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THE ELIMINATION AND DIFFERENTIATION OF CHROMOSOMES IN THE GERM LINE OF SCIARA*

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Abstract. The germ line chromosomes of *S. coprophila* have been followed from the time of origin of the germ cells up to the time of meiosis in the male and up to first larval molt in the female. The mechanism which prevents the accumulation of L (limited) chromosomes in the germ line is a unique process of chromosome elimination: it occurs in male and female embryos after the germ cells have migrated from the pole plasm to the definitive gonad site, and it involves the movement of whole L chromosomes through the nuclear membrane into the cytoplasm. The extra paternal X chromosome is eliminated from the germ cells at the same time and in the same manner. Following this elimination there is a cytological differentiation of the chromosomes remaining inside the nucleus. First, the 4 paternal homologues of the regular complement undergo a loosening of coils and become light-staining whereas the maternal homologues remain condensed like the L's. Next, the L chromosomes undergo a process of extreme attenuation and dispersion following which they return to the condensed state. H^3-thymidine autoradiography on gonial and premeiotic cells in the testis reveals that the L chromosomes undergo DNA replication at the end of the S period, also that there are asynchronies in DNA synthesis among the regular chromosomes. The phenomena of differential chromosome staining and asynchronous DNA replication are considered in the light of current theory regarding heterochromatization and gene inactivation, also in relation to the phenomenon of chromosome imprinting encountered in this genus (Summary see p. 274).

A. Introduction

The unusual patterns of chromosome inheritance, elimination, and differentiation which are found in *Sciara* have been studied by a number of investigators; genetic as well as cytological methods have been utilized (for reviews see METZ, 1938; BERRY, 1941; CROUSE, 1960a,

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1960b, 1965). There remains, nevertheless, one important gap in our knowledge of chromosome inheritance in this genus, namely the mechanism which prevents the accumulation of limited chromosomes in the germ cells.

In the female of *S. coprophila*, where meiosis follows the classical schema, the limited chromosomes (usually 2) pair and undergo reduction (SCHMUCK and METZ, 1932). In the male, however, during the monocentric first meiotic division all of the limited chromosomes (usually 2) move to the single pole with the 4 maternally-derived regular chromosomes and thus become included in *unreduced number* in the secondary spermatocyte and subsequently in the spermatid nucleus (METZ, MOSES, and HOPPE, 1926). Thus, it becomes necessary to postulate an elimination of these chromosomes; otherwise, with successive generations they would increase in number.

The behavior of the limited chromosomes at the first spermatocyte division presents another far more interesting problem which is related to the phenomenon of reversible chromosome imprinting manifest in this genus (see CROUSE, 1960b for discussion). At the monocentric meiotic mitosis the four regular chromosomes that move to the pole bear the maternal imprint (METZ, 1927). Once these chromosomes are inherited through the sperm, however, they give evidence — in the embryonic soma and germ line (DUBOIS, 1933; BERRY, 1941) and during spermatogenesis in the next generation of males — that the maternal imprint has been replaced by the paternal one. The fact that all of the limited chromosomes move to the pole in the monocentric division raises the question as to their origin. Because these chromosomes bear no known markers, it has not been possible to provide a genetic answer to the question. But if they are *not* exclusively of maternal origin — as certain evidence suggests (CARSON, 1946) — this would imply that the imprinting on the regular chromosomes takes place in nuclei in which the limiteds are inactive. In other words, the cytological behavior of the limited chromosomes might provide valuable clues for the study of imprinting. Thus, the experiments reported in this paper were undertaken with at least two objectives in mind: to determine the mechanism which regulates the number of limited chromosomes and to look for evidence of asynchronous behavior between limited and regular and between maternal and paternal chromosomes.

Theoretically there are a number of stages in the development of the germ line at which chromosomes could be eliminated, but only two were given serious consideration. The first study was on spermiogenesis (unpublished experiments by H. V. CROUSE and E. HOLTZMAN), but when it yielded negative results, the more difficult task was undertaken, namely, an investigation of the embryonic germ cells.

The embryonic germ cells of *S. coprophila* had been examined by previous investigators but with negative results (see reference to studies in SCHMUCK and METZ, 1932). In *S. ocellaris*, a species which completely lacks limited chromosomes, BERRY (1941) had undertaken a study of the early germ line of male and female embryos in the attempt to find the elimination of the extra X chromosome, which, in this genus, is characteristically transmitted to each zygote by way of the sperm as a result of the asymmetric second meiotic division. BERRY discovered the elimination of a chromosome from the early germ cells of both sexes. He found that the extra X is eliminated at a stage when the nuclear membrane is intact and when the chromosomes are in the form of fairly discrete bodies (prochromosomes). Moreover, he observed that immediately following elimination in both sexes the chromosomes inside the germ cell nuclei become differentially stained, 4 light-staining and 4 dark-staining. Although BERRY had no rearrangements with which to identify chromosome complements, he noted that of the two large prochromosomes inside the nuclei, one was always light and the other dark; and on this evidence he correctly deduced — as our experiments will demonstrate — that at this stage in germ cell development the two sets of homologues are clearly out of step with each other.

In view of BERRY's interesting findings it was deemed important to examine the germ cells of *S. coprophila* at a comparable stage of development, also to follow the male germ cells from the time of their formation up to first meiotic division, and, by means of H^3-thymidine autoradiography, to examine the premitotic and premeiotic S (synthesis) periods for evidence of asynchronous patterns of DNA replication. By way of perfecting cytological methods and as a prelude to the study on *S. coprophila*, we decided to repeat BERRY's work on *S. ocellaris*.

B. Material and Methods

I. Sciara Stocks

a) S. ocellaris COMSTOCK: Because the strains of flies used by Berry were no longer available, new collections had to be made. Through the kindness of Dr. UZI NUR wild type females were collected in the vicinity of Rochester, New York during the spring of 1963; from these we obtained the unisexual strain of *S. ocellaris* which was used in this study (species identification by H. V. CROUSE on basis of salivary gland chromosomes). Like BERRY's this strain had 3 rods and a V, the V being nearly twice as long as each of the rods. Because there were no sex-linked markers available, sex of progeny could not be predicted from the mother's phenotype; hence the sex of embryos had to be diagnosed from a group of siblings which were allowed to develop beyond the embryonic stage. In *Sciara* the ovary and testis can be distinguished from each other just before the larva hatches out of the egg (BERRY, 1941).

b) S. coprophila LINTNER: The various stocks described below were derived from the monogenic flies collected originally by Professor C. W. METZ and kept in

culture since 1925. Invaluable for the study were the two non allelic sex-linked mutants, Wavy and swollen. The former is a dominant located on an X′ chromosome, the latter a recessive located on X. Swollen females are thus XX in constitution and consequently male-producers (see Metz, 1938). Females heterozygous for both Wavy and swollen are phenotypically Wavy, non-swollen; they are X′X and consequently female-producers. By using these two mutants it was possible to predict sex of progeny prior to egg collection; in all cases, however, some embryos were allowed to develop as a double check on sex of progeny.

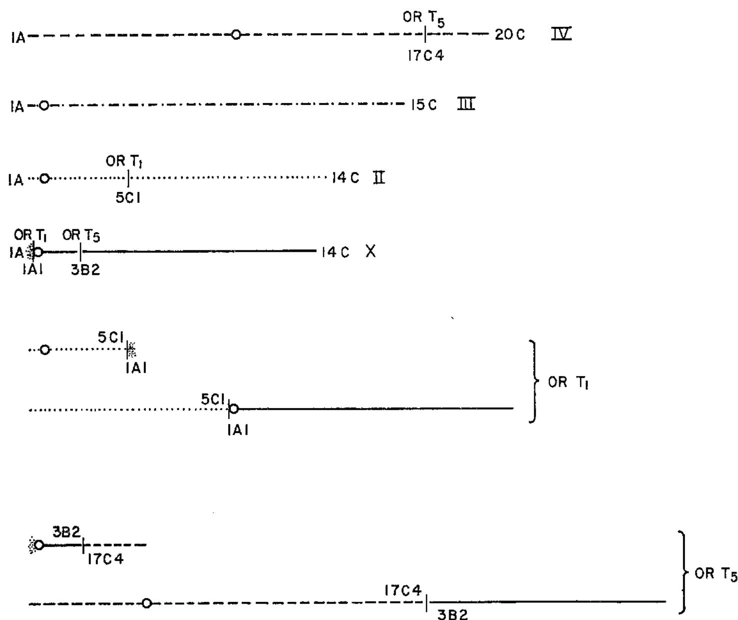


Fig. 1. Diagram showing relative lengths of salivary chromosomes in translocations OR T 1 and OR T 5. The nomenclature is according to the revised maps of *S. coprophila* (Gabrusewycz-Garcia, 1964)

Two different X-translocations were used, Oak Ridge T 1 (ORT 1) and Oak Ridge T 5 (ORT 5). The points of exchange in terms of the revised salivary chromosome maps for this species have just been published (Crouse, 1966). The translocation chromosomes, drawn to scale, are represented diagrammatically in Fig. 1. In this species the X and two of the autosomes, II and III, are rod-shaped and approximately equal in length; the other autosome, IV, is v-shaped and approximately 1.7 times as long as the rods (Crouse, 1943). As seen in Fig. 1, ORT 1 involves chromosomes X and II whereas ORT 5 involves X and IV. The important feature regarding each of these translocations is that two very unequal chromosomes are created. The small chromosome in each case is a rod. The large chromosome in ORT 1 is v-shaped and bears the centromere of X. The large chromosome in ORT 5 is a very large J and bears the centromere of IV. The inheritance of ORT 1 through the male and the female germ line been fully described (Crouse, 1960b); that of ORT 5 has been studied extensively but remains unpublished. The only statement which needs to be made here is the following: through the female germ line the translocations are inherited in an orthodox fashion (as in *Drosophila* or corn); but in both

cases during spermatogenesis — and also at the time of chromosome elimination in the embryo — the small translocation chromosome behaves like the X and the large one behaves like an autosome. Because the small chromosomes in ORT1 and ORT5 are so very small in comparison to the normal X and to the limited chromosomes, they are often referred to in the present paper as "dots".

II. Life Cycle of Sciara

The adult flies of *Sciara* live only a few days at room temperature. During this period copulation occurs, and the sperm are stored in the spermathecae until oviposition begins two or three days later. At oviposition each egg is in first meiotic metaphase and is fertilized as it is laid (Schmuck and Metz, 1932). By gently crushing the thorax, one can force mature females to deposit their eggs on sterile agar plates. In this manner all the eggs (100 to 150) from a single female can be collected within a 30 min period. Throughout this paper the age of an embryo at the time of egg deposition will be considered "0 hours".

It was noted in both species that adult flies derived from eggs collected within a 30 min period might be as much as 48—72 hours behind each other at the time of emergence. To reduce this asynchrony as much as possible and to prepare an accurate time-schedule of developmental events, the larvae were kept well-fed, crowding was avoided, and a selection of synchronized individuals was made at the definitive stages of development, i. e.; at each larval molt (3), at eye-spot formation in 4th instar, and finally at prepupation. Molting in *Sciara* is characterized by loss of the large black head capsule. Thus newly-molted larvae have colorless heads (for 30 min) and can be easily recognized, transferred to fresh agar, and timed from this point. For staging 4th instar larvae the anlagen of the adult eyes were most useful; these eye spots increase in size and pigment density, and their migration laterally to the sides of the head marks the beginning of the prepupal stage. In order to determine the number of days required for each developmental stage at 20° C many batches of eggs were collected and kept under nearly continuous surveillance by a team of two. The duration time of a particular stage (see Tables 2 and 16) was considered to be the number of days taken by those individuals *which completed that stage the most rapidly.*

III. Cytological Techniques

Different cytological methods were required for the different embryonic and larval stages. For the study of 0—16 hour-old embryos, the "eggs" were placed in a small drop of distilled water on an albuminized slide and the tough chorion pricked when the water had almost completely evaporated, so that the embryo would adhere to the slide. The slide was then immersed in Kahle's fixative (1 part glacial acetic acid: 6 parts formalin: 15 parts absolute ethanol: 30 parts distilled water) for at least four hours. After several rinses in 70 percent ethanol, the slides were dehydrated through 95 percent ethanol and 100 percent ethanol, and then extracted for twelve hours at 37° C in a 1:1 methanolchloroform solution. They were then rehydrated through an ethanol series (100 percent ethanol, 95 percent, 70 percent, 50 percent, 30 percent, distilled water), hydrolyzed in 1N HCl at 60° C for twelve minutes, and Feulgen-stained for 30 min in the Schiff reagent prepared according to Darlington and La Cour (1947, p. 116). They were then bleached in SO_2 water (1 part 1N HCl: 1 part 10 percent $K_2S_2O_5$: 20 parts distilled water), dehydrated, and mounted in euparal or, preferably, in balsam.

Older embryos, which contained fewer fat droplets, did not require such treatment. They were pricked forcefully in a drop of Shen Ringer on an albuminized slide, the excess solution removed, and a drop of 45 percent acetic acid applied. For permanent slides, the tissue was squashed directly in the 45 percent acetic acid, the

coverslip removed by freezing on dry ice, and the slide post-fixed overnight in Kahle's fixative. After rinsing several times in 70 percent ethanol the slides were rehydrated and Feulgen-stained as described above. For temporary slides, after the initial fixation in 45 percent acetic acid, the tissue was squashed in lactic orcein (100 mg. G. T. Gurr's natural orcein, 2.5 ml. glacial acetic acid, 2.5 ml. lactic acid).

Squashing of the larval and pupal stages differed only slightly from that of the older embryos. The gonads could be dissected out free of all surrounding tissues except in the case of the early first instar larvae. The initial fixative for temporary slides was Carnoy's (3 absolute ethanol: 1 glacial acetic acid), followed by 45 percent acetic acid. Both temporary and permanent procedures were otherwise identical to those described above. Liquid nitrogen as well as dry ice was used for removing the coverslips; the slide was dipped into liquid nitrogen until frozen, the coverslip was quickly removed, and then the slide was post-fixed in Carnoy's fixative carried out at the temperature of dry ice.

IV. Autoradiography

In the early experiments, late fourth instar larvae or young prepupae were injected with a very small quantity of a solution containing 50 μc/ml. tritiated thymidine (Schwarz Biological Laboratory, sp. act. 1.88 c/mM), and kept until mid-pupal life when spermatogenesis occurs. At that time the testes were dissected in Shen Ringer, fixed and squashed in 45 percent acetic acid on a gelatinized slide, and Feulgen-stained in preparation for filming.

Because the mortality was so high following injection, in later experiments the gonads were given a 30 min exposure in a drop of tissue culture medium containing 10 μc/ml. tritiated thymidine (New England Nuclear Corporation, sp. act. 5 c/mM), rinsed briefly in Shen Ringer, fixed and squashed in 45 percent acetic acid on a gelatinized slide, and Feulgen-stained in preparation for filming. The culture medium was one developed for *Sciara* tissues (CANNON, 1964); it was found to produce no striking cytological alterations in testes which had been cultured up to 30 hours.

All the slides were coated with Kodak autoradiographic stripping film AR-10 and stored for two to three weeks before development. For developing, the slides were immersed in half-strength Kodak D 19 at 17° or 18° C for four minutes, rinsed for 30 seconds in cold water, fixed in half-strength Kodak acid fixer for 10 min, rinsed in three changes of cold water, then cleared in half-strength Kodak hypo clearing agent for two minutes. Finally, the slides were washed for five minutes with three changes of cold water, dried, and examined without coverslips.

V. Drawings and Photographs

Some photographs were taken with a 35 mm. Exakta camera attached to a Wild research microscope, using either the 50 × or 100 × fluotar oil immersion objective. Other photographs were taken on a Tiyoda research microscope equipped with a Leitz apochromat 90 × oil immersion objective, using 4 × 5 sheet film at a plate magnification of 2000—3000 ×.

The drawings were made by use of a Zeiss drawing apparatus on a Zeiss research microscope equipped with a 100 × neofluar objective.

C. Observations

I. Pilot Study on S. ocellaris

We were able to confirm BERRY's observations in all the important details; the only discrepancy was in regard to the time at which the X

chromosome is eliminated. We agree that the elimination occurs after the germ cells have migrated from the pole plasm to the definitive gonad site, but instead of 33—34 hours post-oviposition, we found it to occur

Table 1. *X chromosome elimination and chromosome differentiation* in germ cells of Sciara ocellaris*

Age of embryos in hours (20° C)	Number embryos in study	Number cells in study	Number cells showing no elimination and no differentiation	Number cells showing differentiation and no elimination	Number cells showing elimination and no differentiation	Number cells showing elimination and differentiation	Percent cells cells showing elimination
65	6	13	4	1	4	4	62
66	7	13	1	0	3	9	92
67	7	23	2	0	5	16	91
Total	20	49	7	1	12	29	

* See text.

at 65—66 hours (Table 1). This discrepancy may reflect genetic differences between the two strains of *S. ocellaris*; it is due in part to the fact that our material was kept at 20° C, BERRY's at "room temperature".

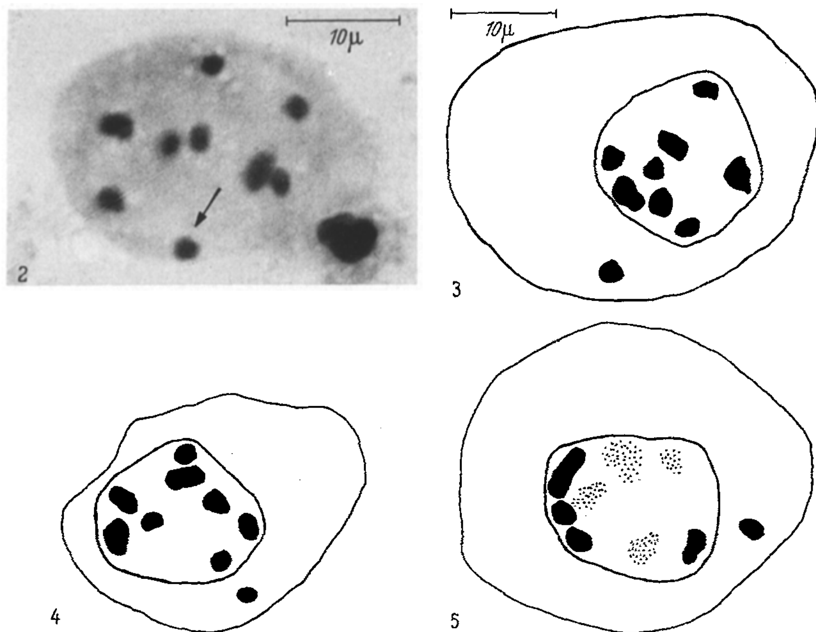
Four germ cells at the time of, or shortly after, X chromosome elimination are shown in Figs. 2—5. In each cell two chromosomes are clearly larger than the rest of the complement. In Fig. 2 the X is located below at the 6 o'clock position, caught just as it is passing out through the nuclear membrane. In the post-elimination stage in Fig. 3 one set of chromosomes is slightly more diffuse than the other, and in Fig. 5 the loosening of one set of homologues has gone to completion. This visible differentiation of chromosomes continues into late first larval instar. The eliminated X persists in the cytoplasm for a variable length of time, usually for several days.

Table 2. *Duration of the developmental stages of Sciara coprophila at 20° C*

Stage	Period of duration	
	males	females
Embryo	5 days	5 days
First instar	3 days	3 days
Second instar	2 days	2 days
Third instar	3 days	3 days
Fourth instar		
no eye-spots	4 days	6 days
eye-spots	2 days	2 days
Prepupal stage	16 hours	16 hours
Pupal stage	5 days	5 days
Imago	3 days	3 days

A careful study was made of the onset of differential staining in relation to X chromosome elimination (Table 1). Clearly, in this species elimination of the X comes first. Of the 41 post-elimination cells recorded, 29 showed differential staining. Of the 8 pre-elimination cells, only one

displayed differential staining; whether cells such as this eventually eliminate the extra paternal X remains unknown. By the 4th day of embryonic life the differentiation of homologues is visible in all germ cells, and it persists until late first larval instar.



Figs. 2—5[1]. Germ cells of *S. ocellaris* at 65—67 hours post-oviposition. Lactic orcein squashes. Fig. 2. The X chromosome (arrow) has just passed through the nuclear membrane. Fig. 3. X eliminated; 4 chromosomes in nucleus have become slightly more diffuse. Fig. 4. X eliminated; remaining 8 chromosomes all similarly stained. Fig. 5. X eliminated; the 2 sets of chromosomes have reached maximum differentiation

II. Studies on S. coprophila

Obviously, in the study on the limited (L) chromosomes of *S. coprophila*, it would be desirable to know precisely the number of L's contributed to the zygote by the egg and by the sperm. That this number varies seemed certain from the data already on record (cited above); nevertheless, exploratory studies were conducted on a variety of stages

[1] Figs. 2—71. With the exception of Figs. 2—5 all the germ cells were taken from *S. coprophila*. In the drawings, loosening of the regular chromosomes, i.e., "differentiation", is denoted by stippling; the condensed ones are represented in solid black. It was not possible to represent the state of condensation of the L-chromosomes accurately and at the same time identify them for the reader; consequently in all cases except Figs. 34—45, the L's have been represented arbitrarily by dense stippling. The embryonic germ cells shown in Figs. 2—38 were taken from 60—70 hour embryos except those in Figs. 6, 7, and 16.

including the spermatid, the zygote nucleus shortly after fertilization, first meiotic division in the female, and both meiotic divisions in the male. On the basis of these studies the following appraisal could be made: (1) a variable number of L's enters the nucleus of the spermatid and the secondary oocyte (2) the oocyte chromosomes are so small that a determination of the exact complement in more than a few ootids would be impractical (3) except for a very brief interval following the second spermatocyte division, the identity of separate chromosomes is lost throughout spermiogenesis, and only a Feulgen-positive nucleus can be followed (4) the chromosomes of the zygote show no differentiation between L's and regulars and they are so long and slender as to make precise identification of chromosomes almost impossible. In view of the insights gained through the exploratory studies, we decided to determine the range in number of L's in the primary spermatocyte, the stage when these chromosomes are most conspicuous and the counts, therefore, least susceptible to error; also to follow the development of the germ cells from the moment of their entry into the pole plasm.

1. The number of limited chromosomes in primary spermatocytes

In the primary spermatocyte the L's are prominent heterochromatic entities whereas the regular chromosomes are light, diffuse, and overlapping. The counts made in this study on 2800 cells are recorded in Table 3. They show that even within the same testis the number of L's may vary from zero to 4 and that spermatocytes with 2 L's are most common constituting 78% of the sample.

2. The morphology and division cycle of the embryonic germ cells

The germ cell nuclei undergo their first division 4—6 hours after oviposition, i.e. at the time when the somatic nuclei are in 5—8th cleavage. Table 4 shows the number of cells in the male germ line at various stages of development. Five embryos examined prior to fifth cleavage had from 2—6 germ cell nuclei, whereas 35 embryos examined during or just after eigth cleavage had 3—18 nuclei and an average of 10. Of these 40 early embryos, only 5 showed germ cell nuclei in metaphase or anaphase (Fig. 7); in the remainder the germ cells were in resting stage (Fig. 6) or prophase.

The counts recorded in Table 4 show the divisions in the early germ line are completed by 16 hours after oviposition. Overlapping of the cells at these early stages makes counting very difficult; hence few counts were obtained. Five embryos, however, had 23—31 germ cells and an average of 26. The average number of germ cells per testis in later embryonic and early larval stages was found to be 14. Since there are two gonads

Table 3. *The frequency of limited chromosomes in primary spermatocytes of Sciara coprophila*

Slide number*		Percent spermatocytes containing				
		0 limiteds	1 limited	2 limiteds	3 limiteds	4 limiteds
E-1	(1)	0	9	69	20	2
	(2)	0	0	91	9	0
E-2	(1)	0	1	82	17	0
	(2)	0	0	98	2	0
E-4	(1)	0	37	59	4	0
	(2)	2	11	64	22	1
W-9	(1)	0	0	93	7	0
	(2)	0	1	71	28	0
W-17	(1)	0	18	45	21	15
	(2)	0	24	71	5	0
H-9	(1)	0	7	93	0	0
	(2)	0	5	88	3	4
F-3	(1)	0	1	75	24	0
	(2)	0	5	80	15	0
T-3		1	2	63	33	1
T-4		1	2	93	4	0
W-14		0	2	97	1	0
W-16		3	21	74	2	0
H-2		0	1	91	7	1
H-3		0	1	84	15	0
H-4		0	11	88	1	0
H-5		0	10	84	6	0
H-7		1	72	27	0	0
H-8		0	14	81	5	0
H-11		0	2	93	5	0
H-13		0	25	72	3	0
H-14		0	6	92	2	0
H-15		0	29	65	6	0
Totals		8	317	2183	268	24
Totals expressed in percentages		0.3	11.3	78.0	9.5	0.9

* Each slide represents one larva or pupa. Both testes could be counted on the first seven slides, and are designated (1) and (2). On the remaining 14 slides, only one testis could be counted.

per animal, the average number of germ cells at these stages is 28, which falls within the range of cell counts found at 16 hours. This observation

Table 4. *Germ cell number per testis at various developmental stages of S. coprophila*

Stage (20° C)	Number testes examined	Range in cell number per testis	Average number cells per testis
Embryo			
4—6 hours			
pre-soma elimination	5	2—6*	4*
post-soma elimination	35	3—18*	10*
16 hours	5	23—31*	26*
48 hours-hatching	14	10—18	14
First instar			
0—50 hours	35	9—20	14
51 hours-first molt	27	9—35	19
Second instar			
0—23 hours	19	15—38	24
24—47 hours	9	33—82	51
48—71 hours	20	40—114	69
72—96 hours	2	80—100	90
Third instar			
0—23 hours	8	100—160	134
24—47 hours	14	199—325	243
48—71 hours	20	219—554	386
72—96 hours	9	405—715	593
Fourth instar			
0—23 hours	1	678	678
72 hours and later	13	600—1285	972

* These counts are for total number of germ cells; the individual gonads are not yet formed.

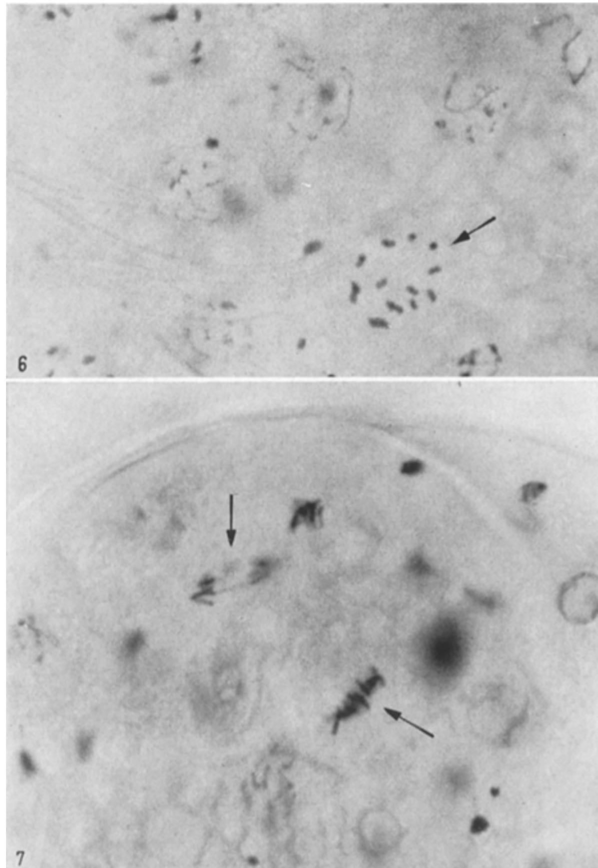
shows that not only are the first divisions of the germ cells completed by 16 hours, but also that the next series of divisions does not begin until late first instar *approximately 6 days later. During this long interval the chromosomes remain in a resting stage.*

The morphology of the germ cell nuclei is very distinctive. They are larger than the somatic nuclei (Fig. 7). Their resting stage, unlike that of the soma, is of the prochromosomal type, and each chromosome forms a compact, slightly irregular body (Fig. 6). All the chromosomes. L's and regulars, remain dark-staining (Figs. 6 and 16) until 48 hours post oviposition at which time one set of the regular chromosomes becomes diffuse and light-staining. This visible differentiation of homologues will be considered in detail below.

3. The Number of Limited Chromosomes in Early Embryonic Germ Cells

Considerable care had to be exercised in determining the number of chromosomes in embryonic germ cells. Prior to 60 hours there are several sources of error which make chromosome counts unreliable. The best

early germ cell nucleus (6 hour embryo) which was obtained is shown in Fig. 6. Even in this figure the number of prochromosomes cannot be stated unequivocally; that it contains at least 12 is certain. Counts were made, therefore, on 60—70 hour embryos. Although chromosome elim-



Figs. 6 and 7. Feulgen-stained eggs of *S. coprophila*, 6 hours post-oviposition. Fig. 6. Germ cell nucleus marked by arrow contains at least 12 prochromosomes. Fig. 7. Arrows mark germ cell nucleus in metaphase and somatic nucleus in anaphase

ination had just occurred in some of the germ cells studied, the eliminated chromosome(s) was (were) still clearly visible in the cytoplasm. The results from these counts are presented in Table 5. Of the 170 embryos studied, 56 percent had twelve chromosomes, i.e. 3 L plus 9 regular (Fig. 8), 18 percent had eleven, 2 L plus 9 regular (Fig. 9), 22 percent had thirteen, 4 L plus 9 (Fig. 10), and a small percentage of the cells had ten or fourteen chromosomes, i.e. 1 or 5 L chromosomes respectively. As

Table 5. *Chromosome number in the germ cells of individual 60—70 hour embryos of Sciara coprophila**

Type of embryos	Number of embryos in each class					
	Number of chromosomes	10	11	12	13	14
	Number of limiteds	1	2	3	4	5
Female embryos from several mothers		0	11	30	10	2
Female embryos from one mother		2	4	24	7	3
Total female embryos		2	15	54	17	5
Male embryos from several mothers		1	8	21	12	0
Male embryos from one mother		0	7	21	7	0
Total male embryos		1	15	42	19	0
Total male and female embryos		3	30	96	36	5
Totals expressed in percentages		2	18	56	21	3

* These counts include any chromosomes already eliminated and lying in cytoplasm.

indicated in Table 5 similar frequencies of chromosome numbers were obtained among the embryos derived from single or from multiple mothers and among embryos of opposite sex. Barring mitotic nondisjunction, the chromosome number in all germ cells within a given embryo is expected to be the same. Although this was found to be the case, it should be noted that only thirteen embryos were suitable for counting more than three germ cells per embryo (Table 6). In 70 hour embryos there are approximately 30 germ cells, but most of them are lost or rendered otherwise unsuitable for counting by the squashing process.

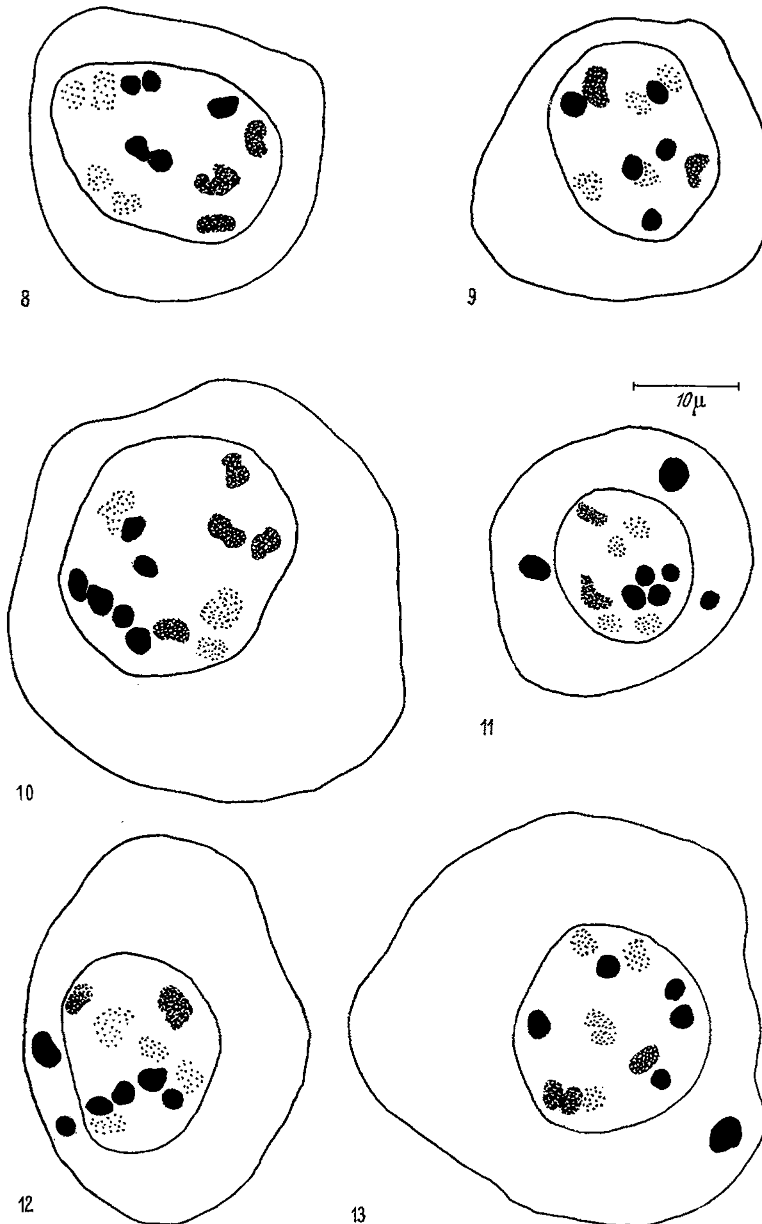
Table 6. *Chromosome number in the germ cells of thirteen 70 hour male embryos of S. coprophila in which counts could be obtained from at least four cells **

Chromosome number	10	11	11	11	12	12	12	12	12	12	13	13	13
Number of germ cells used for counts	4	7	4	4	8	7	10	5	7	5	4	4	6

* These counts include any chromosomes already eliminated and lying in cytoplasm.

4. Elimination of Chromosomes from the Embryonic Germ Cells

All but two L chromosomes are eliminated from the resting germ cell nuclei at the same stage of development as the extra paternal X (Figs. 12 and 15). As shown in Table 7, elimination usually occurs in both male and female embryos 65—72 hours post-oviposition, i.e. *at least* 48 hours after the last mitotic division of the germ cells. In this study the criterion for determining whether an embryo had begun elimination was the observation of *at least one germ cell* in which elimination of a chromosome, limited or X, had taken place. The earliest elimination was observed at



Figs. 8—13. Lactic orcein squashes of germ cells from normal stock. Fig. 8. Three L-chromosomes; the 5 dark chromosomes include the uneliminated paternal X. Fig. 9. Same as Fig. 8 except nucleus contains only 2 L's. Fig. 10. Four L's; only 3 regular chromosomes show differentiation. Fig. 11. The X and 2 L's have been eliminated at well-separated sites along the nuclear membrane; 2 L's remain in the nucleus. The 4 dark homologues show spatial aggregation. Fig. 12. The X and one L have been eliminated; 2 L's remain in the nucleus. Fig. 13. One L has been eliminated, leaving 3 L's and the uneliminated X inside the nucleus

Table 7. *Chromosome elimination in the embryonic germ cells of Sciara coprophila*

Age of embryo in hours (20° C)	Number male embryos in study	Number male embryos which had begun elimination	Percent male embryos which had begun elimination	Number female embryos in study	Number female embryos which had begun elimination	Percent female embryos which had begun elimination	Total number embryos in study	Number embryos which had begun elimination	Percent embryos which had begun elimination
48	5	0	0	—	—	—	5	0	0
50	2	0	0	—	—	—	2	0	0
53—56	13	0	0	7	1	14	20	1	5
57—59	2	1	50	—	—	—	2	1	50
63—65	8	3	38	11	10	91	19	13	68
66—68	15	10	67	7	5	71	21	14	67
69—71	16	12	75	15	12	79	30	23	77
72—74	4	4	100	15	13	87	19	17	89
75—79	15	15	100	17	17	100	32	32	100

56 hours and the oldest embryo in which elimination had not yet occurred was 74 hours. Moreover, within the germ cells of a given embryo there was not complete synchrony. For example, although all of the embryos examined 75—79 hours post-oviposition (Table 7) had begun elimination, 7 of the 90 cells had not yet eliminated any chromosomes (Tables 8 and 9).

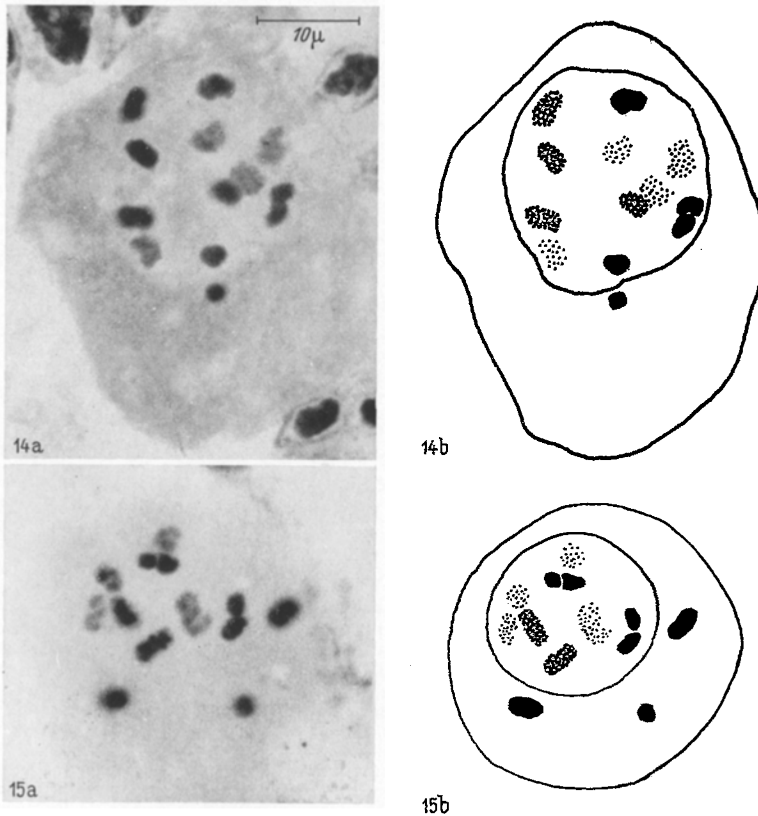
Table 8. *Chromosome elimination and chromosome differentiation* in germ cells of female embryos of Sciara coprophila*

Age of embryos in hours (20° C)	Number embryos in study	Number cells in study	Number cells showing no elimination and no differentiation	Number cells showing differentiation and no elimination	Number cells showing elimination and no differentiation	Number cells showing elimination and differentiation	Percent cells showing elimination	Percent cells showing no differentiation
53—56	7	44	0	42	0	2	5	0
63—65	10	54	0	20	0	34	63	0
66—68	7	19	0	10	0	9	47	0
69—71	15	45	4	21	0	20	44	9
72—74	15	60	0	9	0	51	85	0
75—79	17	40	0	3	0	37	93	0
Total	71	262	4	105	0	153		

* See text, p. 247 for description of differentiation.

As in *S. ocellaris*, elimination occurs after the germ cells have migrated from the pole plasm at the posterior end of the egg to the site of gonad formation. The germ cells at this time are grouped into two loose clusters which become organized into distinct gonads at approximately 80 hours post-oviposition. The elimination process apparently involves the migra-

tion of pro-chromosomes directly through the nuclear membrane into the cytoplasm. The nuclear membrane seems undisturbed and shows no visible gaps or projections (Figs. 12, 14, and 15).



Figs. 14 and 15. Photomicrographs of germ cells from normal stock; lactic orcein squashes. Fig. 14. X has been eliminated; 4 L's remain in nucleus; one set of homologues is clearly differentiated. Fig. 15. Nucelar membrane cannot be followed in photomicrograph; X chromosome and 2 L's have been eliminated. In the nucleus there are 2 L's, 4 light-staining regulars and 4 dark-staining regulars which show clustering

The elimination of the extra L chromosomes may or may not coincide with that of the X in a given germ cell (Figs. 13 and 14); nor do all germ cells within a given cluster exhibit the same elimination pattern (Figs. 17 and 18). Some cells have eliminated only the L chromosome(s) (Fig. 17); others have eliminated only the X (Fig. 18); still another group has eliminated both. The eliminated chromosomes are generally arranged at random in the cytoplasm as though they passed through the nuclear membrane at different points (Figs. 11 and 15).

Table 9. *Chromosome elimination and chromosome differentiation* in germ cells of male embryos of Sciara coprophila*

Age of embryos in hours (20° C)	Number embryos in study	Number cells in study	Number cells showing no elimination and no differentiation	Number cells showing differentiation and no elimination	Number cells showing elimination and no differentiation	Number cells showing elimination and differentiation	Percent cells showing elimination	Percent cells showing no differentiation
48	5	15	15	0	0	0	0	100
50	2	11	5	6	0	0	0	50
53—56	13	38	8	30	0	0	0	20
57—59	2	5	0	4	0	1	20	0
63—65	8	19	1	13	0	5	26	5
66—68	15	47	0	23	0	24	51	0
69—71	16	44	1	15	0	28	64	2
72—74	4	44	0	3	0	11	79	0
75—79	15	50	0	4	0	46	92	0
Total	82	249	30	114	0	105		

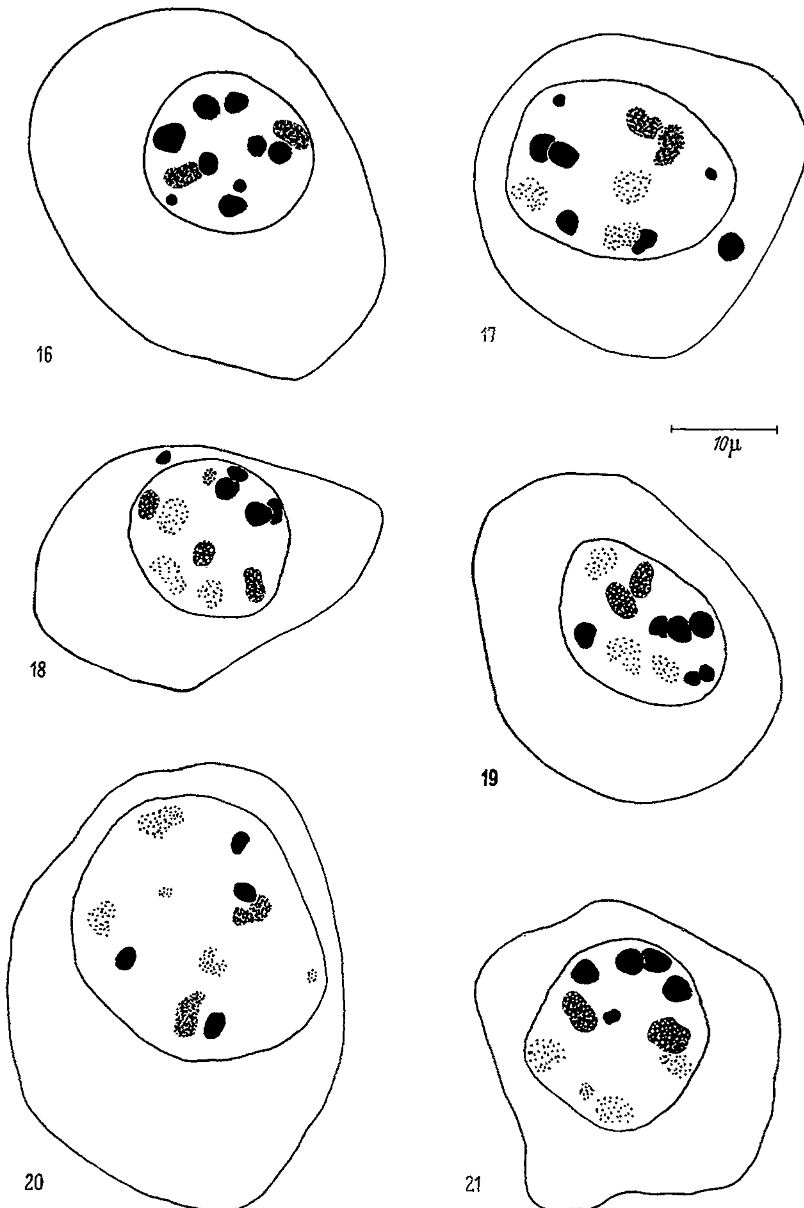
* See text for description of differentiation.

The eliminated chromosomes remain in the cytoplasm as *very dark-staining bodies similar to prochromosomes.* They usually persist in the cytoplasm until the middle of first instar, i.e. for 4—5 days. They were never observed in a process of disintegration; they seem to disappear suddenly.

The number of L chromosomes eliminated depends on the number originally present in the zygote. In every germ cell examined all but two L chromosomes were eliminated. Thus, in germ lines containing either two, three, four, or five L chromosomes, an elimination of zero (Fig. 22), one (Fig. 12), two (Figs. 11 and 15), or three L chromosomes respectively takes place.

5. Differential Staining of the Embryonic Germ Cell Chromosomes

Unlike the regular chromosomes of *S. ocellaris* those of *S. coprophila* become diffuse and light-staining *prior to* chromosome elimination in both female (Table 8) and male (Table 9) embryos. This differentiation first becomes visible 2 days after oviposition; it involves a gradual destaining and loosening of 4 chromosomes. Prior to elimination, cells are found in which 3 of the 4 chromosomes have become light (Fig. 10); less often 4 light-staining chromosomes are observed (Figs. 8 and 9); never in normal (non-translocation) material were 5 light-staining regular chromosomes found in pre-elimination germ cells. Once the X chromosome had been eliminated, the nuclei regularly contained 4 light-staining chromosomes (Figs. 11—15).



Figs. 16—21. Germ cells of embryos derived from OR T 1 males; lactic orcein squashes. Fig. 16. Germ cell from 48-hour embryo prior to chromosome differentiation and elimination. All 11 chromosomes are condensed and dark-staining. Fig. 17. One L has been eliminated; both paternal sex chromosomes (the dots) still remain dark-staining. Three of the paternal homologues have undergone differentiation and are light. Fig. 18. Germ cell from the same embryo as preceeding figure. One dot has been eliminated; the remaining 4 paternal homologues inside the nucleus are light-staining. Fig. 19. Both dots remain dark-staining. The other 3 paternal homologues are light. Fig. 20. All 5 paternal homologues, including the 2 dots, are light-staining. Fig. 21. Four of the paternal homologues are light-staining; the fifth (one of the dots) remains dark

That the 4 light chromosomes of *S. ocellaris* represent a set of homologues was deduced by BERRY (1941). In *S. coprophila* by using translocations OR T 1 and OR T 5 (described above) it was possible to demonstrate that the regular chromosomes which become light-staining are of paternal origin. The germ cells of the F_1 progeny derived from reciprocal crosses between the translocations and normal stock will be described below.

The limited chromosomes undergo an uncoiling process somewhat later and become as light-staining and fluffy as the four paternal homologues. At the time of elimination, however, they are generally dark-staining, sometimes slightly diffuse, but only occasionally quite light-staining (Fig. 22). The staining behavior of the L chromosomes varies among germ cells from the same embryo and even within the same germ cell. In some cells a limited chromosome may appear partially light-staining and partially dark-staining (Fig. 35). By 96 hours post oviposition all L chromosomes, were found to be light-staining and diffuse; at this time the regular chromosomes still retained their differential staining (Fig. 39).

Aggregation of the dark-staining regular (maternal) chromosomes into groups of two, three or four is often observed. Although some cells exhibit this clustering at the time of elimination (Fig. 26), it is most pronounced in germ cells from older embryos (Fig. 39) and first instar larvae (Fig. 40). Such clusters remain until the middle of the first instar at which time the chromosomes become less condensed. Similar aggregations were not found in *S. ocellaris.*

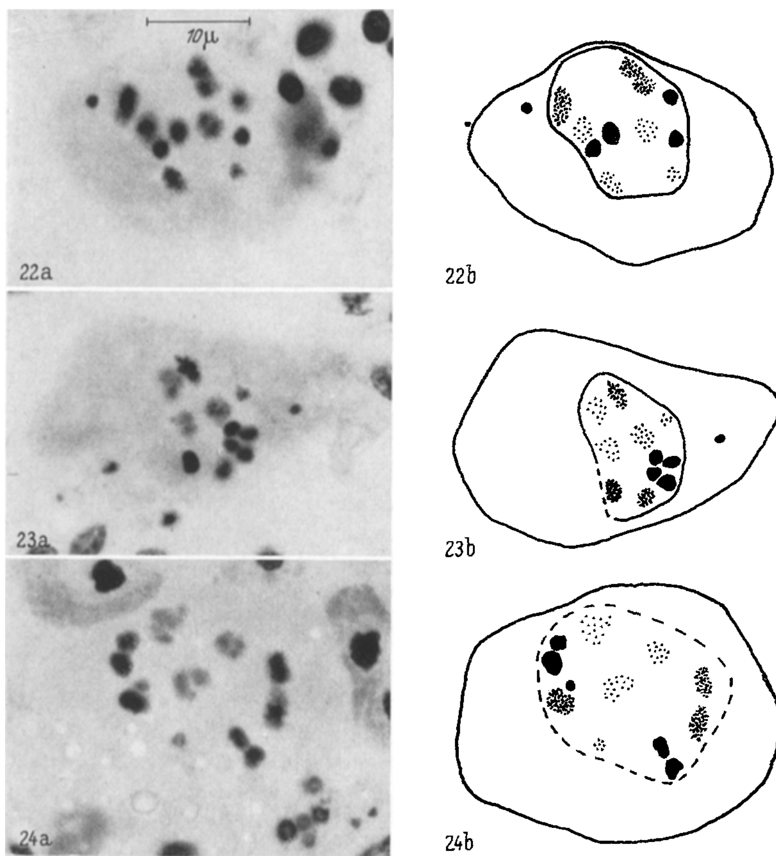
6. Use of Translocations OR T 1 and OR T 5 in Identifying the Differentially-Stained Chromosome Sets

The effects on chromosome morphology produced by the two reciprocal translocations have been described above and are shown in Fig. 1 (p. 234). The significant point for the present studies is that as a result of each translocation the two new chromosomes produced were so unequal as to be distinguishable from the normal complement even in the non-dividing germ cell nuclei; in both cases the very small translocation chromosome will be referred to as the "dot".

In setting up the reciprocal crosses, one point had to be borne in mind, namely, that normal male-producing females (XX) bred to translocation males yield no progeny (CROUSE, 1960b); consequently female-producers (XX′) had to be used in the crosses with the OR T 1 and OR T 5 males. For the reciprocal matings, theoretically either male-producers (XX^t) or female-producers ($X'X^t$) could be used; both were used and yielded essentially the same results.

a) Normal Female-Producers (XX′) Bred to OR T 1 Males: Of the various crosses, the one studied most thoroughly involved OR T 1 males

bred to normal female-producers (XX′). There were several reasons for this. In the first place, OR T 1 males are far more fertile than males from OR T 5. Second, the "sex" chromosome (dot) transmitted in duplicate through the sperm can be recognized as such in the germ cells of all the



Figs. 22—24. Photomicrographs of germ cells of embryos derived from OR T 1 males; lactic orcein squashes. Figs. 22 and 23. One of the paternal sex chromosomes (the dots) has been eliminated; the other four paternal homologues have undergone differentiation and are light-staining. Fig. 24. Both dots still remain in nucleus; one is light-staining, the other dark-staining. The other 3 paternal homologues are light

F_1 embryos. This is unlike the situation in normal stock where, prior to elimination, there are 7 small chromosomes, two of which are the paternal X's. It is also unlike the situation in the reciprocal cross in which OR T 1 is introduced through the egg; here *only half* of the F_1 embryos are expected to inherit the translocation chromosomes.

All of the germ cells shown in Figs. 16—31 came from 40 F_1 embryos derived by breeding normal female-producers (XX′) to OR T 1 males.

In all of the cells (except the one in Fig. 28) 2 dot chromosomes can be seen. This picture is in accordance with, and provides cytological confirmation of, the mode of inheritance outlined previously for this translocation (CROUSE, 1960b).

Theoretically, one of the paternally-derived dots should be retained and the other one eliminated from the nucleus. This pattern of behavior

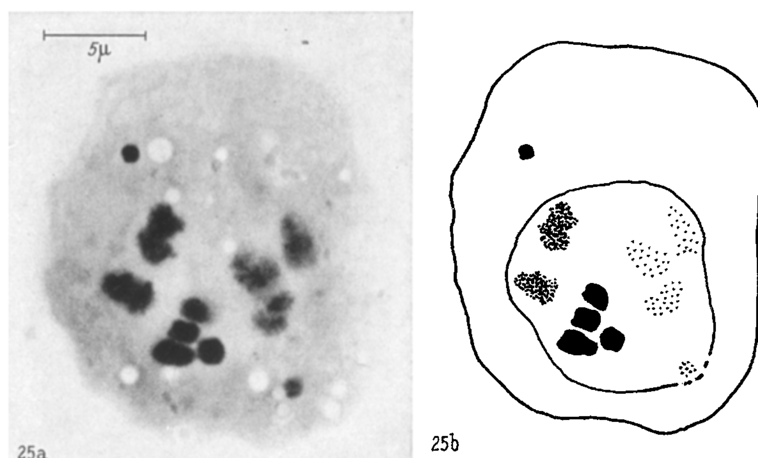
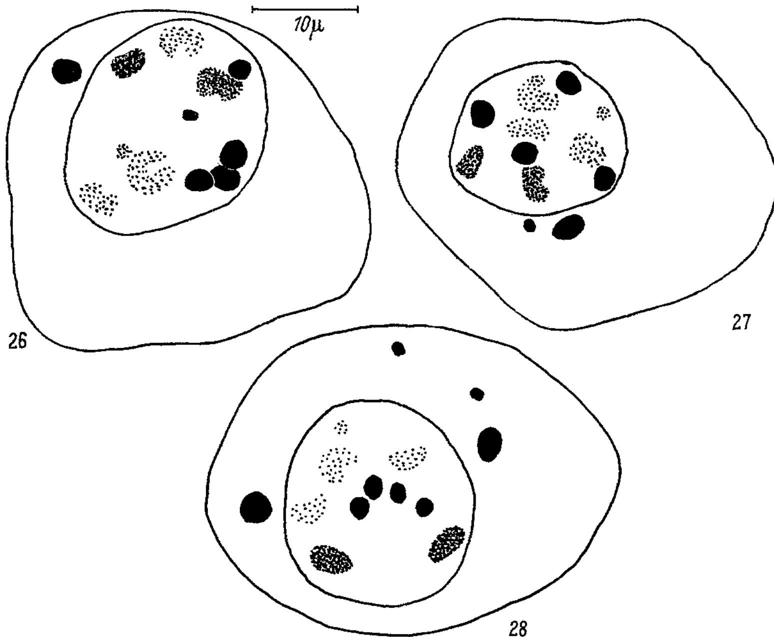


Fig. 25. Photomicrograph of a germ cell of embryo derived from OR T 1 male; lactic orcein squash. One paternal sex chromosome (dot) has been eliminated. The 4 paternal homologues remaining in the nucleus have undergone differentiation and are light-staining. The 4 maternal homologues remain darklystained and show clustering

was observed in 33 of the 40 embryos; in the remaining 7, abnormal patterns of dot behavior were found. From some of the germ cells in 6 embryos, both dots had been eliminated (Fig. 30); and in two of the embryos which showed such cases of erroneous double elimination, a third dot was present inside the nucleus (Fig. 28). In the 7th embryo — although the germ cells contained no eliminated dot — there was only one dot in the nucleus. These abnormalities suggest that errors in elimination are made by this tiny translocation chromosome, also that in pre-elimination germ cells the dot most likely undergoes occasional mitotic non-disjunction. Except for the abnormalities cited, elimination of the OR T 1 dot was on schedule (developmental stage) and was otherwise indistinguishable from the elimination of a normal, non-translocated X.

As pointed out previously, the embryos derived from OR T 1 males presented a unique opportunity for examining the precise relationship between sex chromosome elimination and the onset of differential staining of this chromosome. It will be recalled that in normal, non-translocated material prior to elimination, only 3 light chromosomes were found in most of the germ cell nuclei; ocassionally 4 but never 5 light-staining

chromosomes were observed. The essential question is, given a much larger sample of germ cells, would an appreciable number be found at the pre-elimination stage in which both paternally-derived sex chromosomes were light-staining. In embryos derived from OR T 1 males, any nucleus would qualify for this study if the 2 dots could be observed; in normal

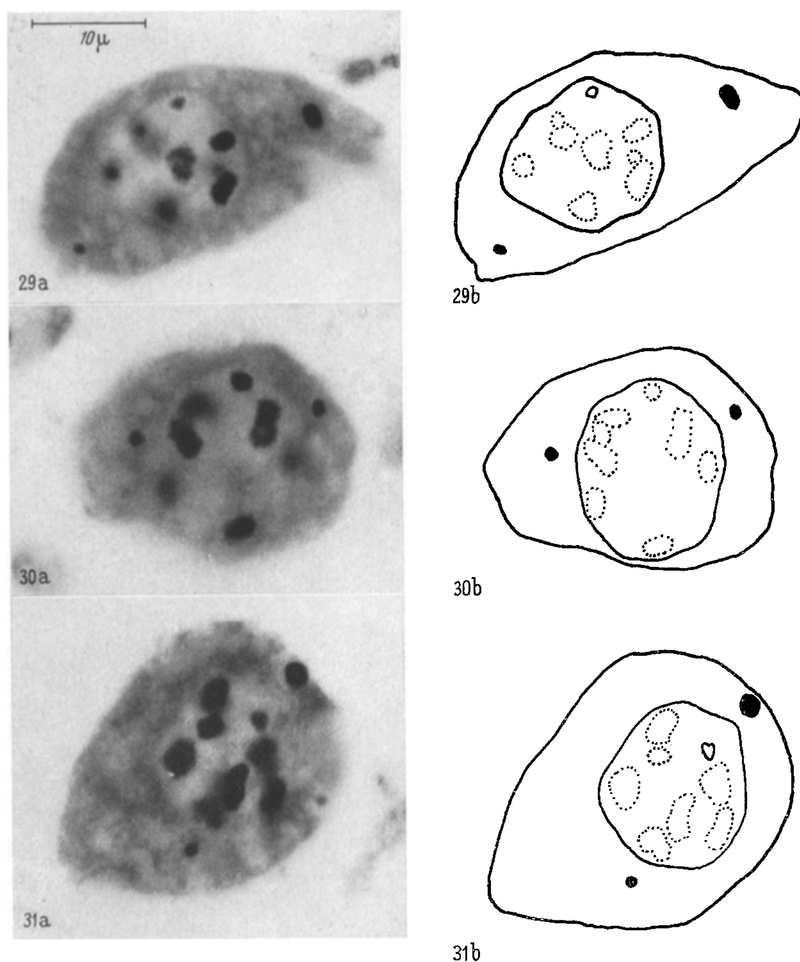


Figs. 26—28. Germ cells of embryos derived from OR T 1 males; lactic orcein squashes. Fig. 26. One L has been eliminated. One of the paternal sex chromosomes (dots) is still dark-staining; the other dot and the 3 remaining paternal homologues have undergone differentiation and are light-staining. Three of the 4 dark-staining maternal homologues show clustering. Fig. 27. One L and one dot have been eliminated. The 4 paternal homologues in the nucleus are light-staining. Fig. 28. Two L's have eliminated. This cell contains 3 paternal sex chromosomes (dots), 2 of which have been eliminated; the third remains inside the nucleus and is light-staining like the other (3) paternal homologues

material, on the other hand, only those nuclei would qualify in which identification of all the regular chromosomes was possible.

Among the 40 F_1 embryos derived from the OR T 1 males, a variety of conditions was found in the germ cells undergoing chromosome elimination and differentiation (Figs. 17—25). Taken together, they establish the fact that *it is the paternal set of homologues which becomes light-staining.* As to the staining properties of the dot chromosomes prior to elimination from the nucleus, three conditions were found: both dots were dark (Figs. 17 and 19); one was dark and the other light (Figs. 21 and 24); and both were light (Fig. 20). This third condition — the counterpart of which was never observed in normal, non-translocation material —

apparently occurs only very rarely; of the entire population of germ cells examined in the 40 embryos under consideration, *only four cells were found to manifest this condition.* Thus, it appears that of the two paternal sex chromosomes only one becomes light-staining; the uncoiling of this chromosome seems to lag slightly behind that of the paternally-derived



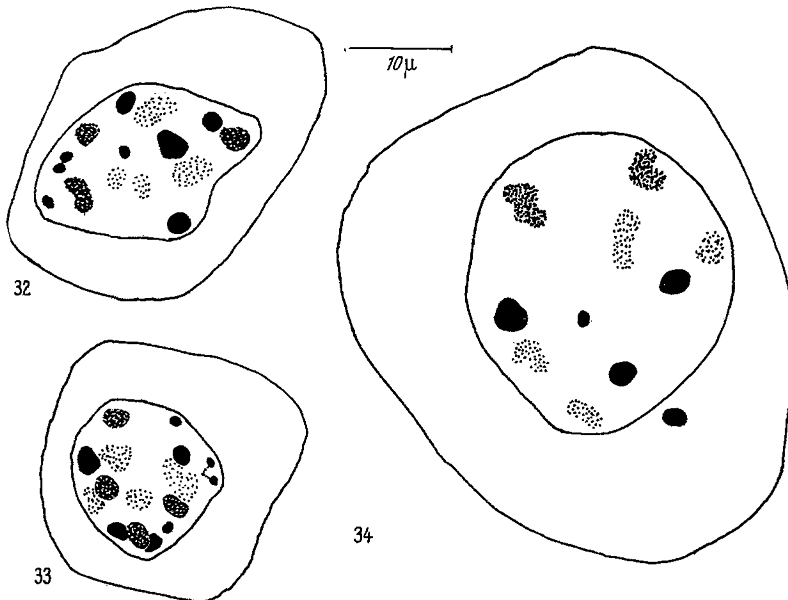
Figs. 29—31. Photomicrographs of germ cells of embryos derived from OR T 1 males; lactic orcein squashes. Fig. 29. One L and one paternal sex chromosome (dot) have been eliminated. Fig. 30. Both dots have been eliminated. Fig. 31. One L and one dot have been eliminated

autosomes and to nearly coincide with the elimination of the other (dark) paternal sex chromosome.

The cells shown in Figs. 29—31 are included in spite of the fact that the chromosomes inside the nucleus cannot be identified. They form a sharp contrast to those

in Figs. 22—25 in which all the chromosomes can be identified but whose nuclear membranes have been excessively stretched or ruptured in the squash procedure. In other words, in order to obtain a good photomicrograph, cells had to be used whose nuclei had been severely flattened.

b) OR T 1 Females (XX^t or $X'X^t$) Bred to Normal Males. Because the females in this cross were heterozygous for OR T 1, only half of the F_1

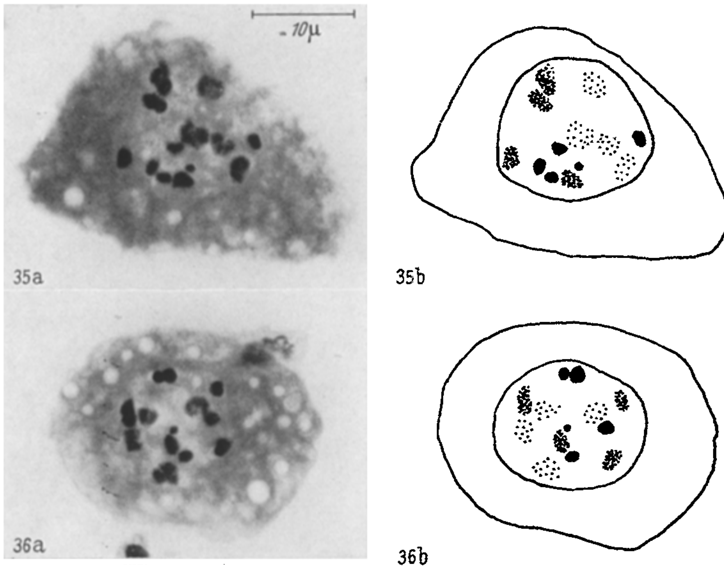


Figs. 32—34. Germ cells of embryos derived from OR T 1 females; lactic orcein squashes. Fig. 32. Nucleus contains 4 maternal sex chromosomes (dots); 4 of the paternal homologues are light-staining; the paternal X has not yet been eliminated and is dark-staining. Fig. 33. Like Fig. 32 except that 2 of the dots are physically connected to each other. Fig. 34. One of the paternal X's has been eliminated, leaving 4 light-staining paternal homologues inside the nucleus; the 4 maternal chromosomes including the dot and the large translocation chromosome, are dark-staining

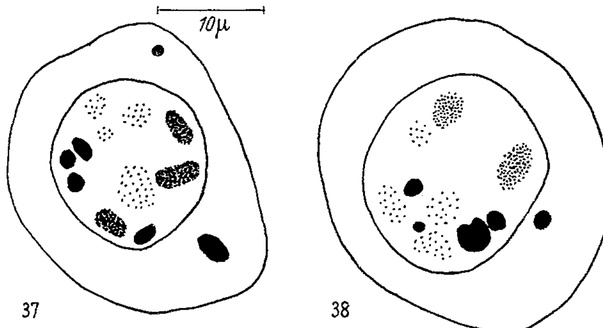
embryos inherited the translocation chromosomes. The germ cells of only a few such embryos were examined. They confirmed the point made above, namely, that the set of regular chromosomes which remain dark-staining are the maternal ones. In the two pre-elimination cells shown in Figs. 35—36 there are 4 fluffy (paternal) autosomes, one dark-staining paternal X (which will undergo elimination), and 4 dark-staining maternal chromosomes. Included among the dark-staining homologues are the dot, the other (large) translocation chromosome, and the two medium-sized autosomes (II and III). In Fig. 34 the paternal X has been eliminated, and the 4 dark-staining regular chromosomes inside the nucleus include the dot.

That the tiny translocation chromosome undergoes mitotic non-disjunction during the early divisions of the germ cells is indicated by the

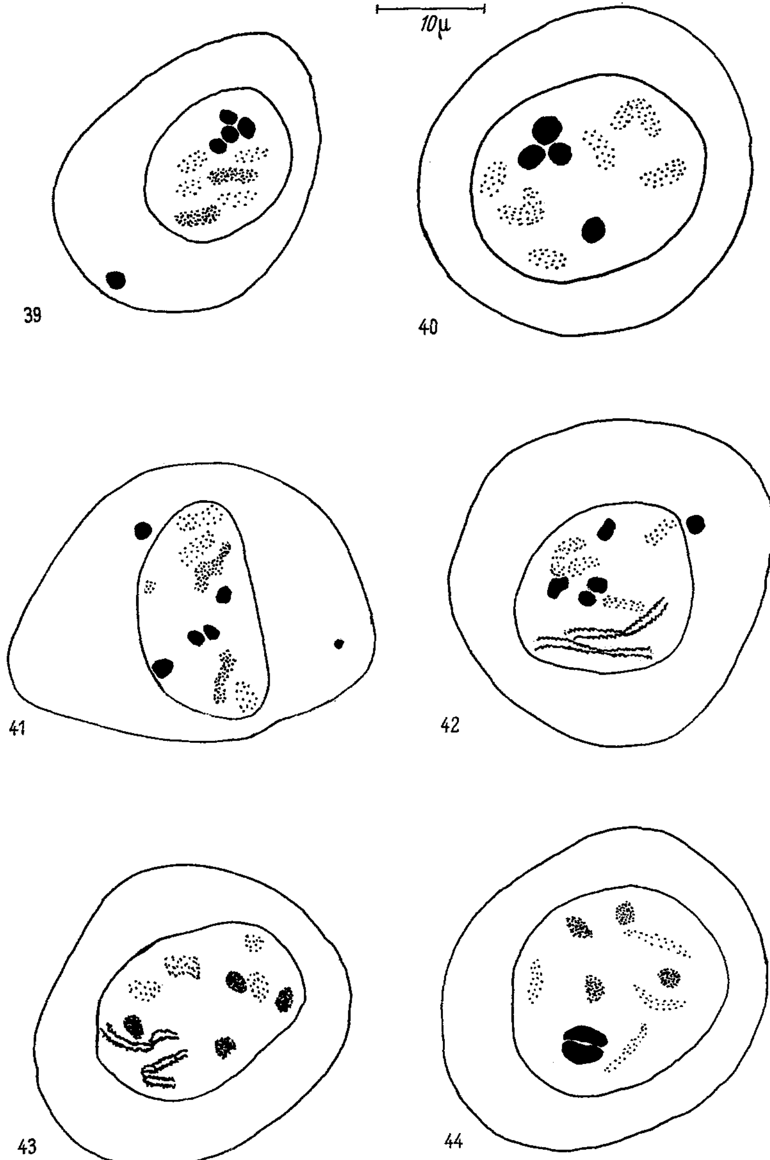
nuclei in Figs. 32—33, each of which contains 4 dots. In Fig. 33 two of the dots are connected to each other. In one embryo a cluster of germ cells was observed in which one nucleus contained 3 dark-staining dots, another 4, and the remainder of the nuclei had the expected one. Loss of germ cells in the squashing process prevented a rigorous study of this phenomenon.



Figs. 35 and 36. Photomicrographs of germ cells of an embryo derived from OR T 1 female; lactic orcein squash. Fig. 35. The maternal sex chromosome (dot) is dark-staining. Also within the nucleus are the 3 other dark-staining maternal homologues, a dark-staining paternal homologue (presumably the X which will be eliminated), 4 light-staining paternal homologue, and 4 L's. The L at the 11 o'clock position is partially light-staining. Fig. 36. Same chromosome complement as preceeding Figure



Figs. 37 and 38. Lactic orcein squashes. Fig. 37. Germ cell of embryo derived from OR T 5 male. One L and one paternal sex chromosome (dot) has been eliminated. The 4 remaining paternal homologues, including the other dot and the large translocation chromosome, are light-staining. Three L's and the 4 dark-staining maternal homologues remain in the nucleus. Fig. 38. Germ cell of embryo derived from OR T 5 female. The normal X chromosome has been eliminated. The dark-staining maternal homologues include the dot and the large translocation chromosome

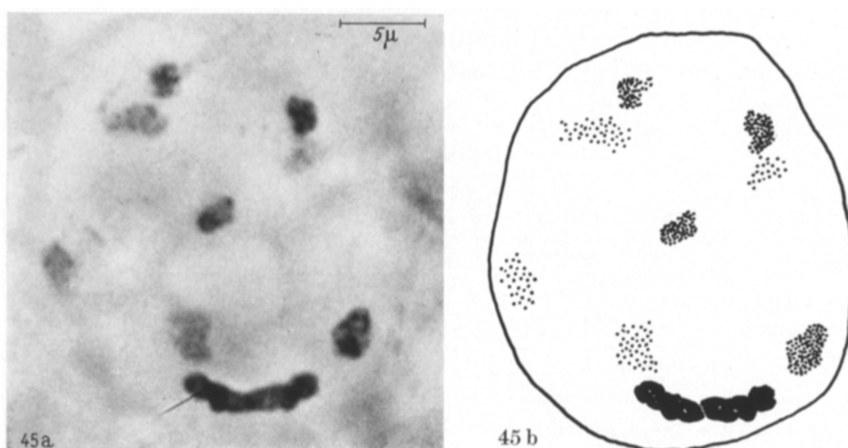


Figs. 39—44. Feulgen-stained germ cells derived from normal and OR T 1 material showing the L chromosomes as they undergo uncoiling and their return to the condensed state by 48 hours of first larval instar. Fig. 39. Four-day embryo; the 2 L's are almost as uncoiled as the 4 paternal homologues. The eliminated X chromosome is in the cytoplasm. The 4 maternal homologues are still dark-staining and clustered. Fig. 40. Two-hour larva; the 2 L's are as uncoiled as the 4 paternal regulars. The eliminated chromosomes have completely disappeared. Fig. 41. Two-hour larva derived from OR T 1 male. Eliminated L and eliminated paternal sex chromosome (dot) still visible in cytoplasm. The 2 L's in nucleus are uncoiled and light-staining. Fig. 42. Twenty-four-hour larva; an eliminated chromosome still visible in cytoplasm. The 2 L's are very long, clearly double, and loosely paired. Fig. 43. Thirty-six-hour larva. Like Fig. 42, but the eliminated chromosomes have completely disappeared. The 4 maternal homologues are beginning to uncoil. Fig. 44. Forty-eight-hour larva. The 2 L's are completely condensed and paired at both ends. The 4 maternal homologues are almost as light staining as the paternal homologues

c) Reciprocal Crosses with OR T 5: The use of OR T 5 actually contributed nothing new to the study; it merely served to corroborate the point already established regarding differential staining. When the dot chromosome was introduced through the sperm, the germ cells were found to contain 2 dots — one of which was eliminated and the other became light-staining (see Fig. 37). Embryos from the reciprocal cross had germ cells with only one dot; this chromosome was retained in the nucleus and remained dark-staining while a non-translocated X was eliminated (see Fig. 38). An unusually large translocation chromosome characteristic of OR T 5 can be seen inside the nuclei of both of the germ cells in Figs. 37 and 38.

7. Chromosome Behavior in the Testes of First Instar Males

It was stated above (p. 249) that the L chromosomes, at the end of embryonic life, become as diffuse and light-staining as the paternally-



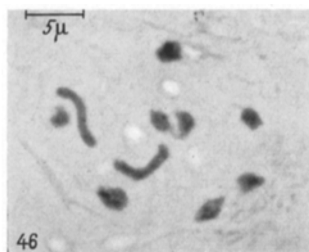


Fig. 45a and b. Photomicrograph of germ cell from 48-hour larva, normal stock; lactic orcein squash. The 2 L's are quite long and paired at one end. The 4 maternal homologues are beginning to uncoil and to become as light-staining as the 4 paternal ones

Fig. 46. Photomicrograph of germ cell from 72-hour larva, normal stock; Feulgen squash. The terminal association of the 2 L's has been disrupted in the squashing. The 2 sets of regular chromosomes are indistinguishable

derived regulars. This condition persists through hatching of the larva (Fig. 40). Shortly thereafter the L chromosomes begin to elongate (Fig. 41), and by the second day of first instar they are quite long and clearly double (Figs. 42 and 43). This stage is succeeded by a sharp condensation of the L chromosomes into dark-staining compact entities in which the doubleness is no longer visible (Fig. 44). At this time (48 hours in first instar) the L's are not so contracted as in the early embryo; actually they are relatively long (Fig. 45) and always quite closely paired either at one end (Fig. 45) or both (Fig. 44).

Changes in the regular chromosomes begin to appear at 36 hours of first larval instar. The maternally-derived set gradually becomes less contracted and no longer shows clustering (Figs. 43—45). By 72 hours the differential staining of homologues has disappeared (Fig. 46). First larval instar lasts 3—4 days and culminates in the first gonial division. After this division the differentiation between homologues is not resumed.

An attempt was made to study the first gonial division and to follow DNA replication with H^3-thymidine. Because of the poor cytology of these cells, it was not possible to get a complete picture of events. It was found, however, that the division is not synchronous within a testis; only a few cells exhibited the same mitotic stage. Autoradiographs were made on the testes of 4-day-old embryos and first instar larvae (various ages) which had been dissected directly into the H^3-thymidine tissue culture medium and fixed after a 30 min exposure (Table 10). Only those germ

Table 10. *The incorporation of H^3-thymidine into male germ cells of S. coprophila during late embryonic and early larval life*

Age of animals (20° C)	Number animals exposed to isotope	Number with well-labeled germ cells	Number with slightly labeled germ cells	Number with mitotic figures in germ cells	Number with labeled soma
4 days after oviposition	2	0	0	0	2
1 day after hatching	4	0	0	0	4
2 days after hatching	6	0	1	0	6
3 days after hatching	3	3	0	0	3
1 hour in second instar; 3 days after hatching	1	1	0	1	1

cells showed incorporation of label which had come from larvae in late first instar. The period of DNA synthesis coincides with the stage at which the regular chromosomes are light and diffuse and the L chromosomes are dark and paired. Because of the poor cytology of these preparations, further information could not be obtained.

8. Sequence of Gonial Division in the Testis

Following the first post-elimination gonial mitosis there is a series of divisions, the last of which is completed by the fourth day of 4th instar.

The divisions cover a period of 9—10 days, but it was not possible to correlate them with gross developmental changes. As shown in Table 11 there are always a few larvae of any age group in which some germ cells are undergoing division. The number of germ cells per testis increases from an average of 24 cells on the first day of 2nd instar to an average of 972 cells on the fourth day of 4th instar. In order to

Table 11. *Relation of gonial divisions to developmental stage in the male of S. coprophila*

Age of Larvae (20° C)	Number of larvae	Number with germ cells in anaphase or metaphase	Percent with germ cells in anaphase or metaphase
Second instar			
0—23 hours	14	5	36
24—47 hours	8	2	25
48—71 hours	16	8	50
72—95 hours	2	1	50
Third instar			
0—23 hours	11	5	45
24—47 hours	11	6	55
48—71 hours	12	10	83
72—95 hours	12	3	25
Fourth instar			
0—23 hours	5	3	60
24—47 hours	1	1	100
48—71 hours	3	1	33
72—95 hours	18	6	33
96 hours-prepupa	38	2*	5

* Both of these larvae were in the 96—108 hour interval.

account for this increase in cell number, five to six divisions must be postulated. The cell counts are based on squashed preparations of whole testes. No testis was counted if some of its cells had obviously been lost in squashing. Even so, some cells undoubtedly were lost from each testis; hence, these counts probably represent the minimum number of germ cells per testis. The number of germ cells observed in larvae of the same age varies considerably.

Only a few cells in a testis were found to be in the same stage of division. Generally such synchronized cells were in the same area of the squashed testis and hence probably located in the same cyst. The two testes of the same individual were not necessarily synchronous with respect to division. Table 12 gives the percentage of

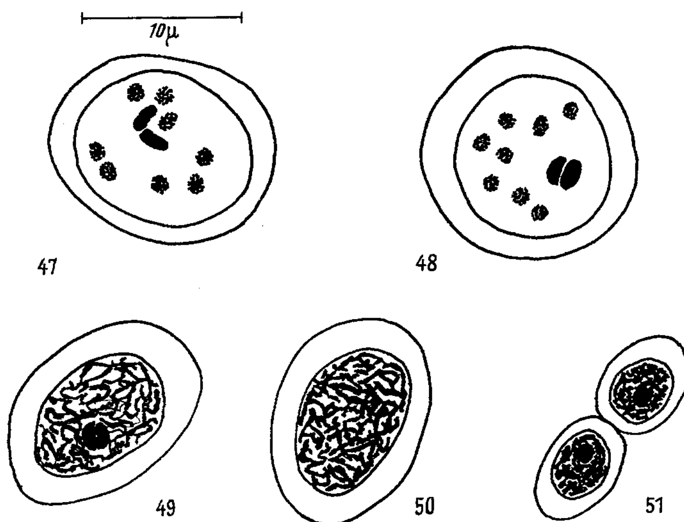
Table 12. *Percentage of metaphase plates in the testes of S. coprophila 24 hours after 2nd molt at 20° C*

Larva number	1	2	3		4		5		6	
Testis	(1)	(1)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Number of cells	250	199	324	236	215	253	241	258	280	200
Number of metaphase plates	0	0	13	3	29	0	8	26	15	0
Percent metaphase plates	0	0	4	1	13	0	3	10	6	0

metaphase plates observed in testes of six larvae which had been in 3rd instar for 24 hours. The highest percentage of metaphase plates in any one testis was 13 percent in larva number 4. The other testis of this larva had no cells in metaphase.

Throughout the succession of gonial division the most characteristic type of germ cell nucleus found was a resting stage with 10 prochromosomes (Figs. 47 and 48). The L chromosomes in this typical resting nucleus are longer, thicker, and more darkly stained than the regular

chromosomes and are usually paired either at one end (Fig. 47) of at both ends to form a doughnut-like structure (Fig. 48). The paired L chromosomes are presumed to constitute the chromocenter of the early prophase nucleus (Fig. 49). As division progresses, this dark region becomes diffuse and indistinguishable from the rest of the chromatin (Fig. 50). The telophase nuclei resemble early prophase except that they are considerably



Figs. 47—51. Germ cells of fourth instar male larvae; Feulgen squashes. Fig. 47. Resting nucleus with 10 prochromosomes. The 2 L's are heteropycnotic and paired at one end; the 8 regular chromosomes are all equally condensed and unpaired. Fig. 48. Resting nucleus showing 2 L's paired at both ends. Fig. 49. Early prophase; the chromocenter is believed to be made up of the 2 L's. Fig. 50. Late prophase; no chromocenter. Fig. 51. Late telophase; chromocenter present in each daugther nucleus

smaller (Fig. 51). Following telophase, the chromatin is again separated into prochromosomes.

Presented in Table 16 is a complete summary of the development of the male germ cells. There are two sets of divisions in the testis: at least 5 divisions in the embryo 4—16 hours post oviposition; and a set of 6 divisions which begins at the time of first larval molt and extends through a period of 9—10 days.

9. DNA Replication in Third Instar Males; Asychrony between Regular and L Chromosomes

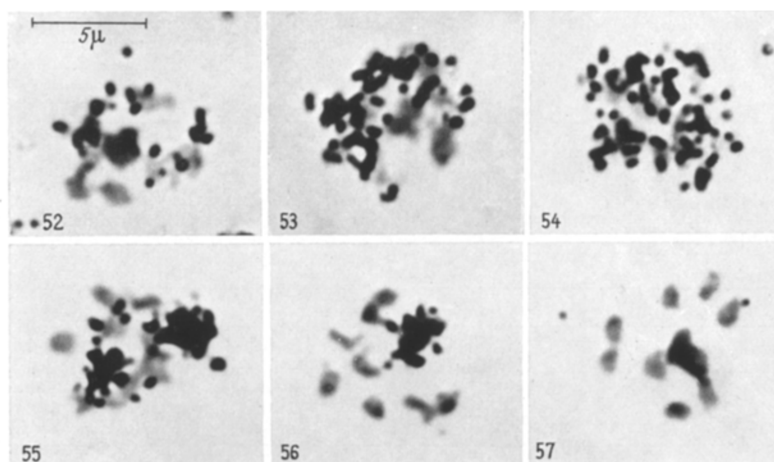
As a prelude to the study on pre-meiotic DNA synthesis, the spermatogonial divisions in late third instar males were examined. Larvae were selected 72 hours after second molt and made to eviscerate by decapitation in culture medium containing H^3-thymidine. After 30 min the

Table 13. *Percentage of labeled and partially-labeled germ cells in larval testes of S. coprophila 72 hours after 2nd molt at 20° C*

Percent cells with label over	Larva number*								
	Thy-1	Thy-2		Thy-3		Thy-4		Thy-5	
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
limiteds only	35	20	2	9	4	14	9	2	26
limiteds and some regulars	8	11	11	28	22	0	21	4	12
a few regulars only	17	14	48	23	6	0	38	57	55
regulars only	20	27	7	19	16	12	16	7	2
all chromosomes	8	28	30	20	37	0	1	4	0
no chromosomes	12	0	2	1	15	74	12	26	0
no chromosomes (telophase)	0	0	0	0	0	0	3	0	5

* Both testes could be counted in four of the five larvae and are designated (1) and (2). Only one testis was counted in Thy-1.

testes were dissected and prepared for autoradiography. Approximately 100 cells in each testis were selected randomly for study. The data are recorded in Table 13. The percentage of labeled cells ranged from 26



Figs. 52—57. Autoradiograms of male germ cells (late 3rd instar) showing premitotic DNA synthesis; Feulgen squashes. Fig. 52. L's unlabeled; silver grains over some regulars. Fig. 53. Only the L's remain unlabeled. Fig. 54. Totally labeled nucleus. Fig. 55. L's and some of regulars labeled. Fig. 56. L's labeled; the 8 regulars unlabeled. Fig. 57. Totally unlabeled nucleus

(Thy-4-1) to 100 (Thy-2-1); the two gonads taken from the same larva were not necessarily equally labeled (Thy-4). In all of the testes a similar range of labeling patterns was found (Figs. 52—57). Some cells had only the regular chromosomes labeled (Fig. 53), whereas others had only the L chromosomes labeled (Fig. 56). In larva Thy-1, for example, 35 percent of the germ cells showed label over the L chromosomes exclusively, and

20 percent showed label only over the regulars. Partial labeling over the regular chromosomes was also found. Some cells (8% in larva Thy-1) had silver grains over the L chromosomes and over a few regulars (Fig. 55); others (17% in larva Thy-1) had grains only over some of the regulars (Fig. 52). Because of chromosome overlapping, it was impossible to determine how many regular chromosomes remained unlabeled. Totally labeled nuclei comprised from zero to 37% of the cells selected for study (Fig. 54). At the time of DNA synthesis the L chromosomes were found to be heteropycnotic and more contracted than the regular chromosomes and usually to be closely paired (see totally unlabeled nucleus in Fig. 57).

10. Premeiotic DNA Synthesis in the Male

The premeiotic synthesis of DNA was found to occur in the male just after completion of the last mitotic division, i.e. 4—5 days following third molt and prior to eye spot formation. Table 14 presents the analysis of autoradiographs prepared from larvae which were either injected with

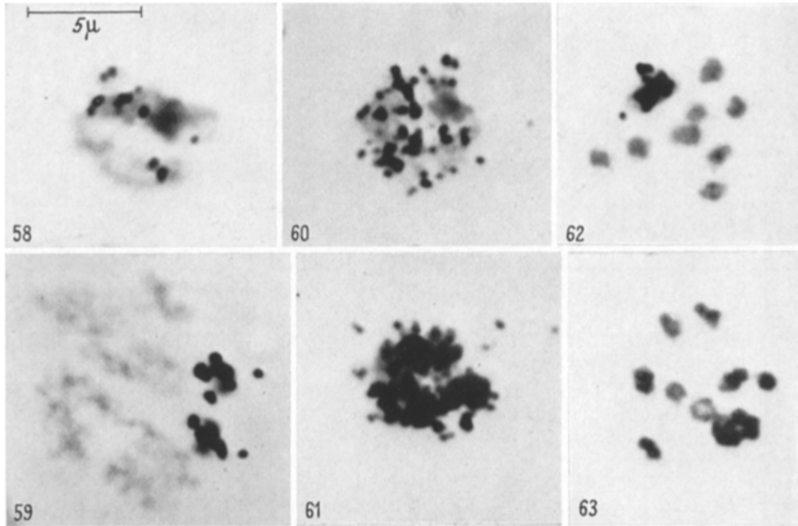
Table 14. *Time of pre-meiotic DNA synthesis in male germ cells of S. coprophila*

Stage at which injected with H^3-thymidine	Number of animals	Number with more than 5% labeled germ cells	Number with less than 5% labeled germ cells	Number with totally unlabeled germ cells	Percent with more than 5% labeled germ cells
Fourth instar					
no eye-spots	4	1	1	2	25
medium eye-spots	4	0	0	4	0
Prepupa	11	0	0	11	0
Young pupa	5	0	0	5	0

Stage at which dissected into medium containing H^3-thymidine	Number of animals	Number with more than 5% labeled germ cells	Number with less than 5% labeled germ cells	Number with totally unlabeled germ cells	Percent with more than 5% labeled germ cells
Fourth instar no eye-spots					
72—95 hours*	1	0	1	0	0
96—119 hours	15	10	2	3	67
120—143 hours	10	6	1	4	60
144—169 hours	2	0	1	1	0
small eye-spots	6	0	2	4	0
medium eye-spots	8	0	2	6	0
large eye-spots	3	0	0	3	0
Prepupa	3	0	0	3	0

* Hours after 3rd molt.

isotope or whose gonads were exposed directly by dissection into isotope. The testes of the former were fixed at an early pupal stage; the latter after a 30 min exposure. Only two of the 24 injected animals showed labeled germ cells; one of these had somewhat more than 5% of its germ cells labeled, the other had even less. Both of these individuals had been injected in 4th instar prior to eye spot formation. In view of these results,



Figs. 58—63. Autoradiograms of male germ cells (late 4th instar) showing premeiotic DNA synthesis; all except Fig. 59 were fixed immediately after exposure to isotope; the cell in Fig. 59 was not fixed until first meiotic prophase; Feulgen squashes. Fig. 58. L's unlabeled; silver grains over some regulars. Fig. 59. Early first meiotic prophase; only the 2 L's are labeled. Fig. 60. Only the L's are unlabeled. Fig. 61. Totally labeled nucleus. Fig. 62. Only the L's labeled; the 8 regulars are unlabeled. Fig. 63. Totally unlabeled nucleus

4th instar larvae without eye spots were selected for use in the direct exposure experiments. These larvae yielded very good results (Table 14); in the two groups, 96—119 hours and 120—143 hours, 67% and 60% of individuals respectively had many labeled germ cells. The older stages had completely unlabeled germ cells except the larvae with tiny eyes; among these a very low level of labeling was observed.

Asynchronous replication of limited and regular chromosomes was first observed in the injected larva which had incorporated the label. This animal was fixed in early prophase of the first spermatocyte division, at which stage the L chromosomes are distinct from the regulars and from each other. The two L chromosomes were found to be labeled exclusively in one group of cells (Fig. 59); and all chromosomes except the L's were found labeled in another group.

Table 15. *The percentage of labeled and partially-labeled premeiotic male germ cells in S. coprophila*

Number of hours after third larval molt at 20° C	Larva number*	Diffuse regulars							Prochromosome stage					
		limiteds labeled only	limiteds and some regulars labeled	some regulars labeled	all regulars labeled	totally labeled	totally unlabeled	telophase cells (unlabeled)	limiteds labeled only	limiteds and some regulars labeled	some regulars labeled	all regulars labeled	totally labeled	totally unlabeled
75	W-6	0	0	60	0	3	4	4	14	0	0	0	0	15
	W-11a	0	0	3	0	0	0	0	18	28	0	0	2	49
	W-11b	0	0	4	8	0	0	0	17	21	1	0	0	49
	W-13a	0	0	2	13	6	0	0	6	42	0	0	0	31
	W-13b	0	0	0	2	57	0	0	77	19	6	0	0	9
96	Thy-1-3a	0	0	0	0	0	0	78	0	0	0	0	0	22
	Thy-1-3b	0	0	0	0	0	0	6	0	0	0	0	0	94
	Thy-1-4a	0	0	1	17	3	0	0	45	1	0	0	0	33
	Thy-1-4b	0	0	0	17	0	0	0	43	2	0	0	0	38
	Thy-1-5a	0	0	0	14	13	0	0	34	7	0	0	0	32
	Thy-1-5b	0	1	2	34	28	0	0	12	16	0	0	1	6
	Thy-1-6	0	0	0	5	4	0	15	12	21	1	0	0	42
96—120	Thy-1-1	0	0	0	20	0	1	0	0	5	0	0	0	74
	Thy-1-2a	0	0	1	14	25	0	0	21	14	0	0	0	25
	Thy-1-2b	0	0	9	2	0	16	0	21	4	0	0	0	48
	Thy-1-7a	0	0	3	24	1	0	6	5	1	0	0	0	60
	Thy-1-7b	0	0	0	0	0	1	0	21	5	0	0	0	73
113	W-14	0	0	0	3	18	0	0	0	25	1	0	0	53
117	W-16	0	0	6	3	1	1	0	1	4	0	0	0	84
118	W-17a	0	0	0	2	1	0	0	0	1	1	0	0	95
	W-17b	0	0	0	6	2	0	0	1	0	0	0	0	91
	W-18a	0	1	3	0	1	0	0	0	0	4	0	0	91
	W-18b	0	0	0	1	1	0	0	4	16	5	0	0	73
137	W-9	0	0	0	0	0	0	0	17	0	0	0	0	83

* If not followed by an *a* or *b*, this number refers to only one larval testis. Otherwise, the two testes of a larva are designated *a* and *b*.

Table 15 presents the detailed results of the autoradiographic study made on testes dissected directly into labeled medium. Approximately 100 cells in each testis were selected at random and scored not only for labeling pattern but also for type of nucleus (diffuse chromatin *versus* prochromosomes). In many instances both testes from the same larva could be used for study, but in some cases the loss of cells in squashing,

or poor cytology, made one testis unsuitable. It was found that cells within a testis are not synchronous with respect to synthesis; also that the two testes of a pair vary in the distribution of labeling patterns.

The labeling patterns found in the premeiotic synthesis (Figs. 59—63 and 65) are similar to those in the premitotic one. There are two types of labeled nuclei — those with prochromosomes and a class of smaller nuclei with diffuse regular chromosomes. The latter stage immediately follows telophase of the last mitosis and is succeeded by the prochromo-

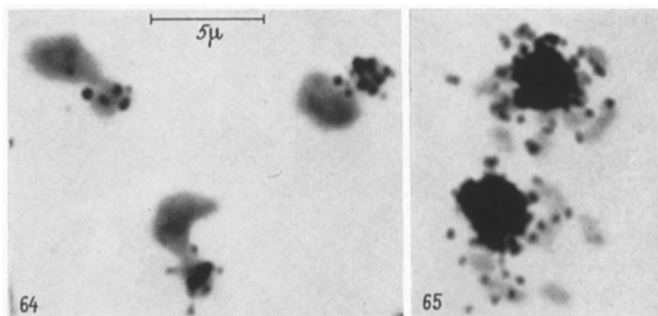


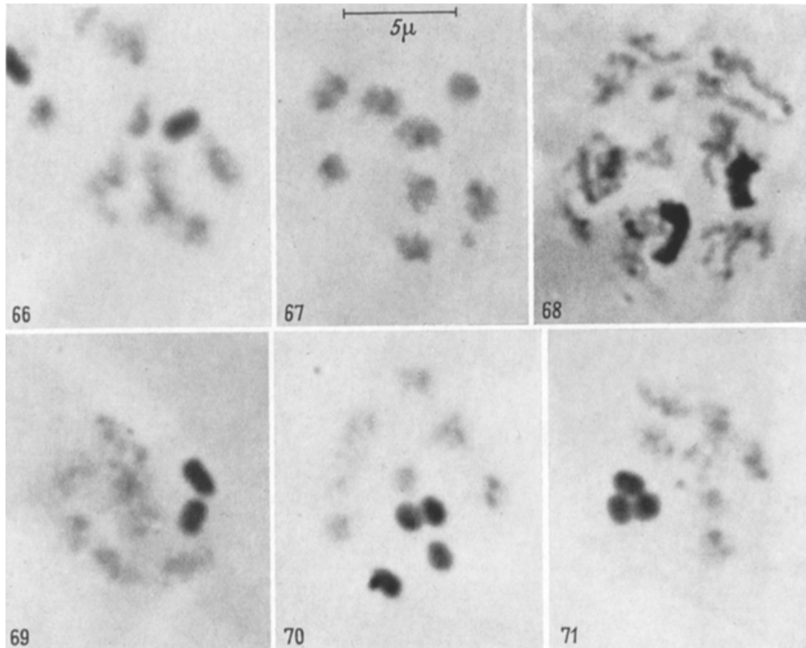
Fig. 64. Autoradiogram of oocytes during premeiotic DNA synthesis (third instar); Feulgen squash. In each of the 3 nuclei the L's form a distinct cluster, separate from the rest of the nucleus, and are exclusively labeled

Fig. 65. Autoradiogram of 2 male germ cells during premeiotic DNA synthesis; Feulgen squash. The L's and some of the regulars are labeled

some (resting) stage which persists until prophase of the primary spermatocyte, a few days later. In both types of nuclei the limited chromosomes are dark-staining and closely paired. Only in nuclei with prochromosomes were the L's exclusively labeled (Fig. 62), and only the nuclei with diffuse regular chromosomes contained the regulars exclusively labeled (Fig. 60). The nuclei with labeled L's and partially labeled regulars belonged mainly to the prochromosome class (Fig. 65); the nuclei with unlabeled L's and partially labeled regulars belonged principally to the diffuse chromatin class (Fig. 58). Most of the unlabeled nuclei contained prochromosomes (Fig. 63), whereas the totally labeled nuclei contained diffuse chromatin (Fig. 61). Since the prochromosome state is characteristic of the germ cells *after* synthesis, these observations suggest strongly that *the L chromosomes begin and end replication later than the regular chromosomes.*

Although a detailed study was not made of DNA synthesis in the female germ line, a cursory examination at the ti ne of premeiotic synthesis revealed some oocytes in which only the L chromosomes were labeled (Fig. 64) and others in which all except the L's were labeled. At

this stage in the ovary the limiteds are clustered and form a distinct entity which is slightly separated from the other chromosomes (Fig. 64), thereby making the asynchrony in labeling unmistakably clear.



Figs. 66—71. Male germ cells during prophase of the first spermatocyte division showing varying number of L's; Feulgen squashes. Fig. 66. Eight regulars; no L's. Fig. 67. One L. Fig. 68. Two L's. Fig. 69. Later prophase. Two L's. Fig. 70. Four L's. Fig. 71. Three L's

11. Early Prophase of the Primary Spermatocyte

The L chromosomes become less tightly paired as the spermatocyte passes from the period of DNA synthesis to early prophase. At this time the L chromosomes can be counted without error. Although — as pointed out above — the most common number is 2 (see Table 3), spermatocytes at this stage were found in which the number of L's ranged from zero to 4 (Figs. 66—71).

D. Discussion

I. History of the Limited Chromosomes

1. Parental Origin

For the problem of chromosome imprinting — as explained in the introduction to this paper — it is important to know whether the limited chromosomes in the primary spermatocyte are exclusively of maternal origin. On the basis of the evidence presented above the L chromosomes retained by the germ cells of most, if not all, embryos are derived from

both parents. This conclusion is reached by the following line of reasoning: (1) Most embryos (95%) inherit 2, 3, or 4 L chromosomes (Table 5) of which 2 are retained by each germ cell. (2) Assuming that the sperm transmits to the zygote the same complement of limited chromosomes found in the primary spermatocyte, nearly all embryos (98.8%) inherit at least one L chromosome from their father (Table 3). (3) Approximately 18% of all embryos inherit (and retain) exactly 2 L chromosomes (Table 5); one of these must be paternally derived unless it is assumed that this entire class of embryos receives no L chromosome from the sperm. Such an assumption seems to be totally unwarranted in view of the fact that only 0.3% of the primary spermatocytes are found to be completely lacking in L chromosomes (Table 3). Moreover, there is some doubt as to whether such spermatocytes produce functional sperm. Compared to spermatocytes which contain L chromosomes they are smaller, develop more slowly, and have never actually been observed to undergo either one of the meiotic divisions (unpublished studies by H. V. CROUSE).

Essential to the above line of argument is the assumption that there is no elimination of L chromosomes from the male germ cells subsequent to meiosis. Two lines of evidence support the validity of this assumption. (1) the number of L chromosomes in the early germ cells (Table 5) is larger than the number in the primary spermatocytes (Table 3); and (2) a cytological study on sectioned testes stained with Feulgen-fast-green failed to reveal evidence of chromatin diminution during spermato-histogenesis (unpublished studies by H. V. CROUSE and E. HOLTZMAN).

2. Variable Number in the Testis

Subsequent to the elimination of chromosomes from the germ line at 60—70 hours post-oviposition, most embryos (98%, Table 5) possess a cluster of approximately 30 germ cells, each containing 8 regular and 2 L chromosomes. In the remaining 2% of embryos the germ cells contain only one L chromosome. The question arises, then, as to the variability in number of L chromosomes observed within a testis at the primary spermatocyte stage (Table 3). Is this variability to be explained in terms of erroneous chromosome elimination in the embryo, or is it caused by nondisjunction of chromosomes during one or another of the 6 consecutive gonial mitoses (see Table 16)? Nondisjunction of the supernumerary B-type chromosomes in corn, rye, and a variety of other plants is known to take place only at very specific mitoses during the course of development. In corn the nondisjunction occurs only at the 2nd microspore division whereas in rye it occurs only at the 1st (see ROMAN, 1947). It is interesting to note that the B-types resemble the limited chromosomes of *Sciara* in being largely heterochromatic, in containing no known genetic factors, and in occurring at variable frequency in the genom. If mitotic nondisjunction is solely responsible for the variable number of

Table 16. *Summary of male germ line development in S. coprophila at 20° C*

Age	Developmental stage	State of the germ cells	Number of germ cells
0—1	Egg; metaphase I-telophase II of oogenesis		
1—$1^1/_2$ hours	Zygote nucleus		
4 hours	Differentiation of the germ cell nuclei		1 or 2*
4—6 hours	5th-8th cleavages; elimination of limiteds and X's from the somatic cells	Mitotic divisions	16
6—16 hours		One more mitosis, followed by a long resting stage	30
24—48 hours	Migration of germ cells to gonad site	Resting stage	30
60—72 hours		Resting stage; elimination of the extra X and all but two limiteds	30
72—80 hours	Formation of gonads	Resting stage	15**
120 hours (6th day)	Larva hatches; start of first instar	Resting stage	15
8th day	First instar	DNA synthesis	15
9th day	First molt; start of second instar	Larval mitosis I	15—30
10th day	Second instar	Some mitosis II	30—60
11th day	Second molt late in the day; start of third instar	Mitosis II, some mitosis III	30—90
12th day	Third instar	End of mitosis III	120
13th day	Third instar	Some mitosis IV	120—240
14th day	Third molt late in the day; start of fourth instar	Mitosis IV, some mitosis V	240
15th day	Fourth instar	End of mitosis V	480
16th day	Fourth instar	Start of mitosis VI	480—960
17th day	Fourth instar	End of mitosis VI; start of a long resting stage	960
18th day	Fourth instar	Start of the premeiotic DNA synthesis	960

* Data of DUBOIS (1933).

** From this point on, the number of germ cells will refer to the number of germ cells per testis.

Table 16 (Continued)

Age	Developmental stage	State of the germ cells	Number of germ cells
19th	Beginning of eye-spot development	End of DNA synthesis	960
20th day	Light eye-spots	Resting stage	960
21st day	Medium-dark eye-spots	Resting stage	960
22nd day	Prepupa formation	Resting stage	960
23rd day	Pupation	Start of meiotic prophase	960
24th day	Pupal stage	Spermatogenesis	960
25th—27th days	Pupal stage	Spermiogenesis	960
28th day	Emergence of imago	Mature sperm	960

L chromosomes observed within a single testis, one would except the frequency of spermatocytes containing a single L to equal the frequency of those containing 3 L's. This is certainly not the case in the two testes designated W-16 and T-3 in Table 3. There appears to be no obvious way to determine how much of this variability results from nondisjunction and how much from faulty chromosome elimination. Perhaps the problem could be resolved if it were possible to obtain reliable chromosome counts on *entire* clusters of germ cells in the early embryo.

3. Behavior during Oogenesis

It was stated previously that a determination of L chromosome number in the ovary at first meiotic metaphase could be made only at the expense of exacting, painstaking study. We did not undertake such a study; cursory examination, however, of 6 Feulgen-stained ovaries (squash preparations) failed to reveal an accumulation of L chromosomes comparable to that encountered in the testis. Like Schmuck and Metz (1932) we found invariably 5 "chromosomes" on the M I plate; 4 of these were the bivalents formed by the regular complement; the 5th entity was one or a pair of L chromosomes. In their study on oogenesis in *S. coprophila* Schmuck and Metz were of the opinion that a pair of L chromosomes undergoes normal disjunction at AI and equational division at AII; and that single L chromosomes divide equationally at AII. A similar pattern of behavior for single L chromosomes during oogenesis in *Sciara impatiens* was established by Carson (1945). In view of these cytological studies on the ovary, one would expect the egg of *S. coprophila* to transmit to the zygote either zero, 1, or 2 L chromosomes. The data recorded in Tables 3 and 5 are not in disagreement with this expectancy.

4. Elimination from Germ Cells

The elimination of the extra limited chromosomes from the germ cells of *S. coprophila* has been fully described and illustrated in this paper. It is significant that the chromosomes are removed from the germ cell nuclei at the same time and in exactly the same manner as the extra paternal X chromosome, also that this process of elimination differs markedly from the one which removes these same chromosomes from the somatic nuclei.

The chromatin diminution in the embryonic soma of *Sciara* was the first recorded case of *whole chromosome* elimination (DuBOIS, 1933). It is very similar in principle to the eliminations reported subsequently in a number of nematocerous dipterans including several members of the *Cecidomyiidae* (REITBERGER, 1934; KRACZKIEWICZ, 1935; METCALFE, 1935; GEYER-DUZYNSKA, 1959) and *Chironomidae* (BAUER and BEERMANN, 1952). This type of chromosome elimination occurs during early cleavages of the egg. The chromosomes to be eliminated fail to complete their anaphase separation and remain on the equator of the spindle, thereby becoming excluded from the daughter nuclei. In all of these dipterous forms the elimination of chromosomes during cleavage marks the separation of soma from germ line. In *Sciara coprophila* the separation occurs at the 5th cleavage; all of the nuclei (potentially somatic) except those located in the pole plasm undergo an elimination of the limited chromosomes (DUBOIS, 1933). The second and final elimination of chromosomes from the somatic nuclei occurs at the 7th or at the 8th cleavage and involves the X chromosome; thereby the definitive male and female soma become XO and XX respectively (DUBOIS, 1933).

The germ cells of *Sciara* form a sharp contrast with the somatic nuclei (see Table 16). They retain the 3 X's and all of the L's until after their migration from the pole plasm to the future site of the gonads. Then each one eliminates the extra paternal X and all but two of the limited chromosomes. Unlike the somatic nuclei there is no prescribed sequence for the elimination of the two kinds of chromosomes; the X may precede or follow the L's through the nuclear membrane. We estimate that a cluster of germ cells completes the elimination process in approximately five hours (65—70 hours post-oviposition). Once the chromosomes enter the cytoplasm they become very smooth and round in contour and remain dark-staining until they disappear. They look very much like the acentric chromosomes of maize which come to lie in the cytoplasm of the microspore instead of the nucleus and which are known to be lacking in genetic activity as compared to the intranuclear ones (McCLINTOCK, 1938).

The discovery, that the number of L chromosomes in the germ line of *S. coprophila* is regulated by the elimination that takes place in the

embryo, raises the question as to the validity of the work on *Plastosciara* (FAHMY, 1949). The author claims that regulation of L chromosome number in the germ cells of this species is accomplished by a seemingly fortuitous elimination of L's in less than half of the secondary spermatocytes. The presumed elimination is said to occur either at prometaphase or at anaphase; the author's description of the process, however, is vague and confusing. We are of the opinion that the claim is erroneous. Such an elimination of L chromosomes does not occur in the secondary spermatocyte of *S. coprophila.* Indeed, the outstanding feature of all the chromosomal "unorthodoxies" in *Sciara* is that they are *not* fortuitous; they are regulated with remarkable precision as to tempo, mode, and locale.

II. The Limited Chromosomes and the Problem of Heterochromatin

The L chromosomes are "heterochromatic" in the sense of the original definition given by HEITZ (1928); they stain darker than the other chromosomes of the complement and contain no known genetic factors. COOPER (1959) emphasized that many heterochromatic chromosomes are composed of blocks of material which have a genetic function and which are not heteropycnotic at all stages of the life cycle. He suggested that euchromatin and heterochromatin are reversible states and that chromosomal regions traditionally termed heterochromatic are merely cases of chromosome differentiation having a very long time span. The L chromosomes belong to this category, for although they are briefly light-staining during early larval life, they are heteropycnotic at all succeeding interphases.

In view of the recent investigations on the relation of chromosome differentiation to gene activity (BEERMANN, 1961; LYON, 1961; CLEVER, 1962; OHNO and CATTANACH, 1962; GRUMBACH, MORISHIMA and TAYLOR, 1963; BROWN and NUR, 1964; TAYLOR, 1964; BERLOWITZ, 1965), it seems likely that the L chromosomes are functioning genetically when they are diffuse. These studies suggest that heterochromatin is the cytologically-visible counterpart of genetic inactivity. Conversely, euchromatin should represent the stage at which the genetic information carried in the chromosome is being conveyed to the cell. The limited chromosomes exhibit in the embryonic germ cells a complex staining pattern before they finally become heteropycnotic. Prior to the cytological differentiation of the regular chromosomes the L's are distinguishable in the nucleus only by their larger size. As differentiation of the paternal homologues begins, the L chromosomes at first remain condensed but soon they too become diffuse. During the first day of larval life the L chromosomes are very long and less condensed than the light-stained paternal homologues. Shortly

thereafter — while the two sets of regulars are still differentiated — the L chromosomes become heteropycnotic and condensed. If one can extend to these chromosomes the theory that genetic activity is reflected in staining capacity, then whatever genetic messages the L chromosomes possess must be transcribed while the chromosomes are in the diffuse state.

That the L chromosomes of *S. coprophila* have a genetic function is suggested by their staining behavior and also by the fact that they have been retained during the course of evolution. It seems unlikely that the germ cells would retain these chromosomes so regularly if they had no function in gonad development. In support of this idea is the work on the *Cecidomyiidae* which demonstrates that the E chromomosmes are necessary for normal development of the ovary (GEYER-DUSZYNSKA, 1959; BANTOCK, 1961). The occurrence of at least three species of *Sciara* which lack limited chromosomes indicates that whatever genetic function the L's may have can be transferred to the regular complement.

III. Differential Staining of the Regular Chromosomes

We have no explanation for the difference in stainability and condensation manifested by the regular chromosomes in the embryonic germ cells at the time of *(S. coprophila)*, or shortly after *(S. ocellaris)*, chromosome elimination. It is a very interesting phenomenon, however, because of its possible relation to chromosome imprinting.

According to the line of argument pursued above, the L chromosomes are not involved in the reversible process of imprinting. The chromosomes, then, which have to be considered are the set of regulars in the sperm. At some point between the first spermatocyte division and the time of fertilization, the imprint on these chromosomes must be changed from "maternal" to "paternal". In the germ line of male offspring the "paternal" imprint has to be retained until after the first meiotic division. In the germ line of female offspring, it can be removed any time after the extra paternally-derived X chromosome has been eliminated. The interval of time during which the paternal homologues in the embryonic germ cells are diffuse and light-staining would theoretically be an appropriate one for removing the "paternal" imprint. In order to preserve the imprint in male embryos and remove it in females one could postulate an episomal-like entity — located exclusively in the cytoplasm of female-producing eggs — which triggers the genetic mechanism for removing the "paternal" imprint from the light-staining set of chromosomes.

Quite obviously there are a number of difficult tasks to be carried out in connection with the analysis of chromosome imprinting. These include two kinds of studies on the embryonic germ cells: a comparison of the cytoplasmic fine structure in males and females; and an examination

(autoradiographic) of RNA and protein synthesis at the time of differential chromosome staining. In connection with the idea of a molecular exchange between cytoplasm and nucleus, it should be recalled that in the autoradiographic studies on labeled amino acid incorporation into *Amoeba*, evidence was obtained that there is a non-random migration of protein between nucleus and cytoplasm (GOLDSTEIN, 1958).

IV. Asynchronous DNA Synthesis

As expected from the behavior of heterochromatic chromosomes in other organisms, the L chromosomes synthesize DNA out of phase with the regular complement, apparently beginning and ending synthesis later. The heterochromatic X chromosomes in the grasshopper is late-replicating (LIMA-DE-FARIA, 1959); so is the X chromosome which is heterochromatic in the female mammal (see review by TAYLOR, 1964). At premeiotic synthesis in the *Sciara* male most nuclei which have the L chromosomes exclusively labeled are in the prochromosome stage. On the other hand, when the L's alone are unlabeled, the regular chromosomes are in the form of diffuse chromatin. This latter state is typical of post-telophase nuclei and thus corresponds to G 1. The prochromosome stage follows DNA synthesis and thus corresponds to G 2. The chromosomes follow a definite pattern of synthesis: soon after telophase of the last mitosis the regular chromosomes begin replication although not entirely synchronously. Somewhat later the L chromosomes begin synthesis; for a certain period of time, therefore, all the chromosomes are replicating together. The end of the S period is devoted exclusively to L chromosomes.

The exclusive labeling in either the L chromosomes or in the regular complement can best be seen at premeiotic synthesis in the ovary (see Fig. 64). In the oocyte at this stage the L's are so clustered as to form a distinct entity, slightly separated from the regular chromosomes.

It is not clear which or how many regular chromosomes are out of phase with each other, but there are a few labeled cells which show totally unlabeled regular chromosomes. It would be of interest to determine which of the regular chromosomes are early labeling and which are late, and how the asynchrony is related to the imprint they bear.

The fact that the L chromosomes remain heteropycnotic throughout the S period is in disagreement with the belief that replication can occur only when a chromosome is very diffuse and highly uncoiled. The L chromosomes are closely paired, dark-staining and very condensed entities at the time of replication; so is the heterochromatic X in the human female (GRUMBACH, MORISHIMA, and TAYLOR, 1963). Thus it appears that although a chromosome appears very condensed in the microscope, it is actually diffuse enough for DNA synthesis to take place.

Summary

A cytological study was made of the chromosomes in the male germ line of *S. coprophila* from the time of oviposition to first meiotic anaphase and in the female germ line from oviposition to first larval molt. H^3-thymidine autoradiography was used to supplement differential staining in the search for asynchronies between limited and regular chromosomes and between maternal and paternal homologues. X-autosome translocations were used to identify chromosome complements. The significant findings include the following:

1. In male and female embryos at 60—72 hours post-oviposition an elimination of chromosomes takes place from each non-dividing germ cell nucleus. It involves a paternal X chromosome and a variable number (0—3) of limited chromosomes. Prior to elimination the germ cell complement includes 1—5 limited chromosomes; following elimination, 2 limited and 8 regular chromosomes remain. During the process of elimination the chromosomes apparently pass through the intact nuclear membrane into the cytoplasm where they usually persist for a few days before disappearing.

2. Although the data are not unequivocal, they suggest that the elimination of limited chromosomes is not based on origin and consequently that those retained in the germ line may be derived from either parent.

3. Immediately preceding or concurrent with chromosome elimination, in male and female embryos alike, the chromosomes within the nucleus become differentially stained. By using reciprocal translocations it could be established that the 4 maternally-derived regular chromosomes remain dark-staining; 4 of the 5 paternal (regular) chromosomes, on the other hand, undergo a loosening of coils and become light-staining. In normal (non-translocation) material the 5th paternal chromosome, the extra X, was never found to be light-staining. In all cases where this chromosome was observed outside the nuclear membrane, it was dark-staining. The differentiation between the two sets of regular chromosomes persists for 4—5 days post-elimination.

4. In all stages examined the limited chromosomes are highly condensed and dark-staining except for a brief period beginning the last day of embryonic life and extending through the second day of larval life (both sexes). At that time the limiteds become as diffuse and light-staining as the paternal set of regular chromosomes.

5. The fully developed testis (late 4th instar) is composed of approximately 1000 primary spermatocytes. In the embryo at 6—16 hours post-oviposition there are 30 germ cells. These migrate from the pole plasm to the gonad site (24—48 hours) and later (72—80 hours) form two gonads, each composed of approximately 15 germ cells. Between first molt and 4th instar there is a succession of 6 gonial mitoses. The germ cells do not divide synchronously except within a cyst.

6. Pre-meiotic DNA synthesis begins during late 4th instar and ends at the time the eye spots first become visible. H^3-thymidine autoradiography shows that the limited chromosomes are in a condensed state when they replicate and that they are late-replicating with respect to the other chromosomes of the complement during the pre-meiotic synthesis period. Asynchronous replication was also observed among the regular chromosomes, but the late-replicating ones could not be identified.

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