Temporal relationship between premeiotic DNA synthesis and heat-induced recombination in oocytes of Drosophila melanogaster

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Temporal relationship between premeiotic DNA synthesis and heat-induced recombination in oocytes of Drosophila melanogaster

by

John Warren Day

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INTRODUCTION

One of the main problems remaining in cellular biology is the molecular mechanism by which recombination of linked genes is accomplished during meiosis in higher organisms. The various models proposed to account for genetic recombination at the molecular level fall into two major categories: those involving breakage of non-sister chromatids with subsequent re-healing in cross-wise fashion ("breakage-reunion"), and those involving switching of templates during chromosome duplication ("copying-choice"). Most of the models of the former type assume an enzymatic breakage of DNA strands, formation of heteroduplex DNA regions, and a repair system to correct mis-matched bases and fill in single-stranded regions so that two complete double-helices result. The models further propose that these events occur during the zygotene-pachytene stages of meiosis, clearly separated from the bulk of DNA synthesis occurring during premeiotic interphase (RHOADES 1968; HOLLIDAY 1964; HOWELL and STERN 1971; PASZEWSKI 1970; STAHL 1969; WHITEHOUSE and HASTINGS 1965; WHITEHOUSE 1966). On the other hand, the copying-choice models considered chromosome duplication and crossing-over to be interrelated events (BELLING 1933; LINDEGREN 1964; LEDERBERG 1955; PRITCHARD 1960). In the past decade many experiments have been designed to distinguish between these two major recombination mechanisms. As a result, some favor a separation of crossing-over and chromosome duplication while others are consistent with the copying-choice models (see reviews of GRELL 1969; HENDERSON 1970; PEACOCK 1971).
Evidence favoring separation of crossing-over and chromosome duplication: ROSSEN and WESTERGAARD (1966) measured DNA doubling during the meiotic cycle in the Ascomycete fungus Neottiella rutilans using Feulgen cytophotometry. They interpreted their results as indicating that DNA replication takes place in haploid cells before sexual fusion and that no further replication occurs during the ensuing meiotic process. However, GRELL (1969) has pointed out several ambiguities in their work including (1) the possibility that their material, which was collected in the field over an undisclosed period of time, consisted of a mixture of species with different chromosome numbers, making their DNA determinations invalid; (2) their disagreement with earlier observations as to which nuclei are the prefusion nuclei; and (3) their failure to demonstrate convincingly that DNA replication does not occur subsequent to karyogamy.

A similar interpretation was made by SUEOKA, CHIANG and KATES (1967) of their findings with an octet strain of Chlamydomonas reinhardi. These investigators determined the time of DNA replication using density gradient centrifugation of DNA isolated at various times during the course of the meiotic cycle. Zygotes formed by the union of gametes with $^{15}N$-labelled DNA were transferred to $^{14}N$ medium in which to mature and germinate. Only one semiconservative DNA replication, resulting in the complete replacement of the $^{15}N$-$^{15}N$ band with a hybrid $^{15}N$-$^{14}N$ band, occurred between fusion of gametes and the release of eight zoospores from the germinating zygote. Since the eight zoospores were genetically four sets of identical pairs, these authors interpreted their results to
mean that a premeiotic DNA replication takes place prior to fusion of

gametes and that the DNA replication occurring during zygote germination
is a mitotic replication similar to that in Ascomycetes such as

Neurospora. However, they pointed out that, since the zygospore nucleus
does not divide until after DNA replication, there was still the possi­

bility that this DNA replication was occurring concomitantly with
crossing-over. Using a tetrad-forming strain of Chlamydomonas, DAVIES
and LAWRENCE (1967) found two periods, both preceding diplotene, during
which recombination frequencies could be altered by DNA synthesis
inhibitors. Thus, the situations in Neotieila and Chlamydomonas indicate
that chromosome duplication takes place in separate nuclei prior to
fusion, chromosome pairing, and crossing-over, but do not rule out the
possibility that DNA synthesis and crossing-over occur simultaneously.

A series of investigations in Orthopteran insects using heat shocks
to alter chiasma frequencies and tritiated thymidine to label chromosomes
at the premeiotic DNA synthesis period have also been cited as evidence
that the two events are separate. HENDERSON (1966) applied heat shocks
to male desert locusts, Schistocerca gregaria, immediately after
injecting them with tritiated thymidine and compared the time of
appearance of labelled cells at metaphase I with the time of modification
of chiasma frequency. He found that a reduction in chiasma frequency
preceded the arrival of labelled cells at metaphase I by 1-2 days,
indicating a separation by at least that much between the premeiotic
DNA synthesis period and crossing-over. Although HENDERSON concludes
from his data that the heat is effective at late zygonema or early pachynema, this is most assuredly not the case if heat is affecting chiasma frequency directly. Considering his data on the durations of various phases of meiosis at 40°C and 30°C, there must be at least 7 days between premeiotic DNA synthesis and pachytene, yet there is only a span of 2 days between chiasma reduction and the appearance of labelled chromosomes at metaphase I. This would place the time of the heat effect closer to interphase and leptotene than pachytene. Furthermore, as pointed out by GRELL (1969), HENDERSON used the values obtained for normal chiasma frequencies in an earlier study (HENDERSON 1963) as control values for his 1966 studies, which sheds some doubt as to which decreases in chiasma frequency are really significant.

In a nearly identical experiment using the Australian grasshopper Goniea australasiae, PEACOCK (1970) found that reduced chiasma frequency at diplotene preceded arrival of labelled cells at metaphase I by 5-6 days. Further studies by HENDERSON (1970) have indicated a separation between premeiotic DNA synthesis and the time of crossing-over (chiasma reduction) of 2½ days for Locusta migratoria, 3 days for Schistocerca gregaria, and 7 days for Melanoplus differentialis.

In a series of experiments dealing with the biochemistry of meiosis in cultured microsporocytes of Lilium, the relationship of premeiotic DNA synthesis to crossing-over has been intensively studied (HOTTA, ITO and STERN 1966; HOTTA and STERN 1971; HOWELL and STERN 1971). Following bulk, premeiotic DNA synthesis, two distinct periods of DNA synthesis occur during meiotic prophase. A small amount of synthesis
at zygotene has been shown by hybridization studies to represent a part of the normal round of semi-conservative replication, suggestive of late replicating DNA species. Similar studies of DNA synthesized at pachytene indicated that this DNA was not late replicating nor did it represent net synthesis of DNA. Furthermore, the pachytene DNA was shown to be of heterogeneous base composition, indicative of repair synthesis operating over the entire genome. All of the necessary enzymes for DNA breakage and rejoining have been shown to be present at the zygotene-pachytene stages in extracts of microsporocytes. A single endonuclease not found at premeiotic interphase, but with peak activity at pachytene, requires double-stranded DNA as substrate and produces single-strand breaks with 5'-hydroxyl and 3'-phosphoryl end groups. Phosphatase and kinase activities capable of modifying the endonuclease-produced end groups to 5'-phosphoryl and 3'-hydroxyl were also found, the kinase having peak activity at zygotene-pachytene. A polynucleotide ligase, with peaks of activity at premeiotic interphase and zygotene-pachytene, was also detected and shown to require 3'-hydroxyl and 5'-phosphoryl end groups. This evidence taken collectively has led to the suggestion that crossing-over in Lilium is accomplished by enzymatic breakage and rejoining during the zygotene-pachytene stages of prophase, well separated from the premeiotic DNA synthesis.

B. C. LU (1969) has studied the effects of high and low temperature treatment on genetic recombination in the fungus Coprinus lagopus. Significant increases in recombination induced by the treatments occurred at the zygotene-pachytene stages of meiosis with maximum effects at late
zygotene-late pachytene for cold treatments and mid zygotene-late pachytene for heat treatments. Preliminary evidence indicated that premeiotic DNA synthesis occurred prior to karyogamy in separate pre-fusion nuclei. In a later study LU (1970) correlated these observations with electron microscope studies of the appearance of synaptonemal complexes, which have been implicated in zygotene-pachytene pairing and crossing-over (MOSES 1968). The conclusion was that genetic recombination could be affected during the entire time course of the presence of synaptonemal complexes. The sequence of events during meiosis in Coprinus was concluded to be premeiotic DNA synthesis, karyogamy, delayed DNA replication, synaptonemal complex formation and, finally, recombination.

Evidence consistent with temporal coincidence of premeiotic DNA synthesis and crossing-over: ABEL (1968) applied heat treatments to the liverwort Sphaerocarpos donellii at various times during meiosis. He found that the most sensitive period for a heat-induced increase in recombination occurred at a time when 80% of the spore mother cells were at interphase and 20% were at leptotene. As long as 24 hours after this most sensitive period, only 10% of the cells were in pachytene, 43% were in leptotene, and 47% were in interphase. ABEL concluded that crossing-over did not occur at pachytene but at an earlier stage close to premeiotic DNA synthesis.

In similar studies in maize, MAGUIRE (1967, 1968) heat shocked developing microsporocytes of an interchange heterozygote involving the nucleolus-organizer chromosome. Changes in recombination frequencies
in the interstitial region of chromosome 6 were determined by observing the nucleolar distribution in tetrads. Increase in recombination frequency in this region decreases the frequency of adjacent-II segregation at anaphase I which gives rise to abnormal tetrads in which two cells have two nucleoli and the other two have none. Normal tetrads in which all cells have one nucleolus are the result of alternate and adjacent-I segregation. MAGUIRE concluded that the greatest effect induced by heat treatment corresponded to premeiotic interphase and early synizesis, a stage which, in maize, includes leptotene and zygotene.

GRELL and CHANDLEY (1965) administered 24-hour heat shocks to Drosophila melanogaster females and determined recombination frequencies among their progeny in successive 1-day broods. They found that significant increases in recombination frequency occurred between days 6½ and 12½, with the peak increase on day 8½. In order to determine the time of premeiotic DNA synthesis they injected females with 
\(^3\)H-thymidine and followed the progression of labelled oocytes through meiosis. They observed that the majority of labelled oocytes appeared 8-9 days after injection of label, corresponding, at least roughly, with the oviposition time of eggs exhibiting peak sensitivity to heat shock.

In an attempt to more critically resolve the heat sensitive period, GRELL (1967') studied heat-induced recombination in oocytes of developing pupae, using 12-hour heat shocks administered every 6 hours during the pre-pupal and pupal periods. The significant increase in recombination now occurred over a much shorter period (36 hours as opposed to 5 days
in the earlier studies), due presumably to the better synchrony of oocytes sampled from the pupal ovaries. In addition, GRELL determined the appearance of eight-cell oogonal metaphase figures in ovary squashes from developing pupae, equating this with the appearance of oocytes. The increase in proportion of eight-cell metaphases just preceded the increase in recombination due to the heat shocks, again indicating at least a partial coincidence between premeiotic DNA synthesis and recombination.

McNELLY-INGLE, LAMB and FROST (1966) studied the effect of temperature alteration on recombination in *Neurospora crassa* and found results closely paralleling those obtained with *Drosophila* by PLOUGH (1917, 1921). In both organisms temperatures above or below 25°C increased recombination in regions near the centromeres. In the *Neurospora* studies temperature changes affected recombination when applied during protoperithecial development prior to meiosis as well as during meiosis. McNELLY-INGLE, LAMB and FROST concluded that the striking similarity between *Neurospora* and *Drosophila* with respect to temperature/recombination frequency curves is suggestive of a possible direct effect of temperature on the recombination processes. The common effect in these organisms is, therefore, a premeiotic one.

**Development of the Problem**

The conclusions reached in those experiments utilizing heat shocks and alteration of chiasma frequency as a measure of recombination frequency have been criticized on the basis that heat shock may cause a
loss of chiasmata which may be masked by redistribution of the remaining chiasmata. Thus, alteration in chiasma frequency may not be a true measure of alteration in recombination frequency. Also, the results of investigations indicating premeiotic effects of heat treatments have been criticized on the tenet that they are delayed effects and actually, some process which occurs prior to crossing-over was altered. Regardless of the validity of these criticisms, the evidence cited above is contradictory and can not easily be resolved. One could, for instance, attribute the premeiotic effects of heat shock in *Sphaerocarpos*, maize, *Drosophila*, and *Neurospora* to delayed effects and postulate that recombination itself actually occurs at the pachytene stage of meiosis in all organisms. Consequently, one would have to accept direct effects in some organisms and indirect effects in others, such as, *Goniaea*, *Schistocerca*, *Melanoplus* and *Coprinus*. On the other hand, one could entertain the possibility that recombination is a complex process involving premeiotic initiation, at least in some organisms, and finalization at pachytene. In organisms which have premeiotic initiation events there may be some which show sensitivity to agents, such as temperature change, which cause alterations in recombination frequencies and others which do not, due to physiological differences between various organisms. Thus, it does not seem unreasonable to expect that among higher organisms diverse means of accomplishing genetic recombination have evolved as a function of varying degrees of complexity of the meiotic mechanism as a whole. That is to say, that, although there indeed may be only one molecular mechanism of formation
of recombinant DNA double helices, the logistics of accomplishing this end within complex nucleic acid-protein organelles, along with duplication and segregation of these organelles, may differ from organism to organism. It is the aim of this investigation to examine some aspects of recombination in an organism which appears to have a premeiotic recombination component process in order to further test the existence of such a process.

**Meiosis in Drosophila melanogaster females:** Drosophila melanogaster females are exceptionally well suited for studies on meiosis and the mechanism of genetic recombination. Their genetic system is the best-characterized of all higher organisms. Experimental alteration of recombination frequencies by intra- and interchromosomal effects of innumerable inversions and translocations are possible. There are mutants available which affect recombination, such as crossover-suppressor-on-the-third-chromosome-of-Gowen, symbolized as c3G (GOWEN and GOWEN 1922), and those recently isolated by SANDLER, LINDSLEY, NICOLLETTI and TRIPPA (1968). Many external agents, such as irradiation, antibiotics, and temperature changes, are known to alter recombination frequencies and have been well characterized. In addition, the ovary has been the object of extensive cytological studies with respect to development, structure, and function at both the light and electron microscope levels. Finally, a powerful experimental tool is available in that Drosophila ovaries can be transplanted into appropriate hosts or cultured in vitro. One major disadvantage to studies on meiosis in
Drosophila females is the difficulty in analyzing the chromosomes during meiotic prophase. However, in view of the array of tools which can be brought to bear on the problem of meiosis and recombination in Drosophila melanogaster females, it appears that they are organisms of great promise in studies of this nature.

**Description of the Drosophila ovary:** The Drosophila ovary consists of a variable number of parallel egg strings called ovarioles (Figure 1). Each ovariole is subdivided into an anterior portion, the germarium, and a posterior portion, the vitellarium. The anterior-most portion of the germarium, the terminal filament, consists of several cells shaped like flat cylinders stacked on one another in single file. Just posterior to the terminal filament the germarium widens out to resemble an elongate pear. The cavity of the germarium contains germ cells in various stages of development and follicle cells which will eventually become associated with the germ cells to form egg chambers which enter the vitellarium portion of the ovariole. A stem cell oogonium (or possibly more than one) occupies an area adjacent to the terminal filament. This cell divides to give rise to two cells, one of which remains adjacent to the terminal filament and behaves again like a stem cell, the other of which undergoes four synchronous divisions to form a cluster of 16 cells interconnected by canals. These divisions occur as the growing group of cells moves posteriorly in the germarium. The clusters of 16 cells become surrounded by a single layer of follicle cells in the posterior-most portion of the germarium. In the
Figure 1. - One ovariole of an ovary from a late pupal stage of Drosophila melanogaster. tf = terminal filament; sco = stem cell oogonium; 2-, 4-, 8- & 16- cys = 2-, 4-, 8-, and 16-cell clusters of interconnected cystocytes; ic = incipient cyst; fc = follicle cell; bs = basal stalk. (Redrawn from ABOIM 1945)
terminology of KING, RUBINSON, and SMITH (1956) the group of 16 cells enclosed in a follicular envelope in the posterior portion of the germarium is called a Stage 1 egg chamber. When this chamber is budded off into the vitellarium by constriction of follicle cells between it and the next anterior group of 16 cells, it is called a Stage 2 egg chamber. The egg chambers proceed posteriorly in the vitellarium while 15 of the 16 cells within, called nurse cells, manufacture large amounts of nutrients which are poured into the 16th cell, the oocyte, via the interconnecting canals. The various stages of development of the egg chamber as it proceeds through the vitellarium have been designated stages 2 through 14, on the basis of changes in cellular morphology and size. Stage 14 is the mature egg ready to be fertilized and oviposited.

The developing ovary begins to differentiate individual ovarioles during the larval stages. At this time they consist only of the germarium portion, which grows in size during the late larval and early pupal periods. It is not until the mid-pupal stage that the vitellarium portions become evident. The ovariole of an adult female contains several egg chambers in the vitellarium in various stages of development and a large germarium. The adult germarium can be divided into three portions: (1) the anterior third (Region 1) containing oogonia in various stages of division; (2) the middle third (Region 2) containing 16-cell groups moving posteriorly; and (3) the posterior third (Region 3) containing the developing Stage 1 egg chamber.

Studies in adults: PLOURGH (1917, 1921) was the first to examine
the effect of temperature on recombination in *Drosophila* and he con-
cluded that the stage affected was either the oogonia or early oocytes
in the germarial region of the ovariole. The later studies of GRELL
(1966) demonstrated genetically that temperature-induced recombination
was entirely of meiotic origin. As mentioned above, the experiments
of GRELL and CHANDLEY (1965) indicated a rough coincidence between the
time of heat-induced recombination and premeiotic DNA synthesis in the
eyearly oocyte. The labelling studies of CHANDLEY (1966) showed that
oocytes incorporate injected tritiated thymidine during their pre-
meiotic DNA synthetic period in the anterior region of the germarium.
KOCH, SMITH and KING (1967) in their studies at the electron microscope
level have shown that synaptonemal complexes are first found in paired
cells (the pro-oocytes) within 16-cell clusters in the anterior region
of the germarium, approximately the same region where CHANDLEY (1966)
found thymidine uptake by early oocytes. They further determined that
the complexes grew to maximum length in no less than 4 days, from which
they concluded that crossing-over may occur near the time of premeiotic
DNA synthesis and for several days thereafter. KING (1970) has
extrapolated from observations in other organisms, in which the synap-
tonemal complex has been observed to be present only in the zygotene-
pachytene stages of meiotic prophase, to propose a sequence of events
in *Drosophila*. According to his sequence, leptonema occurs immediately
after premeiotic DNA synthesis in 16-cell clusters in the anterior region
of the germarium, the oocytes in clusters moving posteriorly through the
germarium are in zygonema, and pachynema is finished in oocytes residing
in the anterior portion of the vitellarium.

The conclusions one can reach from these studies in adults are limited, especially with respect to a clear temporal sequence of meiotic events. It is apparent that premeiotic DNA synthesis, early formation of synaptonemal complexes, and heat-induced recombination may occur simultaneously in oocytes residing in the anterior region of the germarium or, at least, that these events take place sequentially over a rather short period of time.

Studies in larval and pupal stages: A clearer picture of meiotic events can be pieced together from various investigations using larval and pupal gonads mainly because valid comparisons can be made due to the presence of several landmarks during the developmental stages. Thus, if the temporal relationship between various meiotic events examined in several different investigations and some common stage in development, such as pupation, is known, then these meiotic events can be arranged in proper sequence providing culture conditions are the same. Furthermore, the synchrony of stages from ovariole to ovariole within larval and pupal ovaries is somewhat better than in adult ovaries (Grell 1967).

Buchner (1957) examined the relative proportions of 2-, 4-, and 8-cell metaphase figures in squash preparations of ovaries from pupae between the ages of 7 and 31 hours after puparium formation. She also determined the first appearance of nuclei of the proper diameter to represent those of a 16-cell cyst. According to her determinations
the first 8-cell metaphase figures were seen at 9 hours after puparium formation and the first 16-cell nuclei appeared between 7 and 12 hours after puparium formation. She also calculated the combined duration of metaphase plus anaphase to be no more than 8 minutes in the oogonial divisions. Thus, according to this work the first appearance of an oocyte containing the 2c amount of DNA is 9-10 hours after puparium formation. GRELL (1967) also determined the first appearance of 8-cell metaphase figures, equating them with the appearance of oocytes, and found a small percentage present at 120 hours of development post egg laying. By comparing BUCHER's (1957) timetable of development and her illustrations (her Figure 2) with the results obtained in the present investigation, the time of puparium formation in her material can be set at approximately 118 hours post egg laying. Therefore, the first appearance of the oocyte is set at 120 hours by GRELL (1967) and 127 hours by BUCHER (1957).

A third investigation bearing on this point is that of KING, AGGARWAL and AGGARWAL (1968) who studied the development of the ovary at the electron microscope level. In their material puparium formation occurred at 120 hours and the first 8-cell groups (recognized by the presence of interconnecting canals between individual cells) appeared between 144 hours and 150 hours post egg laying and 16-cell groups first appeared between 150 hours and 156 hours. In addition, they first observed synaptonemal complexes in nuclei of pro-oocytes at 156 hours of development and concluded that the first oocytes to undergo crossing-over do so between 144 and 156 hours post egg laying, although they did
not observe 16-cell clusters in 150 hour ovaries. In ovaries of 168 hours of development they show mostly ovarioles with two egg chambers in the vitellarium, the oldest being comparable to a Stage 3 egg chamber. According to these statistics then, the time needed for progression from a newly formed oocyte (i.e. first formation of a 16-cell group) to Stage 3 must be no more than 18 hours in their material.

Thus, the time of first appearance of the oocyte has been determined to be 120, 127, or 150 hours post egg laying, the major discrepancy with respect to this event being between KING, AGGARWAL and AGGARWAL's (1968) work and that of GRELL (1967) and BUCHER (1957). Also, BUCHER (1957) indicates the time of appearance of ovarioles with two egg chambers in the vitellarium to be later than 178 hours post egg laying. Consequently, the time needed for an oocyte to progress from first appearance to Stage 3 in her material is not less than 50 hours, compared to not more than 18 hours claimed by KING, AGGARWAL and AGGARWAL (1968). These discrepancies are much greater than can be expected on the basis of genotype differences between wild-type strains grown on standard culture media at a constant 25°C. Obviously, a clear temporal sequence of meiotic events is needed before meaningful conclusions regarding their individual roles in recombination can be reached.

The best description of chromosome morphology in *Drosophila melanogaster* oocytes during meiosis is that of GUYENOT and NAVILLE (1933). They describe a sequence of events progressing from early oogonial stages in the anterior region of the germarium, through a
long pachytene stage which they observed in the middle and posterior region of the germarium and as late as a Stage 2 egg chamber, and ending in a condensation of chromosomes into a dense knot of chromatin called a karyosome. Their sequence also includes an early stage in which the homologous chromosomes are paired and condensed into chromatic bodies called pro-chromosomes (they observed 3, which most likely represent the X chromosomes and the major autosomes). This stage was immediately followed by a diffuse stage and after this the classical stages of meiotic prophase, beginning with leptotene, were observed. Unfortunately, this sequence was determined by observations of the relative positions of the stages within ovarioles in pupae of ill-defined ages, at the mid-pupal period (before coloration in the eyes), and a later pupal period (pigmentation of the wings). Only approximate timing can be inferred from the morphology illustrated in their Figure 1 (pg. 219), and no decision as to coincidence of the prophase stages described in this study with the other meiotic or premeiotic events discussed in the previously mentioned studies can be made with any degree of confidence.

From the evidence cited in the above four investigations on pupal ovaries, it seems possible that premeiotic DNA synthesis, pairing of homologous chromosomes, initiation of synaptonemal complexes, and crossing-over could all be occurring, at least to some extent, concurrently. It was the purpose of this investigation to determine as precisely as possible the duration of, and temporal relationship between, premeiotic DNA synthesis and heat-induced recombination in oocytes of *Drosophila melanogaster* females during their pupal development.
The interrelationship between heat-induced alteration of recombination and the interchromosomal effect on recombination was studied in an attempt to gain additional information on the nature of these two phenomena, especially with respect to the question of a direct or delayed effect of heat on recombination. In addition, careful observations on the timing of developmental events was made, which will enable meaningful comparisons to be made with other investigations using similar material.
MATERIALS AND METHODS

Synchronous cultures: In any study dealing with temporal relationships among events occurring during the development of an organism, it is desirable to sample from a synchronously developing population. Unlike some systems in microorganisms, obtaining samples of synchronously developing Drosophila in pupal or later stages requires a great deal of physical effort. An often used technique involves the observation of larvae which are ready to pupate and collection of all those which evert their anterior spiracles during a short period of time. This technique is best suited for studies in which relatively small samples are needed. The present studies, however, required many large samples for both genetic and cytological studies. A method for obtaining large synchronized samples, which is a compromise between exactness of synchrony and physical effort involved, is the collection of eggs over a short period of time.

This method requires one to be careful in the manner in which the eggs are collected. Inseminated Drosophila females are inhibited in their egg-laying by mechanical disturbances such as being shaken over into another culture bottle and by a dry surface condition of the culture medium. Under such adverse conditions eggs which are ready to be deposited can be retained and fertilized in the ovariole by stored sperm (KING and SANG 1958). Subsequent oviposition thus introduces a degree of asynchrony into the population, since some of the zygotes
will be in advanced embryonic stages while others would be just
beginning. Furthermore, even if eggs retained by females are
fertilized as they are laid, they may not develop at the normal rate.
In order to eliminate these possible sources of asynchrony within the
samples the following two methods of egg-collecting were employed.

Females collected over an 8-hour period were aged for one day
in quarter pint culture bottles containing standard cornmeal-sugar-
brewer's yeast-agar medium. This standard medium was used throughout
all experiments. The surface of the medium was sprayed with a
suspension of live yeast and supplemented with a small amount of
Fleischmann's or Red Star baker's yeast. Males aged for 1-2 days
in the absence of females were placed in the bottles with appropriate
females at a ratio of 1-2 males per female. After 8-24 hours these
parents were transferred to special containers at a density of 25-
30 females with males at the aforementioned ratio. The containers
were discarded wide-mouth plastic dry-chemical bottles of approxi-
mately one pound capacity. A hole approximately 1/4" in diameter was
cut in the side of each container near the bottom and closed with a
cotton plug to provide air exchange. The mouth of the container was
covered by a single piece of cheesecloth held in place by a rubber
band and pulled taut over the rim of the mouth. Food cups in which
the eggs were collected consisted of aluminum weighing pans (60mm in
diameter; 16mm deep) filled with approximately 3/8" of standard
medium. The surface of the medium was scored several times with a
dissecting needle and brushed with a thin paste of baker's yeast, in order to provide moist depressions in which the females lay eggs readily.

The containers of flies were inverted and placed on the food cups so that the entire rim of the mouth and the cheese cloth contacted the surface of the medium. All the flies could feed and the females could lay eggs through the cheesecloth quite easily. Consecutive samples of eggs could be collected by gently raising the container off of one food cup and placing it onto the next. The cheesecloth was changed after a few transfers in order to minimize carryover of eggs sticking to the cheesecloth. The food cups were stored in plastic boxes provided with air-exchange holes covered with cheesecloth to keep out possible contaminating females and containing in the bottom a moistened paper towel to keep the medium from drying out. The food cups were generally examined 22-24 hours later at the time of hatching of the larvae and early hatching larvae and unhatched eggs were discarded. In large experiments a portion of the food cups had to be examined 2 days later and larvae which were larger or smaller than the majority were discarded. The food cup medium was then cut into pieces and transferred to culture bottles so that the larval density did not exceed 75 larvae per bottle. A folded Kim-wipe (Kimberly-Clark Corp.) or Micro-wipe (Scott Paper Corp.) was pressed into the medium to provide surface
area on which the larvae later pupated.

The second method differed from the first in that the parent flies were kept in quarter pint culture bottles instead of the special containers. They were transferred from bottle to bottle by gentle shaking. Also, there were only 15-20 females with 1-2 times as many males per bottle. Preparation of the surface of the medium and larval selection were the same as in the first method. Adjustment of larval density was accomplished by scooping up excess larvae from one bottle and placing them in another or discarding them.

The first method was more tedious and time consuming, although it was possible to obtain large numbers of eggs laid during one hour periods. The second method was much less time consuming and if the transfers were as gentle as possible and 1½ hours apart, a sufficiently large number of eggs were laid by the females in each collection period. There was a noticeable difference in the rate of oviposition of females when the transfer periods were increased from 1 hour to 1½ hours. In both methods eggs were collected from the parental females from their 3rd through their 7th day after eclosion, after which they were discarded. Constant surveillance of the condition of the culture medium was maintained and dry yeast and moisture was added as necessary to insure optimum growth conditions for the larval stages.
Genetic studies: The early work of PLOUGH (1917) and the more recent studies of GRELL (1967) indicated that regions near or spanning the centric heterochromatin were most responsive to elevated temperatures. In the present experiments three such regions were studied: forked-3 to carnation ($f^3$ - car) on the X-chromosome, Tuft to vestigial ($Tft$ - vg) on the second chromosome, and scarlet to Stubble ($st$ - Sb) on the third chromosome. In order to facilitate rapid scoring of the mutant scarlet, the stocks involved in this series of experiments were made homozygous for brown (bw). Scarlet and non-scarlet flies were white-eyed and brown-eyed, respectively, due to the interaction of scarlet and brown. The interaction of elevated temperature and the inter-chromosomal effect of inversion heterozygosity on recombination was studied utilizing the complex autosomal inversions SM5 (second chromosome inversion carrying the dominant marker curly wings, Cy) and TM2 (third chromosome inversion carrying the dominant marker ultra-bithorax, Ubx$^{130}$). A more complete description of the mutants and chromosome rearrangements used in this study can be found in LINDSLEY and GRELL (1968).

In one series of experiments dealing with recombination in the $f^3$ - car region of the X-chromosome, heterozygous females of the genotype $f^3$ car/+$+$ were obtained by mating Canton-S wild-type females to $f^3$ car/Y males from an attached-X stock, C(1)DX, yf/Y?$ x f^3$ car/Y$. The mutants forked bristles, $f^3$, and carnation eye color, car, are located at 56.7 and 62.5, respectively, on the X-chromosome. In another series, females of the same X-chromosome constitution, but differing in
their autosomal make-up were generated and recombination studied according to the breeding scheme outlined in Figure 2.

Recombination in the Tft - vg and st - Sb regions were studied in females of the genotype Tft + bw/+ vg bw ; st +/- Sb obtained from the mating of + vg bw ; st + homozygous females to Tft + bw ; + Sb/\(T(2;3)\) ap males. The mutants tuft bristles, Tft, vestigial wings, vg, and brown eye color, bw, are located at 53.2, 67.0, and 104.5, respectively, on the second chromosome. The mutants scarlet eye color, st, and stubble bristles, Sb, are located at 44.0 and 58.2 respectively, on the third chromosome. The translocation was used so that the second and third chromosomes just described would be inherited as a unit.

Females of these various genotypes were given heat shocks during their pupal development by placing the culture bottles in which they were developing in incubators maintained at 35° ± 1°C and 80-85% relative humidity. At all other times the cultures were kept at 25° ± 0.5°C and 90-95% relative humidity. In any one experiment, the total number of bottles was divided into a few smaller groups which were placed at the elevated temperature at 114, 120, 126, etc. hours for periods of 8 hours and then returned to 25°C. The bottles were placed on their sides with the cotton plugs removed in order to allow rapid equilibration at the elevated temperature. Just prior to transfer to the elevated temperature, 5-10 pupae were removed from every bottle in each experiment and placed in separate bottles which remained at 25°C. These pupae constituted the control groups for each experiment.

After appropriate treatments during pupal development the adult
Figure 2.— The breeding scheme for obtaining females having identical X-chromosome constitution but differing in the number and type of autosomal inversions present. The mutants vermilion eye color, v, and a recessive lethal, 1(1)64, are located on the X-chromosome, as is the recessive lethal, 1 (non-allelic to 1(1)64), which is associated with the complex X-chromosome inversion, FM3. Plum eye color, Pm, and stubble bristles, Sb, are dominant mutations associated with inversions of the 2nd and 3rd chromosomes, respectively. Also see the text for description of the remaining symbols.
females were collected 0-4 hours after eclosion and mated to appropriate tester males either in vials (2 ♀♀ and 3-4 ♂♂) or in bottles (3-5 ♀♀ and 5-10 ♂♂). An average of 20-30 eggs per female, as ascertained by direct count, were collected before the parents were discarded. The adults arising from these eggs were scored for recombination rate in the various regions under investigation. Preliminary ovariole counts of females of the genetic constitutions used in these experiments indicated that each female contained 30-40 ovarioles. Hence, the eggs collected correspond to the first maturing oocytes in each ovariole. Ovariole counts of several females of each of the different genotypes studied were made during the present experiments in order to verify the preliminary data.

The results of several experiments, each providing data for a few heat shock periods, were pooled in order to provide data for all heat shock periods from 114 to 198 hours. The validity of pooling data was determined by a chi-square variance test for homogeneity using the formula:

\[ x^2 = \sum \frac{n_i (p_i - \bar{p})^2}{\bar{pq}} \]

where \( n_i \) = the total number of progeny in the \( i \)th experiment, \( p_i \) = the proportion of recombinant progeny among the total in the \( i \)th experiment, \( \bar{p} \) = the proportion of recombinant progeny among the total progeny of all experiments, and \( \bar{q} = 1 - \bar{p} \). The degrees of freedom for \( x^2 \) in this case is one less than the total number of experiments pooled.

The effect of heat shock is expressed as the recombination rate among the heat treated females minus the recombination rate among their
controls \( (p_{ht} - p_c) \). The standard error of this difference was calculated according to the formula

\[
S.E. (p_{ht} - p_c) = \sqrt{\frac{(p_{ht})(q_{ht})}{n_{ht}} + \frac{p_c q_c}{n_c}}
\]

where \( p_{ht} \) = the proportion of recombinants among the progeny of the heat treated group, \( p_c \) = the proportion of recombinants among the progeny of their control group, \( q_{ht} = 1 - p_{ht} \), \( q_c = 1 - p_c \), \( n_{ht} \) = the total number of progeny in the heat treated group, and \( n_c \) = the total number of progeny in their control group. The 95% confidence limits were calculated as \( \pm 1.96 \) (S.E.). (SNEDECOR and COCHRAN 1967)

**Cytological studies:** The number of ovarioles contained in the ovaries of the females of the various genotypes studied was determined in orcein-stained squashes. The ovaries were removed from 6-8 females and placed in pairs in small drops of 2% acetic-orcein (2 gms. of Gurr's synthetic orcein in 100 cc. 45% acetic acid) on microscope slides. After staining for approximately 10 minutes, the ovaries were flooded with 45% acetic acid in order to dilute the stain and render the ovaries more easily visible. The ovaries were then teased apart and a coverslip placed on the slide so that the ovaries were lightly squashed by capillary pressure. This technique resulted in a monolayer of ovarioles, allowing a quick and accurate count of the ovariole number by observation of the slide at \( \sim 16X \) using a binocular microscope.

A timetable of pupal development of \( f_{car/+} \) and \( Tft + bw/+ vg bw; st +/+ Sb \) females was made by observing the time of puparium formation,
pupation, formation of two egg chambers in the vitellarium, and deposit of pigment in the wing pads. The time of puparium formation, as signalled by eversion of the anterior spiracles by a pupating larva, and deposition of pigment in the wing pads were observed with the unaided eye directly through the glass culture bottles. The time of pupation was determined by removing samples of pupae from the bottles and observing them under a binocular microscope. Whether the larval form or imago form is present can be observed directly through the pupa case. The time of appearance of two vitellarial chambers in an ovariole was determined by observing orcein-stained ovaries using a compound microscope at 200X magnification.

General observations of ovariole development during pupal stages were made on both orcein- and Feulgen-stained ovaries. In both cases ovaries were dissected in isotonic saline (0.75% NaCl solution) and transferred to the next solution according to the staining technique to be used. If orcein stain was used, the ovaries were transferred to a small amount (~1/3 drop) of 45% acetic acid on a coverglass and teased apart. Immediately, a small drop of 2% acetic-orcein was added to the fixative and the two mixed. A slide was then placed on the drop so that capillary forces drew the coverglass to the slide and provided a "light squash" of the tissue. The edges of the coverslip were then sealed with melted paraffin. Slides made in such a manner were observed within 3-4 days with phase contrast optics. In the case of Feulgen staining, the ovaries were transferred to a small drop of saline on a subbed slide and teased apart into its constituent ovarioles. The excess saline was
then absorbed with absorbent paper and the preparation was blown to near
dryness with air. The slide with its adhering tissue was placed in 3:1
ethyl alcohol: acetic acid for a fixation time of 10 minutes. The slide
was passed through descending alcohols to 50% alcohol, hydrolyzed in 1N
hydrochloric acid for 1 minute at room temperature, then in 1N HCl at
60°C for an additional 5 minutes. The tissue was stained for 1-2 hours
in Schiff reagent at room temperature. After rinsing in tap water to
develop color the tissue was dehydrated through ascending alcohols,
cleared in toluene and a coverslip mounted with Permount. A fast green
counterstain was applied at the 70% alcohol step.

The time of premeiotic DNA synthesis in the first maturing oocytes
of $f^3$ car/+ females was determined by autoradiography of ovaries which
were labelled with tritiated thymidine. Ovaries of pupae ranging in age
from 120 to 186 hours were dissected in Robb's Superior Buffered Saline,
hereinafter referred to as S.B.S., (ROBB 1969) under aseptic
conditions. The ovaries were teased apart, the pieces sucked up into a
micropipette and transferred to a solution of tritiated thymidine
consisting of 1 part aqueous thymidine-methyl-H$^3$ solution (6.7c/mM, New
England Nuclear) and 11-15 parts S.B.S. After an incubation period of
1-2 hours at room temperature (22-24°C) the ovary pieces were again
sucked up into a micropipette and transplanted into the abdomens of
young females (2-6 hours after eclosion). The host females were stored
in 20-dram shell vials containing standard culture medium for 6-48 hours
(donor ovaries from 120-132 hour pupae) or 2-5 days (donor ovaries from
132 hour or older pupae) after which they were sacrificed and the donor
ovaries prepared for autoradiography. All operations were performed under a binocular stereo microscope at a magnification of 20X.

Figure 3 illustrates the micropipetting instrument used in these transplant experiments. It consisted of a glass capillary tube connected by polyethylene tubing to a 25 ul syringe (Hamilton Company) held in a micro-buret holder. The glass capillary (1.0 mm outside diameter, Aloe Scientific) was drawn out to a diameter of approximately .1-.2 mm and broken in such a fashion as to form a beveled "hypodermic" tip. The other end of the capillary tube was fire polished and inserted into one end of a 1½ foot length of polyethylene tubing (Intramedic PE 60 (.030 inch O.D.), Clay-Adams). The fit of the capillary into the polyethylene tubing was so tight that leaks never developed. The other end of the polyethylene tubing was pushed onto the steel needle of the syringe and sealed with clear fingernail polish. The syringe was clamped into the holder of a Model SB2 Syringe Micro-buret (Micro-metric Instrument Co.) and the end of the plunger clamped to the stem of the machine screw. Since clockwise rotation of the machine screw could advance the plunger into the syringe but counterclockwise rotation could not withdraw it, rubber bands were placed over the plunger end and the Micro-metric dial of the machine screw in such a way as to supply the force for withdrawing the plunger as the machine screw was rotated counterclockwise. The entire pipetting system was filled with mineral oil except during the transplanting operations at which time a small amount of S.B.S. was sucked into the micropipette. Advancing the machine screw a distance of 0.001 inch (1 division on the dial) resulted
Figure 3. - Micropipetting instrument used in transplanting pupal ovary fragments into adult hosts.
(a) Knobs for advancing machine screw. (b) Dial indicating travel of machine screw. (c) Hamilton 25 µl syringe. (d) Polyethylene tubing connecting syringe and capillary micropipette. (e) Petri dish containing 3 drops of H-thymidine solution on siliconized coverslips; small evaporating dishes were inverted over the drops to prevent excessive evaporation. (f) Adult flies awaiting implantation of ovary fragments.
in delivery of 0.01 μl of liquid from the tip of the micropipette. Routinely, several ovary pieces were sucked up into the micropipette and then transplanted one or two at a time along with 0.05 to 0.10 μl of liquid into a series of host females.

Donor ovary pieces were removed from host females in 0.75% NaCl solution and prepared for autoradiography in one of two ways depending on whether or not they continued development in the host. Transplants which were well-developed after 2-5 days, as indicated by the presence of several egg chambers in the vitellarium of at least some of the ovarioles, were treated according to the following schedule. The transplants were transferred to a drop of fixative on a siliconized coverslip and gently teased apart in order to separate the individual ovarioles. Many variations of fixatives, fixation time, and subsequent squashing were tried in order to develop a squash technique in which all cells were flattened sufficiently for autoradiography yet as much structural detail of individual ovarioles as possible was retained. The most successful of these were (1) 2-3 minutes fixation in 45% acetic acid followed by gentle squashing, and (2) 3-5 minutes fixation in formalin: acetic acid: ethanol (1:2:2 diluted in 5 parts distilled water) followed by moderate squashing. After squashing the tissue between coverslip and slide, the preparation was frozen on a block of dry ice and the coverslip removed. The slide was immediately placed in 3:1 ethanol: acetic acid for 5-10 minutes of further fixation followed by 10 minute rinses in 70%, then 50% ethanol. After hydrolysis in 1N HCl for 1 minute at room temperature and 5 minutes at 60°C, the
tissue was stained in Schiff reagent for 1-2 hours at room temperature. The slides were rinsed in tap water for a few minutes to develop color and then placed in warm distilled water just prior to dipping them in emulsion.

The technique used for transplants which failed to develop beyond the germarium stage included the following steps. The ovary pieces were transferred from saline to a partial drop of 45% acetic acid on a siliconized coverslip and teased apart as much as possible. A small amount of 2% acetic orcein stain was added and the two liquids mixed, the total amount of liquid on the coverslip being just enough to spread out between the slide and coverslip without any excess. This provided enough capillary pressure between the slide and coverslip to gently flatten the tissue. The preparation was sealed with melted paraffin and examined 1-2 days later. After a photographic record of the posterior-most cysts in each ovariole was obtained, the preparation was frozen on a block of dry ice and the coverslip and paraffin removed. The slide was immediately rinsed for a few seconds in 95% ethanol, air dried, and stored until it was to be coated with emulsion. Just prior to dipping in emulsion slides prepared in this manner were warmed to 40°C in an incubator.

Slides prepared according to these two techniques were dipped, either wet or dry, in a 1:1 mixture of melted Kodak Nuclear Track emulsion NTD and a 0.1% solution of Detergent at 42°C. They were then allowed to drain and dry standing upright in test tube racks for one-half hour, after which they were stored in black bakelite
slide storage boxes (Clay-Adams) sealed light-tight with black electrician's tape. Exposure time for most autoradiograms was from 10-20 days at room temperature. The autoradiograms were developed in Kodak D-19 developer for 2 minutes, fixed for 5 minutes in Kodak Acid Fixer, treated with Hustler's hypo-clearing agent for 2 minutes, and rinsed for 5 minutes in running water. Feulgen-stained preparations were subsequently dehydrated through ascending ethanol solutions, with a fast-green counterstain being applied at the 70% ethanol step, cleared in toluene, and a coverslip applied using Permount mounting medium. Orcein-stained preparations were air-dried and stored without coverslips; before examining, coverslips were mounted with a drop of 5% glycerol in distilled water. All slides were examined and photographs taken with a Zeiss Photomicroscope using either bright-field or phase-contrast optics.
RESULTS AND DISCUSSION

**Synchrony:** The validity of the conclusions reached in experiments of this type depends to a great extent on the degree of synchrony within any one experiment and between separate experiments. Throughout all of the experiments, both genetic and cytological, it was observed that puparium formation occurred slightly before 120 hours post egg laying and eclosion occurred at 9-9½ days post egg laying. Samples from both genetic and cytological studies were taken over a period of several generations in order to make more quantitative observations on synchrony of development.

Figure 4, (a)-(d), summarizes the results of these observations on $f^3_{\text{car}^+/+}$ and $Tft^+_{\text{bw}+/\text{vg} \text{bw} ; st^+/+_{\text{Sb}}}$ females. The observations recorded in (a) and (b) as representing the latter genotype are actually based on a mixture of that genotype and its sibling genotype, $+_{\text{vg} \text{bw} ; st^+/+_{\text{T} (2;3) ap}}$. These two types of pupae are difficult to distinguish until pigment is deposited in the bristles at approximately 186 hours of development, at which time they can be distinguished easily on the basis of the difference between $\text{Sb}$ and $\text{Sb}^+$. The observations recorded in (c) and (d), on the other hand, are based almost entirely on the genotype $Tft^+_{\text{bw}+/+_{\text{vg} \text{bw} ; st^+/+_{\text{Sb}}}$. The near congruency of the sets of curves in (c) and (d) indicate that synchrony in the $f^3_{\text{car}^+/+}$ genotype reflects that in the $Tft^+_{\text{bw}+/+_{\text{vg} \text{bw} ; st^+/+_{\text{Sb}}}$ genotype. Consequently, the near congruency of the sets of curves in (a) and (b) means that synchrony in this latter genotype is reasonably accurately reflected by
Figure 4. - Measure of the degree of synchrony of females of the genotypes $f^3_{\text{car}}/+\,\, (\cdots\cdots)\quad$ and $\text{Tft}+/\text{bw}+\text{vg} \quad \text{st}+/\text{Sb}\,\, (\cdots\cdots)$ during pupal development. Each point on the transition portions of the curves is based on at least 250 observations in (a), 76 in (b), 184 in (c), and 75 in (d).
100 (c) 2 egg chambers in vitellarium
50
25
0

(a) puparium formation
(c) 2 egg chambers in vitellarium

(b) pupation
(d) pigment in wings

Percent completing developmental stage

Age of pupae (hours post egg laying)
the (dashed) curve based on a mixture of two genotypes.

Puparium formation, as signaled by eversion of the anterior spiracles was accomplished by 99% of the $^{3}\text{car/}++$ larvae and 97% of the $^{+}\text{Tft+/+ \text{vg bw}; st+/+ Sb}$ larvae over a 6-hour period between 114 and 120 hours post egg laying. Pupation was completed by 98% of the former genotype and 90% of the latter genotype within a 9-hour period from 123 to 132 hours. In later pupal stages, pigment deposition in the wings was completed by 100% of the former and 93% of the latter between 190 and 198 hours. These three criteria indicate a reasonable synchrony in the organism as a whole, but provide no direct evidence for synchrony among ovarioles within the ovary. Toward this end observations were made on the production of egg chambers in the vitellarium. The transition from one to two egg chambers was observed, the criterion for production of an egg chamber being the constriction of the stalk between the germarium and the egg chamber to a diameter one-half that of the germarium. Part (c) of Figure 4 shows that 80% of the former genotype and 87% of the latter pass through this developmental stage within a period of 12 hours, indicating somewhat less synchrony within the ovary than for the pupa as a whole. These observations not only serve to estimate the degree of synchrony of development of these two genotypes, but, since the two curves are nearly coincident in all four graphs, they suggest that comparison of genetic data from one genotype with cytological data from the other is valid.

Observations of oocyte nuclei directly during the time period studied only served to confirm the fact that female Drosophila
melanogaster meiotic chromosomes are nearly impossible to analyze. However, it does not seem unreasonable to assume that meiotic events are proceeding with approximately the same synchrony as the other developmental events examined. Based on Figure 4, it is probably a conservative estimate that no more than 25% of the oocytes sampled in any 6-hour period represent overlap from earlier or later segments of the meiotic cycle. Thus, the exact extents of the period of sensitivity to heat-induced recombination and the DNA synthesis period are in doubt only by a few hours due to asynchrony imposed by the sampling technique.

In the genetic studies, the degree of synchrony estimated above is based on the assumption that only the posterior-most oocytes were being sampled. Routinely, only 20-30 eggs per female were collected. Table 1 indicates that all the females studied in this investigation contained at least 30 ovarioles, with averages ranging from 34.8 to 42.8. Although accurate egg counts were not made and none recorded, the average was closer to 20 than 30. Therefore, it is highly unlikely that any oocytes sampled were other than posterior-most ones.

It is interesting to note the variation in average ovariole number among the females of genotypes 1-4, which differ only by the presence or absence of the two balancer chromosomes SM5 and TM2. Females of genotypes 2 and 4, both heterozygous for SM5 contained fewer ovarioles than females of genotypes 1 and 3, neither of which carried SM5. The data for these two types of females were pooled and the difference tested by a $\chi^2$ test using a 2x2 contingency table. The difference was significant at the .05 level (one degree of freedom). This may indicate that a dominant
TABLE 1

Number of ovarioles in the ovaries of females sampled from the genetic experiments.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of ♀♀ observed</th>
<th>Total ovarioles</th>
<th>Av. number of ovarioles per ♀ ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{f^3 \text{car}}{+} ; \frac{\text{+}}{+} ; \frac{\text{+}}{+} ) (1)</td>
<td>63</td>
<td>2383</td>
<td>37.8 ± 3.4</td>
</tr>
<tr>
<td>( \frac{f^3 \text{car}}{+} ; \frac{\text{SM5, Cy}}{+} ; \frac{\text{+}}{+} ) (2)</td>
<td>63</td>
<td>2191</td>
<td>34.8 ± 3.3</td>
</tr>
<tr>
<td>( \frac{f^3 \text{car}}{+} ; \frac{\text{+}}{+} ; \frac{\text{TM2}}{+} ) (3)</td>
<td>63</td>
<td>2388</td>
<td>37.9 ± 4.5</td>
</tr>
<tr>
<td>( \frac{f^3 \text{car}}{+} ; \frac{\text{SM5, Cy}}{+} ; \frac{\text{TM2}}{+} ) (4)</td>
<td>63</td>
<td>2232</td>
<td>35.4 ± 3.3</td>
</tr>
<tr>
<td>( \frac{T+t}{+} ; \frac{\text{bw}}{vg} ; \frac{\text{+}}{+} ; \frac{\text{st}}{sb} ) (5)</td>
<td>126</td>
<td>5409</td>
<td>42.8 ± 4.4</td>
</tr>
</tbody>
</table>
factor or factors are located on the SM5 chromosome which have the effect of lowering the ovariole number.

**Sensitive period for heat-induced recombination:** Table 2 summarizes the results of heat shock studies on females of genotype $f^3_{\text{car}}/+ +$, with normal sequence 2nd and 3rd chromosomes. A graphical representation of the values in col. 5 (but with 95% confidence limits instead of standard errors) appears in Figure 5. The first significant alteration in recombination rate in the $f^3_{\text{car}}$ region occurs in females treated between 138 and 146 hours of development post egg laying. This is immediately followed by a short period of lowered sensitivity. The main period of heat sensitivity appears to be between 150 and 170 hours of development, during which the greatest increases in recombination rate were obtained. A final period of significant response occurred between 180 and 188 hours. The short period of lower sensitivity at 144-152 hours may be a spurious result and a continuous sensitive period from 138-170 hours may actually exist for this region. GRELL (1970) studied the heat response of the entire X-chromosome including the region $f^3_{\text{car}}$. She obtained a continuous sensitive period for this region from 132-186 hours, with peak effects for initiation of 12-hour heat shocks at 138 hours and 162 hours. The significance of the late effect, at 180 hours, is, at the moment, obscure. No other such late effect has been observed, either in these investigations or in those of GRELL.

Table 3 represents the results obtained for the Tft-vg region of
TABLE 2
Alteration in recombination rate (f3 - car region) in the first maturing oocytes by heat shocks (8 hours, 35°C.) administered to females at 6-hour intervals during their pupal development.

<table>
<thead>
<tr>
<th>Time of heat shock†</th>
<th>Number of progeny</th>
<th>% Recomb.</th>
<th>Difference ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recomb.</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>990</td>
<td>20,199</td>
<td>4.90</td>
</tr>
<tr>
<td>120</td>
<td>160</td>
<td>4,846</td>
<td>4.31</td>
</tr>
<tr>
<td>126</td>
<td>217</td>
<td>4,473</td>
<td>4.85</td>
</tr>
<tr>
<td>132</td>
<td>352</td>
<td>6,979</td>
<td>5.04</td>
</tr>
<tr>
<td>138</td>
<td>380</td>
<td>6,134</td>
<td>6.19</td>
</tr>
<tr>
<td>144</td>
<td>325</td>
<td>5,876</td>
<td>5.53</td>
</tr>
<tr>
<td>150</td>
<td>410</td>
<td>6,340</td>
<td>6.47</td>
</tr>
<tr>
<td>156</td>
<td>551</td>
<td>8,101</td>
<td>6.80</td>
</tr>
<tr>
<td>162</td>
<td>254</td>
<td>4,307</td>
<td>5.90</td>
</tr>
<tr>
<td>168</td>
<td>213</td>
<td>3,780</td>
<td>5.63</td>
</tr>
<tr>
<td>174</td>
<td>170</td>
<td>3,977</td>
<td>4.27</td>
</tr>
<tr>
<td>180</td>
<td>261</td>
<td>4,536</td>
<td>5.75</td>
</tr>
<tr>
<td>186</td>
<td>92</td>
<td>1,968</td>
<td>4.67</td>
</tr>
<tr>
<td>192</td>
<td>323</td>
<td>6,515</td>
<td>4.96</td>
</tr>
<tr>
<td>198</td>
<td>147</td>
<td>3,045</td>
<td>4.83</td>
</tr>
</tbody>
</table>

† Time of initiation in hours post egg laying (approximately ± 1 hour).

* 95% confidence limits do not overlap 0.
Figure 5. - Alteration in recombination rates in the $f_3$-car region of the X-chromosome by heat shocks of 35°C and 8 hours duration. The heat shocks were initiated at 6-hour intervals between 120 and 198 hours from the time of egg laying. At all other times the temperature was maintained at 25°C. Vertical bars represent the 95% confidence limits.
Heat treated - control (Morgans x 10^-2)

Age of pupae (hours post egg laying)
TABLE 3

Alteration in recombination rates in the Tft - vg region of chromosome II and the st - Sb region of chromosome III, by heat shocks of 35°C and 8 hours duration. The heat shocks were initiated at 6-hour intervals between 114 and 198 hours from the time of egg laying. At all other times the temperature was maintained at 25°C.

<table>
<thead>
<tr>
<th>Time of Heat Shock</th>
<th>Number of Progeny</th>
<th>% Recombination</th>
<th>Heat-Control ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recombinants</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tft-vg st-Sb</td>
<td>Totals</td>
<td>Tft-vg st-Sb</td>
</tr>
<tr>
<td>Control</td>
<td>1236 1533</td>
<td>8390</td>
<td>14.73 18.27</td>
</tr>
<tr>
<td>114</td>
<td>414 478</td>
<td>2559</td>
<td>16.18 18.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+1.45±0.82 +0.41±0.88</td>
</tr>
<tr>
<td>Time of initiation (in hours post egg laying)</td>
<td>Mean (±SEM)</td>
<td>95% Confidence Limits</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td><strong>120</strong></td>
<td>15.20</td>
<td>±0.47±0.92</td>
<td>+2.77±1.04*</td>
</tr>
<tr>
<td><strong>126</strong></td>
<td>16.77</td>
<td>±2.04±0.85*</td>
<td>+6.97±0.97*</td>
</tr>
<tr>
<td><strong>132</strong></td>
<td>17.38</td>
<td>±2.65±0.88*</td>
<td>+7.02±1.00*</td>
</tr>
<tr>
<td><strong>138</strong></td>
<td>20.55</td>
<td>±5.82±1.10*</td>
<td>+4.42±1.37*</td>
</tr>
<tr>
<td><strong>144</strong></td>
<td>17.56</td>
<td>±2.83±1.05*</td>
<td>+4.00±1.15*</td>
</tr>
<tr>
<td><strong>150</strong></td>
<td>18.58</td>
<td>±3.85±1.44*</td>
<td>+6.28±1.59*</td>
</tr>
<tr>
<td><strong>156</strong></td>
<td>16.97</td>
<td>±2.24±0.98*</td>
<td>+4.74±1.10*</td>
</tr>
<tr>
<td><strong>162</strong></td>
<td>22.57</td>
<td>±7.84±1.20*</td>
<td>+5.40±1.23*</td>
</tr>
<tr>
<td><strong>168</strong></td>
<td>17.98</td>
<td>±3.25±1.11*</td>
<td>+3.95±1.20*</td>
</tr>
<tr>
<td><strong>174</strong></td>
<td>15.81</td>
<td>±1.08±0.81</td>
<td>-0.87±0.86</td>
</tr>
<tr>
<td><strong>180</strong></td>
<td>14.82</td>
<td>±0.09±0.95</td>
<td>+1.39±1.05</td>
</tr>
<tr>
<td><strong>186</strong></td>
<td>16.09</td>
<td>±1.36±0.84</td>
<td>+0.21±0.89</td>
</tr>
<tr>
<td><strong>192</strong></td>
<td>13.96</td>
<td>±0.23±0.76</td>
<td>+0.39±0.85</td>
</tr>
<tr>
<td><strong>198</strong></td>
<td>15.70</td>
<td>±0.97±0.99</td>
<td>+0.54±1.06</td>
</tr>
</tbody>
</table>

*Time of initiation in hours post egg laying (approximately ± 1 hour).

* 95% confidence limits do not overlap 0.
Figure 6. - Alteration in recombination rates in the Tft – vg region of chromosome II and the st – Sb region of chromosome III by heat shocks of 35°C and 8 hours duration. The heat shocks were initiated at 6-hour intervals between 114 and 198 hours from the time of egg laying. At all other times the temperature was maintained at 25°C. Vertical bars represent 95% confidence limits.
Age of pupae (hours post egg laying)
the 2nd chromosome and the *st-Sb* region of the 3rd chromosome. The values in cols. 7 and 8 of table 3 are presented graphically in Figure 6 (95% confidence limits replacing standard errors). Continuous heat sensitive periods in the form of significant increases in recombination were obtained for both regions: 126 - 176 hours for *Tft-vg* and 120 - 176 hours for *st-Sb*. The peak effects occurred at heat shock initiation times of 138 hours and 162 hours for the *Tft-vg* region and at 126, 132 and 150 hours for the *st-Sb* region. Although these two regions seem to be slightly more sensitive over a longer period of time to heat shock than the *f^3-car* region, the peak effects in all three regions are elicited during the intervals 126 - 138 hours and 150 - 162 hours. Since the *Tft-vg*, *st-Sb* regions were studied simultaneously in one genotype, the fact that the first significant increase in recombination in the *st-Sb* region precedes that of the *Tft-vg* region by six hours, very likely indicates a true temporal sequence of initiation of some heat-sensitive component of recombination in the centromeric regions of the 2nd and 3rd chromosomes.

Thus, the temperature-sensitive period for heat-induced recombination in oocytes of *Drosophila melanogaster* females treated during pupal development, based on the combined results of this investigation and the more extensive ones of GRELL, appears to be between 120 and 176 hours post egg laying. The question now arises as to whether temperature affects recombination directly or affects some earlier pre-condition for recombination, such as pairing of homologues. In the absence of direct evidence pertaining to the nature of heat effect on recombination an
effort was made to obtain data pertaining to this question. The inter-chromosomal effect of inversion heterozygosity, assuming its effect is on pre-conditions for recombination (LUCCHESI and SUZUKI 1968), was examined to see if the sensitive period for heat-induced recombination would be delayed, indicating a direct effect of heat on the recombination process itself. Table 4 summarizes the results obtained for recombination in the $f^3$-car region of the X-chromosome with various combinations of autosomal inversion heterozygosity. The effect of the complex inverted chromosome SM5, when present alone, was nearly identical to that of TM2 alone. Consequently, the data from these two genotypes were pooled and appear in columns 2, 3, and 4 of Table 4. The effect of both inverted chromosomes present in the genotype simultaneously is presented in the last three columns of the table. Graphical representation of the values in columns 4 and 7 is found in Figure 7, along with the heat response curve for the $f^3$-car region with normal sequence autosomes (from Figure 3).

The response curve is radically altered by the presence of one autosomal inversion. The first significant alteration occurred at 138 hours in the form of a reduction in recombination, whereas, in the situation where no autosomal inversions were present the first significant increase in recombination occurred at this time. The main peak of increased recombination has been shifted in time and lengthened and includes the period from 156 hours to 192 hours. The effect of the presence of both autosomal inversions is, again, to shift the time of the significant increase in recombination, but in this case the duration of
TABLE 4

The interchromosomal effect of inversion heterozygosity on heat-induced alteration in recombination rates in the f^3-car region of the X-chromosome. Females which were heterozygous for one autosomal inversion, either SM5 or TM2, or heterozygous for both, were given heat shocks of 35°C and 8 hours duration at 6-hour intervals between 120 and 198 hours after egg laying.

<table>
<thead>
<tr>
<th>Time of Heat Shock</th>
<th>SM5, Cy ; + or + ; TM2</th>
<th>SM5, Cy ; TM2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14,646 8.90</td>
<td>4470 14.88</td>
</tr>
<tr>
<td>120</td>
<td>936 7.91</td>
<td>601 12.15</td>
</tr>
<tr>
<td></td>
<td>-0.99±0.91</td>
<td>-2.73±1.43</td>
</tr>
<tr>
<td>126</td>
<td>4467 9.27</td>
<td>937 14.19</td>
</tr>
<tr>
<td></td>
<td>+0.37±0.49</td>
<td>-0.69±1.26</td>
</tr>
<tr>
<td>Time</td>
<td>egg</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>132</td>
<td>1954</td>
<td>9.83</td>
</tr>
<tr>
<td>138</td>
<td>1668</td>
<td>7.25</td>
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<tr>
<td>144</td>
<td>2581</td>
<td>9.26</td>
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<tr>
<td>150</td>
<td>5721</td>
<td>8.86</td>
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<tr>
<td>156</td>
<td>6795</td>
<td>10.02</td>
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<tr>
<td>162</td>
<td>5457</td>
<td>10.65</td>
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<tr>
<td>168</td>
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<td>174</td>
<td>1871</td>
<td>10.48</td>
</tr>
<tr>
<td>180</td>
<td>4158</td>
<td>8.44</td>
</tr>
<tr>
<td>186</td>
<td>2638</td>
<td>9.17</td>
</tr>
<tr>
<td>192</td>
<td>4427</td>
<td>8.13</td>
</tr>
<tr>
<td>198</td>
<td>4082</td>
<td>8.62</td>
</tr>
</tbody>
</table>

†Time of initiation in hours post egg laying (approximately ± 1 hour).

*95% confidence limits do not overlap 0.
Figure 7. - Heat-induced alteration in recombination rates in the $f^3$-car region of the X-chromosome in females carrying no autosomal inversions (---●---), heterozygous for one inversion (---□---), or heterozygous for both inversions (---△---). See Table 4 for details of the three different genotypes. Heat shocks of 35°C and 8 hours duration were given at 6-hour intervals between 12:00 and 198 hours after egg laying.
the peak has been shortened and includes the period from 162 hours to 176 hours. Thus, if attention is focused on the significant increases in recombination in the $f^3_{car}$ region induced by heat-treatment, the presence of autosomal inversion heterozygosity delays by 6 - 12 hours the major period of heat sensitivity.

One possible interpretation of this delay is based on the postulate of LUCCHESI and SUZUKI (1968) that interchromosomal effects are due to the lengthening of the time during which recombination can take place. Their model suggests that "interchromosomal effects are mediated by factors which affect the prerequisites for the occurrence of crossing-over, rather than the mechanism of crossing-over itself." Their model incorporates the proposal of COOPER* that termination of the stage during which pairing occurs, and thus, crossing-over may occur, depends on having a certain fraction of the genome synapsed and factors interfering with pairing of homologous chromosomes tend to prolong this stage. In the present studies the delayed onset of the major period of heat-induced recombination in the $f^3_{car}$ region due to autosomal inversion heterozygosity could be accounted for on the basis of interference with the pairing of the X-chromosomes by factors such as the complex loop formation required for pairing of the autosomes and heterologous pairing of unpaired autosomes with the X-chromosomes. The fact that increases in recombination are obtained at later times can be explained as due to an increase in time needed for attaining a

critical amount of pairing in the entire genome, resulting in an extension of the total time available for recombination to occur. Thus, the effect of heat shocks in these studies is consistent with a direct effect on some component of the recombination process subsequent to pairing.

It has been well established that DNA synthesis takes place almost entirely during interphase (TAYLOR 1957; and others). If the heat sensitive period for recombination and premeiotic DNA synthesis are coincident, the suggestion just outlined requires homologous chromosomes to be paired during premeiotic interphase. There are numerous descriptions in the early cytological literature of organisms, both animal and plant, which exhibit condensed, paired homologous chromosomes, called "prochromosomes", at pre-leptotene stages of meiosis (GRELL 1969; STACK and BROWN 1969). More recently, MOENS (1964) has suggested a new interpretation of meiosis in the tomato, Lycopersicon esculentum, in which he observed paired, pachytene chromosomes immediately following interphase. On the basis of his observations, the sequence of meiotic events in the tomato is interphase-pachynema-diffuse stage-diplonema-diakinesis, rather than the classical sequence. Observations of pre-leptotene pairing more pertinent to the present study are those in Drosophila melanogaster by GUYENOT and NAVILLE (1933) who observed "prochromosomes" in the two pro-oocytes of a 16-cell cluster just after the last oogonal division prior to meiosis. Thus, in some organisms, including Drosophila, homologous chromosomes may be paired during premeiotic interphase. Therefore, the definite possibility
exists that DNA synthesis and recombination may overlap to some extent while chromosomes are paired in premeiotic interphase.

**Development of transplants:** The degree of development attained by ovary pieces from pupal donors transplanted into adult hosts is highly dependent on their age at the time of transplantation (BODENSTEIN 1947). This dependence of transplant development on age is summarized in Table 5, columns 1 and 3. The youngest ovary pieces transplanted were from 120-126 hour old donors. Very few donor pieces of this age were recovered from hosts after the usual 3-5 day development period. Those that were recovered appeared in gross dissections as though they had been completely arrested in development at the time of transplantation, but had maintained themselves. Microscopic examination of some of these revealed that the individual ovarioles consisted only of the germarium region, within which there was no indication of 16-cell groups being enveloped by follicle cells (incipient cysts). A frequent characteristic of these undeveloped ovarioles, however, was an increase in the number of oogonial cells compared to the number present at the time of transplantation, indicating that at least some cell division continued after transplantation, even though differentiation of egg chambers apparently did not occur. This type of developmental failure occurred in all transplanted ovary pieces from donors of age 120-126 hours and in about three quarters of those from 126-138 hour old donors. In the case of 126-138 hour old donors approximately one quarter of all transplants recovered developed quite well. The individual ovarioles contained
TABLE 5

Summary of transplant experiments and autoradiographic analysis of premeiotic DNA synthesis in the first maturing oocytes in ovaries removed from \( f^3 \text{car/+} \) females and incubated in \(^3\text{H-thymidine.}

See MATERIALS and METHODS for details of the techniques.

<table>
<thead>
<tr>
<th>Age of Donor</th>
<th>Number of Transplants Recovered</th>
<th>% Transplants Developed</th>
<th>Number of Ovarioles Analyzed</th>
<th>% Posterior Oocytes Labelled</th>
</tr>
</thead>
<tbody>
<tr>
<td>120-126</td>
<td>23</td>
<td>0</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>126-132</td>
<td>16</td>
<td>25</td>
<td>232</td>
<td>0</td>
</tr>
<tr>
<td>132-138</td>
<td>21</td>
<td>23</td>
<td>100</td>
<td>3.0</td>
</tr>
<tr>
<td>138-144</td>
<td>17</td>
<td>53</td>
<td>149</td>
<td>24</td>
</tr>
<tr>
<td>144-150</td>
<td>40</td>
<td>97</td>
<td>251</td>
<td>25</td>
</tr>
<tr>
<td>150-156</td>
<td>53</td>
<td>100</td>
<td>85</td>
<td>25</td>
</tr>
<tr>
<td>156-162</td>
<td>25</td>
<td>100</td>
<td>87</td>
<td>4.6</td>
</tr>
<tr>
<td>162-168</td>
<td>37</td>
<td>100</td>
<td>81</td>
<td>1.2</td>
</tr>
<tr>
<td>168-174</td>
<td>17</td>
<td>100</td>
<td>122</td>
<td>0</td>
</tr>
<tr>
<td>174-186</td>
<td>42</td>
<td>100</td>
<td>121</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>291</td>
<td></td>
<td><strong>1275</strong></td>
<td></td>
</tr>
</tbody>
</table>
several egg chambers in the vitellarium region, in addition to having incipient cysts in the posterior regions of the germaria. About one half of the ovary pieces from 138-144 hour old donors and essentially all ovary pieces from donors older than 144 hours attained a level of development such that each ovariole contained at least a few egg chambers. In a small number of cases mature eggs, complete with filaments, were observed in a few ovarioles within a transplant.

This age dependency is most likely due to hormone action on the developing ovary. It is well known that the hormones of the ring gland of certain insects are involved in the control of molting, metamorphosis, differentiation, and general metabolic activities (WIGGLESWORTH 1970). The ring gland of Drosophila is a compound gland consisting of the prothoracic gland, which produces the hormone ecdysone, the corpus allatum, which produces the juvenile hormone, and the corpus cardiacum, which is involved in storage of neurosecretory products from the brain. During the larval and prepupal development the cells of the prothoracic gland grow much more than the other ring gland cells, their nuclei attaining a 300-fold increase in volume (AGGARWAL and KING 1969) before they disperse and lyse during the pupal period (VOGT 1942). The cells of the corpus allatum grow to a much lesser extent (50-fold increase in volume) and do not disaggregate and lyse but persist through the pupal and adult stages. Thus the adult ring gland consists only of the corpus allatum and corpus cardiacum (KING, AGGARWAL, and BODENSTEIN 1966). Consequently, the haemolymph of larval, prepupal, and early pupal stages contains both ecdysone and
juvenile hormone, while the adult haemolymph contains only the corpus allatum hormone.

One current hypothesis regarding the interrelationships between ring gland hormones and development postulates that whether the adult or juvenile developmental pathway is followed, depends on the ratio of ecdysone to juvenile hormone (BODENSTEIN 1955; AGGARWAL and KING 1969). They propose that high ecdysone/juvenile hormone ratios favor molting, puparium formation, growth and differentiation of pupal and adult tissues, and histolysis of larval tissues. Low ratios, on the other hand, favor growth and differentiation of larval tissues and inhibition of histolysis. Thus the ovary remains in an immature state (i.e. ovarioles differentiate only the germarium portion) during the larval and prepupal stages while the ecdysone/juvenile hormone ratio is relatively low. During the prepupal and early pupal stages, when large amounts of ecdysone are produced, a critical ratio of ecdysone/juvenile hormone is reached, allowing the ovariole to differentiate the vitellarium portions (AGGARWAL and KING 1969). In the late pupal and adult stages the juvenile hormone of the corpus allatum is necessary for egg maturation (VOGT 1942; BODENSTEIN 1947), including the deposition of yolk (WIGGLESWORTH 1954).

Very little can be found in the literature concerning a detailed account of the transition from an immature ovary to a completely metamorphosed ovary. KING, AGGARWAL and AGGARWAL (1966) have postulated that ovarian metamorphosis results in the transformation of all oogonia to cystoblasts and that this transformation may depend on the same
hormonal stimulus (presumably, the critical ecdysone level) which causes metamorphosis in general. However, the observations in the present study indicate that transformation of oogonia to cystoblasts and the early cystocyte divisions to form 16-cell clusters occurs without differentiation of the vitellarium portion of the ovariole. Numerous transplants in which ovarioles failed to develop beyond the germarium stage contained clusters of 16-cystocytes, as indicated by the medial positions of 8-cell metaphase figures (Figure 8). Although the squash technique used in these studies is not favorable for recognizing formation of incipient cysts in the germarium, this event was never observed in transplants which failed to develop. On the other hand, this was observed distinctly in numerous instances in the germarium of well-developed transplants. Consequently, it can be concluded that the first events characteristic of the metamorphosis of the ovary from immature to adult form is the enveloping of 16-cell clusters of cystocytes by follicle cells and the subsequent budding off of these cysts to form the vitellarium portion of the ovariole. Furthermore, the hormonal stimulus necessary for these events is not required for transformation of oogonia to cystoblasts.

A second factor important in transplant development is oxygen supply. Those ovary pieces which became generously supplied with tracheoles were the most highly developed, some ovarioles containing mature eggs with filaments after 5-6 days. Occasionally, ovary pieces were recovered which consisted of two groups of ovarioles connected only at their basal stalks or the terminal filaments. One group was
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were recovered which consisted of two groups of ovarioles connected
only at their basal stalks or the terminal filaments. One group was
Figure 8. - Several ovarioles of an ovary fragment from a 120-hour old donor after 2 days in an adult host. The anterior ends of three ovarioles are indicated by lines in the lower portion of the photograph. Medial positions of three 8-cell groups of cystocytes are indicated by arrows. Acetic-orcein squash preparations; 960X.
supplied with tracheoles while the other was not. The ovarioles of the group supplied with tracheoles, and presumably with a much greater supply of oxygen, contained more egg chambers in the vitellarium. In addition, the egg chambers in the tracheolated portion were larger than comparable egg chambers in the portion not supplied with tracheoles. This size difference was due chiefly to differences in the size of the nurse cells within the chambers. The total amount of development attained by a transplanted ovary fragment depended, firstly, upon receiving the necessary hormonal stimulus before removal from the donor pupa and, secondly, upon the rapidity with which tracheoles invaded the transplant and their absolute number.

**Analysis of DNA synthesis in the oocyte:** DNA synthesis was detected by means of autoradiography of squashes of ovaries previously incubated in a solution of the radioactive precursor $^3$H-methyl-thymidine. In order to analyze DNA synthesis in the first maturing oocytes it was desirable for the transplants to develop to the extent that the posterior-most egg chamber in any ovariole was between Stages 4 and 12 (according to the staging of KING, RUBINSON and SMITH 1956), at which time the oocyte and nurse cells are morphologically distinct. At these stages the nucleus of the oocyte is considerably smaller than a nurse cell nucleus and the chromosomes are condensed in a compact unit called a karyosome. The nurse cell has very little cytoplasm and the chromatin is dispersed throughout the nucleus (Figure 9). Thus, developed silver grains, resulting from the emissions from incorporated $^3$H-thymidine, in
Figure 9. - Posterior-most egg chamber of an ovariole illustrating the morphological distinction between the oocyte (O) and the nurse cells (NC) at later stages of development.  c = cytoplasm; n = nucleus. Feulgen-stained squash preparation; 1180X.
the emulsion above the karyosome of an oocyte nucleus is an unambiguous indication of DNA synthesis in the oocyte at the time of incubation in $^3$H-thymidine solution. Prior to Stage 3, oocytes are indistinguishable from adjacent nurse cells by the staining techniques used and nuclei of oocytes beyond Stage 12 are occluded in the large amounts of yolk deposited in the cytoplasm.

The degree of development attained by ovarioles which did develop beyond the germarium stage was highly variable, the posterior-most egg chambers ranging from Stage 1 in some cases to Stage 14 in a few extreme cases. Also, among transplants which had the same amount of time to develop in a single series of hosts, there was variability from host to host in the average amount of development which actually occurred. Consequently, the number of ovarioles containing posterior-most egg chambers in Stages 4-12 was considerably less than the total number transplanted. Furthermore, some of these were impossible to analyze because squashing resulted in bursting them and dispersing their constituent cells so that they became mixed with cells burst out of egg chambers of neighboring ovarioles. Consequently, only a small proportion of all ovarioles transplanted yielded analyzable Stage 4-12 egg chambers in the final autoradiograms. Nevertheless, sufficient data were obtained by this means of analysis alone to determine the pattern of DNA synthesis in oocytes from pupae between 150 and 186 hours. Figure 10 shows an autoradiogram of an ovariole squashed in such a way as to allow an analysis of DNA synthesis in the oocyte.

To determine the pattern of DNA synthesis in oocytes from pupae
Figure 10. - Autoradiogram of an ovary fragment from a 162-hour old donor pupa, incubated in $^3$H-thymidine solution, and transplanted to an adult female host for 3 additional days of development. This is typical of the favorable result of squashing, in which the oocyte and nurse cells of the posterior-most egg chamber have burst out but have remained together and are not confused with cells from nearby ovarioles. tf = terminal filament; O = oocyte; PE = posterior-most egg chamber; AE = one of several anterior egg chambers belonging to this ovariole. Acetic-orcein stain; 384X.
between 120 and 150 hours post egg laying, it was necessary to employ
two additional methods of analysis based on the assumption that the
DNA synthesis following the last cystocyte division is synchronous in
all 16 cells of the cyst. This would represent the last, or premeiotic,
replication in the oocyte and the first of a series of endomitotic
replications in the nurse cells. The validity of this assumption is
supported by the observations of GRELL and CHANDLEY (1965) and
CHANDLEY (1966) that 98% of all labelled oocytes were accompanied by
labelled nurse cells, and by the present investigation, in which the
nurse cells of all morphologically recognizable labelled oocytes were
also labelled. Thus, the observation of a group of 16 cystocytes, all
nuclei of which were labelled or not labelled, would indicate that DNA
synthesis was occurring or not occurring, respectively, in the oocyte
nucleus at the time $^3$H-thymidine was available. The first of these
methods consisted of screening autoradiograms for labelled groups of
16 cystocytes located posterior-most in the germaria of transplants
which failed to develop beyond the germarium stage. This type of
analysis was done on transplants from 120-132 hour donor pupae. These
transplants were allowed to develop in host females for 6-48 hours
before the tissue was recovered and autoradiograms prepared. The
second method was the analysis of ovarioles which developed beyond the
germarium stage but not to a stage at which the oocyte could be dis-
tinguished among the 16 cystocytes. Analysis of this type was carried
out only on posterior-most egg chambers in which the cystocytes
remained oriented so that labelling over the nuclei of at least the
posterior two thirds of them could be assessed. Since the oocyte is the posterior-most cell within an egg chamber (BROWN and KING 1964), even if some rearrangement of cells occurs during squashing, the oocyte would still be included in the posterior two thirds of the cells. Analysis of this type was done on transplants from 126-150 hour donor pupae.

Table 5 (columns 4 and 5) summarizes the results of these various means of analysis of premeiotic DNA synthesis in the first maturing oocytes. There was no evidence of DNA synthesis in oocytes of 120-132 hours of development as indicated by the fact that no labelled posterior oocytes were observed among a total of 279 ovarioles analyzed. If the synchrony of cystocyte divisions was poor among ovarioles within an ovary and this were compounded by asynchrony in the development of individual pupae, one would expect to see an overlap in the DNA synthesis periods of the last cystocyte division and premeiotic DNA synthesis in the oocyte. Since no labelled posterior 16-cell groups were observed at 120-132 hours, it appears that synchrony is reasonably good and that these two S-periods are separated by at least 12 hours. An apparent overlap of this type would also have occurred if $^3$H-thymidine remained available within the transplanted piece of ovary for a period of time approximating the interval between the two S-periods. An estimate of the time during which $^3$H-thymidine remains available for incorporation into DNA is given by the difference between the observed interval of time separating the two S-periods and the actual length of this interval, i.e., the lengths of the $G_2$ and mitotic periods of the last cystocyte division plus the $G_1$ period preceding premeiotic DNA synthesis. CHANDLEY (1966)
has estimated the intervals separating the cystocyte and premeiotic DNA synthesis periods as 24 hours. The data of BARIGOZZI et al. (1966) suggest the duration of the DNA synthesis period in Drosophila melanogaster embryonic somatic cells in culture to be approximately 6-8 hours. Assuming an S-period of 6-8 hours, this combined length of $G_2 + M + G_1$ is approximately 16-18 hours, compared to the observed interval of at least 12-18 hours. By this approximation it appears that $^3$H-thymidine is metabolized rapidly and does not remain available to the transplanted tissue for more than a period of 4 hours, possibly for a much shorter time. CHANDLEY (1966) determined that injected $^3$H-thymidine is no longer incorporated by ovaries of adult females after a period of 30 minutes. Consequently, it can be concluded with reasonable confidence that premeiotic DNA synthesis in the first maturing oocytes begins between 132 and 138 hours, probably closer to 138 hours.

The maximum percentage of labelled oocytes (24-25%) was observed in each of the three 6-hour intervals between 138 and 156 hours. Thus, the bulk of the premeiotic DNA synthesis occurs during this 18-hour period. It is surprising that only a maximum of 25% of oocytes examined were labelled at any one 6-hour period. This could be attributed to the fact that the S-period is in reality shorter than 18 hours and considerable asynchrony exists within the oocytes sampled. Another possibility is that some heavily labelled posterior cysts were damaged by the radiation and completely resorbed before the transplant was recovered. Only a few cases were observed of apparent resorption of posterior-most egg chambers and these were confined to transplants of 5 days development.
(Figure 11). However, it is not known whether this was due to radiation damage or nutritional factors. A third contributing factor may have been the differential loss of posterior-most egg chambers during the recovery and squashing of the transplants, with subsequent misclassification of next-to-posterior-most egg chambers as unlabelled posterior-most egg chambers. This was carefully guarded against by discarding any group of ovarioles from which egg chambers were observed to have been torn lose in handling, although it is possible that events of this nature could have escaped detection during the squashing of the tissue. Regardless of the relative contributions of these factors and the actual duration of the premeiotic DNA synthesis period, it is clear that the maximum numbers of oocytes in the S-period were present between 138 and 156 hours. Figures 12, 13 and 14 represent autoradiograms of unlabelled and labelled posterior-most egg chambers.

A small proportion of oocytes sampled after 156 hours were labelled; 4.6% in the 156-162 hour period and only 1.2% in the 162-168 hour period. This could be due to a degree of asynchrony both in the maturation of oocytes within a single ovary and in the development of pupae sampled in this time period. In other words, these may represent, at least in part, the slowest developing posterior-most oocytes in the slowest developing pupae sampled in the 156-168 hour period. It is also possible that labelling in these oocytes represents incorporation in late-replicating regions of the genome. Unfortunately, with this material it is impossible to ascertain whether or not grains are localized over any particular region of the genome. However, it is a well-known fact that
Figure 11. - Autoradiogram of an ovary fragment allowed to develop in an adult host for 6 days. The arrows indicate two prominent posterior-most egg chambers, the cells within which have apparently disintegrated and may be in the process of being resorbed. Acetic-orcein squash; 377X.
Figure 12. - Autoradiogram of an ovary fragment from a 145-hour old donor pupa, incubated in $^{3}$H-thymidine, and transplanted to an adult female host for 3 days additional development. Neither the oocyte nucleus (On) nor the nurse cell nuclei (NCn) are labelled, whereas many cells in more anterior egg chambers are heavily labelled (arrow). Feulgen stain, fast green counterstain; 377X.
Figure 13. - Autoradiogram of an ovary fragment from a 139-hour old donor pupa, incubated in $^3$H-thymidine, and transplanted to an adult female host for 4 days additional development. Both the oocyte nucleus (karyosome) (On) and the nurse cell nuclei (NCn) are labelled. Feulgen stain, fast green counterstain; 731X.
Figure 14. - Autoradiogram of an ovary fragment from a 144-hour old donor pupa, incubated in $^3$H-thymidine, and transplanted to an adult female host for 4 days additional development. Both the oocyte nucleus (karyosome) (On) and nurse cell nuclei (NCn) are heavily labelled. In this preparation the squashing was more moderate than in those illustrated in previous figures, resulting in a completely intact egg chamber. Acetic-orcein stain; 704X.

A - focus on tissue; B - focus on silver grains.
in a wide variety of organisms certain portions of the genome are late-replicating. In *Drosophila*, BARIGOZZA et al. (1966) have shown that the centromeric regions of chromosomes I, II, and III, in addition to the entire small chromosome IV, are late replicating. Therefore, it is conceivable that a much higher proportion of the labelling detected after 150 hours represents DNA synthesis in centromeric regions than that before 150 hours.

According to the findings of this investigation premeiotic DNA synthesis in the first maturing oocytes, allowing for some asynchrony, begins around 138 hours and ends at approximately 156 hours. A similar investigation by GRELL (in preparation) using a different technique is in agreement with this pattern of DNA synthesis. In her study, Feulgen-stained whole-mounts of ovaries incubated in $^3$H-thymidine solution were made immediately after the incubation period and subsequently prepared for autoradiography. DNA synthesis in the oocyte was inferred from the presence of heavily-labelled cysts in the posterior region of the gerarium, rather than direct observation of the oocyte karyosome. It was impossible with this technique to determine whether DNA synthesis in the oocyte continued for a longer period of time than its companion nurse cells or whether they were completely synchronous. This was due to the fact that it was not possible to distinguish between a posterior cyst in which a posteriorly-located nurse cell was beginning its first endomitotic DNA synthesis and a cyst in which the oocyte was completing its premeiotic DNA synthesis. That this potential error is a reality was confirmed by observations in the present investigation of
posterior-most egg chambers in which one posteriorly-located nurse cell was labelled but the oocyte and other nurse cells were not (Figure 15). Nevertheless, within the limits of resolution of the techniques of analysis used, it appears that the oocyte and nurse cells generate the 4C amount of DNA synchronously and this occurs between 138 and 156 hours of development post egg laying for the posterior-most egg chambers.

No oocytes sampled from pupae beyond 168 hours of development were found to be labelled. Periods of DNA synthesis following the bulk premeiotic interphase synthesis have been reported by other investigators. WIMBER and PRENSKY (1963) reported a zygotene-pachytene synthesis in Triturus viridescens amounting to no more than 2% of bulk interphase synthesis. HOTTA, ITO, and STERN (1966) reported a zygotene synthesis, amounting to 0.3% of the interphase synthesis, and a pachytene synthesis, amounting to 0.1%, in the microsporocytes of the lily. However, PEACOCK (1970) in grasshopper spermatocytes and TAYLOR (1959) in lily microsporocytes were unable to detect any prophase DNA synthesis by autoradiographic techniques. It is very likely that the technique used in the present investigation is not sensitive enough to detect such low levels of prophase synthesis. Consequently, these labelling studies are inconclusive regarding prophase DNA synthesis in Drosophila oocytes.
Figure 15. - Autoradiogram of an ovary fragment from a 162-hour old donor pupa, incubated in $^{3}$H-thymidine, and transplanted to an adult female host for 3 days additional development. The oocyte nucleus (karyosome) (On) is not labelled but a posteriorly-located nurse cell nucleus (NCn) is heavily labelled. Acetic-orcein stain; 561X.
CONCLUSIONS AND SUMMARY

Figure 16 illustrates the temporal relationship between heat induced recombination and premeiotic DNA synthesis in the first maturing oocytes of *Drosophila melanogaster* females. The horizontal bars represent the periods during which significant alterations in recombination frequencies were induced by heat treatment. The histogram shows the period of premeiotic DNA synthesis as determined in the autoradiographic analysis. As can be seen, the heat sensitive periods for all three regions studied are largely coincident with the period of DNA synthesis. The most valid comparison is that between the lower horizontal bar, indicating the heat sensitive period for the \( F^3 \)-car region of the X-chromosome, and the histogram of DNA synthesis, since these two sets of data were derived from females of the same genotype. The extent of the major period of heat sensitivity of the \( F^3 \)-car region (138-170 hours) is almost precisely that of the DNA synthesis period (132-168 hours). This coincidence is further strengthened by noting that the peak period of heat sensitivity (delimited by the arrows) of the \( F^3 \)-car region, which is adjacent to centric heterochromatin, corresponds to the end of the DNA synthesis period. This is what would be expected on the basis of the evidence of BARIGOZZI et al. (1966) that the proximal region of the X-chromosome is the latest replicating region of the genome. The short period of heat sensitivity observed between 180 and 188 hours is most likely not associated with recombination in view of the fact that the oocyte is in the diplotene phase at this time.
Figure 16. - Temporal relationship between the sensitive period for heat-induced recombination and premeiotic DNA synthesis. The horizontal bars in the upper portion of the figure indicate the extents of the periods during which heat shocks significantly increase recombination in the three regions indicated. The two arrows on each bar delimit the peak effects for each region. The histogram in the lower portion of the figure indicates the extent of the premeiotic DNA synthesis period as measured by autoradiography of ovary fragments incubated in $^{3}$H-thymidine and transplanted into adult hosts. (See text for details of techniques and methods of analysis.)
Sensitive periods for heat-induced recombination:

- Tft - vg
- st - Sb
- f^3 - car

Prenaeotic DNA synthesis:

Age of pupae (hours post egg laying)
The sensitive period for heat-induced recombination in the $f^{3\text{-car}}$ region of the X-chromosome is shifted in time by the presence of autosomal inversion heterozygosity. This interchromosomal effect is interpreted in light of LUCCHESI and SUZUKI's (1968) model as being due to a delay in the onset of homologous pairing as a result of non-homologous associations, coupled with an extension of the total time available for pairing and recombination. These observations are, therefore, consistent with a direct effect of heat at the time of recombination. On the basis of the interchromosomal effect on heat-induced recombination and the coincidence of the heat-sensitive period with premeiotic DNA synthesis, it is concluded that genetic recombination and premeiotic DNA synthesis occur simultaneously in the oocyte of *Drosophila melanogaster*.

The results obtained in this investigation suggest an additional test of the relationship between premeiotic DNA synthesis and recombination. If the conclusions reached above are correct and premeiotic DNA synthesis and recombination are interrelated events, then the period of premeiotic DNA synthesis should be shifted in time by the presence of inversion heterozygosity similar to the shift in the sensitive period for heat-induced recombination. In this regard, further studies on the temporal relationship between premeiotic DNA synthesis and heat-induced recombination in females differing by the presence or absence of inversion heterozygosity will be enlightening. Studies in this same vein using inhibitors of DNA synthesis would also be of value.
The results of this investigation do not allow a choice between the breakage-reunion and copying-choice models of recombination. These findings do, however, place restrictions on future proposals as to the temporal sequence of events during the meiotic cycle which are associated with recombination. Some form of copying-choice mechanism is certainly not ruled out as having a role in recombination in Drosophila and would be strongly suggested if it is shown in future studies, such as those mentioned above, that certain agents can induce similar perturbations in the temporal sequence of both DNA synthesis and the sensitive period for heat-induced recombination. Findings of this nature would also be consistent with an enzymatic breakage-reunion mechanism, with the restriction that the breakages and initial interactions between non-sister DNA strands, possibly by formation of hybrid DNA regions, occur during premeiotic DNA synthesis. On the other hand, heat may be affecting enzymatic and/or structural proteins which are produced at the time of premeiotic DNA synthesis, but do not realize their roles in recombination until a later time. However, such an indirect effect of heat is difficult to reconcile with the delay and extension of the heat-sensitive period caused by inversion heterozygosity. Definitive proof of the effect of heat on recombination and the molecular nature of the events involved in recombination in eukaryotes must await new insights into the structure of chromosomes and a deeper understanding of the biochemistry of meiosis in general.

The results of studies on the development of pupal ovaries transplanted to adult hosts as a function of age of the donor has revealed
the coincidence of ovarian metamorphosis with the onset of the periods of DNA synthesis and sensitivity for heat-induced recombination in the first maturing oocytes. Consideration of these findings along with the well-documented fact that the first eggs laid by *Drosophila melanogaster* females exhibit much higher recombination frequencies than later eggs (MORGAN, BRIDGES, and SCHULTZ 1931; SCHULTZ and REDFIELD 1951) suggests a causative role of hormones in the alteration of recombination frequency at the time of pupation. It would seem that a biochemical analysis of the influence of hormones on DNA, RNA, and protein synthesis in the *Drosophila* oocyte as a function of time may offer some insight into the molecular mechanism of crossing-over. Since isolation of sufficient numbers of oocytes on which to do biochemical analyses does not appear to be technically feasible at the present time, a cytogenetical analysis of the oocyte during pupation, at the light and electron microscope levels, may prove to be of great value.

In summary, the salient findings and conclusions of this investigation are listed as follows:

1. The period of premeiotic DNA synthesis was found to be largely coincident with the periods of sensitivity of three regions near centric heterochromatin of the X, II, and III chromosomes to heat-induced alteration of recombination frequency in the first maturing oocytes of *Drosophila melanogaster*.

2. The period of sensitivity to heat-induced alteration in recombination frequency in the \( f^3_{\text{car}} \) region of the X-chromosome is
shifted to a later time in development by the presence of autosomal inversion heterozygosity.

3. These two findings are interpreted to mean that heat affects recombination directly during premeiotic DNA synthesis.

4. Heat-induced recombination in the first maturing oocytes is coincident with the hormonal changes attendant on pupation. This finding is interpreted, in light of the age effect on recombination, to implicate hormones in a causative role in altering recombination frequencies.

5. Results of transplantation studies indicate that one of the primary events in the differentiation of the ovary from the immature to the imago form is the enveloping of 16-cell cysts by follicle cells in the posterior region of the germarium and the subsequent budding off of this cyst as the first egg chamber in the vitellarium.
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