DNA SYNTHESIS IN PHYSARUM POLYCEPHALUM

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DNA SYNTHESIS IN PHYSARUM POLYCEPHALUM.

Martin Cunningham. Ph.D. Thesis, 1979. Abstract.

This thesis describes investigations of DNA synthesis in the myxomycete, Physarum polycephalum, using an isotope dilution technique capable of measuring macromolecular synthesis directly, in contrast to methods which follow the incorporation of a radioactive precursor. Using petri dish and large surface cultures, the pattern of either nuclear or total DNA accumulation during the mitotic cycle was determined in three media with cycle lengths varying between 9 and 12 h. In common with previously published results, no G1 phase was detected. Statistical analysis suggested that, within the range examined, changes in cycle length were confined mainly, if not solely, to G2, S phase remaining constant at approximately 120 min. During G2 an amount of synthesis was detected which ranged from 10 to 27% of nuclear DNA, more than could be due to nucleolar DNA. In most cases, DNA content showed less than a doubling between divisions, and possible causes of this and of G2-synthesis are discussed.

Examining DNA synthesis in the presence of cycloheximide which at 20 ug per ml could, within 15 min, inhibit by 95% the incorporation of carbon-14 labelled lysine, it was found that (i) inhibition of replication was detectable after 20 min exposure but, at shorter times, the method employed was insufficiently sensitive to detect inhibition consistently; (ii) residual synthesis of approximately 15 - 20% of total or nuclear DNA can occur during 3 h exposure to cycloheximide (20 ug per ml) and (iii) approximately 50% inhibition of protein synthesis by 0.25 ug per ml cycloheximide produced no detectable effect on the level of DNA synthesis.

Other experiments described are: (i) preliminary attempts to construct an in vitro replicating system from plasmodial homogenates, assaying synthesis by isotope dilution; (ii) measurements in small plate cultures of macromolecular synthesis between inoculation and M1, and DNA synthesis within the inoculum region; (iii) determinations and statistical analyses of the growth rate of microplasmodia and surface plasmodia under various conditions.

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Contents.

List of Figures.

List of Tables.

Chapter 1. General Introduction.

This thesis describes investigations of certain aspects of DNA synthesis using the isotope dilution method, a technique that can measure macromolecular synthesis directly, in contrast to either pulse- or continuous-labelling in which the parameter measured is the incorporation of a radioactive precursor into the macromolecule under investigation. The aspects of DNA synthesis investigated were (i) the pattern by which DNA is replicated during the cell cycle, (ii) certain characteristics of the decay in replication rate upon the inhibition of protein synthesis by cycloheximide and (iii) the amount of DNA synthesis occurring in cell-free homogenates. The organism used for these studies was the myxomycete, Physarum polycephalum, and a number of experiments investigating various characteristics of its growth under laboratory conditions are also described.

1.1. Physarum polycephalum.

The myxomycetes (Greek: myxa = slime; myketes = fungus) are a group of eucaryotic organisms not readily classifiable as plant or animal, having affinities with both protozoa and fungi. They are also referred to as true or acellular slime moulds because of the partially phosphorylated and sulphated galactan slime (Zaar, 1978) they secrete, mainly under adverse conditions. Their natural habitat is the soil, and cool, shady areas in which they feed on rotting vegetation such as leaves or wood. All myxomycetes have a similar life cycle, and that of the most commonly-used species, Physarum polycephalum, was first described by Howard (1931) and has subsequently been outlined many times (for example, Sauer, 1973; Ashworth & Dee, 1975; Dee, 1975).

A structure unique to this group is the plasmodium, a syncytium in which millions of nuclei (approximately 10^8 in a petri

dish culture) co-exist in a common cytoplasm, and which is therefore "acellular". As it lacks a cell wall, it has no permanent stable form although it is usually roughly circular. In Physarum polycephalum this single cell can be several centimetres in diameter but only approximately 1 mm thick, and is of a vivid yellow colour due to the presence of a variety of pigments of quite different chemical composition, at least one of which appears to be capable of behaving as a light receptor (Warmington & Weaver, 1976). The plasmodium is one vegetative stage of this organism but, under starvation conditions in the presence of light, spores are formed from which, on germination, the other vegetative stage emerges, small, uninucleate, haploid myxamoebae which are very similar in appearance and behaviour to protozoan amoebae. Under appropriate conditions, two amoebae of different mating type (Dee, 1973) undergo cell and nuclear fusion to form a diploid zygote, which then grows by repeated nuclear divisions without cell cleavage to form a large plasmodium. There is therefore an alternation of a diploid plasmodial stage with a haploid amoebal stage, but apogamic strains have also been described (Anderson et al., 1976) in which plasmodia arise from a single amoebal cell by repeated nuclear divisions without cell or nuclear fusion, resulting in a haploid plasmodium (Mohberg & Rusch, 1971; Cooke & Dee, 1974). This system should facilitate the production of plasmodial temperature sensitive cell cycle mutants by mutagenesis of uninucleate, apogamic amoebae but, as yet, no such mutants have been isolated.

All the experiments to be described below have used the plasmodial form grown mainly on solid medium and, unless otherwise stated, all subsequent references to Physarum specifically concern this stage. In some experiments, and for the purpose of routine subculturing, the plasmodial stage was used in the form of microplasmodia which are produced when a plasmodium is transferred

to shaken liquid culture in which it breaks up to form small plasmodia approximately 2 mm in diameter.

When starved in the absence of light, sporulation does not occur, but plasmodia encyst to form a hard, resistant sclerotium containing many enwalled, oligonucleate subunits called spherules (Zaar & Kleinig, 1975) which can remain viable for several years (Daniel & Baldwin, 1964). It is the encysted form of the microplasmodia (microsclerotia) that is used for the long-term preservation of strains. When placed in contact with growth medium, genetically unchanged microplasmodia hatch. It may be pointed out that, under adverse conditions, myxamoebae can also encyst.

The single most useful property of Physarum is the very high degree of natural mitotic synchrony that exists between the nuclei of a single plasmodium. Values for the mitotic index have been found to be consistently in excess of 95% (Chin & Bernstein, 1968). This synchrony is probably at least partially due to efficient cytoplasmic mixing resulting from the existence of a complex network of channels within which vigorous streaming occurs. Each microplasmodium within a liquid culture is also synchronous, although there is no such synchrony between different microplasmodia.

Physarum polycephalum was used in the experiments to be described because, with its natural synchrony, it fulfils several criteria that facilitate the investigation of macromolecular synthesis. First, for detailed studies of synthesis in relation to the cell cycle, it is desirable to be able to obtain large quantities of material at specific points in the cycle. This can be achieved by artificially synchronising a random population of cells with the disadvantages that (i) such synchrony tends to show serious decay after less than one cycle, making precise determinations, or unambiguous interpretation, impossible and (ii) the act of synchronisation might produce undetected disturbances in

the state of the cells. Physarum, in contrast, provides a naturally synchronous system, the nuclei of which can be readily seen to divide simultaneously over several division cycles, providing the dimensions of the organism are kept within certain limits. It is, however, possible to grow a single plasmodium up to 14 cm in diameter with only slightly impaired synchrony (Mohberg & Rusch, 1969). Using such a plasmodium, it is possible to extract microgram quantities of DNA several times during a division cycle at intervals that can be accurately related to either the preceding or following mitosis. Similar considerations apply if cycloheximide is to be added or a cell-free homogenate made at a specific point during the division cycle.

Secondly, the absence of a cell wall allows nuclei and cytoplasmic components to be isolated by gentle procedures, a factor particularly important when preparing cell-free replicating systems. Thirdly, the organism can be easily cultivated in a semi-defined axenic growth medium (Daniel & Baldwin, 1964) and fourthly, sensitive assay systems can be devised because radioactively labelled precursors are readily utilised from the growth medium (Nygaard et al., 1960), although incorporation is neither as extensive nor as specific as in, for example, E. coli.

It must be mentioned that the events followed in Physarum do not take place within a true cell cycle, because there is no cell division; it is more strictly a mitotic or nuclear division cycle. Nevertheless, the mitosis itself is fairly typical of those in higher organisms, the only major difference being that the nuclear membrane does not quite disappear during mitosis, presumably to prevent random mixing of nuclear contents. Furthermore, macromolecular syntheses have the same period as the mitotic cycle, and so it seems probable that nuclear division acts as the realignment point for cell processes in the same way that cell

division does in a conventional cycle.

1.2. Disadvantages of pulse-labelling methods.

The pattern of synthesis of a macromolecule can be determined either by finding its rate of synthesis at a series of time points or by following its accumulation with time; the former is the first derivative of the latter. It is possible to define the pattern of synthesis with much greater precision using the first derivative, for example, very different patterns of changes of rate with time can give similar accumulation profiles (Mitchison, 1971; Hall, 1975) and large changes in rate often initially produce only small changes in the corresponding accumulation curve, as can be seen in the transition between S and G2 phases in the DNA accumulation curves in Section 3.3.4. The measurement of rate has therefore been the more widely used approach. It can be accomplished by the technique of pulse-labelling, i.e. by the administration of radioactive precursor for a short time followed by measurement of the amount of radioactivity incorporated into the macromolecule under investigation.

For successive pulse-labelling measurements during the cell cycle to be strictly comparable, the pool of the immediate precursor to the macromolecule must, throughout the cycle, attain the same specific radioactivity after the same period of exposure to radioactive precursor. This will not occur unless certain experimental conditions are fulfilled, for example, the precursor must not become significantly depleted from the medium during the pulse, and the sites of uptake of precursor through the cell membrane must not be saturated, but, more importantly, two further criteria must be met. First, the uptake of labelled precursor into the cell should not vary during the division cycle. This criterion is not invariably fulfilled. In rat hepatoma cells, the number of functional sites for thymidine uptake increases only during S phase (Plagemann et al.,

1975) and, as transport is the rate-limiting step, variation in the incorporation of thymidine into the nucleotide pool results. Experimentally-derived patterns of 'synthesis' can certainly be altered by variations in the rate of precursor uptake, for example two apparently irreconcilable patterns of 'RNA synthesis' measured by radioactive uridine incorporation have been reproduced by changing the growth medium used (Stambrook & Sisken, 1972). The uptake of uridine by both microplasmodia and surface plasmodia of Physarum has been studied in some detail (Birch & Turnock, 1976). Although variation in uptake during the mitotic cycle was not studied, the fact that it occurs by a passive mechanism in surface plasmodia in G2 (the rate of uptake is a linear function of the external nucleoside concentration and is not inhibited by other nucleosides) suggests that regulated changes in uptake characteristics during the cycle are unlikely.

Secondly, the characteristics of the intracellular precursor pool must be known and, if possible, allowed for. Incorporation parallels real synthesis only after a delay during which the specific radioactivity of the pool rises. The length of this delay depends on the size of the pool and, if this varies during the division cycle, an accurate measure of real synthesis by pulse-labelling is possible only if the pulse is long compared with the delay. For large pools, this might require a pulse so long that the sensitivity of the method is drastically reduced. Further problems arise if the pool is expandable. With a fixed pool, total uptake can be used as a measure of real synthesis but, if the pool is expandable, after addition of precursor this parameter rises rapidly while the pool fills up. As with a fixed pool, incorporation shows a delay dependent on the initial size of the pool. Its ultimate size can be either dependent or independent of the external concentration of precursor. If the former, then precursor of high specific radio-

activity can be employed to avoid significant expansion, but in both cases it is difficult to rapidly terminate the pulse because the addition of excess unlabelled precursor expands the pool so that incorporation of radioactivity continues, albeit at a reduced rate.

Investigations into the rates of macromolecular synthesis using pulse-labelling now tend to be complemented by isolation of the immediate precursor pool and measurement of its specific radioactivity. If this is constant throughout the cycle, then it is assumed that isotope incorporation provides a valid measure of the rate of real synthesis while, if it varies, corrections can be applied to normalise pool effects. While this has not been attempted as such in Physarum, the pools of nucleoside triphosphates have been studied throughout the mitotic cycle by anion exchange chromatography (Sachsenmaier et al., 1969) and by [^{32}P]phosphate-labelling followed by separation on thin-layer chromatographs (Bersier & Braun, 1974a; Fink, 1975). Changes in the ribonucleoside triphosphates during the cycle were found to be relatively slight, fluctuating about twofold with accumulations during mitosis, when RNA synthesis in Physarum is known to be greatly reduced (Hall & Turnock, 1976), and at about 1 h and 5 h after mitosis (Fink, 1975).

Deoxyribonucleoside triphosphates are present at much lower concentrations, approximately one fiftieth that of the ribonucleoside triphosphates (Fink, 1975), and show more marked changes during the cycle. The pools of all four dNTPs expand before initiation of DNA synthesis: dTTP by a factor of 2, dCTP 5-fold, dATP 4-fold and dGTP 6-fold, and similar effects have been found in other systems (Fink, 1975). The pools then rapidly contract very early in S phase, around 5-10 min after the uncoiling of the telophase chromosomes, coinciding with the maximum rate of DNA synthesis as determined by pulse-labelling (Braun et al., 1965),

and measurable pools remain throughout G2 phase, during which mitochondrial and ribosomal DNA continue to be made. Substantial expansion of the pyrimidine nucleotide pools has also been found in the presence of exogenous uridine (Birch & Turnock, 1976) so that, at increasing times after the addition of exogenous [^{3}H]uridine, incorporation of freshly added [^{14}C]uridine into cold acid-insoluble material was decreased much more than total uptake.

These points demonstrate the necessity of a knowledge of the specific radioactivity attained by immediate precursor pools throughout the cycle if pulse-labelling is to be used to investigate nucleic acid synthesis in Physarum, but even these measurements may be misleading if there is subcellular compartmentation of nucleotide pools. Plagemann (1971) found that, in animal cells, adenosine and uridine incorporation into nucleic acids attains a constant rate and could be decreased very rapidly despite the presence of a large and highly expandable cytoplasmic pool. It was subsequently shown that there is one nucleotide pool located in the cytoplasm and one in the nucleus. The size of the latter very small pool, which is mainly responsible for the supply of nucleotides for RNA synthesis, is under strict regulatory control, but the two pools are not totally independent since, when labelling with very small amounts of uridine, most of the isotope was eventually incorporated into nucleic acid. Similar compartmentation has been found in 6C3HED lymphoma cells (Goody & Ellem, 1975), but there is no available information on the situation in Physarum. It might also be mentioned that, in Physarum, uridine and thymidine do not specifically label RNA or DNA (see Section 3.3.1.) so that it is not sufficient to simply measure incorporation into total cold acid-insoluble material, but some fractionation should be employed.

Therefore, there is insufficient information available about the processes of precursor uptake and incorporation in Physarum to

allow unequivocal interpretation of experiments involving pulse-labelling followed either by autoradiography or by measurement of isotope incorporation into a cold acid-insoluble fraction. For example, pools of dNTPs appear to be quite small, being equivalent to between 1.5 and 16 min of DNA synthesis (Bersier & Braun, 1974a) but this does not reveal how quickly these will equilibrate with exogenous labelled precursor. The larger, expandable pools derived from uridine take between 30 and 60 min to equilibrate with exogenous precursor (Birch & Turnock, 1976).

1.3. Description and advantages of the isotope dilution method.

Apart from pulse-labelling, other available methods for following macromolecular synthesis measure accumulation. Continuous-labelling, because it measures the incorporation of isotope during the experiment (i.e. like pulse-labelling it is an isotope incorporation method), is subject to all the problems associated with pulse-labelling and is in addition less sensitive. Direct chemical determination of the quantity of macromolecule present is dependent both upon efficiency of extraction and upon sample size, a parameter difficult to standardise in Physarum due to its syncytial nature and flat, irregular morphology. Thus, an isotope dilution method was developed (Hall, 1975; Hall & Turnock, 1976) which, because it is based upon measurements of the change in specific activity of an already radioactive macromolecule, does not require that the incorporation of a radioactive precursor be measured and is also independent of both sample size and efficiency of extraction. Its application to nucleic acid synthesis in Physarum will be described.

A culture of microplasmodia is given a small amount of radioactive nucleoside which is utilised both through incorporation into nucleic acids and by catabolism mainly to the corresponding

base which can be further metabolised or excreted into the medium whence it cannot be reutilised (Birch & Turnock, 1976; Fink & Nygaard, 1978). There then follows a period of growth in effectively 'unlabelled' medium which is sufficiently long to ensure that radioactivity in metabolically unstable nucleic acids is either lost or recycled into stable species. The microplasmodia are used to inoculate a surface plasmodium and the specific radioactivity of the nucleic acid under investigation is determined at intervals, usually from the second or third post-fusion mitosis onwards.

If the specific radioactivity, S_0, of the nucleic acid is given by $\underline{a}x^{-1}$, where $\underline{a}$ is the total amount of radioactivity in nucleic acid ~~culture~~ and $\underline{x}$ is the amount of nucleic acid, then the specific activity, S, at any subsequent time, is given by $S = \underline{a}(x + dx)^{-1}$, assuming no turnover of the nucleic acid where $\underline{dx}$ is the amount of newly synthesised, unlabelled material. Thus S is proportional to $(x + dx)^{-1}$ i.e. the specific activity is simply inversely proportional to the amount of nucleic acid present, so that the relative accumulation curve for the nucleic acid can be readily calculated from its specific activity at various times. The specific activity at one division is generally normalised to 1.00 and that at the following division ~~will equal~~ to 2.00 if the macromolecule has doubled in quantity.

The advantages of this technique can be enumerated as follows:

(i) As the incorporation of a radioactive precursor is not measured, the problems associated with isotope incorporation methods are avoided.

(ii) Precision is increased because the method does not rely on either sample size or efficiency of extraction.

(iii) A reduction of specific activity to one half demonstrates both a doubling in the quantity of macromolecule, and that it is metabolically stable; this can be correlated with microscopically observed mitoses. A fall to a value above one half between divisions

would indicate incomplete duplication and to a value below one half that the molecule is subject to turnover, although this is relatively unlikely to be found because of the long 'chase' periods involved after the original addition of isotope.

(iv) Provided measurements are taken at mitosis, the accumulation of DNA or RNA between any two points in time can be expressed directly as a fraction of the genome or of the amount of RNA at mitosis.

(v) Once the accumulation curve for a molecule has been defined, it can be used to determine the characteristics of synthesis of other components which may not be metabolically stable, as has been achieved for total protein (Birch & Turnock, 1977).

Its disadvantages are:

(i) It is less sensitive than pulse-labelling to changes in rate of synthesis.

(ii) Unlike direct chemical measurements, it does not give a measure of the absolute quantity of material present.

(iii) As precursor is present for long periods, it is probable that a greater proportion than after short pulses will be incorporated via indirect routes into macromolecules other than those under investigation.

These disadvantages are unimportant except for the relatively low sensitivity and this depends to a large extent on the precision with which specific radioactivities can be determined. The evidence from previously published applications of the isotope dilution method suggests that it is possible to reach a degree of precision at which a reliable pattern of synthesis throughout the mitotic cycle can be obtained for several classes of macromolecule.

1.4. Published applications of the isotope dilution method in Physarum.

The pattern of ribosomal RNA synthesis (pooled 19S and 26S

fractions from sucrose gradients) after M2 has been elucidated (Hall & Turnock, 1976). Synthesis was low during the immediate period of nuclear division but was continuous throughout interphase, the rate increasing 5- to 6-fold between the beginning and end of interphase. The accumulation curve could be fitted equally well to either of two equations which, when differentiated, would in one case follow a power curve and in the other would be linear. Either of these is more biochemically feasible than the biphasic rate-graph obtained by measuring the incorporation of short pulses of [^{3}H]uridine *in vivo* (Mittermayer et al., 1964) and in isolated nuclei (Mittermayer et al., 1966b; Grant, 1972) which shows a depressed rate not only during division but also during early G2. As this effect was obtained when assaying total RNA, it may be due to differential transcription, that during G2 being mainly due to nucleolar RNA and that during S being due to both nucleolar and nucleoplasmic RNA (Grant, 1972). However, as a pulse-labelling methodology was employed, such results should be viewed with caution but, as similar results were obtained using whole cells and isolated nuclei, it is likely that the precursor pools involved in RNA synthesis are mainly nuclear.

The isotope dilution method was subsequently applied to the synthesis of transfer RNA during the mitotic cycle (Fink & Turnock, 1977). Like ribosomal RNA, its synthesis appears to be dramatically reduced during mitosis and is followed by continuous synthesis throughout interphase with the rate gradually increasing. More detailed analysis is not really profitable because transfer RNA is composed of a large number of individual molecules specified by different genes.

Determination of the accumulation curve for total protein by a direct application of the isotope dilution technique, which would involve prelabelling with a radioactive amino acid and measuring

the decrease in the specific activity of plasmodial protein, would be unacceptable because many proteins must have half-lives that are significantly short in relation to the intermitotic period. However, isotope dilution can be applied indirectly by prelabelling metabolically stable nucleic acids and by then determining, in samples from a surface plasmodium, the specific activity of the nucleic acid (S) and the amounts of nucleic acid (N) and protein (P). Since S is proportional to N^{-1}, the relative accumulation curve for protein is given by $S.N^{-1}.P$. (Birch & Turnock, 1977). Because this procedure requires the measurement of more parameters than a direct isotope dilution method, it has slightly reduced precision, but has nevertheless provided a reliable pattern of accumulation. It also requires that the ratio of the efficiencies of extraction of nucleic acid and protein is constant for each sample.

The pattern of accumulation of nucleic acid (Birch & Turnock, 1977) showed continual synthesis throughout the cycle, with an increasing rate towards the end of the cycle characteristic of ribosomal RNA, but the occurrence of S phase immediately after mitosis (see Chapter 3) does produce a relative straightening of the line compared to that for ribosomal RNA alone. Protein synthesis between divisions was non-linear in form, the best fit being to a third order polynomial and was again continuous throughout the cycle with a marked increase in rate in G2 phase, the part of the cycle associated with the maximum rate of ribosome production (Hall & Turnock, 1976). It was impossible to compare these results with the biphasic pattern of incorporation previously obtained by pulse-labelling (Mittermayer et al., 1966a), as the earlier results were not corrected for possible variation in the specific activity attained by free lysine in the amino acid pools of different samples.

The isotope dilution method has also been applied, in a

preliminary manner, to the synthesis of nuclear DNA between the second and third mitoses, using [^{3}H]uridine to prelabel nucleic acids (Hall & Turnock, 1976). Only six points were obtained between the two divisions, but these were sufficient to show that approximately 80% of the DNA had replicated by 90 min after mitosis and to suggest the existence of a second, slow phase of DNA synthesis during G2 phase. The aim of much of the present project was to investigate this profile in greater detail, to check whether G2-synthesis was consistently reproduced and to determine the effects on the profile of different lengths of mitotic cycle. It was also decided to attempt to extend the range of the types of experiment involving isotope dilution, and therefore the method was applied to the measurement of the residual synthesis occurring in vivo after addition of cycloheximide and the synthesis possible in cell-free homogenates. In both cases isotope dilution, by giving values directly as a proportion of the genome, should provide a more biologically meaningful measure of DNA synthesis than measurements of isotope incorporation, and also avoids the uncertainties involved in such incorporation procedures.

Chapter 2. General Culture Methods and Growth Experiments.

2.1. Strain.

The strain used throughout this research was M_3cVIII. The original Wisconsin 1 isolate was purified by a migration method (Daniel & Rusch, 1961) from a sclerotium collected in the field by Professor M.P. Backus of the Department of Botany, University of Wisconsin. By repeated subculture a number of plasmodial sublines, including M_3cVIII, were begun, all genetically identical except for variation that might have arisen during repeated subculture. Because M_3cVIII was stored as spherules for the first time shortly after purification, long periods of continuous subculture, with the associated risk of genetic variation, have been avoided.

2.2. Media.

Three media were used: (i) N+C; (ii) SOYA DM; (iii) BP.

(i) N+C. (Table 1). This is a rich, semi-defined medium containing Bacto-Tryptone (Difco), yeast extract (Difco), salts, glucose and haematin and buffered at pH 4.6 by citrate. Originally developed as long ago as 1955 (Daniel & Baldwin, 1964), it gives an intermitotic period of $8\frac{1}{2}$ - $10\frac{1}{2}$ h in surface plasmodia under our conditions of growth. Bacto-Tryptone is a pancreatic digest of Casein USP.

(ii) SOYA DM. (Table 1). This is also a rich, semi-defined medium containing salts, glucose and haematin and buffered at pH 4.6 by citrate, but based on Bacto-Soytone (Difco), an enzymatic hydrolysate of soy bean meal. The essential vitamins, biotin and thiamine replace yeast extract. It was developed here as a possible alternative to MyDM (Plaut & Turnock, 1975) a medium based on Oxoid's mycological peptone which was temporarily unavailable. It routinely gives an intermitotic period of $8\frac{1}{2}$ - $11\frac{1}{2}$ h.

(iii) BP. (Table 1). This has the same recipe as N+C but with

Table 1. Semi-defined growth media.

Component	N+C ($g\ l^{-1}$)	SOYA DM ($g\ l^{-1}$)	BP ($g\ l^{-1}$)
Citric acid.H_2O	4.03	3.45	4.03
$FeCl_2.4H_2O$	0.06	0.039	0.06
KH_2PO_4	2.00	2.00	2.00
$MgSO_4.7H_2O$	0.60	0.60	0.60
$MnCl_2.4H_2O$	0.084	-	0.084
$ZnSO_4.7H_2O$	0.034	0.034	0.034
$CaCl_2.6H_2O$	0.894	1.35	0.894
EDTA $Na_2.2H_2O$	-	0.245	-
Glucose	10.0	10.0	10.0
Yeast extract	1.52	-	-
Biotin	-	0.005	0.005
Thiamine	-	0.04	0.04
Haematin	0.005	0.005	0.005
Bacto Tryptone	10.0	-	-
Bacto Soytone	-	10.0	-
Bacto Peptone	-	-	10.0

All components except haematin were dissolved in distilled water and the pH adjusted to 4.6 with 20% (w/v) NaOH. Medium was stored at $-18^{\circ}C$ and was autoclaved at 15 lb in^{-2} for 15 min before use. Haematin was autoclaved similarly in 1% (w/v) NaOH at a concentration of 0.05% (w/v) and stored at $5^{\circ}C$; before use 1 ml was added per 100 ml of medium.

Bacto-Peptone (Difco) replacing tryptone and with thiamine and biotin replacing yeast extract. It was developed by Sudbery (1974) and was said to give an intermitotic period of 16 - 17 h.

N+C and BP are routinely prepared using 120 ml (per litre of medium) of a concentrated salts solution with the following composition:

citric acid.H_2O	33.66 g l^{-1}
$FeCl_2.4H_2O$	0.50 g l^{-1}
$MgSO_4.7H_2O$	5.00 g l^{-1}
$MnCl_2.4H_2O$	0.70 g l^{-1}
$ZnSO_4.7H_2O$	0.28 g l^{-1}

This solution is stored at -18°C.

2.3. Growth of microplasmodia.

The optimal culture conditions for plasmodia are pH 4.1 - 4.6 and 26°C, but growth is inhibited by exposure to light (Daniel & Baldwin, 1964). Microplasmodia were therefore grown in 50 ml of medium in 500 ml conical flasks and incubated in a dark, constant temperature room on a reciprocating shaker (Adolf Kuhner, Basel) operating at 125 reciprocations per min of 6 cm amplitude. Stocks were routinely maintained in this mode and, twice weekly, were subcultured, around the end of exponential phase, by aseptic transfer of a known volume of culture. Initial concentrations were of the order of 1 mg wet weight ml^{-1}. Sterile manoeuvres, as in most subsequent cases, were carried out in a Pathfinder laminar flow bench (type 64 T) using an air flow rate of 90 ft min^{-1}.

To determine the cell density of liquid cultures, duplicate 1 ml samples were aseptically withdrawn into weighed tubes and centrifuged at 10 000 rpm (approximately 2000g) for 1 min in a bench minifuge. Supernatants were removed using a Pasteur pipette, the inside of the tubes dried carefully with a tissue and the tubes

reweighed. Cell density was expressed as mg wet weight per ml of culture.

This method was used to determine the kinetics of growth in all three media (Fig. 1). During exponential phase the slopes of the curves gave the following times of doubling of wet weight: N+C = 13 h; SOYA DM = 19 h; BP = $14\frac{1}{2}$ h. A further growth curve for SOYA DM gave a doubling time of $14\frac{1}{2}$ h (B.Birch, personal communication). The exponential phase of growth in both N+C and SOYA DM continued through to concentrations of approximately 100 mg ml^{-1}, but in BP the picture was quite different; exponential phase ceased at approximately 20 mg ml^{-1} to be followed by a slower rate of growth and finally a decrease in wet weight, suggesting either that the medium had been depleted of an essential growth factor or that an inhibitor was accumulating.

2.4. Growth of macroplasmodia.

In this work, surface cultures were grown in three different ways which will each be described in turn.

2.4.1. Growth on filter paper in small plates.

The basis of this method has been described a number of times (e.g., Guttes & Guttes, 1964). A plasmodium would be grown inside a glass petri dish on a circle of Schleicher and Schuell grade 576 filter paper, 8 cm in diameter, supported by a stainless steel wire mesh over 15 ml of medium. The medium was in contact with the underside of the paper without flooding onto the surface. Plasmodia were inoculated aseptically from liquid cultures as follows. 50 ml cultures in early exponential phase (approximately 40 mg ml^{-1} for N+C and SOYA DM) were centrifuged at 85g for 20 s. The supernatant was poured off and the pellet gently resuspended in half its own volume of sterile distilled water; a culture at 40 mg ml^{-1} would give a pellet of volume approximately 4 ml. 600 ul of this cell

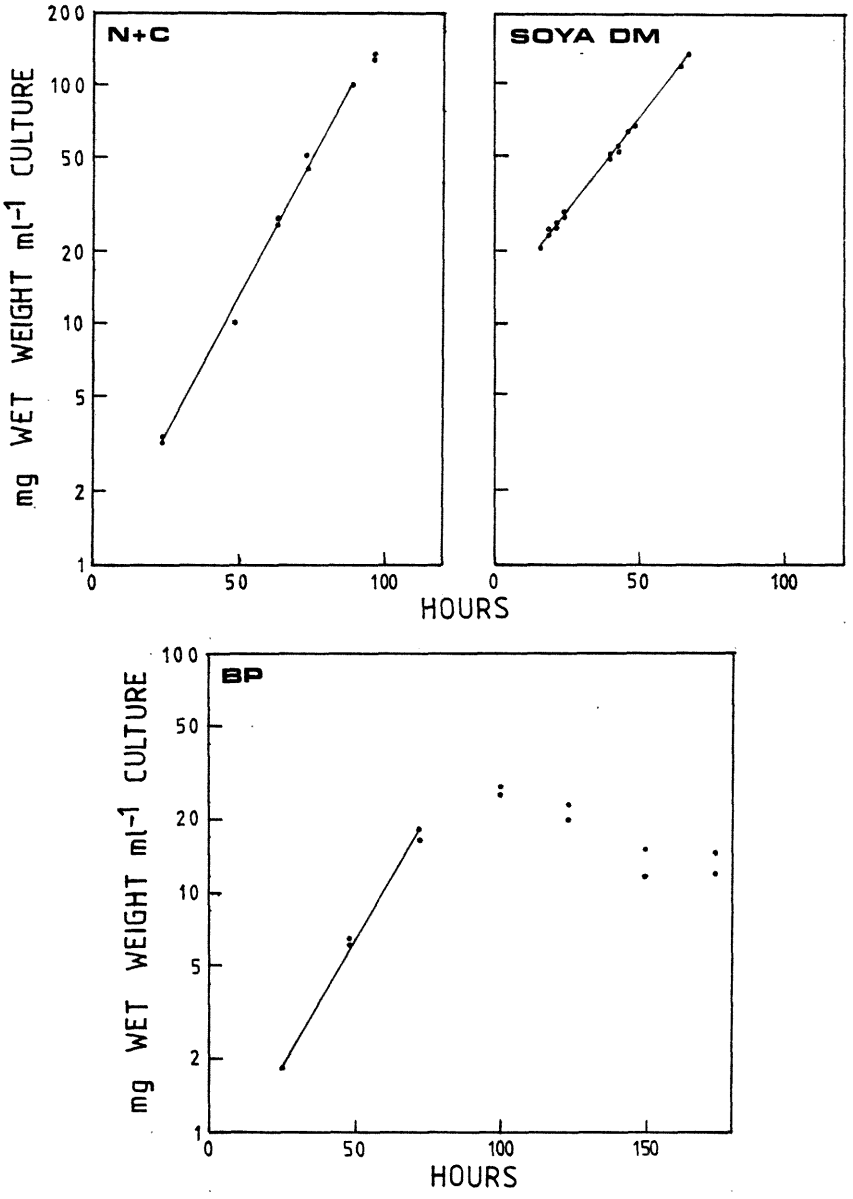

Fig. 1. Growth rate of microplasmodia in N+C, SOYA DM and BP media. Cell density was estimated by the wet weight method (Section 2.3.). Doubling time was calculated by least squares linear regression of values shown graphically to be within the exponential part of the curve (solid line). The time axis refers to hours after inoculation.

suspension was then spread in the centre of the filter paper to a diameter of about 2 cm.

Traditionally the inoculum had been left without medium for a period of up to two hours (Guttes et al., 1961) to allow the microplasmodia to coalesce so forming an integral surface plasmodium, but it has subsequently been found that synchrony is unimpaired if such a period is entirely dispensed with (Holt & Gurney, 1969). In this work a shorter starvation period of 45 min was adopted, because this has been said to give a thinner, slightly faster-growing plasmodium (Mohberg, 1974). As plasmodia can maintain synchrony for at least five mitoses if well-fed (Mohberg, 1974), it seems reasonable to believe that such a relatively short period of starvation would not be essential for synchrony.

Thus, after 45 min in the dark at 26°C, medium was added and a synchronous macroplasmodium then grew slowly outwards from the inoculum spot. In all three media, the first mitosis took place after approximately 5 h, and after 24 h the plasmodia had a diameter of approximately 5 cm. In N+C and SOYA DM, nuclei entered metaphase of M3 within 10 min of each other at the worst, although synchrony in BP was slightly less good (Section 3.3.4.). The difference in the time of M3 between separate plates inoculated at the same time could be as great as 90 min, although most were generally clustered within 30 min.

To avoid the necessity of carrying out experiments in a constant temperature room, an apparatus has been designed to maintain plates at 26°C within a normal laboratory. It consists basically of an insulated wooden cabinet (internal dimensions: 90 cm x 45 cm x 3 cm high) with an aluminium block as base through which water at a constant temperature is pumped. The top is divided into five sections which can be lifted off separately to avoid excessive cooling when handling cultures. Thirty two plates, covered with

aluminium tops to prevent access of light, can be accommodated in an 8 x 4 array, and these are subject to a temperature variation of less than $\pm$ 0.5°C. As 26°C is sometimes close to ambient temperature, the water is pumped through a cooling unit (type FC 15: Grant instruments, Cambridge) as well as through the pump/heating unit (type FH 15: Grant instruments).

2.4.2.a Growth in the large culture apparatus.

For many purposes it is desirable to take all samples from a single plasmodium, yet a small plate culture may provide insufficient material for this. If the petri dish system is simply scaled up by using a larger dish and more inoculum, the plasmodia obtained are very thick, slow-growing and poorly synchronised (Mohberg & Rusch, 1969). Increased thickness presupposes a lower area to volume ratio and a consequently decreased amount of diffusion of oxygen and nutrients into the organism relative to its mass. This problem was originally overcome (Mohberg & Rusch, 1969) by applying the inoculum as an open ring, which gave a thinner plasmodium, and by incubating the culture vessel on a rocker so that the medium was continually agitated to improve aeration. A subsequent modification of this design (Hall & Turnock, 1976) avoided the inconvenient necessity of refeeding the culture after M2 by incorporating a peristaltic pump which continuously circulated the medium between the tray and a reservoir. The apparatus currently in use includes a water jacket in the tray so that there is no need to house the growing culture in a constant temperature room.

This apparatus can be briefly outlined as follows. The plasmodium grows on a double layer of S and S 576 paper, the upper 26 cm in diameter and the lower 34 cm square. These are supported on a metal grid 34 cm square inside a stainless steel tray of internal dimensions 35.5 cm x 35.5 cm x 5.5 cm high. Medium is circulated from a two-litre glass reservoir by a Watson Marlow flow

inducer (model MHRK 55) at a flow rate of 115 ml min^{-1}. It enters the tray below the grid by means of three inlets, one situated at the centre of each of three sides of the tray. On the fourth (front) side, in contact with the double layer of paper by a thin filter paper wick, is an exit weir through which the medium drains directly back to the reservoir. Adjustment of the height of this weir allows the level of the medium to be set so that it maintains uniform contact with the lower side of the filter paper. The stainless steel water jacket is an integral part of the tray and surrounds the inner section, containing medium, grid, etc., on all four sides and underneath. The circulating water is pumped and maintained at 26°C by a pump/heater and cooling unit as above.

Before use the tray was always covered by a steel top and autoclaved at a pressure of 15 lb in^{-2} for 20 min with paper, grid, and inlet and outlet tubing (silicone rubber of 6mm internal diameter, and 10 mm external diameter) in position but with no water in the jacket which, as it has no direct contact with the culture, did not have to be kept sterile. The medium reservoir, also with appropriate inlet and outlet tubing connected and containing two litres of medium without glucose or haematin, was autoclaved at the same time. Sterile haematin and glucose were added to the medium just before the inoculum was to be fed, because the sterilisation conditions used for the apparatus tend to char glucose.

Cultures were inoculated in basically the same way as for small plates except that an inoculum of approximately 4 ml was used and applied as an open ring towards the edge of a circle of S and S 576 filter paper of 9 cm diameter which had been in position immediately on top of the other two layers of paper during autoclaving. Just before inoculation, the steel cover of the tray was replaced by an alcohol-sterilised, transparent plastic lid which could be covered by a larger aluminium lid to prevent exposure of

the growing plasmodium to light. During the 45 min starvation period the jacket was filled with water from a reservoir and the pump switched on. Sterile connections were made between the tubing of the tray and of the medium reservoir, and the outlet tubing of the reservoir was fitted into position in the flow inducer which, because it operates by an externally-applied peristaltic method, did not have to be kept sterile. As it took approximately 15 min for the medium level in the tray to reach the underside of the filter paper, the pump was usually switched on 30 min after inoculation. Air bubbles were removed by lifting the paper with sterile forceps, and the level of medium adjusted by varying the height of the exit weir and the height of the legs on which the tray was placed.

After twenty four hours, most cultures had a diameter of approximately 11 cm, and by M3 nuclei from different areas of the plasmodium could be out of phase by as much as 30 to 40 min in N+C and SOYA DM (with an occasional culture out of phase by 80 min) although, as the total cycle length is approximately 9 h, for most purposes this 5% error does not outweigh the advantages of deriving samples from a single culture. The much less favourable situation in BP medium will be described in Section 3.3.4.

b. Condition of inoculum region.

When sampling from plasmodia growing on filter paper, it is essential to avoid the immediate inoculum area (Mohberg & Rusch, 1969) which contains necrotic material with a high content of slime. That this area behaves abnormally was shown in an isotope dilution experiment which compared, at three successive mitoses after fusion, the specific activity of nuclear DNA in the growing area to that in the inoculum (Table 2.). The experiment was carried out with small plate cultures in SOYA DM medium. Although the specific activities of inoculum and growing areas were almost

Table 2. Comparison of DNA synthesis in growth area and inoculum.

Sample	DNA S.a. ± S.E. (cpm/ug)	DNA accum.rel. to M1 in growth area	DNA accum.rel. to own previous M	Time after fed (h)
(M1)				
growth area	696.4 ± 5.0	1.000	-	7
inoculum	674.7	1.032	-	7
(M2)				
growth area	342.8 ± 2.1	2.031	2.031	17
inoculum	489.3	1.423	1.379	17
(M3)				
growth area	180.3 ± 3.4	3.863	1.901	29
inoculum	408.0	1.707	1.199	29

Small plate cultures in SOYA DM. Specific activities for growing areas are the mean of three samples, with one plate per sample, except at M1 when three plates were pooled for each sample. Specific activities for inocula are from single samples, obtained by pooling the inocula of two or three plates.

Cultures were labelled following the procedure outlined in Section 3.2.1. using 2 uCi [6-^{3}H]thymidine ml^{-1} at a concentration of 1 uM.

S.a. = specific radioactivity of nuclear DNA.

S.E. = standard error.

identical at M1, as might be expected since very little DNA synthesis takes place between inoculation and the first mitosis (Section 3.3.3.), the amount of DNA synthesis in the inoculum between M1 and M2 was only 40% of that in the growing area, and was only 20% between M2 and M3.

This might have occurred because individual nuclei were not replicating their complete complement of DNA during S phase, but is more likely to be due to a lowered frequency of mitosis within the inoculum, which may itself be caused by a loss of synchrony in the inoculum area, by a fraction only of the nuclei entering mitosis, or by synchronous nuclear divisions occurring at longer intervals than normal and thus being out of phase with those in the growing area. These various alternatives are not mutually exclusive. It may be noted that incomplete replication can also be found to a lesser extent in the growing area where it appears to be associated with a fraction only of the nuclei undergoing DNA synthesis and presumably mitosis (Section 3.4.3.).

Although it is difficult to make reliable smears from the inoculum due to the presence of slime, Mohberg and Rusch (1969) obtained results for six small plates at M3 but, as only one smear appears to have been taken per inoculum, it is not clear how representative of the inoculum spot as a whole their results were. Only one smear had synchronous nuclei in mitosis, one had nuclei in interphase only, while the other four had nests of nuclei in interphase and in all stages of mitosis. As the inocula were not followed throughout an intermitotic period, it is impossible to distinguish between the various alternatives given above, although the proportion near mitosis must have been relatively high for them to be consistently observed in all but one of the slides. This suggests that some of the nuclei kept roughly in phase with the growing area, but it is probable that there was a high degree of variation in the

condition of the inoculum between plates.

Although Mohberg and Rusch (1969) found that the inoculum ring of a large plasmodium was synchronous, another isotope dilution experiment has suggested that it is nonetheless abnormal (K. Fink, personal communication). The specific radioactivity of total nucleic acid was determined in segments taken at random from the surface of a large plasmodium in N+C which had been prelabelled with [^{3}H]uridine. Segments containing inoculum material had significantly higher specific activities than those excluding the inoculum.

Such a necrotic area may produce toxic breakdown products, so for this reason, and to avoid contamination of samples with inoculum material, the uppermost circle of paper (9 cm in diameter) containing the inoculum ring and any inwardly growing areas was routinely removed from a large culture during the interphase either before or after M2. As the relative area of contact with inoculum is somewhat less in a small surface culture, it was considered unnecessary to remove the inoculum although sampling from that area was avoided.

2.4.3.a Growth on agar in small plates.

Growth was supported on a base consisting of a 1:1 mixture of medium and 3% Difco Bacto-Agar. Approximately 20 ml was used in a plastic petri dish of 8.5 cm diameter. Surface cultures were inoculated from microplasmodia by transferring 0.1 - 0.2 ml of settled cells from a thick culture to the centre of an agar plate, or they were inoculated directly from another agar culture by transfer of a small square of agar, approximately 1 cm^2, cut from the growing edge of one plasmodium, to the centre of a fresh plate. This provided a method of maintaining stock cultures as surface plasmodia.

b. Maintenance of synchrony.

It is difficult to remove samples for analysis from agar plates without removing some of the base, and so such cultures are

rarely used for biochemical experiments, but they may be inoculated directly onto filter paper for the purposes of experiment by inverting a block of agar about 1 cm square onto the paper. Although inoculation from liquid has the advantages that morphologically healthier cultures are produced and that inoculation can be in the form of an open ring for a large culture, agar to paper transfer can be used to investigate a possible objection to the use of Physarum as a naturally synchronous system. This is that, when a surface culture is inoculated from liquid, synchrony might be artificially induced by the radical change in external conditions between submersion in liquid and support on a solid surface, and possibly also by the manipulations involved in such a transfer. This might act in a similar way to the induction of synchrony in other systems by temperature shock or light exposure, (Mitchison, 1971).

There is much accumulated evidence against this view: e.g., a) the degree of synchrony in Physarum is much higher than that normally produced by a single shock; b) nuclei in a common cytoplasm tend to be synchronous in most systems (Prescott, 1976) and the nuclei within a single microplasmodium are indeed synchronous (Guttes & Guttes, 1964); c)fusion experiments suggest that synchrony is maintained through a positive mechanism (Guttes et al., 1969); d) a large plasmodium can maintain its synchrony at least until the fifth postfusion mitosis (Mohberg, 1974). As an extension of this last line of argument, the degree of synchrony was investigated in filter paper cultures derived from a line of agar cultures which had been inoculated from liquid almost a week earlier. Assuming an intermitotic period of 10 h, approximately fourteen divisions would have occurred since the change from liquid to solid. If synchrony had been induced initially it is very doubtful that it could be maintained for this length of time.

Although less synchronous than usual (Table 3), the cultures were by no means asynchronous, and had intermitotic periods similar to normal, if rather more variable. The poor synchrony at M_B was possibly because the plasmodium had not grown out on all sides from underneath the agar so that communication between distant parts would be hindered. At M_A, although not completely grown out, the plasmodia were smaller so facilitating communication, while at M_C they were roughly circular, the optimum shape for communication.

It might still be argued that synchrony is induced by transfer from agar to paper or that its observed overall increase in the above experiment reflects the loss of an inhibitor of synchrony present in agar. To investigate these objections it was necessary to follow mitosis in cultures actually growing on agar; with care, reasonable smears were possible, although some agar was invariably present. As a synchronous mitosis was observed in cultures two days old (Table 4) derived from a line subcultured in the surface mode for 13 days previously (approximately 30 mitoses), it was clear that synchrony could be maintained in absence of shock. The corresponding cultures on paper were also synchronous and again suggested ease of communication as a determinant of synchrony because plates 2 and 3 were almost circular (asynchrony, 10 and 0 min respectively) at M_A, while 1 (asynchrony, 30 min) was 'U-shaped' with only distant contact between the limits of the arms. By M_B, plate 1 (asynchrony, 3 min) was also circular. Thus, it is likely that synchrony would be impaired in a large or irregularly-shaped plasmodium.

2.5. Preparation of smears.

The procedure adopted was as follows. A small piece of plasmodium (approximately 1 mm^2) was aseptically removed, transferred to a glass slide and spread thinly by smearing with another slide.

Plate	M_A: (h,min) after feeding	Range (min)	M_B: (h,min) after feeding	Range (min)	IMP (h,min)	M_C: (h,min) after feeding	Range (min)	M_B to M_C (h,min)	Estimated mean IMP (h,min) *
1	18,15 - 18,30	15	27,45 - 28,55	70	9,30 - 10,25	51,05 - 51,08	5	22,10 - 23,15	11,05 - 11,35
2	17,30 - 18,00	30	27,00 - 27,50	50	9,30 - 9,50	46,44 - 46,48	5	18,55 - 19,45	9,25 - 9,50
3	17,50 - 18,25	35	25,10 - 25,40	30	7,15 - 7,30	44,50 - 45,05	15	19,20 - 19,55	9,40 - 9,55

Table 3. Synchrony and intermitotic period in long-established surface cultures (1).

Mitoses were recorded in three small surface cultures on filter paper which were themselves all derived from a single plasmodium grown on agar. Because the number of mitoses occurring since inoculation was unknown, microscopically observed divisions were referred to as M_A, M_B etc.

The medium used was N+C, and cultures were refed after M_A.

IMP = intermitotic period.

* Calculated assuming $M_B - M_C$ represents two intermitotic periods.

Culture	M_A: time after feeding (h,min)	Range (min)	M_B: time after feeding (h,min)	Range (min)	Period $M_A - M_B$ (h,min)	Estimated mean IMP (h,min) *
Agar 1	47,28 - 47,43	15				
Agar 2	47,35 - 47,50	20				
Paper 1	17,05 - 17,35	30	50,17 - 50,20	3	32,40 - 33,10	10,50 - 11,05
Paper 2	18,30 - 18,40	10				
Paper 3	18,15	0	50,27 - 50,31	4	32,15	10,45

Table 4. Synchrony and intermitotic period in long-established surface cultures (2).

3 agar cultures (1 - 3) were inoculated on day 1. On day 2, culture 3 was used to inoculate paper cultures 1 - 3. Mitoses were observed in all plates on day 3 and only in paper cultures 1 and 3 on day 4.

The medium used was N+C and the paper cultures were refed before M_A.

* Calculated assuming MA - MB represents three intermitotic periods.

The smear was submerged in ethanol for approximately 15 s, allowed to drain but not dry, and a drop of 1:1 glycerol:ethanol was pipetted onto it. The slide was observed through phase contrast using oil immersion, at a magnification of x1000 using a Nikon binocular microscope, model L-Ke. This process took approximately two minutes and the slides would remain usable for at least a week. Photographs and a clear description of the events of mitosis as seen in alcohol-fixed smears are given by Guttes et al. (1961), and diagrammatic representations are given by Guttes and Guttes (1964), and Mohberg and Rusch (1969).

2.6. A comparison of the growth rate of surface plasmodia under various conditions.

Stock lines were usually subcultured for only 4 - 5 months at the end of which time new microplasmodia were initiated from a stock of spherules stored refrigerated on strips of filter paper. They were hatched into either stationary liquid medium or onto nutrient agar and were themselves replenished by starvation of recently hatched microplasmodia in salts-citrate medium (Daniel & Baldwin, 1964). Such a system minimises any selective pressures that might occur during continued growth in liquid culture (Section 2.7.).

It was thought possible that there might be significant variation in the growth rate of plasmodia from different hatches and under certain other conditions of growth. Therefore, where possible, the average length of the intermitotic period between M2 and M3, and the average time between feeding and M3 were calculated for all experiments in N+C and SOYA DM. In an experiment using small plates, the average time of M3 would be obtained from all those in which M3 was recorded, but the intermitotic period would be derived only from plates in which both M2 and M3 were seen. With large cultures, the average time of metaphase would be used; for example,

if the culture showed a range of 30 min in M3, the time taken would be that at 15 min. These values were used to investigate differences in growth rate under the following conditions:

(i) between different hatches from spherules;

(ii) between N+C and SOYA DM;

(iii) between large cultures and small plates.

Times from feeding to M3 would be expected to show rather more variation than intermitotic period since they depend on the accumulated variation of two such periods together with the variation from inoculation to M1. The latter might be expected to show an additional degree of variability reflecting the initial condition of the inoculum. Thus, although times to M3 will give some indication of growth rate, even if they are significantly different they need not represent a significantly altered intermitotic period.

Tables 5 and 6 summarise all the available data with the three variables mentioned above separated. n is the number of average values (i.e. the number of separate experiments) used to obtain each mean, and S.E. the standard error of the means. In many cases n was too small to allow significance to be reliably determined, but the following tests were carried out.

(a) Calculation of relative variance within experiments and between experiments.

The use of an average value for each experiment in the analyses that follow seems theoretically reasonable; it is to be expected that the variation between experiments would be greater than that within an experiment because of the many uncontrolled variables, such as the precise temperature of the incubator or the density of the inoculating culture, which are the same for all cultures within an experiment but differ between experiments. The use of an average value allows each experiment to be treated

	No. of Hatch	Date strip hatched	Date strip made	Mean IMP (h)	S.E.	n	Observed limits (h)	Mean M3 (h)	S.E.	n	Observed limits (h)
Large Cultures.	N1	Sep 1975	Apr 1974	10.00	1.00	2	9.0 - 11.0	26.46	1.96	2	24.5 - 28.4
	N2	Aug 1976	May 1976	9.36	0.29	5	8.75 - 10.3	26.67	0.66	5	25.25 - 28.5
	N3	Jan 1977	May 1976	8.88	-	1	-	25.58	-	1	-
	N4	Apr 1977	Oct 1976	-	-	-	-	22.72	-	1	-
	N5	Oct 1977	Oct 1977	-	-	-	-	26.80	-	1	-
	N6	Mar 1978	Oct 1977	-	-	-	-	25.41	0.43	3	24.6 - 26.0

	No. of Hatch	Date strip hatched	Date strip made	Mean IMP (h)	S.E.	n	Observed limits (h)	Mean M3 (h)	S.E.	n	Observed limits (h)
Small Cultures.	N1	Sep 1975	Apr 1974	8.75	-	1	-	26.08	0.42	2	25.7 - 26.5
	N2	Aug 1976	May 1976	-	-	-	-	-	-	-	-
	N3	Jan 1977	May 1976	9.60	0.15	5	8.9 - 10.2	26.25	0.17	5	25.8 - 26.7
	N4	Apr 1977	Oct 1976	9.22	0.03	2	9.2 - 9.25	25.21	0.30	9	24.2 - 26.9
	N5	Oct 1977	Oct 1977	-	-	-	-	25.70	0.81	6	23.6 - 28.3
	N6	Mar 1978	Oct 1977	8.8	-	1	-	24.08	0.16	12	23.5 - 24.9

Table 5. Comparison of growth of different hatches in large and small cultures in N+C medium.

	No. of Hatch	Date strip hatched	Date strip made	Mean IMP (h)	S.E.	n	Observed limits (h)	Mean M3 (h)	S.E.	n	Observed limits (h)
Large Cultures.	S1	Jan 1977	Oct 1976	11.12	-	1	-	27.89	1.31	2	26.6 - 29.2
	S2	Apr 1977	Oct 1976	8.67	-	1	-	23.83	0.33	2	23.5 - 24.2
	S3	Sep 1977	Oct 1976	-	-	-	-	-	-	-	-

	No. of Hatch	Date strip hatched	Date strip made	Mean IMP (h)	S.E.	n	Observed limits (h)	Mean M3 (h)	S.E.	n	Observed limits (h)
Small Cultures.	S1	Jan 1977	Oct 1976	10.91	0.77	2	10.1 - 11.7	27.23	0.74	7	24.5 - 29.6
	S2	Apr 1977	Oct 1976	9.38	0.64	2	8.75 - 10.0	24.07	0.80	4	22.4 - 26.25
	S3	Sep 1977	Oct 1976	10.82	-	1	-	25.61	0.59	2	25.0 - 26.2

Table 6. Comparison of growth of different hatches in large and small cultures in SOYA DM medium.

equally, even though there might be different numbers of cultures involved, ranging from one in the case of a large culture, to 10 - 15 for small plates. However, if the variation within experiments was as great or greater than that between experiments, then it would be necessary to analyse each individual determination i.e., in order to treat individual experiments equally, the variation within an experiment must be small relative to that between experiments.

To test this, five experiments were chosen at random from those using the March 1978 hatch in N+C (N6). Only small culture experiments were used.

Experiment	1	2	3	4	5
Mean M3	23.39	23.96	23.60	24.51	23.41
n	10	9	10	14	10
Σx	233.92	215.62	236.02	343.13	234.08
Σx^2	5472.16	5165.76	5570.57	8411.365	5480.59

In this case n equals the number of plates in the experiment for which the time of M3 was determined.

Before applying an analysis of variance, the data were tested for homogeneity of variances (homoscedasticity) and for normality. The former was investigated using Bartlett's test (Sokal & Rohlf, 1969) and the more rapid F_{max}-test. Whilst the latter showed that the variances were not significantly different at $p = 0.05$, Bartlett's test showed that the variances were heterogeneous at a probability between 0.025 and 0.01. Since this test is unduly sensitive to departures from normality in the data, and since there is no obvious theoretical reason why different experiments should produce different variances, it seemed likely that the data were not normally distributed. To test this, the data of experiments 1

and 4 were analysed by the graphical method of ranked normal deviates or rankits (Sokal & Rohlf, 1969) and both showed small but significant deviations from normality (small except for two outliers in the data of experiment 1).

Therefore, the five experiments were analysed in two ways. Firstly a non-parametric method was used, so that no assumptions about the distributions of the data were necessary. This was the Kruskal-Wallis test (Sokal & Rohlf, 1969) which substitutes for a single classification analysis of variance and calculates a constant, H, which is distributed approximately as $\chi^2_{(a-1)}$ where a = the number of experiments. This showed a difference between means significant at p = 0.001, i.e., differences between experiments were markedly greater than differences within them. To gain some idea of the magnitude of this effect, a normal single classification analysis of variance (of Model II type as defined by Eisenhart, 1947) was used, since the deviations from normality were small and normal analyses are generally quite robust. The calculated value of the variance ratio, F, was 42.55, and, since the tabulated value at p = 0.001 was only 5.7, this showed a highly significant added variance component between experiments; this was calculated to be 0.251, while that for within experiments was 0.064. Thus, the percentage of total variation due to that between experiments was 79.7%. This result therefore confirms that it is reasonable to use an average value for each experiment, since the variation within an experiment is only approximately one quarter of that between experiments.

(b) Variation between hatches.

Four N+C hatches, those of January, April and October 1977 and March 1978 (N3 - N6) were investigated with respect to times of M3 in small cultures. The data of N4 and N6 were tested for

deviation from normality by the method of ranked normal deviates which showed that neither were distributed normally. Bartlett's test showed heteroscedasticity between all four hatches significant at p = 0.001 and the F_{max}-test at p = 0.005. The variance of N5 was 5-fold higher than the largest of the others and this was due to a discontinuity in the data, the first three times clustering around 27 h and the second three around 24 h. This has not been traceable to a change in experimental procedure, but it is notable that all M3 values determined after this point were below 25 h (the mean of N6 is only 24.08 h). After removal of N5, the remaining variances were not significantly heterogeneous when tested by Bartlett's and the F_{max}-test.

The data from all four hatches were first analysed by a normal single classification analysis of variance (Model I), since parametric methods are more sensitive than non-parametric, that is they have a greater probability of detecting significant variations between means, and the subsequent comparisons for significance between all four means are simpler using parametric multiple range tests. F_{calc} was greater than F_{tab} (6.565 and 4.57) at p = 0.01 and these significant differences between means were further analysed in two ways. Firstly, the Student-Newman-Keuls (SNK) procedure (Sokal & Rohlf, 1969), which uses the least significant range as the statistic to measure differences between means, grouped the three 1977 hatches together as having no significant difference between their means, with N6 different from each of them at p = 0.05. Secondly, the sum of squares simultaneous test procedure (SS-STP) proposed by Gabriel (1964) was applied and this showed that (i) the three 1977 hatches do not contribute significantly to the sums of squares for hatches; (ii) in contrast to the SNK procedure N6 and N4 were not shown to be significantly different from each other but (iii) N6 is significantly different from the remaining two.

Non-parametric methods were employed to check these conclusions. The Kruskal-Wallis test gave a corrected H value greater than tabulated (12.695 and 11.345) at p = 0.01 showing differences between hatches to be significant. The Wilcoxon two-sample test (Sokal & Rohlf, 1969) demonstrated a difference between N6 and N4 significant at p = 0.01 (in contrast to the SS-STP method above), between N6 and N3 at p = 0.002 and between N6 and N5 at p = 0.05. No difference was significant between any of the 1977 hatches.

Comparison of the January and April 1977 hatches in SOYA DM (S1 and S2; time of M3 in small plates) by the Wilcoxon test showed the difference between means to be just significant at p = 0.05 but not at p = 0.02 which confirms the result of a parametric t-test on the data.

(c) Variation between N+C and SOYA DM.

The data for time of M3 in small plates were again tested both parametrically and nonparametrically. A two-way classification analysis of variance was applied to the hatches of January and April 1977 in both media, and this showed differences significant at p = 0.01 between hatches but not between media. Subsequently, t-tests showed no significant differences between the two media taking each hatch in turn and this was supported by Wilcoxon tests. It may be noted that the hatches of January 1977 (N3 and S1) were derived from a different set of spherule strips for each medium, but from the same set for the April 1977 hatches (N4 and S2).

(d) Variation between large and small cultures.

The three large and twelve small culture results (times of M3) in the March 1978 hatch in N+C were compared by a t-test and the Wilcoxon test, the former showing a difference in means significant at p = 0.01 and the latter at between p = 0.02 and p = 0.05. It is not clear to what extent such a difference would be modified were

more data available; the only hatch used for more than three large cultures (August 1976) was not used for any small cultures, but a comparison of this with that of January 1977 (5 small cultures) can be made actually using intermitotic period as the assay, although both hatch and culture mode are variables. Both a t-test and a Wilcoxon test showed no significant difference between the two means, and this was paralleled by the results using time of M3.

Ignoring the small differences between hatches, and pooling all the available data for M3 in N+C, both a normal t-test and a Wilcoxon test for large sample sizes showed no significant difference at $p = 0.05$ between the mean value of 25.97 h for large cultures (13 values) and 25.10 h for small cultures (34 values), although, in the t-test, the difference was significant at $p = 0.1$.

(e) Summary.

Pooling the intermitotic period results obtained under all conditions for each medium, the following general characteristics for both media were obtained.

Medium	Mean IMP	95% confidence limits	Range (h)	S.D.	n
N+C	9.43	$\pm$0.32	9.11 - 9.75	0.615	17
SOYA DM	10.17	$\pm$1.06	9.11 - 11.23	1.147	7

As they were obtained assuming a normal distribution, the confidence limits are only approximate. Both a Wilcoxon and a t-test showed the difference between these means to be insignificant at $p = 0.05$, although in the latter the value of t_{calc} was very close to that of t_{tab} (2.059 and 2.074).

In summary it appears that small but significant variations in growth rate, as assayed by the time of M3, can occur between hatches, although it is likely that the absolute change in

intermitotic period is minimal. It is also possible that these differences may be wholly or partly due to undetected changes in experimental technique; they do not appear to correlate with the date of origin of the spherule strips. No significant difference either in the time of M3 or in intermitotic period between N+C and SOYA DM was detectable in contrast to their apparently different doubling rates in liquid culture, although SOYA DM appears to produce rather more variable cycle lengths than N+C. Large cultures appear to grow slightly more slowly in general than small cultures, but there was insufficient data to test this comparison fully.

The original reason for using SOYA DM was to induce long intermitotic periods in plasmodia that were, in contrast to previously used media giving low growth rates (e.g., defined medium), healthy and synchronous. The effect on the relative durations of S and G2 phase and on the possible induction of a G1 phase (Section 3.3.4.) could then be investigated. This approach had been suggested by the slow doubling rate observed in cultures of microplasmodia in this medium (Section 2.3., discussed further in Section 3.3.2.). As the above results demonstrate, this aim could not be realised but, as SOYA DM produced more variable intermitotic periods than N+C, it proved useful because periods occurred that were significantly longer than any in N+C. The range of normal cycle lengths over which the durations of S and G2 phase could be investigated was therefore extended.

(f) Time of M1 in SOYA DM.

The time after feeding of the first synchronous mitosis in SOYA DM was recorded in six separate experiments. The results, presented below, are in agreement with observations in other media that M1 occurs at slightly over half the normal length of an intermitotic period (Mohberg & Rusch, 1969). The high degree of variability exhibited is probably due to differences in the

condition of the inoculum and would occur equally in other media. (See Section 3.3.3.).

Culture	Hatch	Time of M1 (h after feeding)	
Small	Jan '77	5.25	Mean = 5.71 h; S.D. = 0.86
"	"	7.0	95% confidence limits = ± 0.91 h
"	"	5.3	Fraction of overall mean
"	Apr '77	5.5	intermitotic period for
"	"	4.7	SOYA DM = 56%; Coefficient of
Large	"	6.5	variation (CV) = 15.1%.

2.7. Effects of continued subculture on growth.

There have been a number of observations of changes in the properties of lines repeatedly cultured over a long period of time. For example, Daniel and Baldwin (1964) found that a subline grown on semi-defined medium for three years lost the ability to sporulate, formed spherules slowly and incompletely, and grew poorly on rolled oats. Different isolates from the same sclerotium developed variations in pigment production, sugar utilisation, spherule formation and sporulation ability after several years of transfer. During four years of cultivation, the subline M_3cIV lost the ability to complete meiosis (Mohberg & Rusch, 1971), thus forming spores with a diploid 4C DNA content rather than the normal 2C, and with a germination rate of only 0.1% (Mohberg et al., 1973). Chromosome spreads of metaphase nuclei of growing plasmodia suggested that the percentage of polyploid nuclei was larger in newly established lines than in those which had been growing in the laboratory for several years, although this was not reflected in a noticeably higher DNA content (Mohberg et al., 1973). In contrast, amoebae of the closely related slime mould Didymium nigripes appear to accumulate nuclei with higher than normal chromosome numbers

during serial subculture, but this was demonstrated with four different strains at four different 'ages' ranging from 9 months to 6½ years (Kerr, 1968).

More recently (Mohberg, 1977) it has been reported that, on several occasions, cultures of M_3 have been observed to drop to 40 chromosomes per nucleus (the normal diploid content is approximately 70) after continuous submersed culture for long periods of time (a year or more). Either a population of microplasmodia with small nuclei slowly outgrows those with diploid nuclei, or diploid nuclei lose chromosomes until they are haploid so that the nuclei with 50 - 55 chromosomes found in diploid plasmodia in a previous study (Mohberg et al., 1973) could be an intermediate in the transition. It has also been pointed out (Collins et al., 1978) that the high chromosome numbers and variability throughout the Myxomycetes are suggestive of natural polyploidy. Polyploids may be unstable during both mitosis and meiosis and this may lead to the observed variability and possibly to changes in chromosome number with time.

Increases in nuclear size and DNA content have been observed to occur over shorter periods of time in ageing Physarum repeatedly cultured on agar (McCullough et al., 1973). In this mode, growth is limited by a process of senescence which leads to death within a few weeks of onset (Poulter, 1969). The exact time of onset is dependent on the genotype of the strain, characteristic life spans ranging from a few weeks to several months (McCullough et al., 1973). In at least two strains, senescence was associated with an increasing proportion of grossly enlarged nuclei with a wide range of DNA contents varying from the normal level to many times that amount. However, the relation, if any, between this process of senescence observed specifically on agar and the occurrence of genetic variation during repeated subculture in liquid is not clear.

Since spherule strips are themselves derived from liquid cultures maintained for a finite period, as time goes on spherules will be prepared from effectively older cultures, perhaps as old as cultures which would have become senescent had they been grown on agar. The growth characteristics of three lines that had been subcultured as microplasmodia in N+C for 12, 6 and 1 month were therefore investigated. One culture of each was inoculated and the increase in wet weight against time measured (Fig. 2). The exponential sections of the curves show doubling rates of 11, 12 and 13 h for the 12, 6 and 1 month cultures respectively. Given the degree of variation in the measurements it is doubtful whether this is significant. All three cultures show little deviation from exponential until 100 mg ml^{-1}, although the oldest line appears to grow more slowly initially than either of the others.

As prolonged growth in liquid might cause adaptation to this mode, possibly with a concomitantly decreased ability to grow as surface culture, the growth properties of small surface plasmodia derived from these lines were also studied (Table 7). Although the lines were from different hatches, the experiment was concerned with changes in growth rate larger than those between hatches. Both a one-way classification analysis of variance and a Kruskal-Wallis test showed that all three means were significantly different from each other at a probability greater than 0.005. However, these differences are no larger than might occur between hatches or even between experiments, with the possible exception of the 6 month-old line, nor does the cycle length show a consistent increase or decrease with the 'age' of the culture. Nuclei, as seen in smears, appeared normal in all cultures, but plasmodia from the 12 month-old line were noticeably smaller than those from the most recent line (4 cm diameter, compared to 5 - 6 cm), with the 6 month-old line showing an intermediate size ($4\frac{1}{2}$ - 5 cm), although all three sets

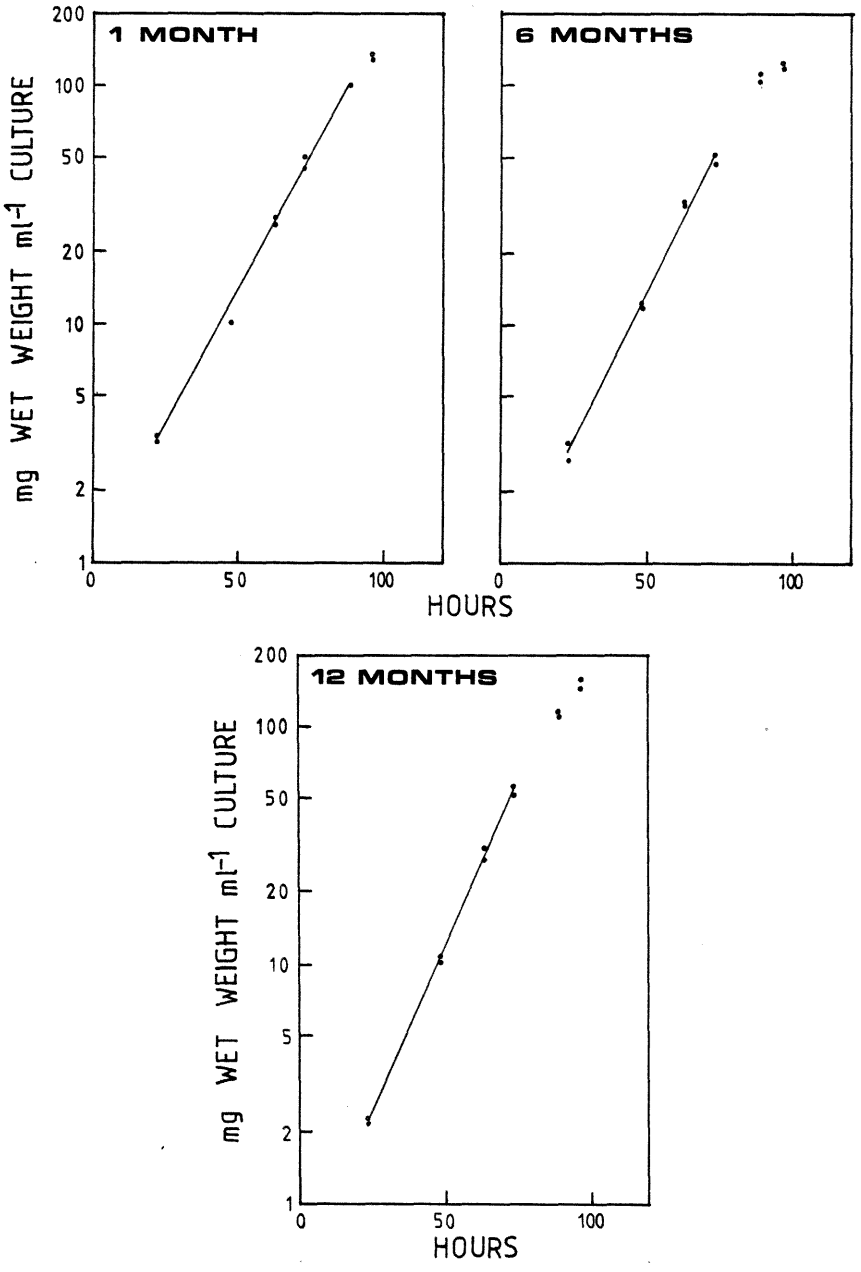

Fig. 2. Growth rate of 1, 6 and 12 month-old lines of microplasmodia. Cultures were inoculated at 0 h from lines subcultured in N+C for the periods shown. Cell density was determined by the wet weight method (Section 2.3.) and doubling rate calculated as for Fig. 1.

Table 7. Intermitotic period in plasmodia from culture lines of different ages.

Line	Plate	M2 (h,min after feeding).	M3 (h,min after feeding).	Intermitotic period (h,min)	Mean ± 95% lim
12 mth	1	14,10	23,30	9,20	
"	2	14,10	23,25	9,15	9,11
"	3	14,10	23,30	9,20	± 0,13
"	4	14,15	23,20	9,05	
"	5	14,20	23,15	8,55	
6 mth	1	14,00	22,03	8,03	
"	2	14,05	21,55	7,50	7,56
"	3	14,05	22,08	8,03	± 0,07
"	4	14,05	21,57	7,52	
"	5	14,05	22,00	7,55	
1 mth	1	14,50	23,41	8,51	
"	2	14,55	23,40	8,45	8,48
"	3	15,10	23,59	8,49	± 0,04
"	4	15,00	23,47	8,47	
"	5	15,05	23,52	8,47	

Small plate cultures in N+C were inoculated following the procedure outlined in Section 2.4.1. and the times of nuclear division determined as in Section 2.5. Cultures were refed after M2. Each line was derived from a different hatch as follows:

12 mth = April 1977; 6 mth = October 1977; 1 mth = March 1978.

appeared morphologically healthy.

Two experiments, one comparing intermitotic period (M2 - M3) in 2 week and 5 month-old lines in N+C and the other with lines of the same ages in SOYA DM, produced similar results i.e., each line gave a mean cycle length significantly different to its partner, but by no more than might be expected between experiments. In both cases the newer line gave the longer period and slightly larger plasmodia. In a further experiment, SOYA DM lines 1 and 4 months old were compared in small plates, together with cultures produced by mixing approximately equal quantities of microplasmodia from both lines immediately before inoculating the plates. In this case, the younger line consistently gave times shorter than the old at M1 and for the periods between M1 and M2 and between M2 and M3. The mixed line was more variable, but generally gave intermediate times except for one shorter than the old line and one longer than the new. Again the new line produced noticeably larger plasmodia than the old, with the mixed showing an intermediate size.

In summary, experiments in both N+C and SOYA DM show that, at least for lines up to 12 months old, the intermitotic period is changed no more than might be expected between different hatches and, possibly for this reason, there is no consistently reproducible correlation between the age of a line and its resulting intermitotic period. However, it was found that older lines tended to give smaller cultures than new, but further experiments would be necessary to see if this was a generally reproducible effect and to see whether more obvious changes in growth rate are observed with cultures over 12 months old. It should also prove possible, using the two-dimensional gel electrophoresis method currently employed in this laboratory, to discover whether chromosome loss in old culture lines (Mohberg, 1977) is associated with changes in individual proteins.

Chapter 3. Patterns of DNA synthesis.

3.1. Introduction.

This chapter presents the results of a number of experiments in which the isotope dilution method has been used to determine in detail the pattern of nuclear DNA synthesis throughout the mitotic cycle in N+C, SOYA DM and BP media. These are preceded by preliminary experiments in which the relative efficiencies of three ^{3}H-nucleosides in radioactively labelling DNA were determined, and by applications of the isotope dilution method to compare growth rate in liquid and surface culture and to measure the synthesis of macromolecules between inoculation and the first synchronous mitosis of a surface plasmodium.

In previously published reports, a number of different methods have been used to determine the pattern of DNA synthesis. Those following the incorporation of pulses of radioactive nucleosides into cold acid-insoluble material (Sachsenmaier, 1964; Braun et al., 1965; Braun & Wili, 1969) are open to the criticisms outlined in Chapter 1 as is the continuous-labelling method adopted by Nygaard et al. (1960). The latter was unusual in that the radioactive precursor used, [^{14}C]orotic acid, is not a nucleoside but a pyrimidine derivative (6-carboxyuracil) and a metabolic precursor of both UTP and CTP. None of the above methods enable the proportion of DNA replicated to be calculated, although measurements of DNA-thymine per unit dry weight (Nygaard et al., 1960) suggested that the DNA content did double.

The more direct method of chemically measuring the DNA content of a plasmodium has also been employed (Sachsenmaier & Rusch, 1964; Mohberg & Rusch, 1969) although, as samples were not normalised, this would be rather imprecise (see Section 1.3.). In one case (Mohberg & Rusch, 1969), DNA content did appear to double by 3 h after mitosis. A more sensitive fluorometric method was used by

Bovey and Ruch (1972) to measure the DNA content of individual nuclei and nucleoli, isolated throughout the cycle. This method avoids problems caused by differential yield of material and requires no normalisation. However, as samples were taken only hourly, the profile obtained contains very little detail, particularly during S phase.

A method closely related to isotope dilution (Mohberg, 1974) employed a [Me-^{3}H]thymidine prelabel to verify that specific activity fell to half during each cycle (i.e., the DNA content doubled) and that each cycle was divisible into one period exhibiting a rapid decline in specific activity followed by another with only a slow decline, if any. The isotope dilution method itself has been used once previously (Hall & Turnock, 1976), to give an outline of nuclear DNA accumulation between M2 and M3 in MyDM medium. However, as only six points were taken during the cycle, the profile obtained again shows little detail, particularly during S phase.

These published methods have all produced the same general outlines, that is, DNA synthesis begins immediately after mitosis with no detectable G1 phase; by the end of S phase, about 2 h later, the majority of synthesis is complete. In some reports a low level of synthesis or incorporation has also been detected during G2. In the early pulse-labelling experiments this is probably attributable to mitochondrial DNA synthesis, but the results of Bovey and Ruch (1972), and Hall and Turnock (1976) exclude cytoplasmic material, and yet the degree of 'G2-synthesis' appears to be in excess of that attributable to the nucleolar satellite. The results of all these experiments are summarised in Table 8 to give an indication of the variation among results although, as very different methods have been used and some values given are only approximations, no quantitative comparison will be attempted.

Report	DNA fraction	Length of cycle (h)	Length of S (min)	% synth. during S	% synth. during G2
1. Nygaard et al., 1960	Total	18.6	90	-	5?
2. Sachsenmaier, 1964	Total	8-10	180	94	6?
3. Braun et al., 1965	Total	8-10	120	-	low
4. Braun & Wili, 1969	Total	8-10	110	85	15
5. Sachsenmaier & Rusch, 1964	Total	9.5	90	-	-
6. Mohberg & Rusch, 1969	Total	8	120 - 180	-	low
7. Bovey & Ruch, 1972	Nuclear	9-10	180	90	10
8. Hall & Turnock, 1976	Nuclear	7.5	90	80	20

Table 8. Summary of published reports on cell cycle parameters in Physarum polycephalum.

Pulse-labelling followed by autoradiography (Braun et al., 1965) has demonstrated that, although all nuclei enter S phase simultaneously, they appear to complete replication at different times, many continuing well after the end of S phase as defined by other procedures. The very long S phase (5 - 6 h) found by Kuroiwa et al. (1978) is at least partially attributable to this phenomenon.

The aims in undertaking the experiments to be described were as follows.

(i) To determine more reliably and in greater detail than hitherto, the pattern of synthesis of nuclear DNA throughout the mitotic cycle.

(ii) To determine whether G2-synthesis is consistently found and what proportion of total synthesis it represents.

(iii) Although all published data show a doubling of DNA content between divisions, this has not always been found in this laboratory (Turnock, 1979 and see Section 3.4.3.). A further aim was, therefore, to discover whether the specific radioactivity of a culture would consistently fall to one half between successive mitoses.

(iv) As there has been no systematic study of the effect of different cycle lengths on the parameters of the mitotic cycle, it was intended to vary the length of the intermitotic period by the use of different media and to investigate the effect of this on the relative lengths of S and G2 phase and, in particular, to discover whether a G1 phase would be induced during long cycles.

3.2. Materials and methods.

3.2.1. Labelling conditions.

Except where stated, the radioactive isotope used was [6-^{3}H]thymidine (Amersham UK TRK.61; 20 - 30 Ci mmol^{-1}, aqueous solution). The following procedure for labelling was adopted primarily to minimise the concentration of nucleosides in the medium at the time of the addition of label (see Section 3.3.1.), and also

to add label as late as possible to maximise the specific activity obtained.

1) The wet weight per ml of a liquid culture was determined as described in Section 2.3. Assuming a doubling rate of 13 h, two cultures were inoculated to be at approximately 40 mg ml^{-1} after two or three days growth.

2) The wet weight of one culture was recorded while [6-^{3}H]thymidine (usually 100 uCi), with 1 uM carrier thymidine, was added to the other. After 5 - 7 h, during which time the nucleoside was taken up by the microplasmodia, the labelled culture was diluted to 10 mg ml^{-1} by using it to inoculate three or four new cultures.

3) After a 20 h chase period, microplasmodia were harvested (at approximately 30 mg ml^{-1}) and surface cultures inoculated as described in Section 2.4. Thus isotope had been diluted from the beginning of the 20 h chase period until the time the first sample was taken, usually around M2 or M3. This total period was sufficient to ensure that all isotope was chased into metabolically stable molecules, (see Section 3.3.1.).

3.2.2. Preparation of nuclei.

Nuclei were prepared from segments, 15 - 20 cm^{2} in area, of large surface plasmodia (approximately eight obtainable from one plasmodium), or from single surface plasmodia (excluding the inoculum centre) by a modification of procedure B of Mohberg and Rusch (1971) as follows.

1) Plasmodia were gently scraped into a 200 ml stainless steel MSE blender cup containing 40 ml of ice-cold homogenising medium (0.25M sucrose, 0.02M $CaCl_2.6H_2O$, 0.01M Tris, 0.1% Nonidet P40, pH 7.0 - 7.2). For interphase nuclei, an Ato-Mix blender, controlled by a 0 - 240V variable transformer, was run at 'low speed' for 15 s at 120V to break up large pieces of plasmodium, then at 'high speed' for 30 s at 200V. For nuclei undergoing mitosis, the blender

was run at 'high speed' for 30 s at 120V.

2) After standing the homogenate on ice for several minutes to allow some of the foam to settle, it was filtered through a triple milk filter (one 'Blow' milk filter medium sandwiched between two 'Johnson and Johnson' regal milk filters) clamped in a two-piece polyethylene Buchner funnel (7 cm diameter). The filters were saturated with 10 ml of homogenising medium before use.

3) Filters were rinsed with 10 ml of homogenising medium, and the total filtrate centrifuged at 300g (2000 rpm in the 4 x 50 ml rotor of an MSE Super Minor bench centrifuge) for 10 min in 50 ml conical-based glass centrifuge tubes.

4) The resulting pellet was resuspended in 40 ml of buffered sucrose (0.25M sucrose, 0.01M $CaCl_2.6H_2O$, 0.01M Tris, pH 7.0 - 7.2) by gentle whirlimixing, and recentrifuged as above. This was then repeated with a further 40 ml of buffered sucrose to obtain the final pellet which was either treated as in Section 3.2.4. or dissolved in a small volume of buffered sucrose and stored in liquid nitrogen for subsequent treatment as in Section 3.2.8.

The initial 50g centrifugation (Mohberg & Rusch, 1971) was omitted, as it led to the loss of nuclei in the initial pellet when it was generally necessary to conserve nuclear DNA.

3.2.3. Isolation of nuclei from microplasmodia.

These were prepared by an adaptation of the method of Mohberg and Rusch (1971). A 10 ml sample from a well-shaken culture of microplasmodia was pipetted into 150 ml of ice-cold medium (0.25M sucrose, 0.01M EDTA, 0.1% Nonidet P.40, pH 7.5) in a 1 litre glass blender cup. This mixture was stirred for 1 min at the lowest speed possible on the Ato-Mix blender, and then centrifuged at 300g (2000 rpm in bench MSE) for 4 min. The pellet was resuspended in 40 ml of the homogenising medium described in the previous section, and homogenised in the 200 ml blender cup for 40 s at 120V

at 'high speed'. After standing on ice to allow foam to settle, the suspension was filtered and centrifuged from this point on as described in the previous section.

3.2.4. Determination of specific radioactivity of DNA.

1) Nuclear pellets or segments of surface plasmodia of approximately 4 cm^2 area were decolourised in 5 ml of ice-cold 5% trichloroacetic acid (TCA) in a 1:1 mixture of acetone and water by being gently broken up with a plastic rod and left for approximately 30 min. The samples were then centrifuged at 300g (2000 rpm in MSE bench centrifuge) for 1 min. Plasmodial segments generally needed to be decolourised again, this time at room temperature, until the pellet was of a cream or white colour.

2) The pellet was dissolved in 4 ml of 0.3M NaOH and incubated at 37°C for 16 h to hydrolyse RNA (see Section 3.3.1.).

3) After cooling on ice, 2 mg of crystallised bovine serum albumin in 100 ul water were added as carrier to the samples originally derived from nuclear pellets, although none was necessary for samples from plasmodial segments. This was followed by 0.34 ml of 10M perchloric acid (PCA), to neutralise the sodium hydroxide and bring the acid concentration to 0.5M. The suspension was left on ice for 30 min, and then centrifuged at 750g (3000 rpm) for 10 min.

4) The pellet was washed twice with 5.0 ml of 0.5M PCA, resuspending with a plastic rod and centrifuging at 750g for 10 min.

5) The washed pellet was resuspended in a small volume, usually 1.2 ml, of 0.5M PCA, and incubated in a 70°C water bath for 40 min, with occasional mixing.

6) After cooling to room temperature, the suspension was centrifuged at 750g for 15 min, and the supernatant gently removed with a Pasteur pipette.

0.5 ml of this solution were used in the diphenylamine reaction, and duplicate 0.2 ml samples were counted in a

Packard 3385 or 3255 liquid scintillation spectrometer in the following manner: 0.2 ml sample, 0.8 ml 0.5M PCA and 4.0 ml toluene-based aqueous scintillant were placed in a plastic insert, mixed well and counted in a glass vial. This method did not allow dpm to be calculated. The scintillant had the following composition per litre: 667 ml A.R.Toluene, 333 ml Triton X-100 or Emulsifier Mix No.1 (a blend of phenoxy polyethoxyethanol surfactants by Fisons), 5.5 g PPO, 0.1 g dimethyl POPOP. In a scintillant : sample ratio of 4:1, a stable emulsion was formed.

3.2.5. Diphenylamine reaction.

The method followed was a modification of the procedure of Giles and Myers (1965) based on the diphenylamine method of estimation of DNA by Burton (1956).

The reagent was prepared as follows:
0.8 g of diphenylamine were dissolved in 20 ml glacial acetic acid to which 2 ml of 70% PCA were then added. Just before use, 1 ml of a 0.16% (w/v) aqueous solution of acetaldehyde was added. This was itself prepared by addition of 0.1 ml of acetaldehyde (at room temperature) to 50 ml of water.

0.6 ml of reagent were mixed with 0.5 ml of sample (consisting of an extract in 0.5M PCA), and the mixture was incubated at 30°C for 16 h. Samples were read at 595 nm in an SP500 spectrophotometer using a 1 ml glass cell of 1 cm light path.

An appropriate series of concentrations of 2-deoxy-D-ribose in 0.5M PCA were included to construct a standard curve for each experiment. Above 6 ug dR ml^{-1} this tended to deviate noticeably from linearity. A conversion factor of 4.58 was used to convert ug dR ml^{-1} to ug DNA ml^{-1} (Hall, 1975).

3.2.6. Determination of total nucleic acid specific activity in microplasmodia and surface plasmodia.

a) Microplasmodia: 5 ml suspensions were removed from a culture

of microplasmodia and centrifuged at 80g for 20 s. The cell pellets were decolourised in 2 x 5 ml of 5% TCA/acetone/water, centrifuging at 300g for 1 min each time. The decolourised pellets were frozen at -18°C in each case, so that all samples could be subsequently treated at the same time.

b) Surface plasmodia: segments of approximately 4 cm^2 area were decolourised in 2 x 5 ml of 5% TCA/acetone/water, and the pellets frozen at -18°C to be subsequently treated with those above.

1) The thawed pellets from both a) and b) were each resuspended in 5 ml of ice-cold 0.25M PCA and left to stand on ice for 15 min. They were then spun for 10 min at 2000g (2500 rpm in the 96 x 15 ml rotor of an MSE Mistral 6L centrifuge) at 2°C, and the supernatants discarded. The cold acid extraction was repeated with a further 5 ml of ice-cold 0.25M PCA, the samples centrifuged as above and the supernatant discarded.

2) Each pellet was resuspended in 4.0 ml of 0.5M PCA, incubated for 20 min at 70°C in a water-bath, and cooled to room temperature in an ice/water-bath.

3) The samples were then centrifuged for 10 min at 2000g, and the supernatants carefully transferred, using Pasteur pipettes, into pre-weighed conical disposable tubes.

4) The hot acid extraction was repeated with a further 4.0 ml of 0.5M PCA and the samples centrifuged as above. The second supernatants were added to the first, and sufficient 0.5M PCA added to bring the weight of each extract to 10.00 g. (This is equivalent to a volume, at room temperature, of 9.77 ml).

5) Triplicate 1000 ul samples from each extract were counted in the aqueous scintillation system described above, while duplicate 100 ul aliquots from each extract were diluted 20-fold in 0.5M PCA and read at 260 nm in an SP500 spectrophotometer, using a 3 ml quartz cuvette with path length of 1 cm.

3.2.7. Distribution of radioactivity in microplasmodia.

Cultures of microplasmodia were harvested when they had reached the point at which they would normally be used to inoculate surface plasmodia. To assay each fraction in triplicate, twelve 2 ml samples were removed, while the cell suspension was kept well-mixed with a magnetic stirrer in order to ensure all samples were comparable. Samples were numbered from 1 to 12.

a) Radioactivity in medium. Samples 1-3.

These were centrifuged at 300g (2000 rpm in MSE bench centrifuge) for 30 s. A 50 ul aliquot was taken from each supernatant and each absorbed onto a small filter paper square (approximately 2 cm square) which was burnt in a Packard Tri-Carb sample oxidiser. The $^{3}H_2O$ so formed was counted in a Packard 3385 or 3215 liquid scintillation spectrometer in 10 ml of the Monophase 40 scintillant system. Use of the external standard ratio allowed the ^{3}H dpm to be calculated for this system, since neither inserts nor membranes were used during counting.

b) Total radioactivity in microplasmodia. Samples 1-3.

The microplasmodial pellets from a) were each resuspended in 5 ml of cold growth medium and filtered immediately through a Sartorius cellulose nitrate membrane filter (2.5 cm diameter, 0.45 mm pore size). Filters were washed with a further 5 ml of cold medium, oxidised as above, and the samples counted.

c) Radioactivity insoluble in cold acid, (RNA, DNA, protein). Samples 4-6.

The samples were centrifuged at 750g (3000 rpm in MSE bench centrifuge) to remove medium and resuspended in 5 ml cold 0.5M perchloric acid and kept on ice for 30 min. They were then recentrifuged at 750g for 5 min, resuspended in a further 5 ml of 0.5M perchloric acid, filtered as above with each filter washed in 2 x 4 ml of cold 0.5M perchloric acid, oxidised and the samples

counted.

d) Radioactivity insoluble in hot acid, (protein). Samples 7-9.

Medium was removed from each sample and the pellets washed with 5 ml of 0.5M perchloric acid as in c) above. Pellets were then resuspended in a further 5 ml of 0.5M perchloric acid and incubated for 20 min at 70°C, with occasional mixing. Samples were recentrifuged at 750g for 5 min, supernatants discarded, and the incubation repeated with a further 5 ml of 0.5M perchloric acid. After recentrifugation the samples were filtered, oxidised and counted.

e) Radioactivity insoluble in cold acid after alkaline hydrolysis, (DNA and protein). Samples 10-12.

Medium was removed, and the samples washed as in c) above. Each pellet was then dissolved in 2 ml of 0.3M NaOH and incubated at 37°C for 16 h to hydrolyse RNA. After cooling, 0.17 ml of 10M perchloric acid was added to each, to bring the acid concentration to 0.5M, followed by 5 mg of crystallised bovine serum albumin (BSA) in 100 ul water to act as carrier. The sample was left on ice for 30 min, then filtered, oxidised and counted as in c) above.

The fractions of total cold acid-insoluble label in protein, RNA and DNA are determined as follows.

Protein = d/c.

RNA = (c - e)/c.

DNA = (e - d)/c.

3.2.8. Distribution of label in isolated nuclei.

Each assay was usually performed in triplicate. Therefore, 9 x 100 ul samples were removed from a concentrated suspension of nuclei in buffered sucrose. For the cold acid-insoluble and hot acid-insoluble determinations each sample was made up to 1.0 ml with distilled water.

a) Radioactivity insoluble in cold acid (RNA, DNA and protein) Samples 1-3.

To each of the three 1.0 ml samples of diluted nuclei was added 1.0 ml of cold 1.0M PCA. 2 mg BSA (crystalline) in 50 ul water were added as carrier, and the samples were left on ice for 30 min, before filtering through membrane filters which were then washed with 2 x 4 ml 0.5M PCA. Filters were oxidised and counted as previously described.

b) Radioactivity insoluble in hot acid, (protein). Samples 4-6.

To each of three 1.0 ml samples of diluted nuclei was added 1.0 ml of 1.0M PCA. 2 mg BSA (crystalline) in 50 ul water were added as carrier and the samples incubated at 70°C for 40 min with occasional mixing. After cooling to room temperature, the samples were filtered and processed as above.

c) Radioactivity insoluble in cold acid after alkaline hydrolysis, (DNA and protein). Samples 7-9.

To each of three undiluted samples of nuclei was added 2 ml of 0.3M NaOH. These were incubated at 37°C for 16 h and cooled on ice. After addition of 2 mg BSA as carrier, 0.17 ml of 10M PCA were added to bring the acid concentration to 0.5M, and the samples were left on ice for 30 min. They were then filtered and processed as above.

Calculations of the distribution of radioactivity were as outlined in the previous section.

3.2.9. Distribution of label in surface plasmodia.

Two plasmodia in the interphase after M2 were homogenised in 10 ml of 5% TCA/acetone/water, and centrifuged at 300g for 5 min. This was repeated until the pellet was decolourised. It was then washed in 0.5M PCA and the resulting pellet resuspended in 4.5 ml of 0.5M PCA. This was divided into 9 x 500 ul aliquots, of which three were recentrifuged to remove PCA and the pellets redissolved

in 2 ml of 0.3M NaOH, while the remainder were made up to 3 ml with 0.5M PCA. Samples were then processed as in the previous section except that (i) no carrier was added, and (ii) two hot acid extractions were included.

3.3. Results.

3.3.1. Comparison of radioactive precursors of DNA.

Nucleosides are readily taken up by microplasmodia, but the proportion that actually enters cold acid-insoluble material is low, because they are extensively catabolised (Fink & Nygaard, 1978). Although thymidine might be considered the ideal precursor for DNA, its incorporation into whole microplasmodia is actually substantially less than that of other nucleosides because it is subject to even greater catabolism. In the mycological peptone-based medium, MyDM, up to 80% of the radioactivity of [2-^{14}C]thymidine (10 uM) had been lost as $^{14}CO_2$ by 24 h (G. Turnock, unpublished observation), although the loss was somewhat reduced at lower concentrations and, for this reason, carrier concentrations of 1 uM have been adopted. In N+C under the same conditions, [2-^{14}C]-thymidine at 10 uM, only 1% of radioactivity was found in acid-insoluble precipitate after 40 min, while 31% had been catabolised to $^{14}CO_2$ (Fink & Nygaard, 1978).

The problem of efficient uptake in N+C was aggravated by an apparently high endogenous concentration of nucleosides in the medium. As there is a common uptake system for nucleosides in Physarum (Birch & Turnock, 1976), these endogenous components effectively diluted radioactive label by competitively inhibiting its uptake and so dramatically slowed its rate of entry into microplasmodia. There was no corresponding evidence for such a high concentration in MyDM. When labelling RNA in MyDM for use in isotope dilution experiments, the procedure that had been followed

was simply to inoculate 12 - 15 ml of an exponentially growing culture of microplasmodia into each of three flasks containing 100 ml of medium with 50 uM [5,6-^{3}H]uridine at 0.5 uCi ml^{-1}. Labelling of nucleic acid would then occur for only 2 - 3 h, the rest of the 24 h before inoculating surface plasmodia effectively constituting a period during which label was chased into stable macromolecular species (Hall & Turnock, 1976). This procedure was repeated using 50 uCi [6-^{3}H]thymidine in 100 ml N+C medium with no added carrier thymidine. The incorporation of label into whole cells and into cold acid-insoluble material was followed over a period of 8 h with a further sample at 24 h (Fig. 3). It can be seen that uptake into nucleic acid continued for well over 8 h. A similar experiment with [5,6-^{3}H]uridine in N+C showed a steady uptake over the period 14 - 22 h after inoculation/labelling (K. Fink, personal communication), in contrast to MyDM in which no incorporation between 16 and 25 h was detected (Hall, 1975).

As these results, together with others, provided evidence for a high concentration of nucleosides in N+C medium, the labelling procedure outlined in Section 3.2.1. was adopted. Growth for 2 - 3 days did indeed deplete the medium of nucleosides before addition of label (K. Fink, personal communication) and, although it became necessary to dilute the culture, and thus grow for slightly longer after labelling than previously, the final specific radioactivity of DNA was nevertheless increased.

This did not overcome the problem of the high catabolism of thymidine by Physarum, which may have been increased in N+C due to the induction of catabolic enzymes by the high endogenous concentration of nucleosides. Two alternative precursors were therefore investigated. [5,6-^{3}H]uridine (Amersham, TRK.410, 40 - 60 Ci mmol^{-1}, aqueous solution) was chosen as a representative pyrimidine ribonucleoside in terms of its uptake and subsequent

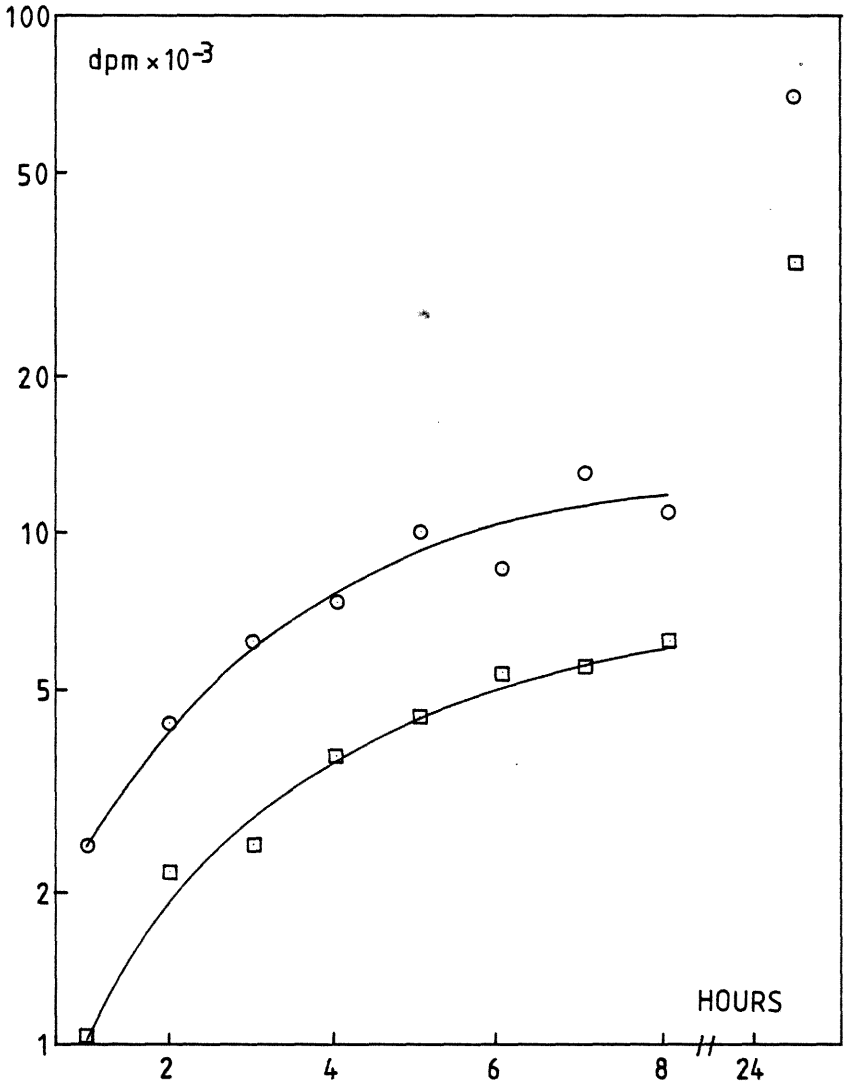

Fig. 3. Incorporation of [6-^{3}H]thymidine by microplasmodia in N+C.

10 ml of a moderately thick microplasmodial culture were inoculated into 50 ml of N+C medium containing 50 uCi [6-^{3}H]thymidine with no added carrier. At hourly intervals 2 ml aliquots were removed and the radioactivity incorporated into whole cells (O) and cold acid-insoluble material (□) determined as in parts b) and c) respectively of Section 3.2.7.

distribution (Fink & Nygaard, 1978), and because a good deal is known about its uptake and metabolism (Birch & Turnock, 1976; Fink & Nygaard, 1978). Deoxy[G-^{3}H]adenosine (Amersham, TRK.78, 10 - 20 Ci mmol^{-1}, aqueous solution) was tested because the uptake and subsequent distribution of deoxyadenosine appear to be typical of the deoxyribonucleosides other than thymidine (K. Fink, unpublished results) and it had been claimed, albeit with few available details, that 84% of its radioactivity entered DNA in Physarum (Evans, 1972, quoted by Mohberg, 1974).

Although determinations of the distribution of label in nuclei from surface plasmodia were subject to some variation, it was apparent that most of the label from [^{3}H]uridine was to be found in RNA, while with thymidine most was in DNA (Table 9. Expt A). There was no apparent improvement in the specific activity obtained.

The distributions of label from [^{3}H]deoxyadenosine and [^{3}H]thymidine were determined twenty-four hours after dilution (Table 9. Expt B). In both cases approximately 30% of the radioactivity was found in the microplasmodia (70% in the medium), and 15 - 20% of the total radioactivity was in cold acid-insoluble material.

These results are in qualitative agreement with those of Fink (Table 9), obtained after only 40 min with ^{14}C-nucleosides at 10 uM; using deoxyadenosine (K. Fink, unpublished results) most label was to be found in RNA, while with thymidine (Fink & Nygaard, 1978), the distribution showed greater relative incorporation into DNA. However, the differences between the two patterns of distribution were more extreme than those of experiment B.

In surface plasmodia prepared from the above microplasmodia, the distributions as percentages of total plasmodial cold acid-insoluble material were relatively unchanged (Table 9. Expt B).

The relative specific activity of DNA in nuclei isolated from

Table 9. Comparison of macromolecular distribution of radioactive DNA precursors.

Expt.	Precursor	Mode of growth	Fraction	Distribution (% cold acid-insol. material		
				Protein	RNA	DNA
A	[5,6-^{3}H]UR	Surface	Nuclei	15	70	14
A	[6-^{3}H]TdR	Surface	Nuclei	21	27	52
B	[G-^{3}H]dA	Microplasmodia	Total	7	80	13
B	[6-^{3}H]TdR	Microplasmodia	Total	12	51	37
B	[G-^{3}H]dA	Surface	Total	7	77	16
B	[6-^{3}H]TdR	Surface	Total	16	37	47
Fink	[8-^{14}C]dA	Microplasmodia	Total	4	91	5
Fink	[2-^{14}C]TdR	Microplasmodia	Total	29	19	52

In all experiments, addition of radioactive precursor was to a culture of microplasmodia in N+C medium and the distribution determined in either a liquid or surface culture derived from it. The procedures employed are outlined in Sections 3.2.7., 3.2.8., and 3.2.9.

UR = uridine; TdR = thymidine; dA = deoxyadenosine.

these plasmodia was 6.5 cpm ug^{-1} uCi^{-1} for [^{3}H]deoxyadenosine and 2.6 cpm ug^{-1} uCi^{-1} for [^{3}H]thymidine, a 2.5-fold higher value for deoxyadenosine. However, as deoxy[G-^{3}H]adenosine was also 2.5 times more expensive than [6-^{3}H]thymidine, appeared to give a less favourable distribution with respect to DNA and had been used much less in the literature, it was decided to continue using [6-^{3}H]-thymidine.

Since a significant proportion of the label is present in species other than DNA in both surface plasmodia and their nuclei, it was considered necessary, whenever obtaining either the amount of radioactivity in DNA or its specific activity, to remove RNA by an alkaline hydrolysis step, and to avoid protein contamination by solubilising DNA into hot perchloric acid (Section 3.2.4.). A straightforward determination of cold acid-insoluble radioactivity, as has been used in several pulse-labelling experiments reported in the literature, might lead to serious inaccuracies, although it has not been determined whether the distribution of label from [^{3}H]thymidine after short pulses is different to that found above after long exposures. After 10 min pulses with [^{3}H]thymidine, Braun et al. (1965) found that ribonuclease treatment of the acid-insoluble fraction removed less than 0.5% of the incorporated label.

That a significant proportion of radioactivity from [6-^{3}H]-thymidine enters cytoplasmic components was demonstrated by an experiment assaying the loss of label at each stage of the preparation of nuclei. The loss between the total homogenate stage after filtration and the initial unwashed pellet is in excess of the loss of DNA throughout the whole procedure as assayed chemically by the diphenylamine reaction, which shows a yield of approximately 45%.

3.3.2. Change in growth rate between liquid and solid culture.

The results of Sections 2.3. and 2.6. suggest there is a large change in doubling time between liquid culture (as measured

by wet weight) and surface culture (measured by intermitotic period); in SOYA DM the intermitotic period (10.2 h) is only approximately 55% of the doubling time in liquid (19 h), and 75% in N+C (9.4 h compared with 13 h), although if the doubling time in liquid culture in SOYA DM is 14.5 h (Section 2.3.), then the time in surface culture is 70% of this value. As this difference might be caused by the use of different assay systems for the two modes of growth, an isotope dilution method was applied in order to measure the rate of synthesis of total nucleic acid in both microplasmodia and the surface cultures derived from these. A 50 ml culture in SOYA DM was labelled with 200 uCi of [5,6-^{3}H]uridine (10 uM) and diluted, to a slightly greater extent than usual, to inoculate six 50 ml lots of medium. Two of these were subsequently used for wet weight and specific activity determinations, while the remaining four were grown for a further two days (one day more than usual), before inoculating a large culture which was sampled at M1, M2 and M3.

The results are illustrated in Fig. 4. In this case, the wet weight values give a doubling time of approximately 14 h, although the points show a good deal of scatter. During the exponential part of the isotope dilution curve the doubling time is 11.9 h, while in the surface culture formed from these it is only 8.6 h. This latter observation correlates well with the observed intermitotic periods: M1 - M2 = 8.9 h; M2 - M3 = 8.7 h. The doubling time in surface cultures thus fell to 72% of that in microplasmodia. Another determination using isotope dilution of total nucleic acid in liquid culture in SOYA DM gave a doubling time of 16.4 h (B. Birch, personal communication). It thus appears that, in SOYA DM, a large degree of variability in the growth rate of microplasmodia can occur. This does not seem to correlate with the initial inoculum concentration, since the culture showing 19 h doubling time

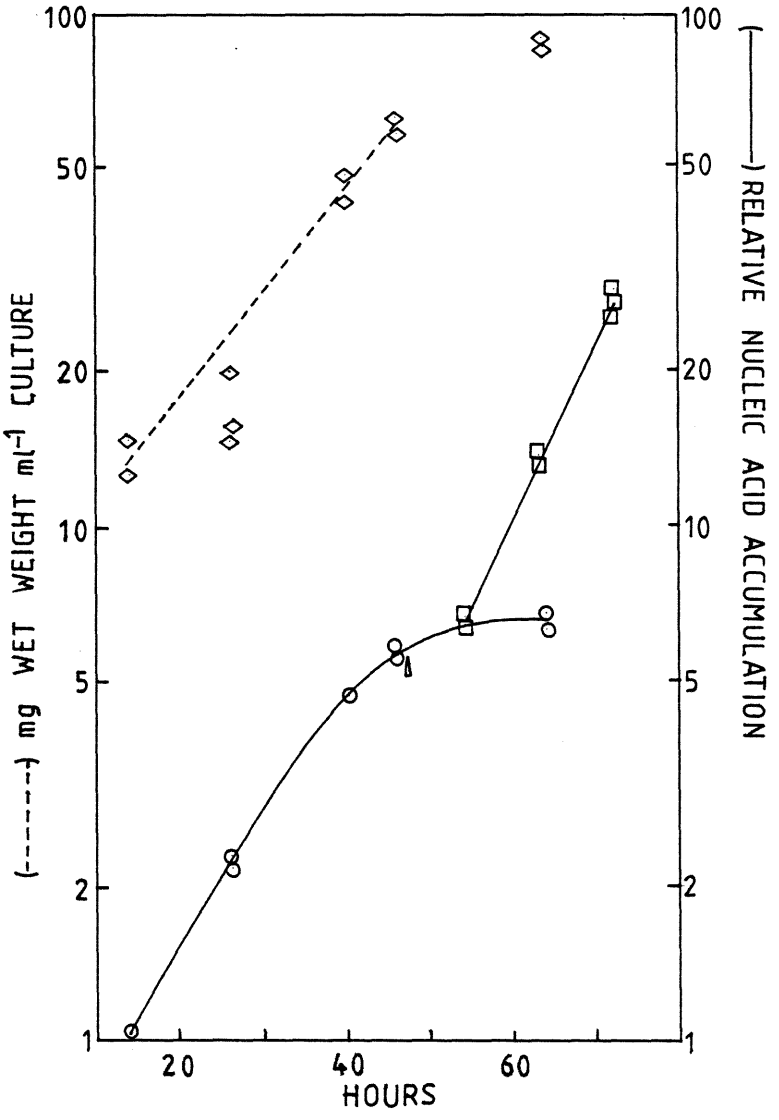

Fig. 4. Growth rate of microplasmodia and subsequent surface culture.

A 3 day-old liquid culture in SOYA DM was labelled with 500 uCi [5,6-^{3}H]uridine (10 uM) for 7 h. 15 ml inoculated a second culture which, after a 24 h chase, inoculated 6 cultures at time 0 h. In two of these mg wet weight ml^{-1} (◇) and nucleic acid accumulation (○) were determined (Sections 2.3. and 3.2.6.). The other four inoculated a large surface culture at the time indicated (△) in which nucleic acid accumulation (□) was determined at M1, M2 and M3.

(Section 2.3.) was inoculated at 10 mg ml^{-1}, that with 16 h was inoculated at 2 mg ml^{-1} and that with 11.9 h at 4 mg ml^{-1}.

It is not clear whether doubling times determined by the wet weight method are systematically different from those obtained by isotope dilution, although it is probable that the former is subject to more sources of variation. For example, when measuring cell density, some error is introduced because slime is not completely removed from the pellet, the proportion remaining probably varying between samples. Furthermore, if slime is produced proportionally to surface area, a change in the mean volume of microplasmodia with cell density will alter the amount of slime per unit of plasmodial material, an effect observed by Sudbery (1974) when measuring the dry weight of whole surface plasmodia. After completing exponential phase, the amount of slime in a plasmodial culture appears to markedly increase, making measurement of high cell densities difficult. It is unknown whether a low growth rate in liquid is reflected in surface cultures derived from it, but it at least seems certain that there is a marked shift in growth rate during the change from growth in liquid to solid mode.

Wright and Tollon (1978) have also observed that, using a range of growth temperatures, the average generation times (measured by intermitotic period) of macroplasmodia grown on SDM agar are shorter and less different from one another than the generation times determined in liquid culture by the variation in optical density at 546 nm. Thus, at 22, 30 and 32°C the average intermitotic periods (M3 - M4) of surface cultures were 11, 13.5 and 14 h respectively, while doubling times in liquid culture were 14, 15 and 23 h.

3.3.3. Macromolecular synthesis between inoculation and M1.

Before discussing the synthesis of DNA between successive synchronous divisions, the accumulation of DNA, nucleic acid and

protein between inoculation and the first postfusion mitosis will be outlined. The use of the isotope dilution technique in this case has the advantage over pulse and continuous-labelling methods that it is possible to express the amount of synthesis that takes place as a fraction of that normally occurring between two successive nuclear divisions.

Cultures were prelabelled by the usual procedure (Section 3.2.1.) with 200 uCi [5,6- H]uridine (10 uM) except that a longer chase-period was employed. One hour before inoculation of surface cultures 10 ml samples, from which nuclei were subsequently isolated, were withdrawn from three out of four cultures, while a 5 ml sample was withdrawn from each culture and the pellet decolourised and frozen. At M1, three surface cultures were pooled for each of two preparations of nuclei, while three other cultures were separately decolourised and frozen. These decolourised, frozen samples were subsequently used for the determination of both total nucleic acid (mainly RNA) and protein accumulation (B. Birch, personal communication) by the isotope dilution method (Birch & Turnock, 1977), and the nuclei were used for DNA accumulation determinations. The following results were obtained, with specific activities normalised to 1.000 in the microplasmodial samples.

Fraction	Microplasmodia			M1			Inoc. to M1
	Relative accum.	S.E.	n	Relative accum.	S.E.	n	(% normal synthesis)
DNA	1.000	0.04	3	1.100	0.02	2	10
Nucleic acid	1.000	0.03	4	1.433	0.03	3	43
Protein	1.000	0.03	4	1.656	0.03	3	66

The values in the final column represent the amount of synthesis between inoculation and M1 expressed as a percentage of the

synthesis expected between two successive mitoses, assuming each species would double in the latter case.

The relative values of DNA, nucleic acid and protein are qualitatively as expected, if it is assumed that the population of nuclei in the inoculum is ~~random~~ non-synchronous and that those early in the cycle at the time of fusion progress quickly to division while those at more advanced stages progress more slowly. Thus, the amount of DNA synthesised is low because, as it is mainly replicated early in the cycle, the majority of nuclei at fusion will already have the 4C content. Between two synchronous mitoses, nucleic acid and protein, as measured by the isotope dilution method (Birch & Turnock, 1977), both show continual synthesis with the rate increasing towards the end of the cycle, an effect more pronounced in the protein accumulation curve. (See Section 1.4.). It is therefore to be expected that both will show substantial synthesis before M1, probably with relatively more protein than nucleic acid accumulated.

It may further be expected that nucleic acid and protein should both show more than 50% of normal synthesis before M1 since the average age of a nucleus in a random population will be less than 0.5 cycles, and because both show an increase of rate late in the cycle. This would not, however, be necessary if there were a change in the ratios of RNA:DNA:protein between liquid and surface culture, possibly related to the apparent change in growth rate (Section 3.3.2). Mohberg and Rusch (1969) consistently observed a somewhat larger DNA:protein ratio in petri dish plasmodia than in rocker cultures, i.e. between two different states of the surface mode of growth, while Plaut and Turnock (1975) demonstrated changes in the ratios of RNA:protein and RNA:DNA within microplasmodia at different growth rates.

However, it is apparent that, in the period during and soon after inoculation, other unknown effects are involved that can lead

to large variations between cultures, such as the differences in the times of M1 recorded in several experiments in SOYA DM (Section 2.6.). In the results presented in the previous section (3.3.2.), an increase of total nucleic acid between inoculation and M1 of only 16% of normal was recorded, while Mohberg and Rusch (1969), using the diphenylamine assay alone, in some experiments recorded an increase in DNA during the period before M1 while in others found either no increase or one of only about 10%. It was suggested that the inoculum transfer schedule sometimes gives partial synchrony of the microplasmodial cultures so that they are mostly in G2 phase at the time they are used to initiate surface plasmodia. Further potential explanations are (i) that the relative proportions of cells at different points in the cycle may alter with the density of a microplasmodial culture, and (ii) that at M1, when plasmodia are small, the possibility of slight contamination with inoculum material cannot be excluded. The mechanism of the induction of synchrony in a macroplasmodium is, however, an important question, and some of the events of the crucial period from inoculation to the first synchronous mitosis would be accessible to more detailed studies using isotope dilution.

3.3.4. Synthesis of DNA during the mitotic cycle.

Experiments were carried out using either one large culture or several small plate cultures. The latter had the advantage that more samples could be taken, a large culture between M2 and M3 containing material sufficient for only approximately eight samples. Unfortunately, this must be weighed against the disadvantage that separate plates do not enter mitosis simultaneously, often exhibiting a variation of up to 45 min, so that a detailed analysis of the DNA synthesis profile obtained from several plates might be impossible. Although a large culture can display a degree of asynchrony, this was found to be generally less than that between

separate plates. However, at the same point in the cycle, there was no significant difference in specific radioactivity amongst plates from the same initial inoculating pellet.

Such imprecisions of synchrony presented the practical difficulty of accurately determining the time of mitosis in all plates or at many points of a large plasmodium, while also preparing nuclei; furthermore, it is impossible to determine precisely the average time of mitosis in a segment of a large plasmodium that is slightly asynchronous. For these reasons, in all graphs presented the time of each sample has been expressed relative to metaphase in the sample isolated closest to division, rather than to its own metaphase which may not have been determined. During the early stages after mitosis, samples were usually taken from those plates or areas of the culture passing through division at times close to that of the specimen used to define metaphase. In this way, errors in the determination of the shape of the S phase curve would he reduced, the greatest errors being concentrated in the relatively unchanging G2 phase. This partially avoided the problem of investigating synthesis over periods of time of the same order as the variation in mitosis either within a culture or between plates.

In N+C and SOYA DM, the experiments describe the synthesis of nuclear DNA between M2 and M3 unless otherwise stated.

a) N+C: large cultures.

Figs. 5 and 6 show results obtained in two experiments, with one large culture used in each. The time axis may be expressed as either 'minutes after metaphase' or '% of the observed intermitotic period'. However, as the latter requires the time of M3 to be determined in a plate or segment which was not used to define M2, the intermitotic period may be somewhat in error, particularly when using small plates. In some cases, the time of the subsequent division was not recorded at all. For these reasons, the abscissa

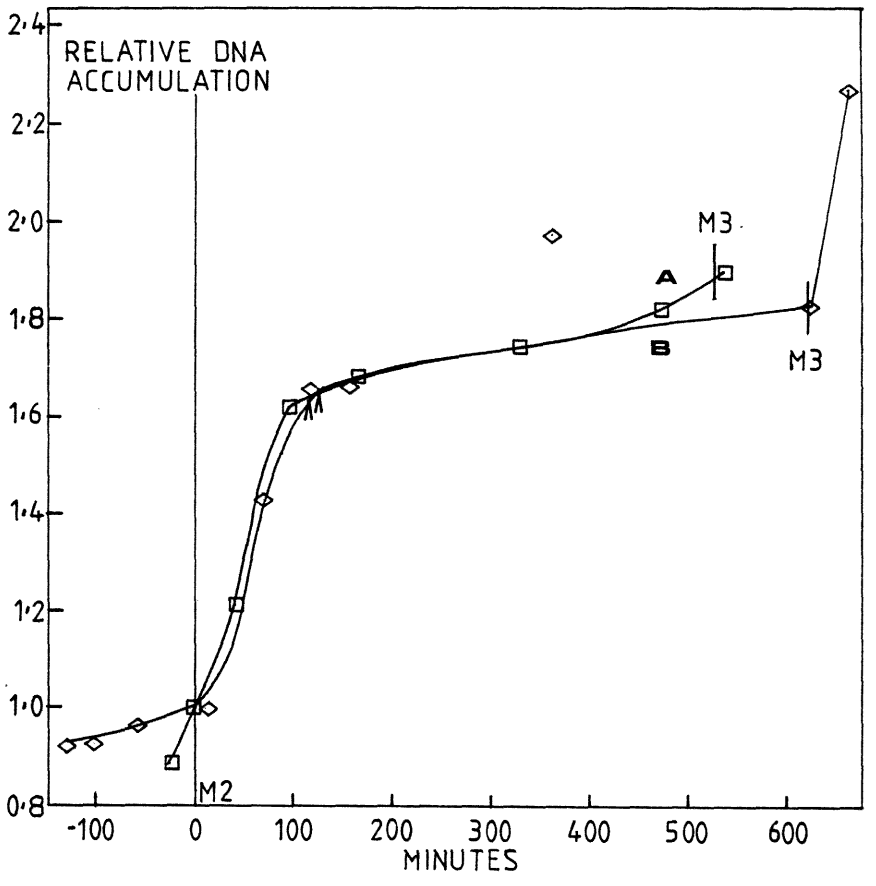

Fig. 5. Nuclear DNA synthesis between M2 and M3 in large surface cultures in N+C (1).

A culture of microplasmodia was labelled with 100 uCi [6-^{3}H] thymidine (1 uM) and diluted as in Section 3.2.1. Large surface cultures were inoculated (Section 2.4.2.a), nuclei were isolated (Section 3.2.2.) and the specific activity of nuclear DNA was determined (Section 3.2.4.). The latter values were normalised to 1.00 at M2 for both experiment A (□) and B (◇). The time of M3 in each case is indicated by a vertical bar and the end of S phase, estimated as the mid-point of the ~~inflexion~~ change between the slopes of S and G2 phase, by ∧. Values on the time axis are relative to M2.

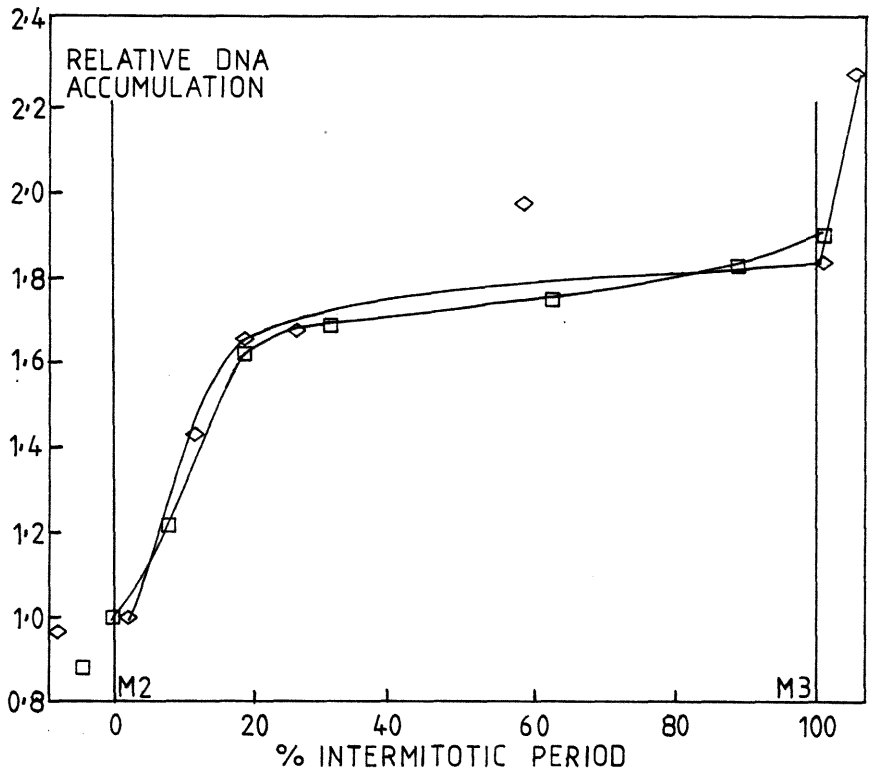

Fig. 6. Nuclear DNA synthesis between M2 and M3 in large surface cultures in N+C (2).

Experimental details as for Fig. 5. For both experiment A (□) and B (◇) values on the time axis are expressed as a fraction of the observed interval between M2 and M3. As the latter therefore coincides for both experiments, it is indicated by a vertical single line. As for Fig. 5., DNA specific activity values are normalised to 1.00 at M2.

in the remaining figures will be expressed as 'minutes after M2'. A comparison of Figs. 5 and 6 shows that the difference between the graphs are slight, although these will depend on the similarity of the cycle length in the experiments involved.

The detailed characteristics of all curves will be discussed in g) below, but it might be noted that those of Figs. 5 and 6 are qualitatively similar to the published results discussed in Section 3.1., i.e. there was a rapid synthesis of DNA beginning immediately after mitosis, demonstrating the absence of a G1 phase, and this was followed by synthesis at a reduced rate until the end of the cycle. This latter phenomenon will be discussed in Section 3.4.2. The quantity of DNA did not double between divisions suggesting an absence of balanced growth, a problem to be discussed in Section 3.4.3. The apparent amount of synthesis at all times is dependent upon the specific activity recorded at M2, so that it was important to take a number of samples before or at M2 to ensure that this value would not be seriously in error. Nevertheless, the final value for the percentage of DNA synthesis between M2 and M3 is subject to error in the specific activity values of both mitoses.

For the analysis in g) below, the end of S phase was estimated as the mid-point of the ~~inflexion~~ change between the gradients of S and G2 and has been indicated by a vertical arrow for each profile. The method of estimation by extrapolation of both gradients and finding the point of intersection was not used because, in most cases, the gradients were not constant.

b) N+C: small cultures.

As in the previous section, two experiments are illustrated (Fig. 7), but in this case each point represents a single small plate culture. Neither set of points is as regular as those obtained with a large culture, particularly the curve representing experiment B in which the plates showed a greater spread in the

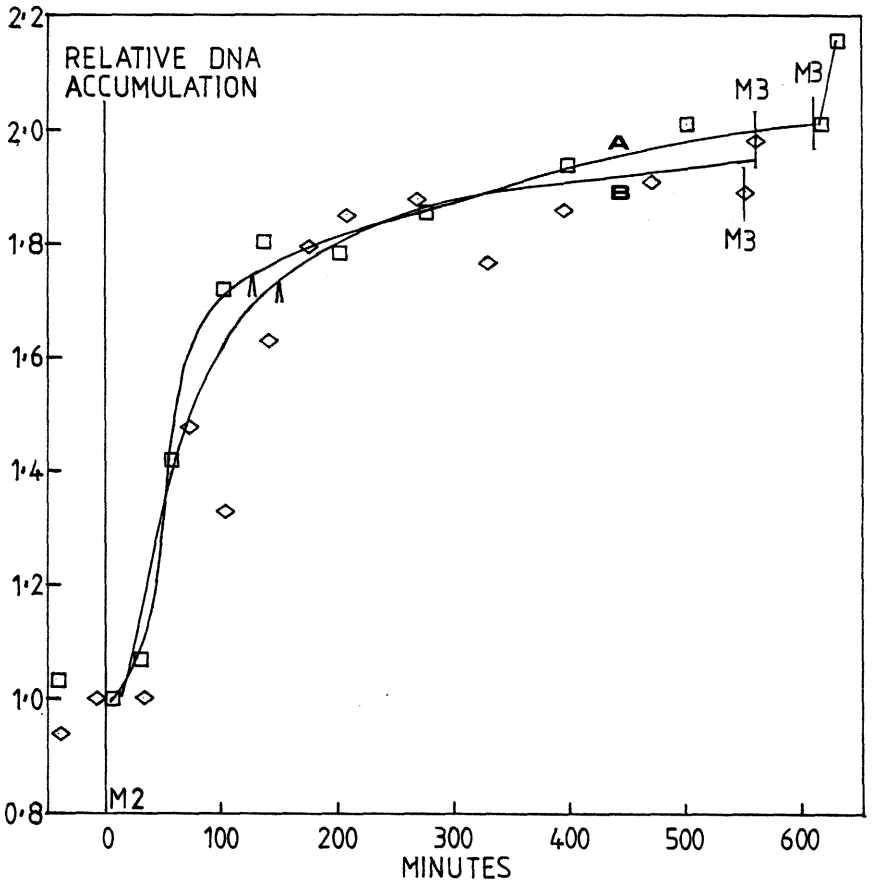

Fig. 7. Nuclear DNA synthesis between M2 and M3 in small plate cultures in N+C.

Small plate cultures were established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.1. Nuclei were prepared (Section 3.2.2.) from cultures taken between M2 and M3, and the specific radioactivity of DNA in each sample determined (Section 3.2.4.). DNA accumulation is plotted as a function of time after M2; the amount of DNA at M2 was normalised to 1.00 for both experiment A (□) and B (◇). The time of M3 is indicated in each case by a vertical bar and the end of S phase by Λ.

times of mitosis than in experiment A (60 min compared to 15 min). Such lack of complete synchrony can produce samples well outside the estimated curve (for example, that at 100 min was from a culture observed to be at a stage approximately 30 min behind most others) and, more importantly, can produce a flattened curve for which it is not possible to estimate the end of S phase with any degree of accuracy. M3 was reached at different times in two plates, one showing 89% synthesis and the other 98%. If such variation was general, this would contribute an extra element of error to the curves, since plates at the same stage could have significantly different specific activities, but no further evidence for this was found.

c) N+C: large culture. S phase.

Fig. 8 shows the accumulation of DNA during S phase in more detail. The stages of nuclear division as seen with phase contrast microscopy are superimposed over the time axis. Very early and early reconstruction were empirically useful distinctions; in the first, the nucleus has divided but shows no internal structure and the two daughter nuclei are still in close proximity, while in the second, the nucleolus appears as a large number of small granules. Mid-reconstruction is taken to begin at about 25 min after metaphase when the nucleolus begins to aggregate into a small number of particles. The transition from telophase to very early reconstruction takes place at approximately 10 min after mid-metaphase.

To fully establish the absence of a G1 phase, it would be necessary to sample at intervals impossibly short both in terms of the experimental manoeuvres required and because the isotope dilution method is not sufficiently sensitive to distinguish between the specific activities of samples taken at times closer together than about 10 min during S phase. However, it is clear that a G1

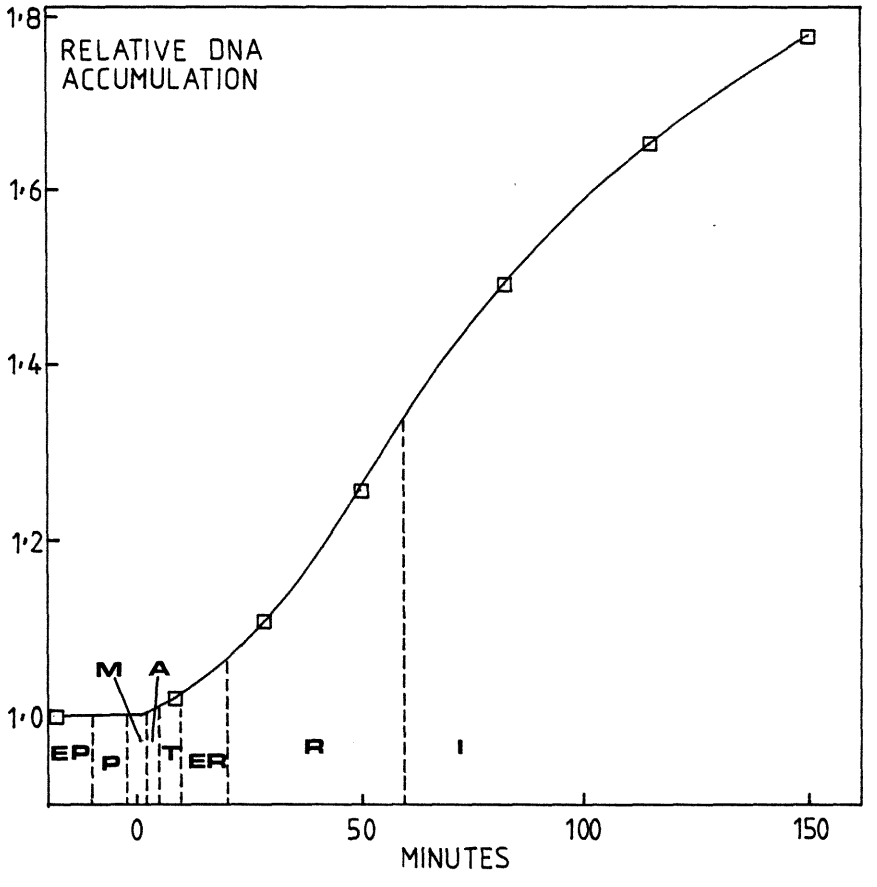

Fig. 8. Nuclear DNA synthesis during S phase in N+C.

A large surface culture was established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.2.a. Nuclei were prepared (Section 3.2.2.) from segments taken before and after M2, and the specific activity of DNA in each sample determined (Section 3.2.4.). DNA accumulation is plotted as a function of time after M2, the amount at M2 being normalised to 1.00. The abbreviations above are as follows: EP = early prophase; P = prophase; M = metaphase; A = anaphase; T = telophase; ER = early reconstruction (including the period of very early reconstruction referred to in the text); R = reconstruction; I = interphase.

phase, if it exists, must be vanishingly short (approximately 10% synthesis has occurred by 20 min after the end of telophase) and would constitute only a minute fraction of the intermitotic period. The rate of DNA synthesis obtained from this curve agrees well with that of the two large cultures shown in Fig. 5 in that, in both cases, after 60 min approximately 35% of the total expected synthesis is complete, and after 120 min this has increased to approximately 65%. This rate may thus be largely independent of both the final length of the cycle and the total amount of synthesis that eventually takes place. The small plates show more variation with one at 40% and 65% synthesis at 60 and 120 min respectively and the other at 50% and 75%.

d) SOYA DM: large cultures.

Two experiments measuring DNA accumulation between M2 and M3 are illustrated (Fig. 9). The specific activity value at M3 was not determined in experiment B. The general features are similar to those in N+C, most synthesis taking place during S phase but with a significant amount also occurring during G2. The transition between the phases appears to be more gradual than in N+C and is difficult to estimate, the figure of 135 min given in Table 10 below being an approximation.

Although SOYA DM was originally investigated because it was expected to give a longer intermitotic period than N+C, it was eventually found to be similar but slightly more variable (Section 2.6.) so that, for example, the culture in experiment A had an intermitotic period of 680 min, longer than ever recorded in N+C. This provided an opportunity to see whether a detectable G1 phase was induced, but this was partly frustrated by relatively poor synchrony. Thus, the 23 min point was taken from a sample recorded as being in only very early reconstruction (approximately M + 15 min) and its value (0.981) could, within the limits

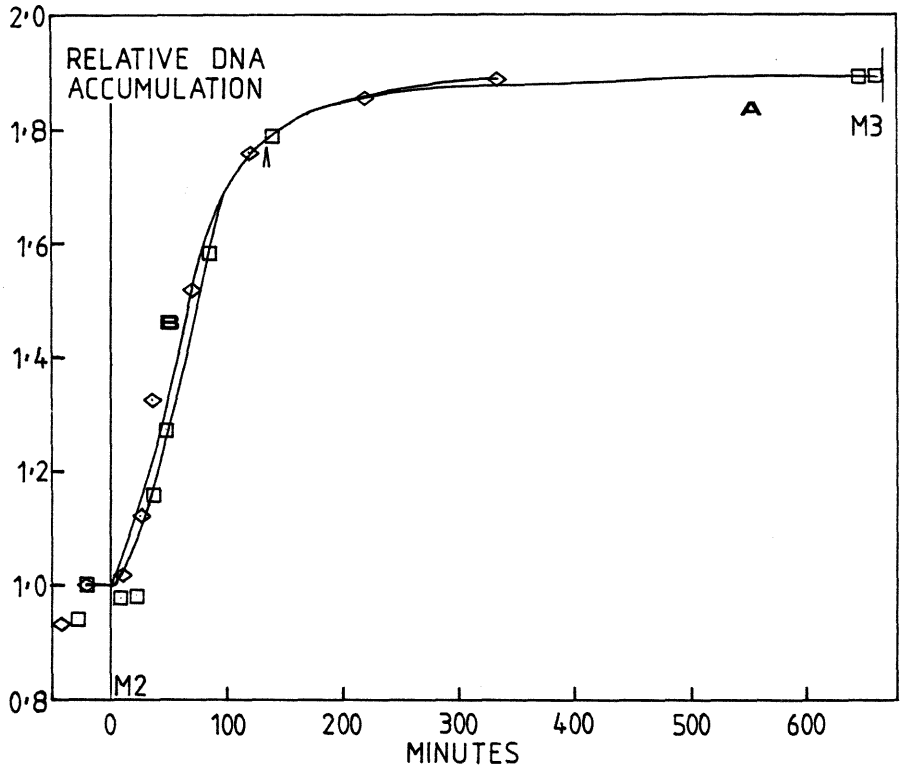

Fig. 9. Nuclear DNA synthesis between M2 and M3 in large surface cultures in SOYA DM.

Large surface cultures were established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.2.a. Nuclei were prepared (Section 3.2.2.) from segments taken between M2 and M3, and the specific radioactivity of DNA in each sample determined (Section 3.2.4.). The latter values were normalised to 1.00 at M2 for both experiment A (□) and B (◇). The time of M3 in one case is indicated by a vertical bar and the end of S phase, which was the same in both experiments, by ∧. Values on the time axis are relative to M2.

of sensitivity of the isotope dilution method, represent a value at or slightly above 1.00. Thus, with the modest increase in cycle length found in this experiment, it is not possible, with so few samples, to detect the very short G1 phase that might result.

e) SOYA DM: small cultures.

Fig. 10 again shows increased scattering caused by poor synchrony between plates, particularly in experiment B for which the range was 45 min at M2; the points at 34 and 54 min, which might suggest the presence of a G1 phase, were, when harvested, at stages of reconstruction only 10 - 15 min after division. The cycle length (700 min) was calculated using different plates for M2 and M3, and so is likely to be inaccurate, while the apparently prolonged S phase (200 min) is likely to be an over-estimate as some of the cultures harvested during S were behind those taken near mitosis. This illustrates how false values for cycle parameters can be obtained if synchrony is poor.

The curve from experiment A was obtained with a group of plates showing considerably less scatter (20 min) except for that at 68 min which was visibly delayed with respect to the rest by about 30 min. It may be noted that this experiment took place between M3 and M4, but its curve has the same form as those illustrated above.

f) SOYA DM: large culture. S phase.

In contrast to that of Fig. 8 in N+C, this S phase profile (Fig. 11) was taken after M3. The specific activity value for M3 was averaged from six points taken at mitosis and during the preceding G2 phase (4 points not shown). The sample at 17 min, which might again suggest the presence of a short G1 phase, was actually at a point of very early reconstruction 10 - 15 min after mitosis. In all of the graphs shown in SOYA DM there has been a point in very early reconstruction which has shown no detectable

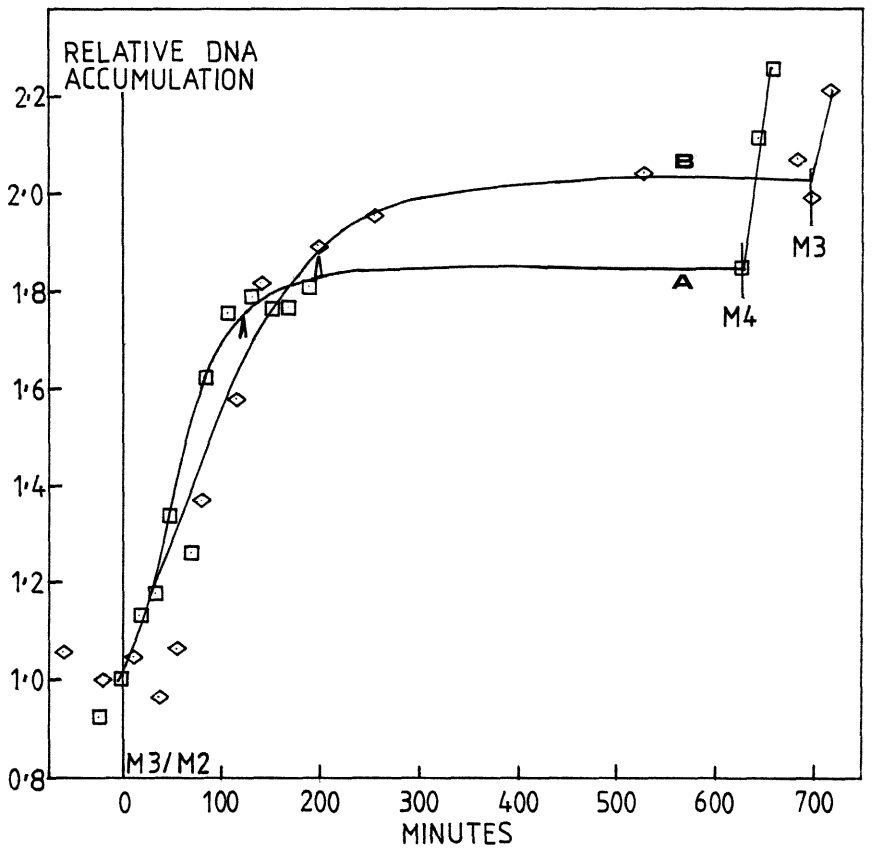

Fig. 10. Nuclear DNA synthesis between successive mitoses in small surface cultures in SOYA DM.

Small plate cultures were established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.1. Nuclei were prepared (Section 3.2.2.) from cultures taken between M2 and M3 (experiment B, ◇) or M3 and M4 (experiment A, □), and the specific radioactivity of DNA in each sample determined (Section 3.2.4.). DNA accumulation is plotted as a function of time after M2 (B) or M3 (A), the amount of DNA at these points being normalised to 1.00. The time of M3 (B) and M4 (A) is indicated by a vertical bar and the end of S phase by ∧.

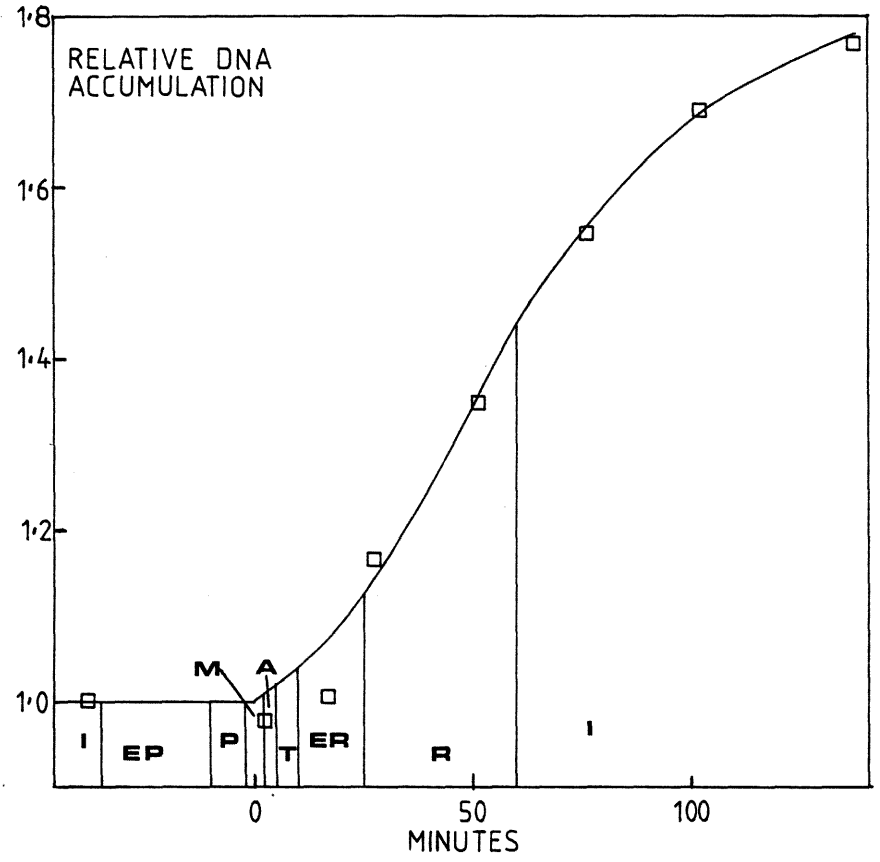

Fig. 11. Nuclear DNA synthesis during S phase in plasmodia in SOYA DM.

A large surface culture was established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.2.a. Nuclei were prepared (Section 3.2.2.) from segments taken before and after M3, and the specific activity of DNA in each sample determined (Section 3.2.4.). DNA accumulation is plotted as a function of time after M3, the amount at M3 being normalised to 1.00 using the mean of six values taken at mitosis and during the previous G2 phase. The abbreviations above are as follows: I = interphase; EP = early prophase; P = prophase; M = metaphase; A = anaphase; T = telophase; ER = early reconstruction; R = reconst.

synthesis, while in N+C (e.g. Fig. 8) there has appeared to be detectable synthesis at about 10 min. It is thus possible that S phase in SOYA DM might be delayed in comparison to that in N+C by about 5 min, but, given the degree of sensitivity of the isotope dilution method, the degree of asynchrony between many samples and the very short time interval involved, it could not be definitely asserted that this is the case without a larger number of more precise measurements being made.

The S phase curve shows 43% synthesis after 60 min and 74% after 120 min, agreeing well with the rates of synthesis in Fig. 9 showing approximately 40% and 75% after 60 and 120 min respectively.

g) Correlation of cycle parameters in N+C and SOYA DM.

The accumulation curves illustrated above were used to construct Table 10. Estimation of the end of S phase as the mid-point of the ~~inflexion~~ change between the gradients of S and G2 is obviously open to a large degree of error. In practice a curve obtained by isotope dilution provides a relatively insensitive measure of such parameters because, being an accumulation curve, it is difficult to detect the small changes in a large quantity of DNA that would be expected to occur as the rate of synthesis slows down between S and G2 phase (see Sections 1.2. and 1.3.). Determinations of synthesis rate would provide a more sensitive means of defining the end of S phase but are subject to the difficulties of interpretation already discussed in Section 1.2. and are thus less accurate than isotope dilution. Having determined this end point, the corresponding value for % DNA synthesised was read off the ordinate and has been expressed both as this value and as a percentage of the total amount of synthesis that actually occurred in the culture. G2 values were obtained by the relevant subtraction.

The data were analysed by testing for correlation in each of

	Medium	Culture mode	IMP (min)	Total % synth.	% synthesis during S	% actual synth. in S phase	% synthesis during G2	% actual synth. in G2 phase	Length of S (min)	% time in S	Length of G2 (min)	% time in G2
1	N+C	Large	530	88	65	74	23	26	125	23.6	405	76.4
2	N+C	Large	620	83	66	80	17	20	115	18.5	505	81.5
3	N+C	Small	610	101	74	73	27	27	125	20.5	485	79.5
4	N+C	Small	550	94	73	78	21	22	150	27.3	400	72.7
5	SOYA DM	Large	680	90	79	88	11	12	135	19.9	545	80.1
6	SOYA DM	Large	-	-	79	-	-	-	135	-	-	-
7	SOYA DM	Small	630	84	76	90	8	10	125	19.8	505	80.2
8	SOYA DM	Small	700	100	(90)	(90)	(10)	(10)	(200)	(28.6)	(500)	(71.4)

Table 10. Cycle parameters measured by isotope dilution.

thirty eight pairwise comparisons, taking 0.05 as the probability level required for significance. Result 6 was excluded because its data were incomplete as was result 8 because of poor synchrony between plates. Since figures expressed as percentages do not generally fall into a normal distribution, the method of analysis chosen was the non-parametric rank correlation due to Spearman (Parker, 1973). Only one comparison, intermitotic period against length of G2 phase, produced a value (0.929) for the Spearman correlation coefficient, r_s, greater than the tabulated value (0.886) that would need to be exceeded to suggest significance at the 0.05 probability level. This method is relatively insensitive since it does not take account of the magnitude of each value and might not detect significance in all cases, so all comparisons showing a coefficient greater than ± 0.7 were further tested parametrically in two different ways:

(i) by calculating the product-moment correlation coefficient, r, and testing the deviation of this value from zero for significance by a t-test, and

(ii) by treating intermitotic period length as an independent variable and calculating the linear regression coefficient, b, by the method of least squares; this was tested for significance by extracting the regression mean square and the residual mean square and calculating the variance ratio, F.

The arcsine $\sqrt{p}$ transformation was used for values expressed as percentages, while the remainder were treated as a normal distribution as the parametric tests used are quite robust.

The results, given below, are consistent with the hypothesis that, with various lengths of cycle, differences are due to alterations in the length of G2 phase, with that of S phase remaining constant. Thus a high correlation between the absolute length of G2 and the intermitotic period would be expected together

with a regression equation with a slope of 1.00 (b = 1.048 in this case). High positive correlation would also be expected between intermitotic period and the proportion of time spent in G2 and high negative correlation between intermitotic period and % of period spent in S (i.e. the inverse value). Both give a probability of significant correlation of only p = 0.1, but this may be due to the relatively insensitive measurement of S phase length and might improve with increased sample size. In this hypothesis, the comparison '% of period spent in S vs absolute length of G2' is essentially the same as 'intermitotic period vs % of period spent in S', and the higher correlation of the former would be a fortuitous outcome of the small sample size. A constant length of S phase would also be expected to lead to a very low correlation between intermitotic period and the absolute length of S phase, and the value of r_s of 0.029 is consistent with this.

Comparison	r	p	b	F	p
IMP vs % time in S	-0.739	0.1	-0.03	4.81	0.1
IMP vs time in G2	0.980	0.001	1.048	95.0	0.001
IMP vs % time in G2	0.739	0.1	0.03	4.81	0.1
IMP vs % synth in S	0.666	0.2	0.04	3.19	0.25
IMP vs rel % synth in S	0.707	0.2	0.07	4.00	0.25
IMP vs % synth in G2	-0.646	0.2	-0.07	2.86	0.25
IMP vs rel % synth in G2	-0.707	0.2	-0.07	4.00	0.25
% tot synth vs % time in S	0.194	0.9	0.05	0.156	0.75
% tot synth vs % time in G2	-0.194	0.9	-0.05	0.156	0.75
% time in S vs time in G2	0.879	0.02	-23.17	13.65	0.025

To examine this further, four sets of results not included in the above figures were analysed in conjunction with those already tested. All were with N+C medium in large cultures and had the

following characteristics:

IMP	Length of S	% time in S	Length of G2	% time in G2
520	140	26.9	380	73.1
565	120	21.2	445	78.8
530	120	22.6	410	77.4
595	135	22.7	460	77.3

The Spearman correlation coefficients for the possible pairwise comparisons were calculated using ten rather than six results and tested for a significant deviation from zero by a t-test. The results are shown below.

Comparison	r_s	Significance level
IMP vs length of S	-0.152	0.9
IMP vs % time in S	-0.815	0.005
IMP vs length of G2	0.958	0.001
IMP vs % time in G2	0.821	0.005
% time in S vs length of G2	-0.906	0.001
length of S vs length of G2	-0.342	0.5
length of S vs % time in G2	-0.612	0.1

These results are in agreement with expectation i.e. length of intermitotic period shows high positive correlation with both length of G2 and % of period spent in G2 and high negative correlation with % of period spent in S phase; comparisons involving the length of G2 give similar results to those with the length of intermitotic period except that that between length of S and % time spent in G2 would be expected to give no correlation; the relatively low degree obtained is most probably fortuitous.

The product-moment correlation coefficient for intermitotic

period against length of G2 was 0.981, significant at a probability level greater than 0.001. Treating intermitotic period as an independent variable (x) and length of G2 as the dependent (y), the linear regression equation was calculated by the method of least squares to be $y = 1.028x - 145.3$. This is plotted in Fig. 12 with the 95% confidence limits. The slope of nearly 1.00 is further evidence that the length of S phase is constant, being given by the intercept on the abscissa when $y = 0$. This was found to be 141 min but does not provide a precise measurement of S phase since its 95% confidence limits, calculated from the regression of x on y, are ± 74 giving a range of 67 - 215 min. The value for the regression coefficient of 1.028 was tested for significant deviation from zero by calculation of the variance ratio which was 206.4, significant at greater than $p = 0.001$.

It might be argued that the length of S phase does alter proportionally with the length of the cycle but that, because S phase is so short, this is beyond the limits of detection. This is unlikely for the following reasons. The shortest S phase recorded was 115 min and, if this were to be correlated with the shortest intermitotic period, 520 min, then the longest period, 680 min, would be expected to give an S phase of 150 min if changes were proportional. This was the longest S phase recorded but it did not correspond with a long intermitotic period. In fact, the very close approximation to 1.00 of the regression coefficient, and the very low and even slightly negative correlation between the length of S phase and cycle length suggest that any change in the length of S due to changes in cycle length must be completely outweighed by the error caused by the inability to measure the length of S phase precisely. This error will only affect estimates of S and G2 phase, since measurement of intermitotic period depends only on the microscopical detection of division.

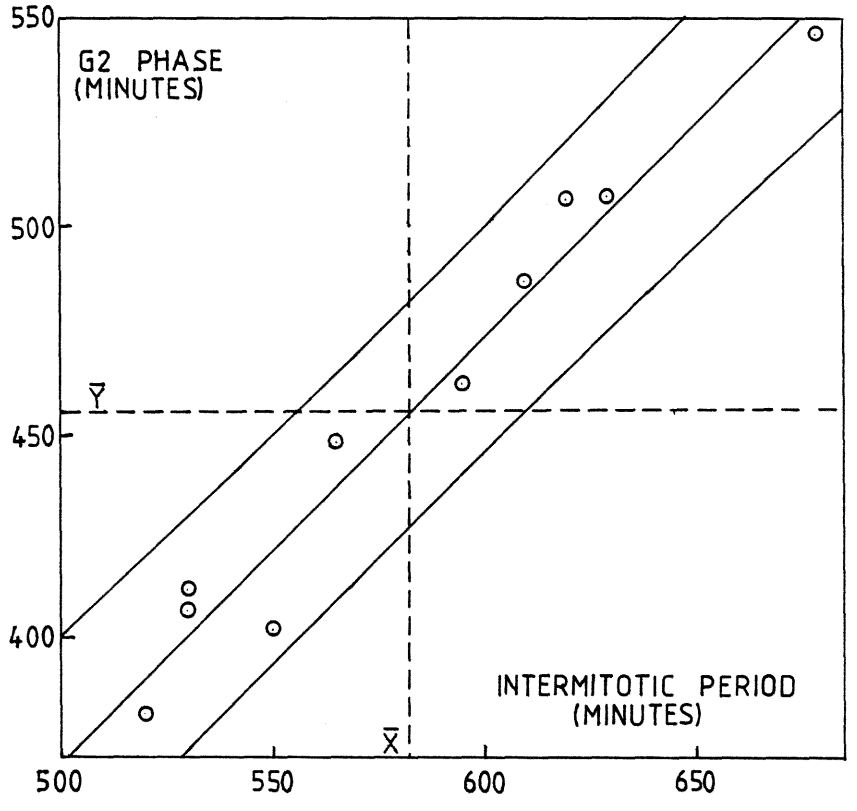

Fig. 12. Linear regression of G2 length upon intermitotic period.

The central line represents the linear regression equation of G2 length upon intermitotic period as determined by a least squares procedure. The 95% confidence limits of values obtained from this equation are shown by the outer pair of lines and values of G2 length and intermitotic period obtained in ten individual experiments summarised in the text are represented by open circles. $\bar{X}$ and $\bar{Y}$ are respectively the overall mean values of intermitotic period and G2 phase.

Thus it seems reasonable to say that the variability in the length of the intermitotic period will depend only on actual changes in the length of the period itself whereas, if both S and G2 change proportionally with the cycle, the variability in these will have components due both to change in cycle length and to the error in measuring the end of S phase. A measure of the relative variability encountered in each is given by the coefficient of variation (CV), i.e. the ratio (standard deviation/mean) x 100. The values obtained are:

	mean length (min)	S.D.	CV
intermitotic period	583	52.56	9.0
S phase	129	10.75	8.3
G2 phase	454	55.07	12.1

It might be expected that the CV of cycle length would be less than that of G2 due to the additional error in defining the length of the latter, and the calculated values of 9% and 12% are consistent with this. If the same held for S phase, an even higher variability would be expected since the error in measuring S phase, expressed as a fraction of S, would be greater than the same error expressed as a fraction of G2. As the value is actually lower it is likely that, if S phase does change with cycle length, it does so proportionally less than G2, so that the relative change in G2 would be greater than the relative change in the cycle length. Without the error in S phase, a change of 9% in an intermitotic period of 583 min with S constant at 129 min would cause a change of 11.6% in a G2 of 454 min.

It is not possible to completely rule out a variable S phase using the data above, particularly if there was an unconscious bias towards choosing a constant length for S which would render

the above argument invalid since this type of error would actually tend to reduce variability in S. To answer the question fully would require either a more sensitive technique for the measurement of S phase or the use of much more variable cycle lengths, although one might then be investigating relative durations under abnormal conditions rather than during normal variation. However, as it is likely that the same controls would operate, the situation in the rather poor BP medium has been investigated.

h) Intermitotic period in BP medium.

Although this medium was reported to give an intermitotic period of 16 - 17 h in the CL strain (Sudbery, 1974), this has not been consistently observed with M_3cVIII. M1 has been, in separate experiments, observed at $5\frac{3}{4}$ h and at $4\frac{3}{4}$ - 5 h, the period from M1 to M2 in one experiment varied between $12\frac{1}{2}$ and $13\frac{1}{4}$ h, while that between M2 and M3 has been separately observed as $10\frac{1}{2}$ - $11\frac{1}{4}$ h, $9\frac{1}{2}$ - 11 h and approximately 12 h, and M3 - M4 in one experiment varied from 15 to 18 h. Thus, the cycle appears to be extremely variable with an average length of about 12 h, the time of any mitosis being generally unpredictable. The difference between these and Sudbery's results may be due to the use of different strains or to variability between batches of Difco Bacto-Peptone, as previously observed with Oxoid's Bacteriological-Peptone (Plaut & Turnock, 1975), although it may be noted that, in at least one case, a series of 12 h intermitotic periods (M3 - M5) was recorded by Sudbery and Grant (1975)

Surface cultures on this medium tended to be pale in colour, very thin but covering an area larger than with other media, slimy often with a prominent, dark inoculum centre, irregularly-shaped and they generally gave a low yield of material. Nuclei sometimes appeared to be smaller than usual when seen under phase contrast, and nucleolar reconstruction seemed to be incomplete or slow in many

nuclei, multinucleolates persisting until well into interphase.

i) Nuclear DNA synthesis in BP medium.

The DNA synthesis profile was initially investigated in sets of small plates with typical results indicated in Fig. 13. The high amount of scatter was due to poor synchrony between plates, experiment A showing a range of an hour and B a range of approximately 80 min, both at a division between 21 and 24 h after feeding. Attempts to repeat these experiments in large cultures were not successful, since the poor growth characteristics evident in small cultures were more pronounced. In particular, synchrony was very low, one culture showing a range of 90 min at M3 and another of 70 min; a large culture in this medium might, therefore, prove a useful 'damaged' system for investigating the mechanism of synchrony. However, although the small plate results gave a broad outline of S phase and its duration, it would not be possible to detect a G1 phase which, if it existed, would probably be of the same order or less than the variation of time between plates.

Nevertheless, although individual cultures usually reached division at widely disparate times, the synchrony within a single plate grown on BP medium was often nearly as good as in the more nutritious media, with a range of only about 15 min. It was estimated that, if the procedure for preparing nuclei were to be omitted so that samples were plunged directly into cold 5% TCA/acetone/water, it would be possible to obtain up to six samples from a plate in BP medium at M3. Since the procedure would be technically less complex, it would also be possible to take samples more frequently and so define more precisely the early stages of S phase to test for the presence of a G1 phase.

j) Comparison of nuclear and total DNA synthesis in N+C.

To check the usefulness of such a procedure, the difference between DNA synthesis profiles obtained with and without isolating

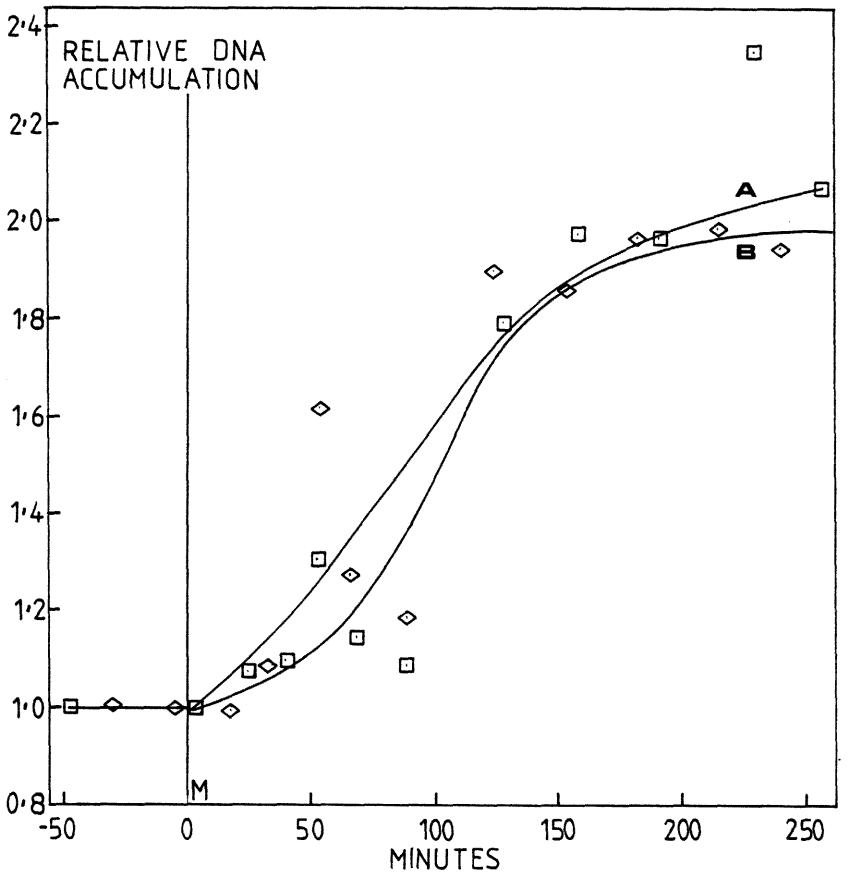

Fig. 13. Nuclear DNA synthesis during S phase in BP medium.

Microplasmodia were labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Section 3.2.1. except that label was added at 25 mg ml^{-1} cell density, was present for 24 h (expt A, □) or 48 h (expt B, ◇), and was chased for 48 h (A) or 72 h (B). Surface cultures were inoculated (Section 2.4.1.) while the cell density of the microplasmodia was near its maximum but before it began to decline. Nuclei were isolated and DNA specific activities determined (Sections 3.2.2. and 3.2.4.). DNA accumulation is plotted against time after a mitosis seen between 21 and 24 h after feeding (M2 or M3) in both A and B, the amount of DNA at this point being normalised to 1.00.

nuclei was therefore determined (Fig. 14) using a large culture in N+C, as large cultures in BP medium were impractical. As mitochondrial DNA is synthesised throughout the cycle and constitutes about 7% of the DNA (Braun & Evans, 1969), its presence in the total DNA samples might be expected to cause a flattening of the S phase slope, with a greater proportion of synthesis thus taking place during G2, so that the inflexion between the two would be less sharp.

This is basically the effect seen, although it is not clear whether the proportion of DNA synthesised in G2 was increased. Other differences were (i) the apparent amount of synthesis from M3 to M4 was less with total DNA, although the difference between nuclear and total DNA was within the limits of experimental error and (ii) the specific activity of total DNA was consistently higher than that of nuclear, the latter being 92% of total at M3 and 88% at M4, while falling as low as 80% during intermediate times. No fully consistent pattern was observed, although the percentage appeared to decrease until the end of S phase and then rise again. Another experiment with four samples at M3, and four at M + 60 min, gave ratios of 82% and 74% respectively, although the difference between these means was not significant.

If the difference in specific activity were due to the presence of mitochondrial DNA then, assuming it constitutes 10% of total, it would have a specific activity 1.9 times that of nuclear DNA at M3 in the experiment illustrated above. This is possible, particularly if it draws on a different thymidine triphosphate pool. The apparent changes in the ratio of specific activities are roughly as would be expected, falling to a minimum at the end of S and then rising again, with the magnitude of such changes being within the limits of experimental error so that a fully consistent pattern would not be found. However, the ratio at mitosis has varied between 92% and 75% in a number of experiments, although it is

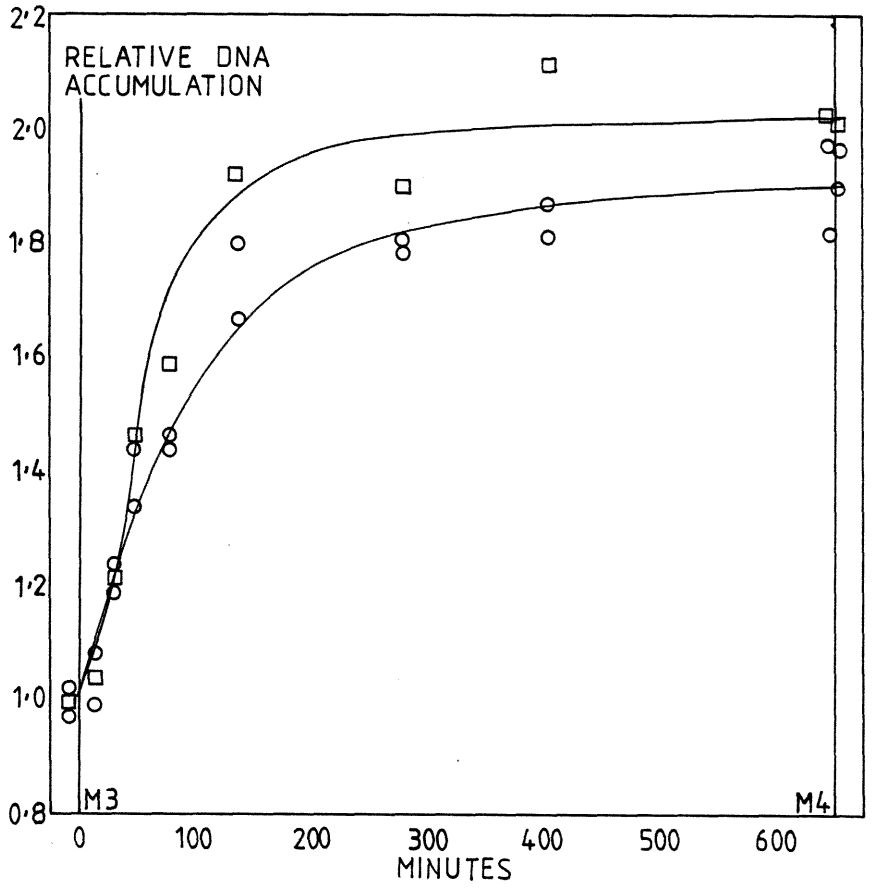

Fig. 14. Comparison of nuclear and total DNA synthesis between M3 and M4 in N+C.

A large surface culture was established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine (1 uM) as in Sections 3.2.1. and 2.4.2.a. At times after M3 indicated above, a segment of filter paper with plasmodium (approx 25 cm^{2}) was cut to provide (i) 2 small samples (approx 4 cm^{2}) homogenised immediately for total DNA specific activity (Section 3.2.4.) and (ii) 1 large sample from which nuclei were isolated (Section 3.2.2.) and their DNA specific activity determined. Total (O) and nuclear (□) values were normalised to 1.00 at M3. Mitoses are indicated by vertical lines.

usually around 85%. This variability might suggest that the difference is actually due to the presence of a non-DNA contaminant in the total DNA fraction, possibly unhydrolysed RNA, which contributes to the radioactive counts but is not detected in the diphenylamine assay. This potentially complex problem was not investigated further since the synthesis profile obtained with total DNA was, in most respects, similar to that of nuclear DNA. In particular, the profiles during early S phase and the approximate durations of S are almost identical.

k) Total DNA synthesis during S phase in BP medium.

Fig. 15 shows the total DNA synthesis in four small plate cultures, each yielding six samples. All cover the period after M3 except for plate A which was harvested in the period after M2, but appears to show the same pattern. M3 occurred at approximately 9 - 10 h after M2 and the range of mitosis between plates was 70 min, the asynchrony within individual plates ranging from 5 - 18 min. The points clearly show the absence of a G1 phase and a rate of synthesis roughly equal to that of the S phase curve in SOYA DM (Fig. 11), with 40% of DNA synthesised after 60 min.

A further experiment was performed under the same conditions in order to define more closely the end of S phase. In this case cultures reached M3 within 40 min of each other, and the asynchrony within cultures ranged from 3 - 20 min. The interval between M2 and M3 ranged from $11\frac{3}{4}$ - 13 h and that between M3 and M4 from $15\frac{1}{4}$ - $18\frac{3}{4}$ h, although by M4 the plasmodia were very pale and slimy and showed poor synchrony with a range of 1 - 2 h in the same culture. Thus, even though cultures had been refed every 24 h, it is unlikely that the latter cycle length was normal for the medium. Nevertheless, the DNA synthesis profiles after M3 do appear to be normal, (Fig. 16), again with no G1 phase and with S phase lasting approximately 130 min. The rate is similar to that previously

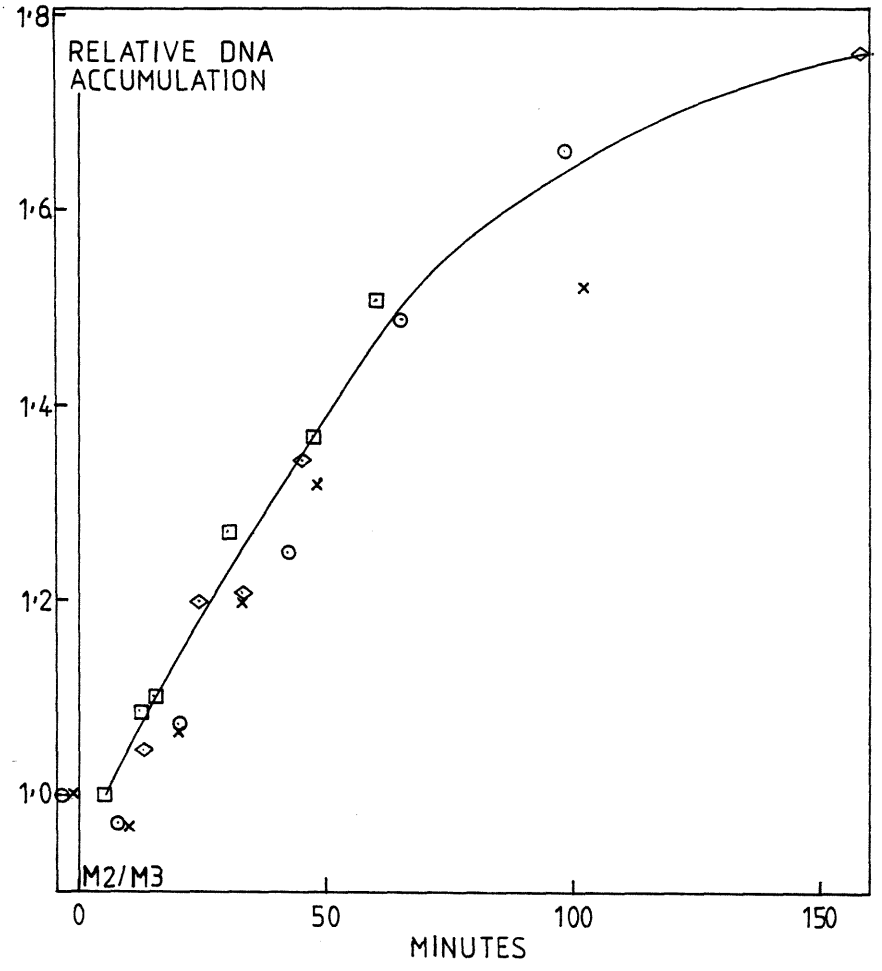

Fig. 15. Total DNA synthesis during early S phase in small plate cultures in BP medium.

Four small plate cultures were established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine as for expt A of Fig. 13. Each plate provided 6 samples which were removed, during and soon after M2 for one plate (x) and M3 for the rest, by scraping material into 5% TCA/acetone/water, and the total DNA specific activity was then determined (Section 3.2.4.). The amount of DNA at mitosis is plotted against the time after mitosis at which the sample was harvested. A different symbol was used for each culture.

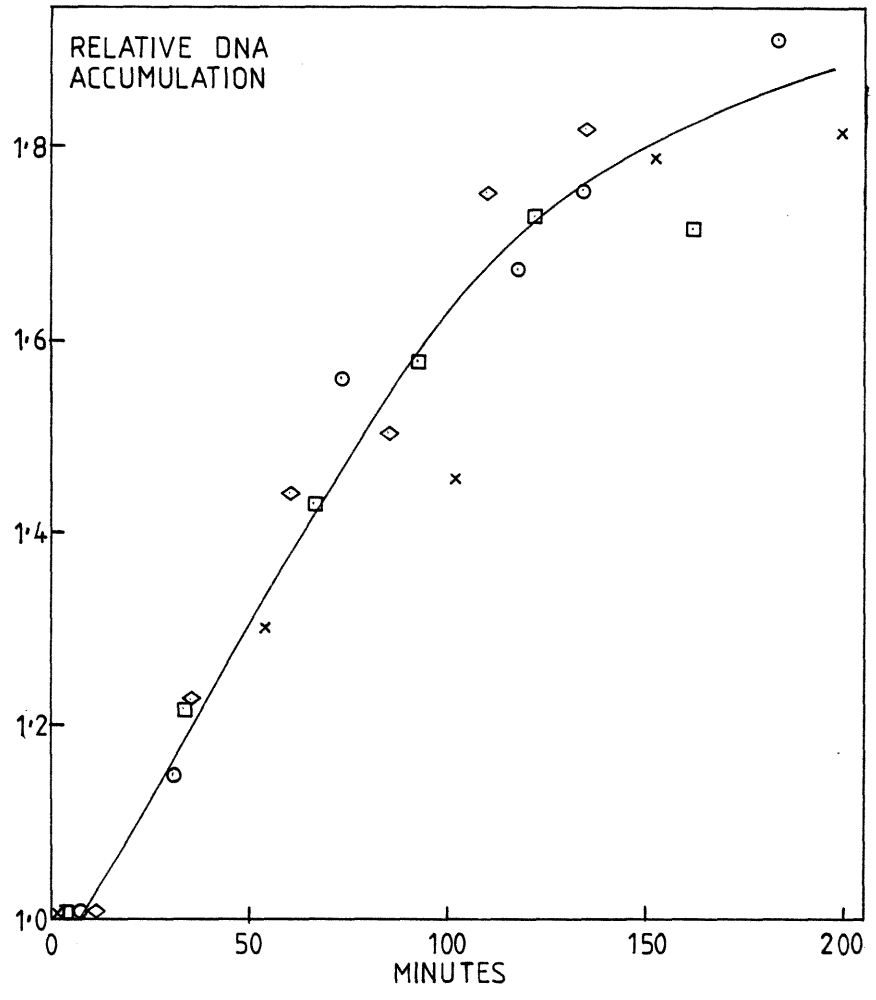

Fig. 16. Total DNA synthesis during the S phase following M3 in BP medium.

Four small plate cultures were established from microplasmodia labelled with 100 uCi [6-^{3}H]thymidine as for expt A of Fig. 13. Each plate provided 6 samples which were removed, during and soon after M3, by scraping material into 5% TCA/acetone/water, and the total DNA specific activity was then determined (Section 3.2.4.). The amount of DNA at mitosis is plotted against the time after M3 at which the sample was harvested. A different symbol is used for each culture.

observed, 37% synthesised after 60 min and 73% after 120 min.

In conclusion, with an extension of cycle length to about twelve hours, the duration of S phase and the rate of DNA synthesis within it seem to be unaltered, even though growth in BP medium tends to be unhealthy. A G1 phase is not introduced in intermitotic periods of this length. The prolongation of the cycle to as much as 18 h appears to leave S phase unaltered with the extension presumably occurring during G2 phase.

3.4. Discussion.

The results presented in the previous section will be discussed under three main headings. First, the absence of a G1 phase and the apparent variability of the length of G2 have implications for theories of cell cycle control. Secondly, the presence of G2-synthesis appears to be confirmed and various possible explanations for its existence will be summarised and, thirdly, the lack of balanced growth with respect to DNA will also be considered.

3.4.1. G1 absence, G2 variability and cell cycle control.

The present results have, in agreement with those outlined in Section 3.1., failed to detect a significant G1 phase, even during the relatively long intermitotic periods in BP medium. That long cycle lengths do not induce a G1 phase in *Physarum* is also suggested by the incorporation of [^{14}C]orotic acid into DNA immediately following mitosis as found by Nygaard et al. (1960) under conditions in which the cycle length was routinely between 13 and 22 h. Experiments using a greater degree of resolution have also failed to detect any gap between telophase and S phase. Kessler (1967), using 5 min pulses of [^{3}H]uridine and [^{3}H]thymidine at 4 min intervals during mitosis, demonstrated by autoradiography that nuclei obtained from samples taken during metaphase and anaphase were unlabelled, but those harvested from samples fixed

after anaphase became progressively more heavily radioactive. Furthermore, the use of inhibitors such as 5-fluoro-2'-deoxyuridine (Sachsenmaier & Rusch, 1964) or cycloheximide (Cummins & Rusch, 1966), or of heat shocks (Brewer & Rusch, 1968; Wright & Tollon, 1978), has failed to induce a G1 phase; either mitosis and the following S phase are both inhibited, or a mitosis is skipped so that DNA synthesis begins without a prior division.

Physarum is by no means unique in lacking a G1 phase. This phenomenon has also been found in Amoeba proteus, Schizosaccharomyces pombe, in the micronucleus of Tetrahymena and Euplotes, in many embryonic tissues such as those of the sea urchin, Xenopus, snail and mouse, and in the subline V79 of cultured Chinese hamster fibroblast cells. Since the majority of cells do, however, possess a G1 phase, the question arises of how organisms are able to exist without one. It seems likely that either one of or a combination of two factors is necessary: (i) events required for the initiation of DNA synthesis, including any that might normally be performed during G1, are completed during G2 and/or (ii) the cells are highly efficient at reorganising condensed chromatin and at synthesising factors such as S phase initiators either during or after mitosis so as to be quickly in a ready state for commencing DNA synthesis.

In support of (i) above, the use of inhibitors such as cycloheximide and actinomycin D in both A. proteus (Prescott, 1976) and Physarum (actinomycin D: Mittermayer et al., 1965; cycloheximide: Cummins & Rusch, 1966) has demonstrated that the synthesis of protein and RNA necessary for the initiation of DNA replication is complete before metaphase at the latest, while, in cells with a G1 phase, inhibition of protein or RNA synthesis during this period can inhibit the following S phase (summarised in Prescott, 1976). Mutants temperature-sensitive for specific G1 events have also been isolated (Hartwell et al., 1974; Liskay, 1978). Complementation

studies involving the $G1^-$-subline, V79-8, (Liskay, 1978) and $G1^+$ cells with lesions during G1 phase have demonstrated that at least two events are involved in G1 phase and that the $G1^-$ cells utilise the same basic cell cycle programme as $G1^+$ cells.

In line with possibility (ii) above, the chromosomes of V79-8 cells have been observed to decondense rapidly after mitosis (Rao et al., 1978), so reaching the extended state of early S phase within 30 min. The lack of a G1 phase is likely to be due to the presence of inducers of DNA synthesis above the critical level for induction even during mitosis (although the chromosomes are not competent to undergo replication in their condensed form), because Sendai virus-induced fusion of mitotic V79-8 cells with G1 phase HeLa cells was found to result in the induction of both DNA synthesis and premature chromosome condensation in the latter (Rao et al., 1978). It is thus possible that the presence or absence of a G1 period in the cell cycle depends on the level of inducers of DNA synthesis at the time of mitosis (and thus on their rate of production). If they are present above a critical level during mitosis, the cell enters S phase immediately after this but, if not, a G1 period is required in which to synthesise them.

The question posed above might also be reversed: if cells can exist without a G1 phase, why do most cells have one at all? Besides the specific G1 functions mentioned above, which seem to be present in the V79-8 line but need not apparently be expressed during this phase, most explanations of the presence of this period have implicated G1 phase in the control of the passage of cells through the division cycle. This idea has arisen from numerous observations (summarised in Prescott, 1976) that changes or differences in the length of the cell cycle are almost wholly attributable to changes in G1 phase. For example, the range of interdivision periods shown by cells within a single culture is attributable to differences in

G1 which are thus responsible for the rapid loss of synchrony in artificially synchronised cell populations. Furthermore, when the mean cycle length of a culture is altered, for example by transfer to different growth media or during the transition from exponential to stationary phase, this is again usually attributable to changes in G1. When cultures arrest growth, this occurs almost invariably during G1 phase, although whether this represents a qualitatively different, G_0, phase is still subject to debate (Baserga, 1978).

Observations of the above type have suggested that, in general, the rate of cell reproduction is governed by the average length of the G1 period, some event or events in this phase being the focus of regulation. Accordingly, G1-variability has been of primary importance in the formulation of theories which attempt to explain the mechanism controlling passage through the cell cycle. It provides the fundamental observation for the transition probability theory which was originally suggested by Burns and Tannock (1970) and proposed in a more formal and general manner by Smith and Martin (1973). In this, the transition probability (P) of passing from the A-state (equivalent to most of G1 phase), in which the cell is not directed towards division, into B phase (equivalent to S, G2, M and possibly part of G1), in which the cell's activities are deterministic and directed towards the following mitosis, remains for each cell a constant with time. Initiation of cell replication processes is thus random, in the sense that radioactive decay is random, and the model quantitatively predicts the wide variation observed in the duration of G1 within populations (Minor & Smith, 1974; Shields, 1977). The value of P is determined by the cell's environment (Brooks, 1975, 1976); high values produce short cycles of homogeneous length, while low values lead to long, variable cycles and, if P is sufficiently low, to the arrest of growth.

The main alternative view of division cycle control is in

terms of a cell-size hypothesis (Wheals, 1977), in which cells are considered to be capable of monitoring their own size and of adjusting the rate of progress through the events of the cell cycle to correlate with their growth rate. The adjustment is thought to occur at only one or two points of the cycle, usually preceding one of the major events, DNA synthesis or mitosis, and takes the form of the maintenance of a constant ratio of DNA to mass at the initiation of S phase or mitosis. Thus, if growth rate slows, an increase in the length of G1 occurs because the cell is unable to begin DNA synthesis until it has reached a particular size. The hypothesis requires that an essential controlling influence originates in the cytoplasm and acts on the nucleus to stimulate or inhibit mitosis or DNA synthesis, its effect being proportional to the mass of the cytoplasm, and a number of more specific models, such as the 'division protein' model of Zeuthen (Mitchison, 1971) or the concentration models proposed by Fantes et al. (1975) and tested in Physarum by Sudbery and Grant (1975, 1976), have been suggested, to fill in these outlines. Thus, changes in environmental conditions alter the mean cycle lengths via effects on growth rate, while differences in cycle length within cultures are attributable, in a less accountable manner than by the transition probability theory, either to the production of daughter cells of unequal size at division or, less clearly, to variability in cell size at division (Prescott, 1976) and hence to variability in offspring, caused by 'random variation' in the length of the preceding cell cycle (Fantes, 1977; Shields et al., 1978).

The use of a number of temperature-sensitive cell division cycle (cdc) mutants in the yeast, Saccharomyces cerevisiae, has allowed the cell-size hypothesis to be formulated in its most precise form. The attainment of a critical cell size is a prerequisite for completion of a point ('Start') in G1 that must

be passed before the cell can begin either of the two parallel pathways of cell cycle events resolved in this organism (Hartwell et al., 1974; Hartwell, 1978). It was proposed that growth (i.e. the doubling of cell mass) is usually rate-limiting in S. cerevisiae cells, and hence that growth and division are coordinated in each cycle because the stage-specific programme pauses at 'Start' until growth, or some substance the amount of which is proportional to cell mass, catches up (Johnston et al., 1977).

Recent investigations (Shields et al., 1978) go some way towards reconciling the kinetically-based transition probability theory with the cell size hypothesis in a manner similar to that suggested by Shilo et al. (1976) and Fantes (1977). Quiescent 3T3 mouse fibroblasts were separated into small and large size classes (volume ratio approximately 2:1) and returned to culture. After a size-dependent lag, cells appeared to enter S phase approximately exponentially, consistent with a random transition while, in the subsequent cycle, cells born of small mother cells had longer cycle times than those from large mothers. The difference in the cycle time of the two classes was traced to that part of G1 situated in the B phase (G1B). Thus, the time spent in G1B is related to cell size but, in the fibroblast system, this is short compared to the time spent in the A-state, so that transition probability remains the major factor controlling cell division.

In more rapidly-growing systems such as cultures of bacteria (Shields, 1978) and yeast (Fantes, 1977; Shilo et al., 1976), the cycle kinetics are also consistent with there being a random transition but with a high value of P, so that the cells would spend a short time on average in the A-state, and a size requirement for the completion of G1B would therefore be the major factor governing cycle times. The finding that the variation in mass at division is less than the variation of cell age may then be explained by a

combination of the size requirement for the completion of G1B and the high value of P. In contrast, results suggesting no effect of cell size on G1 duration in animal cells (Fox & Pardee, 1970) could be due to a small contribution of cell size to G1 duration, with the A-state being, on average, substantially longer than G1B. An advantage of retaining the idea of transition probability is that it can account for the variability of cell size at mitosis which is present even in rapidly growing cultures (see above). Although such explanations do not answer the question of why most cells have a G1 phase, they do give some idea of its function when it is present.

Neither the latter formulation, nor either theory alone, can be applied to cells which lack a G1 phase without assuming that control is exercised over entry into mitosis rather than S phase. The present results show that some variation can indeed occur in the length of G2 phase in Physarum and, in S. pombe, the duration of G2 varies with nutritional conditions (Fantes & Nurse, 1977). Thus a $G1^{-}$ cycle might occur if the transition probability is of a very high value and the size-dependent stage is in G2 rather than G1B.

Investigations into mitotic cycle control in Physarum have therefore been concerned with determining the mechanism by which mitosis is triggered. The experiments have involved either perturbations such as those due to heat shock (Brewer & Rusch, 1968; Kauffman & Wille, 1975a) or protein synthesis inhibitors (Scheffey & Wille, 1978) both of which can delay mitosis, or have employed fusion of plasmodia at different stages of the cycle (Rusch et al., 1966; Sachsenmaier et al., 1972; Kauffman & Wille, 1975a). The results have been interpreted both in terms of a growth-dependent mechanism (Sachsenmaier et al., 1972) and a continuous limit cycle oscillator (Kauffman, 1974; Kauffman & Wille, 1973, 1975a; 1975b,

1976). In the latter, two or more biochemicals interact in such a way as to produce a stable oscillation in cytoplasmic concentrations, mitosis being initiated when the concentration of one of these reaches a critical level. This proposed mechanism is not growth-related, the biochemicals act as timers only, and it has recently been brought under criticism (Tyson & Sachsenmaier, 1978).

Studies into the molecular nature of the mitotic trigger in Physarum have also been undertaken, and the fact that an advance of mitosis has been achieved by administering a solution of growth-associated H1 histone phosphokinase (HKG) from Ehrlich ascites cell chromatin (Bradbury et al., 1974b; Inglis et al., 1976) suggests that the rate-limiting regulatory process is being affected. It has been proposed that an increase of nuclear HKG (Bradbury et al., 1974a) leads to an increase in the phosphate content of H1 histone (Bradbury et al., 1973b) which then causes the initial stages of chromosome condensation (Bradbury et al., 1973a; Inglis et al., 1976). These proposals make no predictions about the levels of protein and nucleic acid synthesis required for the initiation of mitosis (Bradbury et al., 1974a), since the mechanisms regulating the increase of HKG activity are unknown, although it seems to occur through activation rather than synthesis of new enzyme (Mitchelson et al., 1978). It has been suggested (Mitchison, 1976, 1977) that the two components of the limit cycle oscillator might be HKG and phosphorylated H1 histone.

In agreement with a control exercised over entry into mitosis is the observation that cessation of cell reproduction in A. proteus appears to occur in the G2 period (Prescott, 1976). On refeeding, mitosis is missed and a second S phase occurs, providing further evidence that the events preparing for the initiation of DNA synthesis are situated in G2. The most detailed results concerning the mechanism of control of transit through cell cycles with no G1

phase have been found using the fission yeast S. pombe. Although the strains used since 1967 do possess a G1 phase (Mitchison & Creanor, 1971), it is very short and is unlikely to be able to serve as the time in which growth and division are synchronised. Two main lines of investigation have demonstrated the presence of two complementary cell cycle control mechanisms in this organism, first the study of wee mutants (Nurse, 1975; Thuriaux et al., 1978) which are temperature sensitive in the control coordinating cell division with growth and which have the same growth rate as wild type, but divide at a smaller size, and secondly, studies of the kinetics of change of growth and division parameters after shifts to different growth conditions (Fantes & Nurse, 1977).

In normal cells, and in wee mutants at the permissive temperature, there is a cell size requirement for entry into mitosis which is growth-modulated; i.e. the size necessary for mitosis is set by the prevailing growth conditions, being reduced at slow growth rates and increased at more rapid growth rates. In contrast, in wee mutants at the restrictive temperature (Nurse, 1975), and in wild-type cells under conditions in which cell size is small, such as nitrogen-starvation or spore germination (Nurse & Thuriaux, 1977), there appears to be operative a control mechanism ensuring that cells enter DNA synthesis at the same size. As this size is smaller than any wild-type cell in exponential culture, it would normally be cryptic and it simply acts as a mechanism ensuring that cells are not below a minimum size when entering S phase. It is not growth-modulated and, at least in wee mutants, is followed by a minimum incompressible period in G2 which acts as a timer from S phase to nuclear division (Fantes & Nurse, 1978); this control mechanism is therefore similar to that proposed for bacteria (Donachie, 1968; Pritchard et al., 1969).

It is possible that the control over DNA synthesis is

sufficient to explain characteristics such as variable G1 and constant S + G2 in many cell types, while that over mitosis is applicable to organisms such as A. proteus and Physarum with no G1 phase and a variable G2 phase. The hypothesis that, even within a single culture, large cells might be subject to control over mitosis while small cells are controlled at entry to S phase, is capable of explaining a number of apparently contradictory observations (discussed in Nurse & Thuriaux, 1977). The assumption of a minimum DNA:mass ratio control over entry into S phase and a growth-modulated control over mitosis might account for the observation in a number of systems (Prescott, 1976), including S. pombe on depletion of the culture medium for nitrogen (Nurse & Thuriaux, 1977) and phosphate (Bostock, 1970), and Physarum on spherulation (Mohberg & Rusch, 1971), that cells entering stationary phase arrest at G1. However, as the DNA:mass ratio in microplasmodia remains constant at different growth rates (Plaut & Turnock, 1975), the control over mitosis might not be growth-modulated in Physarum, in which case arrest would be expected to occur during G2. It is possible that modulation is only expressed at the low growth rates found in starving plasmodia (Sudbery & Grant, 1976), and it may be feasible to investigate this problem by using the isotope dilution method to determine the position of S phase in both starving and spherulating plasmodia, provided that the cultures are prelabelled to a sufficiently high specific activity.

The two inverse control mechanisms imply both a minimum (or absent) G1 period and a minimum S + G2 and it has been observed that a minimum cycle exists in both Physarum (Sudbery & Grant, 1975, 1976), in which it is 6 - 7 h, and S. pombe (Fantes, 1977). It would be worthwhile to repeat the induction of such a minimum cycle in Physarum by ultraviolet-induced decreases in the DNA:mass ratio, and to determine, by isotope dilution, whether the reduction in

duration does indeed occur completely in the G2 period.

3.4.2. DNA synthesis during G2 phase in Physarum.

The results presented in this thesis suggest that between 10 and 25% of total nuclear DNA synthesis can occur during G2 phase. This is in accord with at least two previously published reports (Bovey & Ruch, 1972; Hall & Turnock, 1976) except that in these, G2-synthesis was reported as 10% of total nuclear DNA synthesis. In no case can it be wholly attributed to the nucleolar satellite since this constitutes only 1% of the total DNA (Zellweger et al., 1972). The extents of synthesis during S phase presented in Section 3.3.4. have a coefficient of variation of 7.8% which is partly due to the insensitivity of the method, but is also likely to reflect a genuine degree of variability in the amount of synthesis during G2 (the standard error in the percentage of synthesis during S or G2 is 2.9 while that in the percentage of time spent in S or G2 is only 0.94). No significant correlation was detected between the amount of synthesis in each cycle phase and its relative length.

It is important to determine whether G2-synthesis is a general phenomenon not detected in other systems because of their variable cycle times or imperfectly retained synchrony, whether it is attributable to a regulatory mechanism involved specifically in the growth of multinucleate plasmodia, or whether it is due to a trivial cause such as incomplete synchrony. The latter seems unlikely because interphase nuclei are not generally detected during mitosis and vice versa, while contamination with inoculum material is also unlikely because the inoculum ring is removed in large cultures and mitotic synchrony with the growing edge is maintained in areas close to the inoculum. Braun et al. (1965) found, using autoradiography, that at least 99% of nuclei incorporate [^{3}H]thymidine at the beginning of S phase, although

the apparent asynchrony they observed in the completion of S phase may account for some of the synthesis in early G2.

Evidence that this phenomenon is not wholly due to heavy satellite was provided by Holt and Gurney (1969), who observed a low variable level of incorporation of radioactive thymidine during G2 into main band nuclear DNA as separated on CsCl density gradients. The time of such incorporation had previously been correlated (Guttes et al., 1967) with the labelling, as seen by autoradiography, of a small fraction of nuclei late in the cycle; these often appeared larger than the majority of nuclei. A subsequent autoradiographic study (Guttes & Guttes, 1969) observed a fraction of nuclei (0.5%) which were large, possibly polyploid, often with several small nucleoli and which continued incorporation into extranucleolar chromatin at a high rate throughout the cycle. During the experiments described in this thesis, large nuclei with amorphous contents were frequently observed, but it is possible that these were produced as an artefact of the smearing procedure. It seems unlikely that a fraction as small as 0.5% could be responsible for all the observed G2-synthesis, and Bovey and Ruch (1972), who observed that about 10% of nuclear DNA was synthesised during G2 phase, carried out measurements on individual nuclei, but did not report a discrete subclass of large nuclei to be responsible.

There have been some clues as to a possible molecular explanation of this phenomenon. Brewer et al., (1974) demonstrated that the initiation of synthesis of DNA subunits takes place throughout at least the first two hours of S phase and, as they then appeared to take approximately 2 h to reach the fully mature size, DNA chain elongation must still be in progress 4 h after the end of mitosis. It was possible that maturation could have continued further into G2, but was not detectable within the limits of resolution used.

Funderud et al., (1978) have recently shown that the maturation of a 70 S species of DNA molecule to one of 130 S occurs from M + 120 min onwards and continues beyond 180 min, possibly for most of G2. As the value of 130 S (approximately one seventh of the single strand length of a Physarum chromatid) was close to the maximum that could be recorded under the experimental conditions no matter how large the DNA molecule, further maturation might also occur during G2 but would be undetectable. It is not possible to estimate the proportion of nuclear DNA synthesis involved but, since only previously-replicated clusters of replicons are joined, it is likely that the fraction is small, although it may account for a significant fraction of the observed G2-synthesis. If it were solely responsible, then the amount of G2-synthesis would be constant for all experiments, but whether wholly or partly responsible, it is probably a general phenomenon detectable only in Physarum because of its extreme synchrony.

~~When radioactive thymidine is used as a precursor during G2, more than 90% of the nuclear DNA labelled derives from the nucleolus (Butler et al., 1978), suggesting that, in contrast to the isotope dilution method, pulse-labelling during G2 would show very little main band incorporation. This apparent discrepancy is explicable if nucleoplasmic and nucleolar DNA synthesis were to draw on physically distinct deoxynucleoside triphosphate pools which are labelled at different rates from exogenous precursor.~~

3.4.3. Unbalanced growth in Physarum.

The concept of balanced growth was originally developed in order to provide a criterion for identifying abnormal changes resulting from experimental manipulations (Anderson et al., 1967). Its first definition was that 'growth is balanced over a time interval if, during that interval, every extensive property of the growing system increases by the same factor' (Campbell, 1957) or

that the ratio A/B is constant, where A and B are any two components of the cell. For synchronous cultures or individual cells this can be redefined as: the ratio A/B is constant only if the time interval between determinations of the ratio is an integral multiple of the cycle time. In practice it is difficult to control the growth conditions of synchronised cultures sufficiently rigorously to ensure that this is always so, and it is also difficult to define the same point in two successive cycles except at mitosis or the beginning of S phase. Furthermore, within a single cell, A/B could show significant variation at the same point in two successive cycles, but be corrected by the next cycle in the same manner as cell size in S. pombe (Fantes, 1977). Thus the concept of balanced growth is often of doubtful use as a criterion of normality when considering synchronous cultures. However, the doubling of DNA content during the cell cycle might be considered an exception to this; if its genome is not fully replicated, a cell is clearly abnormal in some way.

In Physarum the studies of Sudbery and Grant (1975, 1976) suggest that the constancy of the DNA:mass ratio at mitosis is maintained even under quite extreme conditions, while published results using the isotope dilution method show that rRNA (Hall & Turnock, 1976), tRNA (Fink & Turnock, 1977), nuclear DNA (Hall & Turnock, 1976) and total nucleic acid and protein (Birch & Turnock, 1977) can all double in amount between divisions in plasmodia in the large culture apparatus. However, the experiments presented in this thesis demonstrate that the extent of DNA synthesis is often less than 100%, but never significantly more, thus precluding the possibility that the effect is due solely to experimental variation. Values ranged from 83 - 101% with a mean of 91%. It is unlikely that this is due to contamination from the inoculum area which shows reduced synthesis of DNA (Section 2.4.2.), since this is

removed in large plasmodia, and a variable degree of contamination could produce values above 100%.

In two separate experiments in SOYA DM, the increase in nuclear DNA, total nucleic acid (mainly RNA) and protein within the same large culture between M2 and M3 has been recorded, assaying each species by isotope dilution. The values obtained were:
1. DNA = 103.4%; nucleic acid = 58.8%; protein = 79.1%;
2. DNA = 95.3%; nucleic acid = 101.6%; protein = 108.1%.

In 2., growth appeared to be, within the limits of experimental error, balanced with respect to all three components, but in 1., this was not the case for either nucleic acid or protein. It is impossible to derive any general trends from so few experiments, but they do indicate that each species can be synthesised to a different extent within the same morphologically healthy culture. These results parallel others previously obtained in strain CL grown in MyDM in the large culture apparatus; in two cases an increase of rRNA between M2 and M3 of only 56% was seen (Hall, 1975; Turnock, 1979), although the accumulation curve was otherwise very similar to that with M_3cVIII (Hall & Turnock, 1976), while in three other experiments nuclear DNA increased by only 30 - 40% between M2 and M3 (Hall, 1975; Turnock, 1979).

If such an effect were due to nuclei replicating only part of their DNA content, then it could be inferred that the culture was seriously unhealthy or abnormal, i.e. in unbalanced growth, but, in a multinucleate cell, it might also be caused by a fraction of nuclei undergoing no DNA replication, the rest doubling their DNA content normally, a situation which does not necessarily presuppose the culture to be seriously unhealthy. Estimation of the DNA content of individual nuclei by microdensitometry (Hall, 1975; Turnock, 1979) suggested that the latter explanation was true, i.e. a fraction of non-replicating nuclei were responsible for the

reduced degree of synthesis.

It is possible that, in a multinucleate plasmodium, the proportion of nuclei that is to divide and undergo DNA synthesis can be regulated. Such an option would not be available to normal cells and may be unique to Physarum. It would be worthwhile to investigate the conditions under which this effect occurs, and whether it is related to the DNA:mass ratio (the results of experiment 1. above imply a change in this ratio between mitoses). As yet the fate of the non-dividing nuclei is unknown.

Chapter 4. Effects of cycloheximide on DNA synthesis.

4.1. Introduction.

It has been known for many years that inhibition of protein synthesis in eucaryotic cells causes a decline in DNA synthesis (Mueller et al., 1962), and cycloheximide is perhaps the inhibitor that has been most commonly used to examine this phenomenon. Investigations into the effect of this substance on DNA synthesis in Physarum (discussed in Section 4.4.2.) have employed either continuous- or pulse-labelling methods to measure the incorporation of radioactive thymidine or deoxyadenosine into cold acid-insoluble material. It was the aim of the experiments described in this chapter to study some effects of cycloheximide on DNA synthesis assayed by isotope dilution rather than by an experimental labelling method. In particular, the shortest time at which inhibition could be detected was to be determined and compared with published observations (Funderud & Haugli, 1977b) showing inhibition of the mechanism of DNA replication at times as short as 2 min (Section 4.4.2). It is apparent from a number of reports (Cummins & Rusch, 1966; Muldoon et al., 1971; Evans et al., 1976) that some residual incorporation occurs after addition of inhibitor, and it was to be determined whether this is reflected in a residual accumulation of DNA as assayed by isotope dilution. In a number of systems (summarised in Stimac et al., 1977) it has been observed that, under conditions of partial protein synthesis inhibition, the degree of inhibition of DNA synthesis is roughly equal to the degree of inhibition of protein synthesis, and preliminary experiments were undertaken to determine whether this is the case in Physarum.

These are preceded by experiments measuring the effect of cycloheximide on the incorporation of [^{14}C]lysine (normalised by a [^{3}H]uridine prelabel) by surface plasmodia. These were undertaken because published reports on the inhibition of protein synthesis in

Physarum show somewhat varying results (Section 4.4.1.). The use of an experimental labelling procedure was considered justified because the experiments were in the nature of a preliminary study prior to investigating effects on DNA synthesis, and such a procedure would be more rapid than the isotope dilution method developed to assay protein synthesis (Birch & Turnock, 1977). As cycloheximide is a well-established inhibitor of protein synthesis, it was also felt that any observed reductions in incorporation were likely to be due to true protein synthesis inhibition rather than to, for example, an inhibition of uptake alone. The concentration of cycloheximide employed (20 ug ml^{-1}) is higher than that used in most experiments in Physarum (5 - 10 ug ml^{-1}) because the results of Section 4.3.5. suggest that one effect of reducing the concentration of the inhibitor is to slow the onset of maximum inhibition; as it was necessary to inhibit protein synthesis as quickly as possible, a relatively high concentration was therefore employed.

4.2. Materials and methods.

4.2.1. Incorporation of [^{14}C]lysine by ^{3}H-prelabelled cultures.

By experimenting with sections of S and S 576 filter paper with and without overlying plasmodium, it was found that a section of 1 cm^2 area rested comfortably on a drop of medium of 100 ul volume without touching the underlying surface but leaving an area of medium around the edges of the paper to allow for further growth of the plasmodium. Similarly, sections of 2 cm^2 area rested comfortably on 200 ul and early experiments used 1 cm x 2 cm sections on this volume, but it was found that, in most cases, half this area would furnish sufficient material with which to measure incorporation. Besides conserving isotope, this allowed approximately 15 - 20 segments to be taken from a single small plate culture

around M3, so that all points could generally be derived from one plasmodium. The drops of medium were measured using a constriction pipette and were placed in plastic rather than glass petri dishes because in these the liquid spread very little. Usually there were four drops per plate although, if incubation was to be prolonged beyond an hour, a fifth drop which was not covered with a section of paper or plasmodium, was included so as to prevent excessive evaporation of the other drops.

Small surface cultures were established from microplasmodia labelled as in Section 3.2.1. with 20 uCi [5,6-^{3}H]uridine (10 uM carrier). A pencilled grid of squares, 0.5 cm x 0.5 cm, had been ruled on the underside of the filter paper before autoclaving to enable the surface plasmodia to be cut into pieces of approximately 1 cm^2 whenever this was possible. These segments were blotted carefully on absorbent paper to remove excess medium and incubated usually on 100 ul drops of medium containing L-[U-^{14}C]lysine monohydrochloride (Amersham, U.K. CFB. 69; 350 mCi/mmol) with or without cycloheximide (Sigma; No. C-6255) at a concentration of 20 ug ml^{-1}, unless otherwise stated. The amount of [^{14}C]lysine varied and will therefore be stated for individual experiments. All incubations took place at 26°C in the small plate apparatus which is described in Section 2.4.1.

At the end of incubation, samples were scraped into 5 ml of ice-cold 5% TCA/acetone/water to decolourise and were suspended using a small plastic rod. After at least 30 min on ice, samples were centrifuged at 300g (2000 rpm in bench MSE centrifuge) for 1 min, further decolourised in 5% TCA/acetone/water at room temperature for at least 20 min or until pellets were white or cream-coloured, and recentrifuged. The pellets were then suspended in 5 ml of 5% TCA and filtered (details in Section 3.2.7.) through a Sartorius membrane filter of 2.5 cm diameter and 0.45 mm pore size

on which they were combusted in a Packard sample oxidiser (Section 3.2.7.) to allow separate counting of $^{3}H_2O$ and $^{14}CO_2$. The $^{14}CO_2$ was counted in a system containing Carbo-Sorb (Packard), an organic quaternary ammonium compound which renders the radioactive CO_2 soluble in the scintillant Permafluor V (Packard). Assuming that the ^{3}H-prelabelled nucleic acid is of a specific activity uniform throughout the plasmodium, and is itself uniformly distributed, the ^{3}H content of a segment is directly proportional to the mass of that segment at time 0. Thus the incorporation of ^{14}C by different segments can be standardised by calculation of the $^{14}C/^{3}H$ ratio for each segment, provided all samples share the same time 0.

4.2.2. Inhibition of DNA synthesis by cycloheximide (20 ug ml^{-1}).

Specific designs are given for each experiment involving the determination of DNA synthesis in the presence of cycloheximide, this section outlining only those procedures common to all. The basic procedure was, as above, to incubate experimental segments on medium containing cycloheximide at the same time as controls incubated on medium alone. In most cases the specific activity of total DNA was determined, so that more samples could be obtained from a single plasmodium than if nuclei were isolated. Even so, larger segments were required than those used for [^{14}C]lysine incorporation, and their areas were more variable and not measured by use of a grid. Small plate cultures, prelabelled with 100 uCi [6-^{3}H]thymidine (1 uM carrier) by the procedures of Section 3.2.1., were capable of yielding up to nine samples if cut after M3. When nuclei were to be isolated, prelabelled large cultures were used so as to provide sufficient material for a number of samples. Inoculum material was carefully excluded from all the cut segments.

Since larger segments than those for measuring [^{14}C]lysine incorporation were used, and because no radioactive precursors were

present in the medium, sections were incubated not on drops but on grids over 15 ml medium in small plates. Before exposure to inhibitor, the filter paper with plasmodium was cut to provide the requisite number of segments and, at time 0, after removing excess medium by blotting the underside of the filter paper on tissue paper, experimental and control sections were transferred to plates at 26°C containing 15 ml fresh medium with and without cycloheximide at 20 ug ml^{-1}. Three or four sections were incubated per 15 ml when total DNA specific activity was to be determined and one when nuclei were to be isolated.

After incubation, if the specific activity of total DNA was required, the plasmodial material was scraped immediately into ice-cold 5% TCA/acetone/water using a plastic rod and processed as described in Section 3.2.4. If nuclei were to be isolated, plasmodial material was scraped into 40 ml of ice-cold homogenising medium in a 200 ml blender cup and nuclei were prepared as described in Section 3.2.2., before determining DNA specific activity as in Section 3.2.4.

4.3. Results.

4.3.1. Distribution of incorporated [^{14}C]lysine.

The procedure outlined in Section 4.2.1. does not exclude from the sample to be counted ^{14}C radioactivity that might have been incorporated into either RNA or DNA, because the usual hot perchloric acid incubation would remove most of the ^{3}H prelabel from the pellet. Experiments were therefore carried out to determine the proportion of cold acid-insoluble ^{14}C radioactivity that was soluble in hot acid.

(i) 1 cm x 2 cm rectangles from unlabelled small plate cultures were transferred at 60 min after M3 to petri dishes each containing 10 uCi L-[U-^{14}C]lysine (concentration = 0.67 uCi ml^{-1}). Duplicate

samples were removed at intervals, decolourised in 2 x 5 ml 5% TCA/acetone/water, washed in 5 ml 0.25M perchloric acid, incubated in 2 ml 0.5M perchloric acid at 70°C for 20 min and centrifuged. The supernatant, containing label in RNA and DNA, was counted in aqueous scintillant (see Section 3.2.4.), whilst the pellet was resuspended in 5 ml 5% TCA, filtered through a glass microfibre filter (Whatman GF/C : 2.5 cm diameter) and counted in 4 ml of toluene scintillant (composition per litre : 1000 ml A.R.Toluene; 5 g PPO; 0.3 g dimethyl POPOP) in plastic inserts. The procedure was carried out in both N+C and SOYA DM and, expressing the cpm in hot acid-soluble material as a percentage of that in hot acid-soluble and insoluble material combined, the following results were obtained.

	N+C	SOYA DM		N+C	SOYA DM
Time (min)	% total	% total	Time (min)	% total	% total
15	-	8.7	60	7.6	7.7
15	-	10.6	60	7.5	8.1
30	9.5	8.5	120	7.5	-
30	8.3	8.6	120	7.1	-
45	9.5	7.8	400	7.7	-
45	8.0	7.8	400	7.2	-

Mean N+C = 8.0% Mean SOYA DM = 8.5%

2 cultures were used for the N+C results and 3 for the SOYA DM.

(ii) The experiment was repeated at short times in separate experiments in both media, with experimental procedures modified as follows:

N+C: concentration of [^{14}C]lysine = 5 uCi ml^{-1}; time 0 = M3 + 60 - 80 min; 1 cm x 2 cm segments incubated on 200 ul droplets. Four cultures used.

SOYA DM: concentration of [^{14}C]lysine = 6.25 uCi ml^{-1}; time 0 = M3 + 60 min; 1 cm x 1 cm segments incubated on 100 ul droplets. One culture used.

Time (min)	N+C % total	SOYA DM % total	Time (min)	N+C % total	SOYA DM % total
5	19.4	7.5	30	10.3	4.0
5	20.9	6.5	30	11.3	4.5
10	13.4	5.0	45	8.4	-
10	13.7	4.9	45	9.3	-
15	10.5	4.2	60	8.2	-
15	12.5	4.7	60	8.3	-

The results ~~at 5~~ up to 30 min in N+C are likely to be somewhat unreliable because the counts obtained were of the same order as the background.

Thus the results suggest that less than 10% of radioactivity from [^{14}C]lysine is incorporated into cold acid-insoluble/hot acid-soluble material. For the experiments planned, this was considered to be an insignificant source of error but, nevertheless, a corrective factor of 8% was subsequently subtracted from all values of [^{14}C]lysine incorporation into cold acid-insoluble material, although the value of the factor that should be used in the presence of cycloheximide is, in fact, unknown.

4.3.2. Comparison of [^{14}C]lysine incorporation in N+C and SOYA DM.

This experiment was designed to compare the characteristics of lysine incorporation in both media, and to determine the advantages of normalisation of samples using a [^{3}H]uridine prelabel as a standard. Cultures prelabelled with 100 uCi of [^{3}H]uridine were treated as described in Section 4.2.1., except that 200 ul drops were used and an attempt was made to excise segments of identical size (1 cm x 2 cm). Four cultures were used for the N+C curve and two for the SOYA DM. The profiles obtained using ^{14}C

counts alone are illustrated in Fig. 17 and those results normalised using the ^{3}H prelabel in Fig. 18. The results of this experiment demonstrated that 20 uCi of [^{3}H]uridine would suffice for the purpose of normalisation.

For each time point the ratio of incorporation into SOYA DM compared to N+C was calculated.

Time (min)	SOYA DM/N+C ^{14}C alone	SOYA DM/N+C $^{14}C/^{3}H$
15	5.5	5.3
30	9.7	6.5
45	6.2	6.4
60	5.8	5.9

Normalisation appeared to decrease the variability of the estimated ratios. Incorporation in SOYA DM was approximately six times greater than in N+C, as assayed by ^{14}C dpm alone.

At each time point duplicate samples had been taken and the variability between each pair was calculated as 100 x (difference between duplicates/mean of duplicates). The mean and standard error of these values were as follows.

	^{14}C alone		$^{14}C/^{3}H$	
Medium	N+C	SOYA DM	N+C	SOYA DM
Mean	16.9	27.7	17.5	17.2
S.E.	3.8	10.5	5.1	2.7

Normalisation appeared to decrease the variability between duplicates in SOYA DM, but not in N+C. However, its advantage is demonstrated more clearly if no attempt is made to take samples of identical area. This is important because it was not always possible

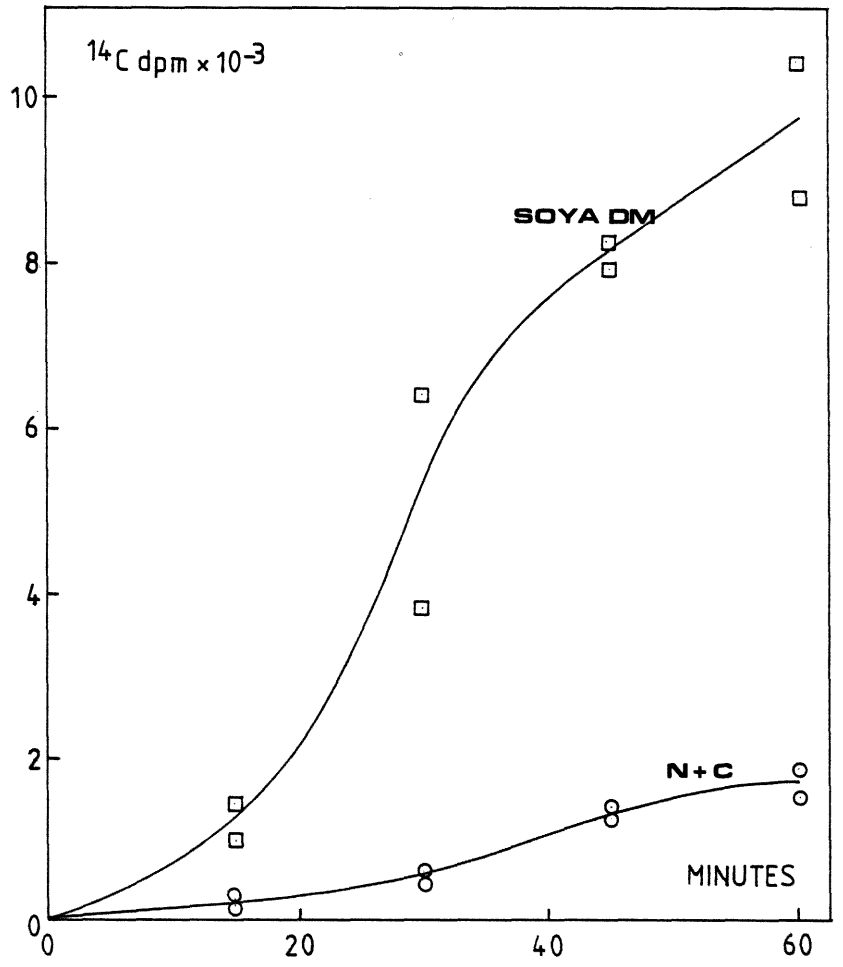

Fig. 17. Incorporation of [^{14}C]lysine by small plasmodia in N+C and SOYA DM: radioactivity due to incorporated ^{14}C alone.

Small plate cultures were established from microplasmodia labelled with 100 uCi [5,6-^{3}H]uridine (10 uM) (Sections 3.2.1. and 2.4.1.). After 25 h, 4 N+C (O) and 2 SOYA DM (□) cultures were cut during interphase into segments approx 2 cm x 1 cm and transferred to 200 ul droplets of medium plus [^{14}C]lysine. At various times segments were harvested and processed as in Section 4.2.1. Counts, converted to dpm, in the ^{14}C channel after combustion are plotted against the length of exposure to [^{14}C]lysine.

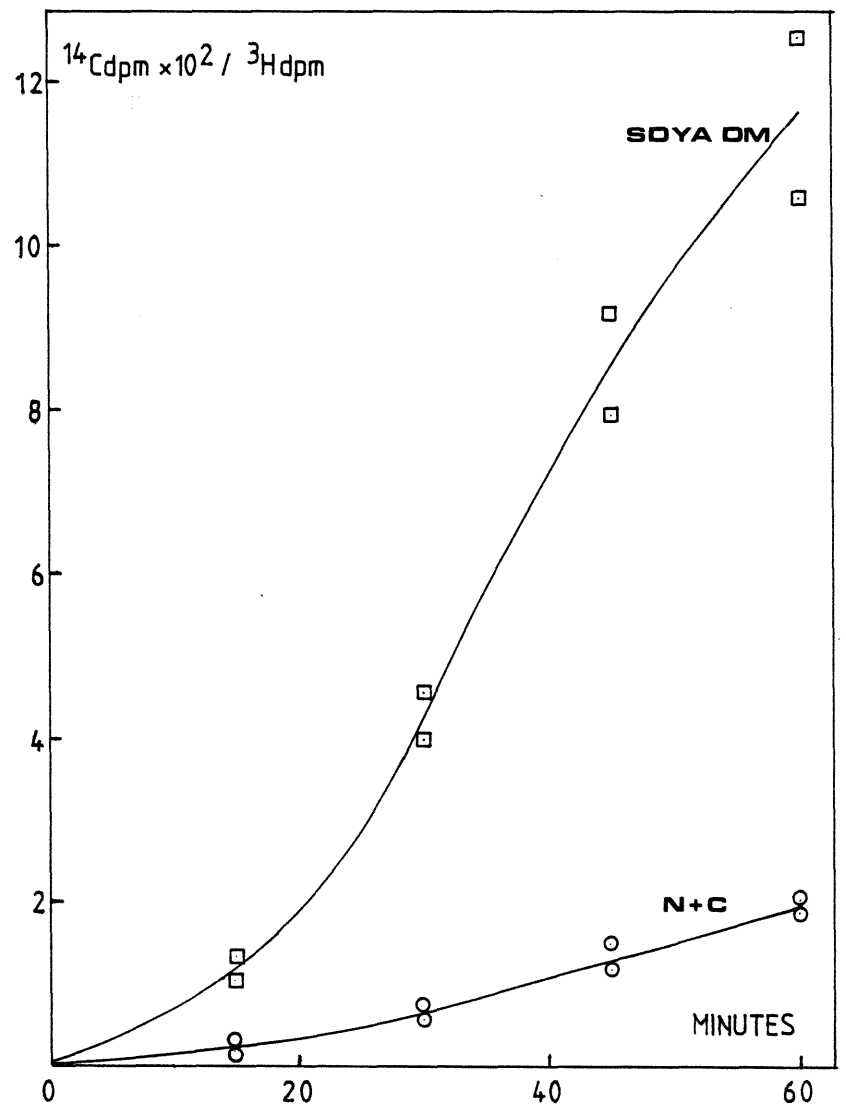

Fig. 18. Incorporation of [^{14}C]lysine by small plasmodia in N+C and SOYA DM normalised by a [^{3}H]uridine prelabel.

Details as in Fig. 17. Counts ~~from in~~ the ^{14}C channel of the oxidiser were converted to dpm and divided by the dpm ~~from in~~ the ^{3}H channel of the oxidiser. This ratio is plotted against the length of exposure to [^{14}C]lysine. A comparison between lysine incorporation in N+C and SOYA DM cannot be made using this ratio as [^{3}H]uridine incorporation may be different in the two media.

to obtain a sufficient number of equal-sized segments when plasmodia were not large. Thus, in a further experiment using plasmodia prelabelled with [^{3}H]uridine in N+C, three samples were harvested at each time point; their areas were 1 cm x 1 cm, 1 cm x 2 cm and 1 cm x 3 cm and they had been incubated on droplets of 100, 200 and 300 ul respectively, each containing [^{14}C]lysine at a concentration of 0.67 uCi ml^{-1}. A separate plasmodium was used at each time point, and the coefficients of variation of the triplicates were as follows:

Time (min)	CV of ^{14}C alone	CV of ^{14}C/^{3}H
30	39.8	1.8
60	45.2	8.0
90	61.7	25.4
120	47.7	3.9
Mean	48.6	9.8
S.E.	4.7	5.4

These results demonstrate a substantial decrease in variability using the normalisation procedure.

4.3.3. Inhibition of [^{14}C]lysine incorporation by 20 ug ml^{-1} cycloheximide.

Besides the degree of inhibition of [^{14}C]lysine incorporation by cycloheximide, it was also determined in these experiments whether there were any differences in [^{14}C]lysine incorporation and its inhibition by cycloheximide between G2 and S phase. The results of two experiments are presented.

(i) N+C medium at times up to 60 min.

The results obtained are illustrated in Fig. 19. The mean percentage inhibition at each time point was:

15 min = 94.4%; 30 min = 96.1%; 45 min = 95.3%; 60 min = 97.2%.

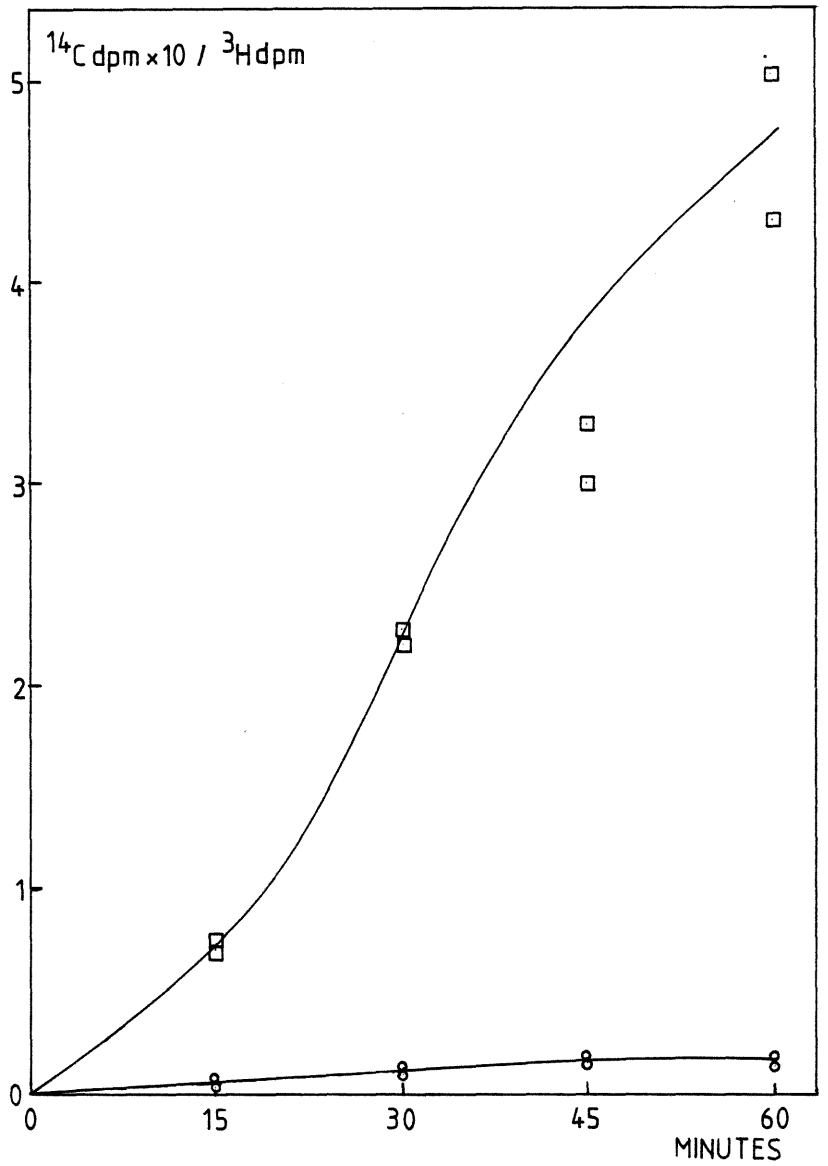

Fig. 19. Inhibition by cycloheximide (20 ug ml^{-1}) of [^{14}C]lysine incorporation by plasmodia in N+C.

Cultures were prelabelled with 20 uCi [5,6-^{3}H]uridine (10 uM). After 27 h growth, interphase segments 1 cm^{2} were transferred to 100 ul drops of N+C + 0.2 uCi [^{14}C]lysine ± cycloheximide. After times as above control (□) and inhibited (o) segments were treated as in 4.2.1.

(ii) SOYA DM medium at times up to 30 min in G2 and S phase.

Since the effects on DNA synthesis of short times of protein synthesis inhibition were ultimately to be determined, it was decided to repeat the above experiment with time intervals of only 5 min. SOYA DM was used in order to conserve label. The results are illustrated in Fig. 20. One culture was used for all S phase points, and another for all those in G2. The latter profile was begun at 135 min before M3 and the S phase profile at 55 min after M3. The mean percentage inhibition at each point was as follows:

Time (min)	G2 % inhib.	S % inhib.
5	83.3	82.2
10	93.8	92.4
15	95.4	94.4
30	97.4	96.9

The results suggest both a similar pattern of lysine incorporation during S and G2 phases (although more sensitive determinations would be required to show whether the two curves were identical; there would be a constant ratio of incorporation between G2 and S for each time point if this were the case), and an almost identical pattern of inhibition by cycloheximide. It was decided that an inhibition of above 90% after 10 min would be sufficient for the purpose of investigating effects on DNA synthesis at short times. The lower values of inhibition at 5 min suggest that, at times soon after 0 min, some radioactive lysine can be incorporated into protein. Even if protein synthesis had completely stopped by 10 min, a figure of 100% inhibition would not be derived by the present method because the value obtained is a summation over the whole 10 min. Complete inhibition would, however, be indicated by a plateau in the incorporation curve. In experiment (ii)

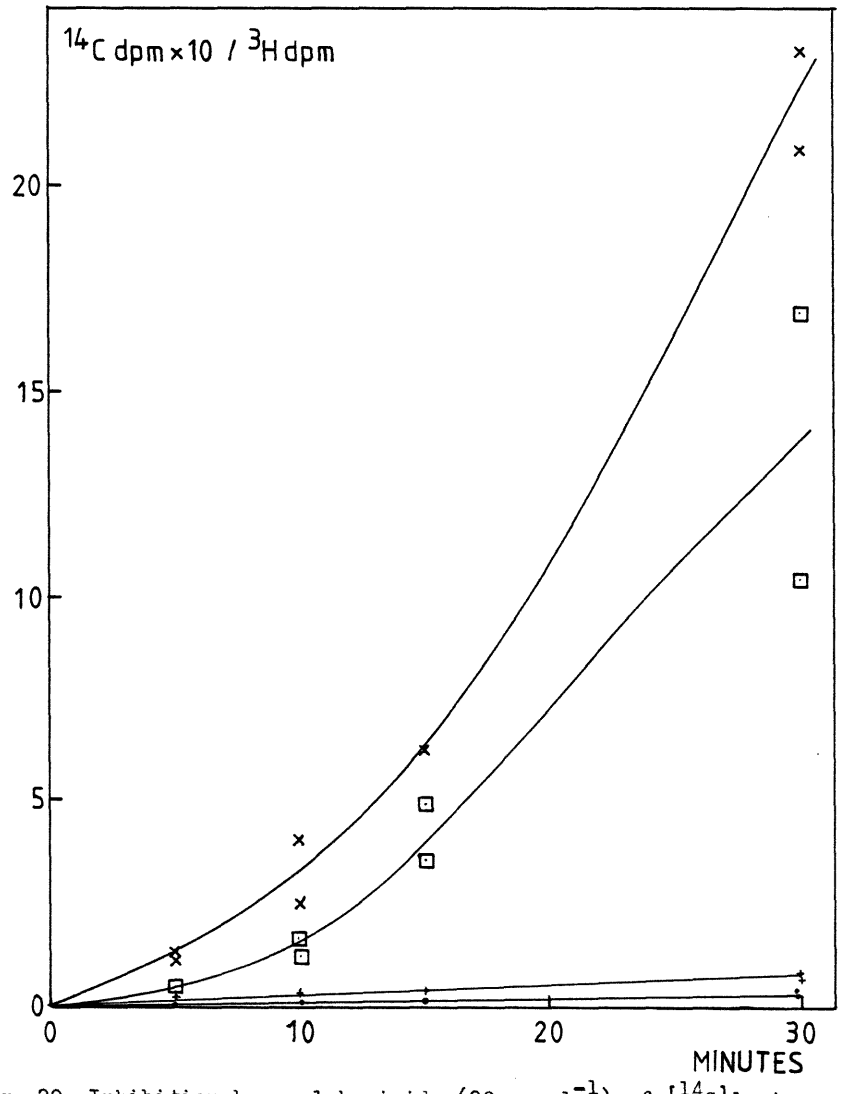

Fig. 20. Inhibition by cycloheximide (20 ug ml^{-1}) of [^{14}C]lysine incorporation by plasmodia in SOYA DM during G2 and S phase.

At 2 h before and 1 h after M3, 1 cm^2 segments of a small plasmodium prelabelled with 20 uCi [5,6-^{3}H]uridine (10 uM) were transferred to 100 ul drops of medium + 0.2 uCi [^{14}C]lysine ± cycloheximide. The $^{14}C/^{3}H$ ratio is plotted against length of exposure to [^{14}C]lysine alone in G2 (□) or S phase (x) or to [^{14}C]lysine + cycloheximide in G2 (·) or S phase (+). Duplicate samples were taken at all points.

the increase in total incorporation between 15 and 30 min in both S and G2 phase suggests that it did occur at a reduced rate at least until after 15 min while in experiment (i) the absence of an increase between 45 and 60 min suggests incorporation was terminated by 45 min.

~~The higher $^{14}C/^{3}H$ ratios during S phase were obtained because, for both the G2 and S phase profiles, segments of approximately 1 cm^2 were taken and, if growth in area had occurred during the intervening period, these segments would represent a smaller fraction of the total plasmodium in the later profile and hence would contain less ^{3}H prelabel, thus increasing the $^{14}C/^{3}H$ ratio. In practice the ^{3}H counts remained approximately unchanged while the ^{14}C counts had increased, suggesting that the difference between the two plasmodia was largely one of thickness rather than area.~~

4.3.4. Inhibition by cycloheximide of mitosis and the following S phase.

A preliminary experiment was carried out to ascertain whether cycloheximide would block both mitosis and the ensuing S phase if added before about 13 min prior to M3 (Cummins et al., 1966; Cummins & Rusch, 1966). Four plasmodia growing in N+C were used; each was cut into quarters and two segments were transferred to medium containing cycloheximide (20 ug ml^{-1}) and two to medium alone during early and very early prophase. The time of mitosis in each control was determined, and smears were also taken from those segments exposed to cycloheximide. The morphological observations summarised overleaf showed that an inhibition of mitosis occurred around prophase/metaphase when inhibitor was added up to 25 min before M, but earlier addition (35 and 50 min) appeared to block progress at a point prior to nucleolar disintegration. The latter effect was not seen by Cummins et al. (1966) using cycloheximide at 10 ug ml^{-1}, but no effort was made to investigate this further

because it was sufficient that mitosis was blocked.

Plasmodium	Time of cycloheximide addn. (min before M3 in control)	Observations (times rel. to M3 in control)
1	25	Resembled metaphase at 5 min
2	50	Still very early prophase by 130 min
3	approx. 15	Remained at a stage resembling prophase/ metaphase up to at least 105 min
4	35	Reached a stage just prior to nucleolar dissolution by 25 min

Control and experimental segments from the above were harvested simultaneously at various times after addition of cycloheximide (0, 5, 10, 20, 40, 60, 120 and 180 min) and the specific activity of the DNA determined. While DNA synthesis did occur in the control segments after mitosis, there was no significant synthesis in any of the segments exposed to cycloheximide, including those apparently blocked during mitosis; this is in agreement with the results of Cummins and Rusch (1966).

A further four plasmodia were treated in a similar manner, except that the inhibitor was added during S phase. The results ~~demonstrated~~ suggested that ~~significant~~ inhibition of DNA synthesis occurred within 20 min and possibly within 10 or 5 min and also suggested that there was a slow residual rate of synthesis continuing at least beyond 20 min.

4.3.5. Effect of partial inhibition of protein synthesis on DNA synthesis.

Using the continuous-labelling method of Section 4.2.1., it is difficult to estimate the relative rate of lysine incorporation at any particular time. Therefore, in order to find a concentration of cycloheximide at which lysine incorporation was only partially inhibited, a pulse-labelling method was employed. The experiment, which took place in N+C medium, utilised 15 min pulses of [^{14}C]lysine at a concentration of 5.3 uCi ml^{-1}, and cycloheximide concentrations of 5, 2, 1 and 0.5 ug ml^{-1}. Lengths of exposure to the inhibitor were 15, 30, 60, 90, 120 and 180 min, thus producing four curves with six points each. Each curve was obtained from a single culture and two control curves (A and B) were also constructed, both of which consisted of points harvested alternately from two of the experimental plasmodia. Throughout the experiment, only one sample was taken at each point. Samples were harvested during mid-interphase after M3. Other factors were as outlined in Section 4.2.1.

For each time point, the fraction of synthesis occurring in the presence of cycloheximide was calculated by dividing the $^{14}C/^{3}H$ ratio for the treated segment by the ratio for the control segment that had been added to label either at the same time or during an adjacent period; the control was always derived from the same plasmodium as the experimental point. Figures for percentage synthesis were converted to percentage inhibition by subtraction from 100 and these values, together with the $^{14}C/^{3}H$ ratios for the two control curves (A and B) are shown overleaf. They indicate that a major effect of reducing the concentration of cycloheximide in the range between 5 and 1 ug ml^{-1} is to slow the onset of maximum inhibition, although the final levels are similar at around 95%.

Time	A	B	5 ug ml^{-1}	2 ug ml^{-1}	1 ug ml^{-1}	0.5 ug ml^{-1}
(min)	$^{14}C/^{3}H$ $\times 10^{3}$	$^{14}C/^{3}H$ $\times 10^{3}$	% inhib.	% inhib.	% inhib.	% inhib.
15	247	194	95.6	53.9	41.3	21.9
30	111	192	94.7	93.7	88.0	73.4
60	205	171	95.3	94.4	94.6	86.6
90	170	192	99.1	100.0	96.1	83.1
120	-	173	90.2	97.2	93.2	80.7
180	307	235	86.9	99.5	80.5	-

A slight residual incorporation of [^{14}C]lysine in the presence of cycloheximide is in agreement with the results of Section 4.3.3. using 20 ug ml^{-1} inhibitor. Such incorporation may not be into protein, and it would be of interest to test whether it is into hot acid-soluble or insoluble material, although this would require the use of [^{14}C]lysine at a high specific activity. In the results obtained with 5 and 1 ug ml^{-1} cycloheximide there is some evidence that the inhibition is partially overcome by 180 min, although more data would be necessary to determine whether this is consistently observed. The final plateau value of inhibition using 0.5 ug ml^{-1} cycloheximide appears to be reduced to between 80 and 85%. Therefore, as inhibition of around 50% was desired, a cycloheximide concentration of 0.25 ug ml^{-1} was decided upon for the following experiment.

The relative degrees of inhibition of protein and DNA synthesis were compared only semi-quantitatively since, in a synchronous culture, the rate of DNA synthesis changes with time so that the precise results obtained will depend upon the point in the cycle at which the measurements are made. It was therefore thought justifiable to use a different method for each parameter, i.e. pulse-labelling with [^{14}C]lysine as a measure of protein synthesis rate, and isotope dilution as a measure of DNA accumulation. For

comparisons more detailed than those envisaged in the present experiment, it would be necessary to use the isotope dilution method for both protein (Birch & Turnock, 1977) and DNA synthesis. Only a [^{3}H]uridine prelabel would then be necessary, but the experimental procedure would be more time-consuming than that employed here. It would then be possible to construct, for both parameters, time courses of the inhibition occurring when a low concentration of cycloheximide is added at different times during S phase.

Cultures in N+C were prelabelled with 100 uCi [^{3}H] thymidine (1uM), and segments of size 1.5 cm x 1 cm (to provide sufficient DNA for the diphenylamine assay) were incubated on drops of 400 ul volume. The periods of exposure to cycloheximide at 0.25 ug ml^{-1} were 0, 15, 30, 45, 60, 90 and 120 min, and pulses were for 15 min with [^{14}C]lysine at a concentration of 3.2 uCi ml^{-1}. Three plasmodia were used altogether, for each of which the DNA specific activity at 0 min (M3 + 20 - 25 min) was subsequently determined to allow the percentage of DNA that had been synthesised in both control and experimental plasmodia between time 0 and the time they were harvested to be calculated. Control and experimental segments at each time point were derived from the same plasmodium, and only one sample of each was taken.

Samples were harvested, decolourised, incubated overnight in 0.3M NaOH to solubilise RNA, and DNA was extracted into hot acid according to the normal procedure (Section 3.2.4.). Its specific activity was determined, and no significant ^{14}C cpm were detected in the extract. After extraction of DNA the pellet is likely to contain ^{14}C-labelled protein, ^{3}H-labelled protein and possibly some residual ^{3}H-DNA, so a further extraction into a larger volume of hot acid was performed to remove any ^{3}H-DNA. The pellet was then suspended in 5% TCA, filtered through a Sartorius membrane and

oxidised. The ^{14}C-channel of the sample oxidiser should contain radioactivity due only to ^{14}C-labelled protein, and the ^{3}H channel of the oxidiser was found to contain on average 18% of the total hot acid-soluble and insoluble ^{3}H; this is presumably due mainly to incorporation of radioactivity from [^{3}H] thymidine into protein.

The ^{14}C counts are subject to error because segments exposed to radioactivity at later times are larger than those at early times, more growth having taken place. The counts are also dependent on yield. However, they can be normalised by dividing by the DNA content of the segment as determined by the diphenylamine assay; incorporation in the experimental segments is then divided by that in the relevant controls to give the percentage of synthesis in the presence of cycloheximide which is converted to % inhibition by subtraction from 100. The following results were obtained for [^{14}C] lysine incorporation at each time-point.

Time (min)	% inhibition ^{14}C alone	% inhibition normalised
15	-13.7	8.5
30	45.7	51.4
45	56.7	52.1
60	59.1	58.3
90	78.7	71.5
120	39.5	46.6

The incorporation of ^{14}C appears to be inhibited to a level of about 50% in less than 30 min of exposure. Since cycloheximide was added at 20 - 25 min after metaphase, the 30 min point is at 50 - 55 min after M at which time only about 25 - 30% of the DNA has normally been replicated (Fig. 8). Thus, a detectable amount of DNA synthesis would be able to occur in the control plasmodia

after the time by which [^{14}C]lysine incorporation has fallen to approximately half its normal rate. The value at 120 min again suggests that inhibition might be partly overcome, but it is equally likely that the value at 90 min is erroneously high.

The DNA accumulation results obtained were as follows:

Time (min)	Culture	Control Sp. act.	Control % Synth.	+CHX Sp. act.	+CHX % Synth.	Synth. in exposed segments as % control
15	C	308	10.2	305	11.2	109.8
30	C	281	21.0	272	25.1	119.5
45	B	275	19.5	261	26.0	133.3
60	B	256	28.5	241	36.1	126.7
90	A	258	40.9	254	42.8	104.6
120	A	231	57.2	242	49.8	87.1

Although the final column contains values that are substantially greater than 100%, suggesting that there was significantly more DNA synthesis in segments exposed to cycloheximide than in the controls, these values are produced by relatively small differences in specific radioactivity between experimental and control samples which may be attributable to experimental error; for example, the value of 133.3% is derived from the 5% difference in specific activity between 274.8 and 260.7 cpm ug^{-1}. However these results do suggest that there is no significant inhibition of DNA synthesis as assayed by isotope dilution while in the same plasmodium the rate of protein synthesis, as assayed by the rate of [^{14}C]lysine incorporation, is inhibited by 50%.

4.3.6. Inhibition of DNA synthesis after short periods of exposure to cycloheximide.

An attempt was made to determine the pattern of DNA synthesis

inhibition by cycloheximide (20 ug ml^{-1}) during the first 40 min of exposure. Soon after M3, a single small plate culture was cut into nine sections and, at M3 + 30 min, three were transferred to fresh medium plus cycloheximide (experimental samples, E), three to medium without cycloheximide (control samples, C) and three were immediately harvested in 5% TCA/acetone/water (time 0 samples, 0). After the required time (t), the three control and the three experimental samples were also harvested. The specific radioactivity of total DNA in those harvested after time t was normalised to the mean value of the time 0 samples. For each normalised triplicate the mean and standard error were determined and plotted graphically. Fig. 21 illustrates the results of an experiment (A) with times between 10 and 40 min after exposure to cycloheximide. Attempts were also made to repeat the procedure using shorter intervals (5 - 20 min) and the results of these are presented in Figs 22 and 23 (experiments B and C, respectively). Time 0 in experiment B was M3 + 60 min, and M3 + 35 min in experiment C.

In both experiments B and C there is no detectable accumulation of DNA in the control samples between 5 and 10 min and in experiment C between 15 and 20 min. This, together with the relatively large error at short times, suggests that significant differences in DNA specific activity were not detected. If so, this might be due to slight asynchrony within cultures, although none was detected morphologically, or more probably to limitations in the sensitivity with which specific activities are measured. To gain a better idea of whether significant differences were detected, the values of specific activity were analysed in more detail.

A non-parametric analogue of a single classification analysis of variance, the Kruskal-Wallis test (see Section 2.6.), was applied to the results for each time-point within the three experiments to discover whether there were differences between the three treatment

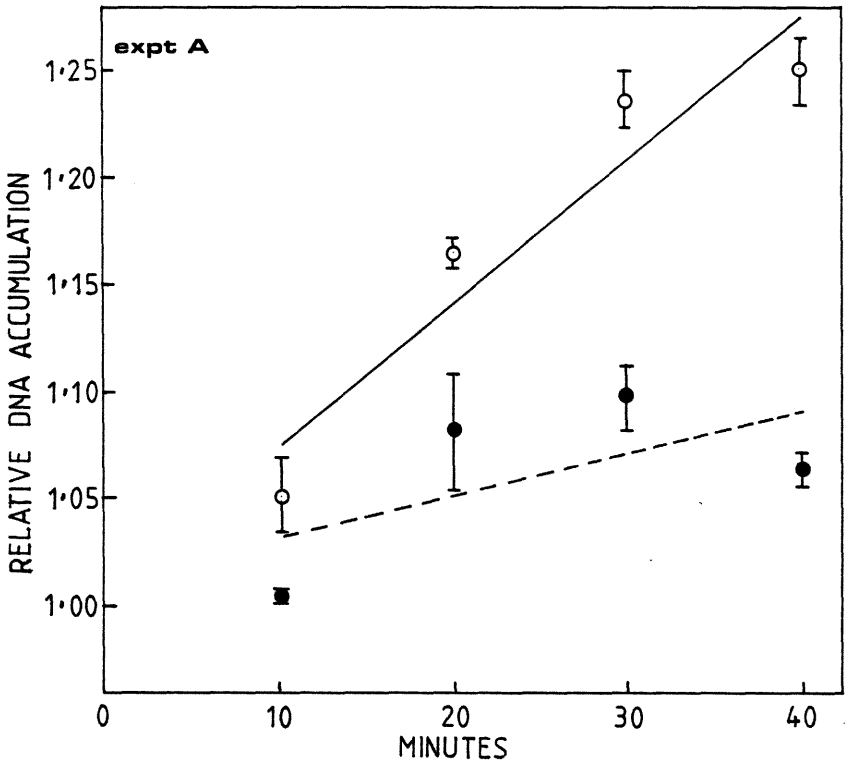

Fig. 21. Inhibition by cycloheximide (20 ug ml^{-1}) of DNA synthesis by plasmodia in N+C at times up to 40 min after exposure.

After M3, 4 cultures, prelabelled with 100 uCi [6-^{3}H]thymidine (1 uM) (Section 3.2.1.), were each cut into 9 segments and, at M+30 min (time 0 above), 3 segments from each culture were transferred to 15 ml medium + cycloheximide, 3 control segments to fresh medium alone, and 3 were harvested. For each time point only samples from one culture were used; after times shown on the x-axis, the 6 remaining segments were also harvested and the total DNA specific activity determined (Section 3.2.4.). For each culture, all values were normalised to a mean of 1.00 for the segments harvested at time 0 and plotted, with standard errors, as a function of the length of exposure to fresh medium ± inhibitor. Exposed = ●; control = ○.

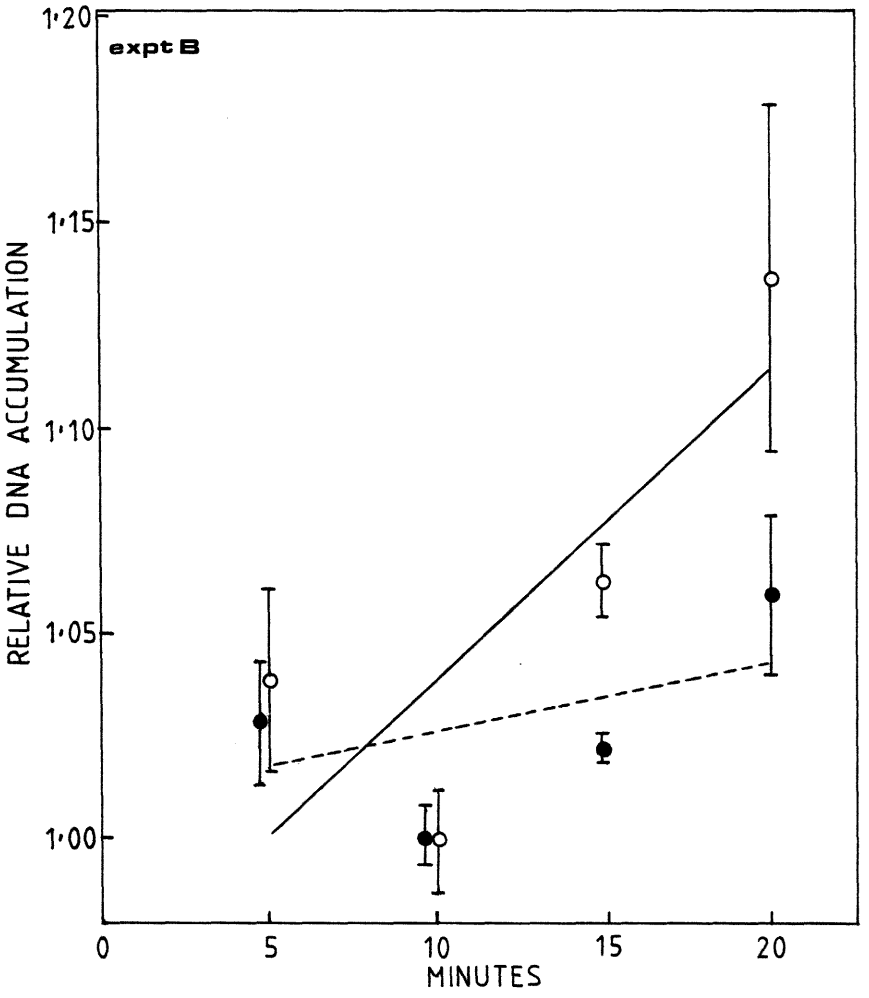

Fig. 22. Inhibition by cycloheximide (20 ug ml^{-1}) of DNA synthesis by plasmodia in N+C at times up to 20 min after exposure. (1).

Details as for Fig. 21. except that time 0 = M3 + 60 min.

Exposed =●; control =○.

As in Figs. 21 and 23, regression lines have been fitted by the method of least squares simply to indicate the overall gradient.

Continuous line = control samples; dashed line = inhibited samples.

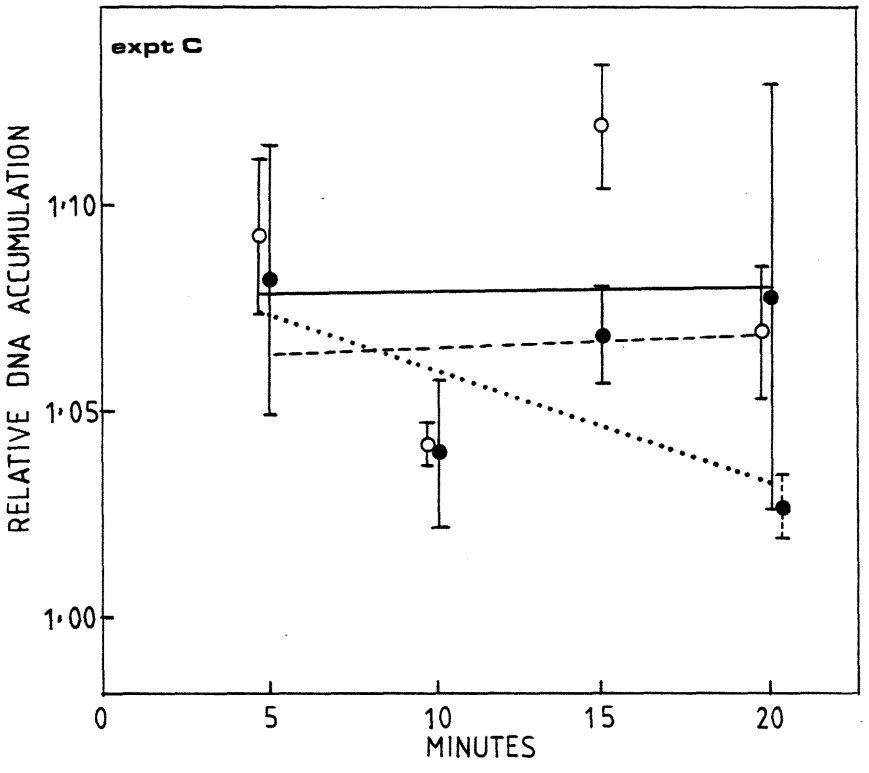

Fig. 23. Inhibition by cycloheximide (20 ug ml^{-1}) of DNA synthesis by plasmodia in N+C at times up to 20 min after exposure. (2).

Details as for Fig. 21. except that time 0 = M3 + 35 min.

Exposed =●; control =○.

The exposed point at 20 min with dotted error bars represents the value obtained when an apparently outlying point is omitted.

Regression lines were fitted as for Fig. 22. Continuous line = control samples; dashed line = inhibited samples; dotted line = inhibited samples if outlying point at 20 min is removed.

means (O, E and C) significant at p = 0.05. It was necessary to use the table derived from the modified distribution which results when only three groups with less than five replicates each are involved (Kruskal & Wallis, 1952). Since the non-parametric STP test which is usually subsequently employed to compare individual pairs of means cannot be carried out if there are only three replicates per group, a one-way classification analysis of variance (ANOVA) was carried out, together with the tests between individual means to be described below. In the following table, the presence of differences between treatment means significant at p = 0.05 is indicated by +.

Expt.	Time (min)	K-W test	ANOVA	Expt.	Time (min)	K-W test	ANOVA
A	40	+	+	B	10	-	-
A	30	+	+	B	5	-	-
A	20	+	+	C	20	-	-
A	10	+	+	C	15	+	+
B	20	-	-	C	10	-	-
B	15	+	+	C	5	-	-

Both tests thus produce the same results. The lack of significance for the 20 min values in experiments B and C was attributable in both cases to one outlying value, the removal of which made the differences between means significant by both tests. However, neither value was classifiable statistically as an outlier by the T test (Grubbs, 1969), presumably because of the small number of samples involved.

Further comparisons between individual means were made using an <u>a priori</u> test, the least significant difference (L.S.D.) (Sokal & Rohlf, 1969), because the means to be compared were nominated before the experiment was carried out. The following

results were found: (+) = difference significant at $p = 0.05$; (-) = difference not significant and bd = borderline.

Expt.	Time (min)	LSD comparison 0 vs C	0 vs E	E vs C
A	40	+	+	+
A	30	+	+	+
A	20	+	+	+
A	10	+	-	+
B	20	+	-	-
B	20*	+	bd-	+
B	15	+	bd+	+
B	10	-	-	-
B	5	-	-	-
C	20	-	-	-
C	20*	+	-	-
C	15	+	bd+	-
C	10	-	-	-
C	5	-	-	-

* results re-analysed after removal of outlying value.

These results suggest that differences of specific activity between samples taken less than 15 min apart beginning at M + 30 min cannot be consistently resolved using only triplicate samples. This is due to experimental variability in the determination of DNA specific activity. The coefficient of variation (CV) was determined for each set of triplicates in the above experiments, and the overall mean value was calculated to be 2.64% (S.D. = 1.7). Using Fig. 8 showing S phase in N+C, the following percentage syntheses and their corresponding percentage changes in specific activity (within the ranges of specific activity usually obtained) were

calculated:

M + 30 to M + 40 = 6.5% synth. = 5.7% change in Sp. act. (10 min);

M + 30 to M + 45 = 10.5% synth. = 9.1% change in Sp. act. (15 min);

M + 30 to M + 50 = 14.5% synth. = 12.3% change in Sp. act. (20 min).

Knowing the CV, it is possible to approximately calculate the number of replicates that would be required within each of two differently treated populations in order to be, say, 80% certain of detecting the percentage differences given above at the 5% level of significance (formula in Sokal & Rohlf, 1969). These were 4 - 5, 3, and 2 - 3 replicates for 6%, 9% and 12% (or 10, 15 and 20 min time intervals) respectively. As the S phase curve is nearly linear up to 100 min, it is likely that similar results would be obtained throughout most of S phase. The results of this calculation are consistent with those of the table above and they suggest that it is unrealistic to expect to detect synthesis of DNA after times as short as 10 min when using only three replicates. It may be noted that the variability of the CVs of the triplicates is itself considerable (CV = 64%), accounting for the finding of a significant difference in experiment A at only 10 min while there were none in experiment C at 20 min, except when the outlier was removed.

If cycloheximide does produce significant inhibition by 15 or 20 min, thus producing intermediate values of specific activity, it might be expected that more than three replicates would be needed to detect, with 80% certainty, differences of specific activity between these treated samples and either the time 0 or control samples; only if no synthesis at all takes place, or if there is no inhibition in the presence of cycloheximide, would there be an 80% certainty of detecting significant differences. However, the use of more replicates creates practical difficulties. The isolation of samples at short times is already subject to error because the harvesting of each sample takes a finite time which is

not negligible compared with short periods of exposure; this error will be increased if more samples are involved. Furthermore, larger plasmodia would be required, and the resulting lower synchrony would increase the variability of specific activity. Therefore, if the isotope dilution method were to be used again to detect small changes in DNA specific activity, it would be necessary to improve its sensitivity. This could probably be most readily achieved by the adoption of a different assay for DNA, possibly a fluorometric assay, since this is at present the least sensitive part of the procedure. Some improvement would probably ensue from the adoption of a method of counting which was more sensitive than the use of ^{3}H in aqueous scintillant, possibly by ~~using a ^{14}C isotope and/or~~ avoiding the use of aqueous emulsions. Nevertheless, even if the assay were to be improved, slight asynchrony in an individual plasmodium could preclude the detection of changes of specific activity over very short periods of time.

While differences between time 0 and control samples in the above results are detectable after 15 min, and in one case after 10 min, differences involving samples exposed to cycloheximide are not consistently detectable at such short times. This, together with the fact that, in all cases except B at 10 and 5 min and C at 20 min (if the outlying sample is included), the mean specific activity of the experimental samples is intermediate between those of the time 0 and control samples, demonstrates that cycloheximide at 20 ug ml^{-1} inhibits DNA accumulation by 15 min; it is, however, impossible to quantify this using the present results. With more replicates, or a more sensitive assay, it is likely that inhibition could be detected at shorter times (see Section 4.4.2.). It is also impossible to determine the pattern of inhibition at short times from the present results; the intermediate values of specific activity obtained could be caused by a delay after exposure followed

by total inhibition, by a constant low level of synthesis after negligible delay or by an effect intermediate between the two.

The percentage inhibition was calculated for later times, using the mean values for DNA accumulation, with the apparently outlying points at 20min in expts B and C removed.

Expt.	Time (min)	% inhibition
A	40	74
A	30	58
A	20	50
B	20	66
C	20	66

These results suggest an increase in the percentage of inhibition with time, as would be produced either by DNA synthesis being absent or continuing at a low rate in the presence of inhibitor. That accumulation at a low rate continues for at least 20 min is suggested by the fact that a significant difference between time 0 and experimental samples occurs only at this time, (and not at 10 min in experiment A), while the results of experiment A (Fig. 21) suggest residual accumulation may occur at least up until 30 min. The following section summarises attempts to detect this residual synthesis over longer periods of exposure to cycloheximide.

4.3.7. Residual synthesis in the presence of cycloheximide.

The results of experiment A above show that the residual synthesis in the presence of cycloheximide (20 ug ml^{-1}) is between approximately 7 and 11% of the total synthesis (assuming 10% synthesis has occurred by time 0) by 20 - 40 min of exposure. An experiment was carried out to determine whether further synthesis does consistently occur by 60 min and whether this amount changes

systematically during the course of S phase. At several times during the S period following M3, four segments were cut from a plasmodium growing in the large apparatus; two were immediately harvested, while the remainder were transferred to small plates containing N+C medium and cycloheximide (20 ug ml^{-1}) in which they were incubated for 60 min before harvesting. Total DNA specific activities were obtained as usual and normalised to that at metaphase. The results are illustrated in Fig 24.

Irregularities in the control S phase profile were due to the relatively poor synchrony of the original culture. The average difference between experimental and control points was 0.1172 i.e. 11.7% of total synthesis (equivalent to a 9.6% change in specific activity) with a range of 8.5 to 15.3% and S.D. of 2.75. There was no correlation between the amount of residual synthesis and the time in S phase, probably partly due to variability in specific activity determination but also because the poor synchrony was such that no samples were taken during late S phase at which time the reduced rate of DNA synthesis might have led to a significantly reduced quantity of residual synthesis, unless a roughly constant amount of synthesis were always to occur after inhibition (Section 4.4.2.).

Thus, if exposure to inhibitor begins in early to mid-S phase, approximately 12% of total DNA is still replicated during the first hour. It is not clear whether this synthesis continues after 40 min (at which time 7% of total synthesis was recorded in experiment A of Section 4.3.6.) because of variability in specific activity determinations, and so attempts were made to follow the time-course of this excess synthesis. In one experiment (Fig. 25) a large plasmodium was cut into sections soon after M2. As the culture exhibited an asynchrony of 25 min in division, time 0 ranged between M + 15 and M + 40 min and at this point sections were transferred to

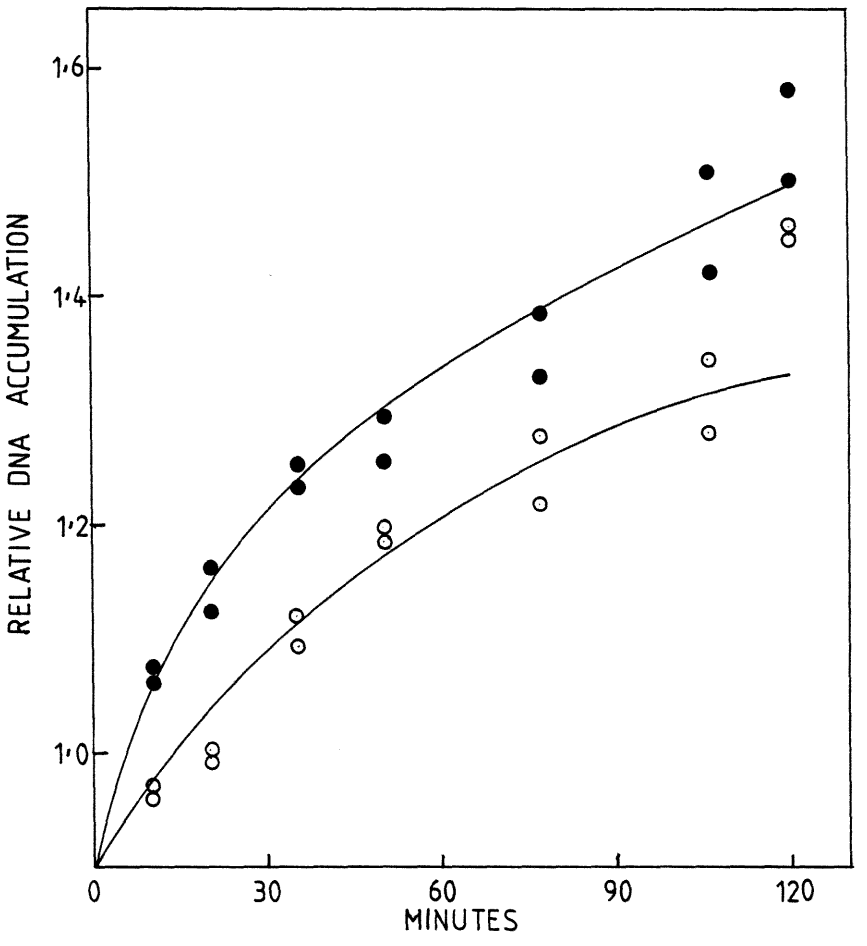

Fig. 24. Residual total DNA synthesis during 60 min exposure to cycloheximide (20 ug ml^{-1}) at various times during S phase.

Total DNA specific activity was determined (Section 3.2.4.) in 4 samples taken at M3 from a large surface culture in N+C prelabelled (Section 3.2.1.) with 100 uCi [6-^{3}H]thymidine. At various times thereafter 4 segments were removed; 2 were harvested immediately and 2 were transferred to fresh medium + cycloheximide and harvested after 60 min. Total DNA specific radioactivity was determined in all samples and normalised to 1.00 at M3. These values are plotted as a function of the time after M3 at which samples were either harvested (O) or exposed to inhibitor (●).

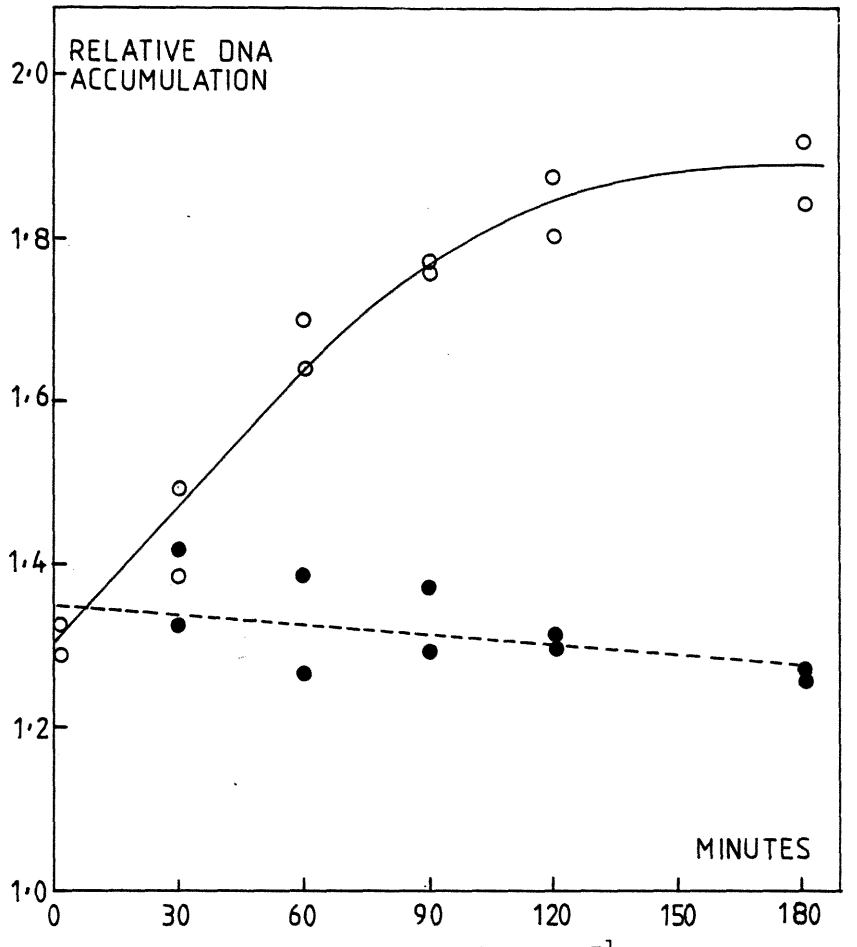

Fig. 25. Inhibition by cycloheximide (20 ug ml^{-1}) of DNA synthesis by plasmodia in N+C at times up to 180 min.

Total DNA specific activity was determined (Section 3.2.4.) in two segments at M2 of a large plasmodium prelabelled with 100 uCi [6-^{3}H] thymidine (1 uM). 15 min later (M+15 to M+40 min due to poor synchrony), 2 more were harvested, 10 were transferred to medium + cycloheximide (●) and 10 remained in place as controls (O). At each time above, 2 exposed and 2 control segments were harvested and total DNA specific activity was ultimately determined in all samples. These values, normalised to 1.00 at M2, are plotted against the length of exposure to the inhibitor for the experimental samples.

plates containing medium ± cycloheximide. Specific activities were normalised to the mean value of two samples harvested at mitosis. At each point, duplicate samples were taken, as shown in Fig. 25. The time 0 samples were from the part of the culture that entered mitosis earliest, with subsequent segments taken from progressively more delayed areas so that the samples shown at later time points on the graph were transferred to inhibitor at effectively shorter times after division than those at earlier points. The only apparent accumulation of DNA in the presence of inhibitor occurs between 0 and 30 min, the remainder of the curve showing a downward trend producing the overall slightly decreasing gradient as fitted by linear regression. This downward trend is presumably an effect of the original asynchrony. These results therefore suggest qualitatively that the majority of the synthesis occurs within the first 30 min after exposure but that, after this time, it is of insufficient magnitude to overcome the effects of the original asynchrony.

Further attempts were made to follow the residual replication of total DNA during long periods of inhibition, and these were compared with the replication of nuclear DNA since, as the synthesis of mitochondrial DNA is generally insensitive to cycloheximide, it is possible that the residual synthesis so far detected is attributable to this component. Similar experimental protocols to that described above were followed using large cultures in N+C except that larger segments were transferred to cycloheximide and these were each used to provide one total and one nuclear DNA sample. The results of two such experiments (A and B) are presented in Figs. 26 and 27. Duplicates were again taken at each time. In A, time 0 was at M3 + 20 - 30 min, the degree of asynchrony within the area of the plasmodium used being only 10 min, while in B, although the asynchrony was approximately 20 min, segments were transferred

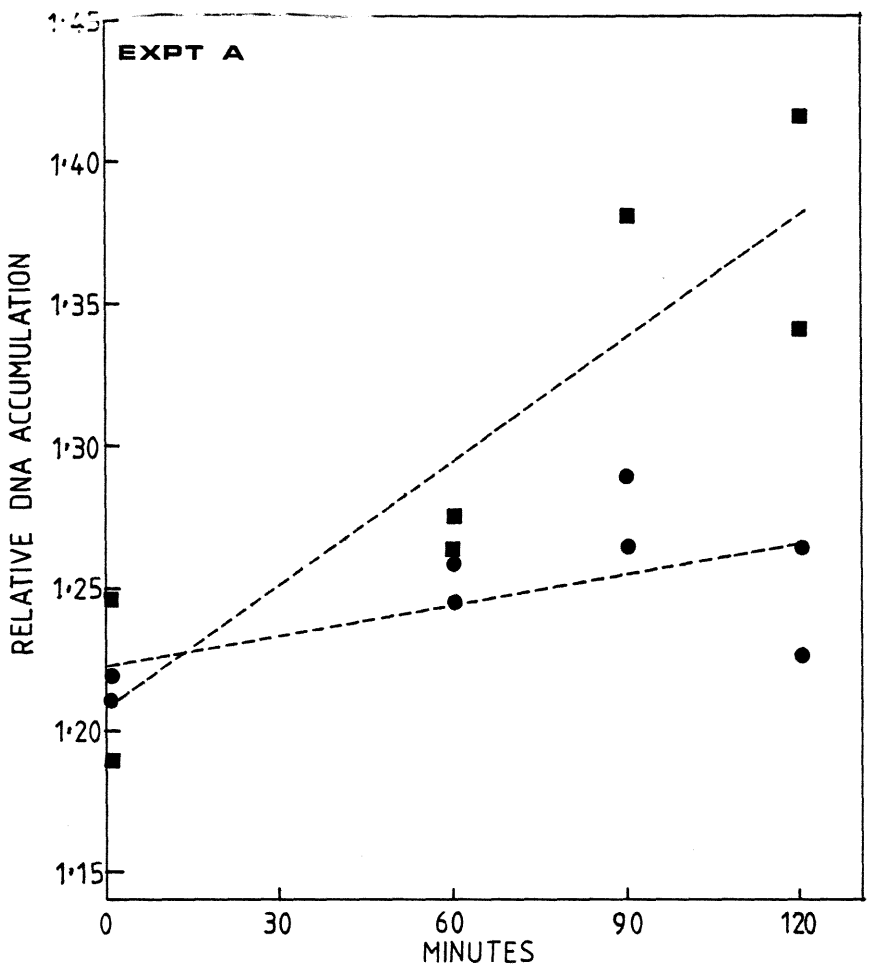

Fig. 26. Comparison of nuclear and total DNA synthesis in the presence of cycloheximide (20 ug ml^{-1}) at times up to 120 min (A). At M3, 2 segments were removed from a large plasmodium prelabelled with 100 uCi [6-^{3}H]thymidine (1 uM). A small section of each was used to determine total, and the remainder, nuclear, DNA specific activity. 6 further segments were transferred to medium + cycloheximide at M+20 to M+30 min and 2 were harvested immediately (time 0). At times shown above, duplicate segments were taken to give values for total (●) and nuclear (■) DNA specific activity. These values, normalised to 1.00 at M3, are plotted against minutes after time 0.

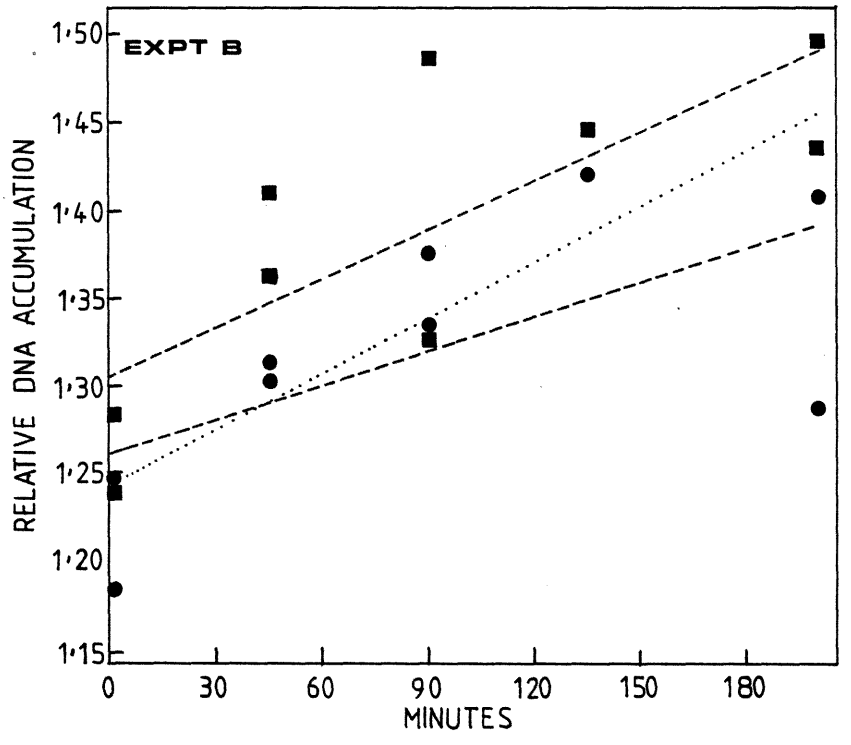

Fig. 27. Comparison of nuclear and total DNA synthesis in the presence of cycloheximide (20 ug ml^{-1}) at times up to 200 min (B). At M3, 2 segments were removed from a large plasmodium prelabelled with 100 uCi [6-^{3}H]thymidine (1 uM). A small section of each was used to determine total, and the remainder, nuclear, DNA specific activity. 7 further segments were transferred to medium + cycloheximide at M+30 to M+38 min and 2 were harvested immediately (time 0). At times shown above, segments were harvested to give values for total (●) and nuclear (■) DNA specific activity. These values, normalised to 1.00 at M3, are plotted against minutes after time 0 at which the samples were harvested. As in Figs. 25 and 26, the dashed lines were fitted by least squares linear regression simply to indicate the overall gradient of DNA accumulation against time. The dotted line is obtained for total DNA if the apparently outlying point at 200 min with low accumulation is omitted.

to cycloheximide at different times so as to effectively decrease this to less than 8 min, time 0 then being approximately M3 + 30 - 38 min.

In all cases the overall gradient, as determined by linear regression, is positive, and in both experiments it is steeper for nuclear than for total DNA, which is the reverse of what would be expected if a significant proportion of residual synthesis were attributable to mitochondrial DNA. Removal of the outlying point at 200 min for total DNA in experiment B (caused by a low reading in the diphenylamine assay) increases the gradient of total DNA synthesis so that it is steeper than that of nuclear DNA, but in both experiments ~~the slopes are so similar that differences could be attributable to experimental error.~~ experimental error is so large that differences between the slopes may not be significant. Thus, within the limits of sensitivity of the procedure used, a component due to mitochondrial DNA in the residual synthesis of total DNA is undetectable. This concords with what might be expected theoretically; if mitochondrial DNA constitutes 10% of total DNA, then approximately 3% of total synthesis will be due to this component during the first 3 h of a 10 h period. This is approximately equivalent to a 3% change in specific activity and so, given a CV of 2.64% for specific activity, 14 replicates would be required in order to be 80% certain of detecting such a difference at the 5% probability level. Thus, mitochondrial DNA synthesis constitutes only a small fraction of the residual synthesis observed in the presence of cycloheximide and is not detectable using the present procedure.

At each time point in experiments A and B the mean residual synthesis was calculated and these values are plotted in Fig. 28. The results of experiment B suggest that synthesis continues beyond 90 min but plateaus beyond 135 min, there being no significant difference between nuclear and total DNA profiles. Nuclear DNA in experiment A shows a similar profile, although the plateau occurs

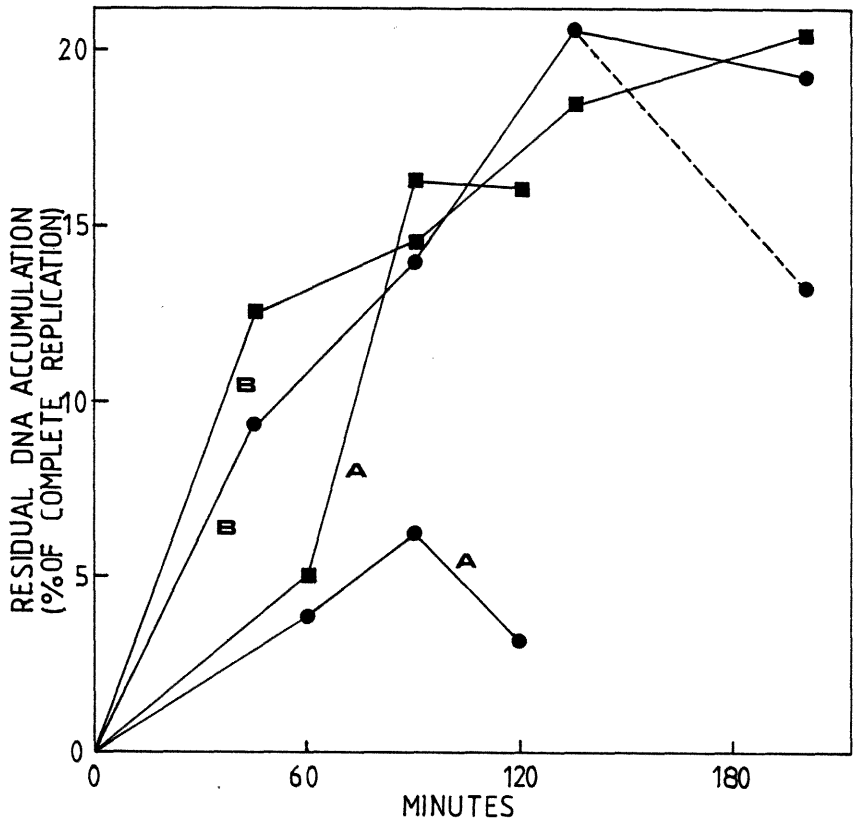

Fig. 28. Residual nuclear and total DNA synthesis in the presence of cycloheximide (20 ug ml^{-1}); experiments A and B.

For each time point in expts A and B (Figs. 26 and 27), the mean amount of nuclear or total DNA was calculated, and from this was subtracted the mean relative amount at time 0. The remainder was multiplied by 100 to express the residual DNA accumulation as a percentage of the accumulation that would occur if the genome were to be fully replicated. These values are plotted as a function of the length of the period of exposure to cycloheximide. The point at 200 min showing 13% synthesis and connected by a dashed line represents the value obtained if the apparently outlying point for total DNA accumulation in expt B is not excluded. Total DNA = ● ; nuclear DNA = ■ .

beyond 60 min, but the total DNA profile shows very much less synthesis. As there is no obvious explanation for this, it is likely that it is at least partly due to intrinsic variability in the determination of specific activity and possibly also to asynchrony in the original plasmodium, although this should affect nuclear and total DNA profiles equally. The results suggest that by 180 min residual synthesis amounting to 15 - 20% of total or nuclear DNA has occurred. This is equivalent to approximately a 14% change in specific activity, and it can be calculated that only two replicates would be required to be 80% certain of detecting such a change at the 5% probability level, but for times shorter than this more replicates would clearly be necessary (for example, 3 replicates for a 10% change). Thus, more replicates than were actually taken would be needed to reveal the profile of residual DNA synthesis in consistent detail and this fact, together with the incomplete synchrony, is probably responsible for the irregularities obtained.

A better experimental design, using the same method for determining specific activity and giving a greater number of replicates with less chance of poor synchrony, would be to use small plate cultures with one culture harvested per time point to give 4 or 5 time 0 and 4 or 5 experimental replicates. It should then be possible to calculate the mean residual synthesis for each time, having normalised each point using the mean specific activity in a separate culture at the preceding mitosis. It would be worthwhile to continue determinations at later times to check in more detail whether synthesis completely stops by 180 min and whether there is a time after which no more synthesis occurs.

4.4. Discussion.

4.4.1. Inhibition of protein synthesis by cycloheximide in *Physarum polycephalum*.

Cycloheximide is an antibiotic, originally isolated from *Streptomyces griseus*, that inhibits translation on eucaryotic cytoplasmic ribosomes. The primary site of inhibition has been variously reported as initiation, elongation and termination, but it has been recently shown (Oleinick, 1977) that all energy-dependent steps in translation are sensitive to the drug; its relative effect on the different steps appears to vary depending on its concentration. Although available evidence suggests that its effect is mediated through the large ribosomal subunit, presumably by binding, it has been shown in *Tetrahymena* that resistance can also be mediated through the small subunit (Sutton et al., 1978).

As in the experiments presented in Section 4.3.3., previous investigations into the inhibition of protein synthesis by cycloheximide in *Physarum* have been in the nature of preliminary measurements to determine whether protein synthesis is inhibited, how rapidly it occurs and what concentration of antibiotic should be used. Either a continuous- or pulse-labelling procedure has invariably been adopted, but quite widely differing degrees of inhibition have been obtained. The present experiments were undertaken for this reason, and also to ensure that significant inhibition had occurred by the short times at which it was planned to investigate the inhibition of DNA synthesis.

In only one previous case (Cummins et al., 1965) has lysine (^{14}C-labelled) been used as precursor. After 60 min labelling and exposure, a similar degree of inhibition (95%) to that in Section 4.3.3. (97%) was obtained, although after 10 min the inhibition was apparently less (80% compared with 92 - 94%), possibly attributable to the lower concentration of cycloheximide

used (10 ug ml^{-1}). [^{14}C]leucine under the same conditions showed only 57% and 85% inhibition after 10 and 60 min exposure, while [^{35}S]methionine showed approximately 99% at all times. It is likely that the use of leucine rather than lysine is responsible for the lower inhibition reported in subsequent papers. Thus, Sachsenmaier et al. (1967) found inhibition of 76% at 20 min and 90% at 40 min (no further incorporation occurred after 20 min with inhibitor concentrations above 20 ug ml^{-1}), while Muldoon et al. (1971) found approximately 73% inhibition after 60 min of continuous-labelling using inhibitor concentrations between 2.5 and 10 ug ml^{-1} (cf. Section 4.3.5.). Using a mixture of [^{3}H]leucine and [^{3}H]valine, Bersier and Braun (1974b) found only approximately 50% inhibition after continuous-labelling for 30 or 60 min, which was less than the inhibition of incorporation of [^{3}H]thymidine under the same conditions. Apart from the presence of mitochondrial protein synthesis and the possibilities of direct effects of cycloheximide on either DNA synthesis or amino acid uptake, it is likely that this discrepancy is due to the presence of large intracellular pools of unlabelled amino acids, possibly with a low rate of turnover. Differences in the extent of incorporation of the various amino acids are probably due to differential effects of cycloheximide on their intracellular pools.

4.4.2. Effects of inhibition of protein synthesis on DNA replication.

A major objective of investigations into the effects of the inhibition of protein synthesis on DNA synthesis in eucaryotes is to elucidate in detail the mechanism by which these two processes are coupled. Most published experiments have been carried out with one or more of the following aims:

(i) to determine the relative kinetics of inhibition of both processes and the tightness of their coupling;

(ii) to decide which factors known to be involved in DNA synthesis

are markedly affected by inhibition of protein synthesis;
(iii) to investigate the structure of the chromatin made during inhibition;
(iv) to determine the amount of DNA made during inhibition;
(v) to discover which stages in the mechanism of DNA replication are inhibited.

Investigations in Physarum have concentrated on the last two of the above categories, invariably using cycloheximide as the inhibitor of protein synthesis. The interpretation of results concerning the amount of DNA made during inhibition is complicated by the fact that synthesis has always been assayed by the incorporation of radioactive thymidine into a cold acid-insoluble fraction so that, if cycloheximide produces additional effects such as inhibition of uptake of precursor, ~~or~~ alterations of DNA precursor or DNA damage resulting in repair synthesis pools, the incorporation relative to controls may be affected to an extent not proportional to the actual inhibition of replication. For this reason, the experiments outlined in Sections 4.3.4. - 4.3.7. were undertaken, since DNA accumulation as assayed by isotope dilution is independent of precursor uptake and pool effects, and can give results directly in terms of the proportion of the genome replicated although repair synthesis would not be distinguished from normal replication. These suggest that synthesis amounting to 15 - 20% of total DNA can occur in the presence of cycloheximide (20 ug ml^{-1}).

An alternative approach is to quantify the effects of cycloheximide on the immediate precursor of DNA derived from the radioactive compound used, so that the proportion of the observed inhibition of incorporation which is actually due to inhibition of DNA replication can be approximately calculated. Using thin-layer chromatography, Bersier and Braun (1974b) showed that cycloheximide (5 and 10 ug ml^{-1}) added at M2 + 20 min caused, after 30 and 60 min, an average expansion of the dCTP and dTTP pools by a factor of 2.98 and 2.90 respectively. The factors of 1.25 and 1.66 recorded for

the dGTP and dATP pools may not have been significant.

If [^{3}H]thymidine is used as precursor, expansion of the dTTP pool by cycloheximide might cause a reduction in its specific radioactivity compared to a control, leading to decreased incorporation of exogenous precursor. Evans et al. (1976), using pulses of [^{3}H]thymidine ± cycloheximide (10 ug ml^{-1}) in early S phase, found that, although pool effects preceded effects on DNA, the expansion of the dTTP pool (approximately double that of a control at times between 10 and 45 min after addition of inhibitor) was insufficient to account for the decrease in specific radioactivity of dTTP (similar to control at 5 min, dropping to 70% at 15 min, 19% at 30 min and 14% at 45 min) and this was, in turn, less than the decrease in the specific activity of DNA (similar to control at 10 min, dropping to 50% at 15 min, 4% at 30 min and 1.6% at 45 min). Significantly smaller effects on all three parameters were found during late S phase and G2. Funderud and Haugli (1977b) found that, when [^{3}H]thymidine was used as precursor, cycloheximide caused a greater decrease of incorporation into cold acid-insoluble nuclear material than when [^{3}H]deoxyadenosine was used, presumably reflecting the additional effects of dTTP pool expansion (Bersier & Braun, 1974b) and possibly feedback inhibition of thymidine kinase (suggested by Evans et al., 1976).

Most experiments in Physarum investigating the amount of DNA synthesis in the presence of cycloheximide were actually carried out before publication of the above results. Using both pulse- and continuous-labelling, Cummins and Rusch (1966) found that treatment with cycloheximide (10 or 50 ug ml^{-1}) at any time from late prophase to late S phase allowed a limited 'burst' of [^{3}H]thymidine incorporation, similar values being obtained with radioactive deoxycytidine and orotic acid and with inhibitor concentrations between 10 and 100 ug ml^{-1}. This DNA appeared to be

replicated semi-conservatively and was conserved through at least one subsequent division. Bersier and Braun (1974b) also detected significant incorporation of thymidine after exposure to inhibitor, with some evidence that it continued after 30 min of exposure.

A more quantitative approach to the number and duration of these bursts or 'rounds' of DNA replication in the presence of cycloheximide was made by Muldoon et al. (1971). At metaphase, plasmodia were transferred to medium containing [^{3}H]thymidine, and at various times thereafter segments were transferred to fresh medium + [^{3}H]thymidine $\pm$ cycloheximide (5 - 10 ug ml^{-1}). Controls were allowed to complete replication, while exposed segments were left with inhibitor for 60 min before harvesting; in previous tests no further incorporation had been detected after 60 min. Results were expressed as 'dpm incorporated into cold acid-insoluble material per mg DNA' and, utilising the values of the controls, were converted to 'percentage of the genome replicated'. The relation between this parameter and the time of cycloheximide addition was not linear, and statistical analyses substantiated the conclusion that the percentage of the genome replicated increased in discrete steps after certain definite times of cycloheximide addition. These steps were referred to as replicative units (RU's) and involved between 7 and 18% of the genome, there being at least ten such units. Density shift experiments indicated that the RU's were unique and were synthesised in the same temporal sequence in two consecutive S periods.

These results were interpreted as evidence that replication of the genome is controlled by regulatory proteins synthesised at specific times during S phase. Such a situation would be most readily explained by assuming that cycloheximide inhibits the production of proteins that initiate the replication of a bank of replicons at specific times, but is less easily accounted for by an

inhibition of DNA elongation within replicons (see below) as has been detected by both Evans et al. (1976) and Funderud and Haugli (1977b). Although inhibition of initiation was not completely discounted in either case, a severe depression of the maturation of replication intermediates was detected after times as short as two and four min (Funderud & Haugli, 1977b), and Evans et al. (1976) could detect no further chain growth after 15 min exposure, a specific inhibition of elongation within replication units being sufficient to explain the observed decrease in strand elongation as measured by alkaline sucrose density gradient centrifugation (see below). Thus, it becomes necessary to postulate the existence of a specific cycloheximide-sensitive point during the elongation of each bank of replicons, and this would be more likely to reflect a periodic production of protein during S phase than an intrinsic property of the DNA.

In contrast to the experiments of Muldoon et al. (1971) in which no further thymidine incorporation occurred after 60 min, the results of Section 4.3.7. (Fig. 28) suggest that the quantity of residual DNA synthesis in the presence of cycloheximide increases with time up to 120 min, although there is insufficient information to determine whether it always increases by the same amount. It is possible that the termination observed by 60 min (Muldoon et al., 1971) is due to effects of cycloheximide on pools or on thymidine uptake rather than on real DNA synthesis. Thus, although unlikely, the pattern of RU's obtained could be due to pool effects, and it might be informative to repeat the above experiments using [^{3}H]deoxyadenosine to at least partially avoid these. It is probable that the values of the RU's in terms of percentage of the genome replicated are inaccurate, because the decrease in the specific activity of the dTTP pool (Evans et al., 1976) will decrease the incorporation of [^{3}H]thymidine beyond that due solely to inhibition

of replication. Attempts to repeat the above experiments using the isotope dilution method so as to avoid such problems proved unsuccessful; no discrete RU's could be detected because the error involved in determining the DNA accumulated was too great.

Investigations into the effect of cycloheximide on the mechanism of DNA replication have been less ambiguous. Analysing DNA on alkaline sucrose gradients, Evans et al. (1976) found that, after a 15 min pulse of [^{3}H]thymidine plus cycloheximide (10 ug ml^{-1}), no elongation occurred during a 90 min chase period in the presence of the drug. The same result was obtained beginning at various times in S phase. The progeny strands produced after 15 min exposure to cycloheximide had an average molecular weight of 69.2% that of the controls and a similar size distribution; if elongation had occurred for only 10 min after exposure to cycloheximide the average molecular weight of the progeny DNA would be approximately 66.7% of the control value. Additional experiments failed to detect an effect of cycloheximide on either the initiation of synthesis of replication units during S, or on their ligation.

Using [^{3}H]deoxyadenosine for periods of labelling as short as 30 s in pulse and pulse-chase experiments, Funderud and Haugli (1977b) found, by alkaline sucrose density gradient analysis, that cycloheximide (5 ug ml^{-1}) inhibited at least three stages of DNA elongation: (i) the formation of 'Okazaki-size', 4.5 S fragments; (ii) the joining of these into 30 - 35 S, 'replicon-size' units and (iii) the slow maturation of these to higher molecular weight DNA. A depression of both the production of 4.5 S pieces and their maturation to 30 - 35 S units was detected after only 2 and 4 min exposure, raising the question of whether such a rapid effect could possibly be mediated through inhibition of protein synthesis. This was answered by repeating the experiments using a strain with a cycloheximide-resistant phenotype known to reside with the ribosome

which presumably allows protein synthesis to proceed in the presence of the inhibitor (Haugli et al., 1972). In this strain, whether or not cycloheximide was present, replication intermediates were found to be almost identical with those in the wild-type in the absence of inhibitor. These results suggest that, if a sufficiently sensitive isotope dilution assay could be devised, a decline in the rate of DNA synthesis would be detected at times much shorter than the 15 min reported in Section 4.3.6.

Further evidence that cycloheximide does exert its effect on DNA replication through an inhibition of protein synthesis has been provided by Stimac et al. (1977) through work on three lines of mammalian cells in culture (mouse L, Chinese hamster ovary and HeLa). [^{3}H]thymidine incorporation was used to assay the overall rate of DNA synthesis, and this was shown to be a valid procedure by directly measuring the size and specific radioactivity of the [^{3}H]dTTP pool; in contrast to Physarum (Evans et al., 1976), nearly complete inhibition of protein synthesis had little effect on pool size and even less on its specific activity in all three cell lines. Eight different methods of protein synthesis inhibition were each used to give varying degrees of inhibition, and all were found to produce within 2 h a reduction in the rate of DNA synthesis to a fairly constant plateau level which was approximately the same as the inhibited rate of protein synthesis. This suggests a very tight coupling between the two processes, and the fact that eight very different inhibitors all produced the same results implies a basic dependence of DNA replication on protein synthesis; one drug alone might always exert its effect by a direct inhibition of DNA synthesis.

Using DNA fibre autoradiography, Stimac et al. (1977) found that the rate of replication fork movement (i.e. DNA chain elongation) was reduced at every fork within 15 min of inhibiting

protein synthesis, and for the first 30 to 60 min this reduction could account for the observed decline in the rate of DNA synthesis as measured by [^{3}H]thymidine incorporation. At longer times, indirect measurements, together with direct measurements by others, suggested that the additional inhibition resulted from a decline in the frequency of initiation of new replicons.

The finding of a tight coupling between protein synthesis and DNA synthesis is in agreement with the majority of previous results (discussed in Stimac et al., 1977), but others have observed, as in the preliminary experiment described in Section 4.3.5., less inhibition of DNA replication than of protein synthesis. Several of these have, however, used cells synchronised by prolonged starvation for deoxynucleotides, a procedure which may cause a temporary uncoupling of DNA synthesis from protein synthesis when the deoxynucleotide pools are restored to normal (Weintraub & Holtzer, 1972). Such an effect cannot be responsible for the results presented in Section 4.3.5. and more experiments are required to show whether the result obtained is consistently repeatable. The use of an isotope dilution method for measuring both protein and DNA synthesis (see Section 4.3.5.) would allow a more detailed determination of the kinetics of inhibition of DNA synthesis, so that the tightness of coupling could be estimated.

Two general coupling mechanisms have been suggested; (i) the overall rate of protein synthesis may be monitored and the rate of DNA synthesis regulated proportionally, or (ii) there may be a specific class of proteins, the synthesis of which is rate-limiting for DNA replication. If the latter mechanism holds, the proteins may be structurally incorporated into the replicating DNA or they may be catalytic in function but, in either case, the rapidity of inhibition of DNA synthesis by cycloheximide implies that the pools of free protein are very small and that, if catalytic, they are

subject to rapid turnover. It has been suggested that these proteins may be histones, but the evidence of Stimac et al. (1977) does not support this idea. There is a precedent for the former type of regulation (i) in the link between protein synthesis and RNA synthesis in bacteria which is mediated by guanosine tetraphosphate (Travers, 1976). Furthermore, it has been suggested (Prescott, 1976) that the rate of passage through the cell division cycle may be governed specifically by the rate of total protein synthesis, which is itself proportional to the growth rate at any time and, under steady state culture conditions, to the size of the cell. In this case however, coupling between the two cycles is thought to occur at the G1 or G2 control points. Thus, the constancy of the duration of S phase with growth rate (Prescott, 1976) might argue against a general coupling mechanism involving the rate of protein synthesis, but the degree of protein synthesis inhibition brought about by an inhibitor is usually much greater than that caused by changes in growth rate, and so different control mechanisms may be operative under such extreme conditions.

Chapter 5. Replication of DNA in vitro.

5.1. Introduction.

As has been amply demonstrated in bacteria (Kornberg, 1974), cell-free replicating systems can provide a valuable tool for the discovery of factors necessary for the synthesis of DNA. Their use with eucaryotes is complicated by the requirement for continued protein synthesis throughout S phase (Section 4.1.) and, probably for this reason, only partial replication of the genome has at present been achieved. To obtain complete replication, it would therefore be necessary to couple the DNA-synthesising system to one synthesising protein. It is possible that replication *in vitro* is equivalent to the residual synthesis in the intact organism in the presence of cycloheximide (Section 4.1.). If so the presence of this substance in the *in vitro* incubation medium would not produce inhibition suggesting that *in vitro* synthesis cannot normally continue beyond a cycloheximide-sensitive point (Muldoon et al., 1971). It would also be informative to apply the methods used in cycloheximide-inhibited cultures by Evans et al. (1976) to discover which of the processes of initiation, elongation or termination of replicons is inhibited *in vitro*. A major advance would be to identify factors that allow cell-free synthesis to proceed beyond points requiring protein synthesis.

In the construction of *in vitro* systems, *Physarum* has certain advantages. Firstly, its natural synchrony allows the replicative activity of an *in vitro* system isolated at a particular point in the mitotic cycle to be compared with that *in vivo* at the same point; a system isolated in S phase should show high activity and one isolated in G2 should show very little. Such a result would suggest that replication both *in vivo* and *in vitro* is of the same nature. This test has been applied to all the published cell-free systems in *Physarum* and has, in each case, given a positive result.

Secondly, because of the absence of cell walls and extensive cell membranes, the isolation of large quantities of nuclei is facilitated and extracts can be prepared by gentle procedures.

The main objective of the preliminary experiments to be reported below was to develop an in vitro replicating system based on that of Brewer (1975) but with synthesis measured by isotope dilution rather than by incorporation of a radioactively labelled deoxynucleoside triphosphate. The proportion of the genome replicated in vitro can thus be directly determined without recourse to a comparison of the rate of radioactive precursor incorporation in vitro with that in vivo, an approach that has been applied in published investigations. Such calculations are likely to be in error because (i) there will probably be differences in the characteristics of precursor uptake between the intact organism and an in vitro system and (ii) radioactive precursor will be diluted to an unknown extent in vivo by endogenous pools which are unlikely to be present in vitro. Another method of calculating the extent of synthesis assayed by incorporation of dNTPs will be discussed in Section 5.4. A direct calculation, by the use of isotope dilution, of the proportion of the genome replicated would also provide a more readily interpretable means of determining whether a cycloheximide-sensitive point has been exceeded and whether cycloheximide causes real inhibition than a calculation based on isotope incorporation, although it would not distinguish between replication and repair synthesis.

Before describing the attempts to assay in vitro DNA synthesis by isotope dilution, published work on cell-free replicating systems in Physarum will be outlined. These have used either isolated nuclei or homogenates of plasmodia. Work on the former system was initiated by Brewer and Rusch (1965) and continued by Schiebel and Schneck (1974) and Funderud and Haugli (1977a). It was originally found that nuclei isolated during S phase by the method of Mohberg and

Rusch (1964) would incorporate [^{3}H]dATP into cold acid-insoluble material (Brewer & Rusch, 1965), and all investigators have confirmed that synthesis requires Mg^{2+} ions and all four dNTPs, suggesting the latter are present within the nuclei at a negligible endogenous concentration. Incorporation was optimally stimulated by 0.25mM spermine, 2.5mM ATP (Schiebel & Schneck, 1974) and 2mM EGTA (Funderud & Haugli, 1977a). The latter appeared to act at least partly by removing Ca^{2+} ions and is thought to stabilise the DNA template by thus inhibiting a Ca^{2+}-dependent nuclease (Brewer & Ting, 1975). The optimal pH and temperature were approximately 7.5 and 37°C respectively.

It may be noted that a dependence of replicative-like DNA synthesis upon exogenously added ATP to produce optimal activity has been widely observed in permeabilised and subcellular procaryotic and eucaryotic systems and also in _in vivo_ DNA synthesis in _E. coli_. In both HeLa and Ehrlich ascites tumour cells the increase in synthesis with ATP has been shown to be due primarily to greater synthesis per active site rather than to initiation at more sites; the size of the short pieces observed in the presence of ATP is thus larger than that of the corresponding pieces in its absence (Hershey, 1977). However, the mechanism of action of ATP is unknown. It may be involved in maintaining the replication enzymes in a form capable of optimum synthesis, or in the preparation of the chromatin for more rapid replication, possibly through participation in DNA-unwinding or in histone phosphorylation. A possible mechanism of participation might be through the DNA-dependent ATPase reported to be present in actively-replicating mouse myeloma (Hachmann & Lezius, 1976).

In their preparative procedure, Funderud and Haugli (1977a) omitted the non-ionic detergent Triton X-100 which had been shown to remove part of the outer membrane of nuclei and decrease

incorporation (Brewer & Ting, 1975). They also adopted a gentler homogenisation procedure involving 4 - 6 strokes with a loose-fitting teflon pestle in a glass homogeniser. In this system incorporation corresponded to approximately 10% of the *in vivo* rate and was linear for 7 - 10 min, continuing up to 60 min, and amounting to approximately 6500 cpm ug DNA^{-1} uCi^{-1} of [^{3}H]dATP.

Neutral and alkaline density gradient sedimentation of DNA that had incorporated bromodeoxyuridine *in vivo* followed by [^{3}H]dTTP *in vitro*, showed that the [^{3}H]DNA was continuous with that made just prior to nuclear isolation and was replicated semi-conservatively (Schiebel & Schneck, 1974; Funderud & Haugli, 1977a). Examination on alkaline sucrose gradients of DNA synthesised *in vitro* after a 60 s pulse of [^{3}H]dATP, or after a 2.5 min pulse followed by a 27.5 min chase period (Funderud & Haugli, 1977b), demonstrated the presence of 4.5 S Okazaki fragments which joined to give 30 - 35 S DNA, a situation identical with that *in vivo*, but occurring at a reduced rate. Thus, although the nuclei were defective in overall DNA synthesis, it was apparent that not all aspects of the replicative process were equally affected.

Mg^{2+} ions and the four dNTPs also appear essential for the incorporation of [^{3}H]dATP by homogenates (Brewer & Ting, 1975), suggesting these also contain negligible pools of the dNTPs. Both ATP and EGTA were again stimulatory, but with higher optimal concentrations of approximately 12 - 14mM and 20mM respectively. Spermine was also stimulatory (optimal concentration, 0.6mM). Inorganic cations did not affect synthesis or were inhibitory, as was Triton X-100. The bulk of the [^{3}H]dATP incorporated into homogenates sedimented on alkaline sucrose gradients at the same position as the growing DNA daughter strands *in vivo*, and little was found in association with parental (^{14}C-labelled) strands. Homogenates of cultures that had been treated for 30 min with

cycloheximide (10 ug ml^{-1}) incorporated [^{3}H]dATP at only about 60% of the level observed in untreated controls.

The procedure adopted (Brewer & Ting, 1975) was to harvest plasmodia in a teflon-in-glass homogeniser in medium containing magnesium acetate, EGTA, glucose and spermine at pH 7.7 and, after removal of plasmodial debris by centrifugation at 50g, a mixture containing ATP, the four dNTPs and [^{3}H]dATP was added, the incubation taking place at 37°C for 60 min. The rate of synthesis obtained was approximately 15% of that *in vivo* (i.e. 0.2% of the genome per minute) during the first 7 - 8 min, the amount of synthesis during 60 min being calculated to correspond to approximately 2% of the genome, a several hundred-fold improvement over that of Brewer and Rusch (1965). The overall incorporation was approximately 500 cpm ug DNA^{-1} uCi^{-1} [^{3}H]dATP.

Following a report of work on rat liver, Brewer (1975) found incorporation to be greatly stimulated by the presence of dextran (12.5% (w/v)) in an homogenising medium containing only magnesium acetate and EGTA. While other preparative details remained virtually unchanged from those of Brewer and Ting (1975), incorporation now occurred at an initial rate of approximately 25% of that *in vivo*, remaining constant for about 40 - 45 min and continuing at a decreasing rate for at least a further 30 - 60 min. It was estimated that approximately 15% of the total genome was replicated, and overall incorporation was approximately 1600 cpm ug DNA^{-1} uCi^{-1} [^{3}H]dATP. In view of the extensive synthesis apparently occurring in this system, it would be of interest to determine whether further elongation and initiation of new banks of replicons does occur and whether synthesis can be inhibited by cycloheximide *in vitro*. Therefore it was this system that was duplicated in the experiments described below (Section 5.3.). As in the system without dextran, Brewer (1975) found that cycloheximide (10 ug ml^{-1}) pretreatment

reduced synthesis to 60% of controls. The DNA formed in this system was separable on alkaline sucrose gradients into two classes, one similar in size to that observed in the intact organism, with a sedimentation rate increasing, presumably by chain elongation, as the organism progresses through S phase (Brewer et al., 1974), and the other of a size (10 S) similar to Okazaki fragments. How these relate to the 4.5 S species found by Funderud and Haugli (1975, 1977a) is unknown.

It had been previously suggested (Lynch et al., 1972) that dextran might act by inhibiting nuclear swelling and leakage of components of the replicative system, but the observation that addition of dextran to dextran-free homogenates led to a substantial recovery of synthetic activity (Brewer, 1975) suggested a more complex mechanism. Dextran might provide an environment that stabilises the replication complex, because synthesis continues for longer in its presence. However, when preparation was in the absence of dextran, maximal activity required both isolated nuclei and the nuclear supernatant (Brewer & Ting, 1975), but when dextran was present, all activity was associated with the nuclear fraction (Brewer, 1975), suggesting that some inhibition of leakage was brought about by dextran. Furthermore, repeated washing of nuclei, even in the presence of dextran, appeared to leach out a stimulatory factor, so that addition of the nuclear supernatant now became necessary for maximal activity (Brewer, personal communication). The factor was heat stable, present throughout the cycle and was unaffected by cycloheximide (Brewer, personal communication). It was purified approximately 20-fold, and appeared to be a glycoprotein of 30,000 molecular weight. It appeared to increase both the rate and overall extent of synthesis, possibly by activating additional replicons i.e. causing replicon initiation. Its activity was not associated with DNA polymerase or

deoxyribonuclease. It did not affect DNA polymerase activity per se or stimulate the joining of Okazaki fragments.

5.2. Materials and methods.

5.2.1. Materials.

1. Dextran T70, mean molecular weight 64,400 (Pharmacia Fine Chemicals).

2. 15 ml Teflon-in-glass homogeniser (Jencons).

3. Deoxy[8-^{3}H]adenosine 5'-triphosphate; ammonium salt in 50% aqueous ethanol. (Amersham UK TRK 347; 5 - 20 Ci mmol^{-1}).

4. 2'-deoxyadenosine 5'-triphosphate; disodium salt (Sigma D6500, 98% pure).

2'-deoxycytidine 5'-triphosphate; Grade 1, sodium salt (Sigma D4635, 99% pure).

2'-deoxyguanosine 5'-triphosphate; Grade 1, sodium salt (Sigma D4010, 98% pure).

Thymidine 5'-triphosphate; sodium salt (Sigma T0251, 98% pure).

5. Adenosine 5'-triphosphate; sodium salt, crystalline Grade I. Produced by phosphorylation of adenosine. (Sigma A2383, 99% pure).

6. Adenosine 5'-triphosphate; disodium salt, crystalline. Prepared from equine muscle (Sigma A3127, 99% pure).

5.2.2. In vitro DNA synthesis assayed by isotope dilution.

Four treatments were involved, signified by the following code:

M = samples harvested directly into 5% TCA/acetone/water at mitosis;

H = samples homogenised, but harvested before *in vitro* incubation;

C = control samples, incubated *in vitro* for 2 h without dNTPs;

N = experimental samples, incubated *in vitro* for 2 h with dNTPs.

The following procedure was adopted, based on that of Brewer (1975).

1. Homogenising medium of the following composition was prepared:

0.028M magnesium acetate.$4H_2O$; 0.06M EGTA; 12.5% (w/v) Dextran. Before adding dextran, the pH was adjusted to approximately 7 with 20% NaOH in order to dissolve the EGTA. Dextran was dissolved using a plastic rod and the pH brought to 7.6. 10 ml of homogenising medium without EGTA was also prepared; the pH was approximately 7.2 without addition of NaOH. Solutions were kept cold throughout these preparations and the procedure below, by leaving on ice whenever possible.

2. Deoxyribonucleoside triphosphates were added to 1.0 ml or 1.5 ml of homogenising medium without EGTA to give the following concentrations: 1.7mM dCTP; 1.7mM dGTP; 1.7mM dTTP; 0.23mM dATP. Various concentrations of ATP were added (Section 5.3.).

3. 100 x 16 mm conical-based disposable tubes (Sterilin) were used for the in vitro incubation. 100 ul of nucleotide mixture were distributed to each N tube, and 100 ul of homogenising medium without EGTA to each H and C. tube. 5 ml of 5% TCA/acetone/water were added to each M tube.

4. M3 was timed in a number of small surface cultures which had been prelabelled by the procedure outlined in Section 3.2.1. One plate was sacrificed at M3 to give, usually, four samples which were processed to obtain the specific activity of total DNA as outlined in Section 3.2.4.

5. At M + 30 min, one plasmodium (excluding the inoculum centre) was homogenised, using 8 - 10 strokes, in 5 or 6 ml of homogenising medium and transferred to a disposable tube. This was repeated with the required number of cultures.

6. Homogenates were centrifuged at 50g at 3°C for 5 min in an MSE Mistral 6L centrifuge (400 rpm using 96 x 15 ml rotor) to remove plasmodial debris.

7. Pellets were removed using a Pasteur pipette. Homogenates were mixed well and 1.0 ml added to each incubation tube, giving a DNA

concentration of approximately 8 ug ml^{-1}. After gentle whirlimixing the C and N tubes were covered and transferred to a water bath at 36°C for a 2 h incubation; the incubates were mixed again after approximately 1 h of this period.

8. After the incubation had begun, 5 ml of 0.5M perchloric acid was added to each H tube and cold acid-insoluble pellets harvested by centrifuging at 750g at room temperature for 10 min (3300 rpm) in an MSE bench centrifuge. The pellets were decolourised and DNA specific activity was determined as in Section 3.2.4. The same procedure was employed to terminate incubation in the N and C tubes after transferring to ice at the end of the 2 h incubation period.

5.2.3. In vitro DNA synthesis assayed by incorporation of [^{3}H]dATP.

The procedure employed was similar to that above, with the following modifications.

1. M and H samples were not necessary, so that only samples incubated with [^{3}H]dATP and controls incubated in its absence were included.

2. Before addition of the nucleotide mixture to the incubation tubes, [^{3}H]dATP (usually 10, 5 or 2 ul) was added to each and the alcohol evaporated off using a gentle stream of N_2.

3. The plasmodia used were unlabelled, except when samples were assayed by [^{3}H]dATP incorporation during an isotope dilution experiment to check that normal incorporation could occur. In this case, the average cpm ug DNA^{-1} due to prelabel was subtracted from the total to give a figure for [^{3}H]dATP incorporation ug DNA^{-1}.

4. After termination of the in vitro incubation by 5 ml 0.5M PCA, samples were processed as above when included in an isotope dilution experiment, but in other cases the quantity of DNA was not assayed.

The [^{3}H]dATP incorporation procedure was substantially shorter than that for isotope dilution, particularly if the diphenylamine assay was not carried out, and it also required fewer samples as it

was unnecessary to employ as many replicates or as many controls. Thus this method was most convenient for testing the effect of changes in experimental procedure.

The procedure used by Brewer (1975) differed from the above in the following particulars.

1. Plasmodia were homogenised in 12.5 ml of homogenising medium. However the concentration of DNA (10 ug ml^{-1}) was similar to that used in the present experiments.
2. Incubation volume was 0.5 ml of homogenate plus 0.05 ml of nucleotide mix. Twice this volume was used in the present experiments to give sufficient DNA to assay by the diphenylamine reaction.
3. [^{3}H]dATP was consistently used at a concentration of 0.75uM with 5 uCi present in 0.55 ml during the incubation.
4. Incubations were terminated by the addition of 2.5 ml of 0.25M perchloric acid, and the resulting precipitates were recovered by centrifugation at 10 000g, dissolved in 1 ml of 0.4M NaOH, and reprecipitated with 2.5 ml of 0.5M PCA. Acid-insoluble material was then collected for liquid scintillation counting on glass fibre filters.

5.3. Results.

The incorporation mixture employed by Brewer (1975) was of the following composition: 0.028M magnesium acetate; 0.06M EGTA; 12.5% (w/v) dextran; 20mM ATP; 0.15mM dGTP, dCTP and dTTP, and 0.02mM dATP (including 5 uCi of [^{3}H]dATP at 0.75uM concentration) at pH 7.6. Approximately 5 ug plasmodial DNA were present in a total reaction volume of 0.55 ml. These conditions were duplicated in an initial experiment, except that a reaction volume of 1.1 ml was used so that there would be sufficient DNA present to be detected by the diphenylamine reaction. Synthesis was measured by isotope dilution (Section 5.2.2.) and by [^{3}H]dATP incorporation, following,

in the latter assay, both the extraction procedure outlined above (Section 5.2.2.) and that used by Brewer (1975) outlined in Section 5.2.3. In no case was significant synthesis detectable, but Brewer's extraction procedure gave counts approximately four times background even though no incorporation had apparently occurred. For this reason, and because of possible incorporation into RNA and protein, the extraction procedure outlined in Section 5.2.2. was adopted, after finding that only a little more DNA, as assayed by the diphenylamine reaction, was recovered by using a 10 000g centrifugation (Brewer, 1975) than by a 750g centrifugation (Section 5.2.2.).

As the DNA concentration (4.5 ug ml^{-1}) in the above experiment was lower than expected, in all subsequent protocols the volume of medium used for homogenisation was decreased from 12.5 ml to 5 or 6 ml (Section 5.2.2.). In an attempt to discover the reason for the lack of synthesis found above, a number of modifications to the procedure were tried in succession.

(i) A more loosely-fitting homogeniser was used and fewer (5) strokes were employed, which gave 3 ug DNA ml^{-1} in the homogenate;

(ii) a normal homogeniser was used and more (18) strokes were employed, giving 10 ug DNA ml^{-1};

(iii) 50g centrifugation was omitted, giving 15 ug DNA ml^{-1};

(iv) dextran was omitted from the homogenising medium to approximate to the medium used by Brewer and Ting (1975);

(v) a glass-in-glass loosely-fitting homogeniser was used.

Synthesis was assayed by either isotope dilution or [^{3}H]dATP incorporation, but in no case was significant synthesis detected (except for some slight incorporation in (v)). Intact nuclei were seen to be present in homogenates (i) - (iv) ((v) was not tested), but many were associated with larger pieces of debris which were found in the discarded pellet after the 50g centrifugation. It is

therefore to be expected that more nuclei would be lost using a less vigorous homogenisation, and this is consistent with the DNA concentrations found in (i) - (iii) above. As it was unknown whether extra homogenisation damaged the nuclei, approximately 10 strokes were subsequently used.

Apart from dextran, both EGTA and ATP were present at high concentration. In a further experimental modification 5mM EGTA plus 50mM MOPS (Funderud & Haugli, 1977a) were found to give slight incorporation of [^{3}H]dATP (equivalent to that in (v) above), while in another the concentration of ATP was reduced to 2.5mM (Funderud & Haugli, 1977a) which gave incorporation of 695 cpm uCi^{-1} of [^{3}H]dATP, 15-fold higher than in reduced EGTA.

The ATP concentration was optimised by assaying [^{3}H]dATP incorporation. With concentrations of 2.5, 5, 10, 15 and 20mM, a maximal incorporation of 700 cpm uCi^{-1} was found at 5mM, while concentrations of 0.5, 1, 2, 4, 6 and 8mM gave an optimal incorporation of 400 cpm uCi^{-1} at both 4 and 6mM. Using an ATP concentration of 5mM, DNA synthesis was then estimated in two experiments using both isotope dilution and [^{3}H]dATP incorporation. In the first experiment samples were harvested at M3 + 30 min, and in the second at M3 + 15 min to determine whether any extra synthesis occurred in the latter case. In both experiments, six replicates were used for H (initial homogenate) and N (experimental) samples and four for the M (mitosis) and C (control) samples. The results obtained using isotope dilution are shown in the table overleaf.

Data were analysed using (i) a simple t-test, (ii) an analysis of variance (which showed differences between treatment means to be significant at $p = 0.05$ in experiment 1 but not in experiment 2), followed by an <u>a priori</u> test using the least significant difference (LSD), (iii) a non-parametric Wilcoxon two-sample test. Dashes

indicate comparisons not tested and lt (less than) 0.05 indicates means not significantly different at p = 0.05.

Expt.	Difference	% total synth.	t-test (p)	LSD (p)	Wilcoxon (p)
1	H,N	5.2	0.02	0.02	0.005
(M + 30)	H,C	2.9	-	lt 0.05	-
	C,N	2.2	-	lt 0.05	lt 0.05
2	H,N	5.0	0.05	0.02	0.025
(M + 15)	H,C	2.6	-	lt 0.05	-
	C,N	2.4	-	lt 0.05	lt 0.05

In both experiments only differences between H and N samples were shown to be significant at p = 0.05 and the magnitude of these indicated that about 5% of the genome was replicated <u>in vitro</u>. However, the incorporation of [^{3}H]dATP was very much lower than that found by Brewer (1975) at the same concentration of dATP, amounting to 80 cpm ug DNA^{-1} uCi^{-1} and 50 cpm ug DNA uCi^{-1} in experiments 1 and 2 respectively. The intermediate values of the C samples, and the absence of significant differences between these and either H or N samples, suggest some synthesis can occur in the absence of added dNTPs, although this does not agree with the results of Brewer and Ting (1975) (see Section 5.4.).

The ATP (Sigma) used in the above experiments had been synthesised by phosphorylation of adenosine and purified by competitive crystallisation, a process that produces ATP less pure than that extracted from equine muscle, the grade used by Brewer and Ting (1975). In their experiments, this latter had an optimal concentration of approximately 12mM, while the ultrapure grade of ATP (Schwarz/Mann) used by Brewer (1975) presumably had an optimum

of 20mM (no optimalisation curve was given). The fact that the ATP used above gave maximal incorporation at only 5mM suggests that, at higher concentrations, an impurity in the ATP might inhibit *in vitro* replication. The optimal concentration of the Sigma equine muscle ATP was therefore determined in an experiment using concentrations of 2, 5, 10, 15, 20 and 25mM, but was again found to be only 5mM, giving an incorporation of 335 cpm uCi^{-1}. A further isotope dilution experiment failed to detect any significant synthesis in the presence of this grade of ATP at 5mM, although the [^{3}H]dATP was incorporated at 60 cpm ug DNA^{-1} uCi^{-1} which is similar to that of experiments 1 and 2 above. Thus synthesis at this level did not appear to be consistently detectable by isotope dilution.

5.4. Discussion.

The figure of 15% of the genome replicated *in vitro* (Brewer, 1975) is presumably derived from a comparison between the incorporation of [^{3}H]dATP *in vitro* and of [^{3}H]thymidine *in vivo* but, as outlined in Section 5.1., this is likely to be in error. However, it is possible to express the incorporated cpm as a fraction of the [^{3}H]dATP present in the incorporation mix. The same fraction of the total dATP present will be incorporated, and this can be converted to an equivalent mass of DNA, as the base composition of main band DNA from *Physarum* is known (Evans & Suskind, 1971). If the mass of DNA in the sample is also known, assuming 15% of the genome has been replicated by M + 30 min (see Fig. 8), this can be converted to the mass of DNA at mitosis. Thus, the mass of DNA synthesised *in vitro* can be expressed as a fraction of the genome (i.e. the mass of DNA at mitosis).

This calculation requires two major assumptions: (i) the pools of endogenous dNTPs are insignificant compared to those added in the incubation mixture and (ii) the base composition of the DNA formed

in vitro does not differ dramatically from that of the total main band DNA. It has been found (Fink, 1975) that the pool size of dATP at mitosis is 11.3 pmol ug DNA^{-1} so that, assuming full recovery, there would be approximately 5.65×10^{-11} moles of dATP associated with 5 ug DNA, that is, in a volume of 0.55 ml, its concentration would be 10^{-4}mM. Exogenous dATP is present at 200 times this concentration and, particularly as the endogenous dATP pool will have decreased approximately 4-fold by 30 min after mitosis (Fink, 1975), it is therefore probable that assumption (i) is justified. Considering that each dNMP has a similar molecular weight, it is unlikely that even quite large deviations from assumption (ii) will cause great inaccuracy. Significant sources of error in the calculation are likely to be (i) the quantity of DNA present, which was presumably not estimated in every experiment by Brewer (1975) and (ii) the efficiency of counting (given as 23% in Brewer & Ting, 1975). It is also important to know the concentration of dATP as accurately as possible.

On the basis of this type of calculation, the maximum equivalent DNA synthesis has been calculated for a number of cases:

Report	% genome replicated
Brewer (1975)	5.1
Brewer & Ting (1975)	1.3
Funderud & Haugli (1977a)	0.6
Expt. 1. (Section 5.3.)	0.4

In view of the above discussion, it is apparent that there are a number of anomalies in the results presented in Section 5.3. that must be resolved by further experiments.

(i) The synthesis during incubation in the absence of exogenous nucleotides suggested by isotope dilution measurements would require

the presence of significant endogenous pools of dNTPs. At mitosis these pools have been found to be sufficient for the synthesis of between 1 and 2% of the genome (Fink,1975) but, as they will have fallen between 2 and 6-fold by M + 30 min and will be diluted in an homogenate, it is unlikely that they would be responsible for significant replication, particularly in the absence of added ATP.

(ii) Without significant endogenous dNTPs, and excluding possible effects due to pool expansion in the presence of added dNTPs, it is likely that the incorporation of [^{3}H]dATP gives a reasonably reliable estimate of <u>in vitro</u> synthesis. If so, the value of 5% obtained by isotope dilution may be attributable to a systematic error, and the failure to detect significant synthesis in the final isotope dilution experiment described in Section 5.3., when incorporation of [^{3}H]dATP was as high as in experiments 1 and 2, also suggests the previous results may be in error. Furthermore, it was noted that the coefficients of variability within replicates were as high as those in Section 4.3.6. (overall mean = 2.62, S.D. = 0.86) and, using these values, the number of replicates required to be 80% certain of detecting the observed difference between means at $p = 0.05$ was calculated to be 7 in experiment 1 and 13 in 2 for H and N samples, and above 15 for all comparisons involving the C samples in both experiments.

(iii) Although the identical grade of ATP to that used by Brewer and Ting (1975) was employed, its optimal concentration (5mM) was less than half theirs (12mM). The difference may be due to the presence of dextran in the experiments described above. Inhibition at higher ATP concentrations may be due to the stimulation of an agent which inhibits replication and which is only present or active in the dextran-containing homogenate. Alternatively, an impurity in the ATP, either active in dextran medium or only present in this batch of ATP, inhibits replication.

Two further experiments would be informative: (i) to reproduce the conditions of Brewer and Ting (1975) and optimise the ATP concentration; if this is still low, then inhibition is more likely to be due to an impurity in the particular batch of ATP used; (ii) to determine the optimal concentration of the ultrapure grade of ATP in the media of Brewer (1975) and Brewer and Ting (1975); if a higher optimal concentration and increased incorporation are not obtained, it is probable that there is an inhibitory agent in the preparation which is stimulated at high ATP concentrations. In this case, it would be necessary to re-optimise each component of the system and to test any available alternatives. However, if the incorporation seen by Brewer (1975) did represent 5% rather than 15% of the genome, it would firstly be necessary to improve the sensitivity of the isotope dilution method in order to rigorously detect this.

During this thesis, it has been emphasised that measurement of macromolecular synthesis by isotope dilution can be interpreted with greater confidence and can be more directly related to the biological events under investigation than measurement by isotope incorporation methods. Nevertheless, it has also become clear, particularly in those experiments involving the inhibition of DNA synthesis by cycloheximide or measuring replication in plasmodial homogenates, that the present isotope dilution procedure, when applied to DNA synthesis, is subject to limitations of sensitivity that become severe when attempting to quantify low amounts of accumulation. This limitation is manifested in an undesirably high degree of variability in the specific activity values of replicate samples and, as this final stage, the determination of specific radioactivity, is the most critical of the procedure, it is probable that any future modifications would bring about the maximum improvement if applied at this stage. In particular, the amounts of

DNA that have to be measured chemically are low compared with the quantities of RNA or protein that would be present in samples of a similar size. If small DNA increments were to be assayed in the future, a more sensitive method for the measurement of DNA quantity would need to be adopted (see Section 4.3.6.).

Further investigations using cell-free replicating systems would make the adoption of such a method desirable. A promising line of enquiry is to attempt to identify cytoplasmic fractions and components that stimulate DNA synthesis by isolated nuclei, particularly any components that might allow synthesis clearly to proceed beyond points that now appear to be indirectly inhibited by cycloheximide. An unambiguous comparison between the amount of DNA synthesised by an intact plasmodium in the presence of cycloheximide and that synthesised by isolated nuclei or a plasmodial homogenate is only possible using isotope dilution, since the characteristics of nucleoside incorporation by intact plasmodia and isolated nuclei or homogenates may be quite different. An improved isotope dilution method should be capable of providing unequivocal values for the fraction of the genome that can be replicated both in vivo in the absence of protein synthesis and in isolated nuclei or in plasmodial homogenates.

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