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THE CELL CYCLE AND SORTING BEHAVIOUR IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

Synchronized cells of the cellular slime mould *Dictyostelium discoideum* were prepared by mitotic wash-off. Cell counts and DNA synthesis measurements indicated a high degree of synchrony. Cells from each phase of the cell cycle were fluorescently labelled and mixed with unlabelled asynchronous cells. Cells that were in *S*-phase and very early G_2 at the onset of starvation demonstrated a strong tendency to sort to the tip of the subsequent slugs. With reference to these results and published evidence, we discuss the possible role of cell-cycle-related adhesion differences in cell sorting.

INTRODUCTION

Differentiation of some biological systems occurs independently of the cell cycle, while in others a particular event associated with differentiation may only be initiated during a specific phase of the cell cycle or after a specified number of cycles. Often differentiation is initiated by starvation. In such systems differentiation may be accompanied by a block in the cell cycle (Reinert & Holtzer, 1975).

In the cellular slime mould *Dictyostelium discoideum* starvation initiates development, which results in the differentiation of two cell types: stalk cells and spores. Cells differentiate while aggregating in response to cyclic-AMP to form a multicellular slug (Bonner, 1967; Gerisch *et al.* 1977). Differentiating cells are randomly distributed in the early aggregate but sort out during late aggregation to form the distinct anterior–posterior prestalk–prespore pattern found in the slug (Durston & Vork, 1979; Tasaka & Takeuchi, 1979; Takeuchi *et al.* 1982). The prestalk and prespore cells give rise to the differentiated stalk cells and spores of the fruiting body.

One of the initial consequences of starvation in *D. discoideum* is the cessation of cell division. Some mitosis is observed during the first few hours of starvation but all division ceases during aggregation. Once the aggregate tip begins to elongate into a slug, mitosis is resumed (Zada-Hames & Ashworth, 1977). By the time the fruiting body is formed there has been a 100% increase in cell number. Bonner & Frascella (1952) were the first to observe mitosis during development and it has subsequently been reported by others (Wilson, 1953; Katz & Bourguignon, 1974; Atryzek, 1976; Marin, 1977; Zada-Hames & Ashworth, 1977).

Evidence has been presented that suggests that *D. discoideum* cells become blocked

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mid-way through the G_2-phase of their cycles during aggregation. Katz & Bourguignon (1974) suggested that starvation triggers some signal that programmes the cells to aggregate at a specific time. This signal may allow cells to continue to move through the cell cycle until they reach the block in G_2. The putative G_2 block is released later, or else is not effective in blocking all cells because cell division (and mitosis and nuclear DNA synthesis) resumes during slug formation (see above).

D. discoideum cells are commonly grown in the laboratory in suspension culture in axenic medium. Under these conditions they divide exponentially until there are about 10^7 cells per ml, then they stop dividing but do not undergo multicellular development. Such non-dividing cells are said to be stationary, and they appear to be blocked in G_2-phase of the cell cycle (Soll, Yarger & Mirick, 1976). If allowed to develop, stationary cells do not undergo any pre-aggregation division (Zada-Hames & Ashworth, 1978). They also appear to be starved (Soll *et al.* 1976).

Stationary-phase cells grown on a variety of media and mixed with exponential-phase cells have been reported to demonstrate sorting tendencies towards the slug tip. Initially, exponential-phase cells grown on glucose sort to the prespore region of the slug if mixed with non-glucose grown cells, but as the cells become stationary they are reported to sort to the prestalk zone (Leach, Ashworth & Garrod, 1973).

The implications from the above are that: (1) starvation may initiate a block in the cell cycle; (2) the block may be in the G_2-phase; and (3) cells at or near the block at starvation may later exhibit sorting tendencies that correlate a particular phase of the cell cycle at starvation with a particular cell type later. The purpose of our investigations is to analyse the sorting tendencies of exponential-phase cells located in the various phases of the cell cycle at the time of starvation. Synchronous cultures are necessary for such a study. Previous investigations have had to rely upon temperature-sensitive mutants, reinoculation of stationary-phase cells or centrifugation to obtain synchronous populations of cells (Katz & Bourguignon, 1974; Zada-Hames & Ashworth, 1978; Woffendin & Griffiths, 1982). These methods do not ensure that the quality of the cells obtained is high and in some the degree of synchrony is insufficient for cell cycle studies. We report on a new method for synchronizing exponential-phase cells and present results that demonstrate that cells in a particular phase of the cell cycle at the time of starvation position themselves in specific regions of the slug.

MATERIALS AND METHODS

Cells of strain Ax-2 were grown axenically in HL-5 medium at 22 °C in shaken culture (Watts & Ashworth, 1970). When cells reached a concentration of 3×10^6 to 6×10^6/ml they were transferred to plastic culture flasks (Costar no. 3150, 150 cm^2) at a final density of $3 \times 10^5/cm^2$ and incubated for 20 min, during which time the cells adhered to the plastic substrate. Synchronous populations of cells were obtained by a wash-off method adapted from that of Terasima & Tolmach (1961). The medium with unattached cells was removed and replaced by 10 ml of conditioned medium (prepared by removal of cells from exponential cultures via centrifugation). In our usual harvesting procedure, the flasks were left for 10 min and then gently shaken at 30 rev./min on a gyratory shaker for 30 s, just long enough for the medium to wash off any loosely attached cells. These cells detach easily because they are mitotic. During mitosis cells round up and no longer adhere to the plastic substrate. The medium containing mitotic cells was removed and the % harvest calculated (no. of wash-off cells/total no. of cells plated, ×100).

Synchronized wash-off cells were cultured axenically at 22 °C as described above. Every hour the following were determined: cell number, DNA synthesis (continuous and pulse labelling) and sorting tendencies. During the initial point the number of nuclei/cell was determined to ensure that any mitosis observed was the consequence of cell division and not cytokinesis of multinucleate cells. Briefly, samples of 10^6 cells were centrifuged at 1000 ***g*** in an Eppendorf centrifuge to remove medium and washed twice with buffer (20 mM-KH_2PO_4/K_2HPO_4 (KK_2) pH 6·9). Cells were pelleted and fixed in 25 μl of 70 % ethanol, then suspended in 3 ml of Hoechst buffer saline (100 μg Hoechst 33258 per litre buffer: 50 mM-KK_2, 2 M-NaCl, 20 mM-EDTA, pH 7·4). Stained nuclei were observed with a fluorescent microscope and the number of nuclei/cell recorded.

Cell growth was monitored by counting 100 μl samples in a haemacytometer. The duration of mitosis was estimated by observing plated cells under a microscope and monitoring the time required for the cells to round up, loosen their attachment to the substrate and undergo cytokinesis.

DNA synthesis was followed by continuous and pulse-labelling of cells with [^{3}H]thymidine (New England Nuclear, sp. act. 60–80 Ci/mmol). Continuous labelling was employed to estimate the total amount of new DNA synthesized after wash-off. [^{3}H]thymidine was added to 30 ml of cells (5×10^5/ml) at a final concentration of 3·3 μCi/ml and incubated at 22 °C with shaking. Samples (1 ml) were removed hourly to determine radioactivity. Pulse-labelling was employed to estimate the synthetic activity of cells at any one time. [^{3}H]thymidine was added hourly to 3 ml of cells (5×10^5/ml) at a final concentration of 25 μCi/ml and incubated for 15 min at 22 °C with shaking. Radioactivity for both labelling procedures was determined by centrifuging cells free of radioactive medium, washing twice with buffer (20 mM-KK_2, pH 6·9) and resuspending them in 100 μl of buffer. Cells were placed on filters (Whatmann 1·5 cm, 3 mm) and air dried. Filters were washed three times in 5 % trichloroacetic acid, once in ethanol/ether (1 : 1), and once in ether alone (20 min/wash). Dried filters were placed in vials with 5 ml of Aquasol-2 and counted in a Packard 3000 scintillation counter.

To determine the sorting behaviour of cells in different phases of the cell cycle, samples of cells (5×10^6) were washed free of medium with 20 mM-KK_2 plus 2 mM-EDTA and labelled for 20 min with tetrarhodamine isothiocyanate (TRITC; Nordic) at a concentration of 10^8 cells/ml stain (0·5 mg/ml 1 % dimethylsulphoxide (DMSO in 20 mM-KK_2 plus 2 mM-EDTA)). Cells were washed three times with EDTA-free buffer and mixed with unlabelled asynchronous cells (similarly incubated in 1 % DMSO and washed) in the following ratios: 10, 50 or 100 unlabelled cells to 1 labelled wash-off cell. Mixtures of cells were plated at a concentration of 10^8 cells/ml (approximately 50 μl) on 1·5 % non-nutrient agar. Plates were incubated for 18 h in the dark at 19 °C (temperatures from 15 °C–23 °C did not appear to affect results). Labelling was analysed by squashing migrating slugs under coverslips and counting the number of fluorescent cells in the anterior third of the slug (prestalk zone) compared to the posterior two thirds of the slug (prespore zone). Controls were labelled in a similar manner as experimental wash-off cells, however, instead of mixing them with unlabelled asynchronous cells they were mixed with unlabelled wash-off cells the same as themselves. Reverse-sorting experiments were also conducted in which 10 % of the asynchronous population of cells was labelled with TRITC and mixed with the remaining 90 % unlabelled asynchronous cells plus the unlabelled wash-off cells in the same ratios as previously described.

RESULTS

Observation of plated *D. discoideum* cells revealed that these required 11 (±2·12) min to complete mitosis (from the time when cells first round up until the time when they complete cytokinesis) (Table 1). This corresponds to approximately 2 % of the (9 h) cell cycle, and we developed a wash-off procedure that gave a 2 % harvest (shaking 30 rev./min for 10 min; see Materials and Methods). These conditions gave excellent synchrony (Fig. 1). Cell number was initially constant; then doubled precisely in one step between 6·0 ± 0·7 and 8·4 ± 0·7 h, then became constant again. [^{3}H]thymidine was incorporated between 0·9 ± 0·4 and 4·5 ± 0·4 h. These figures enable an estimate of the cell cycle phases: namely, $G_1 = 11$ %, $S = 43$ %, $G_2+M =$ 46 % (Table 2). These figures differ somewhat from others in the literature (Katz &

Table 1. *Determination of mitosis and number of nuclei/cell*

Experiment	Mitosis (min)	Number of nuclei 1	2	3
1	9·5	59	6	1
2	7·5	74	8	3
3	14·25	61	9	0
4	10·75	42	1	3
5	11·33	88	11	4
6	8·67	43	5	1
7	8·25	56	11	0
8	9·0	79	13	0
9	14·25	59	3	3
10	12·67	47	10	0
11	10·75	66	11	2
12	12·50	63	8	3
13	11·33	41	7	0
14	13·00	36	0	1
15	12·10	52	11	1
	$\bar{x} = 11{\cdot}06 \pm 2{\cdot}12$	% Mononucleate = 87·5		

Table 2. *Length of individual cell cycle phases*

Cycle phase	Hours ± S.D. (n)*	Percentage of total
M	0·18 ± 0·04 (15)	2
G_1	0·9 ± 0·4 (12)	11
S	3·7 ± 0·40 (12)	43
$G_2 + M$	3·9 ± 0·40 (12)	46

* The number of experiments (n) for each determination is indicated in parenthesis. Estimates were based upon 2% harvest results for DNA synthesis and cell number.

Bourguignon, 1974; Zada-Hames & Ashworth, 1977), although there is agreement that S-phase and G_2 combined occupy most of the cell cycle.

In order to perform sorting experiments (see below), we needed more cells than could easily be harvested from a 2% wash-off. For these experiments, we modified the procedure (shaking at 50 rev./min for 15 min), to give a harvest of approximately 6%. These experiments still gave excellent synchrony (cell number doubles between $5 \pm 0{\cdot}48$ and $9 \pm 0{\cdot}60$ h and [^{3}H]thymidine incorporation occurs between $0{\cdot}67 \pm 0{\cdot}49$ h and $5{\cdot}46 \pm 0{\cdot}48$ h). There was no detectable contamination with randomly phased cells, since there was no continuous rise in the cell number or [^{3}H]thymidine incorporation plots and since the division step was still a precise doubling. The data suggest that harder and longer shaking removes mitotic cells and also cells in an adjoining phase-arc of the cell cycle (e.g., early G_1 ; see below).

Although cells from a 2% harvest were more synchronous than 6% cells, synchrony was still incomplete. Division occurred over 2 h. Since the harvest is now

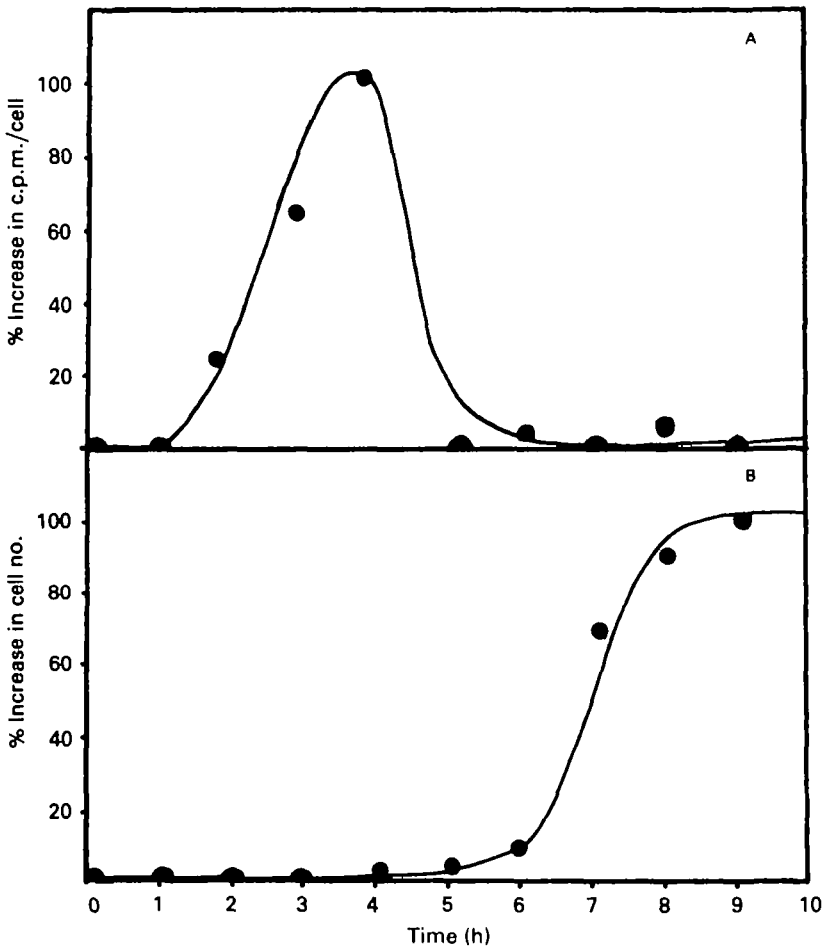

Fig. 1. Synchrony of cells from 2% harvest. A. Incorporation of [^{3}H]thymidine into pulse-labelled cells (c.p.m./cell). B. Increase in cell number with increasing incubation (cells/ml).

reduced to the mitotic percentage, it is likely that wash-off asynchrony is minimal. It is unlikely that wash-off occurs before mitosis, because there is never extensive cell division at the beginning of the experiment. Extreme cases (e.g. see Fig. 2) show about 10% of cells dividing within the first hour and this corresponds to counts of up to about 10% of binucleate cells at time zero (Table 1). Presumably, both reflect mitotic cells that have not completed cytokinesis before the zero point. On the other hand, even the 2% experiments show considerable variation in the apparent G_1, which may even be absent (Fig. 3). This probably reflects intrinsic variation in the cell cycle. We have made films of *D. discoideum* cell division, and these confirm that cycle length varies considerably (between 6 and 10 h).

The synchronized cells were used to examine the developmental fates of different cell cycle phases. Cells were taken at 1-h intervals from 0 h to 9 h after wash-off, washed free of medium, labelled with TRITC as described above, mixed with unlabelled

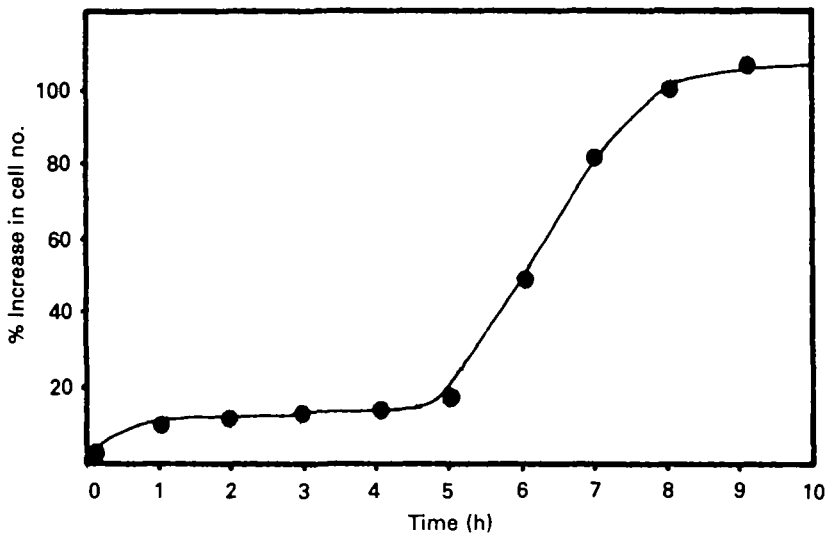

Fig. 2. Increase in cell number due to cytokinesis of multinucleate cells. An initial 10% increase in cell number is observed between 0 and 1 h after wash-off (cells/ml) and can be attributed to the cytokinesis of those cells that are not mononucleate at the time of harvest (87·5% are mononucleate).

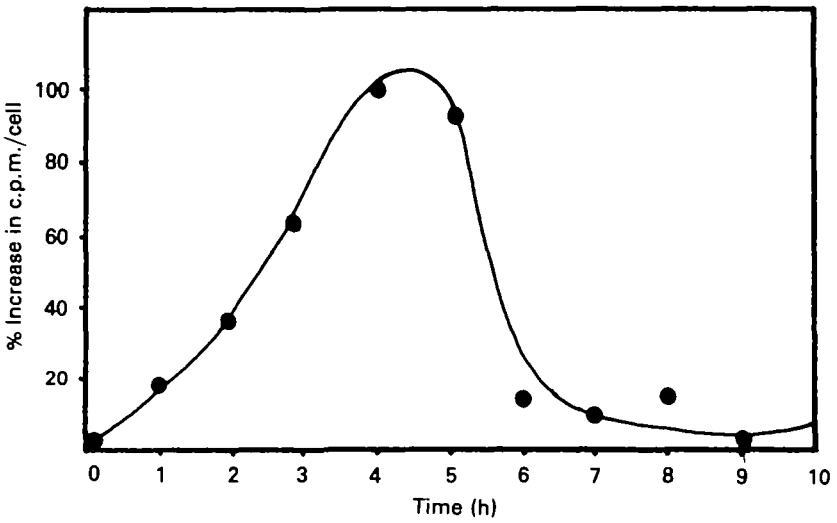

Fig. 3. Lack of G_1-phase during cell cycle. Incorporation of [^{3}H]thymidine into pulse-labelled cells (c.p.m./cell) displays linear increase with time initially as compared to the plateau between 0 and 1 h observed in those cells that do have a G_1-phase.

asynchronous cells and allowed to develop. In these experiments, it was found that cells from early cell cycle phases showed a gradually increasing tendency to sort to the tip of the slug, with a maximum effect at 3, 4 and 5 h after wash-off, when 91% of cells sorted to the slug tip. These times correspond to S and early G_2-phases of the cell cycle, as judged by the analysis above. Cells from later than 5 h or earlier than 3 h showed a strongly reduced tendency for migration to the slug tip (Fig. 4; Table 3).

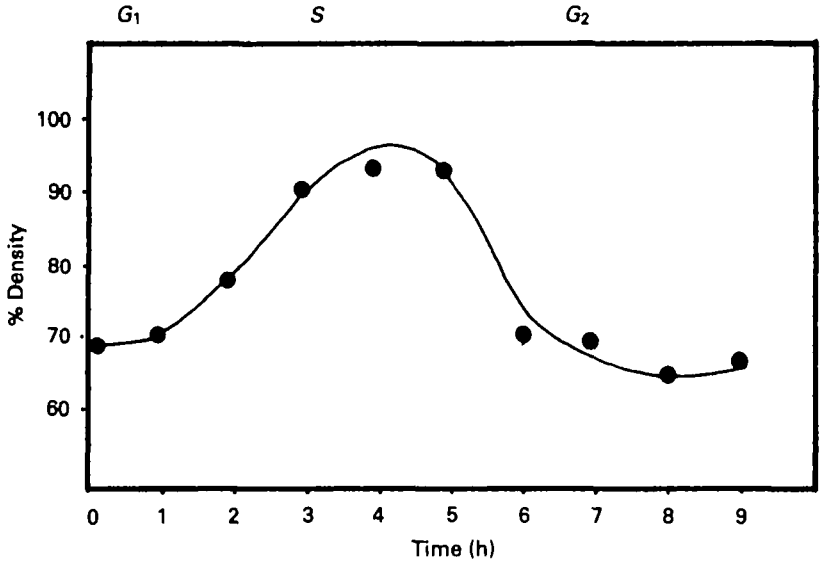

Fig. 4. Incorporation of TRITC-labelled cells into the slug tip (see Table 3). Fluorescently labelled cells were preferentially incorporated into slug tips during *S*-phase and very early G_2. The high background level of incorporation was attributed to overloading of a particular cycle phase at any one time.

Table 3. *% Density of fluorescent cells in prestalk zone of slug*

Hours after wash-off	Experimental	Control	Reverse
0	69·16	53·81	47·34
1	71·33	55·39	50·30
2	77·97	52·49	49·77
3	89·89	51·73	53·33
4	92·34	52·60	50·79
5	91·87	54·97	49·61
6	70·82	54·11	49·60
7	69·04	53·33	47·52
8	66·33	54·82	51·30
9	68·22	53·01	51·24
$\bar{x}$	76·70 ± 10·59	53·63 ± 1·20	50·09 ± 1·78
$\bar{x}_{(0\text{–}2,\ 6\text{–}9)}$	70·40 ± 3·69	–	–
$\bar{x}_{(3\text{–}5)}$	91·37 ± 1·30	–	–
$P = 0{\cdot}001$	$\bar{x}_{(3\text{–}5)}$ significantly greater than $\bar{x}_{(0\text{–}2,\ 6\text{–}9)}$		

Each point represents 100 slugs (10 slugs/time point per 10 experiments) from which % density was calculated as follows:

$$\frac{\text{Density of cells in prestalk}}{\text{Density of cells in prestalk plus density in prespore}} \times 100.$$

There is thus a cell cycle phase-specific tendency for labelled cells to sort to the slug tip in *S* and early G_2. In addition to cell cycle phase-specific sorting, we also observed a general tendency for labelled cells of any phase to proceed to the prestalk zone, if these are mixed with unlabelled cells of random phase (70 %, by density of labelled

cells were found in the tip in points before 3 h and after 5 h). This is not purely a labelling artifact, because appropriate control experiments (see Materials and Methods) show no special tendency for labelled cells to sort to the prestalk zone. This phenomenon is unexplained. It may be an artifact caused by overloading with one cell cycle phase.

DISCUSSION

Our results demonstrate that highly synchronous populations of relatively unperturbed cells can be obtained using a wash-off procedure. Owing to variations in the time required by individual cells to complete their cycles and to variations in the wash-off phase, cultures did not display complete synchrony. On the basis of cell counts and [^{3}H]thymidine incorporation, it was estimated that cells spend approximately 10 % of their time in G_1, 40 % in S-phase and 50 % in G_2. Mitosis accounted for approximately 2 % of the total cycle. G_1 was apparently the most variable phase of the cell cycle, sometimes completely lacking. These values differ from others reported in the literature, although we agree with a previous report (Zada-Hames & Ashworth, 1977) that S-phase and G_2 combined occupy 90 % of the cycle.

Our sorting experiments indicated that there is a strong correlation between a cell's location in the cell cycle at the time of starvation and its subsequent position in the slug. Cells located in S-phase and very early G_2 (3, 4 and 5 h after wash-off) are preferentially incorporated into slug tips, whereas cells in mid-G_2 through mitosis are not.

Investigation of the timing of cell sorting has shown that this normally occurs during late aggregation and formation of the slug; between about 12 h and 15 h of development (Durston & Vork, 1979; Tasaka & Takeuchi, 1979). Our result thus indicates that cell subpopulations at this stage differ, according to their previous cell cycle phase at starvation, in such a way that they now sort out. The nature of their difference is obscure (see below), but it could well still be based on cell cycle differences. Apical and basal regions of the late aggregate differ in the incidence of mitosis (Bonner & Frascella, 1952) and in nuclear [^{3}H]thymidine incorporation (Durston & Vork, 1978); [^{3}H]thymidine incorporation at least is confined to the presumptive prespore region. It is thus possible that cells in different cell cycle phases at starvation are also in different phases during late aggregation or that different phases at starvation later generate cycling and non-cycling cells, respectively. Either circumstance could arise via appropriate temporal regulation of a G_2 block at the early aggregation stage (see Introduction). To suggest a possibility that fits the facts, the block might normally operate with lower probability on cells reaching it later. This is compatible with the asymmetric pattern of sorting cell cycle phases at starvation (cells gradually become more prestalk-destined to a certain point in early G_2, then abruptly lose this tendency), if blocked cells sort tipwards.

The difference leading to cell sorting could involve cell adhesiveness. Electron microscopy studies have shown changes in surface morphology and in cell–substrate adhesion throughout the cell cycle (Lloyd, Poole & Edwards, 1982). Mitotic cells

round up and loosen their attachment to the substrate. G_1 cells reattach via microvilli and blebs appearing on their surfaces. *S*-phase cells flatten and adhere extensively to the substrate, while usually losing microvilli and surface blebs. G_2 cells regain microvilli and blebs and ultimately round up for mitosis. These modulations were first reported for Chinese hamster ovary cells (Porter, Prescott & Frye, 1973), but similar changes have since been reported for many cell types. It is plausible that different cell cycle phases or cycling and non-cycling cells differ in adhesiveness. Differential adhesion has also already been postulated as a mechanism for *D. discoideum* cell sorting (Bonner, Sieja & Hall, 1971; Yabuno, 1971; Sternfeld, 1977; Feinberg, Springer & Barondes, 1979; Tasaka & Takeuchi, 1979; Lam, Pickering, Geltosky & Sui, 1981) and several investigators have concluded that prestalk and prespore cells differ from each other in adhesiveness. It is thus possible that *D. discoideum* cells sort out due to cell-cycle-based adhesion differences, but there is no direct evidence for this.

For completeness, we note that there is evidence that chemotaxis of prestalk cells is important for *D. discoideum* cell sorting (Matsukuma & Durston, 1979; Durston & Vork, 1979). The possibility that chemotactic differences between prestalk and prespore cells underly cell sorting has not been excluded, and there is, in fact, evidence that such differences exist at an early stage of development (Inouye & Takeuchi, 1982). The mechanism of cell sorting and the basis of its relation to the cell cycle remain to be clarified.

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NOTE ADDED IN PROOF

A recent flow cytofluorimetric analysis by one of us (A. J. Durston & C. J. Weijer, unpublished) supports our view (above) that G_1 phase is short or absent in the *D. discoideum* cell cycle. It also indicates that *S* phase is shorter than found here. The period of [^{3}H]thymidine incorporation reported here and in previous synchrony studies is presumably contributed to by a period of mitochondrial DNA synthesis.