

## Meiosis in *Schizophyllum commune*: premeiotic DNA replication and meiotic synchrony induced with hydroxyurea

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### SUMMARY

Hydroxyurea (HU) effectively inhibits meiosis in *Schizophyllum*. The predominant cytological stage in inhibited fruit bodies is fusion. The inhibition is reversible and makes possible synchronization of a naturally nonsynchronous system. Microphotometric determinations of the DNA content in prefusion nuclei treated with HU suggest that premeiotic DNA replication occurs in prefusion nuclei. Synaptonemal complexes are not completed in HU-treated nuclei, suggesting that this event is dependent on premeiotic DNA replication.

### 1. INTRODUCTION

In the last decade much new information has been contributed to our understanding of the molecular events occurring at meiosis. The studies on *Lilium* (Stern & Hotta, 1973) and on the fungus *Coprinus* (Lu & Jeng, 1975) have shown the existence of previously undetected periods of DNA synthesis during the zygotene and pachytene stages of early meiosis, and have correlated peaks of enzyme activity with specific meiotic stages. These achievements were made possible by the exploitation of naturally synchronous meiosis in both *Lilium* and *Coprinus*.

Studies of meiosis in additional species are important for the accumulation of a body of data from which generalizations may be drawn; in addition, other organisms can contribute information about such meiosis-related processes as recombination, mutation and repair. In species where meiosis is not naturally synchronous, experimental means must be found to control initiation or some early meiotic or premeiotic stage. Hydroxyurea, for example, has been shown to inhibit DNA synthesis reversibly at mitotic S phase in many prokaryotic and eukaryotic organisms (Timson, 1975). Since DNA synthesis at meiotic S phase is also inhibited by hydroxyurea in lily (Hotta & Stern, 1971), in *Chlamydomonas* (Chiu and Hastings, 1973), and in yeast (Simchen, Idar & Kassir, 1976), this chemical can serve as a tool for the synchronization of meiosis.

The fungus *Schizophyllum commune* has been utilized for the study of basic genetic phenomena related to meiosis, e.g. the fine control of meiotic recombination, the 'meiotic effect' on spontaneous mutation frequency, and the interrelations between repair and recombination (Simchen & Stamberg, 1969*a, b*; Koltin & Stamberg, 1973; Stamberg & Koltin, 1973; Koltin, Stamberg & Ronen, 1975;

Hundert, Koltin & Stamberg, 1978). Further characterization of meiotic events has been hampered by the lack of natural meiotic synchrony in *Schizophyllum*. We now report an experimental procedure, using hydroxyurea, which synchronizes populations of cells. The effect of hydroxyurea on basidiospore sporulation and germination has been reported elsewhere (Carmi *et al.* 1977). Here we characterize the cytology of hydroxyurea-treated meiotic cells. In addition, the synchronization procedure has provided us with information on the timing of the premeiotic DNA synthesis in *Schizophyllum*.

## 2. MATERIALS AND METHODS

(i) *Strains*. The two haploid strains, both prototrophs, are from our stock collection. The strains are fully compatible and form a fertile dikaryon.

(ii) *Media and growth conditions*. Dikaryons were grown at  $21 \pm 2$  °C. Synchronous fruiting was obtained by the method of Schwalb (1971). This method entails the transfer of mycelium onto a cellophane membrane overlying the solid medium. The standard *Schizophyllum* solid complete medium (Stevens, 1974) was used after treatment with hydroxyurea.

(iii) *Hydroxyurea treatment*. Hydroxyurea (HU) was obtained from Sigma Chemical Co., St Louis. A fresh solution was prepared before each use by dissolving in water at 37–40 °C and filter-sterilizing. It was added to the medium in a final concentration of 0.075 M. Pieces of membrane with adherent young fruit bodies were transferred to Schwalb's medium containing HU.

(iv) *Cytological examinations*. Fruit bodies were removed from HU at varying times and were fixed in ethanol, propionic acid and aqueous chromic acid (modified from Lu & Raju, 1970). Staining was with propionic iron hematoxylin according to Henderson & Lu (1968). Fixation and staining times were as described by Radu, Steinlauf & Koltin (1974).

Microphotometry was performed to determine the relative DNA content of individual nuclei. Fixation of fruit bodies was similar to the description of Rossen & Westergaard (1966) for fruit bodies of *Neotiella*. The fixative was stored at 25 °C and retained its activity for up to 3 months. Hydrolysis was performed in 7.5 N-HCl for 10 min at room temperature. Fruit bodies were stained with Feulgen for at least 45 min. Gills were squashed on a slide and the nuclear mass was determined with a Zeiss scanning microscope photometer Model 05. The two-wave-length method of Patau (1952) for irregular-shaped objects was used. Absorbance was measured at 505 and 570 nm.

Electron microscopy was performed on fruit bodies from untreated and HU-treated cultures after 8 h of treatment. Fixation was in either 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4 °C for 12 h, or in Karnovsky's fixative (Karnovsky, 1965) in the same buffer. Postfixation was in 1% OsO<sub>4</sub> for 10 h; the material was then dehydrated and embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969). The sections were stained in uranyl acetate and lead citrate. Sections were examined in a Jeol Jem T7 electron microscope.

## 3. RESULTS

(i) *Distribution of premeiotic and meiotic stages in untreated fruit bodies*

Previous cytological studies of meiotic stages in *Schizophyllum* concur that the chromosomes are very small, some stages are very brief, and that therefore only a limited number of meiotic stages can be identified (Radu *et al.* 1974; Erlich & McDonough, 1949; Haapala & Nienstedt, 1976). In the present study we have recognized the following identifiable periods, of which periods 2–5 constitute meiosis (Fig. 1): (1) pre-fusion, characterized by cells with two nuclei aligned along the longitudinal axis of the basidium; (2) fusion, characterized by one oval nucleus in the centre of the basidium (we include in this period the stages of prophase I); (3) metaphase I, characterized by contracted chromosomes arranged near the apex of the basidium; (4) telophase I, having two nuclei near the apex of the basidium and aligned along a horizontal axis. Stages in the second meiotic division are very rarely seen, no doubt because they occur in a very short period of time. The next discernible stage is (5) telophase II, characterized by four nuclei in the basidium; (6) sterigma formation, in which four small hyphal branches are seen on the apex of the basidium and the nuclei are migrating into these sterigmata; and (7) mature spores, probably detached from the sterigmata by the process of making the squash preparation.

To characterize the distribution of meiotic steps in the normal fruit body, gills were removed from a series of young fruit bodies at intervals of 1 or 2 h for a total of 9 h. The gills were stained and examined cytologically. Samples of from 100 to 500 cells in meiosis were examined at each time interval for every experiment. Results of all replicate untreated samples were consistent and no significant variation between time intervals was ever found. The data were therefore pooled (Table 1). The distribution of premeiotic and meiotic stages in one such experiment, based on a sample of 1494 cells is as follows: pre-fusion, 26.0%; fusion, 66.9%; metaphase I, 2.9%; telophase I, 2.9%; telophase II, 1.3%. The ratio of cells in postmeiotic stages to those in meiotic stages was calculated, indicating that for every 100 cells in various stages of meiosis there are 4 cells with sterigmata and 9 mature spores (representing slightly more than 2 basidia whose spores were detached by the squash procedure). These spores would presumably have been released from the fruit body within a very short time.

(ii) *Distribution of premeiotic and meiotic stages in HU-treated fruit bodies*

At time 0, young fruit bodies were transferred to medium containing HU. Starting 3 h later, samples of gills were removed every hour for cytological examination. Replicate fruit bodies for each time interval gave consistent results and were therefore pooled, giving samples of from 500 to more than 3300 meiotic cells for each time interval (Table 1, Figs. 2 and 3). The most notable differences occur in the frequency of cells in the pre-fusion and fusion states. Within 3 h of HU treatment pre-fusion cells have dropped from 26% of the total to 16.9%; this trend continues until 7 h, when a stable value of 5 to 6% is reached (as shown by

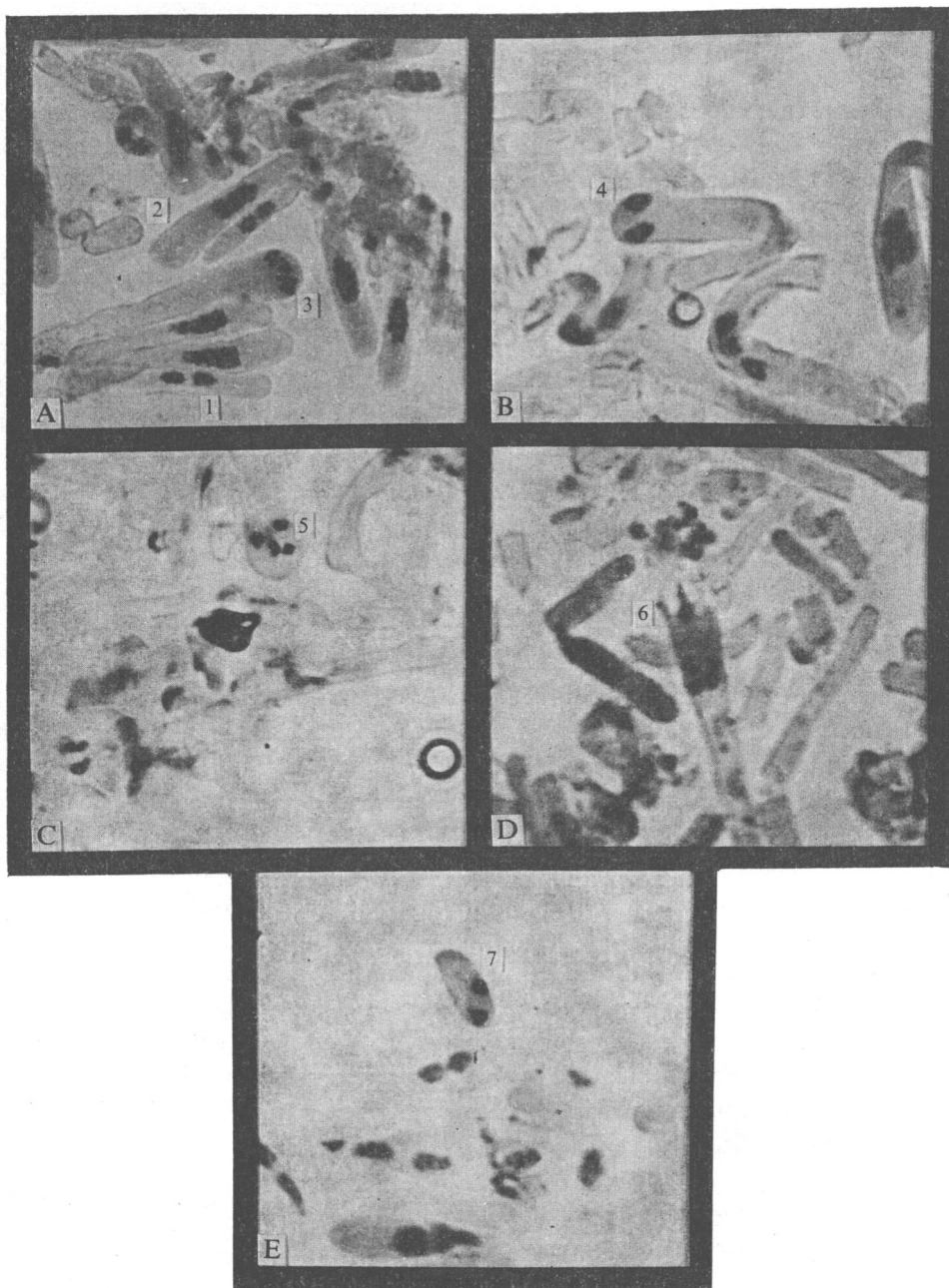


Fig. 1. Cytologically identifiable stages of meiosis: prefusion (A1), fusion (A2), metaphase I (A3), telophase I (B4), telophase II (C5), sterigmata formation (D6), mature spores (E7). For details see text.

Table 1. Distribution of premeiotic, meiotic and postmeiotic stages in HU-treated fruit bodies

Time (h)	Cells in each stage (%)					Total no. meiotic cells	No. cells with sterigmata/no. meiotic cells	No. spores/no. meiotic cells
	Prefusion	Fusion	Meta-phase I	Telo-phase I	Telo-phase II			
Untreated*	26.0	66.9	2.9	2.9	1.3	1494	0.04	0.09
3	16.9	76.5	2.5	2.2	0.9	1863	0.03	0.03
4	19.3	76.3	3.0	0.8	0.6	532	0.03	0.04
5	12.3	79.0	2.8	2.1	0.8	1554	0.04	0.02
6	9.4	87.6	1.5	1.2	0.3	1146	0.02	0.02
7	6.6	87.3	3.0	2.0	1.1	3382	0.02	0.02
8	5.3	90.2	3.1	0.8	0.6	1203	0.03	0.01
9	6.3	91.4	1.5	0.8	0.0	791	0.02	0.01

\* The untreated control represents a pooled sample of cells taken at 1- or 2-hour intervals up to 9 h from untreated fruit bodies, and found to be homogeneous by  $\chi^2$  test.

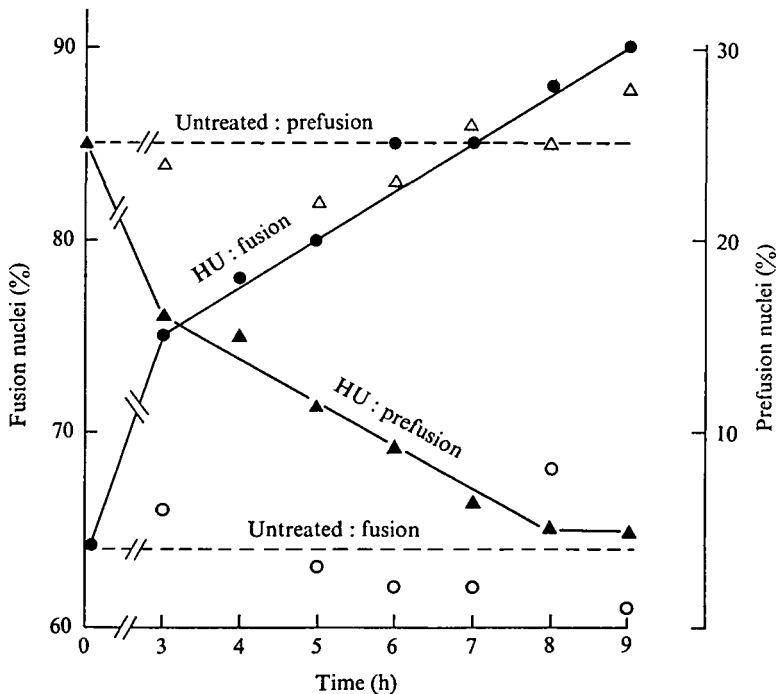


Fig. 2. Frequency of meiotic cells at the prefusion and fusion stages in fruit bodies treated with HU and in untreated controls.

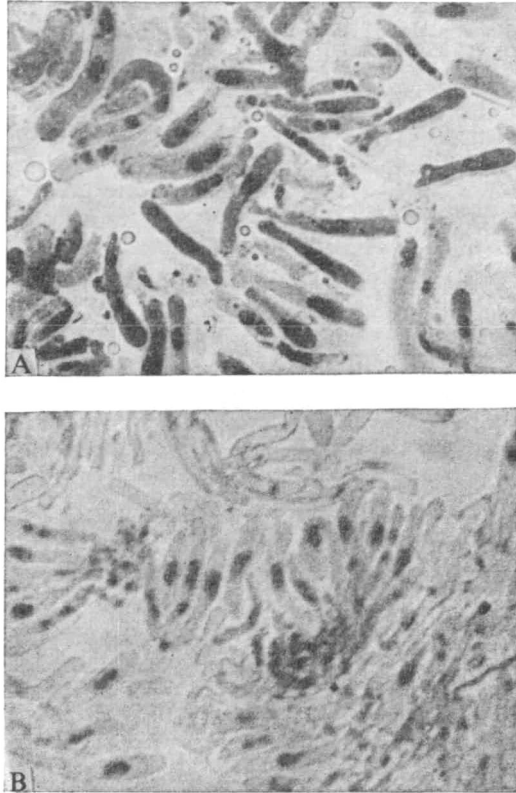


Fig. 3. Basidia from untreated (A) and HU-treated (B) fruit bodies. Note the high frequency of basidia at the fusion stage in the treated fruit body.

a chi-square homogeneity test). For the fusion cells, the trend is exactly the opposite. Within 3 h of treatment fusion cells have risen from 66.9% to 76.5% of the total number of meiotic cells; by 8 h the value is stabilized at over 90%.

The meiotic stages from metaphase I onward collectively account for 7% of the total in untreated fruit bodies. These stages remain essentially constant in frequency in the treated fruit bodies. In contrast the ratio of postmeiotic to meiotic cells changes as a result of the HU treatment. The ratio of cells with sterigmata to meiotic cells drops slightly from 0.04 to 0.02 in about 6 h; the ratio of spores to meiotic cells drops markedly from 0.09 to 0.01–0.02 after 3 h. It should be noted that there is no release of spores from HU-treated fruit bodies; sporulation stops entirely within 30 min after the start of treatment (Carmi *et al.* 1978). Therefore, *a priori*, mature spores could be expected to accumulate in the HU-treated fruit bodies. The data show, however, just the reverse.

(iii) *Distribution of premeiotic and meiotic stages in fruit bodies removed from HU*

At time 0, young fruit bodies were transferred to medium containing HU. After 8 h on HU the fruit bodies were returned to medium free of HU. Samples of gills were removed at various times thereafter and examined cytologically.

As seen in Table 2, the first sample of cells examined after release from HU ( $t = 13$ , or 5 h after the release) shows about the same distribution of stages as did the fruit bodies in Table 1 that were not removed from HU. The percentage of prefusion cells is low and that of fusion cells is very high. This condition is gradually reversed. By 49 h (41 h after removal from HU) the prefusion and fusion cells have returned to the frequencies of these stages in the untreated controls. The later meiotic stages, however, show discontinuities in frequency. Metaphase I, which remained essentially constant at 2–3 % before and during treatment (Table 1), rises to 19 % at 21 h after release from HU, then drops, rises,

Table 2. *Distribution of premeiotic and meiotic stages in fruit bodies after release from HU*

Time (h)*	Cells in each stage (%)					Total no. cells
	Prefusion	Fusion	Metaphase I	Telo-phase I	Telo-phase II	
0	19.0	67.0	3.5	4.4	6.1	113
→ (8)						
13	1.6	93.8	3.0	0.0	1.6	65
29	11.3	75.0	19.3	3.4	0.0	88
33	8.3	83.3	5.2	4.2	0.0	96
45	2.8	70.8	15.1	10.4	0.9	106
49	20.3	71.4	3.6	3.6	1.1	276
56	23.6	62.3	9.5	2.3	2.3	220
58	17.6	71.3	4.6	2.8	3.7	108
62	21.8	65.9	6.8	5.0	0.5	220

\* Fruit bodies were placed on HU at  $t = 0$ . After 8 h (arrow) the fruit bodies were returned to medium free of HU.

and drops again. Telophase I also shows at least one pronounced peak in frequency. The general impression gained from the cytological examination is that the later meiotic stages occur at a slower rate. Metaphase 1 at all times examined is more frequent than in the control. In addition, a meiotic stage never seen in the untreated fruit bodies was consistently seen, at a frequency of 0.02–0.05, among cells following release from HU. This stage is characterized by one roundish nucleus at the apex of the basidium, and is considered to be a late fusion-prophase stage when the nucleus has migrated to its metaphase position but chromosomes are not yet distinguishable.

(iv) *Relative DNA content of individual nuclei*

HU is known to inhibit semi-conservative DNA synthesis (see Introduction). In an attempt to determine the time of premeiotic DNA synthesis relative to the stages of meiosis, young fruit bodies were exposed to HU for 8 h and then immediately fixed and stained by the Feulgen technique. The DNA content of individual nuclei in basidia was determined by microphotometry.

The average relative mass of prefusion nuclei is  $0.034 \pm 0.018$  and the average mass of fusion nuclei is  $0.070 \pm 0.032$  (Table 3). In spite of the large standard

deviation that is mainly due to the small size of the nuclei, the results indicate that the mass of the fusion nuclei is twice that of the prefusion nuclei. The distribution of mass among the prefusion nuclei is bimodal (Fig. 4). Only one peak is found among the fusion nuclei.

The average relative mass of the prefusion nuclei in HU-treated fruit bodies is  $0.023 \pm 0.014$  which is somewhat lower than the average mass of untreated nuclei. The fusion nuclei in the treated fruit bodies are about twice the average mass of the prefusion nuclei in HU-treated fruit bodies. The bimodal distribution of the prefusion nuclei in the control is not found among the treated material. Among the fusion nuclei from treated fruit bodies the distribution is similar to the control but with a lower average mass.

The results suggest that the bimodal distribution of nuclear mass among the

Table 3. *Relative mass of absorbing material in prefusion and fusion nuclei*

Nuclear stage	Treat- ment with HU	No. of nuclei	Average relative mass	Range of mass
Prefusion	—	50	$0.034 \pm 0.018$	0.01 — 0.09
Prefusion	+	30	$0.023 \pm 0.014$	0.01 — 0.07
Fusion	—	47	$0.070 \pm 0.032$	0.02 — 0.17
Fusion	+	50	$0.052 \pm 0.021$	0.01 — 0.11

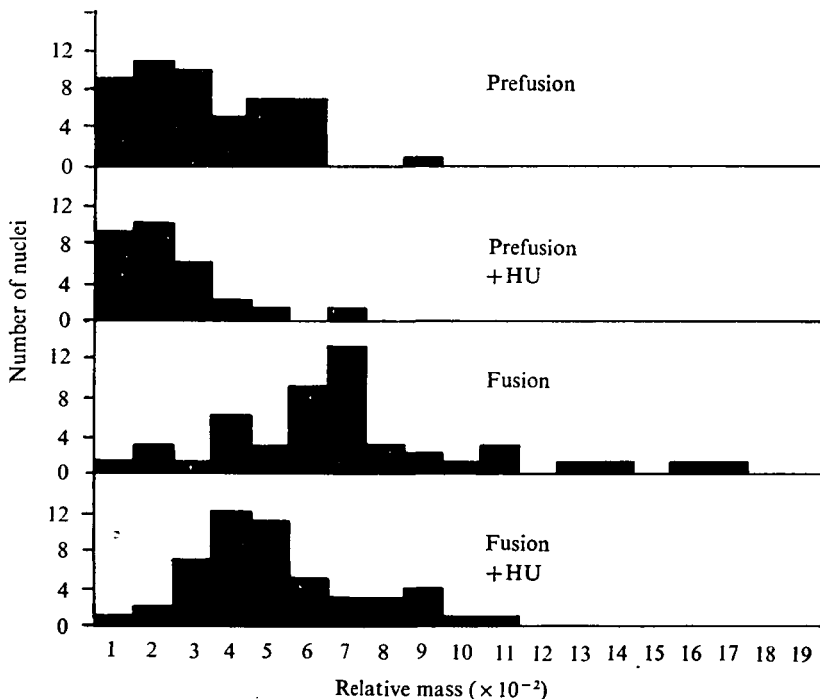


Fig. 4. Relative mass of Feulgen-stained prefusion and fusion nuclei in untreated and treated fruit bodies.



prefusion nuclei reflects the transition from 1C to 2C DNA as a result of premeiotic DNA synthesis. The absence of the peak with the presumed 2C from the treated prefusion nuclei may reflect the inhibition of premeiotic DNA synthesis by HU. The difference in the average mass of the treated and untreated fusion nuclei indicates that nuclear fusion proceeds even in nuclei in which premeiotic DNA synthesis has not been completed, and therefore some of the fusion nuclei from fruit bodies inhibited by HU contain less than 4C DNA.

(v) *Electron microscopy of fusion nuclei*

Untreated fruit bodies and fruit bodies exposed for 8 h to HU were examined by electron microscopy for the presence of synaptonemal complexes in fusion nuclei. (The fusion stage is identifiable in the electron microscope by the large, oval shape of the nuclei.) Among 16 fusion cells from the untreated sample, 13 (81 %) showed normal synaptonemal complexes. In contrast, only 20 fusion cells of the 52 examined in the HU-treated sample (38.5 %) showed normal synaptonemal complexes. In an additional 6 cells (11.5 %) of the 52, only the lateral components of the synaptonemal complex were found. This difference in frequency of complete synaptonemal complexes is significant ( $\chi^2_{(1)} = 7.3$ ;  $P < 0.01$ ).

#### 4. DISCUSSION

The effect of HU on meiosis is complete and reversible. Within 8 h of the beginning of exposure to HU, meiosis is frozen. Nuclei in the prefusion stage at the start of treatment pass to the beginning of fusion and stop at this stage. Nuclei already in fusion at the start of treatment also do not progress. Thus the prefusion category drops from 26 % to 5 %, while the fusion category rises from 67 % to 90 %. Postfusion stages also freeze as a result of the treatment. The percentages of cells in metaphase I and telophase I and II do not change as a result of the HU treatment. *A priori*, it could be argued that the constancy in frequency of postfusion nuclei means that these stages are not affected by HU, and that a small fraction of fusion nuclei 'escapes' from the effect of HU and continues the meiotic process. However, in this case there should be a noticeable accumulation of cells in postmeiotic stages, seen either as cells with sterigmata or as mature spores still in the fruit body. Since spore fall stops completely within 30 min after the exposure of fruit bodies to HU (Carmi, *et al.* 1977), nuclei that complete meiosis could not be released from fruit bodies as mature spores but would have to accumulate therein. Such an accumulation is not seen (Table 1). In fact, the opposite effect is seen. There is a decrease in the ratios of cells with sterigmata and mature spores to meiotic cells. Thus, the conclusion is that nuclei in postfusion stages do not complete meiosis, but are 'frozen' within a short time after the addition of HU.

That the effect of HU on meiosis is reversible is seen by the return to an almost normal distribution of meiotic stages within 41 h after release of fruit bodies from HU (Table 2). At this time prefusion and fusion stages have returned to their pretreatment frequencies. However, the frequencies of postfusion stages are not

constant and as noted in the *Results*, a postfusion stage rarely seen in untreated fruit bodies is consistently seen in the fruit bodies released from HU. This suggests that postfusion stages occur more slowly after HU treatment than in the controls, and this effect of HU is a lingering one. Presumably, this effect disappears when more time has elapsed after the HU release.

The data presented here suggest that, in this species, as in other fungal species examined (Rossen & Westergaard, 1966; Lu & Jeng, 1975; Iyengar, Deka, Kundu & Sen, 1977), premeiotic DNA replication occurs before fusion of the nuclei. This conclusion is based mainly on the bimodal distribution of the mass of the prefusion nuclei and the single peak characteristic of the distribution of mass in fusion nuclei. In recent studies with *Schizophyllum* (Carmi *et al.* 1977) it was estimated that the duration of premeiotic DNA synthesis is about 6–10 h. (Studies with the related species *Coprinus lagopus* give a similar estimate of 8 h for this process (Lu & Jeng, 1975).) Therefore, if premeiotic DNA synthesis occurred after fusion a bimodal distribution would be noticed among the fusion nuclei, rather than among the prefusion nuclei. However, the reverse was found experimentally to be the case. Furthermore, the average mass of fusion nuclei is about twice the average mass of the prefusion nuclei as expected if DNA replication occurred in the prefusion nuclei.

A comparison of the average mass of treated and untreated fusion nuclei indicates that nuclei fuse even if the premeiotic DNA synthesis is only partly completed. Thus, the cytological data may be misleading with respect to the timing of DNA synthesis since fusion nuclei, a stage equivalent to meiotic prophase I, accumulate in HU-treated fruit bodies, yet premeiotic DNA synthesis is affected already in prefusion nuclei. The inhibition at the fusion stage may result from an additional effect of HU, for example, on DNA synthesis in zygotene and/or pachytene (see Introduction).

HU has been reported to inhibit DNA synthesis in premitotic and premeiotic nuclei (see Introduction). Our results agree with this. In addition, the unexpected effect of HU on postfusion nuclei indicates that HU also affects some meiotic process other than DNA replication. The 'freezing' of metaphase and telophase nuclei (Table 1) cannot be due to any effect of HU on DNA replication. HU has been reported to affect RNA and protein synthesis, but at high concentrations only (Timson, 1975). Possibly, such a secondary effect is the explanation here.

It is generally accepted that the synaptonemal complex forms after premeiotic DNA replication, the lateral components being found at leptotene and the complete structure by the end of zygotene (Lu, 1970; Westergaard & von Wettstein, 1972). However, it is claimed that, in *Drosophila* oocytes, synaptonemal complex formation is co-extensive with the DNA replication (Day & Grell, 1976), and in yeast it is suggested that premeiotic DNA replication is not required for formation of the lateral elements (Moens, Mowat, Esposito & Esposito, 1977). We have found components of the synaptonemal complexes in 50% of the HU-treated fusion nuclei, 38.5% with complete complexes and 11.5% with lateral elements only, as compared to complete complexes in 81% of the untreated fusion nuclei.

It was anticipated that among the treated fusion nuclei, about 20% of which entered the fusion state after exposure to HU, a lower percentage of cells with complete synaptonemal complexes would be detected if completion of DNA replication were required for synaptonemal complex formation. The results do show fewer synaptonemal complexes in the HU-treated nuclei. The decrease, however, which should have been from c. 80% to 60%, was more severe, to c. 38%. Therefore we suggest that another stage of DNA replication, perhaps the zygotene replication (Stern & Hotta, 1973), is essential for completion of the synaptonemal complex. HU might well affect this replication, since it is semi-conservative. Some of the fused nuclei when exposed to HU would be blocked at this stage of DNA replication, and, together with the nuclei that fused without completing premeiotic DNA replication, would further decrease the percentage of cells with complete synaptonemal complexes.

Lateral elements are found in a proportion of cells no larger than the proportion of cells already at the fusion stage when exposed to HU. This suggests that in *Schizophyllum*, unlike yeast, formation of lateral elements is dependent on premeiotic DNA replication. We are currently investigating this aspect further, using a mutant defective in DNA replication.

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