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Silver-stained Structures in Mammalian Meiotic Prophase

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Abstract. Silver staining of mammalian spermatocytes revealed, in light microscopy, synaptonemal complex and structures within the sex vesicle. It is feasible to follow the chromosome pairing phenomenon from zygotene to pachytene by examining the behavior of synaptonemal complexes. Nucleolus organizer regions take heavy silver stain in pachytene but are no longer detectable in later stages of meiosis.

Introduction

Since the discovery of the Synaptonemal Complex (SC) in crayfish spermatocytes (Moses, 1956), numerous studies, especially electron microscopic analyses, have been made on the structure and function of the SC. Counce and Meyer (1973) developed the whole mount electron microscopy technique to demonstrate SCs in the spermatocytes of the grasshopper, *Locusta migratoria.* Recently, Moses and his collaborators (1974, 1975, 1976, 1977a, b, c) utilized this technique to study in detail the organization of SC in various mammalian species, including that of man. It is now well known that the two ends of a chromosome (telomeres) are attached to the nuclear envelope (Wettstein and Sotelo, 1967; Solari, 1970; Comings and Okada, 1970) and the homologous pairing starts from these points. Observations of SC have been possible only with electron microscopy.

In the present communication we describe a silver staining technique by which it is now feasible to demonstrate the SCs in mammalian meiosis using a bright field microscope and to analyze the sequence of SC and sex vesicle formation.

Materials and Methods

Although we have used the following mammalian species (a) mouse, (b) Armenian hamster, (c) rat, (d) baboon, and (e) bull for this study, only the mouse spermatocytes will be described in detail because the general phenomena to be described are similar in these materials.

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Meiotic Chromosome Preparation. Seminiferous tubules were processed following the technique described elsewhere (Pathak et al., 1976) with slight modifications. The tubules were combed with two pairs of watchmakers forceps in a hypotonic sodium citrate solution (0.7%) and the cell suspension was allowed to stand vertically in a centrifuge tube filled with approximately 12–14 ml of the hypotonic solution. A 0.5% NaCl solution produced equally satisfactory results. The final preparations were more satisfactory if the hypotonic treatment lasted 30–40 min instead of the usual 10–15 min. The supernatant was aspirated gently in a separate tube and centrifuged at 1,700 rpm for 5 min. The pellet was then fixed in a 3:1 methanol acetic acid mixture, and air-dried slides were prepared.

Silver Staining of Meiotic Chromosomes. The slides were stained following the simple Ag-1 (Bloom and Goodpasture, 1976) as modified by Lau et al. (1978), i.e., they were immersed in a borate buffer solution (pH 9.0) at room temperature for 20 min, washed in deionized water, flooded with freshly prepared aqueous silver nitrate solution (50%), and covered with a 22×50 mm coverglass. The preparations were then placed in a moist chamber and allowed to incubate for 24 to 72 h at 50° C. For meiotic preparations 48 h incubation gave most satisfactory results. At the end of the incubation period the slides were examined under phase objectives. If proper silver-precipitation or yellow staining was visible, the slides were rinsed in deionized water and allowed to air-dry. A slight counter-staining with Giemsa (1.0% in 0.01 M phosphate buffer) improved the preparations for photography.

Results

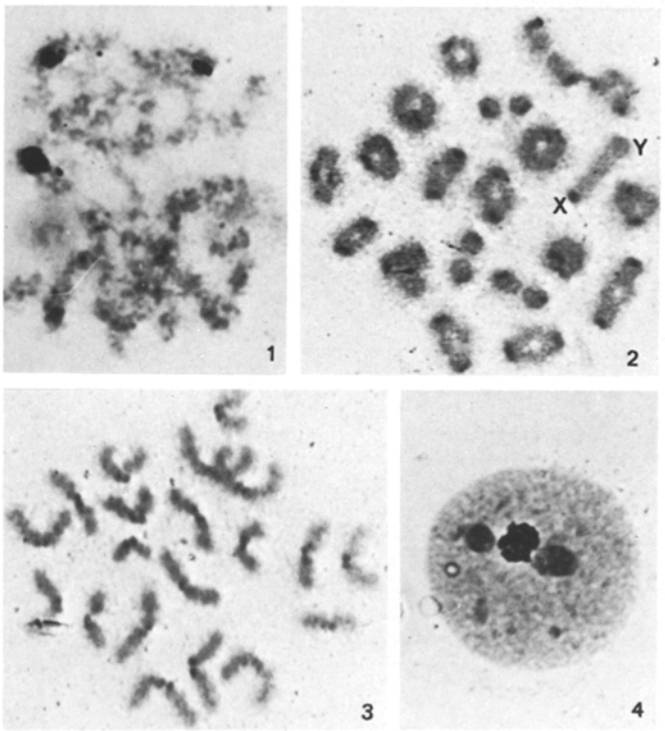
General Patterns of Silver Staining Spermatogenetic Elements

In somatic metaphases following the Ag-1 staining method, the chromosomes are faintly stained with silver, whereas the nucleoli and the nucleolus organizer regions (NORs) are intensely stained (Goodpasture and Bloom, 1975). In meiotic preparations, the chromosomes were also weakly stained, but three structures showed heavy silver deposit: 1) the nucleoli and NOR, 2) the sex vesicle, and 3) thin thread-like structures, which corresponded well to SC observed in whole-mount electron microscopy (Tres, 1977).

The nucleoli were obvious in meiotic prophases up to early pachytene, and the NOR can be identified relatively easily in pachytene. In the mouse genome, usually three pairs of autosomes carry NOR. In pachytene (Fig. 1) we found three bivalents with heavy silver deposit. The structures assumed to be SC can be observed in late leptotene, zygotene, and pachytene. However, all these silver-stained bodies were no longer present in diplotene, diakinesis, MI and MII. In diakinesis (Fig. 2), MI, and MII (Fig. 3) the centromeric heterochromatin areas took more silver staining than the chromosome arms, but Ag-NOR was absent. In the Sertoli cell (Fig. 4) the centrally located nucleolus always showed heaviest silver staining flanked by two pieces of less intensely stained heterochromatin. A complex series of silver-stained structures reappeared during spermiogenesis. These phenomena will be described in a separate report.

Leptotene/Zygotene

A late leptotene or early zygotene stage stained with silver is shown in Figure 5. We consider this stage (Fig. 5a) as leptotene/zygotene because of the following



Figs. 1–4. Silver stained meiotic figures of the mouse (*Mus musculus*) testis. **Fig. 1.** Pachytene, showing three autosomal bivalents with silver deposits. **Fig. 2.** Diakinesis, showing the absence of Ag-NOR. The C-band segments, except the Y chromosome are more darkly stained. **Fig. 3** Metaphase II, showing darkly silver-stained centromeric areas but no Ag-NOR. **Fig. 4.** A Sertoli cell, showing the nucleolus flanked by two pieces of heterochromatin. ×3,900

characteristics: (a) the SC structure had just become noticeable near the nuclear envelope, (b) the SCs were not tightly paired, indicating the process of synapsis, (c) the sex vesicle was not formed and the axial elements of autosomal SC were much longer and thinner compared to those of the pachytene nuclei, and (d) the nucleoli showed heavy silver aggregates. The initiation sites of synapsis at the nuclear envelope is depicted in an enlarged area in Figure 5b (arrowheads). The SCs are attached to the nuclear envelope with a darkly stained tip. A much thickened axial element of a sex chromosome (the X?) is shown in the middle of Figure 5b (arrow). It is not possible to positively identify the other sex chromosome (the Y?) at this stage.

The initiation of pairing is apparently not synchronous among different autosomes. Homologous SCs form an arch-like configuration when they approach each other. Due to the attachment of both ends to the nuclear envelope, sometimes it is possible to observe bouquet-orientation during zygotene/pachytene stages.

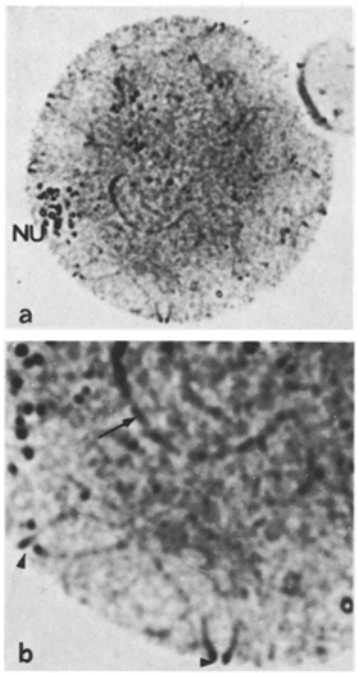


Fig. 5a and b. Silver staining of synaptonemal complexes (SCs) during late leptotene or early zygote. **a** Pairing of SCs near the nuclear envelope. Note the obvious nucleolus (*NU*) but the absence of a well-defined sex vesicle. ×3,900. **b** An enlarged portion of **a**, showing initiation sites of synapsis at the nuclear envelope (arrow heads) and a much thickened axial element, probably the X chromosome (arrow). ×10,900

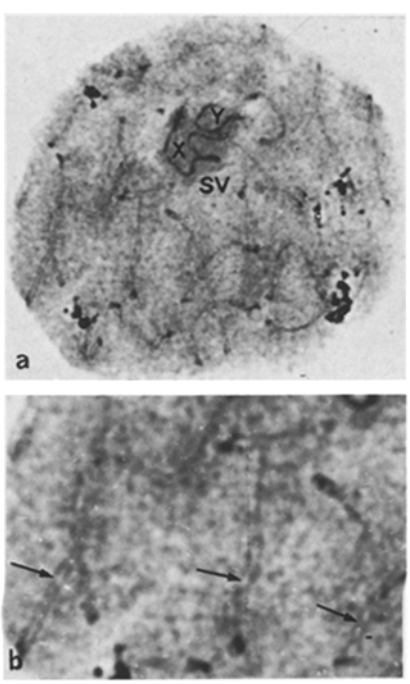


Fig. 6a and b. Silver staining of SC at pachytene. **a** A complete pachytene nucleus, showing fully formed SC and the sex vesicle (*SV*). Note the Ag-NOR staining of nucleoli. ×3,900. **b** An enlarged portion of **a**, in many SCs two axial elements are distinct with random twists (arrows). ×10,900

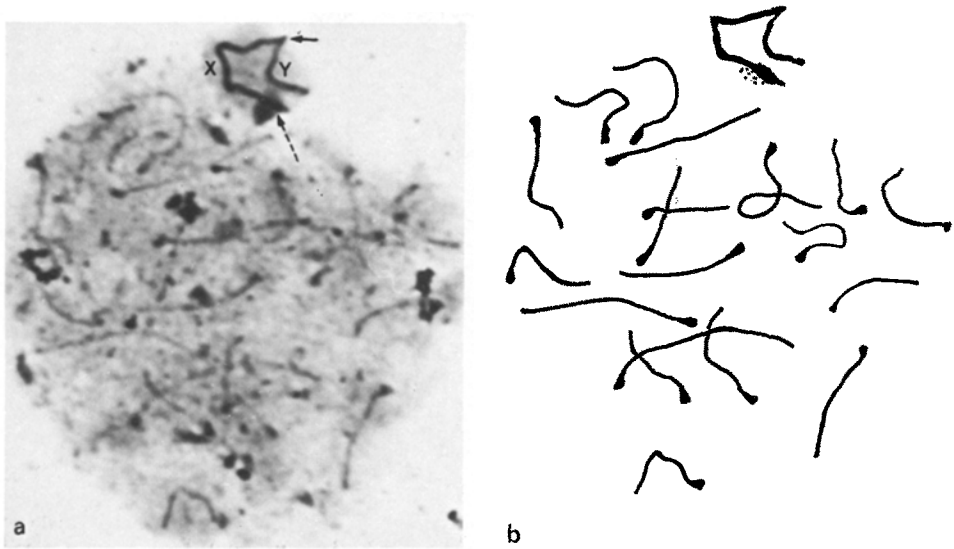


Fig. 7a and b. A late pachytene nucleus, **a** Shows tightly-paired lateral elements. The axial elements of the X and the Y chromosomes form a short SC as marked by a solid arrow. The subterminally located puffed structure in the X chromosome is marked by a dotted arrow. **b** Tracing of SC from **a**. It is possible to count 19 autosomal SCs (bivalents) and an XY pair. ×9,400

Pachytene

The axial elements of SCs of the autosomal bivalents are distinctly visible as paired structures at pachytene (Fig. 6a). The resolution at the light microscopy level is not adequate to demonstrate the central element and transverse filaments of the SC. However, it is possible to demonstrate the random twisting of axial elements in practically every autosomal SC. Paired axial elements are well demonstrated in the enlarged portion of Figure 6b (arrows). The complete pairing at full pachytene is shown in Figure 7. Mature synaptonemal complexes are much shorter and more thickened than those of earlier stages, and it is possible to count 19 SC in Figure 7a, which is diagrammatically sketched in Figure 7b. In some preparations, one end of the SC appears to be much darker than the other. Since in the mouse all chromosomes are acrocentric, it is not positive to conclude whether the thicker regions represented the telomeric or the centromere end. However, in the Armenian hamster where all chromosomes are biarmed, we found thickened tips at both ends. Therefore, the thickened ends of the mouse chromosomes may also represent the telomeric regions.

The Sex Vesicle

In any stage of pachytene, the sex vesicle is prominently differentiated into the X and Y chromosomes (Fig. 6a). The axial elements of the X and Y chromosomes are much thicker compared to those of the autosomal SCs. They are

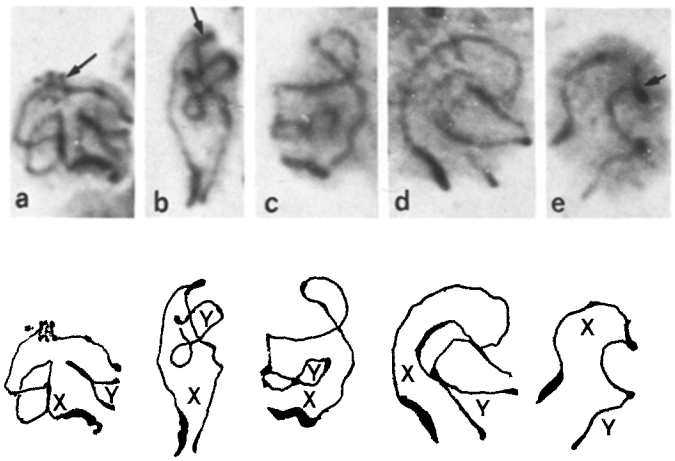


Fig. 8 a–e. Sex vesicles at different stages of pachytene nuclei from mouse testis after silver staining. A diagrammatic sketch is shown below each sex vesicle. Note the thickened segment adjacent to the centromeric area of the X and the presence of "hairpin"-structure in the X chromosome in **a, b,** and **e** (arrows). ×10,900

largely unpaired and only a short segment of each (probably homologous) form the mature SC (Fig. 7a, arrow). The thickening of the axial elements of the sex chromosomes are characteristic. The most obvious feature of the X chromosome axial element is shown at its free end (Figs. 7, 8). In many preparations, this unpaired end of the X showed a large but diffuse structure associated with it. This structure is particularly prominent in Figure 7a (dotted arrow). Since it is well known that the X and the Y chromosomes of mouse spermatocytes attach to each other at the telomeric ends (Kofman-Alfaro and Chandley, 1970; Hsu et al., 1971; Schnedl, 1972; Polani, 1972), the free end of the X is believed to be the centromeric end. This structure varied in size from cell to cell, but was present in most pachytene nuclei. Another characteristic of the X was a "hairpin"-like folding present in the distal part of the X (Fig. 8a, b, e, arrows). Frequently there were structures associated with this twisted area (Fig. 8a) or a thickening of the segment (Fig. 8b, e). The length of the paired region between the X and the Y and that of the "hairpin" segment of the X decreases with the advancement of the pachytene stage.

Discussion

Nucleolus and NOR

It has been postulated (Miller et al., 1976a, b; Schmid et al., 1977) that the silver staining of NOR represents previous transcriptional activity of ribosomal genes. The number of Ag-NOR seldom reaches its maximum number of NOR present in a complement. In leptotene and zygotene, the presence of nucleoli suggests that ribosomal RNA synthesis still takes place, but the individual

chromosomes are not identifiable to ascertain the Ag-NOR regions. In pachytene of the mouse, Ag-NOR are found in three autosomal bivalents. In the cattle, there are five pairs of autosomes with Ag-NOR and in pachytene we observed five bivalents with heavy silver deposit (Pathak and Kieffer, in press, 1979). Schmid et al. (1977 considered that the activity of ribosomal genes are suppressed during meiosis. This conclusion probably applies to pachytene onward, but not to leptotene and zygotene. Thus, the Ag-NOR observed in pachytene also represent the past activity of rDNA. There was no indication of Ag-NOR or nucleoli in any stage after pachytene until early spermatids, at which time silver-stained bodies reappeared, probably indicating a return of rRNA synthetic activity. This activity appeared to be short-lived, however, because developing spermatids contained only extra nuclear silver-stained bodies.

Synaptonemal Complex

The procedure for the demonstration of SC in light microscopy should greatly facilitate studies on meiosis even though the resolution is not to the level of electron microscopy. A number of problems that do not relate to the fine structure of SC can be tackled with increased efficiency. For example, constructing the events of pairing, as shown in Figure 5, may be accomplished. In *Mus dunni*, where each autosome has a short, heterochromatic second arm, we were able to follow the pairing events during zygotene by C-banding (Pathak and Hsu, 1976). In the laboratory mouse, even C-banding offers no assistance. Silver staining in combination with other techniques may prove to be a useful tool for the identification of various stages of early meiotic prophase.

We believe that the success of our preparation was due to two key technical improvements: 1) a prolonged hypotonic solution treatment (30–40 min) and 2) a prolonged Ag-staining period (up to 72 h at 50° C).

It has been postulated that the SC is made primarily of proteins (Coleman and Moses, 1964; Brinkley and Bryan, 1964; Comings and Okada, 1970). Silver probably stains certain proteins more strongly than other. Howard Hubbell (personal communication) found certain proteins, but not others, in isolated nucleoli with special affinity for silver staining. Thus the SC protein(s) also appear to have an affinity for silver. It is possible for the isolation of SC proteins in the future using the silver staining property as a monitor device.

Our data strongly supports the notion (Gillies, 1975) that synapsis between homologous chromosomes is initiated at the telomeres.

The Sex Vesicle

As a rule, the mammalian X and Y chromosomes during early pachytene form an amorphous structure known as the sex vesicle (Sachs, 1954). During leptotene/zygotene stage these chromosomes remain separated from each other, but during late zygotene or early pachytene they come close to each other and form the sex vesicle. A number of electron microscopists have analyzed the structure

of the sex vesicle and the behavior of the sex chromosomes within the vesicle. The tracing of the events in the vesicle using three-dimensional reconstruction with a series of electron micrographs is of course tedious. We believe that the silver staining method can offer a unique tool to analyze the sex vesicle in mammalian meiosis.

The silver staining of the sex elements in the vesicle is considerably heavier than that of the autosomal bivalents in the same nucleus. This is probably the result of transcriptional activity of the autosomes during this stage, while the sex elements are more metabolically quiescent at this time. Nevertheless, the diffuse mass of silver-stained material seen just below the centromere of the X may represent a special gene activity of this stage. Some investigators observed this material in electron micrographs also and consider it to represent the centromere. We do not accept this interpretation. As is clearly shown in Fig. 5a, the diffused mass is a short distance distal to the terminal centromeric end. We have examined the components of sex vesicles of numerous pachytene nuclei and noted a considerable variation in terms of number and size of these diffuse structures along the X and the Y. The largest one near the centromere of the X was present in most cases, but smaller aggregates of similar material were observed in a number of instances other than the one near the tip of the X. It is reminiscent of the Y chromosome activity of *Drosophila* described in detail by Hess and Meyer (1963a and b). Tentatively, we consider that such structures represent the products of gene activities of the sex chromosomes specific for the pachytene stage.

The structure of the sex vesicle as revealed by silver staining suggests that many substages of pachytene may be identified in the future. Several approaches may be useful in tackling problems of the structure and physiology of the sex chromosomes in pachytene: 1) isolating and identifying the SC proteins in purified pachytene cells, 2) combining silver staining and ^{3}H-uridine autoradiography to study the synthetic activities of the sex chromosomes, and 3) analyses of the X chromosome behavior in mice with X-autosome translocation, both homozygotes and heterozygotes.

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References

Bloom, S.E., Goodpasture, C.: An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum. Genet. **34**, 199–206 (1976)

Brinkley, B.R., Bryan, J.H.D.: The ultrastructure of meiotic prophase chromosomes as revealed by silver-aldehyde staining. J. Cell Biol. **32**, 14a (1964)

Coleman, J.R., Moses, M.J.: DNA and the fine structure of synaptic chromosomes in the domestic rooster (Gallus domesticus). J. Cell Biol. **23**, 63–78 (1964)

Comings, D.E., Okada, T.A.: Whole mount electron microscopy of meiotic chromosomes and the synaptonemal complex. Chromosoma (Berl.) **30**, 269–286 (1970)

Counce, S.J., Meyer, G.F.: Differentiation of the synaptonemal complex and the kinetochore in Locusta spermatocytes studied by whole mount electron microscopy. Chromosoma (Berl.) **44**, 231–253 (1973)

Gillies, C.B.: Synaptonemal complex and chromosome structure. Ann. Rev. Genet. **9**, 91–109 (1975)

Goodpasture, C., Bloom, S.E.: Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. Chromosoma (Berl.) **53**, 37–50 (1975)
Hess, O., Meyer, G.F.: Chromosomal differentiations of the lampbrush type formed by the Y chromosome in Drosophila hydei and Drosophila neohydei. J. Cell Biol. **16**, 527–539 (1963a)
Hess, O., Meyer, G.F.: Artspezifische funktionelle Differenzierungen des Y-heterochromatins bei Drosophila-Arten der D. hydei-Subgruppe. Portugaliae Acta Biol. (A), **7**, 29–46 (1963b)
Hsu, T.C., Cooper, J.E.K., Mace, Jr., M.L., Brinkley, B.R.: Arrangement of centromeres in mouse cells. Chromosoma (Berl.) **34**, 73–87 (1971)
Kofman-Alfaro, S., Chandley, A.C.: Meiosis in the male mouse. An autoradiographic investigation. Chromosoma (Berl.) **31**, 404–420 (1970)
Lau, Y.F., Pfeiffer, R.A., Arrighi, F.E., Hsu, T.C.: Combination of silver and fluorescent staining for metaphase chromosomes. Amer. J. hum. Genet. **30**, 76–79 (1978)
Miller, D.A., Dev, V.G., Tantravahi, R., Miller, O.J.: Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Exp. Cell Res. **101**, 235–243 (1976a)
Miller, O.J., Miller, D.A., Dev, V.G., Tantravahi, R., Croce, C.M.: Expression of human and suppression of mouse nucleolus activity in mouse-human somatic cell hybrids. Proc. nat. Acad. Sci. (Wash.) **73**, 4531–4533 (1976b)
Moses, M.J.: Chromosomal structures in crayfish spermatocytes. J. biophys. biochem. Cytol. **2**, 215–218 (1956)
Moses, M.J.: Synaptonemal complex karyotyping in spermatocytes of the Chines hamster (Cricetulus griseus) I. Morphology of the autosomal complement in spread preparations. Chromosoma (Berl.) **60**, 99–125 (1977a)
Moses, M.J.: Synaptonemal complex karyotyping in spermatocytes of the Chinese hamster (Cricetulus griseus) II. Morphology of the XY pair in spread preparations. Chromosoma (Berl.) **60**, 127–137 (1977b)
Moses, M.J., Slatton, G.H., Gambling, T.M., Starmer, C.F.: Synaptonemal complex karyotyping in spermatocytes of the Chinese hamster (Cricetulus griseus) III. Quantitaive evaluation. Chromosoma (Berl.) **60**, 345–375 (1977c)
Moses, M.J., Counce, S.J.: Synaptonemal complex karyotyping in spreads of mammalian spermatocytes. In Symp. Mechanisms in Recombination, (R. Grell, ed.), pp. 385–390. New York: Plenum Press 1974
Moses, M.J., Counce, S.J.: Analysis of synaptonemal complex karyotypes in five mammals. J. Cell Biol. **70**, 131a (1976)
Moses, M.J., Counce, S.J., Paulson, D.F.: Synaptonemal complex complement of man in spreads of spermatocytes with details of the sex chromosome pair. Science **187**, 363–365 (1975)
Pathak, S., Hsu, T.C.: Chromosomes and DNA of Mus. The behavior of constitutive heterochromatin in spermatogenesis of M. dunni. Chromosoma (Berl.) **57**, 227–234 (1976)
Pathak, S., Hsu, T.C., Markvong, A.: Pachytene mapping of the male Chinese hamster. Cytogenet. Cell Genet. **17**, 1–8 (1976)
Pathak, S., Kieffer, N.M.: Sterility in hybrid cattle. I. Dustribution of constitutive heterochromatin and nucleolus organizing regions in somatic and meiotic chromosomes. Cytog. Cell genet. (in press, 1979)
Polani, P.E.: Centromere localization at meiosis and the position of chiasmata in the male and female mouse. Chromosoma (Berl.) **36**, 343–374 (1972)
Sachs, L.: Sex linkage and sex chromosomes in man. Ann. Eugen. (Lond.) **18**, 255–261 (1954)
Schmid, M., Hofgärtner, F.J., Zenzes, M.T., Engel, W.: Evidence for postmeiotic expression of ribosomal RNA genes during male gametogenesis. Hum. Genet. **38**, 279–284 (1977)
Schnedl, W.: End-to-end association of X and Y chromosomes in mouse meiosis. Nature (Lond.) New Biol. **236**, 29–30 (1972)
Solari, A.J.: The spatial relationship of the X and Y chromosomes during meiotic prophase in mouse spermatocytes. Chromosoma (Berl.) **29**, 217–236 (1970)
Tres, L.L.: Extensive pairing of the XY bivalent in mouse spermatocytes as visualized by whole-mount electron microscopy. J. Cell Sci **25**, 1–15 (1977)
Wettstein, R., Sotelo, J.R.: Electron microscope serial reconstruction of the spermatocytes I nuclei at pachytene. J. Microscopie **6**, 557–576 (1967)

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