Sporulation Competence in *Physarum polycephalum* CL and the Requirement for DNA Replication and Mitosis

By AILEEN CHAPMAN AND J. G. COOTE*

Microbiology Department, University of Glasgow, Garscube Estate, Bearsden,
Glasgow G61 1QH, U.K.

(Received 23 September 1981; revised 9 December 1981)

The optimum conditions for sporulation and the requirement for mitosis and DNA replication during the onset of the process have been investigated in plasmodia of the CL strain of *Physarum polycephalum*. A 72 h period of starvation was necessary before a plasmodium became competent to sporulate on exposure to light. The plasmodium became irreversibly committed to sporulation 4 to 5 h after illumination. The commitment point was related to the time of illumination rather than the duration of starvation. Three periods of DNA synthesis were detected during the initial 24 h of starvation, but in an asporogenous derivative of the CL strain only the first two periods were detected. Inhibition of mitosis with nocodazole or of DNA synthesis with hydroxyurea prevented sporulation. Escape of plasmodia from hydroxyurea inhibition of sporulation coincided with the last detectable period of DNA synthesis, but escape from nocodazole inhibition of sporulation occurred 25 to 30 h later.

INTRODUCTION

Sporulation in *Physarum polycephalum* offers a useful system for the study of events which occur during differentiation. The organism has two vegetative phases, the uninucleate amoebae which germinate from spores and the large, multinucleate plasmodium in which as many as 10^8 nuclei undergo DNA replication and mitosis in a synchronous manner (Guttes & Guttes, 1964; Braun *et al.*, 1965). The amoebal to plasmodial transition normally occurs after fusion of haploid amoebae carrying different alleles of the mating type loci *matA* and *matB* (Dee, 1966; Youngman *et al.*, 1979). The diploid heterozygous plasmodium formed then grows by nuclear division in the absence of cell division. Sporulation, whereby a typical plasmodial cell yields dozens of fruiting bodies, takes place after starvation in the dark and subsequent illumination of the plasmodium with visible light (Guttes *et al.*, 1961; Daniel & Baldwin, 1964). During the starvation period the plasmodium changes from a compact mass to a branched network of connected veins. After illumination, nodules appear at intervals on the veins and these quickly form pillars which thereafter develop sporangia at the apical region. The final stages of the sporulation process involve deposition of melanin pigment, cleavage of the apical cytoplasm, meiosis and spore formation (Guttes *et al.*, 1961; Dee, 1975; Laane & Haugli, 1976). The changes associated with sporulation occur synchronously throughout the large plasmodium and this provides ample homogeneous material for biochemical studies (Sauer *et al.*, 1969 *a, b*).

An apogamic strain, Colonia or CL, has been described which carries a mutation at or near the *matA* locus which allows haploid amoebae to form plasmodia in clones (Cooke & Dee, 1975). The multinucleate plasmodium of the CL strain is able to undergo sporulation, but the haploid chromosome content prevents normal meiotic divisions of the nuclei (Laane *et al.*, 1976). Thus, only a fraction of the spores formed are viable and evidence has suggested that these are derived from rare diploid nuclei, contained in the generally haploid plasmodium, which undergo meiosis normally (Laffler & Dove, 1977). The CL strain will

express recessive mutations in the plasmodial phase (Cooke & Dee, 1975) and is therefore ideal for an examination of mutations which interfere with sporulation.

Previous work assessed the requirements for sporulation in the heterothallic diploid strain M_3C (Daniel & Rusch, 1962*a, b*; Sauer *et al.*, 1969*a, b*). A recent report of work with a homozygous diploid variant of the apogamic CL strain suggested that plasmodia release cellular products into the medium during starvation which promote sporulation (Wilkins & Reynolds, 1979). We have attempted to define the optimum conditions for sporulation in the CL strain and have examined the requirement for DNA replication and mitosis during the onset of the process.

METHODS

Organism. The Colonia Leicester (CL) strain was used. It was supplied by T. Laffler (McArdle Laboratory for Cancer Research, Madison, Wisconsin, U.S.A.) as a strain which sporulated consistently well. After sporulation of a plasmodium only about 0.6% of the spores were routinely able to germinate, compared with about 80% spore viability obtained after sporulation of a diploid plasmodium. Low spore viability is characteristic of the CL strain (Laffler & Dove, 1977).

Media. Growth medium (GM) was adapted from Daniel & Baldwin (1964) and contained, per litre: 10 g tryptone (Oxoid), 1.5 g yeast extract (Difco), 10 g D-glucose, 2 g KH_2PO_4 , 4.04 g citric acid, 60 mg $FeCl_2 \cdot 4H_2O$, 600 mg $MgSO_4 \cdot 7H_2O$, 600 mg $CaCl_2 \cdot 2H_2O$, 84 mg $MnCl_2 \cdot 4H_2O$, 34 mg $ZnSO_4 \cdot 4H_2O$. The medium was adjusted to pH 4.6 and hemin (Sigma), prepared in 1% (w/v) NaOH, added to a final concentration of 0.001% (w/v) before use. Sporulation medium (SM), pH 5.0, was prepared according to Daniel & Rusch (1962*a*) and contained, per litre: 24 mg $CuCl_2 \cdot 2H_2O$, 400 mg KH_2PO_4 , 480 mg citric acid, 60 mg $FeCl_2 \cdot 4H_2O$, 600 mg $MgSO_4 \cdot 7H_2O$, 600 mg $CaCl_2 \cdot 2H_2O$, 84 mg $MnCl_2 \cdot 4H_2O$, 34 mg $ZnSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 100 mg nicotinamide.

Growth and sporulation. *Physarum polycephalum* was grown at 24 °C as 20 ml shaken suspensions of microplasmodia in dimpled 250 ml flasks. Subcultures were routinely made every 72 h (late-exponential phase of growth) and only microplasmodia which had been growing exponentially for at least 12 generations were used for sporulation experiments. Growth was assayed by measurement of extracted pigment (Daniel & Baldwin, 1964; Dee & Poulter, 1970). A 1 ml sample of microplasmodia was centrifuged (500 g, 2 min), the pellet washed with water and the pigment extracted with 10 ml TCA/acetone (8 ml 100% (w/v) trichloroacetic acid, 92 ml acetone, 100 ml distilled water) at room temperature for 10 min. After centrifugation at 1000 g for 5 min, the A_{400} of the supernatant was measured. The amount of pigment produced was proportional to the amount of protein present in the microplasmodia throughout the growth cycle. The organism was stored at 4 °C either as amoebae on liver infusion agar (Cooke & Dee, 1975) or as spherules, which develop spontaneously from microplasmodia in aged growth cultures (Daniel & Baldwin, 1964).

To obtain sporulation a 5.0 ml sample of microplasmodia was centrifuged at 500 g for 2 min, and the microplasmodia were resuspended in a volume of distilled water equal to that of the packed microplasmodia. The suspension was then pipetted in a circle on to a filter paper (Whatman no. 50) supported on a wire grid in a 9 cm glass Petri dish. Enough SM (about 20 ml) was introduced beneath the filter to soak the paper, but not flood the surface. After a 72 h period of starvation in the dark at 24 °C, fruiting was initiated by a 30 min exposure to 8 W cool white fluorescent lights, placed 15 to 20 cm from the starved plasmodium. During illumination the temperature in the incubator was automatically lowered to 21 °C to compensate for any local heating effects from the lights. Fruiting body formation was completed within 24 h following illumination. An alternative procedure involved the introduction of GM beneath the filter paper after addition of microplasmodia. The organism was then grown in the dark as a plasmodium for 24 h before the filter was removed to another grid and underlaid with SM.

DNA synthesis in plasmodia. Small pieces (1 cm square approximately) were cut at intervals from a filter paper supporting either a growing or a starving plasmodium. Each piece was transferred to a wire grid in a Petri dish containing either GM or SM with added [*methyl*- 3H]thymidine [$0.2 \mu Ci ml^{-1}$ ($7.4 kBq ml^{-1}$); $47 Ci mmol^{-1}$ ($1.74 TBq mmol^{-1}$); from Amersham] and incubated at 24 °C for 30 min. It was then put into 20 ml cold TCA/acetone for 10 min which removed all the pigment from the section of plasmodium. After centrifugation (1000 g, 5 min) the A_{400} of the supernatant was noted and used to estimate the amount of material collected in order to standardize the radioactivity incorporated into each sample. The precipitate was washed twice in 0.25 M perchloric acid containing thymidine ($100 \mu g ml^{-1}$) and finally solubilized in 2 ml 0.4 M NaOH. The radioactivity in 1 ml of the sample was counted in a Packard Tri-Carb 300C scintillation counter.

Inhibitors. Nocodazole, hydroxyurea, cytosine-1- β -D-arabinofuranoside (Ara-C) were obtained from Sigma. A stock solution ($5 mg ml^{-1}$) of nocodazole was prepared in dimethylsulphoxide and stored at -20 °C. It was diluted in media to the required final concentration and the small amount of organic solvent added had no effect on the organism. Other inhibitors were added directly to media to the required concentration immediately before use.

RESULTS

Sporulation capacity of microplasmodia

When standard 5 ml volumes of microplasmodia were removed at different points in the growth cycle and subjected to starvation, only those collected towards the end of exponential growth produced a plasmodium able to sporulate (Table 1). Microplasmodia taken earlier in the exponential phase were unable to sporulate if exposed directly to SM, but renewed growth as a plasmodium on the filter paper for 24 h prior to starvation allowed the sporulation potential to be expressed. This observation suggested that the amount of microplasmodia used might be an important factor. Accordingly, the volume of culture harvested at intervals during the growth cycle was varied in order to test sporulation capacity with a standard amount of microplasmodia at each stage. Again, however, only microplasmodia taken at the

Table 1. *Sporulation capacity of microplasmodia during the growth cycle*

Samples of microplasmodia were collected at the times shown, distributed on filter papers and either exposed to SM directly or allowed to grow on the filter paper in the presence of GM for 24 h before exposure to SM (see Methods). Fruiting was initiated by illumination for 30 min after a 72 h period of starvation in the dark. Sporulation frequency is the total number of plasmodia which sporulated per number tested. The figures in parentheses are the number of plasmodia which had sporulated 24 h after illumination. Sporulation was only considered successful if fully mature sporangia were formed; any plasmodia showing intermediate stages were disregarded. A and B represent separate experiments where a standard volume of culture or a standard amount of microplasmodia respectively were used.

Age of culture (h)	Pigment (A_{400})	Vol. of culture harvested (ml)	Sporulation frequency	
			No growth on filter	Growth for 24 h on filter
A. 24	0.17	5.0	0/4	1/4
54	0.72	5.0	0/4	3/4
76	1.8	5.0	4/4 (4/4)	4/4
97	1.72	5.0	3/4 (0/4)	3/4
B. 24	0.27	14.0	0/4	2/4
48	0.69	5.4	0/4	4/4
100	1.8	2.1	4/4 (4/4)	3/4
125	1.54	2.4	2/4 (0/4)	4/4

Table 2. *Effect of alteration of starvation conditions on sporulation*

Samples of microplasmodia were collected at the end of exponential growth, transferred to filter papers and exposed directly to starvation medium (SM). For transfer to fresh SM during starvation, filters were transferred first to a grid underlaid with SM as a washing step and then to another grid with fresh SM. Illumination was given after 72 h starvation in the dark and sporulation frequency assessed 24 h later.

Vol. of culture harvested (ml)	Vol. of SM (ml)	Transfer to fresh SM during starvation	Sporulation frequency	Mean no. of fruiting bodies per plasmodium
5.0	2	—	4/4	ND
0.5	20	—	4/4	17
1.0	20	—	4/4	40
2.5	20	—	4/4	60
5.0	20	—	4/4	121
5.0	20	At 24 h	5/5	ND
5.0	20	At 48 h	5/5	ND
5.0	20	At 72 h	5/5	ND
5.0	20	At 74 h	5/5	ND
5.0	20	At 24, 48, 72 h	5/5	ND

ND, Not determined.

Table 3. *Sporulation frequency under different illumination conditions*

In part A, 44 plasmodia were prepared from microplasmodia collected at the end of exponential growth and exposed directly to SM. The Petri dishes were wrapped in aluminium foil and incubated in the dark. At each time interval four cultures were removed from the foil, illuminated for 30 min, rewrapped in foil and incubation continued. Sporulation frequency was noted at 24 h intervals thereafter.

In part B, in each experiment 10 plasmodia were each given five 30 min periods of illumination at the times indicated during starvation. The figures in parentheses are the number out of each set of 10 plasmodia which had newly sporulated 24 h after the point of illumination.

A.	Starvation (h) before illumination	Sporulation frequency	B. Time (h) during starvation when plasmodia illuminated (No. sporulated out of 10 plasmodia 24 h after illumination)			
			Expt 1	Expt 2	Expt 3	Expt 4
	55	0/4				
	60	1/4				
	65	2/4	0 (0)	0 (0)	0 (0)	0 (0)
	69	2/4	24 (0)	12 (0)	18 (0)	7 (0)
	70	3/4	48 (5)	36 (2)	42 (2)	31 (0)
	72	4/4	72 (5)	60 (8)	66 (8)	55 (5)
	74	4/4	96 (0)	84 (0)	90 (0)	79 (5)
	96	4/4				
	120	0/4				
	144	0/4				
	192	0/4				

point of maximum growth sporulated when exposed directly to SM, but as before microplasmodia grown for 24 h on the filter paper prior to starvation had a much wider capacity for sporulation with regard to their origin in the growth cycle (Table 1). Sporulation in microplasmodia exposed directly to SM was most rapidly achieved in those harvested at the end of growth. Those collected later in the stationary phase did eventually sporulate, but formation of sporangia was delayed for at least 24 h (Table 1).

Microplasmodia taken at the end of exponential growth (when sporulation potential was at its greatest) were unaffected by variation of the ratio of medium volume to plasmodial mass, except to form a greater number of fruiting bodies with increased plasmodial mass (Table 2). In addition, no effect on sporulation potential was observed if plasmodia were successively transferred to fresh SM at intervals during the starvation phase.

Optimum starvation and illumination conditions

Several reports had indicated that microplasmodia, after harvesting from liquid medium, needed a period of between 2 and 6 h to fuse or coalesce into a plasmodium before addition of SM (Daniel & Rusch, 1962*a*; Daniel & Baldwin, 1964; Sauer *et al.*, 1969*a*; Wilkins & Reynolds, 1979). We were anxious to reduce this period of fusion in the absence of medium, so as to relate the initiation of starvation more closely to the time of addition of SM. Samples of microplasmodia from late-exponential phase was collected, distributed on filter papers and allowed to fuse for various lengths of time before addition of SM. The shortest period was simply the time between placing the microplasmodia on the filter and underlaying the grid with SM (about 2 min). In separate samples the period of fusion was increased to 15, 30, 60, 120 and 180 min. After starvation and illumination, sporangia formed equally well in all the samples tested, indicating that a prolonged period of fusion prior to addition of SM was unnecessary for successful sporulation.

The duration of starvation and its effect on the sporulation capacity of plasmodia was examined in two ways. First, several plasmodia were starved in the dark and at intervals four were removed, exposed to a single 30 min period of illumination and each batch then returned to the dark. Second, several plasmodia were all illuminated for 30 min periods at 24 h

Fig. 1. Alteration in the point of commitment to sporulation by variation of the time of illumination. Plasmodia were prepared from microplasmodia collected at the end of exponential growth, exposed directly to SM and incubated in the dark. At each time interval, four plasmodia were transferred to another dish and fresh GM or SM supplemented with glucose (1%, w/v) was introduced beneath the filter paper. In separate experiments all plasmodia treated with GM were exposed to a single 30 min period of illumination at 70 h (●), 72 h (○) and 75 h (□) of starvation. Plasmodia exposed to SM supplemented with glucose were illuminated for 30 min at 72 h (▲). Sporulation frequency was noted after a further 24 h incubation in the dark from the point of illumination.

intervals during starvation. The first procedure, where a plasmodium only received one period of illumination, indicated that a minimum of 72 h starvation in the dark was necessary to ensure sporulation in all plasmodia (Table 3). If, however, light pulses were given at intervals during starvation, then some cultures sporulated earlier than others. In one instance 2 out of 10 plasmodia sporulated after illumination at 36 h (Table 3). The asynchrony in fruiting body formation produced by periodic illumination was less satisfactory than allowing all cultures to become competent for morphogenesis by starvation for 72 h in the dark and then initiating the process synchronously on illumination. The optimum period of 72 h starvation was confirmed in a separate experiment where 40 plasmodia were illuminated for 30 min after 72 h starvation in the dark. All sporulated within 24 h. It should be noted that extended starvation in the dark, 120 h or longer before illumination, prevented sporulation (Table 3).

On occasion it was noticed that starved plasmodia would sporulate after only the briefest exposure to room light, but illumination was nevertheless an absolute requirement for sporulation as plasmodia kept continuously in the dark failed to sporulate. Previous work used illumination periods of 2 to 4 h (Daniel & Rusch, 1962*a*; Daniel & Baldwin, 1964; Sauer *et al.*, 1969*a*), but in our experiments light pulses of as little as 5 min were sufficient to promote sporulation (5 out of 5 plasmodia sporulated in each case when exposed to 5, 15 or 30 min periods of illumination after 72 h starvation in the dark). An illumination period of 30 min was used routinely.

Commitment to sporulation

Refeeding starving plasmodia with fresh growth medium or with SM supplemented with D-glucose (1%, w/v) showed that plasmodia became irreversibly committed to sporulate between 4 and 5 h after illumination (Fig. 1), although plasmodia illuminated at 72 h escaped glucose inhibition of sporulation slightly earlier than those supplied with the richer GM medium. Up to the point of commitment the plasmodia would resume growth. The point of no return was related to the time of illumination rather than the duration of starvation, as altering the length of the starvation period had little effect on the time needed for commitment after illumination. In separate experiments it was found that refeeding plasmodia at 48 h or 73 h starvation (illumination given at 72 h) with SM supplemented with L-glutamic acid (0.34%, w/v) also prevented sporulation. L-Glutamine (0.35%, w/v) delayed fruiting body formation

Fig. 2. Growth of microplasmodia in the presence of inhibitors of DNA synthesis and mitosis. Portions (2 ml) of an exponentially growing culture of microplasmodia were subcultured into 20 ml amounts of GM containing either Ara-C ($500 \mu\text{g ml}^{-1}$; ○), hydroxyurea ($500 \mu\text{g ml}^{-1}$; ■), nocodazole ($20 \mu\text{g ml}^{-1}$; □) or no addition (●). During continued incubation, portions of the cultures were assayed at intervals for pigment production (see Methods).

by about 24 h and some sporangia had abnormal morphology. Refeeding plasmodia with $(\text{NH}_4)_2\text{SO}_4$ (0.26%, w/v) had no effect on sporulation.

DNA synthesis and mitosis during sporulation

It was reported that in the M_3C strain of *P. polycephalum* the nuclear division cycle continued during starvation, but at lengthened intervals (Guttes *et al.*, 1961). We wanted to assess the importance of continued DNA replication and mitosis during starvation for sporulation in the CL strain. The benzimidazole derivative nocodazole, an anti-tubulin drug, interferes with mitosis and will prevent the assembly *in vitro* of microtubule protein from *P. polycephalum* amoebae (Quinlan *et al.*, 1981). This drug and hydroxyurea, an inhibitor of DNA replication, prevented the growth of microplasmodia (Fig. 2). Ara-C was also examined as a potential inhibitor of DNA replication, but it had little effect on microplasmodial growth (Fig. 2).

The effect of each drug on DNA synthesis was assessed in a growing plasmodium where mitosis is naturally synchronous and DNA is replicated during an S phase, lasting 2 to 3 h, which starts immediately after the mitotic divisions of the nuclei (Nygaard *et al.*, 1960). In the growing CL plasmodium the interval between rounds of DNA replication was approximately 8 h (Fig. 3*a*). Hydroxyurea, at the concentration which prevented growth of microplasmodia, abolished the peak of DNA synthesis in the surface plasmodium, whereas nocodazole at the growth inhibitory concentration had no effect (Fig. 3*b*). Ara-C also had no effect on the pattern of DNA synthesis.

Addition of nocodazole ($20 \mu\text{g ml}^{-1}$) or hydroxyurea ($500 \mu\text{g ml}^{-1}$) to SM prevented sporulation, but if the drugs were added at intervals during starvation, the plasmodia began to escape the inhibitory effect on sporulation (Table 4). Although there was some variation in escape time of plasmodia prepared on different days, the escape from hydroxyurea inhibition of sporulation was essentially complete between 24 and 48 h whereas escape from nocodazole inhibition was more delayed and not apparently complete until about 55 h into starvation.

Fig. 3. DNA synthesis in growing plasmodia. (a) Pieces of a plasmodium, which had been grown for 24 h on a filter paper in the presence of GM, were cut out at intervals and transferred to fresh GM containing [*methyl*- ^3H]thymidine ($0.2 \mu\text{Ci ml}^{-1}$). Each point represents incorporation of radioactivity into one piece of plasmodium over a 30 min period; incorporation was monitored as described in Methods. Addition of uridine ($100 \mu\text{g ml}^{-1}$) to the GM containing [*methyl*- ^3H]thymidine had no effect on incorporation. (b) In a separate experiment two pieces of a plasmodium were removed at each time interval; one was transferred to GM containing [*methyl*- ^3H]thymidine (●) and the other to the same medium containing hydroxyurea ($500 \mu\text{g ml}^{-1}$) in addition (○). In other experiments a similar pattern of uptake to the control was obtained if either Ara-C ($500 \mu\text{g ml}^{-1}$) or nocodazole ($20 \mu\text{g ml}^{-1}$) were included in the medium with radioactive thymidine.

Table 4. *Effect of inhibitors of DNA synthesis and mitosis on sporulation*

Plasmodia were prepared from microplasmodia collected at the end of exponential growth, exposed directly to SM and incubated in the dark. At the times indicated some plasmodia were transferred to another dish of SM containing either nocodazole ($20 \mu\text{g ml}^{-1}$) or hydroxyurea ($500 \mu\text{g ml}^{-1}$). All plasmodia were illuminated after 72 h starvation and sporulation frequency assessed 24 h later. The figures represent the combined results of four experiments; in two of these the two drugs were examined in parallel.

Time (h) of addition of inhibitor during starvation	Sporulation frequency	
	Nocodazole	Hydroxyurea
0	0/10	0/10
24	0/10	2/10
48	2/10	8/10
50	2/8	—
55	6/8	—
65	4/6	—
72	10/10	10/10
74	4/4	—
No addition	10/10	10/10

Three periods of DNA synthesis were detected during starvation (Fig. 4). The data in Fig. 4 represent the results obtained from typical experiments with the CL strain sporulating normally, together with those obtained from an asporogenous derivative which is discussed more fully in the next section. In the wild-type the first peak of DNA synthesis occurred 3 to 4 h after exposure of the plasmodium to SM, the second between 13 and 15 h and the third between 21 and 24 h. No further peaks of DNA synthesis were detected up to 72 h. This is in agreement with the observation that plasmodia began to escape hydroxyurea inhibition of

Fig. 4. DNA synthesis in starving plasmodia. Plasmodia were prepared from microplasmodia collected at the end of exponential growth, exposed directly to SM and incubated in the dark. Pieces of a plasmodium were cut out at intervals and transferred to fresh SM containing [*methyl*- ^3H]thymidine ($0.2 \mu\text{Ci ml}^{-1}$). Each point represents incorporation of radioactivity over a 30 min period (see Methods); (a), (b) and (c) represent the results obtained from separate plasmodia prepared on different days from different cultures of microplasmodia. The small amount of each surface plasmodium remaining after the removal of pieces for monitoring DNA synthesis was illuminated at 72 h and the sporulation capacity of the plasmodium noted during the following 24 h. ●, CL sporulating normally; ○, asporogenous derivative of the CL strain.

Fig. 5. DNA synthesis in separate plasmodia prepared from the same culture of microplasmodia. Two plasmodia were prepared from a single culture of microplasmodia collected at the end of exponential growth. DNA synthesis was monitored in pieces of each plasmodium during starvation at the times indicated as described in the legend to Fig. 4. The remainder of each plasmodium sporulated after illumination at 72 h.

sporulation after about 24 h in SM (Table 4). In surface plasmodia derived from different liquid cultures of microplasmodia there was generally a slight variation in the times at which the periods of DNA synthesis began during starvation. However, in separate plasmodia prepared at the same time from a single population of microplasmodia, the onset of DNA synthesis occurred in almost perfect synchrony, even at the third round of replication during the starvation period (Fig. 5). Mitotic synchrony in growing plasmodia was noted previously by Guttes *et al.* (1961) who observed that in individual plasmodia prepared from a single culture of microplasmodia, the onset of the first mitosis did not vary by more than 5 min.

Fig. 6. Effect of nocodazole on DNA replication in a starving plasmodium. A plasmodium was prepared from microplasmodia collected at the end of exponential growth, exposed directly to SM and incubated in the dark. At 19 h one half of the plasmodium was transferred to fresh SM supplemented with nocodazole ($20 \mu\text{g ml}^{-1}$; ○) and the other half was left as a control (●). DNA synthesis was monitored in pieces removed from each plasmodium at the times indicated, as described in the legend to Fig. 4.

Table 5. *Sporulation capacity of subcultures of strain CL*

Plasmodia were prepared from microplasmodia collected at the end of exponential growth and incubated in the dark in SM containing the indicated amounts of nicotinamide and CaCO_3 . All plasmodia were illuminated at 72 h and 96 h into starvation and the sporulation frequency noted 24 h after each period of illumination. Subculture A: strain CL sporulating normally. Subculture B: strain CL grown as microplasmodia for about 4 months and beginning to lose sporulation capacity. After further serial transfer in GM the culture became asporogenous.

Additions to SM		Sporulation frequency			
		Subculture A		Subculture B	
Nicotinamide (mg ml^{-1})	CaCO_3 (mg ml^{-1})	Illumination at:		Illumination at:	
		72 h	96 h	72 h	96 h
None	1.0	4/4	4/4	ND	ND
0.1*	1.0*	8/9	9/9	1/13	8/13
0.2	2.0	8/8	8/8	2/8	6/8
0.3	3.0	1/3	3/3	1/3	2/3

ND, Not determined.

* Normal additions to SM (see Methods).

Finally, it was clear that successful completion of mitosis was necessary to ensure a subsequent round of DNA synthesis during starvation. Thus, for example, transfer of a starving plasmodium at 19 h to SM supplemented with nocodazole ($20 \mu\text{g ml}^{-1}$) abolished the third period of DNA synthesis (Fig. 6).

Maintenance of sporulation capacity

It is known that *P. polycephalum* loses the ability to sporulate after a long period of vegetative growth by serial transfer in liquid medium (Daniel & Baldwin, 1964). Subculture of the CL strain for longer than 6 to 9 months promoted a loss in the sporulation potential of the microplasmodia. This was manifest initially by the requirement for a longer period of starvation in the dark before illumination was able to promote sporulation, and even then successful sporulation was not necessarily achieved in all plasmodia (Table 5). It was

reported that sporulation in strain M₃C was dependent upon the addition of nicotinamide to the starvation medium (Daniel & Rusch, 1962*b*). Plasmodia of CL, however, were able to sporulate in its absence, but the reduced sporulation capacity of the continuously subcultured microplasmodia could be marginally improved by increasing the normal levels of nicotinamide and CaCO₃ in the SM medium (Table 5).

Escape from nocodazole inhibition of sporulation was examined in subculture B (Table 5) which was beginning to lose sporulation capacity. In an experiment similar to that presented in Table 4 plasmodia were transferred at intervals during starvation to SM containing nocodazole (20 µg ml⁻¹). All plasmodia were illuminated for 30 min at 72 h and 96 h and the sporulation frequency noted after each period of illumination. In plasmodia not exposed to nocodazole none sporulated after illumination at 72 h, but all did so after illumination at 96 h. Induction of fruiting was therefore delayed by comparison with the normal behaviour of CL (Table 3). Similarly, escape from nocodazole inhibition of sporulation did not occur until between 72 and 90 h of starvation, some 17 to 35 h later than the escape time normally exhibited (Table 4). Subsequently, when subculture B became fully asporogenous, the DNA synthesis during starvation was examined (Fig. 4). Comparison of the pattern of DNA synthesis in the normal CL strain with that of the asporogenous variant shows that the first two peaks of DNA synthesis, at 3 to 4 h and 13 to 15 h of starvation, occurred in both strains to a similar extent, but that the third round of DNA synthesis at 21 to 24 h was not detectable in the asporogenous strain. This difference in the extent of DNA synthesis at the third replication was found in two further experiments.

DISCUSSION

The capacity of CL microplasmodia to produce a surface plasmodium capable of sporulation was only exhibited at the end of exponential growth, if the plasmodium was exposed directly to starvation. However, sporulation potential could be promoted in CL microplasmodia taken at other stages of the growth cycle if growth continued as a surface plasmodium prior to starvation. Supplementary growth of the organism as a plasmodium prior to starvation, although not used routinely here, is useful experimentally for several reasons. It does not require microplasmodia to be collected during a restricted period of the growth cycle; larger amounts of material are available because of an increase in plasmodial mass during the 24 h incubation prior to starvation; starvation can be initiated at a known point in the cell cycle, if this should be required, as a consequence of the synchrony of cell cycle events in the plasmodium.

It was reported by Wilkins & Reynolds (1979) that plasmodia of a diploid variant of CL, constructed by heat shock of the haploid CL strain during plasmodial mitosis (Laffler & Dove, 1977), released into the medium substances essential for the induction of sporulation. They found that if a 4 h period of illumination was given, starting about 50 h into starvation, then for a given volume of SM, larger plasmodia developed sporulation capacity sooner, while increasing the medium volume for a fixed plasmodial mass apparently reduced sporulation. Successive transfers to fresh SM during starvation significantly reduced sporulation. We found that a minimum period of 72 h starvation was necessary before illumination would promote sporulation in all plasmodia. Under these conditions no effect on sporulation capacity was found if the plasmodial mass to medium volume was varied or if the SM was replaced at 24 h intervals during the 72 h starvation period. In individual plasmodia it was clear that some could attain much earlier than others the condition whereby they would subsequently sporulate if exposed to light, but our results are not consistent with the suggestion that this state of competence for morphogenesis is promoted by the release of cellular products into the medium. The asynchrony in the start of each round of DNA synthesis observed in plasmodia derived from separate cultures of microplasmodia might presumably account for the observation that some plasmodia were able to respond to light

and sporulate much earlier than others if illumination was given at regular intervals during starvation.

Starvation and light must promote different metabolic changes within the cell. The former acts to prime the cell so that it is competent to initiate fruiting in response to light. The latter phase, consisting of sporangium formation, will not occur if competent plasmodia are kept in the dark. Once achieved, plasmodia can maintain the competent state for at least 24 h, after which the plasmodium degenerates and will not sporulate if illuminated. The distinction between the creation of conditions within the plasmodium which will allow it to respond to light, and the events of morphogenesis which occur after illumination, was demonstrated by the fact that the point of commitment to fruiting body formation, when cells would no longer revert to growth if resupplied with fresh growth medium, consistently occurred 4 to 5 h after illumination even when the starvation period was varied from 70 to 75 h.

The metabolic events that govern the achievement of the competent state in the plasmodium are not clear. There was a previously reported requirement for nicotinamide (Daniel & Rusch, 1962*b*), but in strain CL we found this requirement to be dispensable, as did Wilkins & Reynolds (1979). During starvation of the M₃C strain there was a considerable fall in the DNA, RNA and protein content of the plasmodium (Daniel, 1966; Sauer *et al.*, 1970; Melera & Hession, 1981). Nuclei were reported to continue to undergo mitosis, but the number of nuclei per unit volume did not increase because of nuclear degeneration (Guttes *et al.*, 1961). Sauer *et al.* (1969*a*) reported that DNA replication occurred late in starvation, but we failed to detect a peak of DNA synthesis after about 24 h, some 48 h before the plasmodia became competent to respond to illumination. Escape from hydroxyurea inhibition of sporulation also occurred at about this time. The possibility that plasmodia became less permeable to radiolabel and inhibitors as starvation progressed cannot be eliminated, but it would seem unlikely as the basal level of [*methyl*-³H]thymidine incorporation into acid-insoluble material did not decrease throughout starvation. In addition, exogenous glucose prevented sporulation if added at any time up to 79 h, and Sauer *et al.* (1969*a*) only detected a decrease in [¹⁴C]glucose uptake by M₃C plasmodia after illumination. After the onset of starvation three rounds of DNA synthesis occurred with a normal 8 to 10 h G₂ period between them. Mitosis was assumed to take place before each period of DNA synthesis as no G₁ phase has been observed either during growth (Nygaard *et al.*, 1960) or starvation of plasmodia (Guttes & Guttes, 1961; Mohberg & Rusch, 1971). Although this was not confirmed by direct microscopic examination, transfer of a starving plasmodium to SM containing the mitotic inhibitor nocodazole prevented any subsequent round of DNA replication. The first mitosis and DNA replication after only 3 to 4 h would represent the arithmetic average for an asynchronous culture of microplasmodia with an 8 h doubling time if the non-uniform distribution of stages in the cell cycle of individual microplasmodia is taken into account (Holt, 1980). After the third round of DNA synthesis the starving plasmodium entered an extended G₂ period from which the capacity to sporulate after illumination was attained some 48 h later.

The escape of plasmodia from inhibition of sporulation by hydroxyurea and nocodazole were not coincident. Plasmodia only escaped nocodazole inhibition some 17 h prior to illumination or between 25 and 30 h after the completion of the last detectable round of DNA replication. These observations might suggest that there was a final mitosis, necessary for sporulation, which differed from previous mitoses in having occurred after an extended interphase period and which was not then followed by a round of DNA replication. Alternatively, the late escape from nocodazole inhibition may reflect the involvement of microtubules in processes necessary for morphogenesis which are not concerned with mitosis. Although non-specific effects of nocodazole on sporulation cannot be ruled out, the mode of action of the drug seems to be restricted to the microtubule system in *P. polycephalum* (Quinlan *et al.*, 1981). If a mitotic division of the nuclei, unaccompanied by DNA replication, occurred about halfway through the extended G₂ period following the third

round of DNA synthesis, it would imply that the plasmodium entered a G1 phase from which sporulation competence developed. Studies on commitment in the eukaryotic cell cycle (Hartwell, 1978; Pardee, 1974; Simchen, 1978; Pragnell *et al.*, 1980; Nurse, 1981) have led to the concept of a critical point early in G1 where the cell becomes committed to growth and division or to an alternative developmental pathway such as sporulation. Whatever the significance, if any, of the late escape from nocodazole inhibition of sporulation to the cell cycle, the importance to differentiation of the function affected by nocodazole was demonstrated in the strain which lost sporulation capacity. In this strain there was a concomitant delay in escape from nocodazole inhibition of sporulation and the ability of the plasmodium to become competent to sporulate on illumination. When the strain finally became asporogenous, the DNA replication which normally preceded the extended G2 period was absent. This presumably meant that the plasmodium was unable to reach the condition where it could complete competence development.

A requirement for a cell cycle dependent event prior to commitment to differentiation is a feature common to many higher eukaryotic systems, e.g. erythroid differentiation (Harrison, 1976; Conkie *et al.*, 1981). The natural synchrony of events in the plasmodium of *P. polycephalum* during growth and sporulation offers an ideal system for investigating regulatory mechanisms associated with the cell cycle during development.

A. C. was the recipient of an SERC research studentship.

REFERENCES

- BRAUN, R., MITTERMAYER, C. & RUSCH, H. P. (1965). Sequential temporal replication of DNA in *Physarum polycephalum*. *Proceedings of the National Academy of Sciences of the United States of America* **53**, 924–931.
- CONKIE, D., HARRISON, P. R. & PAUL, J. (1981). Cell-cycle dependence of induced hemoglobin synthesis in Friend erythroleukemia cells temperature-sensitive for growth. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 3644–3648.
- COOKE, D. J. & DEE, J. (1975). Methods for the isolation and analysis of plasmodial mutants in *Physarum polycephalum*. *Genetical Research* **24**, 175–187.
- DANIEL, J. W. (1966). Light-induced synchronous sporulation of a myxomycete. In *Cell Synchrony*, pp. 117–152. Edited by I. Cameron & G. M. Padilla. New York: Academic Press.
- DANIEL, J. W. & BALDWIN, H. (1964). Methods of culture for plasmodial myxomycetes. *Methods in Cell Physiology* **1**, 9–41.
- DANIEL, J. W. & RUSCH, H. P. (1962*a*). Methods for inducing sporulation of pure cultures of the myxomycete *Physarum polycephalum*. *Journal of Bacteriology* **83**, 234–240.
- DANIEL, J. W. & RUSCH, H. P. (1962*b*). Niacin requirement for sporulation of *Physarum polycephalum*. *Journal of Bacteriology* **83**, 1244–1250.
- DEE, J. (1966). Multiple alleles and other factors affecting plasmodium formation in the true slime mould *Physarum polycephalum* Schw. *Journal of Protozoology* **13**, 610–616.
- DEE, J. (1975). Slime moulds in biological research. *Science Progress* **62**, 523–542.
- DEE, J. & POULTER, R. T. M. (1970). A gene conferring actidione resistance and abnormal morphology on *Physarum polycephalum*. *Genetical Research* **15**, 35–41.
- GUTTES, E. & GUTTES, S. (1961). Synchronous mitosis in starved plasmodia of the myxomycete *Physarum polycephalum*. *Federation Proceedings* **20**, 419.
- GUTTES, E. & GUTTES, S. (1964). Mitotic synchrony in the plasmodia of *Physarum polycephalum* and mitotic synchronisation by coalescence of microplasmodia. *Methods in Cell Physiology* **1**, 43–54.
- GUTTES, E., GUTTES, S. & RUSCH, H. P. (1961). Morphological observations on growth and differentiation of *Physarum polycephalum* grown in pure culture. *Developmental Biology* **3**, 588–614.
- HARRISON, P. R. (1976). Analysis of erythropoiesis at the molecular level. *Nature, London* **262**, 353–356.
- HARTWELL, L. H. (1978). Cell division from a genetic perspective. *Journal of Cell Biology* **77**, 627–637.
- HOLT, C. E. (1980). The nuclear replication cycle in *Physarum polycephalum*. In *Growth and Differentiation in Physarum polycephalum*, pp. 9–63. Edited by W. F. Dove & H. P. Rusch. Princeton, New Jersey: Princeton University Press.
- LAANE, M. M. & HAUGLI, F. B. (1976). Nuclear behaviour during meiosis in the myxomycete *Physarum polycephalum*. *Norwegian Journal of Botany* **23**, 7–21.
- LAANE, M. M., HAUGLI, F. B. & MELLUM, T. R. (1976). Nuclear behaviour during sporulation and germination in the Colonia strain of *Physarum polycephalum*. *Norwegian Journal of Botany* **23**, 177–189.
- LAFFLER, T. G. & DOVE, W. F. (1977). Viability of *Physarum polycephalum* spores and ploidy of plasmodial nuclei. *Journal of Bacteriology* **131**, 473–476.
- MELERA, P. W. & HESSON, C. A. (1981). Starvation phase of *Physarum polycephalum*: characterisation

- of transfer ribonucleic acid. *Molecular and Cellular Biology* **1**, 13–20.
- MOHBERG, J. & RUSCH, H. P. (1971). Isolation and DNA content of nuclei of *Physarum polycephalum*. *Experimental Cell Research* **66**, 305–316.
- NYGAARD, O. F., GUTTES, S. & RUSCH, H. P. (1960). Nucleic acid metabolism in a slime mould with synchronous mitosis. *Biochimica et biophysica acta* **38**, 298–306.
- NURSE, P. (1981). Genetic analysis of the cell cycle. *Symposia of the Society for General Microbiology* **31**, 291–315.
- PARDEE, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 1286–1290.
- PRAGNELL, I. B., ARMDT-JOVIN, D. J., JOVIN, T. M., FAGG, B. & OSTERTAG, W. (1980). Commitment to differentiation in Friend cells and initiation of globin mRNA synthesis occurs during the G1 phase of the cell cycle. *Experimental Cell Research* **125**, 459–470.
- QUINLAN, R. A., ROOBOL, A., POGSON, C. I. & GULL, K. (1981). A correlation between *in vivo* and *in vitro* effects of the microtubule inhibitors colchicine, parbendazole and nocodazole on myxamoebae of *Physarum polycephalum*. *Journal of General Microbiology* **122**, 1–6.
- SAUER, H. W., BABCOCK, K. L. & RUSCH, H. P. (1969*a*). Sporulation in *Physarum polycephalum*. A model system for studies on differentiation. *Experimental Cell Research* **57**, 319–327.
- SAUER, H. W., BABCOCK, K. L. & RUSCH, H. P. (1969*b*). Changes in RNA synthesis associated with differentiation (sporulation) in *Physarum polycephalum*. *Biochimica et biophysica acta* **195**, 410–421.
- SAUER, H. W., BABCOCK, K. L. & RUSCH, H. P. (1970). Changes in nucleic acid and protein synthesis during starvation and spherule formation in *Physarum polycephalum*. *Wilhelm Roux's Archives of Developmental Biology* **165**, 110–124.
- SIMCHEN, G. (1978). Cell cycle mutants. *Annual Review of Genetics* **12**, 161–191.
- WILKINS, A. S. & REYNOLDS, G. (1979). The development of sporulation competence in *Physarum polycephalum*. *Developmental Biology* **72**, 175–181.
- YOUNGMAN, P. J., PALLOTA, D., HOSLER, B., STRUHL, G. & HOLT, C. E. (1979). A new mating compatibility locus in *Physarum polycephalum*. *Genetics* **91**, 683–693.