Changes in Cellular Organisation During Apogamic Development in *Physarum polycephalum.*

by Adrian B. Blindt

ABSTRACT

Amoebae of strain CL of *Physarum polycephalum* undergo apogamic development to form multinucleate plasmodia. During the amoebal-plasmodial transition, large uninucleate cells become irreversibly committed to plasmodium development. The transformation of an amoeba to a plasmodium involves a change in the tubulin isotypes expressed and a radical restructuring of cellular microtubules. During the transition the amoebal cytoplasmic microtubules, centrioles and cytoplasmic MTOC must disappear and the plasmodial-specific tubulin isotypes and intranuclear microtubule organising centre (MTOC) must be acquired.

In developing cultures, amoebae lose the ability to flagellate before they become committed. Enriched suspensions of committed cells can be obtained by inducing asynchronous differentiating cultures to flagellate and passing the cells through a glass bead column. The resulting committed cells can be cultured, with some synchrony, to form plasmodia on bacterial lawns or in axenic liquid medium but cannot be cultured on axenic agar medium. During mating, cells lose the ability to flagellate early in plasmodium development. Committed cells from mating mixtures can be enriched in a similar way to committed cells of CL and have similar growth characteristics.

Uninucleate committed cells of CL have the same DNA content as amoebae and plasmodia but have 6-10 times the amount of RNA. Apogamic committed cells express tubulin isotypes characteristic of amoebae, but after culture in axenic liquid medium, the cells express plasmodial-specific tubulin isotypes. Results suggest that plasmodial-specific tubulin isotypes are switched on in quadrinucleate cells. The amoebal cytoskeleton persists in binucleate and quadrinucleate cells but has disappeared in larger multinucleate cells. Mitosis in uninucleate committed cells is intranuclear (plasmodial-type). The amoebal MTOCs are eliminated during the first few mitotic cycles after commitment and do not become the plasmodial intranuclear MTOCs. Centriole loss apparently occurs before MTOC loss.

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A thesis submitted for the degree of Doctor of Philosophy at the University of Leicester.

April 1987

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ACKNOWLEDGEMENTS

I would like to thank Dr. Jennifer Dee for encouragement and advice during the experimental work and for her endless patience during the writing of this thesis. I would also like to thank Dr. Keith Gull for his help and advice during the time spent in Canterbury.

I am grateful for the technical assistance and instruction received from Jennifer Foxon in Leicester and from Marianne Wilcox, Chris Birkett, Kay Foster, Patrick Hussey, Eileen Paul and Trevor Sherwin in Canterbury.

I am happy to acknowledge financial support for this work from the Science and Engineering Research Council.

I would also like to thank my wife, Annette, for her support, both financial and personal, during the writing of this thesis and for her patience while we sort out our future!

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Chapter 1

General Introduction.

1.1 The life cycle of *Physarum polycephalum*

Physarum polycephalum is a true slime mould or Myxomycete. In the life cycle (Figure 1.1), there are two distinct vegetative growth phases; microscopic, uninucleate, colourless amoebae and macroscopic, multinucleate, pigmented, syncytial plasmodia. Amoebae undergo the amoebal-plasmodial transition to give rise to plasmodia; this may be the result of mating between two heterothallic strains or, in some mutant strains, the result of selfing.

Amoebae, except for mutant AXE strains, can be grown only in monoxenic culture on lawns of bacteria on agar plates. The amoebae divide by binary fission to form colonies which appear as clear plaques on the bacteria lawn. Mitosis in amoebae occurs with breakdown of the nuclear membrane ("open mitosis"). As well as the amoebal-plasmodial transition, amoebae can undergo two other transitions. When amoebae are starved, they encyst to give microscopic cysts; if these microcysts are replaced in nutrient conditions they will germinate to give growing amoebae. If amoebae are suspended in water or some buffers (such as phosphate buffer) they form biflagellated cells (flagellates); these cells lose their flagella when replaced on solid medium and return to normal amoebal growth. Encystment and flagellation both give rise to non-feeding forms; the amoebal-plasmodial transition however, is a change from one metabolically active cell-type to another and is not reversible (although sporulation of the plasmodium will eventually lead back to amoebae).

Plasmodia can be grown in the laboratory axenically on solid surfaces where they appear large, flat, disc-like and yellow in colour. Under

Figure 1.1 Schematic diagram of the life cycle of *Physarum polycephalum*.

m = mitosis

In the life cycle (see 1.1), microscopic amoebae can undergo two reversible transitions. On starvation they form cysts which revert when replaced in nutrient conditions. If amoebae are placed in liquid they form flagellates; these revert when replaced on solid medium. Amoebae give rise to macroscopic plasmodia either as a result of mating or, in certain mutant strains, by apogamy.

If plasmodia are starved in the dark, they form spherules; these hatch to give plasmodia when replaced in nutrient conditions. If plasmodia are starved and exposed to light, they sporulate. Meiosis takes place in spores which hatch to give amoebae.

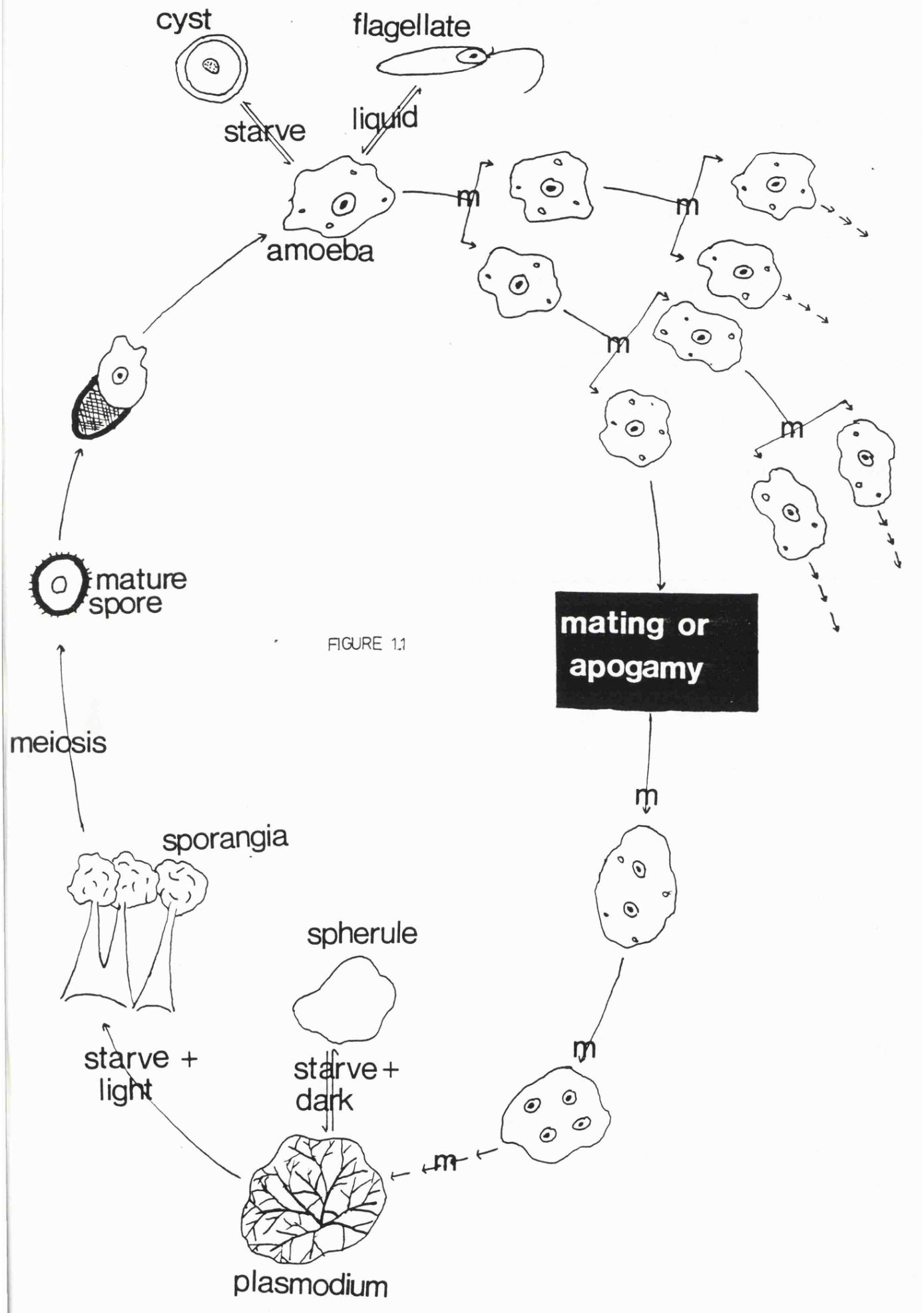

cyst
flagellate
starve
liquid
amoeba
m
m
m
m
mature spore
mating or apogamy
meiosis
m
sporangia
spherule
starve + light
starve + dark
m
m
plasmodium

FIGURE 1.1

such conditions, the plasmodium is ideal for biochemical investigation since it is a single giant macroscopic cell. Rhythmical cytoplasmic streaming can be observed in the prominent veins, the cytoplasm streaming first in one direction for approximately 30 seconds and then in the other; migration of the whole plasmodium occurs when the net movement in one direction exceeds that in the other. Macroplasmodia exhibit synchronous mitoses, thus making the cell ideal for studying the cell-cycle. Mitosis in plasmodia is intranuclear ("closed mitosis"). Pieces of the same plasmodium rapidly fuse with each other when in contact on solid medium. Plasmodia can also be cultured in axenic liquid medium; in such conditions they grow as yellow microplasmodia, each containing several hundred nuclei.

When plasmodia are starved, two transformations may occur. If the plasmodium is starved in the dark, it will form a sclerotium (or spherules); this is a dormant form and can hatch to give a plasmodium when replaced in nutrient conditions. If the plasmodium is starved and then illuminated the plasmodium may sporulate. Spores form on stalks and meiosis takes place within the spore walls to give rise to mature spores. The spore walls become black due to melanisation occurring. In suitable conditions, spores germinate to give amoebae.

1.2 Genetics of mating.

In wild-type strains, the amoebal-plasmodial transition is the result of mating between two different heterothallic strains of haploid amoebae. Two amoebae which differ at a single locus, termed matA (formerly mt) are capable of mating; mating is not possible between strains with the same matA gene (Dee, 1960). Youngman *et al.* (1979) and Dee (1978) noticed

that strains of different origin appeared to have remarkably different crossing efficiency, despite having the same combination of matA alleles in the cross. Genetic analysis by both of these workers led to the discovery of another unlinked locus termed matB (originally designated as rac by Dee, 1978). It has been shown that amoebae that differ at the matB locus can fuse at high frequency to form haploid binucleated cells after reaching a critical density (Shipley and Holt, 1982); cells with the same matB alleles also fuse but at a frequency approximately 1000 times lower. If the fused amoebae differ at the matA locus, development will continue; the nuclei in the haploid binucleate cell fuse to give a diploid zygote, the zygote undergoes mitosis without cytokinesis and becomes a diploid binucleated cell and further rounds of mitosis without cytokinesis lead to a macroplasmodium. If the amoebae have the same matA alleles, the cell will either break up to give two haploid amoebae again or, if the cell goes through mitosis, spindle fusion followed by cytokinesis may occur which leads to two diploid amoebae. Diploid amoebae grow and divide in a similar way to haploid strains and can undergo the same developmental changes. Other genes have now been found, such as imz (termed matC by Anderson, 1986) which also lead to higher mating efficiency; these genes also appear to act at the level of cell fusion (Shinnick *et al.*, 1978).

1.3 Apogamic development

Although in most natural isolates of *Physarum* the amoebal-plasmodial transition occurs only as a result of mating there are mutant strains in which plasmodia can arise in clones. One such strain, CL (Cooke, 1974) has been used extensively in this thesis. The amoebal-plasmodial

transition in CL is temperature sensitive (Adler and Holt, 1977) although amoebae and plasmodia will grow vegetatively at the temperature restrictive for development (30°C). At 30°C, CL amoebae can be cultured in the same way as heterothallic amoebal strains; they grow by binary fission, they will encyst and flagellate in suitable conditions and they will cross with heterothallic strains (see 1.2). At 26°C, the temperature at which heterothallic strains are normally cultured, plasmodia appear in clones of CL amoebae within 3 to 5 days.

Cooke and Dee (1974) showed that the nuclear DNA contents of CL amoebae and plasmodia were the same, indicating that development occurred without nuclear fusion. Anderson *et al.* (1976) analysed time-lapse films of the amoebal-plasmodial transition in CL. After an initial period of amoebal proliferation they observed that many cells grew larger and then became binucleate by mitosis without cytokinesis; they could not trace the origin of these large uninucleate cells, however they did not observe cell fusion occurring between uninucleate cells nor did they observe nuclear fusions. The binucleate cells proceeded to develop into plasmodia by further mitoses and fusions with other multinucleate cells. They suggested that development in CL was apogamic and not homothallic as originally supposed (Wheals, 1970). They also suggested that the increase in nuclear size observed in the large uninucleate cells was due to increased RNA synthesis.

Youngman *et al.* (1977) studied the kinetics of amoebal growth and plasmodium formation in cultures of CL grown at a temperature permissive for the amoebal plasmodial transition. They observed that there was first a period of amoebal proliferation; cells replated during this time formed amoebal colonies. Later, depending on factors such as

temperature, medium and cell density, at a stage when no plasmodia were visible in the cultures, some of the replated cells grew as plasmodia (see also 2.8). These cells are said to be irreversibly committed to plasmodium formation or "committed cells". The number of committed cells rose rapidly until a maximum number was reached; the number then proceeded to fall, presumably due to plasmodial fusions taking place. Youngman *et al.* (1977) also found that they could induce CL amoebae to differentiate faster and at lower cell densities than in control cultures by placing them on the opposite side of a filter to a dense population of amoebae. Thus, there must be an extracellular inducer produced by amoebae which can induce differentiation in apogamic strains. Shipley and Holt, (1982) produced a crude cell extract from amoebae of *Didymium nigripes* that was capable of inducing mating competence in strains of both *Didymium* and *Physarum*; it was also capable of inducing apogamic strains to differentiate. Attempts to purify this extract have to date been unsuccessful (Nader *et al.*, 1984).

Burland *et al.* (1981) performed an experiment which combined assays for replateable plasmodia with microscopic observation of the number of nuclei present in samples of cells from different time-points during the amoebal-plasmodial transition. They discovered that committed cells were detectable before binucleate cells could be observed. Furthermore, from their work it is possible to estimate that the time between commitment and binucleate cell formation is under 9 hours. Collett *et al.* (1983) used similar techniques and estimated that the time between commitment and binucleate cell formation was 6-8 hours. They also estimated that when cells became committed, they were approximately three times the size of amoebae. Anderson *et al.* (1976) estimated that when committed cells

went through mitosis they were approximately four times the size of amoebae. It thus seems likely that the cell cycle during which cells become committed is longer than the normal amoebal mitotic cycle.

Recently, Bailey *et al.* (1987) have managed to trace the cell pedigrees of cells that became binucleate using time-lapse cinematography. Their work confirms that development in CL is apogamic. They have followed cells from the previous amoebal division to binucleate cell formation and concluded that sister cells need not share the same fates. They also estimated that the cell cycle during which commitment takes place was approximately two and a half times as long as a normal amoebal cell cycle.

Kerr, (1967) working on an apogamic strain of the myxomycete *Didymium nigripes*, observed large uninucleate cells become binucleate without cell fusion occurring. The mitosis by which these binucleate cells arose was observed by phase contrast and time-lapse microscopy to be a plasmodial-type "closed" (or intranuclear) mitosis. Kerr thus described these large uninucleate cells as "uninucleate plasmodia". Several unpublished investigations on *Physarum* have utilized phase contrast microscopy and time-lapse cinematography to observe the mitosis by which large uninucleate cells of apogamic strains give rise to binucleate cells (J. Dee, personal communication). The results of these investigations indicate that, in the majority of cases, binucleate cells arise by a "closed" mitosis.

Chainey (1981) carried out temperature shift experiments in which differentiating cultures of CL were incubated at a temperature permissive for the amoebal plasmodial transition and then shifted to a non-permissive temperature. From her results she concluded that there was a

temperature sensitive period (TSP) for commitment to occur and that the TSP ended approximately six hours before commitment. This implies that the TSP ends and commitment begins in the same cell cycle.

1.4 Genetics of apogamic development

Apogamic strains have been isolated from heterothallic strains by several workers (Adler and Holt, 1977: Gorman *et al*, 1979: Honey, 1979: Honey *et al*, 1981). These mutants, designated gad mutants, have been crossed with other strains in an effort to map them and it was found that they all mapped closely to the matA locus. The majority of gad mutations did not recombine with matA suggesting that the mutations may be in the matA locus. There is evidence that the strain CL may have arisen through a gad mutation in a matA2 strain, known as gad-h (Anderson, 1979). Since gad strains are temperature sensitive (e.g. Adler and Holt, 1977), they may be crossed with heterothallic strains or indeed apogamic strains (which have been isolated in a different matA strain) at a non-permissive temperature for selfing.

Mutations have been obtained in gad strains which prevent plasmodium formation; most of these, termed npf mutations, also map at the matA locus. Anderson *et al.* (1986) classified these mutations as "class 1" and "class 2". Class 1 mutants will cross with strains that carry the matA allele of the strain from which the gad npf strain was derived, whereas class 2 strains will not. Class 2 mutations may thus be true revertants since they have the mating type of the heterothallic strain from which they were derived. Anderson *et al.* (1986) showed that class 1 mutants do not express novel matA alleles; it thus seemed probable that they lacked a distinct function required in plasmodium development.

The gad mutations and class 1 npf mutations thus appear to define functions associated with matA but distinct from the mating specificity function. Further study of these mutations will provide a greater understanding of the biochemical and molecular basis of the amoebal-plasmodial transition. However, a major requirement for studying the matA locus in greater detail would be a DNA transformation system. Unfortunately, at present, there is no transformation system available in *Physarum* and further progress on this line of investigation will be delayed until one becomes available.

1.5 Differences between amoebae and plasmodia

The major morphological differences between amoebae and plasmodia could merely be attributed to the difference in cell size; there may not be many differences in gene expression. Researchers have used various techniques to find the extent of the differences in gene expression between the two growth phases. Burland and Dee, (1979) isolated temperature sensitive growth mutants in the strain CLd (gad-h npfC). The mutants were isolated in both the amoebal stage (called ATS mutants) and the plasmodial stage (called PTS mutants). In a sample of ATS mutants, 51% were temperature sensitive in the plasmodial stage and in a sample of PTS mutants, 67% were temperature sensitive in the amoebal stage. Thus, whilst many genes are expressed in both phases, a large proportion of the genes of *P. polycephalum* are phase specific.

Turnock *et al.* (1981) analysed 2-dimensional gels of amoebal and plasmodial proteins of CL, synthesized during growth under identical conditions on bacterial lawns. They judged that among 306 abundant proteins, 12% were amoebal specific and 14% plasmodial specific; a

further 18% showed substantial differences in expression between the two stages. The differences between the two stages of the life cycle are probably far greater than estimated by this experiment as some of the spots present in both cell-types may be due to different proteins migrating to the same place on the gels. So, although it is difficult to list a large number of morphological differences between amoebae and plasmodia, the two cell-types appear to be very different in terms of gene expression.

Monoclonal antibodies have also been raised by injecting whole fusion-competent amoebae into mice (Shipley, reported by Anderson *et al.* 1986). By using this method, antibodies have been raised to amoebal cell-surface antigens and 26 amoebal-specific hybridomas have been found. This work may eventually define a whole new range of amoebal-plasmodial differences.

Differences in expression between amoebae and plasmodia have been looked for at the level of transcription. Several laboratories have attempted to find such differences (Cox *et al*: Pallotta *et al*: Sweeney *et al*). Their work has involved producing cDNA libraries and then probing RNA from amoebae and plasmodia with the resulting clones. Sweeney *et al.* (1987) have been succesful in identifying phase-specific clones as have Pallotta *et al.* (1986). Cox *et al.* (1986) however, did not find any plasmodial or amoebal specific clones in the course of their work although differences in rates of expression were found; this was particularly surprising as different strains of amoebae and plasmodia (originating from different isolates) were used in their investigations.

Havercroft and Gull (1983) utilised monoclonal antibodies (see p.11) with immunofluorescence microscopy to visualise the arrangement of

microtubules during the cell cycle of *Physarum* amoebae and plasmodia. They observed that in plasmodia, microtubules were only present during mitosis where they formed the mitotic spindle. However, amoebae had a microtubule cytoskeleton during interphase which appeared as an array of microtubules coming from a single point, the microtubule organising centre (MTOC). Before mitosis the MTOC appeared to duplicate and then formed the poles of the mitotic spindle. The amoebal mitotic spindle differed from that of the plasmodium as star-shaped projections, called asters, radiated from the poles on the opposite side to the spindle.

Several well documented differences between amoebae and plasmodia are associated with differences in microtubular organisation. Flagella, the mitotic spindle, the microtubule cytoskeleton and the centrioles are all known to be tubulin-containing structures. Burland *et al.* (1983) and Roobol *et al.* (1984) looked for differences in the expression of tubulin isotypes between amoebae and plasmodia. They found that amoebae express two tubulin isotypes ($\alpha1,\beta1$) separable on 2-dimensinal gels, while plasmodia express two α tubulin isotypes ($\alpha1,\alpha2$) and two β tubulin isotypes ($\beta1,\beta2$). This was a surprising result as plasmodia only assemble microtubules during mitosis, whereas in amoebae, tubulin is assembled in cytoplasmic microtubules, the MTOCs, centrioles and also in the mitotic spindle. Green and Dove, (1984) showed that flagellates express a novel tubulin isotype, $\alpha3$, which is a post-translational modification of $\alpha1$ (Sasse *et al*, 1987).

Burland *et al.* (1984) isolated mutants resistant to MBC (an anti-microtubular drug) in an effort to find mutations with altered tubulin protein; these mutants were identified by looking for differences in the position that the tubulin isotypes focussed on 2-dimensional gels. One

such mutant (called an altered spot mutant) was BEN210. Plasmodia of BEN210 express α1, α2, β2 and an altered spot called β210. However, BEN210 amoebae express α1, β1 and β210. Thus the mutation in BEN210 must be in a gene that gives rise to a protein that normally focusses in the β1 position. Furthermore, the result proves that the products of more than one gene must focus at the normal β1 position and that one of these genes is not expressed in plasmodia.

Schedl *et al.* (1984) investigated the organization of the α and β tubulin gene families using Mendelian analysis of restriction endonuclease-generated DNA fragments. They found that there are at least 4 unlinked α-tubulin loci (termed altA, altB, altC, altD) and 3 unlinked β-tubulin loci (termed betA, betB, betC). They also followed the segregation of some MBC resistant mutants (ben mutants) and found that betB is allelic with benD, the locus at which the altered spot mutation ben-210 maps.

Observation of the differences in expression of tubulins has been facilitated by the use of monoclonal antibodies. Antibodies are available which bind specifically to α tubulin isotypes (such as DM1A, YOL 1/34 and KMP-1) and β tubulin isotypes (such as DM1B, DM3B3 and KMX-1). These antibodies have been used in two ways to follow the expression of tubulin. Havercroft and Gull (1983) used immunofluorescence microscopy to follow changes in microtubule distribution during the cell cycle of both plasmodia and amoebae (see p.10). Monoclonal antibodies have also been used to probe Western blots of 2-dimensional gels of amoebal and plasmodial proteins. Birkett *et al.* (1985) probed 2-dimensional gels of amoebal and plasmodial proteins with the antibodies listed above. They found that the amoebal α1 tubulin is

composed of three sub-types and that the plasmodial α1 tubulin is composed of four sub-types. This corresponds with the estimates of Schedl *et al.* (1984).

1.6 *Physarum* as an experimental organism.

All of the differences between amoebae and plasmodia (described in 1.5) make the system particularly promising for use as a model for following changes in gene expression associated with differentiation. Differences exist between the two cell-types both of a cytological and biochemical nature. However, for the system to be ideal as a model system, several features are required which at present are missing.

Other developmental systems (e.g. *S. cerevisiae*) have utilized developmental mutants and DNA transformation systems to investigate developmental switches. The lack of a DNA transformation system in *Physarum* has prevented the developmental mutants which are available being exploited fully (see also 1.4). Another feature that *Physarum* lacks is synchronous development to facilitate biochemical studies (see 3.1.1). The primary aim of this thesis was to synchronise development in the apogamic strain CL.

The amoebal-plasmodial transition of *Physarum* is a particularly suitable system for studying changes in tubulin expression associated with development. As discussed above (1.5), differences between the two cell-types have been described on both the cytological and biochemical level. The transformation of an amoeba to a plasmodium must require radical restructuring of cellular microtubules. It is possible that following these changes through the amoebal-plasmodial transition, could

throw light on the role of microtubules in cell differentiation and possibly on mechanisms involved in the amoebal-plasmodial transition.

CHAPTER 2

Materials and Methods.

2.1 Genetic loci used in this work

matA — involved in plasmodium formation (see 1.2)

matB — involved in amoebal cell fusion (see 1.2)

gad-h — Locus mapping closely to matA which allows apogamic development (see 1.4)

npfC — Locus mapping close to gad-h which prevents selfing (apogamic plasmodium formation) (see 1.4)

fusA — This locus is one of several loci controlling plasmodial fusion. Laboratory strains in Leicester are isogenic at the other fus loci. Two fusA alleles are used, fusA1 and fusA2. Strains that are homozygous at the fusA locus will fuse with either a strain that is homozygous for the same allele or with an apogamic strain that is hemizygous for that allele. However, the heterozygous fusA1/fusA2 will only fuse with another strain that is fusA1/fusA2. fusA is thus a particularly useful tool for checking that a plasmodium is indeed the result of mating.

AXE — The basis of growth in axenic medium is unclear but it is likely that more than one mutations are involved. AXE is used to describe the phenotype of ability to grow in axenic medium (SDM).

leu-1 — causes requirement for leucine.

hts-1 — causes plasmodia to die at high temperature.

2.2 Amoebal strains used in this work

Strain	Refererence or origin	Relevant Genotype
CL	Cooke (1974)	matA2 gad-h npfC$^+$ matB1 fusA2
CLd	Cooke and Dee (1974)	matA2 gad-h npfC matB1 fusA2
CLd-AXE	McCullough *et al* (1978)	matA2 gad-h npfC matB1 fusA2 AXE
LU860	Cooke and Dee (1975)	matA1 matB1 fusA2
LU862	Dee (1978)	matA3 matB3 fusA1
LU213	Dee (unpublished)	matA3 matB3 fusA1 leu-1
LU352	Dee (1986)	matA2 gad-h npfC matB3 fusA1 AXE
LU353	Dee (1986)	matA3 matB1 fusA2 leu-1
LU600	Dee (1986)	matA2 gad-h npfC$^+$ matB3 fusA1 AXE

2.3 Culture and preparation of *Escherichia coli* as a food source for *Physarum* amoebae.

The strain of *E. coli* used during this work was B145. *E. coli* was streaked onto nutrient agar plates and incubated overnight at 37°C. The bacteria were then washed off the following day in 5ml of sterile distilled water and pipetted into a test-tube. The standard bacterial suspension (SBS) was vortexed for at least 5 seconds before use.

Amoebae were cultured on various media (see below and in main text) in monoxenic culture. The addition of 0.1ml of SBS as a food source is designated as B following the medium (e.g. LIAB, DSDMB).

2.4 Preparation of formalin-killed *E. coli* (FKB).

One litre of Oxoid Nutrient Broth was inoculated with 1ml of a dense suspension (10^9 to 10^{10} cells/ml) of *E. coli* and incubated on a rotary shaker at 37°C overnight. The culture was spun down and resuspended in 50ml of buffer (see p.17). The suspension was transferred to a sterile flask, 4ml of formaldehyde (AR) added and the suspension allowed to stand at 4°C overnight. The bacterial suspension was spun down, the pellet resuspended in 50ml of buffer and 5ml of 1M glycine added. The suspension was agitated on a shaker at 30°C or 37°C for 1 hour and then spun down and the pellet washed twice in 50ml of buffer. The pellet was resuspended in 20ml of distilled water (this gave a cell density of 10^9 to 10^{10} cells/ml) and stored in 1ml aliquots in screw-cap bottles at 4°C. To check that no live bacteria were present, some FKB was streaked on nutrient agar plates and incubated overnight at 37°C.

Buffer (pH6.8) used in preparation of FKB.

The following constituents were added to 1 litre of distilled water and sterilized in an autoclave at 15 PSI for 15 minutes..

Na_2HPO_4	50mM	7.0g
KH_2PO_4	20mM	3.0g
NaCl	70mM	4.0g
$MgSO_4.7H_2O$	0.4mM	0.1g

2.5 Media

SDM (Semi-defined medium) liquid.

The following constituents were added to 1 litre of distilled water.

Glucose	10.0g
Soytone (Difco-bacto)	10.0g
KH_2PO_4	2.0g
$CaCl_2.6H_2O$	1.35g
$MgSO_4.7H_2O$	0.6g
$FeCl_2.4H_2O$	0.039g
$ZnSO_4.7H_2O$	0.034g
Citric acid	3.54g
EDTA.Disodium salt	0.224g
Biotin	0.005g
Thiamine	0.04g

The pH was adjusted to 4.6 with 20% NaOH and the medium was sterilized in an autoclave at 15 PSI for 15 minutes. 1ml of 0.05% hematin (in 1% NaOH) was added per 100ml immediately before use.

SDM agar. Molten 3% agar was mixed with an equal volume of SDM liquid just before use.

DSDM (Dilute semi-defined medium). 62.5ml of SDM liquid were added per litre of 1.5% agar.

DSDM5 (DSDM at pH 5). Same as DSDM but buffered with 5ml/litre of 1M pH5 citrate buffer (0.35M citric acid monohydrate (73.5g/l): 0.65M trisodium citrate dihydrate (191g/l))

LIA (Liver infusion agar). 4g of Oxoid liver infusion powder (Oxoid product Nº L26 and 10ml of sodium phosphate buffer (0.5M; pH 6.8) were added per litre of 1.5% agar.

LIA/DSDM agar. The constituents of LIA and DSDM were combined in a single preparation of one litre of 1.5% agar.

LIA + pen/strep. As LIA but penicillin (Glaxo) added at 2.5 x 10^5 units/l and streptomycin added at 250mg/l.

SDM-PS. As SDM but penicillin (Glaxo) added at 2.5 x 10^5 units/l and streptomycin added at 250mg/l.

2.6 Maintenance of amoebal stocks.

Amoebal stocks were maintained by plating 0.1ml of suspensions containing 10^6 cysts/ml with 0.1ml SBS on LIA (LIAB); the suspension was spread over the plate. With heterothallic strains, the plates were incubated at 26°C for 120 hours. With apogamic strains, the plates were incubated at 26°C for 24 hours to allow the cells to excyst and then shifted to 30°C for a further 96 hours to prevent selfing occurring; npfC mutants were treated the same as apogamic strains to prevent the possibility of selfing due to reversion. Using these culture conditions, amoebal cell density reaches approximately 10^7 per plate after 72 hours and then the cells encyst. Plates of cysts can be stored at 4°C for several months. Strains grown on FKB may not survive refrigeration. To maintain AXE strains they must either be frozen or stored on plates with live SBS.

2.7 Transfer of AXE strains to liquid SDM and their subsequent culture.

LIA + pen/strep plates (5cm petri dishes) were inoculated with 0.02ml of FKB to make a small patch. Using a toothpick, amoebae were transferred from a well grown SBS culture and streaked in the middle of the FKB patch. The plates were incubated at 26°C unless apogamic strains were involved in which case plates were shifted to 30°C after 24 hours. The amoebae grew until after several days they formed a distinct patch on the FKB, when they were ready to transfer to liquid SDM. For each sample to be transferred to liquid medium, 1ml of SDM was added to a test-tube. A block was cut from the plate approximately $1cm^2$; this was done under a stereo microscope to avoid transferring a lot of the remaining FKB. Ten samples were treated at a time and when the last block had been cut out, the remainder of the procedure was followed. Each tube was vortexed for 5 seconds and the amoebal suspension was pipetted into a sterile 10ml plastic test-tube with a pasteur pipette. When all of the cultures had been transferred to plastic test-tubes, the cell density of the suspensions were estimated with a haemacytometer. The size of block used usually resulted in a cell density of 10^6 cells/ml. The tubes were placed on a reciprocating shaker at 150 strokes/minute at 26°C. After 5 to 7 days, the cell density of the cultures was estimated again; any cultures showing signs of growth were diluted to between 5×10^5 and 10^6 cells/ml and replaced on the shaker.

2.8 Assaying differentiating cultures for committed cells.

Suspensions containing committed cells were routinely assayed for the proportion of committed cells and the proportion of cells with different numbers of nuclei:

1) The proportion of committed cells. The cell density of suspensions to be assayed was estimated by haemacytometer count and the suspension diluted to approx. 10^3 cells/ml. In some cases, particularly when following the kinetics of amoebal growth and plasmodium formation, more than one dilution were used. 0.1ml of the diluted suspension/s was plated with 0.1ml of SBS on DSDM agar and spread. Plates were incubated at 26°C and scored after 3 days and again after 5 days for amoebal colonies and plateable plasmodia using a Wild binocular microscope at 25X magnification. Plateable plasmodia appear as blobs, many times the size of amoebae, which leave tracks behind them in the bacterial lawn. When serial dilutions were used, amoebae and plasmodia were often scored on different dilutions.

2) The proportion of cells with different numbers of nuclei. Suspensions containing committed cells are a mixture of different cell types. A drop of the suspension was pipetted onto an old DSDM plate (over one week old), the drop allowed to dry in and then the piece of agar was cut from the plate and put on a microscope slide. A coverslip was put on the slide and the proportions of the various cell-types were estimated by observing a sample of cells at 400X magnification by phase contrast microscopy (Wild M20 phase contrast microscope). At least 250 cells on each slide were scored for the number of nuclei/cell.

2.9 Induction of Flagellation (see also 3.1).

The two liquids most commonly used for inducing flagellation are phosphate buffer (pH 6) and sterile double distilled water. Jacobson *et al.* (1976), using phosphate buffer, found that after 90 minutes nearly 100% flagellation could be achieved; Chainey (1981) repeated this

observation. However, in similar conditions, Mir *et al.* (1979) found only 80% of amoebae could flagellate. It was possible that this was due in part to the fact that Mir *et al.* used some inappropriate strains and conditions, such as the apogamic strain CL, cultured at 26°C, at which temperature it could differentiate. One hundred percent flagellation has also been achieved with distilled water (Green and Dove, 1984).

The proportion of flagellates in cultures was estimated by staining with Lugol's iodine which stains flagella. At least 200 cells were observed with phase contrast microscopy (400X magnification)

Lugol's iodine

Potassium iodide (KI)	0.6g
Iodine (I_2)	0.4g
H_2O	10ml

One or two drops of this mixture were added to a 1ml suspension of flagellates; this killed all the cells and stained the flagella (McClung, 1950).

Phosphate buffer (pH6.0)

K_2HPO_4 (25mM)	1 volume
KH_2PO_4 (25mM)	9 volumes

2.10 Percoll density gradient.

Gradients were prepared of 50% Percoll by spinning at 17K in a SS34 rotor for 30 minutes in 10ml centrifuge tubes. Cell suspension was pipetted gently onto the top of the gradient and spun for a further 20 minutes at 5K. Flagellates were found 5mm - 15mm from the top of the gradient, cysts were found 60mm - 70mm from the top and bacteria was found 70mm - 80mm from the top (Burland, 1980).

2.11 Glass bead column to enrich for committed cells.

D. N. Jacobson, working at the McArdle Laboratory for Cancer Research, University of Wisconsin, had previously devised a method for the isolation of mutants in *P. polycephalum* that were unable to undergo the amoebal-flagellate transformation. The method involved suspending *Physarum* amoebae in phosphate buffer and pipetting the suspension onto a column of 200μ glass beads before the cells had begun to flagellate. The principle was that when cells were washed with the buffer, those cells able to flagellate would detach from the beads and swim off, emerging through the bottom of the column and those remaining behind would be cells unable to flagellate (Personal communication of W.F. Dove to J. Dee). The method that follows is based on this observation although it is not known whether the apparatus used below resembles that used in his method.

Glass beads (made of lead glass) approximately 200μ in diameter (Jencons Nº 11) were washed, sterilized wet in an autoclave and dried before use; this resulted in some clumps forming which were separated from the rest of the beads and saved. The clumps were broken up to make smaller clumps of approximately 10 beads; these were used to plug a sterile drawn out pasteur pipette at the neck. Sterile beads were then poured into the pipette until it was half full. The beads did not block the narrow drawn-out part of the pasteur pipette as the plug prevented the beads from entering it.

Before the column was used, 1ml of sterile distilled water was pipetted onto the beads. When the meniscus reached the top of the beads, the water on the column remained attached by capillary action; the water that emerged from the bottom of the column was discarded. A suspension

of cells was then pipetted onto the column. When this had run through, 1ml of water was pipetted onto the column; further 1ml aliquots of water were pipetted onto the column when necessary (to keep the beads wet) until the end of the experiment. The cells remaining on the beads were recovered by breaking the column at the neck, pouring the beads into a test-tube and shaking the beads in the presence of 2ml of ¼ strength SDM (diluted with water).

2.12 Slide cultures.

Slides were obtained with holes in them (10mm diameter). An 18mm x 18mm coverslip was fixed over the hole using molten wax. A 22mm x 22mm coverslip was put on the other side of the hole leaving a small gap through which a drop of molten agar was pipetted. This formed a small round piece of agar which did not touch the sides of the hole. When the agar had set, the coverslip was pulled across the hole to prevent the agar from drying out. A cell suspension was prepared and if appropriate, the suspension was mixed 1:1 with bacteria. The top coverslip was drawn back and a drop of the cell suspension was transferred to the agar block with a sterile loop; when the suspension had dried in, the coverslip was replaced and fixed with molten wax. The slide culture was observed by phase contrast microscopy at 400X magnification (Wild M20 phase contrast microscope).

2.13 Nucleic acid assay

Suspensions of cells were centrifuged briefly and the pellet prepared for chemical assay (Mohberg and Rusch, 1969). A solution was obtained by extraction with 0.5M $HClO_4$ at 70°C. The resulting solution was assayed

for total nucleic acid by measuring the extinction at 260nm (Plaut and Turnock, 1975).

2.14 DNA flow cytometry

Cells were harvested and suspended in SDM at 10^7 cells/ml. To permeabalise the cell membranes, 50µl of 0.2% Triton X-100 in 0.1M NaCl was added to 100µl of cell suspension and incubated for 1 minute at room temperature. To this suspension was added 400µl of mithramycin stock solution. The mixture was incubated for 5 minutes at room temperature and then kept on ice for 1 - 2 hours until analysis. Stained cell suspensions were analysed on a FACS 420 flow cytometer linked to a Consort-30 accessory computer (Becton-Dickinson Immunocytometry Systems). The 4W argon laser was tuned for an excitation wavelength of 457.9nm at 120mW output. The mithramycin emission spectrum was measured through a 530nm bandpass filter with a 30nm band width. Mithramycin binds specifically to DNA guanosine-cytosine base pairs, and fluorescence intensity measured on a linear axis was regarded as proportional to the DNA content of individual cells, of which 10^4 were measured for each histogram.

Mithramycin stock solution

Sigma mithramycin complex	137.5µg/ml
Tris-HCl (pH7.4)	0.1M
NaCl	0.1M
$MgCl_2$	30mM

2.15 Sample preparation for 2-dimensional gels.

2-[N-cyclohexaminol-ethane-sulphonic acid (CHES) was made up to 0.1M and buffered at pH 9.5 with NaOH. This stock solution could be stored at room temperature and had a long life. To 1ml of CHES buffer was added 50mg of SDS (sodium dodecyl sulphate) and 20mg of DTT (dithiothreotol). A cell suspension of known volume and cell density containing approximately 5 x 10^6 cells was spun down in an Eppendorf tube, the supernatant was discarded and 50µl of the CHES buffer (plus SDS and DTT) was added. The Eppendorf tube was vortexed and then inserted in a polystyrene holder which was floated on the top of a beaker of boiling water for at least 5 minutes being removed and vortexed at 1 minute intervals to ensure that the cells dissolved in the boiling buffer. The tubes were allowed to cool and 60mg urea and 100µl solution A were added. The tubes were then stored in a $^{-}80°C$ freezer until they were needed.

Solution A

Nonidet P40	2% w/v
Ampholines pH3.5 - 10	2% v/v
2-mercaptoethanol	5% v/v
Urea (Biorad)	9.5M

2.16 2 Dimensional Gel Electrophoresis

The following methods are based on those of O'Farrell (1975), modified by Burland *et al.* (1983).

1st dimension Isoelectric focussing (IEF).

Glass tubes (140mm x 2mm internal diameter) were soaked in chromic acid for at least 1 hour. They were then rinsed in distilled water

followed by 50% ethanol/50% 0.1M KOH and then again in distilled water. The tubes were dried well in a drying oven. When the tubes were cool, the bases were sealed with a double layer of parafilm and the tubes stood upright in test tubes.

The gel mix was prepared and loaded from the bottom with a 15cm needle to a depth of 12.5cm. The top was overlaid with water and the gel left to polymerise for approx 1 hour. The IEF tank had lower electrode buffer put in the bottom reservoir. The overlay and parafilm were removed from the tubes and they were loaded into the IEF tank making sure that the ends of the tubes were covered by the buffer and that there were no air bubbles. The samples were loaded (20-40µl) and overlayed with 10µl of sample overlay (1 part solution A + 2 parts water). The tubes were then carefully filled to the top with upper electrode buffer, taking care not to mix the samples, and the upper reservoir was filled with upper electrode buffer.

The gels were prefocussed: the voltage was set to 150 volts and then increased to maintain a constant current (approx. 2mA) until 400 volts was reached. The electrophoresis was continued for 16½ hours at 400 volts and then a further 3 hours at 800 volts. The gels were removed from the tubes using a 10ml syringe filled with equilibration buffer and then left in equilibration buffer for 15-20 minutes.

2nd dimension Slab gels (10%).

The glass plates were assembled and sealed with 1% molten agarose. 30ml of water, 18ml of lower gel buffer and 24ml of SDS acrylamide stock were mixed together in a side arm flask and degassed under vacuum. 300µl of ammonium persulphate solution and 40µl of TEMED were added to the

mixture and immediately the gel mixture was pipetted between the glass plates to the required depth. The gels were overlayed with isobutyl alcohol and allowed to polymerize. Once the gel had set, the overlay was poured off and the gels were rinsed with distilled water. The stacking gel was prepared by mixing 7.2ml of water, 3.0ml of upper gel buffer and 1.8ml of SDS acrylamide stock in a sidearm flask and degassed under vacuum. 45µl of ammonium persulaphate and 15µl of TEMED was added and the mixture was then pipetted on top of the separating gel to the top of the glass plates and overlayed with isobutyl alcohol. When the stacking gel had set, the top of the gel was rinsed with distilled water and the gel was placed in the electrophoresis apparatus.

Solutions used in 2-dimensional gel electrophoresis.

IEF gel solutions

Acrylamide stock solution.

acrylamide	28.38g
bisacrylamide	1.62g

Make up to 100ml with water.

Lower electrode buffer.

88% Phosphoric acid	1.11ml
Boiled and degassed water	1l

Upper electrode buffer.

Sodium hydroxide	800mg
Boiled and degassed water	1l

Equilibration buffer.

Trizma base	62.5mM
Glycerol	10% w/v
2-mercaptoethanol	5% w/v
Sodium dodecyl sulphate	2.3%
bromomphenol blue	few grains

IEF gel mix.

Urea	5.5g
Acrylamide stock	1.33ml
10% Nonidet P40	2.0ml
Water	2.0ml
ampholines ph 3.5-10	300µl
ampholines pH 5-7	200µl

Warm to 37°C to dissolve urea, degas quickly and add 20 µl 10% ammonium persulphate and 10µl TEMED

Slab gel solutions.

Lower gel buffer

Trizma (pH8.8)	1.5M
Sodium dodecyl sulphate	0.4%

Acrylamide stock solution

acrylamide	29.29g
bisacrylamide	0.78g

Make up to 100ml with distilled water.

Upper gel buffer

Trizma (pH 6.8)	0.5M
Sodium dodecyl sulphate	0.4%

Agarose

1% in ¼ strength upper gel buffer

Running buffer

Trizma	25mM
Glycine	192mM
Sodium dodecyl sulphate	0.1%

Main gel mix (7.5%)

water	30ml
lower gel buffer	15ml
acrylamide stock	15ml

Degas and add 200µl of 10% ammonium persulphate and 30µl TEMED.

Stacking gel mix

water	6.0ml
acrylamide stock	1.5ml
upper gel buffer	2.5ml

Degas and add 45µl 10% ammonium persulphate and 15µl TEMED.

2.17 Electroblot transfer of SDS gels to nitrocellulose paper (Western Blot).

The following methods for immunoblotting were perfected by Chris Birkett and I am grateful for his instructions.

At the end of the electrophoresis (see above), the gel was removed from the apparatus and immersed in blotting electrode buffer (BEB) for 5 minutes. The blotting "sandwich" was assembled; the assembly was performed in a shallow dish full of BEB and care was taken to avoid air bubbles in the apparatus. The sandwich was transferred to the electroblot tank full of BEB, with the nitrocellulose towards the positive electrode and the gel towards the negative electrode as proteins from SDS gels migrate towards the anode. A current of 100 mA was applied across the electrodes overnight. At the end of the run, the sandwich was carefully disassembled, the nitrocellulose was cut with a clean razor blade to the shape of the gel and the blot was transferred to TBS-Tween. (Towbin *et al*, 1979)

2.18 Immunoperoxidase staining of nitrocellulose blots.

The blot was washed for a further 5 minutes in a fresh change of TBS-Tween. The first antibody was diluted in HST buffer to 1:500 and the blot was placed in the antibody solution for approximately 1 hour on a reciprocating shaker with sufficient agitation to wash the solution over the blots. Unbound immunoglobulins were removed by two 5 minute washes in TBS-Tween followed by a 5 minute wash in HST and two more 5 minute washes in TBS-Tween. The second antibody, peroxidase-conjugated rabbit anti-mouse, was diluted 1:500 in HST and the blot was washed in this solution for approximately 1 hour. Unbound conjugate was removed by three 5 minute washes in TBS-Tween followed by a 10 minute wash in HST and three more 5 minute washes in TBS-Tween. The blot was then transferred to TBS and the substrate solution was prepared. Immediately the substrate solution was ready, the blot was transferred to it. Purple spots (when they were present) usually started to appear within 10 minutes getting gradually darker. When the reaction was complete, the blots were washed with distilled water and then photographed using reflected light.

NOTE Although in this method, only the singular is used, more than one blot could be probed in a single set of solutions.

Solutions used in Western blotting and in immunoperoxidase staining.

BEB

25mM	Tris	3.0g/litre
192mM	glycine	14.4g/litre
20% (v/v)	methanol	200ml/litre

TBS-Tween

10mM	Tris-HCl, pH7.4	1.21g/litre
140mM	NaCl	8.18g/litre
0.1% (v/v)	Tween 20	1.0ml/litre

TBS

10mM	Tris-HCl, pH7.4	1.21g/litre
140mM	NaCl	8.18g/litre

HST buffer

10mM	Tris-HCl, pH7.4	1.21g/litre
1M	NaCl	58.4g /litre
0.5% (v/v)	Tween 20	5.0ml/litre

Substrate solution (Use immediately)

Dissolve 18mg of 4-chloro-1-naphthol (Sigma product C-8890) in 6ml of methanol. Add 94ml of TBS and 25µl of 30% hydrogen peroxide.

2.19 RNA extraction

RNA was extracted by the method of Burland *et al.* (1983). 9ml of lysis buffer was added to 1ml of cell suspension containing 10^7 cells and immediately homogenised with a polytron for 30 seconds. The homogenate was made 4% with respect to *N*-lauroylsarcosine, and solid CsCl was added to 0.15g/ml. After gentle mixing, the suspension was centrifuged at 8000*g* for 10 minutes and the pellet discarded. The resulting solution was layered over a 2.5ml cushion of 5.7M CsCl (density 1.705g/cm^3) and 0.1M EDTA (pH7.6) and centrifuged in a Beckman SW41 rotor at 36000rpm for 24 hours at 20°C. After centrifugation, the supernatant was removed to just above the polysaccharide band (≃ 1 cm from the bottom); the tube was inverted to drain the remaining liquid and the bottom 1cm of the tube was cut off with a razor blade. The RNA pellet was dissolved in water made

0.1M LiCl and precipitated with 2 vol of ethanol. Poly(A)$^+$ RNA was isolated by chromatography of total RNA on oligo-(dT) cellulose.

lysis buffer

guanidine thiocyanate	5.0M
EDTA	10mM
2-mercaptoethanol	5%

2.20 *In vitro* translation of RNA using rabbit reticulocyte lysate (RRL).

7µl of RRL was mixed with 1µl of S^{35} methionine; 1µg of the RNA to be translated was added and made up to 10µl with water. The reaction mixture was incubated at 28°C for 40 minutes and 10µl of cracking buffer was added; the solution was stored at -80°C. To measure the incorporation of label, 4µl of the mixture was spotted onto 3mm filter paper, a mixture with no RNA added was used as a control. The spots were allowed to dry and put into a beaker of 5% Trichloroacetic acid (TCA) on ice for 30 minutes. The TCA was changed for fresh 5% TCA, heated to 90°C for 10 minutes; after this time the TCA was poured off and cold 5% TCA added and put on ice for 10 minutes. Five further washes in 5% TCA on ice were performed and 3 washes in acetone for 10 seconds, 1 minute and 5 minutes. The filter paper was dried and placed in a vial with 3ml of non-aqueous scintillation fluid. The incorporation of label was measured with a liquid scintillation counter. NOTE. All samples were kept in separate containers during all stages of the experiment.

Reservoir buffer

Trizma base	3.0g
Glycine	14.4g
Make up to 1 litre with water	

Cracking buffer

SDS	2%
2-mercaptoethanol	5%
Sucrose	10%
Bromo-phenol blue	0.002%
Reservoir buffer to 100%	

2.21 Acetone precipitation of *in vitro* translated protein samples.

Nine volumes of -20°C acetone were added to the *in vitro* translated RNA sample and left for 2 hours. The suspension was spun down, the supernatant poured off and the pellet air-dried (it was too small to see). 10µl of lysis buffer was added, the pellet resuspended and 10µl of DNase and 2µl RNase added. 10µl of solution A and 20µg. of urea were added and the sample was run as for normal 2-dimensional gel analysis. The gel was dried and exposed to Kodak safety film at -80°C for 3 weeks.

Lysis buffer

$MgCl_2$	5mM
Tris HCl (pH7.6)	20mM
Nonidet P40	1% v/v
leupeptin	50µg/ml

2.22 Immunofluorescence microscopy.

Clean coverslips were coated with poly-lysine. This was done by immersing the coverslips in poly-lysine for 5 minutes and then allowing the coverslips to air dry. Any polylysine remaining on the coverslips was removed by gentle blotting with kitchen paper. Once coated, the poly-lysine coated coverslips can be kept for several months.

All the procedure that follows was performed at room temperature except when specified otherwise. Suspensions to be stained were spun

down and resuspended in SDM at approximately 10^6 cells/ml. Coverslips were placed on a metal grid in a covered tray with a piece of tissue paper soaked in water at the bottom to prevent the slides from drying out. The cell suspension was pipetted gently onto the coverslips and spread with a pasteur pipette. After 30 - 40 minutes at room temperature, the coverslips were transferred to a metal grid over an ice bucket and methanol at -20°C was pipetted gently onto the coverslips; this permeabilised the cells to antibodies. After 10 seconds, the coverslips were rehydrated in PBS and 25µl of the first antibody diluted 1:500 in PBS, was pipetted onto the coverslip and spread with the pipette tip. The coverslip was replaced on the grid over the soaked tissue paper and left for a further hour. The first antibody was then washed off by four 5 minute washes in PBS, 25µl of the second antibody, FITC conjugated rabbit anti-mouse diluted 1:10 in PBS was pipetted onto the coverslip and spread with the pipette tip and the slide was replaced on the metal grid. After an hour the coverslip was washed a further four times for 5 minutes in PBS. The coverslips were dried and mounted on slides with moviol 4-88 (Harco, Harlow Chemical Company). The stained slides were kept in a refrigerator when they were not being observed to help prevent the immunofluorescence fading. Slides were observed and photographed using a Zeiss epifluorescence microscope.

Phosphate Buffered Saline (PBS)

K_2HPO_4 (25mM)	1 volume
KH_2PO_4 (25mM)	9 volumes
NaCl	155mM

2.23 Specificities of antibodies used in this work.

KMX-1 (Gift of Dr. K. Gull) reacts with β-tubulin (Birkett *et al*, 1985).

DM1B (Gift of Dr. S. Blose) reacts with β-tubulin (Blose *et al*, 1984).

DM1A (Gift of Dr. S. Blose) reacts with α-tubulin (Blose *et al*, 1984).

MPM-2 (Gift of Dr. P.N. Rao) reacts with mitosis-specific phosphoproteins (Davis *et al*, 1983) (see 5.1).

2.24 Abbreviations

Genetic symbols are shown under 2.1, strains are shown under 2.2 and media are shown under 2.5; other abbreviations used in this work are shown below.

ARG	Autoradiogram
ATS	Amoebal temperature sensitive
AXE	Strain capable of growth in axenic medium (SDM)
CHES	2-[N-Cyclohexaminolethane-sulphonic acid
DTT	Dithiothreotol
FKB	Formalin killed bacteria
IEF	Isoelectric focussing
MAP	Microtubule associated protein
MBC	Methyl benzimidazole carbamate
mta	Microtubular array
MTOC	Microtubule organising centre
CMTOC	Cytoplasmic microtubule organising centre
IMTOC	Intranuclear microtuble organising centre
PAUF	Precommitted amoebae unable to flagellate
PTS	Plasmodial temperature sensitive
SBS	Standard bacterial suspension
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine
TSP	Temperature sensitive period

CHAPTER 3.

Enrichment for Cells Committed to Plasmodium Formation Based on the Difference in Ability of Cells to Flagellate at Different Stages of Development.

3.1. INTRODUCTION

3.1.1 Synchronising development of *Physarum polycephalum*

The macro-plasmodium of *P. polycephalum* has been used extensively in biochemical research. It is particularly suitable for the study of the mitotic cycle as in the single plasmodium there are millions of nuclei that go through mitosis synchronously. The synchronous cell cycle can be followed by making smears and observing them with phase contrast microscopy to look for signs that are characteristic of the various stages of mitosis; in this way the timing of future mitoses can be predicted as can S and G2. The synchronous cell cycle is probably the major advantage of *Physarum* as an experimental organism; it facilitates biochemical events to be followed since large amounts of material at the same stage of the cell cycle can be obtained. Unfortunately, the synchronous cell cycle is exhibited only by the plasmodium, since it is a syncytium, and is not shared by the amoebae.

When CL amoebae are cultured at a temperature permissive for the amoebal-plasmodial transition, the development does display a small degree of natural synchrony (Youngman *et al.*, 1977). Burland *et al.*, (1981) demonstrated that when populations reached the maximum number of committed cells, approximately 20% of the cells were multinucleate. Clearly, a mixed population of uninucleate and multinucleate committed cells cannot be considered a homogeneous population so, for biochemical

analyses the natural synchrony of the amoebal-plasmodial transition is insufficient.

In transfilter induction experiments (Youngman *et al.*, 1977: see 1.3), although the synchrony was better than in normal differentiating cultures, there was still a time period of approximately 20 hours between the detection of the first committed cells and the time that the maximum number of committed cells was reached. It is possible that the lack of synchrony of development, even in cultures that are induced to differentiate across a filter, is due to cells being receptive to inducer only at a certain part of the cell cycle and to commitment occuring at a specific stage of the cell cycle. This would mean that before a population could be induced to differentiate synchronously it would be necessary to first of all synchronise the amoebal cell cycle. There have been several attempts to achieve this but with no success (K. Gull, personal communication). Cysts are known to be in G1 (Mohberg and Rusch, 1971) and it is possible that a method could be devised to synchronise cells if the cysts could be induced to excyst synchronously. Cysts can transform into flagellates, which are metabolically active yet non-feeding; this transformation may be useful to encourage synchronous excystment. However, even if amoebae could be synchronised it is unlikely that they would go through more than one mitosis synchronously if they were grown on bacterial lawns on agar. It is also unlikely that development could be synchronised due to differences in the microenvironment that exist on bacterial lawns on agar plates.

A different approach to producing a synchronised differentiating population is to take a non-synchronous population and to extract cells that are all at the same stage of the amoebal-plasmodial transition. To

do this it is necessary to have a method of identifying and separating cells that have started (but not finished) the amoebal-plasmodial transition; these cells must then be cultured through their subsequent development. Chainey, (1981) tried to induce flagellation in a differentiating population of the apogamic strain, CL, to find the stage at which ability to flagellate was lost. None of the binucleate or other multinucleate cells observed were flagellated and a significant proportion of the uninucleate cells also failed to flagellate. It is known that cells become irreversibly committed to plasmodium formation while they are still uninucleate, so it was possible that these non-flagellated cells were those that were undergoing the amoebal-plasmodial transition.

Chainey tested the response of flagellates to various factors and found that flagellated cells responded to gravity by swimming upwards. She tried various methods to utilize this response to enrich for non-flagellated cells, but with only limited success. However her results showed a good correlation between enriching for non-flagellated cells and enriching for committed cells, suggesting that ability of cells to flagellate was lost around the time of commitment.

3.1.2 Flagellation of AXE strains of amoebae.

Biochemical study of the amoebal-flagellate transition of *Physarum* has been hampered by the requirement that most strains of amoebae have to be grown in the presence of bacteria. CLd-AXE (see Methods) appeared to be incapable of flagellating (K. Gull, personal communication). This inability to flagellate has two possible explanations. Either CLd-AXE carries an additional mutation which makes it unable to flagellate or the

ability to grow in axenic medium is dependant on being unable to flagellate. The second of these explanations seems plausible since flagellated cells are believed to be a non-feeding form; thus, AXE strains must suppress flagellation to be able to feed and grow in liquid medium. Crosses have now been performed between CLd-AXE and "non-AXE" strains which have given rise to some progeny clones capable of growth in axenic medium; it was possible that, if loss of flagellation ability is due to a mutation in CLd-AXE, some of these strains are capable of flagellating.

The aims of the experiments described in this chapter were:

1) To work out the most efficient method for inducing the amoebal-flagellate transition in strains cultured on LIAB.

2) To attempt to synchronise the amoebal cell cycle by inducing cysts to flagellate and then resume amoebal growth.

3) To provide further evidence regarding the connection between loss of flagellation ability and commitment and to try to establish the sequence and the relative timing of the two events.

4) If the two events were related, to attempt to separate non-flagellated from flagellated cells, thus enriching for committed cells.

5) To test the flagellation ability of some AXE strains which were derived from CLd-AXE.

3.2. RESULTS

3.2.1. Method for inducing the amoebal-flagellate transition in strains grown on bacteria.

The amoebal-flagellate transition of *Physarum* has been studied by several workers and several different methods have been used to induce flagellation in suspensions of amoebae (e.g. Mir *et al.*, 1984 : Green and Dove, 1984). In order to investigate the flagellation of cells in a differentiating culture it was necessary first of all to be able to achieve 100% flagellation of a non-differentiating culture of amoebae. Various conditions were explored in order to do this. The strain used in this study was the apogamic strain, CL. When this strain is grown at temperatures below 30°C, amoebae differentiate to form plasmodia. At 30°C however, this strain behaves in a similar way to heterothallic strains, giving rise to actively growing amoebal cultures. Thus all preliminary experiments on the flagellation of CL amoebae were done on cultures grown at 30°C.

Non-differentiating cultures of CL were washed off in either 5ml of sterile double distilled water or 5ml of phosphate buffer (pH 6.0). The suspensions were allowed to stand at room temperature (21°C) and sampled at regular intervals and the number of flagellates in the suspension observed (see Methods). The maximum proportion of flagellates was reached in phosphate buffer in 90 minutes and was approximately 43%; the maximum proportion was reached in water in 120 minutes and was 64% (Table 3.1). The experiment was repeated but this time the tubes were shaken on a reciprocating shaker at 200 strokes per minute. The maximum proportion of flagellates was reached in both liquids after three hours and in these conditions there were 80% flagellated cells in the phosphate buffer and

Table 3.1. Attempt to induce flagellation in a non-differentiating population of CL amoebae in stationary suspension.

Cell suspensions were made in both phosphate buffer (pH6) and double distilled water and incubated at 22°C. Samples of 100 cells were observed at intervals.

(F = flagellate : A = amoeba : C = cyst)

	PHOSPHATE BUFFER			DISTILLED WATER		
TIME (min)	F	A	C	F	A	C
0	0	87	13	0	88	12
30	3	85	12	2	87	11
60	14	77	9	13	78	8
75	32	57	11	34	59	7
90	43	43	14	45	47	8
120	42	50	8	66	28	6
180	44	45	11	64	31	5

97% in the water (Table 3.2). It was concluded from these results that the best liquid for inducing flagellation was water and that a reciprocating shaker should be used.

3.2.2. Avoiding cysts in growing cultures of amoebae.

As can be seen in the results (Tables 3.1 and 3.2), in both experiments there was a large proportion of encysted cells; these cells take longer to flagellate than amoebae. Thus if it is true that committed cells are unable to flagellate, the presence of cysts could cause problems when attempting to separate non-flagellated committed cells from flagellated amoebae.

It is known that amoebae encyst in unfavourable conditions. In cultures that are plated out to obtain single colonies, the growing edges of the colonies consist of amoebae while cysts form in the centre of the colony where the bacterial lawn has been depleted. Cultures were set up on LIAB at 10^3, 10^4 and 10^5 cells/ plate, incubated at 26°C for 24 hours and then shifted to 30°C. The cells were washed off after a further 2 or 3 days in 2ml of sterile double distilled water. The cell density was measured with a haemacytometer and the proportion of cysts estimated. The results (Table 3.3) indicated that the way to avoid cysts in non-differentiating cultures of CL was to set the plates up at high cell density (10^5 cells/plate) and to incubate the plates for a total of 72 hours. However in these conditions care must be taken not to incubate the plates for any longer than the specified time as after 96 hours, a large proportion of the cells encysted.

Table 3.2. Attempt to induce flagellation in a non-differentiating population of CL amoebae on a reciprocating shaker (200 strokes/min).

Cell suspensions were made in both phosphate buffer (pH6) and double distilled water and incubated at 22°C. Samples of cells were observed at intervals; 100 non-encysted cells were observed in each sample.

(F = flagellate : A = amoeba : C = cyst)

	PHOSPHATE BUFFER			DISTILLED WATER		
TIME (min)	F	A	C	F	A	C
0	0	100	18	0	100	13
30	8	92	16	3	97	15
60	11	89	14	6	94	17
90	29	71	15	21	79	12
180	80	20	12	97	3	14

Table 3.3. Proportion of encysted cells in cultures set up at different cell densities on LIAB and incubated for 3 or 4 days.

Two hundred cells were observed in each sample to estimate the proportion of cysts.

(A = amoeba : C = cyst)

INITIAL CELLS/PLATE	CULTURE PERIOD	FINAL CELLS/PLATE	A	C	%C
10^3	3 days	$2.4x10^5$	191	9	4.5
10^4	3 days	$2.1x10^6$	193	7	3.5
10^5	3 days	$1.8x10^7$	195	5	2.5
10^3	4 days	$2.1x10^6$	174	26	13.0
10^4	4 days	$1.8x10^7$	168	32	16.0
10^5	4 days	$2.5x10^7$	133	67	33.5

3.2.3. Flagellation of CL at high and low cell density.

Thirty plates of CL amoebae were set up on LIA at 10^5 cells/plate and incubated at 26°C for 24 hours and shifted to 30°C for a further 48 hours. Each plate was washed off in 1.5ml of water and the suspension put into a plastic test-tube. The tubes were vortexed and put on a reciprocating shaker at 200 strokes/minute at 26°C. At intervals, 250 cells were observed and classified as flagellated or amoeboid (Figure 3.1). From the results it can be concluded that the method was efficient at inducing flagellation and that it was necessary to shake the suspensions for 3 hours to obtain 100% flagellation.

During these preliminary experiments it was noticed that cultures seemed to flagellate faster if the amoebal suspension was at high cell density. To investigate the effect of cell density, 5 non-differentiating plates of CL amoebae were washed off in 1.5ml of water and a 0.1ml sample of each suspension was diluted with a further 0.9ml of water. All the tubes were put on a reciprocating shaker at 200 strokes/minute. After 60 minutes the cell densities were estimated by haemacytometer count and 100 cells were observed in each tube to find the proportion of flagellated cells. The results (Table 3.4) indicated that high density suspensions flagellate faster than suspensions at low density. Thus, for future experiments, the method used to induce flagellation was to wash off dense cultures in a small volume of water (1.5ml) to produce a suspension at a cell density of at least 10^6 cells/ml.

Figure 3.1. Percentage of flagellated cells present in non-differentiating cultures of CL over a 3½ hour period. Cell suspensions from replicate cultures were allowed to flagellate. At intervals two suspensions were killed and stained with Lugol's iodine and a sample of 250 cells scored for the presence or absence of a flagellum.

FIGURE 3.1

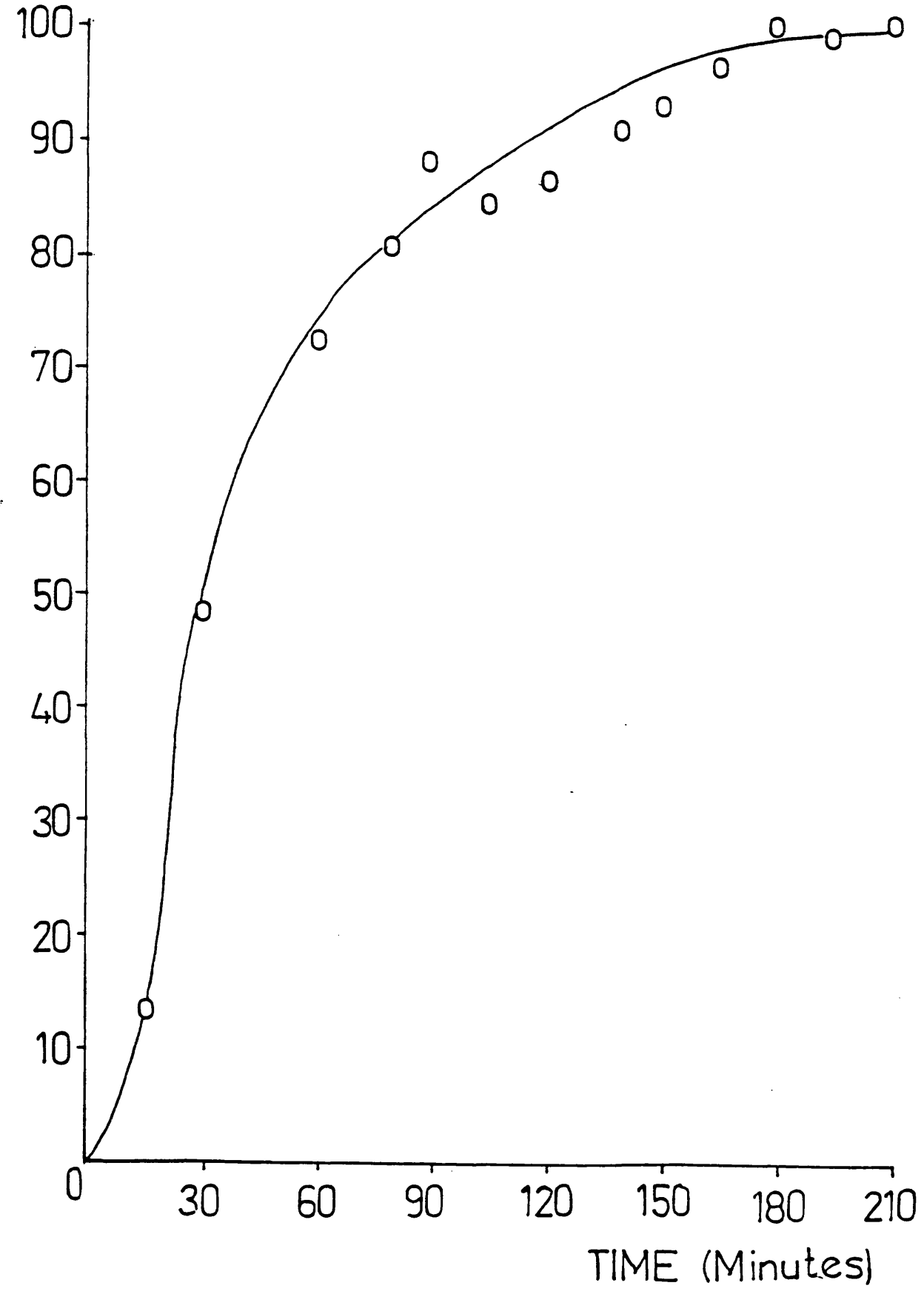

100
90
80
70
60
50
40
30
20
10
0
% FLAGELLATES
30
60
90
120
150
180
210
TIME (Minutes)

Table 3.4. Effect of cell density on the efficiency of flagellation.

Cultures of growing amoebae were washed off plates in distilled water, their cell density measured and 0.1ml from each suspension was removed and diluted with 0.9ml of distilled water; the cell density of this "Low cell density" suspension was measured. All tubes were placed on a reciprocating shaker at 200 strokes/min at 26°C. After 1 hour, all the cultures were killed and stained with Lugol's iodine. One hundred cells were observed from each suspension.

(A = amoeboid : F = flagellated : C = encysted)

CULTURE Nº	HIGH CELL DENSITY				LOW CELL DENSITY			
	A	F	C	Cells/ml	A	F	C	Cells/ml
1	35	61	4	1.2×10^6	74	23	3	1.2×10^5
2	34	62	4	1.3×10^6	71	25	4	1.2×10^5
3	45	54	1	9.0×10^5	68	30	2	1.0×10^5
4	31	66	3	1.1×10^6	70	27	3	9.0×10^4

3.2.4 Flagellation of cysts.

A population of CL was plated out at 10^5 cells/plate on LIAB and put at 26°C for 24 hours. The plates were then moved to 30°C and left for 6 days; at the end of this time period only cysts could be observed on the plates. The plates were washed off in 1.5ml of water and the resulting suspensions put on a reciprocating shaker at 26°C at 200 strokes/minute. The cells were observed at intervals in a haemacytometer and the proportion of flagellated cells was estimated (Table 3.5). Flagellates were first visible after 60 minutes; after this time, the proportion of flagellates gradually increased but unfortunately, there appeared to be a complete lack of synchrony in the excystment of the cells.

3.2.5 Observation of a population of synchronously excysted cells on LIA in slide cultures.

Burland (1980) attempted to isolate cell cycle mutants using Percoll density gradients to separate cells of different sizes. While he was unsuccessful in separating different sizes of amoebal cells, the method was successful in separating cysts from amoebae. The method did not appear to affect the viability of the cells.

A population of cysts was put in the same flagellation conditions as described in 3.2.4 and left for 1½ hours. This was expected to give approximately 20% flagellation (Table 3.5), all of these flagellates should be the result of excystment within the last 30 minutes. The suspension was then cushioned on a Percoll density gradient (see 2.10) and spun at 5000rpm for 20 minutes. The flagellate portion of the gradient was removed and inoculated in a filming slide.

Table 3.5. Flagellation of CL cysts at 26°C.

The flagellation method described in 3.2.3 was used on an encysted population of CL. Samples of 200 cells were observed at intervals.

TIME (min)	NUMBER OF CYSTS	NUMBER OF FLAGELLATES
0	200	0
30	200	0
60	197	3
90	163	37
120	158	42
150	142	58
180	136	64
300	112	88

The cells were observed over a 12 hour period and the slide was scanned at regular intervals to determine the number of cells in mitosis. When the cells were observed in the filming slide it was found that the flagellates had reverted to amoebae; subsequently many of the cells were observed to encyst rather than start active growth. However, within some areas of the slide, cells went through mitosis within an hour of neighbouring cells.

It seems likely that synchronisation is not possible in the conditions described. The main problem would appear to be the heterogeneous nature of the bacterial lawn as within small areas of the slide, in which cells presumably shared the same microenvironment, mitosis was more synchronous than would be expected in a random sample of growing amoebae. It is thus possible that with better culture conditions excystment could be the basis of synchronising the amoebal cell cycle.

3.2.6. Flagellation of CL cultures growing at 26°C and 30°C.

To repeat Chainey's observations on the loss of flagellation ability among cells in differentiating populations, cultures of CL were set up on DSDMB at 10^5 cells/plate. Some were incubated at 26°C to allow plasmodium development and others at 26°C for 24 hours and then shifted to 30°C to prevent plasmodium formation. After 72 hours, by which time multinucleate cells were present at 26°C, each culture was washed off in 1.5ml of water and induced to flagellate. After 3 hours, samples of cells were scored for the number of nuclei and the presence or absence of flagella (Table 3.6). In the non-differentiating culture, 500 cells were scored, all of which were uninucleate. As expected, over 98% of these cells had become flagellated. In the differentiating culture, 500

Table 3.6. Number of cells which became flagellated in non-differentiating and differentiating cultures.

The flagellation method described in 3.2.3 was used and suspensions were shaken for 3 hours. In the non-differentiating population 500 cells were scored and in the differentiating population 500 uninucleate, 100 binucleate and 50 larger multinucleate cells were scored

(A = amoeboid : F = flagellated : C = encysted : N.D.C. = Non-differentiating culture : D.C. = Differentiating culture)

	UNINUCLEATE				BINUCLEATE			MULTINUCLEATE		
	A	F	C	TOTAL	A	F	TOTAL	A	F	TOTAL
N.D.C. (30°C)	1	491	8	500	0	0	0	0	0	0
D.C. (26°C)	114	386	0	500	100	0	100	50	0	50

uninucleate cells were scored; only 77% had flagellated. In the same culture, 100 binucleate and 50 larger multinucleate cells were scored; none of these had flagellated (Table 3.6). It was concluded that the ability to flagellate had been lost in the differentiating culture while the cells were still uninucleate. As suggested by Chainey (1981), it was possible that these uninucleate cells unable to flagellate were committed to plasmodium formation.

3.2.7. Relative timing of commitment and loss of flagellation ability.

Cultures of CL were set up on DSDMB at 10^5 cells/plate at intervals over a period of 90 hours and incubated at 26°C. Five plates were set up at each timepoint and 2 of each set were assayed immediately. At the end of the 90 hour period, the remaining 3 plates from each timepoint were washed off in water (2ml/plate) and the suspensions from each timepoint were pooled. All the suspensions were then assayed for committed cells; Figure 3.2 shows the kinetics of amoebal growth and plasmodium formation during the 90 hour period. The suspensions were allowed to flagellate for 3 hours; a drop of Lugol's iodine was added to each tube and 500 cells scored for the presence or absence of flagella. Figure 3.3 shows the percentages of committed cells plotted with the percentages of non-flagellated cells.

The experiment was repeated (Exp. 2) but this time the starting density of the cultures was estimated by haemacytometer count to be 10^4 cells/plate. The results from this experiment are shown in Figures 3.4 and 3.5.

It is clear that there is a correlation between the number of cells that are unable to flagellate and the number of committed cells. In the

Figure 3.2. Kinetics of amoebal growth and plasmodium formation (Exp. 1). Differentiating plates were set up over a time period of 90 hours at approximately the same cell density estimated to be 10^5 cells/plate (●) and at the end of the time period assayed for amoebae (o) and plateable plasmodia (□). The number of plasmodia directly replateable on axenic SDM agar was also assayed (x) (see 4.2.2)

Figure 3.3. Percentage of committed cells and Percentage of cells unable to flagellate over a time period of 90 hours. Percentages of committed cells (□) were calculated from the numbers in Figure 3.2. Percentages of cells unable to flagellate (o) were calculated by counting 500 stained cells from each timepoint.
(UF cells = Unflagellated cells : LFA = Loss of flagellation ability : C = Commitment : T = Time between the two events.)

FIGURE 3.2

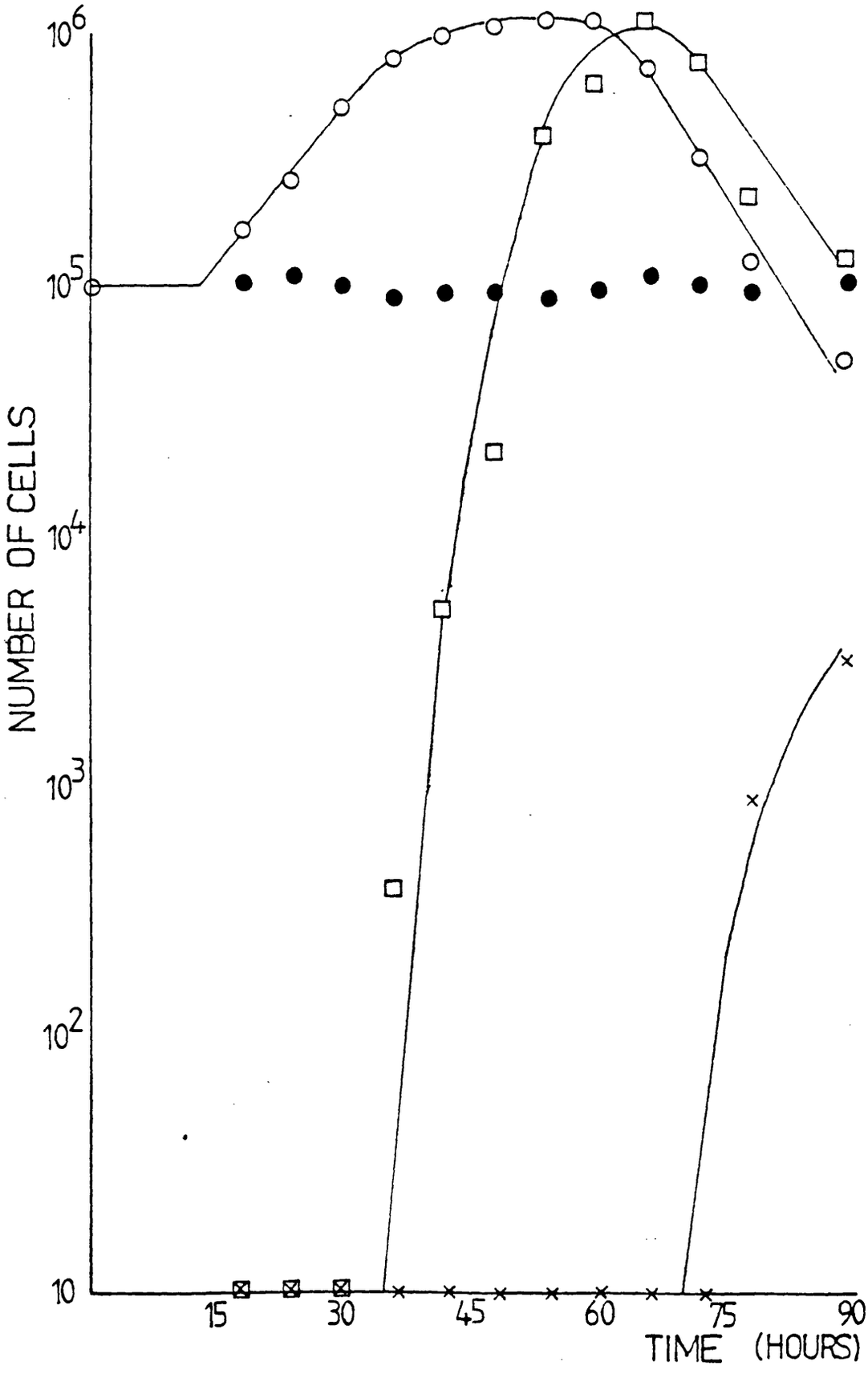

10^6
10^5
10^4
10^3
10^2
10
NUMBER OF CELLS
15
30
45
60
75
90
TIME (HOURS)

FIGURE 3.3

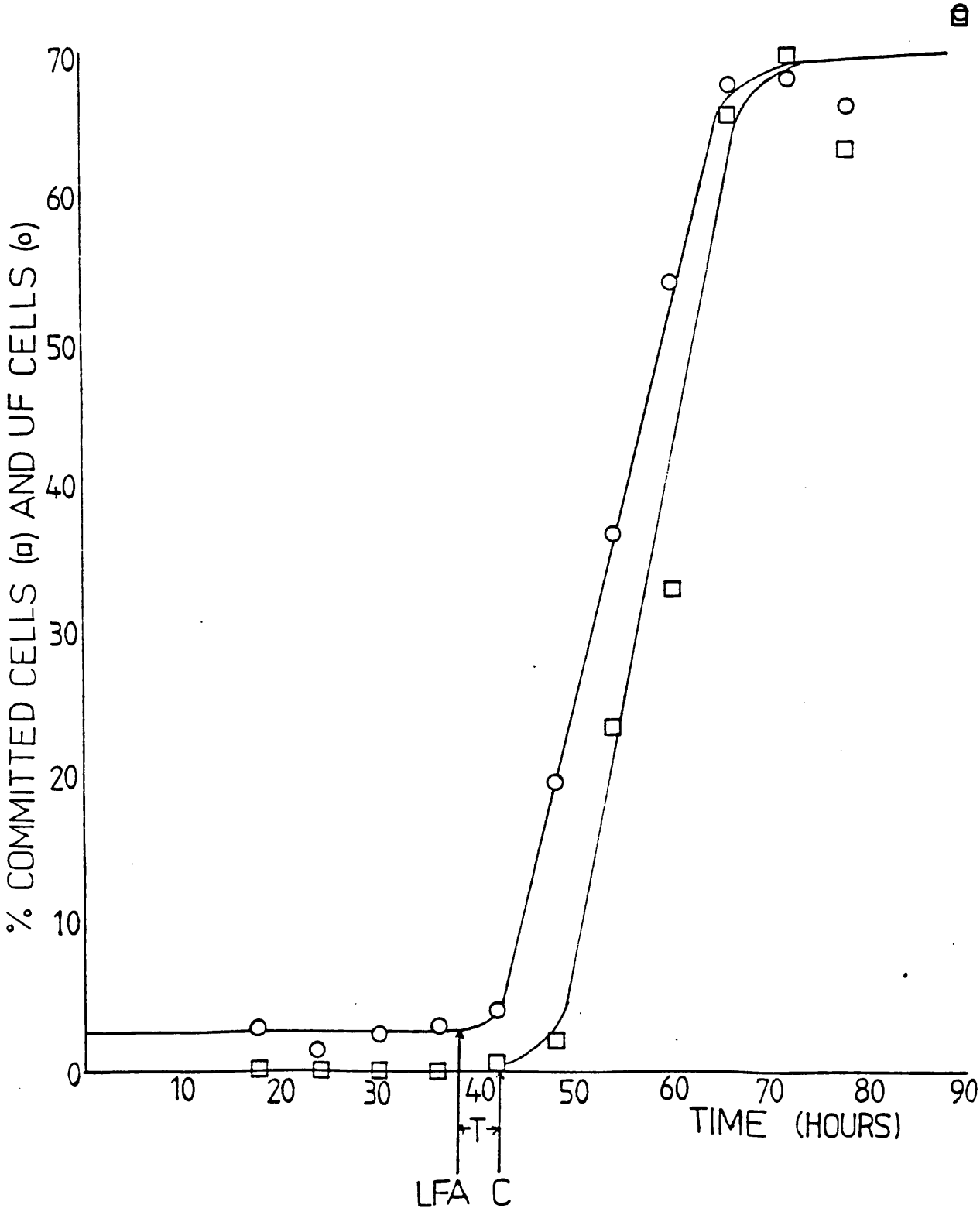

% COMMITTED CELLS (o) AND UF CELLS (o)
0
10
20
30
40
50
60
70
10
20
30
40
50
60
70
80
90
TIME (HOURS)
T
LFA
C

Figure 3.4. Kinetics of amoebal growth and plasmodium formation (Exp. 2). Differentiating plates were set up over a time period of 90 hours at approximately the same cell density estimated to be 10^4 cells/plate (o) and at the end of the time period assayed for amoebae (●) and plateable plasmodia (■).

Figure 3.5. Percentage of committed cells and Percentage of cells unable to flagellate over a time period of 90 hours. Percentages of committed cells (■) were calculated from the numbers in Figure 3.4. Percentages of cells unable to flagellate (●) were calculated by counting 500 stained cells from each timepoint.

(UF cells = Unflagellated cells : LFA = Loss of flagellation ability : C = Commitment : T = Time between the two events.)

FIGURE 3.4

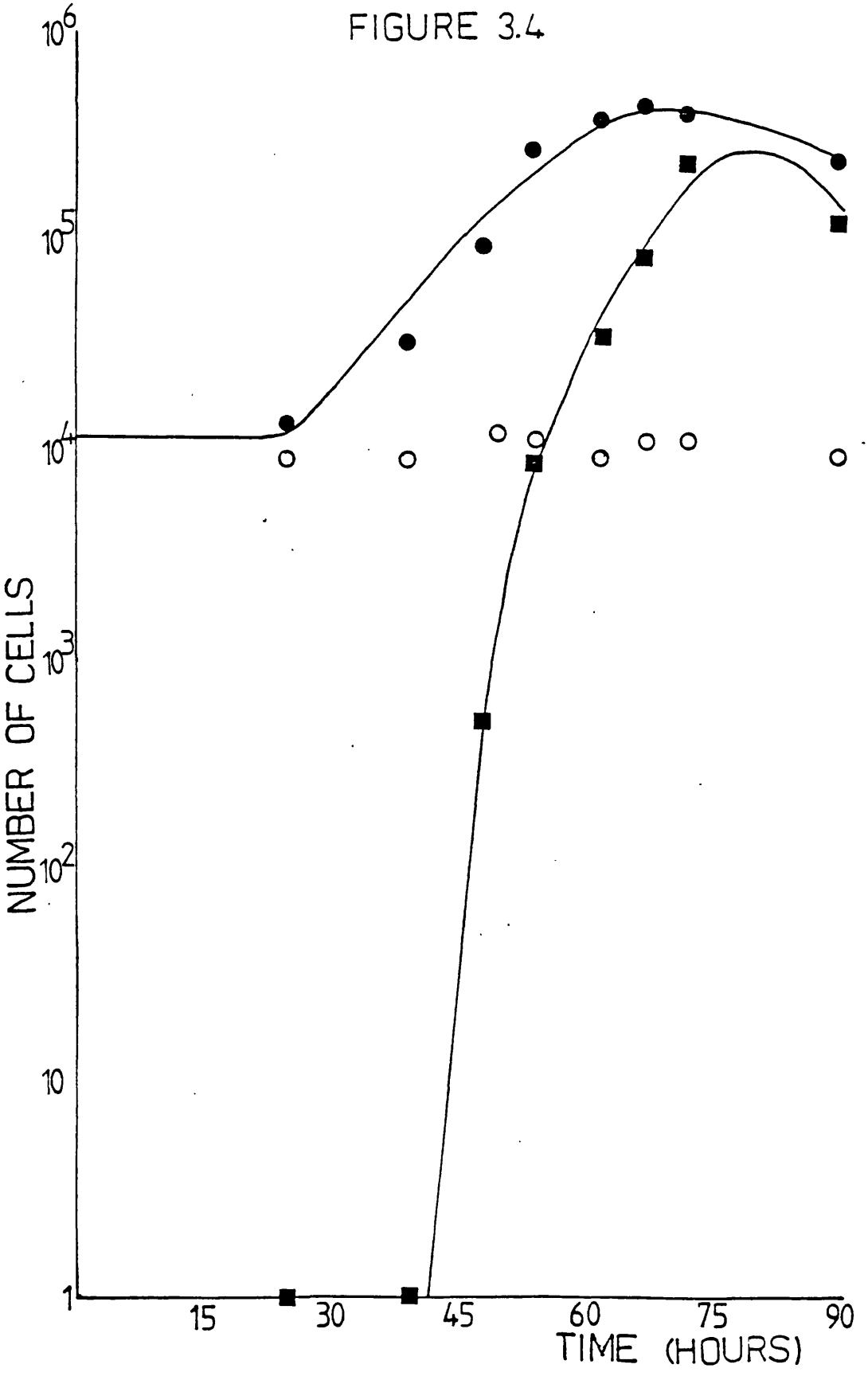
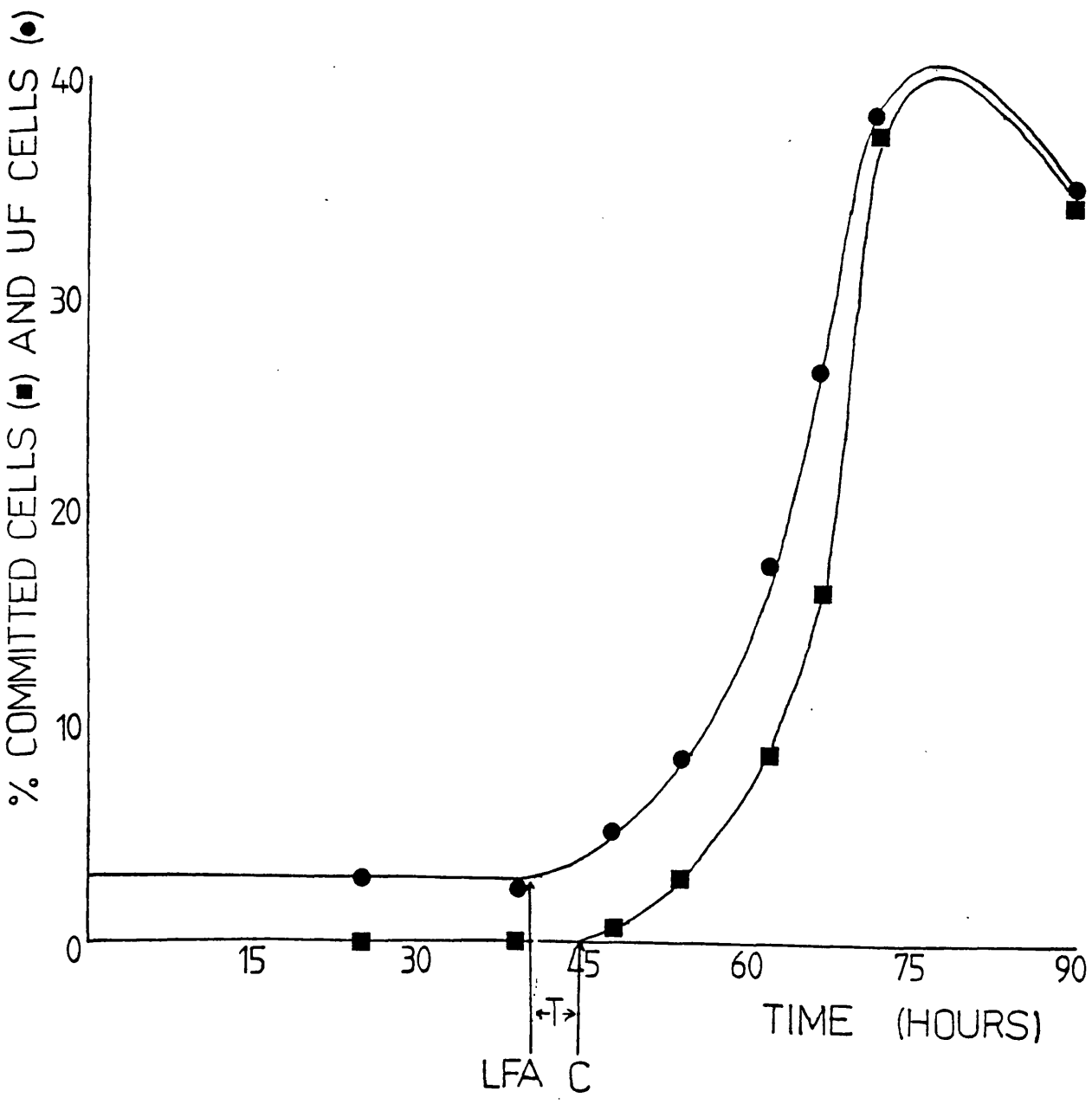

10^6
10^5
10^4
10^3
10^2
10
1
NUMBER OF CELLS
15
30
45
60
75
90
TIME (HOURS)

FIGURE 3.5


% COMMITTED CELLS (■) AND UF CELLS (●)
40
30
20
10
0
15
30
45
60
75
90
TIME (HOURS)
←T→
LFA
C

early samples, before plasmodium formation started, approximately 2% of the cells failed to flagellate in both experiments which is the same proportion as that found in the non-differentiating culture in the previous experiment (Table 3.6). Figures 3.3 and 3.5 show a higher proportion of non-flagellated cells than committed cells at each time point during the early stages of plasmodium formation (42 - 60 hours). It can be concluded that the ability to flagellate is lost before commitment. The time between loss of flagellation ability and commitment (T) was estimated by estimating the gap between the two curves as they start to rise. In Figure 3.3, the gap was approximately 5 hours and in Figure 3.5 the gap was approximately 3 hours. Thus the time between loss of flagellation ability and commitment in these conditions is between 3 and 5 hours.

3.2.8. Enrichment for committed cells using a glass bead column.

D. N. Jacobson, working at the McArdle Laboratory for Cancer Research, University of Wisconsin, had previously devised a method for the isolation of mutants in *P.polycephalum* that were unable to undergo the amoebal-flagellate transformation (Personal communication of W.F. Dove to J. Dee, see 2.11). An attempt was made to reproduce this method using double distilled water instead of phosphate buffer, since it had been found that flagellation could be induced more efficiently in water. The column was set up and run as described in 2.11. Although the column was washed for 2 hours, no flagellates could be observed in the liquid eluting from the column; only bacteria were observed in this suspension. When a suspension was recovered from the beads after the experiment, amoebae could be observed, indicating that they had remained on the

column and failed to flagellate. The experiment was repeated but this time a suspension of amoebae was allowed to flagellate before it was pipetted onto the column. The number of flagellates put on the column was estimated by haemacytometer count to be 4.25×10^6. The liquid eluting from the column was collected in a test-tube and sampled at intervals by allowing a drop to fall on a haemacytometer (Table 3.7). The total volume of the suspension was estimated to be 4ml and the cell density was 1.03×10^6. Thus, in 75 minutes, 4.12×10^6 flagellates came through the column which was 96% of the cells inoculated onto the column. It was concluded that 75 minutes was an effective time to wash the column as by that time, most of the cells had come through and the rate at which cells were eluting by that time was very low (Table 3.7).

As flagellated cells could go through the column and non-flagellates could not, it was possible that a differentiating population could be enriched for committed cells if it was induced to flagellate and then passed through the column. Differentiating populations were assayed for committed cells, induced to flagellate and run through the column; the column was washed through with water for 75 minutes. The suspension emerging through the column and the suspension remaining on the beads were assayed for committed cells. The results (Table 3.8) showed a significant enrichment for committed cells in the sample left on the column. Although some committed cells were lost by elution off the column, later results suggested that this may have been due to the high proportion of committed cells in the initial suspension.

A further experiment was done to determine whether the numbers of committed and uncommitted cells recovered from the column were identical with the numbers of each type in the initial sample. The enrichment

Table 3.7. Flow of flagellates through a glass bead column.

The cell density of the suspension of flagellates was estimated by haemacytometer count to be 4.25×10^6 cells/ml; 1 ml of this suspension was inoculated onto the column and washed through with distilled water (see text). The cell density of the suspension eluting through the column was estimated at intervals by catching a drop on a haemacytometer.

TIME (min)	ESTIMATED NUMBER OF CELLS/ml
0	0
5	0
7½	1.8×10^6
15	3.1×10^6
25	3.6×10^6
30	4.5×10^6
35	2.2×10^5
45	2.0×10^5
50	6×10^4
55	2×10^4
60	7×10^4
65	2×10^4
70	4×10^4
75	1×10^4

The total volume of suspension recovered was 4ml. The cell density of this suspension measured in a haemacytometer was 1.03×10^6 cells/ml.

Table 3.8. Results of assays during enrichment for committed cells using a glass bead column.

The percentage of committed cells in each sample was calculated from the relative numbers of amoebae and plasmodia on 5 replicate assay plates.

(A = amoebal colony, P = plateable plasmodium, %C = Percentage of committed cells)

EXPERIMENT	INITIAL SUSPENSION BEFORE ENRICHMENT			CELLS ELUTED OFF COLUMN			CELLS LEFT ON COLUMN		
	A	P	%C	A	P	%C	A	P	%C
1)	218	97	30.8	453	75	14.2	97	1141	92.2
2)	163	54	24.9	221	51	18.8	33	151	82.1

procedure was repeated but this time the volumes of the initial suspension, the total suspension eluted from the column and the suspension recovered from the glass beads were measured and the cell density of each suspension was counted in a haemacytometer. The three suspensions were also assayed for committed cells by plating as in the previous experiments. The results (Table 3.9) indicated that the number of committed and uncommitted cells in the initial suspension showed good agreement with the number of cells of each type recovered after enrichment.

During the subsequent work reported in this thesis, several changes were made to improve the enrichment method. These changes proved necessary as on occasions, yields from the column were lower than expected. The current version of the enrichment method is given in full in the appendix.

3.2.9. and 10. Preliminary experiments on enrichment for mated cells.

The two experiments that follow were preliminary experiments which must be repeated before any firm conclusions can be drawn from them. They are included as they help to shed light on the relationship between mating and apogamic development and suggest another important use for the enrichment method described in 3.2.8.

3.2.9. Flagellation of a mating mixture of LU 860 and LU 862 amoebae.

Separate suspensions of the heterothallic strains, LU 860 and LU 862 amoebae containing 10^6 cells/ml were prepared: these strains mate rapidly with each other as they have different mat A and mat B genotypes. Cultures were set up on DSDM5B at a cell density of 10^5 cells / plate of

Table 3.9. Measurement of cell density, volume of suspension and relative proportion of committed cells in samples before and after enrichment.

Cell density was estimated both by haemacytometer count and by replating assays.

(A = amoebal colony, P = plateable plasmodium, %C = percentage of committed cells)

	Haemacytometer Count		Replating Assays		
	Nº of cells/ml	Volume	A	P	%C
BEFORE ENRICHMENT	4.1×10^6	1ml	3.04×10^6	1.04×10^5	3.3
AFTER ENRICHMENT					
ELUTED OFF COLUMN	9.8×10^5	3.7ml	2.83×10^6	3.7×10^3	0.2
LEFT ON COLUMN	1.5×10^5	1.5ml	4.35×10^4	1.04×10^5	70.4

Number of cells after enrichmnent (from haemacytometer count) =

$(9.8 \times 10^5 \times 3.7) + (1.5 \times 10^5 \times 1.5) = 3.85 \times 10^6$

This agrees well with the estimate from haemacytometer count of 4.1×10^6 cells before enrichment.

Number of amoebae after enrichment (from replating assays) =

$2.83 \times 10^6 + 4.35 \times 10^4 = 2.87 \times 10^6$

This agrees well with the estimate from replating assays of 3.04×10^6 amoebae before enrichment

Number of committed cells after enrichment (from replating assays) =

$1.04 \times 10^5 + 3.7 \times 10^3 = 1.08 \times 10^5$

This agrees well with the estimate from replating assays of 1.04×10^5 committed cells before enrichment

both strains alone and the two strains together. After 72 hours, by which time mated cells were expected to be present on the crossing plates, the cultures were washed off in 1.5ml of sterile distilled water and the suspensions were put on a reciprocating shaker at 200 strokes/minute to induce flagellation. After 3 hours on the shaker, a sample of the pooled suspension was observed and the number of nuclei in each cell counted and the presence or absence of a flagellum was noted. The results (Table 3.10) showed that, as expected, nearly all of the amoebae in the two amoebal suspensions flagellated but in the mated suspension some uninucleate cells lacked flagella and no binucleate cells had flagella. Uninucleate cells in mating mixtures may be either amoebae or diploid zygotes; binucleate cells may arise both through amoebal cell fusion and through zygote mitosis (see 1.2). Since no attempt was made in this experiment to distinguish between the different types of uninucleate and binucleate cells that were present, the precise stage at which flagellation ability was lost in the mating cultures was unclear. It is probable that all the non-flagellated uninucleate cells were diploid zygotes since other workers have observed fusion occuring between flagellates. Since the binucleate fusion cell is only a transient stage during mating, it is possible that none of these cells were present in the culture. It can thus only be concluded that in mating cultures flagellation ability is lost before diploid binucleate cells are formed; this may be by the binucleate fusion cell or by the diploid zygote.

Table 3.10. Number of cells that became flagellates in 3 hours from cultures of LU 860, LU 862 and a mating mixture of the two strains.

In the non-mated cultures 200 cells were observed and in the mated culture 200 uninucleate cells, 50 binucleate cells and thirty multinucleate cells were scored.

(A = amoeboid : F = flagellated : C = encysted)

	UNINUCLEATE				BINUCLEATE			MULTINUCLEATE		
	A	F	C	TOTAL	A	F	TOTAL	A	F	TOTAL
LU 860	6	191	3	200						
LU 862	5	187	8	200						
LU 860 x LU 862	61	134	5	200	50	0	50	30	0	30

3.2.10. Enrichment for mated cells from a cross between LU 860 and LU 862.

Since the results of 3.2.9. suggested that diploid zygotes, which are committed to plasmodium formation (Shipley and Holt, 1982), had lost the ability to flagellate, it was possible that the enrichment method, which had been successfully used on CL, could be applied to mated cells. Suspensions of LU862 and LU860 amoebae were prepared at 10^6 cells/ml; the two suspensions were mixed together and 0.1ml of the resulting mixed suspension was pipetted onto each of 5 DSDM5B plates and spread. The plates were incubated for 72 hours at 26°C and then each washed off in 1.5ml of double distilled water. The suspensions were pooled, assayed for committed cells, induced to flagellate for 3 hours and then run through a glass bead column. The column was washed repeatedly with water as in 3.2.8. for 75 minutes. The liquid that emerged through the bottom of the column was collected and assayed for committed cells. The beads were collected and shaken gently in 2ml of water and the resulting suspension assayed for committed cells. The results (Table 3.11) indicated that this method was effective at enriching for committed cells resulting from mating although the enrichment was not as effective as it was with apogamic committed cells.

3.2.11 Flagellation of CLd-AXE from agar plates.

CLd-AXE and CLd amoebae were each inoculated on 5 LIAB plates at a cell density of 10^5 cells/ml and incubated at 26°C for 72 hours. Each plate was washed off in 1.5ml of double distilled water, the suspensions from each strain pooled and the tubes put on a reciprocating shaker (200 strokes/min) at 26°C.in conditions to induce flagellation. Samples were

Table 3.11. Results of assays during enrichment for committed cells from a cross between LU 860 X LU 862 using a glass bead column.

The percentage of committed cells in each sample was calculated from the relative numbers of amoebae and plasmodia on 3 replicate assay plates.

(A = amoebal colony, P = plateable plasmodium, %C = Percentage of committed cells)

	A	P	%C
BEFORE ENRICHMENT	143	21	12.8
AFTER ENRICHMENT			
ELUTED OFF COLUMN	231	18	7.2
LEFT ON COLUMN	133	124	48.2

removed at regular intervals and 100 cells observed to estimate the proportion of flagellated cells for each strain (Table 3.12). As expected, CLd was highly efficient at flagellating, whereas CLd-AXE flagellated very poorly; this confirmed the observations of other workers (see 3.1.2).

3.2.12 Flagellation of CLd-AXE from liquid axenic medium.

CLd-AXE was grown in liquid SDM to a density of 10^7 cells/ml. Three cultures were spun down, the cells resuspended in double distilled water and shaken at 26°C at 200 strokes/min. Samples of the suspensions were examined at ½ hour intervals in a haemacytometer for 4 hours but no flagellates were observed in the suspensions. The suspensions were examined after a further 10 hours. The cells were dark and did not appear to be normal; there were still no flagellates observable.

CLd-AXE is thus not a good strain in which to study the amoebal-flagellate transition. Flagellation is poor from plates in monoxenic culture and non-existent from liquid medium.

3.2.13 Attempted flagellation of LU352 from liquid axenic medium.

LU352 is a progeny clone of a cross between CLd-AXE and LU213. LU352 has the genotype *matAh npfC AXE*, so it will self (although not readily) and will grow in axenic medium; it is thus a good AXE alternative for the much used non-AXE amoebal strain, CLd. Some of the major drawbacks of CLd-AXE have been removed in LU352. The plasmodium has good morphology (unlike CLd-AXE) and sporulates readily (unlike CLd-AXE) to give viable spores which hatch to give progeny similar to the parent except that their selfing ability is better (they are *npfC*$^+$ revertants). It was also

Table 3.12. Number of flagellated cells present in suspensions of CLd and CLd-AXE washed off agar plates in distilled water.

Samples of 100 cells were observed at intervals.

(A = amoeboid : F = flagellated)

TIME (min)	CLd		CLd-AXE	
	A	F	A	F
0	100	0	100	0
15	88	12	100	0
30	54	44	96	4
45	36	64	93	7
60	26	74	89	11
75	11	89	87	13
90	3	97	85	15
180	1	99	86	14

noted that flagellates could occasionally be observed during the early stages of liquid culture of LU352 so it was decided to test the flagellation ability of this strain.

Cultures of LU352 were grown in liquid SDM to a density of 10^7 cells/ml. The cultures were spun down in a centrifuge at 2000rpm and resuspended in double distilled water at the same density. The cells were examined in a haemacytometer. The vast majority of the cells rapidly went black and, as they were being observed, started to lyse. Clearly the change from SDM to water was harmful to the amoebae. The tubes were left to shake at 26°C on a reciprocating shaker and were examined again after 12 hours. Nearly all of the cells in the tubes appeared to be flagellated but the cell density had fallen to 2×10^5 cells/ml; there was no sign of the other cells. It is possible that either only some of the cells present in the culture were capable of flagellating or that LU352 was capable of flagellating but the rapid change from growth conditions to distilled water was harmful to the cells. The most likely of these explanations is the latter since all cells in the culture should be identical; in fact it is possible that the majority of the cells which lysed (98%) changed the osmotic potential of the distilled water by releasing their cell contents into solution.

The experiment was repeated but this time instead of distilled water, the cells were put into PBS which is non-nutrient but nearer to the osmotic pressure of SDM. This time the cells did not turn black, however they did not flagellate. The cells appeared to be "healthy" but after 24 hours most of the cells had encysted.

3.2.14 Flagellation of LU352 from liquid axenic medium by dilution of SDM.

It seemed likely that in some way the osmotic shock of moving amoebae straight from SDM to double distilled water was killing the cells and causing them to lyse. To try to avoid this shock, SDM cultures were diluted gradually before finally transferring the amoebae to water.

Four 1ml cultures of LU352 were grown to a density of 9 x 10^6 cells/ml. Two cultures (a and b) were diluted with 3ml of water, spun down and 3ml of liquid removed. The other two cultures (c and d) were spun down and resuspended in water. The cultures were observed at intervals and the number of cells and the proportion of flagellates were estimated in a haemacytometer. After 40 minutes, cultures a and b were rediluted with a further 3ml of water, the cultures left for a minute, spun down and resuspended in 1ml of water. All of the cultures were observed at intervals for a further 200 minutes (Table 3.13).

The cells put directly into water (cultures c and d) went black and swelled up appreciably; by 10 minutes some of the cells were observed lysing. After 40 minutes a small number of the cells had recovered and looked healthy but the majority still looked dark. The total number of cells present also decreased. The cells in the cultures that were diluted (cultures a and b) also turned black although they did not swell up to the same extent. However, after 10 minutes many of the cells appeared to be recovering and by 40 minutes all of the cells appeared to be healthy. When the cells were placed in water they went darker again but did not swell up or lyse. They soon recovered and as can be seen from Table 3.13, over 95% of the cells flagellated after a further 200 minutes. The cultures put directly into water did show a low level of

Table 3.13. Flagellation of LU352 from liquid medium.

Cell suspensions in cultures a and b were diluted gradually with water; cells in cultures c and d were spun down and resuspended in water.

TIME (min)	Cultures a and b	Cultures c and d
0	SDM diluted 1:3 with water Cells appear black Cell density = $9x10^6$/ml	Cells resuspended in water Cells look black and swollen Cell density = $9x10^6$/ml
10	Some cells appear to be healthy; rest black Cell density = $9x10^6$/ml	Many cells observed lysing Cell density = $7x10^6$/ml
40	Cells appear healthy but no flagellates. SDM diluted 1:3 with water, spun down and resuspended in 1 ml water Cell density = $9x10^6$/ml	Most cells still appear black and swollen but some healthy Cell density = $2x10^6$/ml No flagellates visible

Between 75 and 140 minutes, samples of at least 130 cells were observed at intervals (A = amoeboid : F = flagellated)

	Culture a		Culture b		Culture c		Culture d	
	A	F	A	F	A	F	A	F
75	167	1	168	0	134	0	145	0
100	145	5	143	5	138	3	178	0
130	107	36	128	44	142	3	168	2
160	65	87	44	119	137	3	166	2
190	19	141	29	128	161	3	135	3
210	7	152	5	153	146	4	164	7
240	6	151	5	156	143	4	163	7

flagellation (as observed in the 3.2.13) but much lower than those diluted gradually.

3.2.15 Transferring flagellates of the strain LU352 into growth medium.

The method described above was found to be effective at inducing flagellation in LU352. The same observations were made with the strain LU600 which is a derivative of LU352 that has reverted npfC mutation to npfC^{+}. Flagellated cultures of LU600 at a density of 10^{7} cells/ml were obtained by the method described above. After 4 hours (by which time there were virtually 100% flagellates) the cultures were spun down and resuspended in liquid SDM. The cultures were observed in a haemacytometer almost immediately.

All of the cells appeared to have stopped the vigorous flagellate swimmming movements and seemed to be virtually motionless. Additionally, many of the cells appeared to have lost their flagella. Since no detached flagella could be observed in the medium it was concluded that the cells rapidly reabsorbed their flagella on transfer to liquid SDM. The cultures were diluted with SDM to a density of 10^{6} cells/ml and put on a reciprocating shaker at 30°C (this temperature prevents selfing in the apogamic LU600 strain). The cultures were left for 5 days and then the cell density was estimated in a haemacytometer. The 3 cultures observed had cell densities of $8x10^{6}$, $9x10^{6}$ and $1.1x10^{7}$ cells/ml. It was concluded that the flagellation process from liquid medium was totally reversible and that cells could return to normal growth in axenic medium.

3.2.16 Transferring flagellates into simple salt solutions.

It was suspected that the reason for the apparent loss of flagellation when cells were resuspended in SDM was the relatively high osmotic potential of the SDM. To see if this was indeed the case, solutions were prepared of NaCl and KCl which ranged from 2.5M to 0.25mM. Flagellates were prepared from LU 352 as described above. After 4 hours, when there were virtually 100% flagellates present, the cells were spun down and resuspended in the salt solutions. The results (Table 3.14) showed that most of the flagellates reverted in solutions greater than 0.025M. However, the flagellates appeared to be normal in the weaker solutions. It thus seems likely that flagellates of the AXE strains revert as a response to osmotic shock.

Table 3.14. Results of resuspending flagellates of LU 352 in solutions of KCl and NaCL.

Flagellates of the strain LU 352 were obtained by the method described in 3.2.13 and resuspended in salt solutions of differing molarity.

	NaCl	KCl
2.5M	Flagellates appear to revert rapidly	Flagellates appear to revert rapidly
$2.5x10^{-1}M$	Flagellates appear to revert rapidly	Flagellates appear to revert rapidly
$2.5x10^{-2}M$	Flagellates revert within 10 minutes	Flagellates revert within 10 minutes
$2.5x10^{-3}M$	Cells remain flagellated	Cells remain flagellated
$2.5x10^{-4}M$	Cells remain flagellated	Cells remain flagellated

3.3. DISCUSSION

3.3.1. Enrichment method for committed cells.

Effective enrichment for committed cells of *P. polycephalum* can be achieved using separation of flagellated cells from non-flagellated cells with a glass bead column. The method is successful with the apogamic strain, CL and early results indicate that it may work with mated amoebae. During the amoebal-plasmodial transition, ability to flagellate is lost before commitment; thus in cultures at early stages of differentiation, containing a low proportion of committed cells, the proportion of precommitted amoebae unable to flagellate (PAUF) is relatively high. Since it was found that the tendency of cells to remain on the column was a property not only of committed cells but also of non-flagellated amoebae, the PAUF will probably remain on the column and may be expected to reduce the efficiency of the enrichment method when early differentiating cultures are used. In cultures at later stages of differentiation, the proportion of PAUF is relatively low so that the enrichment for committed cells will be more efficient but the population will be a heterogeneous mixture of uninucleate and multinucleate cells at different stages of development. To analyse developmental changes subsequent to commitment, it is thus necessary to consider these factors and use cultures at appropriate stages of development.

There are at least two possible explanations as to why the enrichment method is successful; it could be due to the committed cells adhering to the beads or to the committed cells not being able to pass through the gaps between the beads because the gaps are too small. The smallest gap between the beads will be when they are arranged in such a way that four adjacent beads form a tetrahedron; in two dimensions this gap can be

represented where three beads meet to form a triangle (Figure 3.6). The gaps are clearly much larger than the non-flagellated amoebae which do not pass through the column; in three dimensions, the ratio between the size of the gaps and the size of the cells would be even greater. The gaps would also appear to be very large compared with a committed cell which is thought to be approximately three times the diameter of an amoeba (Collett *et al.*, 1983). It would thus seem likely that non-flagellated cells are not too big to fit between the beads; it is possible that they do not pass through the column because they adhere to the beads. Presumably, flagellates lack this adhering property and can thus be washed through the gaps between the beads.

3.3.2. Other applications of the column.

The enrichment method, although developed for isolating committed cells, may also have other applications. The most obvious of these possibilities is the original aim of the column which was for enrichment for non-flagellating mutants (D.N. Jacobson, personal communication). The method for using the column outlined in this thesis differs from that originally used. In this work, a cell suspension was induced to flagellate, pipetted onto the column and washed repeatedly. In the original experimental design, a suspension of amoebae was pipetted onto the column and then induced to flagellate. It appears that the method used in 3.2.8. is more efficient than that originally used, although even with this improved method, when flagellates were pipetted onto the column, only 96% of the cells came through (Table 3.7). It would thus be desirable to do several rounds of enrichment before screening; however, since during the growth stages of the enrichment procedure each mutant

Figure 3.6. Diagramatic representation of the gaps between the glass beads assuming three beads touching.

Length of the long side of the triangle (A) = 200μ

Length of the short side of the triangle (B) = 100μ

Length of the other side of the triangle (C) = $200^2 - 100^2 = 173.2\mu$

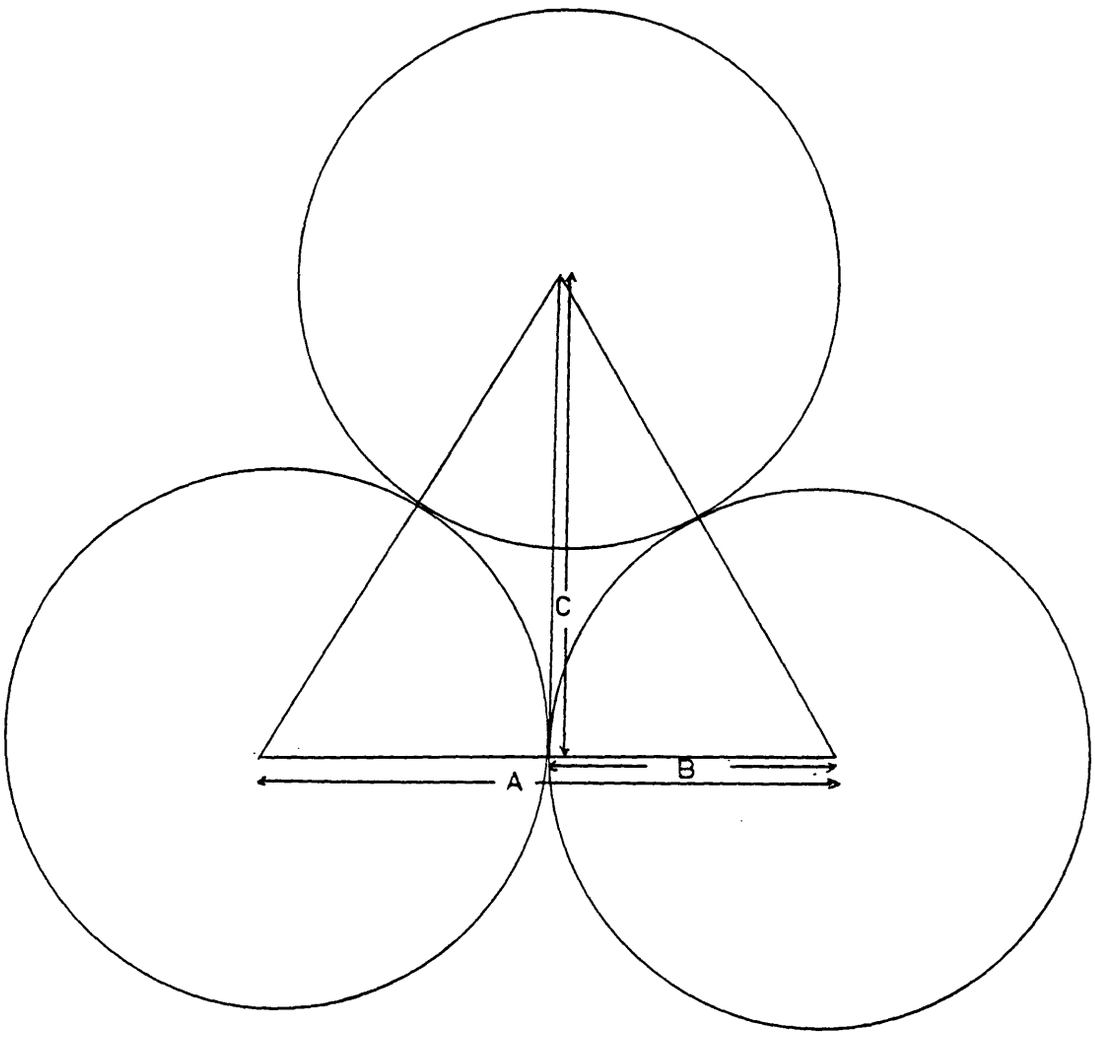
Area of the eguilateral triangle (A, A, A) = 173.2 x 100 = 17320μ^2

Area of the three segments of the beads each with arc of 60° = $\frac{1}{2}\pi r^2$ = $\frac{1}{2} \times \pi \times 100^2 = 15708\mu^2$

$\therefore$ Area of hole = 17320 - 15708 = 1612μ^2

As the area of an amoeba is only approx 100μ^2 the hole is more than big enough for it to fit through.

FIGURE 3.6



will multiply many times, only one mutant of a particular phenotype could be isolated from each experiment. However, the method has the advantage that it is at present the only selection process available for non-flagellating cells in *Physarum* and that many hundreds of thousands of cells could be screened in a single experiment.

It is possible that amoebae of *Physarum* in mitosis are unable to flagellate since centrioles may be involved in both functions. This fact could mean that the glass bead column method would be of use in enriching for mitotic cells. There are however drawbacks for this idea; the immersion of the mitotic cells in water for relatively long periods may upset the mitotic process or indeed kill the cells. Additionally, mitotic cells round up during mitosis and may be less adherent than non-mitotic cells.

Loss of flagellation ability is now the earliest marker for the amoebal-plasmodial transition. As discussed in Chapter 1, many mutants isolated from apogamic strains are unable to develop into plasmodia. The points at which most of these npf mutants are blocked in development are unknown. It would thus be useful to see if these mutants are capable of flagellating when grown at the temperature at which development should occur in the apogamic strain they are derived from since this would indicate whether development is blocked before the stage at which ability to flagellate is lost. If these mutants had lost the ability to flagellate, the column could be used to enrich for cells that had progressed beyond that stage, and this would allow cytological and biochemical experiments on populations of npf strains enriched for early stages of plasmodial development.

3.3.3 Flagellation of axenic strains.

The method developed in 3.2.14 was highly efficient at inducing flagellation of LU352. Furthermore, the method appeared to be completely reversible and did not appear to damage the amoebae. In the biflagellate green alga, *Chlamydomonas reinhardtii*, the flagellar apparatus can be detached by osmotic shock (Hyams and Borisy, 1974). This did not appear to occur in *Physarum* as no detached flagella were observed when flagellates were resuspended in solutions with high osmotic potential. The only drawback to the method is the time taken to achieve full flagellation, which is longer than that taken by strains grown on bacteria. However, despite the drawback, the method is now under routine use to investigate various aspects of flagellation (e.g. Sasse *et al.* 1987).

AXE amoebae and wild-type strains have different flagellation behaviour. When wild-type strains are suspended in SDM some of the cells flagellate whereas AXE strains do not. The reason for this difference in response is clearly not that AXE strains are incapable of flagellating. It is possible that it is because AXE strains perceive SDM as a nutrient whereas wild-type strains do not. However, LU352 did not flagellate in PBS which is not a nutrient medium. It thus seems likely that the difference in flagellation ability between AXE and wild-type strains may be due to a difference in their sensitivity to a change in osmotic pressure; this may be due to a mutation which changes the permeability of the cell membrane. If AXE strains were less permeable than wild-type strains, this could explain why they did not flagellate in SDM; however, it would not explain why the same cells should lyse in water when wild-type strains do not. If AXE strains were more permeable than wild-type

strains it would explain why they lyse when they are immersed in water and would also explain why they were capable of feeding in liquid medium; however, it would not explain why the cells did not flagellate in SDM. The true answer to the reason why AXE strains exhibit different flagellation behaviour to wild-type strains may become clear when the flagellation process in wild-type strains becomes better understood. What is clear is that the difference is necessary for growth in axenic medium since flagellated cells can not feed. Dee (unpublished) has found that even following the method devised in this work some AXE strains will not flagellate. These strains (LU353 and CLd-AXE) are probably the best AXE strains available; this suggests that the relationship between growth in axenic medium and suppression of flagellation may be more complex.

CHAPTER 4.

Growth of committed cells and their RNA/DNA contents relative to amoebae.

4.1. INTRODUCTION.

The method described in chapter 3 was clearly successful in producing an enriched population of committed cells. It is known that committed cells can grow and develop into plasmodia on bacterial lawns, since assays for committed cells are performed in these conditions. However, it would be advantageous if the enriched population could grow, and continue to develop into plasmodia, in axenic medium since this would facilitate following biochemical changes associated with the amoebal-plasmodial transition.

During work on committed cell populations described in Chapter 5, it was noticed that RNA yields were far higher than would be expected from an equivalent number of amoebae. The same observation was made by Sweeney (1987) during investigation of stage-specific cDNA clones. Increase in RNA content in large uninucleate cells during the amoebal-plasmodial transition was first postulated by Anderson *et al.* (1976) (see 1.3), although they had no direct evidence. To investigate RNA content of committed cells collaborative work was done with G. Sweeney.

The aims of the work described in this chapter were:

1) To investigate the behaviour of committed cells in the various growth conditions used to culture amoebae and plasmodia.

2) To investigate RNA and DNA contents of committed cells to see if there is a transitory increase in both or either associated with the amoebal-plasmodial transition.

4.2. Results

4.2.1. Culture of committed cells on bacterial lawns.

To investigate the behaviour of committed cells when cultured by the method routinely used for amoebae and to observe the timing of mitoses subsequent to commitment, enriched suspensions were inoculated on LIAB in slide cultures (see Methods).

Amoebae were present in the enriched suspensions which could not initially be distinguished from uninucleate committed cells. However, in the cultures, the amoebae multiplied to form colonies, while the committed cells underwent mitosis without cytokinesis to give rise to binucleate cells (Figures 4.1.a and b). The cultures were observed by phase contrast microscopy (400X) over a period of 32 hours. The numbers of binucleate and other multinucleate cells were counted at intervals and the number and development of the uninucleate committed cells in the inoculated sample was deduced. There was a period of about 3½ hours before the number of binucleate cells started to increase. After this period, some cells began to go through mitosis without cytokinesis. Within a further six hours the maximum number of binucleate cells was reached. The cultures were observed again between 22 and 32 hours. The results (Table 4.1) indicated that the cells continued to develop with some degree of synchrony; at 10 hours the cells were mostly binucleate, at 22 hours they were mostly quadrinucleate and at 30 hours they were mostly octonucleate. The doubling time was thus approximately 10 hours. By the end of the experiments, large multinucleate cells with plasmodial characteristics were present (Figure 4.1.c).

Figure 4.1 Culture of committed cells. Magnifications are approximate.

4.1.a Enriched culture on LIA plus bacteria after 3 hours (x 1000).

4.1.b Enriched culture on LIA plus bacteria after 9 hours (x 1000). This shows the same cells as in 4.1.a. Note that the cells have either divided as amoebae or become binucleate except for the uninucleate cell which appears to be undergoing closed mitosis; this cell subsequently became binucleate.

4.1.c Enriched culture on LIA plus bacteria after 48 hours (x 200).

4.1.d Enriched culture on solid SDM after 48 hours (x 1000). This culture was set up at the same cell density as the one shown in 4.1.a-c but in the absence of bacteria. After 48 hours very few cells were still alive and those that were had abnormal morphology. "Ghosts" of lysed cells can also be observed.

Figure 4.1

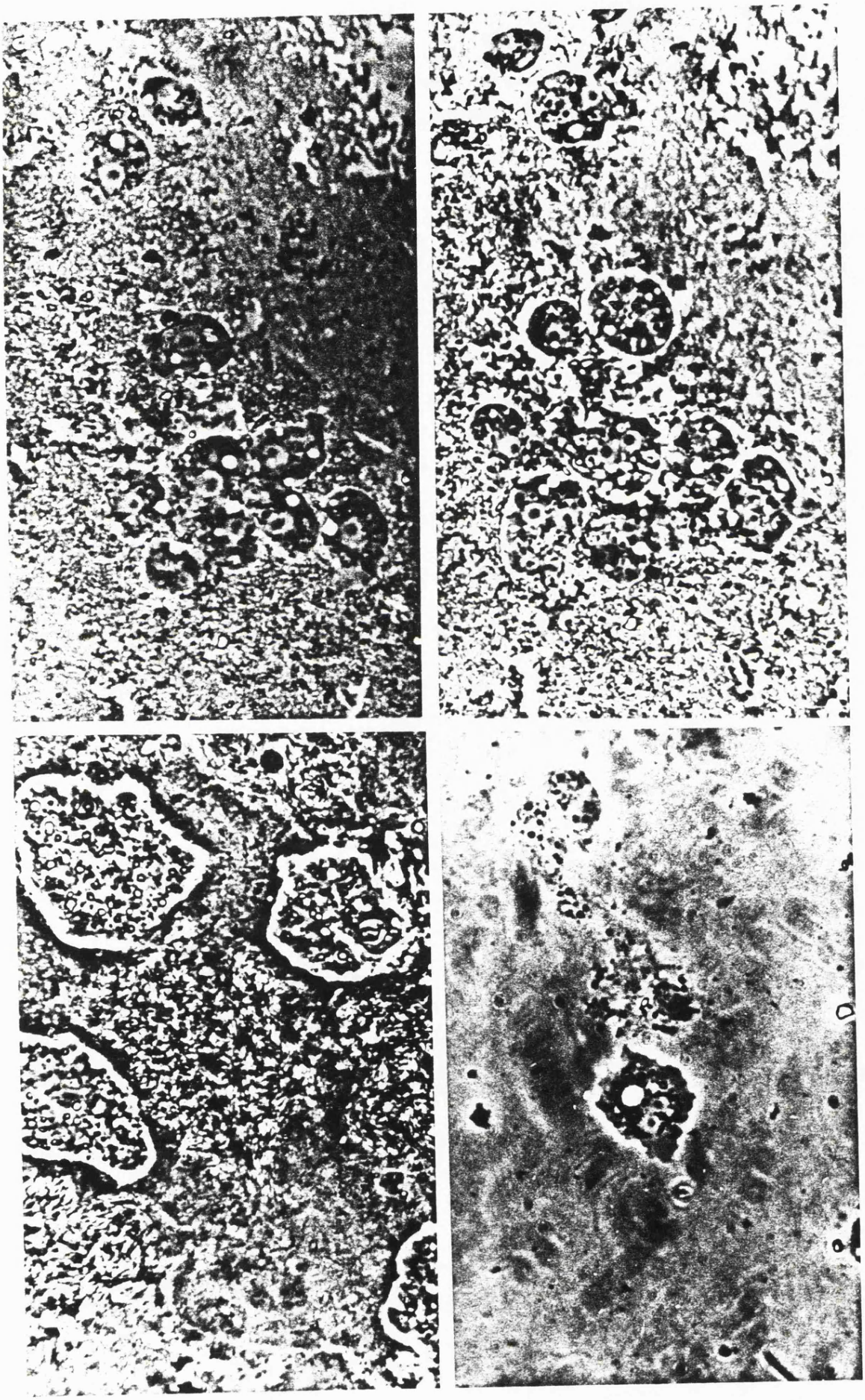


Table 4.1. Growth of committed cells on bacteria.

Samples enriched for committed cells were inoculated on LIAB in filming slides and cultured at 26°C; the slides were scanned at intervals by phase contrast microscopy (400X magnification) for multinucleate cells. The number of multinucleate cells formed during culture are shown for three separate enrichments.

(B = binucleate : Q = quadrinucleate : O = octonucleate : M = more than 8 nuclei)

	EXPERIMENT 1				EXPERIMENT 2				EXPERIMENT 3			
TIME (h)	B	Q	O	M	B	Q	O	M	B	Q	O	M
0	0	0	0	0	1	0	0	0				
½									10	0	0	0
1½	0	0	0	0					11	0	0	0
2					1	0	0	0				
2½	0	0	0	0					10	0	0	0
3					1	0	0	0				
3½	0	0	0	0					10	0	0	0
4					5	0	0	0				
4½	9	0	0	0					12	0	0	0
5	13	0	0	0	19	0	0	0				
5½	20	0	0	0					22	0	0	0
6					38	0	0	0				
6½	35	0	0	0					42	1	0	0
7					58	0	0	0				
7½	52	0	0	0					60	3	0	0
8					80	0	0	0				
8½	62	0	0	0					87	5	0	0
9					88	0	0	0				
9½	62	0	0	0					108	10	0	0
10					88	0	0	0	107	11	0	0
↓												
↓												
↓												
22	2	60	0	0	4	81	1	0	4	103	14	0
25	0	38	24	0	0	68	15	1	0	76	40	3
28	0	16	46	0	0	13	65	3	0	18	96	5
30	0	5	48	6	0	6	69	3	0	14	97	8
32	0	1	51	7	0	4	71	5	0	6	84	12

4.2.2. Acquisition of ability to grow on axenic solid medium

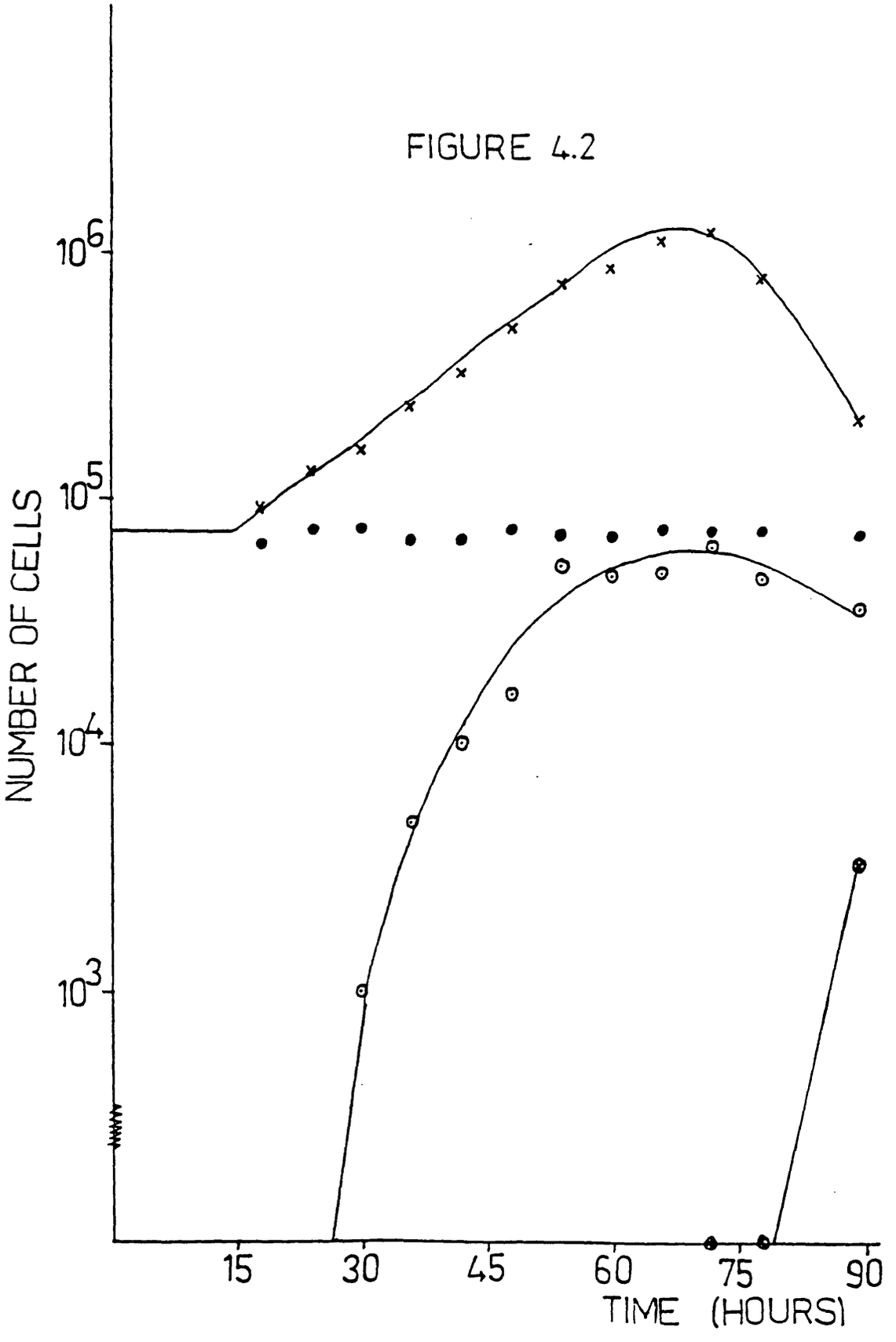
Enriched suspensions of committed cells recovered from the glass beads were also inoculated onto axenic SDM agar (the medium routinely used for plasmodial growth) in slide cultures and were observed at intervals for 24 hours. Many of the cells were observed to lyse; some of the cells became binucleate but subsequently died (Figure 4.1.d). No large multinucleate cells developed in the cultures even after several days. As expected, no amoebal colonies formed in the cultures. It was concluded that neither uninucleate nor binucleate committed cells were able to grow on SDM agar.

The stage at which cells acquire the ability to grow axenically on SDM agar was indicated by the results of assays performed during the experiment described in 3.2.7. to establish the time between loss of flagellation ability and commitment. During this experiment, samples of cells were plated on DSDM plates with bacteria to estimate the numbers of amoebae and committed cells (plateable plasmodia) present. Cell samples were also plated on SDM agar without bacteria to establish the stage at which committed cells could grow and develop on this medium (Figure 3.2). No plasmodia were detected on the SDM agar assay plates until the 78 hour sample, by which time macroplasmodia, which were presumably the product of fusion between many multinucleate cells, were already visible in the original differentiating cultures.

The experiment described in 3.2.7 was repeated with a mating mixture. The strains used were LU862 and LU860, which mate rapidly as they differ at both the mat A and mat B loci. At regular intervals (see Figure 4.2), amoebal suspensions were prepared of both LU860 and LU862 and their cell densities were adjusted to 10^6 cells/ml. Equal volumes of the cell

Figure 4.2 Kinetics of amoebal growth and plasmodium formation in a cross between LU860 and LU862. Differentiating plates were set up over a time period of 90 hours at approximately the same cell density (●) and at the end of the time period assayed for amoebae (x), plateable plasmodia (o) and the number of plasmodia directly replateable on axenic SDM agar (⊠).

FIGURE 4.2



suspensions were mixed together and five DSDM5B plates were inoculated with 0.1 ml of the mixed amoebal suspension and the suspension was spread. Two of the plates were assayed immediately to estimate the starting density and the rest of the plates were incubated at 26°C. Ninety hours after the first cultures were prepared, all the plates were washed off and the suspensions from each time-point were pooled and assayed for committed cells. The assays were done on both DSDM with bacteria (DSDMB) and SDM without bacteria. There was a relatively low level of mated committed cells throughout the experiment. Although the earliest samples in which committed cells were detectable on the DSDMB plates were those taken at 30 hours, no committed cells were detectable on the SDM assay plates until the 90 hours samples, by which time macroplasmodia could already be observed on the original mating cultures.

4.2.3. Culture of committed cells in liquid axenic medium.

An enriched suspension of committed cells, recovered from the glass beads, was spun down and resuspended in liquid SDM at 8 x 10^5 cells/ml. Penicillin was added at 250 units/l and streptomycin at 250 mg/l to prevent growth of any *E.coli* in the suspension. The proportion of committed cells in the liquid SDM-PS suspension was estimated by a plating assay (Table 4.2.a). The suspension was dispensed into 10 ml plastic centrifuge tubes (2 ml per tube) and incubated at 26°C on a reciprocating shaker at 200 strokes/min. At regular intervals, a drop of suspension was pipetted onto a block of agar and observed at 400X by phase contrast microscopy. In each sample at least 200 cells were examined and the number of nuclei per cell was scored; the results of 3 experiments are shown (Table 4.2.b).

Table 4.2. Suspensions cultured in liquid SDM.

4.2.a Assays for committed cells immediately after enrichment (Time = 0 hours) on replicate assay plates (three experiments).

(A = amoebal colony : P = plateable plasmodium)

Percentage of committed cells = (P / (A+P))x100%

Percentage of committed cells at time 0 (exp. 1) = (198/(121+198))x100% = 62.1%

Percentage of committed cells at time 0 (exp. 2) = (331/(156+331))x100% = 68.0%

Percentage of committed cells at time 0 (exp. 3) = (102/(71+102))x100% = 58.0%

4.2.b. Proportion of cells with different numbers of nuclei at different times during culture of committed cells (three experiments).

(U = uninucleate : B = binucleate : Q = quadrinucleate : O = octonucleate : M = more than eight nuclei)

TIME(h)		U	%U	B	%B	Q	%Q	O	%O	M	%M	TOTAL
1)	2	288	95.7	12	4.0	1	0.3	0	0	0	0	301
	22	98	39.5	143	57.7	4	1.6	1	0.4	2	0.8	248
	46	139	41.7	75	22.5	97	29.1	2	0.6	2	0.6	333
	70	148	61.2	12	5.0	27	11.2	39	16.1	16	6.6	242
2)	0	310	92.5	20	6.0	4	1.2	1	0.3	0	0	335
	18	124	41.8	164	55.2	5	1.7	2	0.7	2	0.7	297
	25	122	43.9	125	45.0	24	8.6	4	1.4	3	1.1	278
	45	105	42.5	35	14.2	86	34.4	18	7.3	3	1.2	247
3)	0	232	98.7	3	1.3	0	0	0	0	0	0	235
	24	94	42.9	123	56.2	2	0.9	0	0	0	0	219
	42	107	46.5	35	15.2	84	36.5	4	1.7	0	0	230
	68	65	41.1	4	2.5	32	20.6	50	31.6	7	4.4	158

Starting samples were usually observed at 0 hours; however in experiment 1, the first sample was not observed until 2 hours after enrichment. Preliminary work using immunofluorescence microscopy indicated that in samples cultured in SDM-PS for less than four hours, no cells could be observed in mitosis; thus samples observed within four hours of enrichment should contain the various cell-types in the same proportions as a 0 hour assay.

In the early samples it was not possible to distinguish between amoebae and uninucleate committed cells. A proportion of uninucleate cells did not develop and by 46 hours, most of these had encysted; it was deduced that these cells were uncommitted amoebae, which are known to be unable to grow in liquid axenic medium (see Introduction). Since binucleate and quadrinucleate cells must be committed, the number of uninucleate committed cells can be calculated by deducting the proportions of binucleate and quadrinucleate cells (Table 4.2.b) from the total proportion of committed cells (Table 4.2.a). The deduced proportions of uninucleate committed cells in the starting samples were: 57.9% in experiment 1, 61.5% in experiment 2 and 56.7% in experiment 3. These calculated proportions agree well with the proportions of binucleate cells in the subsequent samples (Table 4.2.b). From the proportions of multinucleate cells in successive samples it was concluded that the cells continued to grow with a nuclear doubling time of approximately 20 hours. Unfortunately, cell fusion occurs among multinucleate cells in the later samples obscuring the relative synchrony of cell growth observed in the early samples.

4.2.4 Culture of mated committed cells in liquid axenic medium.

An attempt was also made to grow an enriched population of committed cells from a mating mixture of LU860 X LU862 in liquid SDM-PS. The suspension was prepared as described in 3.2.10. and the same conditions were used that had been successful in growing committed cells of CL. The suspension was assayed for committed cells and a sample of 300 cells was observed by phase contrast microscopy (400X) to estimate the proportion of cells with different numbers of nuclei. The different types of uninucleate and binucleate cells (see 1.2) were scored separately.

The proportion of committed cells in the enriched culture was 47.1%. There was a relatively low proportion of multinucleate cells in the sample observed 2 hours after the enrichment; only 12% of the cells were large binucleate cells or quadrinucleate cells and no larger cells were observed. However, 22 hours after the enrichment, small yellow microplasmodia were visible in the culture with the naked eye; this suggested that cell fusion had occurred earlier in this mated culture, and possibly between cells with fewer nuclei, than in the cultures of committed cells of CL.

4.2.5. Estimate of nucleic acid content of committed cells of CL.

An enriched suspension of committed cells was obtained and was estimated by haemacytometer count to contain a total of 10^7 cells. A sample was removed from the suspension and assayed for committed cells (Table 4.3.a); the remainder was put on a reciprocating shaker at 26°C. After 2 hours, a sample was removed and observed at 400X by phase contrast microscopy to estimate the proportion of cells with different numbers of nuclei (Table 4.3.b). The enriched culture was then processed

Table 4.3. Suspensions used for nucleic acid estimation.

4.3.a. Assays for committed cells immediately after enrichment (two experiments).

(A = amoebal colony : P = plateable plasmodium)

Percentage of committed cells = (P / (A+P))x100%

Percentage of committed cells in experiment 1 = (118/(113+118))x100% = 51.1%

Percentage of committed cells in experiment 2 = (101/(101+90))x100% = 52.9%

4.3.b. Proportion of cells with different numbers of nuclei at different times during culture (two experiments).

(U = uninucleate : B = binucleate : Q = quadrinucleate)

	Time(hrs)	U	%U	B	%B	Q	%Q
1)	0	500	96.2	20	3.8		
2)	2	295	94.6	17	5.4	0	0
	24	203	52.7	166	43.1	16	4.2

Proportion of uninucleate committed cells in experiment 1

= %committed - %B = 51.1 - 3.8 = 47.3%

Proportion of uninucleate committed cells in first sample in experiment 2

= %committed - %B = 52.9 - 5.4 = 47.5%

for nucleic acid estimation (see Methods). At the same time, samples of CL amoebae (from the cultures at 30°C) and CL plasmodium were processed for nucleic acid estimation. The piece of plasmodium was estimated to be approximately $1cm^2$; a piece of this size is believed to contain approximately 10^7 nuclei (Mohberg, 1982).

The results of the nucleic acid assays (Table 4.4, experiment 1) indicated that as expected CL amoebae and plasmodia had approximately the same amount of nucleic acid per nucleus; the difference could be explained by error in estimating the number of nuclei contained in the piece of plasmodium. Uninucleate committed cells however, appeared to contain between 6 and 10 times the nucleic acid of amoebae or plasmodia.

Another enriched population was prepared. A sample was assayed for committed cells and another sample was observed to estimate the number of nuclei per cell (Table 4.3). One half of the enriched suspension was put on a reciprocating shaker at 26°C (200 strokes/minute) and the other half was processed for nucleic acid estimation (Table 4.4, experiment 2). After 24 hours, the culture on the shaker was removed, a sample was observed to count the number of cells with different numbers of nuclei (Table 4.3.b) and the culture was processed for nucleic acid estimation (Table 4.4, experiment 2).

The measurement of nucleic acid content per 10^7 cells for committed cells agrees well between the two experiments. Binucleate cells have approximately the same amount of nucleic acid per cell as uninucleate committed cells; this means that binucleate cells have only half of the nucleic acid per nucleus as uninucleate committed cells.

Table 4.4. Results of the nucleic acid (NA) assays done on amoebae (CL and LU600), plasmodia, committed cells and binucleate cells. Optical density was measured at 260nm and the figures are the average of two readings.

Experiment 1

Cell-type	Optical density	Nº cells in sample	NA/10^7 cells*
CL amoebae	0.614	$9x10^6$	63.96µg
Committed cells[1]	2.28	$1x10^7$	357.05µg
		Nº nuclei in sample	NA/10^7 nuclei
Plasmodia	0.404	$1x10^7$	37.88µg

Experiment 2

Cell-type	Optical density	Nº cells in sample	NA/10^7 cells*
Committed cells[2]	0.304	$1.6x10^6$	315.69µg
Binucleate cells[2]	0.288	$1.7x10^6$	306.36µg

* The conversion factor used is O.D. x 1000/32 x ml of sample (3ml)

[1] Not all of these cells were committed; for the assays see Table 4.3, experiment 1

[2] Not all of these cells were committed; for the assays see Table 4.3, experiment 2

4.2.6. Estimation of DNA content of committed cells using flow cytometry

DNA content was measured by flow cytometry at the University of Sheffield in collaboration with Dr. R.W. Anderson; the results that will be presented are however only preliminary. The work has now been repeated by groups in Sheffield and Leicester with similar results (Bailey *et al.* 1987).

Two large populations of committed cells were prepared (i and ii). A sample of each culture was removed and assayed for committed cells (Table 4.5.a). The cultures were then taken by car to Sheffield University being shaken gently at regular intervals by hand. Four hours after the cultures were prepared, samples were removed from each culture and 250 cells from each culture were observed at 400X by phase contrast microscopy to estimate the number of cells with different numbers of nuclei (Table 4.5.b). The cultures were then processed for DNA estimation by flow cytometry (2.14).

Unfortunately, no control of CL amoebae was run at the same time however, enriched populations contain amoebae which can be used as an internal control. Another amoebal strain, LU353 was examined at the same time and was considered as an amoebal control. The flow cytometer provides two forms of results; a histogram (Figures 4.3.a - c) and a measure of the proportion of cells in each peak, which is worked out by a computer.

In the amoebal strain (LU353), 89.6% of the cells peaked in channel 49. Both committed cell samples show a distribution of cells mainly in a peak at channel 49 (55.4% in i and 69.5% in ii); in these samples there is also a peak at channel 33/34 (20% in i and 7.2% in ii) and a small peak at channel 89/88 (6.5% in i and 7.3% in ii). It is probable that

Table 4.5. Suspensions used to test the DNA content of committed cells.

4.5.a Results of assays for committed cells.

(A = amoebal colony : P = plateable plasmodium)

	Counts on replicate assay plates				
i)	A	P	ii)	A	P
	25	22		32	26
	21	19		40	29
	34	26		22	25
TOTAL	80	67		94	80

Proportion of committed cells in i) = (67/(80+67))x100% = 45.6%

Proportion of committed cells in ii) = (80/(94+80))x100% = 46.0%

4.5.b. Number of cells with different numbers of nuclei in the suspensions that were used to test the DNA content of committed cells.

(U = uninucleate : B = binucleate : Q = quadrinucleate : O = octonucleate)

	U	%U	B	%B	Q	%Q	O	%O
i)	234	93.6	13	5.2	3	1.2	0	0
ii)	238	95.2	9	3.6	2	0.8	1	0.4

Proportion of uninucleate committed cells

in i) = %committed - (%B+%Q) = 45.6 - (6.4) = 39.2%

in ii) = %committed - (%B+%Q+%O) = 46.0 - (4.8) = 41.2%

Figure 4.3 Flow cytometry results of DNA/cell for:

a) LU 353 grown on bacteria.

1st Peak Channel 49 = 89.6%

2nd Peak Channel 98 = 4.2%

b) Enriched population of committed cells (i)

1st Peak Channel 34 = 20.0%

2nd Peak Channel 49 = 55.4%

3rd Peak Channel 88 = 6.5%

c) Enriched population of committed cells (ii)

1st Peak Channel 33 = 7.2%

2nd Peak Channel 49 = 69.5%

3rd Peak Channel 89 = 7.3%

The units on the bottom axis are arbitrary units of size.

FIGURE 4.3.a

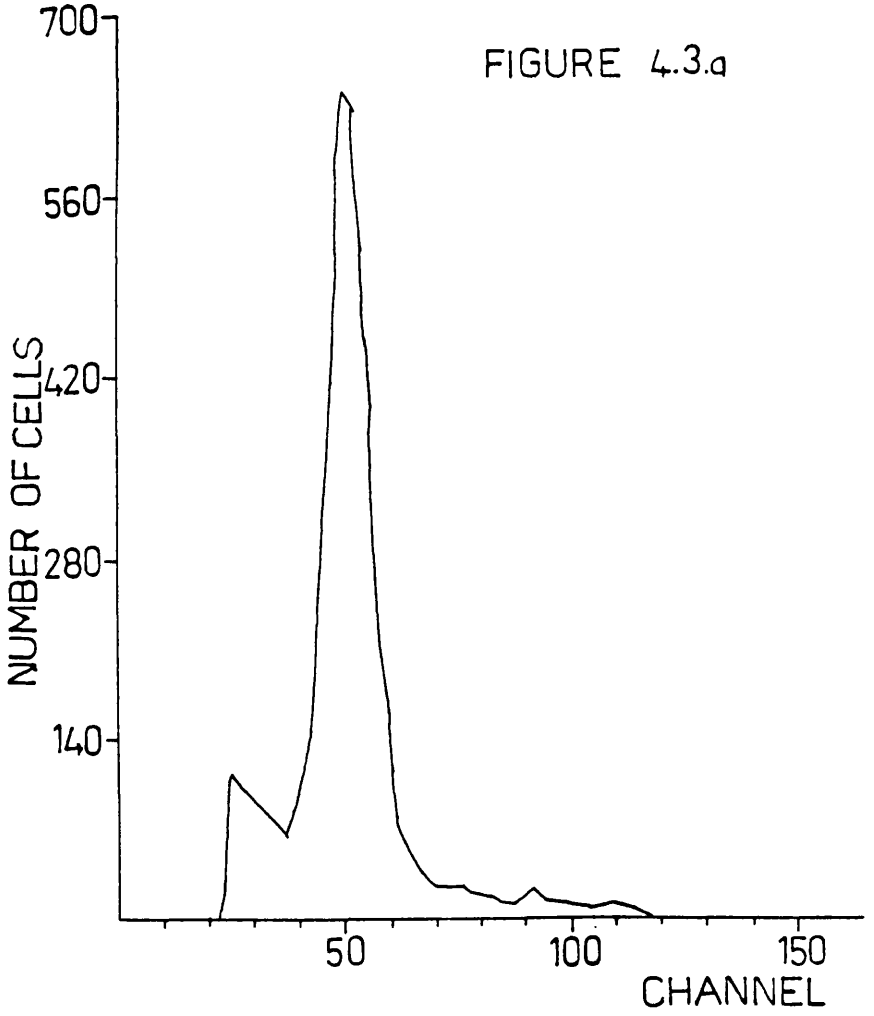


FIGURE 4.3.b

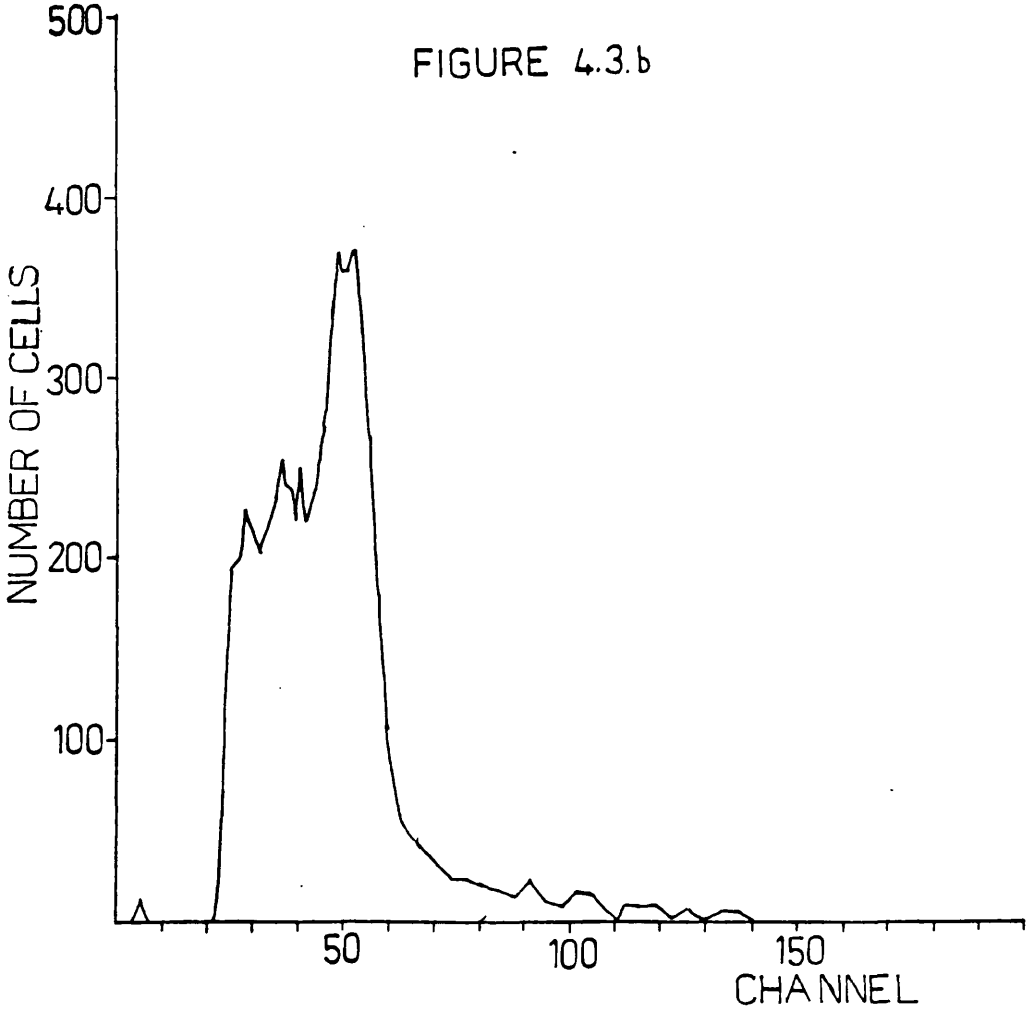
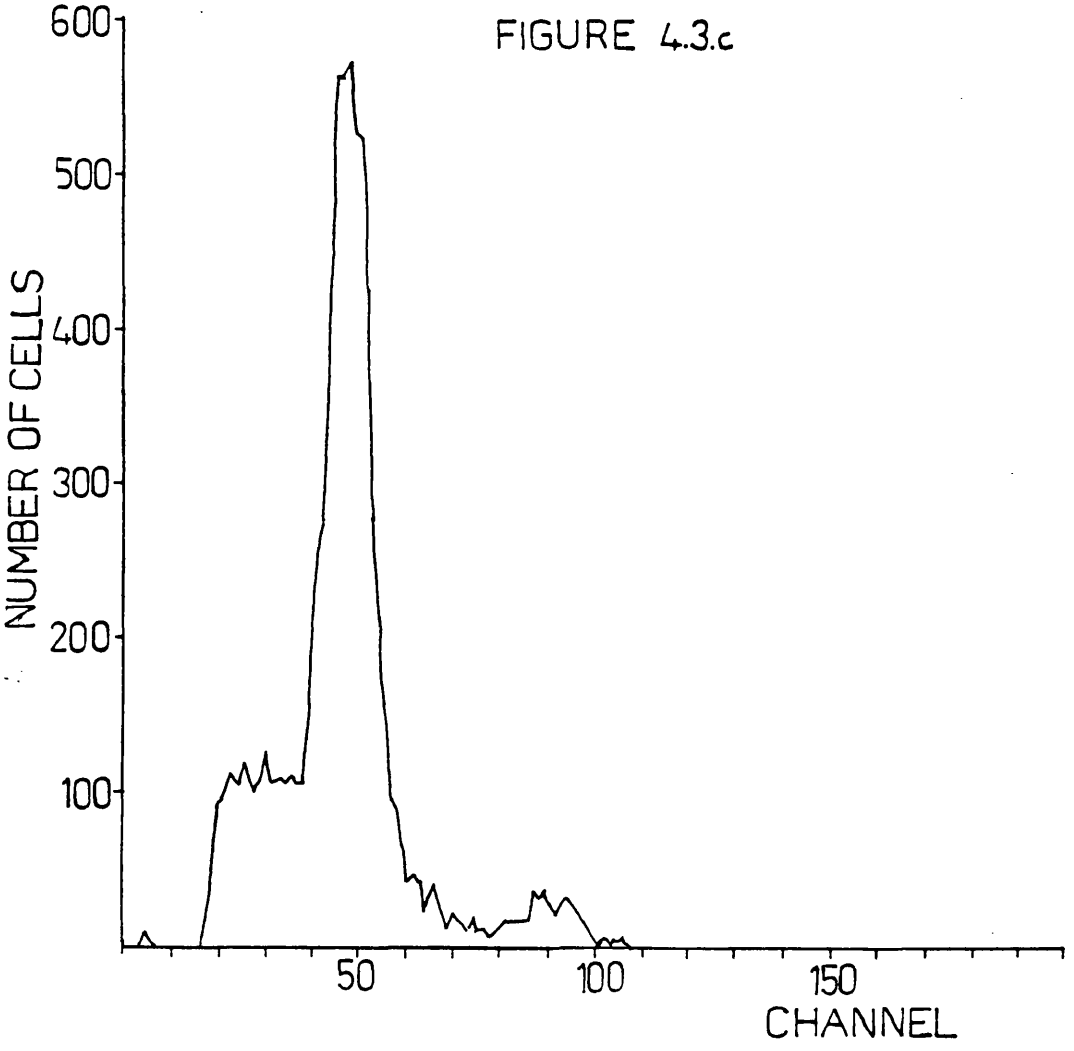


FIGURE 4.3.c



the peak at channel 49 is haploid uninucleate cells, since the amoebal strain peaked in that channel. The additional peak at channel 33/34 in the committed cell samples is probably due to cysts which are in G1 (Mohberg and Rusch, 1971); amoebae do not grow in SDM-PS and in the four hours from the enrichment many of them may have begun to encyst. The proportion of cells in channel 88/89 corresponds well with the number of binucleate cells present in the cultures. When one considers the proportions of various cell-types in the committed cell samples (Table 4.5.b) it seems probable that uninucleate committed cells must have the same DNA content as amoebae. Since there is no increase in DNA associated with differentiation, the increase in nucleic acid observed in 4.2.5. must be due to an increase in RNA.

4.3. DISCUSSION

4.3.1. Growth characteristics of committed cells.

Committed cells from apogamic strains can be cultured both on lawns of bacteria on agar plates and axenically in liquid semi-defined medium. Their growth in this latter medium is of particular use for biochemical studies. Committed cells can be cultured in large quantities in liquid SDM-PS, growth shows a good degree of synchrony and amoebae of CL do not grow in the same medium. Thus, although enrichment for committed cells using the method described in Chapter 3 is not 100% effective, amoebae that contaminate the enriched suspension will fail to grow in the liquid medium, making the population a better subject for the study of committed cells. Apparently, although diploid zygotes from mating mixtures will grow in the same medium as committed cells from apogamic strains, they appear to fuse readily, which leads to a lack of synchrony in their culture in axenic medium.

Amoebae and plasmodia differ in their abilities to grow in different conditions; uninucleate committed cells seem to be intermediate in their growth characteristics (see Table 4.6). They have developed the plasmodial characteristic of ability to grow in liquid SDM, but have not yet acquired the ability to grow on SDM agar. In these respects they resemble mutant AXE strains of amoebae (McCullough *et al.*, 1978). From the kinetics experiment shown in Figure 3.2, it was concluded that differentiating cells did not acquire the ability to grow on SDM agar until they become macroscopic plasmodia; thus, acquisition of this property may be due to a late change in gene expression or may simply be related to cell size. If the reason for lack of growth was the small size, this could also account for the inability of AXE amoebae to grow on SDM agar. Alternatively, if axenic growth on solid medium was genetically determined, AXE strains may express the plasmodial genes which allow growth in liquid axenic medium but not those that allow growth on solid axenic medium.

4.3.2. Timing of commitment in relation to binucleate cell formation.

The cultures that were observed on LIAB slides (4.2.1) came from plates that were incubated for 60 hours; experiments following the kinetics of plasmodium development (see Figure 3.2), show that at this time, cells are still becoming committed. Thus, the cultures that were observed must have contained cells that had recently become committed; these cells would be the last cells to become binucleate in the cultures. If the proportion of multinucleate cells at various time-points after inoculation of slides (Table 4.1) are plotted as a percentage of the total number of cells that became multinucleate (Figure 4.4) it can be seen that there is first of all a lag period, which lasted between 3 and 4 hours during which no cells became binucleate; following this lag, the

Table 4.6. Growth characteristics of various cell types in different media.

	Approximate doubling time (hrs) in different media		
CELL TYPE	BACTERIAL LAWN	LIQUID SDM	SDM AGAR
AMOEBAE	8	-	-
PLASMODIA	?	8	8
AXE AMOEBAE *	8	24	-
COMMITTED CELL **	10	20	-

- = No growth.

? = Doubling time not known but growth is not as good as on SDM.

* = Mutant amoebal strains able to grow in liquid axenic media (McCullough and Dee, 1976).

** = Times given are for binucleate to quadrinucleate and for quadrinucleate to octonucleate cells.

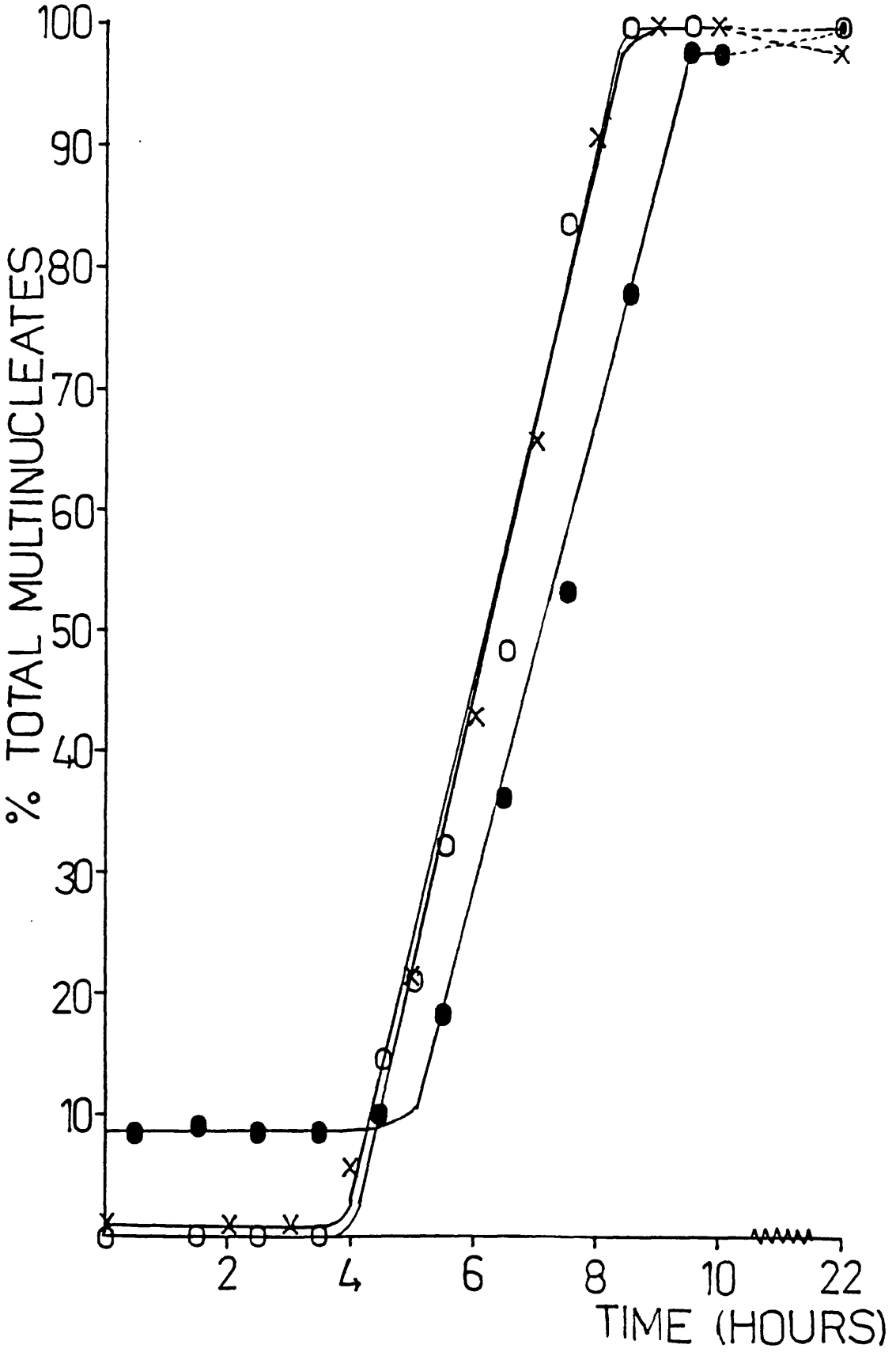
Figure 4.4 Percentage of binucleate cells in enriched cultures. The number of binucleate cells at each timepoint is expressed as a percentage of the maximum number of binucleate cells formed during the experiment (Table 4.1).

o = Experiment 1.

x = Experiment 2.

● = Experiment 3.

FIGURE 4.4


% TOTAL MULTINUCLEATES
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
22
TIME (HOURS)

maximum number of multinucleate cells was reached by 9½ hours. It is possible that this lag was due to a proportion of cells that were close to mitosis being lost during the enrichment procedure; this could have occurred as mitotic cells round up during mitosis and may therefore pass through the column. Alternatively, the lag period may be due to cells taking time to recover from the enrichment procedure. Bailey *et al.* (1987) have performed several experiments in which developing cultures of CL were replated in slide cultures and observed at intervals. In some of their experiments, cultures were enriched for committed cells and in some they were not. They found that often there was a lag, even if an enrichment had not been done. On one occasion there was no lag period; in this case the time from the first uninucleate cell becoming binucleate to the time that the last cell became binucleate was approximately the same as when a lag had been observed. It thus seems likely that the lag is due to time taken to recover from immersion in water and not to losing a proportion of cells. The lag period must thus be deducted from the total time taken for all cells to become multinucleate to obtain an estimate of the maximum time between commitment and binucleate cell formation; in the conditions used in 4.2.1, the time is approximately six hours.

Commitment could be an event that happens at a fixed time in the cell cycle before binucleate cell formation. Alternatively, commitment could occur at any time in the cell cycle between the earliest point (estimated above) and a latest point; as all binucleate cells apparently develop into plasmodia, the very latest time that commitment could occur is just before binucleate cell formation. As the cultures observed in 4.2.1 were not synchronous, it is impossible to say how much of the variation in the time taken for cells to become binucleate is due to variation in timing

of commitment and how much is due to the distribution of cells in the cell cycle.

4.3.3 Increased RNA synthesis in committed cells (see also 6.1.4).

By combining the results of 4.2.5 and 4.2.6 it appears that there is increased synthesis of RNA in uninucleate committed cells. It would thus be useful and relatively easy to measure the RNA content of committed cells directly and to see if the increase in RNA synthesis is general to all RNAs or if it is just in rRNA, mRNA or tRNA.

Hall *et al* (1975) concluded that rRNAs in amoebae and plasmodia were probably transcribed from the same genes. It is thus unlikely that the increase in RNA synthesis in committed cells is due to the cells making different ribosomes although there may be a general increase in the number of ribosomes. Preliminary work by G. Sweeney (personal communication) indicated that cDNA clones expressed at the same level relative to total RNA in amoebae and plasmodia were also expressed at the same level in committed cells. This suggests that during the increased RNA synthesis in committed cells, the ratio of mRNA to total RNA remains constant and thus there is not a massive increase in other RNA species. It is possible that the cell builds up a supply of RNA which is then used through subsequent mitoses; it is interesting that the intermitotic time from binucleate cell to quadrinucleate cell is shorter than the amoebal intermitotic time (Bailey *et al.*, 1987). Another possible explanation for the increased RNA synthesis in committed cells is that the RNA codes for proteins specific for the amoebal-plasmodial transition. If this is true, it would be expected that for genes expressed at the same level in both amoebae and plasmodia the ratio of mRNA to total RNA would be lower in committed cells; this does not appear to be the case.

CHAPTER 5

Changes in Tubulin Gene Expression, Microtubule Organisation and Phosphoprotein Distribution.

5.1. INTRODUCTION

Amoebae and plasmodia of *Physarum polycephalum* exhibit considerable differences in the tubulin isotypes expressed and in the way that the tubulin is organised into microtubules (see 1.5). Now that a method was available to enrich for committed cells and to continue their culture, it was possible to follow changes in tubulin expression and microtubule arrangement associated with the amoebal-plasmodial transition.

In the experiments described in this Chapter, the changes in the expression of tubulin isotypes and microtubule organisation during the amoebal-plasmodial transition were followed using antibodies to tubulin. DM1A is an antibody to α-tubulin and DM1B is an antibody to β-tubulin; they were the gift of Dr. S. Blose and were raised against chick brain microtubules. KMX-1 is an antibody to β-tubulin; it was the gift of Dr. K. Gull and was raised to *Physarum* tubulin. An antibody to phosphoprotein (MPM-2) was also used. MPM-2 is a monoclonal antibody raised to extracts from human mitotic cells and was the gift of Dr. P.N. Rao; it reacts specifically with mitotic and meiotic cells from a variety of species (Davies *et al*, 1983). It recognizes an epitope contained in a family of mitosis/meiosis specific phosphoproteins with molecular weights between 40 x 10^3 to 200 x 10^3. The antibody has also been shown to delay entry into G1 phase when injected into HeLa cells (Davies *et al*, 1983$_2$). This could be due to the bound antibody inhibiting *in vivo* phosphorylase activity that is essential for normal cell division.

CHAPTER 5

Using MPM-2, it should be possible to compare the arrangement of phosphorylated material in amoebae and plasmodia. Differences may be expected in the arrangement of the mitoses since it is known from phase contrast microscopy and electron microscopy that the amoebal and plasmodial mitoses differ considerably. MPM-2 is known to stain centrioles in other cell types (Vandre *et al*, 1984) and preliminary observations on flagellates of *Physarum* indicated that MPM-2 stained amoebal centrioles. Since centrioles are present in amoebae and absent in plasmodia it is one obvious difference to look for. The presence of the nuclear membrane during the plasmodial "closed" mitosis may also be revealed. If these differences are identified then MPM-2 should provide a valuable method for characterizing amoebal-type or plasmodial-type mitosis in cells that are progressing through the amoebal-plasmodial transition.

The aims of this chapter were:

1) To follow changes from amoebal to plasmodial tubulin expression using cultures enriched for committed cells.

2) To observe the change from amoebal to plasmodial microtubule arrangement.

3) To observe the changes in phosphoprotein arrangement and to compare this with the changes in microtubule arrangement.

5.2. Results

5.2.1 Identification of the tubulin isotypes present in committed cells of CL using immunoperoxidase stained blots probed with antibodies to α-tubulin and β-tubulin.

To obtain sufficient cells for protein samples, 20 differentiating plates were washed off in water and the suspensions were enriched for committed cells using 10 glass bead columns. The samples of flagellates emerging through the columns were collected, pooled and processed for analysis by 2-dimensional gel electrophoresis (see 2.15). The cells remaining on the columns were recovered, resuspended in liquid SDM-PS and the proportion of committed cells was estimated by plating assay (Table 5.1.a experiment i). The cell density of the enriched suspension estimated in a haemacytometer was 1.2 x 10^6/ml. The suspension was put into conditions suitable for growth (see 4.2.3) and sampled at intervals. Four hours after the end of the enrichment, the number of nuclei per cell was observed in a sample (Table 5.1.b.i) and 4ml of the enriched suspension was spun down and processed for analysis by 2-dimensional gel electrophoresis. Further 4ml samples were removed from the shaker after 19, 48 and 96 hours and processed for analysis by 2-dimensional gel electrophoresis. At 19 and 48 hours, samples were also observed to estimate the proportion of cells with different numbers of nuclei. However, it was impossible to estimate the number of nuclei per cell in the 96 hour sample since yellow microplasmodia (which contain many hundreds of nuclei) were present; these could be observed in the suspension with the naked eye.

All five samples were separated on 2-dimensional gels. The gels were Western blotted and probed with antibodies to α-tubulin (DM1A) and β-

Table 5.1. Suspensions from which CHES protein samples were made and run on 2-dimensional gels.

Results are shown for two experiments (i and ii).

5.1.a Proportion of committed cells in the initial suspension.

Percentage of committed cells at time 0 deduced from replicate assay plates = (exp. i) = (101/(102+101))x100% = 49.8%

(exp. ii) = (171/(133+171))x100% = 56.3%

5.1.b. Proportion of cells with different numbers of nuclei at different times in enriched population of committed cells grown in liquid SDM-PS.

(U = uninucleate: B= binucleate: Q = quadrinucleate: O = octonucleate M = more than 8 nuclei: M.P = cell with more than 8 nuclei)

TIME(h)	%U	%B	%Q	%O	%M	TOTAL
i) 4	98.6	1.4	0	0	0	431
19	55.5	43.8	0.7	0	0	577
48	58.4	11.7	26.1	3.4	0.3	291
96	Microplasmodia visible in sample with the naked eye.					

Time(h)	%U	%B	%Q	%O	%M	%M.P	TOTAL
ii) 2	94.8	5.2	0	0	0	0	327
22	39.1	56.9	3.4	0.6	0	0	325
48	66.5	3.8	23.1	4.7	1.9	0	316
70	75.9	0	3.8	5.1	5.1	10.1	158
96 and 116	Microplasmodia visible in sample with the naked eye.						

Proportion of uninucleate committed cells in 4 hour sample (exp. i) = 48.4%

Proportion of uninucleate committed cells in 2 hour sample (exp. ii) = 51.1%

tubulin (KMX-1) and first antibody binding was detected using a peroxidase-conjugated second antibody (Figures 5.1.a-e.). The flagellate sample as expected contained α3 tubulin in addition to α1 and β1 (Figure 5.1.a). The 4 hour sample, which contained 48.6% uninucleate committed cells and 1.4% binucleate cells possessed only α1 and β1 tubulin isotypes; this suggested that uninucleate committed cells did not contain α3 tubulin (which is flagellate-specific) or express the plasmodial-specific tubulin isotypes (Figure 5.1.b). The 19 hour sample which contained 43.8% binucleate and 0.7% quadrinucleate cells had very small amounts of α2 and β2 tubulin isotypes visible on the blots suggesting that at least some cells in this sample possessed these plasmodial-specific isotypes (Figure 5.1.c). The 48 hour sample contained more α2 and β2 tubulin than the 19 hour sample (Figure 5.1.d) and by the time microplasmodia were visible in the cultures (96 hours), the tubulin isotypes present were in a similar pattern to that found in plasmodia (Figure 5.1.e).

The experiment was repeated but this time a larger population of enriched committed cells was prepared so that more samples from different time-points could be obtained. The flagellate sample was discarded. The enriched population was resuspended in liquid SDM-PS and assayed for committed cells (Table 5.1.a experiment ii). The cell density of the enriched suspension estimated in a haemacytometer was 1.3 x 10^6/ml. The suspension was put in growth conditions and after 2 hours, the number of nuclei per cell was observed in a sample (Table 5.1.b.ii) and 4ml of the enriched suspension was spun down and processed for analysis by 2-dimensional gel electrophoresis. Further 4ml samples were taken after 22, 48, 70, 96 and 116 hours and processed for analysis by 2-dimensional

Figure 5.1 Immunoperoxidase stained blots probed with DM1A and KMX-1 from different time-points of the same experiment.

5.1.a Flagellate sample. (95% of these cells were flagellated)

5.1.b 2 hour sample. (48.4% of these cells were uninucleate and committed).

5.1.c 19 hour sample. (43.8% of these cells were binucleate and 0.7% were quadrinucleate)

5.1.d 48 hour sample. (11.7% of these cells were binucleate, 26.1% were quadrinucleate, 3.4% were octonucleate and 0.3% had between 8 and 100 nuclei)

5.1.e 96 hour (microplasmodia) sample.

Figure 5.2 Immunoperoxidase stained blots probed with DM1B from different time-points of the same experiment.

5.2.a 2 hour sample. (51.1% of these cells were uninucleate and committed, 5.2% were binucleate)

5.2.b 22 hour sample. (56.9% of these cells were binucleate, 3.4% quadrinucleate and 0.6% octonucleate)

5.2.c 48 hour sample. (3.8% of these cells were binucleate, 23.1% quadrinucleate, 4.7% octonucleate and 1.9% had between 8 and 100 nuclei)

5.2.d 70 hour sample. (3.8% of these cells were quadrinucleate, 5.1% octonucleate, 5.1% had between 8 and 100 nuclei and 10.1 % were classified as microplasmodia)

5.2.e 96 hour sample. (Microplasmodia could be observed with the naked eye)

5.2.f 116 hour sample. (Microplasmodia could be observed with the naked eye)

Figure 5.1

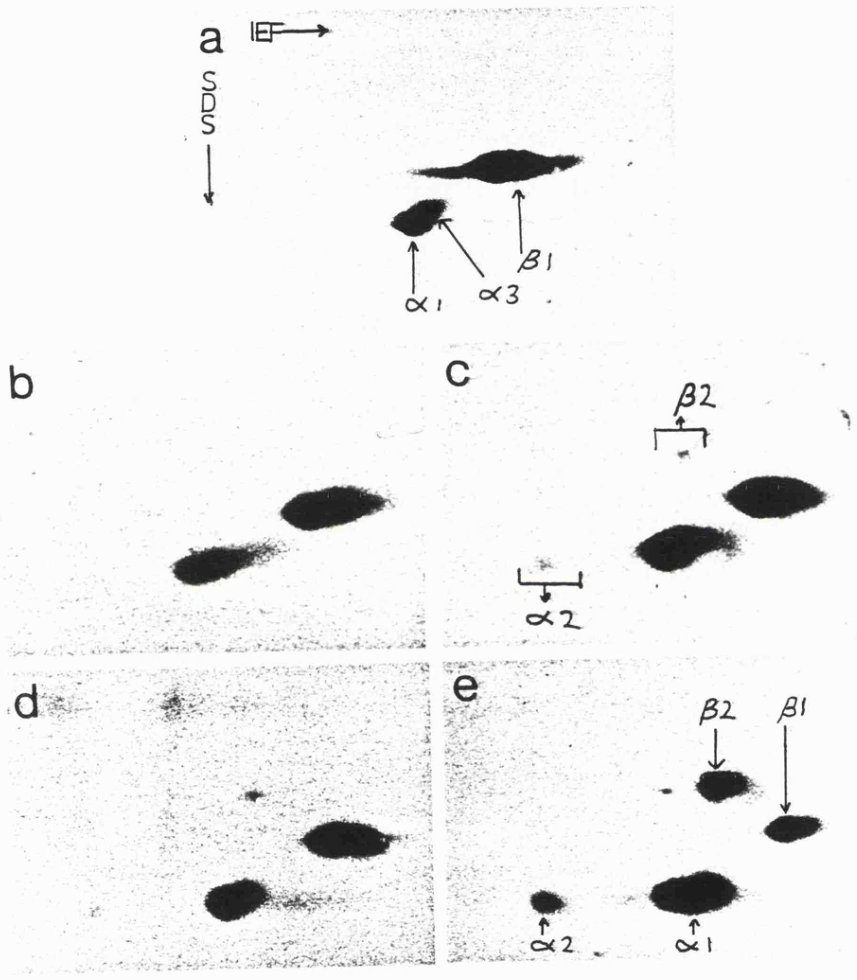
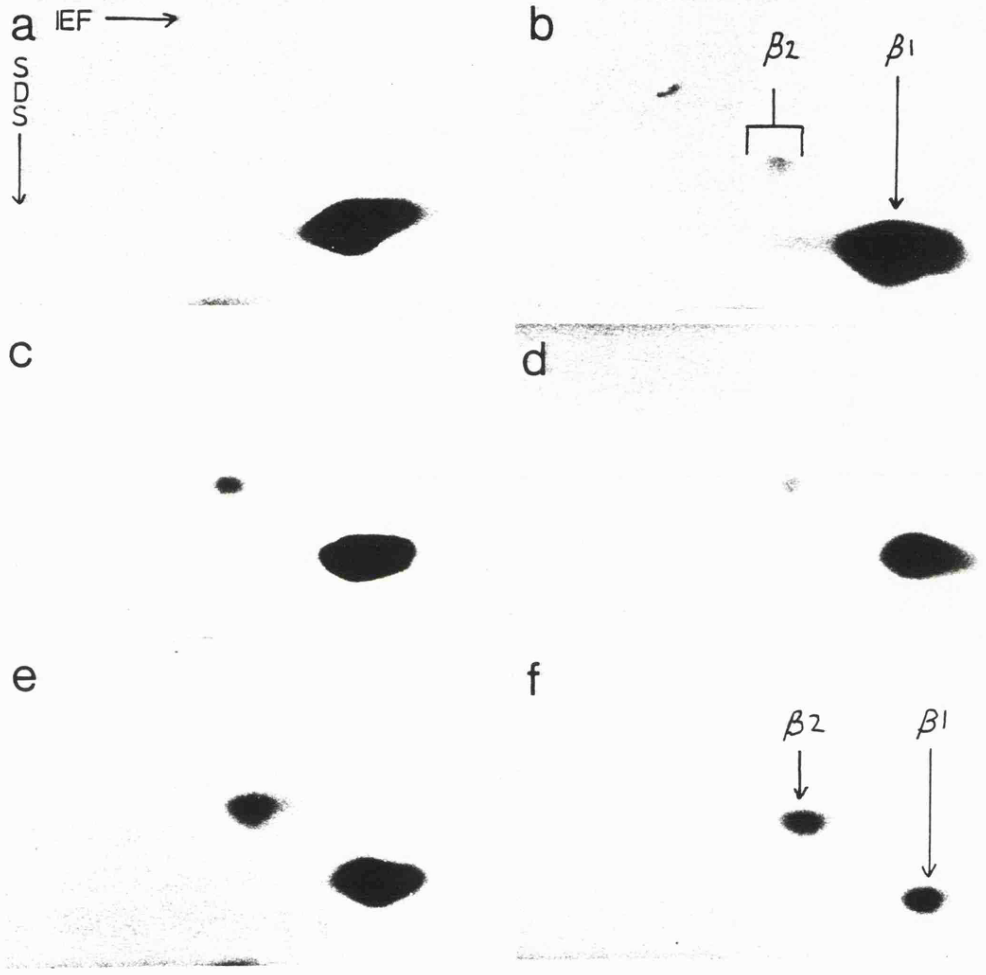


Figure 5.2



gel electrophoresis. Drops of suspension from the 22, 48 and 70 hour samples were observed to estimate the number of nuclei per cell (Table 5.1.b); by 96 hours microplasmodia were present.

All six samples were separated on 2-dimensional gels. The gels were Western blotted and probed with an antibody to β-tubulin (DM1B); only the appearance of β2 was followed since, in plasmodia, the ratio of β2 to β1 is greater than that of α2 to α1 so, there is a greater change to follow during the amoebal-plasmodial transition. First antibody binding was detected using a peroxidase-conjugated second antibody (Figures 5.2.a-f). The 2 hour sample, which contained 51.1% uninucleate committed cells and 5.2% binucleate cells possessed only β1 tubulin. In the 22 hour sample, a small amount of β2 was detectable; in subsequent samples, the β2 spot became darker until by the end of the experiment the relative intensity of β1 and β2 was similar to that observed in plasmodia. The results indicated that in the cell populations there was a gradual change from amoebal to plasmodial tubulin expression.

5.2.2 Identification of the tubulin isotypes expressed in binucleate cells using *in vitro* translation of binucleate cell RNA.

It was possible that protein patterns found in 5.2.1 reflected the protein produced by the cells. Alternatively, the protein patterns may have been modified by residual amoebal protein which masked the actual pattern of protein being produced by the cells. To distinguish between these two possibilities, it was decided to obtain RNA from committed cells, translate it *in vitro* and then run the protein on 2-dimensional gels.

CHAPTER 5

Suspensions from 40 differentiating plates of CL were enriched for committed cells using 20 glass bead columns (to produce sufficient RNA for *in vitro* translation). The enriched suspension was resuspended in SDM-PS and assayed by plating for committed cells; the suspension contained 50.7% committed cells. The cell density of the enriched suspension estimated in a haemacytometer was 9 x 10^5 cells/ml and there were 60ml of enriched suspension. After 5 hours a sample was observed and found to contain 88% uninucleate and 12% binucleate cells (332 cells scored); a 5ml sample was processed for analysis by 2-dimensional gel electrophoresis and a 25ml sample was processed to isolate the RNA. After 22 hours the process was repeated; in this sample there were 46.8% uninucleate cells, 49.7% binucleate cells and 3.4% quadrinucleate cells (348 cells scored).

Since no plasmodial-specific tubulin was detectable on Western blots until binucleate cells were present (Figures 5.1 and 5.2), it was decided to look primarily at binucleate cell RNA translated *in vitro* to see if it gave rise to plasmodial-specific tubulin in greater quantities than was observed in the Western blots. If the binucleate cell sample had given rise to tubulin in a similar pattern to plasmodia, then the uninucleate committed cell sample, that was also obtained, would have been used. The RNA obtained from the binucleate cells was translated *in vitro* and the protein sample resulting from the *in vitro* translation was prepared for 2-dimensional gel electrophoresis. The protein sample from the RNA translated *in vitro* and the protein sample from the same timepoint were run on 2-dimensional gels. The gels resulting from the protein sample were blotted and probed with DM1B. Unfortunately, the binucleate protein control did not produce a blot; however in the previous experiment it was

found that protein samples containing a large proportion of binucleate cells have an abundance of α1 and β1 tubulin with very minor α2 and β2 spots (see Figures 5.1.c and 5.2.b). The gels resulting from the *in vitro* translated RNA were dried and an autoradiogram (ARG) made from them (3 weeks exposure). The resulting ARG is shown in Figure 5.3; the spots were identified by comparing the ARG with those from similar experiments on amoebae and plasmodia that were performed by Burland *et al.* (1983) (see legend to Figure 5.3). Since amoebae do not grow in SDM-PS and binucleate cells contain approximately seven times the RNA of amoebae (see 4.2.5), the majority of the RNA translated in the ARG must have originated from binucleate cells. Surprisingly, the ARG showed a similar pattern of expression to the blots. There is an abundance of α1 and β1 with possibly a small quantity of β2 tubulin; it was not possible to identify an α2 tubulin spot. This indicates that the protein patterns of the blots detected in 5.2.1 may result from the RNA present and leads to the surprising conclusion that binucleate cells either do not express β2 (the β2 present being from the small number of larger cells present) or that they produce it in very small quantities.

5.2.3 Observation of microtubule arrangement through the cell-type transition.

To investigate the microtubule distribution during the amoebal-plasmodial transition, an enriched population of committed cells was prepared. After 7 hours of growth in SDM-PS, the enriched population was assayed for committed cells (Table 5.2.a.i) and was processed for immunofluorescence microscopy using a β-tubulin antibody (DM1B). A

Figure 5.3 Autoradiogram from *in vitro* translated RNA obtained from enriched population that had been allowed to grow in liquid axenic medium for 22 hours.

This population contained 49.7% binucleate cells and 3.4% quadrinucleate cells; the majority of the remainder of the cells were uninucleate amoebae and thus only made a minor contribution to the total RNA pool of the population.

5.3.a shows the complete ARG.

5.3.b is an enlargement of the area marked on 5.3.a. The tubulins are marked as α1, β1 and β2; no α2 could be detected. The other spots i.e. F, P, X, Y and Z are non-tubulin spots that were identified and named by Burland *et al.*, (1983) and T. Burland (personal communication).

Figure 5.3

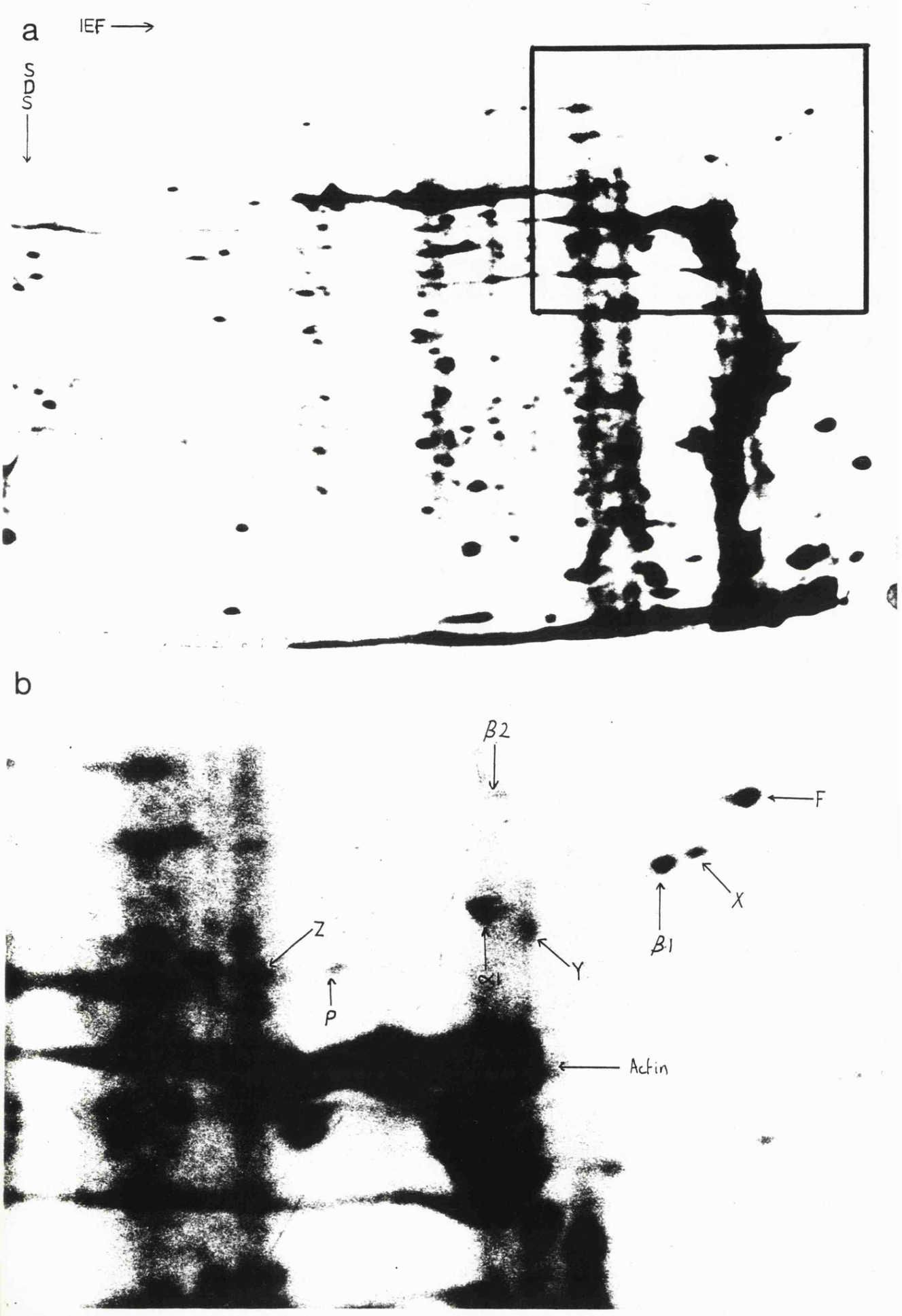


Table 5.2 Suspensions which were processed for immunofluorescence microscopy and stained with DM1B.

Results are shown for two experiments (i and ii).

5.2.a Results of assays for committed cells in the initial suspension.

Percentage of committed cells deduced from replicate assay plates =

(exp. i) = (173/(55+173))x100% = 75.9%

(exp. ii) = (141/(128+141))x100% = 52.4%

Table 5.2.b. Proportions of cells with different numbers of nuclei seven hours after enrichment.

(U = uninucleate : B = binucleate : Q = quadrinucleate : O = octonucleate M = more than eight nuclei)

exp.i	U	%U	B	%B	Q	%Q	O	%O	TOTAL
	166	52.7	125	39.7	20	6.3	4	1.3	315

Proportion of uninucleate committed cells in sample

= %committed - (%B+%Q+%O) = 75.9 - (39.7+6.3+1.3) = 28.6%

Proportion of uninucleate cells that are committed cells

= %uninucleate committed cells / %uninucleate cells = 28.6/52.7 = 54.3%

exp.ii	U	%U	B	%B	Q	%Q	O	%O	TOTAL
	114	50.4	102	45.1	6	2.7	4	1.8	315

population of non-differentiating amoebae was also processed for immunofluorescence microscopy using DM1B.

The enriched population consisted of a mixture of uninucleate cells, binucleate and other multinucleate cells (Table 5.2.b.i). Under phase contrast microscopy, amoebae and uninucleate committed cells are virtually indistinguishable except through a difference in size. The fixation procedure did not allow accurate size assessments. However, no major differences could be observed in the microtubule cytoskeleton between the various uninucleate cells in the enriched population that were in interphase; all of them looked similar to amoebae in interphase (Figure 5.4). It was thus concluded that amoebae and uninucleate committed cells had similar microtubule organization during interphase. Binucleate cells appeared to contain fewer microtubules than the uninucleate cells and quadrinucleate cells had fewer still. Furthermore, among the multinucleate cells there were some with one MTOC (as usually found in amoebae), some with two MTOCs and some which, while possessing microtubules, had no clearly discernible MTOC (Figures 5.5 - 5.7). Samples of amoebae, enriched uninucleate committed cells, binucleate cells and quadrinucleate cells were scored for the number of MTOCs/cell (Table 5.3). Amoebae of the strain used are incapable of growth in axenic liquid medium; so the uninucleate cells observed in mitosis must be committed cells. The mitoses in these cells were different from those observed in amoebae; no asters could be observed. The spindle appeared to be of the plasmodial "closed" type (intranuclear). Many of the mitotic cells had in addition to the spindle, one or two cytoplasmic MTOCs (CMTOCs) which had fewer and shorter microtubules radiating from them than amoebal CMTOCs (Figures 5.9 - 5.11). A sample of uninucleate

Figure 5.4 - 5.8 Interphase cells stained with a β-tubulin antibody, DM1B.

Photographs are shown in pairs; the first photograph is the cell observed with phase contrast, the second with immunofluorescence. Magnifications are approximate.

Figure 5.4 Non-differentiating amoebae (x 5000)

Figure 5.5 Binucleate cells with one MTOC (a-d x 3000: e - f x 2000).

Figure 5.6 Binucleate cells with two MTOCs (a - f x 3000: g - h x 2000).

Figure 5.7 Binucleate cells with no clearly discernable MTOC (x 4000). In the first two immunofluorescence pictures there is possibly an MTOC, but it is not nearly as clear as in Figures 5.6 and 5.7.

Figure 5.8 Large multinucleate cells (x 600). There are no microtubules present in these cells. The microtubules in Figure 5.8.b are in an amoeba just above the multinucleate cell.

Figure 5.4

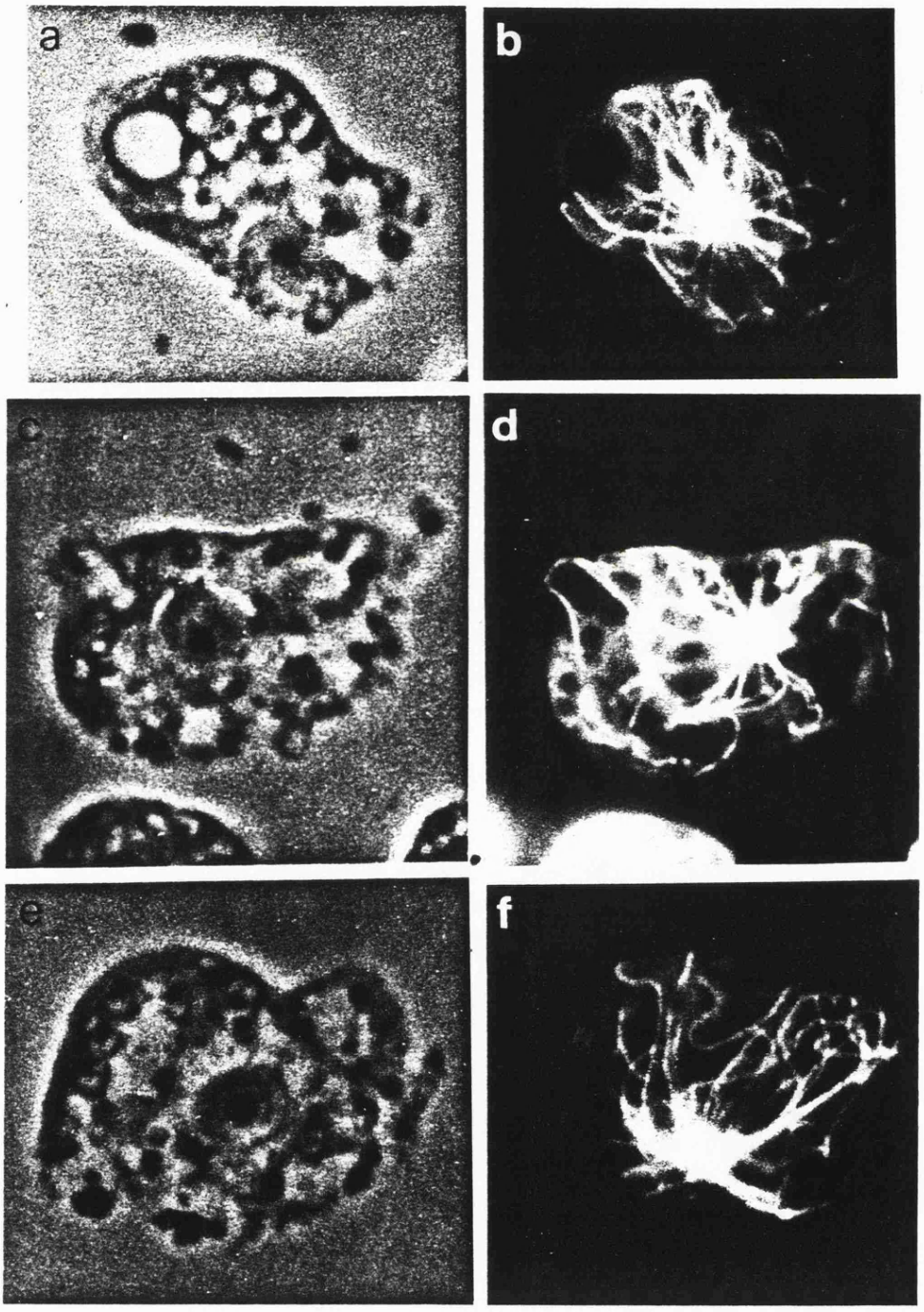


Figure 5.5

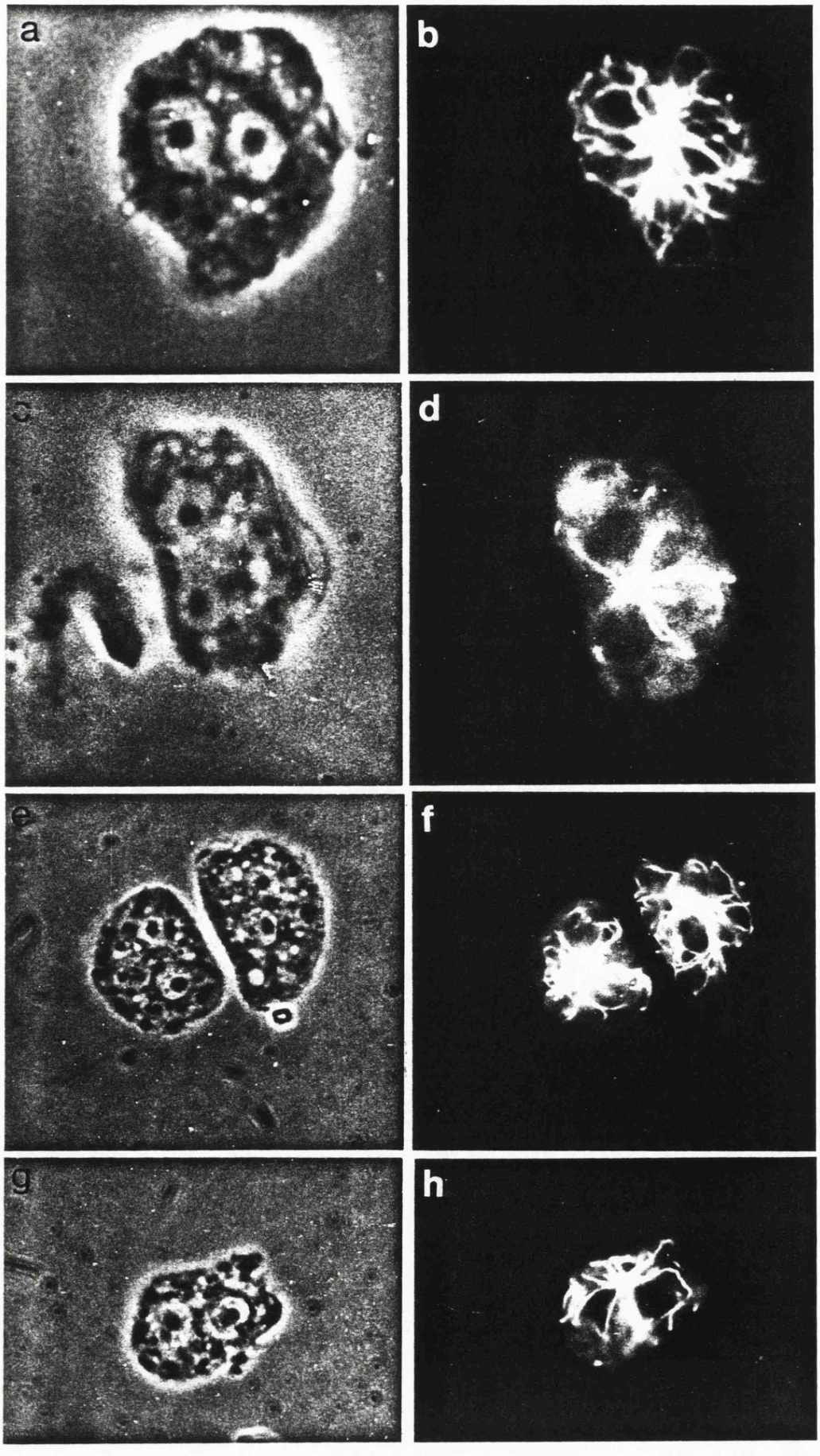


Figure 5.6

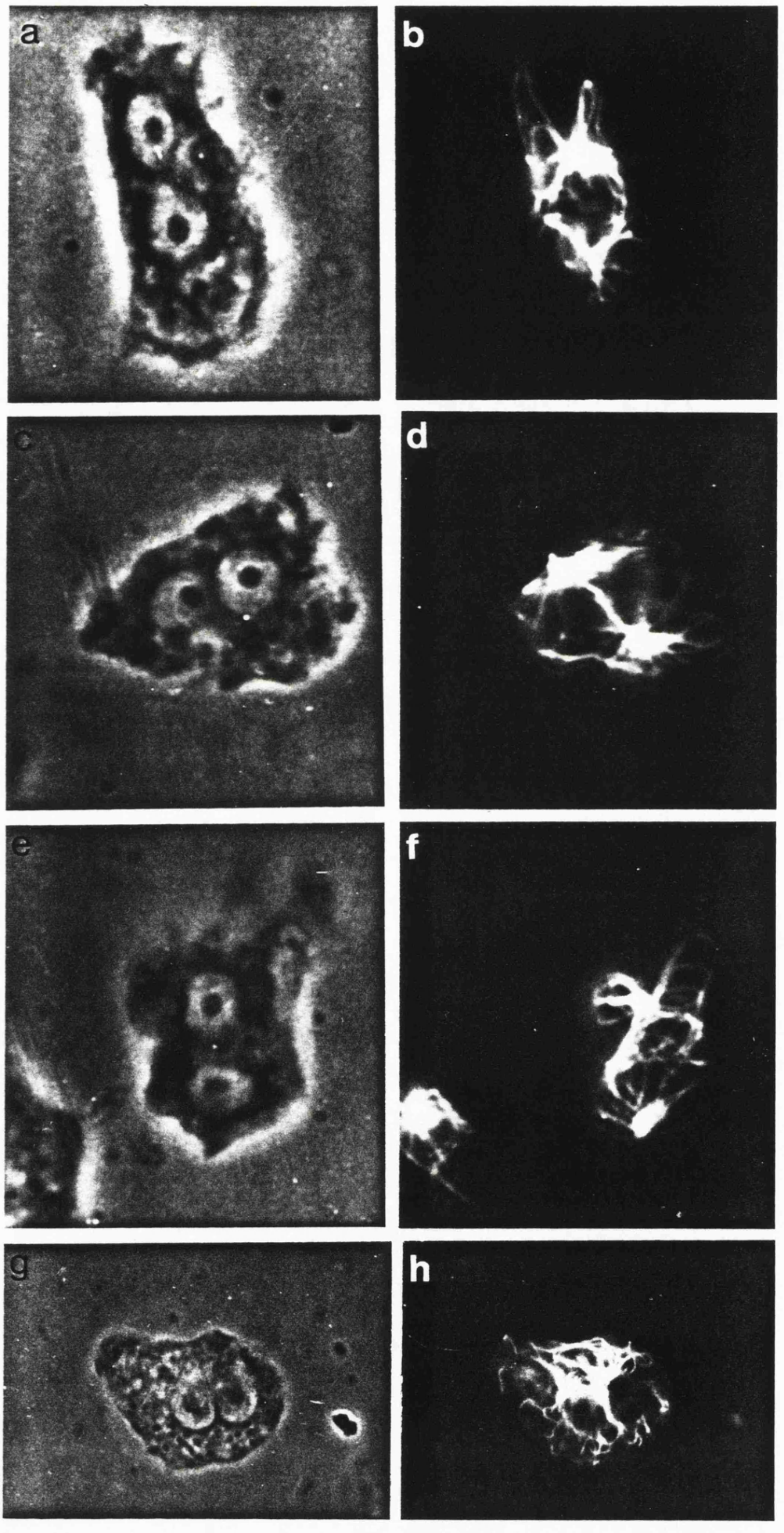


Figure 5.7

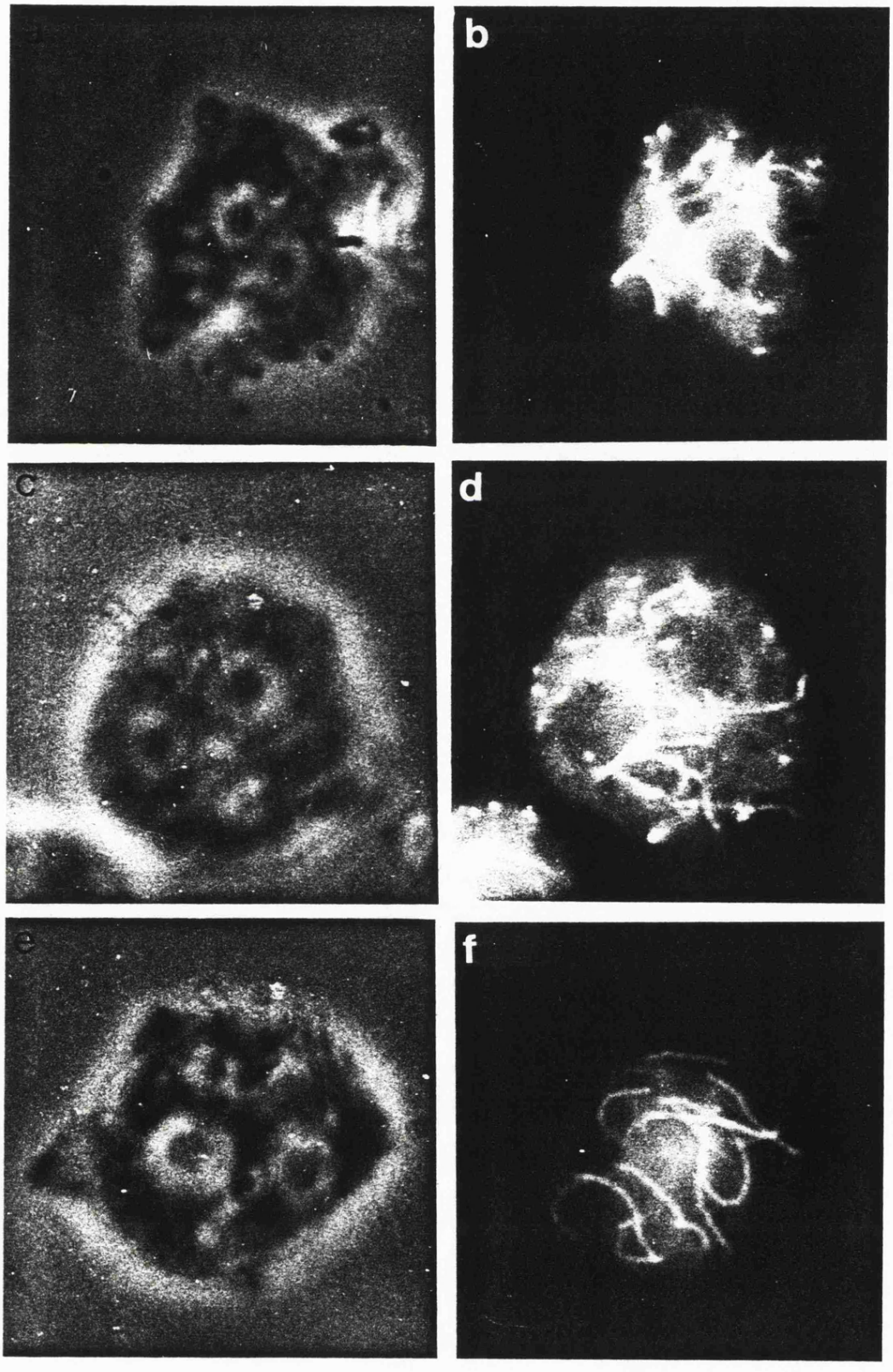


Figure 5.8

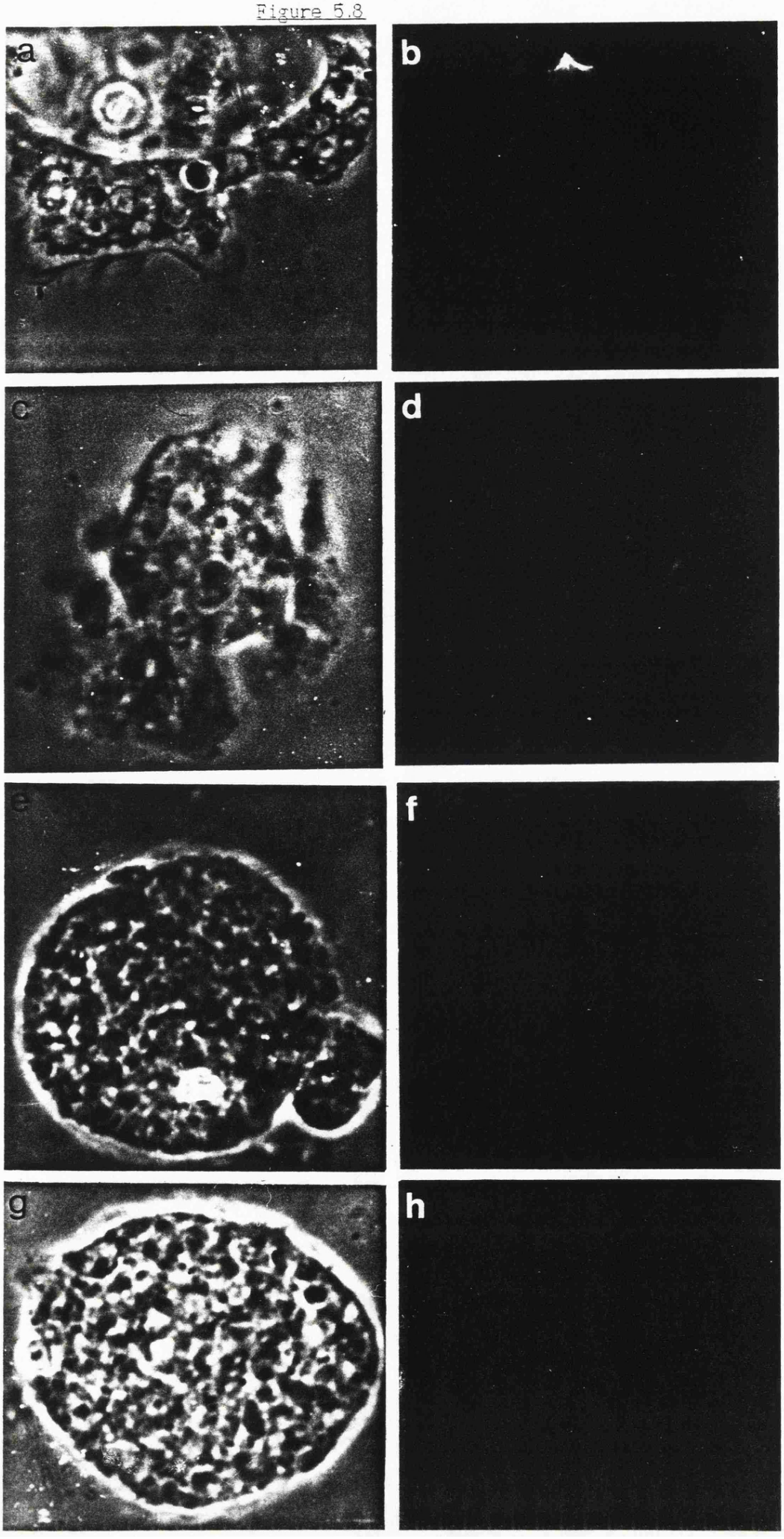


Figure 5.9 - 5.12 Mitotic cells stained with a β-tubulin antibody, DM1B. Photographs are shown in pairs; the first photograph is the cell observed with phase contrast, the second with immunofluorescence. Magnifications are approximate.

Figure 5.9 This shows the different mitoses observed in uninucleate committed cells compared with an amoeba and a microplasmodium.

5.9.a Amoebal mitosis (x 4000); note the asters at the poles of the spindle.

5.9.b Microplasmodium in mitosis (x 600); there are no asters observable.

5.9.c and d Uninucleate committed cell in mitosis (x 4000); no CMTOCs.

5.9.e and f Uninucleate committed cell in mitosis (x 4000); one CMTOC.

5.9.f and g Uninucleate committed cell in mitosis (x 4000); two CMTOCs. Note that in this cell, the CMTOC on the right radiates more microtubules than that on the left.

Figure 5.10 Uninucleate committed cells in mitosis (x 3000)

5.10.a - d have no CMTOC. 5.10.e - f have 1 CMTOC.

Figure 5.11 Uninucleate committed cells in mitosis.

5.11 a - d Cells with 1 CMTOC.

5.11 e - g. Cell with 2 CMTOCs. f and g show different planes of focus.

Figure 5.12 Binucleate cells in mitosis (x 4000).

5.12 a - d have no CMTOC.

5.12 e - f have 1 CMTOC.

Figure 5.9

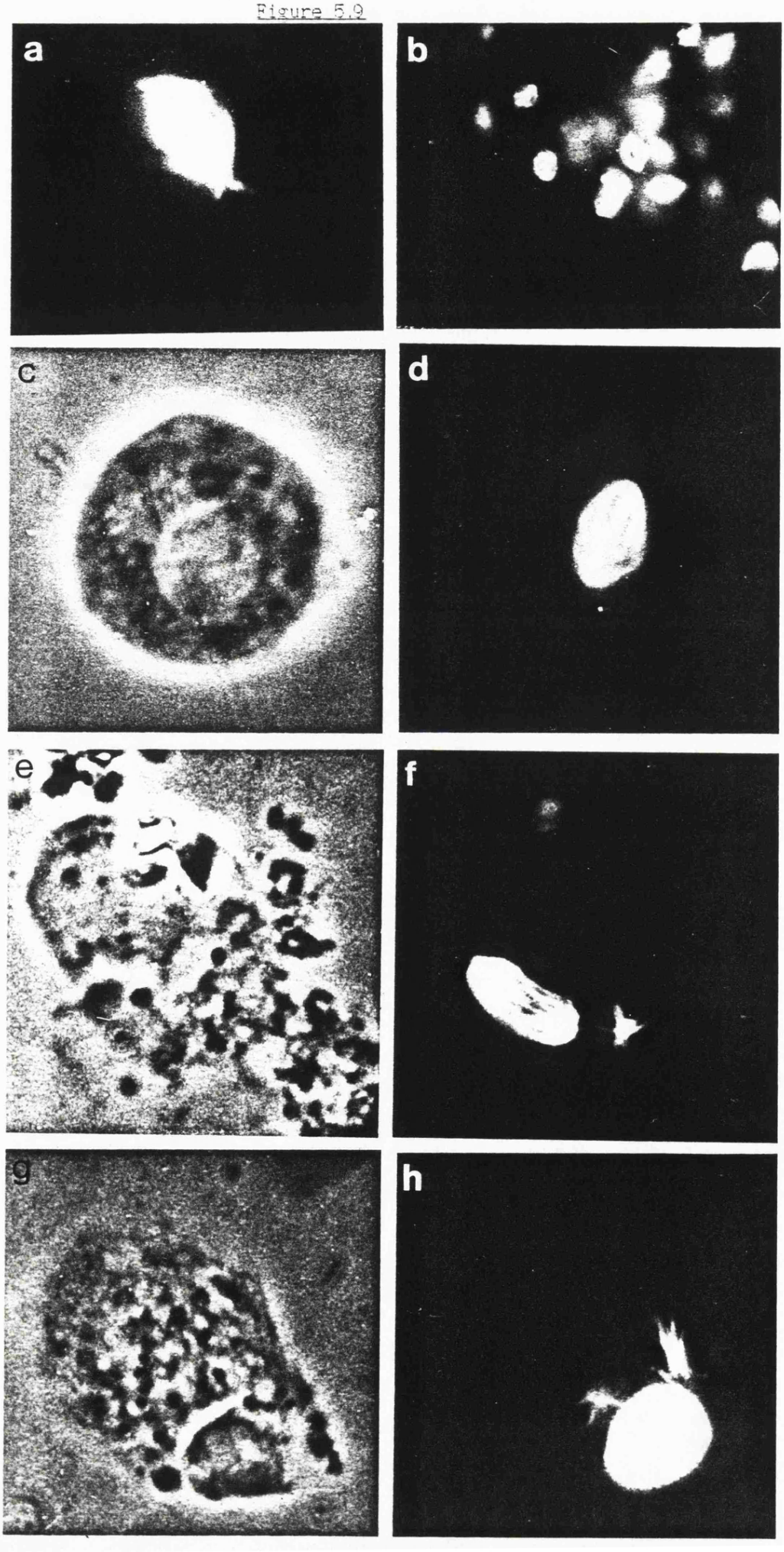


Figure 5.10

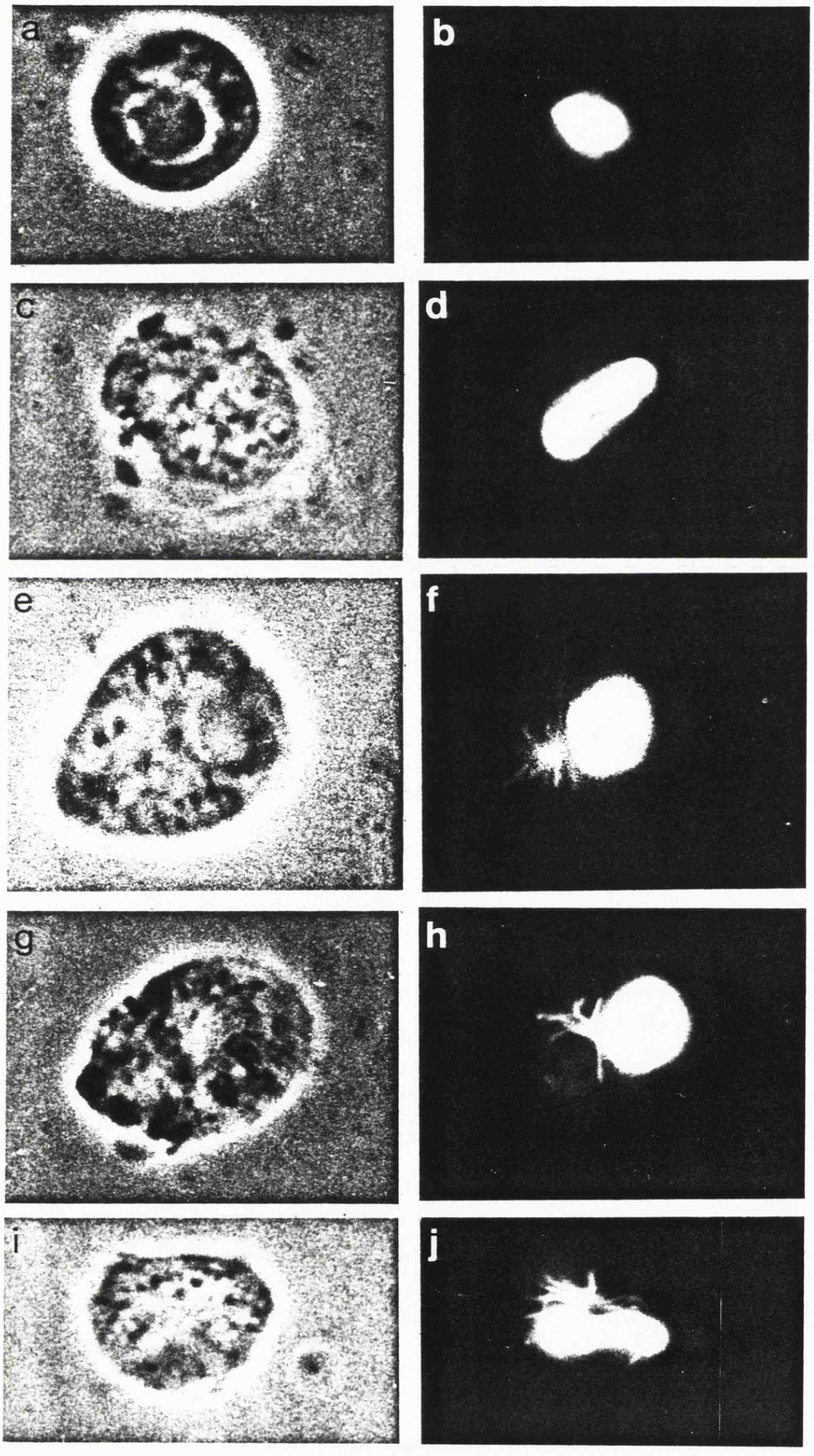


Figure 5.11

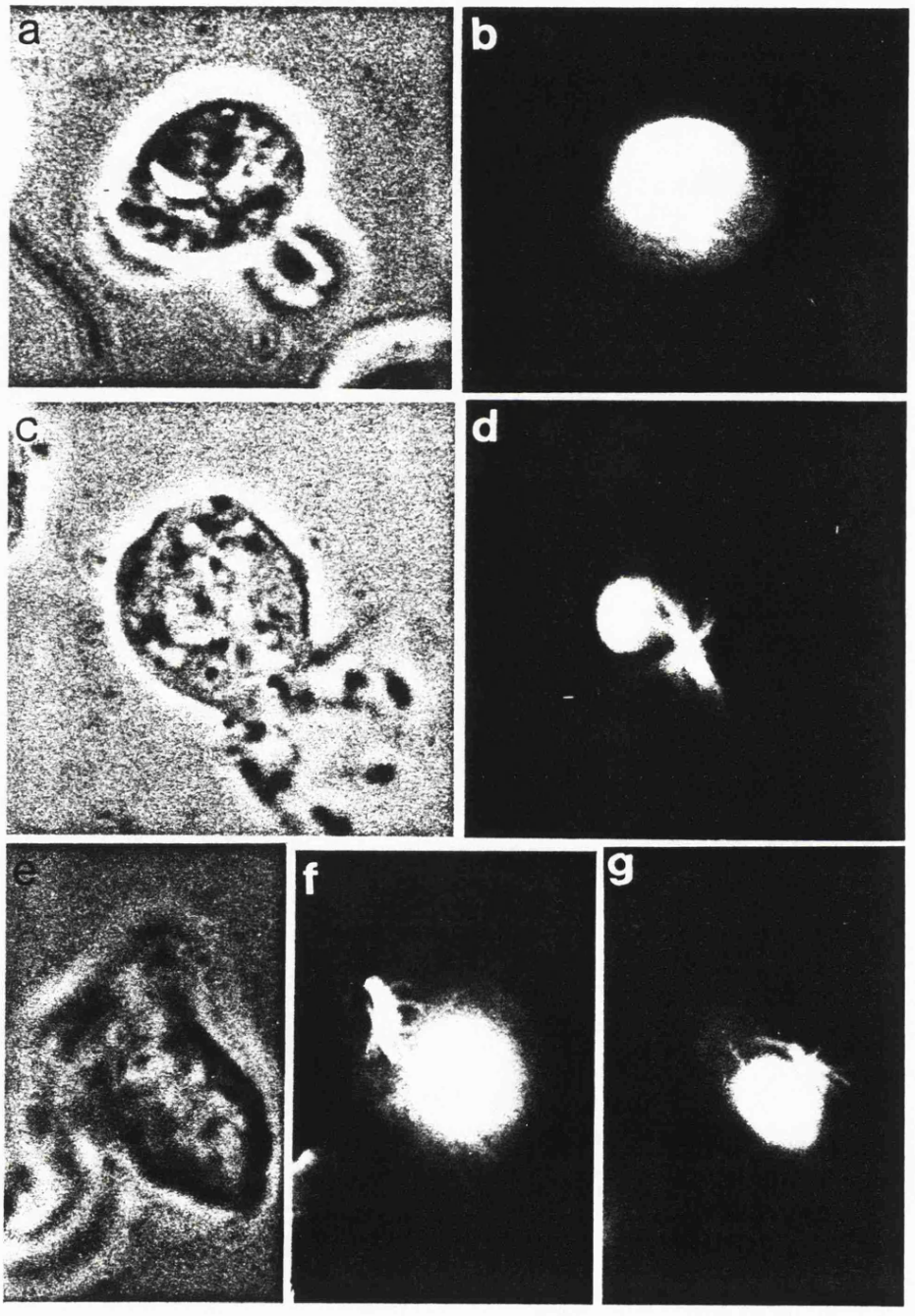
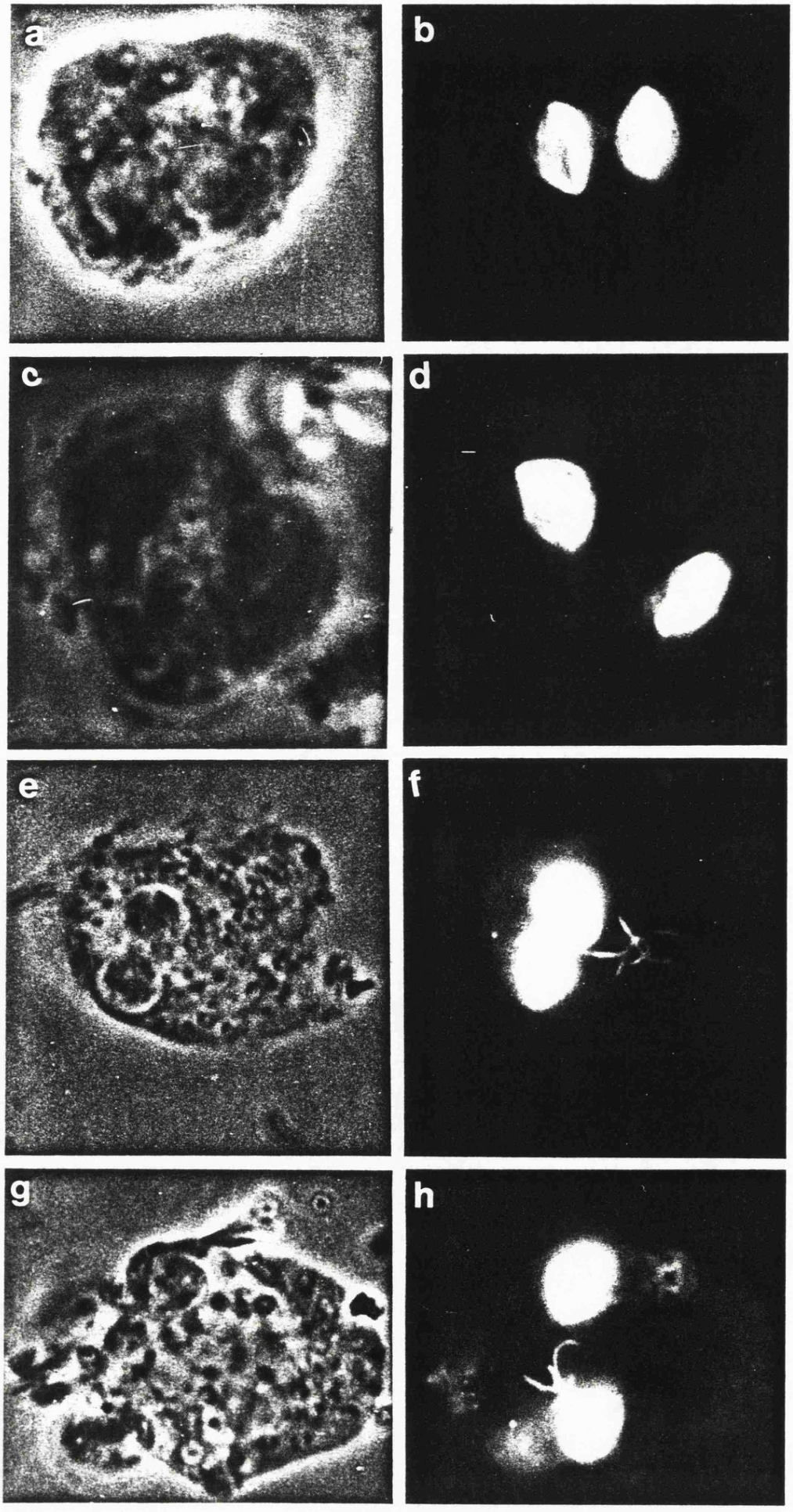


Figure 5.12



mitotic cells was scored for the number of cytoplasmic microtubule organising centres (CMTOCs)/cell (Table 5.3).

The quality of the immunofluorescence also allowed counts to be made of the number of microtubules radiating from the cytoplasmic MTOCs. The cell-types scored were amoebae, mitotic uninucleate committed cells and binucleate cells. Cells with one and with two MTOCs were scored separately (Table 5.4). All of the binucleate cells with two MTOCs appeared to have the same number of microtubules radiating from each MTOC, hence the single figure for number of microtubules per cell (Table 5.4), whereas uninucleate mitotic cells with two CMTOCs usually had different numbers of microtubules radiating from each of their organising centres (Table 5.5).

The experiment was repeated, but this time the population on which the enrichment was performed had been allowed to progress further so that it contained cells at later stages of plasmodium development (Table 5.2.ii). This allowed observation of binucleate cells that were in mitosis, some of which had CMTOCs (Figure 5.12). A sample of these cells was scored for the number of CMTOCs per cell as was a small sample of quadrinucleate cells (Table 5.3). A repeat of the counts on the number of CMTOCs/cell in uninucleate mitotic cells agreed well with the previous experiment. Some large cells, with 16 or more nuclei were observed in which no microtubules were visible (Figure 5.8). It is unlikely that this was due to an inability of the antibodies to enter these cells since in large cells in mitosis, the spindles could be clearly seen (Figure 5.9.b). Some large cells did contain microtubules, but without any clear pattern of organisation it is possible that these cells were the products of fusion between smaller cells.

Table 5.3. Number of MTOCs/cell in various cell types.

	Nº OF MTOC PER CELL				
CELL TYPE	0	1	2	4	TOTAL CELLS COUNTED
AMOEBA	0	97.1	2.9	0	811
UNINUCLEATE (int)*	1.0	96.5	2.5	0	406
UNINUCLEATE (mit)	29.2	51.4	19.4	0	551
BINUCLEATE (int)	26.7	55.1	18.2	0	1264
BINUCLEATE (mit)	84.5	11.6	2.6	1.3	57
QUADRINUCLEATE (int)	81.7	13.4	4.8	0	155

(int = interphase : mit = mitosis)

* These uninucleate cells were a mixed population of amoebae and committed cells, calculated to contain approximately 53% committed cells.

Table 5.4. Number of microtubules per MTOC in various cell types

Nº MT	AM 1 MTOC	UNI(mit) 1 CMTOC	BI 1 MTOC	BI 2 MTOC
4-5	0	12	0	6
6-7	0	32	49	61
8-9	0	15	92	69
10-11	0	5	160	29
12-13	7	1	122	17
14-15	19	3	85	6
16-17	53	1	67	4
18-19	76	0	28	2
20-21	122	0	21	1
22-23	89	0	4	0
24-25	64	0	7	0
26-27	28	0	2	0
28-29	19	0	3	0
30-31	12	0	1	0
32-33	3	0	0	0
34-35	5	0	0	0
36	3	0	1	0
TOTAL	500	69	642	195

(Nº MT = number of microtubules : AM = amoeba: UNI(mit)= uninucleate committed cell in mitosis : BI = binucleate cell)

Table 5.5. Nº of microtubules per cytoplasmic MTOC in uninucleate committed cells with two cytoplasmic MTOCs during mitosis.

Mitotic uninucleate committed cells with two CMTOCs usually had a different number of microtubules radiating from each CMTOC. Each cell was scored for the number of microtubules radiating from the CMTOC with most microtubules (HIGH) and least microtubules (LOW).

HIGH	LOW	Nº of Cells
4	4	1
6	4	3
6	6	2
8	4	3
8	6	2
8	8	1
10	4	3
10	6	1
10	8	1
10	10	1
16	6	1
16	10	1
TOTAL		20

5.2.4 Observation of phosphoprotein distribution through the amoebal-plasmodial transition.

To compare the loss of MTOCs in multinucleate cells with the loss of centrioles, a population enriched for committed cells and a population of amoebae were processed for immunofluorescence microscopy. Some of the slides were stained with DM1B as the first antibody and other slides were stained with MPM-2 as the first antibody. In most of the amoebae, the MPM-2 revealed two bright dots next to each other which were presumed to be the centrioles (see 5.1). There also appeared to be a dappled effect over the nucleus (Figures 5.13 a-d). Some amoebae were observed which had two pairs of bright dots opposite each other with a line between them; these cells had no discernible nucleus when observed under phase contrast microscopy so it was likely that they were undergoing open mitosis, since the centrioles are known to be at the poles of amoebal mitoses (Figures 5.13 e-f). The number of pairs of centrioles (bright dots) per cell was counted for the amoebal population. The sample of amoebae that were stained with DM1B were scored for the number of MTOCs/cell (Table 5.6). The number of MTOCs/amoeba corresponded very well with the number of pairs of centrioles/amoeba suggesting that, as expected, where there was a centriole there was also an MTOC.

In the uninucleate committed cells the MPM-2 produced the same dappled effect over the nucleus that had been observed in the amoebae. However, not all of the committed cells contained the bright dots, suggesting that the centrioles had become dephosphorylated and thus possibly inactivated (Figures 5.14 a-h). There appeared to be very few binucleate cells with centrioles. A sample of binucleate cells was scored for the number of pairs of bright dots per cell. Binucleate cells on the slides that were

Figure 5.13 Amoebae stained with a phosphoprotein antibody (MPM-2) (Magnification approximately x 3000).

Photographs are shown in pairs; the first photograph is the cell observed with phase contrast, the second with immunofluorescence.

5.13.a - d Amoebae in interphase.

5.13 e - f Amoebae believed to be in metaphase. No phase photograph was taken of these cells.

Figure 5.14 Committed cells stained with a phosphoprotein antibody (MPM-2) (Magnification approximately x 3000).

Photographs are shown in pairs; the first photograph is the cell observed with phase contrast, the second with immunofluorescence.

5.14 a - h Binucleate cells in interphase. in 5.14 b and h, centrioles can be seen but not in d and f.

5.14 i - l Uninucleate committed cells believed to be in mitosis.

Figure 5.13

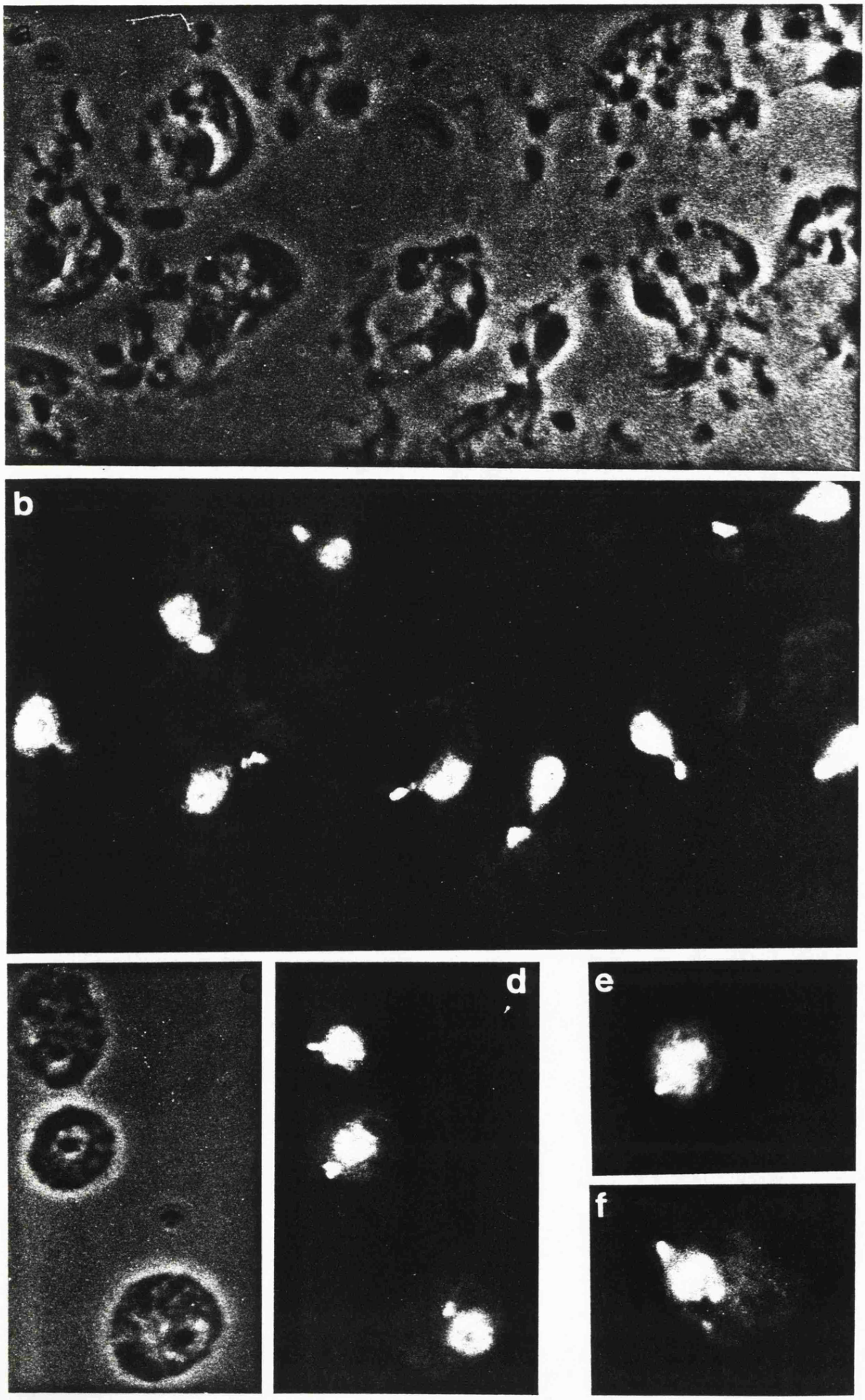


Figure 5.14

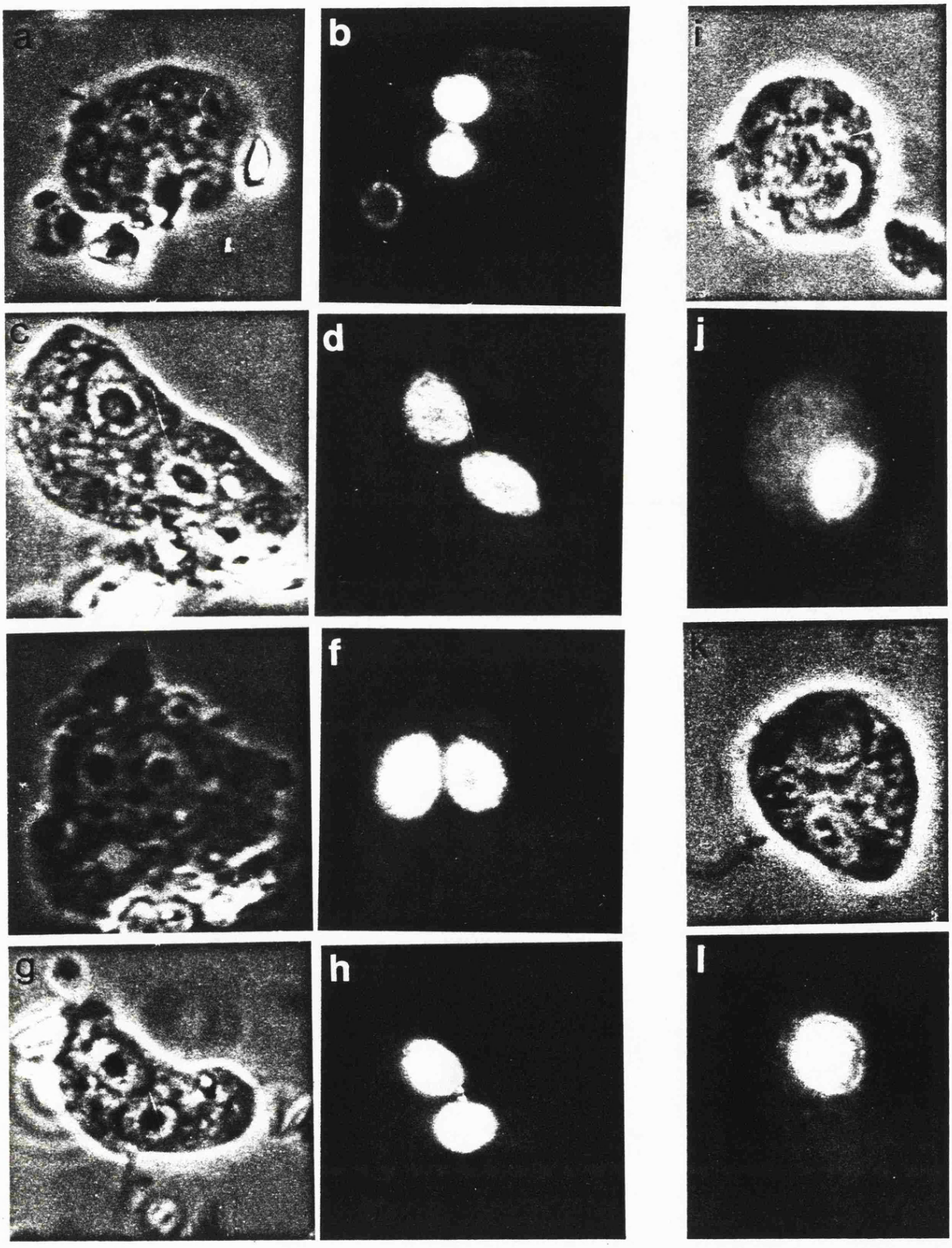


Table 5.6. Proportion of cells with different numbers of pairs of centrioles per cell.

The cells were visualised by immunofluorescence microscopy with MPM-2 and the number of cells in the same population with different numbers of MTOCs per cell as visualised by immunoflourescence with DM1B. The total numbers of cells of each type that were scored are also shown (TOTAL).

	Pairs of Centrioles				CMTOCs				
	0	1	2	TOTAL	0	1	2	4	TOTAL
AMOEBA	0	97.0	3.0	297	0	97.0	3.0	0	271
UNINUCLEATE*(int)	16.1	82.5	1.3	149	0	98.7	1.3	0	149
UNINUCLEATE (mit)	47.2	48.1	4.7	129	26.3	51.9	21.7	0	129
BINUCLEATE (int)	71.8	23.2	4.9	426	26.5	54.6	18.9	0	509
BINUCLEATE (mit)	90.8	7.1	2.0	98	80.6	13.3	4.1	2.0	98

These figures were obtained by staining the same population with either DM1B or MPM-2. Thus, conclusions can only be drawn on a population basis. (int = interphase : mit = mitosis)

* Approximately 55% of the uninucleate cells were committed.

stained with DM1B were scored for the number of MTOCs so that the presence of centrioles and MTOC could be compared (Table 5.6). By comparing the two counts it appeared that centrioles were lost before MTOCs.

Some committed cells were observed in which there was a discrete circle around the nucleus; in some of these nuclei, a line across the equator could be observed. Some of these cells also contained one or more pairs of bright dots but these were not always present and were not in the nucleus. Since no nucleoli were visible in these cells under phase contrast microscopy it was concluded that these cells were undergoing closed (plasmodial-type) mitosis (Figures 5.14 i-l).

5.2.5 Relationship between loss of MTOCs and centrioles.

An enriched population of committed cells was prepared, assayed for committed cells and allowed to grow in SDM-PS for 7 hours. After this time, the enriched population and a population of non-differentiating amoebae was processed for immunofluorescence microscopy. The fixed cells were first stained with MPM-2 followed by a fluorescein-conjugated second antibody. After rigorous washing with PBS, the cells were stained with DM1B followed by a rhodamine-conjugated second antibody. This enabled MPM-2 staining to be observed on the fluorescein channel and DM1B staining in the same cells on the rhodamine channel.

In the amoebae, the effect of the MPM-2 staining was the same as that described in 5.2.4. If the fluorescence was switched to the rhodamine channel the MTOC could be observed in the same position as the centrioles. This is to be expected as the MTOC is associated with the anterior centriole (Wright *et al.*, 1985). In cells where a metaphase

spindle could be observed from the DM1B staining, the MPM-2 revealed bright dots at the poles of the spindle and a bright line corresponding to the metaphase plate. In cells at later stages of mitosis (anaphase and telophase), the only structures visible with MPM-2 were the centrioles at the poles of the spindle (Figures 5.15).

In the committed cells, there were again, cells with varying numbers of centrioles. In these cells where a pair of centrioles could be observed there was also an MTOC (Figures 5.16 d-i). However, it was found that cells with an MTOC did not necessarily have centrioles (Figures 5.16 a-c). Samples of interphase uninucleate cells (which was a mixture of amoebae and committed cells) and binucleate cells were observed and the number of centrioles and MTOCs in each cell was counted (Table 5.7). In both uninucleate and binucleate committed cells, where a spindle could be observed with DM1B staining, there were no bright dots at the poles of the spindle with MPM-2 staining; instead, the nucleus was enveloped by a thin bright line which was also observed in mitotic multinucleate cells (Figures 5.18). In addition to this envelope, some cells in metaphase had a bright line across the nucleus (Figures 5.16 j-o). Cells at later stages of mitosis did not have the envelope so clearly defined. Cells in anaphase (Figure 5.17 a-b) had the entire nucleus stained while cells in telophase (Figure 5.17 c-e) were stained only in the region of the poles of the spindle. Some mitotic committed cells also had a pair of centrioles outside the nucleus (Figures 5.17 f-k); very rarely, two pairs were observed. When these cells were observed on the rhodamine channel, the cytoplasmic MTOCs coincided with the centrioles. However, in common with the interphase committed cells, some mitotic cells were observed where the DM1B staining revealed

Figure 5.15 - 5.18 Cells stained with DM1B and MPM-2.

Photographs are shown in groups of three; the first photograph is the cell observed with phase contrast, the second with DM1B with a rhodamine-conjugated second antibody and the third with MPM-2 with a fluorescein-conjugated second antibody. Magnifications are approximate.

Figure 5.15 Amoebae in mitosis (x 3000).

5.15 a - c. Metaphase

5.15 d - f. Anaphase

5.15 g - h. Telophase/cytokinesis.

Figure 5.16 Committed cells.

5.16 a - c Binucleate cell with MTOC but no centrioles (x 2000)

5.16 d - i Binucleate cells with MTOC and centrioles (x 3000)

5.16 j - o Uninucleate committed cells in metaphase (x 3000).

Figure 5.17 Uninucleate committed cells in mitosis.

5.17 a - b Anaphase (x 2000)

5.17 c - e Telophase (x 2000)

5.17 f - k Metaphase with CMTOC and centrioles (x 3000).

Figure 5.18 Multinucleate cells in mitosis.

5.18 a - c Large multinucleate cell (x 600)

5.18 d - f Binucleate cell (x 3000)

Figure 5.15

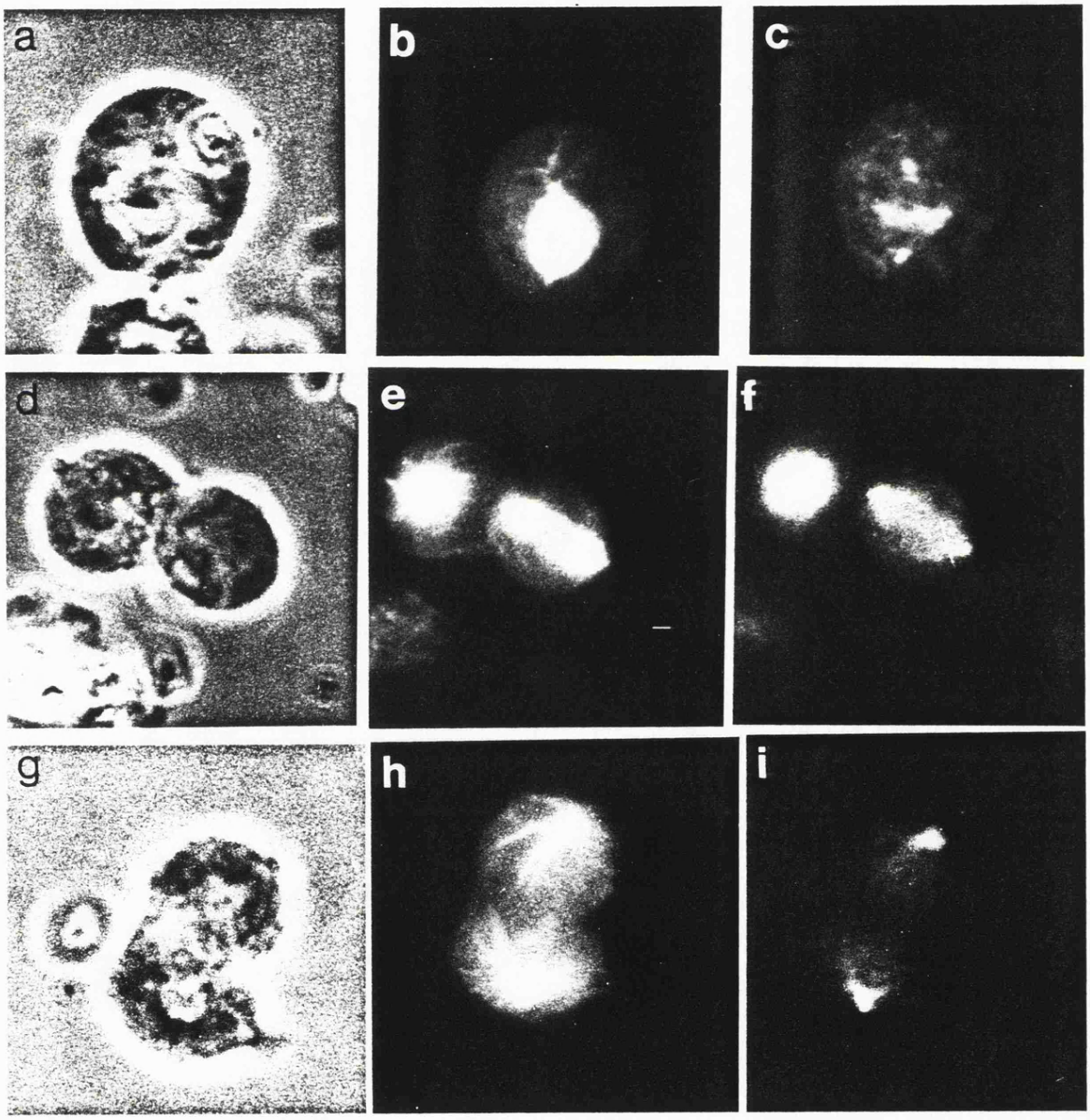


Figure 5.16

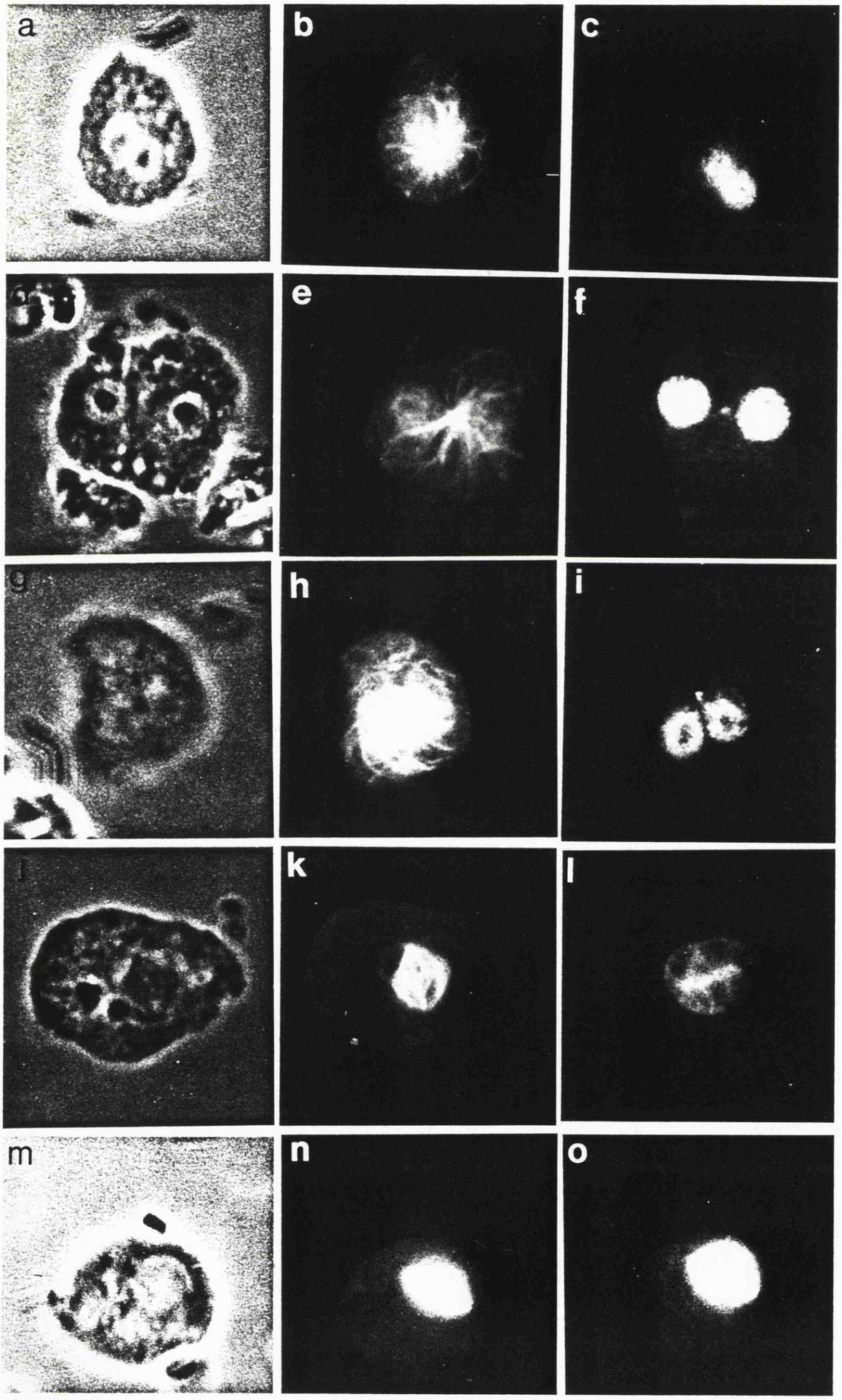


Figure 5.17

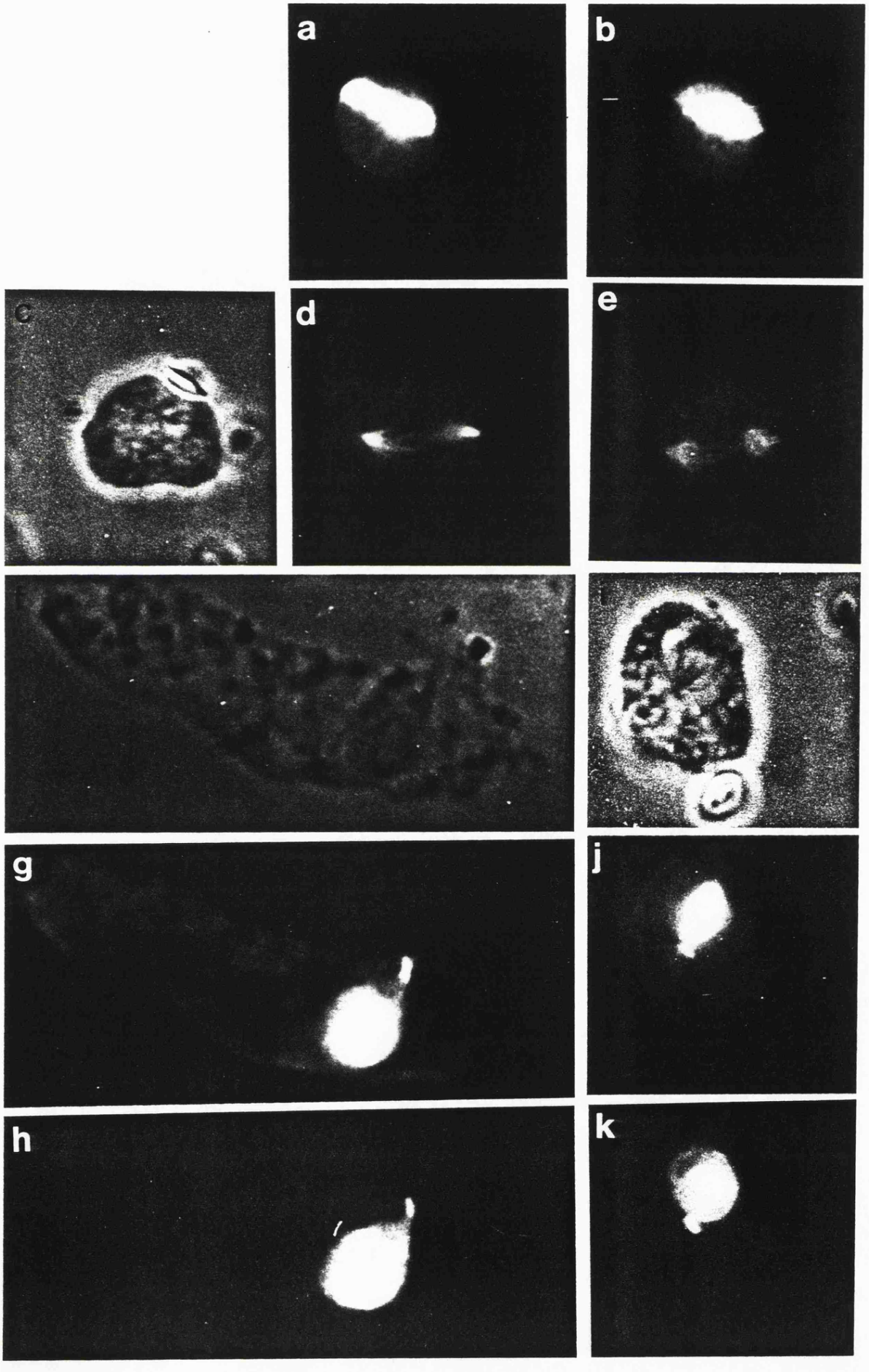


Figure 5.18

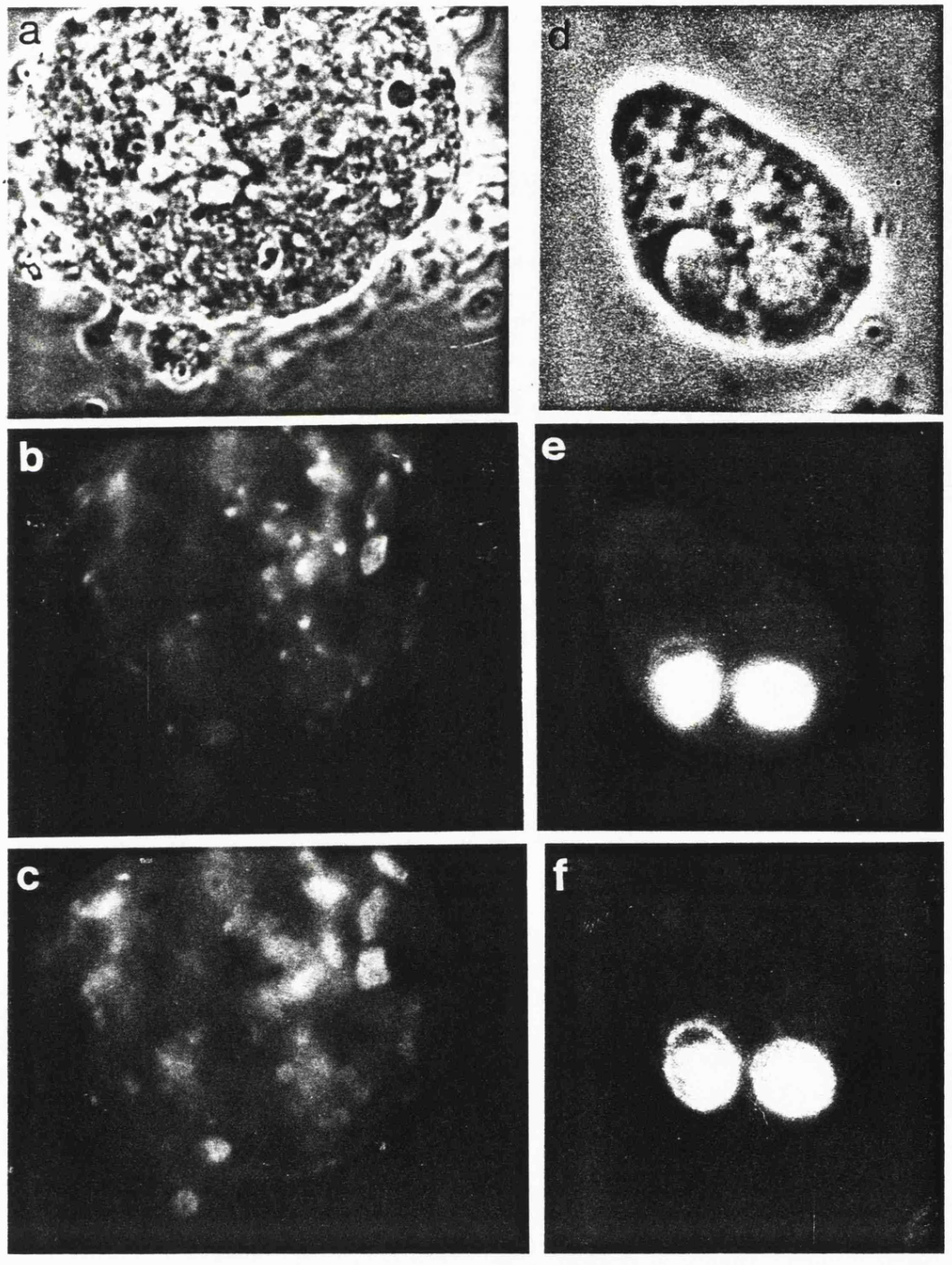


Table 5.7 Number of centrioles per cell and number of MTOC per cell observed using a double stain with DM1B and MPM-2.

It was calculated that 55.1% of the interphase uninucleate cells were committed. The table shows the number of individual cells with x centriole pairs/y CMTOCs. i.e. 1/2 = number of cells with one pair of centrioles and 2 CMTOCs.

	x/y 0/0	x/y 0/1	x/y 0/2	x/y 1/0	x/y 1/1	x/y 1/2	x/y 2/0	x/y 2/1	x/y 2/2	x/y 2/4	TOTAL
UNI	0	16.1	0	0	82.6	0	0	0	1.3	0	149
UNI(mit)	26.4	17.8	3.1	0	31.0	17.1	0	0	4.7	0	129
BI(int)	26.0	45.5	1.3	0	9.7	12.3	0	0	5.2	0	154
BI(mit)	80.6	9.2	1.0	0	4.1	3.1	0	0	0	2.0	98

(UNI = uninucleate cells (55.1% committed) : BI = binucleate cells : int = interphase : mit = mitosis)

cytoplasmic MTOCs but the MPM-2 staining did not reveal centrioles. A sample of uninucleate and binucleate mitotic committed cells was examined and the number of centrioles and MTOCs in each cell was counted (Table 5.7).

5.3 DISCUSSION

5.3.1 Switch from amoebal to plasmodial interphase microtubule arrangement.

The use of immunofluorescence microscopy has enabled the changes in distribution of microtubules to be followed during the cell-type transition from amoeba to plasmodium in *P. polycephalum*. The cell does not switch from the amoebal arrangement to the plasmodial arrangement during a single mitotic cycle but changes over several mitotic cycles. A microtubular cytoskeleton persists in binucleate cells and in quadrinucleate cells but has disappeared in most cells with sixteen or more nuclei.

5.3.2 Switch from Open to Closed Mitosis.

Previous observations, using phase contrast microscopy and time-lapse cinematography, on apogamic strains of Myxomycetes have suggested, that the mitosis in uninucleate committed cells is of the closed plasmodial type (see Chapter 1). The data presented in this thesis from the immunofluorescence work with DM1B and MPM-2 confirms and extends the evidence that mitosis in committed cells is of the plasmodial type. Thus, it appears that a cell born by one mitotic apparatus goes through the following mitosis with a different apparatus although the tubulin isotypes present appear to be the same. A number of authors (e.g. Havercroft and Gull, 1983) have commented that closed mitosis may be necessary in plasmodia to reduce the incidence of spindle fusion; this would not explain why the switch to closed mitosis occurs in uninucleate cells. It is possible that there is a link between the mitotic method and cytokinesis since, at the same time that the first closed mitosis

occurs, cytokinesis does not occur. One way in which the two events could be linked is if a substance were present in the nucleus which, when released, triggers mitosis. This substance would thus be released in open mitosis but not in closed mitosis. It is interesting that in films following the amoebal-plasmodial transition (Bailey *et al.*, 1987) cleavage furrows were observed in some cells for the first mitoses after commitment; this may be due to a residual amount of the hypothetical substance from the previous amoebal division which is sufficient to initiate cleavage but not to complete cytokinesis. It is possible that the mutant amoebal strain, ATS 23 (Burland *et al.*, 1981), which appears to undergo open mitosis but fails to complete cytokinesis is defective in this substance.

Even though there is a clear difference in the expression of tubulin polypeptides in amoebae and plasmodia, it seems unlikely that the phenomenon has functional significance. The amoeba, which has fewer isotypes separable on 2-dimensional gel analysis, appears to have more functional uses for tubulin. The only functional requirement for plasmodial-specific tubulin isotypes would appear to be in relation to closed mitosis since it has been shown that all plasmodial isotypes are incorporated in the mitotic spindle (Roobol *et al.*, 1984). However, the uninucleate committed cell undergoes closed mitosis when there are virtually no plasmodial-specific tubulin isotypes detectable on either the ARG or immunoperoxidase-stained Western blots, suggesting that at the very least, plasmodial tubulin stoichiometry is unnecessary for closed mitosis.

5.3.3 Competitive interaction between intranuclear MTOCs and cytoplasmic MTOCs in the transitionary cell-types.

In the past, it has seemed likely that the amoebal MTOCs become the plasmodial MTOCs since both share a similar structure (Wright, 1982). The major benefit of being able to look at transitionary cell-types becomes obvious as active MTOCs can be observed in the cytoplasm during most committed cell mitoses and some binucleate cell mitoses. This implies that the intranuclear MTOC (IMTOC) must be novel structures and that the cytoplasmic MTOCs (CMTOC) are the amoebal MTOCs which are subsequently lost. Thus, in a single cell, the replication of one MTOC continues while the other is lost; this means that the cell must be able to discriminate between two apparently identical structures. The differential control may be achieved by different "forces" acting in the nucleus and cytoplasm.

As some mitotic committed cells are observed with two CMTOCs, it is probable that the amoebal MTOC is duplicated prior to committed cell mitosis; this is not surprising when one considers the extra length of the cell cycle during which commitment occurs (Bailey *et al.*, 1987). The amoebal MTOC is normally duplicated just prior to mitosis (Wright *et al.*, 1980). It is thus possible that, prior to commitment, the CMTOC is duplicated at the same time that the cell would have gone through normal amoebal mitosis; this may coincide with loss of flagellation ability (Bailey *et al.*, 1987). The reason for only one CMTOC being visible in uninucleate committed cells may be that the CMTOCs are very close together and do not separate until mitosis. Cells with one or no CMTOCs may have lost either one or two of these organising centres.

CHAPTER 5

The number of CMTOCs/cell in mitotic uninucleate committed cells corresponds closely to that in interphase binucleate cells and the number in mitotic binucleate cells corresponds closely to that in interphase quadrinucleate cells. It is thus probable that CMTOCs are lost only during or close to the time of mitosis. It is possible that the reason for CMTOC loss occurring at that time is the pressure exerted on the tubulin pool by the mitotic spindle. Uninucleate committed cells observed in mitosis which had CMTOCs always had fewer and shorter microtubules than binucleate cells. This suggests that the same tubulin was being used in the spindle and in the cytoskeleton and that there was competition between the IMTOC and the CMTOC; clearly the intranuclear MTOC was dominant. If the competitive interaction caused the CMTOC to not nucleate microtubules during mitosis it may then not have survived through to the following cycle. One hypothesis concerning the function of the MTOC is that it acts as an anchor for nucleating elements which can then act as templates for microtubule growth (Tucker, 1984). With this model, if there was demands made on the tubulin pool, the microtubules would shorten, but the microtubule ends could still act as templates for new microtubule growth when fresh tubulin was available. However, if the pressure on the tubulin pool was so great that the ends of the microtubules were depolymerised, no ends would be available as templates to initiate fresh microtubule growth and so the MTOC would disappear. Thus, competitive interaction between IMTOC and CMTOC could be the mechanism for loss of CMTOC.

5.3.4 Loss of CMTOCs and centrioles during the amoebal-plasmodial transition.

During the amoebal-plasmodial transition centrioles must be eliminated. In *Physarum* amoebae, the centrioles become the basal body of the flagella and are also found at the poles of the mitotic spindle. It has already been shown that ability to flagellate is lost some time before commitment and that the committed cell has gone beyond the stage at which it would have gone through an amoebal mitosis; thus, true centriole function must be lost by the time the cell is committed. Preliminary electron microscopy evidence suggested that there is in fact a physical degeneration of the centrioles during the amoebal-plasmodial transition. Phosphorylation/dephosphorylation is known to be an important mechanism for regulation of protein function (e.g. Cohen, 1982). The loss of MPM-2 staining may thus give an early visual marker of centriole degeneration and elimination.

The number of pairs of centrioles visible per cell appears to drop gradually through the first few mitotic cycles after commitment. There does not appear to be any relationship between centriole loss and the stage of the mitotic cycle as there is with CMTOC loss. Furthermore, it is unlikely that the centrioles are usually duplicated prior to committed cell mitosis as very few cells are observed with two pairs of centrioles. Thus, the CMTOC may continue to function, albeit with a lesser activity, in the absence of active centrioles. This evidence agrees with the observation of Wright *et al.*, (1985) that the centrioles are only associated with the MTOC during interphase and that they constitute distinct physiological entitities. In this respect, *Physarum* differs from other systems, such as embryonic myoblast fusion, where loss of MTOC

activity coincides with the loss of centrioles (Connolly *et al.*, 1985). The observation that centrioles are lost before the CMTOCs suggests another hypothesis for CMTOC loss. It is possible that in some way the presence of centrioles stabilises the CMTOC and improves its chances of surviving to the next cell cycle. This factor could operate in addition to the competitive interaction postulated in 5.3.3.

5.3.5 Change in the number of microtubules per CMTOC during the early stages of plasmodium development.

The results of the counts of microtubules/MTOC on amoebae suggests that the structures viewed by immunofluorescence are individual microtubules as the counts correspond well with the number of long microtubules, visualised by electron microscopy (Wright *et al.*, 1980). Wright *et al.* used electron microscopy to count the number of microtubules radiating from each microtubular array (mta) during interphase. mta 1 had 20 to 35 microtubules up to 9000nm in length, mta 2 had 39 very short (approximately 370nm) microtubules, mta 3 had 5 to 6 microtubules which did not go into the cytoplasm, mta 4 had 7 to 9 short (500-1000nm) microtubles and mta 5 had 4 microtubules (length 2000nm). It thus seems likely that the microtubules that will be visible with immunofluorescence microscopy are those radiating from mta 1 and mta 5 which would give a total of 24 - 39. The actual range of 12 to 36 with a mean of 21 is not too far from these figures; the lower counts could be explained by microtubules twisting round each other as observed by Wright *et al.*(1980). Wright (1982) also showed that mta 2-5 were associated with the posterior centriole and disappeared during mitosis. Thus in the mitotic committed cells only microtubules from mta 1 are likely to be

visible; it seems possible that the same would be true of subsequent cell-types.

If one considers the number of microtubules/MTOC in different cell-types, the order is amoebae > binucleate cell (1 MTOC) > binucleate cell (2 MTOC) > committed cell in mitosis. It has already been discussed that the probable reason for fewer microtubules in mitotic cells is likely to be the pressure exerted on the tubulin pool by the spindle. Clearly, the CMTOC in the mitotic uninucleate committed cells is still capable of nucleating more microtubules than it does during mitosis, since the number of microtubules/MTOC increases in the binucleate cells. It is possible that the reason for there being fewer microtubules in binucleate cells than in amoebae is that there is a reduction in the amount of tubulin per cell. An alternative explanation is that there could be a reduction in the amount of a critical microtubule associated protein (MAP).

It is unlikely that the difference in the number of microtubules/MTOC between the two categories of binucleate cells (those with one MTOC and two MTOCs) is due to the amount of tubulin or MAPs present in these cells since it would be expected that both cell-types would have similar quantities of tubulin and MAPs. It seems more likely that the limiting factor is the total length of microtubules that the cell is able to nucleate. Although there are more microtubules per MTOC in the cells with one MTOC (12.1 as opposed to 8.4), there are more microtubules per cell in those cells with two MTOCs (16.8 as opposed to 12.1). If one looks at Figures 5.5 it appears that in cells with 1 MTOC, the microtubules radiate from the MTOC to the cell periphery In Figures 5.6, it appears that in cells with two MTOCs, the microtubules radiate from

one MTOC to either the cell periphery or to the other MTOC. Thus in the cells with two MTOCs the microtubules are shorter; this means that if total length is the restricting factor, there can be more microtubules than in cells with one MTOC.

5.3.6 Competitive interaction between two CMTOCs in a single cell.

There appears from this work to be several categories of transitionary cell-types which can be distinguished on the basis of the number of CMTOCs. Two CMTOCs are observed both in uninucleate committed cells in mitosis (approximately 20%) and in a similar proportion of binucleate cells. There is however a marked difference between these two cell-types both in the length of the microtubules (which is discussed above) and in the way that microtubules are distributed between the two organising centres. In mitotic cells with two CMTOCs there usually appear to be different numbers of microtubules radiating from each organising centre. However, in binucleate cells with two CMTOCs there appears to be the same number of microtubules from each organising centre. It has already been postulated that the CMTOC is duplicated in all committed cells before mitosis and it may be that in mitotic cells there is one mature and one immature CMTOC which leads to the difference in nucleation of microtubules. However, if CMTOC duplication occurred at the time of normal amoebal division, there should be more than enough time for the new CMTOC to mature. A more attractive explanation for the difference in the number of microtubules/CMTOC is that the two CMTOCs compete for the available tubulin in the mitotic cells where the majority of the tubulin pool is incorporated into the spindle. This competition could also be the reason for CMTOC loss occurring at mitosis. The large number of

binucleate cells with one CMTOC could be due to a stabilising effect occurring on the remaining CMTOC when competitive interaction causes the loss of one.

This work thus suggests several different mechanisms which could account for the loss of the CMTOC and it is possible that more than one of these mechanisms could together account for the loss of the CMTOC:

1) Competition between CMTOC and IMTOC.

2) Destabilisation of CMTOC resulting from loss of centrioles.

3) Competition between more than one CMTOC in the same cell.

5.3.7 Assembly of tubulin into microtubules in the absence of a MTOC.

It is possibly surprising that there are many binucleate cells which whilst containing microtubules do not appear to have a distinct CMTOC (Figure 5.7). The evidence presented in this thesis suggests that binucleate cells with no MTOC arise from uninucleate committed cells in mitosis with no CMTOC. Mitotic uninucleate committed cells which have no CMTOC appear to have no assembled tubulin in the cytoplasm. This implies that there is something in binucleate cells which promotes microtubule development that is not present in plasmodia, which have no cytoplasmic microtubules during mitosis or interphase. It is possible that this factor is merely that there is a higher tubulin concentration in binucleate cells than there is in plasmodia. It is also possible that there may be a factor in amoebae, which is still present in binucleate cells, that promotes microtubule formation. This factor could be amoebal-specific tubulin isotypes (which are not separable on 2-dimensional gels). However, this is unlikely since plasmodia assemble microtubules during mitosis. It is more likely that there are one or

more proteins involved in microtubule assembly, that are present in the cytoplasm of amoebae (and early transitionary cells) but are not present in the cytoplasm of plasmodia. Alternatively, nucleation of microtubules could require templates which are usually associated with the MTOC. In these binucleate cells which have lost the CMTOC, nucleation sites, which act as templates, could be present in the cytoplasm unassociated with a MTOC.

5.3.8 Possible method by which cells acquire the plasmodium tubulin isotype pattern during the amoebal-plasmodial transition.

The results from the immunoperoxidase stained blots of committed cell tubulin can be interpreted in different ways. In individual cells there could be a gradual change from amoebal expression to plasmodial expression which would lead to the gradual change in the pattern of expression in a population that was observed. Alternatively the same result could be obtained by a switch directly from amoebal expression to plasmodial expression at a certain stage of development; the reason why a complete switch was not observed could be due to the abundance of cells that had not progressed as far in development and by the proportion of amoebae (that do not grow or develop in SDM-PS) that are always present after an enrichment. The first explanation can of course be made to fit any results simply by defining the rate of change to plasmodial expression accordingly. The second explanation however can, to some extent, be tested.

There are several repeatable pieces of evidence to arise from the Western blot analysis (5.2.1):-

a) Samples of uninucleate committed cells never gave rise to either α2 or β2 spots. The blots appeared to be identical to amoebal blots except that the flagellate α3 spot was not present; there were no flagellates present in the enriched samples.

b) Samples that had grown between 20 and 24 hours (which consisted of a large proportion of binucleate cells and a small number of larger cells) contained only traces of β2.

c) Samples that had grown for 4 to 5 days had a tubulin pattern similar to that found in plasmodia.

The *in vitro* translation of the binucleate cell RNA (22 hour sample containing 49.7% binucleate cells) gave rise to 2-dimensional gels which had a tubulin pattern similar to amoebae (although traces of β2 could be observed). This implies that binucleate cells have not switched to the plasmodial pattern of expression. Thus any model concerned with a switch to plasmodial expression of tubulin must assume that this switch occurs after binucleate cell formation; this could be either late during the binucleate mitotic cycle or during the quadrinucleate cycle.

Any model must also take into account the evidence that amoebae are known to contain approximately three times the amount of tubulin per nucleus that plasmodia contain (Roobol *et al.*, 1984). It is also known that tubulin used in one cell cycle in plasmodia can also be used in the next cycle (E.C.A. Paul, reported by Laffler and Tyson, 1986); while this does not imply that tubulin is completely stable, in this model complete stability will be assumed.

By looking at the intensities of the various tubulin spots visible in silver-stained 2d gels, the proportions of the various tubulin isotypes can be estimated:-

	α1	β1	α2	β2
Amoebae	50	50		
Plasmodia	40	5	15	40

However, Birkett *et al.* (1985) found that different monoclonal antibodies had different specificities to the various α or β tubulin isotypes. DM1B detects the ratio of β1 to β2 in plasmodia as approximately 50:50 and KMX-1, which was raised to amoebal β1 tubulin detects the ratio of β1 to β2 in plasmodia as approximately 80:20. It is thus necessary to use these values to estimate the proportions of the β tubulin present in the various cell samples.

To compare the amounts of each isotype between the different cell-types it is necessary to multipy the amoebal amount by three (as amoebae have 3X the total tubulin of plasmodia); it is also necessary to relate the nuclearity of multinucleate cells with the rate of tubulin production. Using these modified figures it is possible to produce a model of how the relative quantities of the different isotypes should change assuming a complete switch from amoebal to plasmodial expression and assuming that tubulin has a long half life.

cell-cycle	α1	%α1	β1	%β1	α2	%α2	β2	%β2
1	150	50.0	150	50.0				
2	150+40	47.5	150+5	38.8	15	3.8	40	10.0
3	150+120	45.0	150+15	27.5	45	7.5	120	20.0
4	150+280	43.0	150+35	18.5	105	10.5	280	28.0
5	150+600	41.7	150+75	12.5	225	12.5	600	33.3
6	150+1240	40.9	150+155	9.0	465	13.7	1240	36.5
7	150+2520	40.5	150+315	7.0	945	14.3	2520	38.2
		↓		↓		↓		↓
		↓		↓		↓		↓
		↓		↓		↓		↓
		↓		↓		↓		↓
		40		5		15		40

However, for DM1B and KMX-1 it is necessary to substitute the following values.

	DM1B				KMX-1			
cell-cycle	β1	%β1	β2	%β2	β1	%β1	β2	%β2
1	300	100.0			300	100		
2	300+50	80.0	50	20.0	300+80	95.0	20	5.0
3	300+150	66.7	150	33.3	300+240	90.0	60	10.0
4	300+350	58.8	350	41.2	300+560	86.0	140	14.0
5	300+750	54.5	750	45.5	300+1200	83.3	300	16.7
6	300+1550	52.3	1550	47.7	300+2480	81.8	620	18.2
7	300+3150	51.2	3150	48.8	300+5040	80.9	1260	19.1
		↓		↓		↓		↓
		↓		↓		↓		↓
		↓		↓		↓		↓
		↓		↓		↓		↓
		50		50		80		20

To summarize, the assumptions used for this model are as follows:-

a) Amoebae have three times the tubulin per nucleus of plasmodia.

b) The ratio of α to β tubulin is 1:1

c) DM1B and KMX-1 detect β1 and β2 tubulin with the relative efficiency stated.

d) The switch from amoebal to plasmodial protein synthesis occurs as an all or nothing switch at a defined time. It is assumed that this must be either late in the binucleate cycle or early in the quadrinucleate cycle. Until this switch occurs, production of tubulin continues at the amoebal rate.

e) The amount of protein in a cell is directly proportional to its size. Cell size is proportional to the number of nuclei. i.e. Binucleates have twice the protein of uninucleates etc.

f) The intensity of the β tubulin spots can be estimated by eye.

g) Tubulin is completely stable.

To test this model, it is necessary to combine the postulated amounts of tubulin in the various cell-types with the proportions of cells

present in the various experiments. Large multinucleate cells (8 to approximately 100 nuclei) are classed as cells with 32 nuclei and microplasmodia (approximately 100 or more nuclei) are classed as cells with 128 nuclei. In the blots in which KMX-1 was used the proportions of the various cell-types can be seen in Table 5.1.b.i. In the 4 hour sample, no cells with more than two nuclei were observed, thus, according to the model, one would not expect to see any α2 or β2; this is the case. In the 19 hour sample however, there is 0.7% of quadrinucleate cells. One can calculate the proportion of tubulin isotypes expected in this sample by multiplying the proportion of each cell type (%ct) with the postulated amounts of the tubulin isotypes present in these cell-types (see above). The total amount of tubulin can then be estimated for the cell sample (in arbitrary units) by totalling the %ct x tubulin isotype and the proportion of each isotype in the population can be calculated.

	% cell-type (ct)	β1	β1 x %ct	β2	β2 x %ct
uni	55.5	150	83.25		
bi	43.8	300	131.40		
quad	0.7	380	2.66	20	0.14
% isotype			99.93		6.4×10^{-2}

Likewise, the 48 hour sample can be calculated in the same way.

	% cell-type (ct)	β1	β1 x %ct	β2	β2 x %ct
uni	58.4	150	87.6		
bi	11.7	300	35.1		
quad	26.1	380	99.2	20	5.22
oct	3.4	540	18.4	60	2.04
8-100 nuc	0.3	860	2.6	140	0.42
% isotype			96.9		3.1

The same procedure can also be performed on the other set of blots from the samples scored in Table 5.1.b.ii. Once again the 2 hour sample contains only uninucleate and binucleate cells. Thus, no β2 spot would be expected (these blots were probed only with DM1B).

22 hour sample.

	% cell-type (ct)	β1	β1 x %ct	β2	β2 x %ct
uni	39.1	150	58.65		
bi	56.9	300	170.70		
quad	3.4	350	11.90	50	1.70
oct	0.6	450	2.70	150	0.90
% isotype			98.95		1.05

48 hour sample

	% cell-type (ct)	β1	β1 x %ct	β2	β2 x %ct
uni	66.5	150	99.75		
bi	3.8	300	11.40		
quad	23.1	350	80.85	50	11.55
oct	4.7	450	21.15	150	7.05
8-100 nuc	1.9	1050	19.95	750	14.25
% isotype			87.65		12.35

70 hour sample

	% cell-type (ct)	β1	β1 x %ct	β2	β2 x %ct
uni	75.9	150	113.85		
bi	0				
quad	3.8	350	13.30	50	1.90
oct	5.1	450	22.95	150	7.65
8-100 nuc	5.1	1050	53.55	750	38.25
100+ nuc	10.1	3450	348.45	3150	318.15
% isotype			60.14		39.86

The estimated intensities of the spots are as follows:-

	Hours	β1	β2
exp. 1	22	99%	1%
	48	97%	3%
exp. 2	22	98%	2%
	48	90%	10%
	70	80%	20%

The results calculated from the model are close to the estimated intensities of the spots. It must be accepted the method of assessing the intensity of the spots by eye is prone to large errors and errors may also occur in estimating the number of cells with different numbers of

nuclei in the population. Changing certain aspects of the model would change the expected results:

i) Many of the multinucleate cells present are likely to be fusion products originating from cells that are less advanced in the amoebal-plasmodial transition. At present, the model assumes that the nuclearity of a cell directly reflects its maturity. Unfortunately, due to fusion, many of the larger cells, particularly in the later samples, will be less mature than assumed. This factor is virtually impossible to work into the model; it would have the effect of making the estimated intensity of the β2 spots in the later samples less than stated. This would thus help the model to fit the observed results.

ii) It is probable that the tubulin is not completely stable, as assumed in the model. This would mean that a factor would have to be introduced to the model to take into account tubulin degradation and result in a greater proportion of plasmodial-specific tubulins being present.

iii) It is possible that as the amoebae encyst, tbey make a smaller contribution to the tubulin pool of the population than would actively growing amoebae. It may thus be necessary to ignore the contribution of the amoebae to the total tubulin pool.

iv) If the cells start to produce β2 tubulin late in the binucleate cell cycle, it would be necessary to assign plasmodial stoichiometry to a proportion of the binucleate cells.

v) If the cells start to produce β2 tubulin late in the quadrinucleate cell cycle, it would be necessary to assign plasmodial stoichiometry only to a proportion of the quadrinucleate cells.

The model of switching from amoebal to plasmodial expression at the quadrinucleate stage nonetheless does to a large extent fit the results,

particularly when the problem with assessing the developmental stage of multinucleate cells in later samples (i) is taken into account.

CHAPTER 6

General Discussion.

6.1 Cellular events during the amoebal-plasmodial transition of CL.

The abbrevations in brackets after the headings refer to Figure 6.1.

6.1.1 Commitment (com).

Figure 6.1 summarizes current evidence regarding the sequence of events during the amoebal-plasmodial transition. It is clear that changes associated with development begin before commitment and that many changes occur after commitment. Thus, a committed cell is not a uninucleate plasmodium but is a transitionary cell-type. Further evidence that the uninucleate committed cell is an intermediate cell-type was obtained by Sweeney *et al.* (1987), using the enrichment method described in this thesis. They investigated the expression of stage-specific cDNA clones in samples of uninucleate, binucleate and quadrinucleate committed cells and found that some plasmodial-specific mRNAs were present in uninucleate committed cells at a similar level to that found in plasmodia, while other plasmodial-specific mRNAs were only detected at relatively high levels in microplasmodia.

The timings of events relative to commitment from this thesis suggest that the cell cycle leading to binucleate cell formation is much longer than an amoebal cell cycle. This was also suggested by the results of Collett *et al.* (1983) and Anderson *et al.* (1976) who both found that extra growth must occur prior to binucleate cell formation. The extra length of the cell cycle has recently been confirmed by Bailey *et al.* (1987) from direct observation of cell lineages leading to commitment (see Chapter 1).

Figure 6.1. Sequence of events during the amoebal-plasmodial transition.

tsp = End of temperature sensitive period (Chainey, 1981)

lfa = Loss of flagellation ability

com = Commitment

liq = Acquisition of ability to grow in liquid SDM-PS

cys = Loss of ability to encyst

rna = Transitory increase in RNA synthesis.

cent = Loss of centrioles begins

mtoc = First round of MTOC loss

fus = Acquisition of plasmodial fusion characteristics (also Anderson *et al.*, 1976: Bailey *et al.*, 1987)

tub = Approximate time that plasmodial-specific tubulins are switched on.

quad = Quadrinucleate cell.

plas = Macroplasmodium.

X = Unit of area. A committed cell is approximately 3 times the size of an amoeba (Collett *et al.*, 1983). Just before a binucleate cell is formed, the cell is approximately four times the size of an amoeba (Anderson *et al.*, 1976)

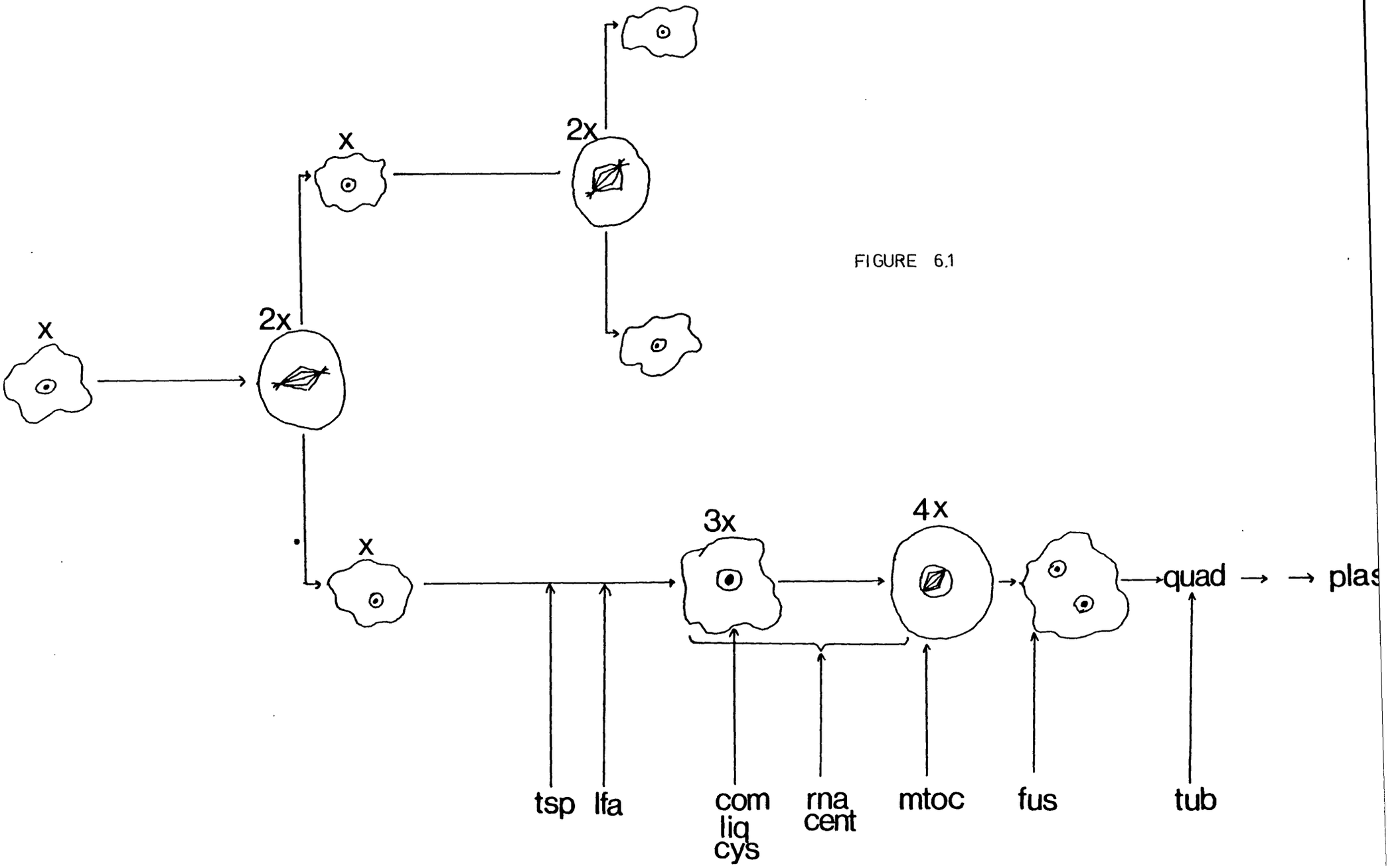


FIGURE 6.1

6.1.2 Loss of flagellation ability (lfa).

The first detectable event in the amoebal-plasmodial transition is the loss of flagellation ability. This means that there must be cells which are not committed but are unable to flagellate (see 3.3.1). Precommitted amoebae unable to flagellate (PAUF) were the major reason why the enrichment method was not 100% effective at separating committed cells from amoebae. However, PAUF appear to be very interesting cells in their own right. The loss of flagellation ability in PAUF could be due to many different factors as it is likely that there are many proteins involved in flagellum morphogenesis in *Physarum*. Loss of flagellation ability in PAUF could thus be due to a switch occurring at any stage of the pathway leading to flagellum formation. It seems likely that there is no α3 tubulin in PAUF, since there is little α3 observable on Western blots of populations enriched for unflagellated cells. This means that the switch in the pathway leading to flagellum morphogenesis must be before the point at which the post-translational modification of α1 to α3 occurs. An attractive explanation for loss of flagellation in PAUF is that the centrioles have become inactivated in these cells. There is circumstantial evidence to support this hypothesis since loss of flagellation ability occurs at approximately the same time as the cell would be expected to undergo amoebal mitosis (if it did not develop). The role of the centriole in mitosis is controversial, however it is clear that in *Physarum* the centriole is associated with the pole of the mitotic spindle in amoebae. It is possible that loss of centriole activity observed in populations stained with MPM-2, begins when flagellation ability is lost. However, since there is a relatively low proportion of uninucleate cells without stained centrioles it is more

likely that loss of centriole occurs later in the cell cycle, presumably after commitment.

Before commitment, changes associated with the amoebal-plasmodial transition are apparently reversible. However, it is possible that cells that have started the transition can not revert to amoebal growth but die when replated. There is however evidence from work in this thesis that precommitted cells can return to normal amoebal growth. When proportions of cells before and after enrichment were measured (Table 3.9), the proportions balanced well. In the population used which contained 3.3% committed cells it would be expected that there was a large proportion of PAUF (see Figures 3.3 and 3.5). If the PAUF had died the two sets of figures would not have balanced. Additional evidence was obtained when enriched populations were plated on LIAB in filming slides (see 4.2.1); since replated cells either became multinucleate or formed amoebal colonies and very few cells died, it seems likely that the PAUF, which were presumably in the enriched population, reverted to amoebal growth.

6.1.3 Acquisition of ability to grow in SDM-PS (liq).

Since development before commitment is apparently reversible, it is not possible to say whether ability to grow in SDM-PS begins at commitment or before commitment. In Chapter 3 it is speculated that suppression of flagellation ability or loss of flagellation ability may be the method by which mutant amoebal strains acquire the ability to grow in axenic liquid medium. It is thus possible that PAUF are also capable of growth in axenic liquid medium. However, there was evidence during experiments following the growth of enriched populations in SDM-PS that there was no increase in cell number during culture. This implies that

there was no sub-population of amoebae which divided in SDM-PS and it is thus unlikely that PAUF go through even one division in liquid axenic medium.

6.1.4 Increase in RNA synthesis (rna).

An increase in nuclear size in uninucleate committed cells was first reported by Anderson *et al.* (1976). They pointed out that the large nuclei could be due to an increase in DNA content. However, they favoured the explanation that the large nuclei were the result of metabolic changes associated with development. The results of the flow cytometry (4.2.6) demonstrates that there is not an increase in DNA content associated with development. There is however a large increase in nucleic acid content of uninucleate committed cells (4.2.5); since DNA content is constant, this increase in nucleic acid must be due to increased RNA synthesis, confirming the hypothesis of Anderson *et al.* This increase in RNA synthesis proved to be a useful phenomenon, since it allowed investigations to be made of committed cell RNA using relatively small numbers of cells.

6.1.5 Loss of ability to encyst (cys).

When either amoebae or plasmodia are placed in poor growth conditions they change into dormant forms, cysts or spherules. When committed cells were plated on solid SDM, conditions in which they could not grow, they failed to encyst and subsequently died. This suggests that committed cells have lost the ability to encyst and are not yet capable of forming spherules. This may be because the committed cells are in a transitionary state and, since so many changes are occurring, it is impossible for them to go into a different developmental pathway. The increased RNA synthesis (discussed above) implies that committed cells are highly metabolically active and it may be impossible for them to go straight from this highly active state to a dormant state. There are no indications from this work when spherulation is acquired, but it would be a reasonable hypothesis that this coincides with the ability to grow on solid SDM; this does not occur until macroplasmodia are present in cultures.

6.1.6 Acquisition of plasmodial fusion characteristics.

The stage at which committed cells acquire the ability to fuse with other cells of the same genotype has been suggested by the results of several investigations. In Chapter 4, when committed cells were observed

in slide cultures they were often close to each other (see Figure 4.1.a). Despite this fact, fusions were only observed involving multinucleate cells but not uninucleate cells. Anderson *et al.* (1976), working on CL, observed that binucleate cells always arose by the nuclear division of a uninucleate cell (without cytokinesis) and never from the fusion of two uninucleate cells. J. Bailey (personal communication) observed a fusion between a large uninucleate cell and a multinucleate cell; however, this was an isolated and thus a rare occurence. These results provide evidence that plasmodial fusion characteristics are acquired either in binucleate cells or just before binucleate cell formation.

6.1.7 Changes in cell movement.

The changes in microtubule organisation (discussed in detail in 5.3), may be related to other events involved in the amoebal-plasmodial transition. The microtubule cytoskeleton of amoebae may be involved in controlling the shape of the cell and may thus be implicated in cell movement. When amoebae enter mitosis, they round up and stop moving (Anderson *et al.*, 1976); it is probably significant that this occurs at the same time that the microtubule cytoskeleton is lost and tubulin is incorporated into the mitotic spindle (Havercroft and Gull, 1983). Cell movement of plasmodia has been studied extensively (e.g. Kamiya, 1959: Allera *et al.*, 1971: Isenberg and Wohlfarth-Bottermann, 1976); it is due to shuttle streaming which is caused by an interaction of actin and myosin. Anderson *et al.*, (1976) found that uninucleate committed cells, observed on filming slides, moved in a similar way to amoebae. Rhythmic contractions, characteristic of plasmodia, did not start until cells had more than 4 nuclei. The microtubule cytoskeleton of uninucleate

committed cells is similar to that of amoebae. However, in subsequent cell-types, the number of microtubules present in the cells diminishes. It is possible that plasmodial movement cannot begin until the microtubule cytoskeleton has been lost, or at least reached a critical level. Alternatively it is possible that it is necessary for a microtubule cytoskeleton to persist in small multinucleate cells until plasmodial-specific proteins are at a sufficient level to initiate plasmodial movement.

6.2. Relationship between mating and apogamy.

It must be remembered that the majority of the work in this thesis was done on the mutant apogamic strain, CL. The relationship between apogamy and mating is not clear, however preliminary experiments in this thesis suggest that the two sorts of development may be similar. A promising line of investigation on *Physarum* may be to investigate more fully the amoebal-plasmodial transition due to mating since, mating may be easier to control than apogamic development.

6.2.1 Commitment.

Shipley and Holt (1982) showed that commitment in mating cells occurred at approximately the time of cell fusion which is controlled by matB. However, development in mating cultures is not controlled by matB since if cells have different matB alleles but the same matA alleles they will fuse but not develop (see 1.2). It is unlikely that commitment in apogamic development is controlled by matB; it is probable that commitment in apogamic strains is controlled by matA, the locus at which gad-h maps. Thus, commitment in relation to mating and commitment in

relation to apogamy appear to be different events in the amoebal-plasmodial transition. Commitment in apogamic development presumably has an equivalent event in mating; it is possible that this event is related to nuclear fusion.

6.2.2 Loss of flagellation ability.

It was concluded that flagellation ability was lost in mated cultures some time after amoebal fusion (3.2.9). If the kinetics of plasmodium formation was compared with loss of flagellation ability in mated cultures it could help to determine the time at which ability to flagellate is lost in mated cultures. However, as discussed above, commitment in mated cultures occurs close to the time of cell fusion. Since loss of flagellation in mated cultures apparently occurs after cell fusion, there should be no, or very few, non-flagellated cells in mating populations that would replate as amoebae. It is possible that the equivalent of the PAUF, observed in CL, in mated populations would presumably be binucleate fusion cells which may continue to develop when replated. It thus seems likely that the enrichment method would be more effective on mating mixtures; the limited success of enrichment on these populations in this thesis may merely have been due to the experiments being of a preliminary nature.

6.2.3 Growth properties of mated cells and acquisition of ability to grow in liquid SDM-PS.

The growth properties of mated cells seem to be similar to those of apogamic committed cells. Clearly, mated cells must be capable of growth on lawns of bacteria as the plating assay for these cells is based on

this fact. The results in 4.2.2 indicated that, in common with apogamic committed cells, diploid zygotes are incapable of growth on solid axenic medium. As with apogamic committed cells, it is only when plasmodia are clearly visible with the naked eye in mating cultures that any cells are directly capable of growth on solid SDM. In common with CL, the late acquisition of ability to grow on solid SDM in mating mixtures could be due to the action of a gene switched on very late during the amoebal-plasmodial transition or possibly cells needing to be a certain size before they can feed on this medium.

It appears that mated cells must also be capable of growth in liquid axenic medium. However, it is not possible to know whether the same proportion of cells replate as plasmodia on bacterial lawns as grow in liquid SDM-PS. It is also impossible to estimate the growth rate of mated cells from the results in 4.2.4 due to the fact that plasmodia developed rapidly in these cultures, presumably due to fusion occurring. The reason for fusion occurring earlier in the enriched mated cultures is unclear. It is possible that there were some large cells in the culture, that were not observed during assays, which were the focus for fusion; however, this never occurred during enrichments and subsequent culture of CL. Another possibility is that plasmodial fusion is switched on earlier in mated populations than in apogamic development. There is however a genetic explanation of this result which does not contradict the comparison between apogamic and heterothallic development. In cultures of CL, all the cells are isogenic and must have the same matB gene. Thus, fusions between amoebae (and if the matB gene is still active) between committed cells, will only occur at a very low frequency in CL. In the enriched mated population, LU860 and LU862 had different matB

genes. Diploid amoebae that are heterozygous for matB mate poorly with cells of either matB type (Youngman and Anderson, 1985) It is possible, however, that diploid zygotes express both matB types and may thus fuse with other diploid zygotes as a result of matB gene action before the plasmodial fusion genes become active (Figure 6.2).

6.3 Prospects for further analysis of development in *Physarum*.

In the General Introduction (section 1.6), it was pointed out that, while *Physarum* was promising as a model developmental system, it lacked some of the properties that would make it ideal for studying differentiation; these were synchronous development and a DNA transformation system. In the course of the work described in this thesis, a method was devised which allowed cells to be cultured through the amoebal-plasmodial transition with a degree of synchrony. This level of synchrony was sufficient for some biochemical analyses however, the level was insufficient for accurate timings of developmental events before and during the transition. It is probable that the only way to achieve more synchronous development is to synchronise the amoebal cell cycle and to use inducer to initiate development. It is possible that amoebal growth could be synchronised using excystment of AXE strains (3.2.5) and further work on this line of investigation could be fruitful. However, at present AXE strains are not available which go through the amoebal-plasmodial transition at high frequency in liquid. Nader *et al.* (1984) analysed the inducer but were not successful at purifying it; possibly with a little more work, inducer will become available and synchronous development will be achieved.

CHAPTER 6

Investigators are now working on a DNA transformation system on *Physarum* but transformation at an acceptable frequency is currently unavailable. However, preliminary results (Haugli and Johansen, 1986) have been encouraging and it is probably just a matter of time before a DNA transformation system becomes available.

APPENDIX

Enrichment Method for Committed Cells.

One of the most useful things related to the work in this thesis is the enrichment method for committed cells which was developed in Chapter 3. In the main text of the thesis however, the full method is not actually described. The method below is a simple step by step instruction on how to achieve enrichment for committed cells of *Physarum* which it is hoped will be of help to other workers in the field.

1) Prepare differentiation plates.

Wash off a plate of CL cysts from the fridge and adjust the cell density to 10^6 cells/ml. Dilute the CL suspension 1:1 with SBS. To each of 10 or more dry DSDM plates (one week old) pipette on 0.2ml of the mixed amoebal/bacterial suspension and spread evenly to cover a central square area of the plate which nearly reaches the sides. Allow to dry in and incubate cultures at 26°C for 60+ hours (Plates set up at approximately 3pm on day 1 should be ready at 9am on day 4. i.e. Friday to Monday etc.) Plates should be observed at 25X with a good stereo microscope at intervals and growth can be followed without contaminating the plates. A "trained eye" should be able to assess the progression of the cultures towards plasmodial formation. During amoebal growth, the cultures appear more spotty (separate cells can be easily distinguished). When the cultures begin to develop, areas of the plate assume a smoother appearance with cells appearing to merge into each other (it is more difficult to distinguish separate cells). It is possible, if cultures are developing too quickly, to delay development by respreading the plates (usually after 40

hours). This is done by pipetting 0.3ml - 0.5ml of water onto the plate, disturbing the culture with a glass spreader, spreading evenly and then allowing the suspension to dry back in.

2) Wash off differentiation plates.

Pipette 1.5ml of sterile distilled water onto each plate and wash off. Transfer pooled wash-off to sterile container, vortex for about 5 seconds and put on a shaker at 26°C. It is preferable to use a reciprocating shaker at 200 stroke/minute although a fast rotary shaker (150 revs/min) will also suffice. However, on a rotary shaker it is more likely that multinucleate cells (binucleate or more) will fuse with one and other. Allow to shake for 2 to 3 hours.

3) Assemble columns.

Take several drawn out pipettes (1 pipette for two plates washed off) and put them into retort stand. Take a lump of glass beads and drop it into the pipette to block the neck. The lump should be of a size that does not enter the thin drawn out part of the pipette but blocks the neck at the top. Lumps of beads result from sterilising the glass beads wet and drying them in an oven. Pour the 200μ glass beads into the pipette until the pipette is approximately half full. The beads should not enter the neck as the lump should prevent them from pouring through. Pipette 1ml of water onto the column. The water should drip through with approx. 1 drop every 2 or 3 seconds; a little slower or faster should not affect the method. At this stage it may well be necessary to discard some of the columns where beads have passed into the neck and blocked it. Pipette 1ml of SDM onto the column.

4) Enrichment.

Once the SDM has passed through the column, pipette on the cell suspension (which has been on the shaker for 2 to 3 hours) so that the suspension reaches the top of the tube. Allow the suspension to pass through the column and then pipette on distilled water to the top of the column. The suspension emerging from the column can be sampled by catching a drop on a haemacytometer and observing by phase contrast microscopy; the suspension should contain a majority of flagellates. It may be useful to compare the proportion of flagellates in the suspension emerging through the column with the proportion in the original suspension to satisfy oneself as to whether the enrichment is working! Continue to add 1ml aliquots of water to the column when the liquid has passed through until only appx. 10^4 cells/ml are in the sampled suspension. At this point, pipette 1ml of SDM onto the column and allow it to run through. It appears that committed cells are very sensitive to the water that is pipetted onto the column, so one should not prolong the method more than is necessary. It has been noticed that occasionally the water causes the cells to explode; this is probably due to impurities in the distilled water (such as detergent used when cleaning the bottles). It is possible that a weak buffer could be used instead of distilled water, however, flagellation has proved to be more efficient in distilled water (see 3.2.1) and solutions with high osmotic strength may adversely affect cells that are already flagellated (see 3.2.16).

5) Recovery of enriched population.

Remove the columns from the retort stands and using a glass cutter, cut the end off the pipette above the "bung". Hold the pipette above a test-tube and pipette on approximately 1ml of water; the beads should wash into the test-tube. Put the contents of two columns into each test-tube. Shake firmly but not vigourously for approximately 10 seconds and decant the liquid into another test-tube taking care not to transfer any beads across; it may be necessary to pour into another fresh tube to avoid transferring beads. Spin down the enriched suspension and resuspend in SDM to a cell density of $5x10^5$ cells/ml.

6) Assaying for committed cells.

There are two important aspects to estimate in the enriched population: i) The proportion of committed cells ii) The proportion of cells with different numbers of nuclei. To estimate i), dilute a sample of the enriched population to 10^3 cells/ml and plate several 0.1ml aliquots of this suspension with 0.1ml of SBS on either DSDM agar or LIA/DSDM agar. Spread over the plate and incubate at 26°C. Observe after 3 days and again after 5 days with a good stereo microscope (25X mag.) and count the number of amoebal colonies and instant plasmodia. To estimate ii)*, put a drop of enriched suspension on a block of agar, allow it to dry in, put on a coverslip and observe at 400X by phase contrast microscopy. Count at least 200 cells. Combining the results of these two experiments, it is possible to estimate the proportion of uninucleate committed cells in the enriched suspension.

* This should be done both after the enrichment and (if the enriched suspension is cultured) before the population is used in an experiment.

7) Growing committed cells in liquid SDM.

To grow the committed cells, take the suspension which is now in SDM and add penicillin to 0.25 units/ml and streptomycin to 0.25 mg/ml (From stock solution). Put on a reciprocating shaker at 200 strokes/min or if not available a rotary shaker at 150 revs/min. In the case of a rotary shaker it is preferable to vortex the solution at 2 hour intervals as this will reduce the probability of cells fusing.

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