# PRE-MEIOTIC DNA SYNTHESIS AND RECOMBINATION IN *CHLAMYDOMONAS REINHARDI*[1]

S. M. CHIU AND P. J. HASTINGS

*Department of Genetics, University of Alberta, Edmonton, Alberta*

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ABSTRACT

The time of the pre-meiotic S-period was determined by $^{32}P$ incorporation in synchronously germinating zygospores of *Chlamydomonas reinhardi* at six and one-half to seven hours after the beginning of germination. Phenethyl alcohol treatment caused death of zygospores at a period one hour before the S-period, and also during meiotic prophase. Recombination between *arg-1* and *arg-2* was increased by treatment with phenethyl alcohol or mitomycin C at a time between the first sensitive period to phenethyl alcohol and the S-period. Actinomycin D caused an increase in recombination at the time of this sensitive period. FUdR, nalidixic acid and hydroxurea all cause a decrease in recombination when applied during S-period, and have no effect earlier. These results are explained by postulating (1) that the units of delayed premeiotic replication are whole replicons, and (2) that the amount of recombination is proportional to the number of replicons in which synthesis is delayed. It is suggested that the control of DNA replication controls the distribution of recombination events.

TREATMENT of zygotes of *Chlamydomonas reinhardi* with various inhibitors of DNA synthesis has been found to affect the frequency of crossing over at two specific periods during germination (HASTINGS 1964; DAVIES and LAWRENCE 1967). The first responsive period is located at the pre-leptotene stage of meiosis, probably corresponding to the time of pre-meiotic DNA replication and the second period at prophase, as judged by cytological observation. The consistency of the results with many DNA inhibitors suggests specificity of the inhibition of DNA synthesis on recombination. Therefore, the synthesis of DNA at both times of meiosis is related to recombination.

Prophase DNA synthesis in *Lilium* has been demonstrated by HOTTA, ITO and STERN (1966). It takes place at zygotene and pachytene of meiosis and consists of 0.3% of total nuclear DNA. Biochemical study and cytological observation of cultured meiocytes have led to the conclusion that it consists of two distinct types of syntheses: the early phase of synthesis is a delayed replication while the later one may represent repair synthesis associated with crossing over.

The relationship of pre-meiotic DNA synthesis to recombination remains unclear. Apparently recombination does not occur during pre-meiotic S-period,

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since it has been shown in *Neotiella rutilans* (ROSSEN and WESTERGAARD 1966) that nuclear fusion occurs after DNA synthesis. This paper reports an attempt to discover the nature of the effect of DNA synthesis inhibitors on recombination during the pre-meiotic S-period by studying the specific effects of inhibitors which affect synthesis in different ways.

## MATERIALS AND METHODS

*Genetic Analysis:* Arginine-requiring mutants of *Chlamydomonas reinhardi* strain 137 C, which releases four zoospores, were originally isolated by EVERSOLE (1956). Zygotes were obtained from the cross of *arg-1 mt+* and *arg-2 mt−* which are on linkage group 1, separated by about six map units (HASTINGS *et al.* 1965). Gametes were induced from vegetative cells growing on TAP medium (GORMAN and LEVINE 1965) supplemented with 0.4% casein amino-acids, and solidified with 1.5% agar, by suspending them in distilled water or nitrogen-free minimal medium. Sexually active gametes were usually obtained within six hours. The gamete suspensions of *arg-1 mt+* and *arg-2 mt−* were mixed and allowed to mate for thirty minutes. At the end of this period, the mating mixture was spread on 4% minimal agar plates. The plates were exposed to light for 24 hours and then put in the dark for at least five days for the maturation of zygotes. Mature zygospores were induced to germinate synchronously by exposure to light for 30 minutes. Unmated cells were killed by exposing the plates to chloroform vapor for 40 seconds. Zygospores were scraped from the plates and suspended in TAP liquid medium. Inhibitors were added at different intervals after the start of germination and were removed by centrifugation 30 minutes later. The pellet of zygospores was resuspended in TAP medium, adjusted to a suitable concentration and spread uniformly on 1.5% TAP agar plates. Each plate received 1000 to 2000 zygospores. The concentrations of inhibitors used were as follows:

nalidixic acid, 10 μg/ml; FUdR, 1 mM; hydroxyurea, 1–5 mM; mitomycin C, 100 μg/ml; actinomycin D, 10–100 μg/ml; phenethyl alcohol, 0.2–1%; cycloheximide, 30 μg/ml.

*Inhibition of DNA, RNA and protein synthesis:* Inhibition of DNA and RNA synthesis was determined by incorporation of $^{32}P$ phosphate or $^{14}C$-adenine for one and one-half or two hours into vegetative cells, followed by differential extraction as described below. Inhibition of protein synthesis was determined by measuring the incorporation of $^{14}C$-glycine, $^{14}C$-arginine or $^{14}C$-alanine into acid-insoluble material after labelling of vegetative cells for two hours. The vegetative cultures were grown in TAP liquid medium in the light (5000 lux) at 21°C.

*Scoring:* The scoring was usually carried out between five to seven days after plating using a dissecting microscope. Since only tetrads containing prototrophic recombinants can grow and form green colonies on this selective plate, they can be easily distinguished from parental auxotrophs, which grow only a very limited extent. The frequency of green colonies indicates the frequency of crossing over between *arg-1* and *arg-2*.

All experiments have been performed several times. The results reported are obtained consistently.

*$^{32}P$-phosphate incorporation and differential RNA and DNA extraction:* The zygospore suspension, prepared as described above, was labeled with 10 μc/ml $^{32}P$-phosphate for thirty minutes at different intervals from three to nine hours after the beginning of germination. Each sample contained about $10^7$ zygospores. The incorporation was stopped by cooling to 0° and the zygospores were then washed twice with cold 0.005 M phosphate buffer.

The procedure described by SMILLIE and KROTKOV (1960) was employed for differential RNA and DNA extraction.

The method was calibrated with wild type vegetative cells of the + mating-type. DNA nucleotides in the extracts were determined by the indole method (KECK 1956) and RNA by the orcinol method (MARKHAM 1955). It was found that no further ribonucleotides could be extracted after 20 hours in 0.3 N KOH at 37°C, and that two washes in 0.3 N perchloric acid removed all hydrolyzed material. DNA was extracted twice with 0.5 N perchloric acid at 70°C

for 30 minutes. The second extraction yielded from 0 to 33% of the first extraction, and no further release of material was detected by radioactive counts or by the indole reaction, after a third extraction. It was found that, after $^{32}$P-phosphate labelling, some count remained in the pellet. The orcinol reaction revealed no ribose in the hot acid extract, while the indole reaction showed 2 to 3% of the material indicated by the orcinol reaction in the alkaline extract. This 2 to 3% indole reaction is also obtained with purified RNA and with ribose. Thus for vegetative cells, the alkaline extract contains RNA and not DNA and no further RNA can be obtained by alkaline hydrolysis, while the hot acid extract contains DNA but not RNA, and no further DNA can be hydrolyzed with hot acid.

The method cannot be calibrated in this way for zygospores, because interfering substances prevent chemical determination of DNA and RNA. However, after $^{32}$P incorporation, no further alkaline hydrolyzable counts are found after the KOH treatment and cold perchloric acid washes, and no further hot acid hydrolyzable counts are found after the two extractions with hot acid.

Similar results are obtained by $^{32}$P-phosphate and 8-$^{14}$C-adenine incorporation.

The extracts were neutralized and dried and their radioactivities were measured with a gas-flow counter.

## RESULTS

*Inhibition of DNA, RNA and protein synthesis:* Nalidixic acid at 10 $\mu$g/ml gives 74% inhibition of DNA synthesis. This is near that found at 100 $\mu$g/ml, which gave 81% inhibition. 10 $\mu$g/ml gives 15% inhibition of RNA synthesis in two hours. There was no inhibition of $^{14}$C-arginine incorporation in two hours at any concentration up to 100 $\mu$g/ml.

FUdR at 1 mM gives 33% inhibition of DNA synthesis in two hours. At $10^{-2}$ M, 89% inhibition is obtained. Cell division is inhibited by both these concentrations, but recovers after two and one-half hours. The effect on recombination described below is obtained by treatment with $10^{-5}$ M or higher concentrations (HASTINGS 1964). No uniform inhibition of RNA synthesis is seen within two hours. This concentration gives 22% inhibition of protein synthesis under these conditions.

Hydroxyurea at 1 and 5 mM gives 71% inhibition of DNA synthesis, which is the maximal amount found. While 1 mM gives no inhibition of RNA synthesis, a 27% inhibition is produced by 5 mM. There is no inhibition of incorporation of $^{14}$C-arginine in two hours at these concentrations.

Mitomycin C gave 88% inhibition of DNA synthesis, when used at 100 $\mu$g/ml, and 90% inhibition at 200 $\mu$g/ml. The residual DNA synthesis occurs in the first 90 minutes. No consistent inhibition of RNA synthesis was found in two hours. Incorporation of $^{14}$C-arginine in two hours was inhibited 12% at 100 $\mu$g/ml and 16% at 500 $\mu$g/ml.

Actinomycin D produces 53% inhibition of RNA synthesis (determined by $^{14}$C-adenine incorporation) at 10 $\mu$g/ml, and an 84% inhibition at 100 $\mu$g/ml. This is the greatest amount obtained, and is seen for all concentrations above 30 $\mu$g/ml. At 10 $\mu$g/ml, 32% inhibition of protein synthesis is seen in two hours. 10 $\mu$g/ml Actinomycin D produces no inhibition of DNA synthesis, while 50 $\mu$g/ml inhibits DNA synthesis by 51%.

Phenethyl alcohol at 0.1% causes a 63% inhibition of DNA synthesis, 75% inhibition at 0.4%, and 81% inhibition at 1% phenethyl alcohol. At 0.4% phenethyl alcohol, the 25% DNA synthesis occurs in 60 to 90 minutes, and

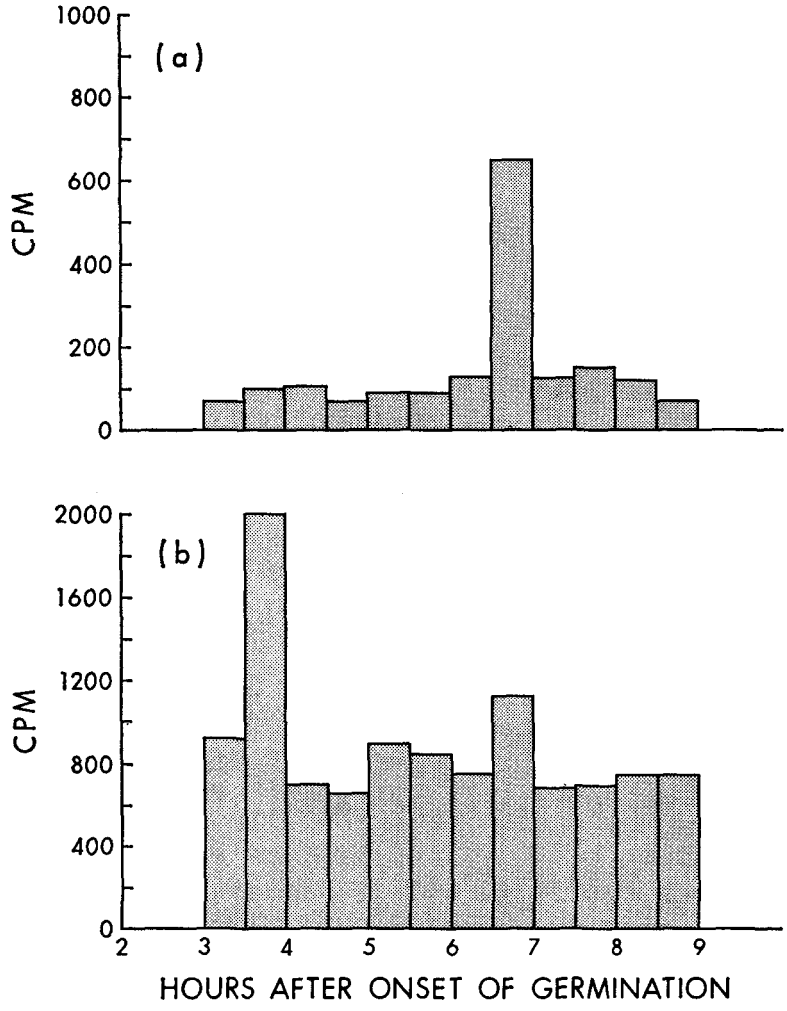


FIGURE 1.—Incorporation of $^{32}P$ into (a) DNA and (b) RNA during one-half hour pulses at different times during the germination of zygospores.

inhibition is complete after this time. All these concentrations showed a 95% inhibition of RNA synthesis as determined by $^{32}P$ incorporation for one and one-half hours. Incorporation of $^{14}C$-arginine in two hours was inhibited 62% by 0.1% phenethyl alcohol, and 99% by the other concentrations used.

Cycloheximide at 30 μg/ml gave 69% inhibition of protein synthesis as determined by $^{14}C$-alanine, and by $^{14}C$-glycine. This was the greatest inhibition of alanine incorporation which was seen, but the maximal inhibition of $^{14}C$-glycine incorporation was 84%, obtained by 50 μg/ml or more of cycloheximide. There was no inhibition of RNA synthesis during one and one-half hours $^{32}P$ incorporation. At 30 μg/ml there was an 11% inhibition of DNA synthesis. At 50 μg/ml and higher DNA synthesis inhibition was 46%.

*Determination of DNA synthesis:* The result of $^{32}P$-phosphate incorporation into the DNA fraction at different times during germination is shown in Figure 1. The main peak of incorporation occurs between six and one-half and seven hours after the start of germination. This is confirmed by continuous labelling experiments in which the main increase in the amount of incorporated $^{32}P$ occurs during this time, with little increase afterwards. Since very little incorporation is observed preceding or following this period, it appears that this period of synthesis lasts only thirty minutes.

*RNA Synthesis during germination:* There is some incorporation of $^{32}P$ into RNA throughout germination (Figure 1). However, incorporation is very high three and one-half to four hours after the start of germination. Two lesser peaks of incorporation are found at a time preceding and during the main period of DNA synthesis.

*Effect of phenethyl alcohol on the survival of germinating zygospores:* When zygospores of *Chlamydomonas reinhardi* were pulse-treated with 0.4% phenethyl alcohol, the germination was blocked almost completely at five and one-half to six hours after germination. A depression in the survival was also found when the treatment was at prophase (Figure 2a and Table 1). The first sensitive period is one hour before the main period of DNA synthesis, as determined by the $^{32}P$ incorporation experiment. Zygospores treated at any time with 1% phenethyl alcohol did not germinate. Treatment with 0.2% phenethyl alcohol had no effect.

*Effect of inhibitors of DNA synthesis on recombination:* The concentration of inhibitors used in this investigation had no significant effect on the survival of treated zygospores. About 90% of zygospores germinated. However, the time between the onset of germination and the release of meiotic products is prolonged after DNA synthesis inhibition.

It has also been found that the responsive time of zygospores to the treatment

TABLE 1

*Germination and recombination between* arg-1 *and* arg-2 *after treatment of germinating zygospores at various times with 0.4% phenethyl alcohol. Contingency $\chi^2$ values have 1 degree of freedom. Control germination was 92.5% and control recombination was 14.3%*

| Time of treatment | Total zygospores counted | Percent germination | Total colonies counted | Percent recombinant tetrads |
|---|---|---|---|---|
| 4–4½ | 567 | 66.0 | | |
| 4½–5 | 460 | 76.5 | 497 | 10.1 ($\chi^2$=5.2)* |
| 5–5½ | 368 | 93.4 | 1792 | 10.8 ($\chi^2$=5.1)* |
| 5½–6 | 411 | 0 | | |
| 6–6½ | 276 | 85.5 | 1010 | 16.73 ($\chi^2$=2.6) |
| 6½–7 | 396 | 86.1 | 1248 | 14.5 |
| 7–7½ | 295 | 65.1 | 1206 | 13.8 |
| 7½–8 | 348 | 73.9 | | |
| 8–8½ | 430 | 33.7 | | |
| 8½–9 | 428 | 76.9 | | |

* $P < .05$.

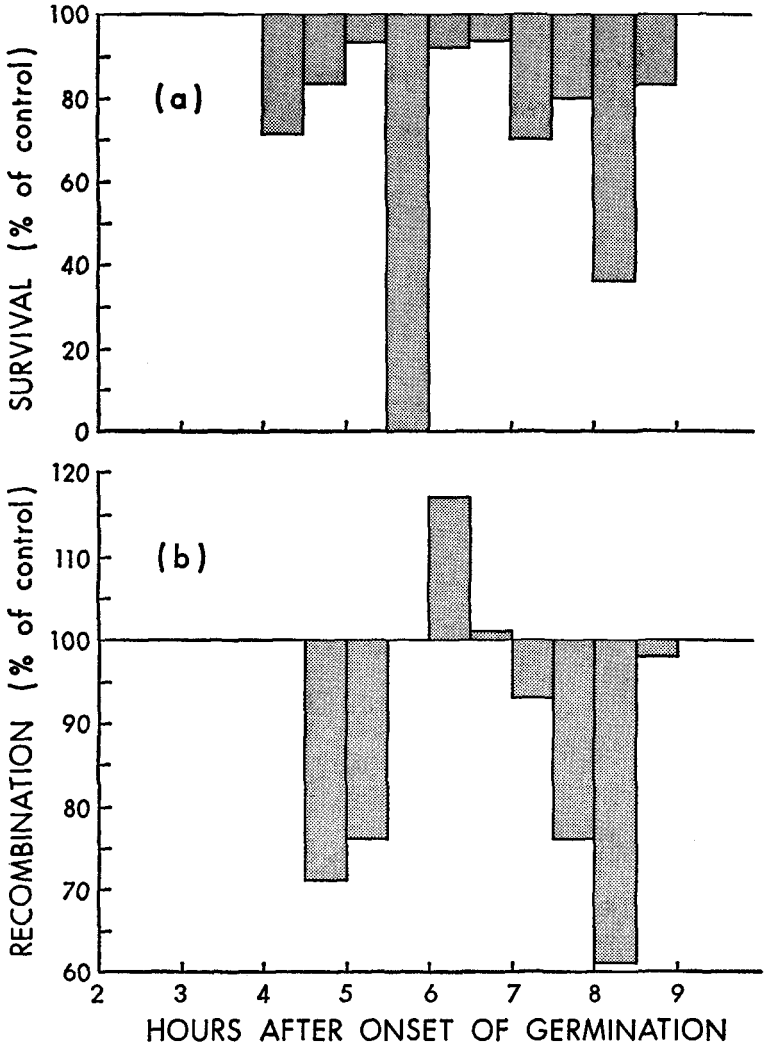


FIGURE 2.—Survival of zygospores and recombination between *arg-1* and *arg-2* following one-half-hour treatments with 0.4% phenethyl alcohol at different times during germination. Both are expressed as a percentage of the control value. (a) survival, (b) recombination. Recombination could not be measured in the five and one-half to six hour sample because of the low germination. Germination in the control was 92.5%.

with a given inhibitor varies from experiment to experiment. Since the time of pre-meiotic DNA synthesis, meiosis, and liberation of meiotic products show similar variation, it is assumed that it is the rate of germination which varies. Hence, for making the comparison of the effective times of different inhibitors possible, an inhibitor of known effect was used as time marker in each experiment. It has been observed that the effective times of various inhibitors are separated by quite constant intervals and the sequence remains the same. Thus

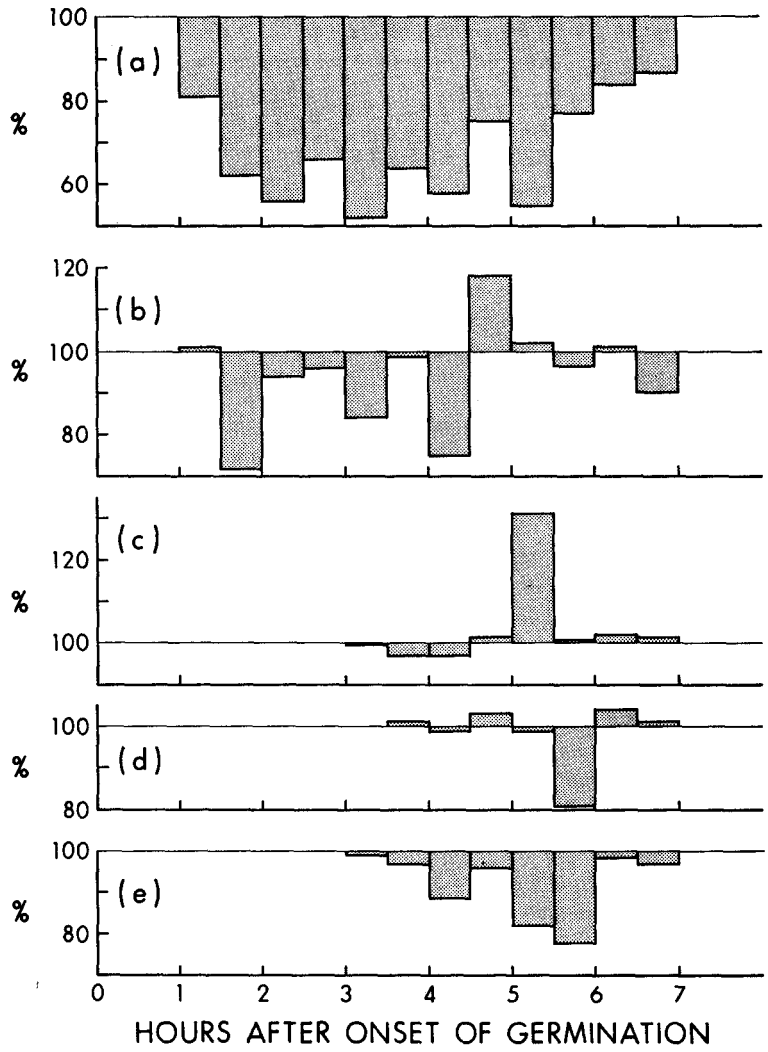


FIGURE 3.—Recombination between *arg-1* and *arg-2* as a percentage of the control value following one-half-hour treatments with various agents during zygospore germination. (a) 30 μg/ml cycloheximide, (b) 10 μg/ml actinomycin D, (c) 100 μg/ml mitomycin C, (d) 1 mM FUdR, and (e) 10 μg/ml nalidixic acid. These results are all from the same experiment.

it is taken that the time when recombination is reduced by nalidixic acid in Figure 3e and in Figure 4b represents the same stage in germination even though the actual time differs.

Figure 3 and Table 2 show that the treatment with mitomycin C gives an increase in recombination at five to five and one-half hours after the beginning of germination and FUdR and nalidixic acid depress recombination 30 minutes later.

The effect of the treatments with nalidixic acid and hydroxyurea is shown in

TABLE 2

*Recombination between* arg-1 *and* arg-2 *in zygospore samples taken at times during germination and treated with various agents. Contingency $\chi^2$ values against the control have 1 degree of freedom*

| Time of treatment | Control | | 30 μg/ml Cycloheximide | | 10 μg/ml Actinomycin D | |
|---|---|---|---|---|---|---|
| | Total colonies counted | Percent recombinant tetrads | Total colonies counted | Percent recombinant tetrads | Total colonies counted | Percent recombinant tetrads |
| 1–1½ | 611 | 24.4 | 1594 | 19.8 | 903 | 24.6 |
| 1½–2 | 674 | 24.6 | 1912 | 15.3 | 1687 | 17.8 |
| 2–2½ | 808 | 24.9 | 1742 | 14.0 | 1421 | 23.4 |
| 2½–3 | 1161 | 24.7 | 1138 | 16.3 | 1658 | 23.7 |
| 3–3½ | 1270 | 24.9 | 1063 | 13.2 | 1285 | 20.9 |
| 3½–4 | 761 | 25.0 | 1346 | 16.1 | 527 | 24.8 |
| 4–4½ | 1621 | 24.9 | 1040 | 14.7 | 1509 | 18.7 ($\chi^2$=17.4)*** |
| 4½–5 | 1578 | 25.1 | 1104 | 18.9 | 1504 | 29.6 ($\chi^2$=8.0)** |
| 5–5½ | 1288 | 24.5 | 986 | 13.5 | 1241 | 25.0 |
| 5½–6 | 1174 | 25.4 | 1258 | 19.9 | 1517 | 24.5 |
| 6–6½ | 1691 | 24.5 | 1770 | 20.5 | 1573 | 24.6 |
| 6½–7 | 1339 | 25.0 | 1323 | 21.7 | 763 | 22.5 |
| | 100 μg/ml Mitomycin C | | 1 mM FUdR | | 10 μg/ml Nalidixic Acid | |
| 3–3½ | 1015 | 24.9 | 1381 | 25.0 | 1170 | 24.8 |
| 3½–4 | 1374 | 24.6 | 1120 | 25.4 | 1304 | 24.3 |
| 4–4½ | 1165 | 24.5 | 1412 | 24.6 | 868 | 22.1 ($\chi^2$=2.4) |
| 4½–5 | 1129 | 25.3 | 1022 | 25.8 | 1609 | 24.1 |
| 5–5½ | 1034 | 32.1 ($\chi^2$=16.0)*** | 1221 | 24.4 | 1108 | 20.1 ($\chi^2$=6.6)* |
| 5½–6 | 1228 | 25.4 | 1090 | 20.5 ($\chi^2$=8.0)** | 1352 | 19.8 ($\chi^2$=11.5)*** |
| 6–6½ | 1433 | 25.0 | 1469 | 25.5 | 1889 | 24.1 |
| 6½–7 | 855 | 25.4 | 506 | 25.3 | 1041 | 24.2 |

* $P < .05$ ** $P < .01$ *** $P < .001$.

Table 3 and Figure 4, b and c. As shown in the figure, hydroxyurea has a similar effect to nalidixic acid. Both reduce recombination between six and seven hours, with the greater effect being produced between six and one-half and seven hours. Since the same population of zygospores was used for the $^{32}P$ incorporation experiment shown in Figure 1, with DNA synthesis between six and one-half and seven hours, it is concluded that all these DNA inhibitors except mitomycin C affect recombination at this period of pre-meiotic DNA synthesis. The mitomycin C effect is 30 minutes earlier than the period of DNA synthesis.

*Effect of phenethyl alcohol on recombination:* During the period between five and one-half and six hours, when 0.4% phenethyl alcohol treatment is lethal, it is not possible to measure recombination. Before this time recombination is depressed by treatment. In the sample treated from six to six and one-half hours,

TABLE 3

*Recombination between* arg-1 *and* arg-2 *after treatment with various agents at different times during zygospore germination. Contingency $\chi^2$ values have 1 degree of freedom. Control recombination was 20.0%*

| | 100 μg/ml Actinomycin D | | 10 μg/ml Nalidixic Acid | | 3 mM, Hydroxyurea | |
|---|---|---|---|---|---|---|
| Time of treatment | Total colonies counted | Percent recombinant tetrads | Total colonies counted | Percent recombinant tetrads | Total colonies counted | Percent recombinant tetrads |
| 3–3½ | 1324 | 16.7 ($\chi^2$=4.0)* | | | | |
| 3½–4 | 1666 | 13.1 ($\chi^2$=27.7)*** | | | | |
| 4–4½ | 1318 | 13.8 ($\chi^2$=10.2)** | 1164 | 20.3 | 1365 | 19.5 |
| 4½–5 | 1424 | 16.0 ($\chi^2$=6.2)* | 1411 | 19.9 | 1828 | 19.6 |
| 5–5½ | 1475 | 20.3 | 992 | 20.4 | 877 | 19.6 |
| 5½–6 | 2440 | 26.2 ($\chi^2$=20.1)*** | 1049 | 21.2 | 1516 | 19.8 |
| 6–6½ | 802 | 14.1 ($\chi^2$=11.1)*** | 1313 | 14.2 ($\chi^2$=14.6)*** | 1154 | 14.4 ($\chi^2$=12.0)*** |
| 6½–7 | 588 | 7.8 ($\chi^2$=46.2)*** | 991 | 13.7 ($\chi^2$=15.7)*** | 2458 | 10.3 ($\chi^2$=78.3)*** |
| 7–7½ | 1260 | 16.1 ($\chi^2$=5:8)* | 1409 | 19.2 | 1696 | 19.8 |

* $P < .05$ ** $P < .01$ *** $P < .001$.

recombination is higher than the control level (Figure 2b and Table 1). This is the same time as the increase induced by mitomycin C, and is 30 minutes before the main period of DNA synthesis. Although the increase caused by phenethyl alcohol is not significant, the same enhancement in recombination has been produced in other experiments, and the pooled data of two experiments show that the increase is significant ($\chi_1^2 = 6.4$, $p < .02$).

*Effect of the inhibition of RNA synthesis on recombination:* The striking result of the treatment with 10 μg/ml actinomycin D was the stimulation of recombination at a time 30 minutes earlier than the effective time of mitomycin C, or 60 minutes before the DNA synthetic period (Figure 3, Table 1). The early effective time, however, varies from experiment to experiment. At a concentration of 100 μg/ml this inhibitor gave a greater increase in recombination at five and one-half hours, but it also strongly depressed recombination at the same effective time as nalidixic acid and hydroxyurea (Figure 4). This is compatible with the observation that DNA synthesis as well as RNA synthesis is inhibited at high concentration.

*Effect of the inhibitor of protein synthesis on recombination:* Treatment with cycloheximide caused a general depression of recombination during the early part of germination with a pattern very similar to that produced by actinomycin D (Figure 3). Recombination was near the control value during the period of DNA synthesis.

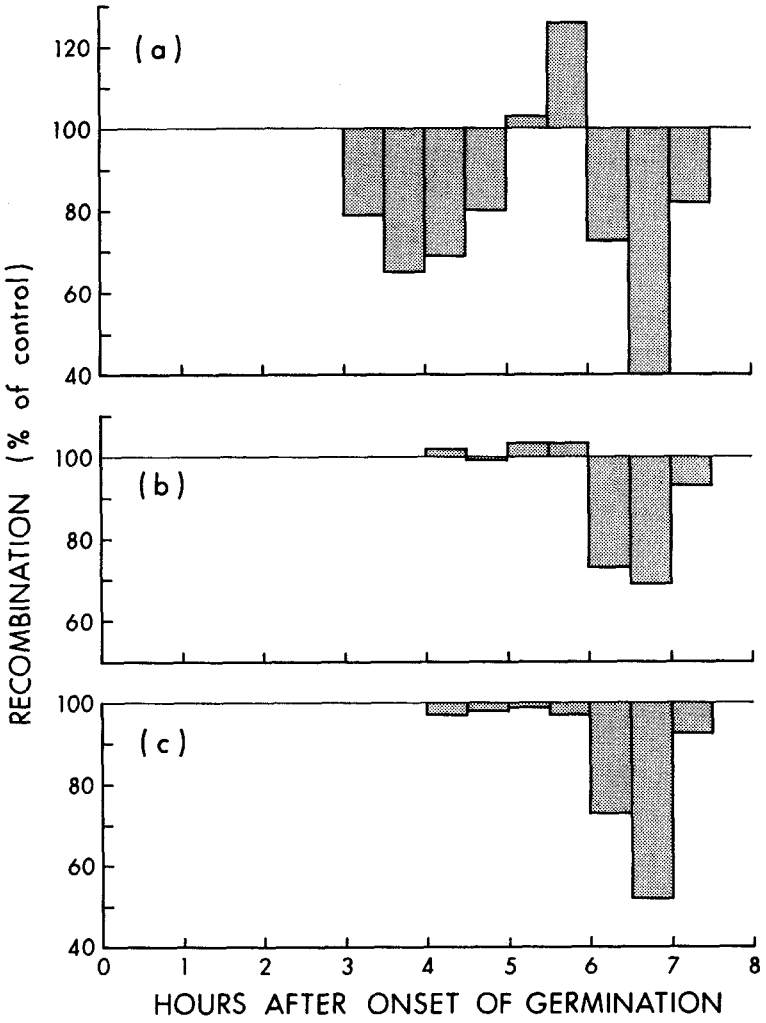


FIGURE 4.—Recombination between *arg-1* and *arg-2*, compared with the control value, following one-half-hour treatments during zygospore germination with (a) 100 μg/ml actinomycin D, (b) 10 μg/ml nalidixic acid, and (c) 3 mM hydroxyurea. These results are from the same experiment.

## DISCUSSION

SUEOKA, CHIANG and KATES (1967) have shown by density labelling experiments that there is no replication of the α band (chromosomal) DNA during zygote maturation, but that it replicates once during germination of the zygospore. During germination the chloroplast DNA (β-band) replicates several times, but it is still a minor fraction of the total DNA (CHIANG 1971). We have repeated CHIANG's experiment (CHIU and HASTINGS, unpublished results) and confirmed the single replication of α-band; we found two replications of β-band.

These events occurred during the period covered by the $^{32}P$ incorporation experiment shown in Figure 1, which extends to after the time of meiosis. Thus it would appear that the main period of DNA synthesis detected by $^{32}P$ incorporation between six and one-half and seven hours is this replication of the nuclear DNA, since this is the only extensive amount of DNA synthesis found during the time in which $\alpha$-band replication occurs. This S-period, occurring before meiosis, and being the only period of $\alpha$-band synthesis in the zygote (SUEOKA, CHIANG and KATES 1967), would appear to be the premeiotic S-period. The premeiotic S-period does not occur in the gametes before they fuse, since gametes have almost the same DNA content as vegetative cells (SUEOKA, CHIANG and KATES 1967). These authors are not specific about the time in the cell cycle when the vegetative DNA content was determined, but KATES, CHIANG and JONES (1968) show that the DNA content of gametes is the same as the minimal DNA content of vegetative cells during the GI phase.

The DNA synthesis inhibitors which we have used show satisfactory specificity at the concentrations used, except phenethyl alcohol which gave almost complete inhibition of RNA and protein synthesis at all concentrations. Actinomycin D was shown to be specific for RNA synthesis inhibition at the low concentration used, but to have some effect on DNA synthesis at the higher concentration. Cycloheximide is specific for protein synthesis inhibition at the concentration used here. These agents have been shown to be specific in other systems (SHIBA *et al.* 1959; SZYBALSKI and IYER 1967; and HOTTA and STERN 1971).

Support for the specific mode of action of inhibitors is found in the observation that actinomycin D produces an effect like that of FUdR, hydroxyurea and nalidixic acid when it is used at a concentration which was found to produce a partial inhibition of DNA synthesis, but not when used at a concentration which inhibits only RNA synthesis. The relationship of the time of the pre-meiotic S-period to the time of these effects supports the assumption that DNA synthesis is the target of the treatment of the DNA synthesis inhibitors.

These experiments confirm the observation that DNA synthesis inhibitors (HASTINGS 1964; DAVIES and LAWRENCE 1967) including $\gamma$ radiation (LAWRENCE 1965, 1968) have an effect on the frequency of crossing over long before meiotic prophase. This, with the observation of ROSSEN and WESTERGAARD (1966) that recombination cannot occur before meiotic prophase, makes it safe to assume that the pre-meiotic effects are all caused before the time at which crossing over actually occurs.

The use of this one region of linkage-group I leaves open the possibility that crossovers have merely been redistributed. However, it has been shown by DAVIES and LAWRENCE (1967) that other regions in *Chlamydomonas reinhardi* respond in the same way as this region.

LARK and LARK (1966) reported that at a specific concentration, phenethyl alcohol inhibits a new round of replication, but allows the completion of already initiated replication, probably by inhibition of initiator production, or by attacking a protein concerned in initiation. We found that phenethyl alcohol is by no means specific for DNA synthesis in this system, inhibiting RNA and protein

synthesis more efficiently than DNA synthesis. However, we saw that there was complete inhibition of DNA synthesis after 60 to 90 minutes. Such a delayed effect would be expected if DNA synthesis were inhibited by preventing initiation. Phenethyl alcohol was found to have an effect on recombination like that produced by mitomycin C, which affects DNA synthesis after a delay, but does not affect protein or RNA synthesis. Neither actinomycin D nor cycloheximide has this particular effect of phenethyl alcohol. It therefore seems likely that inhibition of DNA synthesis initiation is responsible for this effect, and this will be assumed in the discussion which follows.

Since the first period of reduction in viability produced by phenethyl alcohol is one hour before S-period, this is apparently the time of initiator production. Zygospores treated at this time do not recover, so the effect appears to be irreversible in this system. At six to six and one-half hours after the beginning of germination, inhibition of initiation is no longer lethal, but it produces a significant increase in recombination. HOTTA and STERN (1971) have demonstrated that DNA synthesis during zygotene is a part of chromosome replication which has been delayed. Since some eukaryotes have been shown to have many regions of replication per chromosome (PLAUT and NASH 1964) it would appear that in Chlamydomonas, those replicons which are not initiated until late in the initiation period (six to six and one-half hours) need not be replicated in the main S-period. The obligate part of the initiation (when inhibition is lethal) may concern regions which must be replicated in S-period, or it may be that a certain proportion of the DNA must be replicated in S-period for the zygospore to complete germination. From this it would seem that the regions in which DNA synthesis has been delayed comprise whole replicons. This is supported by the observation of a second period of lethality induced by phenethyl alcohol during prophase (eight hours) (Figure 2), implying that initiation is required to replicate the delayed regions.

The increase in recombination produced by phenethyl alcohol at the end of the initiation period is explained by the hypothesis that the amount of recombination is directly proportional to the number of regions in which replication has been delayed.

On this hypothesis, any other agent which causes an increase in recombination just prior to the S-period may also be doing so by increasing the amount of delayed replication. Mitomycin C causes an increase at the same time as phenethyl alcohol. In addition, a similar increase in recombination is produced by treatment with actinomycin D, 30 minutes earlier than the time of effect of mitomycin C (Figure 3). RNA synthesized at this time therefore appears to be implicated in DNA synthesis initiation. If this were a messenger RNA for a protein concerned with initiation of replication, protein synthesis inhibition would be expected to produce an increase in recombination between the time of the actinomycin D effect and that of the phenethyl alcohol and mitomycin C effect. There is a general depression of recombination produced by cycloheximide at and before this time, and no significant increase above this depressed level has been found in several experiments. This is also true when the length of pulse

treatment is reduced to 15 minutes. It may be that the expected increase is masked by the depression in recombination, or that the messenger is translated when the inhibition is released so that there is no permanent effect of the inhibition. Alternatively, it might be concluded that the RNA responsible for this effect is not being translated.

The other three inhibitors of DNA synthesis used in this study, FUdR, nalidixic acid and hydroxyurea, all produce a decrease in recombination during the S-period. These are agents which are expected to produce an immediate inhibition (COHEN *et al.* 1958; GOSS *et al.* 1965; HOTTA and STERN 1971) and which therefore stop synthesis that has already been initiated. The effect of these inhibitors on recombination can be reconciled with the hypothesis above if it is postulated that the availability of DNA precursors, produced by inhibition, induces the initiation of more replicons. This would reduce the number of delayed replicons, and hence the amount of recombination. We cannot explain why, on the proposed mechanism of action of mitomycin C (SZYBALSKI and IYER 1967), mitomycin C should affect recombination like phenethyl alcohol, rather than like FUdR. When mitomycin C is used at 200 μg/ml, there is a 10% reduction in recombination at the time of the FUdR effect, but this reduction is not significant. This raises the possibility that the concentration used has a greater effect on initiation than on the progress of replication, but that an effect like that of FUdR could be found at other concentrations of mitomycin C. Such a difference in concentration requirement might be caused by a difference in GC content of DNA in different regions (SZYBALSKI and IYER 1967). The hypothesis predicts that mitomycin C treatment will increase the amount of prophase DNA synthesis, while FUdR will decrease it. An attempt is being made to test this prediction.

This interpretation of the mitomycin C effect is supported by the observation that mitomycin C at 100 μg/ml causes complete inhibition of DNA synthesis after a delay, as does phenethyl alcohol.

Since all the observations reported here of the effects of agents at or near the pre-meiotic S-period are compatible with the hypothesis that the amount of recombination varies directly as the number of regions of delayed replication, it is worth asking what this relationship might mean.

One possibility is that the effect is indirect. ROTH and ITO (1967) have shown that the zygotene DNA synthesis, which is delayed replication (HOTTA and STERN 1971), is needed for synapsis, since inhibition of this synthesis prevents synapsis. This makes it possible that the degree of synapsis varies as the amount of delayed synthesis, and the amount of recombination shows the same relationship because it is dependent on synapsis.

An interesting possibility is that recombination and delayed replication are directly related. This would be the case if a region in which replication had not yet occurred were the primary structure in a recombination event. The delayed replication would then occur during recombination, and no endonuclease activity would be needed for making the initial nucleotide chain breaks.

PUTRAMENT (1971) has documented the locus specificity of conversion frequencies, and the region specificity of the action of genes modifying recombi-

nation frequency. She attributes these phenomena to structural aspects of chromosomes. However, since MULDOON *et al.* (1971) have shown region specific control of the time of DNA synthesis in the myxomycete *Physarum*, it is possible to attribute locus specific control of recombination to control of replication. Specifically, on the model that a region where replication is delayed becomes the site of a recombination event, the decision as to which replicons are delayed in their replication would control the distribution of recombination events on the chromosomes.

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