DISTRIBUTION OF TRITIUM-LABELED DNA AMONG CHROMOSOMES DURING MEIOSIS*

I. Spermatogenesis in the Grasshopper

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ABSTRACT

Thymidine-H^3 of high specific activity was used to study the distribution of labeled chromatids during meiotic divisions in spermatocytes of a species of grasshopper (Orthoptera). The distribution is regularly semiconservative as has been shown previously for mitosis, i.e., all chromatids are labeled after incorporation of thymidine-H^3 into DNA at premeiotic interphase. If incorporation occurs at the interphase preceding this one, the chromosomes arrive at meiotic divisions with the equivalent of one chromatid of each homologue labeled. Chromatid exchanges occur at a frequency which is very nearly that predicted on the assumption that each chiasma represents an exchange between homologous chromatids. However, the exchanges are randomly distributed among chromosomes in a size group, whereas chiasmata are not. A quantitative analysis of the frequency and pattern of exchanges indicates that most of these result from breakage and reciprocal exchange between homologous chromatids. Sister chromatid exchanges are much less frequent and may be limited to premeiotic stages.

INTRODUCTION

Thymidine-H^3 of high specific activity has been widely used for labeling DNA (deoxyribonucleic acid) since it was first prepared and used for studying DNA distribution during chromosome reproduction (Taylor et al., 1957). It proved useful for demonstrating and following the pattern of sister chromatid exchanges in mitosis (Taylor, 1958; Marin and Prescott, 1964). However, simi-

lar attempts to study exchanges during meiosis have yielded meager returns primarily because it is difficult to label chromosomes at the appropriate premeiotic stages and to find the cells containing these labeled chromosomes at the desired division stages. After some preliminary trials on a variety of plant and animal materials, we selected spermatocytes of the grasshopper as the biological material for our more extended studies. An organism with relatively few, large chromosomes which yield good cytological preparations at late meiotic prophase as well as at the later division stages is a prime requirement. One would also like to have an abundance of meiotic stages in some material which can be grown and treated with isotopes under laboratory conditions. An

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* The author wishes to dedicate this article to Professor Hans Bauer on the occasion of his sixtieth birthday. A special commemorative volume of *Chromosoma* is being published in recognition of his valuable contributions to cytology and cytogenetics. This paper will be listed by title among those presented in that volume.

organism with zygotic meiosis would be highly desirable, so that only one set of chromosomes could be labeled before fertilization, but to date none of the available organisms with this characteristic meets the other requirements. Higher plants provide an abundance of material in developing anthers, but the labeling of appropriate premeiotic stages has not been very successful.

Over the past four years, we have accumulated enough evidence from studies of the distribution of tritium-labeled DNA among chromosomes during spermatogenesis in the grasshoppers to indicate that DNA distribution following replication is semiconservative in meiosis as we have previously reported for mitosis (Taylor et al., 1957; Taylor, 1958). The frequency of sister chromatid exchanges is not excessive; otherwise the semiconservative distribution would be obscured. Only limited correlations can yet be made between the frequency of chiasmata and exchanges. However, the patterns of labeling indicate that homologous chromatids undergo breakage and exchange of relatively large segments during meiosis. In addition, some sister chromatid exchanges occur, but these may be limited to the premeiotic interphases.

MATERIALS AND METHODS

Romalea microptera (Beauvoir) was obtained from the Carolina Biological Supply Company, Elon College, North Carolina, in the spring as nymphs which were in the third or fourth instar. The animals were maintained in the laboratory on a diet of lettuce, spinach, and cereal flakes (Pablum) at 20-25°C with 12 hours of light per day. They survived without the heat and dry conditions required by most grasshoppers.

Males in the fourth or fifth instar were given 15 to 20 μc of thymidine-H^3 (sp. act. 1 to 5 c/mmole) in one injection or, so that the isotope would be available for a longer time, two injections 3 to 6 hours apart. The thymidine in 20 to 40 μliters of distilled water was injected by means of a microsyringe and a 27 gauge hypodermic needle inserted between the scales on the abdomen. In each group of 25 to 30 animals injected at one time, 2 or 3 were sacrificed at intervals of 1 or 2 days for a period extending, in various experiments, from 1 to 63 days.

The testes were fixed in ethanol-acetic acid (3:1) directly after removal from the animal or after incubation for 5 hours in a small volume of one-half strength Ringer's solution containing colchicine (200 μg/ml). In the latter case, incubated testes were transferred to distilled water for 1 hour before fixation to swell the cells and chromosomes. The cells treated with the hypotonic solution yield better-flattened preparations for autoradiography than those fixed directly from the animal. After hydrolysis for 7 minutes in 1 N HCl, the material was stained by the Feulgen reaction. After transfer to 45 per cent aqueous acetic acid for a few minutes, the cells were squashed very flat between a slide and a coverglass. The preparations were frozen on solid CO_2 (dry ice), the coverglass was removed, and the slides with the material attached were placed in ethanol-acetic acid (3:1) for a few minutes. The slides were then transferred to 70 per cent ethanol until stripping film, Kodak AR-10, was applied. All slides were coated, before the squashes were made, by dipping in a gelatin solution of the following composition and then air drying: gelatin, 0.50 gm; chrome alum, $CrK(SO_4)_2 \cdot 12H_2O$, 0.05 gm; water, 100.00 ml. A few crystals of thymol (as a preservative) and a wetting agent, e.g. Photoflo, may also be added to the solution.

After 6 weeks' exposure the film was developed in one-half strength Kodak D-19, rinsed, and fixed in one-half strength Kodak acid fixer for 10 minutes. After being washed and treated with Kodak hypo clearing agent, the slides were rinsed in distilled water and air dried. Since the film forms a protective layer over the cells, the slides keep indefinitely.

RESULTS

The Meiotic Chromosomes of Romalea

The *Romalea* male has 23 chromosomes, all of which have terminal or near terminal centromeres. The X chromosome is easily recognized because it is unpaired and positively heteropycnotic at late meiotic prophase. At metaphase I it is negatively heteropycnotic and usually oriented at the equator of the spindle with the autosomes (Fig. 1). At anaphase I the X chromosome dyad (Fig. 2) resembles the two largest dyads formed by autosomes 2 and 3. Although the X (chromosome
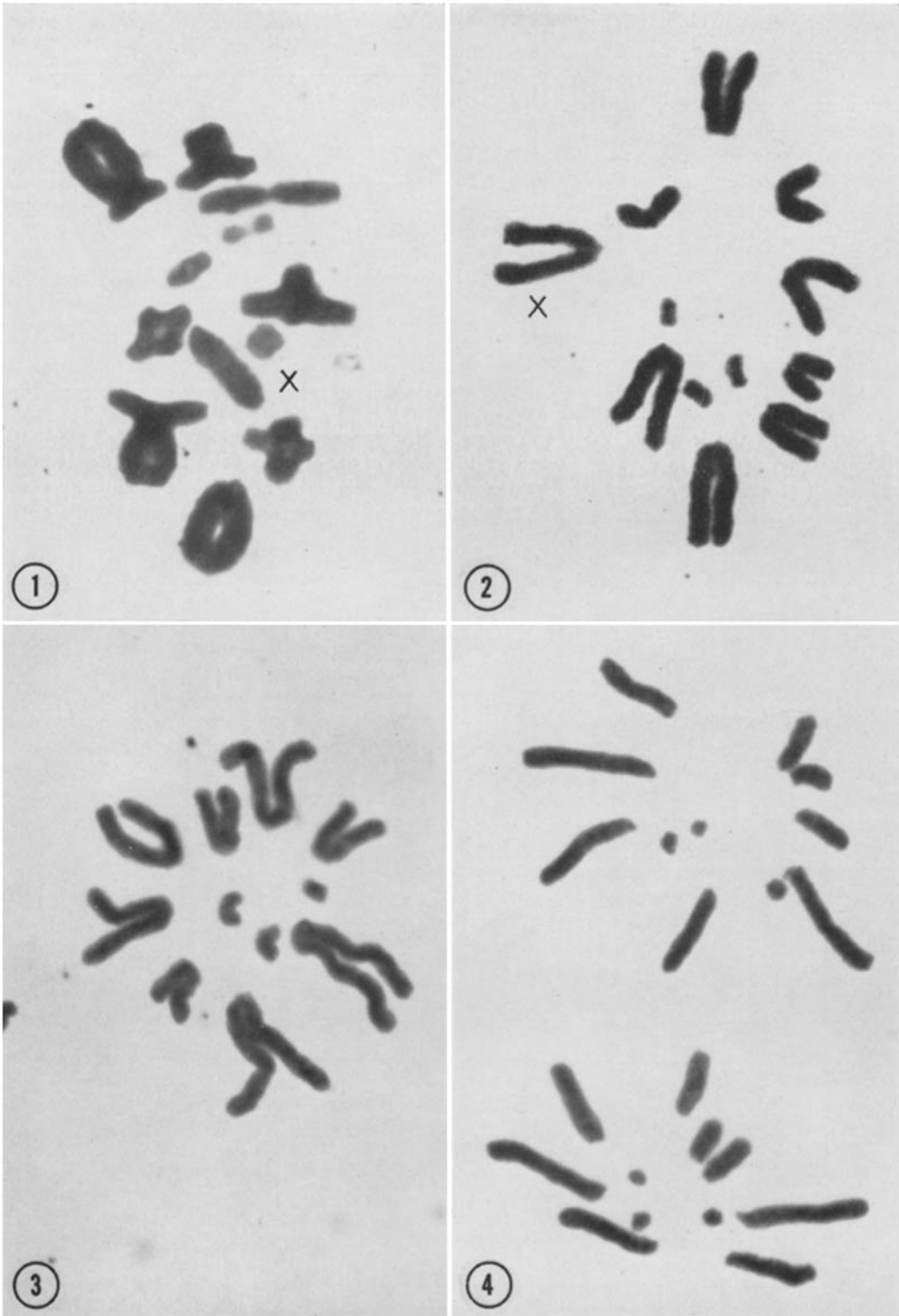
FIGURES 1 TO 4   Meiotic divisions in spermatocytes of *Romalea*.

Fig. 1. Metaphase I. × 2400

Fig. 2. One chromosome group at anaphase I. × 2400

Fig. 3. Metaphase II. × 2400

Fig. 4. Anaphase II. × 2400

1) can sometimes be recognized because it is still slightly heteropycnotic, it and the two large autosomes form a natural size group, A, and are often indistinguishable. The next size group, B, consists of chromosomes 4, 5, and 6 (Figs. 3 and 4). Chromosome 4 is slightly larger than 5 or 6, but is not regularly distinguishable. The next size group, C, also consists of three chromosomes, 7, 8, and 9; 9 is slightly smaller but not consistently recognizable. Group D is composed of three small chromosomes, 10, 11, and 12.

The Cell Cycle and Spermatogenesis

Spermatocytes of Orthoptera develop in elongated sperm tubes which contain the primordial cells at one end and the mature sperms at the other. Usually many intermediate stages can be found along the length of the sperm tubes. Spermatocytes develop in cysts or packets of 16 cells, each of which is produced by synchronous divisions of a single spermatogonial cell. The progress of the cells of each cyst is remarkably uniform throughout the premeiotic and meiotic stages.

The spermatogonia incorporate thymidine-H^3 at certain stages in interphase. Since less than one-third of the cysts are labeled by a single injection, the S (DNA-synthetic) phase is probably shorter than G_1 and G_2 (non-DNA-synthetic periods of interphase before and after S) combined. No further attempts have yet been made to determine the relative lengths of these stages. When it was noted that a single injection would label either all or none of the cells in a cyst, but would often leave some chromosomes or chromosomal sectors unlabeled in each complement, two injections were given at intervals of 3 to 6 hours. Chromosomes of some cysts were then rather uniformly labeled. The most frequently noted exception was the X chromosome. As reported previously for another grasshopper (Lima-de-Faria, 1959), the

X chromosome is considerably out of phase with most of the other chromosomes. In *Romalea* many cells were seen in which the autosomes were labeled without concurrent labeling of the X. The converse was less frequent.

Length of Meiotic Stages and Spermiogenesis

Spermatocytes of *Romalea* develop rather slowly (Table I). Meiosis required about 24 to 26 days under the laboratory conditions, and labeled sperm reached maturity about 60 days after injection. This interval is the time required for spermatocytes to advance through meiosis from premeiotic interphase, when the last DNA synthesis occurs, to the production of very elongated sperms which have the morphology of mature sperms and presumably would be functional.

Patterns of Segregation of Labeled DNA During Meiotic Divisions

The segregation of labeled DNA is regularly semiconservative. The first labeled cells to arrive at anaphase I and metaphase II (24 days for

FIGURES 5 TO 8 Meiotic divisions in spermatocytes of *Romalea*.

Fig. 5. Autoradiograph at metaphase II of a cell which incorporated thymidine-H^3 at premeiotic interphase. × 2400.

Fig. 6. Autoradiograph at anaphase I of a cell which incorporated thymidine-H^3 one cell cycle earlier than those shown in Fig. 5, *i.e.*, preceding the last spermatogonial mitosis. × 2400.

Fig. 7. Autoradiograph of a portion of a metaphase figure from the same cyst as Fig. 5. × 3600.

Fig. 8. Autoradiograph of a cell at anaphase II which probably incorporated thymidine H^3 one cell cycle preceding premeiotic interphase. × 3600.
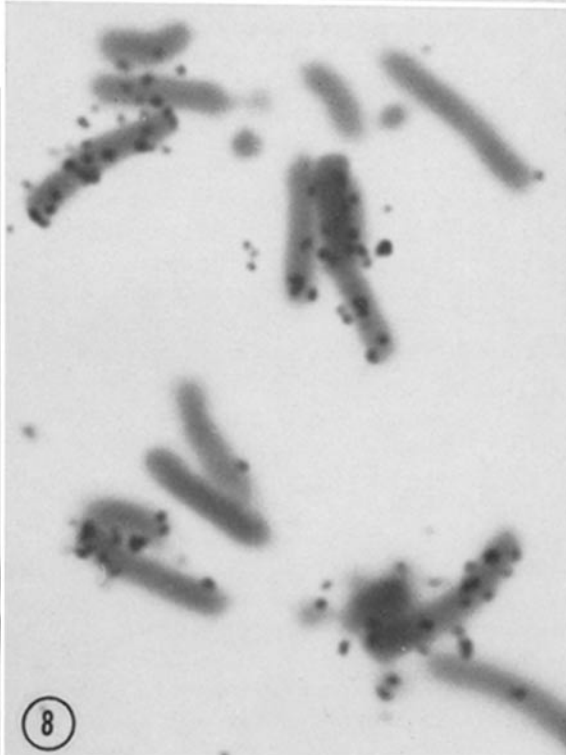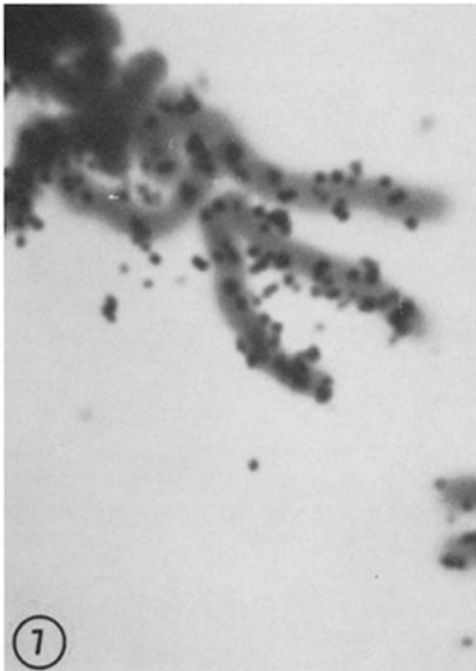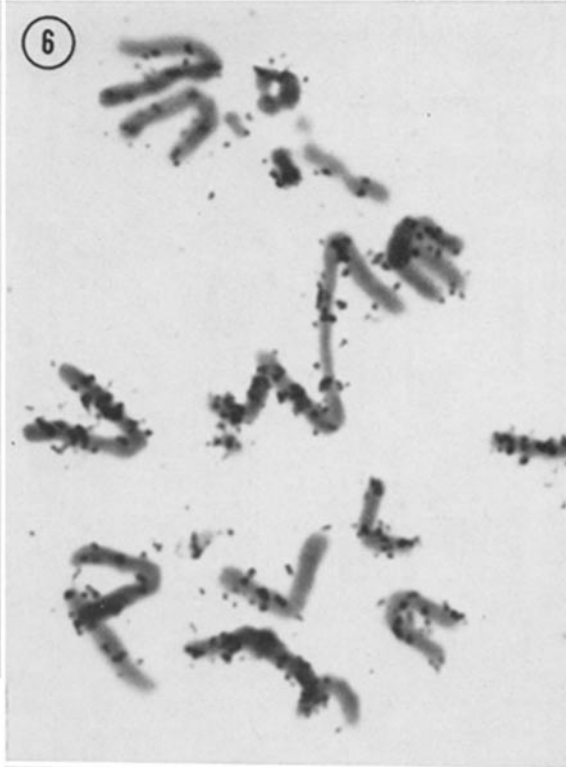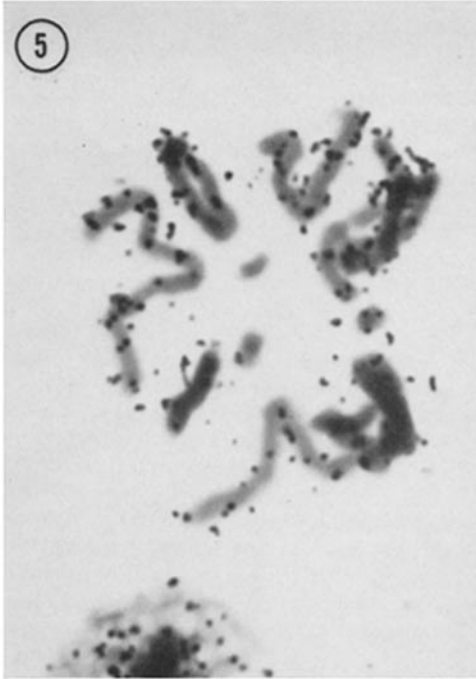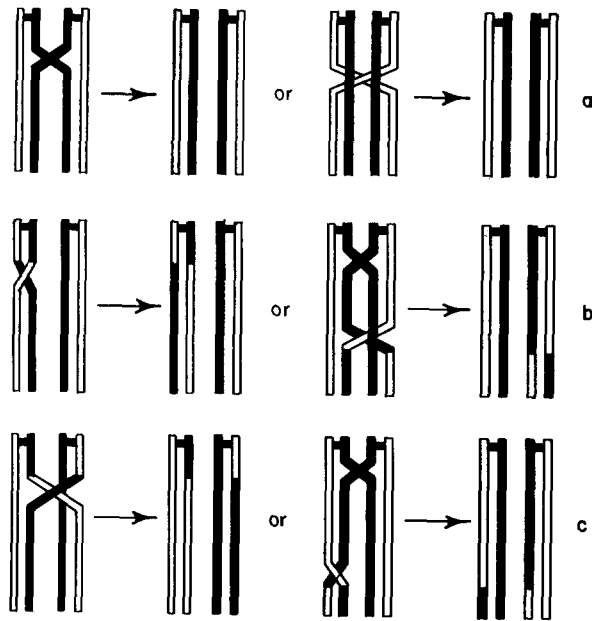
Figure 9 Diagram showing the consequences of various combinations of hidden crossovers (those which do not change the distribution of tritium), visible crossovers, and sister chromatid exchanges. Sister chromatids are shown connected at the centromere. Solid bars represent labeled chromatids, and outlined ones represent those which are unlabeled.

a. No visible exchanges, as in Fig. 10. These cannot have sister chromatid exchanges but may have a hidden crossover.

b. Apparent sister chromatid exchange, as in Fig. 11, but a hidden crossover proximal to a visible crossover would produce the same effect.

c. Apparent crossover, as in Fig. 12 (nonreciprocal switch point within each dyad). However, the same distribution can be produced by a sister chromatid exchange with a proximal hidden crossover.

Romalea) have both chromatids of each diad labeled (Figs. 5 and 7). Although there is considerable asynchrony of labeling among the chromosomes, some cysts have cells in which tritium is present in every chromosome, and most of the chromosomes are labeled along the whole length. If one chromatid has an unlabeled region, the sister or homologous chromatid usually can be seen to have a similar unlabeled gap. These observations are consistent with synchronous labeling of homologous regions of each chromosome. The most striking asynchrony among different chromosomes was noted with respect to the X chromosome, as mentioned previously. This chromosome frequently had little if any tritium even in cells where the other chromosomes were well labeled. This observation is important in view of the pattern of labeling in mature sperm which has been discussed elsewhere (Taylor, 1964).
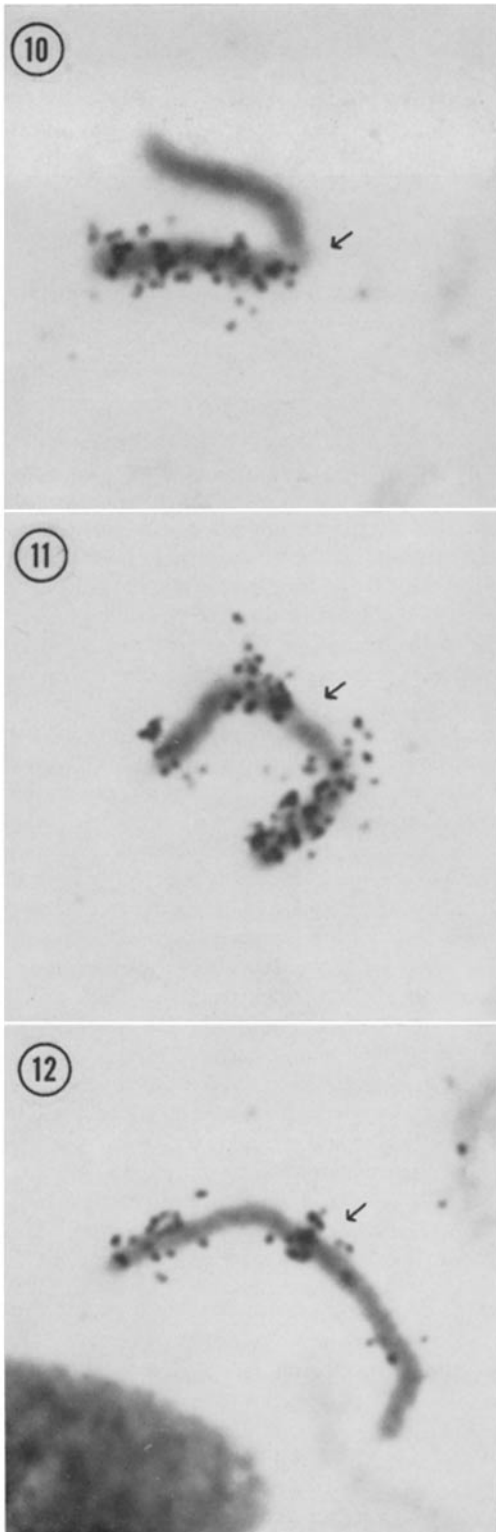
In the cells which are interpreted to have been labeled at the interphase preceding the last premeiotic spermatogonial division, the label segregates and it may be predicted that only two of the four homologous chromatids will be labeled. Of course, one must take into account the sister chromatid exchanges and crossovers which may have occurred; therefore, it is more nearly correct to say that about one-half the total length of each

group of four chromatids of a tetrad will be labeled. Cells observed in anaphase I on the 28th day following injection, when labeled, appeared to have this type of segregation of tritium (Fig. 6).

By the 28th day after injection a few well labeled cells were also found in metaphase II and anaphase II. Since these had about one-half of the total chromosome length labeled (Fig. 8), they were considered to have incorporated tritium at the second interphase preceding meiotic prophase.

The impression gained is that segregation follows the semiconservative pattern and that exchanges occur between labeled and unlabeled chromatids. However, the most convincing evidence for the semiconservative nature of segregation comes from the detailed analysis of dyads presented below with representative examples shown in Figs. 10, 11, and 12. According to this pattern, which has been observed in mitosis (Taylor *et al.*, 1957; Taylor, 1958; Prescott and Bender, 1963), all chromatids are labeled at the first division following one interphase labeling. This would be comparable to the labeling of meiotic chromosomes at the last premeiotic interphase in spermatogenesis.

When cells are labeled one cell cycle earlier, *i.e.*, in the interphase preceding the last spermatogonial mitosis, the chromosomes arrive at meiotic pro-

phase with the equivalent of one chromatid of each homologue labeled. However, the patterns of labeling will not be revealed until the chromatids separate at anaphase I. Even then one must take into account the sister chromatid exchanges and crossovers (exchanges between homologous chromatids) in interpreting the patterns of segregation. Fortunately some of the dyads are apparently not involved in exchanges, and the segregation of label is easily interpreted in these (Fig. 10). However, when exchanges occur the considerations illustrated in Figure 9 must be kept in mind. It should be noted that each sister chromatid exchange will be revealed by two switch points in separate chromatids, whereas one-half of the exchanges between homologous chromatids (crossovers) will involve chromatids both of which are labeled or unlabeled, and therefore will not be revealed. However, when exchanges are observed, they cannot be readily classified as sister chromatid exchanges or as crossovers either on the basis of an analysis of dyads or from analysis of the distribution of label in complete tetrads (pairs of homologous chromosomes).

With these limitations in mind, dyads from cells blocked at metaphase II by colchicine were analyzed for distribution of label. Most of the cells were broken by squashing, and the chromosomes scattered. Although such chromosomes do not provide a complete picture of the labeling pattern within a single cell or even a complete tetrad, they have the advantage that contact with the film will usually be close enough to prevent artifacts produced by shielding of the low energy beta particles of tritium. Examples of these dyads, all of which were apparently from two cysts of a male injected with $20 \mu c$ of thymidine-H^3 (6.7 c/mmole) 26 days before fixation, are shown in Figs. 10 to 12. The testis from which these chromosomes were derived was incubated for 6 hours in an aqueous solution of colchicine before fixation.

FIGURES 10 TO 12   Autoradiographs of dyads from cells which incorporated thymidine-H^3 one cell cycle before the premeiotic interphase (arrows indicate the terminal centromeres).

Fig. 10. Dyad with no visible exchanges. × 3600

Fig. 11. Dyad with proximal reciprocal switch points for labeled and unlabeled segments, and a distal non-reciprocal switch point. × 3600.

Fig. 12. Dyad with two non-reciprocal switch points for labeled and unlabeled segments. × 3600.

The dyads (half tetrads) could be classified into three groups: (a) those with one chromatid labeled over the entire length and the other one completely unlabeled (Fig. 10); (b) those with the first visible exchange, reading from the centromere toward the ends, reciprocal with respect to labeled segments (Fig. 11); and (c) those with the first exchange non-reciprocal with respect to labeled segments (Fig. 12).

The first group have no visible exchanges. However, they may have been involved in a crossover (exchange between homologous chromatids) in which two labeled or two unlabeled arms were exchanged (Fig. 9 a). Sister chromatid exchanges can be ruled out for this group; these would have produced visible switch points in two chromatids. The second group appear to be the result of sister chromatid exchanges, but the same distribution of labeled segments can be achieved by a hidden crossover (one involving both labeled or both unlabeled chromatids) proximal to a visible crossover (Fig. 9 b). The third group is produced either by a crossover between a labeled and an unlabeled chromatid or by a sister chromatid exchange accompanied by a proximal hidden crossover (Fig. 9 c). The most important characteristic of this latter group of exchanges is that they cannot be produced except by exchanges that involve homologous (non-sister) chromatids, unless separation of centromeres and proximal segments is equational at division I. Evidence presented below excludes this possibility. *Therefore, breakage and exchange between homologous chromatids must be the most frequent event producing the visible exchanges of labeled segments.*

The results of the analysis of 32 dyads of group A chromosomes and 38 of group B chromosomes are shown in Table II. The number of switch points was counted in each dyad. To show how these were scored, reference is made to Figs. 10, 11, and 12. The dyad in Fig. 10 has no switch points, but the dyad in Fig. 11 has one in the right chromatid at the same level as the first one in the left chromatid. There is also one near the end of the left chromatid. In Fig. 12 the right chromatid has no switch point but the left chromatid has two. The arrows show the position of the near terminal centromeres which join the two chromatids in each of the three photographs.

An analysis of the 70 diads used to obtain the data in Table II also indicates that the segregation of the centromeres and the portion of the chromo-

some arms proximal to the first crossover is reductional at the first division. This conclusion is, of course, consistent with genetic analysis in ascomycetes, but the conclusion can be arrived at independently from these autoradiographs. If the separation of centromeres and their proximal parts were randomly reductional or equational, one-half of the dyads would have no label or would have both chromatids labeled adjacent to the centromere. Out of 70 dyads only 5 show the above-mentioned pattern of equal segregation of label. Since such apparent equational segregation of label can also be produced by a crossover too near the centromere to leave a detectably labeled proximal segment, the low frequency of exceptions is easily accounted for on this basis. We may conclude that segregation is regularly reductional proximal to the first crossover.

An analysis of the frequency of exchanges per chromatid indicates that the distribution of exchanges is random. In Table III is given the frequency of chromatids with 0, 1, 2, 3, and 4 visible exchange points. Each crossover between a labeled chromatid and a non-labeled chromatid and every sister chromatid exchange produces two visible exchange points, one in each chromatid involved. The frequency of chromatids with 0, 1, 2, 3, and 4 exchanges fits a Poisson distribution with probabilities in a chi square test of 0.35 for group B chromosomes and 0.71 for group A chromosomes. The frequency of exchanges among the two groups is also approximately proportional to length of the chromatids, as might be expected of such random events.

Chiasma Frequency

The chiasma frequency for the chromosomes of groups A and B is given in Table IV. For group A

No. dyads analyzed	Visible switch points	Classification of dyads; refer to Fig. 10	Fig. 11	Fig. 12	Mean switch points per dyad
32 (group A)	57	7	7	18	1.8
38 (group B)	49	10	5	23	1.3

TABLE III

Comparison of Observed Switch Points per Chromatid with the Number Expected if the Distribution Is Random (Poisson Distribution)

No. chromatids analyzed	Mean switch points per chromatid	Switch points per chromatid					P value in a chi square test
		0	1	2	3	4	
64 (group A)	0.890	25	21	12	4	0 (observed)	0.71
		26	24	10	3	1 (expected)	
76 (group B)	0.644	36	31	9	0	0 (observed)	0.35
		40	25	9	2	>1 (expected)	

TABLE IV

Comparison of Observed Chiasma Frequency with the Number Expected if the Number per Bivalent is Random (Poisson Distribution)

No. bivalents analyzed	Mean chiasmata per bivalent	Chiasmata per bivalent								
		0	1	2	3	4	5	6	7	8
88 (group A)	3.67	0	0	0	35	47	6	0	0	0 (observed)
		2.3	8.3	15.1	18.5	17.0	12.5	7.7	4.0	1.8 (expected)*
74 (group B)	2.62	0	0	30	42	2	0	0	0	0 (observed)
		5.4	14.4	18.4	16.1	10.5	5.6	2.4	0.9	0.3 (expected)*

* P value in chi square test for both group A and group B is less than 0.01.

(chromosomes 2 and 3 only) the frequency is 3.67 chiasmata per bivalent at late diplotene. For group B the frequency is 2.62 at a similar stage. These data were collected to compare the number of observed chiasmata with the frequency predicted on the basis of the observed switch points. The chiasmata per bivalent, unlike the switch points, do not appear to be events occurring completely at random. When the frequency is compared with a distribution predicted on the basis of the Poisson equation, the fit is poor, and the conclusion is reached that some mechanism operates to maintain a minimum and perhaps a maximum number of events. The minimum number could be maintained by some pairing force or adhesion of telomeres which is active when a crossover fails to form within a certain distance of the distal end of the chromosome. A factor limiting the number of chiasmata is more difficult to visualize, if it actually occurs.

If, however, we ignore any such factors and assume that all chiasmata represent exchange between two homologous chromatids, we see that the frequency is nearly that predicted from the observed switch points. Each crossover produces two switch points per chromosome pair (tetrad). For group A the number of switch points per dyad is 1.8, or 3.6 per tetrad. However, one-half would be expected to be hidden, and the frequency would be 7.2. Since there are two switch points per crossover, this corresponds to 3.6 crossovers per tetrad. The chiasma frequency of 3.67 is almost a perfect fit. However, the switch points were determined among a group of chromosomes where the X dyad could not be distinguished from dyads of chromosomes 2 and 3. Therefore, the frequency observed may be lower than the actual frequency for chromosomes 2 and 3 only.

For the B group this correction is not necessary. Here the switch points indicate 2.6 crossovers per tetrad, and the chiasma frequency is 2.62. The correlation is exceedingly good if all switch points are assumed to be crossovers, i.e., if sister chromatid exchange does not contribute significantly to the observed switch points. To the extent that sister chromatid exchange may occur, therefore, the observed frequency of switch points would be too low to account for the number of chiasmata observed.

DISCUSSION

Since the distribution of exchange points is random, some conclusions may be drawn concerning the distinction between exchange between homologous chromatids (crossing over) and that between identical chromatids (sister chromatid exchange). Autoradiographic evidence has clearly shown that sister chromatid exchange occurs during the mitotic cycle in all the cells with large chromosomes which have been examined (Taylor, 1958; Marin and Prescott, 1964). However, information on the grasshopper is not available except the following observation which was made in this study. Chromosomes at diplotene may be seen to have unlabeled gaps when cells labeled two cycles before the last premeiotic interphase are examined. These cells have time to segregate labeled and unlabeled chromosomes before the premeiotic replication. Therefore, they go into meiosis with only a fraction of the chromosomes labeled. If there were no sister chromatid exchanges during spermatogonial divisions, each chromosome would arrive at meiosis with either one completely labeled chromatid or no labeled chromatids. All diplotene pairs (tetrads) would then be labeled along one chromosome or along both when pairing occurred between two labeled chromosomes. However, diplotene figures have been observed with unlabeled gaps which are not explainable on the basis of asynchronous replication. Therefore, some sister chromatid exchange occurs. No quantitative data are yet available. In any case there is no way to distinguish clearly individual sister chromatid exchanges from homologous exchanges during meiosis. However, a quantitative analysis even with the limited data available shows that sister chromatid exchange during meiosis is of low frequency as compared with homologous exchange and could be entirely absent.

The question to be asked is whether exchanges like those in Fig. 11 may be accounted for by double crossovers as shown in Fig. 9 c, or whether they are indeed sister chromatid exchanges. With an exchange frequency of 3.6 for the A group of chromosomes, one would expect a hidden crossover to precede a randomly placed visible crossover with a frequency of 0.9. Such a crossover has a mean distance from the centromere of one-half the length of a chromosome, and one-half of the crossovers are hidden. According to the Poisson equa-

tion, 23 per cent of the visible crossovers will be preceded by one or more hidden crossovers. When there are two (5 per cent of the time), the effect of the first will be canceled and the chance for more than two is about 1 per cent. Therefore, about 5.4 (17 per cent of the 32 diads) would be expected to have a visible crossover preceded by a hidden crossover. The observed frequency of this type (Fig. 11) was 7 for the A group. Therefore, the conclusion is that hidden crossovers can account for most of those observed, and that sister chromatid exchange occurs at a low frequency as compared with crossing over. As stated previously, it may be restricted to premeiotic stages.

The close correlation between the chiasma frequency and the observed switch points also fits with the idea that sister chromatid exchanges may be infrequent. As pointed out previously, the number of exchanges made visible by labeling with thymidine-H^3 will account for the observed frequency of chiasmata if all exchanges are assumed to be crossovers rather than sister chromatid exchanges. However, this type of reasoning becomes circular, because one would like to establish that exchanges produce the observed chiasmata rather than argue that a certain chiasma frequency indicates a comparable amount of exchange.

The observation that switch points made visible by tritium labeling are randomly distributed among chromatids, whereas chiasmata are not, indicates that there is not a necessary one-to-one relation between chiasmata and reciprocal exchanges. Though there is still no direct evidence that reciprocal exchanges produce the observed chiasmata, the idea is strongly supported by evidence reviewed in various textbooks (Darlington, 1937; Swanson, 1957). Perhaps the best rationalization of the present findings is the one mentioned in reporting the results, namely, that reciprocal exchanges result in chiasmata, but that other mechanisms have also evolved to ensure regular segregation in meiosis when crossovers fail to occur (see, for example, Cooper, 1949, 1964). The situation is better illustrated by chromosomes which regularly have one chiasma. If crossovers were distributed at random, many of these small tetrads (chromosome pairs) would have no chiasmata and segregation would not be normal at division I. Any mechanism which ensured terminal pairing would then have a high survival value.

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