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EFFECTS OF INHIBITORS OF DNA SYNTHESIS ON RECOMBINATION IN *CHLAMYDOMONAS REINHARDI*

by

© SONG-MAO CHIU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

SPRING, 1973

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Effects of Inhibitors of DNA Synthesis on Recombination in *Chlamydomonas reinhardi*" submitted by Song-Mao Chiu in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In *Chlamydomonas reinhardi* strain 137C, which releases four zoospores at the end of germination, the period of pre-meiotic DNA replication (main S period) was shown to occur during the germination of zygospores. This period in wild type zygospores and those from the cross *arg*-1 x *arg*-2, take place between 6½ and 7½ hours after the beginning of germination. In addition, minor incorporation of ^{32}P into DNA was detected in the early germination period and at prophase.

Several inhibitors with different modes of action on DNA synthesis were selected to study the roles of DNA synthesis in recombination. Survivals and recombination frequencies in the 6 map-unit region (*arg*-1 - *arg*-2, linkage group I) were studied.

Phenethyl alcohol at 0.4% reduced the germination of zygospores at two periods, the first one 60 minutes before the main S period and the second one at prophase. It enhanced recombination during the interval between the first survival sensitive period and the main S period..

Treatments with the inhibitors nalidixic acid, FUdR, hydroxyurea and adenine affected recombination at two short periods, corresponding respectively to the main S period and the minor ^{32}P incorporation period. At the main S period

all of these inhibitors depressed recombination, but at the prophase period nalidixic acid and FUdR enhanced, but hydroxyurea and adenine depressed it. Mitomycin C, showed unique effects on recombination, enhancing it at two periods, one 30 minutes before the main S period like phenethyl alcohol and the other at prophase as other inhibitors.

The repair inhibitors, acriflavine and caffeine, both depressed recombination at prophase, while actinomycin D enhanced recombination 60 minutes before the main S period.

In vegetative cells, PEA and mitomycin C treatments blocked DNA synthesis after a delay of 90 minutes, which is interpreted to mean that they inhibit the initiation rather than its continuation. Nalidixic acid, FUdR and hydroxyurea, in contrast, produced a partial blockage only, and treated cells were able to recover.

The significance of the data and the modes of action of the DNA inhibitors are discussed. The phenethyl alcohol survival data suggest that delayed replication occurs in *Chlamydomonas* and that replicating units involved are whole replicons. The recombination data are interpreted by the hypothesis that the average number of delayed replicons per cell determines the recombination frequency and the regions of delayed replication are the regions of crossing-over.

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I would like to express grateful acknowledgement to Dr. P. J. Hastings for his guidance, criticism, support, and patient understanding throughout the course of this investigation. Without his help this study would not have been possible. I am also grateful to Dr. G.W.R. Walker for his support and encouragement during the course of this study. Also his assistance and suggestions in the preparation of this manuscript were extremely helpful. Appreciation is also extended to Mr. R. Storms and Mr. D. Morrison for their valuable discussion, Mrs. C. Dimsdale for help in the preparation of material for light microscopy, and Dr. J.E. Kinnear, Department of Microbiology, for the operation of analytical untracentrifuge. Finally, I would like to thank Mrs. E. Ford for her skillful typing.

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CHAPTER I

INTRODUCTION

Although some aspects of recombination in prokaryotes and eukaryotes have been worked out quite well, the basic mechanism remains unclear. Genetic recombination in eukaryotes occurs at prophase of meiosis and rarely at mitosis. The determination of the exact nature of the mechanism by which it is accomplished, although one of the most attractive problems in genetics and physiology, is largely unsolved for several reasons. First, the chromosome of eukaryotes is composed not only of DNA but also of protein and RNA. Second, the structural organization of eukaryotic chromosomes, which is extremely important to the understanding of recombination, is not clear at present. The strandedness of chromosomes, for instance, is still under debate (see Whitehouse, 1969; Prescott, 1970; and Wolff, 1969 for review). Third, no experimental organism has yet been found in which both the biochemical, the cytological and the genetic study of recombination can be made. Thus large synchronous populations of meiotic cells, essential to biochemical analysis are not readily obtained in organisms having chromosomes of a suitable size for cytological observation; and genetic study demands an available number of suitable mutants.

For the above reasons, many approaches have been

utilized in the study of recombination in eukaryotes. All of these have yielded data important to an understanding of the mechanism.

1. *Microscopy*

Light microscopy (LM) for the direct observation of chromosomal behavior during meiosis was one of the first techniques employed in the study of recombination. Many meiotic events (*e.g.* duplication, condensation, and segregation of chromosomes) are seen to occur in mitosis, but some features, such as pairing of homologs and crossing-over, are unique to meiosis. Pairing of chromosomes, shown to begin at early zygotene, is completed before pachytene, and the synapsed chromosomes dissociate at early diplotene. Although these studies have not determined the exact time of chromosome duplication, synapsed chromosomes at the time of exchange are thought to consist of four strands (see Lindegren, 1933) so that duplication may already have taken place before this stage. LM autoradiographic studies combined with genetic studies have shown that crossing-over takes place at pachytene and involves a physical exchange of chromosomes (see Taylor, 1958, 1965; Peacock, 1968, 1970; Whitehouse, 1969; and Henderson, 1970 for review). Chiasmata found at diplotene are believed to be the cytological evidence of crossing-over, since a high correlation between them has been demonstrated (Taylor, 1967; Peacock, 1968;

see Whitehouse, 1969 for review).

LM cytophotometric studies of meiocyte nuclear DNA content (Swift, 1953) and autoradiographic studies of germ cells (Taylor, 1953; Moses and Taylor, 1955; Lima-de-Faria and Borum, 1962) have shown that DNA replication is completed before early prophase. Rossen and Westergaard (1966) have shown that DNA synthesis in the ascomycete *Neottiella* takes place before nuclear fusion. This indicates that crossing-over takes place after replication.

Autoradiographic studies have detected prophase DNA synthesis in meiosis in many species (Wimber and Prensky, 1963; Lima-de-Faria *et al.*, 1968; Mukherjee and Cohen, 1968; Riley and Bennett, 1971). However, negative results have also been reported (Callan and Taylor, 1968; Hofman-Alfro and Chandley, 1970; Peacock, 1970).

The use of electronmicroscopy (EM) has revealed an unique structure called the synaptonemal complex located along the synapsed regions of bivalents (see Moses,1968 for review). This ribbon-like structure has a diameter of about 1000A^{o} and consists of two darkly stained lateral elements and a less dense central element. It has been suggested that each lateral element contains two subelements attached independently to the chromatin fibrils of one sister chromatid (Comings and Okada, 1970; von Wettstein, 1971).

The chemical constitution of the synaptonemal complex (SC) is not very clear. Cytochemical study indicates that it is composed entirely of basic protein (Sheridan and

Barrett, 1969) although the presence of nucleic acids in the inner part of the lateral elements and perhaps the central element has been suggested (see Moses, 1968, 1969). The formation and development of the SC have been studied quite extensively in recent years. It has been shown that the axial core, attached at multiple sites to the chromatin fibrils of unpaired leptotene chromatin, is the precursor of the lateral element of mature tripartite SC (Moens, 1968; von Wettstein, 1971). The origin of the central element, on the other hand, remains unknown. Von Wettstein (1971) suggests that central elements are originally stacked inside the nucleolus and are released from this organelle. Comings and Okada (1970) propose that it is formed from the pairing of the protein loops of lateral elements of homologs. Moens (1968), on the other hand, believes that the central element consists of the master genes of the cycloid-model type (Whitehouse, 1967) and therefore is part of chromatin fibrils. Since the SC has been found attached to the nuclear membrane. (Comings and Okada, 1970; Giles, 1972), it has been suggested that gross pairing is determined or facilitated by such attachments (Sotelo and Wettstein, 1969).

The molecular basis of homolog recognition is still not known. Coded information carried by the basic protein of lateral elements (von Wettstein, 1971) and the base sequence of single-strand DNA (Comings and Okada, 1970; Stern, 1969) have been proposed for this role.

Lu (1970) has studied the relationship between the SC

and genetic recombination,utilizing cold and heat treatments to induce changes in the frequency of recombination in *Coprinus*. It is found that both treatments cause an increase in recombination if applied during zygotene and pachytene when SC is present. When the treatments are applied after this period, when the SC cannot be seen, no effect on recombination is observed. This suggests that the SC is related to recombination although its real function in recombination is not known (see Moses,1968,1969 for review).

Studies of the structural organization of eukaryotic chromosomes include electronmicroscopic examination of chromosomes spread on thin films, cytochemical and autoradiographic studies , combined cytological and radiation studies (see Prescott 1970 for review). Polytene, lampbrush and metaphase chromosomes are used very often for this purpose. These studies have been concerned with both macromolecular structure of strands, and the number of strands per chromosome, and many models of eukaryotic chromosomes have been proposed (Taylor, 1963, 1967; Uhl, 1965; Callan, 1967; Crick, 1971; du Praw, 1970).

2. *Direct Biochemical Analysis*

Considerable contributions to the understanding of the underlying mechanism of recombination have been provided by direct biochemical analysis of meiosis, especially macromolecular synthesis. The biochemistry of plant meiocytes has been studied very intensively by Stern and associates (Stern, 1969). The most striking finding is the

demonstration of discrete DNA syntheses occurring at zygotene and pachytene respectively. The amount of DNA synthesized during this second period of DNA synthesis is about 0.3-0.4% of total nuclear DNA (Hotta *et al.*, 1966). It is interesting that DNA synthesized at zygotene has a higher buoyant density (and therefore higher GC content) than the bulk of nuclear DNA, whereas that synthesized at pachytene has the same density.

By the utilization of the DNA-DNA hybridization technique, it has been shown that at saturation, the amount of labelled and fragmented, denatured zygotene DNA which hybridizes with denatured DNA obtained from the stages between the pre-meiotic S period and zygotene is approximately half that of DNA's obtained from stages after zygotene or from somatic nuclei. No such difference is observed for pachytene DNA (Hotta and Stern, 1971a). This clearly indicates that certain regions of the genome, which have a high average GC content, remain unreplicated until zygotene.

Protein synthesis during meiosis has been studied in *Lilium* (Taylor and McMaster, 1954; Taylor, 1959; Hotta *et al.*, 1968; Parchman and Stern, 1969) and *Trillium* (Hotta and Stern, 1963a and b) and in yeast (Esposito *et al.*, 1969). It was found that synthesis of protein throughout prophase was essential to the meiotic process since the addition of amino acid analogues or antibiotics known to upset protein synthesis during this period result in the disturbance of the process and the production of cytological abnormalities

(Hotta and Stern, 1963a; Kemp, 1964; Parchman and Stern, 1969). In addition, a progressive change in the pattern of protein synthesis accompanying progress through meiosis has been shown by DEAE chromatography (Hotta *et al.*, 1968).

Recently, some functions of proteins synthesized at meiotic prophase have been demonstrated. Acid-fraction proteins synthesized at zygotene have been found to be essential to delayed replication (Hotta *et al.*, 1968). It has been shown that this fraction is associated with zygotene DNA in the form of a protein-DNA complex attached to the nuclear membrane (Hotta *et al.*, 1968; Hecht and Stern, 1971). The complex possesses DNA polymerase activity and appears to have properties similar to the replicating complex of microorganisms. More interesting, a DNA binding protein has been found in *Lilium* and many mammalian species by Hotta and Stern (1970, 1971b), which appears only during chromosomal pairing and crossing-over and is absent in the somatic cells of these organisms. It possesses high binding affinity for single-stranded DNA and has the unusual property of catalyzing the renaturation of denatured DNA at room temperature. Since these properties are similar to the gene-32 protein of T_4 bacteriophage (Albert and Frey, 1970) which is essential to genetic recombination, a possible role of this protein in recombination is suggested.

Sheridan and Stern (1967) have compared the basic proteins of meiocytes and the other tissues of *Lilium* utilizing gel electrophoresis. It is found that a band of

basic protein appears only in meiotic cells. Although the location and the function of this basic protein is not known, it appears very likely that it is derived from the SC since only meiotic cells possess this structure, and cytochemical study indicates that the SC consists mainly of basic protein (Sheridan and Barrett, 1967; Moses, 1968, 1969 for review). If this is true this basic protein must be produced before leptotene since lateral elements of the SC, which consist mainly of basic protein, are already present at this time.

Endonuclease, polynucleotide kinase and ligase activities during zygotene and pachytene have been shown to be higher than before or after these stages (Howell and Stern, 1971). This implies that the increase in activity of repair enzymes is associated with the genetic recombination occurring during this period.

Little progress has been made in the study of RNA synthesis during meiosis, although a significant variation in synthetic activity during the meiotic process has been shown (Hotta and Stern, 1963b; Stern, 1969a; Esposito *et al.*, 1970).

3. *Meiotic and Recombination Mutants*

Perhaps the best approach to the study of meiosis and recombination is the isolation and characterization of mutants affecting meiosis and recombination. The difficulty with this approach lies in the fact that mutants are usually sterile or lethal. Nevertheless, in yeast, several

conditional meiotic mutants have been obtained (Bresch *et al.*, 1968; Esposito and Esposito, 1970), and preliminary investigations with these mutants indicate that either DNA replication or recombination is affected (Esposito *et al.*, 1971). The evidence that recombination is under genetic control is derived from the observation in *Neurospora* (Catchside, 1968), that the several *rec* genes so far discovered, when present in the homozygous condition, affect the recombination of specific sites that are not linked to them. The mechanism of regulation is still under investigation.

Recent studies on radiation-sensitive and recombination-deficient mutants in prokaryotes and eukaryotes provide valuable information for the understanding of the mechanism of recombination. The conclusion drawn from this work is that recombination may share some features with the mechanism of radiation-damage repair since most of the recombination-deficient mutants are sensitive to radiation treatment and many radiation-sensitive mutants are recombination-deficient (see Witkin, 1968; Whitehouse, 1970 for review). It is also suggested that repair enzymes such as endonucleases, exonucleases, polymerases, and ligases are involved in recombination.

4. *Genetic Fine Structure Studies*

Tetrad analysis of intragenic recombination in fungi has provided important information for the understanding of

the mechanism of recombination. Several features have been found in the studies of intragenic recombination (Holliday, 1964, 1968; Whitehouse and Hastings, 1965; see also Emerson, 1967; Whitehouse, 1970; and Fincham, 1971 for review). These are as follows:

a) Gene conversion

Allele segregation in intragenic recombination is frequently non-reciprocal, *i.e.* one allele is present in more copies than the other within a given ascus. In a two-point cross for studying intragenic recombination, a wild type recombinant is not accompanied by the appearance of a double mutant recombinant, and *vice versa*. The process leading to the occurrence of a non-mendelian ratio is called conversion.

b) Co-conversion

Alleles located close to each other often tend to have converted together. In yeast, two alleles separated by 100 base pairs are found to convert together more often than not, whereas those separated by 1000 base pairs are converted independently.

c) Negative interference

Conversion is associated very frequently with the exchange of flanking markers on one of the two strands involved in a conversion. This exchange is independent of the distance between mutant sites and flanking markers. This tendency for multiple exchanges over a short segment of genetic material is called negative interference.

d) Map expansion

A map based on the frequency of recombination within the gene is often not additive. Instead the map distance between distant markers is greater than the sum of the intervening distances.

e) Polarity

The conversion frequencies of mutant sites or markers located within a gene show a gradient from the ends.

f) Post-meiotic segregation

Separation of alleles may take place at the first mitosis after meiosis as shown by non-identical pairs of spores within the 8-spored ascus. This indicates that each chromatid consists of two subunits or half-chromatids, which are the actual units of recombination.

Many models of recombination have been proposed based on these studies (Whitehouse, 1963, 1963, 1966, 1967; Whitehouse and Hastings, 1965; Holliday, 1964; Stahl, 1969; Fogel and Hurst, 1967; Paszevski, 1970).

5. *Inhibitors*

Inhibitors may be used to block specific biochemical processes *in vivo* (*e.g.* protein or nucleic acid synthesis) in order to determine their physiological function. Therefore, in principle, this technique is as powerful as that involving biochemical mutants deficient for these biochemical processes. This technique has its greast use in preliminary testing since it obviates the necessity of isolating mutants

and is applicable to a wide range of organisms.

Deoxyadenosine, an inhibitor of DNA synthesis, has been used to study the function of prophase DNA synthesis (Ito *et al.*, 1967). Cultured meiocytes of *Lilium* at various stages of prophase were treated with this inhibitor. It was found that this inhibitor, added between leptotene and midzygotene, arrested meiocytes at zygotene. Treatment at zygotene (after prophase DNA synthesis has started), produced fragmented chromosomes, which are observed at prophase and anaphase II and treatment at pachytene produced chromatid breaks at metaphase I and II. Such differences in the effect of treatment at zygotene and pachytene were interpreted as the result of differences in the nature of two discrete DNA syntheses during two stages. A synthesis at zygotene was represented as being a delayed replication of sections of the "axial element", randomly located along the chromosomes, whereas pachytene synthesis was represented as a repair synthesis associated with crossing-over. Their conclusions have been substantiated by their later direct analysis of zygotene and pachytene DNA molecules described earlier. In similar studies, Roth and Ito (1967) and Sen (1969) have observed that the formation, progress, and dissociation of the SC depend on zygotene DNA synthesis, since treatment of cultured meiocytes with deoxyadenosine to inhibit this synthesis resulted in the disturbance of these processes.

The function of pachytene DNA synthesis has been resolved by treatment with hydroxyurea, a specific inhibitor

of semiconservative replication (Painter and Cleaver, 1967). In *Lilium* meiocytes, it has been shown that at 1 mM it preferentially inhibits pre-meiotic S period replication, whereas at 5 mM it inhibits repair synthesis as well (Hotta and Stern, 1971). It is found that pachytene DNA synthesis is greatly inhibited by 5 mM hydroxyurea but little affected by 1 mM, which inhibits zygotene synthesis almost completely. This indicates that the synthesis at pachytene is repair synthesis.

Cycloheximide, an inhibitor of protein synthesis, has been used to study the function of prophase proteins. It has been found that zygotene protein synthesis is essential for zygotene DNA synthesis, since its inhibition by cycloheximide prevents zygotene DNA synthesis (Hotta *et al.*, 1968).

Treatment with cycloheximide at late zygotene and early pachytene, when synapsis has taken place causes a reduction in the frequency of chiasmata, sometimes to zero (Parchman and Stern, 1969; Sen, 1969). Since a chiasma is believed to be the visible consequence of a crossover, it has been suggested that synapsis alone is not sufficient for crossing-over to occur and crossing-over must take place after this period. Roth and Parchman (1971) have reported that the structure of the SC is disturbed after such treatment. The cause is still not known. In a similar study, in which a lower concentration (0.25 µg/ml) of inhibitor was used, Sen (1969) reported that the inhibition of protein synthesis

did not affect the structure of SC.

The synchronous meiosis of yeast and *Chlamydomonas* are the best systems for the combined biochemical and genetic study although cytological examination is difficult. In the work described below, *Chlamydomonas* was chosen since it has better synchronization of meiosis than yeast and considerable genetic and biochemical data are available.

The unicellular green alga *Chlamydomonas* is a eukaryote having a clearly visible nuclear membrane (Johnson and Porter, 1968), defined chromosomes and normal pattern of cell division (Levine and Folsome, 1959; Levine and Ebersold, 1960 for review; Johnson and Porter, 1968). It also shows the characteristic features of the eukaryotic life cycle: mitosis, cell growth, fertilization, and meiosis. Moreover, it can be grown in a simple chemically defined medium, and different stages of the life cycle can be experimentally controlled (Sager and Granick, 1954; Levine and Folsome, 1959; and Kates and Jones, 1964).

Cytological examination indicated that vegetative cells have eight chromosomes (Levine and Folsome, 1959). Recently, however, 16 chromosomes have been demonstrated (Loppes and Matagne, 1972) - this agrees with the 16 linkage groups constructed from genetic study (Hastings *et al.*, 1965).

A method of inducing the synchronous division of vegetative cells by the imposition of a dark and light cycle has been developed by Kates and Jones (1964). This naturally induced synchrony provides a very useful tool for

the study of cell division.

A very important feature of *Chlamydomonas reinhardi* is that its cellular DNA is composed of three components, each with a distinctive bouyant density. The CsCl density gradient of total DNA thus reveals three bands: the main α band (1.723 gm/cm^3) is nuclear DNA, the β band (1.692 gm/cm^3) is chloroplast DNA, and a minor band γ (1.715 gm/cm^3, is presumably mitochondrial DNA (Chiang and Sueoka, 1967a). Separation and isolation of these organelles is therefore not required for the investigation of these individual DNAs. Chiang and Sueoka (1967a, b) Kates *et al.* (1968), and Sueoka *et al.* (1967) have taken advantage of this to study nuclear and chloroplast DNA synthesis during cell cycle in synchronous culture and sexual cycle by employing the ^{14}N-^{15}N density transfer technique.

Davies (1966, 1968) studying the UV-irradiation survival of germinating zygospores of *Chlamydomonas* has found that photoreactivation activity is quite constant throughout germination whereas dark-repair activity varies during meiosis. The period between late interphase and early prophase has the lowest dark repair capacity. The author suggests that the decrease in dark repair capacity at pre-prophase may be significant to genetic recombination.

Recently, Chiang (1971) has performed an elegant experiment to study the recombination of chloroplast and nuclear DNAs in which DNAs from different parents are differentially labelled with density-and radioisotopes and

therefore can be distinguished after CsCl density centrifugation. DNA samples are taken at various times during the sexual cycle and subjected to CsCl density gradient centrifugation. In this way, it has been shown that recombination of nuclear DNA takes place during germination. Recombinant DNA molecules are evident as the cosedimentation of both parental DNA molecules (the presence of both kind of radioisotopes) at a density lighter than heavy parental DNA and heavier than light parental DNA. They are formed undoubtedly from the physical exchange of heavy and light DNA molecules.

Genetic studies of recombination in *Chlamydomonas* have shown that the frequency of recombination can be altered by various kinds of physical and chemical treatments at different times during the sexual cycle (Levine and Ebersold, 1958; Eversole and Tatum, 1956; Hastings, 1964). Lawrence and Davies (1967) have studied the effects of protein inhibitors including chloramphenicol, RNase, fluorouracil, and azaguanine on recombination. These inhibitors give a general depression in recombination especially when treated at the early germination period. However, inconsistency among the treatments of these inhibitors is found. An interesting observation coming from these studies is that γ radiation (Lawrence, 1965), and inhibitors of DNA synthesis such as mitomycin C, adenine, deoxyadenosine (Davies and Lawrence, 1967) and fluorodeoxyuridine (Hastings, 1964), affect recombination at two specific

periods during germination. The first is at pre-leptotene, presumably at the pre-meiotic S period, and the second is in prophase. Since the treatment of microsporocytes with γ radiation also produces similar effect on chiasma frequency at these two periods, it is therefore of interest to know what relation these periods of sensitivity to inhibitors bears to prophase and S-phase DNA synthetic periods in *Lilium*.

Purpose of Thesis Study

The last approach, that is, the application of inhibitors, was chosen for this investigation. Several factors may affect the results of the treatment with inhibitors. These include the specificity, concentration, penetrability, and the survival of treated cells. Moreover, since a selective plating technique was used for the scoring of recombination on which only prototrophs are allowed to grow, any inhibitor affecting the growth or the propagation of zoospores may result in an apparent reduction in recombination frequency. Another problem associated with pulse treatment is the removal of the inhibitor. Residual effect of the inhibitors may be present if the inhibitor cannot be removed completely or to a noneffective level. Nevertheless, if quite a number of inhibitors affecting the same kind of synthesis are tested, this difficulty can be minimized.

The following work was an attempt to elaborate the mechanism of recombination through an investigation of the

roles DNA, RNA, and protein syntheses play in recombination, by utilizing various inhibitors of these syntheses.

Pulse treatment rather than continuous treatment was used since it not only allowed determination of the sensitive period but also ensured that the effect of the treatment observed has resulted directly from the inhibition of specific synthesis and not by means of a secondary or tertiary effect of the inhibitor.

Phenethyl alcohol (PEA) specifically inhibits the initiation of DNA replication in *E. coli*, but the mechanism by which it acts is not clear (Lark and Lark, 1966; Treick and Konetzka, 1964). It has been shown to affect DNA-membrane association (probably affecting the replicating complex) (Masker and Eberle, 1972), phospholipid metabolism (Nunn and Tropp, 1972), and enzyme induction presumably through the inhibition of m-RNA synthesis (Rosenkranz *et al.*, 1965).

In *B. subtilis*, on the other hand, PEA does not show specific inhibition of a new round of replication (Zyskind and Pattee, 1972). It is possible that the contradiction in the results may be due to differences in the dosage-response curves of different organisms. For this reason in tests of PEA effects on *Chlamydomonas* PEA-concentration studies are necessary.

Mitomycin C has been shown to inhibit DNA synthesis specifically, with no effect on RNA or protein synthesis, in bacteria and mammalian cells (Shiba *et al.*, 1959). A

production of interstrand cross-links or an alkylation of bases, especially in regions of DNA with high GC content (Szybalski and Iyer, 1967) may be responsible for inhibition. Kersten and Kersten (1969), on the other hand, have shown that the quinone ring of mitomycins may also play a role in the inhibitory effects on nucleic acid synthesis.

Adenine inhibits DNA synthesis in bacteria (Henderson, (1962) and higher cells (Odmark and Kihlman, 1965) through the repression of the enzyme glycinamide ribonucleotide (GAR) synthetase (Nierlich and McFall, 1963).

Fluorodeoxyuridine (FUdR) has been shown to inhibit DNA synthesis by binding competitively with thymidylate synthetase to block the single-step conversion of dUMP to TMP (Cohen, 1958).

Nalidixic acid (NA) inhibits DNA synthesis preferentially, allowing RNA and protein syntheses to continue (Boyle *et al.*, 1969). Both replication and repair syntheses are blocked immediately (Eberle and Masker, 1971). The mechanism of inhibition remains unclear. It has been demonstrated that DNA polymerase and some of the enzymes for precursor biosynthesis such as thymidine and thymidylate kinases, and deoxyribosyl transferase are not affected. However, since DNA polymerase mutants are more sensitive to treatment than wild type (Winshell and Rosenkranz, 1970), it has been suggested that NA may alter DNA structure, producing a lesion that can be repaired by DNA polymerase. Indeed, Cook *et al.* (1966) have shown that NA causes the

degradation of DNA to acid-soluble fragments.

Hydroxyurea (HU) has been shown to inhibit semiconservative replication specifically with no effect on repair synthesis (Painter and Cleaver, 1967). In *Lilium* meiocytes, however, Hotta and Stern (1971a) report that at 1 mM it inhibits semiconservative replication preferentially, but at 5 mM about 70% of repair synthesis is inhibited as well.

Actinomycin D blocks DNA-dependent RNA synthesis by forming a complex with DNA, preferentially at high-guanine regions (Reich and Goldberg, 1964).

Cycloheximide, preferentially inhibits protein synthesis by the 80 S ribosomal system but not of the 70 S (Ennis and Lubin, 1964; Vazquez and Monro, 1964). It has been shown that the release of inoganic phosphate from GTP catalyzed by transferase TF-2 is blocked by this inhibitor (Munro *et al.*, 1968).

Acriflavine and caffeine have been demonstrated to inhibit excision repair (Lieb, 1961; Shankel, 1962; Witkin, 1963; Alper, 1963; Davies, 1966).

CHAPTER II

MATERIALS AND METHODS

Strain:

Wild type and arginine-requiring mutants of *Chlamydomonas reinhardi*, strain 137C, which releases 4 zoospores, were obtained from Professor P. R. Levine, Harvard University. Arginine-requiring mutants were originally isolated by Eversole (1956).

Strain 137F, which releases 8 zoospores, and was used only in the experiment of DNA determination was obtained from Dr. K. S. Chiang.

Media:

The following media used in this study, are described in Levine and Ebersold (1958) and Hastings (1964) with some minor modifications.

A. Minimal Medium

Minimal medium is composed of 5% modified Beijerinck's solution (Bold, 1942), 5% phosphate buffer, and 0.1% trace element solution in distilled water.

B. Nitrogen-Free Medium

N-free medium has the same composition as minimal medium except that Beijerinck's solution was replaced by N-free Beijerinck's solution, which does not contain NH_4Cl.

C. TAP Medium (Gorman and Levine, 1965)

TAP medium has the composition as follows:

Tris (hydroxymethyl) Amino methane	2.42 gms
Beijerinck's solution	50 ml
Trace element solution	1 ml
1 M KH_2PO_4 (pH 7.4)	1 ml

Distilled water to 1 litre. Finally 1 ml of glacial acetic acid was added.

Note: For the growth of arginine-requiring mutants, TAP medium is supplemented with 0.4% of casein amino-acids (TA medium).

Solid media were prepared by adding 1.5% or 4% of agar to the above liquid media.

The composition of solutions used above is as follows:

Beijerinck's solution

NH_4Cl	8 gms
$MgSO_4 \cdot 7H_2O$	2 gms
$CaCl_2 \cdot 2H_2O$	1 gm
Distilled H_2O	1 litre

Phosphate buffer

K_2HPO_4	4.34 gms
KH_2PO_4	7.26 gms
Distilled H_2O	1 litre

Trace element solution

EDTA	50 gms
$ZnSO_4 \cdot 7H_2O$	22 gms
H_3BO_3	11.4 gms

$MnCl_2 \cdot 4H_2O$ ..... 5.06 gms

$FeSO_4 \cdot 7H_2O$ ..... 4.99 gms

$CoCl_2 \cdot 6H_2O$ ..... 1.61 gms

$(NH_4)_6MoO_4 \cdot 4H_2O$ ..... 1.10 gms

$CuSO_4 \cdot 5H_2O$ ..... 1.57 gms

Distilled H_2O to 1 litre and pH adjusted to 6.5-6.8 by adding freshly prepared 20% KOH.

Vegetative Culture

A. Synchronous Culture

The method for synchronous culture is described by Kates and Jones (1964) and Sueoka *et al.* (1967).

Wild type vegetative cells were grown in liquid minimal medium in 500 ml Erlenmeyer flasks. The cultures were aerated with 5% CO_2 in air, shaken at 100 rpm, illuminated with overhead fluorescent lamps at an intensity of about 5000 lux, and maintained at 21°C. An alternation cycle of 12 hours light and 12 hours dark was imposed to induce synchronous division.

B. Asynchronous Culture

Wild type cells were grown on 1.5% TAP agar plates and arginine-requiring mutants on 1.5% TA agar plates under fluorescent lamps.

Induction of Gametes

Synchronous gametes were induced from synchronously growing vegetative cells by the nitrogen-withdrawal

technique described by Kates and Jones (1964) and Sueoka *et al.* (1967). Vegetative cells growing in minimal medium at a final concentration of about 10^6 cells per ml were harvested around the 6th hour of the light period. These were centrifuged and resuspended in N-free medium, and adjusted to a final concentration of about $3x10^6$ cells per ml. The culture conditions were identical to those of synchronous vegetative cultures except that continuous illumination was given. Gametes were produced about 16 hours after transfer resulting in a 3-fold increase in cell concentration.

Alternatively, gametes were induced from asynchronous vegetative cells. Vegetative cells grown on TAP or TA plates for about 4-5 days were suspended in either distilled water or N-free minimal medium. The suspension was illuminated with light and shaken at about 100 rpm. Gametes were usually obtained within 6 hours.

Mating of Gametes

The cell concentration of the gamete suspension was determined by a Coulter counter, and two suspensions, one containing mating type + (mt^+) gametes, and the other an approximately equal number of mt^- gametes, were mixed together. The mixture was returned to the light, without shaking, for 30 minutes. During this time drops of this mixture were withdrawn and examined under a light microscope to ensure the occurrence of mating.

Maturation of Zygotes

The suspension of young zygotes was plated uniformly, with the aid of a glass spreader to avoid clumping, on 4% minimal agar plates, poured at least one week previously to ensure a dry surface. The zygote plates were put under fluorescent light for 24 to 36 hours, then wrapped in aluminum foil and kept in the dark for at least 5 days for the maturation of zygotes. During this period zygotes expand, become brownish, and develop a thick cell wall. The mature zygotes are called zygospores.

Germination of Zygospores

Germination of zygospores was induced by exposing plates to light for 30 minutes. The plates were then inverted over chloroform vapor for 40 seconds to kill unmated cells. Germinating zygospores were scraped from the plates and suspended in either minimal or TAP medium. The suspension was returned to the light and the concentration of zygospores was determined with a haemocytometer.

Genetic Method

The region between arg-1 and arg-2, which are separated by 6 map units on linkage group 1, was chosen for the measurement of recombination.

Vegetative cells of arg-1 mt^+ and arg-2 mt^- were cultured on 1.5% TA agar plates separately. The techniques for gamete induction, mating, maturation of zygotes, and the stimulation of germination were as described above.

Germinating zygospores were suspended in TAP medium.

Inhibitors were added at different intervals following the start of germination and removed 30 minutes later by pelleting (at 2000 rpm) with an International Clinical Centrifuge. The pellet was resuspended in TAP medium, adjusted to a suitable cell concentration, and spread uniformly on 1.5% TAP plates. Each plate received 1000-2000 zygospores to avoid the overgrowth of the colonies. The scoring was carried out with the aid of a microscope usually between five to seven days after plating. Since only those tetrads containing prototrophic recombinants show growth and form green colonies on TAP plates, they can be easily distinguished from parental auxotrophs, which do not grow, or grow only to a very limited extent, and do not form green colonies. The frequency of green colonies indicates the frequency of crossing-over between arg-1 and arg-2.

The selective plating technique was employed here since the crossing-over frequency as determined by this technique has been shown by Hastings (1964) to be no different from that determined by the zygote plating technique of Ebersold and Levine (1958) in which zygospores were first grown on non-selective plates then replica-plated on selective ones.

The concentration of inhibitors used were as follows: phenethyl alcohol (PEA), 0.1-1.0%; fluorodeoxyuridine (FUdR), 1-2 mM (Hastings, 1964); mitomycin C, 50-200 µg/ml (Davies and Lawrence, 1967); hydroxyurea (HU), 1-5 mM; nalidixic

acid (NA), 10-30 µg/ml; adenine, 200 µg/ml (Davies and Lawrence, 1967); actinomycin D, 10-100 µg/ml; cycloheximide (CH), 2-100 µg/ml; acriflavine (Davies, 1966), 10-15 µg/ml; caffeine, 0.05%.

For the comparison of the relation between the pre-meiotic S period and the effective period of the unknown inhibitors, DNA inhibitors that had been previously tested and found to affect recombination specifically at the S period and at prophase (see Section II, Results) were used in recombination experiments as time markers.

Determination of DNA Contents of Vegetative Cells and Gametes.

To ensure the stage-homogeneity of the cell population, the vegetative cells or gametes used were obtained from synchronous cultures as described in Chapter II. Cell concentration was determined by a Coulter counter and a haemocytometer. Gametes were identified by their uniform and small size and the capacity for mating (Sueoka *et al.*, 1967). Vegetative cells were harvested at the 6th hour of the light period (that supposed to be at G_1 of the cell cycle; Hastings, personal communication). The procedures for the extraction of nucleic acids and for the indole method of DNA determination were as described below.

Total Nucleic Acid Extraction

The procedure described by Sueoka *et al.* (1967) was followed for total nucleic acid extraction. A sample of

about 10^8 cells was harvested from the culture and washed once with saline-EDTA (0.15 M NaCl plus 0.1 M-EDTA, pH 8). This was followed by several extractions with acetone until no green color was present. The pellet was extracted twice with cold 0.3 N perchloric acid (PCA). 2 ml of 0.5 N PCA was then added to the residue and the temperature kept at 70°C for 25 minutes with constant shaking. The extraction was repeated once and the combined supernatants were saved for DNA determination described below.

DNA Determination

The indole method (Keck, 1956) was employed for DNA determination. 1 ml of unknown was mixed with 1 ml of freshly prepared indole reagent, made by mixing an equal volume of 2.5 N HCl and 0.06% aqueous indole solution. The mixture was boiled in a water bath for exactly 10 minutes, cooled, and extracted twice with an equal volume of chloroform. The aqueous phase was decanted after centrifugation and was read at 490 nm against a PCA blank with a Zeiss spectrophotometer. A standard curve was prepared from calf thymus DNA (Calbiochem.).

RNA Determination

The quantity of RNA was determined by the orcinol method (Markham, 1955). 1 ml of unknown was mixed with an equal volume of freshly prepared orcinol reagent made by dissolving 0.1% of $FeCl_3 \cdot 6H_2O$ and 0.1% of orcinol in concentrated HCl. The mixture was heated for 8 minutes in

a water bath, cooled, and read at 670 nm. A standard curve was prepared from yeast RNA.

Incorporation of ^{32}P into Germinating Zygospores

The procedures for obtaining zygospores and the induction of germination were as described above. Wild type zygospores were obtained from synchronous culture whereas heterozygous zygospores of arg-1 and arg-2 were obtained by suspending cells grown on plates.

1. Pulse labelling

Zygospore suspensions prepared by the procedure described above were labelled with 10 μCi/ml of ^{32}P-phosphate for 30 minutes at different intervals after the start of germination.

2. Continuous labelling

^{32}P-phosphate was added to a zygospore suspension at the 3rd hour after the start of germination. Samples were fixed 3, 4 and 5 hours later.

Incorporation of both pulse and continuous labelling was stopped with cold 0.005 M phosphate buffer (pH 6.8) and then washed twice with the same buffer. The pellets were subjected to differential RNA and DNA extraction as described below.

Differential RNA and DNA Extraction

The procedure of differential extraction described by Smillie and Krotkov (1960) was employed with minor modifications. Approximately 10^7 cells were required for

the extraction. Labelled zygospores or vegetative cells were washed twice with cold 0.005 M phosphate buffer (pH 6.8), extracted several times with 80% acetone, twice with cold methanol/0.05 M formic acid, and finally with cold methanol until no green color was extracted. The residue was further extracted three times with cold 0.3 N PCA for 25 minutes with frequent shaking to extract the acid soluble fraction. Lipids were removed by extracting the residue with 95% ethanol, then ethanol/ether (3:1), and finally ether. Each extraction was carried out at 55°C for 2 minutes. The dried pellets were suspended in 0.3 N KOH at 37°C for 20 hours to hydrolyze RNA. Cold PCA was then added and adjusted to 0.3 N, and the mixture was centrifuged. The precipitate was washed twice with cold 0.3 N PCA and supernatants were combined and neutralized with KOH. After a further centrifugation, the supernatant was decanted and the radioactivities were counted as described below.

The dry residue remaining after RNA extraction was subjected to DNA extraction with 0.5 N PCA at 70°C for 30 minutes to hydrolyze the DNA. The extraction was repeated once and the combined supernatants were neutralized, centrifuged, and counted as described below.

The method was calibrated with wild type mt+ vegetative cells. The quantities of RNA and DNA were determined by the orcinol and indole methods respectively. It was found that no further ribonucleotides could be extracted after 20 hours of alkaline hydrolysis, and the third extraction with 0.5 N

PCA at 70°C for 30 minutes caused no additional release of indole reactive material. It was also found that no orcinol reactive substance was present in hot PCA extracts, while 2 to 3% of alkaline extract determined by orcinol reaction showed indole reaction. However, the same percentage of indole reaction was also found in purified RNA and ribose (Hastings, personal communication). Thus for vegetative cells, this method is capable of extracting both RNA and DNA completely from the cells. Moreover, alkaline extract contains only RNA but not DNA and hot acid extract contains only DNA but not RNA.

In zygospores labelled with ^{32}P, it was found that no further alkaline hydrolysable counts were found after the KOH digestion and cold PCA washes, and no further hot acid hydrolysable counts were found after the two extractions with hot acid (Hastings, personal communication).

For the measurement of radioactivities, about 200 µl of either RNA or DNA extracts were put on planchettes, dried, and counted with a Picker gas-flow counter.

Density Transfer Experiment

A zygospore suspension prepared according to the procedure described above was spun down. The zygospore pellet was resuspended in $^{15}NH_4Cl$-substituted minimal medium at the 5th hour after the start of germination. Incorporation was stopped 6 hours after transfer. Samples were harvested by centrifugation, washed twice with 0.1 SSC,

frozen and stored at -20°C.

DNA extracts were prepared from the frozen material and subjected to CsCl density gradient centrifugation according to the procedures described below.

Preparation of DNA

Samples of about 10^8 cells were washed twice with cold 0.1 SSC (.015 M NaCl, .0015 M sodium acetate, pH 7.0) and pellets were frozen at -20°C. The phenol extraction method of Kirby *et al.* (1967) modified by Hastings and Surzycki (1968, unpublished method) was employed. The frozen pellet was ground with sea sand with a pestle and mortar cooled with dry ice. The powder was dissolved in the extracting mixture, consisting of 0.5 ml of saline plus EDTA (SSC plus 0.1 M EDTA), 0.3 ml of 25% sarkosyl NL97 and 20% sodium lauryl sulfate, plus 4 ml 8% sodium para-amino salicylate. An equal volume of double-distilled phenol saturated with SSC and containing 0.1% hydroxyquinoline was added to the above mixture. Extraction was continued for 2-4 hours. After centrifugation in a Sorval refrigerated centrifuge at 11000 rpm for 15 minutes, the aqueous layer was removed and shaken with an equal volume of phenol, and the lower (phenol) phase was re-extracted with extracting mixture. The combined supernatant was then extracted several times with phenol until no protein was present at the interface. The aqueous layer of the final extraction was carefully removed; 2.5 volumes of cold 98% ethanol was then added slowly and the mixture was left at -20°C overnight to precipitate nucleic

acids. The precipitate was dissolved in 1 SSC, and reprecipitated, dried and dissolved in 1 SSC.

RNA and protein were further removed from the resulting solution by enzyme digestion in two steps. The solution was treated with T_1 and pancreatic RNases previously heated at 80°C for 10 minutes to inactivate possible DNase contaminants. Pronase solution, which had been kept at room temperature for autodigestion of any DNase present, was then added and the mixture was further incubated at 37°C for 4 hours. The solution was then extracted with an equal volume of phenol, and the aqueous phase was carefully removed and dialyzed sequentially against the following outside dialyzing solutions: 1 SSC plus 0.05% sarkosyl, 1 SSC, and 0.1 SSC, the dialyzed DNA solution was adjusted to 1 SSC and kept at -20°C.

CsCl Density Centrifugation

DNA prepared by the method described above was transferred to CsCl solution, and the density of the solution was adjusted to 1.710 gm/cm^3. The solution was run in a Spinco model E analytical ultracentrifuge at 44000 rpm for 48 hours according to the technique of Meselson *et al.* (1957). Several photographs were taken when equilibrium was reached. The negative photographs were scanned with a Joyce-Loebl double-beam recording microdensitometer or a chromoscan.

Studies on the Effects of Inhibitors on Macromolecular Synthesis

Exponentially growing wild type mt+ vegetative cells were used to test the effects of inhibitors on macromolecular synthesis.

1. Effect on DNA and RNA syntheses

Vegetative cell cultures were treated for 1½ to 2 hours with various concentrations of inhibitors, with ^{32}P-phosphate added to a final concentration of 10 μCi/ml. Samples collected after treatment were centrifuged. The pellets were washed several times with cold 0.005 M phosphate buffer and subjected to differential RNA and DNA extraction as described above. The amounts of ^{32}P present in the DNA and RNA fractions were measured with a gas-flow counter.

2. Time-course study of the effect on DNA synthesis

The procedure was identical to (1) except that samples were collected every 30 minutes after the beginning of treatment with inhibitors. The amount of ^{32}P incorporation into DNA fraction was measured after differential extraction.

3. Effect on protein synthesis

Vegetative cell cultures were treated 1½ to 2 hours after various concentrations of inhibitors, with ^{14}C-alanine or arginine (sp. act. 5-10 μCi/mM) added to a final concentration of 1 μCi/ml. The incorporation was stopped by adding an equal volume of cold 10% TCA (trichloric acid). After staying for 30 minutes in an ice-water bath, the filter technique was employed to determine the amount of

amino acid incorporation into the acid insoluble fraction. The mixture was filtered through a glass fiber filter paper (Whatman, Grade GF/A) by suction. It was then followed by several washes with 5% TCA, water, and ethanol. The filter paper was then counted either in a liquid scintillation counter or a gas-flow counter.

Cytological Method

The method of staining the nucleus described by Hastings (1964) was followed, except that the entire procedure was carried out in centrifuge tubes. Zygospores were collected by centrifugation, fixed in Carnoy's fixative (alcohol/glacial acetic acid, 3:1) for 90 minutes, rinsed in distilled water, hydrolyzed with 1 N HCl at 60°C for 10 minutes, and stained with basic fuchsin for two hours. After dehydration through an alcohol series, followed by 1 day in xylene, zygospores were removed from the tube, spread on microscopic slides and mounted in euparal. A Leitz phase-contrast microscope with oil-immersion objective was used for cytological observation.

CHAPTER III

OBSERVATIONS AND RESULTS

Section 1. Studies on Meiotic Pattern and Nucleic Acid Synthesis during Germination in *Chlamydomonas reinhardi* Strain 137C

A. *DNA Content of Vegetative Cells and Gametes*

The calculated DNA contents of gametes and vegetative cells of both mating types obtained from DNA determination experiments are shown in Table 1. As can be seen from the table, both gametes and vegetative cells have a DNA content of about 2.5×10^{-7} µg/cell. This confirms the observation of Sueoka *et al.* (1967) on *C. reinhardi* strain 137F. Moreover, the result also indicates that strain 137F and C possess equal amounts of DNA per cell no matter whether it is a vegetative cell or a gamete. It is to be noted that the DNA content of vegetative cells and gametes determined here is twice that as determined by Sueoka *et al.* The reason is not known.

B. *Density Transfer Experiment*

Germinating zygospores previously grown in normal (^{14}N) medium, were grown in ^{15}N medium from 5 to 11 hours after the start of germination. DNA prepared from this experiment was subjected to CsCl density gradient centrifugation. The microdensitometer tracing of the UV absorption photograph

is shown in Figure 1a. It reveals a single major peak. However, when the DNA sample was sedimented in a lower density gradient, two minor lower density peaks were observed in addition to this main peak (Figure 1b). Apparently, the minor peaks are chloroplast DNA as judged from their quantities and densities compared with the main peak. Since the two minor peaks are more or less equal in quantity, it appears very likely that they are hybrid and heavy-heavy respectively, suggesting that two rounds of chloroplast DNA replication took place between 5 and 11 hours after the start of germination. If the density values obtained for these two peaks by Sueoka and co-workers (1.703 and 1.710, respectively) as a density reference and use of the equation of Mandel *et al.* (1968) as follows:

$$\rho = \rho_0 + 4.2\ \omega^2\ (\gamma^2 - \gamma_0{}^2) \times 10^{-10}\ \text{gm/ml}$$

where ρ is the density of unknown DNA

ρ_0 is the density of reference DNA

γ^2 is the distance from the center of rotation to the peak of unknown DNA

γ_0 is the distance from the center of rotation to the peak of reference DNA

ω is the speed of rotation in radians/sec.

the major peak has a calculated density of 1.734. This value is very close to the hybrid density (1.731) of nuclear DNA indicating that in strain 137C there is only one round of chromosomal DNA replication during 5-11 hour period after the beginning of germination.

C. *DNA Synthesis During Germination*

Because of the low labelling efficiency of *Chlamydomonas* nucleic acid with precursors such as ^{14}C-adenine, ^{32}P-phosphate was used for this purpose. It was found that both DNA and RNA were well labelled by this isotope.

1. ^{32}P-phosphate pulse incorporation

The results of ^{32}P incorporation into the DNA fraction of wild-type zygospores at different times during germination are shown in Figure 2. These zygospores were suspended in minimal medium similar to density transfer experiments. The figure shows only one major peak of incorporation, at 6½-7 hours after germination (other peaks are seen, but these are minor ones). The previous density transfer experiment showed that there was only one round of nuclear DNA replication during a period almost completely covered by this pulse incorporation experiment, and since zygospores fixed at different times during this pulse incorporation experiment showed that meiotic prophase began at 8½ hours and the second division began at 11 hours after germination, the main peak observed here must represent pre-meiotic DNA synthesis. Since very little incorporation is observed preceeding or following this period, it appears that pre-meiotic replication lasts only thirty minutes.

It is interesting to note that a minor incorporation occurs at 8 to 9 hours, which is during the meiotic prophase stage. This may parallel the prophase DNA synthesis demonstrated in *Lilium* meiocytes by Hotta *et al.* (1966).

A minor amount of incorporation early in germination, *i.e.* 4½-5½ hours, which appears consistently in every experiment, on the other hand may represent chloroplast DNA synthesis since several rounds of chloroplast DNA replication occurring during germination have been shown by Chiang and Sueoka (1967).

Zygospores germinated in TAP medium instead of minimal showed an essentially identical pattern of incorporation except that the S period was delayed 30 minutes (*i.e.* at 7-7½ hours after germination; Figure 3).

The results of experiment 3 in which zygospores were obtained from the cross between arg-1 and arg-2 and germinated in TAP medium are shown in Figure 4. A pattern of ^{32}P incorporation very similar to that of wild-type is shown. The pre-meiotic S period is located at 6½-7 hours.

2. ^{32}P-phosphate continuous incorporation

This method was employed to confirm the time of the pre-meiotic S period determined by pulse labelling. The experiment was carried out at the same time as experiment 3 using the same population of zygospores. ^{32}P-phosphate was added at three hours after the start of germination. The results (^{32}P incorporation into DNA fraction at different times after labelling) are shown in Figure 5. An abrupt increase is observed between 6 and 7 hours with very little incorporation prior to or after this period. The results thus indicate that the main peak and S period is located at 6½ to 7 hours.

D. *RNA Synthesis During Germination*

The pattern of RNA synthesis differs greatly between the wild-type and heterozygotes for the arginine loci. Wild-type zygospores, grown in either minimal or TAP medium, show a strong peak of incorporation of ^{32}P into RNA both prior to and during the main S period, and also during meiotic prophase (Figures 2b and 3b). In arginine heterozygotes (Figure 4b), some incorporation of ^{32}P into RNA occurs throughout germination. However, the incorporation was very high during very early germination, i.e. at 3½ to 4 hours after the start of germination. Two lesser peaks of incorporation were found at the time preceding S period and mid S period.

The results of continuous incorporation experiments shown in Figure 5b indicate that the incorporation of ^{32}P into RNA occurs linearly as germination proceeds.

Section 2. Effects of Several DNA Synthesis Inhibitors on Survival of Germinating Zygospores and Recombination

A. *Effects on Macromolecular Synthesis*

FUdR at 2 mM inhibited 33% of total DNA synthesis in the 1½ hour treatment. Moreover, it inhibited 15% of DNA synthesis at 0.1 mM and 72% of DNA synthesis at 10 mM in two hour treatment (Tables 2 and 3). The

effect of FUdR on RNA synthesis was not consistent (Tables 2 and 3). At 1 mM it caused a 22.5% reduction of ^{14}C-alanine incorporation in 2 hours (Table 14).

In 1½ hour treatment, mitomycin C inhibited 86 and 90% of DNA synthesis at 50 µg/ml and 200 µg/ml, respectively (Table 2). The effect on RNA synthesis was not uniform (Table 2). Mitomycin C caused no appreciable effect on the incorporation of ^{14}C-arginine at 50 µg/ml, and 12% inhibition at 100 µg/ml (Table 4).

NA gave 70% inhibition of DNA synthesis when used at 3 µg/ml and 78% inhibition at 30 µg/ml in 2 hours (Table 3). It inhibited about 10% of RNA synthesis at any concentration up to 300 µg/ml. HU at 1 mM and 5 mM inhibited 56 and 71% of DNA synthesis respectively in 2 hours (Table 3). 1 mM gave no effect on RNA synthesis while at 5 mM caused 28.6% inhibition (Table 3). Both NA and HU showed no effect on ^{14}C-arginine incorporation in 1½ hour treatment at any concentration up to 100 µg/ml in the former and 10 mM in the latter (Table 4).

Adenine at 50 µg/ml inhibited 43% of DNA synthesis and at 200 µg/ml gave 66% inhibition in 1½ hours (Table 2). RNA synthesis was indifferent to adenine treatment at the concentration up to 200 µg/ml (Table 2).

PEA at concentrations of 0.1 to 1.0% inhibited all forms of macromolecular synthesis very strongly in 1½ hour treatment (Tables 2 and 4). The degree of inhibition of DNA synthesis varies with the concentration of PEA since at 0.1%

it inhibited about 60% of total DNA synthesis, whereas 0.4 and 1.0% inhibited 75% and 85% respectively of DNA synthesis. Inhibition of protein synthesis is also concentration-dependent since 0.1% inhibited 60% of control synthesis whereas 0.4 and 1.0% inhibited 99% of protein synthesis. The effect on RNA synthesis on the other hand, is independent of PEA concentration in this range. At the concentrations tested here, about 95% of RNA synthesis was inhibited.

B. *Time-course Study of the Effect on DNA Synthesis*

The time-course of the inhibitory effect of 0.4% PEA on DNA synthesis is shown in Figure 6. It indicates that PEA has a delayed effect on DNA synthesis, which is completely blocked 90 minutes after the treatment. Although the effect on RNA synthesis is not presented here, the pattern is different. In spite of great reduction in RNA synthesis by the treatment, linear increase in the incorporation of ^{32}P into RNA throughout the treatment was observed.

The results of the time-course study of the effect of mitomycin C, NA, FUdR, and HU are shown in Figures 6 and 7. As can be seen from the figures, mitomycin C is similar to PEA in its effects but is distinct from FUdR and HU. Mitomycin C causes a delayed effect on DNA synthesis, which is blocked almost completely 90 minutes after the treatment. In contrast, DNA synthesis continues throughout the treatment of other DNA inhibitors with the maximum reduction in synthesis at 30 to 60 minutes after the treatment.

C. *Effect of PEA on Survival*

The data for survival of germinating zygospores after the treatment with PEA in various concentrations and at different times are shown in Table 5 and Figure 8. As can be seen from the data, the effect of PEA on the survival of zygospores varies with the concentration of PEA used. 0.1% PEA shows no effect on survival; 1.0% kills zygospores at all stages beyond the early germination period. This may be due to low penetrability at the early period. It is striking to find that 0.4% PEA reduces the germination of zygospores almost to zero at 5½ to 6 hours after the start of germination. The main S period as determined by the inhibitors of DNA synthesis, FUdR and adenine is located at 6½ to 7 hours. In other words, the sensitive period of PEA is 60 minutes prior to the main S period.

A depression in survival of germinating zygospores at prophase was found. The sensitive period was from 7 to 8½ hours.

D. *Effect of Other DNA Synthesis Inhibitors on the Survival of Germinating Zygospores*

Apart from PEA, none of the inhibitor concentrations used in this recombination study had any effect on the survival of treated zygospores (Table 6) with few exceptions (adenine, 4-5 and 7½-8 hours; HU, 2 mM at 8-8½; 5 mM at 8-9 hours). In most of the experiments, about 90% of the zygospores germinated.

E. *Effect on Recombination*

When data of recombination experiments in which several inhibitors were carried out at the same time were compared, it was found that the effective times of inhibitors were separated by quite constant intervals and their sequence remained the same although for a given inhibitor the effective times varied slightly from experiment to experiment. The variations in the responsive times of zygospores to the treatment with inhibitors are probably caused by differences between experiments in the rate of germination since the times of the pre-meiotic S period, meiosis, and the liberation of meiotic products showed a similar variation.

Figures 9 and 10 and Tables 7 and 8 show that treatment with mitomycin C gives an increase in recombination at 5 to 5½ hours after the start of germination and FUdR depresses recombination 30 minutes later, *i.e.* 5½ to 6 hours. It is to be noted that both also enhance recombination at prophase (7½ to 8 hours after the beginning of germination in Figure 9 and 7-7½ hours in Figure 10).

In Figure 10 and Table 8, NA is seen to be similar to FUdR in its effect, giving a reduction in recombination at 5½ to 6 hours, *i.e.* 30 minutes after the first effective period of mitomycin C, and an increase in recombination at 7 to 7½ hours. Again, all three inhibitors have the same second effective period. The depression of recombination by NA at 5 to 5½ hours may be due to a residual effect of NA which may remain inside the zygospores or in the

suspension not removed by centrifugation.

The effects of adenine, PEA and FUdR on recombination is shown in Figure 11 and Table 9. The responsive periods for FUdR are at 6½ to 7 and 8 to 8½ hours after the onset of germination. Adenine causes a depression in recombination from 6½ to 8½ hours. Apparently the first and second responsive periods for adenine, like those for FUdR, are at 6½ to 7 hours and 8 to 8½ hours respectively. The continued depression in recombination between these two periods may also be due to a residual effect of adenine.

Based on the observation described above that 0.4% PEA gave a specific effect on the survival of zygospores, only this concentration of PEA was investigated for effect on recombination. The lethal effect of PEA at 5½ to 6 hours made scoring of recombination during this period impossible. Before this sensitive period, the treatment gave a depression in recombination, and immediately after (at 6-6½ hours, or 30 minutes before the first sensitive period of FUdR and adenine), the treatment gave an increase. Treatment between the first effective period of FUdR and prophase again caused depression in recombination.

In another experiment in which the PEA survival sensitive period was determined to be located at 5-5½ hours and the first recombination effective period of nalidixic acid was at 6-6½ hours, PEA treatment at 5½ to 6 hours (*i.e.* 30 minutes after the survival sensitive period of PEA and 30 minutes before the effective period of NA) enhanced

recombination (Figure 12, Table 10). The pooled data of this and the former experiment indicate that the increase in recombination with the treatment at 30 minutes before the first responsive period of NA or FUdR is significant (x^2 = 6.4, $p < 0.02$). PEA also gave a depression in recombination at prophase ($7\frac{3}{4}$ -$8\frac{1}{4}$ hours). However, since the viability of treated zygospores was so low at this period, the measured recombination frequency is therefore not very reliable.

The effects of the NA and HU treatments carried out in experiment R6 are shown in Figure 13 and Table 11. As can be seen from the figure, HU has the same effective periods as NA i.e. 6 to 7 hours and 8 to $8\frac{1}{2}$ hours. However, the pattern of its effects on recombination is different. Whereas NA depresses recombination during the first period and enhances it during the second, HU (like adenine) depresses recombination at both periods.

These experiments show that all inhibitors of DNA synthesis , except PEA, have two effective periods on recombination during germination. The first one is before prophase and second one is during this stage of meiosis. It was also found that all these DNA synthesis inhibitors, except mitomycin C, have effects at the same time at both responsive periods. This inhibitor responds during the first period 30 minutes sooner than the other inhibitors.and at the same effective period as PEA.

Since the same population of zygospores of experiment R6 was used for a ^{32}P incorporation experiment (shown in Figure 4) which shows a main S period at 6½ to 7 hours, it is concluded that all these DNA inhibitors, except mitomycin and PEA, affect recombination at the main S period.

The experiment R7 shown in Figure 12 and Table 10, in which the treatments of inhibitors at prophase were reduced to 15 minutes, was an attempt to resolve the second responsive period of DNA inhibitors. Nalidixic acid and hydroxyurea were chosen for comparison since they affect recombination in different directions at this period. As may be seen from the figure, both inhibitors have the effective period at the same time at prophase (at 7¼-7½ hours) although they have different effects on recombination. It is noted that both 1 mM and 5 mM of HU show the same effect on recombination.

Since mitomycin C and the other group of DNA synthesis inhibitors show different effects on recombination at premeiotic period, it was interesting to know the effect when a mixture of mitomycin C and one of these inhibitors was used for the treatment. The result of this experiment in which a mixture of mitomycin C and FUdR was used as the treatment is presented in Table 19 and Figure 17. Mitomycin C alone gave an increase in recombination at 6-6½ hours and 8-9 hours. The combined treatment, however, caused an increase in recombination only at the second responsive

period (8-9 hours) although the enhancement on recombination was greater than the treatment with mitomycin C alone.

Section 3. Effects of Actinomycin D on Recombination

The dose response of actinomycin D on RNA and DNA synthesis is shown in Table 12. The relative inhibitory effects on these two syntheses depends on the concentration of inhibitor used, and only with 4 µg/ml is an approach to specificity for RNA seen. At higher concentrations the inhibition of DNA synthesis is very strong. At 100 µg/ml, it inhibits 37.5% of protein synthesis (Table 14).

The concentration of actinomycin D, used in the genetic study, did not affect the survival of treated zygospores even when the concentration used was as high as 100 µg/ml (Table 14).

The effects on recombination are shown in Figures 9 and 10 and Tables 7 and 8. Strong stimulation of recombination is seen with treatment 60 minutes before the main S period (as determined by DNA inhibitors NA, HU, and FUdR) or 30 minutes before the first recombination responsive period of mitomycin C. Actinomycin D also affected recombination at other periods. However, such effects varied from experiment to experiment. At 100 µg/ml it gave greater stimulation of recombination 60 minutes before the main S period but also caused a strong depression in recombination at the main S period (Figure 13 and Table 11). In addition,

this high concentration of inhibitor depresses recombination at the early germination period as well as the period between the main S period and the second effective period of NA and HU.

Section 4. Effect of Cycloheximide on Recombination

A. *Dose Response of CH on Macromolecular Synthesis*

Chlamydomonas reinhardi was very sensitive to cycloheximide. Protein synthesis was strongly inhibited by a very low concentration of inhibitor (Table 14). At 0.1 µg/ml it inhibited more than 40% of protein synthesis. However, maximal inhibition appeared to be reached when the concentration of CH was higher than 50 µg/ml and more than 70% of protein synthesis was inhibited. Here the residual protein (about 27% that of the control) probably represents mitochondrial and chloroplast proteins since it has been shown that CH specifically inhibits protein synthesis of the 80 S ribosomal system but not of the 70 S (Ennis and Lubin, 1964; Vazquez and Monro, 1964).

RNA synthesis was indifferent to all concentrations of CH except 100 µg/ml (16% inhibition; Tables 15 and 2).

At concentrations higher than 10 µg/ml, CH inhibited DNA synthesis. 46% of DNA synthesis was inhibited when the concentration was greater than 50 µg/ml.

The results thus reveal that CH is a specific inhibitor for protein synthesis only at concentrations lower than 10 µg/ml.

B. *Effect of CH on the Survival of Zygospores*

CH at 30 µg or less per ml had no effect on the survival of zygospores. At 100 µg/ml, it caused a reduction in germination, especially with treatment after the main S period (Table 13).

A higher concentration of CH (100 µg/ml) was used in an attempt to inhibit the initiation of DNA replication since this effect has been demonstrated in *Physarum* by Muldoon *et al.* (1971). Failure to find a specific killing period for treatment before the main S period such as that observed with PEA treatment suggests that CH either does not inhibit the initiation of replication or inhibits it reversibly.

C. *Effect of CH on Recombination*

CH in the concentration range 2 µg/ml - 100 µg/ml depressed recombination for all treatment times. The maximum reduction occurred with treatment before the main S period (5½-6 hours, as determined by DNA inhibitors; Figure 10 and Table 8). The result of various concentrations of CH on recombination is presented in Figure 14 and Table 16. As may be seen from the figure, the concentrations in this range have very similar effects on recombination at the early germination period.

The result of the experiment shown in Figure 15 and Table 17 was the attempt to test whether the RNA synthesized at the time (prior to the S-phase), when actinomycin D stimulates recombination, contains messenger for the

initiation of replication. If so, and if the messenger was translated into an initiator protein, it was reasoned that the inhibition of protein synthesis between the responsive periods of actinomycin D and mitomycin C would be expected to stimulate recombination. As can be seen from the result, no significant increase above the normal was obtained with 15 minute treatments at the period between the responsive periods of actinomycin D and mitomycin C (*i.e.* 4¾-5¼ hours).

Section 5. Effects of Acriflavine and Caffeine on Recombination

Acriflavine and caffeine, at the concentratons used, did not affect the survival of zygospores in several replications of this experiment (Table 6). Their effects on recombination especially for treatments before or during the S period were not very uniform (Tables 18 and 19, Figures 16 and 17). Their effects on prophase were, however, quite consistent. Both inhibitors consistently reduced recombination with a maximum reduction at the second recombination responsive period of DNA inhibitors (8-8½ hours in Figure 16, and 8-9 hours in Figure 17).

TABLE 1. DNA Content of Vegetative Cells and Gametes of *Chlamydomonas reinhardi*

Strain		DNA Content (μg x 10^{-7}/cell) Vegetative Cells	Gametes
Strain 137 C			
Wild type	mt-	2.56	2.67
Wild type	mt+	2.44	2.44
Strain 137 F			
89	mt±	2.46	2.42
90	mt∓	2.55	2.55

TABLE 2. Effects of Inhibitors of DNA Synthesis on RNA and DNA Syntheses

Exponentially growing vegetative cells were treated with inhibitors, with ^{32}P-phosphate added to a final concentration of 10 µCi/ml. Samples were collected 1½ hours after treatment and subjected to differential RNA and DNA extraction. The radioactivities of either RNA or DNA extracts were measured with a gas-flow counter.

Expt. No.	Inhibitor	Concentration (µg/ml)	^{32}P in DNA CPM	% Inhibition (DNA)	^{32}P in RNA CPM	% Inhibition (RNA)
P 21	Control	0	3,370	0	121,608	0
	Mitomycin C	10	603	82	102,663	16
		50	476	86	99,891	18
		200	333	90	123,831	2
	FUdR	500	2,273	33	102,348	16
	Hydroxyurea	760	603	82	112,545	7
P 11	Control	0	122	0	4,664	0
	Phenethyl	1,000	45	63	192	96
	Alcohol	4,000	30	76	256	95
		10,000	23	81	152	97
P 12	Control	0	155	0	4,811	0
	Adenine	50	89	43	5,473	0
		200	52	66	4,505	6

TABLE 3. Effects of Inhibitors of DNA Synthesis on RNA and DNA Syntheses

The experimental procedure was identical to that described in Table 2, except that treatments were for 2 hours.

Expt. No.	Inhibitor	Concentration (µg/ml)	^{32}P in DNA CPM	% Inhibition (DNA)	^{32}P in RNA CPM	% Inhibition (RNA)
P 31	Control	0	1,891	0	48,216	0
	FUdR	30	1,615	15	56,578	0
		300	1,080	43	38,480	20
		1000	1,080	43	52,644	0
		3000	531	72	33,558	30
	Nalidixic acid	3	564	70	36,562	24
		30	411	78	44,320	8
		300	249	87	42,708	11
	Hydroxyurea	76	827	56	46,716	3
		380	550	71	34,438	29
		760	360	81	32,862	32

TABLE 4. Effects of Inhibitors of DNA Synthesis on Protein Synthesis

Exponentially growing vegetative cells were treated with inhibitors, with ^{14}C-arginine (sp. 5-10 μCi/mM) added to a final concentration of 1 μCi/ml. Treatments were stopped by adding with an equal volume of 10% TCA. The amount of incorporation into the acid-insoluble fraction was determined by filter paper technique using a liquid scintillation counter.

Expt. No.	Inhibitor	Concentration (μg/ml)	C^{14}-arginine Incorporation CPM	%Inhibition
C-21	Control	0	970,437	0
	Phenethyl alcohol	1000 (0.1%)	378,130	61.0
		4000 (0.4%)	9,307	99.0
		10000 (1.0%)	9,156	99.1
	Nalidixic acid	10	961,859	0.9
		30	987,417	0
		100	967,801	0.3
	Mitomycin C	50	956,294	1.5
		100	851,180	12.2
		500	815,317	16.0
	Hydroxyurea	76 (1 mM)	981,620	0
		380 (5 mM)	996,501	0
		760 (10 mM)	927,155	4.5

TABLE 5. Survival of Zygospores Following Treatments with Various Concentrations of Phenethyl Alcohol at Different Times during Germination

The zygospores used here were obtained from the cross between arg-1 mt+ and arg-2 mt-.

	0.1%		0.4%		1.0%	
Hour treatment	Total Counted	% Germination	Total Counted	% Germination	Total Counted	% Germination
4-4½	238	88.23	567	65.96	294	15.3
4½-5	163	84.66	460	76.52	182	85.16
5-5½	190	86.31	368	86.41	373	0
5½-6	256	92.18	311	0	260	0
6-6½	238	87.81	276	85.5	284	0
6½-7	260	91.53	396	86.1	383	0
7-7½		88.21*	295	65.08	310	0
7½-8		88.21*	348	73.85	204	5.88
8-8½		88.21*	430	33.72	334	0
8½-9	297	88.21*	428	76.86	397	0
Control	406	92.50				

*Treatment from 7-9 hours.

TABLE 6. Survival of Zygospores Following ½ to 1 hour Treatments with Various Agents at Different Times during Germination.* Control germination was 92.49%.

Hour treatment	Acriflavine (10 µg/ml) % Germination	Caffeine (0.05%) % Germination	Mitomycin C (0.2 mg/ml) % Germination	Hydroxyurea (1 mM) % Germination	Hydroxyurea (5 mM) % Germination
5-5½	90.55	91.09	93.33	90.35	90.16
5½-6	92.14	91.74	92.62	90.39	89.74
6-6½	92.74	90.84	92.24	89.92	89.71
6½-7	92.43	92.02	92.10	90.79	90.25
7-7½	92.75	93.51	92.33	93.86	90.40
7½-8	93.37	90.86	90.54	90.88	89.32
8-9	92.05	91.78	91.91	91.81	84.58 (x^2=17.3)
9-10	91.69	88.36	90.11	90.63	90.96
10-11	92.40	91.47	91.15	90.14	89.82

*Number of zygospores examined per sample ranged from 215 to 467 with an average of 315.23.

TABLE 6. Continued.

Hour treatment	FUdR (2 mM) % Germination	Nalidixic Acid (20 µg/ml) % Germination	Adenine (500 µg/ml) % Germination	Hydroxyurea (2 mM) % Germination
4-4½	89.7	91.9	85.9	90.0
4½-5	92.4	90.8	86.3	89.5
5-5½	88.5	92.0	88.4	92.0
5½-6	92.2	92.9	90.4	92.3
6-6½	92.1	91.5	90.5	90.5
6½-7	92.3	91.4	91.6	90.7
7-7½	92.5	92.1	95.2	88.5
7½-8	89.7	89.3	86.3	91.5
8-8½	89.5	89.8	90.2	85.4
8½-9	90.1	90.0	91.1	92.3

TABLE 7. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Control		Actinomycin D (10 µg/ml)		Mitomycin C (200 µg/ml)		FUdR (1 mM)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
3½-4	984	25.2	1312	24.5	532	25.5	1022	24.0
4-4½	561	24.1	869	25.9	1064	25.2	991	26.1
4½-5	714	23.8	803	30.3 (x^2=8.3)**	865	27.5 (x^2=3.4)	646	25.9
5-5½	696	25.0	1156	25.1	1001	30.9 (x^2=11.0)***	946	25.5
5½-6	716	26.7	1213	27.9 (x^2=0.3)	823	23.8 (x^2=1.7)	1025	16.0 (x^2=29.6)***
6-6½	656	24.0	1100	21.3 (x^2=1.4)	1341	25.8 (x^2=0.98)	1881	24.3 (x^2=0.1)
6½-7	913	27.3	1401	27.4	1274	24.1	1374	24.9 (x^2=0.1)
7-7½	925	25.4	1079	25.2	1170	25.0	1248	24.5
7½-8	1129	25.4	784	25.9	1697	30.6 (x^2=8.8)**	1051	30.7 (x^2=7.1)**
8-8½	952	25.9	1813	25.0	860	27.2 (x^2=0.9)	2193	27.5 (x^2=0.7)
8½-9	1242	27.1	1285	23.3 (x^2=4.6)*	1336	24.6 (x^2=2.1)	1288	25.0 (x^2=1.5)
9-9½	834	25.6	952	24.6 (x^2=0.5)	1508	25.6	1298	24.1 (x^2=0.6)

* - $\rho < .05$; ** $\rho < .01$; *** $\rho < .001$

TABLE 8. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Control		Actinomycin D (10 µg/ml)		Cycloheximide (30 µg/ml)		Mitomycin C (100 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
1-1½	611	24.4	903	24.6	1594	19.8	---	---
1½-2	674	24.6	1687	17.8	1912	15.3	---	---
2-2½	808	24.9	1421	23.4	1742	14.0	---	---
2½-3	1161	24.7	1658	23.7	1138	16.3	---	---
3-3½	1270	24.9	1285	20.9	1063	13.2	1015	24.9
3½-4	761	25.0	527	24.8	1346	16.1	1374	24.6
4-4½	1621	24.9	1509	18.7 (x^2=17.4)***	1040	14.7	1165	24.5
4½-5	1578	25.1	1504	29.6 (x^2=8.0)**	1104	18.9	1129	25.3
5-5½	1288	24.5	1241	25.0	986	13.5	1034	32.1 (x^2=16.0)***
5½-6	1174	25.4	1517	24.5	1258	19.9	1228	25.4
6-6½	1691	24.5	1573	24.6	1770	20.5	1433	25.0
6½-7	1339	25.0	763	22.5	1323	21.7	855	25.4
7-7½	1631	24.7	1928	29.3 (x^2=10.0)**	1756	19.6	925	29.0 (x^2=5.6)*
7½-8	1286	23.9	1406	23.8	1716	19.1	1145	24.7
8-8½	1291	24.7	1482	24.1	1281	17.9	938	25.0

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 8. Continued.

Hour treatment	FUdR (1 mM) Total Colonies Counted	FUdR (1 mM) % Recombinant Tetrads	Nalidixic Acid (30 µg/ml) Total Colonies Counted	Nalidixic Acid (30 µg/ml) % Recombinant Tetrads
1-1½	---	---	---	---
1½-2	---	---	---	---
2-2½	---	---	---	---
2½-3	---	---	---	---
3-3½	1381	25.0	1170	24.8
3½-4	1120	25.4	1304	24.3
4-4½	1412	24.6	868	22.1 (x^2=2.4)
4½-5	1022	25.8	1609	24.0
5-5½	1221	24.4	1108	20.1 (x^2=6.6)**
5½-6	1090	20.5 (x^2=8.0)**	1352	19.8 (x^2=11.5)**
6-6½	1469	25.5	1889	24.1
6½-7	506	25.3	1041	24.2
7-7½	1725	28.9 (x^2=8.2)**	1639	32.9 (x^2=25.9)***
7½-8	1068	25.6	1121	23.8
8-8½	1048	25.[illegible]	1889	25.3

TABLE 9. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Control		Phenethyl Alcohol (0.4%)		FUdR (2 mM)		Adenine (500 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
4½-5	1115	14.17	497	10.07 (x^2=5.2)*	---	---	---	---
5-5½	591	14.21	1792	10.77 (x^2=5.1)*	---	---	---	---
5½-6	---	---	---	---	---	---	---	---
6-6½	1274	1428	1010	16.73	1008	14.19	1543	11.35 (x^2=5.3)*
6½-7	---	---	1248	14.50	1488	11.35 (x^2=5.3)*	1963	9.86 (x^2=14.6)***
7-7½	1304	14.42	1085	10.03	14.98	13.75	2328	12.35 (x^2=3.1)
7½-8			1474	10.79 (x^2=8.3)**	1400	14.21	1707	9.02 (x^2=21.4)***
8-8½	1068	14.51	317	8.83 (x^2=6.9)**	1157	18.06 (x^2=5.1)*	1019	9.81 (x^2=10.7)***
8½-9	2692	14.56	1174	13.97	1193	14.41	1962	13.60

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 10. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Control		Nalidixic Acid (30 µg/ml)		Hydroxyurea (1 mM)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
4-4½	2173	17.02	1796	16.84	---	---
4½-5	1455	16.00	593	16.35	953	16.20
5-5½	1415	16.74	712	16.53	1467	16.92
5½-6	807	16.72	621	15.31	1753	14.77
6-6½	660	16.81	1444	11.37 (x^2=19.7)***	554	14.80 (x^2=1.2)
6½-7	690	16.66	1136	16.55	1290	17.55
7-7¼	746	16.48	1692	16.92	2695	16.71
7¼-7½	520	16.73	1602	22.04 (x^2=7.6)**	1548	12.89 (x^2=4.9)*
7½-7¾	---	---	1616	16.87	1461	16.07
7¾-8			2055	16.52	2381	16.63
8-8¼			1610	13.35 (x^2=3.6)	2015	16.37
8¼-8½			1416	14.20 (x^2=1.9)	1468	16.75

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 10. Continued.

Hour treatment	Hydroxyurea (5 mM) Total Colonies Counted	Hydroxyurea (5 mM) % Recombinant Tetrads	Phenethyl Alcohol (0.4%) Total Colonies Counted	Phenethyl Alcohol (0.4%) % Recombinant Tetrads
4-4½	---	---	---	---
4½-5	759	16.04	---	---
5-5½	1150	16.44	---	---
5½-6	1378	14.97	1587	20.03 (x^2=3.8)
6-6½	1229	14.00	1452 (x^2=4.1)*	17.76
6½-7	1472	16.18	1672	15.55
7-7¼	1740	16.87	1336	14.29
7¼-7½	1066	11.16 (x^2=9.5)**	---	---
7½-7¾	1528	16.21	1134	13.22 (x^2=3.5)
7¾-8	--	--	2001	12.56 (x^2=7.6)**
8-8¼	2193	16.73	950	11.05 (x^2=9.5)**
8¼-8½	---	---	---	---

TABLE 11. Recombination between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Control		Actinomycin D (100 µg/ml)		Cycloheximide (100 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
3-3½	2158	19.41	1324	16.69 (x^2=4.0)*	1221	8.01 (x^2=78.06)***
3½-4	---	---	1666	13.01 (x^2=27.7)***	---	---
4-4½	---	---	1318	13.80 (x^2=10.2)	---	---
4½-5	---	---	1424	15.99 (x^2=6.2)*	---	---
5-5½	1485	19.73	1475	20.33	---	---
5½-6	2619	20.44	2440	26.16 (x^2=20.1)***	---	---
6-6½	1479	19.55	802	14.03 (x^2=11.1)**	630	8.47 (x^2=40.2)***
6½-7	1996	19.82	588	7.82 (x^2=46.2)***	1792	11.37 (x^2=45.6)***
7-7½	1314	19.75	1260	16.13 (x^2=5.8)*	1266	13.24 (x^2=19.5)***
7½-8	553	20.25	1490	11.86 (x^2= 10.2)**	1519	8.91 (x^2=51.2)***
8-8½	432	19.67	1974	20.53	---	---
8½-9	723	21.00	845	17.97	618	10.43 (x^2=24.9)***

TABLE 11. Continued.

Hour treatment	Nalidixic Acid (30 µg/ml) Total Colonies Counted	Nalidixic Acid (30 µg/ml) % Recombinant Tetrads	Hydorxyurea (3 mM) Total Colonies Counted	Hydorxyurea (3 mM) % Recombinant Tetrads
3-3½	---	---	---	---
3½-4	---	---	---	---
4-4½	1164	20.29	1365	19.46
4½-5	1411	19.89	1828	19.56
5-5½	992	20.41	877	19.57
5½-6	1049	21.15	1516	19.83
6-6½	1313	14.23 (x^2=14.6)***	1154	14.44 (x^2=12.0)**
6½-7	991	13.72 (x^2=15.7)***	2458	10.41 (x^2=78.3)***
7-7½	1409	19.24	1696	19.77
7½-8	1138	19.79	1588	20.17
8-8½	1686	23.13	1071	14.91 (x^2=5.5)*
8½-9	777	20.20	601	19.41

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 12. Effect of Actinomycin D on RNA and DNA Synthesis

The experimental procedure was identical to that of Table 2.

Expt. No.	Concentration of Actinomycin D (µg/ml)	^{32}P in DNA	% Inhibition (DNA)	^{32}P in RNA	% Inhibition (RNA)
P-22	0	2,908	0	143,883	0
	4	2,890	6	102,600	29
	20	2,585	11	91,494	36
	50	1,423	52	48,294	67

TABLE 15. Effect of Cycloheximide on RNA and DNA Syntheses

The experimental procedure was identical to that of Table 2.

Expt. No.	Concentration of Cycloheximide	^{32}P in DNA	% Inhibition (DNA)	^{32}P in RNA	% Inhibition (RNA)
P 11	0 (µg/ml)	122	0	4,664	0
	10	129	0	4,417	5
	30	109	11	4,813	0
	50	66	46	4,480	4
P 21	0	3,370	0	13,512	0
	100	1,811	46	11,320	16

TABLE 13. Survival of Zygospores Following ½ to 1 hour Treatments with Actinomycin D and Various Concentrations of Cycloheximide at Different Times during Germination.

Hour treatment	Cycloheximide 10 µg/ml Total Counted	% Germination	Cycloheximide 30 µg/ml Total Counted	% Germination	Cycloheximide 100 µg/ml Total Counted	% Germination	Actinomycin D 100 µg/ml Total Counted	% Germination
Control	958	82.46						
3-3½	---	---	---	---	407	79.85	---	---
3½-4	---	---	---	---	608	80.42	---	---
4-4½	501	80.20	523	83.94	387	80.36	556	81.47
4½-5	412	83.01	450	81.56	848	82.85	528	82.95
5-5½	617	82.49	385	82.60	403	79.90 (x^2=1.2)	462	82.25
5½-6	403	80.40	418	81.56	365	74.79 (x^2=9.8)**	351	80.34
6-6½	520	82.90	393	81.89	605	72.39 (x^2=22.3)***	489	82.23
6½-7	414	80.43	435	83.45	427	74.74 (x^2=11.1)**	631	83.36
7-7½	258	81.01	435	82.09	393	54.09 (x^2=84.6)***	403	81.88
7½-8	476	81.72	519	81.31	455	77.36 (x^2=5.1)*	475	82.52
8-8½	444	81.53	495	80.60	473	68.49 (x^2=35.8)***	323	81.73

TABLE 13. Continued.

Hour treatment	Cycloheximide 10 µg/ml Total Counted	% Germination	30 µg/ml Total Counted	% Germination	100 µg/ml Total Counted	% Germination	Actinomycin D 100 µg/ml Total Counted	% Germination
8½-9	586	80.54	520	80.38	466	62.01 (x^2=71.3)***	412	81.79
9-9½	---	---	---	---	620	60.64 (x^2=92.8)***	---	---
9½-10	---	---	---	---	443	70.42 (x^2=26.1)***	---	---
10-11	---	---	---	---	439	69.93 (x^2=28.0)***		

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 14. Effects of Various Agents on Protein Synthesis

The experimental procedure was identical to that described in Table 4, except that treatments were 2 hours and vegetative cells were labelled with ^{14}C-alanine instead of arginine. The radioactivities were measured with a gas-flow counter.

Expt. No.	Inhibitor	Concentration (µg/ml)	^{14}C-Alanine Incorporation CPM	%Inhibition
C-11	Control	0	835	0
	Cycloheximide	0.1	476	43.0
		0.5	354	57.6
		1.0	386	53.8
		3.0	416	50.2
		5.0	344	58.8
		10.0	335	59.9
		30.0	259	69.0
		50.0	213	74.5
		100.0	239	71.4
	Actinomycin D	100.0	522	37.5
	FUdR	300.0	647	22.5

TABLE 16. Recombination Between arg-1 and arg-2 after Treatment with Actinomycin D and Various Concentrations of Cycloheximide at Different Times during Zygospore Germination.

Hour treatment	Control		Cycloheximide (2 µg/ml)		Cycloheximide (10 µg/ml)		Cycloheximide (50 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
2½-3	807	24.4	422	15.4 (x^2=13.7)***	563	17.4 (x^2=9.4)**	623	17.01
3-3½	793	25.9	244	18.9 (x^2=5.5)*	483	16.3 (x^2=17.7)***	545	16.1 (x^2=19.24)***
3½-4	613	24.9	547	19.6 (x^2=5.2)*	891	19.2	527	20.1
4-4½	466	25.6	364	22.0	410	18.6 (x^2=6.17)*	---	---
4½-5	953	26.7	636	28.0	601	27.2	---	---
5-5½	595	24.4	388	19.4 (x^2=3.2)	415	20.2	531	20.2
5½-6	251	26.8	368	18.9 (x^2=5.5)*	392	15.6 (x^2=11.9)***	287	15.0 (x^2=12.0)***
6-6½	605	25.3	---	---	115	19.1 (x^2=2.0)	107	16.8 (x^2=3.6)
6½-7	315	24.4	---	---	103	20.4	---	---
7-7½	156	25.6	---	---	176	27.8	---	---
7½-8	403	24.8	---	---	166	25.9	---	---
8-8½	501	25.2	---	---	186	19.4	---	---
8½-9	153	25.5	---	---	---	---	239	20.9

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 17. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination.

Hour treatment	Control		Cycloheximide (20 µg/ml)		Actinomycin D (10 µg/ml)		Mitomycin C (200 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
3½-3¼	885	24.5	1033	18.5 (x^2=10.3)**	1305	20.7 (x^2=4.5)*	---	---
3¼-4	1305	23.7	669	17.7 (x^2=9.2)**	---	---	---	---
4-4¼	750	22.2	1006	17.0 (x^2=7.6)**	802	22.6	514	22.8
4¼-4½	894	20.3	586	16.9 (x^2=2.6)	774	21.9 (x^2=0.6)	938	22.4 (x^2=1.2)
4½-4¾	775	24.5	1336	24.8	948	26.0 (x^2=0.5)	828	23.9 (x^2=0.01)
4¾-5	808	24.5	1270	26.2	1057	30.7 (x^2=8.4)**	1174	25.4 (x^2=0.2)
5-5¼	893	25.0	1175	26.8	690	25.8	1364	32.2 (x^2=14.0)***
5¼-5½	748	25.0	1047	24.8	776	25.6	1040	25.9
5½-5¾	1037	25.9	951	25.3	1445	24.9	925	25.2
5¾-6	845	24.8	1119	23.0	1018	25.2	846	26.2
6-6½	997	25.2	833	15.0 (x^2=29.2)***	1242	24.8	1140	25.8
6½-7	379	25.1	1219	17.5 (x^2=10.7)**	730	23.6	627	26.0

TABLE 17. Continued.

Hour treatment	Control		Cycloheximide (20 µg/ml)		Actinomycin D (10 µg/ml)		Mitomycin C (200 µg/ml)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
7-7½	964	25.3	1014	15.1 (x^2=32.1)***	1170	25.0	1364	25.8
7½-8	1011	25.9	726	17.0	1076	25.8	1039	25.2
8-8½	867	25.8	1012	17.0	825	25.8	1061	30.4 (x^2=5.0)*
8½-9	1032	25.5	817	13.0 (x^2=44.0)***	926	26.8	1349	26.1

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 18. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Acriflavin (12 µg/ml) Total Colonies Counted	Acriflavin (12 µg/ml) % Recombinant Tetrads	Caffeine (0.05%) Total Colonies Counted	Caffeine (0.05%) % Recombinant Tetrads	FUdR (2 mM) Total Colonies Counted	FUdR (2 mM) % Recombinant Tetrads	Hydroxyurea (1 mM) Total Colonies Counted	Hydroxyurea (1 mM) % Recombinant Tetrads
4½-5	1934	14.53 (x^2=1.2)	---	---	---	---	---	---
5-5½	1024	13.76	1546	12.35 (x^2=9.7)**	1049	13.53 (x^2=2.5)	769	13.78 (x^2=1.5)
5½-6	807	18.33 (x^2=3.6)	1299	12.31 (x^2=7.9)**	920	14.23	663	15.98
6-6½	1388	17.86 (x^2=3.8)	1092	13.73 (x^2=2.1)	862	13.80 (x^2=1.7)	846	14.06
6½-7	990	21.91 (x^2=21.9)***	1633	12.48 (x^2=8.4)**	1366	10.83 (x^2=17.9)***	1397	12.74 (x^2=6.3)*
7-7½	1438	14.12 (x^2=1.7)	2343	12.41 (x^2=11.2)***	1100	14.45	1403	13.75 (x^2=2.6)
7½-8	786	12.72 (x^2=4.0)*	486	15.02	1060	16.79	1941	12.87 (x^2=7.2)**
8-8½	1242	10.70 (x^2=17.6)***	1219	11.48 (x^2=12.1)***	1165	19.39 (x^2=9.2)**	1388	11.52 (x^2=13.1)***
8½-9	1528	12.17 (x^2=9.8)**	1010	12.57 (x^2=5.5)*	981	13.36	956	12.02 (x^2=7.4)**
Control	3397	15.57						

*p <0.05; **p <0.01; ***p <0.001

TABLE 19. Recombination Between arg-1 and arg-2 after Treatment with Various Agents at Different Times during Zygospore Germination. Contingency x^2 values have 1 degree of freedom.

Hour treatment	Mitomycin C		Mitomycin C Plus FUdR		Hydroxyurea (1 mM)	
	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads	Total Colonies Counted	% Recombinant Tetrads
5-5½	1391	20.73	1707	19.93	1295	20.23
5½-6	1498	23.29 (x^2=2.4)	1745	21.43	1302	20.35
6-6½	1323	27.13 (x^2=17.0)***	2024	20.20	1406	17.32 (x^2=8.8)
6½-7	1361	21.63	1373	19.88	1562	16.38 (x^2=14.4)***
7-7½	1090	22.56	1712	19.33	1395	20.78
7½-8	639	23.31	1143	23.62	1377	20.55
8-9	1498	24.09 (x^2=4.5)*	1415	28.26 (x^2=24.9)***	1916	18.31 (x^2=5.7)*
9-10	1234	23.09	1471	24.13	1321	18.62
10-11	1164	21.73	473	22.62	1104	22.37
Control	2536	21.05				

*ρ <.05; **ρ <.01; ***ρ <.001

TABLE 19. Continued.

Hour treatment	Hydroxyurea (5 mM) Total Colonies Counted	Hydroxyurea (5 mM) % Recombinant Tetrads	Acriflavin (10 µg/ml) Total Colonies Counted	Acriflavin (10 µg/ml) % Recombinant Tetrads	Caffeine (0.05%) Total Colonies Counted	Caffeine (0.05%) % Recombinant Tetrads
5-5½	1294	19.31	1174	20.10 (x^2=1.8)	1520	22.43
5½-6	1555	19.93	1794	22.24	1099	24.38 (x^2=4.4)*
6-6½	1092	20.69	1140	21.92	1702	21.85
6½-7	1868	16.27 (x^2=48.9)***	1861	17.08 (x^2=11.6)***	1384	17.26 (x^2=8.7)**
7-7½	1712	19.35	1768	15.95 (x^2=18.7)***	1458	15.22 (x^2=22.5)***
7½-8	2143	16.09 (x^2=22.4)***	1267	22.73	1043	24.23 (x^2=3.9)*
8-9	2068	17.11 (x^2=12.2)***	1982	15.03 (x^2=30.3)***	1787	16.06 (x^2=20.8)***
9-10	1402	20.39	978	18.30 (x^2=3.6)	1400	20.00
10-11	1199	21.76	1139	21.77	895	19.21
Control	2536	21.05				

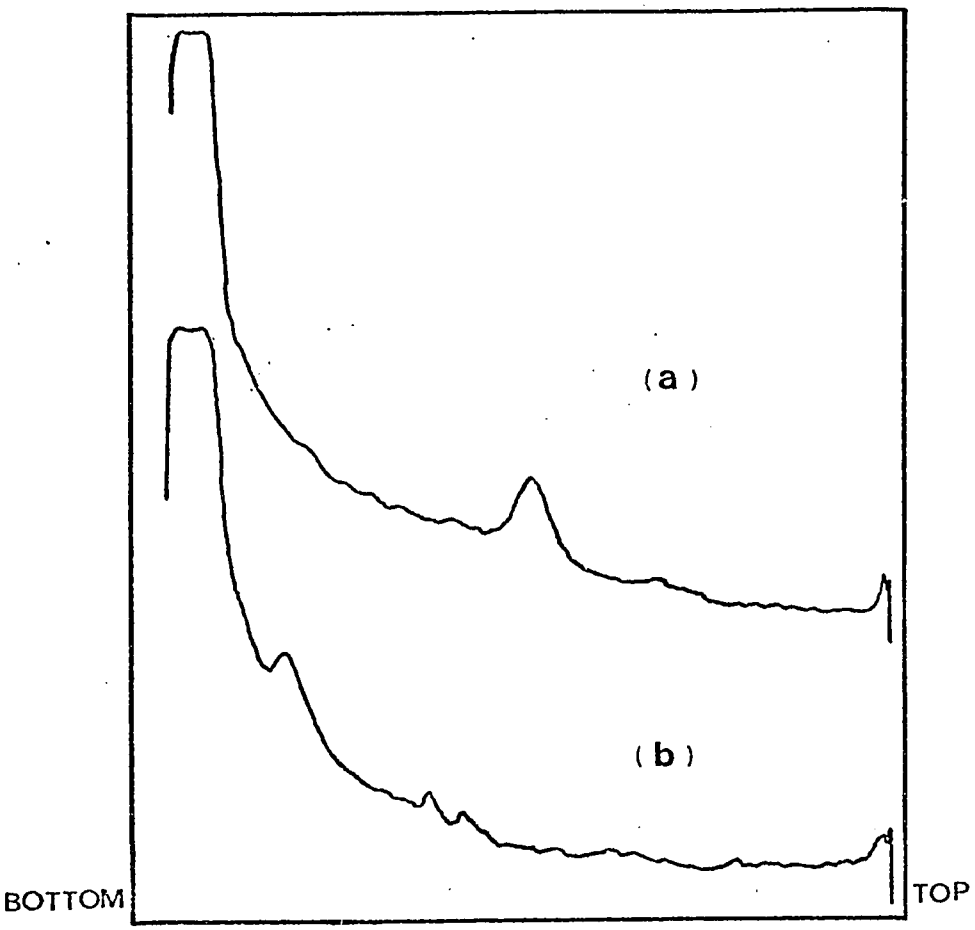


Fig. 1 Microdensitometer tracings of ultraviolet absorption photographs of DNA prepared from germinating zygospores grown in ^{15}N medium. The photographs were taken after 48 hours of CsCl density-gradient centrifugation at 44000 rev/min at 25°C in a Beckman model E analytical ultracentrifuge. A Joyce-Loebl double-beam recording microdensitometer was used for tracing the photographs. (a) centrifuged in high density and (b) centrifuged in low density of CsCl gradient.

FIGURE 2. Incorporation of ^{32}P into (a) DNA and (b) RNA during ½-hour pulses at different times during the germination of wild type zygospores. Zygospores were grown in Minimal medium.

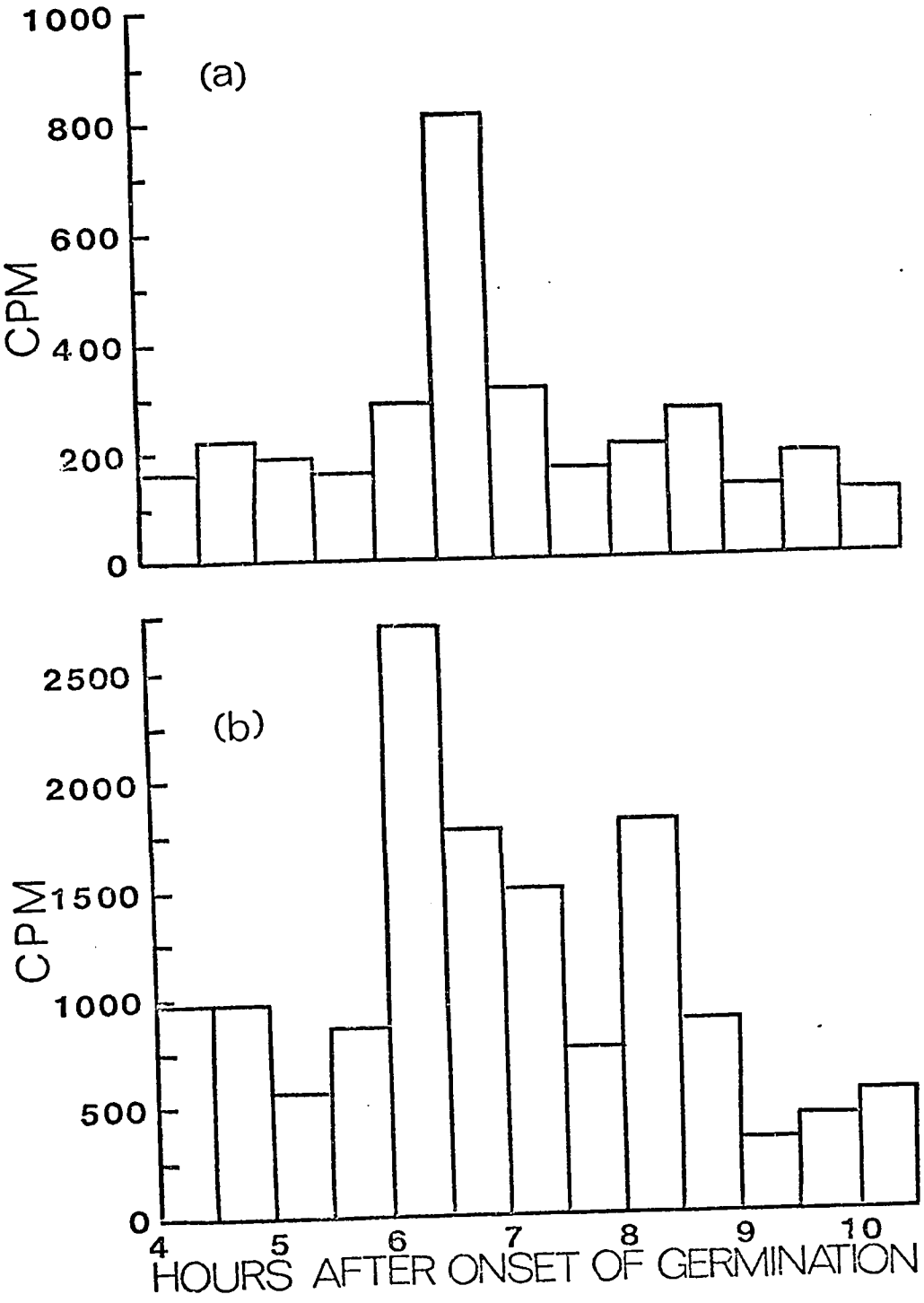

1000
800
600
400
200
0
CPM
(a)
2500
2000
1500
1000
500
0
CPM
(b)
4
5
6
7
8
9
10
HOURS AFTER ONSET OF GERMINATION

FIGURE 3. Incorporation of ^{32}P into (a) DNA and (b) RNA during half hour pulses at different times during the germination of wild type zygospores. Zygospores were grown in TAP medium.

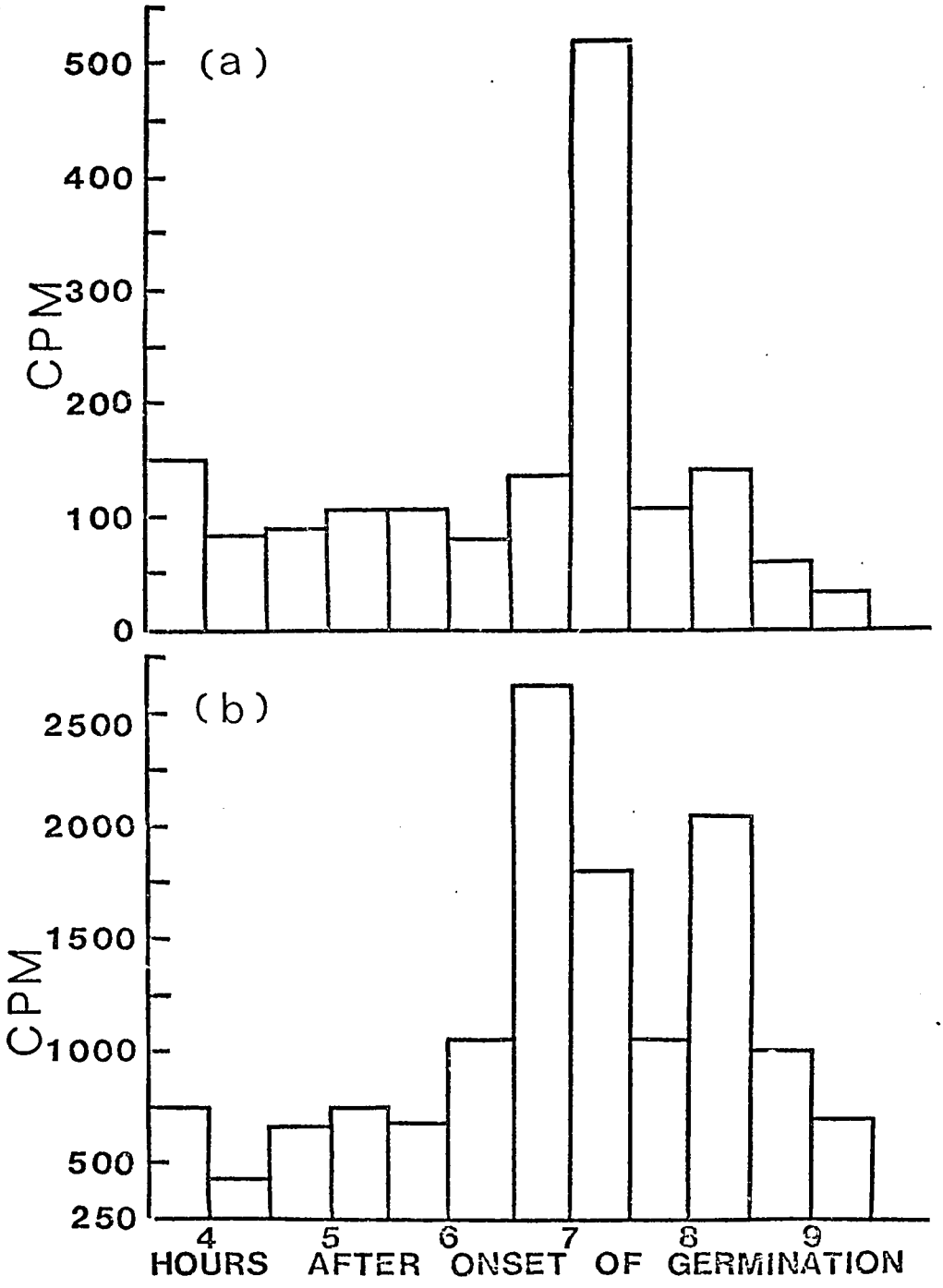

(a)
500
400
300
200
100
0
CPM
(b)
2500
2000
1500
1000
500
250
CPM
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 4. Incorporation of ^{32}P into (a) DNA and (b) RNA during half hour pulses at different times during the germination of zygospores. Zygospores were obtained from the cross between arg-1 and arg-2 and grown in TAP medium.

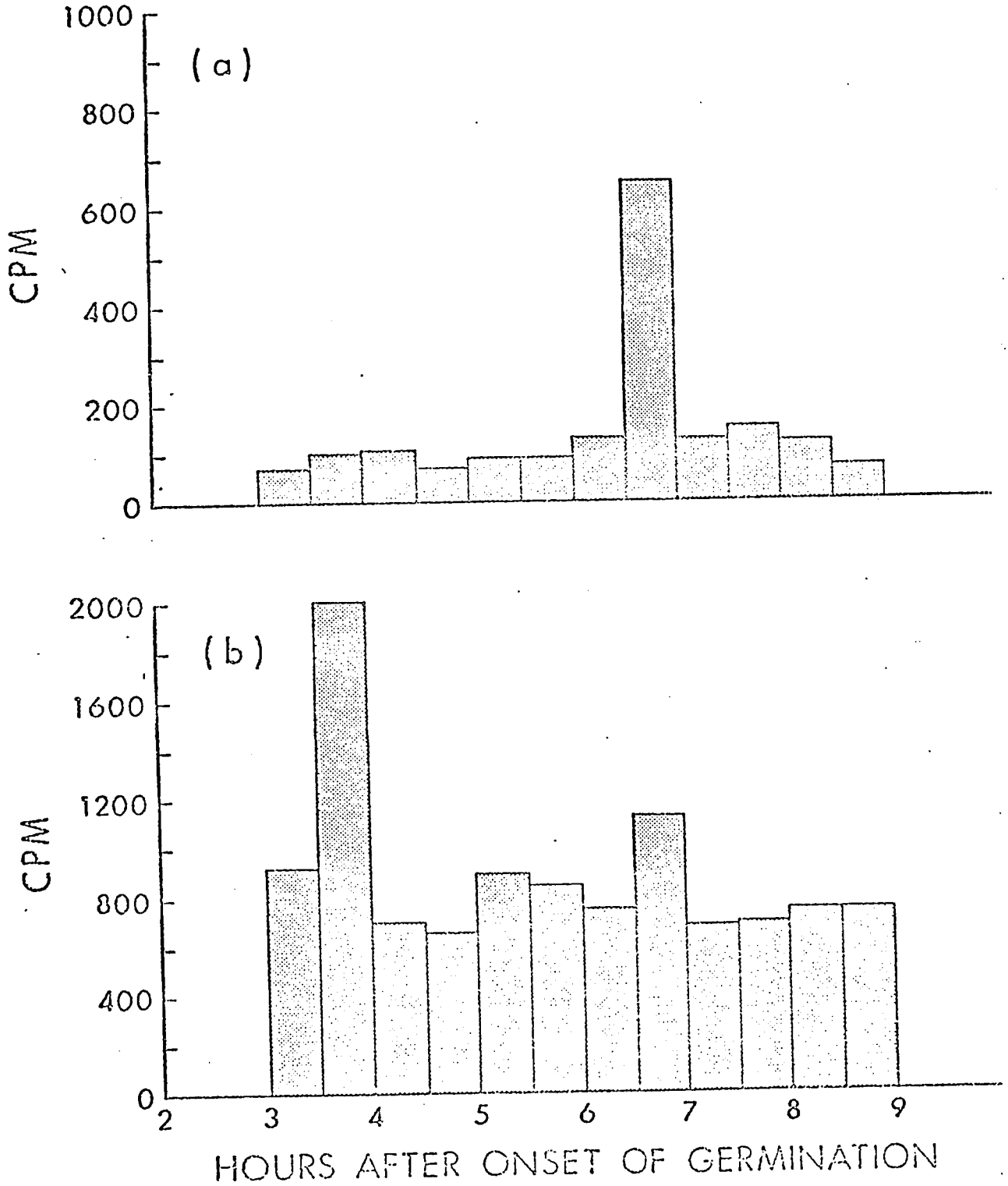

(a)
1000
800
600
400
200
0
CPM
(b)
2000
1600
1200
800
400
0
CPM
2
3
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 5. Continuous incorporation of ^{32}P into (a) DNA and (b) RNA during the germination of zygospores. Zygospores were obtained from the cross between arg-1 and arg-2 and grown in TAP medium. ^{32}P-phosphate was added 3 hours after the onset of germination.

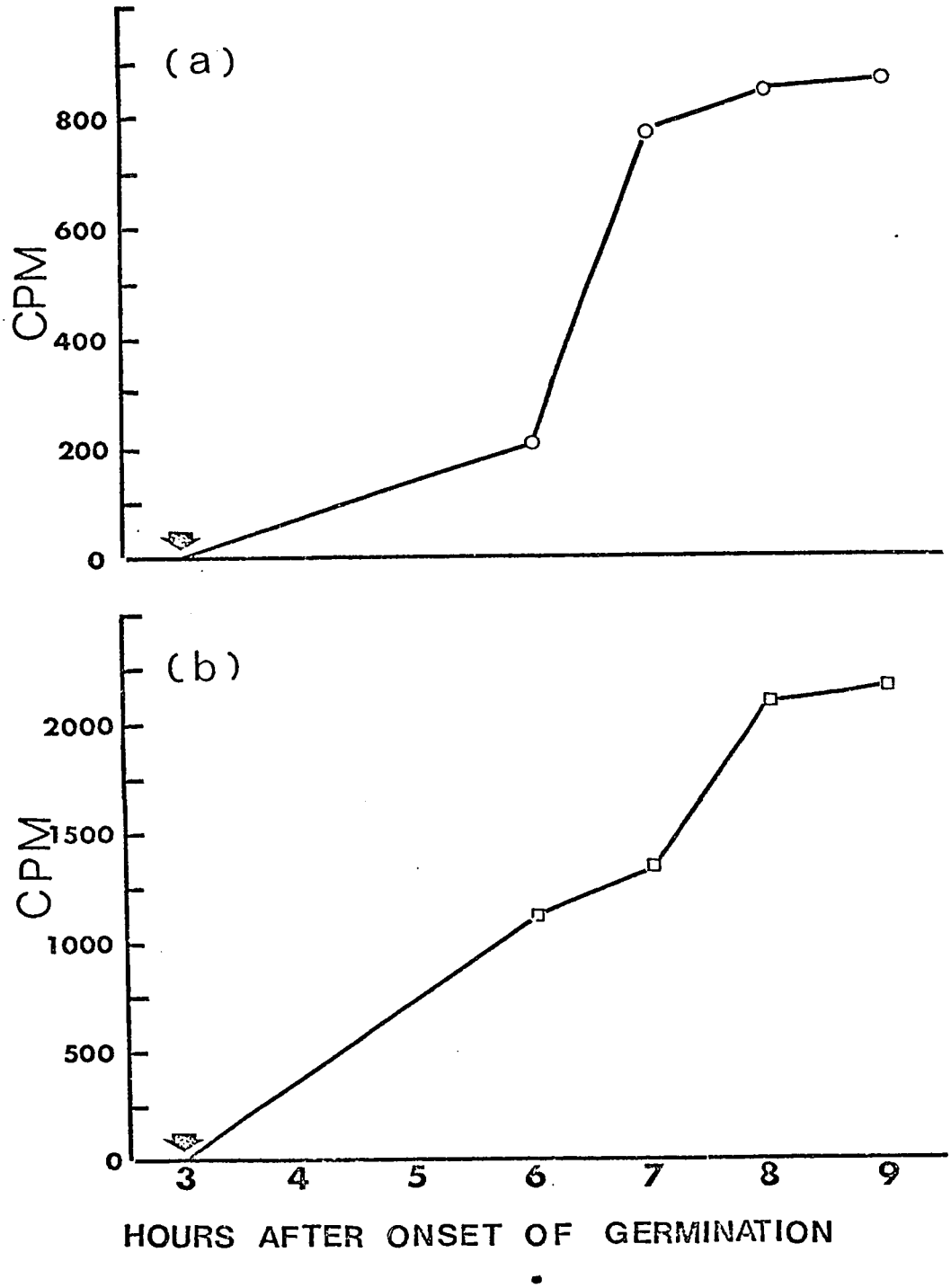

(a)
800
600
400
200
0
CPM
(b)
2000
1500
1000
500
0
CPM
3
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 6. Time-course study of the effects of phenethyl alcohol, mitomycin C and nalidixic acid on DNA synthesis. Exponentially growing wild type vegetative cells were treated with inhibitors and 10 uCi/ml of ^{32}P-phosphate. Samples were collected at different times after treatments and subjected to differential RNA and DNA extraction. Radioactivities in DNA fractions were measured with a gas-flow counter.

■, control; ○, 50ug/ml of mitomycin C;

▲, 10 ug/ml of nalidixic acid;

●, 0.4% phenethyl alcohol.

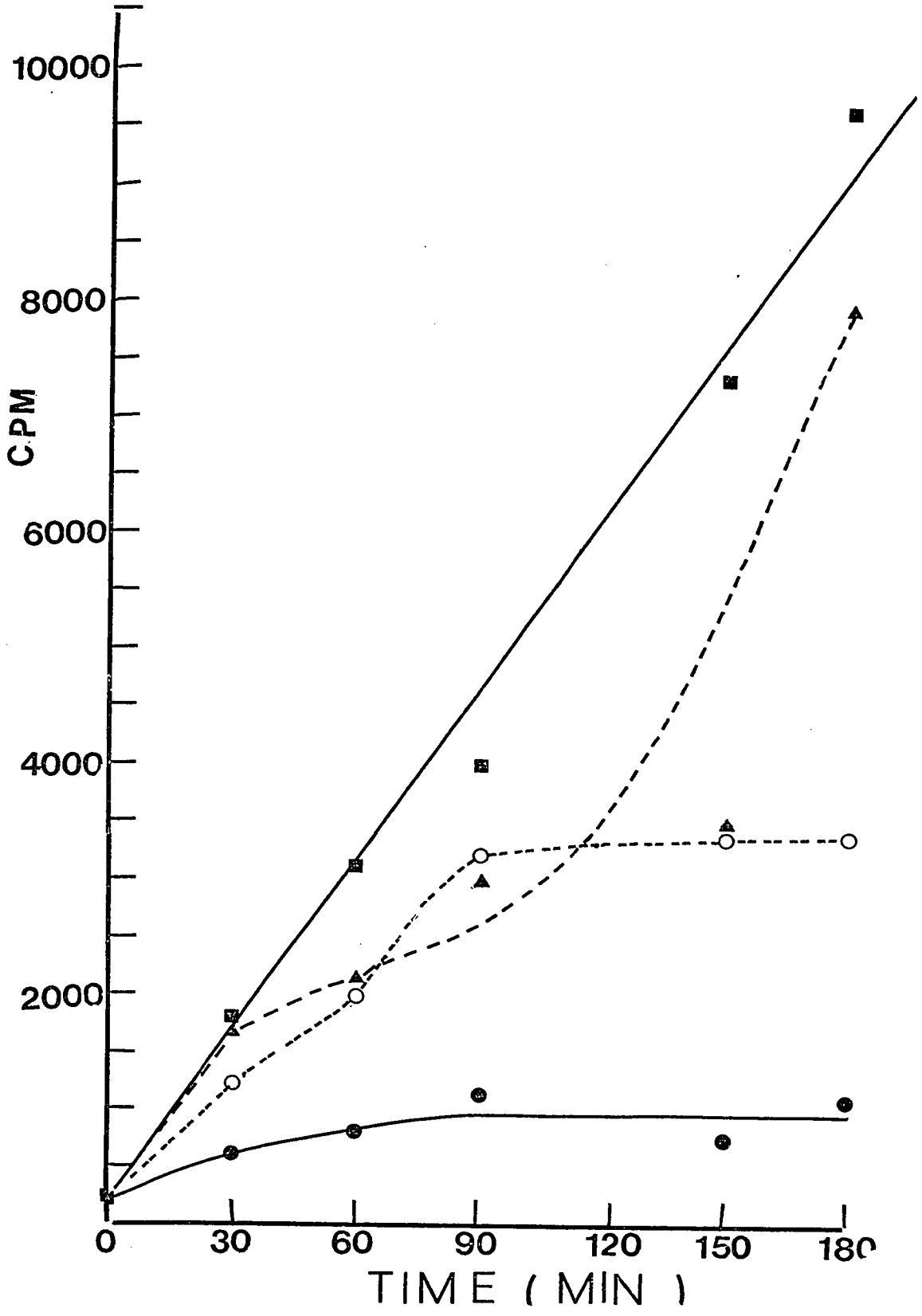
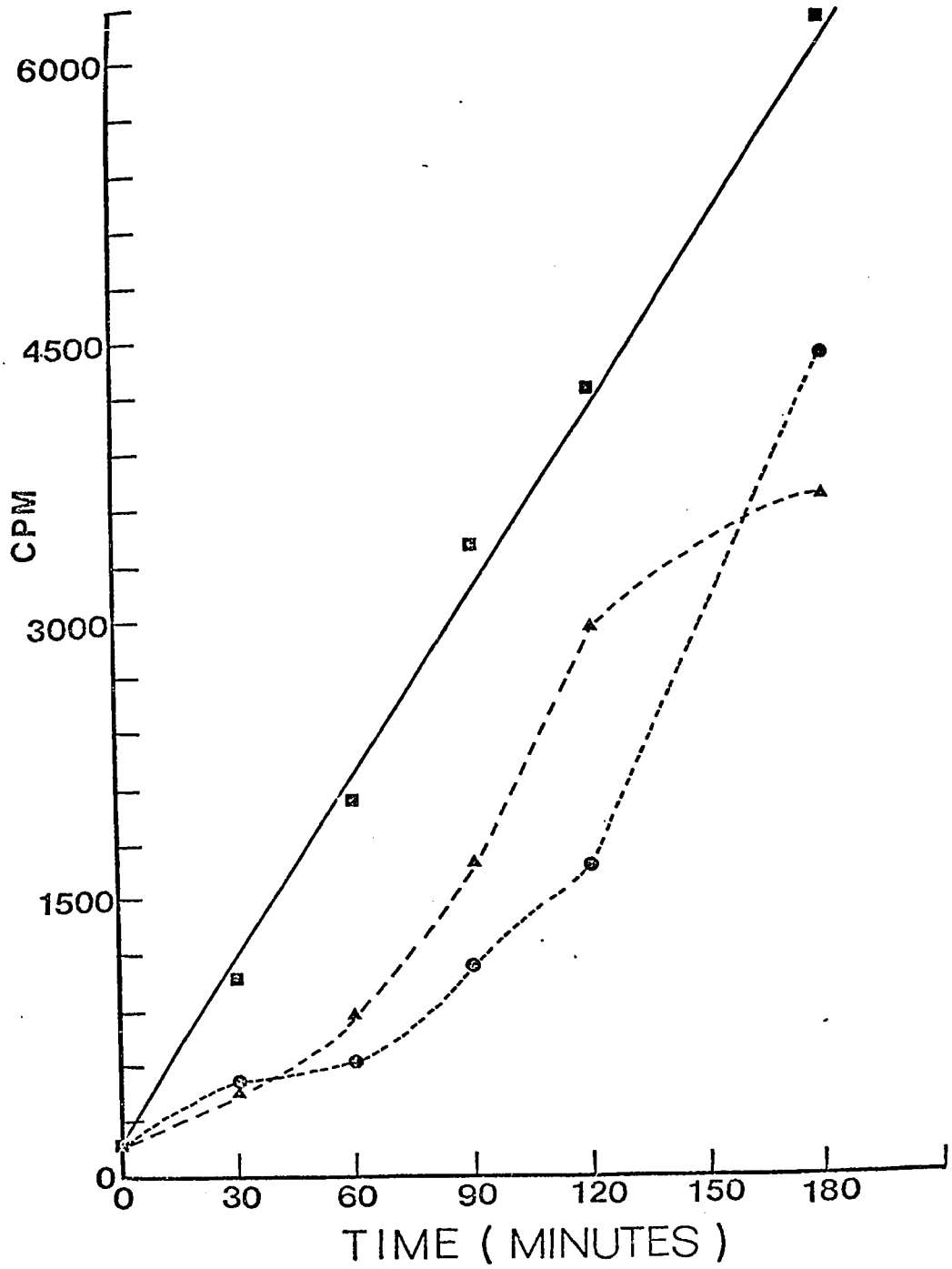

10000
8000
6000
4000
2000
CPM
0
30
60
90
120
150
180
TIME (MIN)

FIGURE 7. Time-course study of the effects of FUdR and hydroxyurea on DNA synthesis. The experimental procedure was as described in Fig. 6.
■, control ; ●, 1 mM FUdR:
▲, 1 mM hydroxyurea.


6000
4500
3000
1500
0
CPM
0
30
60
90
120
150
180
TIME (MINUTES)

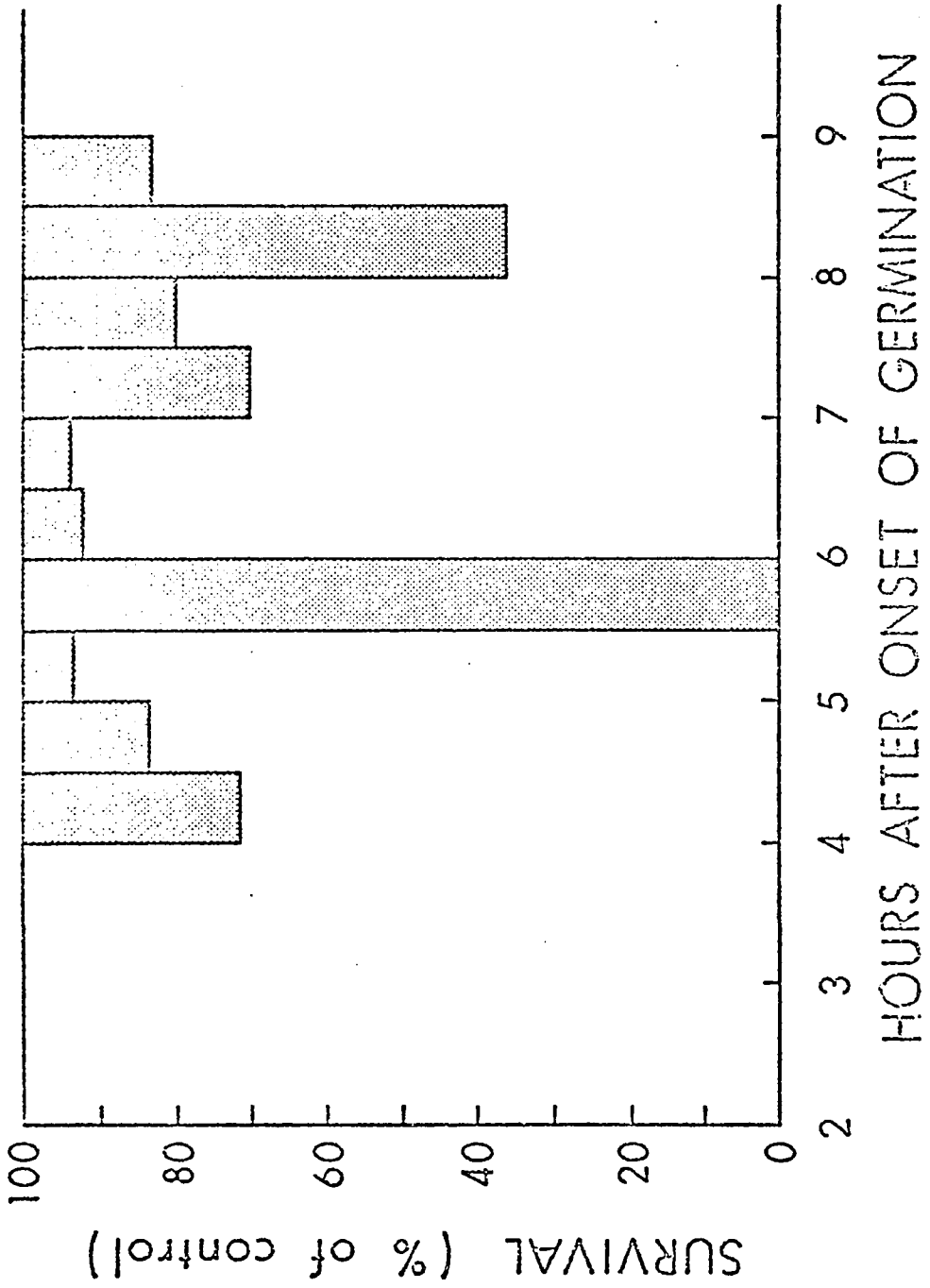


FIGURE 8. Survival of zygospores as a percentage of the control value after 30 minute-treatment with 0.4% phenethyl alcohol at different times during zygospore germination.

FIGURE 9. Recombination between arg-1 and arg-2 as a percentage of the control value following 4-hour treatments with various agents at different times during zygospore germination. (a) 10 ug/ml actinomycin D (b) 200 ug/ml mitomycin C and (c) 1 mM FUdR. These results are from the same experiment.

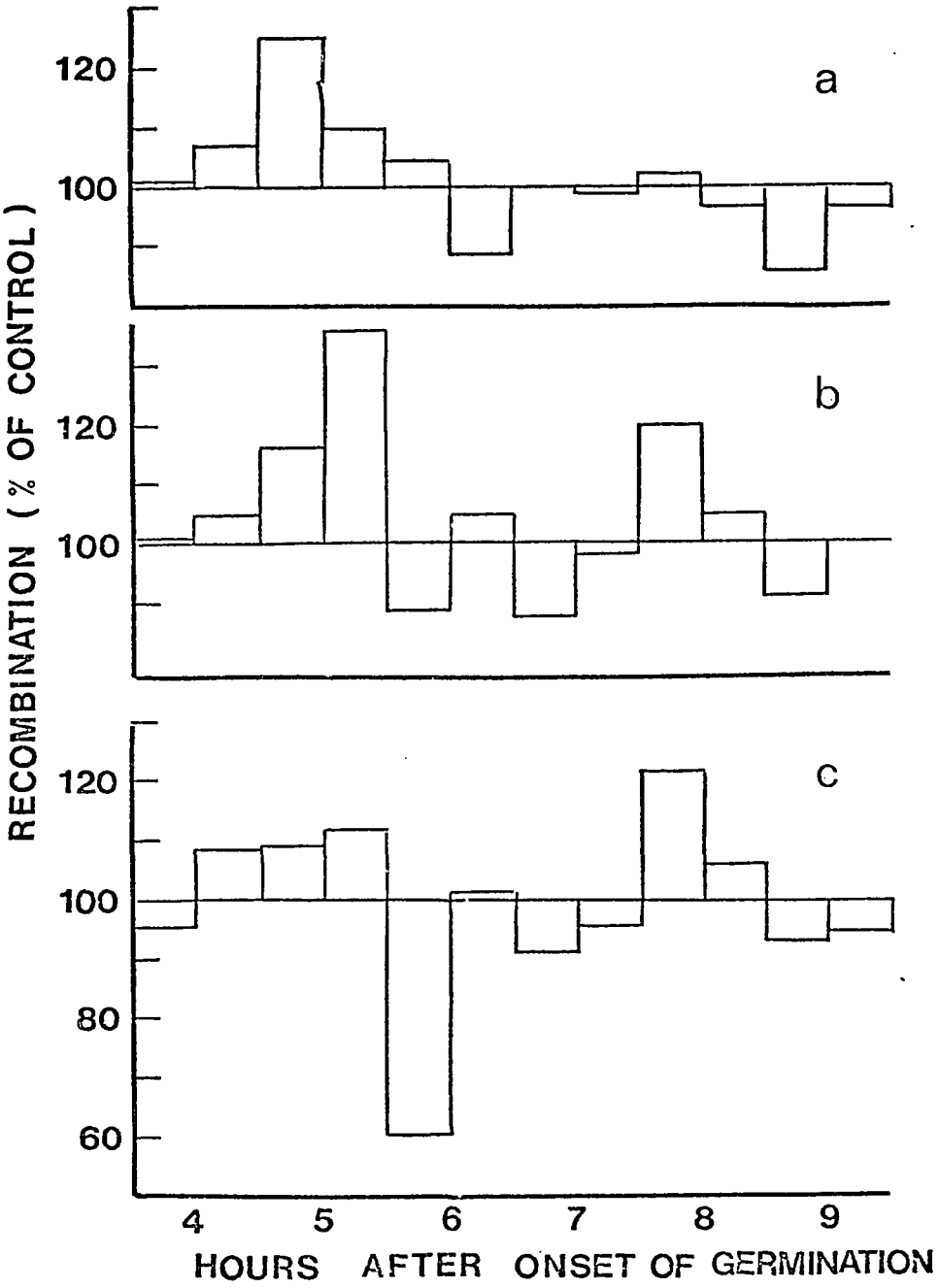

RECOMBINATION (% OF CONTROL)
120
100
a
120
100
b
120
100
80
60
c
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 10 A and B.

Recombination between arg-1 and arg-2 as a percentage of the control value following ½-hour treatments with various agents at different times during zygospore germination. (a) 100 ug/ml mitomycin C, (b) 10 ug/ml nalidixic acid, (c) 1 mM FUdR, (d) 10 ug/ml actinomycin D, and (e) 30 ug/ml cycloheximide.

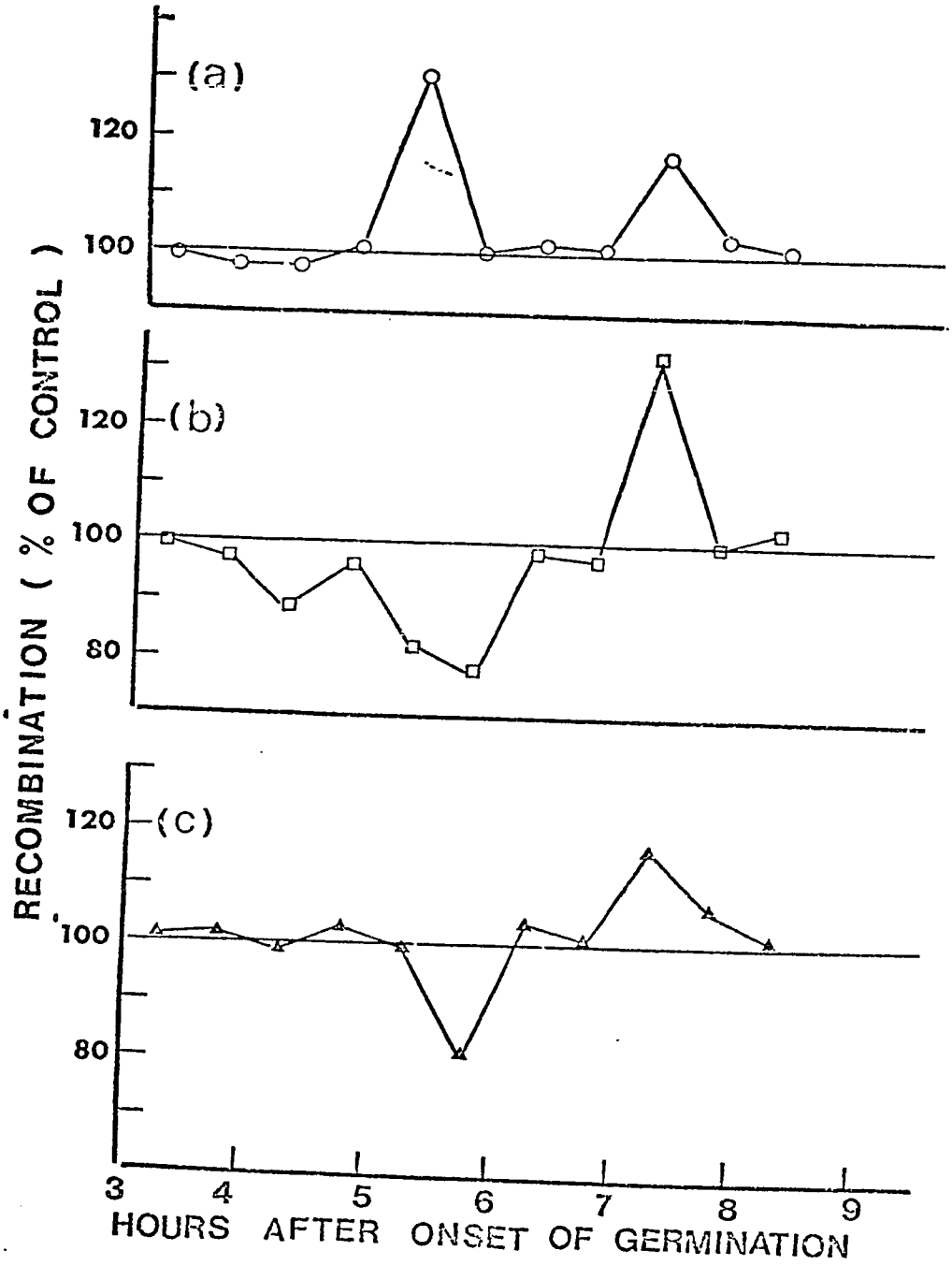

(a)
120
100
(b)
120
100
80
(c)
120
100
80
RECOMBINATION (% OF CONTROL)
3
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 10 A

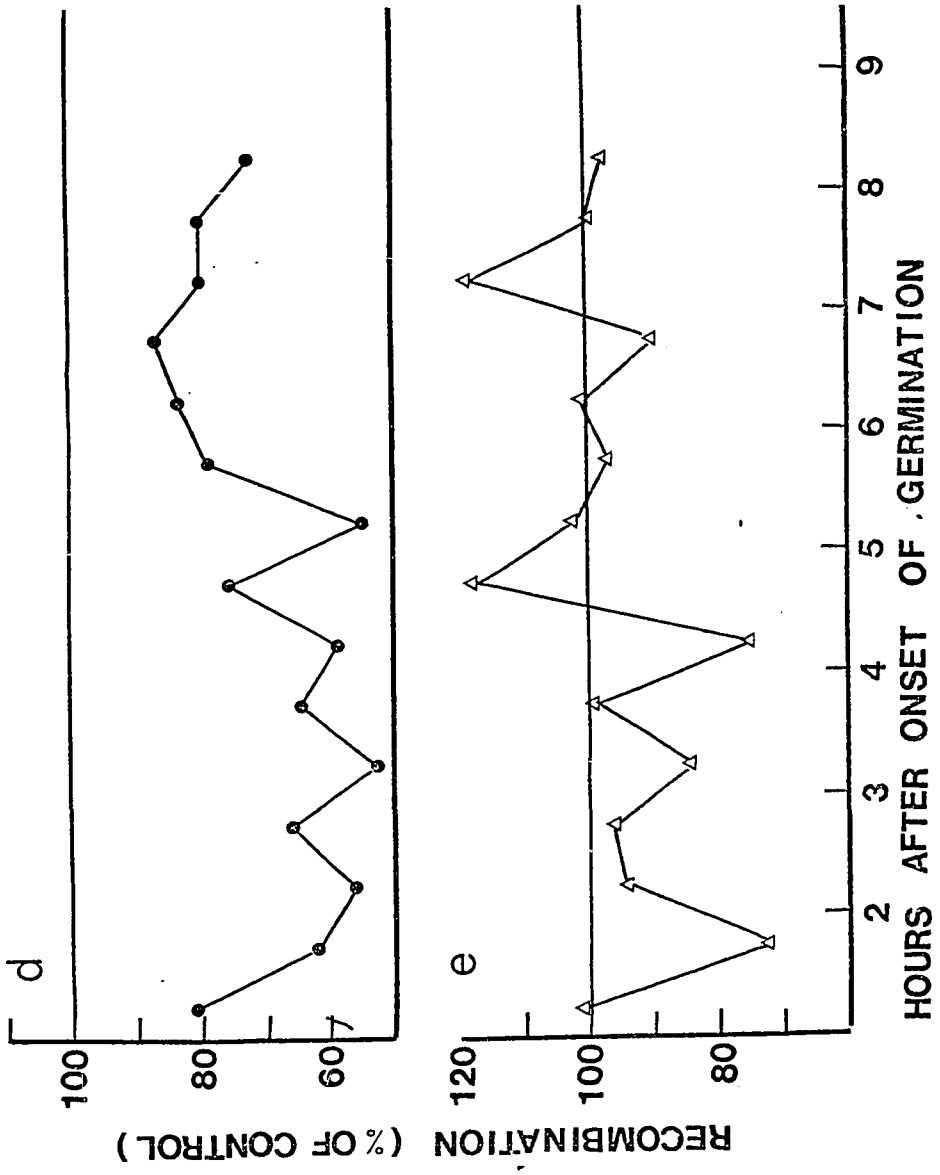

d
e
100
80
60
120
100
80
RECOMBINATION (% OF CONTROL)
2
3
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 10 B

FIGURE 11. Recombination between arg-1 and arg-2 as a percentage of the control value following ½-hour treatments with various agents at different times during zygospore germination. (a) 0.4% phenethyl alcohol, (b) 2 mM FUdR, and (c) 200 ug/ml adenine.

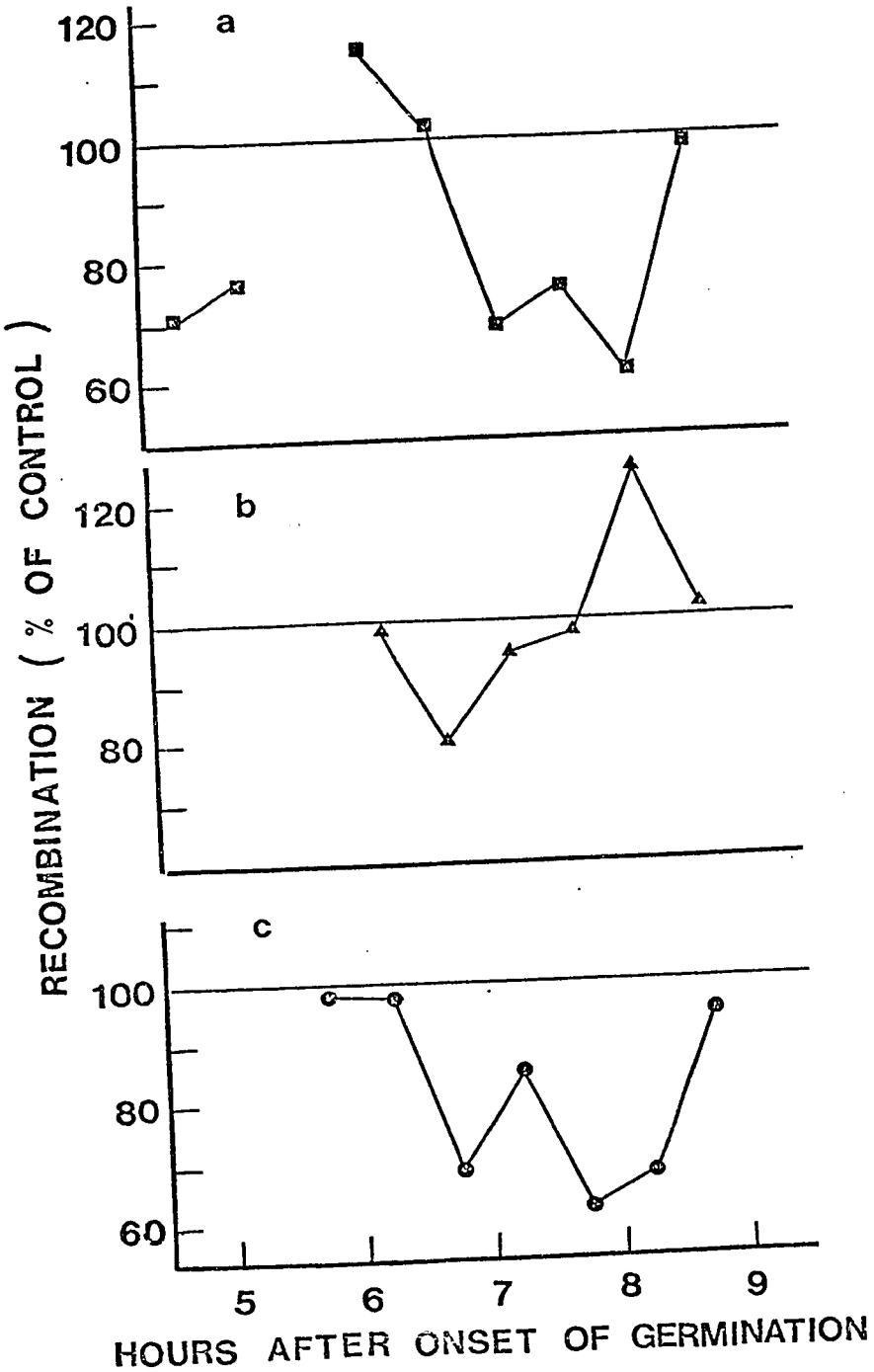

a
b
c
120
100
80
60
120
100
80
100
80
60
RECOMBINATION (% OF CONTROL)
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 12. Recombination between arg-1 and arg-2 as a percentage of the control value following treatments with various agents at different times during zygospore germination. Treatments were 30 minutes except at 7-9 hours after the onset of germination where treatments were reduced to 15 minutes. (a) 0.4% phenethyl alcohol, (b) 30 ug/ml nalidixic acid , (c) 1 mM hydroxyurea, and (d) 5 mM hydroxyurea.

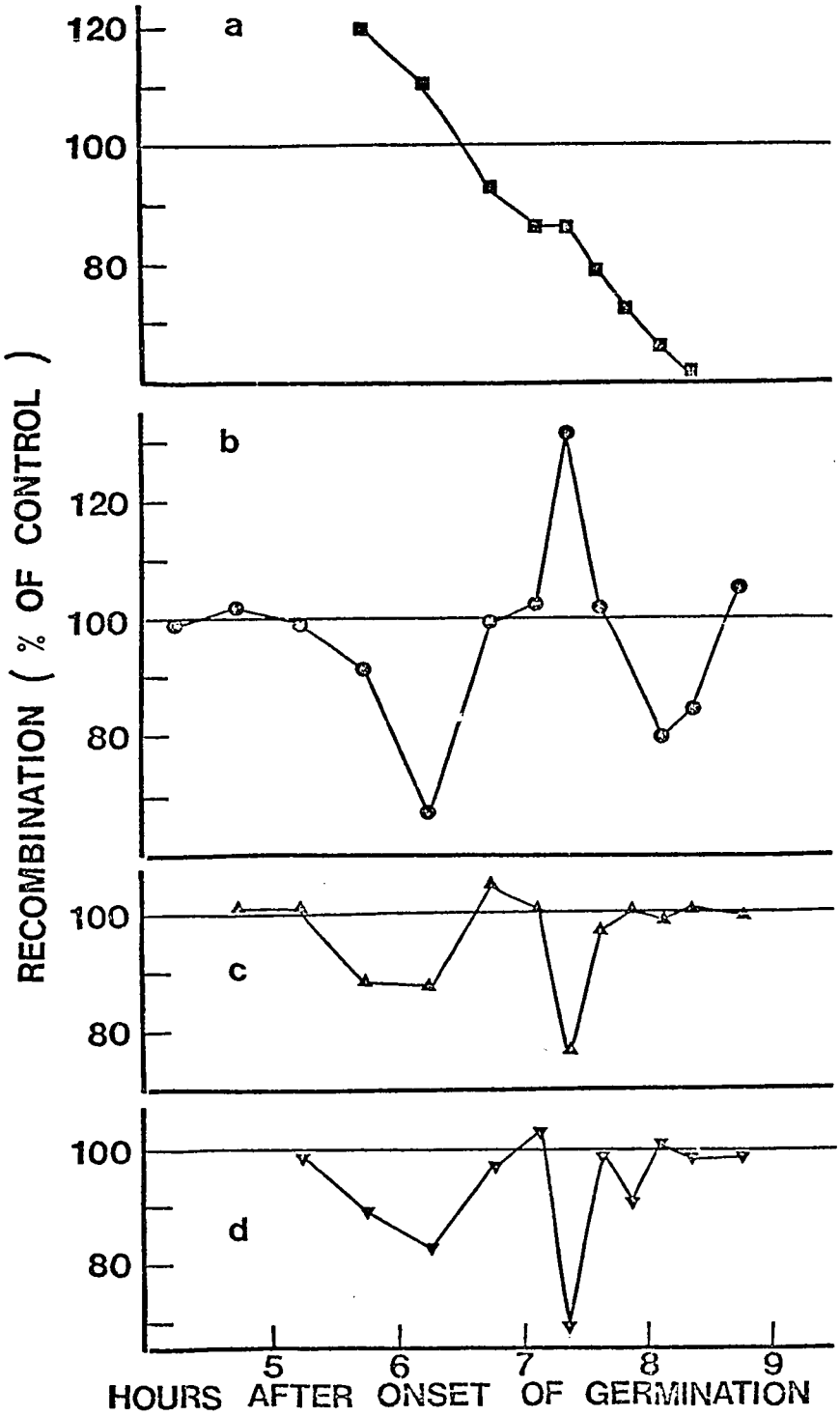

a
b
c
d
120
100
80
120
100
80
100
80
100
80
RECOMBINATION (% OF CONTROL)
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 13. Recombination between arg-1 and arg-2 as a percentage of the control value following ½-hour treatments with various agents during zygospore germination. (a) 100 ug/ml actinomycin D, (b) 10 ug/ml nalidixic acid, and (c) 3 mM hydroxyurea.

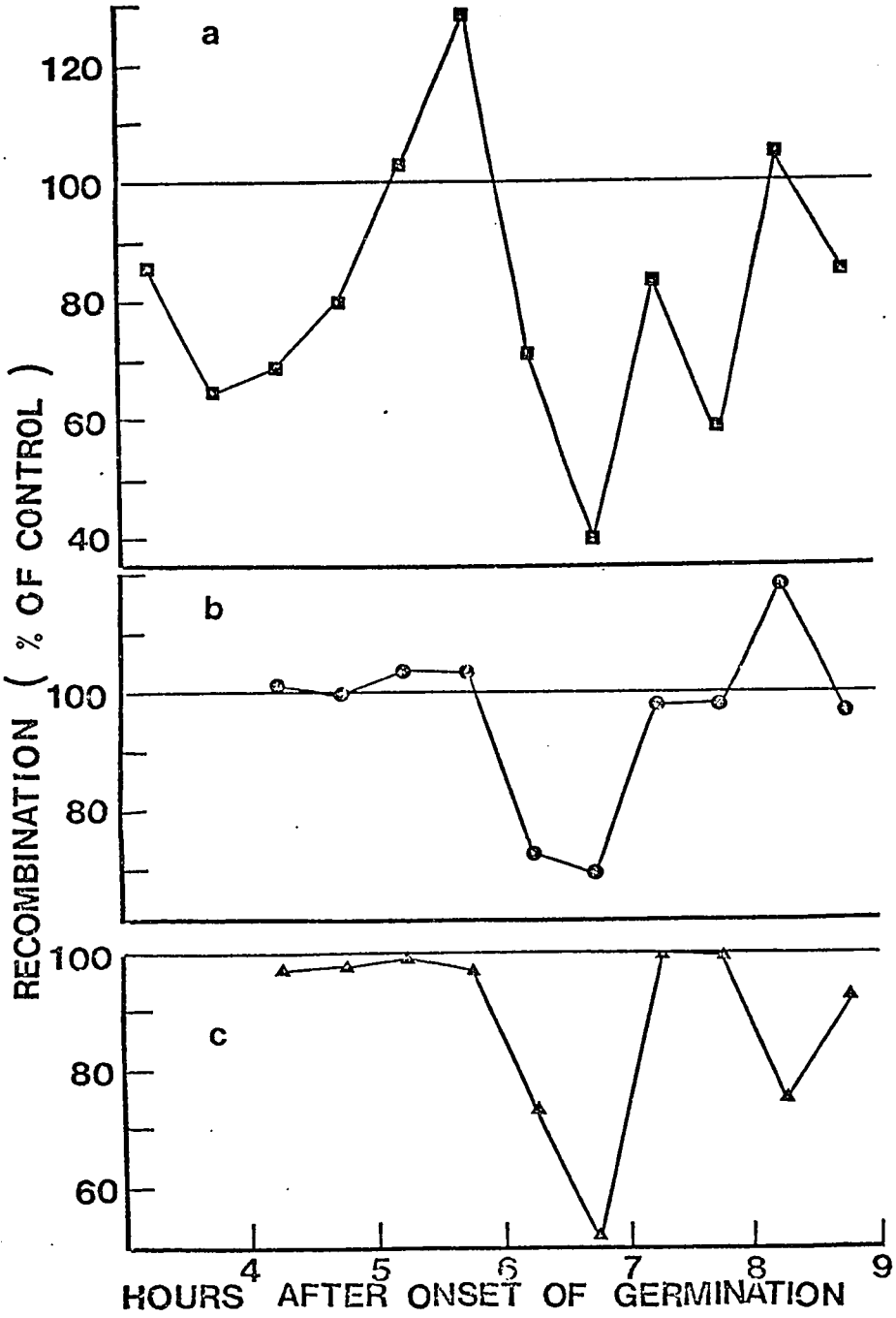

a
b
c
120
100
80
60
40
100
80
100
80
60
RECOMBINATION (% OF CONTROL)
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 14. Recombination between arg-1 and arg-2 as a percentage of the control value following ½-hour treatments with various concentrations of cycloheximide. (a) 2 ug/ml, (b) 10 ug/ml, and (c) 50 ug/ml.

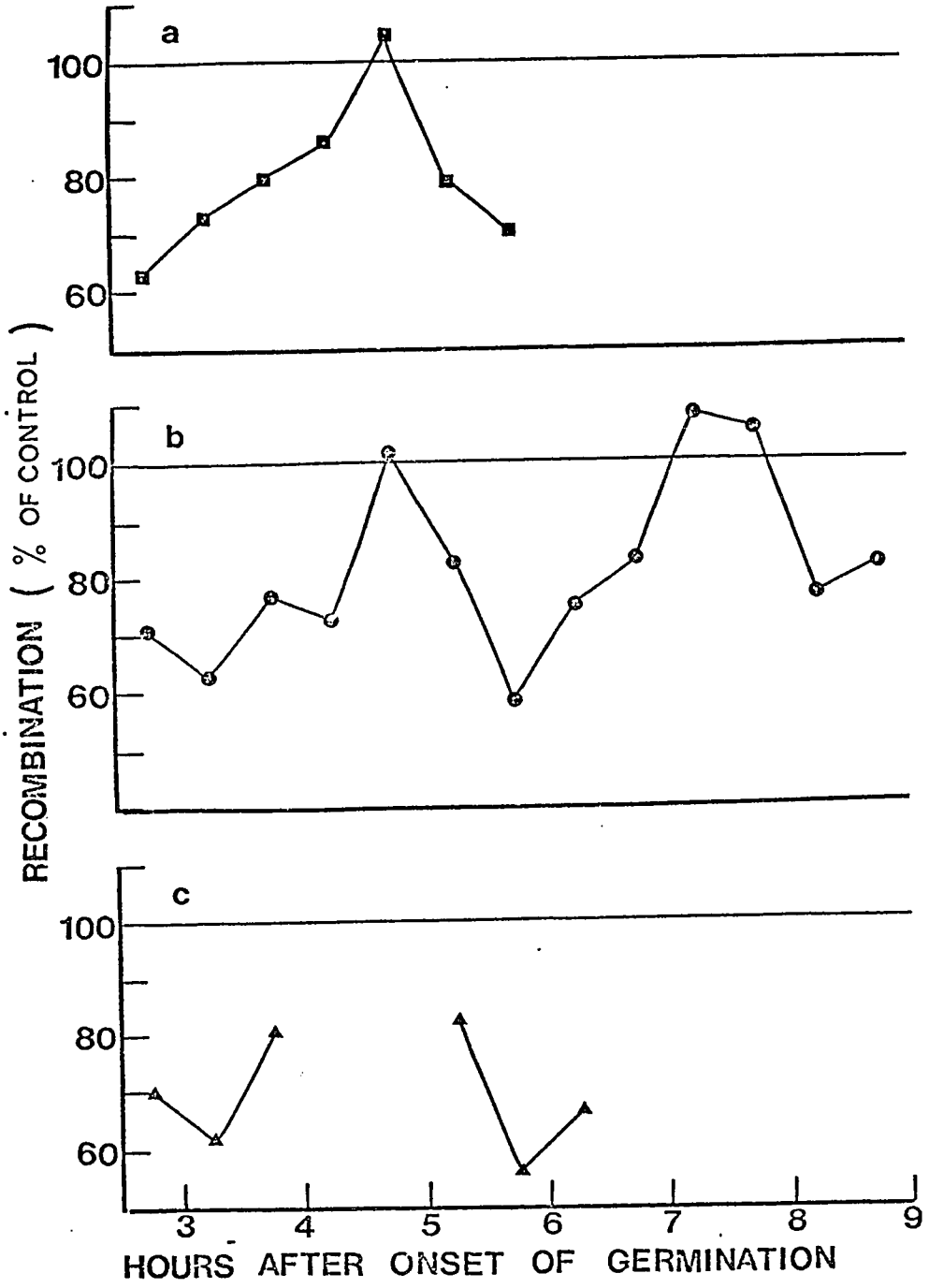

a
b
c
100
80
60
RECOMBINATION (% OF CONTROL)
3
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 15. Recombination between arg-1 and arg-2 as a percentage of the control value following treatments with various agents during zygospore germination. During the period from 3½ to 6 hours after the start of germination treatments of actinomycin D, mitomycin C and cycloheximide were reduced to 15 minutes. (a) 10 ug/ml actinomycin D, (b) 200 ug/ml mitomycin C, (c) 1 mM FUdR, and (d) 20 ug/ml cycloheximide.

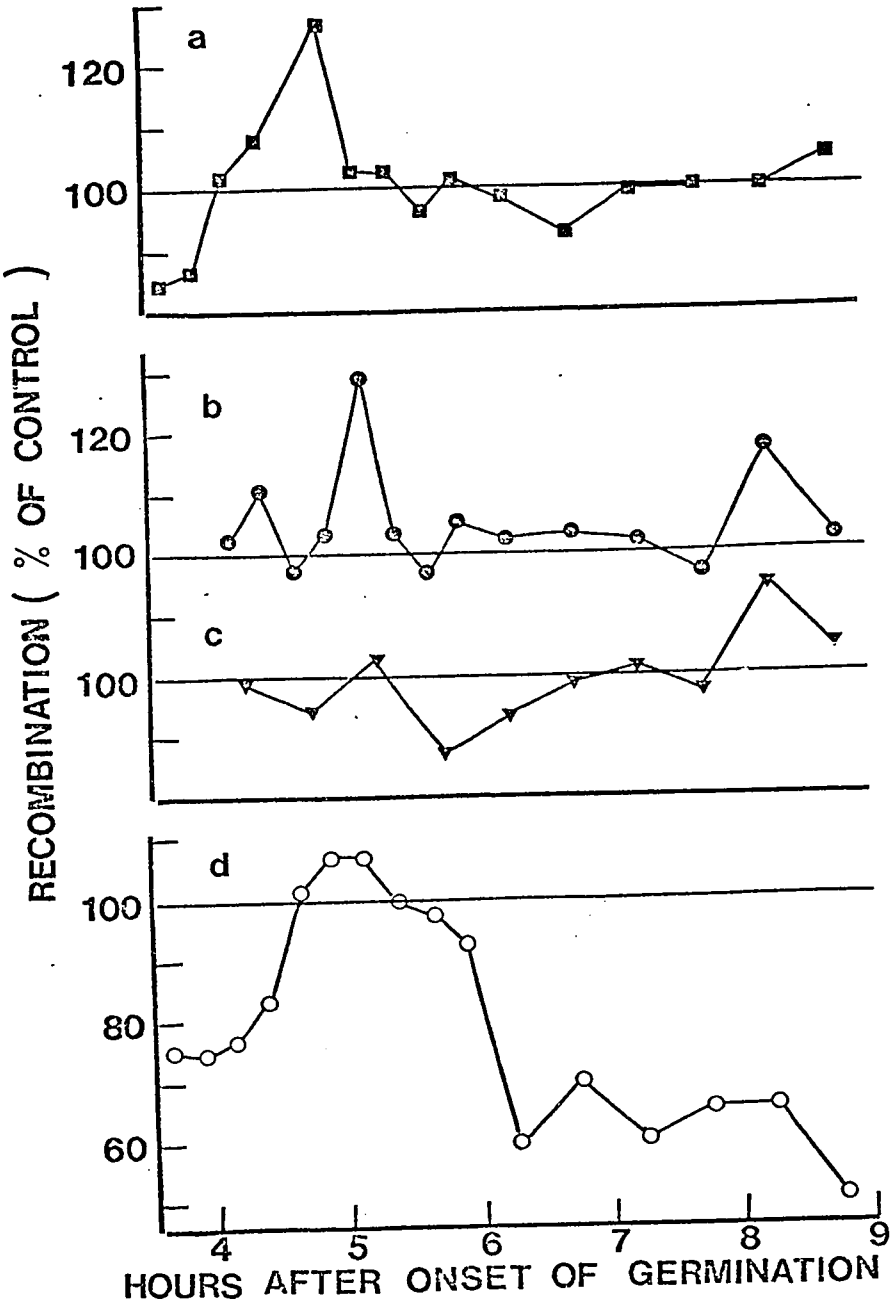

a
120
100
b
120
100
c
100
d
100
80
60
RECOMBINATION (% OF CONTROL)
4
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 16. Recombination between arg-1 and arg-2 as a percentage of the control value following half-hour treatments with various agents during zygospore germination. (a) 2mM FUdR, (b) 12 ug/ml acriflavine, and (c) 0.05% caffeine.

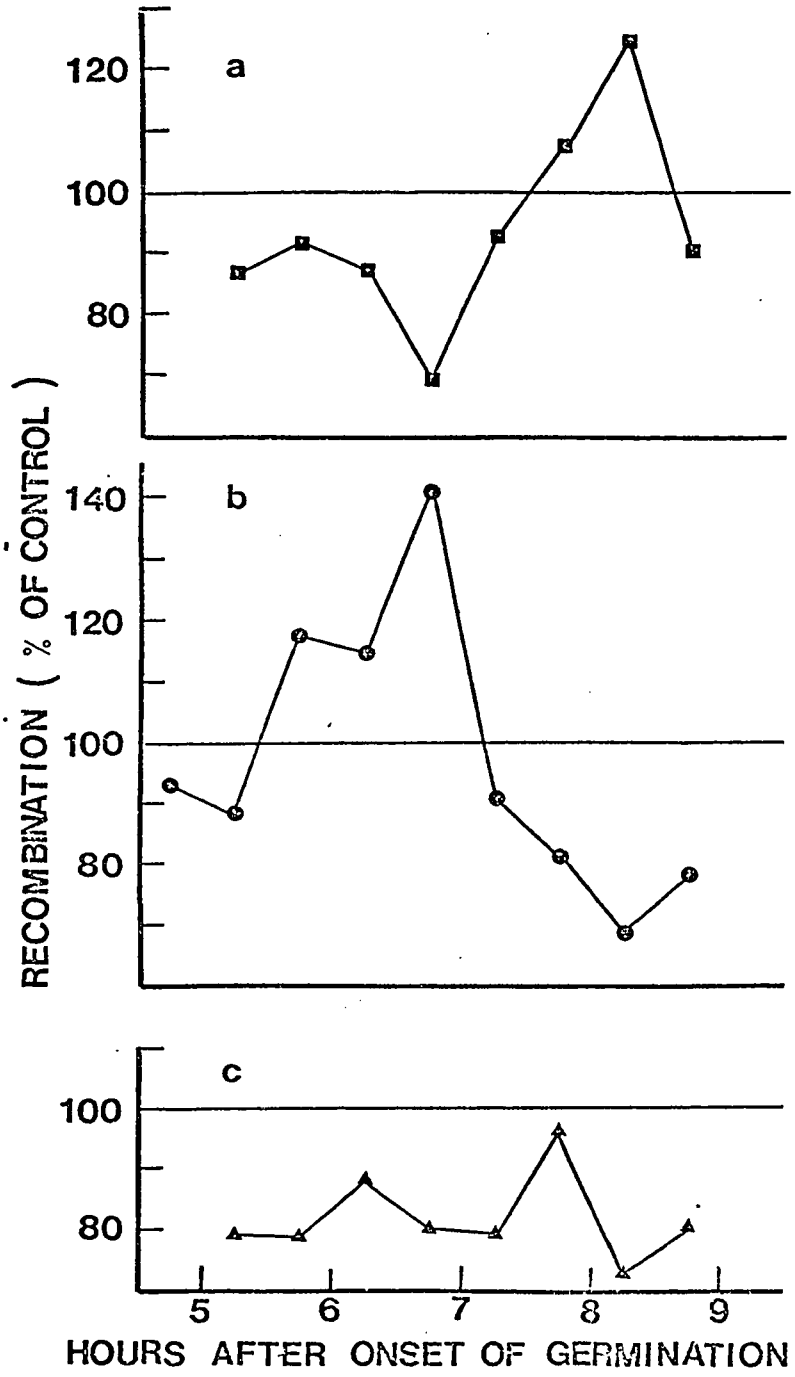

a
120
100
80
b
140
120
100
80
c
100
80
RECOMBINATION (% OF CONTROL)
5
6
7
8
9
HOURS AFTER ONSET OF GERMINATION

FIGURE 17 A and B

Recombination between arg-1 and arg-2 as a percentage of the control value following treatments with various agents during zygospore germination. Treatments were for 30 minutes except for the period from 8 to 10 hours after the start of germination, where treatments were for 60 minutes. (a) 50 ug/ml mitomycin C, (b) 50 ug/ml mitomycin C plus 2 mM FUdR, (c) 1 mM hydroxyurea, (d) 5 mM hydroxyurea, (e) 10 ug/ml acriflavine, and (f) 0.05% caffeine.

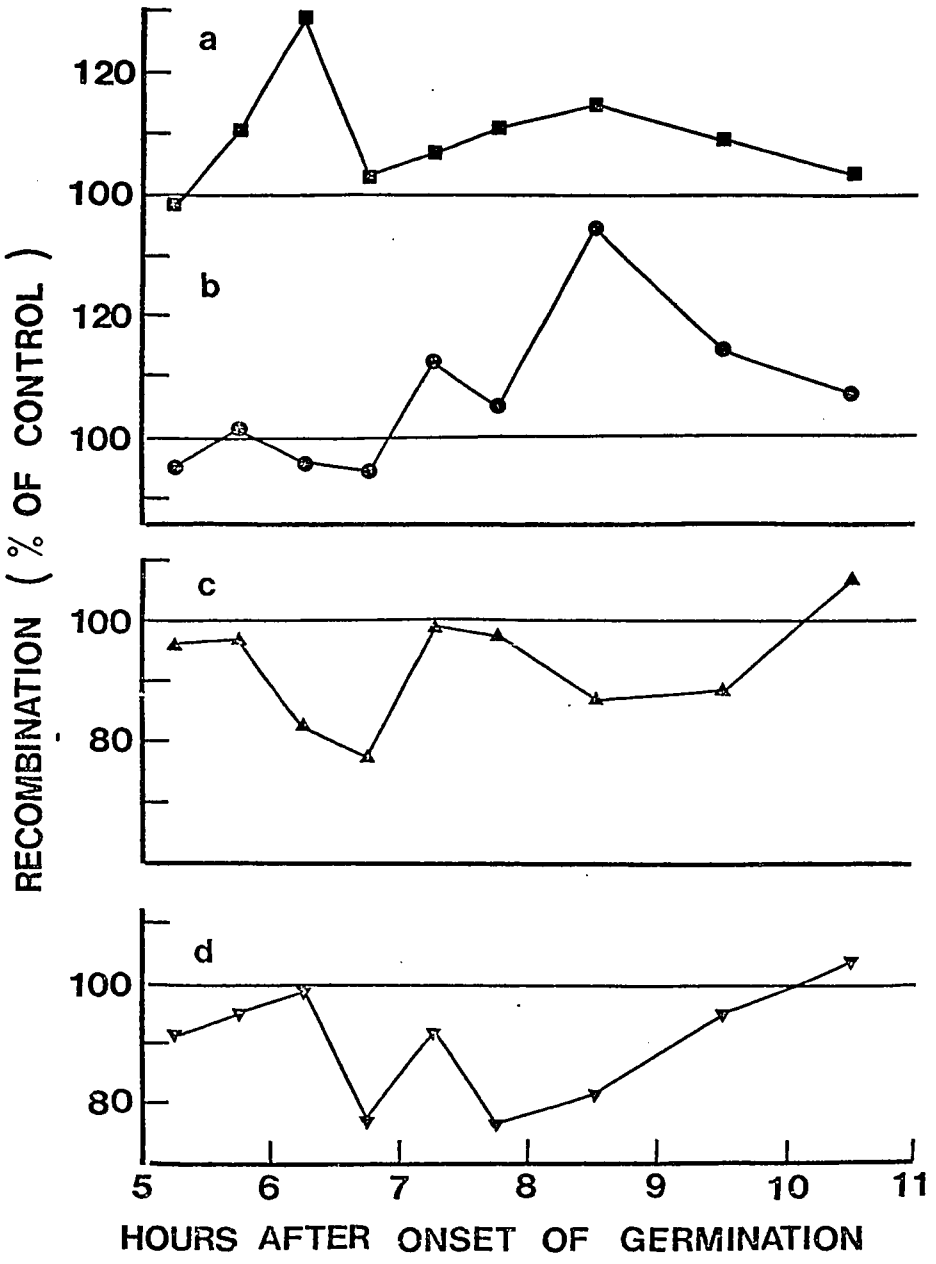

a
b
c
d
120
100
120
100
100
80
100
80
RECOMBINATION (% OF CONTROL)
5
6
7
8
9
10
11
HOURS AFTER ONSET OF GERMINATION

FIGURE 17 A

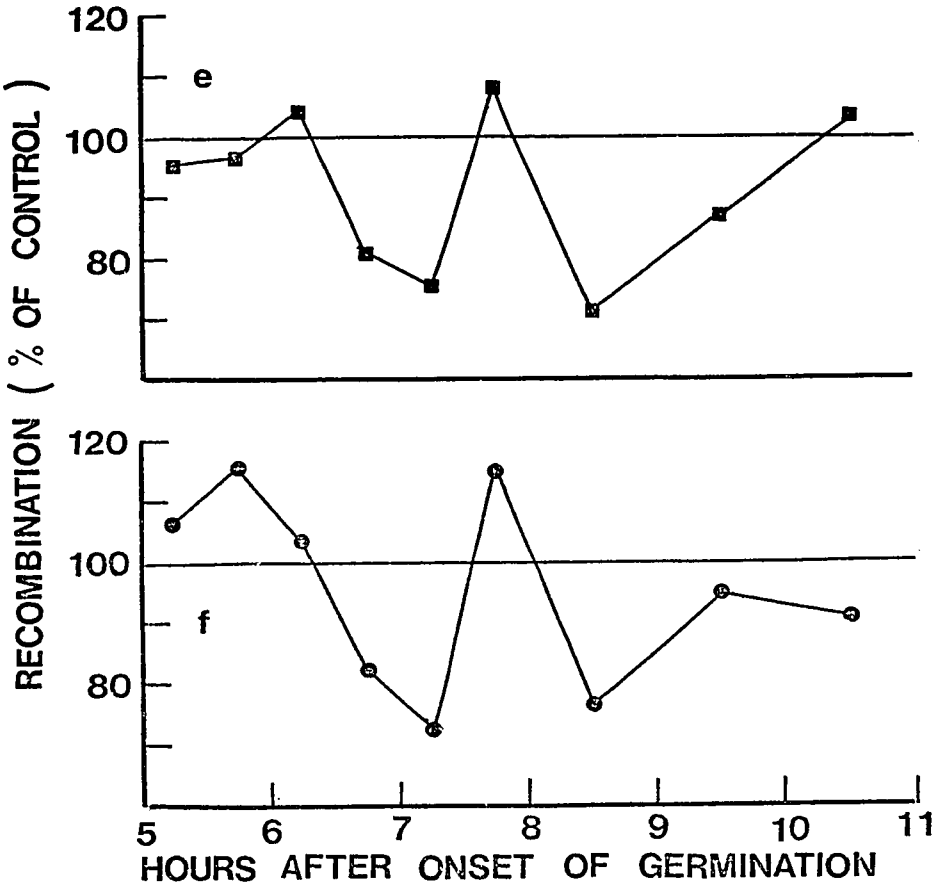

120
100
80
e
120
100
80
f
RECOMBINATION (% OF CONTROL)
5
6
7
8
9
10
11
HOURS AFTER ONSET OF GERMINATION

FIGURE 17 B

CHAPTER IV

DISCUSSION

The study of DNA synthesis in *C. reinhardi* strain 137C has revealed that there is only one round of nuclear DNA replication during the germination of zygospores. This takes place prior to meiotic division. Further, an equal DNA content of vegetative cells and gametes has been demonstrated, and it has been shown by Kates *et al.* (1968) that the DNA content of gametes is the same as the minimal DNA content of vegetative cells at G_1 phase. This suggests that pre-meiotic nuclear DNA replication cannot take place prior to gametic fusion. Sueoka *et al.* (1967) have also shown that there is no nuclear DNA replication during zygote maturation, and therefore the replication of nuclear DNA during the germination of zygospores must be the pre-meiotic replication. The meiotic pattern of *C. reinhardi* strain 137C is therefore basically identical to that of higher plants and animals in that there is one round of DNA replication prior to meiotic division.

In 1963 Jacob *et al.* proposed the replicon model for the regulation of DNA replication. According to the model the replicon consists of a replicator and an initiator gene, analogous respectively to the operator and the regulator gene of their earlier operon model (Jacob and Monod, 1961). The initiator gene produces a diffusible element called the

initiator, which acts on the replicator allowing the beginning of replication. As soon as initiation occurs, the replication proceeds until the whole replicon is replicated. Therefore, it is the initation but not the progress of replication that is under precise control, and any new round of replication requires the presence of initiator.

It is suggested by Jacob *et al.* (1963) that bacterial and phage chromosomes, and sex factors consist of single replicons. This concept of regulation of replication through the control of initiation has been supported with some modifications in bacteria by biochemical and culture studies (see Lark, 1969 and Kuempel, 1970 for review). It has been demonstrated that the initiation is regulated by the production of initiation proteins, which are produced only immediately before initiation.

Lark and Lark (1966) and Treick and Konetzka (1964) have shown that, at specific concentration, phenethyl alcohol specifically inhibits the initiation of DNA replication in *E. coli*. In *Chlamydomonas*, PEA was found not to be specific for the inhibition of DNA synthesis. Instead it was shown to inhibit RNA and protein syntheses more strongly than DNA synthesis. However, assuming that the effects of PEA on zygospore survival depend on inhibited synthesis of specific macromolecules, concentration-dependent differences in zygospore survival must be reflected by related differences in dose-responses for these macromole-

cular syntheses. Since the only concentration of PEA having specific effects on zygospore survival was around 0.4% and inhibition of RNA synthesis was indifferent to concentration in this range, it is unlikely that zygospore survival is related to RNA synthesis. Further, since inhibition of protein synthesis was high at both 1.0 and 0.4%, it is again unlikely that zygospore survival is related directly to protein synthesis. In contrast, the effects on DNA synthesis and on zygospore survival are more or less parallel, and hence it is likely that the effect of PEA on zygospore survival is related to DNA synthesis. The effect on DNA synthesis, as observed in the time-course study with complete inhibition 90 minutes after treatment may be interpreted thus: new rounds of DNA replication are inhibited but already initiated replications go to completion. This suggests that DNA synthesis is being inhibited by an inhibition of initiation.

The genome of higher organisms, according to autoradiographic studies, consists of many replicons (Plaut and Nash, 1964; Huberman and Riggs, 1968). Recent investigation on *Physarum* indicates that the genome of this organism is composed of at least ten replicating units, which initiate their replication at different times during the S period (The addition of cycloheximide at different times during S period leads to a stepwise increase in the amount of DNA synthesized; Muldoon *et al.*, 1971). Therefore, since PEA appears to inhibit initiation only, the killing of zygospores

by treatment 60 minutes before the main S period suggests that replicons initiated at this period are essential to meiosis or germination. Zygospores treated at this time do not recover, so the effect appears to be irreversible in this system.

The depression of survival by PEA treatment at prophase suggests that initiation is also required at this second period. Hotta and Stern (1971) have shown that prophase DNA synthesis in *Lilium* is of two types: delayed replication and repair synthesis. Since the repair process is known to involve a multiplicity of enzymes (exonucleases, endonucleases, ligase, etc.) and is thought to act only in the presence of randomly occurring DNA lesions (Howard-Flanders, 1968; Witkin, 1969), it appears unlikely that the DNA synthesis involved would require initiation sites. In contrast, there are no *a priori* reasons for considering that delayed replication does not require them. Thus, it seems very likely that the delayed replication demonstrated at prophase in *Lilium* (Hotta and Stern, 1971) also occurs in *Chlamydomonas* and replication consists of whole replicons.

The variation in recombination frequencies from experiment to experiment is probably due to the variation in the physiological conditions of zygospores since Hastings (1964) has shown that the age of zygospores may affect the frequency of recombination.

The data of dose-reponse experiments have shown that except for PEA, which inhibits all kinds of macromolecular

syntheses, the inhibitors of DNA synthesis chosen for this study are all fairly specific for the inhibition of DNA synthesis at the concentrations used. Actinomycin D was shown to be a specific inhibitor at low concentration for RNA synthesis but at high concentration it had some effect on DNA synthesis also. Cycloheximide was shown to inhibit protein synthesis specifically except at high concentration, where it also inhibited DNA synthesis.

The effects of inhibitors of DNA synthesis other than PEA confirm earlier observations that recombination in *Chlamydomonas* can be induced to change by various inhibitors of DNA synthesis including γ radiation at two short periods during zygospore germination (Hastings, 1964; Lawrence, 1965, 1968, 1970; Davies and Lawrence, 1967). The first period occurs before meiosis and the second period at prophase. The demonstrated specificity of these inhibitors for DNA synthesis, together with the absence of a two-period effect of recombination by actinomycin D and CH strongly indicates that the effects of these other DNA synthesis inhibitors on recombination are mediated through their effects on DNA synthesis. In addition, the depression of recombination by 100 µg/ml of actinomycin D at the main S period also supports this conclusion since a lower concentration of this antibiotic, which specifically inhibits RNA synthesis, does not show this effect.

The observations that these inhibitors of DNA synthesis have no significant effects on viability of treated zygospores

and that the change in recombination is not accompanied by any concomitant alteration in survival show that the change in recombination caused by these inhibitors does not result from the selective killing of zygospores. Although only the region between *arg*-1 and *arg*-2 was used for measurement, it seems very unlikely that the changes in recombination frequency observed here are due to the effects leading to changes in the distribution of recombination events, since similar responses toward DNA inhibitors have been shown in an other region of the same linkage group (Davies and Lawrence, 1967). Moreover, treatment with γ radiation, which has an effect on recombination similar to that of the DNA synthesis inhibitor FUdR, has been shown to cause a similar reponse in different regions of the genome (Lawrence, 1968). This conclusion is also supported by the effects of γ radiation on chiasma frequency in *Lilium* meiocytes (Lawrence, 1961a). However, it is possible that these inhibitors may cause effects of a different magnitude in other regions of the genome. In fact, in studies of recombination in *Drosophila*, Suzuki (1965a, b) has found that treatments with actinomycin D and mitomycin C give an increase in recombination at the regions spanning the centromere whereas other regions of the same linkage group are unaffected.

The recombination data reveal that the effects of these inhibitors of DNA synthesis on recombination are complex since some treatments may stimulate and others may

depress recombination at either the pre-meiotic period or at prophase. Mitomycin C gives an increase in recombination during both periods, whereas adenine and HU give a depression during both times, and NA and FUdR give a decrease during the first period and an increase during the second period. Thus, there is no trend of similarity between their effects on recombination and DNA synthesis by which their mechanism of action might be clarified. For this reason, the effects of these inhibitors on recombination at two responsive periods will be discussed separately.

Although these inhibitors of DNA synthesis have been shown to affect recombination before or during the main S period, it appears quite unlikely that recombination *per se* takes place during this period, since in *Neottiella* Rossen and Westergaard (1966) have shown that replication of nuclear DNA occurs prior to karyogamy, which precedes meiosis. Thus effects produced by these inhibitors at the pre-meiotic S period are probably due to modifications of the process leading to chromosome synapsis or crossing-over.

The recombination data for treatment with inhibitors of DNA synthesis at the pre-meiotic period reveal an interesting phenomenon. PEA and mitomycin C, with a recombination effect before the main S period, give an increase in recombination, whereas the other group of inhibitors (NA, HU, FUdR, and adenine), with effects at the S period, all cause a depression. In other words, inhibition of DNA synthesis before and inhibition during the main S

period produce opposite effects on recombination. Moreover, actinomycin D, which has an effective time 60 minutes before the main S period enhances recombination.

These observations suggest a hypothesis that the effects of these inhibitors on recombination are indirect, since they depend on the time rather than the nature of inhibition. Thus, the inhibition of either RNA or DNA synthesis prior to the S period invariably gives an increase in recombination, whereas inhibition of DNA synthesis at the S period gives a decrease. According to this hypothesis the opposite effects are due to an interference with each of two opposed crossover control mechanisms. This interference can be caused by the inhibition of both DNA and RNA synthesis. The first mechanism restricts the amount of cross-overs, and acts before the main S period, whereas the second one is required for crossing-over and operates only during this period. The existence of two kinds of control mechanisms at the pre-meiotic period is supported by the observation that treatments with γ radiation at this period produce opposite effects on intergenic and intragenic recombinations (Lawrence, 1965, 1970). However, this hypothesis fails to explain the cancellation of a mitomycin C stimulating effect by FUdR in the experiment in which the two inhibitors were used simultaneously (Results, Sec. 3).

On the other hand, the effects of these inhibitors of DNA synthesis on recombination are consistent with their modes of DNA synthesis in the time-course study. PEA and

mitomycin C, which stimulate recombination 30 minutes prior to the main S period, both have a delayed effect on DNA synthesis in the time-course study, where blockage is not complete until 90 minutes after treatment. In contrast, of the group of inhibitors consisting of FUdR, NA, HU, and adenine which give a decrease in recombination, FUdR and HU caused immediate inhibition.

Some similarities in the effects of PEA and mitomycin C on recombination suggest that they act by a similar mechanism. Both have been shown earlier in the discussion to have similar effects on DNA synthesis, interpreted as being due to a common hibition by both chemicals of initiation. For PEA, in addition, the specific killing of zygospores suggests that the inhibition may be irreversible. Thus it appears likely that treatments with both inhibitors 30 minutes before the main S period may cause the replication of certain replicons to be delayed until too late (*e.g.* until prophase). Since this shift is accompanied by increased recombination, it may be suggested that the number of delayed replicons determines the amount of recombination. Since zygospore viability remains unaffected with PEA treatment at 30 minutes before the main S period, it is possible that their replication at the S period is not an essential requirement for germination.

These observations form the experimental basis of the replicon hypothesis of recombination (Chiu and Hastings, 1973), which suggests that the units of delayed replication consist of whole replicons and the amount of delayed

replication determines the amount of recombination. The depression of recombination with treatments by DNA inhibitors other than PEA and mitomycin C according to this hypothesis is caused by a decrease in the amount of delayed replication. Experimental induction of interphase replication of delayed-replicating regions has been demonstrated in cultured *Trillium erectum* meiocytes (Stern and Hotta, 1969). Meiocytes explanted soon after the pre-meiotic S period are induced to undergo mitosis rather than meiosis. However, this mitotic induction is accompanied by the induction of zygotene DNA replication at the stage prior to cell division. It has been suggested by Chiu and Hastings (1973) that these inhibitors might immediately block S-phase DNA synthesis which has already been initiated and thus provide available precursors for extra replicon initiations at the same S period. This would mean fewer delayed replicons, and hence depressed recombination. Since only FUdR and HU of this group show immediate blockage of DNA synthesis, however, this kind of mechanism does not appear to provide an entirely satisfactory explanation for depressed recombination according to the replicon hypothesis.

The enhancement of recombination caused by some inhibitors prior to the main S period, on the other hand, is explained according to the replicon hypothesis as being caused by the inhibition of initiation. Thus the stimulation of recombination by actinomycin D 30 minutes prior to the recombination-effective period of mitomycin C and PEA would

be caused by the inhibition of initiator production. A minor peak of RNA synthesis at this period is observed (Figure 4), and it would be interesting to know whether this RNA consists of messenger for the production of initiator protein. In tests for this possibility, covering the effective periods of actinomycin D and mitomycin C by a series of 30 minute and 15 minute treatments with CH, however, the expected stimulation of recombination was not observed. One explanation is that RNA synthesized at this period does not contain m-RNA for initiator proteins. This is supported by the recent discovery in *E. coli* that RNA synthesis required for DNA replication does not produce messenger for the production of initiator proteins, since the synthesis is required after these proteins have been produced (Lark, 1972). *In vitro* studies of DNA replication also support this conclusion (Brutlag *et al.*, 1972).

Another explanation, deriving from the fact that the inhibitory effect of CH is reversible (Parchman and Stern, 1969), is that a delayed translation of initiator m-RNA occurring after the inhibitor is removed. A third is that a strong depression in recombination by the inhibition of protein synthesis may mask the stimulus from DNA inhibition.

One way of testing whether two groups of DNA inhibitors act by different processes is to measure the amount of DNA synthesized at prophase in zygospores treated with inhibitors before or during the main S period. However, for technical reasons, this test did not work out very well.

Another test consists of a combined study using mitomycin C with one other inhibitor. In one experiment, treatment with one inhibitor is followed by treatment with the other; in a second experiment, a mixture of the two inhibitors is used for treatment.

The result of such a test with a treatment mixture consisting of mitomycin C and FUdR (Results, Sec. III) shows that FUdR cancels the effect of mitomycin C at its first effective period and mitomycin C cancels the effect of FUdR at the main S period. This suggests that the two inhibitors act in opposite directions on recombination and therefore cancel each other at the pre-meiotic period.

It would be interesting to know how delayed replication functions in recombination. One possibility is that it may act indirectly through synapsis. This is supported by the finding that it is required for the formation, progress, and maturation of synaptonemal complexes (Roth and Ito, 1967; Sen, 1969; Stern and Hotta, 1969). It has been suggested that the base sequence of the unreplicated delayed regions may play an important role in the pairing of homologs (Stern, 1969). Alternatively, the regions of delayed replication may be the regions where genetic recombination takes place. The newly replicated strands of these regions may then be used for genetic exchange following the recombination scheme either of Whitehouse (1963) or of Holliday (1964). Hastings (1972), on the other hand, suggests that the regions of delayed replication are involved in both

synapsis and recombination. It is also suggested that delayed replicating regions are located between structural genes and at the homologous position on both homologs. Since the presence of single-stranded gaps is believed to be essential for recombination (Paszewski, 1970) and is an essential feature of the recombination models of both Holliday and Whitehouse, it is assumed by Hastings that following delayed replication gaps are left between the ends of newly synthesized polynucleotide strands and the strands which are replicated at the S period. These gaps may then initiate the dissociation of newly synthesized strands from their complementary strands for the formation of a heteroduplex according to the scheme of either of the above models. Since the Whitehouse model requires a pair of gaps ("primary breaks") to occur on DNA chains of opposite polarity and Holliday's model demands them on strands of similar polarity, only two pairs of chromatids can be cross-overs from either model. Therefore, for either model alone, two-strand or four-strand double-crossovers are possible, but a three-strand double cross-over is not. Thus in order to satisfy genetic data Hastings suggests the occurrence of a mixture of recombination of both models in equal frequency is required.

The effects of DNA synthesis inhibitors at prophase on recombination are not clear since stimulation is caused by some and depression by others. Apparently the effects are not related to the modes of action of these inhibitors

on DNA synthesis. For instance, HU and FUdR which have the same type of effect on DNA synthesis but have opposite effects on recombination; whereas mitomycin C and FUdR show different patterns of inhibition of DNA synthesis (in the time-course study) but have similar effects on recombination. Moreover, no correlation has been found between their effects on DNA synthesis and on recombination, with regard to specificity of effect or magnitude of effect. In addition, simultaneous, but separate, 15 minute treatments with inhibitors exerting opposite effects on recombination were not able to resolve whether the effects were simultaneous or sequential, and each inhibitor still showed a single peak or trough.

Prophase DNA synthesis in *Lilium* has been demonstrated to consist of two kinds of synthesis (delayed replication and repair synthesis; Hotta and Stern, 1971). The possibility that these inhibitors may preferentially affect delayed replication or repair synthesis has been tested using 1 and 5 mM of HU and repair inhibitors acriflavine and caffeine. Two concentrations of HU, 1 and 5 mM respectively, have been shown in *Lilium* by Hotta and Stern (1971) to inhibit differentially semiconservative replication and repair synthesis. However, since both these concentrations of HU produce similar depressions of recombination in this study, it seems probable that these concentrations do not act specifically on these two kinds of synthesis in *Chlamydomonas reinhardi*. On the other hand, the consistency of the effects

of acriflavine and caffeine on recombination at prophase suggests that they act specifically on excision repair. Since both inhibitors have the same effective period at prophase as do other DNA inhibitors, and both reduce recombination at this time, it appears that the reduction in recombination at prophase caused by some DNA synthesis inhibitors may also result from preferential effects on repair synthesis. If this is true, the stimulation of recombination by other DNA inhibitors is thus due to the preferential inhibition of delayed replication. However, the cause of the enhancement of recombination by the inhibition of delayed synthesis is not known. One possible explanation is that this inhibition may lengthen the time of recombination.

Another attractive explanation is that the inhibition of delayed replication may result in the production of large gaps between the ends of newly replicated strands and the strands which are replicated at the S period. According to Hastings' scheme of recombination (1972) only small gaps, which may be separated by a very few nucleotides, are left after delayed replication, and it is assumed that these small gaps have the alternative possibilities of being competively sealed by ligase and being engaged in recombination. Large gaps, on the contrary, which could not be sealed by ligase would have a relatively greater chance of being engaged in recombination.

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