

RIBOSOMAL RNA SYNTHESIS IN PHYSARUM POLYCEPHALUM

by

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

September 1975.

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PHYSIOLOGICAL REACTIONS IN STRESSFUL AND ANNOYING

by

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Acknowledgements.

I should like to thank Dr. Geoffrey Turnock (my supervisor) and Dr. Joyce Mohberg for their help and encouragement throughout the course of this work. I also acknowledge receipt of a Medical Research Council studentship.

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GENERAL INTRODUCTION

This thesis is primarily concerned with the regulation of ribosomal RNA synthesis in a simple eucaryote, namely Physarum polycephalum.

Part I examines some of the properties of the rRNA genes, and in Part II a detailed study of the synthesis of rRNA during the mitotic cycle is described and its relationship to the replication of the rRNA genes is discussed.

An ideal organism in which to study biochemical processes in relation to the cell cycle should possess the following properties:

- (a) The organism should be easily cultivated in a reasonably defined growth medium.
- (b) The culture should contain a uniform cell population.
- (c) The organism should be easily synchronised, and the synchrony should be maintained for several mitotic cycles without decay.
- (d) It should be possible to synchronise sufficient cells to enable an adequate number of measurements to be made.
- (e) The synchronisation process must not alter the metabolism of the organism in any way.
- (f) Often sensitive assays involve the use of isotopic methods and in these cases radioactively labelled precursors should be readily taken up from the growth medium.

The true or acellular slime mould, Physarum polycephalum is one of the few organisms which fulfil to a large extent the above expectations, and is therefore ideal for this type of study.

Physarum polycephalum is classified as a Myxomycete (Greek: myxa = slime, myketes = fungus), a group which is not readily identified as either an animal or a plant. Their natural habitat is cool, shady, wooded areas, where they feed on rotting wood.

All myxomycetes have a similar life cycle; a unique property of which is the possession of a true plasmodial stage, i.e. a motile, acellular, multinucleate syncytium of indefinite form. The life cycle of Physarum polycephalum was first described by Howard [1931(a)] and is depicted in Fig. 1. The main vegetative stage is the vivid yellow, diploid plasmodium which lacks a cell wall, and contains a complex network of channels containing a rapid oscillatory cyclosis resulting in efficient cytoplasmic mixing. Extensive plasmodial migration (net movement) only occurs under non-growing conditions. As well as growing on a solid support, plasmodia may also be cultured in the laboratory submerged in liquid medium. Such cultures are maintained on a shaker which fragments the plasmodia into small pieces (up to about 2mm in diameter) called microplasmodia.

Under starvation conditions in the presence of light, a plasmodium segregates into many small spheres, each of which forms a fruiting body or sporangium consisting of a supporting stipe and a head containing many black, resistant, uninucleate, haploid spores (Howard [1931(b)]; Guttess, Guttess & Rusch [1961]; Sauer, Babcock & Rusch [1969]). Under moist conditions each spore, which is about 10 μ m in diameter, germinates releasing one or two colourless, uninucleate, haploid amoebae which rapidly multiply to give rise to a large population of cells. On solid media (e.g. an agar plate) they are very similar in both appearance and behavior to soil amoebae, whereas in liquid they temporarily become flagellate. The life cycle is completed when two amoebae of different mating type (Dee [1960]; Dee [1973]) undergo cell and nuclear fusion to form a diploid zygote (Ross [1957]), which grows by repeated nuclear divisions without cell cleavage to form a large plasmodium.

When starved in the dark sporulation does not occur; instead plasmodia encyst to form a hard, resistant sclerotium containing many

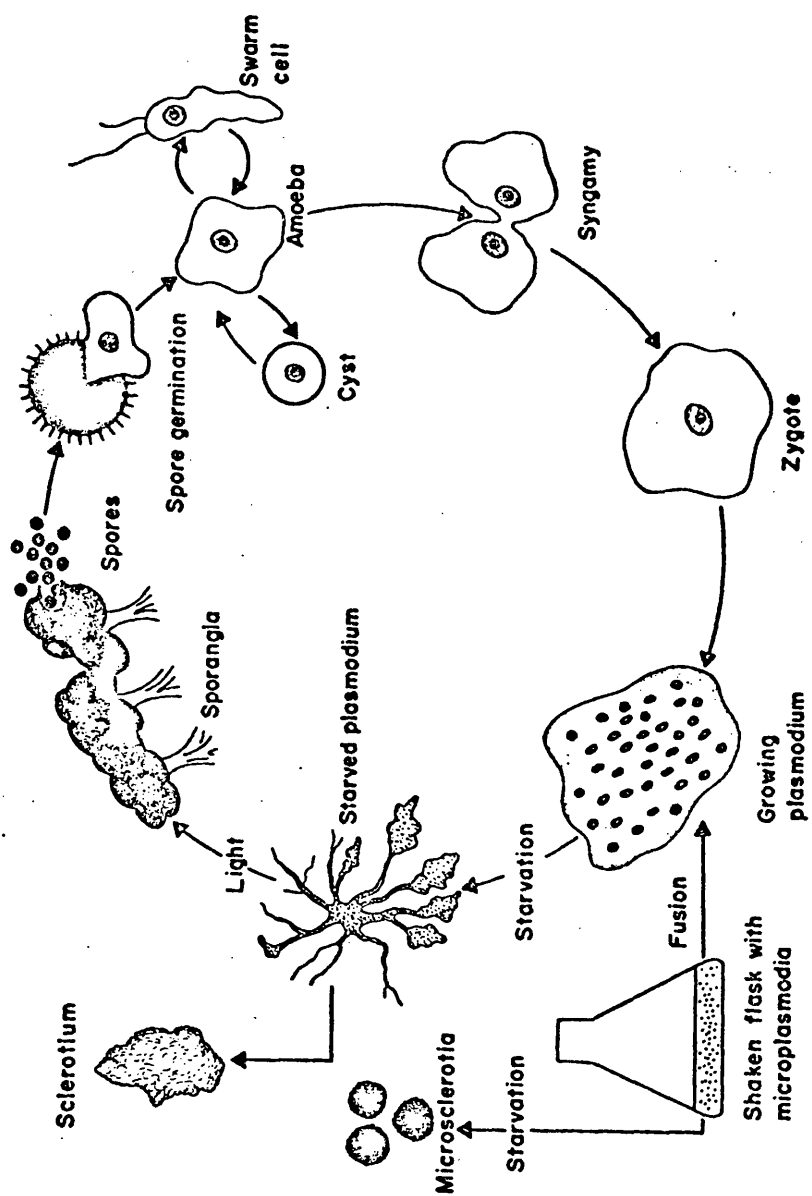


Fig. 1. The life cycle of *Physarum polycephalum*.

spherules, which remain viable for several years (Daniel & Baldwin [1964]). In liquid culture an analogous change can occur if microplasmodia are transferred to a non-nutrient salts medium (Daniel & Baldwin [1964]), resulting in the formation of microsclerotia. This is probably the most convenient and safest way of keeping stock cultures in the laboratory since it avoids prolonged subculturing with its inherent danger of imposing selective pressures on the organism.

The above life cycle is typical of all heterothallic strains of Physarum polycephalum. However, strains which were originally described as "homothallic" (Wheals [1970]), but are now thought to be apogamic (see section I.2.1) have been described and in these strains plasmodia appear to arise from a single amoebal cell by repeated nuclear division without cell or nuclear fusion resulting in a haploid plasmodium (Cooke & Dee [1974]).

The first laboratory grown plasmodial myxomycetes, more than one hundred years ago, were grown in moist chambers on rotting wood or tan bark (de Bary [1864]; Baranetzki [1876]). Since then "growth media" have progressed through fungal mycelia (Miller [1898]; Macbride [1900]), live bacteria (Pinoy [1907]; Cohen [1941]), killed or autolysed yeast or bacteria (Cohen [1939]; Sobels [1950]; Hok [1954]), rolled oats (Howard [1931(b)]; Camp [1936]; Daniel & Rusch [1961]), to the more recently described semi-defined (Daniel & Baldwin [1964]; Dee & Poulter [1970]) and completely defined (Dee, Wheals & Holt [1973]) axenic media.

Perhaps the most striking property of Physarum polycephalum is the high degree of natural mitotic synchrony which exists between nuclei (of which there are about 10^8 in a petri dish culture) within a single plasmodium (Guttes & Guttes [1964(a)]), the mitotic index being greater than 95% (Chin & Bernstein [1968]). This, together with the ability to grow single plasmodia of up to 14cm in diameter (Mohberg & Rusch [1969])

with little decay in synchrony, has led to extensive use of Physarum as a system for studying the cell cycle, the results of which have often been reviewed (Schiebel [1973]; Cummins [1969]; Grant [1973]). It should, however, be remembered that plasmodia of Physarum do not possess a true "cell cycle" but rather a "mitotic cycle" since cell cleavage does not follow mitosis. Nevertheless, as most cell functions (e.g. macromolecular syntheses etc.) exhibit the same periodicity as the nuclear division cycle, I believe that a comparison of the results obtained from Physarum with those from systems possessing a conventional cell cycle, is quite valid.

Physarum polycephalum has also been quite widely investigated (a) as a simple system for studying differentiation (reviewed by Sauer [1973] and Hüttermann [1973]), and (b) as a primitive non-muscular contractile system (reviewed by Wohlfarth-Bottermann & Stockem [1972]; Hatano [1973]; and Komnick, Stockem & Wohlfarth-Bottermann [1973]).

PART ONE

I.1 INTRODUCTION

I.1.1 Summary of published work on the rRNA genes in *Physarum polycephalum*.

Study of the rRNA genes in *Physarum* began in the mid-sixties with the discovery that DNA preparations exhibit three different density bands in isopycnic caesium chloride gradients. The bulk of the DNA (about 90%) has a density of 1.702g.cm^{-3} and a guanosine-cytosine content of 41.5%; and is flanked by a lighter band (density: 1.686g.cm^{-3} , G-C content: 26%; about 7% of the total DNA), and a heavier band (density: 1.714g.cm^{-3} , G-C content: 55%; about 2-3% of the total DNA). Both of these minor DNA components, often known as "satellite DNAs", differ from the major nuclear DNA in two important respects:

(a) they have a narrow band width in isopycnic caesium chloride gradients, suggesting relatively homogeneous species. This has been confirmed by Britten & Smith [1971] who have examined the reannealing kinetics of purified heavy satellite DNA and found it to be highly repetitive.

(b) satellite DNAs may be selectively extracted and enriched by a procedure involving precipitation with 1M NaCl in the presence of sodium dodecyl sulphate (Braun & Evans [1969]).

The light satellite DNA was shown to be a cytoplasmic species by density gradient centrifugation (Evans [1966]; Guttes, Hanawalt & Guttes [1967]); and of mitochondrial origin both by autoradiography (Guttes & Guttes [1964]) and by in vitro synthesis in isolated mitochondria (Brewer, De Vries & Rusch [1967]). The heavy satellite DNA, on the other hand, is located in the nucleolus as indicated by autoradiography (Guttes

& Guttas [1969]) and biochemical evidence (Zellweger, Ryser & Braun [1972]). Nucleoli may also contain some main band DNA as well as heavy satellite DNA (Bovey & Ruch [1972]). Furthermore, it has been shown that the rRNA genes are localised in the nucleolus by cell fractionation (Zellweger, Ryser & Braun [1972]), light microscopic autoradiography (Guttas & Guttas [1969]) and electron microscopic autoradiography (Ryser, Fakan & Braun [1973]); and are a component of the heavy satellite DNA as indicated by the ability of rRNA to preferentially hybridise to this DNA fraction (Zellweger & Braun [1971]; Sonenshein, Shaw & Holt [1970]; Newlon, Sonenshein & Holt [1973]).

Unlike the bulk of the nuclear DNA which is predominantly replicated during the first three hours following mitosis (Nygaard, Guttas & Rusch [1960]; Braun, Mittermayer & Rusch [1965]; Braun & Wili [1969]; Bovey & Ruch [1972]), the heavy satellite DNA, and consequently the rRNA genes, are replicated throughout the mitotic cycle (Guttas & Guttas [1969]; Braun & Evans [1969]; Holt & Gurney [1969]; Zellweger, Ryser & Braun [1972]; Newlon, Sonenshein & Holt [1973]) with the exception of the first hour after mitosis (Zellweger, Ryser & Braun [1972]). Measurements on growing surface plasmodia at the end of the G2 phase, and starved plasmodia suggest that 0.18 to 0.22% of the nuclear DNA codes for rRNA (Newlon, Sonenshein & Holt [1973]; Ryser & Braun [1974]). My own value of 0.16 to 0.18% (depending on the strain used) is in agreement with those published (see section I.3.5).

I.1.2 Aims and reasons for present work.

Physarum polycephalum can exist in two morphologically distinct vegetative forms - as small, colourless, uninucleate amoebae and as a much larger, yellow, multinucleate plasmodium. Very little biochemical information exists on the amoebal stage compared with the great wealth

of work done on plasmodia. This was primarily due to the absence, until very recently, of a suitable axenic medium for the growth of amoebae. Nevertheless, it is obvious that the overall physiology and metabolism of these two forms must be quite different. Therefore it is reasonable to suppose that some genes will only be transcribed in one of the vegetative forms, so that plasmodial-specific genes will be inactive and amoebal-specific genes active, during the amoebal-plasmodial transition. Such a mechanism is likely to operate where a cell function is confined to only one vegetative form, for example the yellow pigment formation in plasmodia. On the other hand, some of the more fundamental cellular functions must operate in both forms, for example rRNA synthesis. It is therefore of interest to see whether the same rRNA genes are transcribed in both vegetative forms, or whether there are separate "amoebal rRNA genes" and "plasmodial rRNA genes". In other words does the cell utilize the same set of rRNA genes, and modify its control mechanisms for rDNA transcription to meet the requirements of a completely different morphological state, or is a new set of genes employed? If we assume that the genes would be different in the latter case, distinction between these two possibilities can be achieved by competitive nucleic acid hybridisation.

A universal property of eucaryotic rDNA is its high degree of redundancy, the number of rRNA genes varying from about a hundred in lower eucaryotes to several thousand in higher plants (see section I.4.1). It seems likely that this multiplicity of rRNA genes is necessary at least to some extent, to maintain the rate of rRNA synthesis needed for cell growth and differentiation, and the way in which the number of genes is maintained or varied under different conditions is being studied in many systems. In view of the existence of strains of Physarum polycephalum with different nuclear ploidy levels (Mohberg, Babcock,

Haugli & Rusch [1973]) it was considered to be of interest to determine whether ploidy has any effect on the number of rRNA genes.

At first it might be thought that the rDNA content is likely to increase in proportion to the nuclear DNA content. However, there are several reasons to suggest that such an assumption may be rather premature in the case of Physarum. Firstly, the replication of rDNA occurs both during and after that of the nucleoplasmic DNA in plasmodia, suggesting the existence of independent control systems which do not necessarily need to be linked. The difference between the control systems for the replication of nucleoplasmic DNA and nucleolar DNA is further illustrated by the ability of cycloheximide to strongly inhibit synthesis of the former, but only slightly that of the latter (Werry & Wanka [1972]). Secondly, only one nucleolus is observed in Physarum whatever the nuclear DNA content, unlike many systems where the number of nucleoli increases with ploidy. There is therefore no a priori reason for supposing that the proportion of rDNA will be invariant with ploidy. In fact, the proportion of rDNA in ten species of the genus Nicotiana has been found to be lower in the tetraploid species (0.27-0.43%) than in the diploid species (0.67-0.90%) (Siegel, Lightfoot, Ward & Keener [1973]). Thus the absolute number of rRNA genes varies much less than the total nuclear DNA content. On the other hand, in a polyploid series of plants (ranging from haploid to hexaploid) of Datura innoxia, produced by in vitro culture of pollen grains, the proportion of rDNA has been shown to be constant (Cullis & Davies [1974]).

Investigations of this kind, however suggestive, are only strictly meaningful if either (a) the proportion of rDNA is constant throughout the cell cycle, i.e. bulk nuclear DNA and rDNA are replicated at the same time, or (b) determinations of gene dosage are all carried out at the same point in the cell cycle, necessitating synchronous cultures. If

neither of these criteria are fulfilled then values from different cultures will not be comparable. Clearly condition (b) was not observed in either Nicotiana or Datura since DNA was isolated from leaves, nor is there any evidence to suggest that condition (a) is true in these species. However Physarum polycephalum, which can be grown as a large surface plasmodium, the nuclei of which maintain a high degree of natural synchrony (Mohberg & Rusch [1969]), is particularly well suited to this type of study.

I.1.3 Nucleic acid hybridisation.

Answers to both of the problems outlined above can be achieved by applying nucleic acid hybridisation techniques. This is now one of the most widely used diagnostic methods in nucleic acid research, yet the detailed mechanism is not fully understood, and there still remains some controversy as to whether the reaction is reversible or not. To further complicate this, it is now known that annealing in solution occurs by a different mechanism to annealing on nitro-cellulose filters. Studies on renaturation of DNA in solution (Wetmur & Davidson [1968]; Wetmur [1971]) and RNA-DNA hybrid formation in solution (Craig, Crothers & Doty [1971]; Pörschke & Eigen [1971]) have indicated that there are several steps in these associations: (a) the initial collision or nucleation, (b) stabilization of the initial collision site by the reversible formation of a small number of base pairs adjacent to it (formation of the helix nucleus), and (c) subsequent helix formation by a very rapid "zippering". During hybridisation in solution the conversion of the helix nucleus to a stable hybrid form is much more rapid than its dissociation to free RNA and DNA. Thus the overall association appears to be essentially a one-step process. However, when the DNA is immobilised on a membrane filter, an intermediate is now known to accumulate (Spiegelman, Haber & Halvorson [1973]). In this case the conversion of the intermediate to

stable hybrid occurs at a rate comparable to its dissociation to free RNA and DNA. The overall reaction, therefore, does not obey single step kinetics since both the formation of the intermediate and its conversion to a stable form are rate determining processes. Furthermore, the rate of intermediate formation during filter hybridisation is virtually identical to the rate of formation of the helix nucleus during duplex formation in solution, strongly suggesting that the intermediate is analogous to the helix nucleus, and is formed in the same way. However, in the case of filter hybridisation, the conversion of the intermediate form to the final stable hybrid must be much slower, since the overall reaction is slower (Kennell [1971]; McCarthy & McConaughy [1968]); and it has been suggested that this reduction in rate is likely to be due to steric effects imposed by immobilising DNA on filters (Spiegelman, Haber, & Halvorson [1973]; Flavell, Birfeld, Sanders & Borst [1974]).

Hence there is no reason to suppose that RNA-DNA hybrid formation in solution cannot be treated as a single step, reversible process. Moreover, the recent observation that intact Escherichia coli rRNA can displace fragmented rRNA from hybrids, and in vitro transcribed tRNA^{tyr}-like sequences (longer than mature tRNA) can efficiently displace tRNA^{tyr} from hybrids (Beckmann & Daniel [1974]) strongly suggests that the reaction is reversible. In fact, the only evidence which suggests that hybrid formation is not reversible (Bishop [1970]), is invalid since it was based on the incorrect assumption that filter hybridisation is kinetically a single-step process.

It is therefore quite clear that we cannot rely on accurate gene dosage values being obtained by allowing hybrid formation to go to a so-called "completion". Instead there is a strong argument for employing a kinetic approach and obtaining saturation values from the time course of the reaction, in an analogous way to determining $V_{\max}$ in an enzyme-

catalysed reaction. For such an approach, hybridisation in solution would appear to be far more favourable than on filters, since the kinetics are much more readily defined. A kinetic approach has several other important advantages including greatly reduced annealing times, thereby eliminating the risk of RNA degradation, and also requiring much less nucleic acid. For these reasons, this approach was adopted whenever an accurate gene dosage value was required.

On the other hand, nucleic acid competition studies do not depend upon the reaction going to completion, and therefore measurements of either the kinetics of hybrid formation, or equilibrium after long term annealing can be employed in this particular case.

It is now appreciated that indiscriminate application of hybridisation techniques can lead to seriously inaccurate estimates of gene frequency. Steps were therefore taken to obtain incubation conditions (especially annealing temperature) which minimize intrastrand base pairing (Straus & Bonner [1972]; Schmeckpeper [1972]; McCarthy & Church [1970]), since this is particularly important when using RNA with a high degree of secondary structure. A good indication of the fidelity of base pairing within the hybrid was obtained from its melting profile, and from a comparison with that of native DNA; since the melting temperature of a duplex is lowered by about 1°C for each 1.5% mispairing of bases (Laird, McConaughy & McCarthy [1969]).

Inclusion of formamide at a high concentration throughout the procedure has several advantageous effects: (a) it reduces the melting temperature of the hybrid (McConaughy, Laird & McCarthy [1969]), allowing the annealing to be performed at a much reduced temperature, thereby eliminating thermal degradation of RNA; (b) it allows the fractionation of the hybrid from unreacted species to be carried out at room temperature without interference either by significant nonspecific binding of RNA to

hydroxyapatite used for the separation (Schmeckpeper [1972]; Goodman, Gulati, Redfield & Spiegelman [1973]), or by hydroxyapatite-catalysed degradation of RNA, shown to occur at the elevated temperatures necessary in the absence of formamide (Gillespie [1966]; Martinson & Wagenaar [1974]); and (c) it destabilises DNA duplex formation (an unwanted side reaction) more than RNA-DNA hybridisation (Bishop [1972]; Birnstiel, Sells & Purdom [1972]) with the result that at high formamide concentrations, the melting temperature of the hybrid may be as high as that of the DNA duplex.

I.2 MATERIALS AND METHODS.

I.2.1 Choice of strains.

Until recently, only heterothallic strains of Physarum polycephalum, in which haploid amoebae of different mating type fuse to form a diploid plasmodium, had been described. Therefore, a laborious series of outcrosses and backcrosses was required to isolate plasmodia homozygous for a particular mutation after amoebal mutagenesis and selection. However, this difficulty was overcome when Wheals [1970] showed that amoebae of the Colonia isolate of Physarum polycephalum, originally described by Von Stosch, Van Zul-Pischinger & Dersch [1964], produced plasmodia within individual clones. Measurements of nuclear DNA contents of these amoebae and plasmodia produced from clones (Cooke & Dee [1974]) have shown that there is no change in ploidy during plasmodial formation, the plasmodia presumably remaining haploid. Therefore the Colonia strains are clearly not homothallic as first thought, but instead plasmodia must arise either by: (a) apogamy - a single amoebal cell developing into a plasmodium by repeated nuclear division without cell or nuclear fusion and consequently without change in ploidy, or (b) coalescence - fusion of two or more genetically identical amoebae, without nuclear fusion, producing a dikaryotic cell which by repeated nuclear division gives rise to a plasmodium.

Recently, time-lapse photography (Cooke & Anderson, personal communication) has revealed that binucleate cells may arise from single uninucleate amoebae without cell fusion, suggesting that apogamy is the more likely mechanism of plasmodial formation. The advantage of using apogamic amoebae for mutant selection is immediately apparent. Mutants detected in the amoebal stage can be easily tested for in the plasmodial stage, since a plasmodium can be produced directly from a single amoebal

clone. Recessive mutants expressed only in the plasmodial stage can also be isolated by screening plasmodia derived from single amoebal clones.

Recently, Cooke & Dee [1975] have derived a strain designated CL (Colonia Leicester) from the Colonia strain C50 (Wheals [1970]) which produces plasmodia in clones with high efficiency and which completes the life cycle reliably and repeatedly in single clones. Two auxotrophic mutants (lys⁻ and leu⁻) have been isolated from this strain (Cooke & Dee [1975]) and have been shown to be due to single gene mutations.

Clearly, the eventual integration of biochemical and genetical studies on Physarum polycephalum is desirable and consequently the majority of my work has been done with the genetically preferred strain CL. Two other strains were used to study the effect of ploidy; M₃CVIII (derived from Daniel's M₃C strain; Daniel & Baldwin [1964]) which is diploid, and RSD5xRSD2 (Haugli [1971]) which is polyploid. Axenically grown amoebae for nucleic acid competition experiments were of strain RSD4 (Mohberg & Rusch [1971]).

I.2.2 Culture methods.

(i) Stock cultures. Stock cultures of all plasmodial strains were kept as spherules, prepared as described by Daniel & Baldwin [1964]. At approximately 3-4 month intervals a new spherule strip was germinated on an agar plate then transferred to liquid culture. These microplasmodial cultures were maintained on a rich Mycological peptone based medium and subcultured routinely every 2-3 days (1ml of culture into 50ml of medium contained in a 500ml Erlenmeyer flask). Flasks were kept on a reciprocating shaker in a 26°C constant temperature room. Experimental cultures were inoculated from exponentially growing stocks.

By returning to a fresh spherule strip every few months selection

arising from prolonged subculturing in liquid is avoided. This is known to occur in some strains which lose the ability to complete meiosis but are able to sporulate, resulting in non-viable spores (Daniel & Baldwin [1964]; Mohberg, Babcock, Haugli & Rusch [1973]).

Mycological peptone medium (MyDM). This complex medium is an adaptation of the semi-defined medium described by Dee & Poulter [1970] in which mycological peptone replaces bacteriological peptone and all additional vitamins except biotin and thiamine are omitted, these being the only two essential for growth (Daniel & Rusch [1964]; Daniel, Babcock, Sievert & Rusch [1963]). Apart from peptone and vitamins, this medium contains an inorganic salts mixture (buffered at pH 4.6 by citrate), glucose, and haematin (Physarum has an obligatory requirement for an added source of haem; Daniel & Baldwin [1964]). Typical doubling times in this medium range from about 8-10 hours (Plaut & Turnock [1975]).

Mycological peptone medium:		g.l ⁻¹
Salts:	KH ₂ PO ₄	2.00
	CaCl ₂ ·2H ₂ O	0.90
	MgSO ₄ ·7H ₂ O	0.60
	FeCl ₂	0.04
	ZnSO ₄ ·7H ₂ O	0.034
	Citric acid (anhydrous)	3.24
	EDTA·Na ₂	0.224
Vitamins:	Biotin	0.005
	Thiamine·HCl	0.04
Peptone:	Mycological (Oxoid Ltd.)	10.00

The salts, vitamins, and peptone are dissolved in distilled water and the pH adjusted to 4.6 with NaOH. The medium is then autoclaved (15 lb.in⁻² for 20 min) in 50ml lots in 500ml Erlenmeyer flasks. Immediately before use 0.5ml of sterile haematin solution (Koch-Light

Labs. Ltd., 0.5mg.ml^{-1} in 1% NaOH) and 2.0ml of sterile glucose solution (25% w/v) are added.

(ii) Petri dish cultures. Small surface plasmodial cultures were grown in petri dishes on the same peptone medium as above with an equal volume of 3% (w/v) agar. Subculturing was achieved by aseptically transferring a small block of the agar (about 7mm x 7mm) containing part of the plasmodial growing front, to the centre of a new plate. A plasmodium will cover a 9cm petri dish in about 48 hours.

(iii) Large surface cultures. Large surface cultures (about 18cm in diameter) were grown in pairs using the rocker apparatus described by Mohberg & Rusch [1969].

(iv) Amoebal cultures. Amoebae were grown in an axenic medium of salts, bovine serum albumin, and beef embryo extract (Goodman [1972]; McCullough, personal communication). Cells were inoculated into 100ml Erlenmeyer flasks containing 20ml of medium to give a density of about 10^6cells.ml^{-1} . They were subcultured when a density of about $8 \times 10^6\text{cells.ml}^{-1}$ had been achieved (about 7 days).

I.2.3 Stages of the mitotic cycle.

Small pieces of the plasmodium were removed with a sterile microspatula, placed on a microscope slide, smeared with the edge of a second slide, and fixed for 10s in absolute ethanol. After adding a drop of 1:1 (v/v) glycerol:ethanol, the preparation was covered with a coverslip and observed through a phase contrast microscope (1000x magnification). Photomicrographs of the mitotic stages have been published many times (Mohberg & Rusch [1969]; Mohberg [1974]; Rusch [1969]).

I.2.4 Preparation of nuclei.

Nuclei from plasmodia grown on agar (10 petri dish cultures were

pooled for each isolation) were isolated according to procedure B described by Mohberg & Rusch [1971] with several minor modifications.

(1) Plasmodia were gently scraped off the agar with a spatula and transferred to a 1 litre Waring blender cup containing 200ml of ice-cold homogenising medium (0.25M sucrose, 0.02M CaCl_2 , 0.01M Tris-Cl, pH 7.0, plus 0.1% Nonidet P40 [B.D.H. Chemicals Ltd., Poole, England]).

(2) The blender (MSE Ato-Mix), controlled by a 0-240V variable transformer was run at "low speed", 150V, for 15s to break up the large pieces of plasmodium; then at "high speed", 200V, for 45s.

(3) After keeping the homogenate on ice for 2-3min to allow some of the foam to settle, it was centrifuged at 50xg for 5min (5°C) to remove large pieces of cell debris.

(4) The supernatant was filtered through a triple milk filter (1 "Blow" milk filter medium sandwiched between 2 "Johnson and Johnson" regal milk filters) clamped in a two piece polyethylene Buchner funnel (13cm diameter). Filters were saturated with homogenising medium before use.

(5) The filter was rinsed with 25ml of homogenising medium and the total filtrate centrifuged at 1000xg for 10min (5°C) in 50ml conical-based glass tubes.

(6) After discarding the yellow supernatant, the pellet was resuspended in 80ml of buffered sucrose (0.25M sucrose, 0.01M CaCl_2 , 0.01M Tris-Cl, pH 7.0) by gentle whirlmixing, and re-centrifuged (1000xg, 10min, 5°C). This was then repeated with a further 80ml of buffered sucrose.

(7) Finally, the nuclear pellets from a total of about 200 petri dish cultures (20 isolations) were pooled, resuspended in an equal volume (typically about 30ml) of buffered sucrose and stored at -20°C.

Nuclei prepared in this way were not entirely free from contaminating

cytoplasm; ideally only 1-2 plasmodia should be pooled for each isolation to obtain clean nuclear preparations. However, as this would entail 100 separate isolations a compromise had to be made between an acceptable degree of contamination and the number of isolations.

Nuclei from large surface cultures (about 18cm in diameter), grown in pairs using the rocker apparatus described by Mohberg & Rusch [1969], were isolated as they entered early prophase of the fourth mitosis after coalescence. In this case gentler homogenisation conditions were used (since the nuclear membrane is more fragile at mitosis), namely 100V on "low speed" for 2min.

1.2.5 Preparation of DNA from isolated nuclei.

(1) Pronase (Protease, type VI; Sigma Chem.) was preincubated at a concentration of 10mg.ml^{-1} for 2h at 37°C to destroy any contaminating nucleases. It was then added to the nuclear suspension to produce a final enzyme concentration of 1mg.ml^{-1} and incubated at 37°C for 1h. This treatment produced efficient lysis of the nuclei and also enhanced the yield of DNA during the subsequent phenol extraction, presumably by partially digesting the chromosomal proteins and thereby releasing DNA which might otherwise have been trapped during deproteinisation. In this way essentially 100% recovery of the DNA could be achieved.

(2) The pronase treated suspension was diluted with an equal volume of 0.4M NaCl , 0.02M EDTA , 0.01M Tris-Cl , pH 7.4; followed by 0.2vol. of a 5% (w/v) solution of tri-isopropyl naphthalene sulphonate and 0.2vol. of a 30% (w/v) solution of 4-aminosalicylate, both dissolved in extraction buffer (0.2M NaCl , 0.01M EDTA , 0.01M Tris-Cl , pH 7.4) and kept on ice for 5min.

(3) Deproteinisation was based on the method of Parish & Kirby [1966]. 1vol. of freshly distilled phenol/10% (v/v) m-cresol,

equilibrated with the extraction buffer and containing 0.1% (w/v) 8-hydroxyquinoline, was added and the mixture shaken at room temperature for 30min. The phases were separated by centrifugation (2000xg, 20min, 15°C) and the upper aqueous layer carefully removed leaving behind the denatured protein at the buffer/phenol interphase.

(4) The DNA was precipitated from the aqueous layer at room temperature by the addition of an equal volume of 2-ethoxyethanol, and collected by centrifugation (26,000xg, 15min, 10°C).

(5) The gelatinous precipitate of DNA was redissolved in a small volume (about 25ml) of standard saline citrate buffer (SSC: 0.15M NaCl, 0.015M sodium citrate, 5mM EDTA, pH 7.0) by gentle mixing overnight on a rotating mixer (Chiltern Instruments Ltd.), then dialysed against 3 x 2litre changes of 0.1 x SSC.

(6) The following enzymes were added: ribonuclease T₁ (EC 2.7.7.26; boiled for 5min to inactivate contaminating deoxyribonuclease), 10units. ml⁻¹; ribonuclease A (EC 2.7.7.16; boiled for 10min), 50µg.ml⁻¹; α-amylase (EC 3.2.1.1), 0.1mg.ml⁻¹; and β-amylase (EC 3.2.1.2), 0.1mg.ml⁻¹; and incubated at 37°C for 1h.

(7) A further phenol extraction was carried out as described in step (3), after the addition of 0.1vol. of 10 x SSC, 0.2vol. of 5% tri-isopropyl-naphthalene sulphonate, and 0.2vol. of 30% 4-aminosalicylate. In this case the phenol/buffer mixture was only shaken for 20min prior to centrifugation.

(8) The DNA was precipitated from the aqueous phase with 2-ethoxyethanol and redissolved in SSC as described in steps (4) and (5); then dialysed against 2 x 2litre changes of 0.01 x SSC.

(9) Polysaccharide was removed by selective precipitation of the DNA with streptomycin sulphate, using a method based on that of Evans [1966], as follows: The concentration of DNA was adjusted to 0.15-0.20

mg.ml⁻¹ with 0.01 x SSC, and streptomycin sulphate (100mg.ml⁻¹) added at room temperature to give a concentration of 5mg.ml⁻¹. 30min later the DNA-streptomycin precipitate was collected by centrifugation (35,000xg, 20min, 4°C). Precipitation of DNA was quantitative (greater than 99.5%), being undetectable in the supernatant. The precipitate was redissolved in SSC containing 1M NaCl (about 15ml) by gentle mixing overnight; then dialysed, first against SSC containing 1M NaCl, then against gradually decreasing salt concentrations (so as not to reprecipitate the DNA-streptomycin) and finally at least 6 x 2litre changes of 0.01 x SSC.

(10) The DNA solution was stored at 4°C over a few drops of chloroform.

DNA prepared in this way contained no detectable protein or RNA (by conventional chemical methods), and the precipitation with streptomycin sulphate increased the DNA:polysaccharide ratio from 1:7 to 10:1, a 70-fold enrichment. $E_{260}:E_{280}$ was greater than 2.0, and $E_{260}:E_{230}$ was greater than 2.0.

Each step in the procedure (after the nuclear isolation) was shown to be capable of quantitative DNA recovery. This is especially important when measurements of gene frequency are to be made since steps with a low efficiency might very easily lead to selective loss of certain DNA sequences.

I.2.6 Chemical assay for DNA and determination of its extinction coefficient.

DNA was determined by the diphenylamine method of Burton [1956], modified according to Giles & Myers [1965]: 0.8g of diphenylamine was dissolved in 20ml of glacial acetic acid, to which 2ml of 70% perchloric acid and 1ml of freshly prepared 0.16% (w/v) aqueous acetaldehyde were added. After hydrolysing the DNA sample in 0.5M perchloric acid at 70°C for

30min, 1.2ml of "diphenylamine reagent" were added to 1.0ml of the acid hydrolysate and incubated at 30°C for 16h. The extinction at 595nm was then determined. The standard curve (using deoxyribose dried over P_2O_5 as the standard) covered the range 0-6µg of deoxyribose; from which the amount of DNA could be calculated (DNA concentration = 4.58x deoxyribose concentration).

From a knowledge of the extinction at 258nm and the DNA content of a solution of Physarum DNA, its extinction coefficient could be determined. A value for $E_{258}^{1\%}$ of 214 was obtained.

I.2.7 Preparation of rRNA from microplasmodia.

(1) Microplasmodia from a 50ml exponentially growing culture were harvested by centrifugation (150xg, 30s, room temperature), resuspended in 25vol. (typically 25ml) of homogenising buffer (0.2M NaCl, 0.01M Tris-Cl, pH 7.4) containing 1% tri-isopropyl-naphthalene sulphonate and 6% 4-aminosalicylate, and homogenised at 0°C for 1min in a glass Potter-Elvehjem homogeniser.

(2) An equal volume of freshly distilled phenol/10% (v/v) m-cresol, equilibrated with the homogenising buffer and containing 0.1% (w/v) 8-hydroxyquinoline, was added and the mixture shaken vigorously for 15 min at room temperature in a 100ml sterile bottle with the aid of a mechanical flask shaker (Griffin & George Ltd.). Phases were then separated by centrifugation (3000xg, 10min, 15°C) and the upper aqueous layer carefully removed.

(3) The phenol layer was re-extracted with 0.5vol. of homogenising buffer, and the aqueous layers combined.

(4) The phenol extraction was repeated a further three times as described in step (2); twice using 1vol. of phenol, and finally with 0.5vol. of phenol.

(5) After the addition of 0.1vol. of 4M NaCl to raise the salt concentration to 0.6M, nucleic acids were precipitated with 2vol. of absolute ethanol at -20°C for at least 1h. The precipitate was collected by centrifugation (7500xg, 15min, 0°C), redissolved in a small volume of homogenising buffer, and precipitated for a second time with ethanol.

(6) The nucleic acid was dissolved in a small volume (typically 4ml) of 50mM KCl, 5mM magnesium acetate, 50mM Tris-Cl, pH 7.8; and treated with deoxyribonuclease I (EC 3.1.4.5; ribonuclease free, $50\mu\text{g}.\text{ml}^{-1}$) at room temperature for 20min. Pronase (preincubated at a concentration of $10\text{mg}.\text{ml}^{-1}$ for 2h at 37°C , to remove traces of nuclease) was added to a final concentration of $0.2\text{mg}.\text{ml}^{-1}$ and incubated at 37°C for 1h, followed by a further extraction with phenol/m-cresol as in step (2).

(7) The RNA was precipitated 4-5 times with 2.5vol. of absolute ethanol after dissolving in 0.15M sodium acetate, pH 6.0 (with acetic acid).

(8) The final precipitate was dissolved in gradient buffer (0.2M NaCl, 1mM EDTA, 10mM Tris-Cl, pH 7.6) and clarified by centrifugation (7500xg, 5min, 4°C).

(9) Ribosomal RNA was obtained by zone sedimentation through 5-20% (w/v) sucrose gradients in 13.7ml of gradient buffer. 0.5mg of total RNA in 0.5ml was loaded onto each gradient and centrifuged at $24,000\text{rev}.\text{min}^{-1}$ for 16h (0°C) in the 6 x 15ml swing-out rotor of an MSE Superspeed 65 centrifuge. Gradients were analysed by means of an ISCO model 180 gradient fractionator and model 222 ultraviolet analyser: The polypropylene centrifuge tubes were punctured close to the bottom, and the gradients underlaid with 40% (w/v) sucrose solution, displacing them at a rate of $2.5\text{ml}.\text{min}^{-1}$ through a 5mm light-path flowcell. The extinction at 254nm was recorded as a function of the gradient volume by a slave recorder, with a full scale reading of 2.5 absorbance units.

Fractions containing 19S and 26S rRNA (see Fig. 2.) were collected, 0.1 vol. of 4M NaCl added, and the rRNA precipitated with 2.5vol. of absolute ethanol at -20°C overnight.

(10) After centrifugation ($34,000\times g$, 15min, 0°C) the rRNA was dissolved in standard saline citrate (SSC: 0.15M NaCl, 0.015M sodium citrate, 5mM EDTA, pH 7.0) and reprecipitated 4-5 times with ethanol.

(11) rRNA was finally dissolved in a small volume of 0.01 x SSC and stored at -20°C .

rRNA prepared in this way contained no detectable protein or DNA.

$E_{260}:E_{280}$ was greater than 2.0, and $E_{260}:E_{230}$ was greater than 3.0. About 2mg of rRNA was obtained from a 50ml microplasmodial culture.

Radioactively labelled rRNA was prepared from 50ml cultures of microplasmodia grown for two days in the presence of either [$6\text{-}^3\text{H}$] uridine ($50\mu\text{M}$, $10\mu\text{Ci.ml}^{-1}$, Amersham); or inorganic [^{32}P] phosphate ($60\mu\text{Ci.ml}^{-1}$, Amersham) with the KH_2PO_4 concentration in the medium reduced by a factor of 20. (This reduced phosphate concentration did not appear to have any deleterious effect on the cultures for at least 50 generation times.)

I.2.8 Preparation of rRNA from amoebae.

(1) Shake cultures of axenically grown amoebae ($6 \times 50\text{ml}$; 10^6 cells. ml^{-1}) were harvested by centrifugation ($1000\times g$, 2min, room temperature) and washed with 2 x 100ml of salts medium to remove as much albumin as possible.

(2) They were then resuspended in a small volume of buffer (0.2M NaCl, 10mM Tris-Cl, pH 7.4) containing 1% tri-isopropyl-naphthalene sulphonate and 6% 4-aminosalicylate, and sonicated in ice at an amplitude of $8\mu\text{m}$ for 3 x 30s (with 15s pauses for cooling in between) with an MSE 100watt ultrasonic disintegrator.

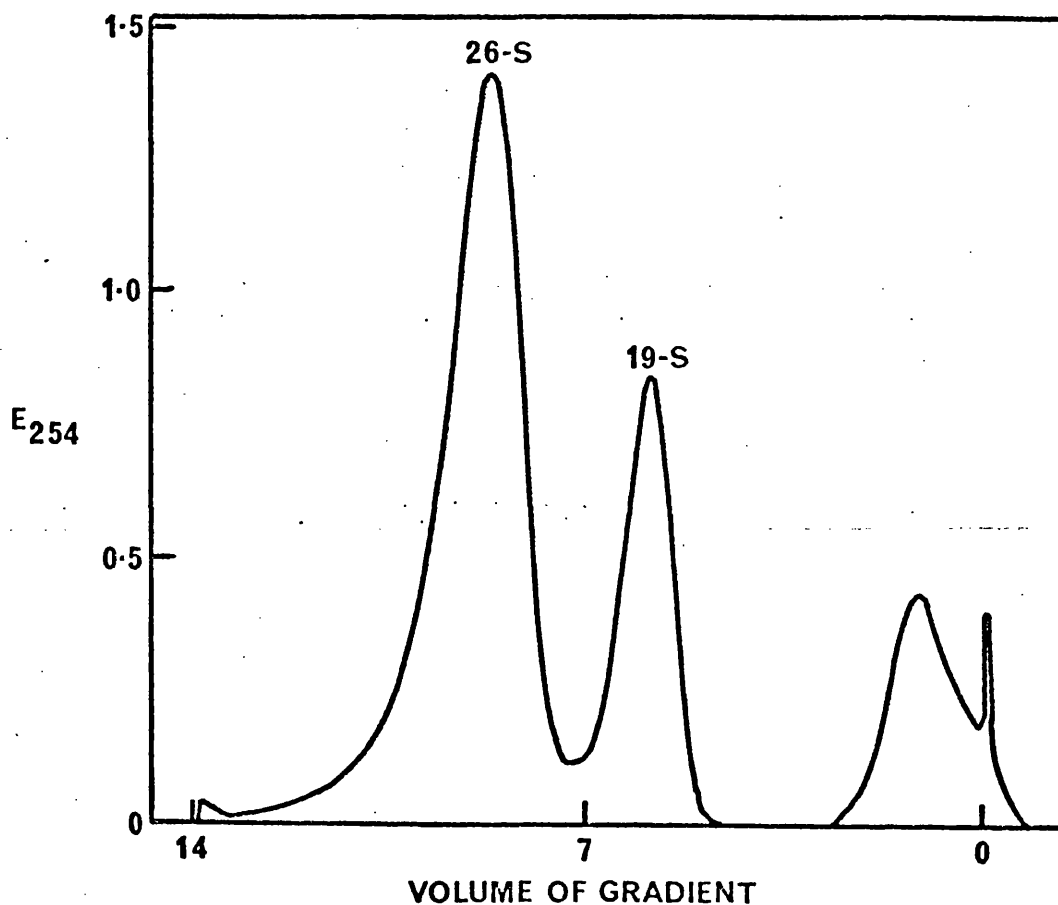


Fig. 2. Zone sedimentation analysis of a preparation of RNA.

This illustrates a typical fractionation of Physarum RNA by zone sedimentation of 500 μ g of total cell RNA in a 5-20% linear sucrose density gradient, routinely used for the preparation of pure ribosomal RNA (26S and 19S species). Sedimentation is from right to left.

(3) rRNA was then prepared as for microplasmodia.

I.2.9 Preparation of rRNA from Escherichia coli.

Total RNA was prepared from E. coli MRE 600 (Frozen cell sludge; Whatman Biochemicals Ltd., Cat. No. 77011) by a conventional method (Pigott & Midgley [1968]) and rRNA obtained by zone sedimentation in sucrose density gradients as described in section I.2.7.

I.2.10 Determination of extinction coefficient of Physarum rRNA.

The extinction at 258nm and phosphate content (Chen, Toribara & Warner [1956]) of a solution of Physarum rRNA were determined. From this a value for $E_{258}^{1\%}$ of 221 was obtained.

I.2.11 Preparation of hydroxyapatite.

Hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ was prepared by the method of Tiselius, Hjertén & Levin [1956] with minor modifications (Levin [1962]; Miyazawa & Thomas [1965]; Bernardi [1969]) essentially as described by Bernardi [1971], as follows:

(i) Preparation of brushite, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$. 2litres of 0.5M CaCl_2 and 2litres of 0.5M Na_2HPO_4 were fed at a flow rate of 150ml.h^{-1} (by means of a twin channel peristaltic pump) into a large flask containing 200ml of 1M NaCl, stirring just sufficiently to stop the precipitate from settling. The coarse, granular brushite was then allowed to sediment, the supernatant decanted off, and the precipitate washed with 2 x 5litres of distilled water.

(ii) Conversion of brushite into hydroxyapatite. Brushite was suspended in 4litres of distilled water, 100ml of 40% (w/v) NaOH added, slowly heated to boiling point with gentle stirring, and boiled for 1h. The precipitate was then allowed to settle and the supernatant siphoned

off. It was washed with 3 x 5litres of distilled water, siphoning after about 5min of sedimentation in each case to eliminate the "fines". The precipitates from two preparations were pooled at this stage, suspended in 2litres of 10mM NaP_i (see footnote) and boiled for 5min. This was repeated once with 10mM NaP_i and again with 1mM NaP_i , boiling for 15min in each case. About 500ml of packed precipitate, in the form of blade-like crystals, was obtained from two pooled preparations. Material prepared in this way and stored at 4°C in 1mM NaP_i retains its chromatographic properties for several months, after which flow rates become greatly reduced by the gradual breakdown of the crystals and their aggregates. Therefore, to ensure optimum chromatographic properties a new batch of hydroxyapatite was made approximately every 3 months.

I.2.12 DNA-RNA hybridisation.

(1) DNA at a concentration of $200\mu\text{g}.\text{ml}^{-1}$ was sheared to a molecular weight of about 3×10^5 , suitable for nucleic acid hybridisation, by sonicating in ice at an amplitude of 4 μm (Collins [1971]) for 2 x 1min with a 30s pause for cooling in between (MSE 100watt ultrasonic disintegrator, fitted with a titanium vibrator micro-probe[end diameter: 0.125 inches]).

(2) 1.0ml mixes containing sonicated DNA (20-40 μg), [^{32}P]-rRNA or [^3H]-rRNA (1-2 μg) and competitor rRNA (where applicable) in 0.24M NaP_i -50% (v/v) formamide buffer were incubated in acid cleaned glass scintillation vials at 85°C for 10min to denature the DNA, then quickly transferred to the annealing temperature of 47°C. For hybridisation to saturation, the vials were incubated for up to 60h; whereas for

Footnote: NaP_i = equimolar mixture of NaH_2PO_4 and Na_2HPO_4 , e.g.

10mM NaP_i is equivalent to 5mM NaH_2PO_4 plus 5mM Na_2HPO_4 .

determining the kinetics of annealing samples were normally removed at hourly intervals for about 5h. After removal they were quickly chilled on ice to stop any further reaction, and stored at -20°C until processed. Control mixes were treated in the same manner, but contained no DNA.

(3) 3.0ml of 0.24M NaP_i buffer were added to each vial (to lower the formamide concentration for efficient ribonuclease activity) followed by 40 μl of a mixture of ribonuclease A (EC 2.7.7.16; 2mg.ml^{-1} ; boiled for 10min) and ribonuclease T_1 (EC 2.7.7.26; $4000\text{ units.ml}^{-1}$; boiled for 5min). It can be seen from Fig. 3. that whereas 50% formamide leads to a substantial reduction in ribonuclease activity, 12% formamide has very little effect.

In the case of competition experiments, where the concentration of the competing RNA species may vary by as much as two orders of magnitude, unlabelled plasmodial rRNA was added prior to enzyme treatment, so that all mixes contained the same RNA/ribonuclease ratio.

After addition of the enzyme mix, the vials were kept on ice for 10min, during which time unreacted rRNA was rendered too small for further hybrid formation, then incubated at room temperature for 70min to complete the RNA hydrolysis.

(4) Escherichia coli rRNA was added to give a concentration equivalent to ten times the initial Physarum rRNA concentration, and incubation at room temperature continued for a further 10min. Inclusion of this step greatly reduced the level of non-specific RNA binding to hydroxyapatite during the subsequent fractionation, resulting in much lower "backgrounds". An analogous effect was obtained if the enzyme treated mixes were passed through a Dowex 50 anion exchange column before fractionation. A feasible explanation is that labelled rRNA-ribonuclease (i.e. the enzyme-substrate complex), binds to the hydroxyapatite column and is eluted with the hybrid (proteins do bind to hydroxyapatite and

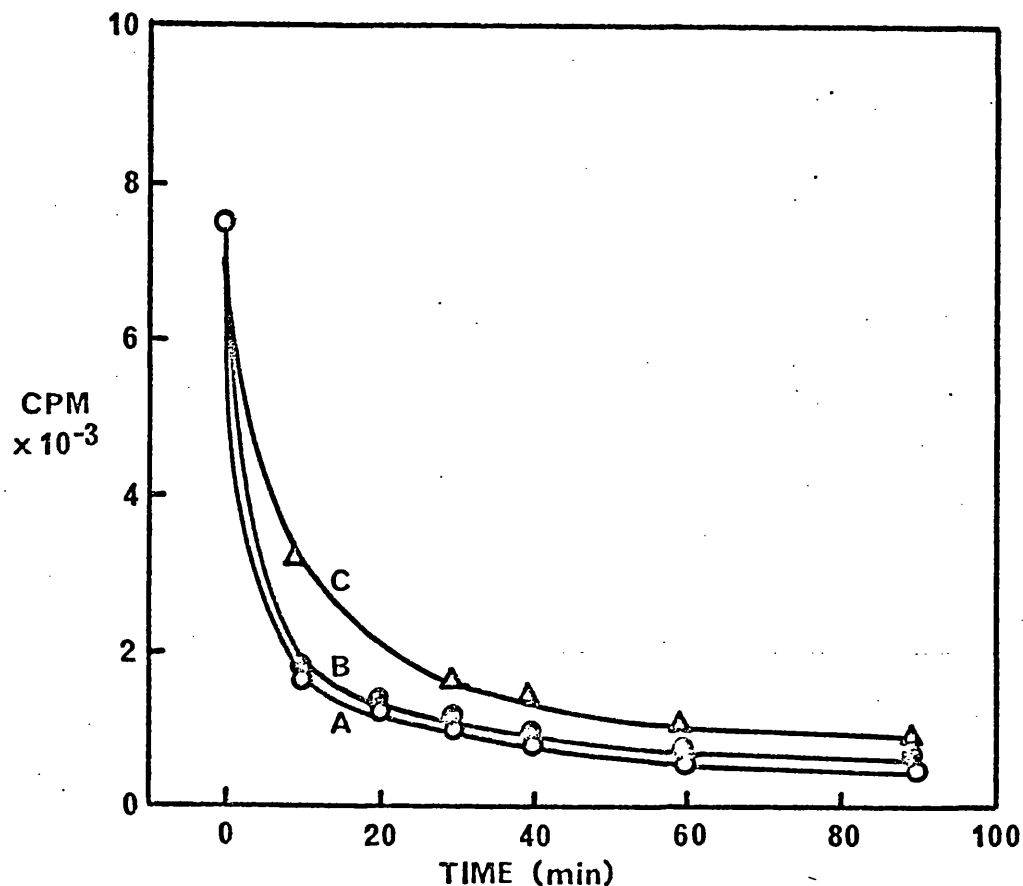


Fig. 3. Ribonuclease activity in the presence of formamide.

2.0ml mixes containing 100 μ g of [32 P]-rRNA (about 750cpm. μ g $^{-1}$) in 0.2M NaP $_i$ buffer containing either 0, 12 or 50% formamide (v/v) were incubated with a mixture of ribonuclease A (20 μ g.ml $^{-1}$) and ribonuclease T $_1$ (40 units. ml $^{-1}$). 200 μ l samples were removed before addition of the nucleases and at intervals during the 90min incubation. Each sample was added at once to 200 μ l of cold 10% trichloroacetic acid and left on ice for 30min to precipitate. They were then filtered through Millipore membrane filters, washed, dried and counted after the addition of 10ml of scintillation fluid. A, no formamide; B, 12% formamide; C, 50% formamide.

are eluted over a range of salt concentrations including that used for eluting hybrid). This explanation is not unreasonable when one considers that "non-specific binding" may only amount to perhaps 300cpm from an input of 150,000cpm, i.e. 0.2%. A Dowex anion exchange column would remove such a protein-RNA complex, and addition of excess unlabelled RNA would effectively "chase" the labelled RNA residues from the active site of the enzyme.

(5) After cooling on ice, 3.0ml of formamide were added to increase the formamide concentration to 50% and at the same time reduce the NaP_i concentration from 0.24M to 0.14M. The samples were stored at -20°C at this stage if necessary.

I.2.13 Chromatography of DNA-RNA hybrids on hydroxyapatite.

Hydroxyapatite was prepared as described in section I.2.11, since commercially available hydroxyapatite was found to give very poor flow rates (due mainly to the small particle size of these preparations). Formamide (Fisons; laboratory grade was used for reasons of economy, since large quantities were required) was passed through Dowex chelating resin (Sigma Chem. Co; No. I-4751; dry mesh 50-100) before use. Aqueous buffers containing formamide were prepared fresh each day and stored in dark bottles. Columns for chromatography were 10 x 40mm microfilters containing a sintered glass support (Baird & Tatlock; type 232/1200-23; no. 3 porosity).

The procedure used for the fractionation of hybrids was a modification of that described by Goodman, Gulati, Redfield & Spiegelman [1973]:

(1) Hybrid mixes were brought to room temperature, 2ml of a thick slurry of hydroxyapatite, freshly equilibrated with 0.16M NaP_i -50% (v/v) formamide buffer, added and shaken gently at 22°C for 1h.

(2) The columns were then poured in batches of ten and each washed

with 80ml of 0.16M NaP_i -50% formamide at a rate of $40\text{ml}\cdot\text{h}^{-1}$ by means of a ten channel Watson & Marlowe peristaltic pump, to remove single-stranded material.

(3) DNA•rRNA hybrid was then eluted with 10ml of 0.30M NaP_i -50% formamide and collected in a glass scintillation vial. $[\text{}^{32}\text{P}]$ -rRNA•DNA hybrid was assayed by measuring the Cerenkov radiation in a Packard 3385 scintillation spectrometer (optimum gain setting: 42%, optimum sample volume: 10ml). For $[\text{}^3\text{H}]$ -rRNA•DNA hybrid, the 10ml fraction was collected in a vial containing 0.5ml of Escherichia coli rRNA ($1\text{mg}\cdot\text{ml}^{-1}$) as carrier and precipitated with 2ml of cold 50% (w/v) trichloroacetic acid at 0°C for 30min (0.8ml of acid to neutralise the 0.3M NaP_i and 1.2ml to increase the acid concentration to 0.3M for precipitation of RNA). Carrier RNA must be added before collecting the hybrid fraction rather than after, since in the latter case up to 50% of the hybrid may be lost, presumably by irreversible binding of the small amount of hybrid (about 40ng) to the sides of the glass collecting vial.

(4) Mixes were then filtered through glass fibre filters (Whatman GF/C), washed with 6 x 2ml of cold 5% trichloroacetic acid, 4 x 2ml of cold absolute ethanol and dried at 80°C overnight. Finally, the filters were placed in 2.5ml of toluene-PPO-POPOP scintillant (5g PPO, 0.3g POPOP, 11litre toluene) contained in a small glass insert within a conventional glass scintillation vial, and counted in a Packard 3385 scintillation spectrometer (optimum gain setting: 55%).

I.2.14 Melting profile of rRNA•DNA hybrid.

(1) A 5.0ml mix containing 200 μg of sonicated DNA and 10 μg of $[\text{}^3\text{H}]$ -rRNA (from strain CL) in 0.24M NaP_i -50% formamide was hybridised and processed as described above (section I.2.12), all additions being increased 5-fold. A control mix without DNA was treated in the same way.

(2) After ribonuclease treatment the mixes were incubated with 10ml of hydroxyapatite slurry, then poured into large jacketed columns. The circulating fluid (60% glycerol) was controlled to within 0.2°C by a thermostated heater/circulating pump (Haake [Berlin] FJ).

(3) The columns were first washed at room temperature with 160ml of 0.16M NaP_i -50% formamide, then clamped off and equilibrated at the starting temperature of 35°C .

(4) At equilibrium a 10ml fraction was collected. The length of the column was such that a head of at least 10ml of buffer could be maintained above the hydroxyapatite and within the heated jacket, so that unequilibrated buffer need not be added during each elution step.

(5) After collecting a 10ml fraction the column was topped up with buffer, re-equilibrated at a higher temperature, and a further 10ml fraction collected. A total of 10 fractions were taken in steps of about 5°C , then precipitated with acid in the presence of carrier RNA and counted (as described in the previous section). Results were expressed as a percentage of the total strand separation.

I.2.15 Melting profile of native Physarum DNA.

The melting curve for Physarum DNA (50pg.ml^{-1}) in 0.16M NaP_i -50% formamide (the buffer was de-aerated by boiling for a few minutes) was obtained in a Unicam SP800 spectrophotometer equipped with an SP876 temperature programme controller and an SP20 external recorder. The temperature of the DNA sample was raised at a rate of 1°C/min and the absorbance at 258nm monitored continuously. Results were expressed as a percentage of the total hyperchromicity.

I.2.16 Presentation of kinetic data.

RNA-DNA hybridisation in solution is a second order reaction

(Nygaard & Hall [1964]). However, if DNA renaturation is prevented and one of the reactants is in large excess, the reaction becomes essentially pseudo first order (Bishop [1972]). If RNA is the excess species, then:

$$\frac{D_0}{D} = \exp^{k_h R_0 t} \quad \text{-----} \quad (1).$$

where: D_0 = concentration of DNA homologous to rRNA (i.e. rDNA) at zero time; D = concentration of rDNA at time t ; R_0 = concentration of rRNA at zero time; and k_h = rate constant of hybridisation.

Expanding the exponential term we get:

$$\frac{D_0}{D} = 1 + k_h R_0 t + \frac{(k_h R_0 t)^2}{2} + \frac{(k_h R_0 t)^3}{6} + \dots \quad \text{-----} \quad (2).$$

If terms to the second and higher powers are negligible:

$$\frac{D_0}{D} = 1 + k_h R_0 t \quad \text{-----} \quad (3).$$

Now, $D_0 = H_s$ (where H_s = the saturation value, i.e. the amount of DNA which codes for rRNA), and $D = (H_s - H)$ (where H = concentration of hybrid at time t).

Hence from equation (3):

$$\frac{H_s}{H_s - H} = 1 + k_h R_0 t$$

and: $1 = 1 - \frac{H}{H_s} + k_h R_0 t - \frac{k_h R_0 t H}{H_s}$

Re-arranging we get:

$$k_h R_0 t = \frac{H}{H_s} + \frac{k_h R_0 t H}{H_s}$$

$$\frac{1}{H} = \frac{1}{(k_h R_0 H_s) t} + \frac{1}{H_s} \quad \text{-----} \quad (4).$$

It can therefore be seen that there is a linear relationship between the reciprocal of the hybrid concentration and the reciprocal of time; the saturation value (H_s) being given by the reciprocal of the intercept on the H^{-1} axis.

To arrive at this simple relationship three assumptions have been made:

- (1) DNA renaturation does not occur.
- (2) RNA is in large excess.
- (3) The second and higher power terms in equ. (2) are negligible.

In my experiments there was normally at least a 40-fold excess of rRNA to rDNA. Also, in the presence of a high concentration of formamide an RNA•DNA hybrid is as stable as a DNA•DNA duplex (Birnstiel, Sells & Purdom [1972]; Bishop [1972]; see section I.3.2). Therefore rDNA renaturation should not have exceeded 2.5%, and assumptions (1) and (2) are justified. Finally, it is reasonable to ignore the second and higher power terms in equ. (2) as long as $k_h R_o t$ is equal to, or less than 1.0. From equ. (1) this is true until the reaction is more than half over, and deviations will be most severe in the later stages of the reaction.

The conventional double reciprocal plot as given in equ. (4) has several disadvantages, the main one being the reciprocal time scale which puts increased weighting on the potentially less accurate, short time measurements. However, this can be overcome by a simple transformation of equ. (4), known as the Scatchard plot (Marsh & McCarthy [1973]):

Multiplying equ. (4) throughout by $\underline{t}$ we get:

$$\underline{\frac{t}{H}} = \frac{1}{(k_h R_o H_s)} + \frac{t}{H_s}$$

Therefore a graph of $\frac{t}{H}$ against t will yield a straight line and the saturation value (H_s) will be given by the reciprocal of the slope.

All saturation values from kinetic data were obtained by linear, least-squares analysis using a Hewlett-Packard calculator (model 9100B), with programme Stat-Pac IV-10.

I.3 RESULTS AND DISCUSSION.

I.3.1 Effect of temperature on hybrid formation.

The effect of temperature on the hybridisation of plasmodial rRNA to homologous DNA is shown in Fig. 4. and Table 1. Fig. 4A illustrates the time course of hybrid formation, and Fig. 4B the linear relationship of the double reciprocal transformation, i.e. (amount of hybrid)⁻¹ and (annealing time)⁻¹. It is clear that both the rate of hybridisation and the apparent saturation value (derived from the intercept of the double reciprocal plot) increased with increase in annealing temperature, although the effects were less pronounced at the higher temperatures.

These observations are in agreement with those of Straus & Bonner [1972], who showed that the experimentally determined gene frequency increases with increasingly stringent criteria of sequence matching. They, and Studier [1964], suggest that this is due to single-stranded rRNA and rDNA forming short, base-paired intrastrand regions (formed increasingly more as the temperature decreases) which suppress the rate of true hybrid formation by reducing the concentration of sites available for interstrand base pairing.

Increasing the annealing temperature above 47°C would bring it dangerously close to the melting temperature of the hybrid of 60°C (see section I.3.2) with the risk of increased hybrid dissociation and consequent deviation from pseudo first order kinetics. In view of this, and also the general observation that the optimum annealing temperature is normally 10-15°C below the melting temperature of the hybrid (Bishop [1972]), it was decided to adopt 47°C as the standard hybridisation temperature.

In conclusion, therefore, with the possibility of obtaining an error of up to 100% in the saturation value (see Table 1.), simply by using a

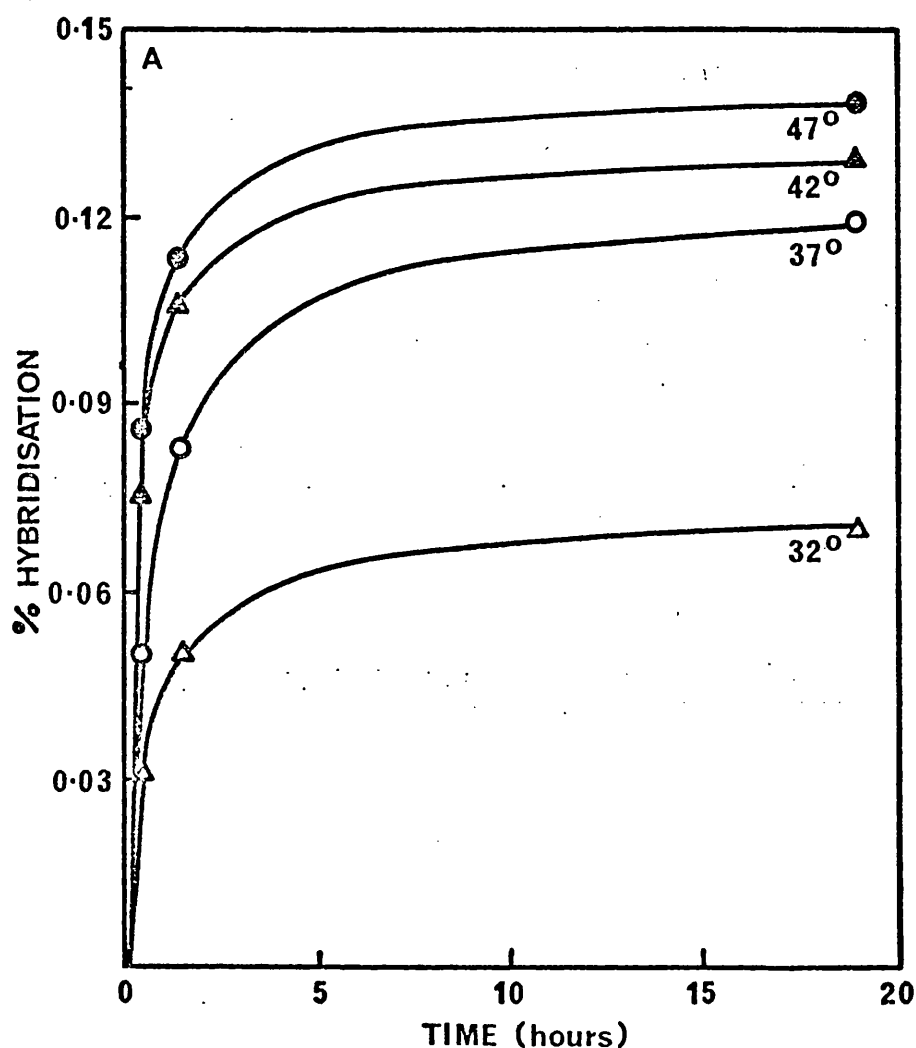


Fig. 4. Effect of temperature on hybrid formation.

The kinetics of hybrid formation were followed in reaction mixes containing 20 μ g of plasmodial DNA and 1.2 μ g of [32 P]-rRNA at four different annealing temperatures - 32, 37, 42 and 47°C. Fig. 4A illustrates the time course of hybrid formation and Fig. 4B (overleaf) the double reciprocal transformation i.e. $\frac{1}{H}$ versus $\frac{1}{t}$. Saturation values are given by the reciprocal of the intercept on the $\frac{1}{t}$ axis.

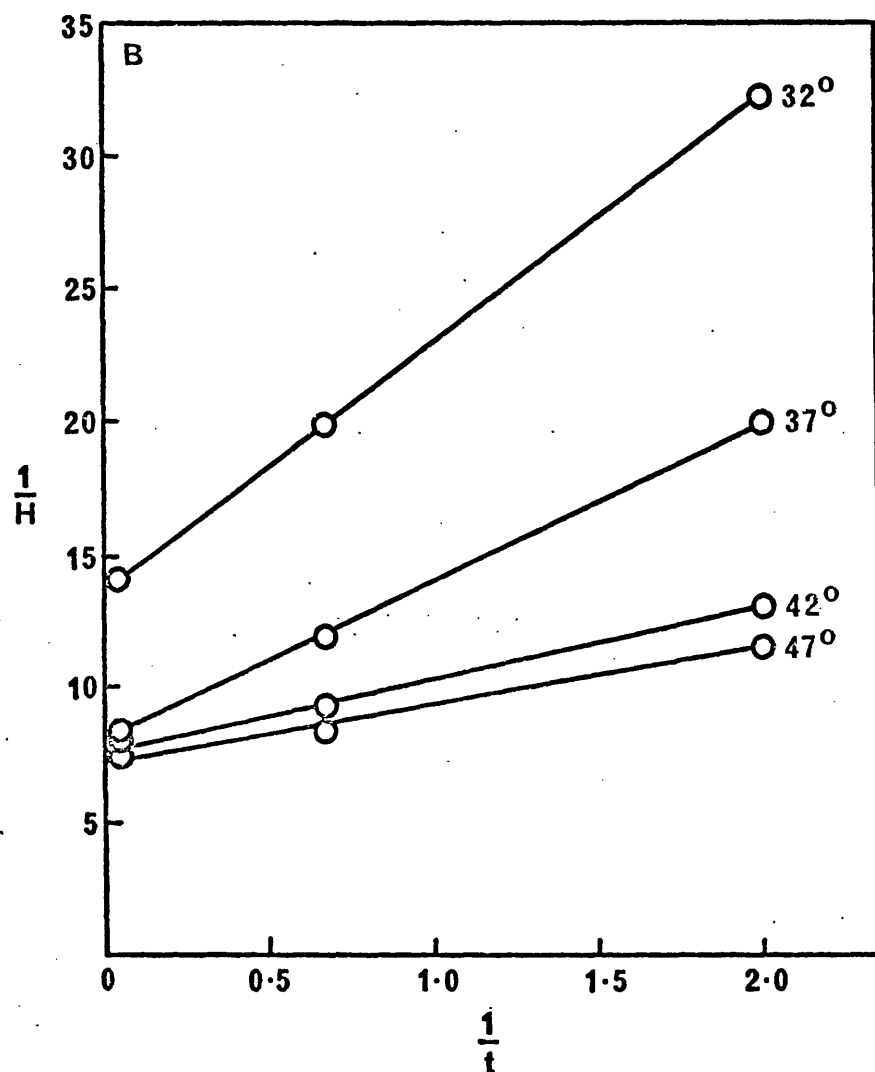


Table 1. Effect of temperature on hybrid formation.

Summary of apparent saturation values derived from Fig. 4B.

Annealing temperature	H _s (%)
32°	0.072
37°	0.123
42°	0.132
47°	0.139

sub-optimal annealing temperature, the need to check and optimise this parameter when studying a new system is obvious.

I.3.2 Melting temperature of Physarum rRNA•DNA hybrid.

It is relatively easy to optimise the amount of rRNA•DNA hybrid produced, by varying the annealing temperature. However, it is more difficult to obtain a quantitative measure of the fidelity of base pairing produced by a particular set of conditions. Nevertheless, some indication (although mainly of a qualitative nature) can be obtained from a study of the shape of the melting profile and $T_{m,i}$ (see footnote) of the rRNA•DNA hybrid, compared with those of native Physarum DNA, since the melting temperature is lowered by about 1°C for each 1.5% mispairing of bases (Laird, McConaughy & McCarthy [1969]).

Fig. 5. illustrates the melting curves of plasmodial rRNA•DNA hybrid and native DNA, both in 0.16M NaP_i-50% formamide buffer. The comparatively narrow temperature range over which the melting transition of the hybrid occurs would imply that the preparations are free from small "duplexes" of unstable hybrid formed between similar but far from identical base sequences, since these would appear as a shoulder on the lower part of the melting curve, due to their low stability. However, we can not rule out the possibility of a uniform distribution of mismatching within regions of accurate base pairing, since this would simply shift the entire

Footnote: T_m is the mean temperature of dissociation measured optically. i.e. the temperature by which 50% of the total hyperchromic shift has occurred.

$T_{m,i}$ is the mean temperature of strand separation. i.e. that temperature at which half of the duplex molecules have completely separated into their component single strands.

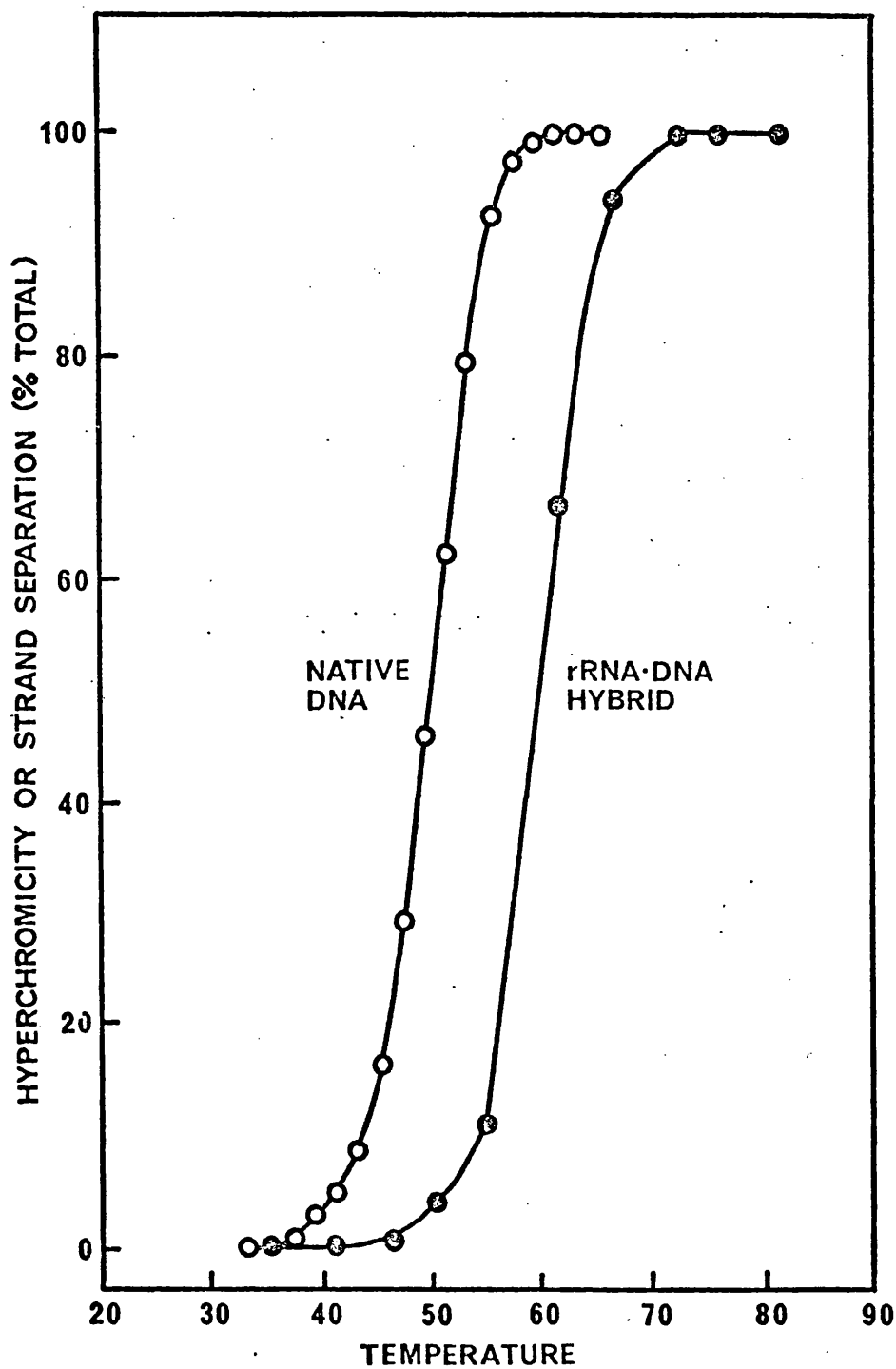


Fig. 5. Melting temperatures of native DNA and rRNA-DNA hybrid
in 0.16M NaP_i-50% formamide buffer. See Materials and Methods
 (sections I.2.14 and I.2.15). T_m of DNA = 50.5°C ; $T_{m,i}$ of hybrid
 = 60°C .

curve to a lower temperature.

It has been generally observed that in the presence of 50% formamide (Birnstiel, Sells & Purdom [1972]),

$$T_{m,i}(\text{hybrid}) - T_m(\text{rDNA}) = 2-6^{\circ}\text{C}.$$

Although we are not in a position to test this relationship directly in Physarum, since the T_m of rDNA is not readily obtainable, a reasonably accurate value can be predicted. Total nuclear DNA from this organism has a G + C content of 43% (Braun, Mittermayer & Rusch [1965]) whilst the rDNA has a value of 54% (Cummins, Weisfeld & Rusch [1966]). With the aid of the data presented by Birnstiel, Sells & Purdom [1972] it can be predicted that the rDNA from Physarum should have a T_m 5°C higher than the bulk DNA (based on the difference in G + C contents of these two DNA classes), i.e. 55.5°C under the conditions described in Fig. 5. Therefore:

$$T_{m,i}(\text{hybrid}) - T_m(\text{rDNA}) = 4.5^{\circ}\text{C}. \quad (\text{for } \underline{\text{Physarum}})$$

- a figure well within the range quoted above for other systems.

However, it must be emphasised that although this figure is useful for comparative purposes its physical significance is debatable. It does not necessarily mean that RNA•DNA hybrids are more stable than the corresponding DNA duplexes in the presence of 50% formamide (as is frequently stated in the literature) since strand separation is almost invariably used to determine melting temperatures of hybrids, whereas hyperchromicity is used for DNA duplexes. This is unfortunate since melting temperatures obtained by these two methods, designated $T_{m,i}$ and T_m respectively, may not be directly comparable. In fact Bolton & McCarthy [1964] have shown that the $T_{m,i}$ of Escherichia coli DNA on DNA-agar columns is about 4°C higher than the T_m of the same DNA duplex measured spectrophotometrically. Therefore, in the absence of similar data for DNA duplexes and RNA•DNA hybrids dissociated on hydroxyapatite columns (in the presence of formamide), we must assume that the two

methods may not be directly comparable. If the 4°C difference between the methods is found to apply to the present system, then:

$$T_{m,i}(\text{hybrid}) - T_{m,i}(\text{rDNA}) = 0$$

or in other words RNA•DNA hybrids and DNA•DNA duplexes have very similar stabilities in the presence of 50% formamide. In contrast, when formamide is absent RNA•DNA hybrids have a $5\text{--}10^{\circ}\text{C}$ lower melting temperature than the corresponding DNA duplexes (Birnstiel, Sells & Purdom [1972]; Chamberlin & Patterson [1965]; Walker [1969]). Whether this reflects a different destabilising effect by formamide on the two types of duplex, or if more accurately base paired hybrids are formed in its presence, is not known. However, a means of preferentially destabilising DNA duplexes during RNA-DNA hybridisation in solution is a valuable asset since it reduces DNA renaturation.

In conclusion, therefore, I believe that the lack of heterogeneity in the melting curve of the hybrid, the high $T_{m,i}$ of the hybrid, and its similarity to values obtained with other systems and hybridisation methods, are all strongly suggestive of a high degree of fidelity in base pairing within the hybrid, but are by no means definitive proof.

I.3.3 Proportion of nuclear DNA that codes for rRNA in Physarum.

The kinetics of hybridisation in reaction mixes containing $1\mu\text{g}$ of $[^{32}\text{P}]$ or $[^3\text{H}]$ -labelled plasmodial rRNA and $40\mu\text{g}$ of plasmodial DNA (strain CL) were measured during the first few hours of annealing, and the results are shown in the form of Scatchard plots in Fig. 6. The same batch of sonicated DNA was used in both cases. The excellent agreement between the two values suggests that the two preparations of rRNA were essentially free of labelled, non-RNA contaminants, since the likelihood of two quite different radioactive precursors (i.e. inorganic $[^{32}\text{P}]$ -phosphate and $[6\text{-}^3\text{H}]$ -uridine) giving the same labelling pattern in an impurity is

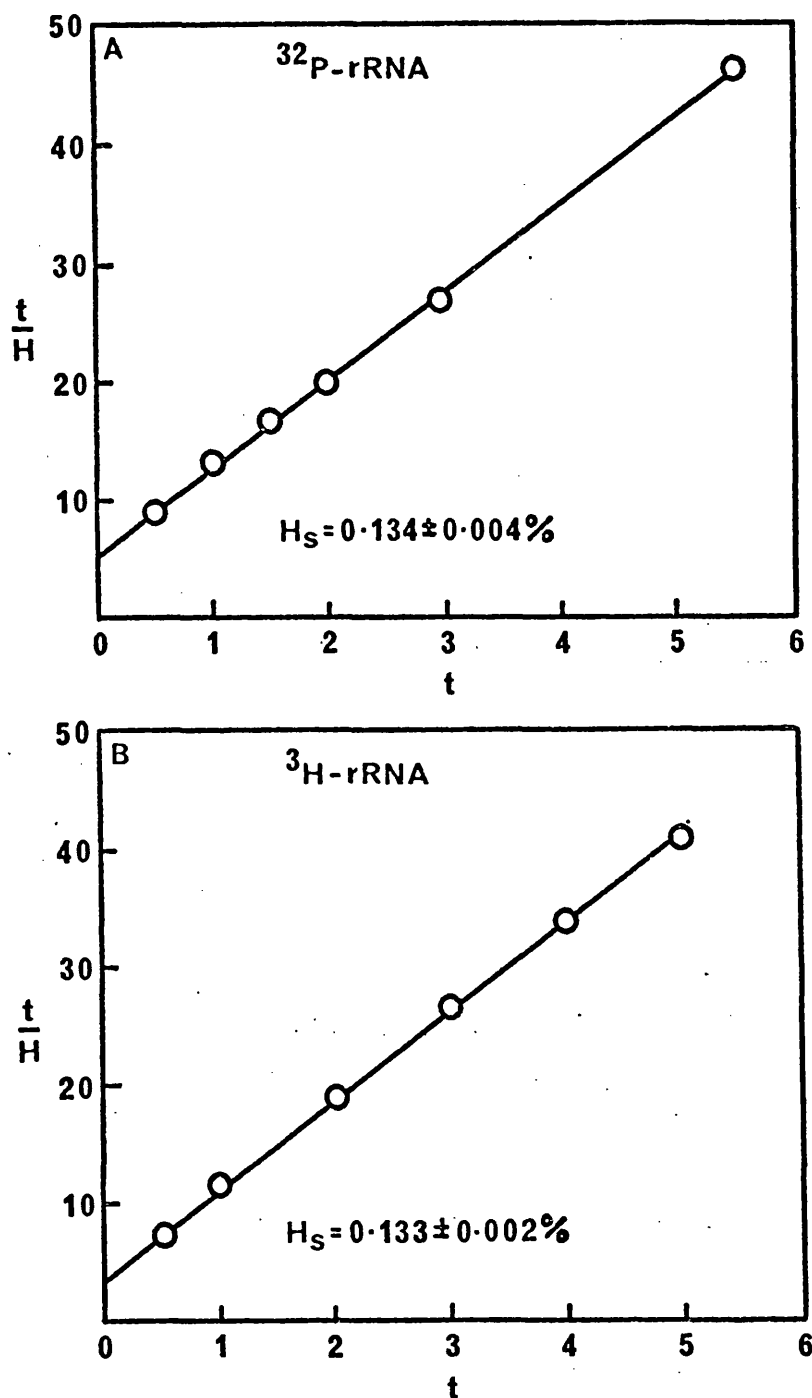


Fig. 6. Proportion of nuclear DNA that codes for rRNA in Physarum.

The kinetics of hybridisation were followed for about 5h in reaction mixes containing 1 μ g of [^{32}P] or [^3H]-rRNA and 40 μ g of DNA (strain CL).

Results are shown in the form of Scatchard plots. Each point is the mean of two determinations.

rather remote. This is particularly important since accurate determinations of saturation values are dependent upon reliable measurements of the specific radioactivity of the labelled species.

The saturation value obtained, 0.13%, represents an average value for the amount of rDNA, as the DNA was prepared from an asynchronous population of plasmodial nuclei. It agrees reasonably well with the saturation value of 0.10-0.11% obtained by Newlon, Sonenshein & Holt [1973], and with the more recent figure of 0.22% (a slightly high value since it refers to DNA isolated from plasmodia in late G2 phase) obtained in Braun's laboratory (Ryser & Braun [1974]). (An earlier report from Braun's laboratory had suggested a value of 1.3%.)

A value of 0.134% gives an average of 260 copies of the ribosomal RNA genes per CL nucleus assuming a nuclear DNA content of 0.71pg (Mohberg, personal communication)

I.3.4 Competitive hybridisation between plasmodial rRNA and amoebal rRNA.

Competitive hybridisation between two RNA species and a single DNA species does not rely on the reaction going to completion and can therefore be successfully studied by either: (a) allowing hybrid mixes to reach an equilibrium by having a long annealing period, or (b) by following the kinetics of hybrid formation, if the two RNA species are of a similar size. If the sizes vary significantly only the kinetic approach can be employed, since the larger RNA species can gradually replace the smaller species in the RNA-DNA hybrids, if the annealing period is long, by forming larger and consequently more stable hybrids (Beckmann & Daniel [1974]). However, the rRNAs of amoebae and plasmodia of Physarum are not likely to differ much in size so both methods have been applied.

It was necessary in the following experiments to use axenically grown amoebae as a source of amoebal rRNA, as it would probably be

impossible to isolate pure amoebal rRNA from cells grown on a bacterial lawn in the conventional manner (Dee [1962]). Admittedly a large proportion of the bacteria could be removed by differential or density gradient centrifugation. However, a lot of bacteria in various stages of digestion can be seen within amoebae grown on bacteria, and these would contaminate amoebal rRNA preparations with partially degraded, heterogeneously sized bacterial RNA. As a result accurate determination of the amoebal rRNA concentration (essential if competition experiments are to be quantitative) would be impossible.

It was therefore necessary to use amoebae of strain RSD₄ (Mohberg & Rusch [1971]) instead of CL in this particular study, since this was the only axenically grown strain available at the time. However, one would not expect the rRNA to vary from strain to strain.

(i) Hybridisation to equilibrium. If two RNA species, one of which is radioactively labelled, are hybridised to DNA and do not compete for the same site, then the amount of labelled hybrid formed is constant (i.e. independent of the unlabelled RNA concentration) and is equal to the saturation level. If, on the other hand, the two RNA species compete for the same site on the DNA, then the amount of labelled hybrid formed will decrease as the concentration of the competing species increases, from the saturation value in the absence of competitor to zero at infinite competitor concentration.

Duplicate reaction mixes containing 3µg of plasmodial [³H]-rRNA, 16µg of plasmodial DNA (strain CL) and varying amounts of amoebal rRNA up to 21µg were hybridised for 60h. As published competition curves sometimes deviate from ideal behavior at high competitor RNA concentrations, control reaction mixes containing unlabelled plasmodial rRNA or E. coli rRNA in place of the amoebal rRNA, were also analysed. However, the results illustrated in Fig. 7. clearly show that with this system these

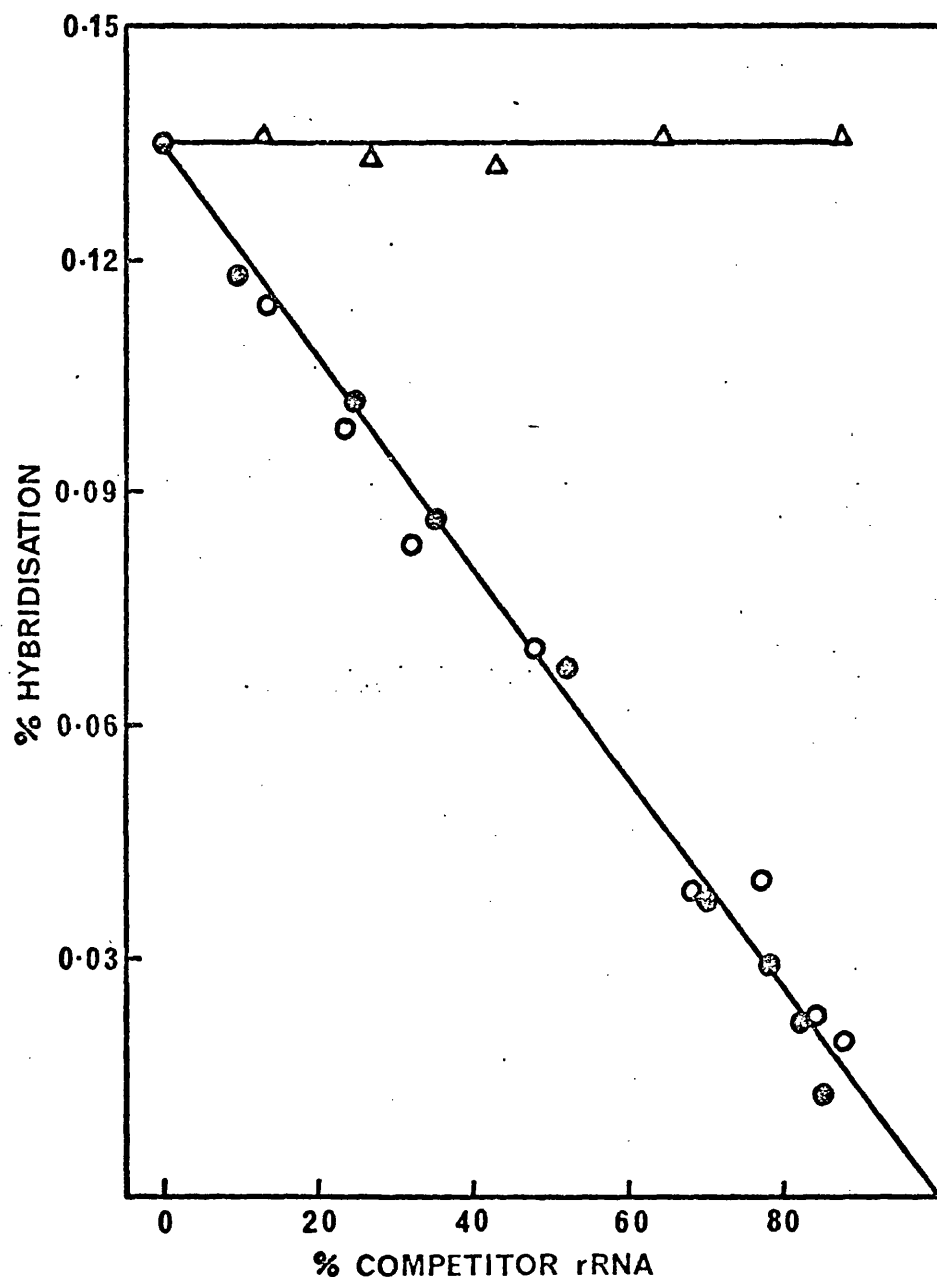


Fig. 7. Competition between plasmodial rRNA and amoebal rRNA for hybridisation to homologous DNA. I. Hybridisation to equilibrium.

Mixes containing 3 μ g of plasmodial [3 H]-rRNA, 16 μ g of plasmodial DNA and varying amounts of (a) amoebal rRNA (O), (b) plasmodial rRNA (●), and (c) *E. coli* rRNA (Δ) were hybridised for 60h; and the amount of [3 H]-labelled hybrid formed was determined in each case. Each point is the mean of two determinations.

two controls behave in the predicted manner over the entire competitor concentration range; least squares analyses of the data giving values of $102\% \pm 2\%$ for competition between unlabelled plasmodial rRNA and plasmodial [^3H]-rRNA, and $1.5\% \pm 3\%$ for competition between E. coli rRNA and plasmodial [^3H]-rRNA.

Complete competition ($99\% \pm 2\%$) was clearly obtained between amoebal rRNA and plasmodial [^3H]-rRNA, strongly suggesting that the RNAs from these two vegetative stages of the life cycle exhibit a very high degree of homology.

(ii) Kinetics of hybrid formation in the presence of competitor.

As an alternative method, the kinetics of hybrid formation between plasmodial [^3H]-rRNA and plasmodial DNA were followed during the first 5h of annealing, in the absence of competitor and in the presence of two different concentrations of amoebal rRNA, as shown in Fig. 8. The decrease in the amount of labelled hybrid formed (or "apparent saturation value"; derived from the slopes of the Scatchard plots) with increase in competitor RNA concentration indicates that the two rRNAs are competing for the same site on the DNA.

For complete (100%) competition, the experimentally observed "apparent saturation values" should be proportional to the percentage of labelled rRNA in the different reaction mixes, that is:

$$\frac{\text{apparent } H_s}{\text{percentage labelled rRNA.}} = \text{constant.}$$

It can be seen from Table 2. that within the standard errors of the measurements, this relationship is valid.

Both of the above methods for studying competition between amoebal rRNA and plasmodial rRNA clearly show that there is a very high degree of homology between the rRNAs from the two vegetative forms of Physarum polycephalum. This result strongly suggests that the rRNAs are transcribed

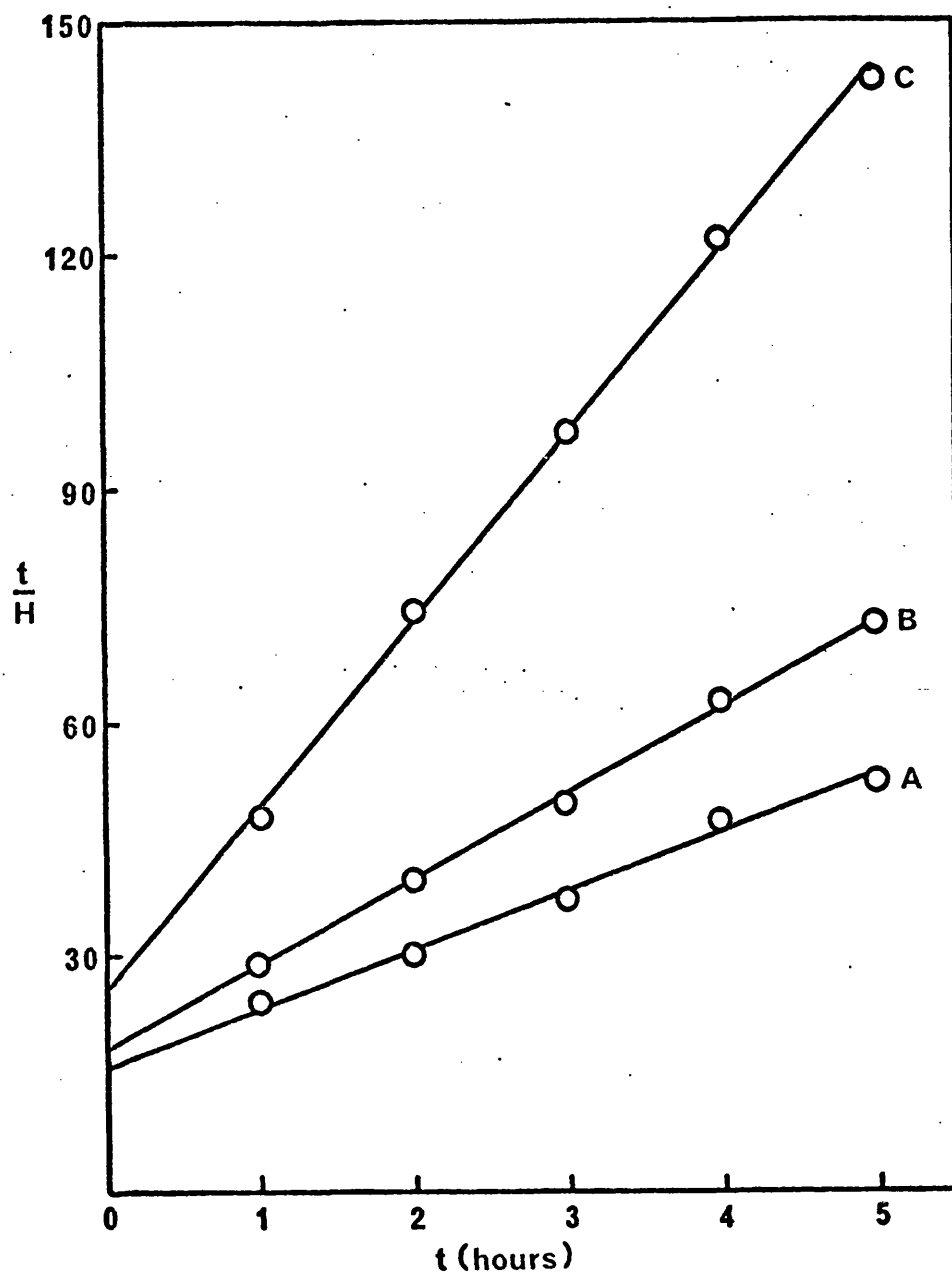


Fig. 8. Competition between plasmodial rRNA and amoebal rRNA for hybridisation to homologous DNA. II. Kinetics of hybrid formation.

The kinetics of hybridisation were followed in mixes containing 32.9 μ g of DNA, 0.93 μ g of plasmodial [3 H]-rRNA and 0, 0.40 or 1.79 μ g of amoebal rRNA. Each point is the mean of two determinations.

A. 100% plasmodial rRNA. B. 70% plasmodial rRNA, 30% amoebal rRNA.

C. 34% plasmodial rRNA, 66% amoebal rRNA.

Table 2. Competition between plasmodial rRNA and amoebal rRNA for hybridisation to homologous DNA.

Apparent saturation values ($\pm$ standard error) were calculated from the data presented in Fig. 8. and related to the percentage of labelled (plasmodial) rRNA in the reaction mixes as described in section I.3.4.

Observed H_s	Labelled rRNA	$A/B \times 10^4$
% (A)	% (B)	
0.130 ± 0.007	100	13.0 ± 0.7
0.089 ± 0.003	70	12.7 ± 0.4
0.042 ± 0.001	34	12.4 ± 0.3

from the same set of genes in these two, quite different stages of the life cycle.

It is important, however, to emphasize that 100% competition between two RNA species is not necessarily a definitive indication of complete homology, particularly when studying eucaryotic systems. Even after optimising annealing conditions to minimise cross-reacting (see section I.3.1) there will still be a limit beyond which sequences will be sufficiently similar to compete. Perhaps a useful extension to the competition work would be to compare melting temperatures of rRNA·DNA hybrids formed between plasmodial DNA and plasmodial rRNA (as described in section I.2.14) with those between plasmodial DNA and amoebal rRNA, under the same conditions. A small degree of heterology, low enough to allow cross-reaction, may then be reflected in a slight $T_{m,i}$ difference within the hybrid. In view of recent improvements in the development of a completely defined axenic growth medium for amoebae (Henney, Asgari & Henney [1974]; Henney & Asgari [1975]), without the need for beef embryo extract and liver infusion, it is probably feasible now to obtain amoebal rRNA of a sufficiently high specific radioactivity to carry out the above experiment. Unfortunately at the time when the competition experiments were done this was not the case.

An entirely different approach which may be used to test for homology between rRNA species is to examine the pattern of methylation in the mature rRNAs. This involves pulse labelling with radioactively labelled methionine, ribonuclease T_1 digestion of the isolated rRNA, and finally two dimensional chromatography and autoradiography of the oligonucleotides (Sanger, Brownlee & Barell [1965]). A difference in the labelling pattern of the rRNAs would be indicative of either a difference in the post-transcriptional processing of the two RNA species, or more likely, a difference in the genes for rRNA. This method is dependent on

specifically labelling the methyl groups of RNA, since any catabolism of the labelled methionine and subsequent incorporation into nucleosides would increase the background on the autoradiographs rendering them uninterpretable. Unfortunately it is doubtful whether such a high degree of specificity is readily achieved in Physarum; an organism which is capable of substantial catabolic activity.

I therefore believe that further confirmation of the homology between plasmodial and amoebal rRNA would be both difficult and time-consuming and in view of the competition results obtained, I do not think that the extra work involved would be very worthwhile.

Finally, Haugli, Dove & Jimenez [1972] have recently isolated eight cycloheximide-resistant mutants in amoebae of Physarum polycephalum after treatment with UV and caffeine (Haugli & Dove [1972]) or with N-methyl-N'-nitro-N-nitrosoguanidine (NMG), and all were found to be expressed in the plasmodium. Two unlinked loci, actA and actB were shown to be responsible for cycloheximide resistance. Ribosomes from actA mutants, when incubated in an in vitro polypeptide synthesising system, were resistant to cycloheximide, while ribosomes from wild type or actB mutants were not. Clearly, the actA locus operates through the ribosomes and, since it is expressed in both the amoebal and plasmodial stages of the life cycle, it would suggest that the ribosomes in these two stages may be identical.

I.3.5 rDNA in strains of Physarum polycephalum with different ploidy.

As outlined in the introduction, it was considered to be of interest to determine whether the amount of rDNA per nucleus is regulated independently of the remainder of the genome, by measuring the number of rRNA genes in strains of different ploidy.

DNA was prepared from nuclei, isolated in early prophase, from large

surface plasmodia of strains CL, M₃CVIII and RSD5xRSD2 that encompass a 5-fold range of nuclear DNA contents (Mohberg, Babcock, Haugli & Rusch [1973]; Mohberg, personal communication). The proportion of rDNA in each preparation was determined from the kinetics of hybridisation with plasmodial [³H]-rRNA (from strain CL in all cases), and the results (Fig. 9. and Table 3.) clearly show that the saturation values do not vary with ploidy. The final column in Table 3. gives the calculated number of rRNA genes per late G2 nucleus, i.e. after replication of both the nucleoplasmic and nucleolar DNA.

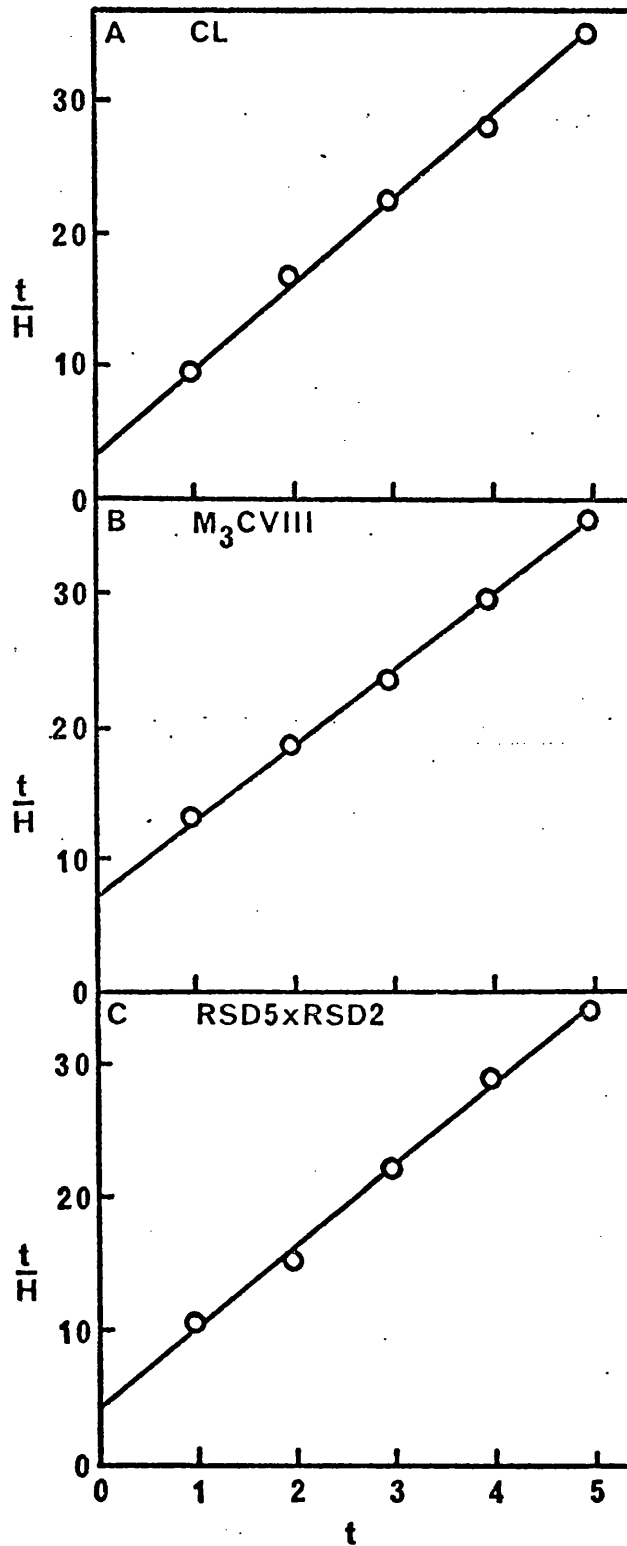


Fig. 9. rDNA in strains of different ploidy. The kinetics of hybrid formation between $1\mu\text{g}$ of $[^3\text{H}]$ -rRNA and $20\mu\text{g}$ of DNA, prepared from prophase nuclei of plasmodial strains CL, M_3 CVIII and RSD5xRSD2; were followed during the first 5h of annealing. Each point is the mean of two determinations.

Table 3. Ribosomal DNA in strains of different ploidy.

DNA was prepared from prophase nuclei isolated from plasmodia of strains CL, M₃CVIII and RSD5xRSD2, and the amount of ribosomal DNA determined from the kinetics of hybridisation with plasmodial [³H]-rRNA as presented in Fig. 9.

Strain	Nuclear DNA content	Ribosomal DNA content	
	pg	%	No. of genes
CL	0.71 ± 0.01	0.162 ± 0.004	320
M ₃ CVIII	1.23 ± 0.08	0.176 ± 0.004	600
RSD5xRSD2	3.64 ± 0.19	0.164 ± 0.005	1660

I.4 GENERAL DISCUSSION OF THE CONTROL OF rDNA REDUNDANCY.

I.4.1 Control of rDNA redundancy in other organisms.

The rRNA genes are almost universally present as multiple copies, there being approximately 5-10 copies/genome in bacteria, 100 to several hundred copies/haploid genome in lower eucaryotic cells and animal cells, and greater than 1000 copies in higher plants (see Table 4.). There appears to be no direct correlation between the rDNA content and the size of the genome in eucaryotic cells.

In all reported cases, the rRNA genes are clustered; in the case of eucaryotic cells, at the nucleolar organiser (i.e. the DNA associated with the nucleolus). The most obvious explanation for this high degree of redundancy is that more than one gene is required to maintain the rate of rRNA synthesis needed for cell growth and differentiation. The gene activity in Escherichia coli has been calculated from the rRNA chain growth rate and the values obtained indicate that at maximum growth rates (e.g. 3 doublings/h) the rRNA genes become almost saturated with RNA polymerase molecules. (Dennis & Bremer [1973]). Similar calculations in HeLa cells have indicated that at least 100 rRNA genes would have to be continuously transcribed at maximum rate during the 24h cell generation time to achieve a doubling of rRNA (Attardi & Amaldi [1970]). It is therefore clear that at least some degree of redundancy in the rRNA genes is required to meet the cell's requirements for rRNA synthesis. To test this idea further we must look for examples where the number of rRNA genes is reduced and see if this has any effect on the phenotype, and also look for systems with a high rRNA synthetic capacity to see if they also have an increased rDNA content.

Perhaps the best known example of a decrease in rDNA redundancy is in the "bobbed" mutants of Drosophila. In this organism the rRNA genes

Table 4. Size of genome and number of rRNA genes in procaryotic and eucaryotic cells.

Organism.	Number of rRNA genes.	Size of genome (haploid).	Reference.
<i>Escherichia coli</i>	6	2.8×10^9	Spadari & Ritossa [1970]
<i>Bacillus subtilis</i>	9-10	3.9×10^9	Smith, Dubnau, Morell & Marmur [1968]
<i>Saccharomyces cerevisiae</i>	140	1.25×10^{10}	Schweizer, MacKechnie & Halvorson [1969]
<i>Neurospora crassa</i>	125	2.4×10^{10}	Wood & Luck [1969]
<i>Tetrahymena pyriformis</i>	170	8.25×10^{12}	Engberg & Pearlman [1972]
<i>Physarum polycephalum</i> CL	260	4.3×10^{11}	See section I.3.3
<i>Ascaris lumbricoides</i>	255	2.8×10^{11}	Tobler, Zulauf & Kuhn [1974]
<i>Drosophila melanogaster</i>	150	1.2×10^{11}	Graziani, Boncinelli, Malva & Gargano [1974]
Chick	100	7.2×10^{11}	Ritossa, Atwood, Lindsley & Spiegelman [1966]
<i>Xenopus laevis</i>	450	1.8×10^{12}	Brown & Weber [1968]
HeLa cells	280	3.1×10^{12}	Jeanteur & Attardi [1969]
<i>Vicia sativa</i>	3750	2.4×10^{12}	Maher & Fox [1973]
Tobacco	3450	3×10^{12}	Tewari & Wildman [1968]
Flax (large stable form)	3500	0.9×10^{12}	Timmis & Ingle [1973]
Maize (<i>Zea mays</i>)	3100	3.8×10^{12}	Ingle & Sinclair [1972]
Wheat (<i>Triticum vulgare</i>)	2100	5×10^{12}	Ingle & Sinclair [1972]
Cucumber (<i>Cucumis sativum</i>)	4400	1×10^{12}	Ingle & Sinclair [1972]
Pea (<i>Pisum sativum</i>)	3900	5×10^{12}	Ingle & Sinclair [1972]
Onion (<i>Allium cepa</i>)	6650	16×10^{12}	Ingle & Sinclair [1972]

are repeated about 150 times in the nucleolar organiser of wild type stocks, and are located at the bobbed (bb) locus. Wild bobbed (bb⁺) is that locus which, even without a partner, gives rise to a normal phenotype. The locus which in similar conditions is incapable of giving rise to a normal phenotype is called a bobbed mutation (bb) and contains a reduced number of rRNA genes (Ritossa, Atwood & Spiegelman [1966]). If a bobbed mutant is kept associated with a wild type locus (Xbb/Ybb⁺), the bb locus is stable and the flies do not show any adverse effects as a result of the reduced rDNA content. If, however, a strongly bobbed phenotype is made (Xbb/Ybb⁻) the flies exhibit slow development, short bristles, low viability, and low fecundity; a phenotype which is suggestive of a defect in the protein-synthesising machinery (Attardi & Amaldi [1970]). It would seem that transcriptional activity is no longer adequate with this greatly reduced number of rRNA genes. However, such a mutation does not perpetuate; instead the bb locus becomes "magnified" (bb^m) and increases its rDNA content to yield phenotypically normal individuals (Ritossa [1968]; Boncinelli, Graziani, Polito, Malva & Ritossa [1972]). This newly magnified bb^m locus becomes stable only if it remains in combination with the Ybb⁻ chromosome (Ritossa [1968]). If instead it is associated with a wild bobbed (bb⁺) locus its rDNA content decreases immediately, and this decrease is a function of the rDNA contributed by the partner locus (Henderson & Ritossa [1970]; Malva, Graziani, Boncinelli, Polito & Ritossa [1972]).

We therefore have a situation where the organism can tolerate a loss of some of its rDNA, but if this becomes excessive, rRNA transcriptional activity is seriously impaired and a compensatory mechanism becomes operative to regenerate a normal phenotype.

A second example where a reduction in the number of rRNA genes may be responsible for a change in phenotype can be seen in flax. Heritable

changes in the weight of this plant can be induced according to the fertilizers applied, to produce a large stable form (L) or a small stable form (S) (Durrant [1962]; Durrant [1971]), the former containing 16% more DNA than the latter (Durrant & Jones [1971]). Timmis & Ingle [1973] have shown that the L form contains 47-63% more rDNA than the S form and suggest that the four fold difference in plant weight may be due to this difference in rDNA content. The redundancy of the rRNA genes in flax is relatively low compared with other plant species (Ingle & Sinclair [1972]) and may therefore be rather sensitive to a loss of even a small amount of rDNA.

In the case of flax, the small form is stable and no compensatory mechanism has evolved to overcome the rDNA deficiency. However, in another plant species, wheat, such a mechanism has evolved. Four different chromosomes, namely 1A, 1B, 6B and 5D, of hexaploid Triticum aestivum are capable of forming nucleoli under suitable conditions (Crosby [1957]), and these chromosomes contain the rRNA genes (Mohan & Flavell [1974]; Flavell & Smith [1974(a)]; Flavell & Smith [1974(b)]). However, the amount of rDNA/nucleolus organiser is not constant, as shown by substitution, one at a time, of each of the above four chromosomes into a common genetic background (Flavell & Smith [1974(b)]). Also, doubling the number of 1B chromosomes increases the rDNA content by 31-49% (indicating that 1B contains 31-49% of the rRNA genes), but deleting the 1B chromosomes decreased the number by only 15-23% (Flavell & Smith [1974(a)]). This suggests that changes may occur in rRNA gene multiplicity at other nucleolus organisers to partially compensate for a deficiency of rDNA.

The above are examples where the rRNA gene redundancy has been reduced artificially (by deletions etc.) and they seem to have deleterious effects on the organism (if a compensatory mechanism is not devised). However, there are cases where the rRNA gene redundancy is reduced

"naturally", without any apparent adverse effect on the organism; although the reason for such a reduction is quite obscure.

The first example is polytenisation of the salivary gland of Drosophila, during which euchromatin undergoes a series of DNA replications resulting in as many as 1024 DNA fibres (Swift & Rasch [1954]); the heterochromatin clumps together to form the chromocentre and replicates little or not at all (Gall, Cohen & Polan [1971]; Rudkin [1969]), and the rRNA genes (at the bobbed locus) replicate to some extent, but not as much as the euchromatin (Rodman [1969]; Nash & Plaut [1965]). The relative amount of rDNA therefore decreases during polytenisation (Hennig & Meer [1971]; Spear & Gall [1973]).

A second example of preferential loss of rDNA is demonstrated in Nicotiana species with different ploidy levels. (N.B. In this context we are loosely calling polytenisation a "natural" event, as it is a much different situation to the artificially induced deletions or mutations described above.) Siegel, Lightfoot, Ward & Keener [1973] have shown that in ten Nicotiana species the proportion of rDNA varied from 0.27% to 0.9%, with tetraploid species having lower values (0.27-0.43%) than the diploid species (0.67-0.9%). Therefore the absolute number of rRNA genes in these species varies much less than the total DNA content. However, in another plant species, Datura innoxia, where a polyploid series (haploid to hexaploid) was produced by in vitro culture of pollen grains, the proportion of rDNA was shown to be constant, i.e. the number of rRNA genes increased as the ploidy increased (Cullis & Davies [1974]). A possible explanation for the difference between Datura and Nicotiana (suggested by Cullis & Davies [1974] and Siegel, Lightfoot, Ward & Keener [1973]) is that a mechanism for regulating the amount of rDNA only evolves after polyploid formation and is therefore not present in the newly formed polyploids of Datura. The fact that the tetraploid Nicotiana

species have arisen as amphidiploids (Goodspeed [1954]) further supports this.

If rRNA gene redundancy is necessary for adequate transcriptional activity, we might expect to see an increase in multiplicity in situations where a high rate of rRNA synthesis is required. Perhaps the best known example of this is amplification during oogenesis in amphibia. Somatic cells of amphibia normally contain one nucleolus per haploid chromosome complement. Each nucleolus is derived from a nucleolar organiser region which, in the case of Xenopus laevis contains a cluster of 450 rRNA genes (Brown & Weber [1968]). However, over a period of about 20 days during the extended pachytene of early oogenesis, this rDNA is amplified to such an extent that it constitutes about 70% of the total cell DNA, necessitating eleven rounds of replication (Perkowska, Macgregor & Birnstiel [1968]). This leads to a multinucleolate condition (about 1000 nucleoli/nucleus) (Gall [1968]; Macgregor [1965]; Callan [1966]), each nucleolus being extra-chromosomal, containing rDNA (Brown & Dawid [1968]) and capable of autonomous rRNA synthesis. rRNA and consequently ribosomes are synthesised and accumulated throughout oogenesis until the egg is mature and ready for ovulation. At the first meiotic reduction division, rRNA synthesis ceases and the extra-chromosomal DNA is discarded into the cytoplasm where it is either degraded or diluted out. rRNA synthesis does not recommence until the onset of gastrulation (Brown & Littna [1964]) at which stage the expected diploid number of two nucleoli appears for the first time during embryogenesis.

Admittedly amplification is a clear example of a very high degree of rDNA redundancy being associated with a high transcriptional activity, but are the two really linked? The presence of multiple nucleoli during oogenesis in amphibia is somewhat exceptional, and the oocytes of most animals contain a single prominent nucleolus. In the oocytes of the

echiuroid worm Urechis caupo, and the surf clam Spisula solidissima, only a five fold "amplification" of rDNA occurs (Brown & Dawid [1968]), considerably less than in amphibian oocytes. A second hypothesis has therefore been suggested by Brown & Dawid [1968] to explain the need for amplification in amphibia, and is based on the observation that oocytes store large numbers of ribosomes. In bacteria ribosome production is a function of the rate of protein synthesis (Neidhardt [1964]; Maalqé & Kjeldgaard [1966]) so that active monosomes do not accumulate (Morris & De Moss [1966]); and somatic cells of higher organisms appear to regulate ribosome synthesis in a similar way. It has therefore been suggested that the production of extra-chromosomal rDNA may be a way of escaping these control mechanisms to enable the oocytes to store ribosomes for use later during embryogenesis.

Finally, Tetrahymena illustrates in a much less dramatic way, the way in which rRNA gene redundancy may alter with transcriptional activity. Exponentially growing cells contain about 170 rRNA genes/haploid genome. However, this figure is reduced by 30-40% in starved or stationary growth phase cells (Engberg & Pearlman [1972]). Furthermore, when starved cells are shifted to nutrient medium, a preferential initiation of rDNA replication is induced (Engberg, Mowat & Pearlman [1972]). However, the increase is small and is not likely to solely account for the subsequent rapid synthesis of rRNA in response to the nutritional shift-up.

In conclusion, therefore, very few generalisations can be made with regard to rRNA gene redundancy and its control. In some examples relatively large decreases in multiplicity may be made without much effect on the phenotype, while in other cases the consequences are much more serious and compensatory mechanisms have often evolved to ensure survival. Perhaps one of the most important observations is that in many of the systems examined, the replication of bulk nuclear DNA and rDNA would

appear to be independently controlled with the result that the genomic proportion of rDNA may vary under different conditions.

I.4.2 Control of rDNA redundancy in *Physarum polycephalum*.

The relationship between the amount of rDNA and ploidy has been investigated in three strains of *Physarum polycephalum* which encompass a five-fold variation in the amount of DNA per nucleus. The proportion of rDNA was found to be constant at 0.16-0.18% of the total genome in the three strains; a result analogous to that obtained in a polyploid series of plants of *Datura innoxia* (Cullis & Davies [1974]). It would therefore appear that the replication of rDNA and bulk nuclear DNA are co-ordinately controlled during polyploid formation. It was argued in the previous section that the difference between *Datura* and *Nicotiana* (where the proportion of rDNA did vary with ploidy: Siegel, Lightfoot, Ward & Keener [1973]) may suggest that a mechanism for regulating the amount of rDNA only evolves after polyploidisation and is therefore not present in the newly formed polyploids of *Datura*. The RSD5xRSD2 strain of *Physarum polycephalum* is a relatively "new" polyploid. It was formed in 1969, but has been kept as spherules until very recently. The actual subline I used had therefore only been growing in culture for 2-3 months. Furthermore, the polyploid strain RSD5xRSD2 was produced spontaneously by mating haploid amoebae and was not derived from diploid cultures. It therefore has more in common with *Datura* than with the much older *Nicotiana* polyploids which have arisen as amphidiploids (Goodspeed [1954]). However, whether differences in the age and derivation of a strain have any bearing on the rRNA gene redundancy is still a matter of conjecture.

Further evidence that the replication of rDNA and bulk nuclear DNA in *Physarum* would appear to be co-ordinately controlled has been obtained by Ryser & Braun [1974], who showed that there was no difference in the

amount of rDNA in growing and starved plasmodia. It is clear that there is no additional rDNA synthesis during favourable growth conditions, as was observed in Tetrahymena (Engberg & Pearlman [1972]).

Therefore, although the control systems for the replication of rDNA and nucleoplasmic DNA in Physarum polycephalum would appear to be independent, since (a) the replication of rDNA during the mitotic cycle of plasmodia occurs both during and after the replication of the rest of the nuclear DNA, and (b) cycloheximide strongly inhibits the synthesis of nuclear DNA, but only slightly that of nucleolar DNA (Werry & Wanka [1972]); nevertheless it appears that these two control systems are co-ordinately linked, unlike many of the other systems discussed in the previous section.

The only situation in Physarum in which there may be an over-production of rDNA has recently been reported by Guttess [1974]. However, this was a rather abnormal situation involving transplantation of late interphase, highly polyploid nuclei into early interphase host plasmodia containing small nuclei. The nucleolar DNA of the polyploid nuclei which had been allowed to bypass mitosis continued to incorporate label from [³H]-thymidine (as observed by autoradiography), along with the nuclei of the recipient, until they entered prophase one mitotic period later. However, it must be emphasised that distinction between rDNA and the remainder of the nucleolar DNA was not possible in these experiments. All these experiments really suggest is that nucleolar and nucleoplasmic DNA replication are separately controlled. No conclusions about the way in which these two control systems may be linked under normal conditions can be made from what is clearly an unnatural system.

In conclusion, therefore, there is strong evidence to suggest that the control mechanisms for the replication of rDNA and bulk nuclear DNA are co-ordinately linked. The inability to vary the amount of rDNA under

different conditions indicates that rRNA synthesis is not controlled solely by the number of genes from which it is transcribed. Further evidence to support this is presented in Part II where the rate of rRNA synthesis during the mitotic cycle is related to the rDNA content.

PART TWO.

II.1 INTRODUCTION.

II.1.1 Summary of published work on RNA synthesis during the mitotic cycle in *Physarum polycephalum*.

A great deal of information about the regulation of RNA synthesis can be obtained by studying organisms or cultures of cells in which the events of the cell cycle occur in synchrony. It is therefore hardly surprising that research in this area has been pursued in *Physarum polycephalum*, an organism which possesses a very high degree of natural synchrony.

The first attempt at studying RNA synthesis during the mitotic cycle in *Physarum* was carried out by Nygaard, Guttus & Rusch [1960], who followed the incorporation of short pulses of [6-¹⁴C]-orotic acid into RNA pyrimidines during the mitotic cycle. They concluded that RNA is synthesised continuously throughout interphase but is markedly depressed during division and early S phase. A more detailed study of RNA synthesis during and immediately after mitosis was subsequently carried out by Kessler [1967] using short pulses of [³H]-uridine and observing the incorporation into RNA by autoradiography. RNA synthesis was readily observed during early prophase, but rapidly decreased to an undetectable level during metaphase and anaphase. Both RNA and DNA synthesis were resumed immediately after anaphase.

The pattern of total RNA synthesis during the whole mitotic cycle has been determined by measuring the rate of incorporation of short pulses of [³H]-uridine (Mittermayer, Braun & Rusch [1964]; see Fig. 10.) and is unusual in that it suggests RNA synthesis is depressed not only during nuclear division, but also during the early part of the G2 phase. A similar

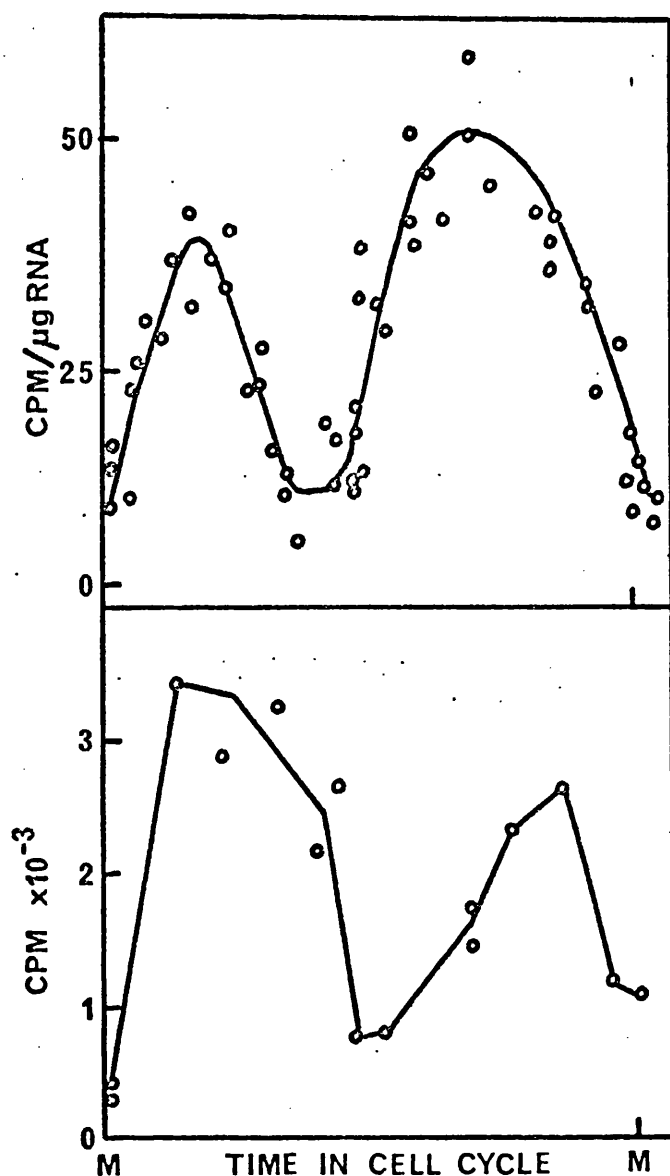


Fig. 10. Rates of RNA synthesis during the mitotic cycle in intact plasmodia and isolated nuclei. Upper figure: Incorporation of 10min pulses of [³H]-uridine into the RNA of intact cells during the mitotic cycle; each point being derived from a single whole plasmodium (from Mittermayer, Braun & Rusch [1964]). Lower figure: In vitro incorporation of 10min pulses of [³H]-UTP into RNA of nuclei, isolated at different times during the mitotic cycle (from Mittermayer, Braun & Rusch [1966]).

biphasic pattern of RNA synthesis, although less pronounced, has been demonstrated during in vitro studies with isolated nuclei (Mittermayer, Braun & Rusch [1966]; Grant [1972]; see Fig. 10.). As this biphasic pattern refers to total RNA synthesis, several attempts have been made, both in intact cells and in isolated nuclei, to see whether the two peaks of transcriptional activity reflect the syntheses of different classes of RNA, and there is a great deal of evidence which suggests that differential transcription during the mitotic cycle does occur (In intact cells: Mittermayer, Braun & Rusch [1964]; Fouquet & Braun [1974]; Fouquet, Bohme, Wick, Sauer & Braun [1974]; Cummins, Weisfeld & Rusch [1966]. In isolated nuclei: Grant [1972]; Cummins & Rusch [1967]; Cummins [1969]). In general, the G2 peak of transcriptional activity is mainly due to nucleolar RNA synthesis (i.e. rRNA synthesis) whereas the S peak contains a substantial proportion of both nucleolar and nucleoplasmic RNA synthesis.

In conclusion, therefore, RNA synthesis in Physarum polycephalum is continuous throughout the mitotic cycle except for a very short period during metaphase and anaphase. It has been suggested that the rate of synthesis fluctuates in a biphasic manner and there is some evidence that the two peaks may reflect different classes of RNA synthesis. Unfortunately this biphasic pattern of synthesis has only been demonstrated by pulse-labelling, a method which is based on many assumptions, some of which have not been tested and may well be invalid. The indiscriminate application of pulse-labelling techniques has frequently produced completely misleading results (see section II.1.3). Therefore, in the absence of independent data on the synthesis of RNA, such results should be viewed with extreme caution.

II.1.2 Aims and reasons for present work.

Current published data on the synthesis of RNA during the mitotic

cycle in Physarum polycephalum only relate to total RNA synthesis. Whilst this gives an insight into the overall pattern of gene transcription, it yields very little information about the synthesis of individual RNA species, and their control mechanisms. I therefore decided to investigate the way in which a single RNA species, namely rRNA, is synthesised during the mitotic cycle, and in particular see whether rRNA gene dosage has any effect on the rate of rRNA synthesis. If the number of rRNA genes is a rate determining element in the regulation of rRNA synthesis, then one would expect to observe a two-fold increase in the rate following gene replication. Alternatively, synthesis might be unaffected by gene dosage and instead be limited by the activity of cytoplasmic elements, perhaps by a feedback mechanism at the level of transcription, or by selective degradation of newly synthesised rRNA. However, a cytoplasmic control system could, but need not necessarily, nullify any gene dosage effect if both are operative. It was suggested at the end of Part I that the lack of variation in the amount of rDNA in Physarum, under different conditions is a strong indication that rRNA synthesis is not controlled solely by the number of genes from which it is transcribed. Therefore, a study of the pattern of rRNA synthesis during the mitotic cycle, in relation to rDNA replication, provides an ideal opportunity to test this hypothesis further.

Strictly speaking, rRNA is not a single RNA species, but two, i.e. 19S and 26S rRNA. However, they are derived from a common precursor and there is no evidence to suggest that they are not processed in equal amounts, as judged by their relative proportions and estimated molecular weights (Melera & Rusch [1973]). Therefore, for the purposes of this study, they can conveniently be considered as a "single" RNA species.

Finally, it should be borne in mind that mature rRNA is not the primary product of transcription and it is possible that a further

regulatory mechanism may operate during the processing of precursor RNA. Although detailed studies on precursor processing in Physarum have not yet been done, available data (Jacobson & Holt [1973]; Melera & Rusch [1973]) suggest that the maturation sequence is very similar to that found in higher eucaryotic cells (Burdon [1971]; Weinberg & Penman [1970]; Wellauer, Dawid, Kelley & Perry [1974]) as shown in Fig. 11. A comparison of the kinetics of rRNA maturation in Physarum and HeLa cells (Melera & Rusch [1973]) has suggested that rRNA precursors in Physarum have a shorter half-life, i.e. mature rRNA is produced at a faster rate than in HeLa cells.

II.1.3 Measurements of rates of macromolecular syntheses in synchronously growing cultures.

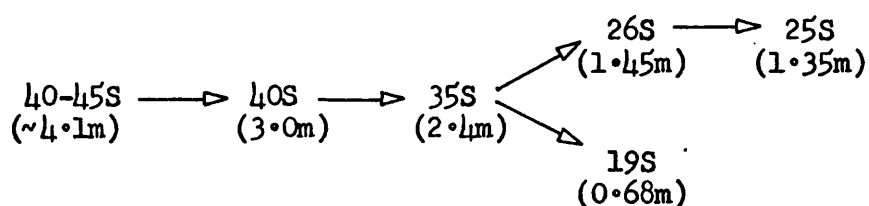
Rates of synthesis may be obtained in two ways:

- (a) From direct measurements of rates by pulse-labelling. The incorporation of isotope during a short exposure to a specific labelled precursor will be proportional to the rate of synthesis of the macromolecule.
- (b) Indirectly from measurements of accumulation of the macromolecule. Accumulation can be measured in several ways including direct chemical analysis which relies on quantitative extraction of the macromolecule, or by an "isotope dilution" method where only a representative sample of the macromolecule is required.

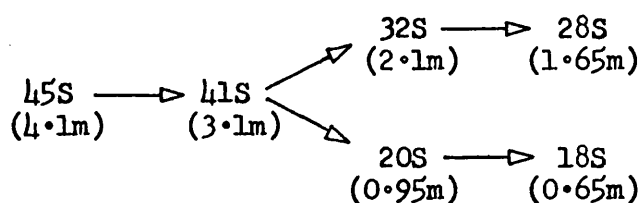
Unfortunately, quite large changes in rate are often reflected as only small changes in the corresponding accumulation curve of a compound as can be seen from the three simple models in Fig. 12. Unless the synchrony and accuracy of the data are very good, these small changes in accumulation may not be detectable. Therefore, it is hardly surprising that direct measurements of rates by pulse-labelling has been the more widely used approach. It was stated earlier that isotope incorporation

Fig. 11. Processing of rRNA precursors in Physarum polycephalum, HeLa cells and mouse L-cells.

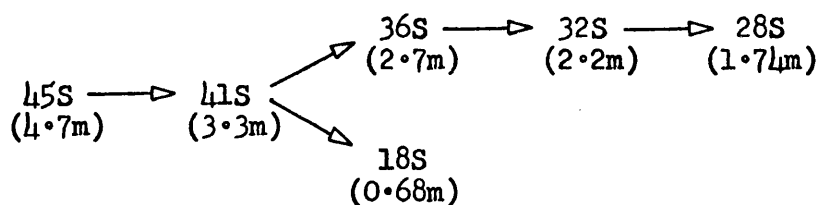
(a) Physarum polycephalum (from Jacobson & Holt [1973])



(b) HeLa cells (from Weinberg & Penman [1970])



(c) Mouse L-cells (from Wellauer, Dawid, Kelley & Perry [1974])



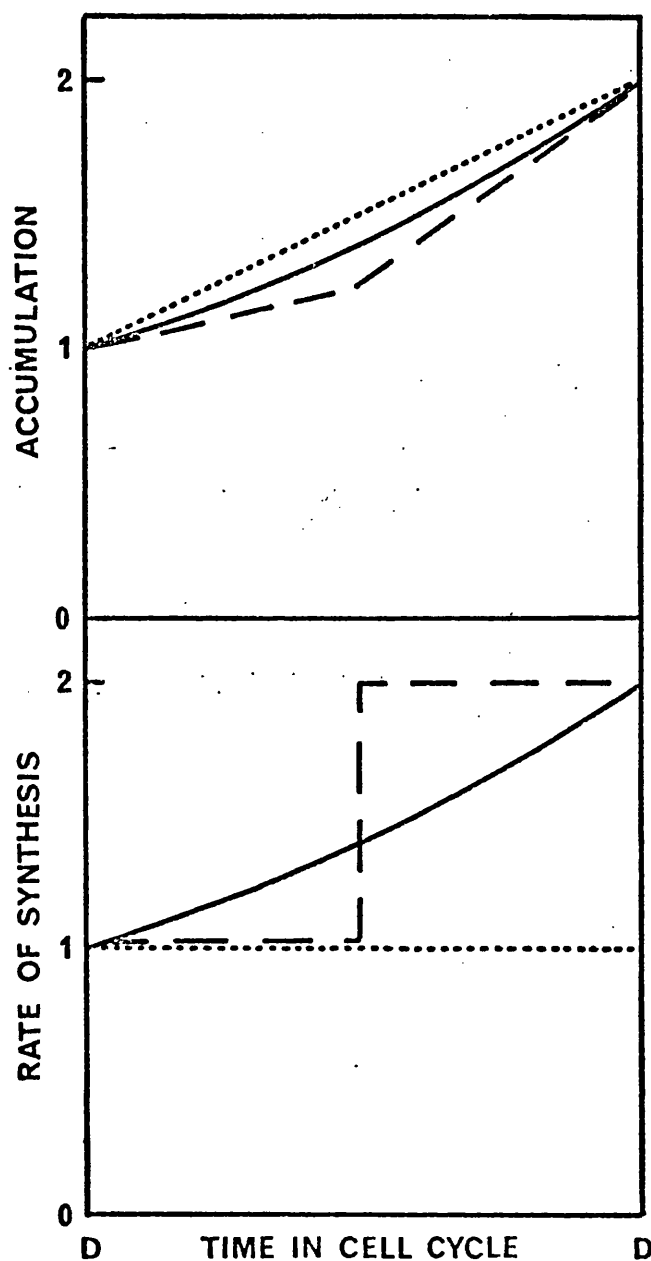


Fig. 12. Patterns of synthesis of a macromolecule during the cell cycle.

Upper graph: continuous line, exponential increase in accumulation; dots, linear increase; dashes, linear increase with a doubling in rate in mid-cycle (e.g. a gene dosage effect). D denotes division. Note that the corresponding rate curves in the lower graph show much greater differences than the accumulation curves. (Re-drawn from Mitchison [1971])

during a pulse will be proportional to the rate of synthesis. However for comparisons between successive measurements during the cell cycle to be valid, the specific radioactivity of the pool of the immediate precursor to the macromolecule must attain the same value in the same time, throughout the cell cycle. There are two important criteria which must be obeyed for this to apply:

(a) The uptake of labelled precursor into the cell must not vary during the cell cycle.

It has been shown that this is not obeyed for several uptake systems. For example, the number of functional transport sites for thymidine increases only during the S phase in Novikoff rat hepatoma cells (Plagemann, Richey, Zylka & Erbe [1975]) and Chinese hamster cells (Everhart & Rubin [1974]), resulting in a variation in the rate of thymidine uptake during the cell cycle. This then leads to variation in incorporation of thymidine into the nucleotide pool since transport has been shown to be the rate limiting step (Plagemann, Richey, Zylka & Erbe [1975]). Similarly, the amino acid permeases of Saccharomyces cerevisiae double over a restricted part of the cell cycle (Carter & Halvorson [1973]) giving rise to periodic changes in the rate of amino acid uptake. Clearly variations in uptake may alter pool specific radioactivities during the course of the experiment and consequently invalidate rate measurements. A good example of the way in which this has led to erroneous conclusions is illustrated in animal cells. Two conflicting patterns of the way in which the rate of RNA synthesis increases during the cell cycle have been described. Several authors have reported a linear increase in rate during G1 and S phase (Kim & Perez [1965]; Scharff & Robbins [1965]; Enger & Tobey [1969]), whereas others have demonstrated only a slight increase at the beginning of the S period, strongly suggesting a gene dosage effect (Terasima & Tolmach [1963]; Klevecz & Stubblefield [1967]; Pfeiffer &

Tolmach [1968]). However, this dilemma was resolved when Stambrook & Sisken [1972] showed that both patterns of RNA synthesis could be obtained when [5-³H]-uridine is used as the precursor, depending on the growth medium used. They subsequently showed that the difference in rates of synthesis were simply a reflection of differences in the rates of precursor uptake into the cells.

(b) A detailed knowledge of the intracellular precursor pool and its dilution by endogenous synthesis is required. The intracellular precursor pool can be either fixed in size, or expandable in the presence of exogenous precursor (Mitchison [1971]). In both cases the isotope incorporation will parallel the total activity (i.e. pool + incorporated activity) after an initial short delay while the specific radioactivity of the pool is rising. The larger the pre-existing pool, the longer the delay will be. When the length of the pulse is short compared with the time taken to reach a constant specific radioactivity, the size of the pre-existing pool becomes very important. Providing the pool increases throughout the cell cycle in proportion to the increase in the rates of uptake and synthesis, the amount of incorporation will be a valid measure of rate. Unfortunately, pool sizes are known to fluctuate and one way of partially overcoming this problem is to increase the length of the pulse, although this also decreases the sensitivity of the rate measurements. Nevertheless, pool changes can seriously affect pulse-label incorporation experiments. For example, changes in the rate of thymidine incorporation in human cells after virus infection (Newton, Dendy, Smith & Wildy [1962]) or irradiation (Smets [1969]) are primarily due to changes in pool size. Also, the rate of RNA synthesis in Chinese Hamster cells increases during interphase by a factor of about two when pulse-labelled with methionine or four with uridine (Enger & Tobey [1969]). Once again this is likely to be caused by changes in intracellular pool sizes.

Finally, difficulty in terminating a pulse may be experienced if the precursor pool is expandable. At first it might seem advisable to pulse-label with a high specific radioactivity precursor under these circumstances, so as not to expand the pool (which would delay it from reaching a constant specific radioactivity). However, if unlabelled precursor is then added to terminate the pulse, the pool will simply expand and isotope incorporation will continue. This effect has been observed during thymidine labelling of DNA in Vicia (Evans [1964]).

An appreciation of possible variations in precursor uptake and intracellular pool sizes, and their effects on isotope incorporation have led to more careful studies in recent years. Almost invariably investigations of rates of RNA synthesis during the cell cycle (by pulse-labelling), now include isolation of the nucleoside triphosphate pool and measurement of its specific radioactivity, as a necessary control. A precursor pool of constant specific radioactivity during the cell cycle is then used as evidence that isotope incorporation following a brief exposure to the precursor is a valid measurement of rate. On the other hand, if the pool varies, corrections have been applied to the rates of synthesis, based on the pool specific radioactivity, to "normalise" the pool effects.

However, there is now an increasing amount of evidence which suggests that nucleosides, in particular uridine and adenosine, may be incorporated into at least two different intracellular pools in animal cells, only one of which serves as a primary source of nucleotides for RNA synthesis. Plagemann [1971(b)] was the first to suggest this when he showed that uridine and adenosine incorporation into nucleic acids attains a constant rate within a few minutes of their addition to the medium despite the presence of a large, highly expandable cytoplasmic pool. Similarly, upon their removal from the medium, or after the addition of a large excess of unlabelled nucleoside, the incorporation into RNA decreases very rapidly.

Plagemann subsequently showed that one uridine nucleotide pool is located in the cytoplasm and the other in the nucleus. It is the latter very small pool which mainly supplies nucleotides for RNA synthesis. Further experiments suggested that the size of the nuclear pool (20-25% of the phosphorylated uridine) is under strict regulatory control, unlike the expandable cytoplasmic pool. However, the two pools are not entirely independent since most of the isotope is eventually incorporated into nucleic acid when labelling with very small amounts of uridine. It is not known whether this is due to diffusion or transport of nucleotides from cytoplasm to nucleus, or simply mixing of the pools during mitosis. Plagemann's data also confirm his earlier suggestions (Plagemann & Roth [1969]; Plagemann, Ward, Mahy & Korbecki [1969]; Plagemann [1971(a)]) that the rate of uridine incorporation into nucleotides determines to some extent its rate into RNA, and hence the latter is only a semi-quantitative measure of the true rate of RNA synthesis.

Very recently, Goody & Ellem [1975] demonstrated a similar compartmentation of nucleotide pools in 6C3HED lymphoma cells. The primary source of uridine nucleotides for RNA synthesis in these cells is a small pool constituting no more than 5% of the total cellular UTP pool, which is in rapid equilibrium with exogenously supplied nucleosides but only equilibrates relatively slowly with the cytoplasmic pool.

In conclusion, a total cell precursor pool of constant specific radioactivity throughout the cell cycle is not valid evidence that isotope incorporation into a macromolecule during a brief exposure to the precursor will be proportional to its rate of synthesis. Furthermore, measurements of the specific radioactivity of the total cell precursor pool can not be used to correct rates of isotope incorporation, when the pool size appears to vary. Therefore, although pulse-labelling is potentially the more sensitive method for determining rates of synthesis, I believe its

disadvantages far outweigh its advantages and hence we must re-examine the indirect method for obtaining rates from accumulation curves. As mentioned earlier, quite large changes in rate are often reflected as only small changes in the corresponding accumulation curve. Therefore the accuracy of the data must be very good if the latter approach is to be employed. Ideally the measurements of accumulation should not be dependent upon sample size (which is a particularly difficult parameter to measure in Physarum due to its syncitial nature) or efficiency of extraction, both potential sources of inaccuracy. I therefore developed an "isotope dilution" technique to investigate the synthesis of rRNA during the mitotic cycle in Physarum, based on measurements of specific radioactivity and hence independent of sample size or efficiency of extraction.

As a general control of the isotope dilution technique I also decided to measure the duration of the S phase in plasmodia of Physarum by exactly the same method as that used to measure RNA synthesis. Previous measurements of the length of the DNA synthetic period in this organism have been mainly based on isotope incorporation during pulse-labelling with a radioactive precursor (Nygaard, Gattes & Rusch [1960]; Braun, Mittermayer & Rusch [1965]; Braun & Wili [1969]) and consequently are subject to the same uncertainties as those already described for measurements of RNA synthesis by the same method. Therefore, as well as providing a general control of the method, measurement of DNA synthesis by the isotope dilution technique will also provide a useful comparison with published results obtained by pulse-labelling.

II.1.4 Principle of the isotope dilution technique used to measure the synthesis of rRNA during the mitotic cycle.

As the name implies, this method involves prelabelling of the culture,

chasing all of the isotope into metabolically stable products, and then following the decrease in specific radioactivity of the rRNA as it is diluted by newly synthesised, unlabelled rRNA. Thus, if at the time at which labelling ceases, the specific radioactivity of the rRNA, s_0 , is given by $\frac{a}{x}$ (where a is the total amount of radioactivity in the culture, and x is the amount of rRNA) then, at any subsequent time, $s = \frac{a}{(x + dx)}$ (where dx is the amount of newly synthesised, unlabelled material). Therefore s is proportional to $\frac{1}{(x + dx)}$; or in other words, the accumulation of rRNA is inversely proportional to its specific radioactivity. Clearly this method does not rely on a knowledge of the precursor uptake mechanism or intracellular pool sizes etc.

II.1.5 Suitability of Physarum polycephalum for proposed research.

Physarum is particularly well suited to the study of rRNA synthesis during the mitotic cycle for the following reasons:

(a) If rates of synthesis are to be obtained indirectly from measurements of rRNA accumulation, then very good synchrony is essential. As this is rarely achieved with artificially synchronised cultures, we are left with a small number of naturally synchronous systems of which Physarum polycephalum has probably been the most widely exploited. Within a single large plasmodium the synchrony is excellent. Metaphase times at opposite edges of a 15-20cm plasmodium rarely differ by more than about 10-15min, which represents very good synchrony for an organism with an intermitotic period of 8-9h.

(b) A large amount of material can be obtained from a single large plasmodium, using the specially constructed culture apparatus described in section II.2.2. This enables up to 15 samples to be taken for the preparation of rRNA, (or 6-8 samples for DNA), enabling a complete accumulation curve to be constructed from a single plasmodium.

(c) Since plasmodia lack a cell wall, nucleic acids can be readily extracted with a high efficiency of recovery.

(d) Physarum polycephalum possesses a specific transport mechanism for nucleosides (Birch & Turnock, unpublished work). Radioactively labelled nucleic acid precursors, for example [^3H]-uridine, are therefore rapidly taken up from the medium, enabling nucleic acids of reasonable specific radioactivity to be readily obtained.

II.2 MATERIALS AND METHODS.

II.2.1 Choice of strains.

Initially, the haploid plasmodial strain CL (Colonia Leicester), described by Cooke & Dee [1975], was chosen for the reasons outlined in section I.2.1. However, it was found that this strain was unable to maintain a balanced state of growth (see section II.3.7) under the conditions used, and hence subsequent work involved use of the diploid strain M_3C VIII (derived from Daniel's M_3C strain; Daniel & Baldwin [1964]). This strain exhibits fast growth and good sporulation, spore germination, and spherulation. Furthermore there was some evidence to suggest that M_3C strains can maintain a balanced state of growth (when grown as surface plasmodia) for several mitotic cycles (Mohberg & Rusch [1969]; Mohberg [1974]).

II.2.2 Culture methods.

(i) Stock cultures. Stock cultures were maintained as described in section I.2.2.

(ii) Large surface cultures. It was decided from the beginning to grow surface plasmodia of a size large enough to permit a complete rRNA (or DNA) accumulation curve to be derived from a single plasmodium, thereby eliminating the need to correct for slight differences in intermitotic time between cultures etc. A method for growing plasmodia of up to 14cm in diameter had been described previously by Mohberg & Rusch [1969], and consisted of a large stainless steel tray containing a Millipore membrane supported by filter paper on a stainless steel screen. Plasmodia were started from an inoculum ring to allow outward and inward growth, and incubated on a rocker with nutrient medium flowing back and forth, wetting the underside of the paper. The apparatus was primarily

designed to enable large quantities of histones to be prepared at a particular stage in the mitotic cycle. Consequently, no attempt had been made to obtain a steady growth rate, as this was unnecessary, and instead the growth medium was simply aspirated from the trays and replaced with fresh medium after about 15h of growth. However, I felt that such nutritional fluctuations, apart from altering the growth kinetics of the organism, might also induce transitory changes in the pattern of rRNA synthesis, and were therefore to be avoided in the present study. Instead, a much larger ratio of medium to culture volume is to be preferred, thereby eliminating the need to change the medium during the course of the experiment. Unfortunately this is not readily achieved with the apparatus described by Mohberg & Rusch, so an alternative system where medium could be circulated from a large reservoir was developed.

Description of culture apparatus.

The general construction of the apparatus can be seen in Fig. 13. It consisted of a stainless steel tray (35cm x 30cm x 5cm deep) with medium inlets (level with the bottom of the tray) on two opposite sides. Medium left by means of a central weir which could be screwed up or down to alter the level of the medium in the tray. A stainless steel screen (5mm mesh size) was supported, about 3-5mm above the base of the tray, on the inlet tubes and slightly tapered sides. This screen in turn supported two sheets of 576 chromatography paper (Schleicher & Schuell Co., Keene, N.H., Conn., USA), the lower sheet measuring 35cm x 26cm and the upper one slightly smaller. A 1.5cm x 1.5cm hole was cut in the centre of the screen and both sheets of paper, directly above the outlet weir. A narrow strip of paper, one end of which was trapped between the two sheets of paper, and the other end passed a few millimeters down the outlet tube, acted as a wick and permitted better medium level control. Medium was circulated through the tray from a one litre reservoir at a

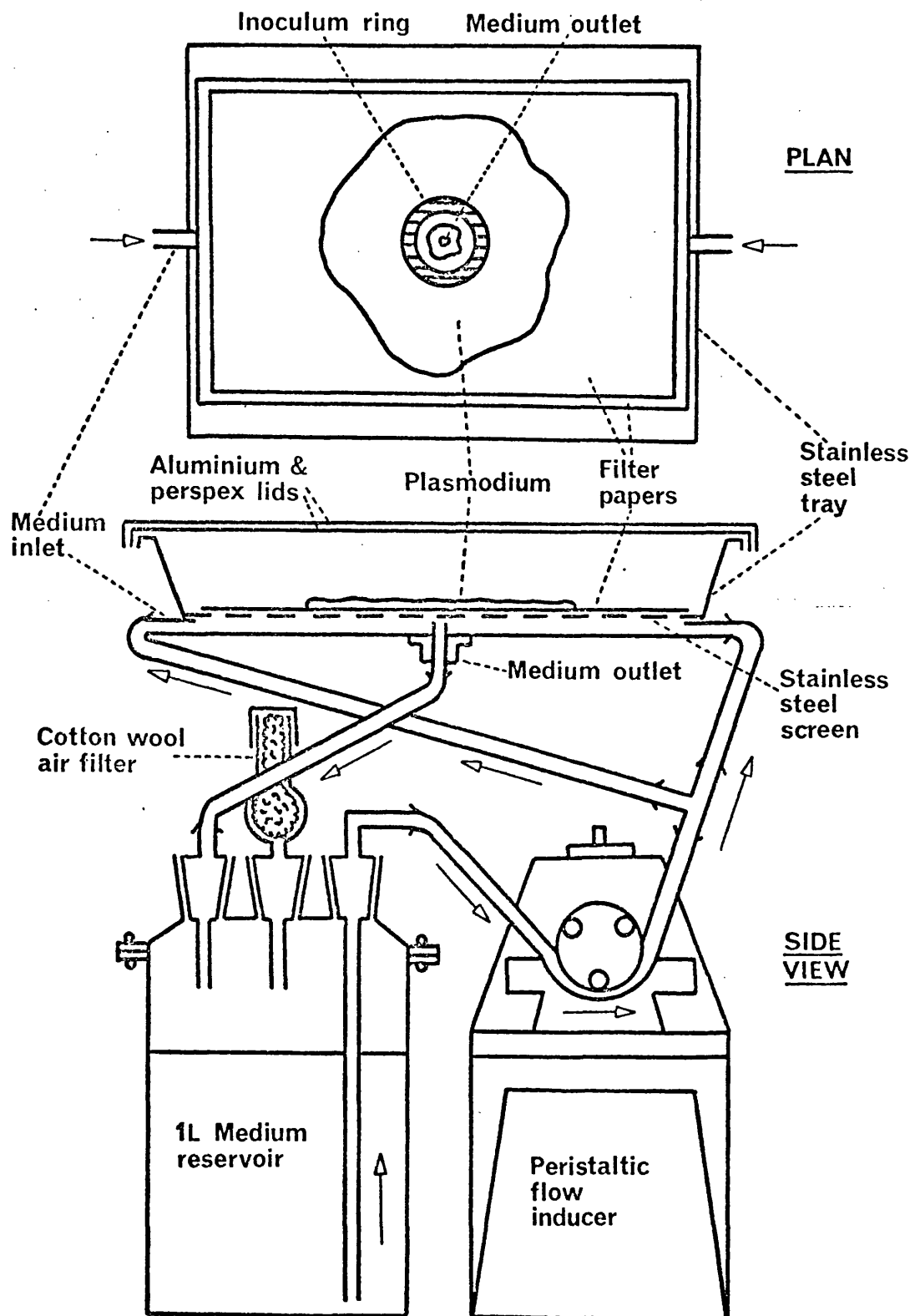


FIG.13. CULTURE APPARATUS.

rate of about $100\text{ml}.\text{min}^{-1}$ with the aid of a Watson-Marlow H.R. Flow Inducer (type MHRK 55). The level of medium in the tray was adjusted by altering the height of the weir so that uniform contact with the underside of the filter paper was obtained. Forced aeration of the one litre reservoir was found to be unsatisfactory since it caused a small positive pressure within the vessel (despite the loosely packed air outlet filter), thereby preventing proper functioning of the outlet weir. However, it was thought that forced aeration was not required in view of the large exposed surface area of filter paper.

Growth of large plasmodium.

(1) The entire apparatus was assembled, except for the flow inducer (which was of a peristaltic type and therefore did not require any aseptic connections to be made after autoclaving the apparatus), and the reservoir filled with one litre of Mycological peptone medium (except for the glucose and haematin; see section I.2.2).

(2) A screen was placed over the filter papers to prevent subsequent "wrinkling" during autoclaving, and the tray was covered with a close fitting aluminium lid.

(3) The entire apparatus was autoclaved for 20min at $15\text{lb}.\text{in}^{-2}$.

(4) Immediately before use, 40ml of sterile glucose solution (25% w/v) and 10ml of sterile haematin solution ($0.5\text{mg}.\text{ml}^{-1}$ in 1% NaOH) were added aseptically to the medium reservoir.

(5) The weighting screen was removed from the top of the filter papers with sterile forceps and the aluminium lid replaced by a transparent perspex inspection cover (sterilised by rinsing with absolute ethanol, then dried overnight at 37°C in a pre-sterilised shallow aluminium box.). The perspex cover was covered by an aluminium lid to exclude light from the culture.

(6) Inoculum for large surface cultures was obtained by inoculating

12-15ml of an exponentially growing microplasmodial culture into each of 3 x 500ml Erlenmeyer flasks containing 100ml of Mycological peptone medium, and incubating at 26°C for 20-24h.

(7) The cultures were then transferred to sterile, conical-based 50ml glass centrifuge tubes capped with aluminium foil and centrifuged in a bench centrifuge (MSE Super Minor) at 150xg for 20s (room temperature).

(8) The medium was discarded, the pellets pooled, and diluted with 0.5vol. of sterile water. The microplasmodia were resuspended by gentle whirlmixing. (It is essential that all clumps of microplasmodia are thoroughly dispersed.)

(9) A 7ml inoculum ring (about 7cm in diameter and 1.5cm wide) was added with a wide-tipped pipette to the centre of the filter paper (see Fig. 13.) and left at 26°C to allow the microplasmodia to coalesce.

(10) 60min later medium circulation was started by activating the flow inducer. Any air bubbles trapped below or between the filter papers were allowed to escape by carefully lifting the paper with sterile forceps.

(11) Mitotic stages were determined from alcohol-fixed smears as described in section I.2.3. Intermitotic times (from the second to third metaphase after coalescence, usually referred to as MII and MIII) varied from 8-9h. Surface plasmodia prepared in this way reached a diameter of approximately 20cm by MIII, that is, about 22-24h after coalescence.

(12) Sampling was achieved by aseptically removing segments of the plasmodium, cutting through the upper filter paper only, and discarding material from the vicinity of the initial inoculum ring.

Radioactively labelled surface plasmodia for studying nucleic acid synthesis by the isotope dilution method, were prepared from 20-24h microplasmodial cultures which had been grown in the presence of [5,6-³H]-uridine (50μM, 0.5μCi.ml⁻¹; Amersham).

(iii) Petri-dish surface cultures. Small surface cultures (up to

6cm in diameter) were grown in 9cm glass petri-dishes containing a single disc of 576 chromatography paper supported by a stainless steel screen. 0.2-0.3ml of inoculum (prepared as described above) was pipetted onto the centre of each disc, and allowed to coalesce for 60min. 15ml of Mycological peptone medium were then added and incubated at 26°C. The medium was changed after 12-15h if growth was maintained for much longer than this.

II.2.3 Distribution of radioactivity in microplasmodial cultures labelled with [³H]-uridine.

Each of the following assays is based on a 2.0ml sample of a microplasmodial culture.

(a) Radioactivity in medium. Samples were briefly centrifuged (1000xg, 30s, room temperature) and the medium retained. 50µl aliquots were combusted in a Packard 306 Sample Oxidizer (after being absorbed onto small filter paper squares) and counted in a Packard 3385 liquid scintillation spectrometer.

(b) Total cell radioactivity. The microplasmodial pellets from (a) were resuspended in 5ml of cold growth medium and filtered immediately through Sartorius membrane filters (Sartorius, 34 Göttingen, W. Germany). The filters were washed with a further 5ml of cold medium, then oxidized (after wrapping in filter paper and moistening with 0.1ml of water to control the combustion) and counted.

(c) Radioactivity insoluble in cold acid. i.e. nucleic acid and protein. After centrifuging to remove the medium, the pellets were re-suspended in 5ml of cold 5% (w/v) trichloroacetic acid solution containing 1mg.ml⁻¹ of uridine, and kept on ice for 30min. They were then centrifuged and the procedure repeated with a further 5ml of acid. Finally they were filtered through membrane filters, washed with 4 x 2ml of cold 5% trichloro-

acetic acid, oxidized and counted as above.

(d) Radioactivity insoluble in hot acid. i.e. protein. Samples were extracted once with cold acid as in (c), then resuspended in 5ml of 0.5M perchloric acid and incubated at 70°C for 20min with occasional mixing. After centrifugation, the pellets were re-extracted with a further 5ml of acid at 70°C for 20min, then filtered and processed as above.

(e) Radioactivity insoluble in cold acid after alkaline hydrolysis. i.e. DNA and protein. Samples were extracted once with cold acid as in (c), then dissolved in 2ml of 0.3M NaOH. After incubation at 37°C for 18h to hydrolyse the RNA, they were cooled, 0.2ml of 100% (w/v) tri-chloroacetic acid added, followed by 0.1ml of Escherichia coli RNA (5mg.ml⁻¹) as carrier, and kept on ice for 30min. They were then filtered and processed as above.

(f) Radioactivity insoluble in hot acid after alkaline hydrolysis. i.e. protein. Samples were processed as in (e) but incubated at 90°C for 30min (with occasional mixing) prior to filtering.

II.2.4 Preparation of rRNA from samples of surface plasmodia, and determination of its specific radioactivity.

(1) Up to 15 samples (each approximately 5cm x 3cm) may be taken from a single large surface plasmodium for the preparation of rRNA.

(2) Samples were quickly scraped from the filter paper segments with a spatula, transferred to small polyethylene vials, and stored in liquid nitrogen until required.

(3) Frozen plasmodial samples were each homogenised in 15ml of homogenising buffer and rRNA prepared as described in section I.2.7, with the omission of the pronase treatment.

(4) Combined fractions containing 19S and 26S rRNAs were obtained

from sucrose density gradients, each containing approximately 0.3mg of total RNA.

(5) The concentration of rRNA was determined from the absorbance at 258nm ($E_{258}^{1\%} = 221$; see section I.2.10), after diluting the fractions with 2vol. of gradient buffer.

(6) 1.0ml aliquots of the diluted fractions were mixed with 10ml of aqueous scintillation fluid (5.5g PPO, 0.1g dimethyl POPOP, 333ml Triton X-100, 667ml toluene) and counted in a Packard 3385 liquid scintillation spectrometer (optimum gain setting: 55%), enabling the specific radioactivity to be determined.

II.2.5 Specific radioactivity of DNA in labelled nuclei.

(1) Approximately 6-8 samples (each about 6cm x 5cm) may be taken from a single large surface plasmodium for DNA estimation.

(2) Immediately after sampling, nuclei were prepared from each segment as described in section I.2.4 with the following changes to compensate for the smaller amounts of plasmodium: The sample was homogenised in a 250ml Waring blender cup containing 40ml of homogenising medium, for 15s at 170V on the "high speed" setting. Nuclei were washed with 2 x 40ml of buffered sucrose medium.

(3) The final nuclear pellet was resuspended in 5ml of cold 5% (w/v) trichloroacetic acid in acetone/water (1:1 by volume) and kept on ice for 20min to remove any traces of pigment (Mohberg & Rusch [1969]). After centrifugation (4000xg, 5min, 4°C) the nuclear pellet was stored at -20°C until required.

(4) The pellet was dissolved in 4ml of 0.3M NaOH and incubated at 37°C for 16h. The solution was then cooled on ice, 10mg (in 100µl) of bovine serum albumin added as carrier, followed by 0.34ml of 10M perchloric acid to give an acid concentration of 0.5M.

(5) After 30min at 0°C, the precipitate (containing DNA and protein) was collected by centrifugation (4000xg, 10min, 4°C) and washed twice with 7ml of cold 0.5M perchloric acid.

(6) The pellet was resuspended in 2ml of 0.5M perchloric acid and incubated at 70°C for 30min (with occasional mixing). After cooling to room temperature protein was removed by passing through a membrane filter.

(7) The filtrate was diluted with 0.5M perchloric acid to give an absorbance at 258nm of about 0.5. 1.0ml aliquots were used to determine the DNA concentration by the diphenylamine reaction (see section I.2.6); and the radioactivity after mixing with 10ml of aqueous scintillation fluid.

II.2.6 Estimation of nuclear DNA content of plasmodia by microdensitometry.

(1) Nuclei were isolated from segments (each approximately 3cm x 2cm) of surface plasmodia as described in section II.2.5, and stored in liquid nitrogen.

(2) When ready for use, nuclei were rapidly thawed and one drop placed on a microscope slide together with one drop of chicken erythrocyte nuclei to provide a standard (Rasch, Barr & Rasch [1971]). Nuclei were mixed, spread evenly with the side of a pasteur pipette, and allowed to air dry.

(3) Slides were fixed in acetic-alcohol (1vol. of glacial acetic acid + 3vol. of absolute ethanol,), rinsed well in distilled water, and stained by the Feulgen method (Darlington & La Cour [1962]), with the modification that hydrolysis was by 5M HCl at room temperature for 45min (Itakawa & Ogura [1954]).

(4) Stain intensity was measured with a Vickers M85 Scanning Microdensitometer (Vickers Instruments Ltd.), using the following settings:

slit width 30, wavelength 560nm and spot size 2. Fifty Physarum nuclei from each preparation were scanned together with ten chicken erythrocyte nuclei, the latter being taken to have a DNA content of 2.45pg/nucleus (Rasch, Barr & Rasch [1971]).

II.3 RESULTS AND DISCUSSION.

II.3.1 Distribution of radioactivity in microplasmodial cultures labelled with [^3H]-uridine.

A 50ml culture of microplasmodia in the early logarithmic phase of growth was labelled with [5,6- ^3H]-uridine (50 μM , 1 $\mu\text{Ci.ml}^{-1}$; Amersham) for 20h. The distribution of radioactivity was then determined by taking 20 x 2ml samples of the culture and performing each of the assays described in section II.2.3 in quadruplicate. The results are shown in Table 5. 99% of the acid insoluble radioactivity appeared in nucleic acids, only 1% entering proteins. The ratio of radioactivity in RNA to DNA was about equivalent to their relative amounts in the cell.

Physarum polycephalum possesses a specific transport mechanism for nucleosides, and uridine is rapidly taken up from the medium by microplasmodia (Birch & Turnock, unpublished work). The medium is therefore depleted of uridine in 2-3h under these conditions although not all of the radioactivity is incorporated into the cells since, in addition to being used as a precursor for nucleic acid synthesis, uridine is also catabolised to uracil which accumulates in the medium (Birch & Turnock, unpublished work). Therefore, nucleic acids will only be labelled during the first 2-3h of growth in the presence of [^3H]-uridine, followed by an 18h period during which isotope will be "chased" from metabolically unstable species. Consequently, assuming that the extracellular labelled uracil does not re-enter the cells during the chase period, only metabolically stable nucleic acid species, i.e. rRNA, tRNA and DNA will be significantly labelled after 20-24h of growth. Indeed, reutilisation of uracil does not occur as indicated by the constant level of radioactivity in the medium from 16-25h after the onset of labelling (Fig. 14.). Furthermore, the addition of unlabelled uridine after 19.2h of growth in

Table 5. Distribution of radioactivity in microplasmodial cultures labelled with [5,6-³H]-uridine.

A 50ml culture of microplasmodia in the early logarithmic phase of growth was labelled with [³H]-uridine (50 μ M, 1 μ Ci. ml⁻¹) for 20h. The distribution of radioactivity was then determined by taking 20 x 2ml samples of the culture and performing each of the assays described in section II.2.3 in quadruplicate. 73% of the isotope was found in the cells and 27% in the medium. Results are expressed as a percentage of the radioactivity insoluble in cold acid (2.13×10^6 dpm).

Fraction.	%
(a) Radioactivity insoluble in cold acid	
(nucleic acid + protein)	100.0
(b) Radioactivity insoluble in hot acid	
(protein)	1.3
(c) Radioactivity insoluble in cold acid after	
alkaline hydrolysis (DNA + protein).	4.2
(d) Radioactivity insoluble in hot acid after	
alkaline hydrolysis (protein).	1.3
(e) DNA [(c) - (d)]	2.9
(f) RNA [(a) - (c)]	95.9

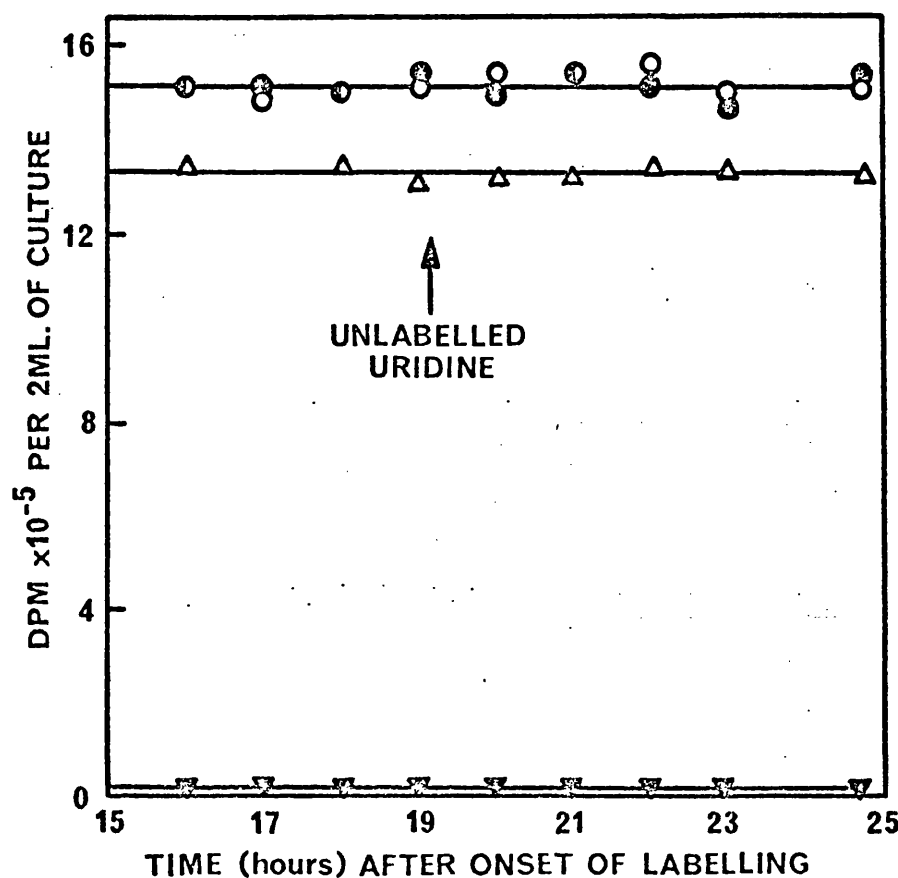


Fig. 14. Distribution of radioactivity during the "chase period" in a microplasmodial culture labelled with $[^3\text{H}]$ -uridine. Microplasmodia were labelled with $[^3\text{H}]$ -uridine ($50\mu\text{M}$, $1\mu\text{Ci.ml}^{-1}$), and samples taken for analysis (as described in section II.2.3) from 15h to 24.75h after the onset of labelling. At 19.2h unlabelled uridine was added to give a concentration of $50\mu\text{M}$. (○), total cell radioactivity; (●), radioactivity insoluble in cold acid, i.e. nucleic acids and proteins; (▼), radioactivity insoluble in hot acid, i.e. proteins; (Δ), radioactivity in the medium.

the presence of isotope does not result in any further uptake of radioactivity, as would be expected if labelled uridine is no longer present in the medium. It can also be seen from Fig. 14. that the amount of intracellular radioactivity insoluble in cold acid, 95% of which is RNA, remains constant from 16-25h after the onset of labelling. This would suggest that these labelled nucleic acid species are metabolically stable, although it should be emphasized that breakdown and reutilisation of the products specifically for nucleic acid synthesis (since the radioactivity in protein does not increase during this period) would produce the same result.

II.3.2 Synthesis of rRNA during the mitotic cycle in strain M₃CVIII.

Microplasmodia of strain M₃CVIII, grown for 22h in the presence of [³H]-uridine, were used as inoculum for the formation of a large surface plasmodium as described in section II.2.2(ii). Under these conditions radioactivity in RNA would be present only in rRNA and tRNA as discussed in the previous section.

Segments of the large plasmodium were removed for analysis from shortly before the second post-fusion mitosis (MII) until several hours after the third mitosis (MIII). RNA was isolated from the segments, fractionated on sucrose density gradients, and the specific radioactivity of the combined 19S and 26S rRNAs determined as described in section II.2.4. Fig. 15. shows a typical sucrose density gradient from which the 19S and 26S rRNAs were obtained, and illustrates the close correspondence between the profiles of radioactively labelled rRNA and tRNA (which will only constitute 3% of the total rRNA and tRNA after five doublings) and the more recently synthesised unlabelled RNA, represented by the absorbance at 254nm. Furthermore, it confirms the absence of any appreciable labelling of other (metabolically unstable) RNA species.

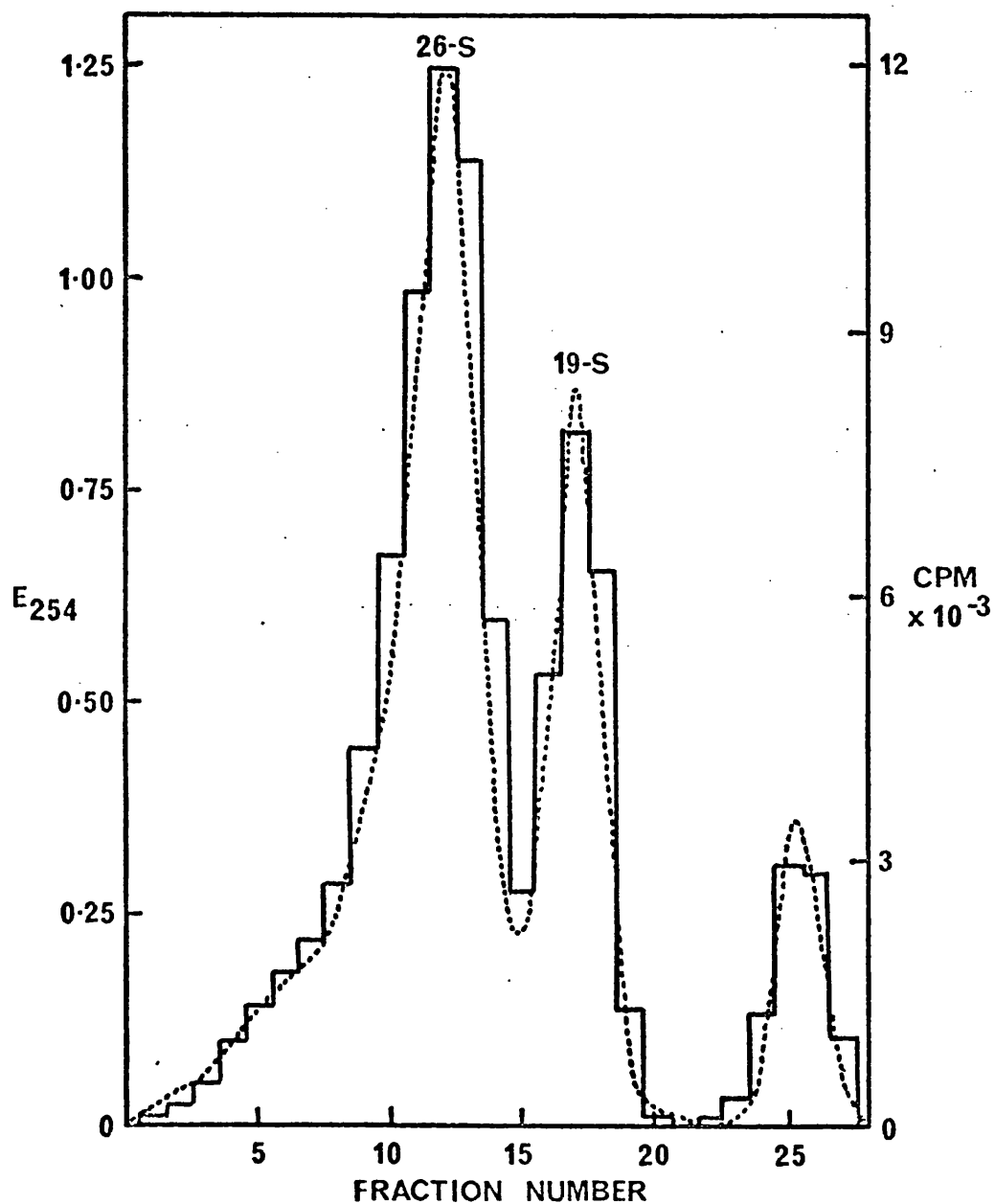


Fig. 15. Zone sedimentation analysis of a preparation of RNA.

300 μ g of RNA, purified from a plasmodium that had been prepared from microplasmodia labelled with [³H]-uridine (see section II.3.2), were analysed by zone sedimentation in a sucrose density gradient. Sedimentation is from right to left.

(—), radioactivity; (....), absorbance at 254nm.

The change in specific radioactivity of the rRNA as a function of time during the mitotic cycle and the corresponding accumulation curve for rRNA (given by the reciprocal of the specific radioactivity) are shown in Figs. 16 and 17. Several points are apparent from the results obtained:

(a) The synthesis of rRNA is continuous throughout the mitotic cycle, except for a brief cessation or very low rate during mitosis, of more specifically between prophase and telophase-early reconstruction, a period of about 30min. This is in agreement with the results of Kessler [1967] who demonstrated that, following a pulse-label with [^3H]-uridine for 5min, incorporation of isotope into nuclear RNA could not be detected by autoradiography during metaphase and anaphase in Physarum polycephalum.

(b) The amount of rRNA doubled between mitosis II and III which suggests that the plasmodium was in a balanced state of growth, and also that the turnover of rRNA during this period was very low; since a net doubling in the amount of rRNA with concomitant turnover and dilution of the labelled breakdown products with unlabelled nucleotides would produce a decrease in specific radioactivity (or apparent increase in rRNA accumulation) of greater than a factor of two. In fact, to obtain a two-fold reduction in specific radioactivity in the presence of rRNA turnover, would require the labelled products of breakdown to be re-incorporated without dilution by endogenous nucleotides, a process which is extremely unlikely. Therefore, an additional advantage of the isotope dilution method is its ability to provide an index of metabolic turnover under conditions of balanced growth. Turnover of rRNA has been measured in bacteria (Kaempfer, Meselson & Raskas [1968]; Kaempfer & Meselson [1968]), in yeast (Kaempfer [1969]) and in animal cells (Weber [1972]; Kolodny [1975]; Wiegers, Kramer, Klapproth, Rehpenning & Hilz [1975]), and has been found to be very low during active growth.

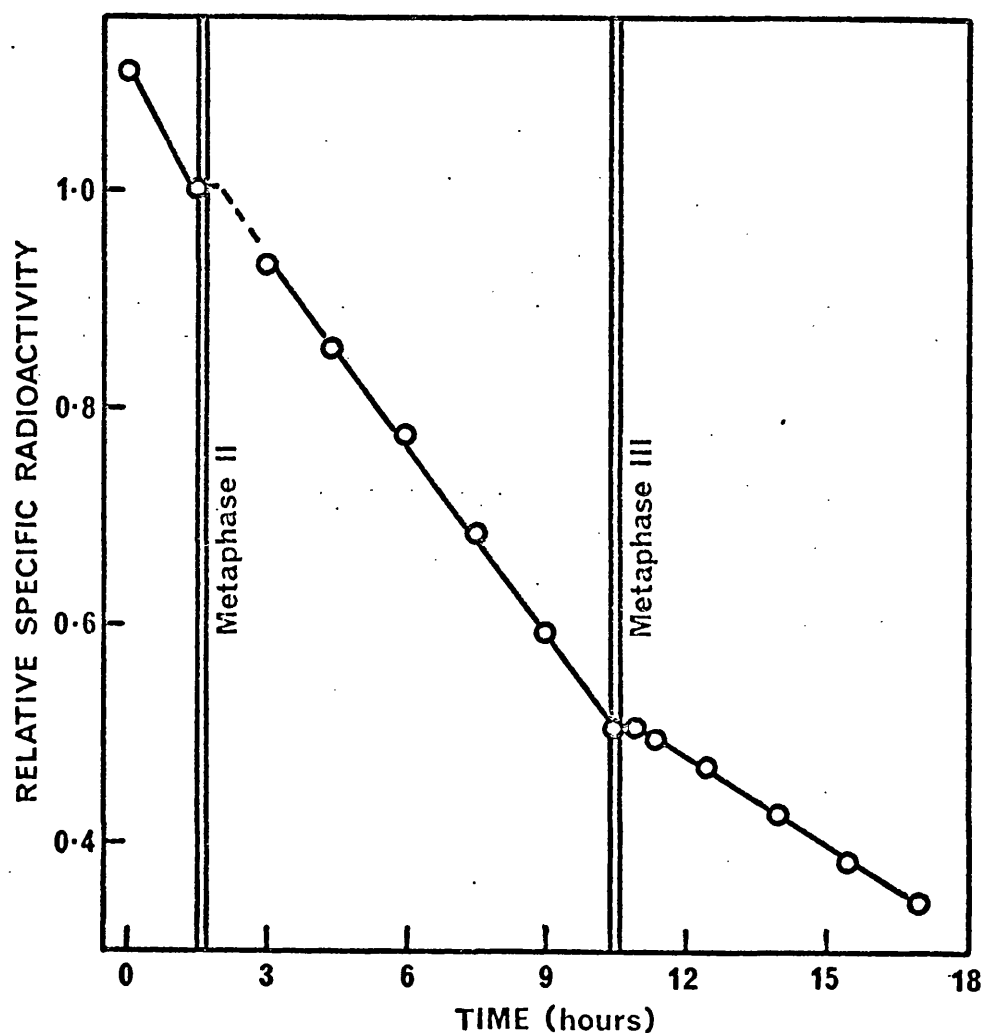


Fig. 16. Synthesis of rRNA during the mitotic cycle in strain M₂CVIII.

I. Decrease in specific radioactivity of rRNA as a function of time.

A large surface plasmodium was established from microplasmodia labelled with [³H]-uridine (see section II.2.2), and rRNA was isolated from samples taken from just before MII until several hours after MIII. The specific radioactivity of the rRNA is given as a function of time in relation to MII and MIII. The data are normalised to a specific radioactivity of 1.0 at MII (726cpm.µg RNA⁻¹).

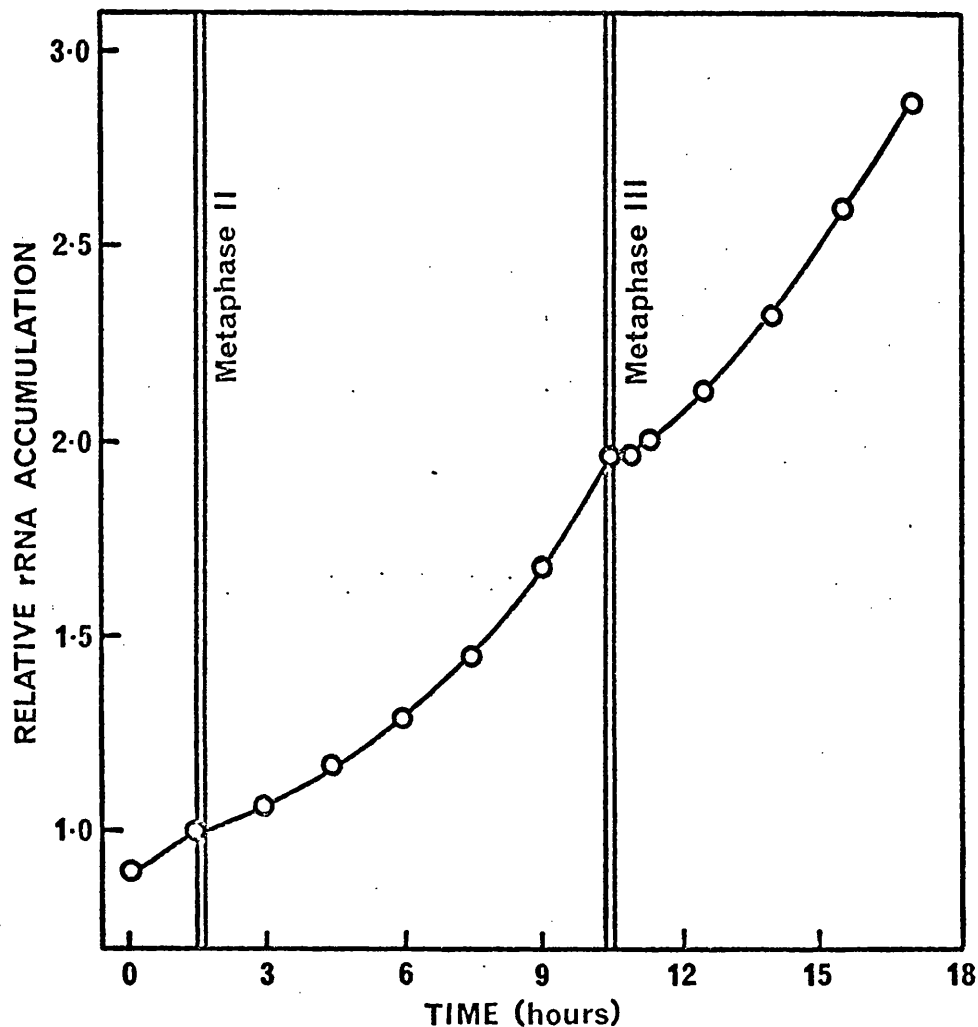


Fig. 17. Synthesis of rRNA during the mitotic cycle in strain M₂CVIII.

II. Accumulation of rRNA. The accumulation curve for rRNA is given as a function of time during the mitotic cycle; each point being the reciprocal of the corresponding specific radioactivity in Fig. 16. Since the specific radioactivity at MII was normalised to 1.0, then the amount of rRNA at MII is also 1.0.

(c) The rate of rRNA synthesis clearly increases throughout the mitotic cycle, from a minimum during nuclear division to a maximum at the end of the G2 period.

The crucial question is whether this increase in rate is due to regulation of rRNA synthesis at the level of transcription (and/or precursor processing), or whether it is simply a gene dosage effect due to replication of the rRNA genes. To resolve this a rate graph must be derived from the accumulation data and there are two ways of doing this:

(i) A smooth line may be drawn through the experimental accumulation data, and the rates of synthesis determined from tangents drawn to this line at suitable intervals, or,

(ii) A mathematical equation may be found which closely describes the experimental data, and rates then determined from the differential.

In view of the difficulty and perhaps subjectivity involved in constructing tangents to a non-symmetrical curve I decided to apply the second method. Three relatively simple general equations which may be used to describe a non-linear relationship between two variables, in this case the amount of rRNA (given by $\underline{x}$) and time ($\underline{t}$) are as follows:

(i) A power relationship:

$$x = (at + c)^b \dots\dots\dots(1)$$

(ii) A quadratic relationship:

$$x = 0.5\alpha t^2 + \beta t + c \dots\dots\dots(2)$$

(iii) An exponential relationship:

$$x = c.e^{kt} \dots\dots\dots(3)$$

The constants in these equations ($a, b, c, \alpha, \beta, k$) may be adjusted until the equation most closely describes the experimental data. Clearly a great deal of time could be spent finding a precise and probably very complex equation which fits the experimental data perfectly. However, in view of the potential errors in the data, this would be no more precise

than applying the above three "simple" equations, two of which give forms which are so close to the experimental data as to be indistinguishable.

To obtain the best mathematical interpretation of the results, the period from 20min after metaphase II to metaphase III in Fig. 17. was analysed (since rRNA synthesis ceased or was reduced to a very low rate during the first 20min following metaphase). The duration of this period was therefore 8.51h (see Fig. 18.). The initial amount of rRNA at MII was arbitrarily set at 1.00 (as in Fig. 17.) and the amount at MIII was consequently 2.00. Hence when, $\underline{t} = 0$, $\underline{x}$ (amount of rRNA) = $\underline{x}_0 = 1.00$, and when, $\underline{t} = 8.51$, $\underline{x} = 2.00$.

The three general equations (1), (2) and (3), may now be simplified since, $\underline{c} = \underline{x}_0 = 1.00$.

(i) The power relationship:

$$\underline{x} = (a\underline{t} + 1)^b \dots\dots\dots(4)$$

(ii) The quadratic relationship:

$$\underline{x} = 0.5\alpha\underline{t}^2 + \beta\underline{t} + 1 \dots\dots\dots(5)$$

(iii) The exponential relationship:

$$\underline{x} = e^{k\underline{t}} \dots\dots\dots(6)$$

A value for $\underline{a}$ in equation (4) was chosen, and the corresponding value for $\underline{b}$ calculated by applying the constraint: when $\underline{t} = 8.51$, $\underline{x} = 2$. Values for $\underline{x}$ were then calculated from this equation for each of the six $\underline{t}$ values at which experimental data were available. This was then repeated with further pairs of values for $\underline{a}$ and $\underline{b}$, until a minimum value for the sum of the squares of the residuals in $\underline{x}$ had been achieved, i.e. a minimum value for $\sum (\underline{x}_{\text{calc}} - \underline{x}_{\text{exptal}})^2$

The optimum values for $\underline{\alpha}$ and $\underline{\beta}$ in equation (5) were arrived at in a similar manner. However, in equation (6) there is only one constant, $\underline{k}$, which must therefore have a unique value if the constraint: when $\underline{t} = 8.51$, $\underline{x} = 2$, is to apply. A summary of the optimum values for $\underline{a}$ and $\underline{b}$ in

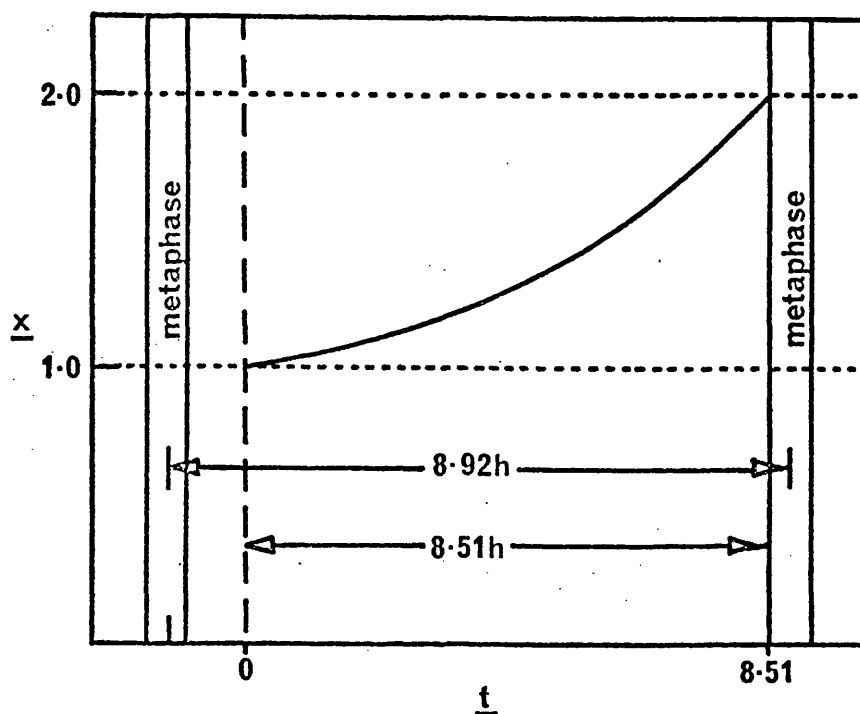


Fig. 18. Parameters used to derive mathematical interpretations of the pattern of rRNA synthesis during the mitotic cycle in M_3 CVIII.

Table 6. Constants used in equations which best describe the pattern of rRNA synthesis during the mitotic cycle in M_3 CVIII.

Type of equation.	Constants.	$\sum (x_{\text{resid}})^2$
Power $[x = (at + 1)^b]$	$a = -0.06679, b = -0.825$	1.0×10^{-3}
Quadratic $[x = 0.5\alpha t^2 + \beta t + 1]$	$\alpha = 0.01980, \beta = 0.03283$	4.3×10^{-3}
Exponential $[x = e^{kt}]$	$k = 0.08143$	31.6×10^{-3}

equation (4), α and β in equation (5), and k in equation (6), together with their corresponding $\sum (x_{\text{residual}})^2$ values is given in Table 6. The accumulation curves described by these three optimum equations are shown in Fig. 19. (as continuous lines), together with the experimental data. Clearly rRNA synthesis during the mitotic cycle does not follow an exponential pattern, but the best fits to the other two equations cannot be distinguished.

II.3.3 Rate of rRNA synthesis during the mitotic cycle.

Having obtained equations which fit the experimental data very closely it is now possible to derive a rate graph for rRNA synthesis during the mitotic cycle, simply by differentiating the equations with respect to time:

(i) For the power relationship:

$$x = (at + 1)^b$$

$$\frac{dx}{dt} = ab(at + 1)^{b-1} \dots\dots\dots(7)$$

(ii) For the quadratic relationship:

$$x = 0.5\alpha t^2 + \beta t + 1$$

$$\frac{dx}{dt} = \alpha t + \beta \dots\dots\dots(8)$$

(iii) For the exponential relationship:

$$x = e^{kt}$$

$$\frac{dx}{dt} = k \cdot e^{kt} = kx \dots\dots\dots(9)$$

(This latter equation does not fit the data but is given for comparative purposes.)

The rate curves given by equations (7), (8), and (9) are shown in Fig. 20. The increase in the net rate of rRNA synthesis is linear for equation (8) and non-linear for equation (7). However, the two lines intersect in two places, therefore at any time in the mitotic cycle the difference in rate between the two equations will not be very large.

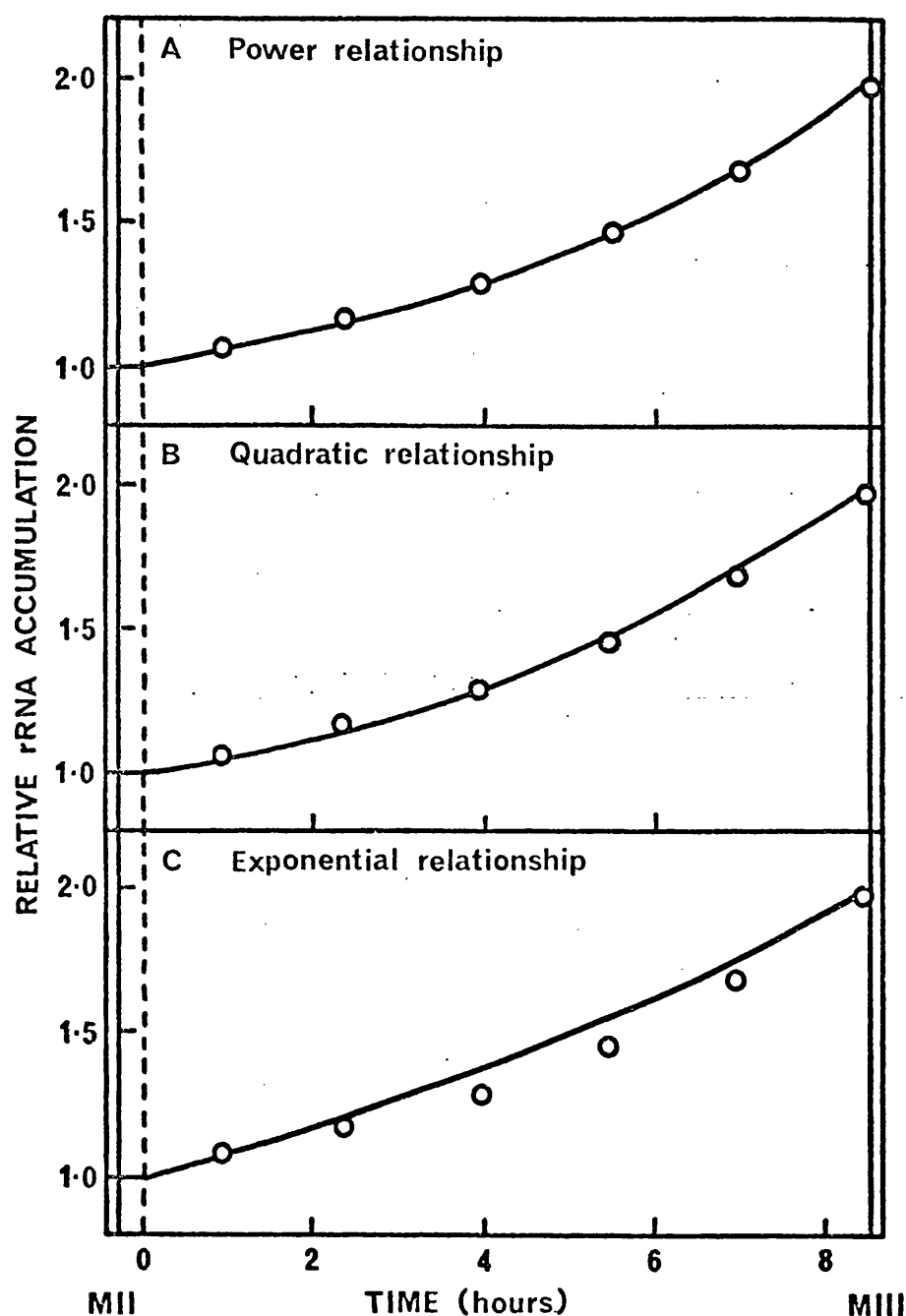


Fig. 19. Possible mathematical descriptions of the accumulation curve for rRNA between successive mitoses. Power, quadratic and exponential equations were fitted to the accumulation data given in Fig. 17., as described in section II.3.2. The accumulation curves described by these three equations, giving the best fit to the experimental data (O), are shown as continuous lines in each case.

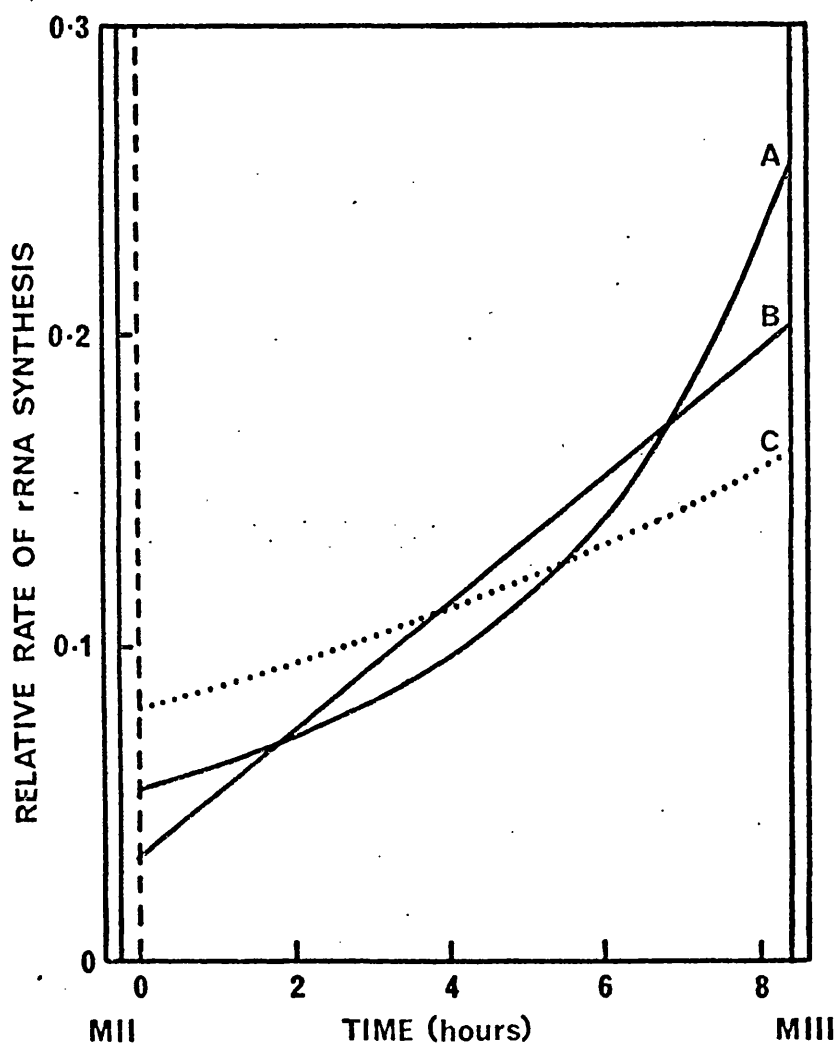


Fig. 20. Variation in the rate of rRNA synthesis during the mitotic cycle. To determine the rate of synthesis, $d[rRNA].dt^{-1}$ as a function of time during the mitotic cycle, the power and quadratic equations described in Fig. 19. were differentiated, yielding respectively a curve (A) and a straight line (B). [The exponential relationship (C) is given for comparative purposes only since the data was not a close fit to this equation.]

Furthermore, the overall increase in the rate of rRNA synthesis between the beginning and end of the mitotic cycle, ignoring the very low rate during and immediately after mitosis, is 5 to 6 fold in both cases.

Clearly the pattern of rRNA synthesis in Physarum polycephalum (illustrated in Fig. 20.) is very different to the biphasic pattern reported for total RNA synthesis, obtained by pulse-labelling, in both intact cells (Mittermayer, Braun & Rusch [1964]; see Fig. 10.) and isolated nuclei (Mittermayer, Braun & Rusch [1966]; Grant [1972]; see Fig. 10.). However, there is a great deal of evidence which suggests that the two peaks of transcriptional activity reflect the syntheses of different classes of RNAs. Grant [1972] has studied the effect of high salt concentration and α -amanitin on RNA synthesis in isolated Physarum nuclei. Nucleoplasmic RNA synthesis is known to be stimulated by high salt concentration (Maul & Hamilton [1967]; Pogo, Littau, Alfrey & Mirsky [1967]; Younger & Gelboin [1970]), and is completely inhibited by α -amanitin (Roeder & Rutter [1969]; Jacob, Sajdel & Munro [1970]); whereas nucleolar RNA synthesis is unaffected in both cases. Grant concluded that the G2 peak of synthesis in Physarum is almost entirely due to nucleolar RNA (i.e. essentially rRNA) synthesis, but the S peak contains a substantial proportion of both nucleoplasmic and nucleolar synthesis. Further evidence that rRNA may be preferentially synthesised during the G2 phase of the mitotic cycle and depressed in early S phase has been obtained by Fouquet, Böhme, Wick, Sauer & Braun [1974], who measured the ratio of rRNA to poly(A)-rich RNA during the mitotic cycle, after separation by chromatography on oligo-(dT)-cellulose or Sigma Cell 38 columns.

The overall pattern is therefore a relatively low rate of rRNA synthesis during the early part of the mitotic cycle (S phase) increasing to a higher rate during G2; a result which is qualitatively similar to that obtained in the present study.

II.3.4 Relationship between rRNA synthesis and replication of the rRNA genes in Physarum polycephalum.

In Physarum polycephalum the rRNA genes are a component of the "heavy satellite" DNA fraction of the genome (Zellweger & Braun [1971]; Sonenshein, Shaw & Holt [1970]; Newlon, Sonenshein & Holt [1973]). Unlike the bulk of the nuclear DNA, this heavy satellite DNA is replicated throughout the mitotic cycle (Guttes & Guttes [1969]; Braun & Evans [1969]; Holt & Gurney [1969]; Zellweger, Ryser & Braun [1972]) with the exception of the first hour after mitosis (Zellweger, Ryser & Braun [1972]). Recently Newlon, Sonenshein & Holt [1973] have shown directly that the rRNA genes are replicated throughout the G2 period. The amount of DNA, isolated from synchronous cultures, which hybridised to rRNA was 0.135% just after the S phase, 0.147% half way through G2, and 0.186% by the end of G2. Unfortunately, as this is the only published data on the replication of the rRNA genes in Physarum, it is not possible to make a detailed comparison of the patterns of gene replication and transcription, although clearly they are similar in that they both increase throughout most of interphase. However, the 5-6 fold overall increase in the net rate of rRNA synthesis during interphase, compared with a 2-fold increase in the number of rRNA genes, suggests that the increase in transcriptional activity is not solely due to gene replication, but that regulation at the level of transcription and/or precursor processing may also operate.

II.3.5 Rates of rRNA synthesis and effect of gene dosage in other systems.

In general, rRNA synthesis is continuous throughout the cell cycle except for a brief cessation (or reduction to a very low rate) during mitosis in animal cells (Prescott & Bender [1962]; Terasima & Tolmach [1963]; Doida & Okada [1967]; King & Barnhisel [1967]; Zylber & Penman [1971]). The gap in synthesis starts in late prophase and continues

into telophase. On the other hand, in systems which exhibit amitotic chromosome separation, such as the macronuclei of ciliates, rRNA synthesis is continuous throughout nuclear division (Prescott [1964]; Rao & Prescott [1967]). Similarly no gap in synthesis is found during nuclear division in the fission yeast, Schizosaccharomyces pombe (Mitchison & Walker [1959]) and the budding yeast, Saccharomyces cerevisiae (Sogin, Carter & Halvorson [1974]), which have a somewhat aberrant mitosis and lack fully condensed chromosomes during nuclear division. In view of the above observations it has often been suggested that the cessation in RNA synthesis during mitosis in animal cells is a result of the highly condensed structure of the metaphase chromosomes preventing transcription. The observation that RNA synthesis in cytoplasmic organelles, e.g. mitochondria, is continuous during mitosis (Fan & Penman [1970]) adds further support to this hypothesis. However, there are reasons to believe that this interpretation may not be correct. Firstly, transcription in animal cells ceases in early prophase, before the disintegration of the nucleolus and the nuclear membrane, and is resumed late in telophase before nucleolar reformation. The precise timing of the gap in RNA synthesis therefore suggests it is not simply due to chromosome condensation, but instead may be regulated by a specific control mechanism. Secondly, although nucleolar rRNA synthesis ceases during metaphase in HeLa cells, 4S tRNA synthesis continues at about one third of the interphase rate, and 5S rRNA synthesis is virtually unaffected (Zylber & Penman [1971]). Clearly some loci can still be transcribed on condensed metaphase chromosomes. A possible explanation proposed by Lewin [1974], is that the small RNA species (4S and 5S) are synthesised by a third RNA polymerase which is not inhibited during nuclear division. However, whatever the mechanism is which causes a cessation in rRNA synthesis in higher eucaryotes, clearly it also operates in Physarum.

In general, the rate of rRNA synthesis increases throughout interphase, although there is no general pattern to this increase. Furthermore, no conclusive correlation has yet been demonstrated between the rate of rRNA synthesis and gene replication. In Escherichia coli the rate of rRNA synthesis increases exponentially during the cell cycle and there is no discernible effect due to gene replication (Dennis [1971]). A similar pattern of transcription has been observed in several lower eucaryotes including the budding yeast, Saccharomyces cerevisiae (Sogin, Carter & Halvorson [1974]); the fission yeast, Schizosaccharomyces pombe (Mitchison, Cummins, Gross & Creanor [1969]); and Chlorella pyrenoidosa (Herrmann & Schmidt [1965]). The way in which the rate of rRNA synthesis increases during interphase in animal cells is not clear since two conflicting patterns have been described. Several authors have reported a linear increase in rate during the G1 and S phases of the cell cycle, with no sharp increase during the DNA synthetic period (HeLa cells: Kim & Perez [1965]; Scharff & Robbins [1965]. Chinese hamster cells: Enger & Tobey [1969]. Mouse L cells: Fujiwara [1967]. Mouse P815Y cells: Warmesley & Pasternak [1970]); whereas others have observed only a slight increase in rate during G1 followed by a pronounced increase at the beginning of or during the DNA synthetic period, strongly suggesting a gene dosage effect (HeLa cells: Pfeiffer & Tolmach [1968]; Terasima & Tolmach [1963]. Chinese hamster cells: Klevecz & Stubblefield [1967]; Stubblefield, Klevecz & Deaven [1967]; Crippa [1966]. Mouse fibroblast cells: Zetterberg & Killander [1965]). The reason for this variation in Chinese hamster cells at least has been resolved by Stambrook & Sissen [1972], who showed that both patterns of RNA synthesis could be obtained in the same cell line with the same precursor ([5-³H]-uridine), depending on the growth medium used; the different patterns being simply due to variations in the rate of precursor uptake into the cells, in the different

media. Consequently the precise pattern of RNA synthesis during the cell cycle in animal cells and the effect, if any, of gene replication has yet to be established. Clearly the suitability of pulse-labelling techniques (as employed in most of the above studies) for the measurement of rates of syntheses in animal cells is extremely doubtful in view of the above results, and alternative methods which are not based on comparisons of rates of precursor uptake (such as the isotope dilution method) should be reconsidered.

In conclusion, therefore, rRNA synthesis in Physarum polycephalum is similar to most other systems studied in that; (a) synthesis is continuous throughout the mitotic cycle except for a brief pause during mitosis itself (ciliates and yeasts are exceptions), and (b) the rate of synthesis increases throughout interphase. However, Physarum differs from several other lower eucaryotes (and also Escherichia coli) in that the rate of rRNA synthesis does not increase exponentially during interphase, but instead exhibits a 5-6 fold overall increase during this period.

II.3.6 Nuclear DNA synthesis during the mitotic cycle in Physarum polycephalum.

The accumulation curve for DNA synthesis during the mitotic cycle was determined by following the decrease in specific radioactivity of nuclear DNA after pre-labelling with [5,6-³H]-uridine, in an analogous way to that used for measuring rRNA synthesis in section II.3.2. This served both as a general control of the isotope dilution method, and as a useful comparison with published results obtained by pulse-labelling. Measurements were made on isolated nuclei to avoid any contribution by mitochondrial DNA synthesis. [³H]-uridine was used in preference to the more commonly used DNA precursor, thymidine, for the following reasons:

(a) The uptake of uridine into the cell and its subsequent metabolism have been well characterised (Birch & Turnock, unpublished work). Upon addition of isotope, incorporation is completed in 2-3h under the conditions used, and is followed by a "chase" period of 2-3 doublings after which only metabolically stable nucleic acids will be labelled, i.e. rRNA, tRNA, and DNA; and intracellular pools will have become entirely depleted.

(b) The cost of [^3H]-uridine is considerably less than that of [^3H]-thymidine.

(c) Preliminary results had indicated that both uridine and thymidine label proteins to some extent and consequently a purification procedure for DNA is required, whichever isotope is used. Radioactively labelled RNA is readily removed from nuclei, which have a DNA:RNA ratio of 1.2:1 (Mohberg & Rusch [1971]; nuclear RNA should be unlabelled in any case after the chase period), by alkaline hydrolysis. To check the efficiency of alkaline hydrolysis, 70 μg of purified [^3H]-rRNA (21,149cpm. μg^{-1}) was added to a sample of unlabelled Physarum nuclei and processed as described in section II.2.5. 99.995% of the radioactivity was converted to a form soluble in cold trichloroacetic acid.

Microplasmodia of strain M₃CVIII, grown for 24h in the presence of [5,6- ^3H]-uridine (50 μM , 0.5 $\mu\text{Ci.ml}^{-1}$, Amersham), were used as inoculum for the formation of a large surface plasmodium as described in section II.2.2(ii). Segments of the large plasmodium (excluding material from the region of the initial inoculum ring) were removed for analysis from early prophase of the second post-fusion mitosis (MII) to early prophase of MIII. Immediately after sampling, nuclei were prepared from each segment and subsequently processed as described in section II.2.5. The accumulation of DNA (given by the reciprocal of the specific radioactivity) as a function of time during the mitotic cycle is shown in Fig. 21.

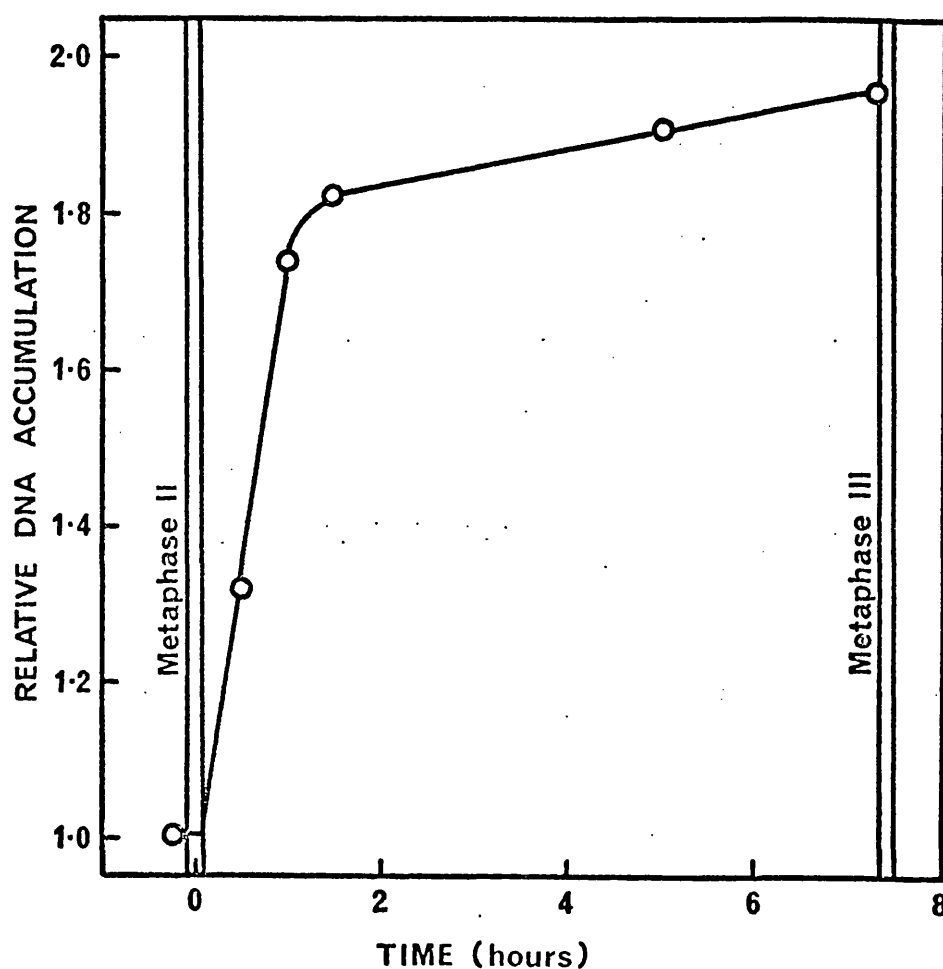


Fig. 21. DNA synthesis during the mitotic cycle in strain M_3CVIII .

A large plasmodium was established from microplasmodia labelled with [3H] uridine (see section II.2.2). Nuclei were prepared from segments taken between MII and MIII, and the specific radioactivity of DNA in each sample was determined. DNA accumulation (given by the reciprocal of the specific radioactivity) is plotted as a function of time during the mitotic cycle. The amount of DNA at MII was normalised to a value of 1.0.

The amount of DNA essentially doubled (96% increment) between MII and MIII clearly demonstrating that the plasmodium was in a balanced state of growth. Synthesis of DNA was rapid during the first 60-90min of the cycle, by which time 80% of the DNA had replicated. Following this the rate decreased substantially during the remainder of the cycle. This is qualitatively very similar to the results obtained by pulse-labelling (Nygaard, Guttess & Rusch [1960]; Braun, Mittermayer & Rusch [1965]; Braun & Wili [1969]) and chemical measurements (Mohberg & Rusch [1969]; Bovey & Ruch [1972]); and clearly confirms the absence of a significant G1 phase in this organism (Kessler [1967]; Braun, Mittermayer & Rusch [1965]), a property which is not uncommon in rapidly dividing cell types (Mitchison [1971]).

The 10% increase in DNA content during the G2 phase will correspond in part to the replication of the nuclear satellite DNA (Guttess & Guttess [1969]; Braun & Evans [1969]; Zellweger, Ryser & Braun [1972]; Newlon, Sonenshein & Holt [1973]) which includes the genes for rRNA (Zellweger, Ryser & Braun [1972]; Newlon, Sonenshein & Holt [1973]), although this satellite DNA only constitutes about 2% of the total nuclear DNA (Zellweger, Ryser & Braun [1972]). However, the presence of a second, slow phase of DNA synthesis during G2 is in agreement (a) with recent work on the units of replication of Physarum DNA (Brewer, Evans & Evans [1974]), which has indicated that synthesis may continue for a longer period than that deduced from pulse-labelling experiments, and (b) with cytofluorometric measurements of the DNA content of nuclei isolated at different times during the mitotic cycle (Bovey & Ruch [1972]). It should be emphasised that the second, slow phase of DNA synthesis is unlikely to be due to asynchrony within the plasmodium. The degree of synchrony, even within large plasmodia is normally very good (Mohberg & Rusch [1969]) except for nuclei in the inoculum ring, and in the cultures used for these experiments nuclei in

smears from different parts of a plasmodium were at the same stage of post-fusion mitosis III within 10-15min of each other.

The advantages of the isotope dilution technique are clearly seen in that (a) it does not involve the inherent uncertainties of pulse-labelling, (b) it is much more sensitive than direct chemical measurements which are particularly difficult with surface plasmodia due to their syncytial nature, and with isolated nuclei which have a rather low DNA content in Physarum, and (c) it indicates whether or not the culture is in a balanced state of growth.

II.3.7 Synthesis of rRNA during the mitotic cycle in strain CL.

Initially, the haploid plasmodial strain CL (Cooke & Dee [1975]) was chosen for the reasons outlined in section I.2.1. However it was found that this strain did not maintain a balanced state of growth up to mitosis III, although subsequent experiments with strain M₃CVIII handled in precisely the same way did exhibit balanced growth. The synthesis of rRNA from several hours before mitosis II until several hours after mitosis III (determined as described in section II.3.2) is shown in Fig. 22. The increase in the amount of rRNA between nuclear divisions is only 56% (an identical increment of 56% was also obtained in a second experiment). However, despite the lack of a doubling in the amount of rRNA between mitoses, the form of the accumulation curve is very similar to that found with strain M₃CVIII (see Fig. 17.), the rate of synthesis being very low just after nuclear division and increasing throughout interphase to a maximum at the end of the G2 phase. The approximate difference in the rate of rRNA synthesis between the beginning and end of interphase in CL was obtained by drawing tangents to the accumulation curve and found to be about 5-fold, again very similar to strain M₃CVIII.

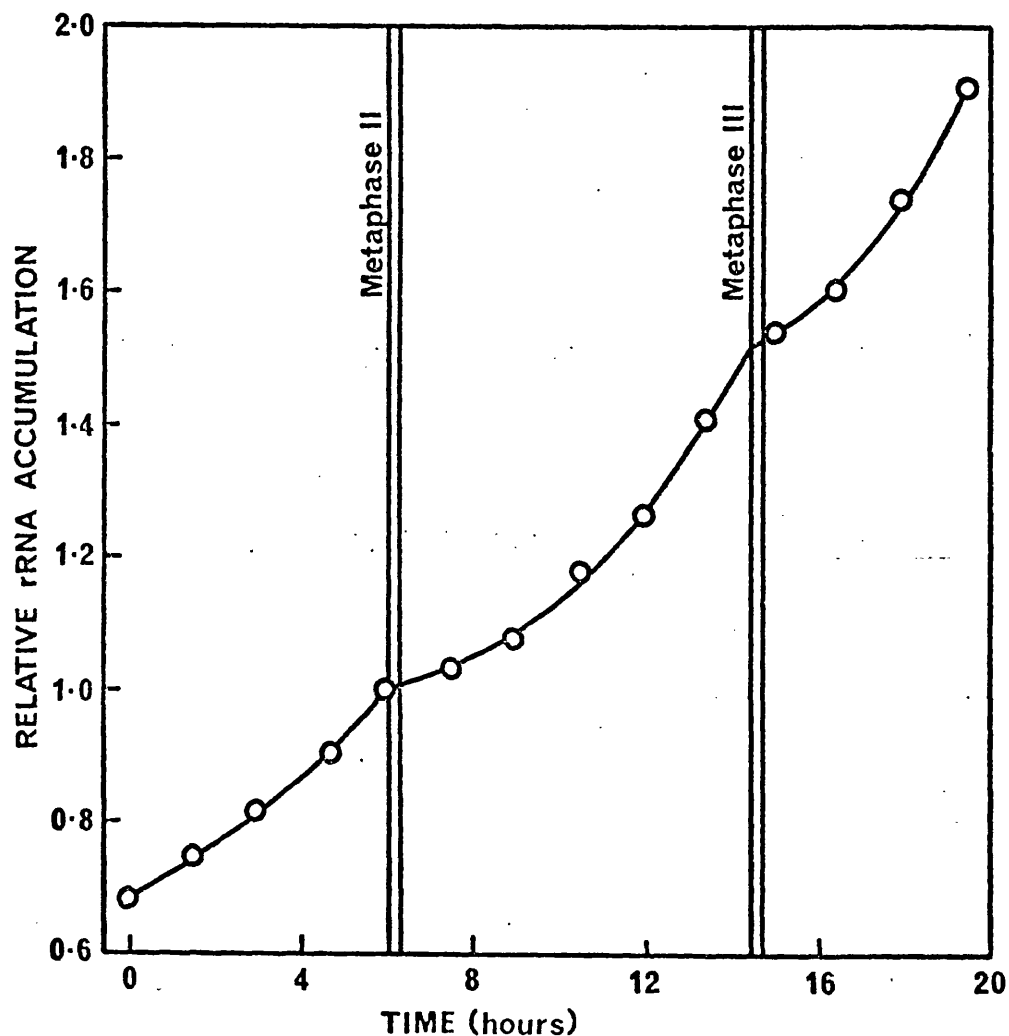


Fig. 22. Synthesis of rRNA during the mitotic cycle in strain CL.

A large surface plasmodium was established from microplasmodia labelled with [^3H]-uridine (see section II.2.2), and rRNA isolated from samples taken from a few hours before MII until a few hours after MIII. The accumulation of rRNA (given by the reciprocal of its specific radioactivity) is plotted as a function of time in relation to MII and MIII. The data are normalised to a value of 1.0 at MII.

II.3.8 Synthesis of DNA during the mitotic cycle in strain CL.

The results of three experiments in which the increment in nuclear DNA between early prophase of post-fusion mitoses II and III in plasmodia of strain CL was measured, are given in Table 7. The increase in DNA in large plasmodia ranged from 30-42%, confirming that balanced growth is not maintained up to mitosis III in strain CL. However, in experiment 3, in which small petri-dish cultures (up to 6cm in diameter) were established from the same suspension of microplasmodia used to prepare the large, tray culture, the DNA content of the small plasmodia did double in amount between MII and MIII. Hence the sub-line of strain CL being used was at least capable of balanced growth under some conditions. Furthermore, at mitosis II nuclear DNA from both large and small plasmodia had the same specific radioactivity, indicating that up to that point the large culture had grown normally.

Two possibilities can be considered, to explain the inadequate increase in DNA content between MII and MIII in strain CL. One is that only 30-40% of the nuclei double their DNA content in the normal way, and the other is that some or all of the nuclei replicate their DNA only partially. To distinguish between these two possibilities the DNA contents of individual nuclei, isolated at various times up to post-fusion mitosis III, were determined by microdensitometry and the results are shown in Fig. 23. Small petri-dish cultures have a mean G2 phase DNA content of about 0.6pg/nucleus (apart from about 10% which appear to be diploid), being slightly higher in late G2 (graph [B]) than in early G2 (graphs [A] and [C]) as expected. This is also found up to early prophase of mitosis II in large plasmodia (graphs [D] and [E]) but at early prophase of MIII two nuclear sizes are clearly present (graph [F]); the larger one having the expected late G2 phase DNA content of approximately 0.6pg/nucleus, and the smaller one about half of this. Following nuclear division only

Table 7. Increase in nuclear DNA between post-fusion mitoses II and III in plasmodia of strain CL.

Plasmodia, pre-labelled with [5,6-³H]-uridine, were set up either in the large stainless steel tray (inoculum size, 7ml) or in petri-dishes (inoculum size, 0.3ml). The specific radioactivity of the DNA in nuclei isolated during early prophase of mitoses II and III was determined as described in section II.2.5, and the increment in DNA calculated (from the reciprocal of the specific radioactivity). In experiment 3(ii), in which small plasmodia were established from the same inoculum used for 3(i), nuclei from 2-3 cultures were pooled for each nuclear isolation.

Experiment.		Specific activity of DNA.		Increment
		Mitosis II.	Mitosis III.	in DNA.
		cpm.µg ⁻¹		%
1	Large plasmodium	612	432	42
2	Large plasmodium	479	362	32
3(i)	Large plasmodium	251	193	30
3(ii)	Small plasmodia	249	125	99

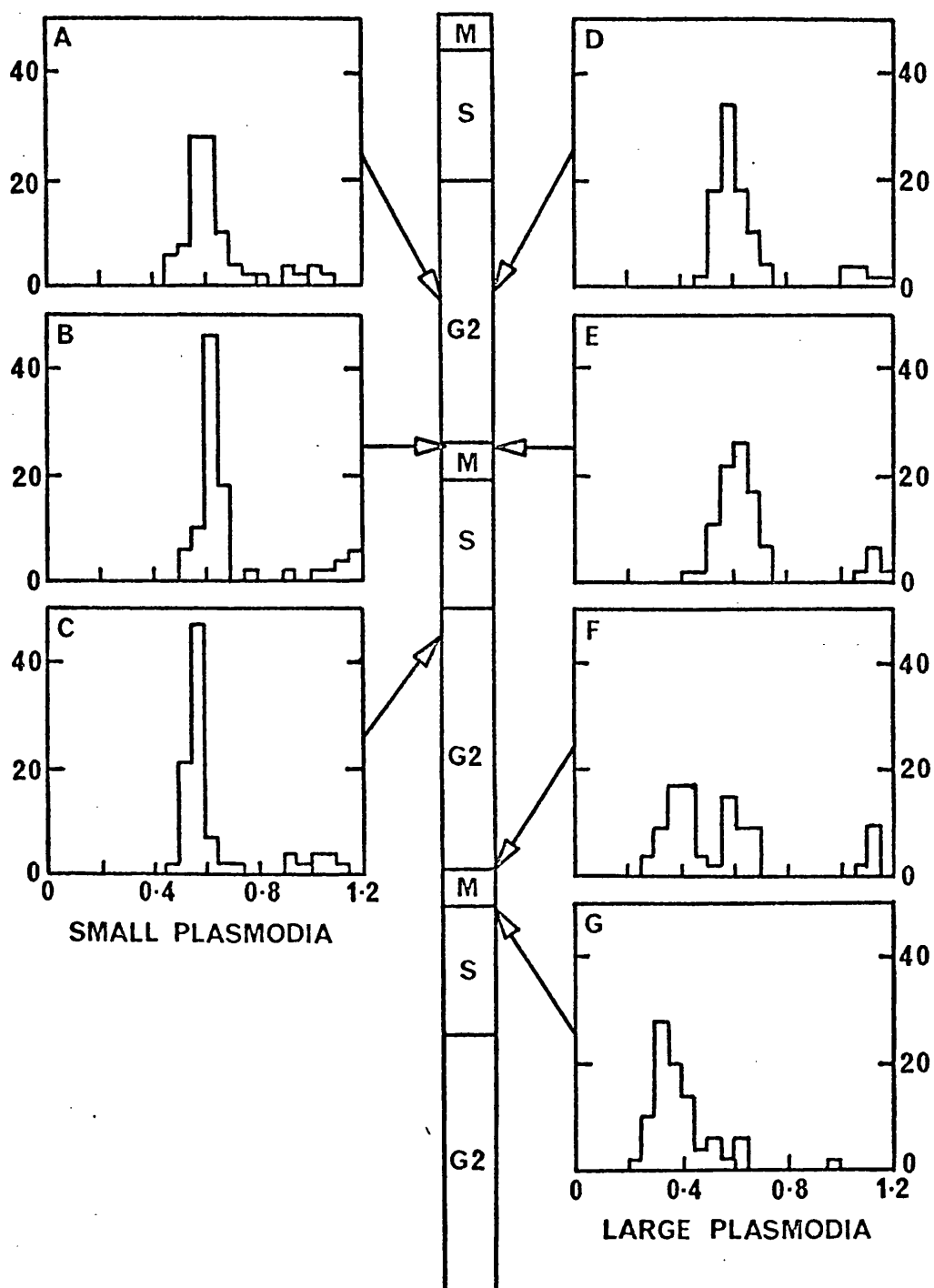


Fig. 23. DNA contents of nuclei isolated from small and large surface plasmodia between post-fusion mitoses I and III.

A and D, 3h before MII; B and E, early prophase of MII; C, 3h after MII; F, early prophase of MIII; G, early reconstruction of MIII.

Abcissa: pg of DNA. Ordinate: nuclei (% of total).

a single peak is found (graph [G]) with a mean DNA content of about 0.3 pg/nucleus (the few nuclei containing 0.6pg probably arise from division of the small number of diploids). The most reasonable explanation to account for this aberrant behavior is that nuclei divide normally during post-fusion mitosis II, but only about 30-40% of them replicate their DNA during the subsequent S phase. This would then give rise to two peaks as seen in graph [F] of Fig. 23. During mitosis III only nuclei which have completely replicated their DNA will divide, resulting in a single peak (graph [G]) with a mean DNA content of about 0.3pg/nucleus.

It should, perhaps, be pointed out that CL was one of the three strains used to observe the effect of ploidy on the number of rRNA genes in Physarum, described in Part I. During this study large plasmodia were grown to post-fusion mitosis IV before harvesting; yet the saturation value obtained for hybridisation between rRNA and DNA was identical to that from the other two strains. This suggests that either (a) the sub-line used for the ploidy experiments, which was obtained from a different amoebal stock to that used in the present study, was capable of balanced growth up to post-fusion mitosis IV, or (b) during imbalanced growth the stringent control mechanisms for DNA synthesis still operate so that nuclei either completely replicate their DNA or do not synthesise DNA at all. This latter hypothesis is compatible with the observation of two peaks in DNA content at early prophase of MIII as seen in Fig. 23[F].

Although the microdensitometric measurements on isolated nuclei may provide a reasonable explanation as to what is happening in strain CL during its period of imbalanced growth, it does not give any indication as to why it occurs. Departure from a steady state of growth would appear to occur suddenly, sometime after post-fusion mitosis II; yet it is not observed in smaller (petri-dish) cultures. An unusual observation is that the intermitotic period between MII and MIII in these large cultures

is often significantly shorter (by about 10-15%) than in the smaller petri-dish cultures, although no explanation can be offered as to why this should happen.

Certainly the importance of checking the growth characteristics of a new strain or condition of culture, before using it to investigate biochemical parameters in relation to the mitotic cycle, is clearly seen. Measurement of the increment in DNA between successive mitotic divisions, using the assay described above, would appear to be a useful and sensitive index of the growth of a culture. However, it should be emphasised that at no time has the strain M_3CVIII been found to deviate from a balanced state of growth, both as small and large surface plasmodia. Furthermore, in experiments with a related M_3C strain, Mohberg & Rusch [1969] carried out chemical measurements of RNA, DNA and protein both on large rocker cultures and on petri-dish cultures, and demonstrated balanced growth beyond post-fusion mitosis III. In further work in the same laboratory (Mohberg [1974]), a doubling in the amount of DNA in each successive mitotic cycle up to post-fusion mitosis V was observed in a rocker culture, although it should be noted that the plasmodium was reduced in size by appropriate surgery a few hours after mitosis III, thereby preventing it from reaching a very large size. Thus the possibility of obtaining a balanced state of growth for several mitotic cycles in a reasonably large plasmodium would appear to be well established. The strain CL is somewhat unusual in that it is haploid in both the amoebal and plasmodial stages of the life cycle (Cooke & Dee [1974]), plasmodia arising by apogamy (Cooke & Dee [1974]; Cooke & Anderson, personal communication). It may be that the aberrant growth behavior of strain CL is just another peculiarity of this unusual strain.

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