA, after which four drops of tubulin antiserum diluted 1:4 in PBS-BSA were placed on the cover slips which were incubated in petri dishes containing moist filter paper for 10 - 30 minutes at 37°C. Next, cover slips were washed in PBS-BSA and incubated in PBS-BSA at 37°C for 10 - 30 minutes. They were then stained in four drops of fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum (Miles Biochemicals), diluted 1:20 in PBS-BSA, for 10 - 30 minutes at 37°C. Finally the cover slips were rinsed, incubated again in PBS-BSA, mounted on slides in a drop of pH 8.1 borate buffer, sealed with nail polish, and viewed with the fluorescence microscope.

In some cases, double staining with tubulin antiserum and Hoechst 33258 was done. Cover slips were treated as above, but placed in Hoechst stain, 0.5 µg/ml in H_2O, after the last wash in PBS-BSA. Hoechst staining was then carried out as described above. Different fluores-

cence excitation maxima for fluorescein isothiocyanate and Hoechst 33258 permitted distinction of the fluorescence of these two dyes.

Fluorescence Microscopy A Leitz Diavert microscope equipped with a Ploem epifluorescence illuminator and an HBO 100W mercury lamp was used for fluorescence microscopy. For fluorescein isothiocyanate, excitation was at 480 nm and suppression at 525 nm (Leitz "K" filter package); for Hoechst 33258, excitation was at 365 nm and suppression at 430 nm (Leitz "A" filter package); and for acridine orange excitation was at 365 nm and suppression at 430 nm (Leitz "I-2" filter package). Black and white photographs were taken on Kodak Tri-X or Ilford HP-5 35 mm film, exposed for 30 seconds and developed at ASA 1600. Color photographs were taken on 35 mm GAF 500 film, at exposures of less than 1 second, and developed at ASA 800.

Treatment of Chromosomes with Enzymes

All experiments with enzymes involved the addition of enzyme solutions to unfixed chromosomes in the microsurgery chamber. Reactions were terminated with 1% glutaraldehyde, and, in some cases, preparations were stained with Hoechst 33258 or acridine orange.

Deoxyribonuclease I (DNase I) Sterile, crystalline DNase I (Worthington, DPFF, 1,976 u/mg) was dissolved in sterile H_2O at a concentration of 1 mg/ml. This solution was frozen in 1 ml aliquots. For use, an aliquot was thawed and diluted 1:100 in MSM. The final concentration of enzyme was determined spectrophotometrically to be 10.4 µg/ml. In some experiments, to control for possible contaminating protease activity, phenylmethylsulfonyl fluoride (PMSF, 40 mM in 95% ethanol) was added to the DNase I in MSM to a final PMSF concentration of 2 mM. In other experiments, 1 M hexylene glycol was added to the DNase solution to stabilize chromosomal proteins during digestion.

Protease-Free DNase I A sample of protease-free DNase I was obtained from Dr. Dalton Wang. Commercial DNase I (Worthington DPFF) was purified by affinity chromatography with lima bean protease inhibitor bound to Sepharose (Wang and Moore, 1978). Stability of the enzyme for

10 days in pH 8 buffer at 37°C in EDTA indicates freedom from protease activity. For use on extracted chromosomes, this enzyme was diluted to 15.1 µg/ml in autoclaved MSM.

Micrococcal Nuclease Micrococcal nuclease (Worthington, NFCP, 32,657 u/mg) was dissolved in sterile H_2O at a concentration of approximately 100 µg/ml and frozen in 1 ml aliquots. For use, an aliquot was thawed and diluted 1:100 in MSM (final concentration determined spectrophotometrically, 1.44 µg/ml). In some experiments, PMSF was added to 2 mM.

Ribonuclease A (RNase A) RNase A (Worthington, RASE, 3133 u/ml in 0.1 M PO_4 buffer at 21.35 mg/ml) was diluted 1:50 to 0.42 mg/ml in MSM. After addition of enzyme to the microsurgery chamber, preparations were incubated at 37°C for one hour and then fixed with 1% glutaraldehyde.

Trypsin Trypsin, (Worthington, TRL, 247 u/mg) was dissolved in H_2O at 4 mg/ml and frozen in 1 ml aliquots. For use, this was diluted 1:1,000 in MSM with 10 mM $CaCl_2$ (final enzyme concentration, 3.29 µg/ml).

Pronase Pronase (Sigma, type UI, 5 u/mg) was dissolved in H_2O at 10 mg/ml and frozen in 1 ml aliquots. For use this was diluted 1:50 in MSM to 0.2 mg/ml.

Scanning Electron Microscopy

After extracting chromosomes and marking their positions on the cover slip by clearing away most surrounding cells, preparations were fixed in 1% glutaraldehyde. In early experiments, glutaraldehyde was dissolved in culture medium or MSM, and cover slips were exposed for 1 - 72 hours at 4°C. These preparations were post-fixed in 1% OsO_4 for 1 hour. Later experiments employed 1% glutaraldehyde in pH 8.1 borate buffer for 5 minutes, with no difference in the quality of preparations. Preparations were dehydrated in an ethanol series, placed in acetone, and critically point dried from CO_2 (Anderson, 1951). They were then

coated either with gold or gold-palladium, the latter by the direct current sputter method (Echlin, 1974). Scanning electron microscopy was done either with an ETEC (20 kV electron source, 8 mm working distance) or Jeol JFM 35 (30 kV electron source, 15 mm working distance) instrument. Micrographs were made with Polaroid type 55 p/n film.

IV. RESULTS

Properties of Extracted Chromosomes

Muntjac chromosomes extracted either with microneedles or by the application of silicone oil were spread onto the surface of the cover slip for analysis (figure 7). Thin fibers which interconnect the chromosomes were responsible for the consistent isolation of the entire complement. One or two chromosomes in an extracted group were occasionally distorted by contact with the microneedle. If the mitotic cell was pressed with a microneedle too forcefully during chromosome extraction, the chromosomes were caught inside the cell. Pulling on the chromosomes already extracted in such cases resulted in distortion of those chromosomes and stretching of interconnecting fibers to chromosomes still inside the cell.

Each chromosome can be identified on the basis of morphology (figure 8). Phase contrast photomicrographs of unfixed and unstained chromosomes from several extracted metaphase groups are shown in figure 9a. In figure 9b, Giemsa-stained muntjac chromosomes, prepared from a cell treated with colcemid and hypotonic solution, are shown for reference. Features such as centromeres and secondary constrictions are clearly visible on extracted chromosomes. The greater length of extracted chromosomes compared with fixed chromosomes pictured in figure 9 is due to the lack of colcemid treatment of the cells used for microsurgery and to stretching of chromosomes during micromanipulation. As demonstrated in figure 10, extracted chromosomes are extremely elastic, withstanding stretching to greater than twice their normal length before becoming permanently deformed. Following identification, specific chromosomes or parts of chromosomes can be isolated from the metaphase aggregate by using a microneedle to cut interconnecting fibers or chromosome regions (figure 11).

Radial Array of Extracted Chromosomes

The microsurgically extracted chromosomes were arranged in a radi-

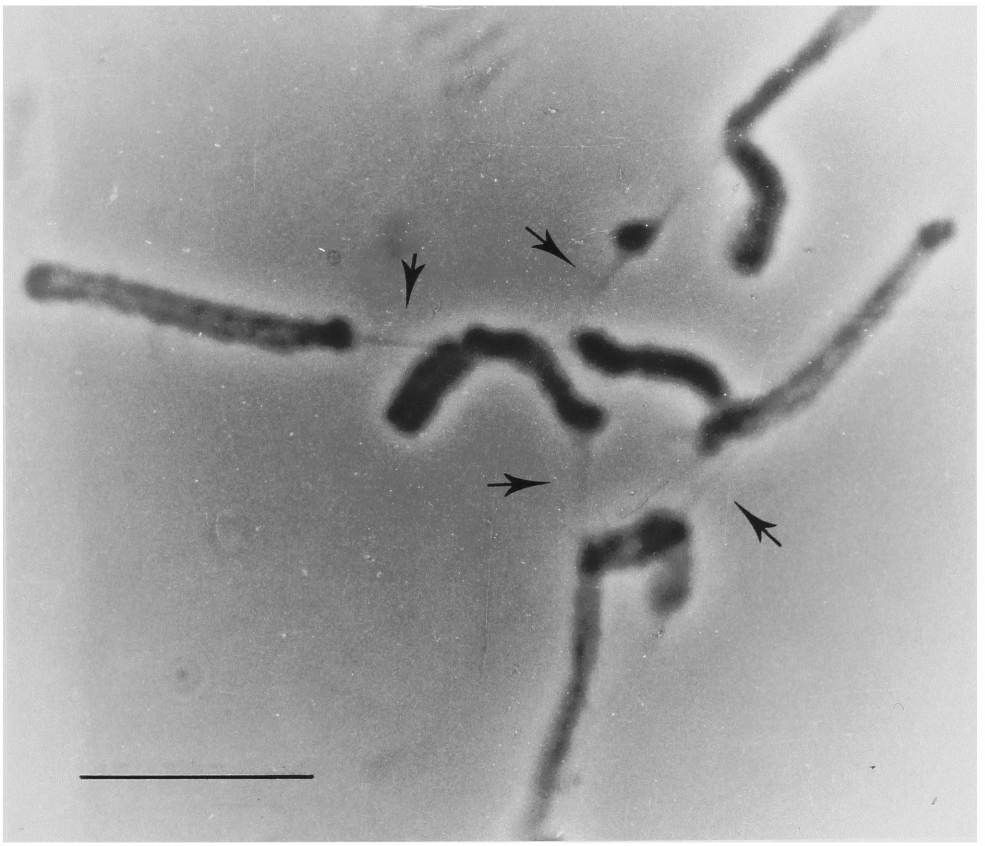


Figure 7. Extracted chromosomes displayed on the cover slip. Interconnecting fibers are indicated by arrows. Bar, 10 μm.

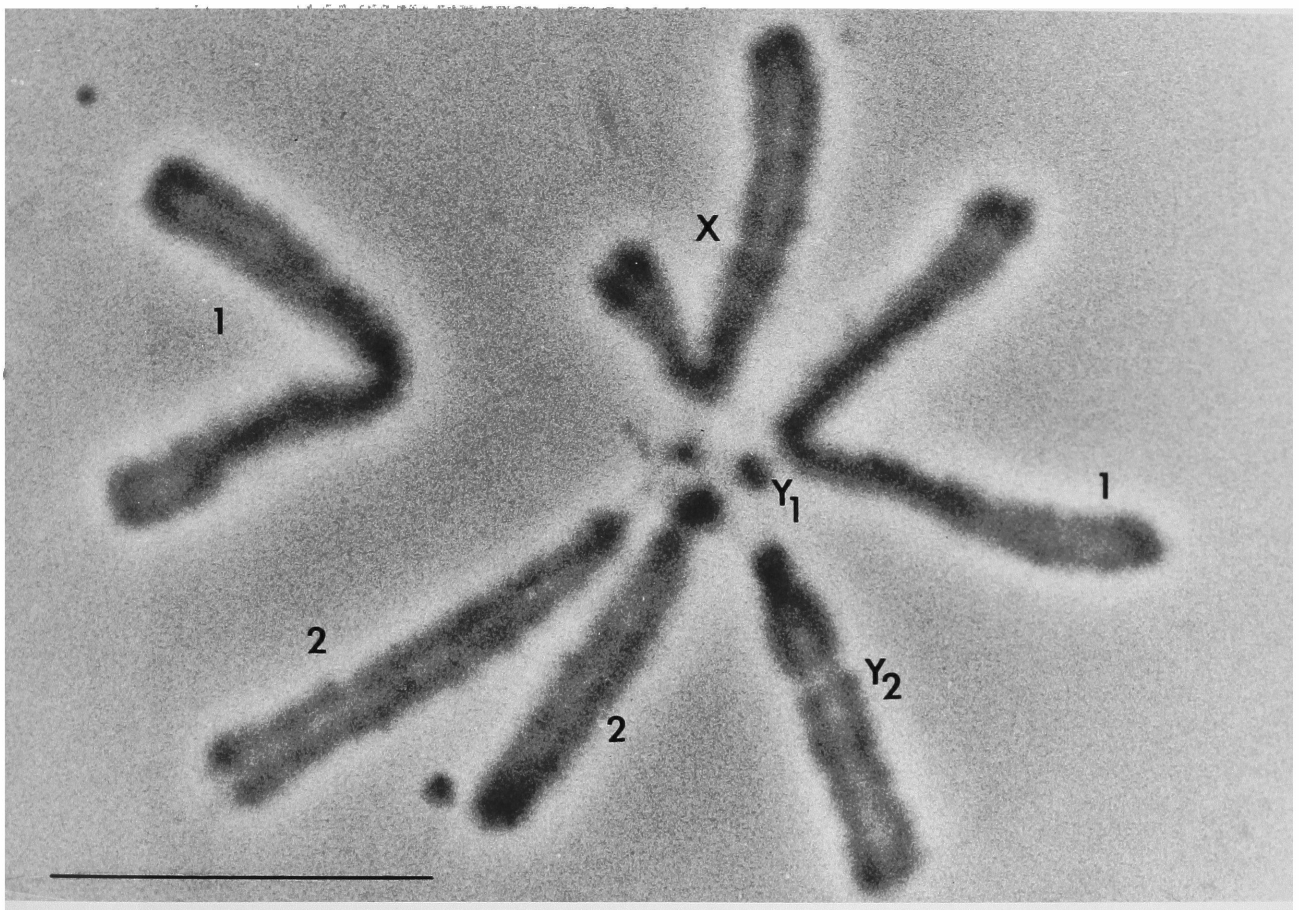


Figure 8. Phase contrast micrograph of extracted chromosomes with chromosome identifications. Bar, 10 μm.

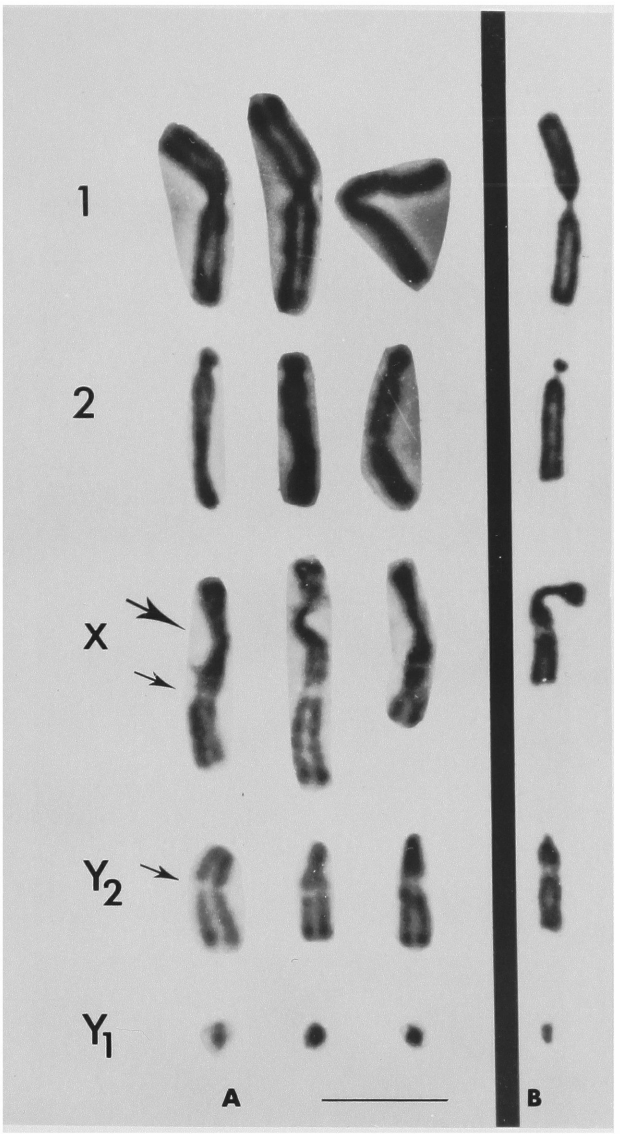


Figure 9. Morphology of extracted chromosomes. (A) Unfixed and unstained extracted chromosomes. Photographs of individual chromosomes were cut out from photomicrographs of extracted metaphase groups; chromosomes in the same column were not necessarily from the same cell. Note the elongated centromeric region of the X chromosome (thick arrow), and the secondary constrictions of the X and Y_2 (thin arrows); (B) Giemsa-stained fixed chromosomes obtained from a muntjac cell treated with colcemid and hypotonic solution, provided for reference to usual muntjac chromosome morphology. Bar, 10 μm.

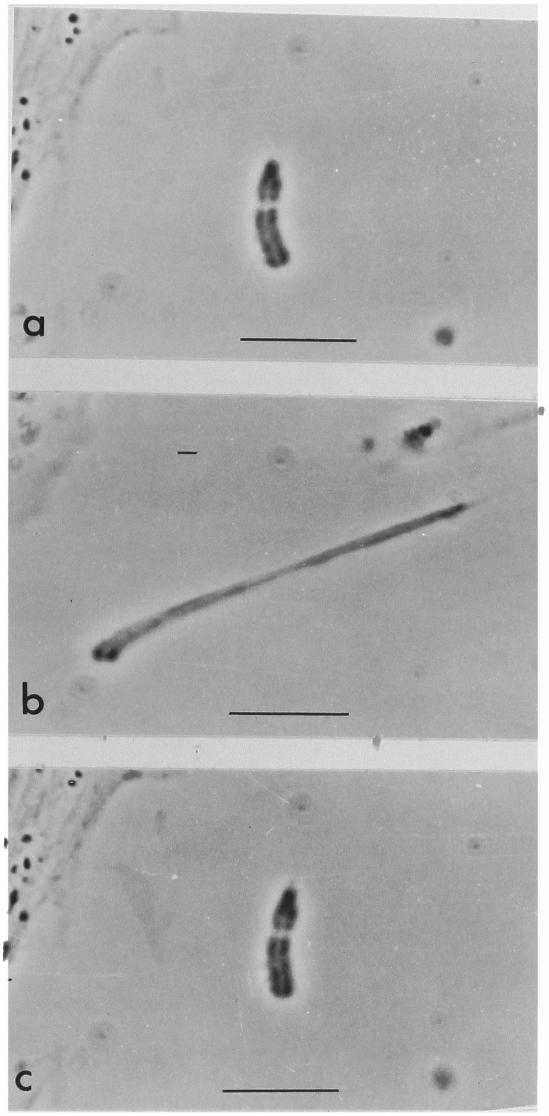


Figure 10. Chromosome Y_2 is shown (a) relaxed; (b) stretched between microneedles (microneedles beyond field of micrograph). Tension was applied to this chromosome via interconnecting fibers which are barely visible in the micrographs; (c) relaxed subsequent to stretching. Bars, 10 μm.

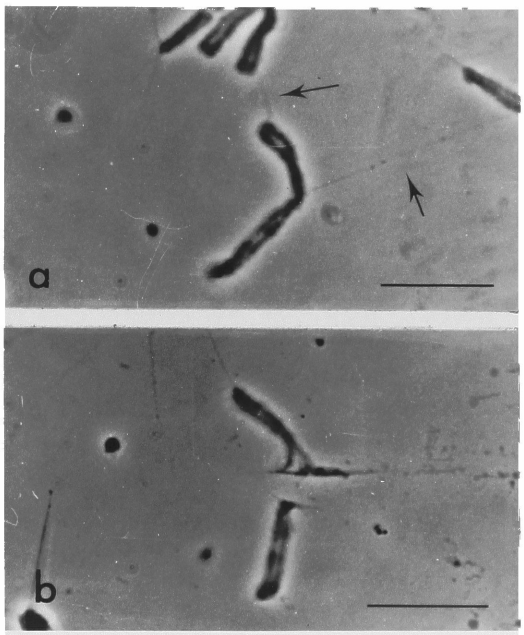


Figure 11. Isolation and dissection of an extracted chromosome. (a) Chromosome no. 1 is shown connected by fibers (arrows) to other chromosomes extracted from the same cell. Note the position of the chromosomes in relation to two dark pieces of debris on the cover slip; (b) chromosome no. 1 has been isolated from the other chromosomes and cut with a microneedle near the centromere. The interchromosomal connecting fibers are visible, but have been severed, and other chromosomes have been removed from the field (note position of the chromosome in relation to debris compared with (a). Bars, 10 μm.

al configuration (figure 12). Each centromere faced the interior of the array, while the chromosome arms pointed outwards. The chromosomes were bound in the radial array by a fibrous network that interconnected the centromeres. The fibers had high tensile strength. It was not possible to pull a chromosome out of the radial array; the interconnecting fiber would merely stretch as the chromosome was displaced. In some cases, a chromosome arm also appeared to be connected to its neighbor by a fiber. However, such connections did not involve any particular chromosomes, nor any particular regions of the arms. Isolated chromosomes are very sticky (figure 13), and if neighboring chromosomes in the radial array were allowed to touch one another, they would adhere. By avoiding contact between neighboring chromosome arms, the formation of such connecting fibers could be prevented. On the other hand, it was not possible to avoid the formation of interconnecting fibers between centromeres and the center of the radial array.

The radial array is not an artifact induced by micromanipulation. It is also seen in both live and fixed metaphase cells of the muntjac (figure 14). An analysis was undertaken to determine whether the chromosomes occupy specific positions around the radial array. Microsurgically isolated chromosomes were not used for this analysis due to the possibility of altering the positions of chromosomes during micromanipulation.

Analysis of Chromosome Position at Metaphase

Criteria for analysis of living cells were: 1) all seven chromosomes could be unequivocally identified (both the nonterminal centromeres of the No. 2 chromosomes and the secondary constrictions on the X and Y_2 chromosomes had to be visible for identification to be unequivocal); and 2) no chromosomes overlapped. An advantage of micromanipulating cells was that each cell could be oriented so that the clearest possible view of each chromosome was obtained. A representative cell is shown in figure 14a. A total of 101 living cells, about half of those checked, met these criteria.

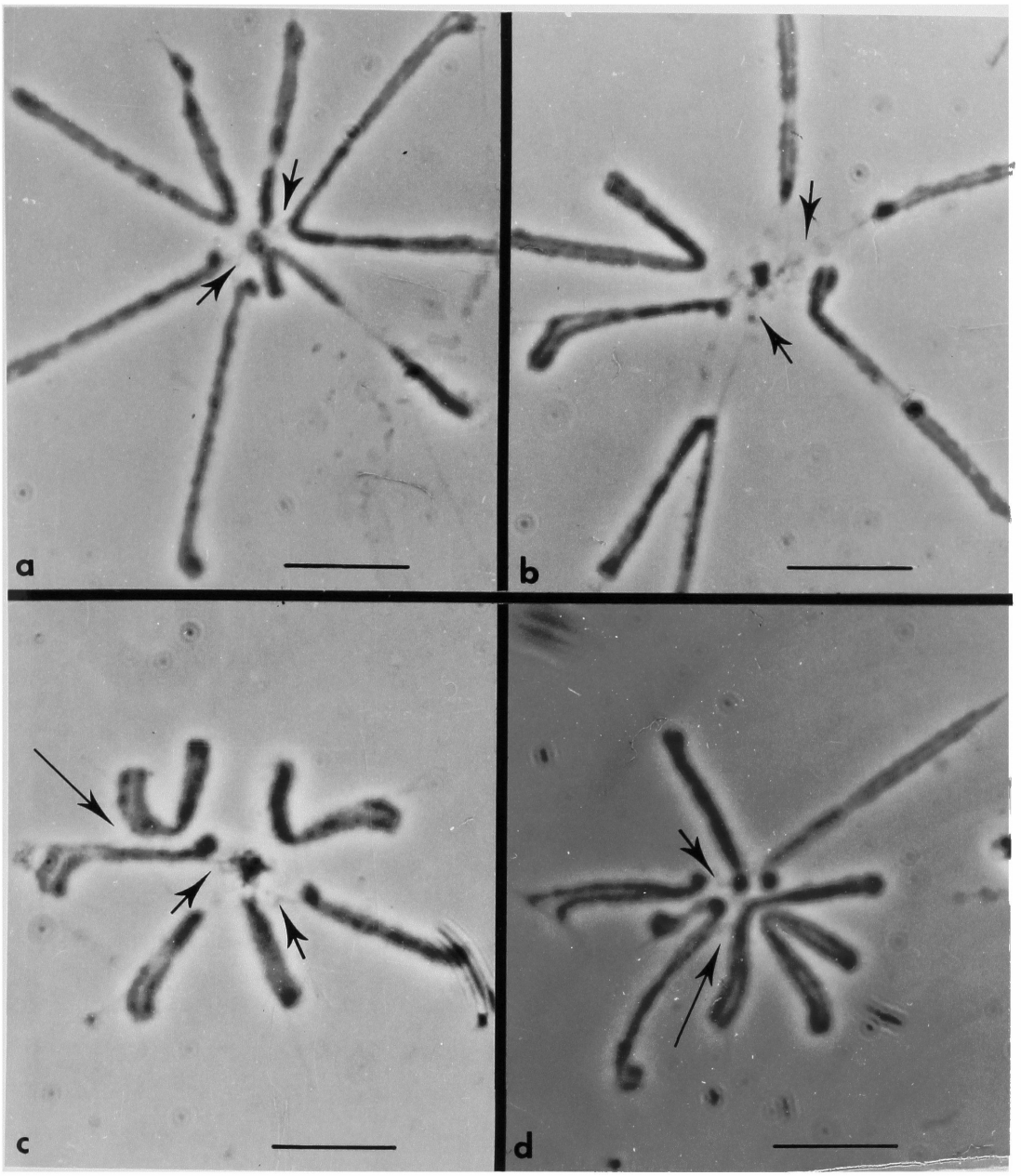


Figure 12. Radial arrays of extracted chromosomes. The centromeres face the center of the array and are connected to a fibrous network (short arrows). In some cases, adjacent chromosome arms are connected (long arrows). Bars, 10 μm.

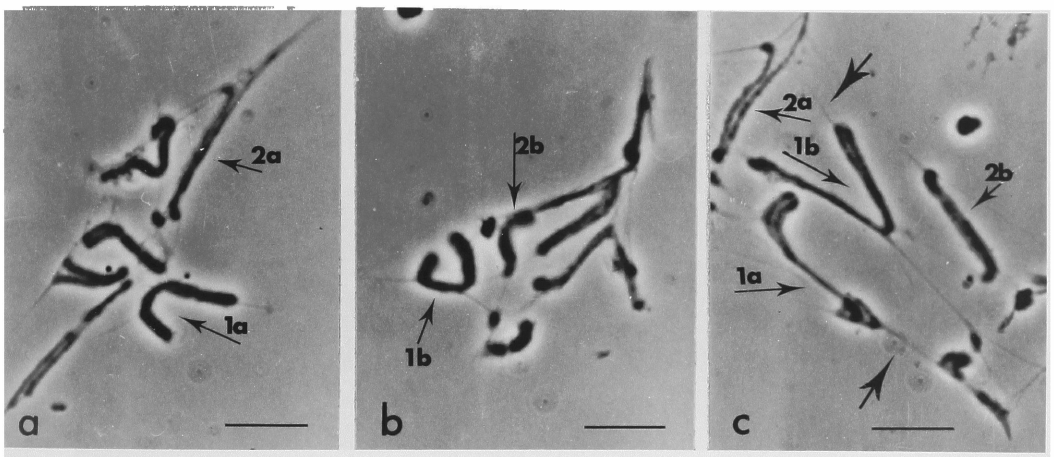


Figure 13. Two sets of extracted chromosomes (a,b) extracted from two different cells were brought into contact using microneedles. In (c) the chromosomes from the two cells are seen to be interconnected by fibers (arrows). Labeled arrows distinguish particular chromosomes in each chromosome set. Bars, 10 μm.

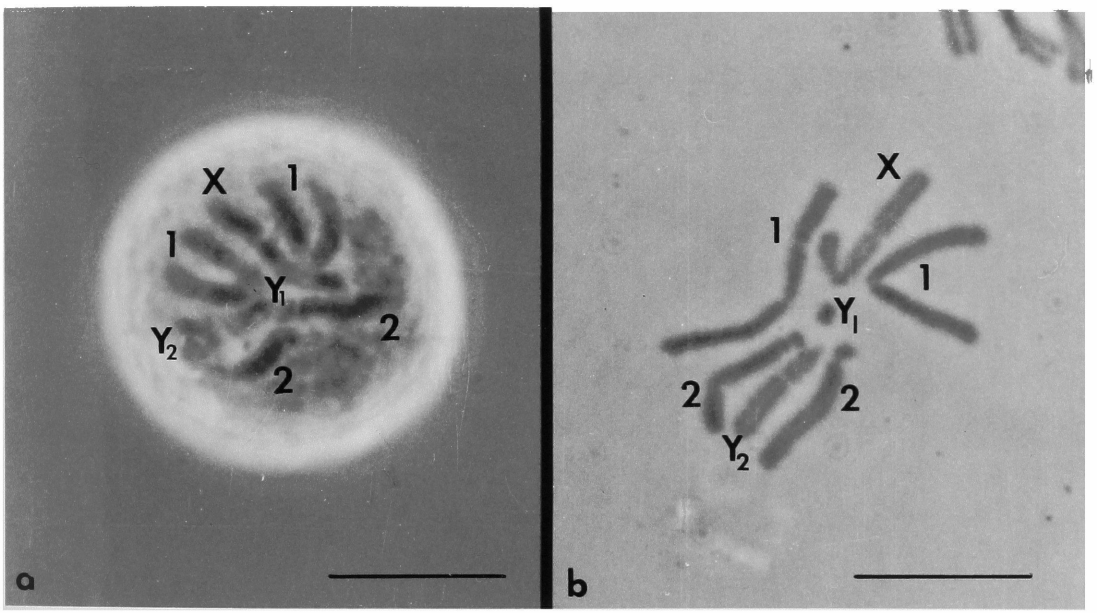


Figure 14. (a) Phase contrast micrograph of a living muntjac cell oriented so that the radial array of chromosomes is visible. Chromosomes are identified by number. (b) Muntjac radial metaphase obtained by mitotic shake-off, treated with hypotonic solution, fixed, and Feulgen-stained. The seven chromosomes are identified. Bars, 10 μm.

Clear radial metaphase figures comprised less than 10% of the metaphase cells obtained by mitotic shake-off. The remainder were either clumps of cells, broken metaphases, or metaphases that had landed on the slide at an angle to the plane of the chromosomes, resulting in chromosome overlap. Only metaphases satisfying the following criteria were analyzed for chromosome position: 1) presence of all seven muntjac chromosomes with normal morphology; 2) radial arrangement of chromosomes; and 3) no overlap of adjacent chromosomes involving the centromere-proximal halves of the long or short arms. An example of a metaphase meeting these criteria is shown in figure 14b. A total of 567 such metaphases was analyzed.

The model by which the data are analyzed is essentially that of Heneen and Nichols (1972). The small Y_1 chromosome is disregarded, since it lies at the center of the radial array. The X and Y_2 chromosomes are treated as indistinguishable homologues. The 11 possible arrangements of the three pairs of chromosomes are diagrammed in figure 15.

The expected frequencies of the 11 arrays are the same as reported by Nur (1976) and are indicated in Table III. The frequencies of the four classes of arrays (zero, one, two, and three pairs of homologues adjacent, respectively) are computed in a paper by Lacadena *et al.* (1977). The expected frequencies for patterns within each class are equal to one another. Thus patterns 1, 5, 6, and 7 are each expected with a probability of 2/15, whereas patterns 2, 3, 4, 8, 9, 10, and 11 are each expected with a probability of 1/15.

The results of the analysis of fixed and living cells according to this model are presented in Table III. The null hypothesis of a random arrangement of chromosomes in the radial array is not rejected in either case. As noted by Heneen and Nichols (1972), configurations 1, 5, 6, and 7, as well as 8, have non-superimposable mirror images. As expected, approximately equal numbers of each image were seen (Table IV).

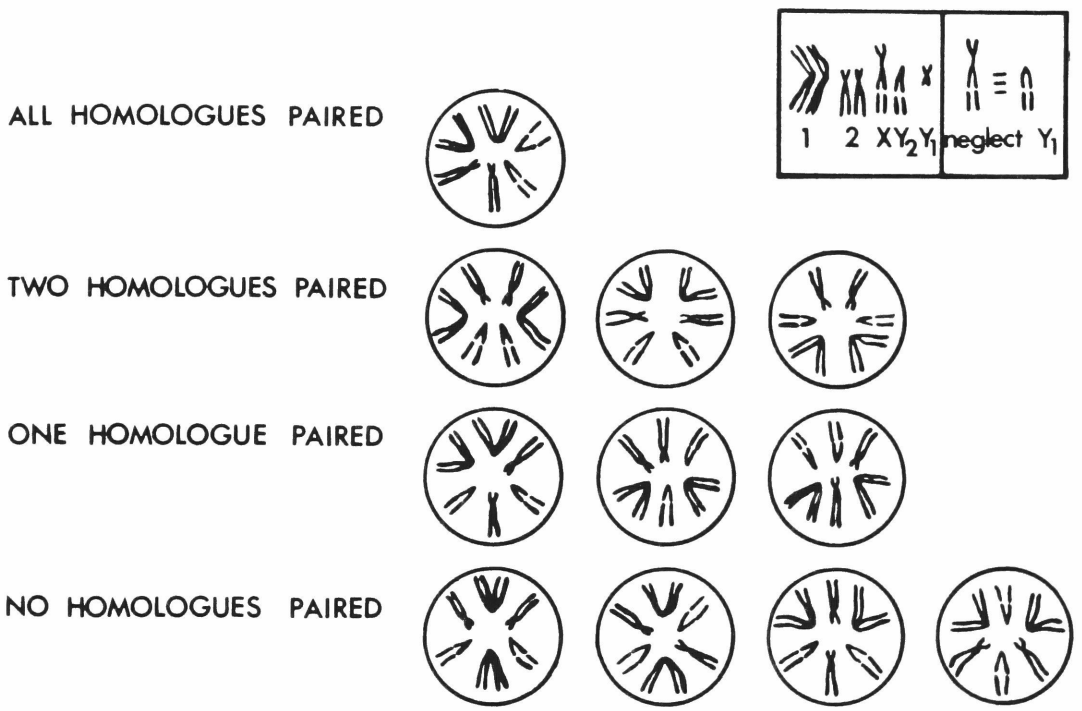


Figure 15. Eleven radial arrangements of three pairs of homologues. The Y_1 chromosome is neglected and the X and Y_2 are treated as homologues.

TABLE III

Observed and expected frequencies of 11 possible types of configurations of 3 pairs of muntjac chromosomes from living and fixed cells.

CLASS		ALL HOMOLOGUES PAIRED	TWO HOMOLOGUES PAIRED			ONE HOMOLOGUE PAIRED			NO HOMOLOGUES PAIRED				TOTALS
TYPE*		2 1 2 1 3 3	3 1 2 1 2 3	2 1 3 1 3 2	1 2 3 2 3 1	2 1 3 1 2 3	1 2 3 2 1 3	1 3 2 3 1 2	1 2 2 3 3 1	1 2 3 3 2 1	2 1 1 3 3 2	3 1 1 2 2 3	
LIVING CELLS	OBSERVED	11	4	4	9	18	11	18	10	6	4	6	101
	EXPECTED	13.5	6.7	6.7	6.7	13.5	13.5	13.5	6.7	6.7	6.7	6.7	100.9
	χ^2	0.46	1.09	1.09	0.79	1.50	0.46	1.50	1.63	0.07	1.09	0.07	9.75
													p>0.70 14df
FIXED CELLS	OBSERVED	85	38	35	41	74	76	64	33	37	46	38	567
	EXPECTED	75.6	37.8	37.8	37.8	75.6	75.6	75.6	37.8	37.8	37.8	37.8	567.0
	χ^2	1.17	0	0.21	0.27	0.03	0	1.78	0.61	0.02	1.78	0	5.87
													p>0.95 14df

* Numbers [1] and [2] correspond to chromosome pairs [1] and [2], respectively. Number [3] corresponds to the X and Y_2 chromosomes.

TABLE IV

Observed and expected number of mirror image configurations among living and fixed cells.

CONFIGURATION	LIVING CELLS$_2$ OBS.**	EXP.	χ^2	p*	FIXED CELLS$_2$ OBS.**	EXP.	χ^2	p*
1 2 / 1 3 / 3 2	6 5	5.5	0.10	>0.70	39 46	42.5	0.58	>0.30
1 3 / 1 2 / 3 2	11 7	9.0	0.88	>0.30	38 36	37.0	0.06	>0.95
2 3 / 2 1 / 3 1	3 8	5.5	1.14	>0.20	38 38	38.0	0	>0.99
3 2 / 3 1 / 2 1	12 6	9.0	2.0	>0.10	31 33	32.0	0.06	>0.95
2 3 / 3 2 / 1 2	1 5	3.0	2.66	>0.10	14 23	18.5	2.18	>0.10

* 1 df

** Observed number of each of the two possible mirror images of the configuration shown at the left.

Role of Mitotic Spindle in Maintaining the Radial Array

The persistence of the radial array after removing chromosomes from the cell suggests that the mitotic spindle might be extracted along with the chromosomes and might be vital in maintaining the array. Two means were employed to test this: 1) chromosomes were removed from cells treated with spindle poisons, and 2) extracted chromosomes were stained with tubulin antiserum.

Extraction of Chromosomes from Cells Treated with Spindle Poisons

In preliminary experiments, it was found that cells incubated for 7 hours with 50 μM colchicine or 0.5 μM vinblastine sulfate displayed no trace of the radial array of chromosomes (figure 16). Rather, the chromosomes were arranged haphazardly, often overlapping one another and with centromeres facing various directions.

The results of extracting chromosomes from 10 colchicine-treated cells and 7 vinblastine sulfate-treated cells are shown in Table V and figure 17. In most cases, individual chromosomes having no connections with other chromosomes could be obtained. The absence of interconnecting fibers was verified by moving the chromosome with a microneedle, which caused no motion of adjacent chromosomes. In addition, groups of 2, 3, or more chromosomes were extracted. Interconnecting fibers among chromosomes within a group were attached to various sites, and were not restricted to the centromeres. These interconnections probably represent adhesions, since the chromosomes overlapped one another in treated cells. Finally, some chromosomes were left behind in treated cells and were not connected to extracted chromosomes. Note that the chromosomes extracted from treated cells display the contraction of chromatids typically seen in metaphase arrested preparations.

Thus, the radial array is maintained by a colchicine- and vinblastine sulfate-sensitive structure which is also necessary for the presence of centromeric interconnections. These drugs bind to tubulin, the principal component of microtubules (Wilson *et al.*, 1974, 1976; Margolis and Wilson, 1977), and inhibit formation of the mitotic spindle (Levan, 1938; Barber and Callan, 1943; Gaulden and Carlson, 1951; Palmer *et al.*,

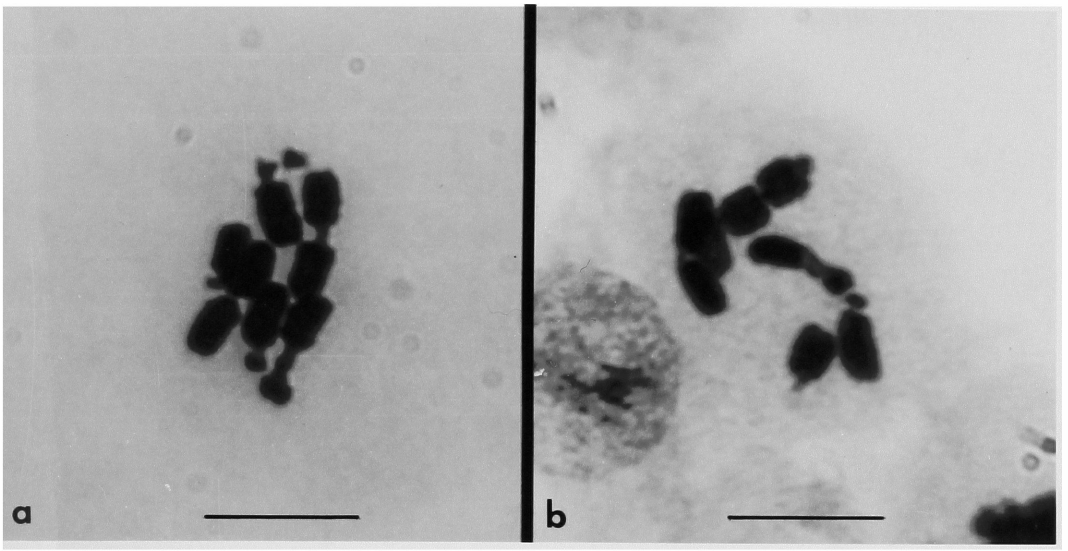


Figure 16. Mitotic cells arrested in 50 μM colchicine (a) or 0.50 μM vinblastine sulfate (b). Cells on glass cover slips were incubated for 7 hours with either drug, fixed with 3:1 methanol:acetic acid, air dried, and stained with Giemsa. Note haphazard arrangement of chromosomes, lack of radial array, overlaps of chromosomes, and contraction of chromatids. Bars, 10 μm.

TABLE V

EXTRACTION OF CHROMOSOMES FROM CELLS TREATED WITH MITOTIC POISONS

DRUG	EXTRACTION NO.	INDIVIDUAL CHROMOSOMES	CHROMOSOME GROUPS	LEFT IN CELL
COLCHICINE	1	5	0	2
	2	2	3	2
	3	1	2 & 3	1
	4	1	2	4
	5	1	2	4
	6	5	0	2
	7	0	6	1
	8	6	0	1
	9	1	4	2
	10	3	0	4
VINBLASTINE SULFATE	1	0	5 & 2	0
	2	1	3	3
	3	0	2 & 2	3
	4	6	0	1
	5	5	2	0
	6	0	6	1
	7	0	6	1

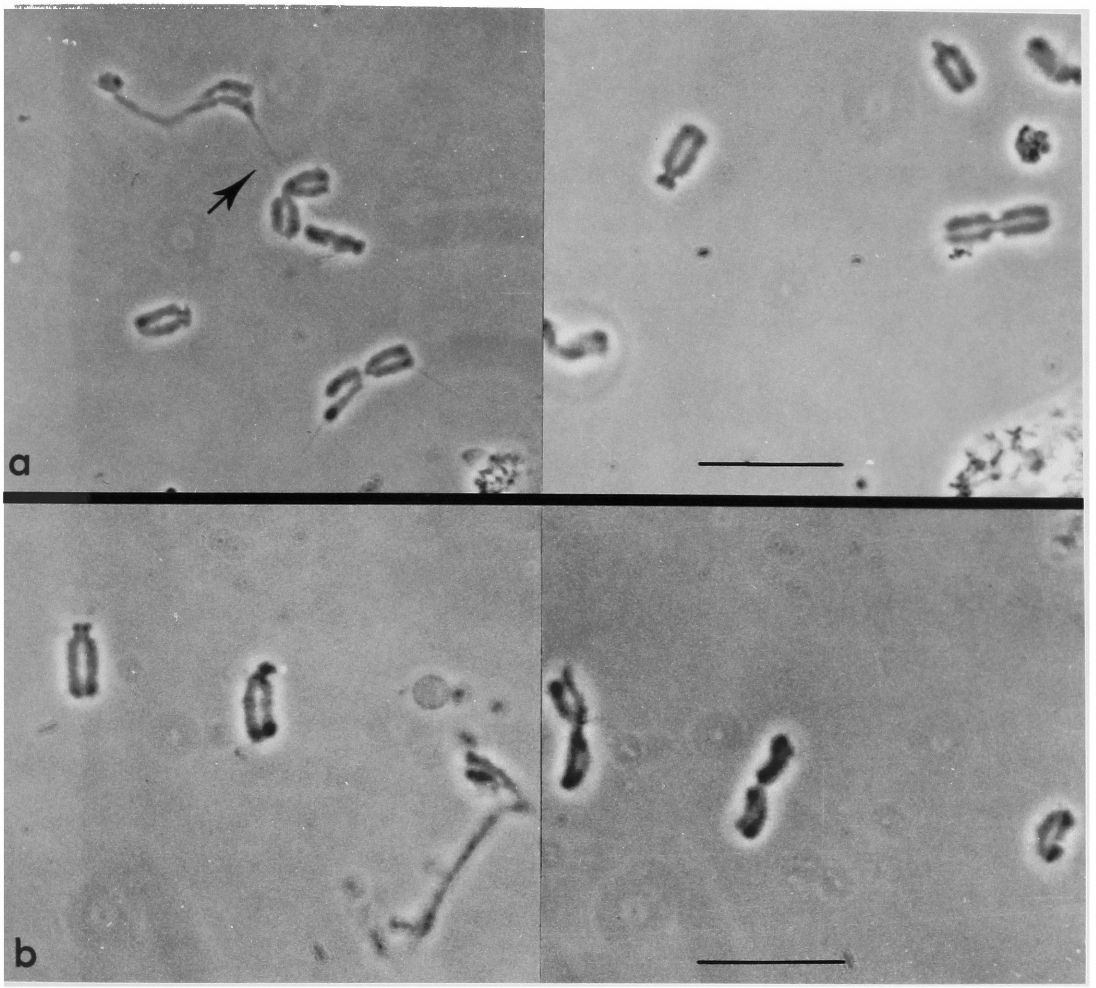


Figure 17. Extracted chromosomes from (a) colchicine- or (b) vinblastine sulfate-treated cells. Some chromosomes were not interconnected to others, while others were interconnected by fibers between regions other than centromeres (arrow). In (b), six of the seven muntjac chromosomes were removed and arranged side by side on the cover slip; lack of interconnecting fibers permitted this manipulation. Note contraction of the chromatids (cf. figure 16). Bars, 10 μm.

1960). While other cell processes are affected by colchicine and vinblastine sulfate, the two drugs have in common the binding of tubulin. Thus it is most likely that the radial array was disrupted due to absence of the mitotic spindle. To strengthen this conclusion, an effort was made to demonstrate the presence of the spindle among extracted chromosomes.

Staining for Tubulin In figure 18, extracted mitoses are shown with phase contrast (18a,c) and fluorescence after staining for tubulin (18b,d). Chromosomes did not fluoresce. However, at the center of each extracted array, a pair of brightly fluorescent structures was seen. Careful focusing revealed that each structure consisted of a pair of bright spots surrounded by fibrous material. The pair of structures often overlapped one another (e.g., 18b), and sometimes contacted a chromosome. However, the bright fluorescence did not fill the center of the radial array and did not contact all of the chromosomes.

The fluorescent structures appear to represent the mitotic spindle. No fluorescence was seen when pre-immune rabbit serum was used in the indirect immunofluorescence reaction in the place of tubulin antiserum. The antiserum was derived from electrophoretically purified tubulin and forms a single band by immunoprecipitation with mouse tubulin. While it is still not certain that the antiserum is monospecific, it specifically stains microtubules (Brinkley et al., 1976) in interphase (figure 19a), the spindle in metaphase cells (figure 19b), and only the pair of structures at the center of an extracted array of chromosomes. Finally, the stained structures have the position and appearance expected for the mitotic spindle.

With phase contrast (figure 20), the spindle appears as a pair of dark spots surrounded by fibers. It was not seen in extracted arrays from colchicine- or vinblastine sulfate-treated cells. Thus, the mitotic spindle is microsurgically removed from cells along with chromosomes, and is probably responsible for maintaining the radial array after extraction of the chromosomes. To determine whether the fibers which connect centromeres of extracted chromosomes to the center of the array ac-

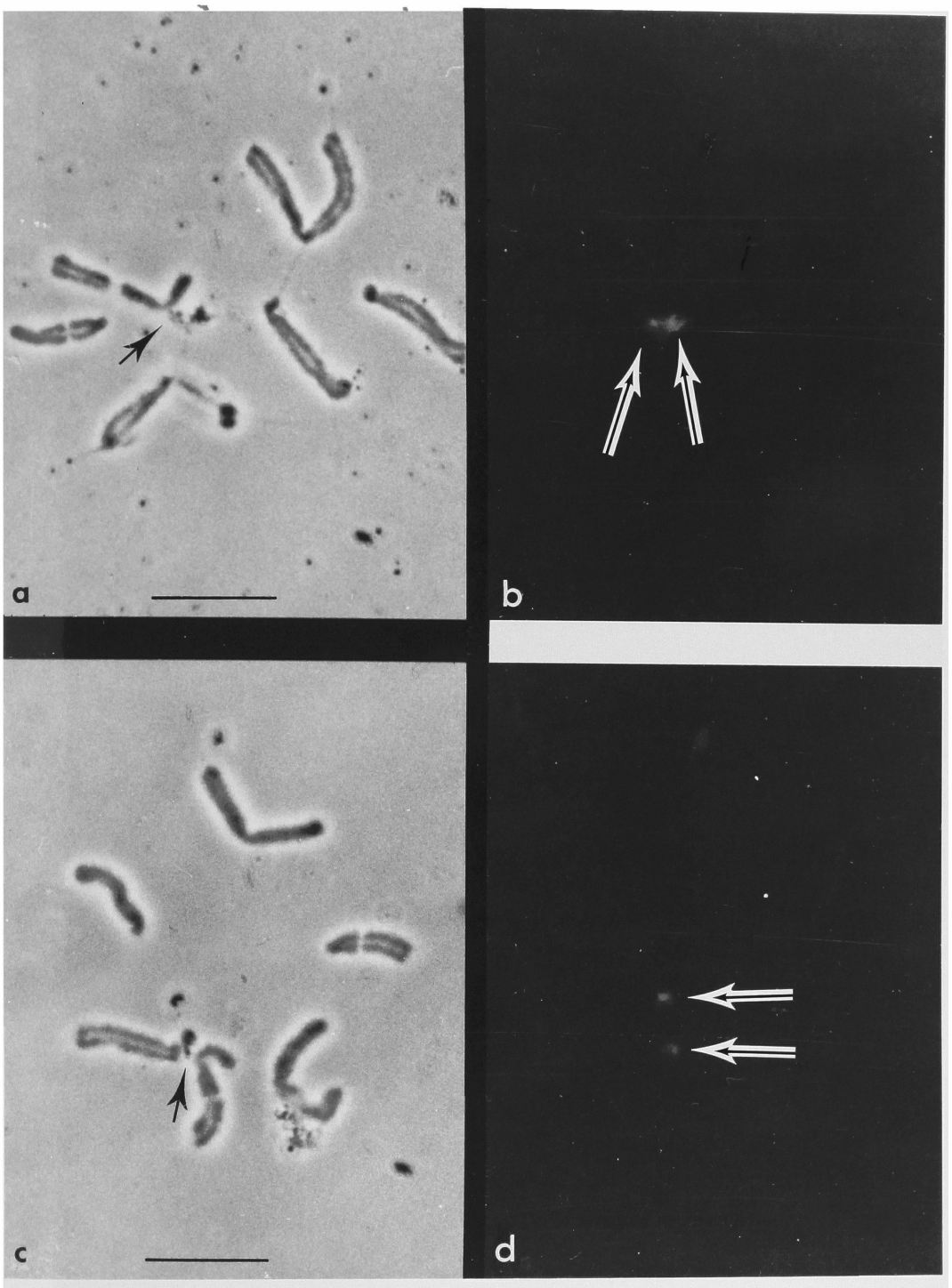


Figure 18. Indirect immunofluorescent staining of extracted chromosomes with tubulin antiserum. Phase contrast micrographs of arrays are shown at left (a,c) and fluorescence micrographs of the same arrays after staining are at right (b,d). The pair of fluorescent structures (white arrows) are the only fluorescent parts of the extracted arrays and are visible as dark regions with phase contrast (black arrows). Bars, 10 μm.

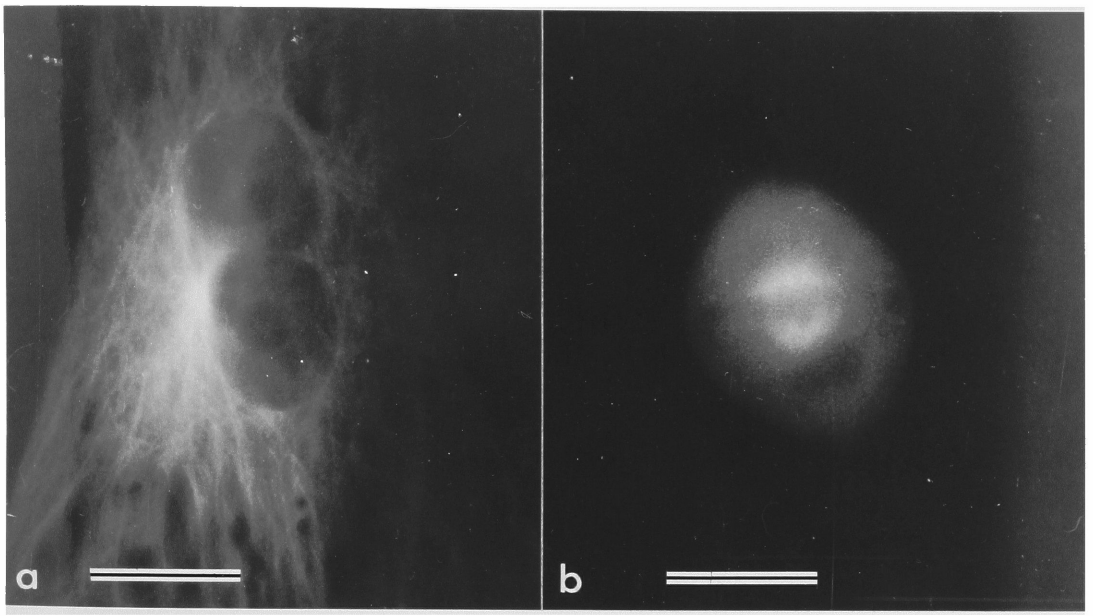


Figure 19. Glutaraldehyde-fixed (a) interphase and (b) metaphase muntjac cells stained with tubulin antiserum. This interphase cell was binucleate. Bars, 10 μm.

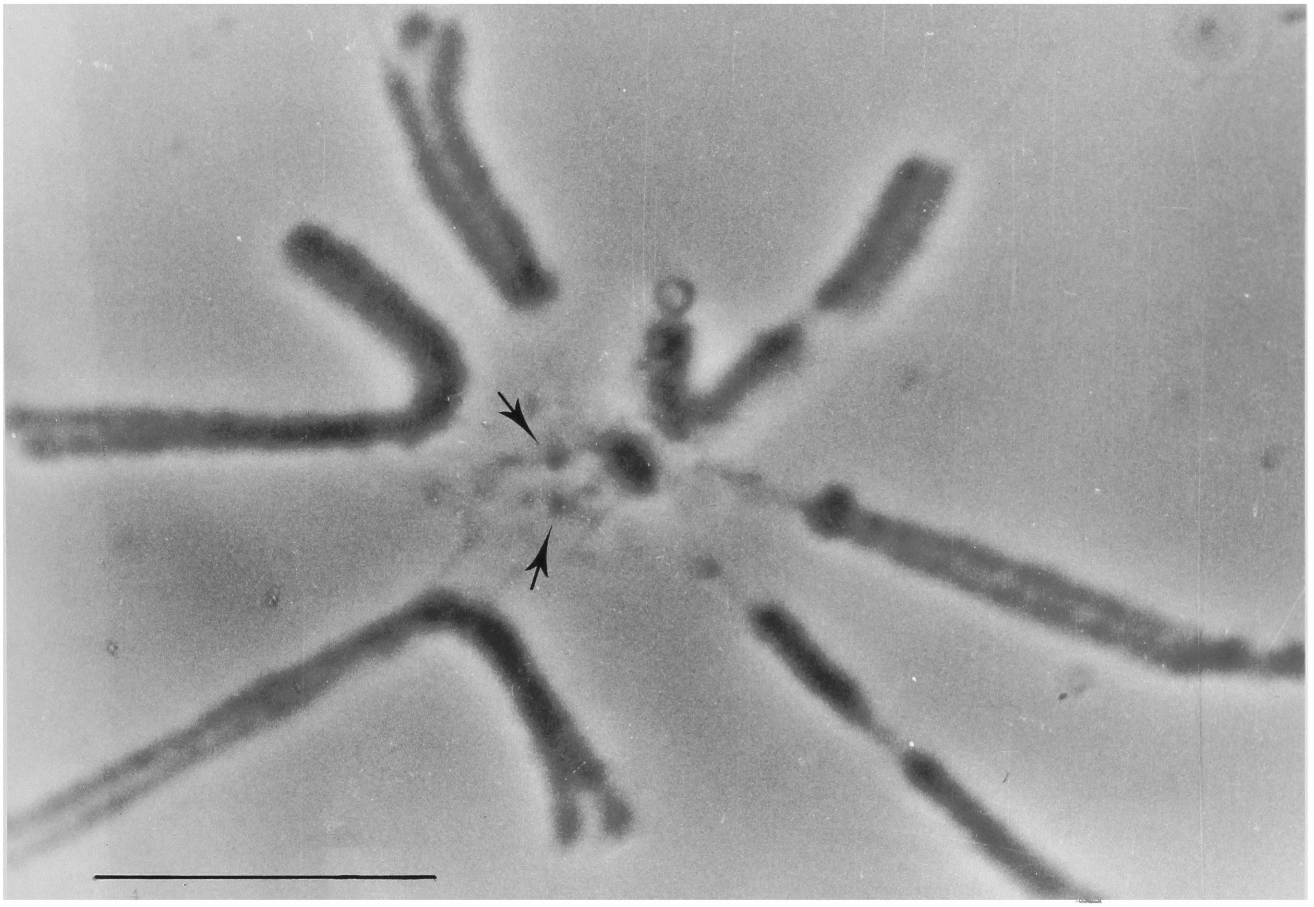


Figure 20. Phase contrast micrograph of extracted chromosomes. At the center of the array is a pair of dark structures enmeshed in fibers (arrows). This probably corresponds with the mitotic spindle. Bar, 10 μm.

tually represent spindle fibers, further information about the chemical nature of chromosome interconnections was sought.

Cytochemical Investigations of Chromosome Interconnections

Staining for DNA The bisbenzimidazole Hoechst 33258 binds to DNA and fluoresces brightly (Lämmler and Schutze, 1961; Hilwig and Gropp, 1972). When glutaraldehyde-fixed extracted chromosomes were stained with Hoechst, both chromosomes and some interconnecting fibers fluoresced (figure 21). The photographs were over-exposed to show the thin interconnecting fibers, and thus the great intensity of chromosome fluorescence. As expected for adhesions, fibers between adjacent chromosome arms fluoresced brightly. The spindle region at the center of the radial array did not fluoresce with Hoechst. However some chromosomes were displaced from the spindle region during micromanipulation, and the fibers which connected these chromosomes to the spindle region were Hoechst-fluorescent.

To verify that Hoechst-fluorescent structures actually contain DNA, cells were grown for one cycle in the presence of BrdU. BrdU in the place of thymidine in one strand of DNA quenches Hoechst fluorescence when a low concentration of dye is used for staining (Latt, 1973; Craig-Holmes and Shaw, 1976). In figure 22, the chromosomes at the top (22a,b) were extracted from a control cell, while those at the bottom (22c,d) were obtained from a BrdU-treated cell. Both preparations were stained with Hoechst 33258 at 0.025 µg/ml, photographed, and printed under identical conditions. All Hoechst fluorescence of chromosomes and interconnecting fibers was quenched in the BrdU-extraction, confirming the DNA-specificity of Hoechst staining. An incidental finding was the occurrence of chromosome gaps in BrdU-treated cells (figure 22c). Although small numbers of cells were examined, the lesions tended to involve the heterochromatic neck of the X chromosome. The fragments were not displaced from the chromosome and were still connected to the spindle.

DNase Treatment DNase I and micrococcal nuclease caused disso-

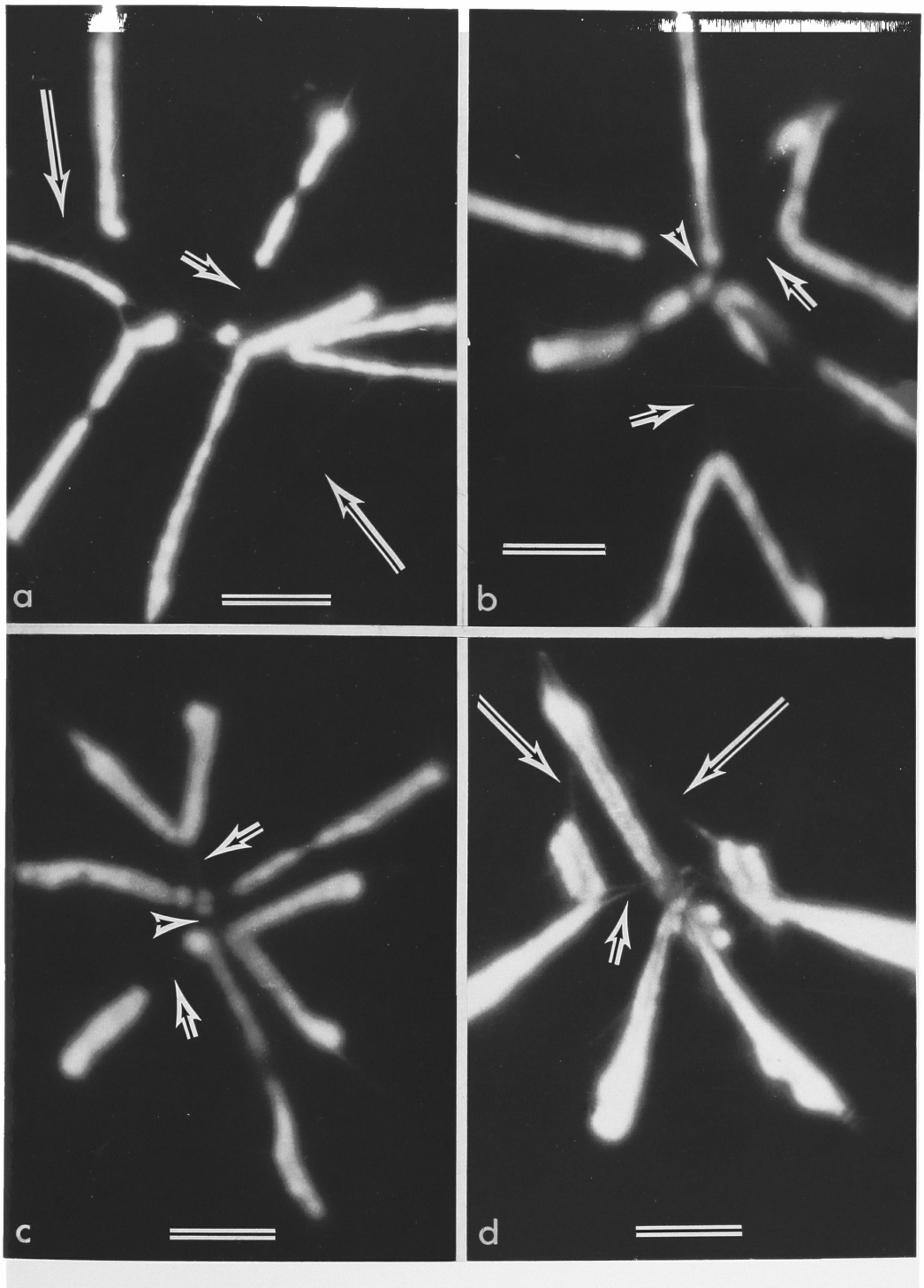


Figure 21. Hoechst 33258 stained extracted chromosomes. Adhesions are brightly fluorescent (long arrows). Chromosomes which have been displaced from the spindle region are connected to it by fluorescent fibers (short arrows). Arrow-heads point to centromeres which are adjacent to the spindle region and, though attached to the region, the connecting fibers are not fluorescent. Bars, 10 μm.

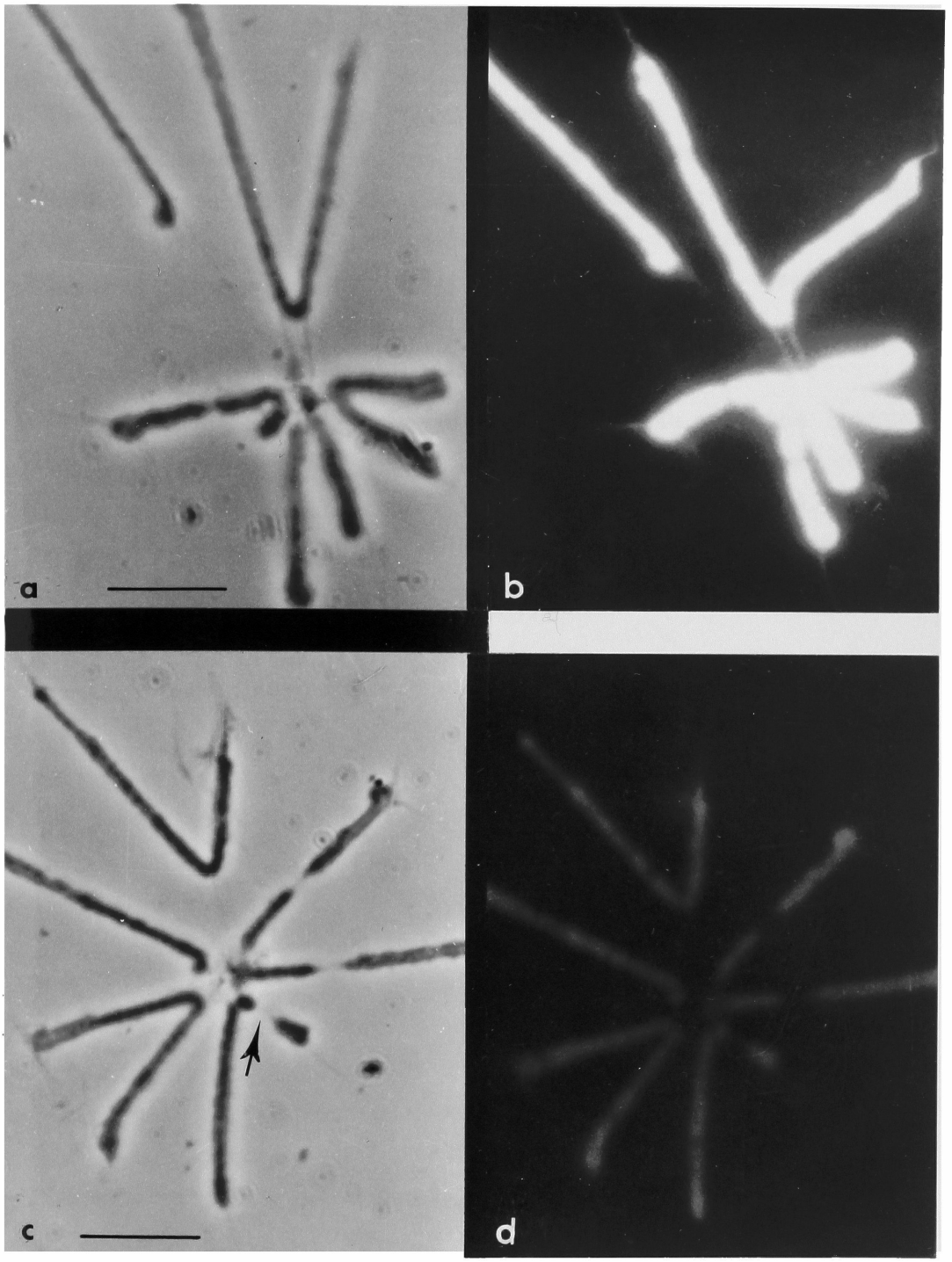


Figure 22. Hoechst staining of control (a,b) and BrdU-treated (c,d) cells. Phase contrast micrographs are shown in (a) and (c), fluorescence micrographs in (b) and (d). Note the gap in the heterochromatic neck of the X chromosome in the BrdU-extraction (arrow). Bar, 10 µm.

lution both of chromosomes and of some interconnecting fibers (figure 23). The reactions were terminated with glutaraldehyde after the effects were clear but before the chromosomes were completely lost from the cover slip. Some fibers had not broken by the time of glutaraldehyde addition. These fibers generally ran between the center of the array and chromosomes which had not been displaced from this region. Breakage of interconnections was verified by staining the enzyme-treated chromosomes with Hoechst 33258 (figure 24). However it was not possible to determine the exact point at which interconnecting fibers broke.

The first chromosome regions to break in DNase were the secondary constrictions of the X and Y_2 (figure 23a). If digestion was continued for several minutes, the chromosome arms entirely dissolved. However, the centromere regions remained visible and were still attached to the spindle region in cases where they were not displaced during micromanipulation (figure 25).

In addition to nuclease activity, DNase I binds to and depolymerizes actin (Mannherz et al., 1975). However, micrococcal nuclease has no effect on actin, yet had an effect equal to that of DNase I on extracted chromosomes. Thus, nuclease activity is probably responsible. Three controls insure that DNase activity was not the result of a protease contaminant. First, a specially purified protease-free DNase I was used (figure 26a,b) and found to dissolve chromosomes and interconnecting fibers in a similar manner to regular DNase I. Second, the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to DNase I and micrococcal nuclease solutions. The presence of 5% ethanol caused contraction of the chromosomes. However, PMSF did not interfere with the ability of deoxyribonucleases to dissolve chromosomes and interconnecting fibers (figure 26c,d). Finally, 1 M hexylene glycol, an alcohol which is often included in chromosome isolation media to stabilize chromosomes (Wray and Stubblefield, 1970), was added to the DNase solution. Again the chromosomes were contracted, but enzyme activity was not impeded (figure 26e,f). Still, it is possible that non-serine proteases, perhaps secreted by the cells, were present in the microsurgical chambers. Experiments with purified protease and RNase A provide

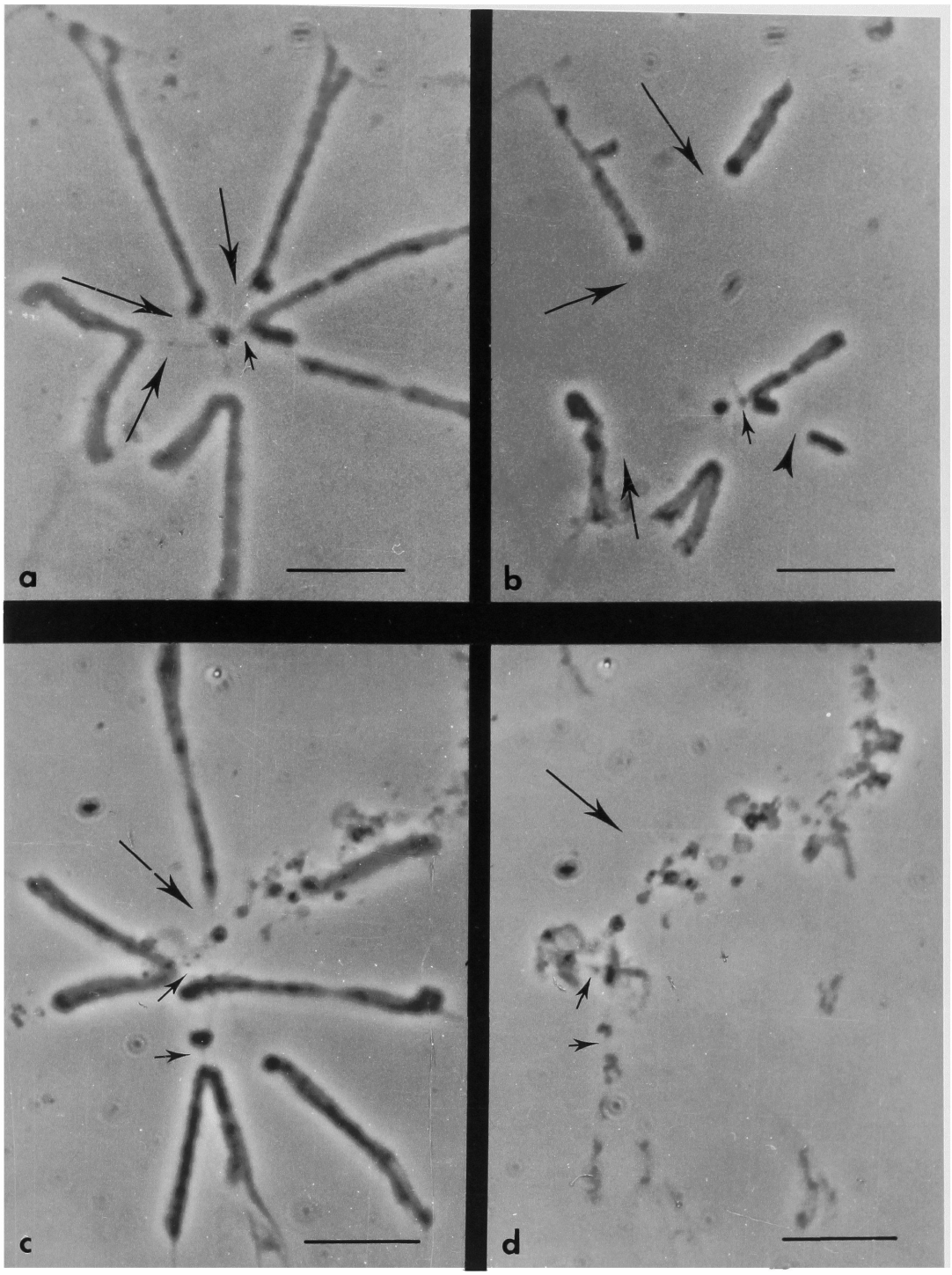


Figure 23. DNase I (a, before, b, after treatment) and micrococcal nuclease (c, before, d, after treatment) treatment of unfixed extracted chromosomes. Sites where interconnections have broken are indicated by long arrows, while chromosomes remaining attached to the center of the array are indicated by short arrows. The arrow head in (b) indicates the severed secondary constriction of the Y_2 chromosome. Bars, 10 μm.

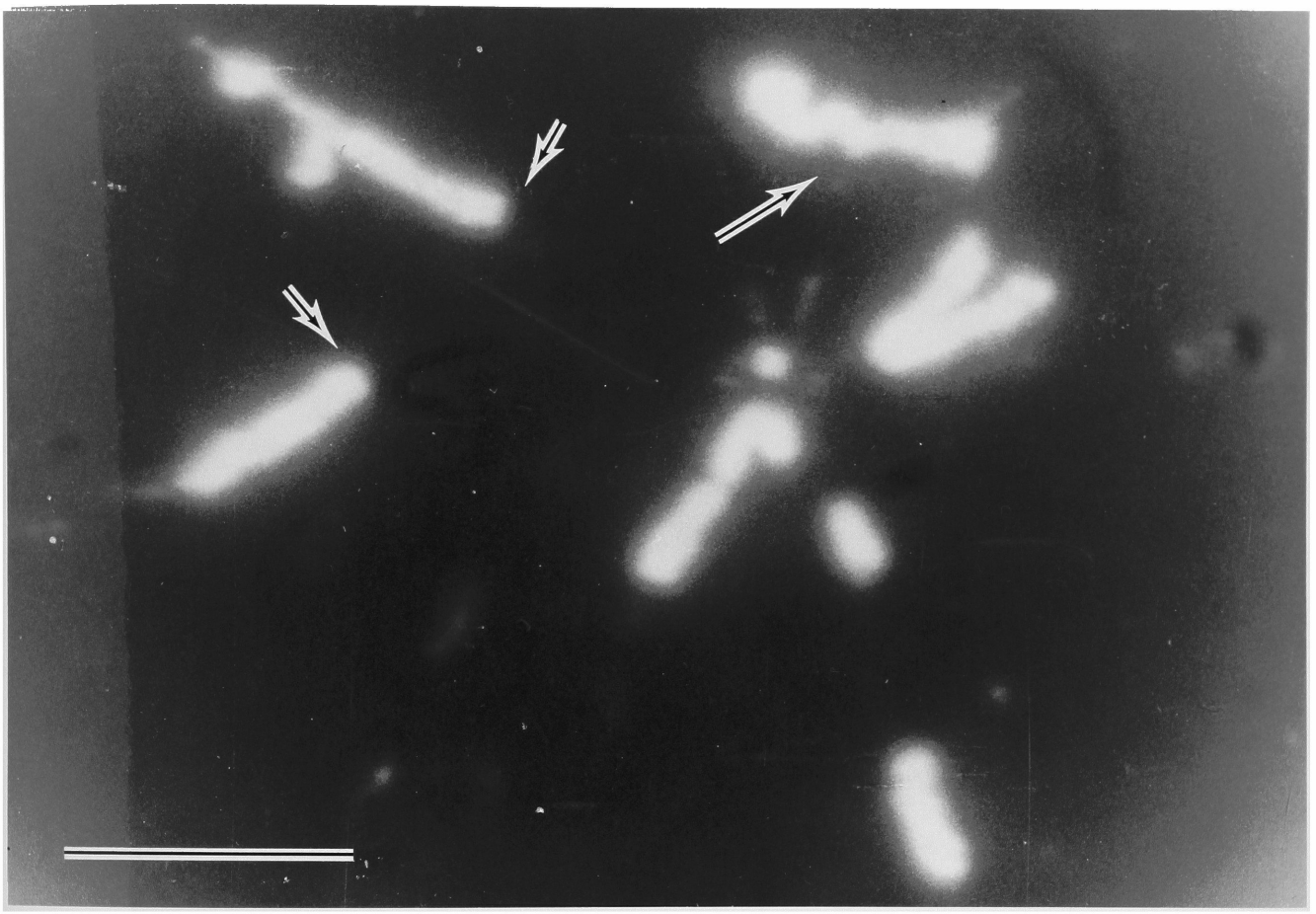


Figure 24. Hoechst 33258 fluorescence of the DNase I treated array shown in figure 23a,b. Chromosomes no longer connected to the center of the array by fluorescent fibers indicated (arrows). Bar, 10 μm.

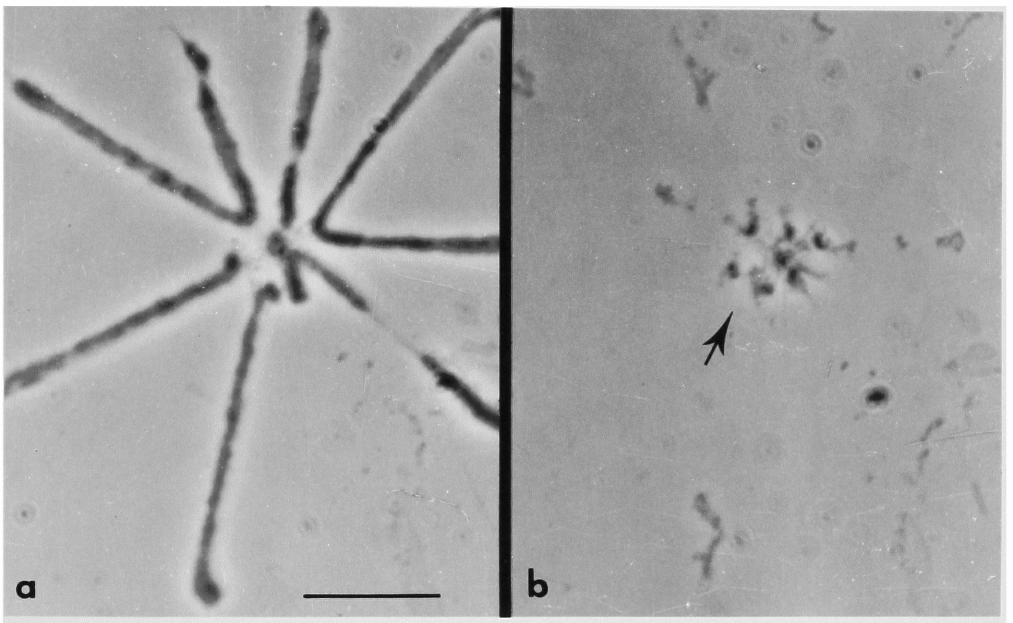


Figure 25. Extracted metaphase before (a) and after five minutes of treatment with micrococcal nuclease (b). In (b), centromeres are clustered around the center of the array and still connected to it (arrow). Bar, 10 µm.

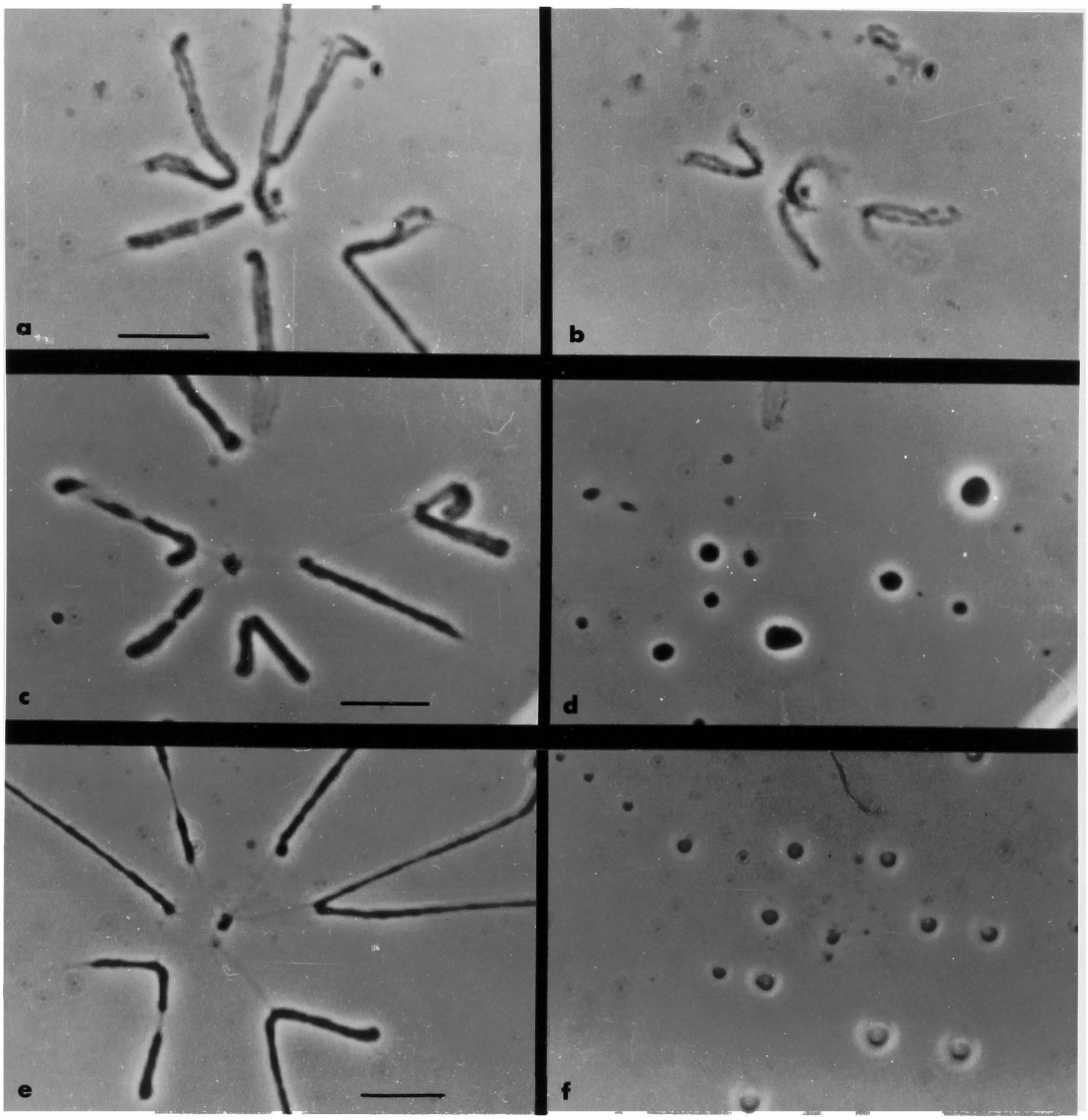


Figure 26. (a,b) Treatment of chromosomes with protease-free DNase (a, before, b, after); (c,d) treatment with DNase I containing 2 mM PMSF and 5% ethanol (c, before, d, after). The ethanol induced contraction of the chromosomes, leading to the dense globules representing remnants of chromosomes after digestion was terminated; (e,f) treatment with DNase I with 1 M hexylene glycol. Hexylene glycol was added to MSM before addition of DNase (e), leading to contraction of chromosomes. Bars, 10 μm.

evidence that such contaminating proteases, or possible RNase activity were not responsible for the effects of DNase.

Protease Treatment Shortly after the addition of trypsin or pronase, the distinctness of chromosomes as seen with phase contrast decreased (figure 27). Eventually the chromosomes appeared as only faint outlines, except for centromeric regions, which remained dark. Therefore, it was not possible to determine if interconnecting fibers were broken by protease. However, in contrast to DNase-treated preparations, these chromosomes did not suddenly snap away from the radial array, indicating breakage of an interconnecting fiber. Thus, while it is unclear whether interconnecting fibers are sensitive to protease, it is clear that the activity of DNase is different from that of protease.

RNase Treatment Incubation of chromosomes for one hour in RNase A produced no visible effect (figure 28). Chromosome structure remained intact, and interconnecting fibers did not break. To insure that the RNase was active, cells were torn with a microneedle to render them permeable to the enzyme, exposed to RNase, and then stained with acridine orange. The acridine orange staining pattern of a intact cell is shown in figure 29a. Green fluorescence in the nucleus is due to DNA, while red fluorescence of the nucleoli and in the cytoplasm is largely attributable to RNA (Rigler, 1966). In control cells which were torn and then incubated for one hour in MSM at 37°C, red staining was less intense but still present (figure 29b). However, in torn cells incubated with RNase, all red fluorescence was lost (figure 29c), showing that the RNase was active. Thus, the effects of DNase are due to DNase activity, not protease or RNase activity.

Staining for Histone The presence of protein in chromosome interconnections was demonstrated by staining for histones H1 and H2B. Indirect immunofluorescent staining patterns are shown in figure 30. Chromosome fluorescence appears intense in photographs which were intentionally over-exposed to demonstrate the interconnecting fibers. Adhesions between chromosome arms are stained, as are fibers running from centromeres which were displaced from the center of the array. Staining

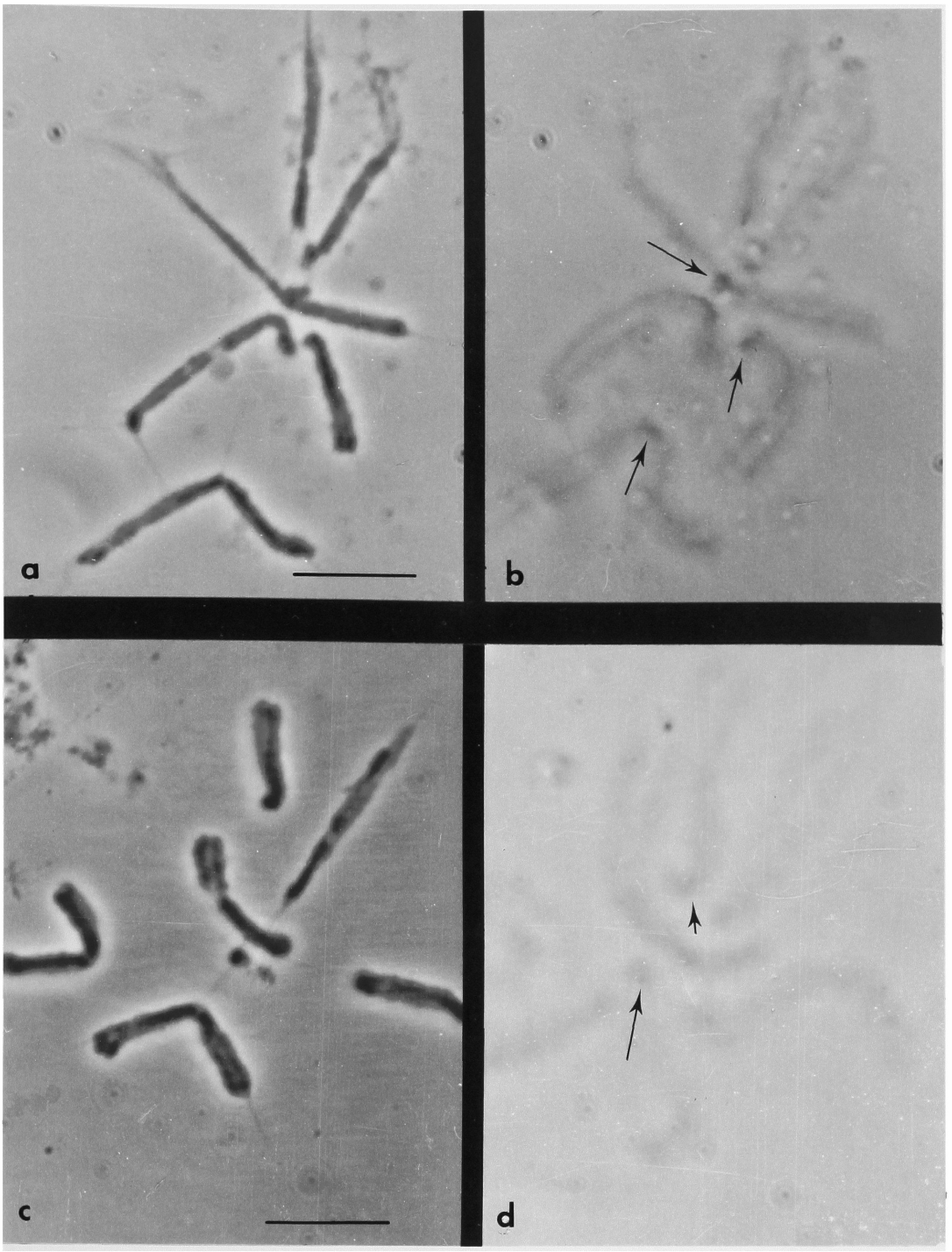


Figure 27. Trypsin (a, before, b, after) and pronase (c, before, d after) treatment of unfixed extracted chromosomes. Dark centromere regions remaining after digestion indicated by arrows. Note that the chromosomes became less visible, but are structurally intact after treatments. Bars, 10 μm.

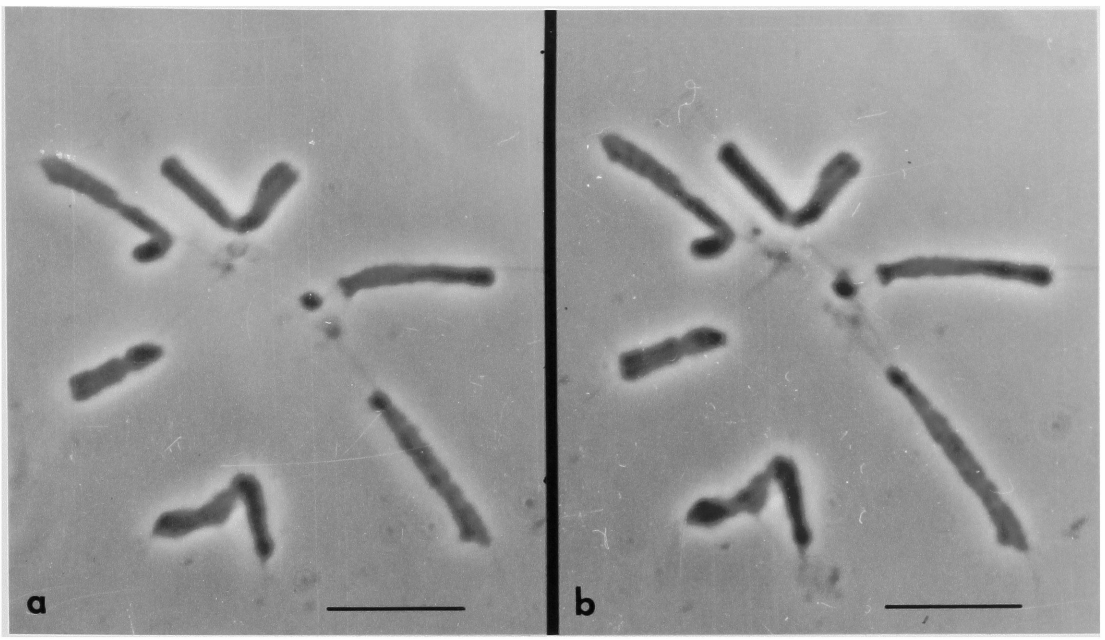


Figure 28. Phase contrast micrographs of extracted chromosomes before (a) and after (b) RNase treatment. Bars, 10 μm.

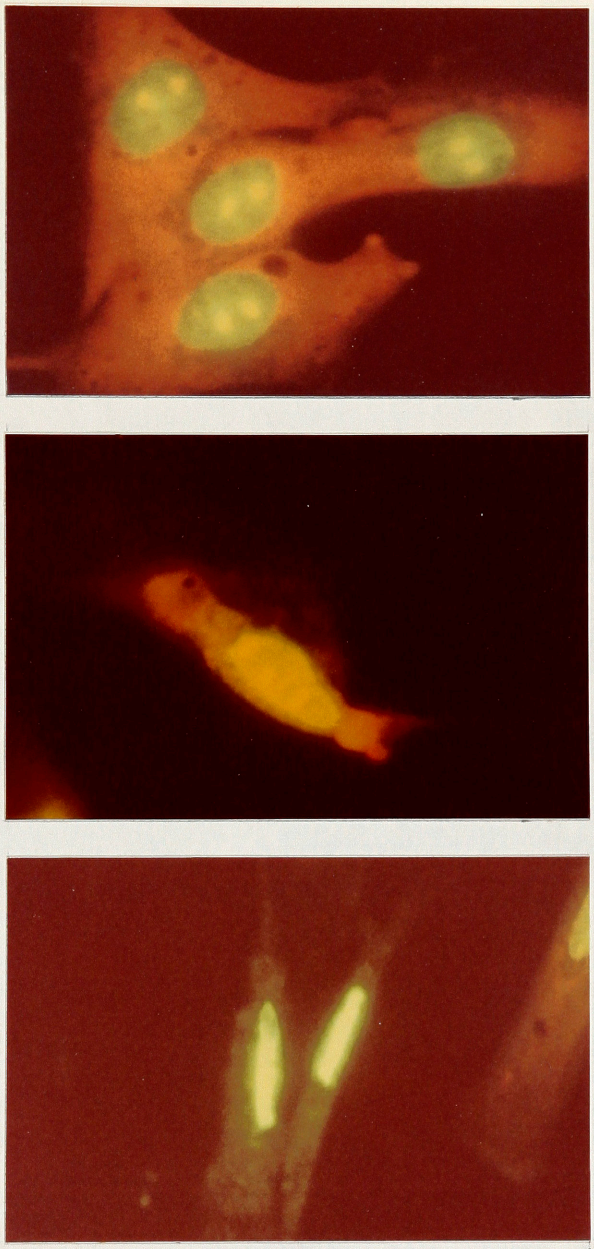


Figure 29. Fluorescence micrographs of acridine orange stained cells. (a) Intact cell; (b) cell torn with microneedle and incubated in MSM for one hour; (c) torn cell incubated with RNase.

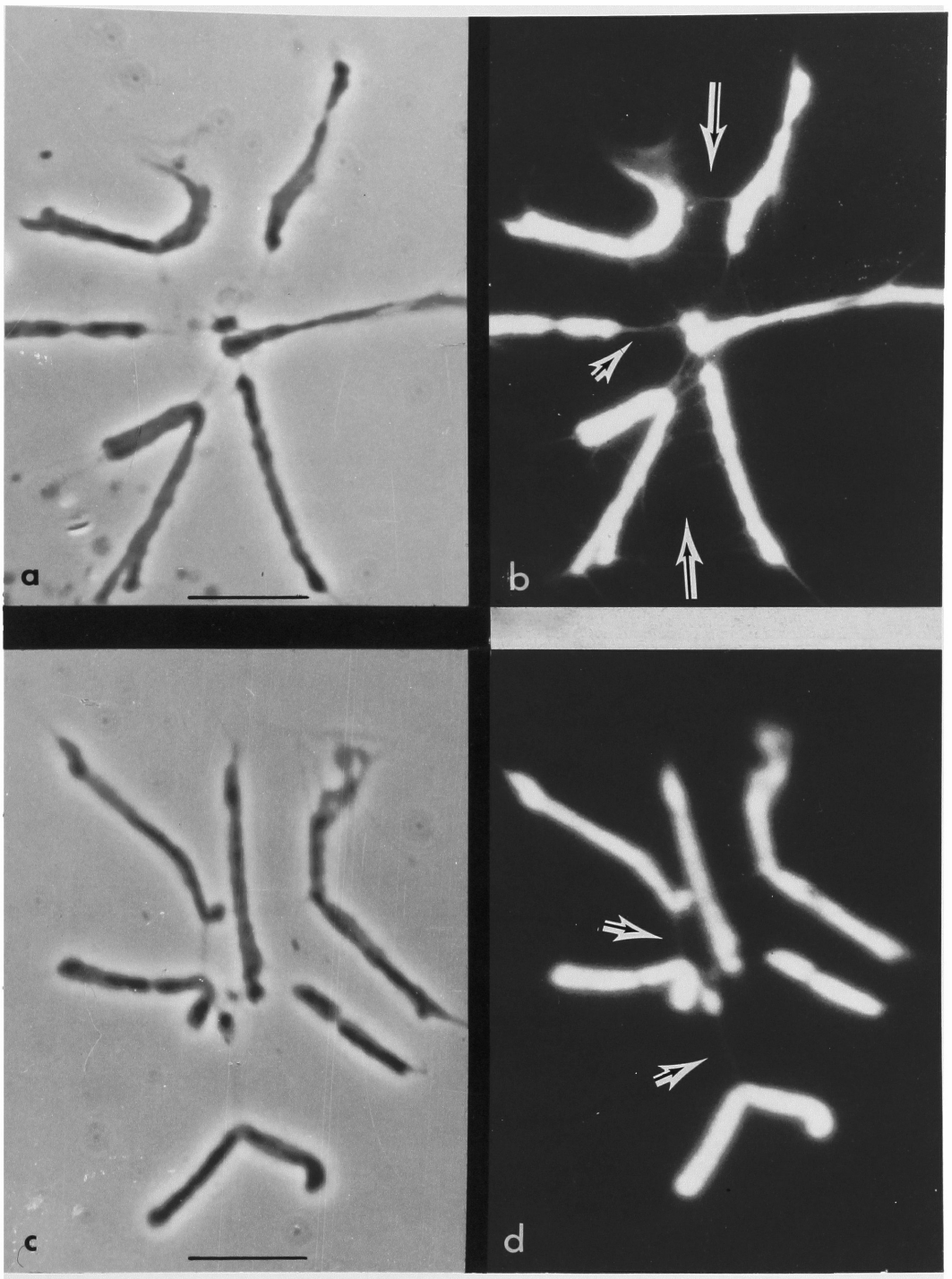


Figure 30. Extracted chromosomes stained with antiserum to histone H1 (b) and H2B (d). Corresponding phase contrast images shown in (a) and (c). Adhesions (long arrows) and centromeric interconnections (short arrows) are indicated. Bars, 10 μm.

with pre-immune rabbit serum produced no fluorescence, either of chromosomes or interconnections.

The specificity of histone antisera was confirmed by staining methanol fixed interphase cells with antisera to H1 or H2B which had been absorbed with purified H1 or H2B. As shown in figure 31, absorption of histone antisera with the appropriate histone type, and that type only, caused a marked reduction in fluorescent staining of interphase nuclei.

Absence of Hoechst-Fluorescent Interconnections in Metaphase Cells

The results presented so far confirm that interconnections between arms of adjacent chromosomes are adhesions, since they contain DNA and histone as chromosome fibers. The presence of the spindle is necessary for the radial array and for centromeric interconnections. Yet it is unclear if the centromeric interconnections are actually part of the spindle, part of the chromosomes, or a separate structure.

Since Hoechst 33258 clearly stains interconnecting fibers in extracted metaphase arrays, an attempt was made to visualize such fibers *in situ*. Cells on cover slips were fixed with glutaraldehyde and stained with Hoechst 33258. The mitotic cells which were oriented so that the radial array was visible were examined. No centromeric interconnections were seen in over 25 such cells (figure 32). In addition, metaphase cells were collected by mitotic shake-off, treated with hypotonic solution, fixed with 3:1 methanol:acetic acid, spread on slides, air dried, and stained with acridine orange. In this case, too, DNA-containing interconnecting fibers were never seen in over 100 cells examined, even though the radial arrays were intact.

DNA-containing fibers running between centromeres and the spindle region are thus only seen when microsurgically extracted chromosomes were displaced from the spindle region. Similarly, only after such displacement is the connection of the chromosome to the spindle region sensitive to DNase. DNA-containing fibers are not found connecting chromosomes to the spindle *in situ*. Two possible explanations for the origin

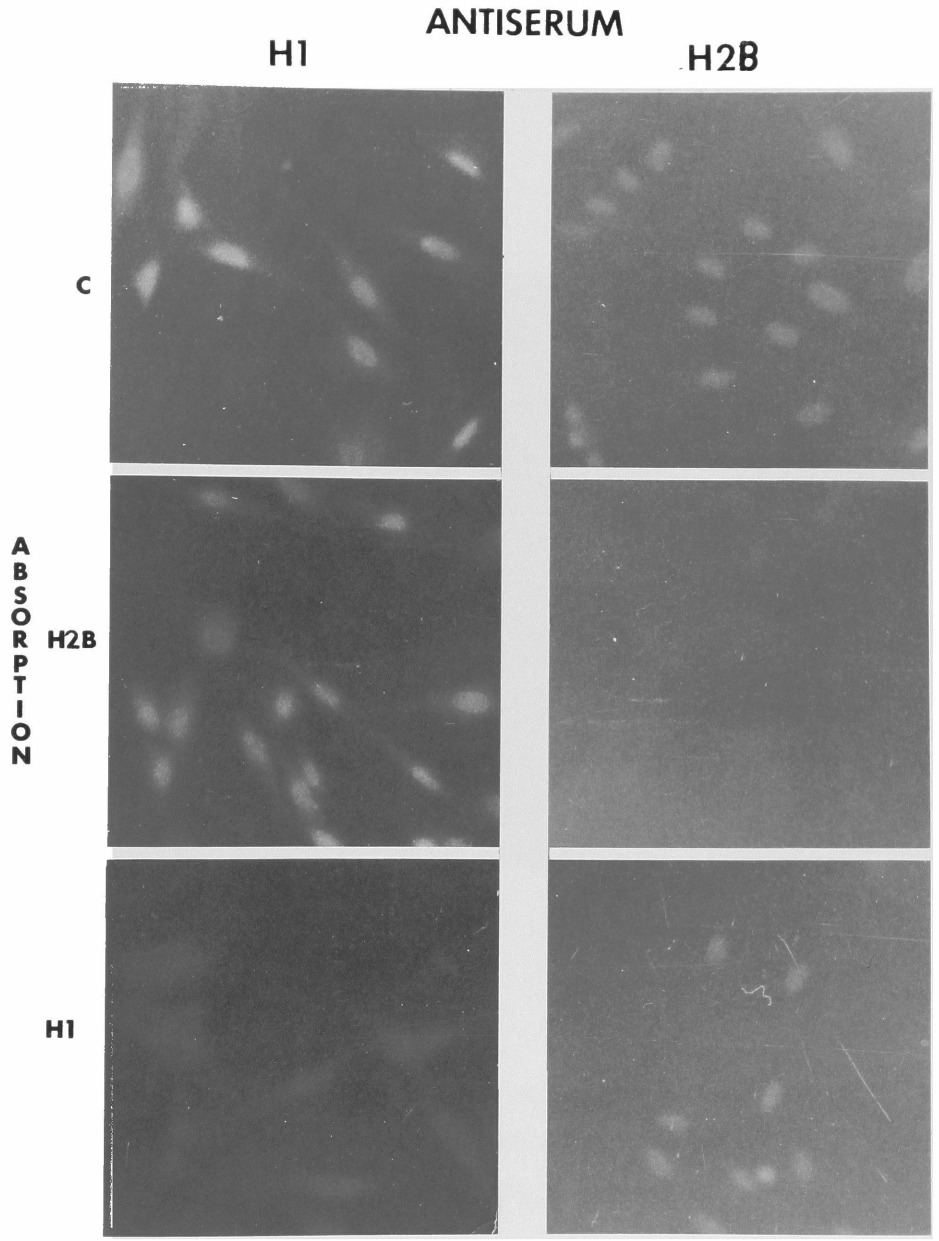


Figure 31. Absorption of histone antisera with purified histone. Pictures at left taken of cells stained with H1 antiserum, at right with H2B. Sera were unabsorbed (c, top pair), or absorbed with histone H1 (middle) or H2B (bottom). Fluorescence with H2B antiserum was generally less bright than with H1 antiserum.

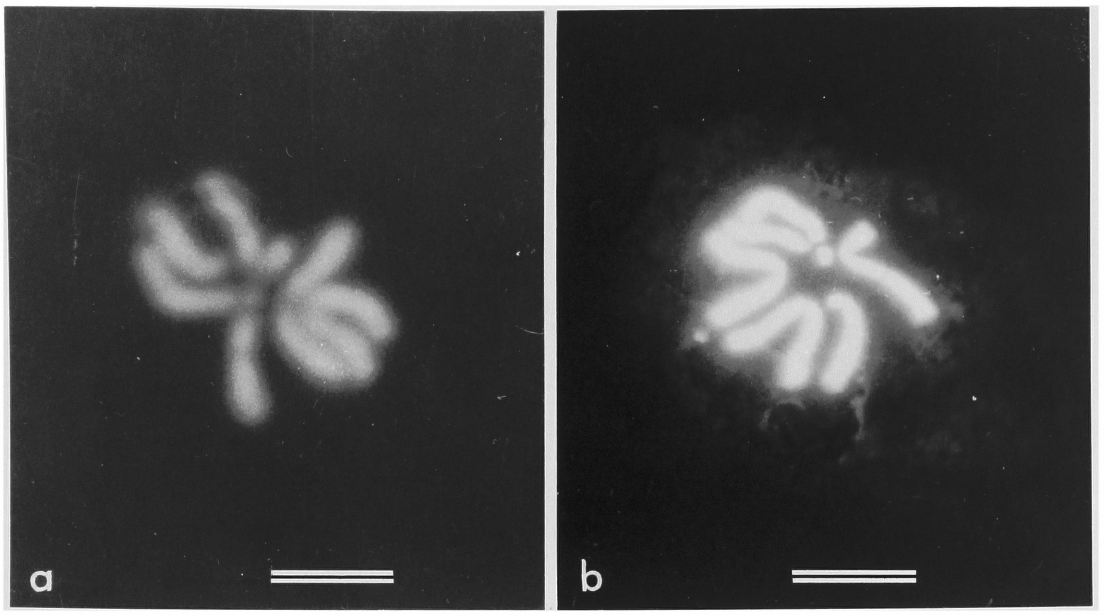


Figure 32. (a) Glutaraldehyde-fixed metaphase stained with Hoechst 33258; (b) methanol:acetic acid-fixed metaphase stained with acridine orange. No connections are seen between centromeres. Bars, 10 μm.

of DNA-containing centromeric fibers are: 1) adhesions are formed between adjacent centromeres during micromanipulation; or 2) DNA-containing fibers attaching the chromosome to the spindle are generated by stretching the chromosome when it is being pulled.

Staining with Hoechst 33258 and Tubulin Antiserum

To resolve whether DNA-containing interconnecting fibers run between adjacent centromeres or between centromeres and the spindle, glutaraldehyde-fixed extracted chromosomes were stained sequentially with tubulin antiserum and Hoechst 33258. Micrographs were first taken at the fluorescein isothiocyanate excitation wavelength (480 nm), and then at the Hoechst 33258 excitation wavelength (365 nm). Background fluorescence at these two wavelengths was negligible. As shown in figure 33, some chromosomes are connected directly to the spindle. However, when chromosomes have been pulled away from the spindle, at least in some cases, a DNA-containing fiber extends from the chromosome to the spindle. In other cases, centromeric fibers do connect adjacent chromosomes and are probably artifacts. Nine sets of extracted chromosomes were examined in this way and apparent continuity between spindle and DNA fibers was seen in six cases. Of course, the resolution of fluorescence microscopy does not permit determination of whether the two types of fibers were actually connected.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) provides a high resolution view of chromosome interconnections, which are near the limit of light microscopic resolution. An extracted metaphase is shown in figure 34 with SEM (34a) and with phase contrast (34b). The spindle region, identified on the phase contrast view, appears to be a fibrous network as seen with SEM. Most of the chromosomes are connected to the spindle by fibers attached to the centromere regions. In one case, the fiber became entangled with another chromosome. It is not known whether these interconnecting fibers contained DNA. Yet it is clear that the only fibers run-

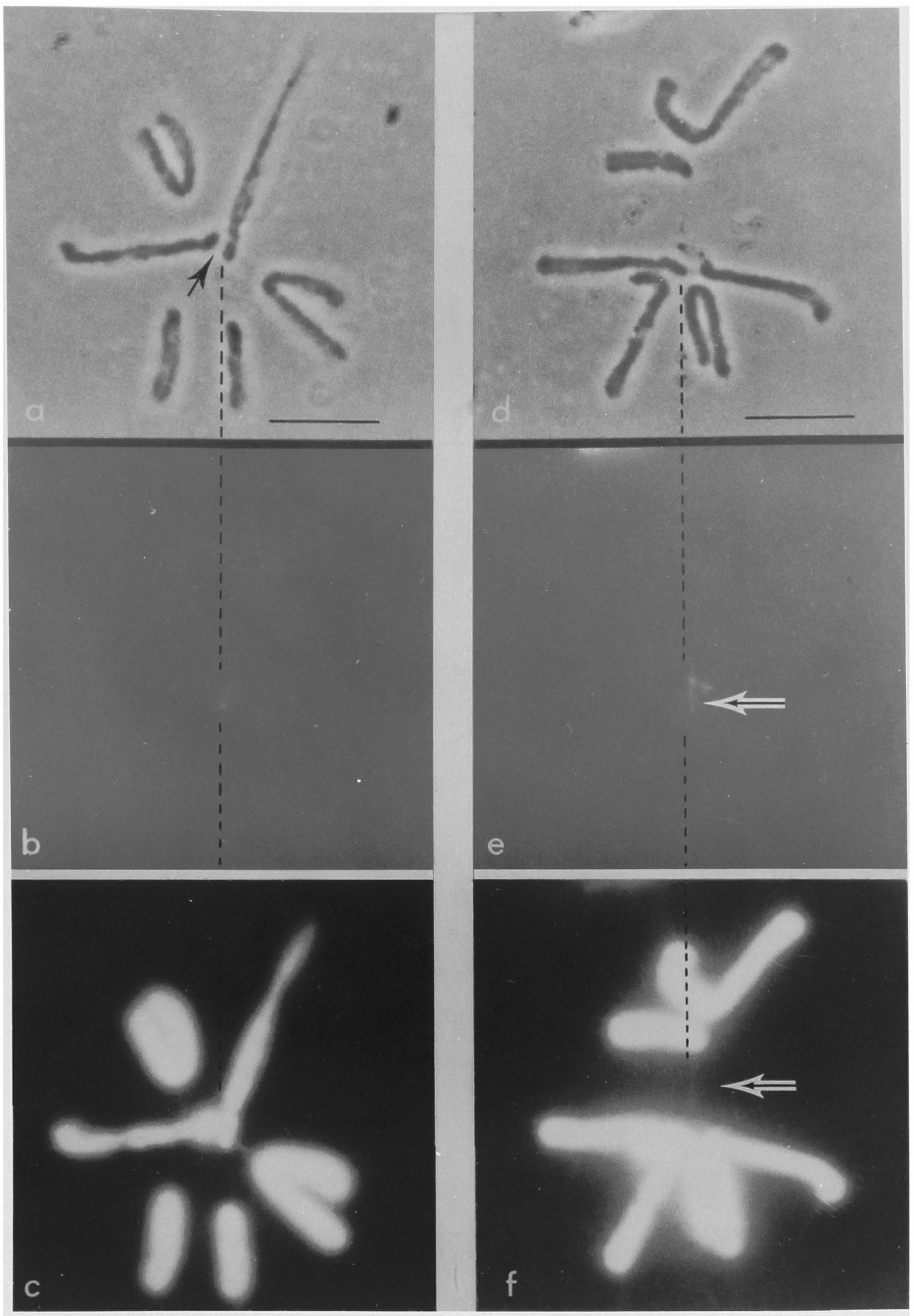


Figure 33. Two extracted arrays stained with tubulin antiserum (b,e) and Hoechst 33258 (c,f). Dashed line provides reference for comparing the pictures. In the left hand set (a,b,c), Hoechst-fluorescent fibers do not appear to contact the spindle. The arrow in (a) points to two chromosomes with centromeres contacting the spindle. At the right (d,e,f), the fibers indicated by the arrows coincide in the Hoechst and tubulin-stained images. Bars, 10 μm.

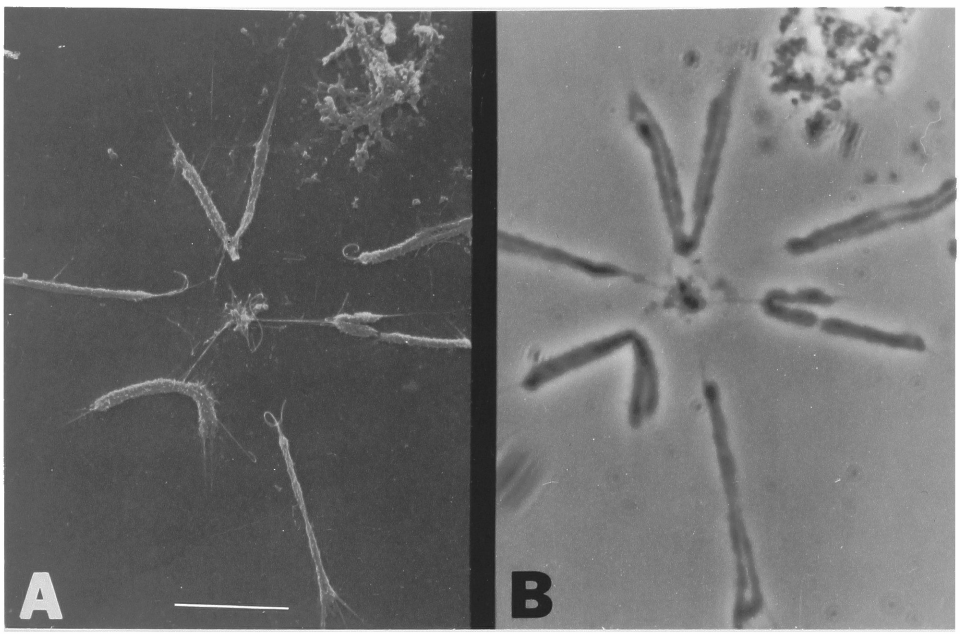


Figure 34. (a) Scanning electron micrograph of extracted metaphase showing chromosomes attached by fibers to the spindle region at the center of the array. Some fibers have broken during preparation. (b) Corresponding phase contrast micrograph. Bar, 10 μm.

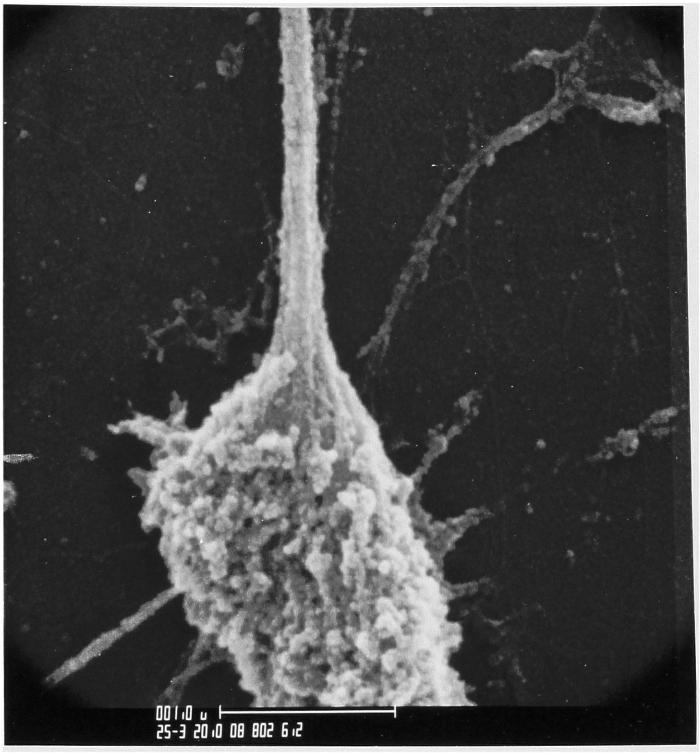
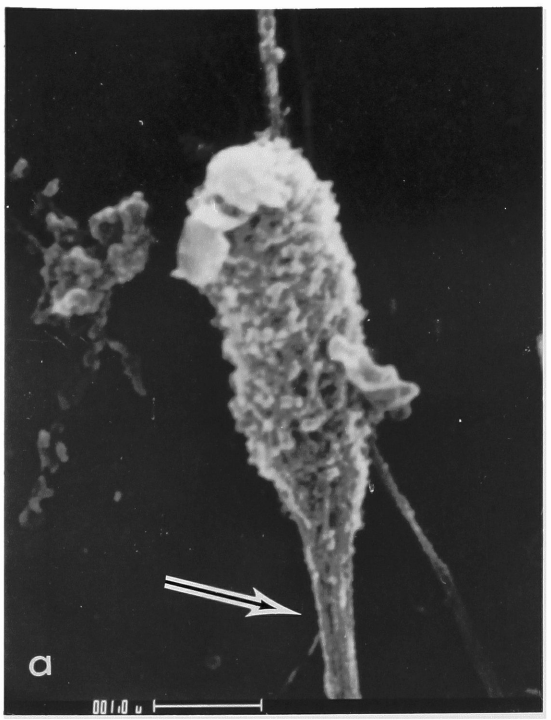


Figure 35. Scanning electron micrograph of interconnecting fiber attached to centromere region of Y_2 chromosome. The surface of the chromosome consists of looping fibers which appear to be continuous with the interconnecting fiber. The interconnecting fiber is multi-stranded. Bar, 1.0 μm.

ning from the centromeres attached the chromosomes to the spindle region.

A higher magnification scanning electron micrograph of a centromere region with interconnecting fibers is shown in figure 35. Interconnecting fibers appear to be continuous with chromosome fibers and often are multi-stranded. This is consistent with their being extensions of chromosome fibers due to stretching or adhesion. No effort was made to measure fiber diameters due to uncertainty of the thickness of the gold coating on the specimens.

The surface of the chromosomes appears to consist of looping fibers (figure 36). An exception to this morphology is the secondary constriction of the X and Y_2 chromosomes. Here, only a small number of parallel fibers was seen (figure 36b). This is best appreciated in chromosomes which were stretched during micromanipulation, but is apparent in unstretched chromosomes as well. Centromeres also consist of parallel fibers, but, in this case, the packing of the fibers is very tight (figure 36a).



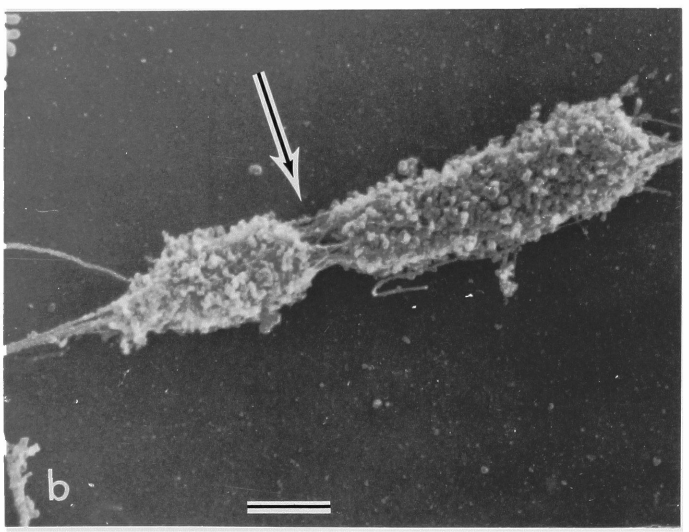


Figure 36. (a) Centromere region (arrow) and short arm of X chromosome viewed with SEM. Note fibrous chromosome surface and tightly packed centromere fibers; (b) Y_2 chromosome, showing loosely packed longitudinal fibers at secondary constriction (arrow). Bars, 1.0 µm.

V. DISCUSSION

Arrangement of Chromosomes at Metaphase

Radial Array Chromosomes of the Indian muntjac are arranged in a radial array on the mitotic spindle. All centromeres are clustered at the center, with the arms pointing outward. The small Y_1 chromosome is also located at the center of the array. A similar configuration was noted in the Indian muntjac by Heneen and Nichols (1972).

The radial array is maintained by the mitotic spindle both within the cell and among the microsurgically extracted chromosomes. Cells treated during interphase with the spindle poisons colchicine or vinblastine sulfate never developed a radial array. Rather, chromosomes in these cells were arranged haphazardly and could be removed individually from the cells. On the other hand, when chromosomes were removed from cells with intact spindles, the radial array was maintained even outside the cells, and could be seen at the center of the array by fluorescent staining with tubulin antiserum. The term "mitotic spindle" is used here to refer to a colchicine and vinblastine sulfate-sensitive structure that binds tubulin antiserum. The exact relation of this structure to the mechanism of chromosome movement at anaphase is unknown. It would be interesting to attempt to stimulate the separation and movement of extracted chromosomes. Forer *et al.* (1977) have provided evidence that the isolated mitotic apparatus of sea urchin zygotes is able to complete division if injected into sea urchin eggs. It is not known why division does not proceed *in vitro*, but the ability to precisely control the chemical environment of microsurgically isolated chromosomes might permit the obstacle to be overcome.

The role of the spindle in maintaining the radial array must be borne in mind when analyzing the positions of chromosomes at mitosis. In cells with intact spindles these positions are determined by the spindle and do not necessarily reflect the distribution of chromosomes during interphase. On the other hand, in cells treated with spindle poisons, interphase positions are more likely to be preserved (unless these, too, are dependent on microtubules). Rodman *et al.* (personal

communication) have recently shown that in human cells certain chromosome associations, such as between acrocentrics, are stable to colcemid treatment, while others, such as the tendency for larger chromosomes to be at the periphery of metaphase spreads, are labile. Similarly, in muntjac cells Cohen et al. (1972) employed colcemid and found that chromosomes were randomly arranged, while Heneen and Nichols (1972) did not use a spindle poison and analyzed radial arrays.

Position of Chromosomes in the Radial Array Analysis of both live cells and fixed metaphases has revealed that chromosomes are positioned at random in the radial array. The use of mitotic shake-off enabled the examination of a large number of radial metaphases, but the preparation procedure is subject to the criticism of possible distortion of metaphases. Treatment of these cells with hypotonic solution was necessary to obtain clear distinction of individual chromosomes and did not disrupt the radial array. That hypotonic treatment does not change the position of chromosomes in the radial array is evidenced by similar results of random positioning of chromosomes obtained with fixed cells and with living cells. The latter are assumed to be minimally disrupted. It should be noted that there is no internal rearrangement or movement of chromosomes when the cell is oriented by microneedles.

Another potential criticism of this work is possible bias in the selection of radial arrays for analysis. Among fixed cells, it seems unlikely that a radial configuration of a particular type would be especially prone to disruption so that its frequency would be decreased to agree so nearly with random expectation as was found. And, again, the data from living cells, which are not subject to this possible bias, support the validity of the analysis. The main limitations in the data from the living cells are due to cases in which chromosomes could not be unequivocally identified or in which overlap of chromosomes occurred. Difficulties in identification, however, were almost always due to excessive granularity of cells and are unlikely to represent a particular type of radial configuration. Moreover, of eight metaphases in which overlap of chromosomes occurred, only one of these involved overlap of homologues.

The conclusion that chromosomes are arranged randomly at metaphase differs slightly from the conclusion based on the analysis of Heneen and Nichols's (1972) data, which showed a slight tendency for homologous pairing of chromosome number 1. Among the factors that might account for this discrepancy are different culture conditions, possible errors in chromosome identification, bias in selection of mitoses, and differences in sample size. It is possible, as suggested by Heneen and Nichols (1972), that in some of the metaphases examined the chromosomes had not yet reached their final positions prior to anaphase. In this case, the present study has not revealed the trend toward a non-random arrangement seen by those authors.

These findings are perhaps the clearest indication of a lack of homologous paring at mitosis in any mammal yet studied. The small number of chromosomes and the fact that each could be identified morphologically enabled the use of a simple and direct statistical analysis. The finding of a random position at metaphase, of course, does not imply a random arrangement of chromosomes at all times in the cell cycle. An ordered pattern might well exist in interphase or at prophase, but become disrupted as the chromosomes are arranged on the equatorial plate at metaphase. Yet it is striking that in Dipteran insects, where somatic crossing over occurs regularly, homologous pairing persists through mitosis, whereas, in mammals, somatic crossing over occurs rarely, if at all, and homologous pairing is absent at mitosis. It therefore seems likely that neither homologous pairing nor somatic recombination are frequent in mammals. However, this does not rule out the rare occurrence of somatic recombination and its possible important consequences. Moreover, conditions such as Bloom's syndrome, which enhance the probability of exchange given even a low frequency of chance pairing of homologues, might lead to a relatively high frequency of somatic crossing over.

Another matter of importance in interpreting the random radial arrangement is that the data presented herein were derived from populations of cells. It is possible that the arrangement is fairly stable from division to division in a cell lineage, becoming random only after

a large number of mitoses. This would be expected, for example, if the chromosomes were polarized in the nucleus, with the centromeres remaining clustered near the nuclear membrane. Preliminary experiments in C-banded muntjac cells (Korf et al., unpublished data) do indicate a tendency for muntjac centromeres to remain clustered together during interphase. This could lead to certain homologues being paired in some cells for a number of generations, during which time genetic exchange might take place between those chromosomes.

Chromosome Interconnections

The studies of microsurgically extracted muntjac chromosomes are the first in which the nature and significance of chromosome interconnections could be evaluated with precision. As chromosomes were removed from the cell it was possible to note whether they had contacted one another, and thus artifactual adhesions could be identified and even avoided. Rather than a network of chromosomes enmeshed in interconnecting fibers, the microsurgically extracted chromosomes were arranged in the distinctive radial configuration, just as inside the cell, and only centromeres were connected to fibers running to the center of the metaphase array. The adhesiveness of unfixed chromosomes, as demonstrated by micromanipulation, offers great opportunity for the formation of artifactual interconnections. The potential for formation of adhesions must lead one to interpret with caution reports of interconnections among unfixed chromosomes.

The origin of interconnections among extracted chromosomes is illustrated in figure 37. Artifactual adhesions (figure 37a) result from contact between neighboring chromosome regions occurring during micromanipulation. Such contacts usually involve chromosome arms, but may also explain some of the fibers running between adjacent centromere regions. Analysis of intact metaphase cells stained with Hoechst 33258 did not reveal any cases where chromosomes were in contact with one another. It is unlikely that chromosomes are as adhesive inside the cell as they are after extraction. Glancy d'Angelo (1950) demonstrated that Chiro-

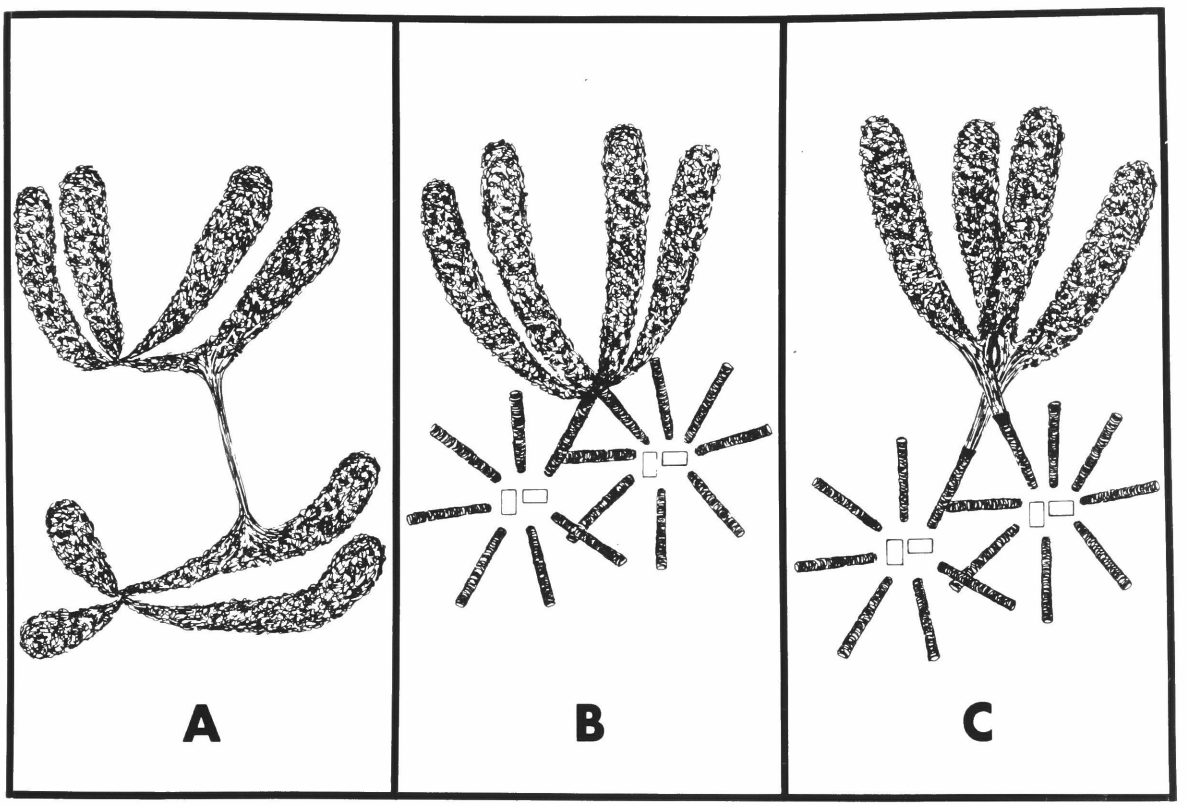


Figure 37. Model for chromosome interconnections. (a) Adhesions; (b) connection of centromeres to spindle; (c) possible origin of DNA-containing interconnections between spindle and chromosomes displaced from the spindle region.

nomous polytene chromosomes are sticky when removed from the cell but not within the nucleus. Stickiness is thus probably due to the ionic medium in which extracted chromosomes are bathed.

Figure 37b represents the attachment of chromosomes to the mitotic spindle. Immunofluorescent staining for tubulin revealed such direct contacts of spindle fibers to centromeres which had not been displaced from the spindle region during micromanipulation. These fibers did not stain for DNA and were DNase-resistant. The exact point of attachment of spindle fibers might be further analyzed with transmission electron microscopy of extracted chromosomes.

Figure 37c presents a possible explanation for the occurrence of DNA-containing fibers connecting displaced centromeres to the spindle region. Since the connections of these chromosomes to the spindle were DNase-sensitive and Hoechst-fluorescent fibers appeared to contact tubulin-stained fibers, it is proposed that the connections are stretched chromosome fibers. The kinetochores would be located at the points of contact of chromosome and spindle fibers. This hypothesis is consistent with the relative rigidity of spindle fibers (Nicklas and Staehly, 1967), and the extensibililty of microsurgically isolated chromosomes. An alternative hypothesis is that pulling a chromosome away from the spindle breaks its spindle fibers but the formation of artifactual adhesions between the centromere and other chromosomes prevents the complete separation of the chromosome from the radial array. Nicklas and Staehly (1967) noted that pulling chromosomes away from the spindle did not cause the breakage of spindle fibers, but merely stretched the chromosomes. Breakage of spindle fibers occurred only when the chromosome was rapidly moved back and forth in the cell.

The hypothesis in figure 37c does not contradict the fact that anaphase movements involve whole chromatids, not merely stretching of centromere regions towards the poles. Anaphase movements are probably initiated by separation of sister chromatids and centromeres, which are tightly attached during metaphase. At metaphase, poleward forces would be expected to stretch centromeric fibers, while at anaphase resistance

of chromatids to movement is reduced, and movement occurs.

Support for this hypothesis might be provided by examining with transmission electron microscopy the position of the kinetochores in relation to the spindle and stretched chromosome fibers. The hypothesis is of interest since if correct, the chromatin immediately surrounding the kinetochore could be studied.

In any case, it is clear that mitotic chromosomes of the Indian muntjac are not connected to one another by DNA. Interconnecting fibers have also been seen among microsurgically isolated human, mouse, and Chinese hamster chromosomes (Hoskins, 1965, 1968, 1969; Diacumakos et al., 1971). These species have relatively small chromosomes tightly packed on the mitotic spindle, making adhesions between chromosomes difficult to avoid. Hoskins's (1968) interpretation that interconnecting fibers are part of the mitotic spindle is not supported. The present studies permitted spindle fibers and adhesions to be distinguished and did not reveal spindle fibers running between chromosomes or the presence of DNA within spindle fibers.

DuPraw's hypothesis is also not supported by the evidence obtained from muntjac cells. The chromosomes are arranged at random in the radial array in a population of cells, sister chromatids disjoin randomly to the two poles (Mayron and Wise, 1976), and chromosomes are not interconnected at mitosis. Moreover, doubt is cast on the reality of interchromosomal connectives in any mammalian system, due to the stickiness of chromosomes. On the other hand, the present studies do not rule out that chromosome interconnections exist at other stages of the cell cycle. The telomeric fusions postulated by the chromosome replication model of Dancis and Holmquist (1977) would be expected to disappear before mitosis. Also, it is possible that interconnecting fibers exist in cell types other than fibroblasts. However, the bulk of evidence negates the existence of permanent connections between mammalian chromosomes.

Chromosome Structure

In the course of studying microsurgically isolated muntjac chromosomes, several observations on chromosome structure were made. The significance of these findings will now be discussed.

The Role of Protein and DNA in Chromosome Structure Current evidence suggests that each chromosome consists of a single DNA molecule (see Thomas, 1971) which runs continuously from one end of the chromosome to the other (Kavenoff and Zimm, 1972). At interphase, DNA is complexed with histones H2A, H2B, H3, and H4 to form structures known as nucleosomes (Kornberg, 1977; Finch et al., 1977). DNA runs along the outside of the histone core, and adjacent nucleosomes are connected by DNA. Histone H1 is thought to bind to this spacer DNA and may be involved in the higher order packaging of nucleosomes into clusters or coils (Oudet et al., 1975; Finch and Klug, 1976; Worcel and Benyajati, 1977; Thoma and Koller, 1977; Renz et al., 1977; Hozier et al., 1977), leading to 250 Å fibers.

Electron microscopy and nuclease digestion of metaphase chromosomes have revealed that nucleosomes are present at mitosis as well (Rattner et al., 1976; Wigler and Axel, 1976; Howze et al., 1976). The predominant fiber in the metaphase chromosome has a diameter of around 250 - 500 Å (Dupraw, 1970; Daskal et al., 1976; Rattner and Hamkalo, 1978a,b), similar to that obtained by a higher order packaging of nucleosomes at interphase. It is unknown how this fiber is packed into the mitotic chromosome. DuPraw (1966, 1970) formulated a "folded-fiber" model, in which a long chromatin fiber is both transversely and longitudinally folded along the length of the chromosome. The main evidence for this model is the observation of parallel chromatin fibers in whole mounted mitotic chromosomes examined with transmission electron microscopy (DuPraw, 1966). Comings (1972) presented an alternative interpretation of his electron micrographs of chromosomes, namely that chromatin fibers are arranged in a network. Parallel fibers might occur artifactually by stretching. A special problem with the folded fiber model is that it is difficult to envision the mechanism of chromosome arrange-

ments such as inversions (Bahr, 1977).

Some models for chromosome structure have stressed a role for an axial core. Stubblefield and Wray (1971) disrupted Chinese hamster chromosomes by shearing or by treatment with urea or 2 M NaCl and noted the persistence of a ribbon-like core of fibers. They suggested that each chromatid consists of a pair of cores, around which chromatin is bound. Sorsa (1975) presented a model for the eukaryotic chromosome where a chromatin axis runs through each chromatid. The axis would determine the sites of chromatin folding and would mediate the separation of sister strands during replication. The problem with these models is that the evidence for the existence of a core depends on examination of partially disrupted chromosomes, where there is significant risk of induction of artifacts.

Recently, electron microscopical and biochemical studies of isolated chromosomes have revealed a set of proteins that appear to be involved in metaphase chromosome structure (Adolph et al., 1977a,b; Paulson and Laemmli, 1977). These proteins have been referred to as "scaffold proteins" and appear to bind loops of DNA to a central axis. The axis, however, is not a continuous fiber but rather a region about which scaffold proteins interact and aggregate. According to this model, the scaffold proteins bind to chromatin and then to each other to fold the chromatin at mitosis.

The dissolution of arms of microsurgically extracted chromosomes by DNase suggests that chromosome structure is not determined by protein cores serving as a skeleton for chromatin assembly. Consistent with this, protease treatment does not cause a loss of chromosome structure, although the density of the chromosome as seen with phase contrast decreases. While it is possible that a protein core exists yet is inaccessible to protease, this does not explain the lability of the chromosome to DNase even in the presence of protease inhibitor. It is unlikely that all of the chromosomal DNA is accessible to nuclease. The nucleosome consists of regions of greater and lesser sensitivity to nuclease attack (Sollner-Webb et al., 1978). The extent of DNA digestion

in microsurgically extracted chromosomes is unknown since fragments of chromosomes float away in the medium. The important observation, however, is that metaphase chromosome integrity is disrupted by nuclease but not by protease.

Adolph et al. (1977a) have found that metaphase chromosomes retain their shapes after nearly complete removal of DNA. This appears to be at variance with the results obtained with microsurgically isolated chromosomes. However the conditions of treatment in the fomrmer study differ from those of the present work. Chromosomes were collected from colchicine-treated cells in the presence of hexylene glycol, exposed in suspension at neutral pH to staphylococcal nuclease, treated with heparin and dextran sulfate to remove histones, and centrifuged through a sucrose gradient. The enzyme treatment takes place at relatively low ionic strength. Although the residual chromosome structure is stable in 2 M NaCl, it is possible that this is due to aggregation of proteins after histone and DNA were removed. In the present studies, digestion takes place at higher ionic strength, which may promote the dissolution of chromosomal proteins after removal of the DNA. Rattner et al., (1978) have also observed dissolution of the arms of metaphase chromosomes treated with nuclease at high ionic strength. While hexylene glycol might be suspected to increase the stability of nuclease treated-chromosomes, its addition to DNase I did not inhibit the dissolution of microsurgically isolated chromosomes.

Thus the present results are consistent with the view that scaffold proteins bind loops of chromatin to a central axis. However this axis should not be thought of as a rigid skeleton which forms a chromosome-shaped framework to which chromatin binds. The shape of the chromosome is conferred by interactions among chromatin-bound proteins, and under appropriate conditions, can be disrupted by breakage of the DNA. It should be noted that failure to observe a residual chromosome-like structure after DNase treatment of microsrgically isolated chromosomes is not due to the invisibility of such a structure with light microscopy. The fragmentation of chromosomes could be clearly seen with phase contrast, whereas chromosome integrity was observed to be retained

in the study of Adolph et al. ((1977a).

One region of the chromosome was resistant to both nuclease and protease digestion. This was the paracentromeric region, which contains constitutive heterochromatin (Comings, 1971; Hsu and Arrighi, 1971). Comings et al. (1972) have shown that constitutive heterochromatin is resistant to treatments which remove either DNA or protein, and Rattner et al. (1978) found that centromeres of isolated Chinese hamster chromosomes persist after prolonged DNase treatment has caused dissolution of the remainder of the chromosome. SEM reveals that the centromere regions of muntjac chromosomes consist of tightly packed parallel fibers. The basis for the tight packing of heterochromatin is not known. Studies of condensed and extended chromatin fractions from interphase cells have variously implied a role of histone (Littau et al., 1965; Reeck et al., 1972; Simpson and Reeck, 1973; Berlowitz, 1974) or non-histone (Frenster, 1965; Murphey et al., 1973; Comings et al., 1977) proteins in maintaining condensed chromatin. The problem with most such studies is that the condensed chromatin fractions include both heterochromatin and transcriptionally inactive chromatin. An exception is the preparation of Musich et al. (1977), which was derived by cleavage of African green monkey chromatin with an endonuclease to which only the repetitive DNA in heterochromatin is sensitive. In this study, a set of non-histone proteins of similar molecular weight to H1 were found in the place of H1 in heterochromatin. In other species where isolation of heterochromatin by this means is not possible, the stability of these regions to nuclease and protease might provide a basis for their purification.

Surface Fibers of Chromosomes The SEM examination of microsurgically isolated chromosomes reveals a coarse, fibrous surface. This has also been reported by Golomb and Bahr (1971) and Daskal et al. (1976), among others. Unlike these studies, however, microsurgical isolation does not require the accumulation of metaphase cells with spindle poisons. Such treatments result in pronounced condensation of chromatids and obscure certain features. The lack of such condensation of microsurgically isolated chromosomes enables the secondary constrictions of the X and Y_2 to be viewed clearly. In contrast to the looping fibers

seen elsewhere along the chromosome, fibers in this region are loosely packed and longitudinally-oriented. They were also the first regions of the chromosomes to break in DNase, indicating that this loose packing confers greater accessibility of chromatin fibers to enzyme. The secondary constrictions are distinctive in that they are associated with the nucleolus until late prophase. The presence of the nucleolus may delay or prevent condensation of the associated chromatin, or, alternatively, this transcriptionally active material might be organized differently from the rest of the chromosome.

Histone Staining Indirect immunofluorescence staining with antisera to histones H1 and H2B reveals that these histones are distributed more or less uniformly along the metaphase chromosome. The photographs were over-exposed to enhance the visibility of interconnecting fibers, and thus minor variations in the histone distribution might have been overlooked. However, significant differences in histone staining along methanol:acetic acid-fixed chromosomes have been reported by Pothier et al. (1975) and Bustin et al. (1976). Acetic acid is known to extract histone from chromatin (Dick and Johns, 1967; Retief and Rüchel, 1977), and since uniform staining is seen in glutaraldehyde-fixed chromosomes (present studies and Bustin et al., 1976), the patterns seen in acid-alcohol fixed preparations are probably artifactual.

H2B is a component of nucleosomes and therefore occurs throughout the chromosome. The presence of H1 indicates that this histone (or one which cross reacts antigenically) is incorporated in the condensed metaphase chromosome. H1 is thought to be involved in the packing of nucleosomes into 250 Å fibers (see above). The 250 A fiber is the basic fiber of the mitotic chromosome. A great increase in the proportion of phosphorylated H1 is found in cells entering mitosis (Gurley et al., 1975), and it has been proposed that this is important for chromosome condensation. If antisera could be obtained which react specifically with phoshorylated H1, the distribution of this modified histone along microsurgically isolated metaphase chromosomes might be examined.

VI. FUTURE PROSPECTS

The studies reported in this thesis have exploited certain advantages of microsurgically isolated chromosomes to provide new insights into some long standing problems. Micromanipulation offers a source of material which is ideal for morphological and chemical analyses. The chromosomes are not subjected to harsh chemical treatments during isolation, and the cells need not be exposed to spindle poisons. Three-dimensional arrangements of chromosomes are preserved on removal from the cell, and it is possible to correlate electron and light microscopical observations. While not necessarily immune to artifacts, these preparations can be precisely monitored so that artifacts can be identified and, to some extent, avoided.

There are many possible future applications for microsurgically isolated chromosomes. As previously mentioned, biochemical studies of chromosome fractions are plagued by the uncertain correlation of these fractions to cytologically defined entities such as heterochromatin. Microsurgically isolated chromosomes may help bridge the gap between cytological and biochemical observations. It is possible to dissect chromosomes by cutting them with a microneedle. The collection of specific regions from several chromosomes might permit analysis of DNA and proteins by sensitive micromethods (see Neuhoff, 1973).

Dissected chromosomes also may be of use in studying DNA replication. When DNA is spread from tritiated thymidine pulse-labeled cells, DNA regions that replicated during the pulse appear as tracks of silver grains on autoradiography (Huberman and Riggs, 1968). DNA replicates bidirectionally from multiple sites of origin. Several studies indicate that replication is initiated synchronously in certain regions of DNA (Hand, 1975; Jasny *et al.*, 1978). At the level of the metaphase chromosome, patterns of replication visualized by autoradiography (Taylor, 1960) or staining chromosomes from BrdU pulse-labeled cells (Latt, 1975) also are discontinuous. Latt (1975) has suggested that individual chromosomal regions replicate as units: within a region, replication begins synchronously, but different regions begin replication at dif-

ferent times. It is not known if clusters of replicating DNA are contained in the same regions of the chromosome at metaphase. By microsurgically isolating chromosomes from tritiated thymidine-pulsed cells, dissecting particular regions, and spreading the DNA, it may be possible to address this question.

Finally, an exciting potential application of microsurgically isolated chromosomes is in chromosome transplantation. Diacumakos et al. (1970) have already shown that it is possible to implant metaphase chromosomes in metaphase cells, although markers to demonstrate activity of the transplanted chromosomes in the recipient cells were not available. The most often used means of achieving chromosome mediated gene transfer is addition of metaphase chromosomes to the medium of cultured cells (McBride and Athawal, 1976). Chromosomes are taken up by phagocytosis, and only small regions escape enzymatic destruction and become associated with the genome (Simmons et al., 1978). Moreover, of those which become associated, only some become stably integrated in the recipient nucleus (McBride and Athawal, 1976). Also, it is possible to select for a limited number of genetic traits in vitro. Microsurgical transplatation may be exempt from many of these limitations. Specific chromosomes can be selected and placed in any region of the cell, including the nucleus, bypassing the destructive phagocytic process. The recipient cell can be cloned and tested for genetic activity of the transferred material without the need for selective conditions to separate recipients of the transferred chromosome from non-recipient cells. Such studies would be a valuable adjunct to somatic cell genetic analysis, both in mapping of chromosomes and in studying gene regulation.

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