SOME ASPECTS OF RNA SYNTHESIS DURING THE CELL CYCLE OF THE FISSION YEAST, *SCHIZOSACCHAROMYCES POMBE*

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DECLARATION

With the exception of Chapter 4, all the work in this thesis has been composed and carried out by me alone. In Chapter 4, the development of the method and the measuring of the rates of stable RNA synthesis were carried out in conjunction with Dr W.H. Wain, as stated at the beginning of that Chapter. The results of our collaboration have been published, and a copy of the article is found in Appendix 1.

For Christina and Samuel.

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CHAPTER I

GENERAL INTRODUCTION

In this thesis I wish to present some information about the cell cycle patterns of RNA synthesis and uridine uptake in the fission yeast, Schizosaccharomyces pombe. The synthesis of RNA and the uptake of metabolites used in its synthesis have been examined during the cell cycles of a variety of cellular material by a number of workers. Much of this work has been reviewed by Mitchison (1971), but relevant work has been published more recently (e.g. Eikhom, Abraham & Dowben, 1975; Sogin, Carter & Halvorson, 1974; Wolfe, 1972, 1975). The work generally falls into two major categories: measurements of the accumulation of RNA or nucleotide pool material often by colorimetric or cytological assays (Mitchison & Walker, 1959; Zetterberg & Killander, 1965; Stebbing, 1971); and of the rates of precursor uptake or RNA synthesis using pulse treatments of radioactively labelled tracers (Pfeiffer, 1968; Stambrook & Sisken, 1972,a,b; Wolfe, 1972, 1975). There are two important distinctions between these types of study. First, accumulation is not synthesis. It is the difference between synthesis and degradation, an important point in light of the rapid rates of turnover associated with some types of RNA (Brandhorst & Humphreys, 1972; Fraser & Creanor, 1974). And second, the rate of synthesis or uptake is the first derivative with respect to time of the amount synthesized or taken up. This allows the pattern of synthesis to be described much more precisely than by measurements of accumulation. But if pulses of radioactive tracer are to be used to measure rates of synthesis, care must be taken in designing the experiment to ensure that the specific activity of the pool of the immediate precursor to the macromolecule

being synthesized remains constant during the experiment (see Mitchison, 1971, for an extensive discussion of these and related problems).

The cell cycle of a growing cell is commonly considered to be the sequence of events that a freshly divided cell passes through until it has itself divided into two daughter cells. During the cell cycle the constituents of the cell increase in mass in an ordered way in preparation for the coming division. The activities of the cell cycle are usually divided into an initial gap period (G_1), a period of DNA synthesis (S), a second gap period (G_2), and mitosis (M). The length of a cell cycle and the relative lengths of its constituent parts is constant and characteristic for a given cell type in a given environment. When the fission yeast, Schizosaccharomyces pombe, is grown at 32°C in minimal medium, it has a 140 min cell cycle composed of a 30 min G_1, a 15 min S, a 90 min G_2, and a 5 min M (Mitchison & Creanor, 1971,b). But such a listing does not present the entire picture of events because in S. pombe nuclear division occurs approximately 30 min before cell division. Cell division, therefore, occurs about the time of DNA synthesis in these cells.

RNA SYNTHESIS

The manner in which the rate of RNA synthesis changes during the cell cycle varies considerably depending on the type of cell examined. Generally, however, RNA synthesis doubles in rate either by a continuous, smooth increase throughout the cycle (Tsai & Green, 1973; Stambrook & Sisken, 1972,a; Gorman, Taruo, LaBerge & Halvorson, 1964; Sogin, Carter & Halvorson, 1974) or by a sharp, step-like increase during a restricted part of the cycle (Eikhom et al., 1975; Pfeiffer, 1968; Pfeiffer & Tolmach, 1968; Wolfe, 1972). In those cells which condense

their chromosomes during mitosis, the rate of RNA synthesis is greatly reduced during the mitotic period (Stenman, 1971; also Mitchison, 1971, p. 116 lists 16 further references). Stein and Farber (1972) were able to mimic this mitotic cessation of RNA synthesis *in vitro* by adding mitotic phase chromosomal non-histone protein to S phase chromatin. In the slime mold, *Physarum polycephalum*, the rate of RNA synthesis not only decreases at the time of nuclear division. There is a second decrease in rate during G_2, but the reason for its occurrence is unknown (Mittermayer, Braun & Rusch, 1964).

In many systems, the rate of RNA synthesis appears to be proportional to the DNA content of the cells, ranging from bacteria (Dubnau, Smith & Marmur, 1965; Donnelan, Nags & Levinson, 1965) to sea urchin eggs (Flickinger & Humphreys, 1974; von Ledebur-Villiger, 1972) and cultured mammalian cells (Pfeiffer & Tolmach, 1968; Novi & Baserga, 1972; Eikhom *et al.*, 1975). The rates of synthesis of the major RNA species in HeLa cells may be under such a gene dosage control (Pfeiffer, 1968). The rates of non-ribosomal RNA synthesis in mouse fibroblasts (Mauck & Green, 1973) and possibly in the budding yeast *Saccharomyces cerevisiae* (Sebastian, Takano & Halvorson, 1974) could also be under similar control. Furthermore, some evidence suggests that the rate of synthesis of ribosomal RNA (r-RNA) in *S. cerevisiae* doubles early in the S period (Fraser & Carter, 1976). But on the other hand, this rate may vary throughout the cell cycle without reflecting gene dosage changes (Sogin *et al.*, 1974). Both the RNA content and the rate of total RNA synthesis in this yeast do, however, reflect cell size and overall rate of growth (Schweizer & Halvorson, 1969; Weiss, Kukora & Adams, 1975). Other cell types which show continuous increases in their rates of RNA synthesis may also be pacing these rates to increases in either cell mass or

volume (e.g. Cattolico, Senner & Jones, 1973; Edwards, Stratham & Lloyd, 1975).

Membrane associated phenomena also play a part in the regulation of RNA synthesis in many cells. The synthesis of either total- or selected types of RNA can be stimulated by treating cells with hormones (Luck & Hamilton, 1972; O'Malley, Rosenfeld, Comstock & Means, 1972) and cyclic AMP (c-AMP) and its dibutyryl derivative, db-c-AMP (Rosenfeld, Abrass, Mendelsohn, Roos, Boone & Garren, 1972; Foury & Goffeau, 1975). Furthermore, colicins and inhibitors known to act at the cell surface may also inhibit RNA synthesis (Luria, 1975; Crifò, Bozzi, Mondoví & Strom, 1973).

Most of these possible regulators of RNA synthesis are not necessarily mutually exclusive. One type of control may set the overall level of RNA synthesis while another modulates its pattern about that level (Pfeiffer, 1968; Krider & Plaut, 1972).

PRECURSOR UPTAKE

The active uptake of metabolites by cells can be mediated by general carriers, such as the non-specific deoxypurine transport system suggested for Novikoff rat hepatoma cells (Plagemann & Erbe, 1974,b) and the common nucleoside diphosphate kinase found in human glial cells (Ågren, Pontén, Ronquist & Westermark, 1974). On the other hand, uptake is often mediated by substrate specific transport sites. In budding yeast separate permeases and kinases have been demonstrated for such closely related compounds as uracil and uridine (Grenson, 1969). Uridine and adenine uptake by S. pombe is also considered to proceed by way of substrate specific transport systems (Cummins & Mitchison, 1967; Foury & Goffeau, 1975).

Although metabolite uptake is ultimately dependent upon use of the metabolite by the cell, the rate of uptake can vary independently of the rate of use. The amino acid pools of Candida (Cowie & McClure, 1959) and the adenine pool of S. pombe (Cummins & Mitchison, 1967) both expand when there is an external supply of adenine. During the period of this pool expansion, the rate of uptake is temporarily faster than the rate of adenine incorporation. On the other hand, if the pool remains constant and the endogenous supply is also constant, then the rates of uptake and incorporation must be the same. Either rate, however, may be rate limiting.

The cell cycle patterns of change in the rates of uptake vary with both the substrate being transported and the cell type studied. However, a majority of the patterns observed fall into one of three classes:

1. intermittent uptake activity with peaks in the rate;
2. continuous uptake activity, but with the rate doubling sharply at a point in the cycle; or
3. continuous increase in rate throughout the cycle.

In both Tetrahymena (Stone, Miller & Prescott, 1965) and mouse L cells (Adams, 1969) ^{3}H-thymidine is only taken up during the period of DNA synthesis. During the remainder of the cell cycle a small nuclear pool of TTP appears to be maintained, but not turned over. Uridine uptake in Tetrahymena (Wolfe, 1975) in mouse L cells and in Chinese hamster ovary cells (Sander & Pardee, 1972), on the other hand, occurs throughout the cycle. But the rates of uptake double sharply once per cycle. In the hamster cells the rates double late in the G_1 phase, while in Tetrahymena the rates begin to double with the onset of DNA synthesis. Furthermore, Sander and Pardee reported that the rate of thymidine uptake paralleled that of uridine. Different culture con-

ditions may account for the difference between these results and those of Adams (1969).

By altering the growth medium, Stambrook and Sisken (1972,a) were able to convert the pattern of uridine uptake from one of periodic rate changes to one of a continuous rate increase throughout the cell cycle. This change was fully reversible and could be induced by growing the cells for one generation in the new medium. A linear increase in the rates of uridine and 2_deoxy-D-glucose uptake have also been reported to occur in synchronous cultures of Novikoff rat hepatoma cells, although the rates of choline, hypoxanthine and thymidine uptake appear to increase discontinuously (Plagemann, Richey, Zylka & Erbe, 1975).

A number of possible mechanisms have been described by which cells may control their rates of metabolite uptake. By far the most common type of these involves some sort of feedback from the intracellular metabolite pool. Feedback mechanisms have been suggested for the control of valine uptake in sea urchins (Mitchison & Cummins, 1966), and thymidine uptake in both mouse L cells (Adams, 1969) and Tetrahymena (Stone et al., 1965). In budding yeast, permeases specific for uridine, uracil and histidine have also been shown to be under feedback control (Grenson, 1969; Crabeel & Grenson, 1970), as has the adenine transport system in S. pombe (Cummins & Mitchison, 1967). Carter and Halvorson (1973,a) noted that in budding yeast a number of amino acids were transported by separate sites. Furthermore, the rates of uptake of these amino acids doubled sharply at different points in the cell cycle. They suggested that these sharp, step-like rate doublings were produced by periodic synthesis of uptake sites in a manner analogous to the periodic synthesis of enzymes.

Fluctuations in the energy supply to the active transport sites

could also produce changes in the rate of metabolite uptake. This energy may be supplied either by direct coupling to an energy-yielding reaction, or by harnessing the free energy present in an electrochemical gradient of some second molecular species (Stein, 1967). Eddy and Nowacki (1971) presented evidence that the active uptake of amino acids in Saccharomyces carlsbergensis is driven by the active transport of either potassium ions or protons. Edmunds and Cirilla (1974) argue that fluctuations in these ion exchange mechanisms could not only generate oscillations in the amino acid transport patterns, but may be the basis of many other cyclical biological events as well.

RNA SYNTHESIS AND PRECURSOR UPTAKE BY S. POMBE

In the fission yeast, Schizosaccharomyces pombe, RNA synthesis and precursor uptake have been examined in a series of papers by Mitchison and his co-workers. Mitchison and Walker (1959) demonstrated that RNA accumulated continuously throughout the cell cycle, with no interruption during division. But their methods were too imprecise to distinguish between a linear increase and an exponential one. The rates of RNA synthesis and precursor uptake were then examined by autoradiography (Mitchison & Lark, 1962; Mitchison, 1963; Cummins & Mitchison, 1964; Mitchison, Cummins, Gross & Creanor, 1969). Two distinct patterns of rate change emerge from these studies. A sudden doubling in the rate of ^{3}H-adenine uptake is apparent in the data of Cummins and Mitchison (1964). In this study the rate appears to remain constant until the second half of the cell cycle, and then doubles sharply. The data of Mitchison and Lark (1962) suggest that the rate of ^{3}H-adenine incorporation also increases during the first two-thirds of the cell cycle. The rate then remains constant until cell division.

But there are similar patterns of change in cell volume and dry mass (Mitchison, 1957) and these two patterns may be related.

After examining the rates of uptake and incorporation of all four RNA bases and two nucleosides, Mitchison et al. (1969) concluded that the rates of RNA synthesis and initial precursor uptake, as well as the sizes of the pre-existing precursor pools and of the expanded adenine pool, steadily increase through the cell cycle following roughly the increase in cellular dry mass. Stebbing (1971) further reinforced these latter conclusions by presenting evidence from synchronous cultures suggesting that both total RNA and pool nucleotide material accumulate exponentially during the S. pombe cell cycle.

While we were devising a method to measure the rates of ribosomal (r-) and heterogeneous nuclear (Hn-) RNA synthesis, Dr W.H. Wain and I noted that the rate of ^{3}H-uridine incorporation in synchronous cultures of S. pombe doubled sharply at the end of the cell cycle. Further experiments using radioactively labelled uridine and adenine established that our results reflected changes in the rate of total RNA synthesis (Wain & Staatz, 1973). This doubling in the rate of RNA synthesis occurs at the time of both cell division and DNA synthesis (Mitchison & Creanor, 1971,b) and may reflect a gene dosage control of transcription. These findings and the corresponding changes in the rates of precursor uptake became the basis for Chapter IV, and the relationships of the rates of RNA synthesis and precursor uptake to each other and to DNA synthesis and cell division are the topic of investigation of this thesis.

2'-DEOXYADENOSINE

One agent which has been used to both induce division synchrony and to lengthen the cell cycle of selection synchronized cultures of S. pombe is 2'-deoxyadenosine (AdR) (Mitchison & Creanor, 1971,a). In the presence of this drug, DNA synthesis is inhibited within about an hour. Since DNA is normally made just after cell division in S. pombe, virtually all the cells are able to divide once after AdR has been added. In log phase cultures cell numbers continue to increase exponentially for about a generation, while in selection synchronized cultures there is one burst of synchronous division. Thereafter cells continue to grow without dividing for at least 4 hrs. However, neither of these cell number increases is accompanied by DNA synthesis. About an hour after removing the AdR there is a burst of DNA synthesis in both types of culture. This is followed immediately by a synchronous round of cell division which is accompanied by a normal S period. A second, apparently normal synchronous division follows 60 min after the first.

Deoxyadenosine, like thymidine, is a normal metabolite in the DNA synthetic pathway. Also like thymidine, at high concentrations AdR inhibits DNA synthesis in a variety of cells (Klenow, 1959; Lark, 1960; Young & Hodas, 1965). It may, therefore, produce side effects similar to those reported in cells which have been given a thymidine block (Churchill & Studzinski, 1970; Meyn, Hewitt & Humphrey, 1973). In fact Xeros (1962) showed thymidine to be less harmful to mammalian cells than either AdR or deoxyguanosine. AdR also inhibits dihydroorotase activity (Bresnick & Blatchford, 1964) and deoxyriboside transport (Plagemann & Erbe, 1974,b) in Novikoff ascites cells. In cell free extracts of chick embryos the synthesis of dCMP is inhibited and its use stimulated by the AdR/derivative dATP (Reichard, Canellakis & Canellakis, 1960).

And it may well be at this latter point that AdR interferes with the cells mechanism for governing DNA synthesis.

In S. pombe, three enzymes whose activities increase by periodic steps in selection synchronized cultures show patterns of smooth, continuous patterns of increase in induction synchronized cultures (Sissons, Mitchison & Creanor, 1973). Two further enzymes in these cultures showed steps in their potential following either type of synchronization. The timing of these steps in potential appears to be related to the time of DNA synthesis and independent of the time of cell division. Such differences in the patterns and timing of events led Mitchison (1973) to discuss the possibility of two sequences of events occurring during the normal cell cycle: the "DNA-division cycle" or "DD-cycle" and the "growth cycle". In cultures which have been synchronized by selection, the relationship between these two cycles remains unaltered from what it was in exponential phase cultures. But AdR induces synchrony only in the DD-cycle, leaving the growth cycle still exponential. This is supported by exponential increases in optical density, and in the accumulation of total RNA and protein in induction synchronized cultures (Mitchison & Creanor, 1971,a). When a selection synchronized culture is treated with AdR, the relationship between these two cycles is simply shifted in proportion to the length of treatment. By examining rates of precursor uptake and incorporation in such cultures and comparing the results to those obtained in normal selection synchronized cultures, it should be possible to assign changes in these rates to one or the other cycle.

PHENYLETHANOL

Another possible inhibitor of DNA synthesis in S. pombe is phenylethanol (PE), often reported in the literature as either benzylcarbinol or phenethyl alcohol. Lilley and Brewer (1953) first reported its growth inhibiting properties: 0.3% PE inhibited growth of most bacteria, yeasts and moulds tested. PE was at first thought to preferentially inhibit the synthesis of either DNA (Berrah & Konetzka, 1962; Treik & Konetzka, 1964) or RNA (Monoyama & Ikeda, 1964; Prevost & Moses, 1966). But further work indicated that the primary site of action was in cellular membranes (Silver & Wendt, 1967; Normark, 1971; Nunn & Tropp, 1972; Wright, Ceri & Lewis, 1973), and most of the other effects of the drug can be traced to the involvement of membranes in such aspects of growth and metabolism as DNA synthesis (Masker & Eberle, 1972), respiration and photosynthesis (Knutsen, 1966; Terenzi & Storck, 1969) and protein synthesis (Plagemann, 1968,a,b).

Budding yeast has two classes of response to PE (Burns, 1968). One of these is based upon a primary inhibition at the cell membrane and includes both DNA and RNA syntheses. The other class requires equal inhibition at both the membrane and some internal sites and includes glucose uptake. At concentrations of 0.5% or greater, PE also upsets the cytoplasmic sol-gel balance in this yeast (Burns, 1971).

The one report of PE being used on S. pombe (Bostock, 1970,b) demonstrated that although 0.3% PE completely inhibits DNA synthesis, cells grow in 0.2% PE with a generation time of about 4-4.5 hr. The increase in generation time is primarily due to a 2hr G_1 period being inserted into an otherwise unperturbed cell cycle. Furthermore, when 0.2% PE is added to a log phase culture, DNA synthesis ceases for 2 hr while cell division and cell growth continue at a reduced rate.

Thereafter DNA synthesis resumes at a rate comparable to that of cell growth.

From these reports it is clear that PE is not likely to be a selective inhibitor of DNA synthesis in S. pombe. But by allowing the cells to equilibrate and grow in its presence it should be possible to make a culture of selection synchronized cells in which DNA synthesis is separated from cell division by about half a cell cycle. In such a culture, events which occur near the end of the cell cycle in normal cells will still occur there if they are related to the growth cycle or to cell division. But if they are associated with DNA synthesis they may occur in the middle of the cell cycle. If such DNA-related events occur in proportion to the amount of DNA present, they too should stop increasing during the 2 hr following PE addition to an exponential culture. In this latter case, however, a more direct effect of PE on precursor pools have to be ruled out.

STEP-DOWN

Previous work suggested that in S. pombe the rate of RNA synthesis increased more or less exponentially during the cell cycle. When this thesis was begun, it was, therefore, decided to see if the rate at which a particular species of RNA was made during the cycle varied from that of total RNA. Since total RNA is mainly ribosomal, a minor species was considered more likely to show some sort of variation. Since heterogeneous nuclear (Hn-) RNA was being widely discussed in the literature, I decided to examine its synthesis. Hn-RNA is generally described as very large (20 to 300 S), rapidly labelled RNA of hetero-disperse size and with a short half-life (Armelin & Marques, 1972; Aronson, 1972; Darnell, 1968; Shearer & McCarthy, 1970). It contains

sequences of polyadenylic acid (poly(A)) and polyuridylic acid (poly(U)) (Molloy, Thomas & Darnell, 1972; Greenberg & Perry, 1972,a,b; Jelinek, Adesnik, Salditt, Sheiness, Wall, Molloy, Philipson & Darnell, 1973), but its sequences and secondary structure are considered to be more similar to DNA than to r-RNA (Jelinek & Darnell, 1972; Smith, Hough, Chamberlin & Davidson, 1974). It has been found in mammalian cells (Darnell, 1968), echinoderms (Aronson, 1972), amphibians (Loening, Jones & Birnstiel, 1969; Sommerville & Hill, 1973), insects (Armelin & Marques, 1972) and higher plants (Loening et al., 1969). On the other hand, Prescott, Stevens and Lauth (1971) were unable to find any high molecular weight "Hn-" RNA in either Amoeba proteus or in the literature concerning any other unicellular eukaryote. Later work has, on the whole, continued to support this claim (Sillevis-Smitt, Vermeulen, Vlak, Rozyjn & Molenaar, 1972; Prescott, Bostock, Gamow & Lauth, 1971). However, Melera and Rusch (1973) reported that the myxomycete, Physarum, may contain a giant heat labile RNA with a uridine/methyl ratio different from that of r-RNA. In S. pombe, Taber and Vincent (1969) found no RNA larger than 38 S. It and all other RNA fractions larger than 16 S were shown to be ribosomal. Udem and Warner (1972) also found no mammalian-like high molecular weight Hn-RNA in budding yeast. But they argued that analogous RNA could exist as smaller species which underlie the ribosomal peaks in gels or gradients. Hn-RNA as small as 20 S has, in fact, been reported to occur in mammalian cells (Darnell, 1968).

To test their hypothesis in S. pombe, I decided to try to examine the types of RNA made when rRNA was suppressed. One method which was considered for inhibiting ribosomal synthesis selectively was a step-down first devised for bacteria (Hayashi & Spiegelman, 1961). The

rationale behind this procedure was that since the RNA content of a cell is proportional to its growth rate and since most RNA in a cell is ribosomal, by rapidly decreasing the growth rate the cells would be left with too many ribosomes. The cells should, therefore, stop making ribosomes until the ribosomal concentration has been adjusted to the new growth rate. Since at that time it was thought that all m-RNA was rapidly turned over, Hayashi and Spiegelman felt that mRNA synthesis should be relatively unaffected. With S. pombe a rapid decrease in growth rate was produced by transferring an exponentially growing culture from complete medium to a minimal medium containing 4.75% enthanol (Gross & Mitchison, 1962). During the half hour following such a step-down, many growth processes in this yeast were either stopped or greatly reduced. The RNA that was made did not appear to be ribosomal. It had DNA-like base ratios and heterodisperse sedimentation characteristics (Mitchison & Gross, 1965). In an autoradiographic study of uptake and incorporation of all four RNA-bases following step-down, Mitchison et al. (1969) found about half of the step-down RNA (SD-RNA) was located in the cytoplasm after a short pulse label. As various RNA fractions became better understood, interpretations of results from step-down experiments have reflected these changes. Thus, in discussing their results, they suggested that step-down RNA "may be similar to cytoplasmic RNA associated with ribosomes" (i.e. m-RNA), or it "may equally well be a mixture of several types of RNA" (Mitchison et al., 1969, p. 421). By relating cells to their position in the cell cycle according to their length they also demonstrated a cell cycle stage-dependant change in the base ratios of SD-RNA. This change did not appear to be caused by changes in uptake rate or pool size.

In following up this work, I became aware of the complexity of SD-RNA. It is likely that many gene products are affected in an analogous way to r-RNA. Also, because of its heterodisperse nature, SD-RNA proved difficult to analyse. On the technical side, the step-down was superseded by the much more powerful methods for isolating m-RNA and Hn-RNA based on their polyadenylate residue (e.g. Swan, Aviv & Leder, 1972). At the same time our experiments began to indicate that total RNA synthesis might double in rate discontinuously during the cell cycle. Because of the technical difficulties in the further analysis of SD-RNA and because the work on total RNA merited further work, the work on SD-RNA was abandoned. The step-down now appears to be a useful procedure mainly for the study of how cells control their ribosome concentration.

INTENT OF THE THESIS

The objective of this thesis is to examine the rate of RNA synthesis during the cell cycle of the fission yeast, Schizosaccharomyces pombe. An attempt has been made to establish the specifity of ^{3}H-uridine in labelling RNA in this organism. This has been followed by measurements of the relative amounts of the different RNA species which are labelled after pulses of different lengths. The rates of RNA synthesis and ^{3}H-uridine uptake have been monitored in cultures which were synchronized by gradient selection and by induction using AdR. Similar rate measurements were also made in selection synchronized cultures treated with AdR or PE. The results were compared to changes in DNA content, cell number, total RNA content, cell division and dry mass of the cultures. Suggestions have been made as to how the cell might control the rates of RNA synthesis and precursor uptake.

CHAPTER II

MATERIALS AND METHODS

THE ORGANISM

The fission yeast, Schizosaccharomyces pombe, Lindner (1893), strain N.C.Y.C. 132, is a rod-shaped Ascomycete which, during exponential growth, is between 6 and 20 μm long and about 4 μm in diameter. It has a mean generation time of 2 hr 20 min at 32°C, normally growing in length only. Division is by formation of a median septum usually called a "cell plate" in the literature. Cell separation occurs by splitting this cell plate. Strain 132 is haploid and normally has a 2C value of 28.2 fg of DNA per cell (Mitchison & Creanor, 1971,a).

CULTURE CONDITIONS

Edinburgh Minimal Medium 2 (EMM2), the medium used routinely for this work, is a defined minimal medium described by Mitchison (1970). It uses 10 g/l of glucose as the main carbon source, 5 g/l NH_4Cl as the nitrogen source and 300 mg/l NaH_2PO_4 supplies the phosphate. The pH is maintained by 1 g/l sodium acetate which may act as a secondary carbon source as well. Cells enter stationary phase at a density of about 10^8 cells/ml because of limiting supplies of glucose and sulphate.

For experiments involving the incorporation of ^{32}P into nucleic acids, and for step-down experiments, another medium, EMM 1, was used. This medium is identical to EMM2 except that it contains 1/30 of the phosphate, with the result that phosphate is the growth limiting factor and cells begin to enter stationary phase at about 1×10^7 cells/ml.

Cells used for the step-down experiments were grown up in 2% malt extract broth (Oxoid).

Stock cultures of S. pombe were maintained in 10 ml of EMM2 in universal bottles or McCartney bottles. Stocks were sub-cultured every few days by inoculating a bottle of fresh medium with a loopful of stationary culture (10^8 cells/ml). After inoculation the cultures were maintained at 32°C for 2 to 3 days, then stored at room temperature and either used within a week or discarded. Reserve cultures were also kept on Malt Extract Agar (Oxoid). These were sub-cultured every few months and stored at 4°C after the initial 2 to 3 day incubation at 32°C.

For experiments, only cultures in mid-log phase (0.5 to 8 x 10^6 cells/ml) were used. These cultures were inoculated with about 2½ ml of stationary culture per liter. While they were growing up over night at 32°C they were either stirred continuously by a magnetic stirrer or they were shaken at 200 rpm on an orbital shaker. During the progress of the experiments the culture flasks were kept in a 32°C water bath and shaken vigorously every 10 min.

Routine sterile techniques were used during the handling of cells, except during experimental manipulations.

OPTICAL DENSITY, CELL NUMBER AND CELL PLATE INDEX

The optical density (OD) of a culture has been used as a measure of the dry mass of that culture (Mitchison, 1970). With a calibration curve of OD vs cell number the optical density can be used as a measure of the cell numbers in a normal, exponentially growing culture. Optical densities were routinely measured at 595 nm using a Unicam SP 600 spectrophotometer and a 1 cm pathlength cuvette.

Cell numbers were determined directly with a Coulter Counter Model B. Samples, either 0.5 or 1.0 ml, were collected in glass tubes containing 1 ml of ice cold electrolyte (0.5% formaldehyde, 1% NaCl) and

stored on ice for processing either later the same day or on the next day. Before being counted the samples were diluted to 6 ml with electrolyte, mixed thoroughly and sonicated for 90 sec at 20 kHz and 2 μm amplitude with an MSE Ultrasonic Disintigrator. This broke up clumps and separated loosely attached pairs of cells. The cells were then diluted to 50 or 100 ml with electrolyte and counted using a 70 μm aperture.

The cell plate index is the percentage of cells containing a cell plate at any one time. Like the mitotic index it can be used as a measure to monitor the proportion of cells in a culture which are dividing at any given time. Cell plates were counted in a fresh drop of culture using dark field illumination on a Nikon S-KE microscope. Cell plates first appear as a faint line, but as they mature they increase in thickness and brightness. The end of the cell plate stage is taken to be when the plates begin to notch at the beginning of cell separation. By these criteria (Faed, 1959) or by a simple calculation (Mitchison & Wilbur, 1962), it has been estimated that cells contain cell plates for only 15 to 20 min of a 140 min cell cycle.

ESTIMATION OF DNA AND RNA

After extracting total nucleic acids from cells by the method of Schneider (1945), the DNA content of the extract was determined with a colorimetric assay based on diphenylamine (see Appendix A for detailed method). The method, taken from Polanshek (1973), was originally devised by Burton (1956) and later modified by Giles and Meyers (1965) and by Bostock (1970,a).

RNA was estimated by diluting an aliquot of the Schneider extract 100-fold and measuring its absorbance at 260 nm. Since DNA constitutes only 1 to 2% of the total nucleic acids in S. pombe (Mitchison & Lark,

1962), its contribution to the OD_{260} was ignored. For conversion purposes it was assumed that 1.0 OD_{260} = 31 µg RNA/ml.

CELL SYNCHRONIZATION

Cultures of synchronously dividing cells were usually prepared by the selection method of Mitchison and Vincent (1965). Approximately 5 l of culture were harvested on a Whatman No. 50 filter paper, the cells were resuspended in 3-5 ml of medium, and then given 3-4 strokes in a homogenizer with a loose-fitting pestle to break up clumps and separate pairs of cells. The cells were then loaded on top of a 10 to 40% sucrose gradient (80 ml) made up in medium and centrifuged at room temperature in an MSE 4L for 5 min at about 800 x g. The top 1-3 ml of the band of cells were removed from the gradient with a syringe and resuspended in fresh medium at 32°C.

The work in Chapter 6 was done using the deoxyadenosine induction method of Mitchison and Creanor (1971,a). This involved pulsing an exponential phase culture with 2 mM 2'-deoxyadenosine for 3 hrs. At the end of the pulse the cells were collected on a membrane filter (Oxoid) and washed with and then resuspended in fresh medium at 32°C.

RADIOISOTOPES

Stocks of radioactive tracers from the Radiochemical Centre, Amersham, were used at the following specific activities: ^{32}P as orthophosphate (10 mCi/ml), ^{35}S as sulphate (Carrier free), uridine-5-^{3}H (29 Ci/m mol), uridine-2-^{14}C (56 mCi/m mol), adenine-8-^{3}H (24 Ci/m mol), and adenine-8-^{14}C (27.7 mCi/ml).

SCINTILLATION COUNTING

Non-aqueous samples were counted under 6 ml of toluene containing 0.5% butyl PBD (Ciba Ltd.), while aqueous samples were counted after being dissolved in a cocktail of 40% methoxyethanol and 60% toluene with 0.5% butyl PBD added.

Samples were counted in glass vials for either 1 or 10 min in a Packard Tri-Carb 2002 or a Packard Tri-Carb 2425 Scintillation Spectrometer.

KINETIC MEASUREMENTS ON PRECURSORS

To determine the uptake and incorporation kinetics of uridine and adenine, cells were labelled with either ^{3}H-uridine or ^{3}H-adenine. Samples of 1.0 ml were withdrawn on alternate minutes for uptake and for incorporation measurements. Uptake samples were filtered immediately through a Whatman GF/A glass fiber filter disk and washed with 4 x 10 ml of water. Incorporation samples were collected in tubes containing 1 ml of cold 10% trichloroacetic acid (TCA) and stored in an ice bath until the end of sampling. They were then collected on GF/A filters, washed with 3 x 10 ml of 5% TCA and 2 x 10 ml of water. All the filters were dried *in vacuo* for an hour before being counted as above. The difference between the uptake and the incorporation data was used as an estimate of the kinetics of filling of the acid soluble pool of radioactivity.

RATES OF PRECURSOR INCORPORATION

Rates of radioactive precursor incorporation into acid-insoluble components of the cell were measured by shaking 5 ml of cells at 32°C in the presence of the appropriate precursor. The pulse was stopped by adding 5 ml of ice cold 10% TCA to the flask, which was then placed

on ice for 20 min to allow for the extraction of small molecules. Triplicate 1.0 ml samples were collected on GF/A filter disks which had been soaked in 5% TCA. The filtered cells were washed with 3 x 10 ml 5% TCA and 2 x 10 ml water. They were then dried for 1 hr *in vacuo* before being counted as above.

In synchronous cultures, rates were followed for 1½ to 3 generations. The 5 ml aliquots of culture were pulse labelled at either 10 or 20 min intervals beginning about 40 min after making the synchronous cultures. To examine the effect of external uridine concentration upon the rate pattern, uridine concentrations (and specific activities) were varied from 7 ng (1 µCi) per ml to 500 µg (20 µCi) per ml. Adenine was used at 1.5 µg (1 uCi) per ml except in the experiments with a prefilled adenine pool: in these, cells were maintained in adenine (20 µg/ml) starting 4 hrs before synchronizing the cells and were labelled with either ^{3}H-adenine at 5 or 20 µCi/ml or ^{14}C-adenine at 2 µCi/ml).

RATES OF PRECURSOR UPTAKE

The rates of uridine and adenine uptake were determined by the same basic method used to measure the rates of incorporation, but the filters were soaked in water containing 1 mg non-radioactive precursor per ml and the pulses were stopped by adding 5 ml of iced water with 1 mg non-radioactive precursor per ml. The samples were filtered within 5 min of stopping the pulse and washed with 4 x 10 ml of water only.

RIBONUCLEASE DIGESTION

Ribonuclease (RNase) was made up to 2 mg/ml in 0.15 M NaCl and 0.015 M tri-sodium citrate (SSC). Extracted total nucleic acid samples were dissolved in 0.5 ml of the RNase solution for digestion.

Samples of whole cells were collected on GF/A filters, washed 3 x with 10 ml of 5% TCA and rinsed twice with 10 ml of water before being digested with 200 µl of the RNase solution. Digestion was carried out at 37°C for at least 3 hrs. After digestion the filters were washed 3 x with 5% TCA and 2 x with water before being dried. Bulk samples were loaded directly onto polyacrylamide gels for electrophoresis.

STATISTICAL TREATMENT OF RATE DATA

When uptake and incorporation rate experiments were repeated several times the data were pooled and analysed in the following manner: For selection synchronized cultures, results from several experiments were normalized to show a common mean level of either uptake or incorporation during the plateau between the first and second steps in cell number. This plateau was chosen as a baseline for normalization because it was completely included in all experiments. The data were then pooled and the means (X) and 95% confidence limits (95% CL) were calculated for all the values within each 0.1 of a cell cycle. For induction synchronized cultures the plateau used as a normalization baseline was that from 1 hr before removing the AdR to 1 hr after removing it. And in exponential phase cultures treated with phenethyl alcohol the plateau was that from 1 hr to 3 hr after adding the inhibitor. In both of these last 2 cases the data were pooled for each 10 min period, and treated statistically as above.

The rates of filling of the acid-soluble precursor pools were estimated by subtracting the incorporation rate data from the uptake rate data. In order to do this, the ratio of label taken up to that incorporated under the particular experimental conditions was determined. During the normalizing procedure, above, the mean plateau levels of the

pooled uptake rate and incorporation rate data were then set that ratio apart. The mean values were subtracted to give the mean rates of pool filling. The 95% CL for the rate of pool filling was calculated by the formula:

$$95\%\ \text{CL pool} = 1.96 \times \left(\frac{95\%\text{CL}_{upt}}{1.96}\right)^2 + \left(\frac{95\%\text{CL}_{inc}}{1.96}\right)^2 .$$

SPECIFIC ACTIVITIES OF TOTAL RNA

The amount of total RNA was estimated using a modification of the assay described by Hutchison and Monro (1961). 5 ml of culture at a density of 1 to 8 x 10^6 cells/ml were frozen in tubes immersed in a dry-ice/ethanol bath. After all the samples had been collected they were thawed at 0° in the presence of 5 ml of 10% perchloric acid (PCA), extracted at 0° for 30 min, and centrifuged (10 min at 1500 x g, 0°C). The pellets were resuspended in 5 ml of 5% PCA, left for 10 min at 0°C and recentrifuged as before. After resuspending the pellets in 1.0 ml of 5% PCA the samples were digested at 70°C as for the DNA estimation (see Appendix A, No. 3). The amount of RNA present in each sample was determined as above.

The radioactivity in an aliquot of each supernatant was then counted and the specific activities (cpm/µgRNA) were calculated.

FRACTIONATION OF RNA ON POLYACRYLAMIDE GELS

Cells were labelled as for rate measurements using ^{32}P at 1 to 10 µCi/ml, ^{3}H-uridine or ^{3}H-adenine at 1 to 20 µCi/ml. Labelling was stopped by adding an equal volume of ice-cold extraction buffer (TNT) containing 2% triisopropylnaphthalene sulphonic acid (Eastman), 2% NaCl and 0.01 M Tris at pH 7.8 (Loening, 1967). The cells were immediately

collected on a GF/A filter disk and rapidly frozen in 2-4 ml of TNT buffer. The samples were homogenized either by passing the frozen cells through the Eaton press (Eaton, 1962), or alternatively the samples were thawed and an equal volume of 40 mesh glass beads was added to each tube. The cells were then disrupted by grinding them for 10 min using a Vibro Mixer, (Braun, A.G.).

The homogenate was deproteinized using the method of Fraser, Creanor and Mitchison (1973): it was thrice emulsified with 50% v/v chloroform, 50% v/v of the phenol/m cresol mixture of Kirby (1965). After adding NaCl to the aqueous phase to a final concentration of 0.3 M, the nucleic acids were precipitated in the cold by mixing with 2.5 volumes of ethanol. To remove traces of phenol and other contaminants, the nucleic acids were reprecipitated twice from a buffer containing 0.15 M sodium acetate, and 0.5% sodium dodecyl sulphate (SDS) at pH 6.0 (Loening, 1969).

The nucleic acids were fractionated by electrophoresis on 2.2 to 2.6% polyacrylamide gels according to the method of Loening (1967) (see Appendix B for details of the procedure). Gels were then scanned for absorbance at 265 nm in a Joyce-Loebl Gel Scanner, frozen with dry-ice, and sliced into 0.4 mm disks with a Mickel Gel Slicer. RNA in the slices was hydrolysed over night at 60°C in 10% piperidine, 1 mM EDTA; then the slices were dried to remove the piperidine. The slices were rehydrated and the nucleotides in them dissolved with 0.5 ml of water, after which the radioactivity was counted.

OLIGO(dT) CELLULOSE AFFINITY CHROMATOGRAPHY OF RNA

A polyadenylic acid sequence (poly(A)) containing fraction was isolated from labelled total S. pombe RNA by affinity chromatography on a column of oligodeoxythymidylic acid-cellulose (oligo(dT) cellulose)

(Swan, Aviv & Leder, 1972). To do this, 0.1g of oligo(dT) cellulose was suspended in a binding buffer containing 0.4M NaCl, 10 mM Tris, 1 mM EDTA and 0.1% SDS (pH 7.4). After a column (0.4ml) was packed, an RNA sample was dissolved in 1 ml of binding buffer and passed twice through the column. The column was then washed with 2 ml of fresh binding buffer. The poly(A) sequence containing material was eluted from the column with 2-3 ml of a buffer identical to binding buffer except that it contained no NaCl. The RNA was then precipitated with ethanol and stored at -20°C.

CHAPTER III

THE MEASUREMENT OF RNA SYNTHESIS IN EXPONENTIALLY GROWING CULTURES

Several reports in the literature describe the uptake of labelled nucleosides and their incorporation into RNA by fission yeast (Cummins & Mitchison, 1964, 1967; Foury & Goffeau, 1973, 1975; Mitchison, 1963; Mitchison & Lark, 1962; Mitchison et al., 1969), and by budding yeast (Burns, 1968, 1971; Sogin, Carter & Halvorson, 1974; Sillevis-Smitt, et al., 1972). Much of the earlier work on S. pombe involved quantitative autoradiographic studies of single cells labelled mainly with adenine. But some comparisons were made between rates of uptake and incorporation of the ^{14}C-bases adenine, uracil, guanine and cytosine (Mitchison et al., 1969). Foury and Goffeau (1973, 1975) examined the effects of c-AMP on uptake and incorporation of ^{3}H-uridine in several mutants of S. pombe. They demonstrated that uptake proceeds at a constant rate for at least 8 min after adding the labelled precursor and has an apparent affinity constant (K_m) of 1.4×10^{-5}M. They also described a slow incorporation which started around 3 min after uptake. By 5 min this incorporation accounted for only 10% of the uptake. Both uptake and incorporation were stimulated by c-AMP.

In the present work ^{3}H-uridine has been used as the primary radioactive label, although both ^{3}H-adenine and ^{32}P (as phosphate) have also been used. Labelled uridine was initially selected because of the previous kinetic studies (Foury & Goffeau) and because it was readily and cheaply available in the laboratory. ^{3}H-uridine is commonly used as a specific label of RNA in eukaryotes. In certain cases, however, its rate of incorporation is not proportional to the rate of RNA synthesis (Stambrook & Sisken, 1972,b). So, before using ^{3}H-uridine incorporation to measure RNA synthesis the following three things must

be known: Whether ^{3}H-uridine incorporation selectively labels RNA in proportion to its synthesis; if so, what is the optimal uridine concentration; and what sorts of RNA are preferentially labelled for a given length of precursor pulse.

KINETICS OF ^{3}H-URIDINE UTILIZATION

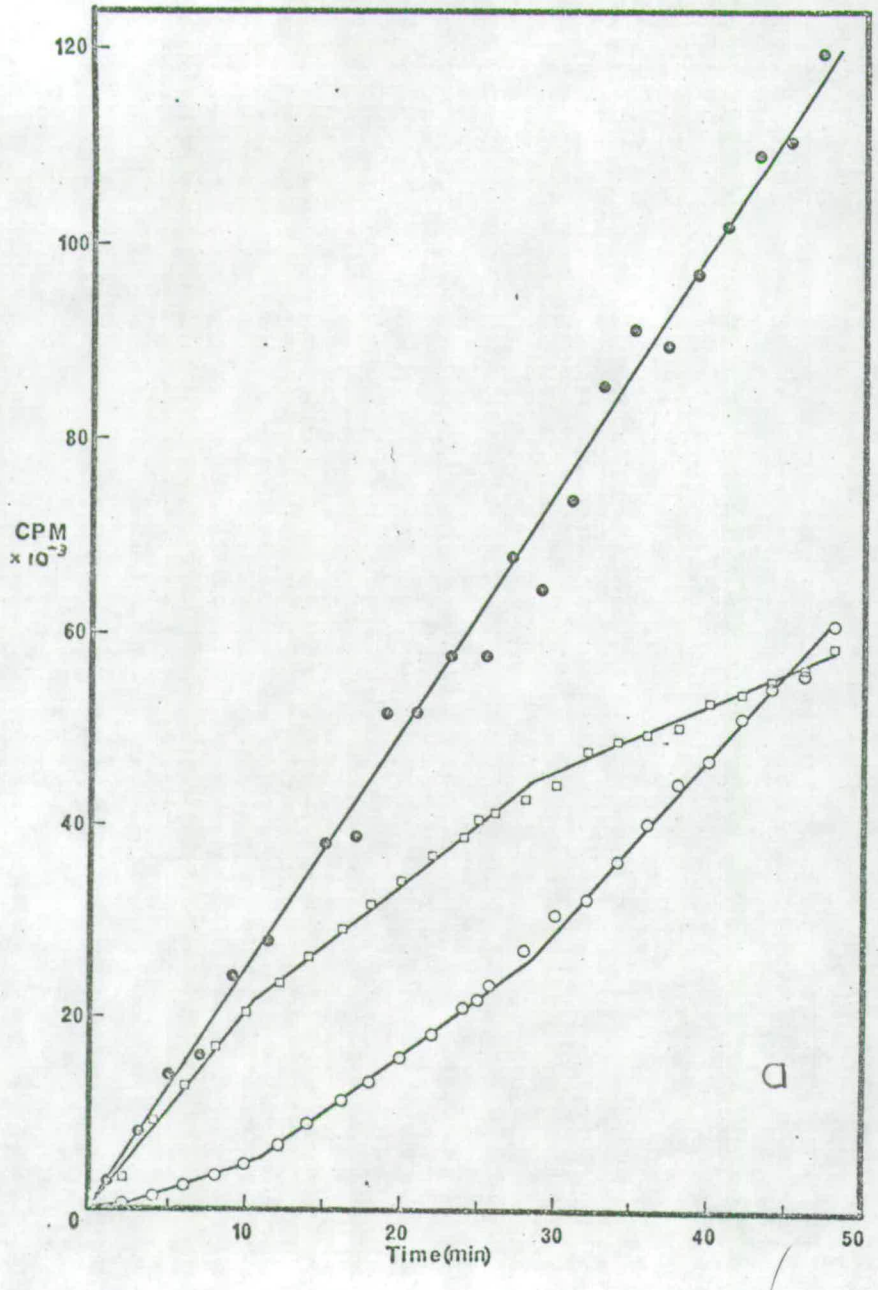
To examine the kinetics of ^{3}H-uridine utilization in S. pombe, a log phase culture was labelled at 3.75 µCi/ml and 3.75 µg/ml unlabelled uridine was added with the label. Samples were withdrawn on alternate minutes to measure the amount of radioactivity the cells had either taken up from the medium or incorporated into TCA-insoluble material. Uptake of ^{3}H-uridine proceeded linearly for the entire 48 min of labelling (Fig. 3/1a). Incorporation, on the other hand, increased in rate in an apparently discontinuous manner. In figure 3/1a the incorporation data are presented connected by straight line segments which meet at 11 min and 25 min. For the first 25 min, the data fit these straight lines perfectly. Thereafter the data are more scattered and the pattern of increase could be exponential. When these same incorporation data are plotted on semi-logarithmic axes (Fig. 3/1b), they produce a curve which rises rapidly at first and then slows its rate of increase. By 30-35 min it is increasing at a nearly constant rate. If incorporation were progressing linearly with rate changes at 10-11 min and again at 25 min, then the semi-log plot should show cusps at these times. Suggestions of such cusps can be seen in Fig. 3/1b, but they are more in the form of exponentially increasing stretches from 6 to 14 min and from 20 to 30 min. These are by no means proof of incorporation proceeding linearly and periodically changing rate. But the data do exclude the possibility of an exponentially increasing incorporation of ^{3}H-uridine.

Figure 3/1.

The kinetics of ^{3}H-uridine utilization in an exponential phase culture. Cultures were labelled with 3.75 μCi/ml, with uridine added to a final concentration of 15.7 μM at the time of labelling. The results are given as cpm x 10^{-3} per ml culture. The difference between uptake (●) and incorporation (○) is given as a measure of the entry of radioactive precursor into the acid soluble uridine pool (□).

(a) linear axes;

(b) semi-logarithmic axes.


120
100
80
60
40
20
0
CPM
x 10⁻³
10
20
30
40
50
Time(min)
a

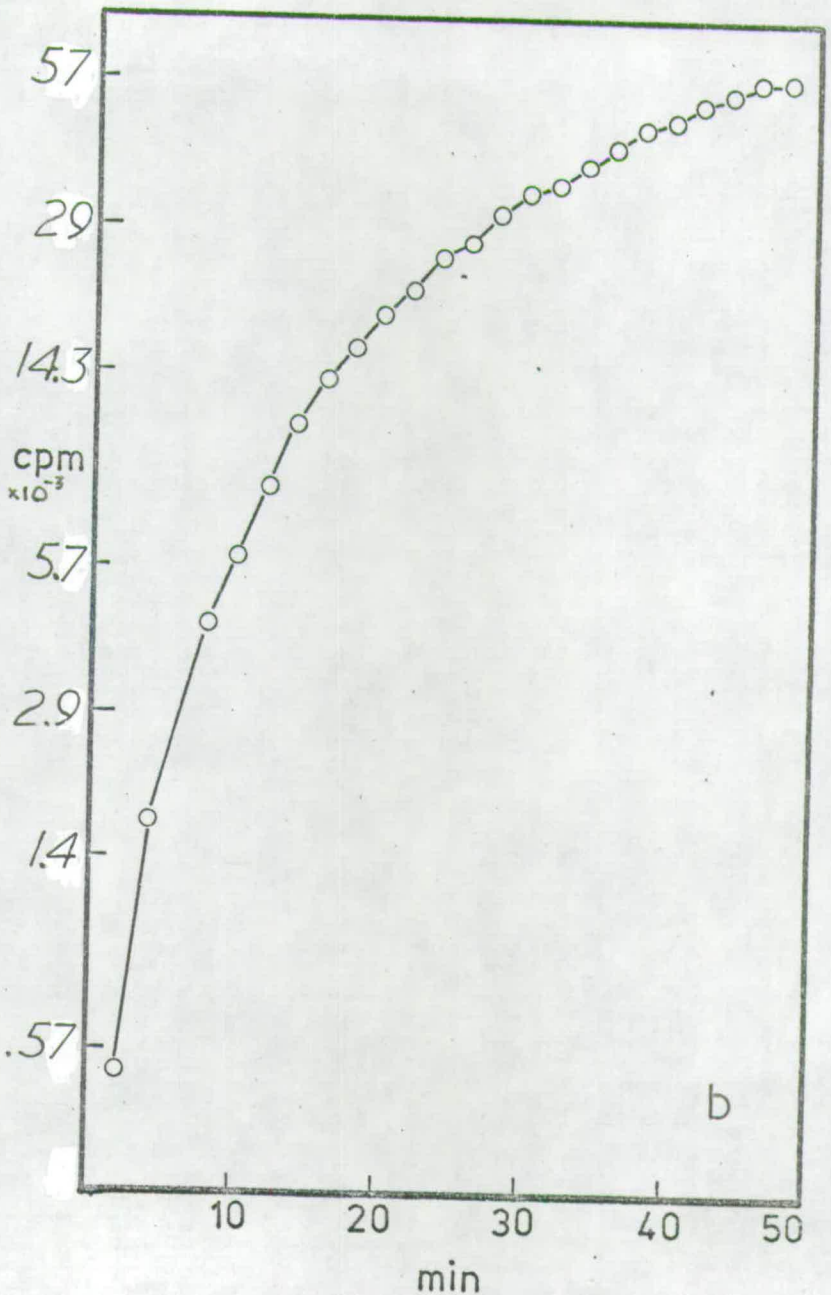
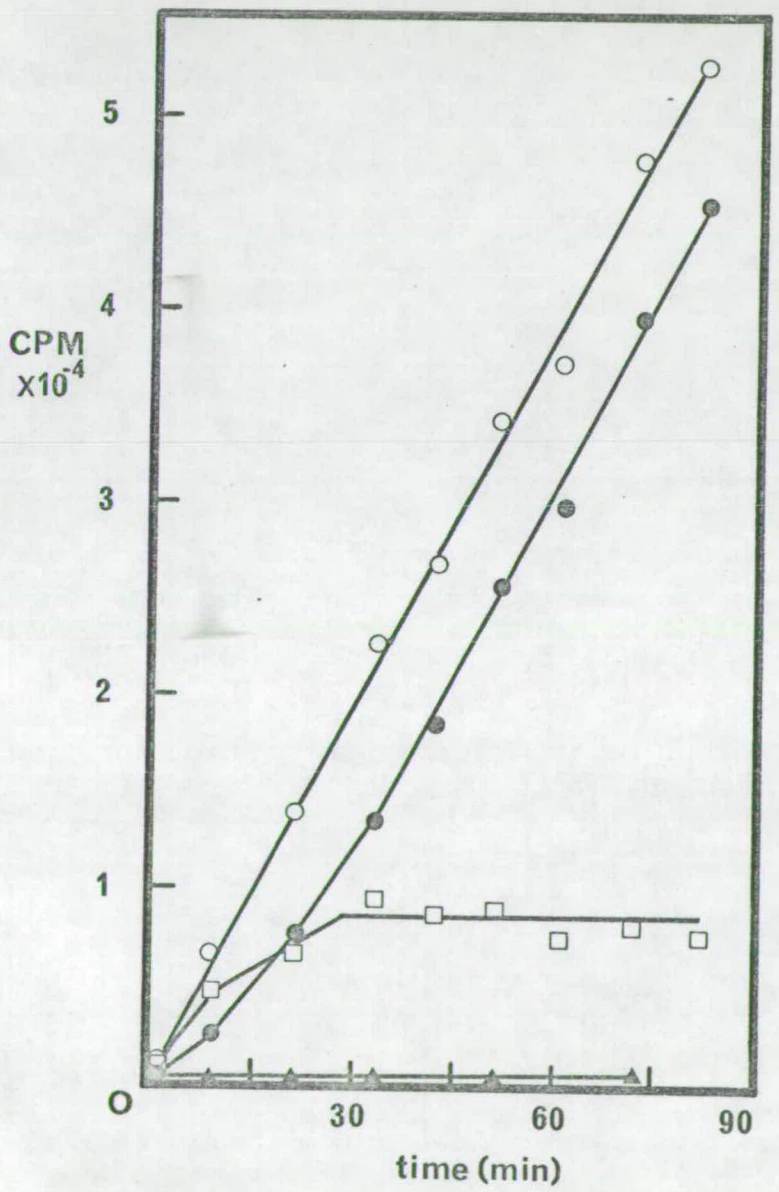

57
29
14.3
5.7
2.9
1.4
.57
cpm
x10⁻³
10
20
30
40
50
min
b

Figure 3/2.

The kinetics of ^{3}H-uridine utilization per cell.

A culture was labelled as in Fig. 3/1. Results are given as relative cpm/10^{7} cell. Uptake (O); incorporation (●); radioactive pool filling (□); ribonuclease resistant incorporation (▲).


5
4
CPM
X10-4
3
2
1
O
30
60
90
time (min)

The difference between the uptake and the incorporation data reflects the behaviour of the pool of ^{3}H-uridine and its acid-soluble, radioactive derivatives.

Initially ^{3}H-uridine enters these pools rapidly. But as the specific activity of the label in these pools approaches that of the medium the rate of entry into the pools decreases. Because of the shapes /of the parent curves, the radioactive pool curve can also be presented as a series of linear segments. But in this case each segment has a slower rate of increase than its predecessor. As with the incorporation data, the rate changes in the pool filling data appear to occur at about 10-11 min and 25-30 min. After the rate change at 25-30 min, radioactivity enters the pool about as rapidly as the culture is growing (the mean doubling time for both is 140 min). So the specific activity of the radioactive pool may be equilibrated with that of the label in the medium after 25 to 30 min of labelling. Such discontinuous changes in the kinetics of incorporation and soluble pool filling are usually considered to represent the filling of bypass pools (C.H. Sissons, pers. comm.). In yeast the two direct metabolites of uridine are uracil and UMP (Grenson, 1969). And uracil, in turn can be converted to cytosine. Uracil and cytosine may, therefore, act as a bypass pool, diverting some of the ^{3}H-uridine from the shorter route to RNA directly through UMP.

To determine how long the radioactive pool took to equilibrate when ^{3}H-uridine was added to the medium, the above experiment was repeated. This time, however, labelling was continued for 80 min, samples were withdrawn every 8 min to measure uptake and incorporation, and the results were plotted on a per cell basis (Fig. 3/2). As in the previous experiment, uptake is linear for the entire 80 min of labelling. The ^{3}H-uridine pool, as suggested in the last experiment, now clearly

equilibrates with the medium during the first 30 min of labelling and then remains at a constant size per cell for the remainder of the experiment. Incorporation increases in rate for the first 30 min, then becomes linear and increases at the same rate as uptake. The relatively long time between each sample makesit impossible to distinguish between a linear and an exponential initial increase in the cellular rate of incorporation.

After the radioactivity in the incorporation samples had been determined, the filters were washed free of scintillant and incubated with ribonuclease (RNase) for 4 hr. This treatment reduced the level of radioactivity in all the samples to the background level, thus indicating that virtually all of the incorporated ^{3}H-uridine is in RNA.

PROLONGED GROWTH IN URIDINE

The work described so far involved adding ^{3}H-uridine and non-radioactive uridine together to cultures which had never previously experienced uridine. But adding uridine to such cultures might temporarily disturb the kinetics of uridine utilization. To test whether such was the case, a culture of *S. pombe* was grown in the presence of uridine (20 µg/ml) for 3 hr. A trace amount of ^{3}H-uridine was then added to the culture and the kinetics of uptake and incorporation were followed for 40 min. For the first 34 min the results (Fig. 3/3) are virtually identical to the data presented in Fig. 3/1 for cells without previous experience of uridine. Uptake proceeds linearly for the entire 40 min, but incorporation increases its rate discontinuously at about 10, 22 and possibly 34 min. The last change in rate of incorporation is the one difference between these data (Fig. 3/3) and those in Fig. 3/1. Further data beyond 40 min would be needed to confirm the existence of

Figure 3/3.

Kinetics of ^{3}H-uridine utilization after growth in uridine. Uridine pools were filled by growing the culture in EMM2 containing 82 µM uridine for 3 hr before labelling at 6 µCi/ml. The difference between total uptake (●) and incorporation (○) is given as a measure of the filling of the acid soluble uridine pool with ^{3}H-uridine (□).

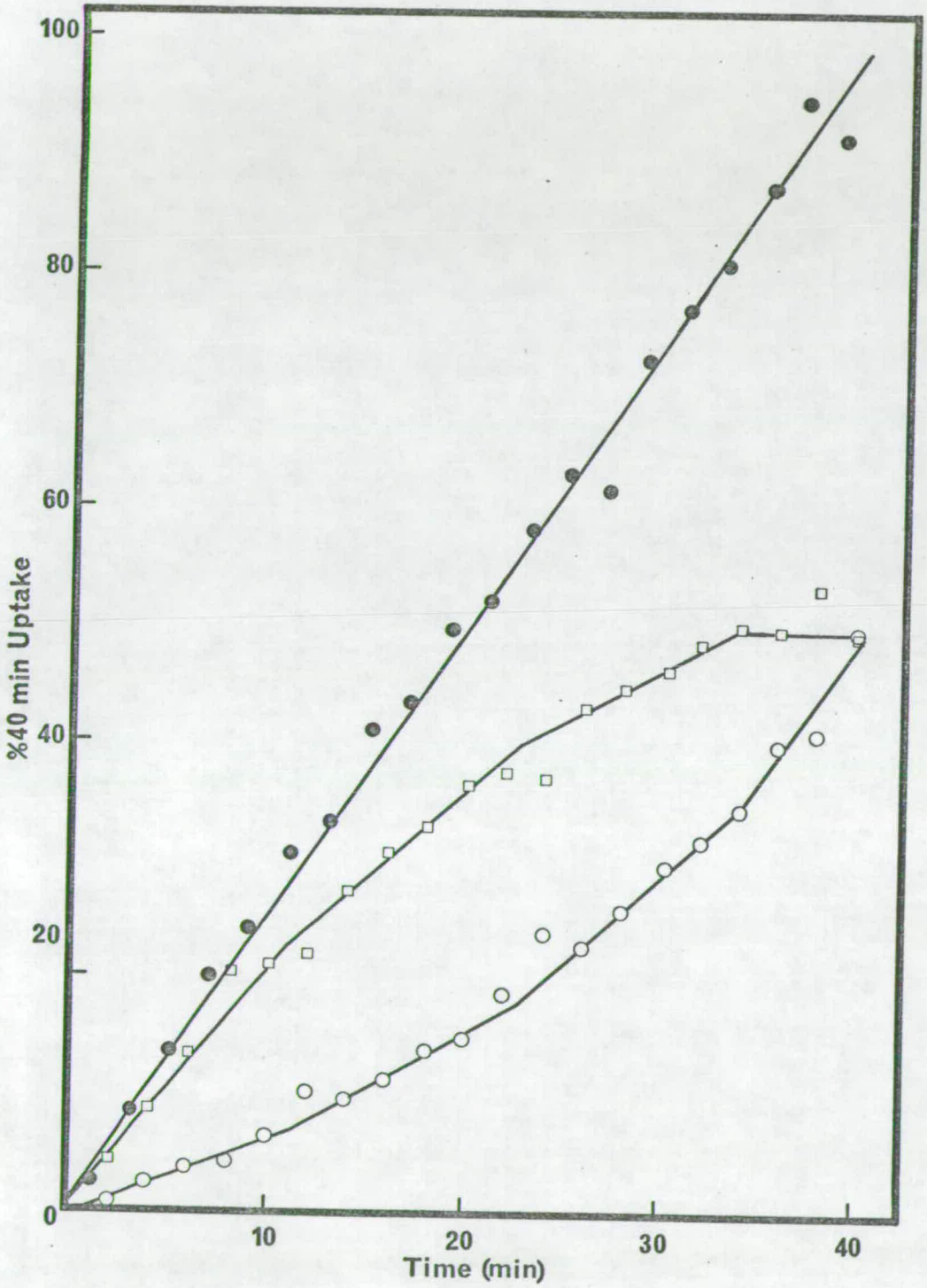
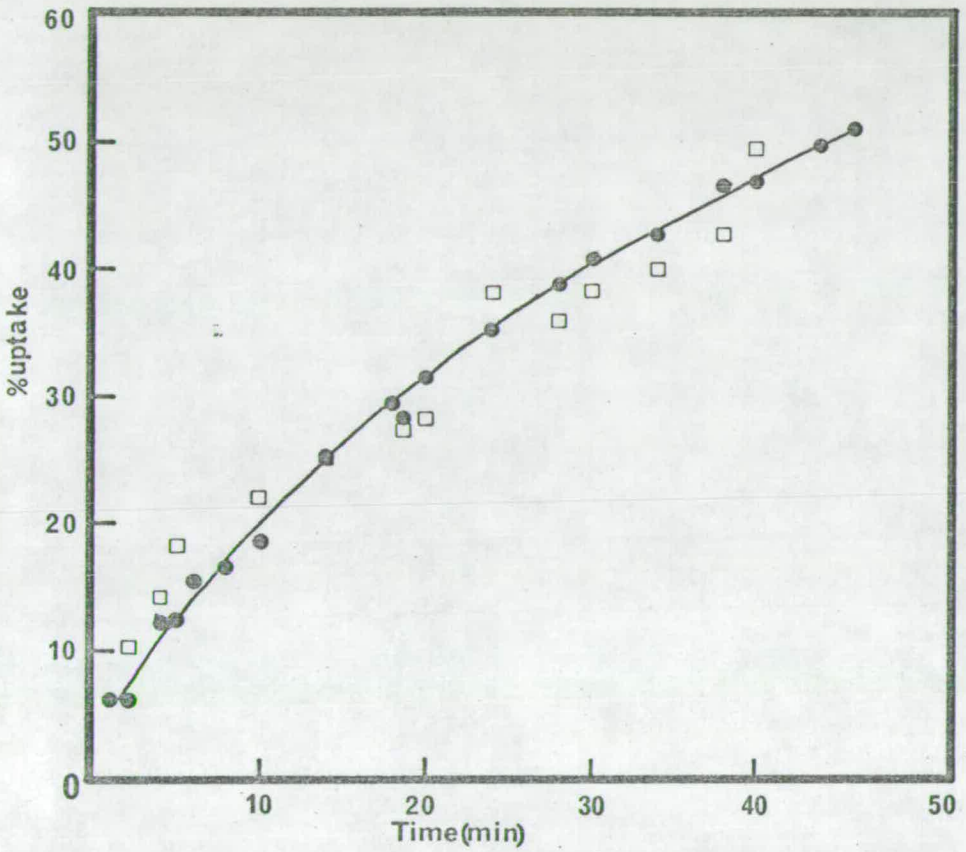

100
80
60
40
20
0
%40 min Uptake
10
20
30
40
Time (min)

Figure 3/4.

Incorporation of ^{3}H-uridine as a percentage of the total uptake of labelled precursor during normal growth and after filling the uridine pools. Data for normal growth (●) is from Fig. 3/1b; data after growth in uridine (□) is from Fig. 3/3.


60
50
40
30
20
10
0
%uptake
10
20
30
40
50
Time(min)

a rate change at about 34 min. In any case, pulses of label as long as 34 min would be too long to be useful in synchronous cultures with a generation time of 2.5 hr. So this possible change in the kinetics was not pursued.

When the amount of ^{3}H-uridine incorporated is presented as a percentage of the label taken up in the same time, a smooth curve results (Fig. 3/4). This curve increases from about 5% after 1-2 min of labelling to about 50% after 46 min of labelling. After about 30 min the rate of increase appears to remain constant. This again suggests that the specific activity of the radioactive pool has equilibrated with that of the medium by around 30 min. It is also clear from this figure that growing S. pombe in 20 µg/ml uridine for 3 hr does not noticeably affect the kinetics of ^{3}H-uridine incorporation. It is, therefore, unlikely that adding a moderate amount of uridine to a culture at the time of labelling should change the patterns of uptake or incorporation.

RNA SPECIES LABELLED BY DIFFERENT PULSE LENGTHS.

Pulses of labelled precursor are commonly used to measure rates of synthesis. But different types of RNA are preferentially labelled by pulses of different durations. To determine how pulse time is related to the labelling specificities of different RNA fractions, cultures were labelled with ^{3}H-uridine for 1, 5, 10 and 19 min. Total nucleic acids were then extracted and separated electrophoretically on 2.2 and 2.4% polyacrylamide gels. For comparison, cells were also grown overnight in the presence of $^{32}PO_4$, after which the nucleic acids were fractionated in a similar manner. The major species of RNA described previously for S. pombe were also found on these gels: the 25-S and 18-S ribosomal

Figure 3/5.

Patterns of labelling of total nucleic acids by ^{3}H-uridine pulses of various lengths. For comparison a culture was grown for 16 hr in EMM1 containing ^{32}P. Total nucleic acids were extracted and separated electrophoretically on polyacrylamide gels run for 3 hr at 50 V. Gels were cut into 0.8 mm slices and the amount of radioactivity in each slice was determined. Labelling conditions and gel concentrations were as follows:

(a) 1 min, 20 µCi/ml, 2.4%;

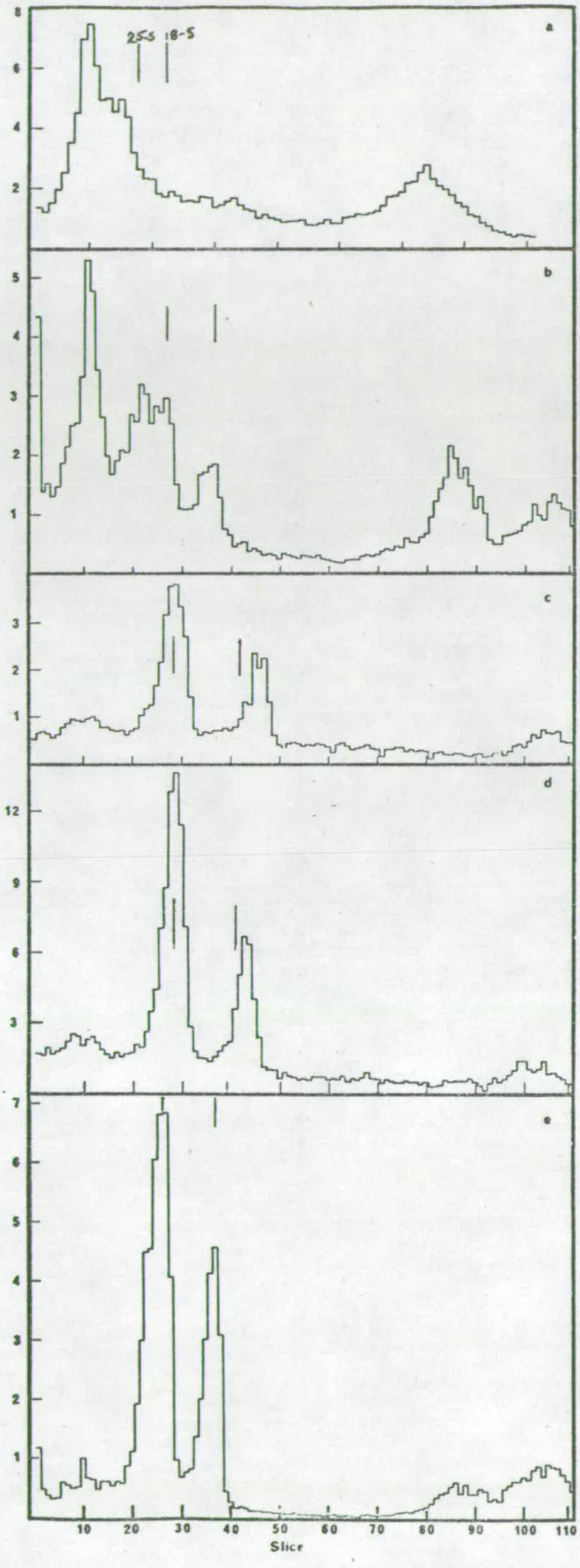
(b) 5 min, 5 µCi/ml, 2.4%;

(c) 10 min, 1 µCi/ml, 2.2%;

(d) 19 min, 1 µCi/ml, 2.2%;

(e) ^{32}P 16 hr, 0.5 µCi/ml, 2.4%.

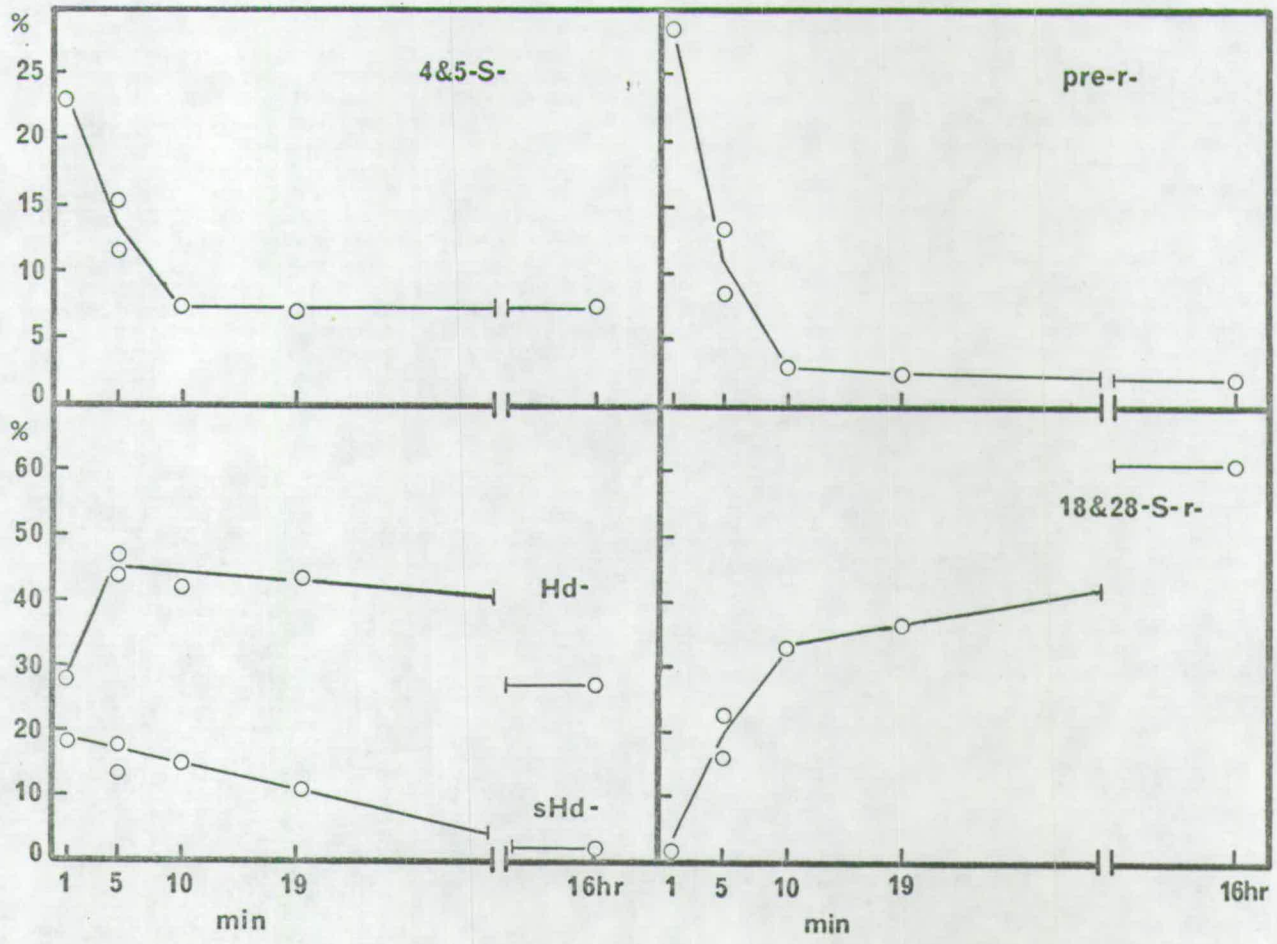
Vertical lines represent peaks in OD_{260} at 25-S and 18-S.


25-S
18-S
a
b
c
d
e
Slice

RNAs, the 38-40-S ribosomal precursor RNA, 5-S ribosomal RNA, and 4-S transfer RNA (Taber & Vincent, 1969). As the labelling time is increased, there is a parallel increase in the relative amount of label located in the stable, 25-S and 18-S ribosomal fractions of the RNA and a decrease in the amount of label found in the ribosomal precursor- (pre-r-) and the underlying heterodisperse fractions (Fig. 3/5). The relative contributions of the label in each RNA fraction to the total label on each gel was estimated by determining the proportion of the total area under each curve in Fig. 3/5 occupied by each RNA fraction. As a base line under the peaks, a line was arbitrarily drawn from the lowest point of the curve on one side of the peak to the lowest point on the other side of the peak. The area above the base line was considered to represent the specified peak fraction (e.g. 25-S), while the area below the base line was considered to represent heterodisperse RNA (Lee, pers. comm.). This Hd-RNA was arbitrarily subdivided into two broad size classes: from the top of the gel to the 15-S contains the high molecular weight heterodisperse (Hd-) sequences; from about 15-S to the bottom of the gel contains smaller heterodisperse sequences (sHd-) located between the mature ribosomal fractions and the 4-5-S RNA peaks. The relative contributions of both pre-r- and 4-5-S RNAs decrease rapidly with time, so that a 10 min pulse labels them to about the same extent as an overnight label (Fig. 3/6). Mature high molecular weight r-RNA, on the other hand, increases its contribution rapidly from 1% at 1 min to about 34% after 10 min labelling. Thereafter the percentage of label in this fraction slowly increases to the steady-state level of around 63%. The Hd-fraction shows a rapid increase in its relative contribution from 28% at 1 min to 40 to 45% between 5 and 19 min. Its contribution eventually decreases to about 27% for steady-

Figure 3/6.

The relative contributions of several species of RNA to the overall patterns of labelling seen with different lengths of ^{3}H-uridine pulse. The contribution of each fraction is given as the percentage of the total counts which that fraction contained. Data is taken from Fig. 3/5 and the various fractions are described in the text.


%
25
20
15
10
5
0
4&5-S-
pre-r-
%
60
50
40
30
20
10
0
Hd-
sHd-
18&28-S-r-
1
5
10
19
16hr
min
1
5
10
19
16hr
min

state labelling. There is an apparently linear reduction in the relative contribution of the smaller heterogeneous fraction of RNA as pulse-length is increased.

The rapid initial decrease in the pre-r- and the concomitant rapid increase in the mature r-RNAs are a result of the rapid processing of the ribosomal RNA fractions. This rapid processing and accumulation of large amounts of stable RNA is a major reason for the relative decrease in the amounts of label found in the other RNA fractions. The contribution of the Hd- fraction decreases slowly because it contains nearly half the RNA labelled in 5 min, while the 4 and 5-S-fraction decreases much more rapidly since it contains a smaller proportion to the RNA initially labelled. Although the sHd- fraction is also small, its rate of decrease is moderately slow. But this fraction is likely to contain partial break-down products from the larger fractions, both Hd- and pre-r-, so its relative contribution may not accurately represent either synthesis of new material or storage of stable material.

The initial intention of using different lengths of ^{3}H-uridine pulse was to preferentially label RNA fractions of different stabilities with each pulse length. But the rapid processing rate makes this intention only partially possible. Thus, 10 min is the minimum pulse-length that could be used to get a distribution of label similar to that in steady state labelling. If pulses are extended to 19 min, the labelling pattern remains almost unchanged, but the cells are able to incorporate over twice as much label as they do at 10 min. By using 19 min pulses it would, therefore, be easier to measure the rate of incorporation accurately while still allowing up to 7 successive measurements to be made in a cell cycle. To measure rapidly-labelled RNA synthesis,

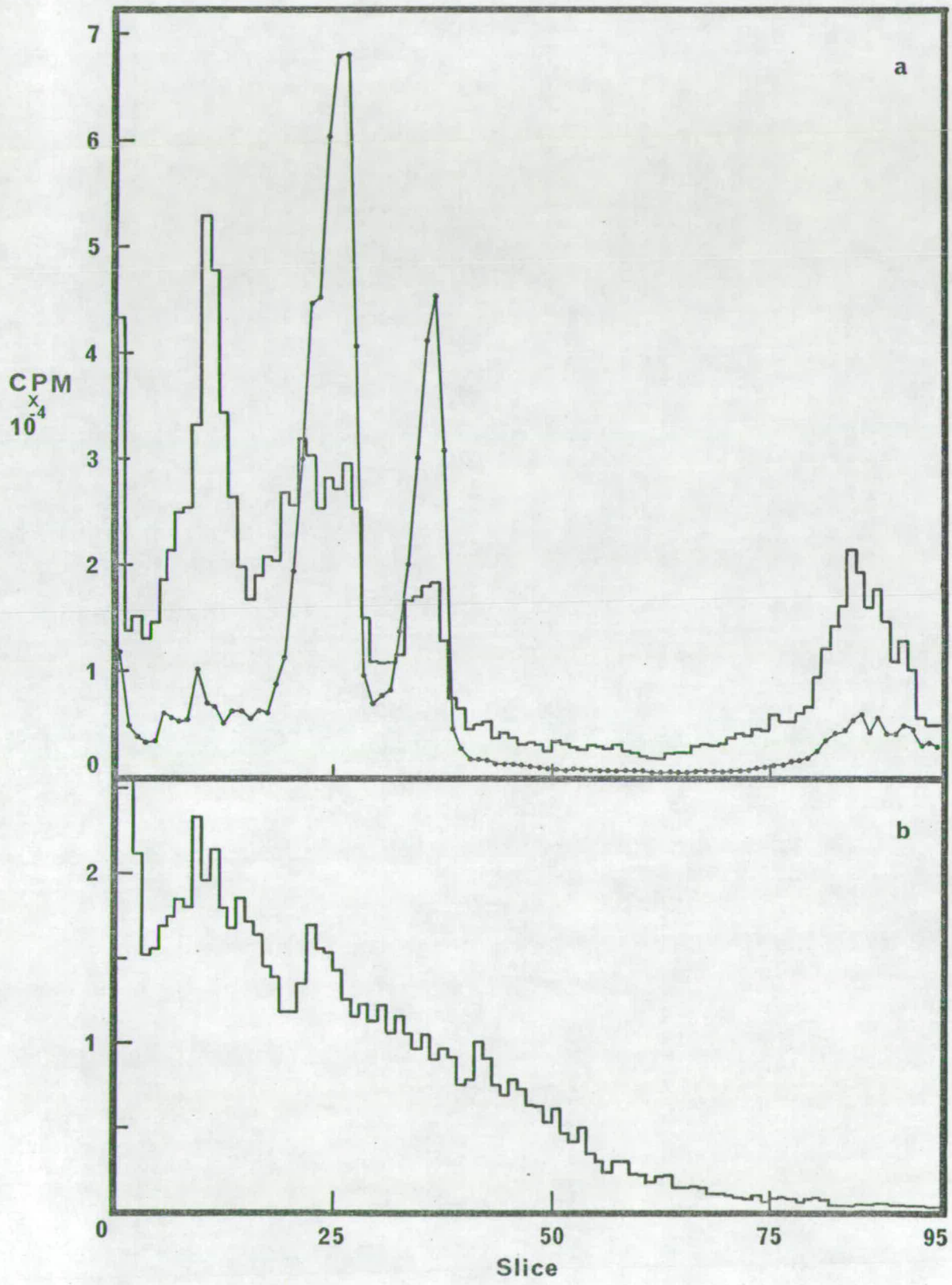
on the other hand, 1 min pulses appear to be necessary because even 5 min pulses label a significant amount of 28- and 18-S-r-RNA.

HETERODISPERSE RNA

The Hd-RNA fraction is at best an ill-defined collection of large RNA molecules ranging from the size of the large ribosomal precursor (38-S in S. pombe according to Taber & Vincent, 1969) to just smaller than material in the 18-S ribosomal peak. Within this size range there is a "wedge" of labelled material underlying the peaks, while below this size range there is very little labelling except in the 4- to 5-S region. One reason for considering this "wedge" material to be different from the RNA fractions in the various ribosomal peaks comes from the pattern of label distribution following a 1 min pulse (Fig. 3/5a). Although the "wedge" of Hd- RNA is labelled, of the ribosomal fractions only the pre-r-RNA peak contains label. A similar pattern of labelling occurs if the processing of pre-r-RNA is inhibited by cycloheximide (Taber & Vincent, 1969) or by step-down (See Chapter 8). Udem and Warner (1972) suggested that similar RNA found in S. cerevisiae could be analogous to the giant heterogeneous nuclear (Hn-) RNA found in higher eukaryotes. Since such Hn-RNA contains polyadenylic acid (poly(A)) sequences (Greenberg & Perry, 1972), it was decided to see if similar poly(A) sequences were present in the Hd-RNA region of S. pombe RNA gels. To do this cells were pulse-labelled with ^{3}H-uridine for 5 min and the RNA was extracted in the usual manner. The poly(A) sequence-containing RNA was then separated from the total RNA by binding it to an oligodeoxythymidylic acid-cellulose (oligo(dT) cellulose) column. After thorough washing, the poly(A) containing RNA was released from the column, concentrated, and separated electrophoretically on a 2.4% polyacrylamide

Figure 3/7.

The distribution of poly(A)-containing sequences in RNA labelled with ^{3}H-uridine for 5 min. Poly(A)-containing RNA sequences were isolated from total nucleic acids by binding them to an oligo(dT)-cellulose column. RNA was fractionated electrophoretically on a 2.4% polyacrylamide gel as in figure 3/5. (a) ^{32}P (0.5 µCi/ml) steady-state labelled total nucleic acids (●), ^{3}H-uridine (5 µCi/ml) 5 min labelled RNA (histogram); (b) poly(A)-containing sequences labelled 5 min with 5 µCi/ml ^{3}H-uridine.


a
b
CPM
x
10^-4
0
1
2
3
4
5
6
7
0
1
2
25
50
75
95
Slice

gel (Fig. 3/7b). The control gel (Fig. 3/7a) contains a mixture of the ^{3}H-uridine labelled RNA which had not been fractionated on the oligo(dT)-cellulose column and RNA which had been stead-state labelled with ^{32}P. The steady-state-labelled RNA was included to provide markers for the major peaks of stable RNA at 25-S, 18-S and 4 to 5-S. The poly(A)-containing RNA is heterodisperse in size and has a wedge-like distribution with a maximum at the top of the gel and tapering off to nothing at about 7-8-S. Minor peaks appear in the regions of the ribosomal precursor and 25-S r-RNA, but whether they are ribosomal in origin is unknown. They do appear to contain poly(A) sequences, however, since the peaks are present in RNA which has been bound to a second oligo(dT)-cellulose column after having been removed from total RNA by a first.

But Hn-RNA is not the only species to contain poly(A) sequences. Many m-RNAs also contain such sequences (McLaughlin, Warner, Edmunds, Nakazato & Vaughan, 1973; Reed & Wintersberger, 1973), and much of the Hd-fraction is likely to be messenger. Such a broad mixture of RNA molecules will most likely have an equally broad range of stabilities. The messenger component probably has an average half-life of 40-45 min (Fraser, 1975), whereas some unstable RNAs may have a half-life of no more than 4 min (Zusman & Rosenberg, 1971).

URIDINE CONCENTRATION AND RATE OF UPTAKE

In our joint paper (Wain & Staatz, 1973) we described the rates of ^{3}H-uridine incorporation in synchronous cultures labelled at uridine concentrations ranging from 0.03 µM to 820 µM and found no significant differences in pattern throughout the concentration range. This finding contrasts with the results of similar experiments using Chinese hamster cells (Stambrook & Sisken, 1972a). In these

Figure 3/8.

The rate of ^{3}H-uridine uptake at different external uridine concentrations. Cultures were labelled for 19 min at 6.5 µCi/ml in the presence of .03 µM to 3.7 mM uridine. Results are given as cpm x 10^{-3} per 1.0 OD_{595} of culture labelled with 10^{7} cpm/µg uridine. Data from 2 parallel experiments are shown.

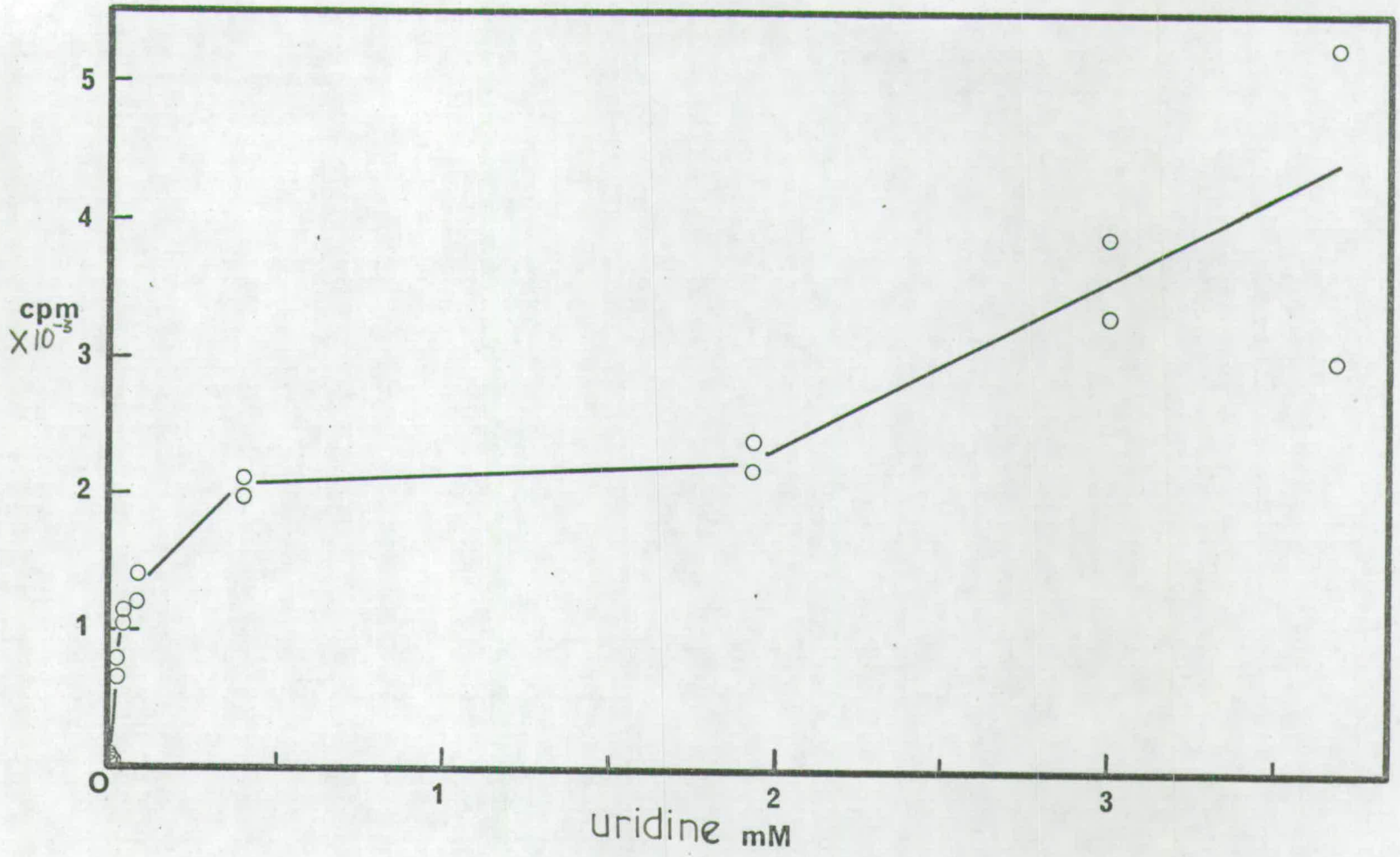
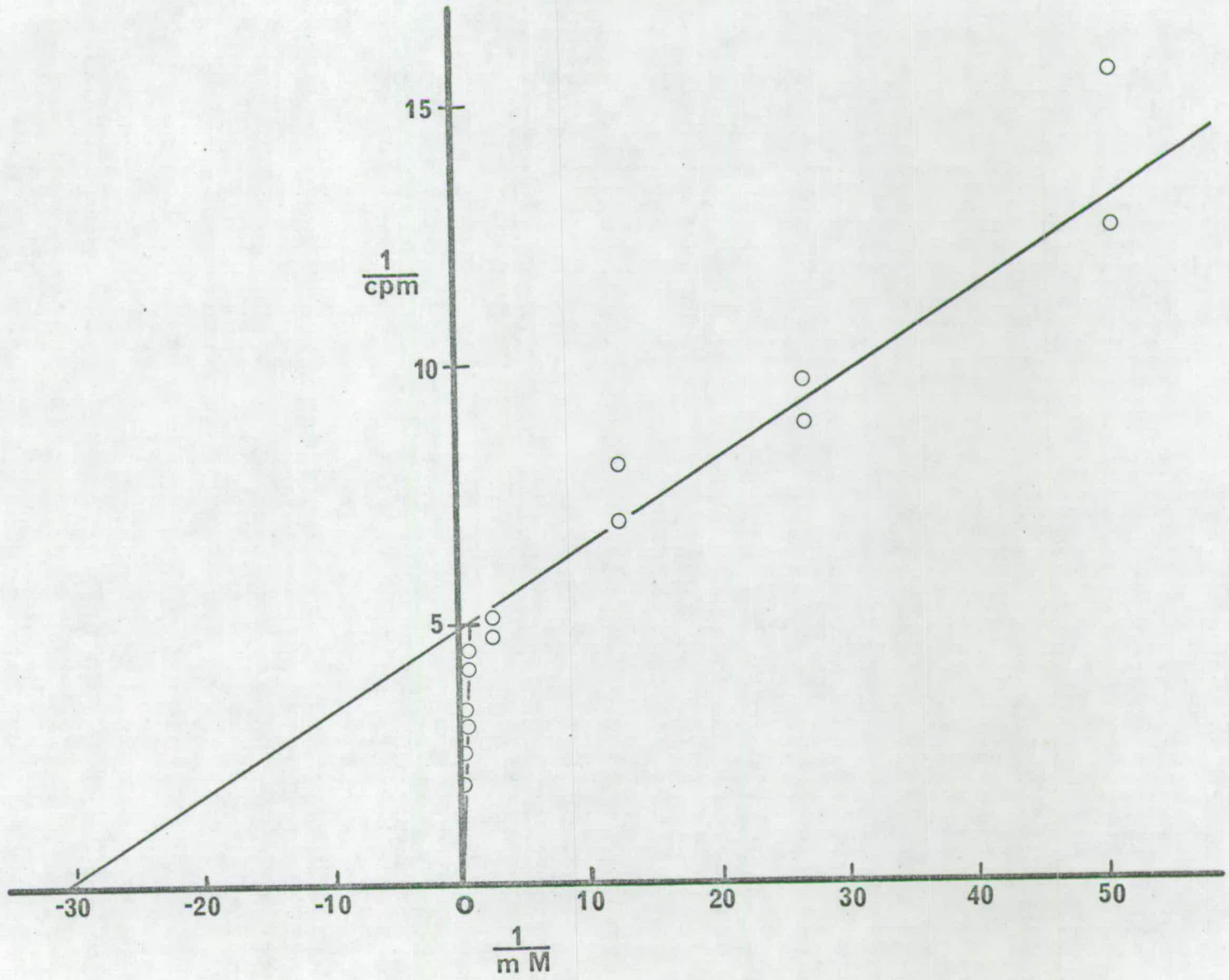

cpm
X 10⁻³
5
4
3
2
1
O
1
2
3
uridine mM

Figure 3/9.

Lineweaver-Burk plot of ^{3}H-uridine uptake rate against uridine concentration. Culture and labelling conditions are the same as for Fig. 3/8. The apparent affinity constants (K_m) for the two kinetic functions are 3.15×10^{-5} M and 15.8 mM respectively.


15
10
5
1/cpm
-30
-20
-10
0
10
20
30
40
50
1/m M

latter cells, a step-like increase in the rate of ^{3}H-uridine incorporation can be converted to a linear increase by raising the uridine concentration of the medium to 200 µg/ml. The reason for the change was shown to be a change from active uptake of label at low uridine concentrations to non-regulated diffusion at high uridine concentrations. Unlike mammalian cells, in nature micro-organisms are likely to be subjected to vast changes in their supplies of metabolites. Cummins and Mitchison (1967) demonstrated that S. pombe is able to maintain a constant final size of its intracellular pool of adenine metabolites in medium containing 0.1 to 20.0 µg/ml adenine. This yeast may, therefore, be able to regulate the entry of metabolites at concentrations as high as 820 µM. The rate of ^{3}H-uridine uptake was, therefore, examined as a function of the external uridine concentration over the range of 0.03 µM to 3.7 mM. At low uridine concentrations the rate of uptake increases rapidly with small increases in concentration (Fig. 3/8). Between 0.5 and 1.0 mM the increase in rate appears to taper off, and between 1 and 2 mM it remains more or less constant. Above about 2 mM the rate of uptake again increases with increases in uridine concentration. This implies that below 2 mM uridine uptake follows normal saturation kinetics, but that the kinetics may change above this exogenous concentration of uridine. A Lineweaver-Burk double reciprocal plot of this data has two separate and approximately linear segments (Fig. 3/9). A similar two-segment plot for uracil uptake in S. cerevisiae was interpreted by Grenson (1969) as two separate kinetic functions. A similar kinetic pattern from uridine uptake studies on rat hepatoma cells has been taken as evidence that, at high concentrations, uridine enters these cells by simple diffusion (Plagemann & Erbe, 1973). From the present results, the apparent affinity constants (K_m) have been calculated to be

Figure 3/10.

Kinetics of ^{3}H-uridine uptake and incorporation in the presence of 2 mM uridine. Cultures were labelled at 20 µCi/ml and sufficient uridine was added at the time of labelling to produce a final uridine concentration of 2 mM. Results are given as cpm x 10^{-2} per 1.0 OD_{595} of culture. Uptake (o), incorporation (•).

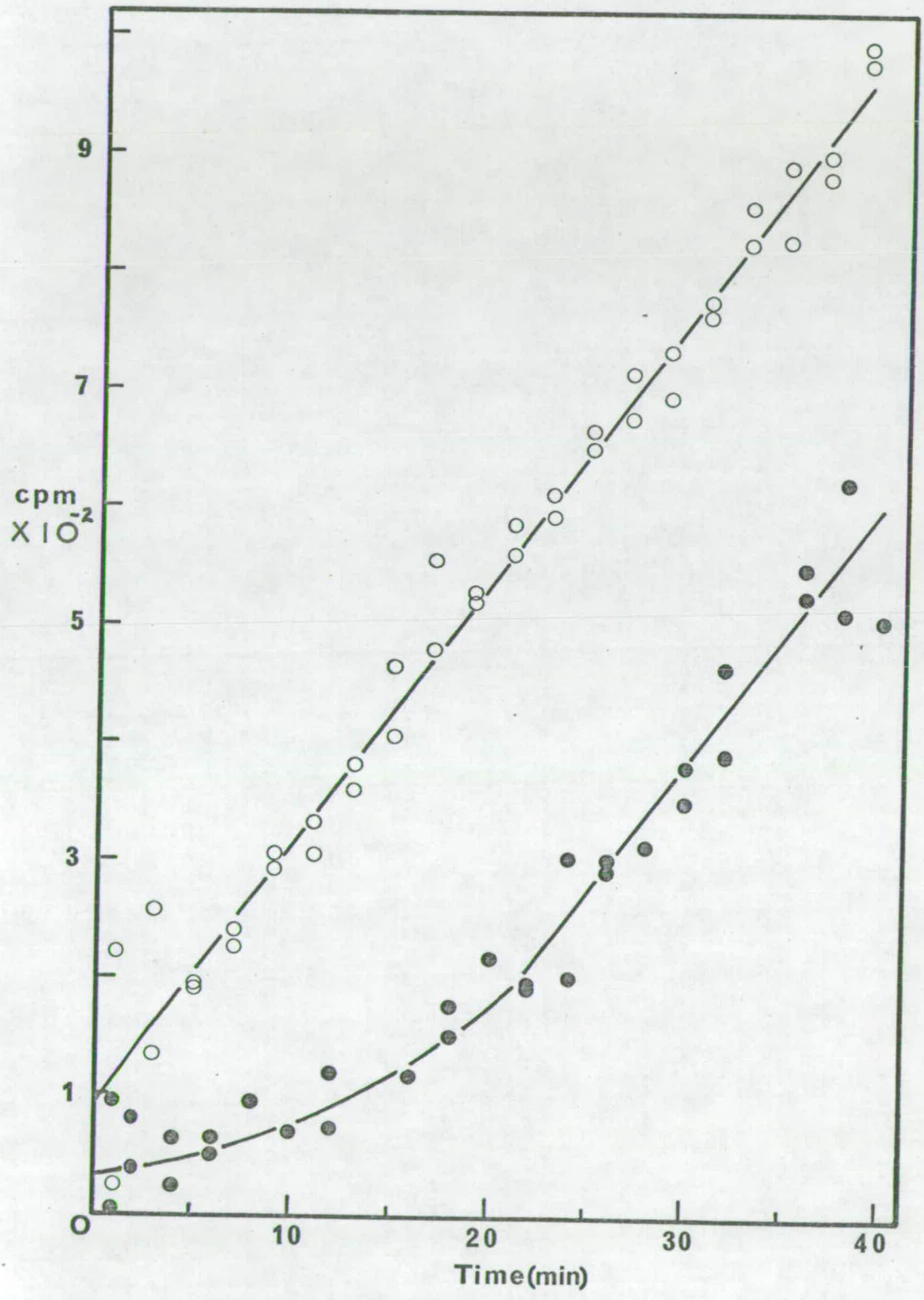
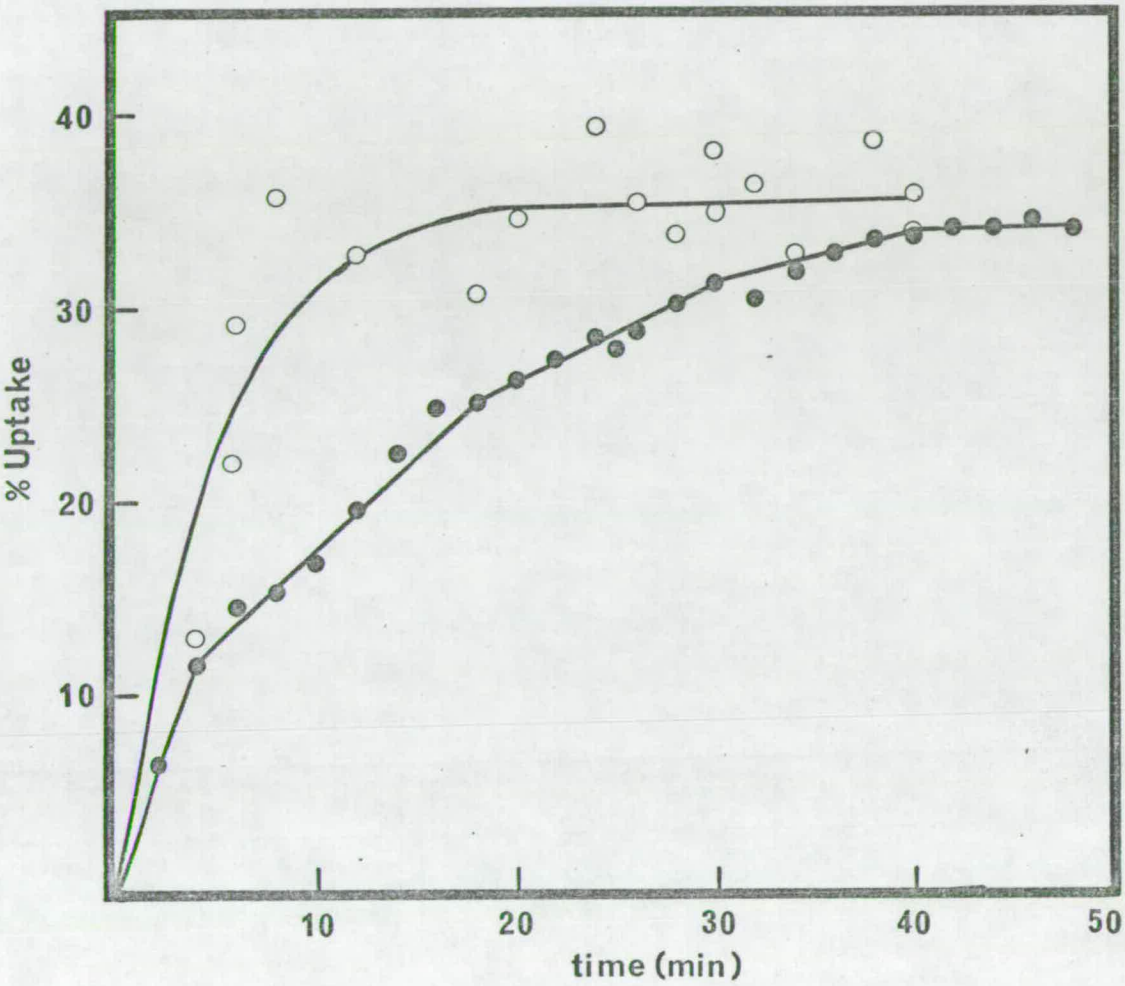

9
7
cpm
X 10^-2
5
3
1
0
10
20
30
40
Time(min)

Figure 3/11.

Incorporation of ^{3}H-uridine (per cell) as a percentage of the total cellular uptake in the presence of 82 µM (●) and 2 mM (o) uridine. Data taken from Figures 3/1a (control), and 3/1O (2 mM).


40
30
20
10
% Uptake
10
20
30
40
50
time (min)

3.15×10^{-5} M and 15.8 mM. The change in kinetics appears to occur at just under 2 mM uridine. The most probable explanation for this change is that at concentrations of 2 mM or greater, ^{3}H-uridine enters the cells mainly by diffusion, whereas at lower uridine concentrations it enters mainly by active uptake. In their report on ^{3}H-uridine uptake in several mutants of S. pombe, strain 972^{h-}, Foury and Goffeau (1975) did not demonstrate these two kinetic functions. But their results appear to have been obtained at uridine concentrations of 1 mM or less. They determined the K_m for ^{3}H-uridine uptake in glucose-starved cells to be 1.4×10^{-5} M, or about half of the smaller K_m reported here for respiring cells.

EFFECTS OF 2 mM URIDINE

Because of these bifunctional kinetics, ^{3}H-uridine uptake and incorporation were examined in the presence of 2 mM uridine (Fig. 3/10). In the first few minutes of labelling, the data points are too scattered to interpret. But from 5 min onwards uptake increases linearly, as it does at lower uridine concentrations (Fig. 3/1). Incorporation increases in rate for about the first 20-25 min. Thereafter it increases at the same rate as does uptake. The data points are too scattered to discern whether the rate of incorporation increases continuously or discontinuously during the first 20 min. But when cells are labelled in the presence of 2 mM uridine, their uridine pools equilibrate with ^{3}H-uridine in the medium much more rapidly than if they had been labelled at a lower uridine concentration. This more rapid pool equilibration is reflected in the percentage of the label that a cell incorporates from what it has taken up (Fig. 3/11). In control cultures, cells take 40 min to reach a constant 35% incorporation, whereas in cultures grown

Figure 3/12.

The effects of 4 mM uridine on growth (OD_{595}) and cell plate index. Uridine was added to a log phase culture to a final concentration of 4 mM at time = 0. Open symbols, 4 mM; closed symbols, control.

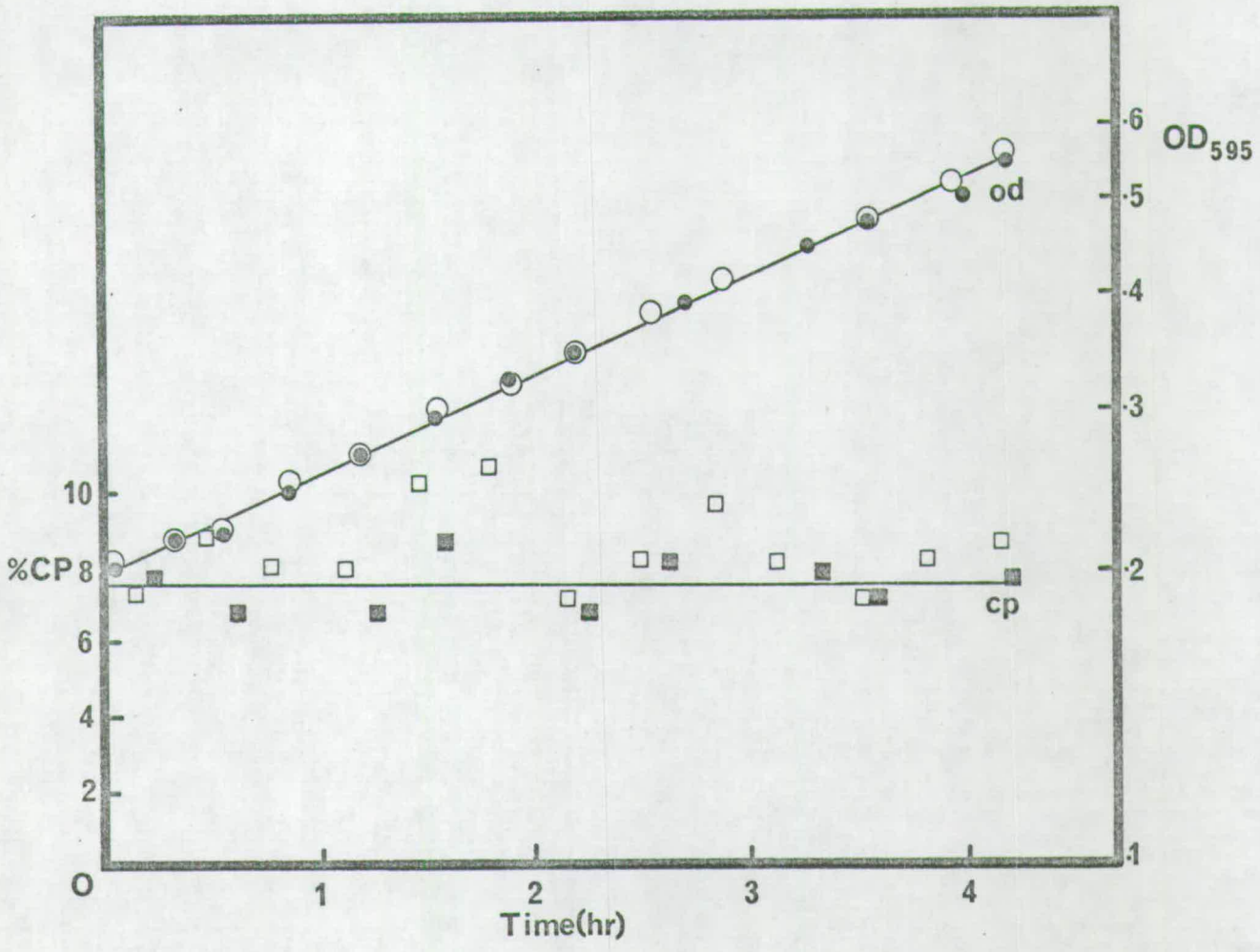
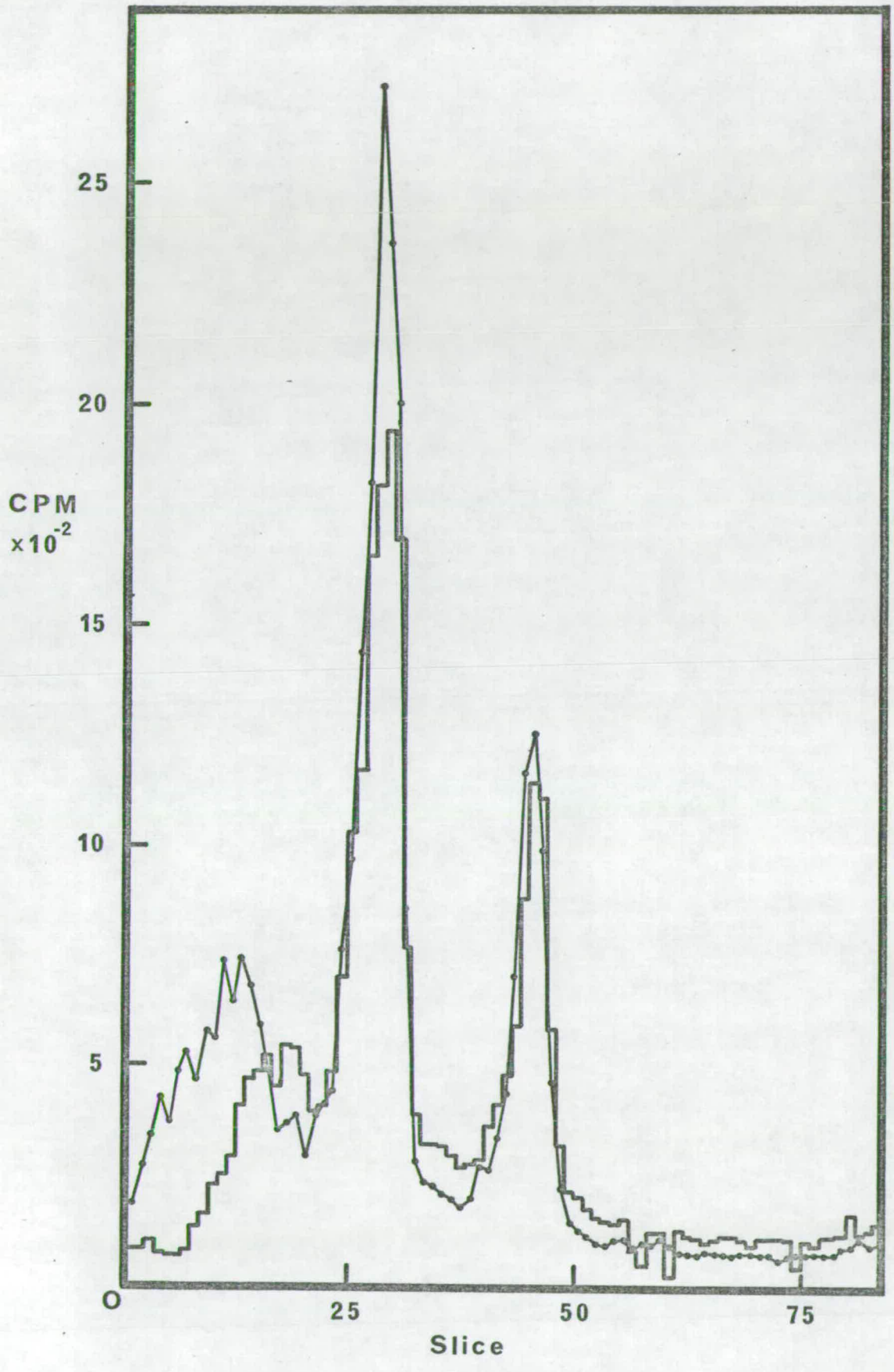

OD595
od
cp
%CP
10
8
6
4
2
0
1
2
3
4
Time(hr)
·6
·5
·4
·3
·2
·1

Figure 3/13.

The effect of 2 mM uridine on the distribution of ^{32}P in various fractions of total nucleic acids. Cultures were labelled with 3 μCi/ml ^{32}P for 19 min in the presence (histogram) and absence (●) of 2 mM uridine added 5 min before the label was added. Total nucleic acids were extracted and separated electrophoretically on a 2.4% polyacrylamide gel run for 2.5 hr at 50 V. Gels were cut into 0.8 mm slices and the radioactivity in each slice was determined separately.


25
20
CPM
x10-2
15
10
5
O
25
50
75
Slice

in 2 mM uridine, cells reach this constant level after about 20 min. The initial saturation kinetics seen in Fig. 3/8 thus appear to be overcome by labelling cells in the presence of 2 mM uridine. By overcoming a similar uridine uptake restriction in Chinese hamster cells Stambrook and Sisken (1972,a) were able to convert periodic doublings in the rates of ^{3}H-uridine uptake and incorporation during the cell cycle into continuous increases.

Since a high external uridine concentration affects the kinetics of uridine utilization, it may also have other effects upon growth or nucleic acid metabolism. In Tetrahymena, high uridine concentrations have been used to induce synchronous cell division in much the same way that thymidine block is used on mammalian cells (Villadsen & Zeuthen, 1971). In S. pombe, however, uridine concentrations as high as 4 mM have no effect upon the rate of increase in cellular dry mass (Fig. 3/12). The cell plate index of such a culture also appears similar to that of the control culture, although there is a marginally greater spread in the data points.

To test whether RNA synthesis was affected by high uridine concentrations, a culture was labelled for 19 min with ^{32}P (3 µCi/ml) in the presence of 2 mM uridine. A control culture was labelled without uridine in the medium. Total nucleic acids were then extracted and separated electrophoretically on 2.4% polyacrylamide gels. Two things stand out in these gels (Fig. 3/13): the amount of label in the ribosomal precursor and in the 25-S r-RNA fractions is less following uridine treatment than in the control; and the ribosomal precursor has migrated further in the treated sample than in the control. More quantitatively, Table 3/1 shows the percentage contributions of various RNA fractions in these gels.

Table 3/1: The effect of 2 mM uridine upon the percentage of counts in each fraction of RNA labelled with ^{32}P.

Fraction	Control	2mM	(2mM-Control)
Pre-r	27.58	18.28	-9.30
25-S	34.44	27.11	-7.33
18-S	11.26	11.61	0.35
Hd-	21.22	34.59	13.37
sHd-*	5.48	8.41	2.93

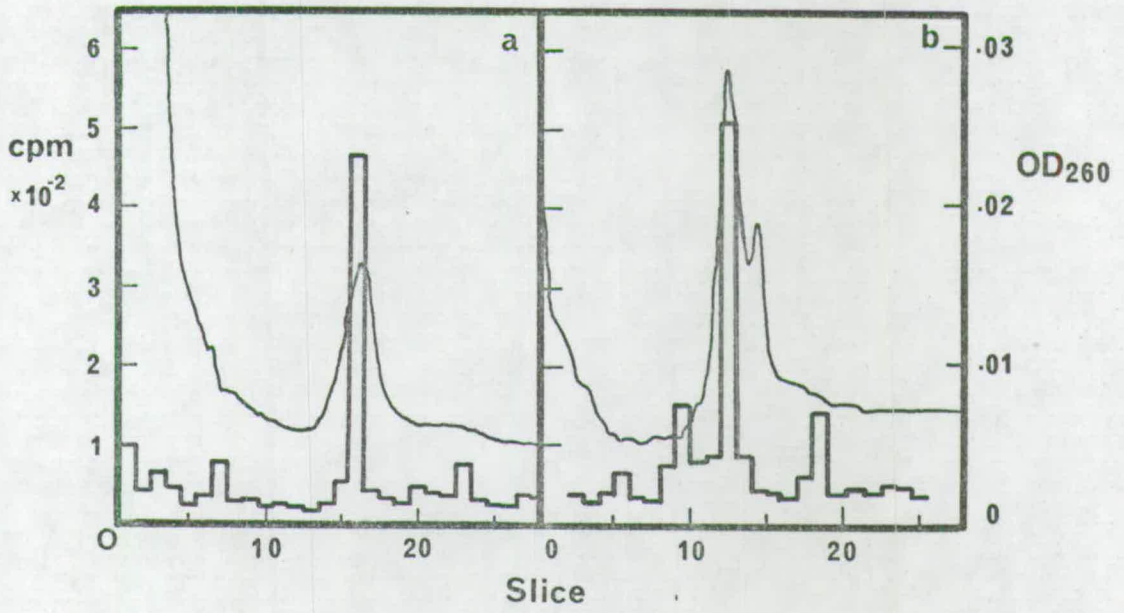
* sHd-RNA: counts present from slice 57 to the bottom of the gel.

This data is taken from the relative contribution of each fraction to the overall labelling patterns seen in Fig. 3/13.

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Figure 3/14.

The effect of 2 mM uridine on the labelling of DNA with ^{32}P. Total nucleic acids were labelled and extracted as for Fig. 3/13 and then digested with RNase for 4 hr at 37°C. The digests were separated electrophoretically on 2.4% polyacrylamide gels as in Fig. 3/13. Continuous line is OD_{260}; histogram is cpm; (a) control; (b) 2 mM uridine.


a
b
cpm
x10-2
6
5
4
3
2
1
0
10
20
0
10
20
Slice
.03
.02
.01
0
OD260

The decrease in the relative contributions of ribosomal precursor and 25-S r-RNA upon treatment with 2 mM uridine is approximately balanced by an increase in the two heterodisperse fractions of RNA. Only 18-S r-RNA appears to be labelled at the same rate in the two cultures. But both 18-S and 25-S r-RNA are produced from a single precursor molecule (Taber & Vincent, 1969), and such processing produces equimolar amounts of the two ribosomal species (Grierson, Rogers, Sartirana & Loening, 1970). Any change in their relative contributions to the overall labelling patterns must include a change in the stability of one fraction. It is also likely that if pre-r- and 25-S-r-RNA are degraded more rapidly, some of the degradation products would be detected in the heterodisperse RNA fractions. Such a decrease in stability might also account for the smaller size of the pre-r- peak detected on the gel.

A portion of the ^{32}P-labelled total nucleic acids extracted in the above experiment were digested with RNase for 4 hr (37°C) in SSC before being fractionated on the2.4% polyacrylamide gel. The only fraction of high molecular weight material which absorbed strongly at 260 nm was assumed to be DNA (Padilla, Creanor & Fraser, 1974). From the specific activities of the label in these fractions, the cells labelled in the presence of 2 mM uridine incorporated 17.1% less ^{32}P into their DNA in 19 min than did the control cells (Fig. 3/14). This reduction in labelling could be caused by either reduced DNA synthesis or interference with the incorporation of exogenous ^{32}P into DNA. In either case, the normal pathways to DNA synthesis appear to be affected by 2 mM uridine.

These experiments with 2-4 mM uridine indicate that high external uridine concentrations alter the normal pathways or control systems for

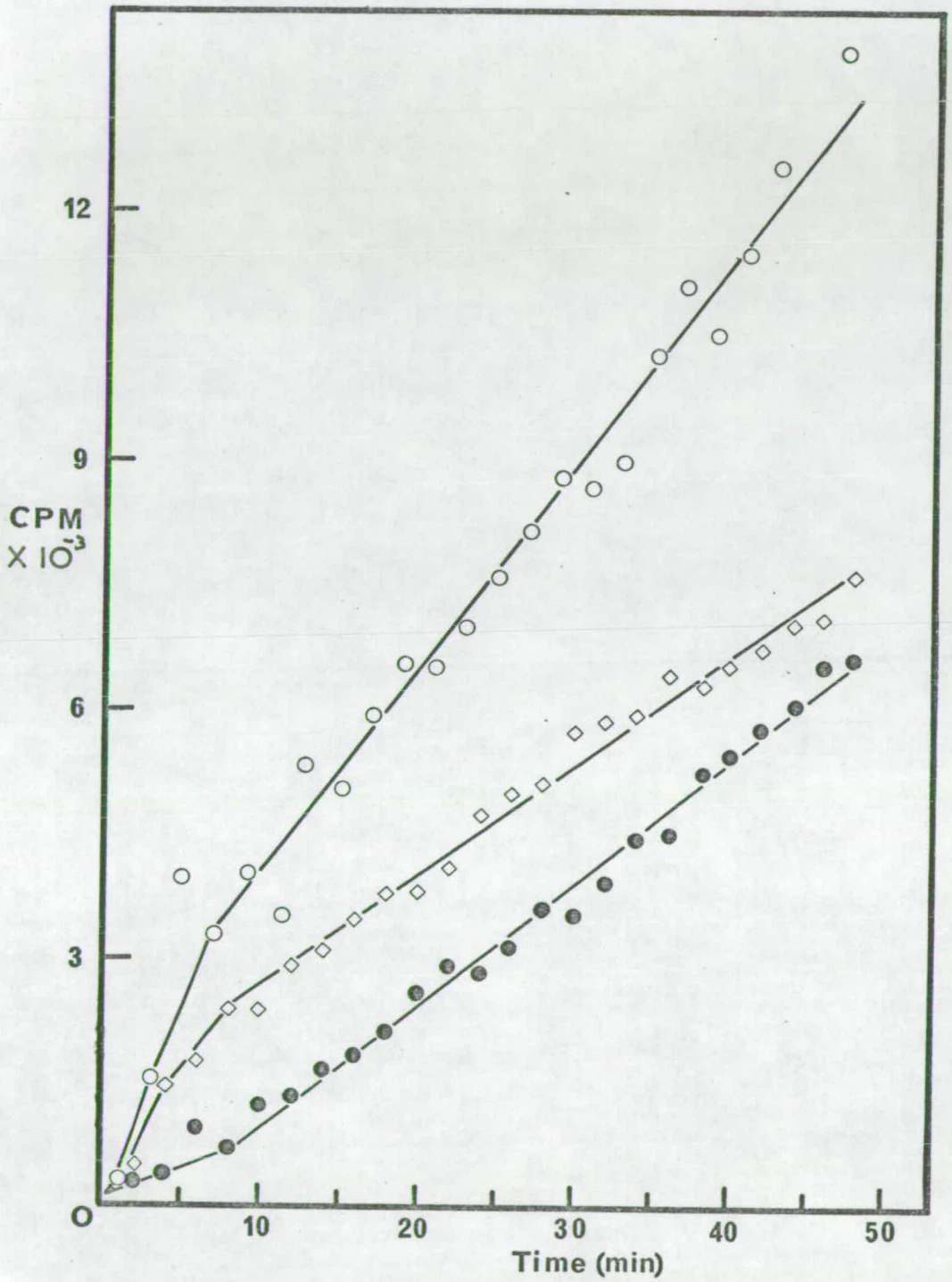
nucleic acid synthesis in S. pombe without appreciably affecting overall growth for at least 1.5 generations. Experiments at such uridine concentrations may be useful to test whether the rate of ^{3}H-uridine incorporation is limited by the rate of uptake. But precise information about timing of rate changes or absolute values for rates of synthesis would have to be carefully corrected for these metabolic perturbations. Another disadvantage of these experiments is purely technical. In order to get significant radioactive material taken up or incorporated by cells in 2 mM uridine, up to 20 µCi/ml ^{3}H-uridine must be used to label them. This, in turn, increases the background level of radiation to 20-30% of the counts incorporated by the cells in a 1 min pulse. Scatter of the data points inevitably is large in these experiments, especially those involving incorporation at short pulse times.

^{3}H-ADENINE

Although most of the work in this thesis was done using radio-labelled uridine, some comparative data was also obtained with ^{3}H- and ^{12}C-adenine. In S. pombe, ^{3}H-adenine incorporation can be used directly as a measure of RNA synthesis because there is a 100-fold excess of RNA over DNA (Mitchison & Lark, 1962). A thorough study of adenine uptake and pool formation has already been made for S. pombe (Cummins & Mitchison, 1967). But after their work was done the strain N.C.Y.C. 132 of S. pombe used in this laboratory, was replaced with fresh stocks because of genetic drift. Although the replacement stocks were also strain N.C.Y.C. 132, significant differences have been noted between the two (Mitchison & Creanor, 1971,b; Creanor, pers. comm.). The kinetics of ^{3}H-adenine uptake and incorporation also appear to be slightly

Figure 3/15.

Kinetics of ^{3}H-adenine utilization in an exponential phase culture. Cells were labelled with 1.7 µCi/ml in the presence of 124 µM adenine which was added at the time of labelling. Results are given as cpm x 10^{-3} per 1.0 OD_{595} of culture. The difference between the uptake (o) and the incorporation (•) is presented as a measure of the filling of the pool of adenine and its soluble derivatives (◇).


12
9
6
3
O
CPM
X 10^-3
10
20
30
40
50
Time (min)

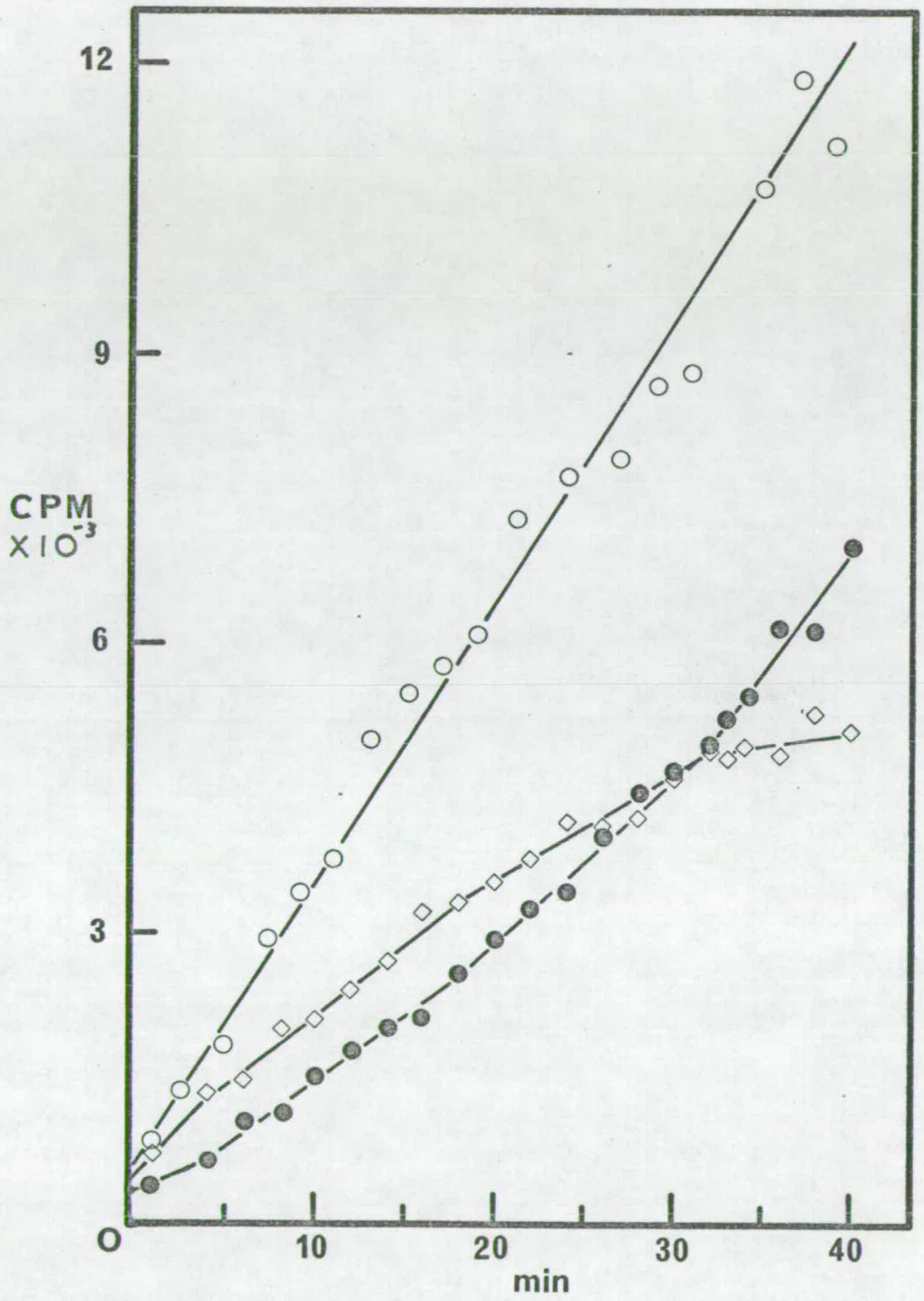
different in the "old" and the "new" strains. As reported by Cummins and Mitchison, when the cells are first presented with adenine, their adenine pools expand, causing a rapid initial rate of uptake (Fig. 3/15). The rate of uptake then decays to a constant value after 5-10 min in the new strain, rather than the 15-20 min reported for the old strain. Incorporation of ^{3}H-adenine in the present cultures starts slowly and increases in rate during the first 8-12 min before becoming constant. This, again, contrasts with the earlier work in which the rate of incorporation reached a constant value after 2-3 minutes. The present results indicate that the pool of ^{3}H-adenine and its soluble derivatives fills at a constant rate after about 10 min of labelling, but before this time the rate increases rapidly.

These adenine pools can be filled prior to labelling by maintaining the culture in medium containing adenine (20 µg/ml) for 3 hr before adding the label (Cummins & Mitchison, 1967). When these pools have been prefilled, the kinetics of ^{3}H-adenine uptake and incorporation (Fig. 3/16) appear to be much more like those of ^{3}H-uridine (Fig. 3/1). Uptake proceeds at a constant rate from the start of labelling, whereas incorporation increases in rate for at least 40 min. As in the case of uridine, the incorporation data may be interpreted as being either an exponential increase or a series of linear increases with periodic rate changes. But in the case of this present precursor, it appears to be much more continuous than with ^{3}H-uridine. With the adenine pool expanded and prefilled it takes about 30 min for the specific activity of the pool to equilibrate with that of the medium when ^{3}H-adenine is subsequently added.

Thus, the differences in the kinetics of ^{3}H-adenine uptake and

Figure 3/16.

Kinetics of ^{3}H-adenine utilization after filling the adenine pools. Pools were filled by growing the culture in the presence of 148 µM adenine in EMM2 for 3 hr before labelling with 4 µCi/ml ^{3}H-adenine. Results are given as cpm x 10^{-3} per 1.0 OD_{595} of culture. The difference between uptake (o) and incorporation (●) is presented as a measure of the filling of the pool of adenine and its soluble metabolites (◇).


12
9
6
3
0
CPM
X 10⁻³
10
20
30
40
min

incorporation between the "old" and "new" strains 132 appear to be in degree rather than in overall pattern. The main conclusions reached by Cummins and Mitchison (1967) should, therefore, remain valid for the strain of S. pombe used in this work.

DISCUSSION

In this Chapter I have examined some aspects of the labelling of RNA with ^{3}H-uridine and made a few comparative observations using ^{3}H-adenine. The object of this work was to choose conditions of labelling for pulse-labelling experiments which would preferentially measure the rates of synthesis of different types of RNA. Previously RNA synthesis had been measured in S. pombe using radio-labelled adenine (Mitchison & Lark, 1962), but the expandable adenine pool (Cummins & Mitchison, 1967) could cause problems of interpretation.

The ^{3}H-uridine pool, on the other hand, does not expand, and uptake of the precursor proceeds at a constant rate for at least 82 min after adding the label (Fig. 3/2). Although after 25 to 30 min the specific activity of the radioactive pool appears to be nearly equilibrated with the label in the medium (Figs. 3/1, 3/2), this equilibrium is only reached on a cellular basis after about 40 min of labelling (Fig. 3/11). All of the label which is incorporated appears in RNase sensitive material, but not all of the ^{3}H-uridine taken up passes directly through UMP into RNA. At least one, and possibly two, bypass pools are also labelled by this tracer. The rate of ^{3}H-uridine incorporation therefore increases periodically as each bypass pool is filled (Fig. 3/1). These rate changes occur at 10-12 min and again at 25-30 min after adding the labelled precursor (Fig. 3/1). After the

second rate change, incorporation and uptake proceed at the same rate per cell (Fig. 3/2). From the work of Grenson (1969) the most likely bypass pools for ^{3}H-uridine to enter are uracil and cytosine. Since both of these compounds are precursors to RNA, their incorporation products meet the criterion of RNase sensitivity.

At low or moderate uridine concentrations, ^{3}H-uridine uptake appears to follow first order kinetics (Fig. 3/9), and growth in uridine prior to labelling does not affect the patterns of uptake or incorporation. But at uridine concentrations of 2 mM or greater, the uptake kinetics change (Fig. 3/9). Uridine enters the cells much more rapidly at these high concentrations, and the K_m increases from 3.15×10^{-5} M to 15.8 mM. Grenson (1969) has reported similar functional kinetics for ^{3}H-uridine uptake in budding yeast. The simplest explanation for this increase in the K_m would be that high concentrations of uridine overcome an uptake restriction. Diffusion would thereby become the main uptake mechanism at high concentrations, whereas an active transport of uridine would be the main one at lower concentrations. Stambrook and Sisken (1972,a) report that such a change in mechanism occurs in Chinese hamster cells at uridine concentrations above 0.82 mM.

However, the possibility that at very high concentrations, uridine is actively transported by an additional set of permeases cannot be ruled out.

In either case, in the presence of 2 mM uridine, the rate of incorporation of the radioactive precursor equals that of uptake in half the time it requires to do so in control cultures.

Although uridine concentrations as high as 4 mM do not have any noticeable effect on either the CPI or the growth rate (from OD) of a log-phase culture (Fig. 3/12), 2 mM uridine causes a reduction in the

amount of radioactivity incorporated into DNA and into high molecular weight RNA following a 19 min pulse of ^{32}P (Figs. 3/13 & 3/14). The RNA incorporation pattern appears to be caused by a more rapid degradation of ribosomal precursor and 25-S RNAs, rather than by reduced synthesis. But the 17% less label found in DNA is unlikely to be caused by degradation. DNA synthesis could be reduced, but a 17% reduction should manifest itself rather quickly by a change in either the CPI or growth rate. A second possibility is that a high intracellular uridine concentration causes a breakdown of polyphosphate granules in the cell. And this internal supply of phosphate might be preferentially used in DNA synthesis.

The length of the ^{3}H-uridine pulse determines to a very great extent the type of RNA labelled. After 1 min of labelling, most of the label is found in the unstable ribosomal precursor and heterodisperse fractions, but some label is also found in the 4-5 S fractions (Figs. 3/5 & 3/6). Following a 10 min pulse, the distribution of label is very similar to that in steady state labelled RNA. From a practical point of view, however, 19 min pulses were convenient, because they could easily be started either 10 or 20 min apart, thereby allowing either 7 or 14 samples to be taken during a 140 min cell cycle. And they allowed twice as much radioactivity to be incorporated as in a 10 min pulse with no significant change in the pattern of labelling (Fig. 3/5).

Since S. pombe contains 100-fold more RNA than DNA, ^{3}H-adenine can be used as a second radio-label for RNA (Mitchison & Lark, 1962). As reported by Cummins and Mitchison (1967), the ^{3}H-adenine pools in S. pombe expand when the cells are first given adenine, causing a rapid initial uptake of the precursor (Fig. 3/15). The rate of uptake then

decays to a constant value after 10 min. During this same period the rate of incorporation increases, and by 10 min after adding the adenine, uptake and incorporation increase at the same rate. To bypass the effects of the expansion of the adenine pools, cultures can be maintained with adenine in the medium for a generation before adding labelled adenine. When this is done, ^{3}H-adenine uptake increases at a constant rate and incorporation increases in rate for at least 30 min (Fig. 3/16). So to use that rate of ^{3}H-adenine incorporation as a measure of the rate of RNA synthesis, both the prefilled and the empty-pool conditions must be examined.

From the above considerations, it was decided to label cells with ^{3}H-uridine in the presence of 1-2 µg/ml (4.2-8.4 µM) unlabelled uridine. Rates of stable RNA synthesis and of precursor uptake would be followed using 19 min pulses, whereas rates of unstable RNA synthesis would be followed using 1 min pulses. For comparative purposes cultures would also be labelled with ^{3}H-adenine. Some of these latter cultures would have their adenine pools filled prior to labelling, while others would not.

Before beginning experiments on synchronous cultures, 7 parameters of culture growth and ^{3}H-uridine metabolism were determined in log phase cultures (Fig. 3/17). As expected, cell numbers, OD_{595} (cellular dry mass), RNA content, DNA content, and the rates of ^{3}H-uridine uptake and incorporation (19 min pulses) all increase exponentially for at least 8 hr (about 3 generations). During this time the cell plate index (CPI) ranged from 6 to 9% with a mean of 7%. The slight differences in the slopes of the various lines in Fig. 3/17 can be accounted for by the small differences in the generation times of individual cultures. These times ranged from 135 min for ^{3}H-uridine uptake rate to 150 min for ^{3}H-

Figure 3/17.

Some parameters of growth in an Exponential Phase Culture.

(a) ^{3}H-uridine uptake at 1 µCi and 2 µg/ml, in cpm x 10^{-3};

(b) ^{3}H-uridine incorporation at 1 µCi and 2 µg/ml, in cpm x 10^{-3};

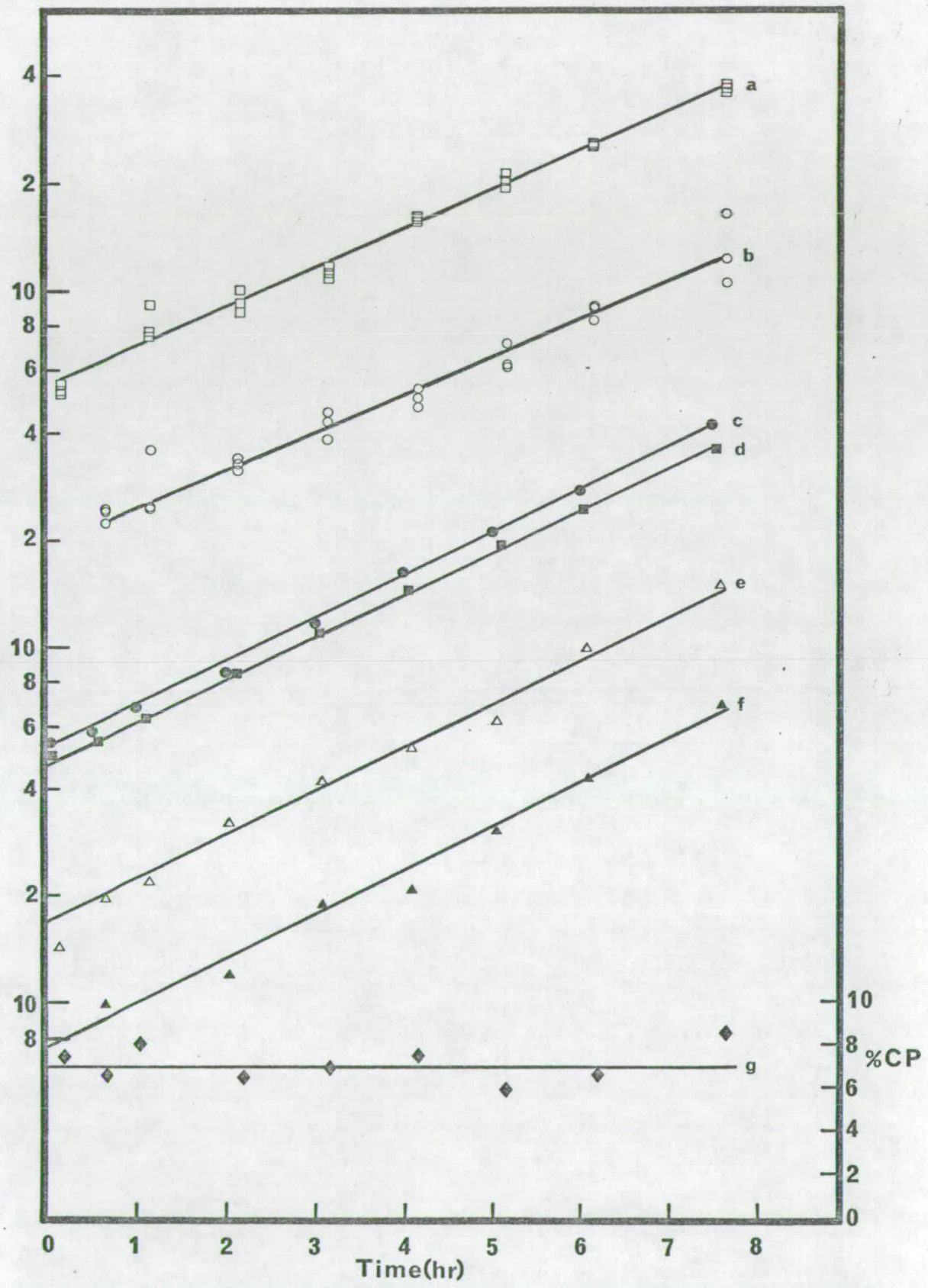
(c) cell numbers x 10^{-6}/ml;

(d) OD_{595} x 10;

(e) RNA/ml in OD_{260} of PCA extract;

(f) DNA/ml as corrected OD_{595} of Diphenylamine reaction;

(g) cell plate index, from right hand abscissa.


4
2
10
8
6
4
2
10
8
6
4
2
10
8
a
b
c
d
e
f
g
10
8
6
4
2
0
%CP
0
1
2
3
4
5
6
7
8
Time(hr)

uridine incorporation rate. In all of the above 7 curves the scatter is relatively minor.

These results show that the methods are reasonably accurate and give a log phase growth base line with which further experiments can be compared. In the remainder of this thesis I wish to examine the relationships between changes in the rate of RNA synthesis and various other cell cycle events.

CHAPTER IV

SELECTION SYNCHRONIZED CULTURES

Earlier work on RNA accumulation (Mitchison & Walker, 1959) and on rates of precursor uptake and incorporation into RNA in S. pombe (Mitchison & Lark, 1962; Mitchison, 1963; Cummins & Mitchison, 1964; Mitchison et al., 1969) has been interpreted as showing that the rates of RNA synthesis are continuous and approximately exponential during the cell cycle. Most of this work is based upon autoradiographic measurements of single cells positioned in the cell cycle according to their size. On the whole, these data appear to indicate that rates increase either exponentially or parallel to dry mass and cell volume changes. Cummins and Mitchison (1964), however, presented data indicating a step-like doubling of the rate of adenine uptake during the cell cycle. Data from bulk measurements of Mitchison et al. (1969) suggest that the rate of adenine incorporation may also step in selection synchronized cultures. None of these experiments gave data for more than 5 points during the cell cycle or examined several successive cell cycles.

Because of the conflicts in the earlier work it was decided to measure the rate of RNA synthesis at up to 14 points during the cell cycle and to follow the rate for at least two generations in selection synchronized cultures. Since the rate measurements were made with relatively short pulses of radio-labelled uridine and adenine, care had to be taken to account for any cell cycle specific changes in the specific activities of the internal pools. Such changes could alter the rate of precursor incorporation while the rate of RNA synthesis remained constant. Factors which could affect the specific activity of an

internal precursor pool are: (1) a change in the rate of precursor uptake at a point in the cell cycle; (2) a change in the size of the pool when the labelled precursor is added, especially if the precursor concentration in the medium changes appreciably (expandable pool); (3) an alternation of the size of the pool at a point in the cell cycle; and (4) a change in the ratio between the contributions of the external and internal supplies of precursor to the synthetic pathway at some point in the cell cycle. The rates of uridine and adenine uptake and incorporation were, therefore, measured under a variety of conditions.

Part of the work presented in this Chapter was done with Dr W.H. Wain and has been published as a joint paper with him (Wain & Staatz, 1973; See Appendix I). Of the material presented both here and in the paper Dr Wain and I each contributed about half the work on the following: Development of the method for measuring rate of incorporation; the measurement of the 19 min pulse-rates of incorporation of uridine, adenine and ^{35}S; and the determination of specific activities of the PCA-extracted RNA after 19 min uridine pulses (Figs. 4/1, 4/2 & 4/3). The ribosomal protein patterns and the specific activity of ^{3}H-uridine incorporated into isolated ribosomes described in the paper were wholly the work of Dr Wain and are not presented here.

By slight modification of the methods devised for the above work, the rates of uptake and the short-pulse rates of incorporation were measured after Dr Wain had left this Department. The rate of uridine incorporation at 500 μg/ml was likewise examined after publication of the above paper, the highest concentration used in our joint paper being 200 μg/ml. Rates of RNA synthesis and of precursor uptake are examined here at up to 14 points per cell cycle and for at least 2 cell cycles in

selection synchronized cultures. Cells were pulse-labelled with radioactive precursors over a wide range of precursor concentrations (7 ng to 500 µg/ml) added at the time of labelling. Some cultures were also grown for 3 to 4 hours in the presence of precursor (20 µg/ml) before synchronization, to see if pre-filling the precursor pools had any effect upon the rates of precursor use during the cell cycle. To compare the rates of synthesis of rapidly-labelled RNA synthesis with that of stable RNA separate cultures were given pulses of 1, 5, 10 or 19 min.

RATES OF ^{3}H-URIDINE, ^{3}H-ADENINE and ^{35}S INCORPORATION

To get detailed information about the rate of precursor incorporation into stable RNA during the cell cycle, aliquots from a selection synchronized culture (Mitchison & Vincent, 1965) were pulse-labelled for 19 min with ^{3}H-uridine (1 µCi/ml) and $Na_2{}^{35}SO_4$ (carrier-free) or with ^{14}C-uridine (0.5 µCi/ml). Uridine concentrations in the labelling medium ranged from 0.03 µM to 820 µM and successive pulses were started either 10 to 20 min apart. The data from 6 uridine and 2 ^{35}S experiments were normalized as described in Chapter 2, and presented as means ± 95% confidence limits for all points within each tenth of a cell cycle. The rate of uridine incorporation doubles in a sharp step-like manner at the time of cell division (Fig. 4/1a). The rate of sulphate incorporation into the same cells increases exponentially (Fig. 4/1c) demonstrating that the step in the uridine pattern is not a product of differential loss of cells at different times during the cell cycle. The two most likely things that could cause step-like changes in the rate of uridine incorporation are either a change in the rate of RNA synthesis or a change in the specific activity of the internal uridine pool as described above. To see if the step in rate of incorporation was limited to the

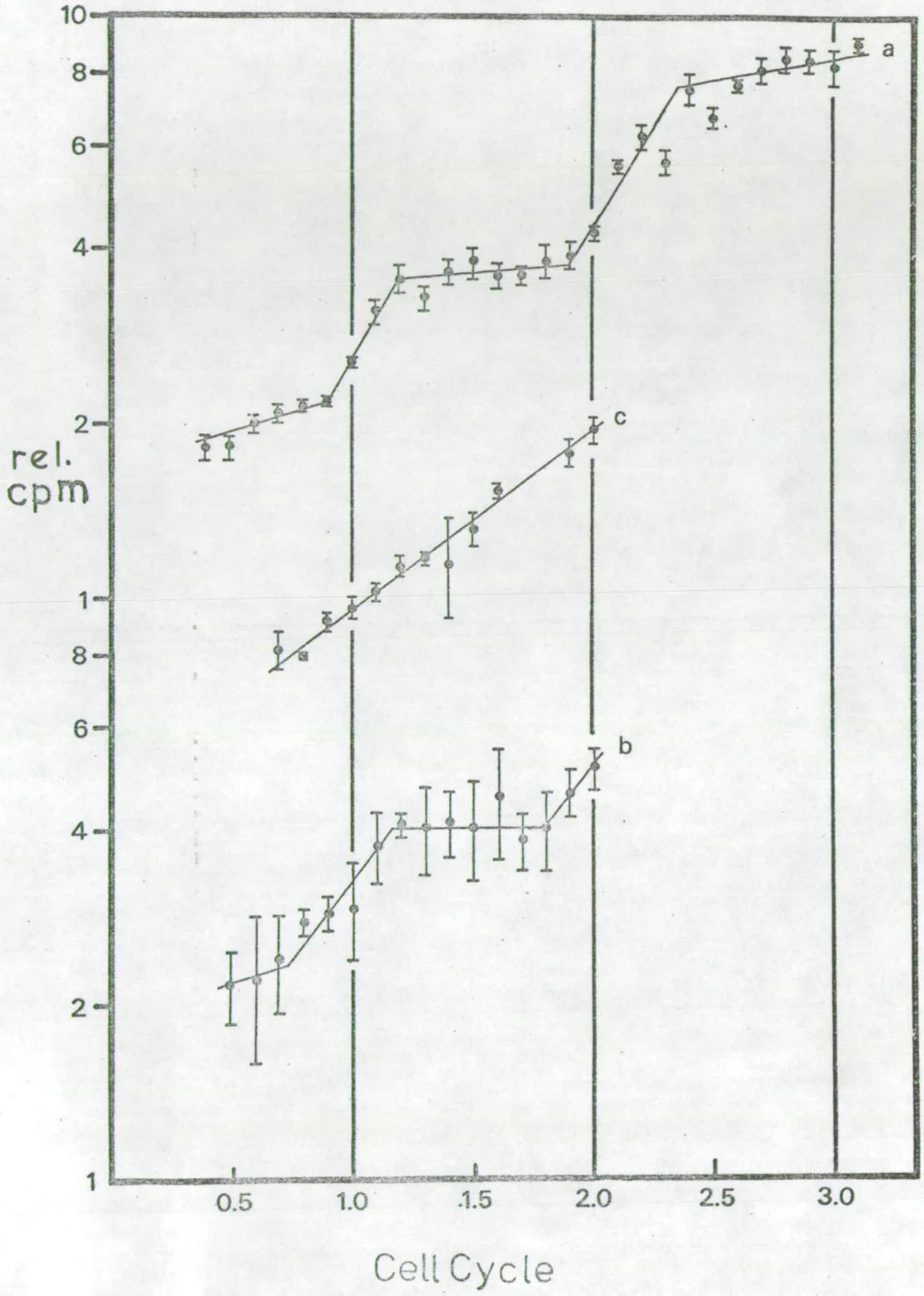
Figure 4/1.

Rates of incorporation of uridine, adenine and sulphate into the acid-insoluble fraction of whole cells during the cell cycle. Cells were pulse-labelled for 19 min. The mean value of the plateau between the first and second peaks in the cell plate index of each experiment was normalized at 4.0. The data from repeat experiments were then pooled and the means and 95% confidence limits were calculated for all points within each 0.1 cell cycle.

(a) from 6 synchronous cultures labelled with ^{14}C-uridine or ^{3}H-uridine, with uridine concentrations in the labelling medium ranging from 0.03 µM to 820 µM in separate experiments;

(b) from 2 synchronous cultures labelled with ^{3}H-adenine in the presence of 1.5 µg adenine/ml of culture;

(c) from 2 synchronous cultures labelled with $Na_2{}^{35}SO_4$ in the presence of 10 µg Na_2SO_4/ml medium. Data given as relative cpm.


10
8
6
4
2
1
8
6
4
2
1
rel.
cpm
a
c
b
0.5
1.0
1.5
2.0
2.5
3.0
Cell Cycle

uridine pathway in S. pombe, the rate of ^{3}H-adenine incorporation was measured in experiments analogous to those just described for ^{3}H-uridine. In this case, data from 2 synchronous cultures which had been labelled in the presence of 1.5 µg adenine/ml culture were normalized and pooled. The rate of ^{3}H-adenine incorporation clearly doubles in a discrete step at the time of cell division (Fig. 4/1b). Since both the purine base, adenine, and the pyrimidine nucleoside, uridine, are incorporated in a similar manner, the step-like rate doublings are almost certainly a general feature of nucleic acid biosynthesis in S. pombe.

PREFILLED ADENINE POOLS

Since the adenine pool in S. pombe is expandable, the influences it might have upon the adenine data can be eliminated by prefilling it with unlabelled adenine before synchronizing the cells and beginning the labelling experiments. A log phase culture was therefore grown in the presence of adenine (20 µg/ml) for 4 hr prior to harvesting. During and after synchronizing all media used also contained adenine. Aliquots of the culture were labelled for 19 min with ^{14}C-adenine (2 µCi/ml) and the rate of its incorporation was determined. In cultures of cells with prefilled adenine pools the rate of adenine incorporation had the same pattern (Fig. 4/2a) as it did in cultures which first experienced exogenous adenine at the time of labelling (Fig. 4/1b). This result and the small, non-expandable uridine pool (Chapter 3) make it unlikely that an expandable pool causes major changes in the rate of precursor incorporation into RNA.

Figure 4/2.

Effects of precursor concentration upon the pattern of the rates of total RNA synthesis during the cell cycle.

(a) ^{14}C-adenine (2 µCi/ml) incorporation after prefilling the adenine pool by growing the cells in the continuous presence of 20 µg adenine/ml of culture from 4 hr before synchronizing the cells;

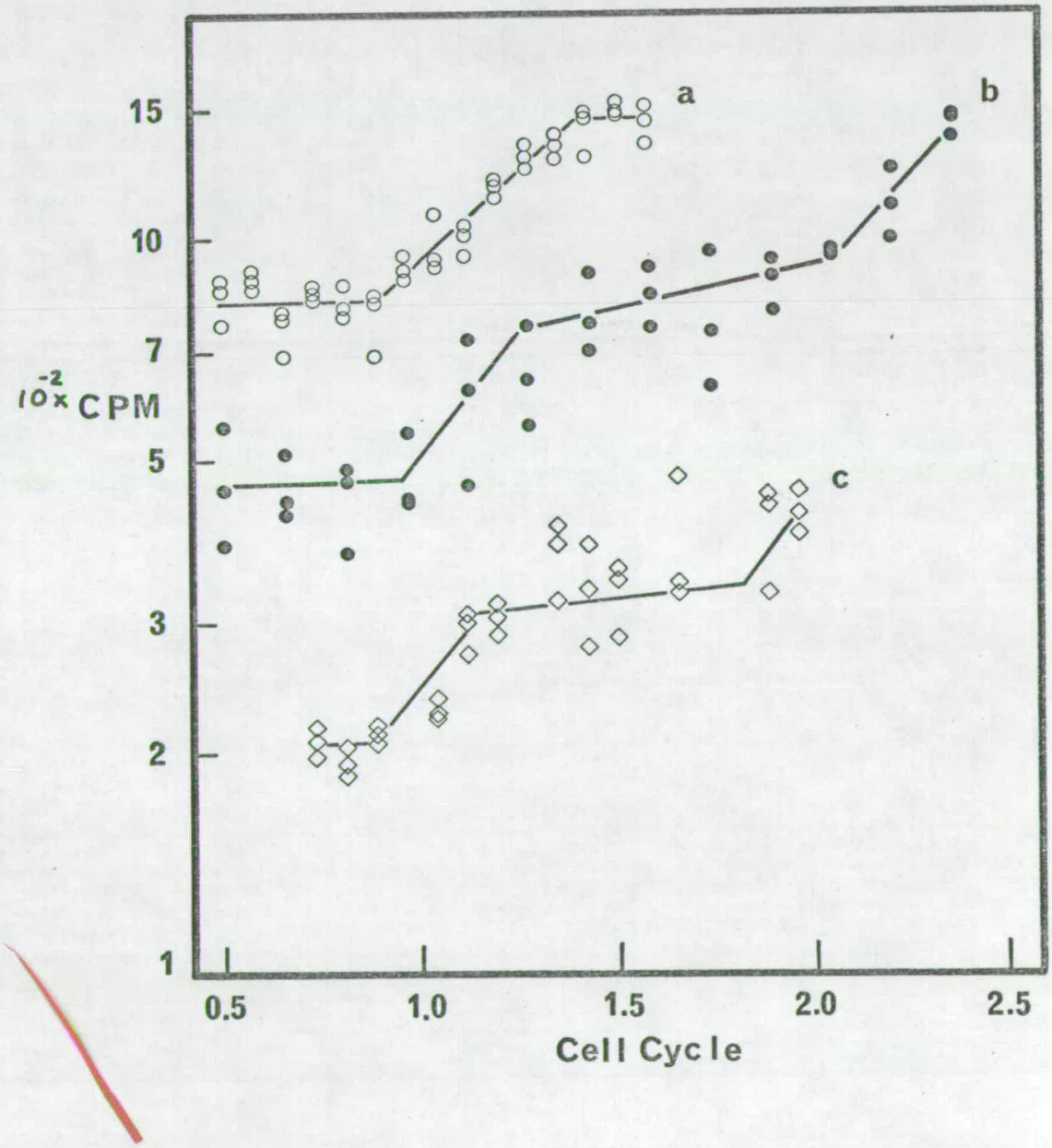
(b) ^{3}H-uridine (20 µCi/ml) at a final concentration of 2 mM;

(c) ^{3}H-uridine (1 µCi/ml) at a final concentration of 0.03 µM.

a =

b = 2.05 mM UTP

c =



THE EFFECT OF 2 mM URIDINE

As shown in Chapter 3, when S. pombe is labelled in the presence of low concentrations of uridine, uptake of this precursor appears to occur chiefly by way of facilitated diffusion or active transport. At concentrations of 2 mM or greater, however, uridine enters the cells mainly by diffusion. If the rate of transport of the precursor into the cells were rate limiting it could impose its own pattern of rate changes on the rate of incorporation. For just such reasons Stambrook and Sisken (1972a) were able to convert step-like increases in the rate of uridine incorporation during the cell cycle in Chinese hamster cells into a linear pattern of increase by simply raising the uridine concentration in the labelling medium from 0.25 µM to 200 µM. For S. pombe we reported no change in the cell cycle pattern of incorporation in cultures labelled at external uridine concentrations ranging from 0.03 µM to 820 µM in separate experiments (Wain & Staatz, 1973). But further considerations of the impermeability of this yeast to many drugs known to be effective in mammalian cells led to the permeability experiments at very high uridine concentrations (See Chapter 3). Following on from these experiments I remeasured the rate of uridine incorporation during the cell cycle. This time, however, to overcome the effects of any possible uptake limiting factors, unlabelled uridine was added to a final concentration of 2 mM. The pattern of change in rate of incorporation remains the same as in earlier experiments (Fig. 4/2b). But the midpoint of the rise in the doubling occurs at 0.175 of a cell cycle as opposed to 0.04 cell cycle for the midpoint of the change in the mean rate of incorporation (Fig. 4/1a). This delay in the doubling of the rate of incorporation may reflect the normal range over which these rates may double. It may equally well be an effect of the high uridine concentration upon RNA synthesis.

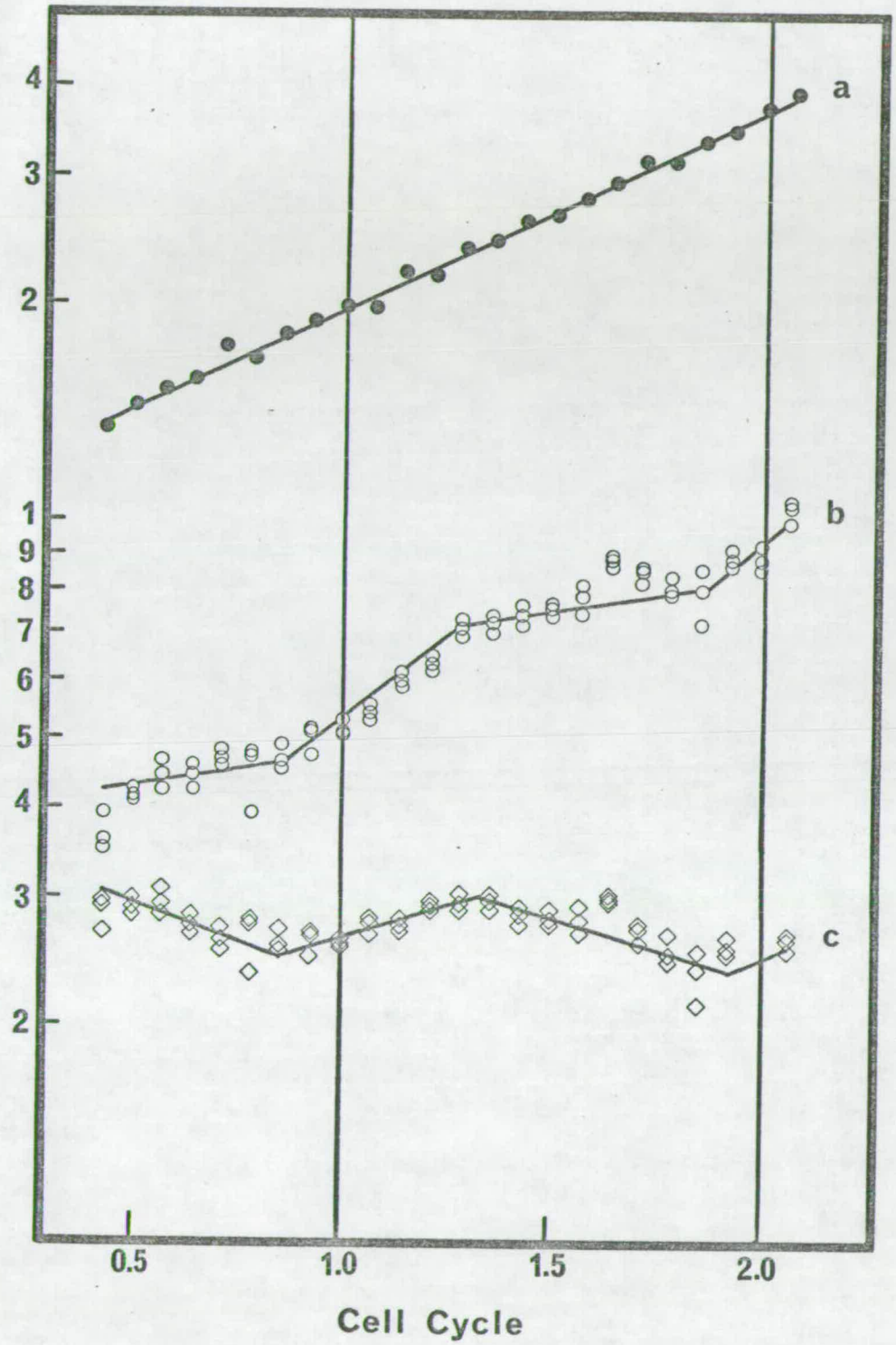
Figure 4/3.

Specific activity of extracted total RNA labelled by 19 min pulses of ^{3}H-uridine started every 10 min throughout the cell cycle.

(a) total RNA extracted from 5 ml of cells, measured as the OD_{264} of a hot PCA digest of acid-washed cells;

(b) radioactivity counted in 50 µl of the PCA digest, given as cpm x 10^{-3};

(c) specific activity of the PCA digest, presented as cpm x 10^{-3} µg RNA in 5 ml culture.


4
3
2
1
9
8
7
6
5
4
3
2
a
b
c
0.5
1.0
1.5
2.0
Cell Cycle

SPECIFIC ACTIVITY OF EXTRACTED RNA

A final method used to demonstrate the relationship between precursor incorporation and RNA synthesis was to measure the specific activity of RNA extracted after pulse labelling cells with ^{3}H-uridine at different times during the cell cycle. Although the total RNA/ml culture, measured as the OD_{264} of the hot 5% PCA-digest, increases exponentially (Fig. 4/3a), the rate of uridine incorporation into the PCA-extracted RNA clearly doubles with a step (Fig. 4/3b). By dividing the counts incorporated by the amount of RNA, we get the specific activity of the RNA (Fig. 4/3c). This corrects for RNA losses during the extraction procedure. The pattern is similar to that for the specific activities of the RNA in purified ribosomes of S. pombe (Wain & Staatz, 1973), with minima about the time of cell division and a maximum at about mid-cycle.

The experiments described so far indicate that for 19 min pulses, variations in pool size and the rates of uptake are probably not major factors controlling or limiting the rates of precursor incorporation into RNA during the cell cycle of S. pombe. Changes in the relative contributions of external and internal sources of precursors are also unlikely, as they would have to occur in identical fashion in both uridine and adenine pathways. In contrast to results from Chinese hamster cells (Stambrook & Sisken, 1972,a,b), in this yeast the rates of both uridine and adenine incorporation do reflect the rate of RNA synthesis. This rate of synthesis doubles at the time cells both divide and make their DNA. Whether these two parts of nucleic acid synthesis are directly linked remains to be investigated. But gene dosage control of RNA synthesis has been proposed for organisms ranging from bacteria (Dubnau, Smith & Marmur, 1965) to mammalian cells (Pfeiffer, 1968).

Figure 4/4.

The rates of ^{3}H-uridine uptake, incorporation and soluble pool filling during the cell cycle. Cells were pulse labelled for 19 min. Data were normalized as described in the text and presented as means ± 95% confidence limits. The difference between the uptake and the incorporation data is given as the rate of soluble pool filling.

(a) rate of uptake from 4 separate experiments;

(b) rate of soluble pool filling;

(c) rate of incorporation and the same data as given in Fig. 4/1a;

(d) rate of pool filling per cell. The cell numbers used to calculate the per cell rate were the mean of those from 2 uptake- and 2 incorporation experiments.

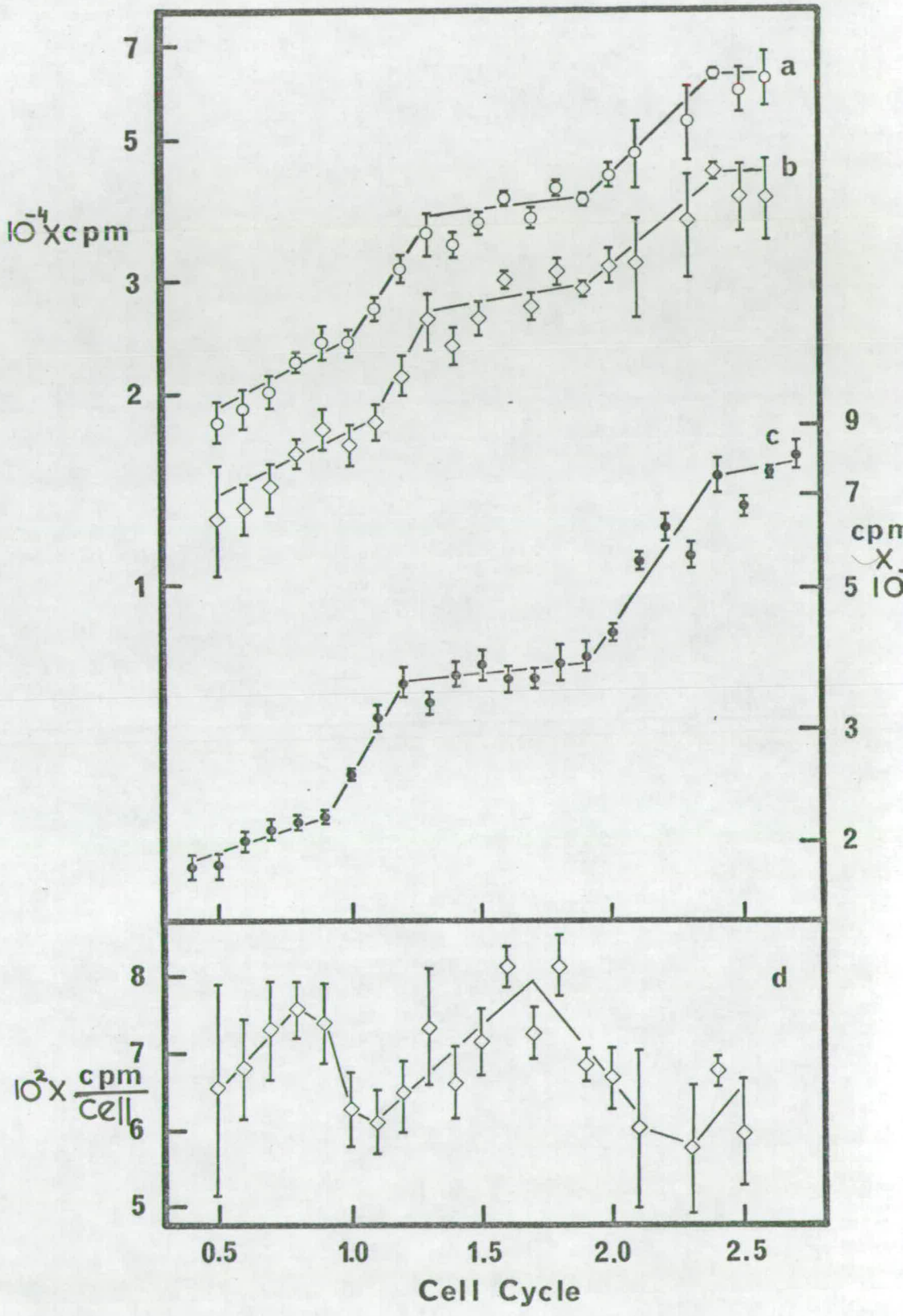

7
5
3
2
1
10^{-4} X cpm
a
b
c
9
7
cpm
X
5 10
3
2
8
7
6
5
10^{2} X cpm / cell
d
0.5
1.0
1.5
2.0
2.5
Cell Cycle

Figure 4/5.

The rates of ^{3}H-adenine uptake incorporation, and soluble pool filling during the cell cycle. The methods used were as reported for Fig. 4/4. Cultures were all labelled at 1.6 µg adenine/ml culture and 1 µCi/ml.

(a) rate of uptake from 2 experiments;

(b) rate of soluble pool filling;

(c) rate of incorporation and the same data as presented in Fig. 4/1b.

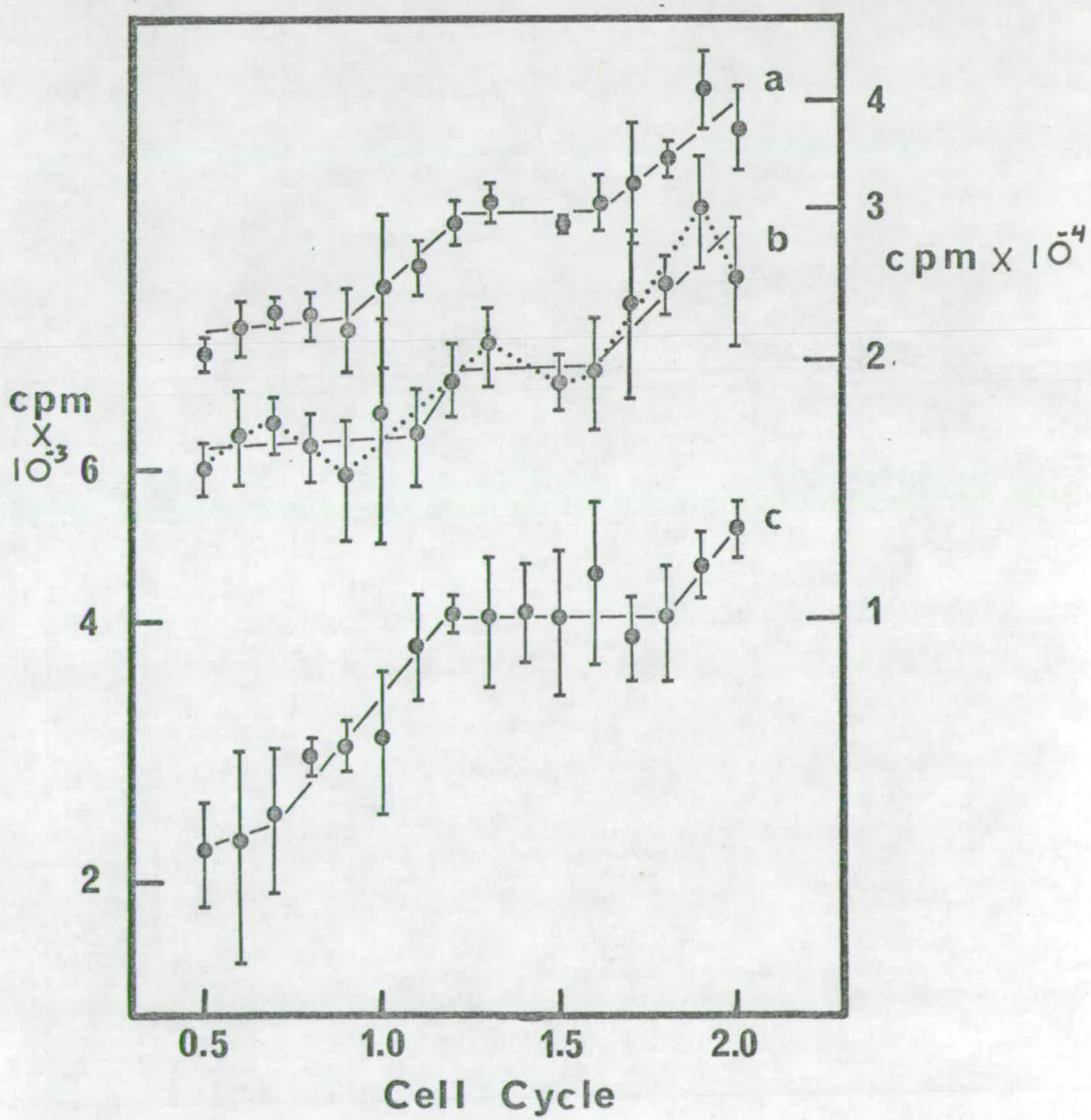
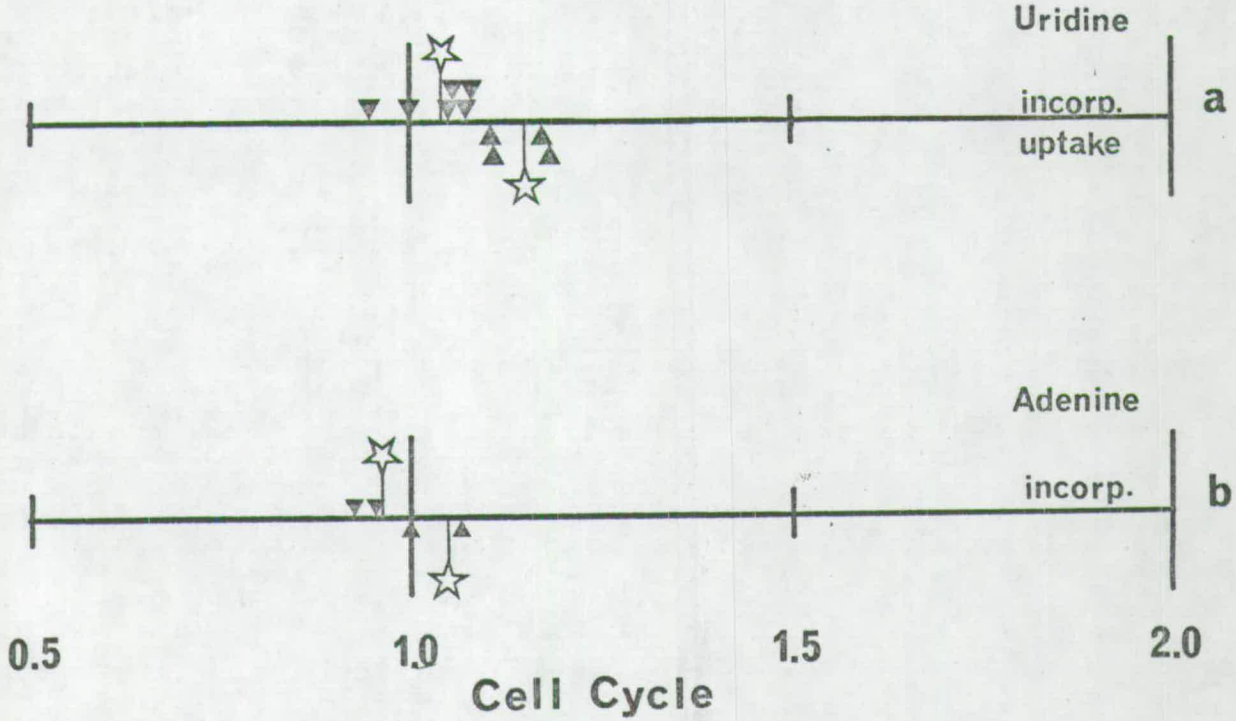

a
b
c
cpm X 10⁻³
6
4
2
cpm X 10⁻⁴
4
3
2
1
0.5
1.0
1.5
2.0
Cell Cycle

Figure 4/6.

Cell cycle maps of the mid-points of the increases in the rates of ^{3}H-uridine and ^{3}H-adenine uptake and incorporation during the cell cycle. Triangles represent individual experiments and stars represent the normalized, pooled data presented in Figs. 4/4 and 4/5.


Uridine
incorp.
uptake
a
Adenine
incorp.
b
0.5
1.0
1.5
2.0
Cell Cycle

RATES OF UPTAKE AND POOL FILLING

The results above are only indirect measures of the rates of precursor uptake and soluble pool filling. Like the rates of incorporation, the rate of uptake was directly measured using 19 min pulses of ^{3}H-labelled precursors, but in this case the total amount of radioactivity in water-washed cells was measured. The difference between the rates of uptake and incorporation is presented as a measure of the rate of soluble pool filling and was determined as described in Chapter 2. The rates of uptake and soluble pool filling for ^{3}H-uridine (Fig. 4/4) and ^{3}H-adenine (Fig. 4/5) double sharply during a short interval following cell division. The ^{3}H-adenine pool filling data, however, is open to a second interpretation represented by the dotted line in Fig. 4/5. The rate could rise until mid-cycle and then fall. Then, as uptake rate increases the pool again fills at an increased rate. Because of scatter in the adenine data, both of these interpretions fit within the 95% confidence limits. No such doubts arise from the ^{3}H-uridine data (Fig. 4/4); a step-like rate doubling is the only pattern consistent with the measured rates of uptake, incorporation and pool filling. A similar rapid increase in the rate of uridine uptake occurs in Tetrahymena at the start of DNA synthesis (Wolfe, J., 1975). In S. pombe a possible step in the rate of adenine uptake has also been reported about half way through the cell cycle (Cummins & Mitchison, 1964). The present data, however, indicates that the rates of uptake of both uridine and adenine double about 0.1 cell cycle after the similar increases in the respective rates of incorporation. The range over which the mid-points of these rate doublings occur is shown on the cell cycle maps, Figure 4/6. These mid-points range over more than 0.1 cell cycle for

each type of experiment. But all of the uptake steps occur after their respective rates of incorporation have doubled. The mid-points for the normalized pooled data show a 0.1 cell cycle delay from the time of doubling of the incorporation rate until its respective uptake rate doubles. This lag between the two mid-points reinforces the earlier conclusion that neither uptake nor pool filling are likely to cause the observed changes in the rate of incorporation. If this had been the case, the rates of uptake would have doubled either before or at the same time as the rate of incorporation.

The similarity between the rate patterns of uridine and adenine uptake and incorporation suggest that a feedback mechanism similar to the one known to control adenine uptake (Cummins & Mitchison, 1967) may be acting upon the rate of uridine uptake. This feedback hypothesis is further supported by the changes in the cellular rate of ^{3}H-uridine pool filling (Fig. 4/4d). When the rate of incorporation begins to double, the rate of uridine pool filling per cell decreases to a minimum value. The rate of uridine uptake then increases and the cellular rate of pool filling rises. The missing link is further evidence to show that the decrease in the rate of pool filling causes the rate of uptake to increase.

SHORT PULSES

So far, all experiments described have been done using 19 min pulses of labelled precursors. But the pattern of rate changes may not be the same for rapidly-labelled, unstable RNA and for stable RNA.

The relative amounts of the different fractions labelled by pulses of ^{3}H-uridine of 1 to 19 min is described in Chapter 3 (See Figs. 3/5 & 3/6). Comparable rates of incorporation were determined using

Figure 4/7.

The rates of RNA synthesis during the cell cycle measured by ^{3}H-uridine pulses of 1, 5, 10 and 19 min, and the rate of ^{3}H-uridine uptake measured by 1 min pulses. The data are from one of at least 2 repeats of each experiment. The rates of RNA synthesis from (a) 1 min pulses at 10 µCi/ml; (b) 5 min pulses at 2 µCi/ml; (c) 10 min pulses at 1 µCi/ml; and (d) 19 min pulses at 0.5 µCi/ml. The rate of ^{3}H-uridine uptake (e) from 1 min pulses at 3.75 µCi/ml. All labelling was carried out with 1 µg/ml unlabelled uridine added with the radioactive tracer.

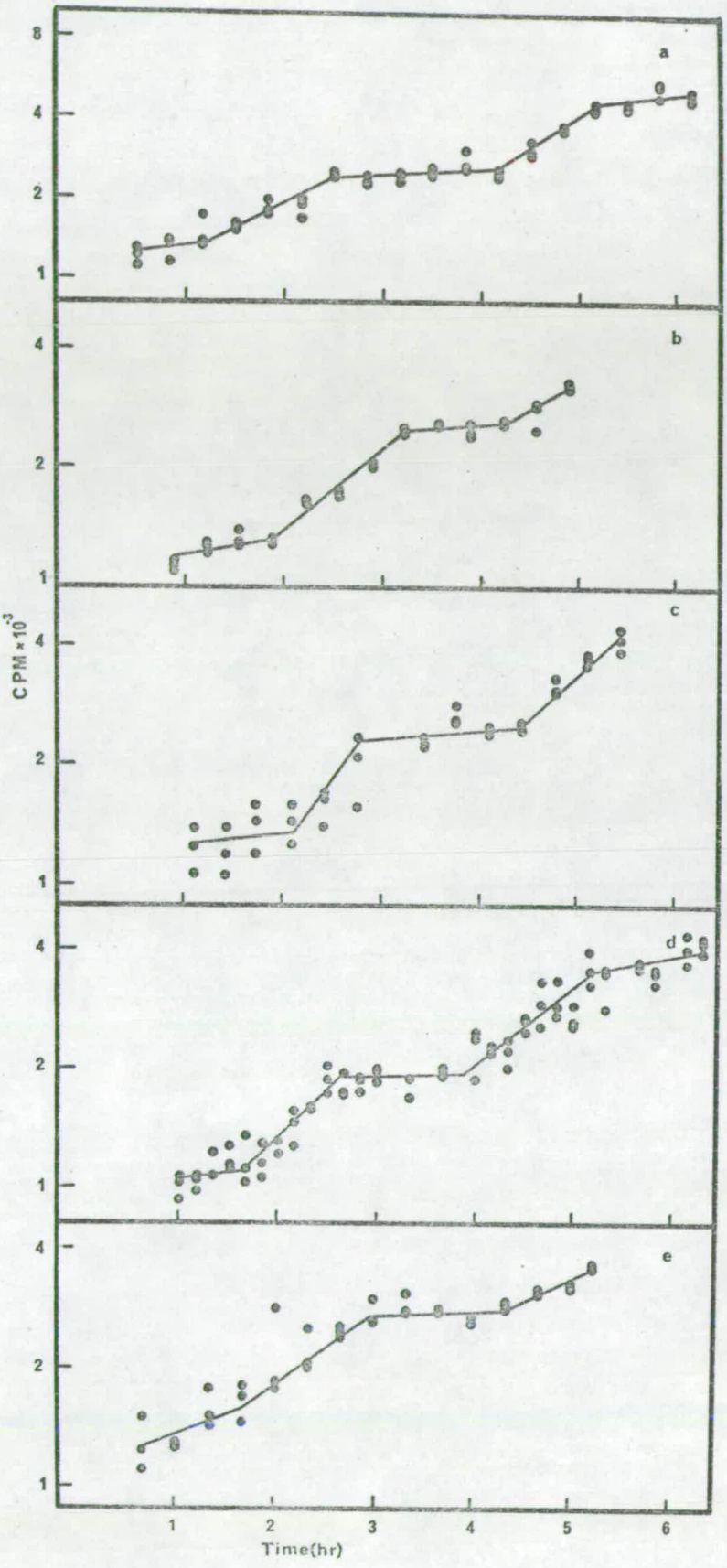

a
b
c
d
e
CPM×10^-3
Time(hr)
1
2
3
4
5
6
8

Figure 4/8.

The rates of ^{3}H-adenine uptake and incorporation during the cell cycle, measured by 1 min pulses. Cultures were labelled with 2 µCi/ml ^{3}H-adenine and 2 µg/ml unlabelled adenine was added with the radioactive tracer.

(a) rate of uptake;

(b) rate of incorporation.

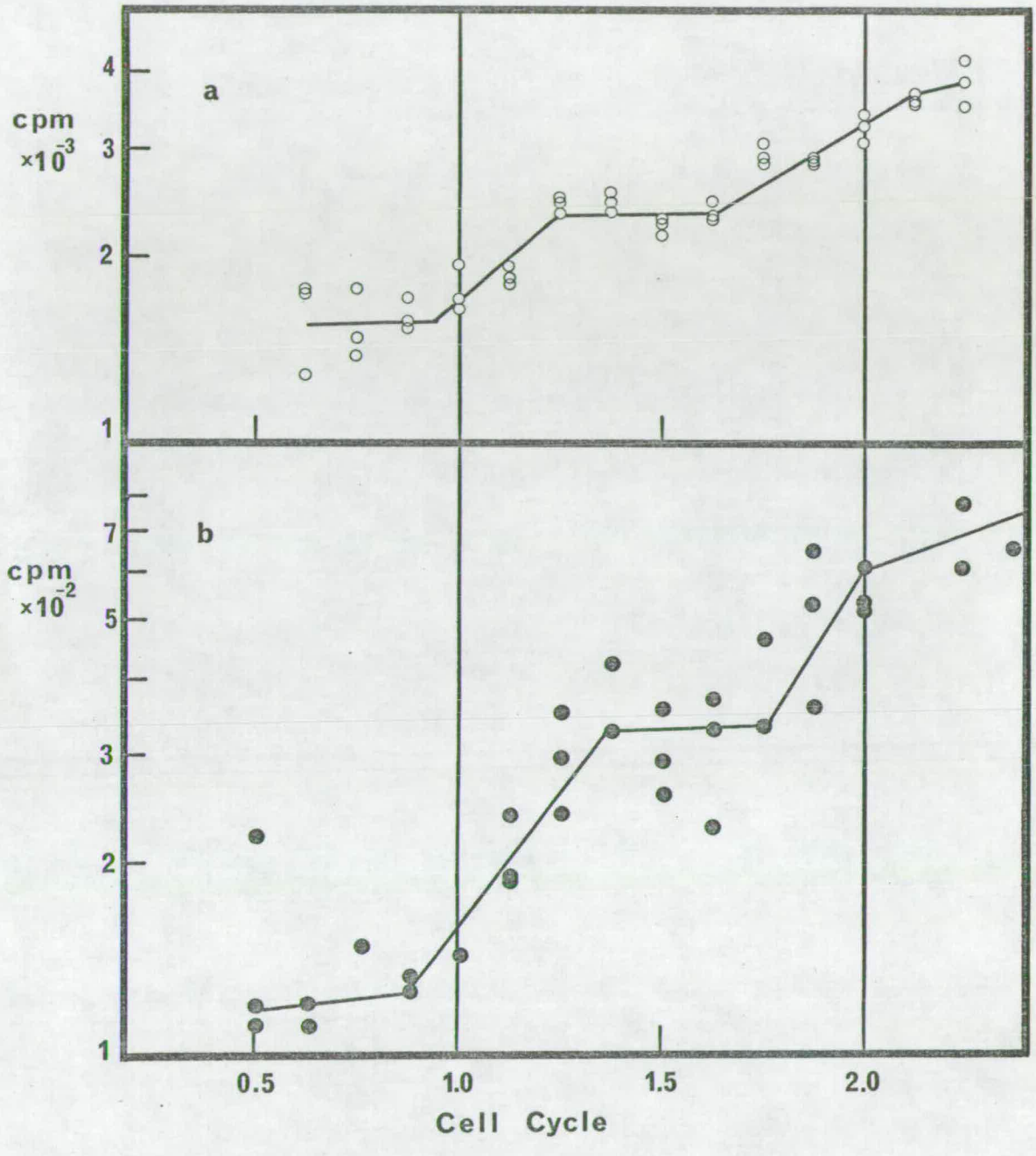

a
b
cpm
×10⁻³
cpm
×10⁻²
4
3
2
1
7
5
3
2
1
0.5
1.0
1.5
2.0
Cell Cycle

Figure 4/9.

The rates of ^{3}H-adenine uptake and incorporation from 1 min pulses throughout the cell cycle of cells with pre-filled adenine pools. Adenine pools were pre-filled by growing the culture in 20 µg adenine/ml from 3 hr before synchronization. Cultures were labelled with 20 µCi/ml.

(a) rate of uptake in cpm x 10^{-3}/ml culture;

(b) rate of incorporation in cpm x 10^{-2}/ml culture.

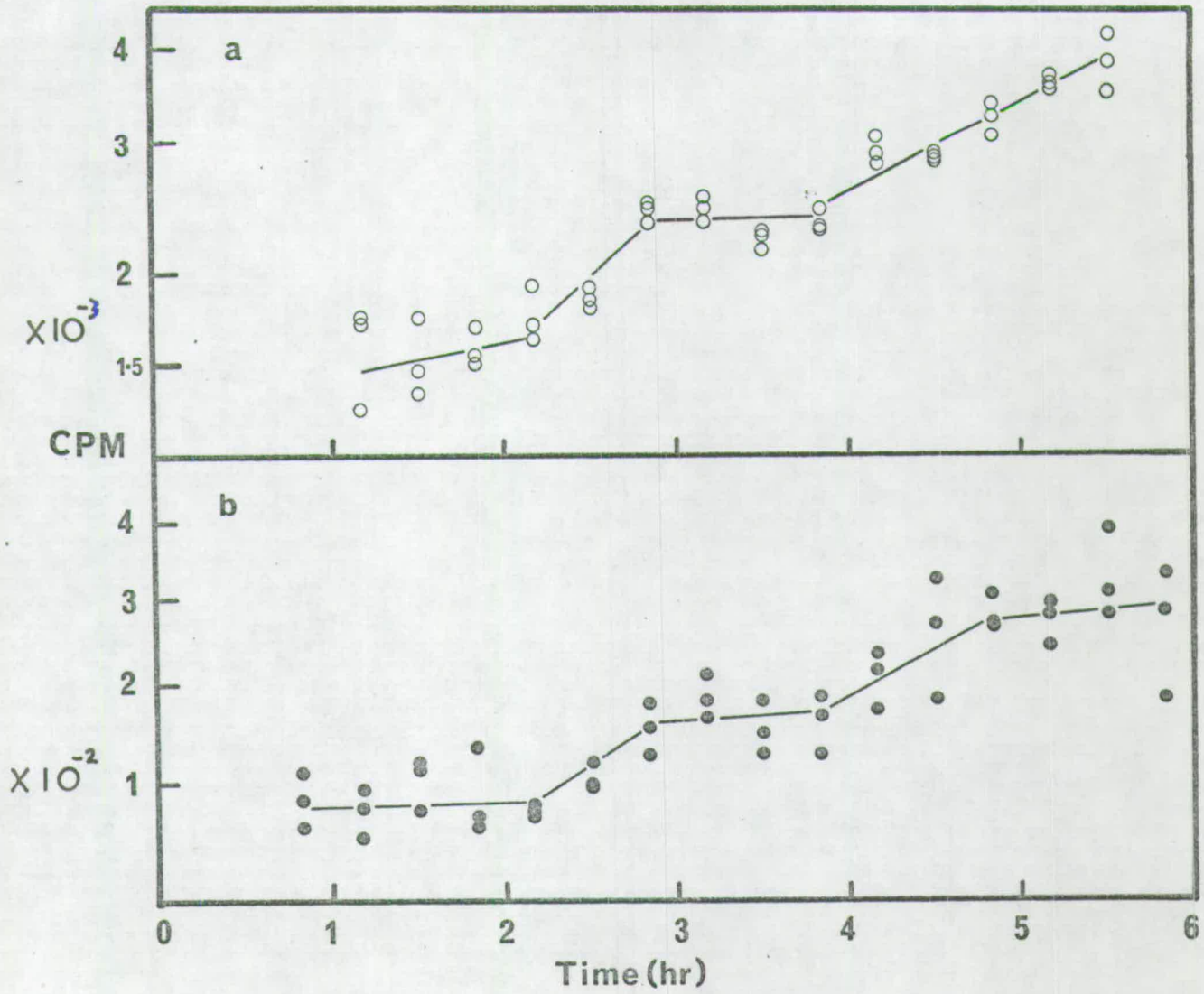
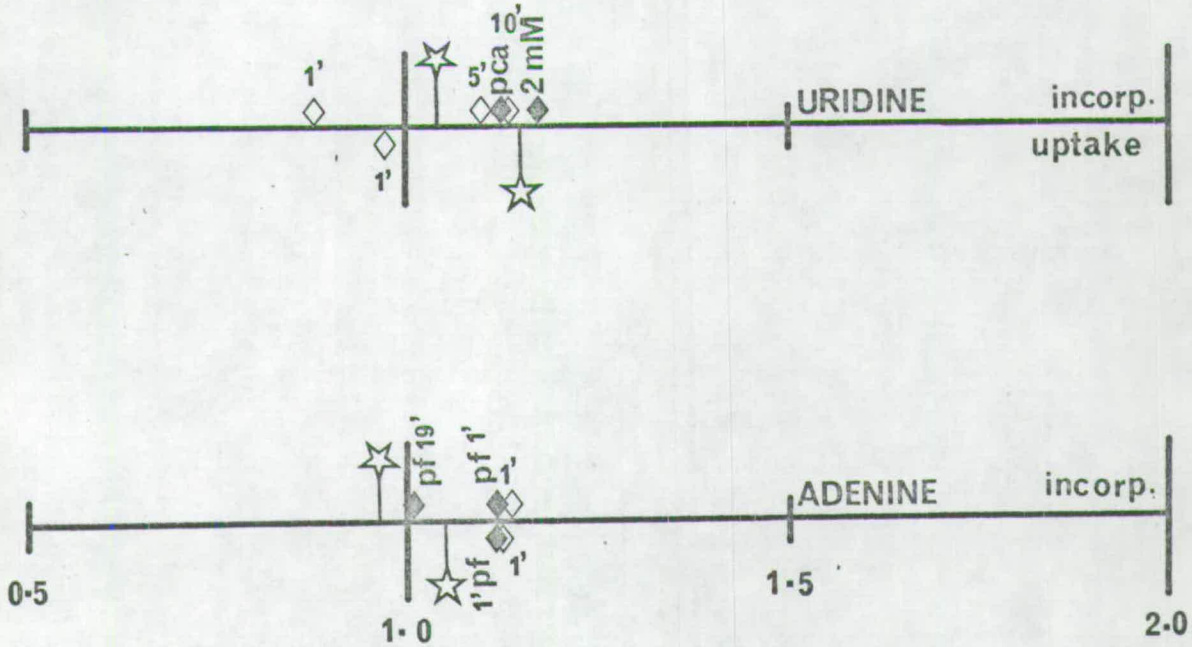

a
b
4
3
2
1·5
X IO⁻³
CPM
4
3
2
1
X IO⁻²
0
1
2
3
4
5
6
Time (hr)

Figure 4/10.

Cell cycle map of the mid-points of the increases in the rates of ^{3}H-uridine and ^{3}H-adenine uptake and incorporation measured under various conditions. (☆) pooled normalized data from 19 min pulses (Figs. 4/4 & 4/5): (◇) short pulses, numbers above the symbols give pulse lengths in minutes (Fig. 4/7): (◆) other treatments: (PCA) specific activities of PCA-extracted RNA (Fig. 4/3): (2 mM) labelled in the presence of 2 mM uridine (Fig. 4/2b): (pf) prefilled adenine pools, numbers indicate pulse lengths (Figs. 4/2a & 4/9).


1'
1'
5'
pca
10'
2 mM
URIDINE
incorp.
uptake
pf 19'
pf 1'
1'
1'pf
1'
ADENINE
incorp.
0·5
1·0
1·5
2·0

^{3}H-uridine pulses of 1, 5, and 10 min in separate experiments and compared to previously measured rates of incorporation from 19 min pulses and to rate of ^{3}H-uridine uptake measured with 1 min pulses (Fig. 4/7). Regardless of the length of uridine pulse used, the rates double rapidly at the time of cell division and DNA synthesis. Even with 1 min pulses, where the scatter of points is the greatest, both the rates of uptake and incorporation double in a step-like manner at this time. Likewise, the rates of ^{3}H-adenine uptake and incorporation, measured with 1 min pulses have the same pattern of rate doubling (Fig. 4/8), even when the cells' adenine pools have been prefilled (Fig. 4/9). In this latter case, the steps in both rates appear to have occurred at the same time (0.12 cell cycle). But the scatter in this data is too great to determine the precise timing of the rate increases. The important point is that the rates continue to double at about the time of cell division after the adenine pools have been prefilled. In Fig. 4/10 the mid-points of the rate increases for ^{3}H-uridine and ^{3}H-adenine uptake and incorporation measured by short pulses are compared with each other and with the corresponding mid-points for rates measured with 19 min pulses. No matter what the treatment, the rates continue to double at about the same time. Those treatments which increase scatter in the data show rate-steps slightly later in the cell cycle, but the mid-points of these steps generally occur within the range of those from experiments using 19 min pulses(Fig. 4/6). Therefore, the relationships between rates of precursor uptake and incorporation and between incorporation and stable RNA synthesis which were demonstrated using 19 min pulses of labelled precursors appear to also hold for short pulses and the rate of rapidly labelled RNA synthesis.

DISCUSSION

The rates of ^{3}H-uridine and ^{3}H-adenine incorporation have been shown to double sharply at the end of the cell cycle and then remain constant until the end of the next cycle. Since both the purine, adenine and the pyrimidine, uridine, are incorporated in this manner such step-like rate doublings may well be a general feature of nucleic acid synthesis in S. pombe. Certainly, the exponential increase in the rate of sulphate incorporation in the same cultures eliminates the possibility of cell cycle stage specific losses of cells as a trivial explanation for the patterns. Changes in the specific activities of the internal pools has also been eliminated as a possible cause of these rate patterns by several types of data. First, if the specific activities of such precursor pools were to change, both purine and pyrimidine pools would have to change in identical ways. Second, prefilling the expandable adenine pool prior to labelling does not alter the rate pattern of adenine incorporation. And third, when ^{3}H-uridine labelling is carried out in the presence of 2 mM unlabelled uridine, uptake is forced to occur primarily by diffusion but the pattern of incorporation remains unaltered. Furthermore, the rates of ^{3}H-uridine and ^{3}H-adenine uptake double in a step-like manner, but these rate doublings are about 0.1 cell cycle after the rate doublings for incorporation of the respective precursors. Thus, changes in the rates of precursor uptake and the specific activities of the internal precursor pools do not appear to produce the step-like changes in the rates of uridine and adenine incorporation. That these rates of incorporation do reflect the rate of RNA synthesis is shown by the specificity of the ^{3}H-uridine label for RNA (Fig. 3/2) and by their similarity to the rate pattern of uridine incorporation into extractable RNA (Fig. 4/3).

Thus, the present results show that the rates of RNA synthesis appear to have the same cell cycle pattern, regardless of whether it is rapidly labelled RNA (from 1 min pulses) or stable RNA (from 19 min pulses). This rate of synthesis doubles at the same time that cells both divide and make their DNA (Mitchison & Creanor, 1971,b). And the rates of uptake of both ^{3}H-uridine and ^{3}H-adenine show similar step-like doublings which occur approximately 0.1 of a cell cycle after that of RNA synthesis. From autoradiographic evidence Cummins and Mitchison (1964) suggested that the rate of adenine uptake might sharply double during the cell cycle of S. pombe. Their results indicate that the rate of uptake may remain fairly constant until the second half of the cycle and then double sharply. Similar, but not as clear cut evidence that the rate of RNA synthesis might also double discontinuously during the cell cycle can be found in two sources. First, an autoradiographic study showed that the rate of ^{3}H-adenine incorporation increased during the first/two-thirds of the cycle, and then remained constant (Mitchison & Lark, 1962). But cellular dry volume and mass was also found to have the same pattern (Mitchison, 1957), so the results were interpreted as reflecting these mass and volume changes (Mitchison, 1963). And second, the rate of ^{3}H-adenine incorporation in a synchronous culture appears to remain constant during the middle of the cell cycle (Fig. 4 of Mitchison, et al., 1969). But only six points were taken during one cell cycle so the pattern could be due to sampling error in one point. In this same paper, the rates of uptake and incorporation of uracil and adenine were also examined by autoradiography, as were those of guanine and cytosine and their nucleosides. Log phase cultures were pulse labelled with the appropriate radioactively labelled precursor. After

grain counting, the cells were placed in the cell cycle according to their lengths. The general conclusions reached from these studies were that the rate of RNA synthesis increases in a roughly exponential pattern during the cell cycle, and that the rates of precursor uptake rise through the cell cycle following roughly the incorporation rates.

One major drawback of all this previous work is the failure to examine rate changes in more than one successive cell cycle. Rate changes at the ends of the cycles may only become obvious if followed from one cycle to the next. But in the autoradiographic work this was, of course, not possible. In the only measurements made with synchronous cultures only the first cell cycle was used. Present data (Figs. 4/4 & 4/5), however, suggests that the rates of uptake and incorporation of both uridine and adenine may be suppressed immediately after selection synchronizing. Such rate suppression has also been recently demonstrated by Fraser and Moreno (1976). A second important source of error in the autoradiographic studies may well be the lack of precision in placing cells in their correct cell cycle stage. The lengths of cells in any cycle stage appear to fit a fairly normal distribution (Mitchison & Wilbur, 1962). Furthermore, at least with ^{3}H-leucine labelled cells, there is a wide range of grain counts for cells of any given length. Both of these distributions should cause enough scatter of data points to obscure details of the rate patterns. The combined effects of the rates doubling at the ends of the cycle and the above obscuring of detail appears to have produced uptake and incorporation patterns which are easily interpreted as continuous rate increases throughout the cell cycle.

The temporal coincidence between the S period and the time of doubling of the rate of RNA synthesis in *S. pombe* suggests that the rate

of transcription may be proportional to the number of genes available. Such a gene dosage control of transcription has been proposed for a number of other organisms (Dubnau et al., 1969; Pfeiffer, 1968).

In order to establish such a hypothesis for S. pombe, however, several other possibilities would have to be eliminated. The doubling in the rate of RNA synthesis might be associated with cell division. Or it might be the product of a perturbation the cells received during the gradient synchronization procedure.

The rate of adenine uptake is known to be regulated by a feedback mechanism involving the amount of adenine in the internal adenine pool (Cummins & Mitchison, 1967). The similarity between the patterns of uridine and adenine uptake rates, therefore, implies that a similar feedback control may be acting upon the rate of uridine uptake.

CHAPTER V

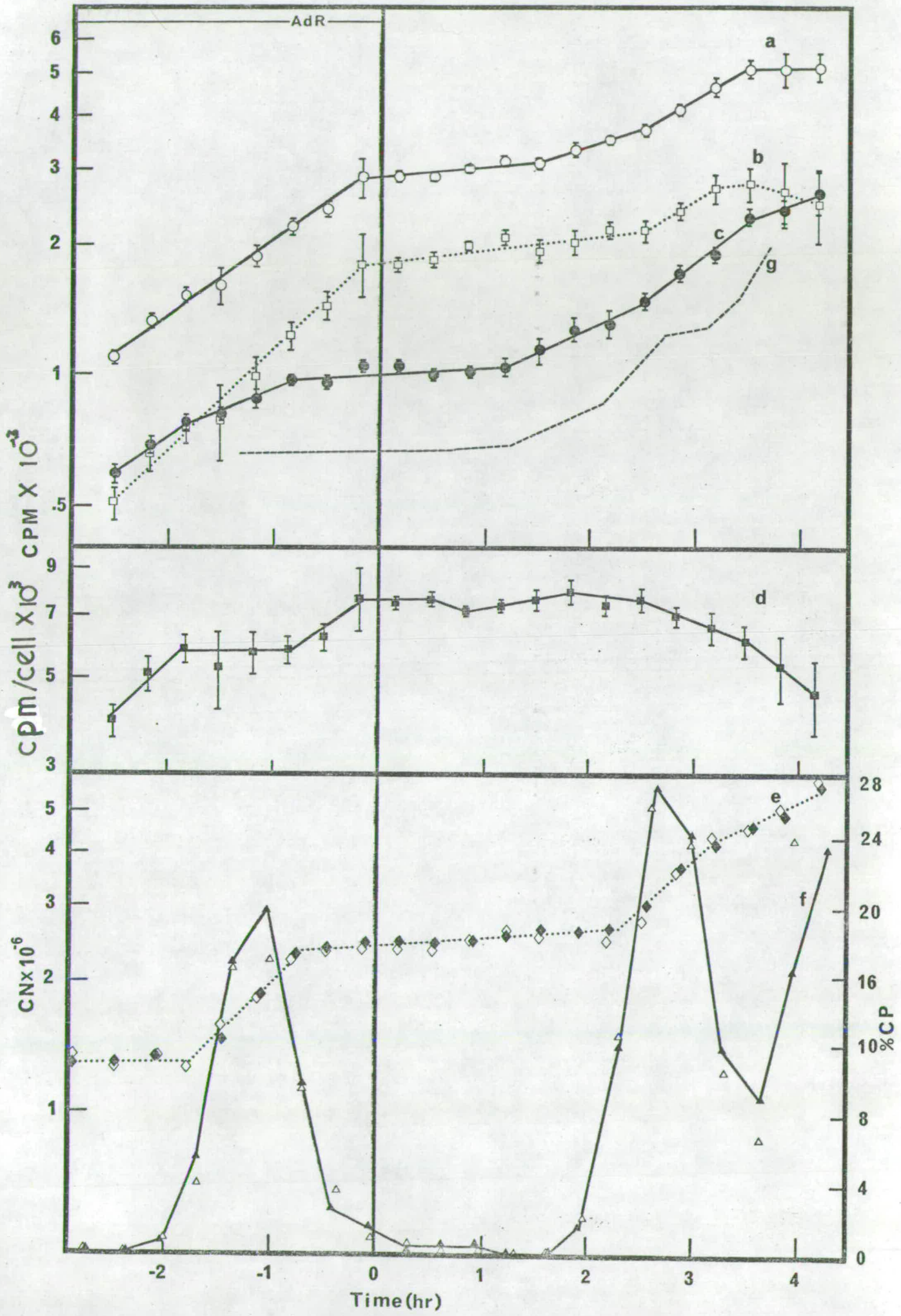
THE EFFECTS OF 2'-DEOXYADENINE PULSES UPON SELECTION SYNCHRONIZED CULTURES

In the last Chapter the rate of total RNA synthesis was shown to double sharply at the end of one cell cycle and the beginning of the next cycle. DNA synthesis also occurs at the same time in S. pombe (Mitchison & Creanor, 1971,b). To determine if these two parts of nucleic acid biosynthesis were directly linked, it was decided to measure the rate of RNA synthesis under conditions which separated the time of DNA synthesis from that of cell division. Such a separation can be achieved by growing cultures in 2 M glucose (Duffus & Mitchell, 1970) or by inhibiting one of the events and allowing the other to proceed.

A range of inhibitors of DNA synthesis in fission yeast have been reported (Bostock, 1970,b; Mitchison & Creanor, 1971,a; Herring, 1974). Mitchison and Creanor (1971,a) demonstrated that if a 3 hr pulse treatment of the DNA inhibitor, 2'-deoxyadenosine (AdR), is given during the first cell cycle of a selection synchronized culture of S. pombe, cell division occurs at the normal time, but DNA synthesis is delayed until 2 hr after drug removal. Immediately following this round of DNA replication the cells divide synchronously, and this second cell division is accompanied by a normal round of DNA synthesis. But there is a further advantage in using AdR. Because the inhibitor is easily reversible, exponentially growing yeast cultures can be induced to divide synchronously by AdR treatment (Mitchison & Creanor, 1971,a). In this Chapter I shall describe experiments using selection synchronized cultures which were treated for 3 hr with 2 mM AdR. I shall put off until Chapter 6 a discussion of work done using induction synchronized cultures.

Figure 5/1.

The rates of ^{3}H-uridine uptake, incorporation, and pool filling in a selection synchronized culture treated with 2 mM AdR for the first 3 hr after synchronizing. Values are presented as means $\pm$ 95% confidence limits of normalized data from 2 uptake and 3 incorporation experiments. The rates were measured using 19 min pulses of label and are given in arbitrary cpm. Rates of (a) uptake, (b) pool filling, and (c) incorporation per ml culture and (d) pool filling/cell. For reference (e) cell number/ml x 10^{-6} and (f) cell plate index are also included, as is (g) the DNA/ml from Mitchison and Creanor (1971,a).


AdR
a
b
c
g
d
e
f
cpm/cell X10³
CPM X 10⁻³
CN X 10⁻⁶
%CP
Time (hr)
6
5
4
3
2
1
.5
9
7
5
3
28
24
20
16
10
8
4
0
-2
-1
0
1
2
3
4

RATES OF ^{3}H-URIDINE UPTAKE AND INCORPORATION

The rates of ^{3}H-uridine uptake and incorporation were examined using 19 min pulses, both during and after AdR treatment (Fig. 5/1). Immediately after synchronizing, the cells were suspended in EMM2 containing 2 mM AdR. Samples were removed every 20 min and labelled as described previously. After 3 hr the culture was collected on a membrane filter (Oxoid), washed with warm EMM2, and resuspended in fresh, warm EMM2. Sampling was then continued for another 4 hr. Because the timing of subsequent events is most closely related to the time of AdR removal, the data are plotted using this time as 0-hr. As in Chapter 4, the results are presented as means $\pm$ 95% confidence limits of normalized data from 2 uptake and 3 incorporation experiments. In every experiment, triplicate samples were taken at each sampling time. The mean of the plateau in the uptake experiments was normalized at 2.95, while that of the incorporation was set at 1.0. This was the ratio between these two means when corrections were made for cell number and specific activities. The incorporation curve (Fig. 4/1c) was then subtracted from the uptake curve (Fig. 4/1a) to produce the curve for rate of soluble pool filling (Fig. 5/1b). Culture density and cell plate index are included to show the timing of the rate changes in relation to these cell cycle markers. The pattern of DNA synthesis (Fig. 4/1g) is also included from Figure 4 of Mitchison and Creanor (1971,a). The repeatability of this type of experiment is indicated both by the small differences in the cell numbers and CPI's of separate cultures and by the small size of the 95% confidence limits of the uptake and incorporation data.

Two things should be noted about these results. Firstly, in the presence of AdR, the rates of both uptake and incorporation increase before

the cells start to divide. Although the rate of incorporation reaches a plateau after about 2 hr, the rates of both uptake and soluble pool filling continue to rise exponentially until the drug is removed. And secondly, 1 hr after removing the AdR, the rate of incorporation rises rapidly. This increase in the rate of ^{3}H-uridine incorporation begins an hour before cells begin to divide. According to Mitchison and Creanor (1971,a) DNA synthesis stops 30 to 40 min after removing the drug. Cells begin to divide synchronously 2 hr after drug removal, and this cell division is accompanied by a normal S period. Thus, by the time of the first cell plate peak, the DNA content of the culture is twice what it was at the end of AdR treatment, and it is beginning to double a second time. The rate of RNA synthesis (uridine incorporation) is also doubled during this time, but whether it does so a second time is unknown.

These results suggest two very different cellular reactions: one in the presence, the other after the removal of AdR. Both uptake and pool filling rates increase as long as the drug is present. The rate of incorporation also appears to be stimulated during the first 2 hr of AdR treatment. Thereafter, the incorporation rate parallels the DNA content of the culture, and the rate of uptake follows suit, at least until the cells divide.

THE EFFECT OF AdR ON THE KINETICS OF ^{3}H-URIDINE USE.

The relationship between the rates of uptake, incorporation and soluble pool filling in the presence of AdR were examined by following the kinetics of ^{3}H-uridine use in a selection synchronized culture to which 2 mM AdR had been added (Fig. 5/2). As before, the inhibitor was added immediately after synchronization, and starting 10, 90 and 180 min after adding it, the labelling was begun (Fig. 5/2 inset). Samples were

Figure 5/2.

The kinetics of ^{3}H-uridine uptake, incorporation and pool filling by a selection synchronized culture in the presence of 2 mM AdR. Insert shows the times at which parts a, b, and c were measured in relation to changes in cell number (◆) and the cell plate index (Δ). Uptake (o), pool filling (▲), and incorporation (●) are given as cpm/ml culture adjusted to 1.0 OD_{595} and labelled at 5.7 µCi/ml with 1.4 µg/ml non-radioactive uridine added at the time of labelling.

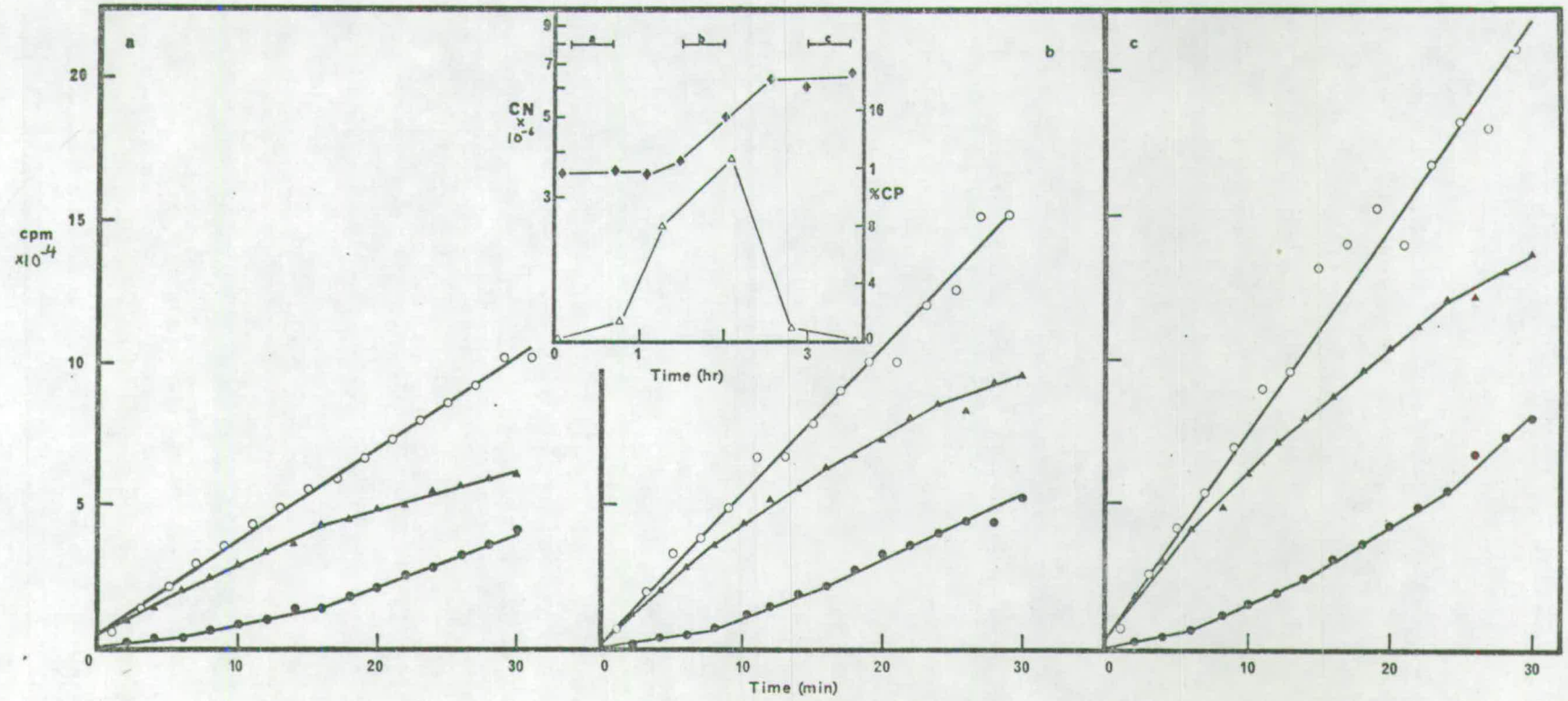

a
b
c
cpm
x10⁻⁴
CN
x
10⁻⁴
%CP
Time (hr)
Time (min)
0
10
20
30
5
10
15
20
1
3
4
7
8
9
16

Figure 5/3.

The kinetics of ^{3}H-uridine incorporation during AdR treatment. The data is the same as in Fig. 5/2, but it is presented here plotted on semi-logarithmic axes instead of linear ones. Length of AdR treatment before labelling: (●) 10 min; (◇) 90 min; (○) 180 min.

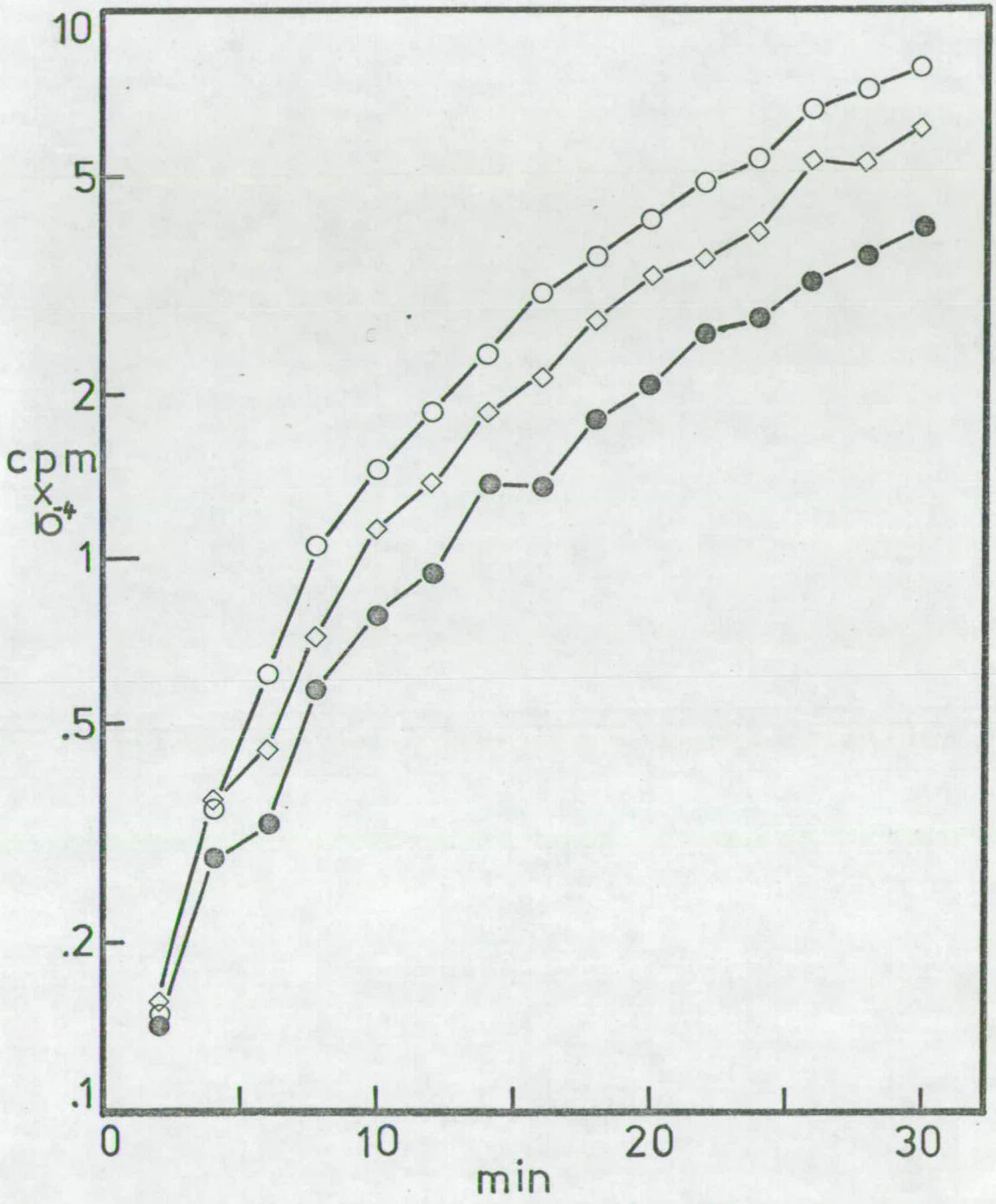
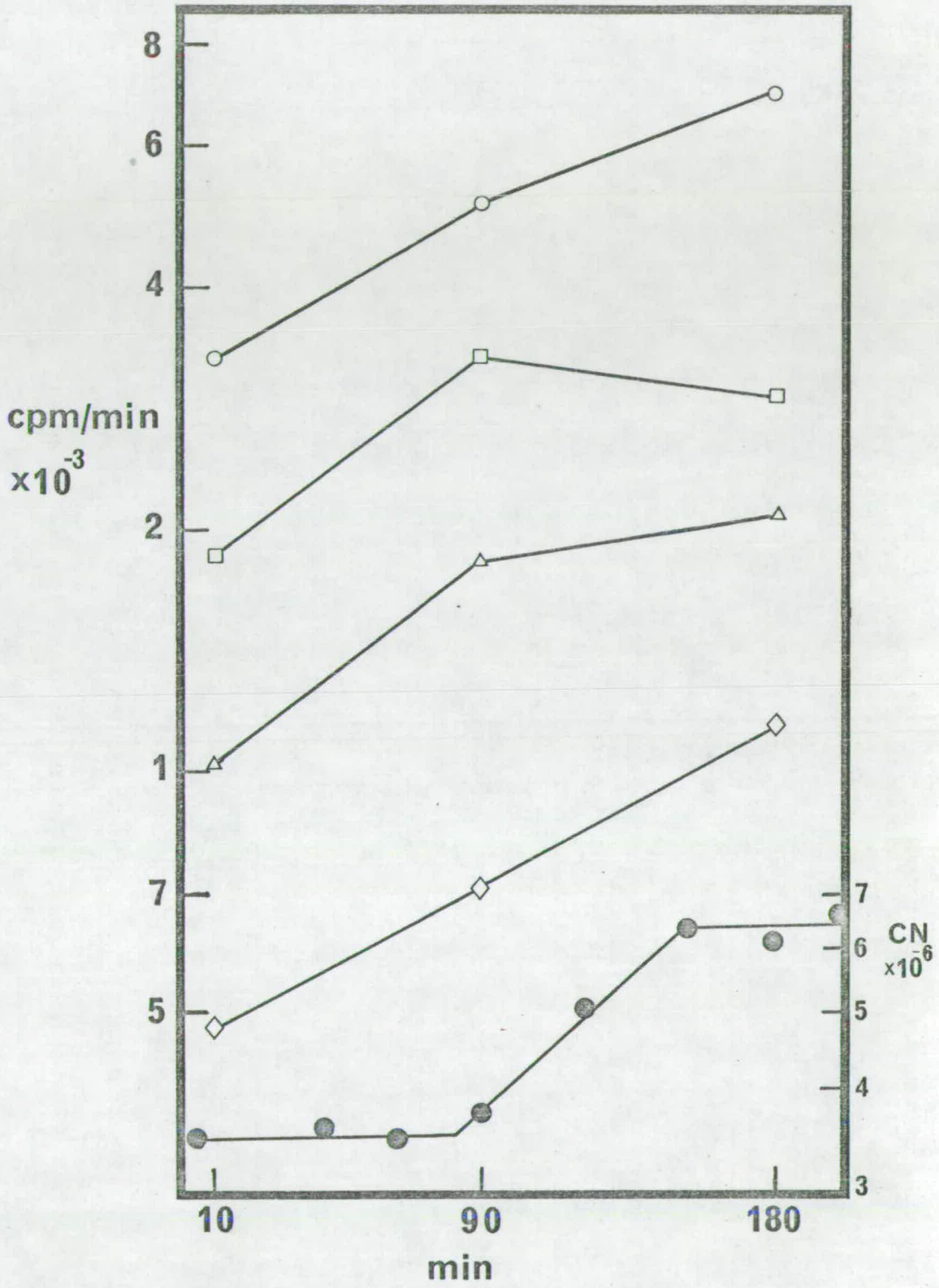

10
5
2
1
.5
.2
.1
cpm
x
10-4
0
10
20
30
min

Figure 5/4.

The rates of ^{3}H-uridine uptake and incorporation in a selection synchronized culture in the presence of 2 mM AdR. Rates were obtained from the slopes of the linear segments of the appropriate kinetic curves in Fig. 5/2. Uptake (o), incorporation from the first (◇), second (Δ) and third (□) segments, and cell number (●).


8
6
4
cpm/min
x10-3
2
1
7
5
7
6
5
4
3
CN
x10-6
10
90
180
min

taken on alternate minutes for uptake and incorporation measurements respectively. The general kinetic patterns are similar to those of the log phase cultures shown in Chapter 3. Uptake of ^{3}H-uridine increases linearly for at least 30 min after adding the labelled precursor. During the 3 hr of AdR treatment, the rate of uptake doubles, as it did in Fig. 5/1a. Incorporation, on the other hand, increases in rate in a way much like the three sequential rate increases shown in Fig. 3/1. If these incorporation data are plotted on semi-log axes (Fig. 5/3), it becomes apparent that they do not increase exponentially until 20 min of labelling at the earliest. The filling of the soluble pools with ^{3}H-uridine, likewise, appears to proceed via a series of discontinuous rate changes. In this case, though, the rates decrease with time.

The changes in rate of incorporation and pool filling appear to take place at about 6 and 15 min after adding the label. After 3 hr of AdR treatment a third rate change may also occur after 24 min of labelling. By comparing the changes in the rates of incorporation taken from the slopes of each segment of the incorporation curves, we see that the rates from the two initial segments double (Fig. 5/4). The first segment requires 3 hr to do so, thus doubling at the same rate as uridine uptake. The second increases more rapidly during the first 90 min but still needs 3 hr to double in rate. The third segment of the incorporation curve only increases in rate by about 65% during the first 90 min of labelling. Thereafter it remains constant. AdR, thus, appears to preferentially inhibit increases in the rate of ^{3}H-uridine incorporation into more stable RNA species. The overall picture from this set of experiments is an increase in the soluble ^{3}H-uridine pool size during AdR treatment.

DISCUSSION

The data from this Chapter suggest two very different types of behaviour are being studied: one during, the other after AdR treatment. In the presence of AdR the rates of ^{3}H-uridine uptake, incorporation and pool filling appear to be stimulated to increase. Uptake and pool filling rates increase for the entire period of treatment, while the incorporation rate increases for the first two hours of it. In this last case during the period of the first cell number doubling, from 80 to 140 min, the incorporation rate rises less rapidly than during the initial 80 min of AdR treatment. This might indicate that after an initial drug-induced stimulation the rate of incorporation is linked to cell numbers rather than to DNA content, as previously suggested. On the other hand, about an hour after AdR has been removed from the culture, the rate of uridine incorporation begins to increase even though cell division is not resumed until just over 2 hr after drug removal. But DNA synthesis has been reported to occur at just that time (Mitchison & Creanor, 1971,a). The rate of uridine uptake parallels the rate of incorporation at this time. When uridine incorporation increases in rate, the rate of uptake speeds up within 20 min. This part of the data suggests a feedback control of the rate of uridine uptake. On a per-cell basis the rate of ^{3}H-uridine pool filling (Fig. 5/1b) may be the regulator of this control. In the presence of AdR, this rate of filling increases during the first hour of exposure, then remains constant during cell division. Thereafter it again increases for the final hour of drug treatment. This initial increase in the cellular rate of pool filling is balanced by a similar sized decrease when the cells again start dividing, 2.5 hr after AdR removal. AdR might, therefore, cause the cells to reset

the rate at which material moves through their uridine pools. This, in turn, would cause the increase in the rate of uptake.

From the time of removing the AdR until the end of the experiment, the rate of uridine incorporation parallels the DNA content of similar cultures described by Mitchison and Creanor (1971,a). This parallel pattern again fits with the gene dosage model suggested in Chapter 4. But when the cells again start to divide, they readjust the size of their uridine pools to the level they were before adding AdR. This readjustment, in turn, may reduce the specific activity of the uridine pool and cause the rate of uridine incorporation to decrease in relation to the rate of RNA synthesis. Thus, unless corrections were made for such changes in pool specific activity, only for the first 2.5 hr after removing the AdR is the rate of ^{3}H-uridine incorporation likely to accurately reflect the rate of RNA synthesis.

These changes in uridine pools noted here may also alter the contribution of the internal supply of uracil products. Several of the enzymes of the uracil biosynthetic pathway show peculiar changes in activity during AdR induction synchrony (Sissons, Mitchison & Creanor, 1973). In the light of these findings and those presented in this Chapter, I decided to examine the rates of uridine uptake and incorporation in induction synchronized cultures. This work along with the associated kinetic studies of uridine utilization during induction synchrony are presented in Chapter 6.

CHAPTER VI

AdR INDUCTION SYNCHRONY

In the last two Chapters, cultures of synchronously dividing cells were produced by selecting from a log phase culture a small proportion of the cells which normally divide together. In this Chapter, synchronously dividing cultures are produced by inducing all the cells of a log phase culture to divide at the same time. A good review of both types of synchrony is given by Mitchison (1971). Induction methods generally involve blocking either DNA synthesis or cell division in a log phase population of cells for about one generation. During this time, cells from all stages accumulate at the blocking point. When the block is then removed, the entire population proceeds towards division together. The timing and even the pattern of synthesis of cell products may be different in induction synchronized and in selection synchronized cultures and these differences have led Mitchison (1971, 1973) to suggest that the normal cell cycle consists of a growth cycle and a DNA-division cycle running in parallel. During induction synchrony, however, these cycles appear to become temporarily dissociated. By examining events in both types of synchronous culture one should, therefore, be able to assign the events to either the growth or the DNA-division cycle.

As mentioned at the beginning of the last Chapter, S. pombe was first induced to divide by treatment with 2'-deoxyadenosine (Mitchison & Creanor, 1971,a). This drug has been similarly used to synchronize bacteria (Lark, 1960) and mammalian cells (Xeros, 1962). When a log phase culture of S. pombe is given a 3-3.5 hr pulse of 2 mM AdR the cells divide synchronously 2.5 hr after the end of the inhibitor pulse. DNA synthesis is inhibited within 30-90 min of adding the AdR, but resumes

slowly after about 2.5 hr of inhibition (Sissons et al., 1973). About an hour after removing the AdR, there is a burst of DNA synthesis which is followed immediately by two synchronous divisions spaced 60 min apart. Each of these divisions is accompanied by an apparently normal S period. During the entire experiment, RNA and protein content and the OD_{595} of the culture increases exponentially, as they did prior to AdR treatment (Mitchison & Creanor, 1971,a). The rate of RNA synthesis has not been previously examined during AdR induction synchronization.

RATES OF ^{3}H-URIDINE UPTAKE AND INCORPORATION

The rates of ^{3}H-uridine uptake and incorporation were measured from the beginning of the induction period until the cells had completed a synchronous division about 7 hr later. As in previous Chapters, the rates were measured using 19 min pulses of labelled precursor started 20 min apart, and in each experiment 3 estimates of rate were made at every sampling time. The results (Fig. 6/1) show a marked similarity to the analogous results from selection synchronized cultures treated with AdR (Chapter 5, Fig. 5/1). The rates of ^{3}H-uridine uptake and radioactive pool filling increase exponentially during the first 2.5 hr of AdR treatment. But, unlike the results of Chapter 5, in the present experiment, the cell numbers also increase during this initial 2.5 hr period. Thereafter all three of these parameters remained constant until at least 40 min after removing the inhibitor. The rate of incorporation also increases exponentially after adding 2 mM AdR to the culture, but by about 30 min before removing the AdR, the rate of increase has decayed and incorporation occurs at an approximately constant rate until 20-40 min after the drug is removed. Immediately after removing AdR from the culture, the rates of ^{3}H-uridine uptake and incorporation drop for 20-40 min.

Figure 6/1.

The rates of ^{3}H-uridine use in AdR induction synchronized cultures. Cultures were treated with 2 mM AdR for 3.5 hr, then resuspended in AdR-free EMM2. Labelling was done with 19 min pulses at 1 µCi/ml. 4 µM unlabelled uridine was added at the time of labelling. Data was normalized and pooled as described in the text and the results are presented as means ± 95% C.L. of triplicate data points from each of 2 uptake and 2 incorporation experiments. Rates per ml culture:

(a) uptake, as 10^{-4} x cpm/ml;

(b) incorporation, as 10^{-3} x cpm/ml;

(c) pool filling, as 10^{-4} x cpm/ml.

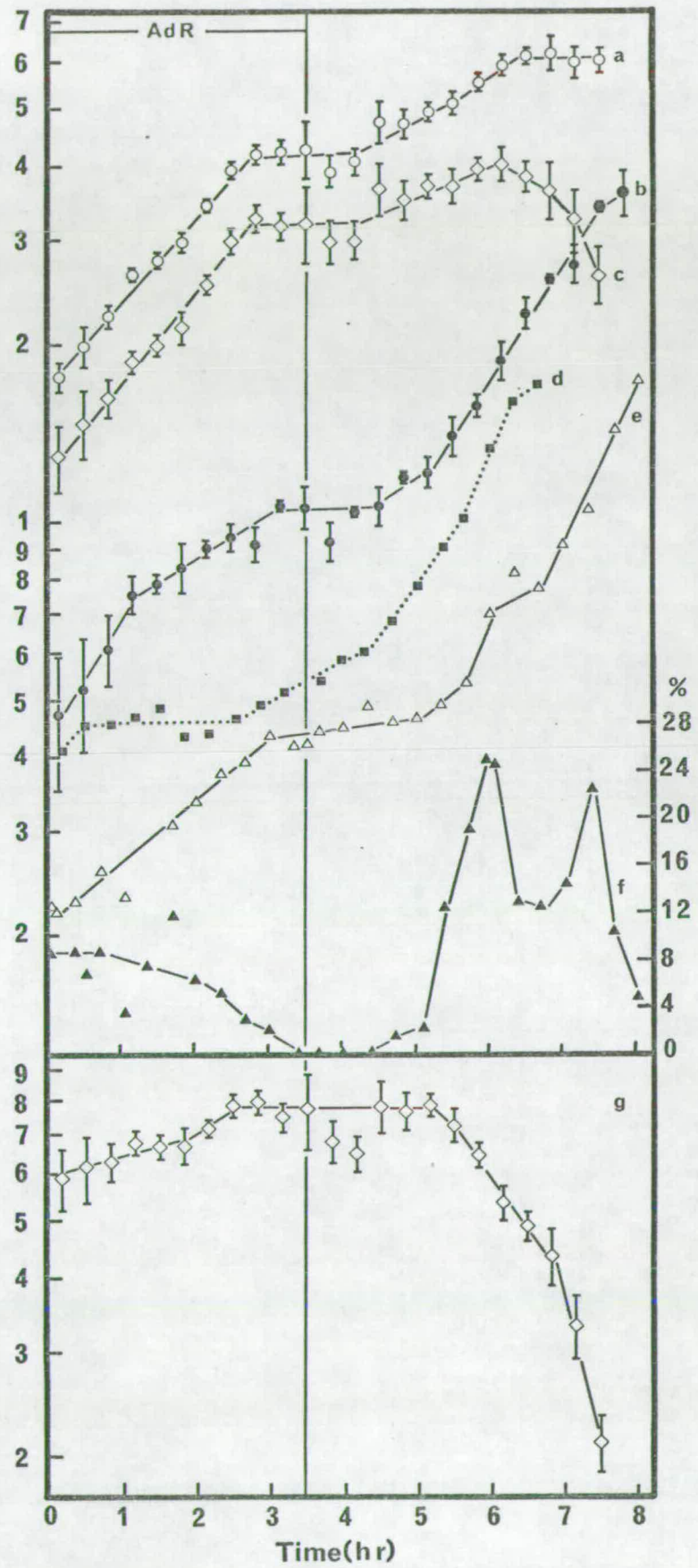
Also,

(g) rate of pool filling per cell, as 10^{2} x cpm/cell;

(e) cell numbers x 10^{-6};

(f) CPI;

(d) DNA/ml culture as OD_{595} of diphenylamine reaction.


AdR
a
b
c
d
e
f
g
%
28
24
20
16
12
8
4
0
Time(hr)

These temporary rate reductions are reflected by a similar drop in the rate of pool filling. These rate reductions may be caused by the handling necessary to remove the AdR from the cultures. But other small rate reductions at this time cannot be ruled out. Further work bearing on the pattern of rates at this time of resuspension will be presented later in this Chapter. About 40 min after removing the AdR, the rate of ^{3}H-uridine incorporation starts to slowly increase. An hour later it begins to increase much more rapidly and continues to do so until the end of the experiment. The rate of ^{3}H-uridine uptake also begins a slow, 2 hr increase approximately 40 min after removing the AdR. But it does not increase as rapidly as the rate of incorporation, and after the first synchronous division it remains constant.

The cellular rate of pool filling has an interesting pattern during induction synchrony. For the first 2 hr of AdR treatment it remains constant or rises only very slowly. Then it increases rapidly for 40 min, only to become constant again at a higher rate. Except for the 40 min immediately following resuspension, the rate of pool filling per cell remains at this constant level until the onset of the first synchronous division. At that time it decreases sharply and exponentially. And at the start of the second synchronous division this rate decreases even more rapidly, to a level at least 3 times less than it was when AdR was first added to the culture.

In agreement with Sissons et al. (1973), the DNA content of the culture remains fairly constant for the first 2.5 hr of AdR treatment. Then it increases slowly for another 2 hr. An hour before the first synchronous burst of cell division the rate of DNA synthesis increases sharply, and a further increase in the rate of DNA synthesis accompanies

this division. Between the time DNA synthesis restarts and the beginning of the synchronous division, the DNA content of the culture doubles. It doubles again during the division itself. These doublings in DNA content are matched by increases in the rate of ^{3}H-uridine incorporation. But until 1 hr after removing the AdR, DNA synthesis and the rate of incorporation do not parallel each other. This state of affairs is most likely caused by the AdR induced increase in the radioactive uridine pool size demonstrated in Chapter 5.

THE EFFECT OF ADDING AdR TO A LOG PHASE CULTURE

To determine how rapidly AdR acts on the various aspects of growth and uridine metabolism, the rate of RNA synthesis was followed for 1.5 hr in a log phase culture, using 19 min pulses of ^{3}H-uridine. Comparative data were collected every 20 min for the patterns of increase in cell numbers and DNA content, and the CPI was also monitored. AdR was then added to the culture to a final concentration of 2 mM and the measurements were continued for a further 3.5 hr. The rate of RNA synthesis, DNA content and cell numbers increase exponentially before the addition of AdR, while the CPI ranged between 6 and 10% (Fig. 6/2). For some unknown reason the DNA content of this culture increased much faster than either cell number or rate of RNA synthesis. Following the addition of the inhibitor,cells continued to divide at much the same rate for almost 3 hr. This is evident from both the continued steady increase in cell numbers and the CPI,which only began to decrease from the 7% level after about 2 hr of treatment. DNA accumulation, on the other hand, stopped within the first 20 min of treatment. Then, as described in the previous section, it began to increase again slowly about 1 hr after it had stopped. The

Figure 6/2.

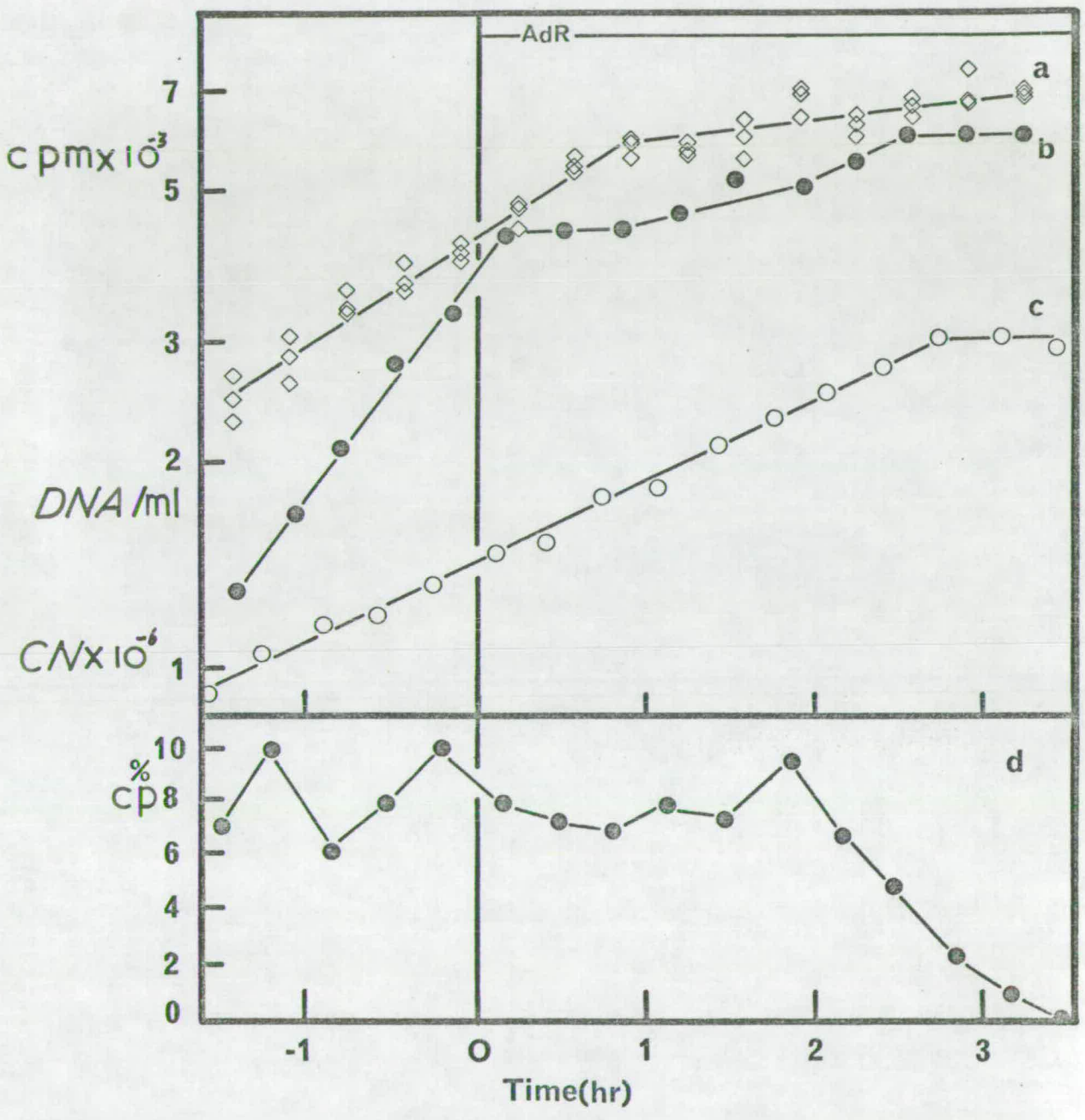
The rate of ^{3}H-uridine incorporation during the transition from log phase to inhibition of growth by 2 mM AdR. Cultures were labelled with 19 min pulses of precursor at 1 µCi/ml, with 4 µM unlabelled uridine added at the start of labelling.

(a) rate of incorporation in cpm x 10^{-3}/ml culture,

(b) DNA content/ml culture as OD_{595} of the diphenylamine reaction,

(c) cell number x 10^{-6},

(d) CPI.


AdR
a
b
c
d
cpmx 10⁻³
DNA/ml
CVx 10⁻⁶
%cp
7
5
3
2
1
10
8
6
4
2
0
-1
0
1
2
3
Time(hr)

rate of RNA synthesis decelerated to a nearly constant rate approximately 45 min after DNA synthesis was inhibited.

Such rapid inhibition of DNA synthesis is not always the case with AdR: in Fig. 6/1 there was a lag of 110 min, and in Fig. 6/3 there was a lag of 45 min between the time AdR was added and when DNA synthesis ceased. Mitchison and Creanor (pers. comm.) have also found that AdR can take anywhere from 30 min to 2 hr to inhibit DNA synthesis. The reason for such variance is unknown, but perhaps the short lag found in this experiment is related to the unusually rapid rate of DNA synthesis. The time AdR took to affect the rate of RNA synthesis ranged from 40 to 60 min in different cultures. Thus, in most instances the rate of RNA synthesis became constant between 20 and 60 min after DNA synthesis stopped. In one instance, however, RNA synthesis reached a constant rate 50 min before DNA synthesis ceased (Fig. 6/3). Fraser and Moreno (1976) found a variable delay of 20 to 40 min between the time of inhibition of DNA synthesis by hydroxyurea and the time at which the rate of RNA synthesis becomes constant in log phase cultures of S. pombe. With hydroxyurea, however, RNA synthesis always reached a constant rate after DNA synthesis had been blocked. The AdR results suggest that the rate of RNA synthesis does not necessarily have to parallel the DNA content of the culture. However, AdR is known to alter the metabolism of deoxynucleotides (Plagemann & Erbe, 1974,b; Reichard, Canellakis & Canellakis, 1960), and it also appears to increase the size of the ^{3}H-uridine pool (Chapter 5). This increase may be independent of its effect on DNA synthesis. Hydroxyurea, on the other hand, is known to act as a specific inhibitor of DNA synthesis (Fraser & Moreno, 1976). So, results obtained with it may reflect the true relationship between these two aspects of

nucleic acid metabolism.

CONTINUOUS AdR TREATMENT

According to Sissons et al. (1973), after 2 hr in AdR, S. pombe resumes DNA synthesis at a very slow rate. Mitchison and Creanor (1971,a) report that by 2-3 hr the OD of a culture treated continuously with AdR increases at about half the rate of a normal culture. But this OD increase probably reflects growth without accompanying cell division until after 7 hr of AdR treatment. The relationship between DNA synthesis and the rate of ^{3}H-uridine incorporation was examined in such a culture during 6.5 hr of exposure to AdR. This allowed examination, under constant culture conditions, of the period when cells are normally being transferred and adjusting to AdR-free medium.

During the first 3 hr of continuous AdR treatment, cells continued to divide with a mean generation time of 2.5 to 3 hr (Fig. 6/3). DNA synthesis in this culture was found to fit the pattern described above. It was exponential for the first 110 min, with a mean doubling time of approximately 90 min. It then increased very slowly until about 3-4 hr of AdR treatment, at which time it remained constant. About 5.5 hr after adding AdR, DNA again began to be synthesized at an accelerating rate until the end of the experiment. RNA accumulated in an approximately exponential manner for the first 4 hr of this experiment (Fig. 6/3a). It then remained at nearly a constant level for 1.5 hr. At about the time when DNA began to increase, after 5.5 hr in AdR, RNA began to accumulate rapidly again. The changes in the RNA content of the culture were not reflected by changes in the rate of ^{3}H-uridine incorporation. During the first hour of AdR treatment, the rate of incorporation from 19 min pulses (Fig. 6/3c) increased exponentially with a mean generation time of

about 60 min. Thereafter the rate of increase slowed until the mean doubling time was around 7.5 hr. There was no further increase in the rate of incorporation when both DNA and RNA accumulation rates increased sharply at the end of the experiment.

If the rate of RNA synthesis at any given time is assumed to be the slope of the accumulation curve at that time, then it should increase slowly and exponentially for the first 4 hr of AdR treatment. Thereafter it should decrease slightly to a new, almost constant rate until DNA synthesis begins after 5.5 hr of treatment. At this time the rate should again increase. The rate changes at both 4 hr and 5.5 hr should be rapid and step-like.

The rate of ^{3}H-uridine incorporation coincides with this derived pattern of the rate of RNA synthesis only during the period from 1 to 4 hr of AdR treatment. This coincidence suggests that during this 3 hr period the rate of incorporation can be used as a measure of the rate of RNA synthesis. Furthermore, during this period the rate of incorporation does not decrease if the cells are not resuspended. The slight reduction in the rate of incorporation following resuspension (Fig. 6/1) may, therefore, be induced by the resuspension.

RATES OF ^{3}H-URIDINE INCORPORATION FROM 1 MIN PULSES

To get an estimate of how AdR affects the rate of rapidly-labelled RNA synthesis, cells were pulse labelled with ^{3}H-uridine for 1 min at 20 min intervals during induction synchrony. The rate of incorporation (Fig. 6/4) increases for the first 1.5 -2 hr in AdR, then remains more or less constant until about 1 hr after removing the AdR. Thereafter it increases in an approximately exponential fashion, perhaps increasing its rate of increase again when the cells divide synchronously about 2.5 hr

Figure 6/3.

Effect of continuous 2 mM AdR treatment on the rate of ^{3}H-uridine incorporation. Cultures were labelled with 19 min pulses at 1 µCi/ml with 4 µM unlabelled uridine added at the start of labelling.

(a) RNA content/ml culture as OD_{260} of PCA extracts $\times 10^{-1}$,

(b) DNA content/ml culture as OD_{595} of diphenylamine reaction,

(c) rate of ^{3}H-uridine incorporation as cpm x 10^{-3}/ml culture,

(d) cell number x 10^{-6}/ml.

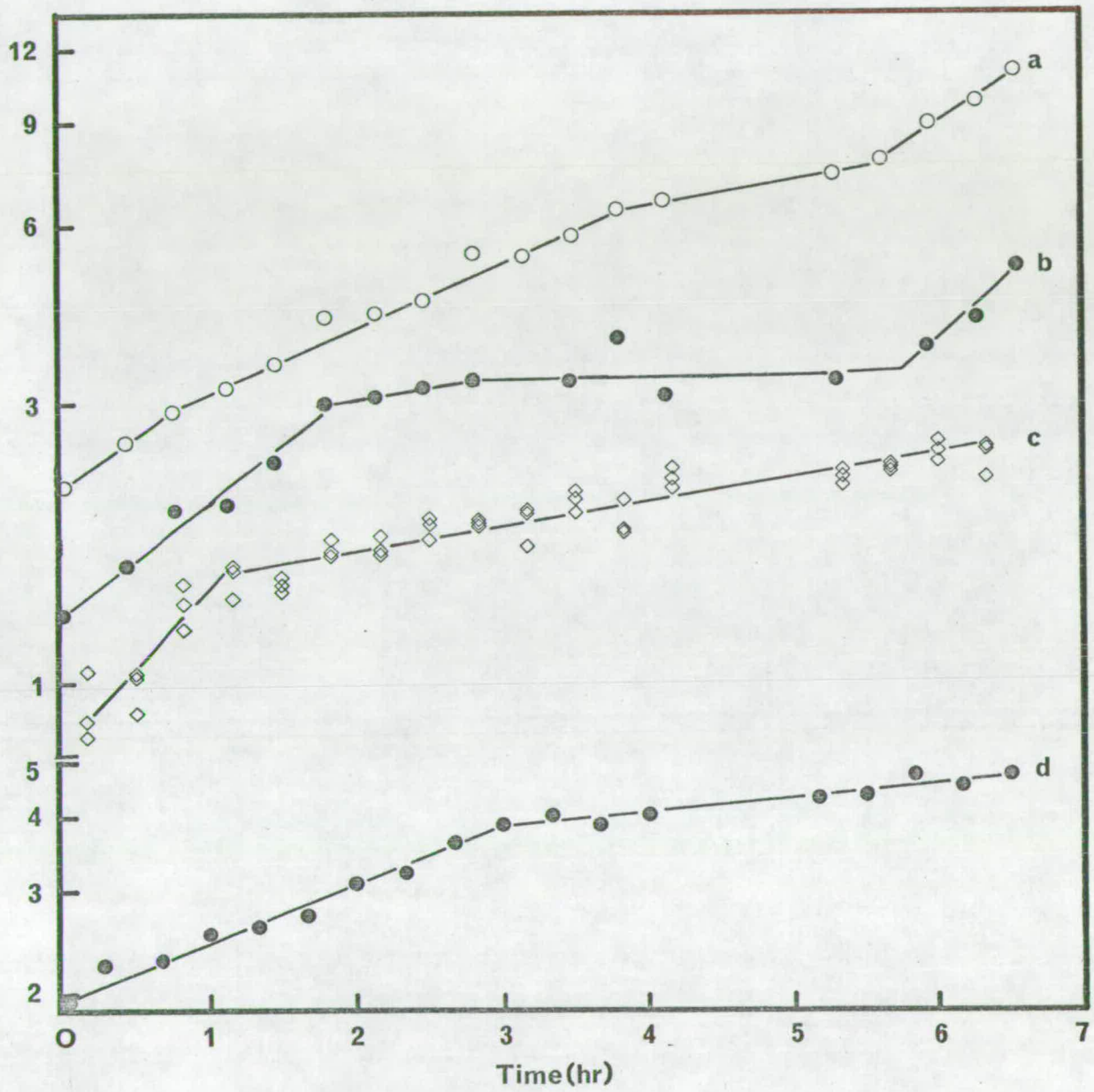
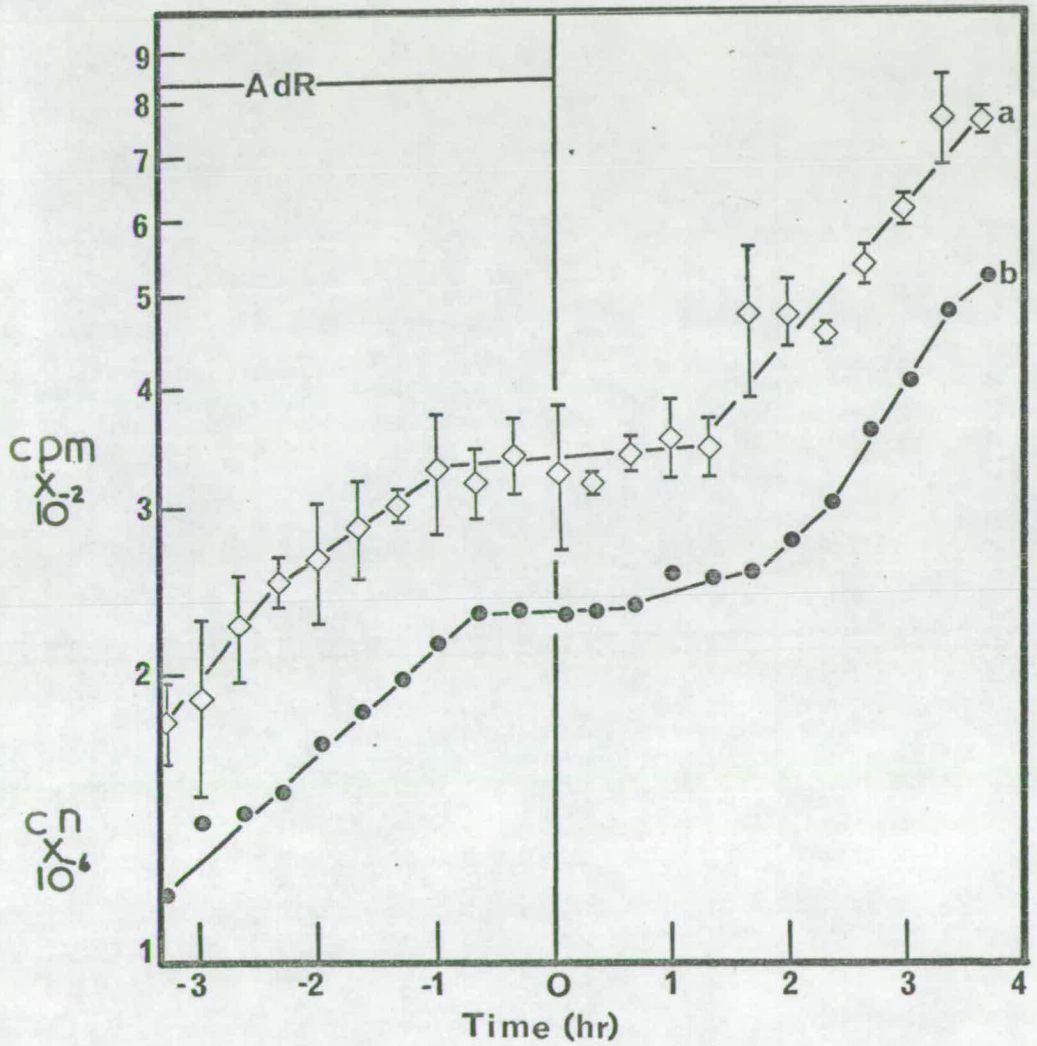

12
9
6
3
1
5
4
3
2
O
1
2
3
4
5
6
7
Time (hr)
a
b
c
d

Figure 6/4.

The initial rate of ^{3}H-uridine incorporation during AdR induction synchrony, as measured with 1 min pulses. Cultures were labelled at 10 µCi/ml at 0.3 µM.

(a) rate of incorporation as cpm x 10^{-2} / ml culture;

(b) cell number x 10^{-6}.


AdR
a
b
cpm x 10⁻²
cn x 10⁶
9
8
7
6
5
4
3
2
1
-3
-2
-1
O
1
2
3
4
Time (hr)

after removing the drug. The similarity between these results and the rates of incorporation measured with 19 min pulses (Fig. 6/1), suggest that changes in the specific activity of the uridine pool between the first and the 19th min of labelling play a minor role in determining the pattern of rates of incorporation. But AdR may well have longer term effects on the kinetics of uridine utilization, as suggested by results in Chapter 5.

KINETICS OF ^{3}H-URIDINE UTILIZATION DURING AdR TREATMENT

In Chapter 5 the kinetics of ^{3}H-uridine utilization were described for selection synchronized cultures exposed to AdR. Similar kinetic experiments have also been carried out using log phase cultures treated with 2 mM AdR. The data from these latter experiments do not contain cell cycle specific and selection procedure induced effects. As in previous chapters, uptake remained linear and incorporation increased in rate for at least 30 min after adding ^{3}H-uridine (Fig. 6/5). Both uptake and incorporation continued to increase during the entire 3.5 hr of AdR treatment. When the incorporation data from Fig. 6/5 are plotted on semi-logarithmic axes (Fig. 6/6) it becomes apparent that cultures treated with AdR for either 1 min or 1.5 hr incorporated ^{3}H-uridine exponentially only after 20-22 min of labelling. Before this time, and during the entire 30 min labelling period of the culture treated for 3 hr, the patterns of incorporation consist of a series of arch-like increases, characteristic of linearly increasing segments plotted on semi-logarithmic axes. The control data points from 15 to 25 min are, unfortunately, too scattered to deduce any specific pattern. They do not, however, increase exponentially during this period.

Results of similar experiments reported in Chapter 3 (Fig. 3/1)

Figure 6/5.

Kinetics of ^{3}H-uridine use during 2 mM AdR treatment of log phase cultures. Cultures were labelled at 1 µCi/ml in the presence of 4 µM uridine. Length of AdR treatment: (a) control; (b) 1 min; (c) 1.5 hr; (d) 3 hr AdR treatment. (◆) uptake; (●) incorporation.

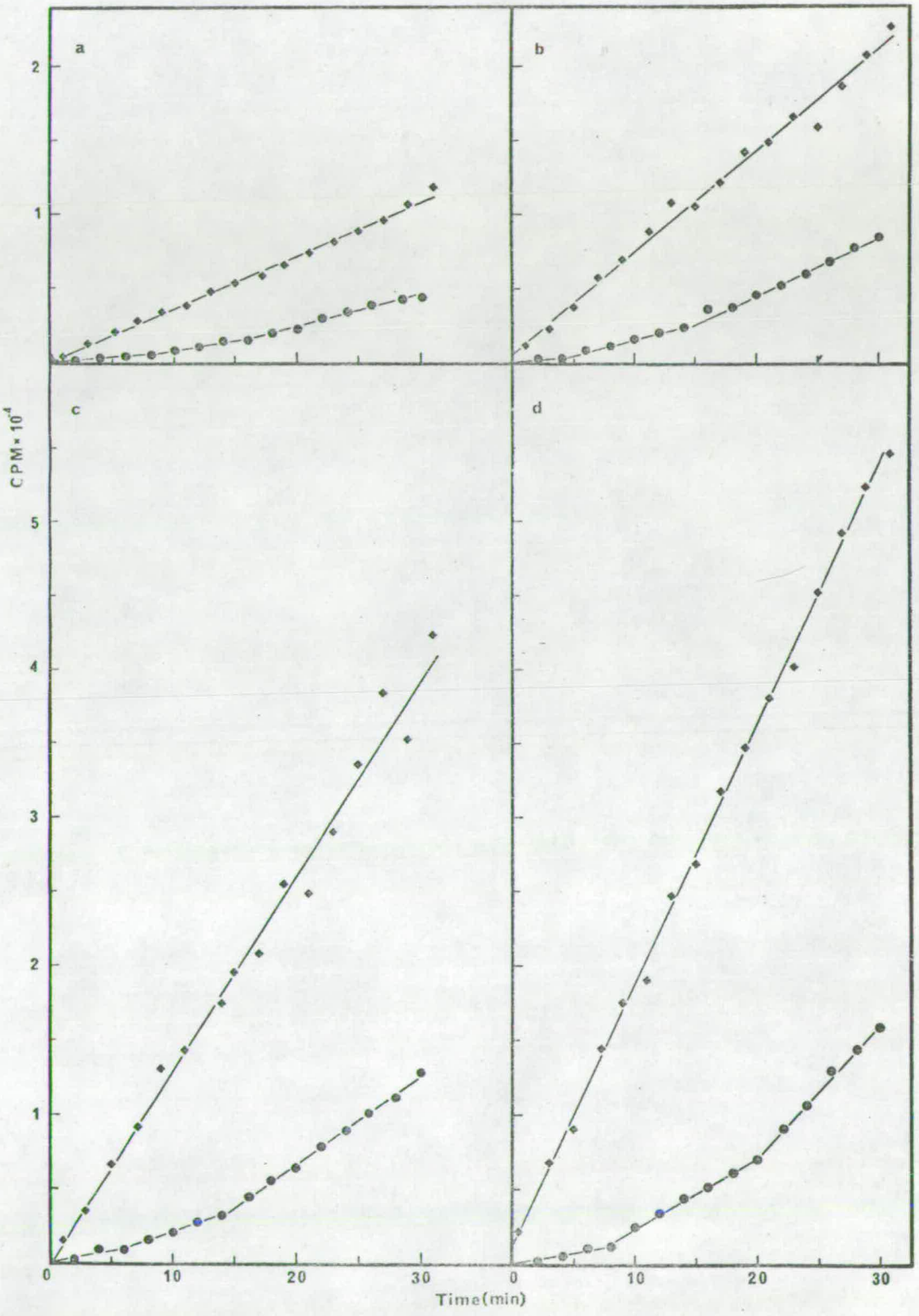

a
b
c
d
CPM × 10⁻⁴
Time(min)
0
10
20
30
1
2
3
4
5

Figure 6/6.

Effect of 2 mM AdR on the kinetics of ^{3}H-uridine incorporation in log phase cultures. This data is the same as in Fig. 6/5, but here it is presented on semi-logarithmic axes instead of linear ones. AdR treatment for :

(a) control,

(b) 1 min,

(c) 1.5 hr,

(d) 3 hr.

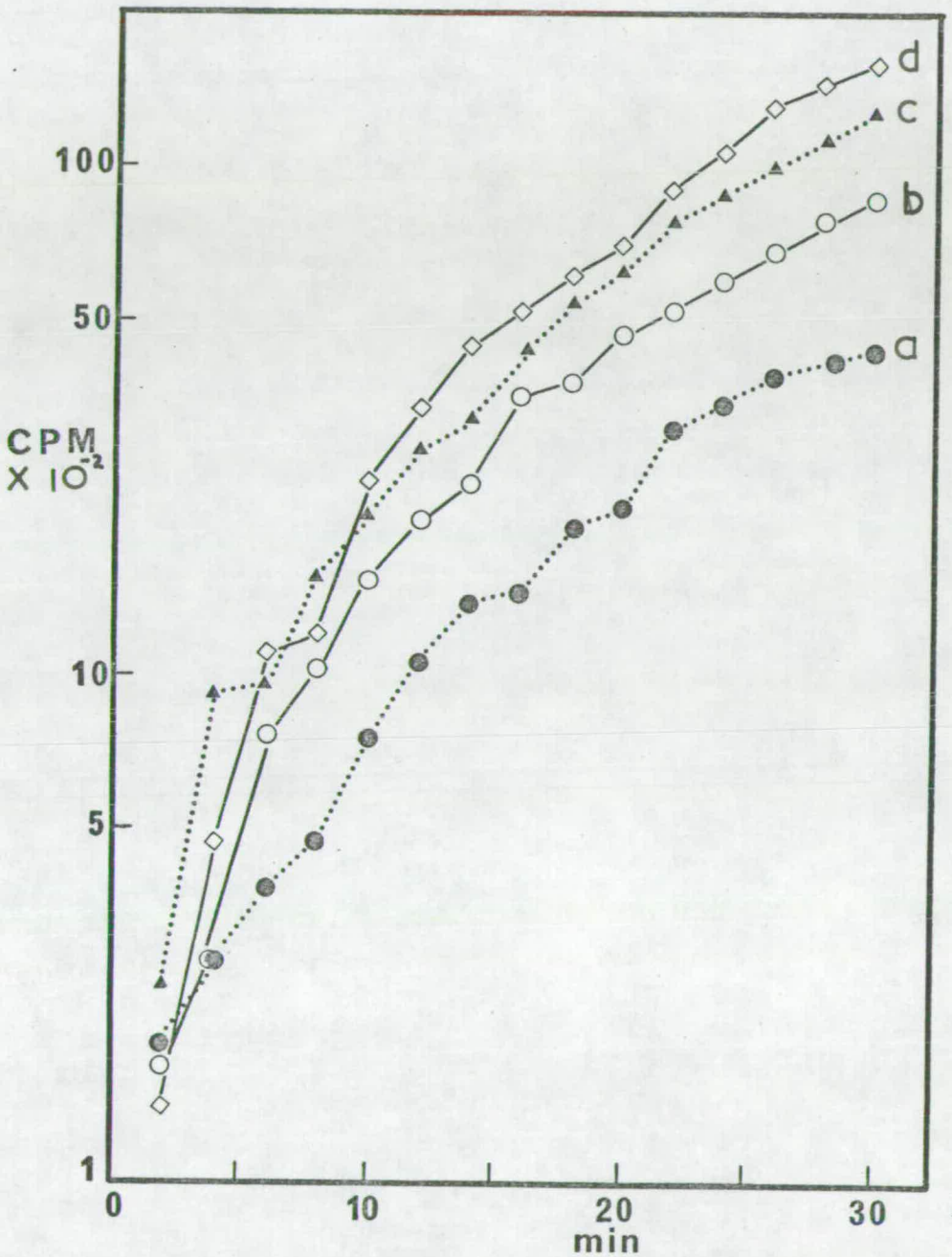
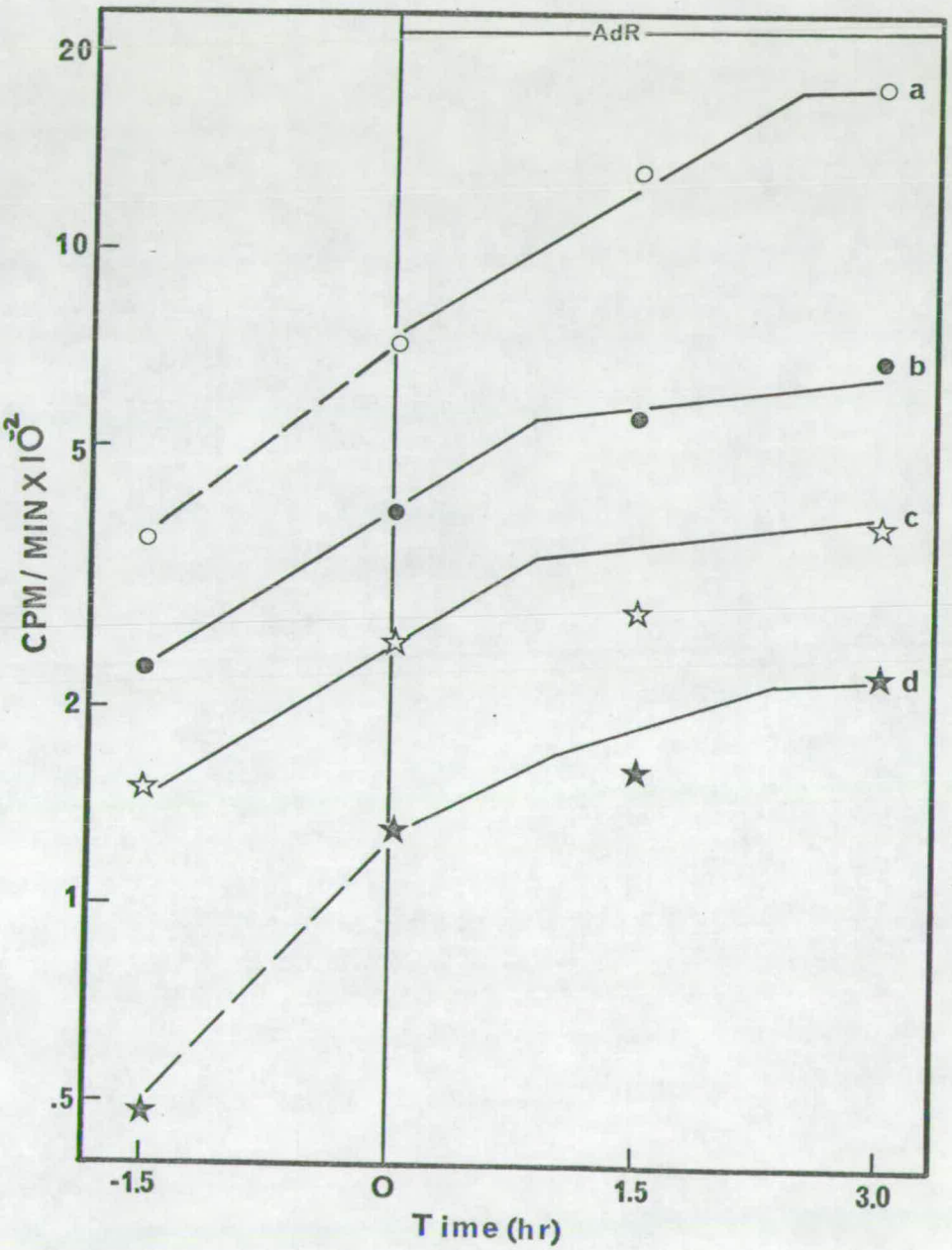

d
c
b
a
100
50
10
5
1
CPM
X 10⁻²
0
10
20
30
min

Figure 6/7.

Rates of ^{3}H-uridine uptake and incorporation from the slopes of the kinetic curves presented in Fig. 6/5. (a) Rate of uptake. Rates of incorporation derived from the slopes of: (d) 1st segment; (c) 2nd segment; (b) 3rd segment of the incorporation curves as described in the text. Dotted lines from rates measured by pulse labelling measurements in (a) Fig. 6/1a; (b & c) Fig. 6/1c; (d) Fig. 6/4.


AdR
20
10
5
2
1
.5
CPM/ MIN X IO-2
a
b
c
d
-1.5
O
1.5
3.0
Time (hr)

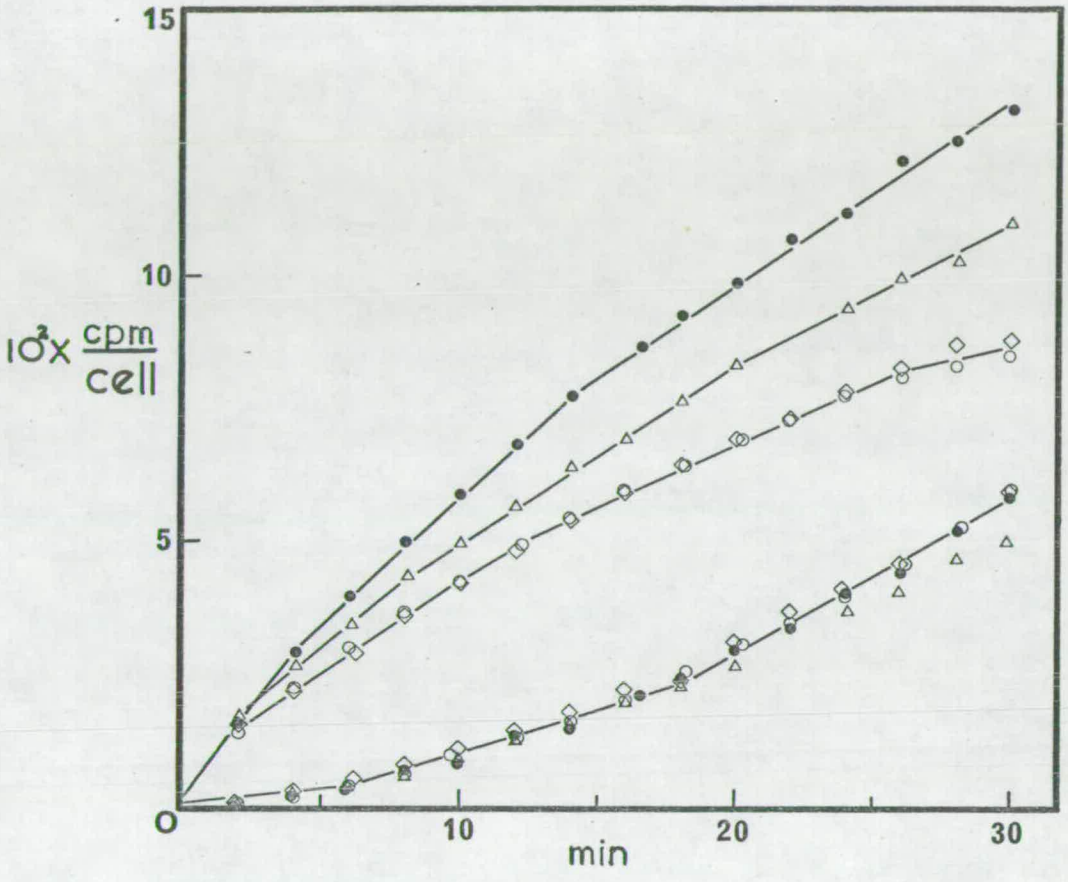
indicate that ^{3}H-uridine incorporation proceeds linearly for at least 50 min after adding the labelled precursor. The rate of incorporation in these untreated, log phase cultures appears to change at 8 min and again around 26 min after adding the label. The present results from AdR treated cultures also indicate a change in the rate of incorporation between 4 and 8 min after adding ^{3}H-uridine. But the second rate change appears to occur between 14 and 20 min in these cultures. The time difference for the second rate change between the untreated cultures and the treated ones may be caused by the AdR. But it may equally well reflect the normal time range over which this rate change can take place. That the control incorporation in Fig. 6/5 appears to change its rate of increase between 16 and 20 min argues in favour of the latter interpretation.

The rates of ^{3}H-uridine uptake and incorporation were determined by measuring the slopes of the curves in Fig. 6/5. They were then plotted as a function of the duration of AdR treatment (Fig. 6/7). The lines connecting the experimental points are taken from the rate measurements made by pulse labelling cells (Figs. 6/1 & 6/4). The close agreement between the two independent rate measurements serves as an internal check on the reliability of the methods and demonstrates that the kinetic and the pulse experiments are measuring the same system. The lack of fit between the 19 min pulse rate and the rate measured from the slope of the second segment of the incorporation curve for 1.5 hr AdR treatment is to be expected since this second segment lasted from 6 to 14 min and the rate measured from its slope was an average rate for that period.

An important effect of AdR noted in these experiments is the expansion of the pool(s) of ^{3}H-uridine and its TCA-soluble derivatives. This

Figure 6/8.

Kinetics of ^{3}H-uridine pool filling and incorporation per cell during 2 mM AdR treatment of a log phase culture. Labelling is the same as for Fig. 6/5. Top 3 curves, kinetics of pool filling/cell following AdR treatments of (o) 3 hr; (◇) 1.5 hr; (△) 1 min; (●) control. Bottom curve, kinetics of incorporation /cell symbols as above.


15
10
5
0
10
20
30
min
10³x cpm/cell

expansion is shown most unambiguously when the kinetics of pool filling are presented on a per-cell basis (Fig. 6/8). Brief exposure to AdR had no noticeable effect on pool size. But after a 1.5 hr exposure, the pool has expanded by 14% compared to the control when measured after 20 min of labelling. And after a 3 hr exposure, the pool is just over 40% larger than the control. This pool expansion can be linked to a comparable increase in uptake during AdR treatment (Fig. 6/8), but which is the cause and which the effect is not known. In either case, incorporation of ^{3}H-uridine per cell remains constant throughout the drug treatment.

DISCUSSION

The rates of ^{3}H-uridine uptake, incorporation and radioactive pool filling were followed in AdR induction synchronized cultures of Schizosaccharomyces pombe. The rate patterns obtained using 19 min precursor pulses (Fig. 6/1) show a marked similarity to the analogous patterns from selection synchronized cultures of S. pombe which had been treated with AdR (Fig. 5/1). During the first 3 hr of AdR treatment, the rates of both uptake and pool filling increase exponentially. Thereafter they remain approximately constant until 50-60 min after the inhibitor has been removed. Uptake then increases exponentially in rate until the start of the second synchronous wave of cell division, after which it remains constant. Although the rate of ^{3}H-uridine pool filling also starts to increase at the same time as uptake, the rate of increase decays rapidly, so that when the rate of uptake becomes constant the rate of pool filling is decreasing. The rate of ^{3}H-uridine incorporation also increases at the start of AdR treatment, but it becomes constant after only 2 hr of treatment. After the AdR is removed, the rate of

incorporation increases slowly for 40-50 min. It then increases rapidly and exponentially, parallelling the DNA content of the culture from at least 20-40 min before removing the inhibitor until the end of the experiment. This same pattern was also found for the initial rate of uridine incorporation measured with 1 min pulses.

Before the rate of incorporation can be equated with the rate of RNA synthesis, the effect of AdR treatment on this rate must be examined. As mentioned in Chapter 5, this inhibitor appears to alter the size of the ^{3}H-uridine pool in *S. pombe*. But on a per-cell basis the kinetics of uridine incorporation are unchanged by up to 3 hr of drug treatment, even though the uridine pool has nearly doubled (Fig. 6/8). The rate of ^{3}H-uridine pool filling/cell increases sharply for 40-60 min following the first 2 hr of AdR treatment. This rate of increase appears to be analogous to a similar one in selection synchronized cultures treated with AdR (Fig. 5/1). Similarly, both types of AdR culture show a decrease in the cellular rates of uridine pool filling during the synchronous cell divisions which occur after the inhibitor has been removed. These data suggest that AdR causes an expansion of the ^{3}H-uridine pool and that this expanded pool requires a higher rate of filling to maintain its level. The initial expansion appears to occur mainly after about 2 hr of AdR treatment and the cells recover from this expanded pool state with the onset of normal cell divisions. The increase in the rate of pool filling/cell noted during the first hour of AdR treatment in selection synchronized cultures (Fig. 5/1), was not noted in log phase cells treated in the same way. It may, therefore, be caused by either the gradient selection technique, or by the combined action of that technique and the AdR, rather than by the AdR alone. At the other end

of the experiment, the cellular rate of uridine pool filling in induction synchronized cultures decreased to about a third of its initial value. The most likely explanation of this is that after the first synchronous division, cells were at a high enough density to begin entry into stationary phase. In doing so, they may reduce the size of their metabolic pools to a level far below that required for log phase growth. Except during these periods of adjustment, the radioactive pools appear to remain relatively constant. Thus, from about 60 min before, until 80 min after removing the AdR, the rate of ^{3}H-uridine incorporation most likely reflects the rate of RNA synthesis. Indeed, the lack of change in the cellular kinetics of ^{3}H-uridine incorporation in the face of increasing rates of soluble pool filling during AdR treatment suggests that the rate of incorporation may reflect the rate of RNA synthesis during the entire experiment. If this is the case, then the parallel changes in the rate of RNA synthesis and the DNA content of the culture lend further support to the gene dosage model suggested in Chapter 4.

CHAPTER VII

THE EFFECTS OF 1-PHENYLETHANOL

The second inhibitor of DNA synthesis used in this study was 1-phenylethanol (1-PE). Bostock (1970,b) reported the inhibitory action of its isomer, 2-phenylethanol (2-PE or phenethyl alcohol), on DNA synthesis in *S. pombe*. At a concentration of 0.3%, 2-PE blocks the initiation of DNA synthesis. In 0.2% 2-PE, on the other hand, cells grow with a generation time of 4.5 hr, due to a 2 hr G_1 being inserted into an otherwise apparently undisturbed cell cycle. Thus, cells grown in 2-PE have their S period in the middle of the cell cycle. If 0.2% 2-PE is added to a log phase culture, the DNA content of that culture remains constant for the first two hours and then increases with a mean doubling time of 4-5 hr. Cell numbers and dry mass (OD_{595}) of such cultures change within 20-30 min to the new rate of increase, without an intervening constant period. With such an inhibitor there are, therefore, two separate types of situation in which cell division can be separated from DNA synthesis. The rate of RNA synthesis was measured in selection synchronized cultures of cells maintained in PE and in log phase cultures to which PE was added at the start of the experiment.

The experiments presented here were, in fact, planned to be carried out using 2-PE. But since the results of several preliminary studies done with 1-PE left behind in the laboratory by Bostock agreed with his earlier work using the 2-isomer, 1-PE was used for the remainder of these experiments. The change in the site of attachment of the aromatic ring to the ethanol backbone makes 1-PE slightly less polar and less dense than 2-PE (Weast, Selby & Hodgeman, 1964). Allowing for that, the two isomers should have very similar chemical properties.

Figure 7/1.

The effects of several concentrations of 1-phenylethanol upon exponential growth of S. pombe. OD_{595} measurements of cells growing in 0.1% (Δ), 0.2% (□), 0.3% (▲), and 0.4% (●) 1-PE added at 0 hr. Control culture in EMM2 (o).

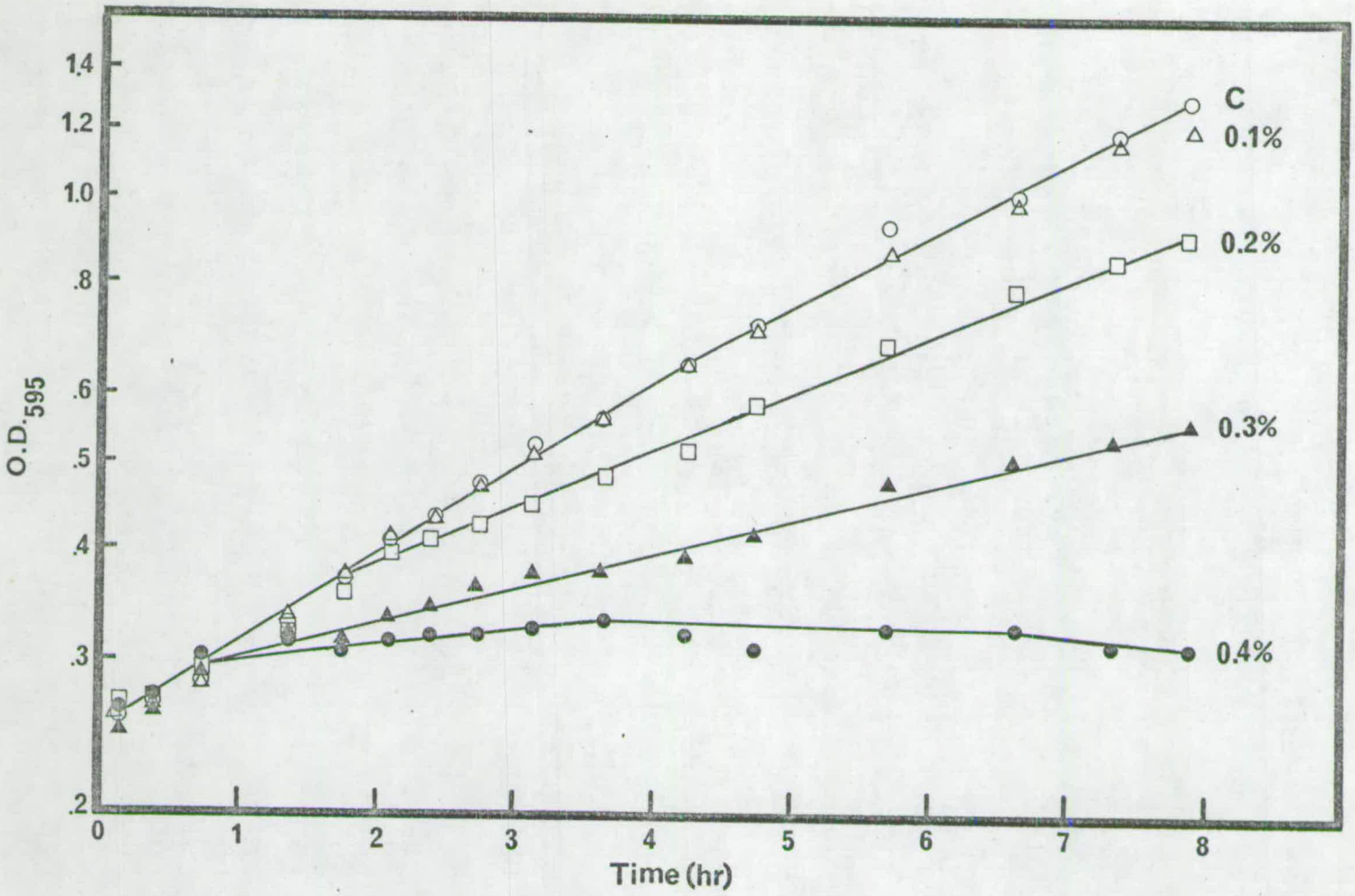

1.4
1.2
1.0
.8
.6
.5
.4
.3
.2
O.D.595
0
1
2
3
4
5
6
7
8
Time (hr)
C
0.1%
0.2%
0.3%
0.4%

Figure 7/2.

Some effects of adding 0.2% 1-phenylethanol to an exponentially growing culture.

(a) DNA/ml as OD_{595} of diphenylamine assay $\times 10^{2}$;

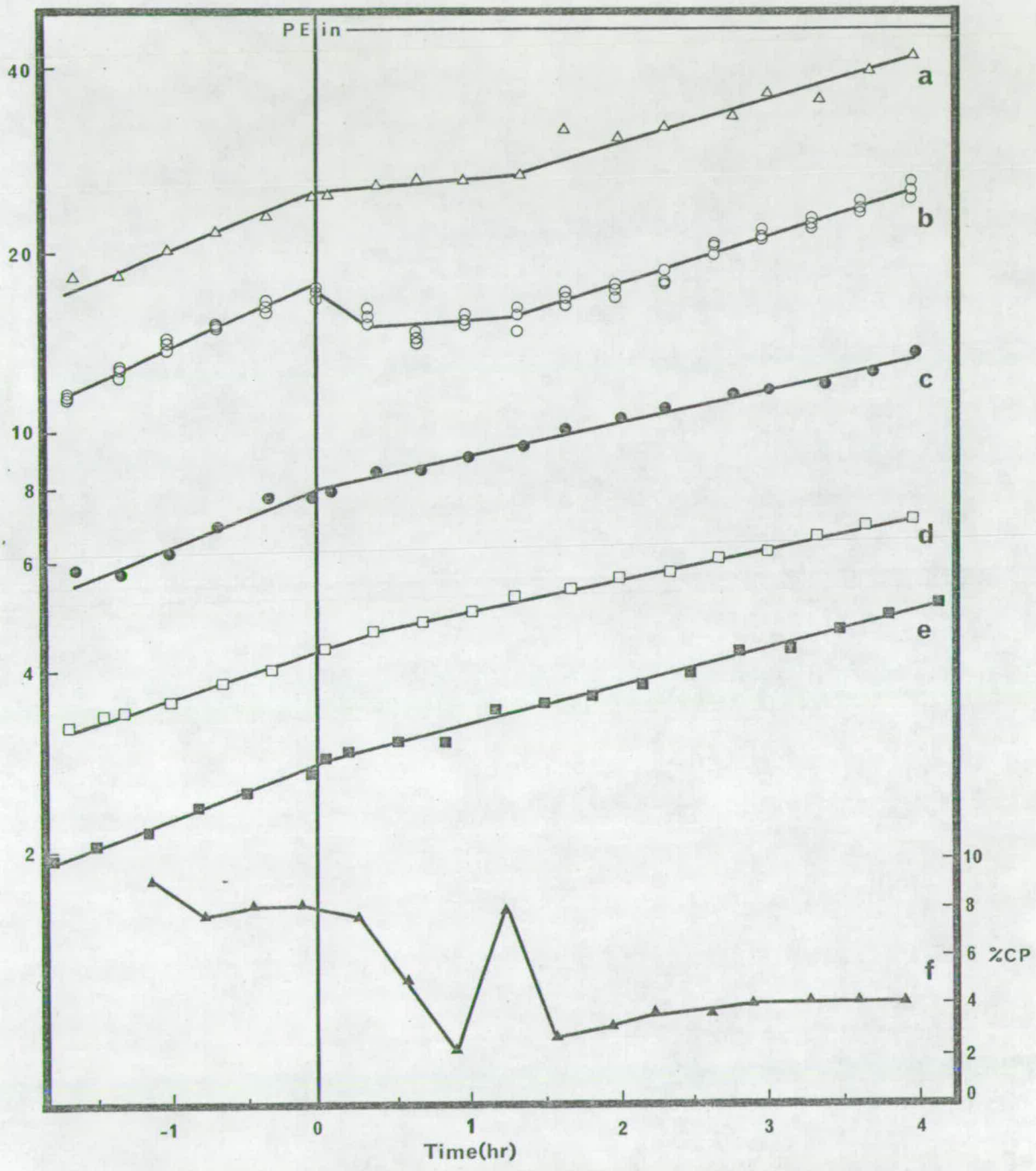
(b) rate of ^{3}H-uridine incorporation as $10^{-2} \times$ cpm/ml culture, from a 19 min pulse of 1 µCi/ml;

(c) RNA/ml as $OD_{260} \times 10^{-1}$;

(d) cellular dry mass as OD_{595};

(e) cell number x 10^{-6};

(f) cell plate index.


PE in
40
20
10
8
6
4
2
a
b
c
d
e
f
10
8
6
4
2
0
%CP
-1
0
1
2
3
4
Time(hr)

DOSE-RESPONSE

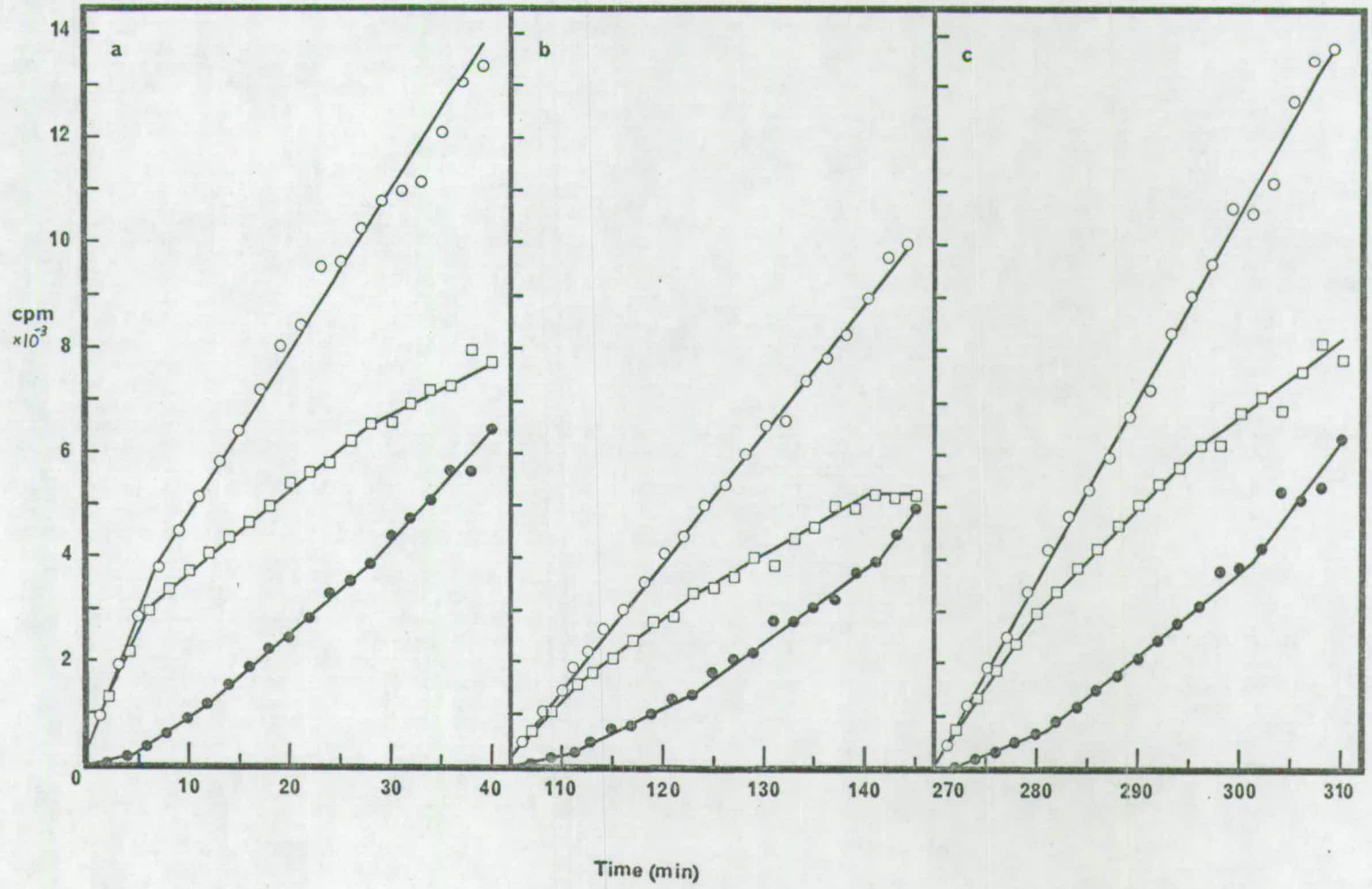
To check that 0.2% was the optimal concentration of 1-PE, as Bostock (1970,b) reported for the 2-isomer, a log phase culture was divided into 5 parts. Each part was grown with a different concentration of 1-PE and the rates of growth were monitored by OD_{595} (Fig. 7/1). Concentrations of 0.3% or higher produced generation times in excess of 8 hr, while cells treated with 0.1% 1-PE grew at the same rate as the control culture. Cells growing in the intermediate concentration of 0.2% had a generation time of about 4-4.5 hr. Cultures appeared to adjust to this new rate of growth within 20 min, and they grew exponentially at this new rate for at least six hours. All further experiments were, therefore, carried out using 0.2% of the inhibitor.

SOME EFFECTS OF ADDING 0.2% 1-PE TO A LOG PHASE CULTURE

Parallel log phase cultures were set up to monitor a selection of growth parameters both before and after adding 0.2% 1-PE to each culture. The rate of growth changed to the new 4 hr generation time within 20-30 min of adding the 1-PE, as seen in the cell number and OD_{595} curves (Fig. 7/2d,e). This change in growth rate is also reflected in the cell plate index (Fig. 7/2f) which decreases from about 8% to 4%. About 1-1.5 hr after adding the inhibitor, however, the CPI returns briefly to its original 8% level. Such a temporary increase in the CPI has been noted on all four separate occasions when it was determined. It most likely represents the small proportion of cells which were in a PE-insensitive stage of the cell cycle when the inhibitor was added. Since their cell cycles were therefore not extended like those of all other cells, they all divided at their normal time. DNA synthesis in these cells was delayed 1-1.5 hr (Fig. 7/2a) rather than the 2-5.5 hr

Figure 7/3.

The effect of different lengths of 1-phenyl ethanol treatment on the kinetics of ^{3}H-uridine use by exponentially growing cultures of S. pombe. Cultures were labelled with 2 µCi/ml ^{3}H-uridine at a final concentration of 8.2 µM. Labelling started (a) 1 min; (b) 105 min; (c) 270 min after adding 0.2% 1-PE. Results are presented as cpm x 10^{-3}/ml culture (o) uptake; (●) incorporation; (□) pool.


a
b
c
cpm
×10⁻³
0
2
4
6
8
10
12
14
10
20
30
40
110
120
130
140
270
280
290
300
310
Time (min)

delay reported for the 2-isomer (Bostock, 1970,b), and this accounts for the cell cycle of 1-PE treated cells being up to an hour shorter than those of Bostock's cells. The accumulation of RNA (Fig. 7/2c) paralleled changes in cell mass and cell number, and changed to the new growth rate without an intervening constant period. The rate of ^{3}H-uridine incorporation from 19 min pulses, on the other hand, decreased when the 1-PE was first added, then paralleled the DNA content of the cells. The rate remained constant until 80-90 min after the inhibitor was added to the culture. It then began to increase with a mean doubling time of about 4-4.5 hr. This pattern suggests that 1-PE causes an initial decrease in the rate of incorporation, after which a gene dosage control may take over. If, as is the case with 2-PE (Plagemann, 1970), 1-PE causes a reduction in the rate of metabolite uptake, the reduced rate of incorporation may not reflect an actual reduction in the rate of RNA synthesis.

EFFECTS OF 1-PE ON THE KINETICS OF ^{3}H-URIDINE USE.

The kinetics of ^{3}H-uridine uptake, incorporation and radioactive pool filling were followed at 1 min, 1.75 hr and 3 hr after adding 0.2% 1-PE to a log phase culture. These times correspond to the periods of decrease, plateau and recovery of the rate of uridine incorporation in Fig. 7/2. The overall patterns of uridine use appear much the same as in untreated cultures described in Chapter 3 (Fig. 7/3). The one exception is that, although uptake proceeds linearly, 5-7 min after adding the drug, the rate of uptake sharply decreases. Thereafter it appears to continue at a constant rate, but the rate may, in fact, continue to decrease very slowly. After 100 min of 1-PE treatment, the rate of uptake is indeed less than that between 7 and 40 min of treatment, but it

Figure 7/4.

Kinetics of ^{3}H-uridine incorporation in the presence of 0.2% 1-PE. Data is the same as in Fig. 7/3, but here it is presented on semi-logarithmic axes instead of linear ones. Length of 1-PE treatment before labelling : 1 min (o); 105 min (●); 270 min (◇).

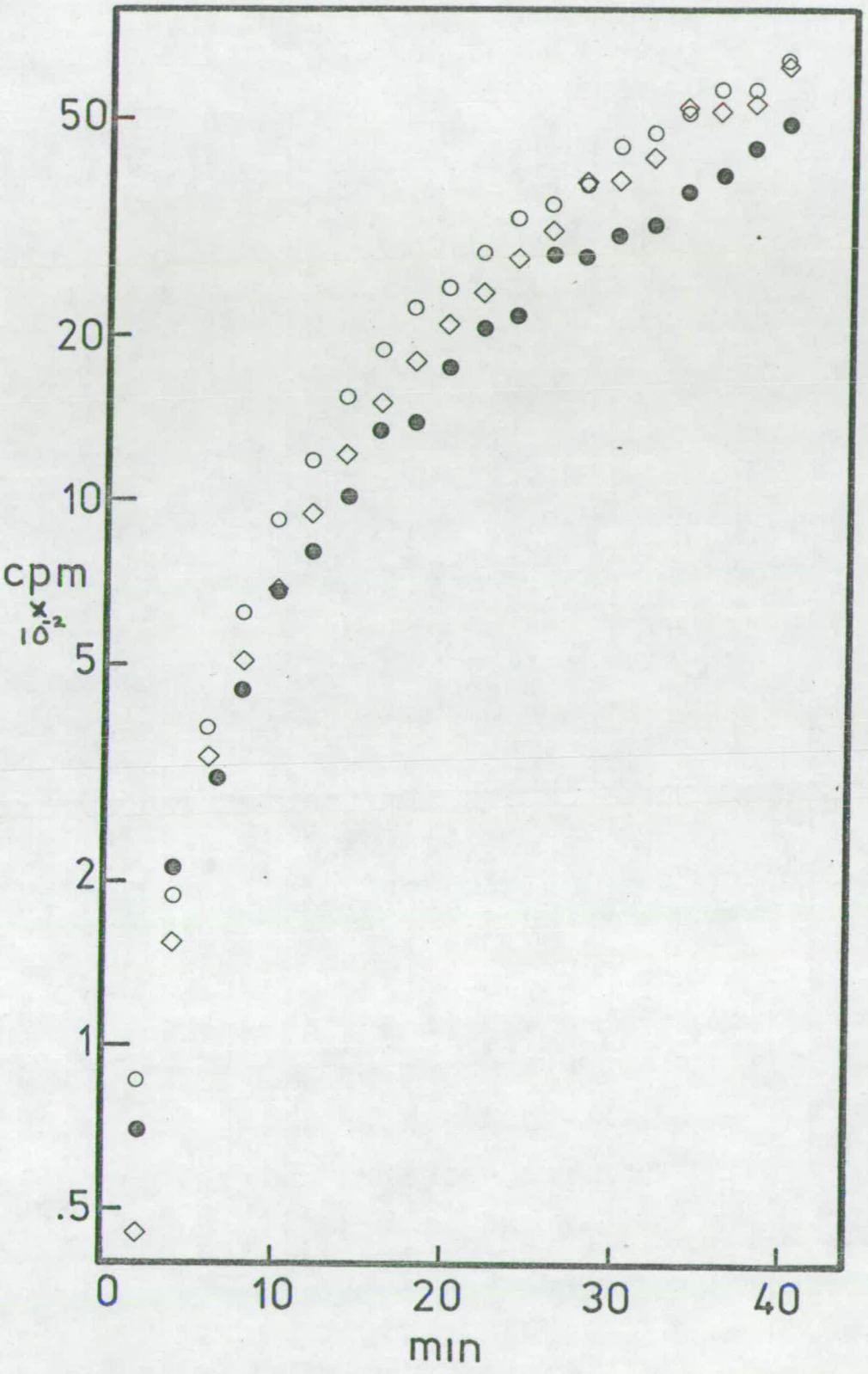
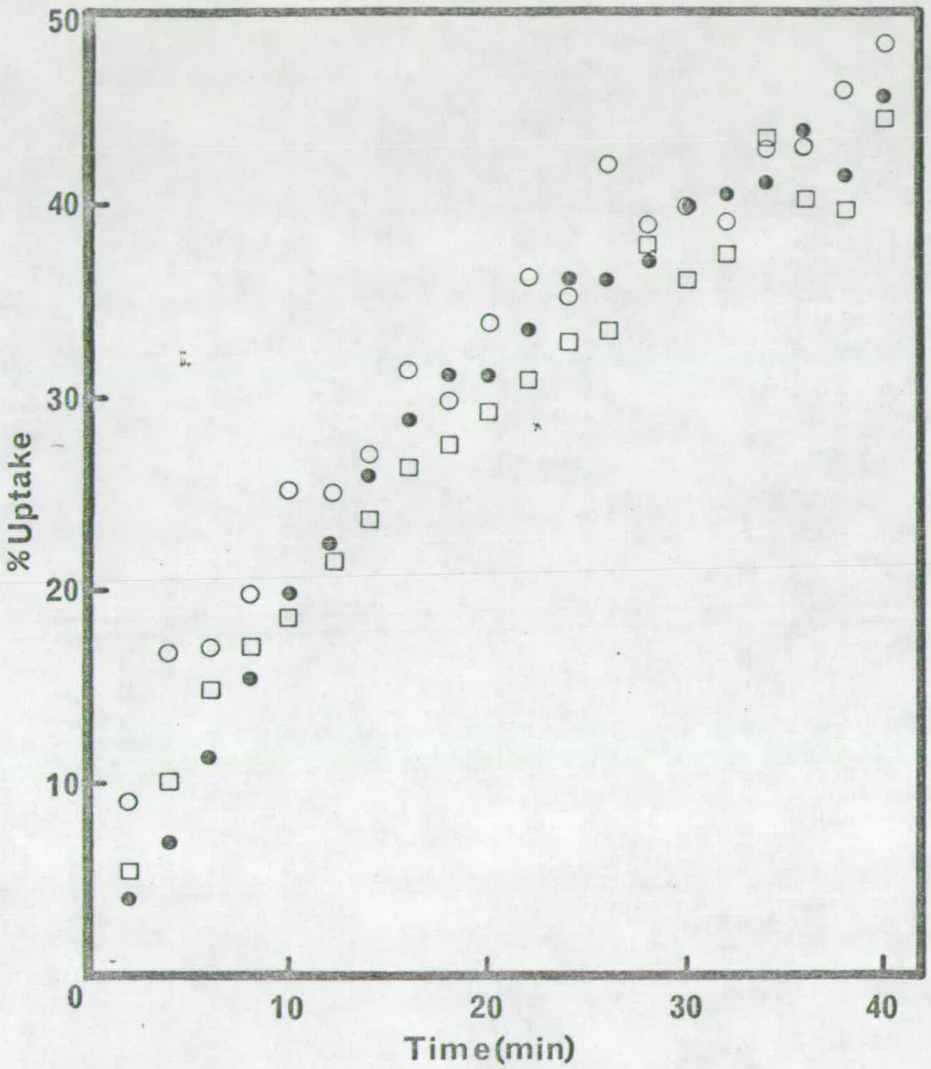

50
20
10
cpm
x
10⁻²
5
2
1
.5
0
10
20
30
40
min

Figure 7/5.

The effect of different lengths of 1-phenyl ethanol treatment upon the percentage of ^{3}H-uridine taken up by cells which they incorporate into TCA-insoluble material. Labelling conditions are the same as for Fig. 7/3. Length of treatment: 1 min (●), 105 min (○), 270 min (▽).


50
40
30
20
10
0
% Uptake
10
20
30
40
Time (min)

now remains constant during the entire 40 min labelling period. After 3 hr of labelling, the rate of uptake has again increased to almost the same level as during the first 5-7 min of drug exposure.

The kinetics of ^{3}H-uridine pool filling were followed by subtracting the incorporation data from the uptake data in Fig. 7/3. As these pools filled, their rates of filling decayed. But as in the case of incorporation, it is not possible to definitely establish the pattern of this decay. However, after 6 min of 1-PE treatment and again after 3 hr 10 min of treatment, the evidence strongly suggests sharp decreases in the rates of uridine pool filling.

Incorporation of ^{3}H-uridine in these cultures increases in rate, but the data is insufficiently precise to determine whether the rates increase continuously or discontinuously. Only in Fig. 7/3c does the data strongly suggest at least two constant rates with a sharp rate change after approximately 10 min of labelling. In either case, incorporation does not increase exponentially during the first 20-25 min of labelling (Fig. 7/4). The most likely pattern for these incorporation kinetics to follow may be the segmental pattern seen in untreated cells (Fig. 3/1). But 1-PE might alter the kinetic patterns as well as the rates.

The overall depression of the rates of use may be simply due to the reduced rate of radioactive precursor uptake, which in turn presents the cell with less radioactivity to incorporate. In support of such an hypothesis, the percentage of the precursor taken up by cells which is incorporated into RNA, appears to be virtually independent of the length of 1-PE treatment (Fig. 7/5). After 3 hr of 1-PE treatment, however, relative incorporation may be slightly reduced. In no case is it more

than 4% less than the other data in Fig. 7/5. Furthermore, 1-PE treatment does not lower the percentage of precursor incorporated from the level in untreated cells shown in Fig. 3/4. The means by which 0.2% 1-PE reduces the rate of ^{3}H-uridine incorporation in S. pombe is, therefore, most likely to be by reducing the amount of precursor taken into the cells. Similar inhibitory effects on RNA precursor uptake have been noted for 2-PE acting on mammalian cells (Crifò, Bozzi, Mondovì & Strom, 1973; Bruchovsky & Till, 1967,a). And there is evidence that at least this 2-isomer causes cells to leak nucleic acid precursors back into the medium (Richards & McBride, 1973). Such leakage could further lower the apparent rate of uptake.

EFFECT ON SYNTHESIS OF VARIOUS RNA FRACTIONS.

If, as suggested above, ^{3}H-uridine incorporation decreases because of a reduction in the amount of labelled precursor taken up, then the distribution of label in the various RNA species should remain unaltered. But, on the other hand, RNA synthesis may be inhibited separately and all RNA species may not be affected equally. Log phase cultures were therefore labelled for 19 min with ^{3}H-uridine after 45 and 180 min of 1-PE treatment. An untreated control culture was similarly labelled and the RNA from all three cultures was then extracted and separated electrophoretically on 2.2% polyacrylamide gels. The amount of radioactivity incorporated into each species of RNA was then determined. The patterns of distribution all appeared very similar to each other, except in the relative amounts of the ribosomal RNA species labelled. Table 7/1 presents the ratios of the label in each peak to the other peaks. During 3 hr of drug treatment there is a 30% relative increase in the ribosomal precursor and a 70% increase in the 18-S RNA labelling in relation to the level of label in 25-S r-RNA. A much greater

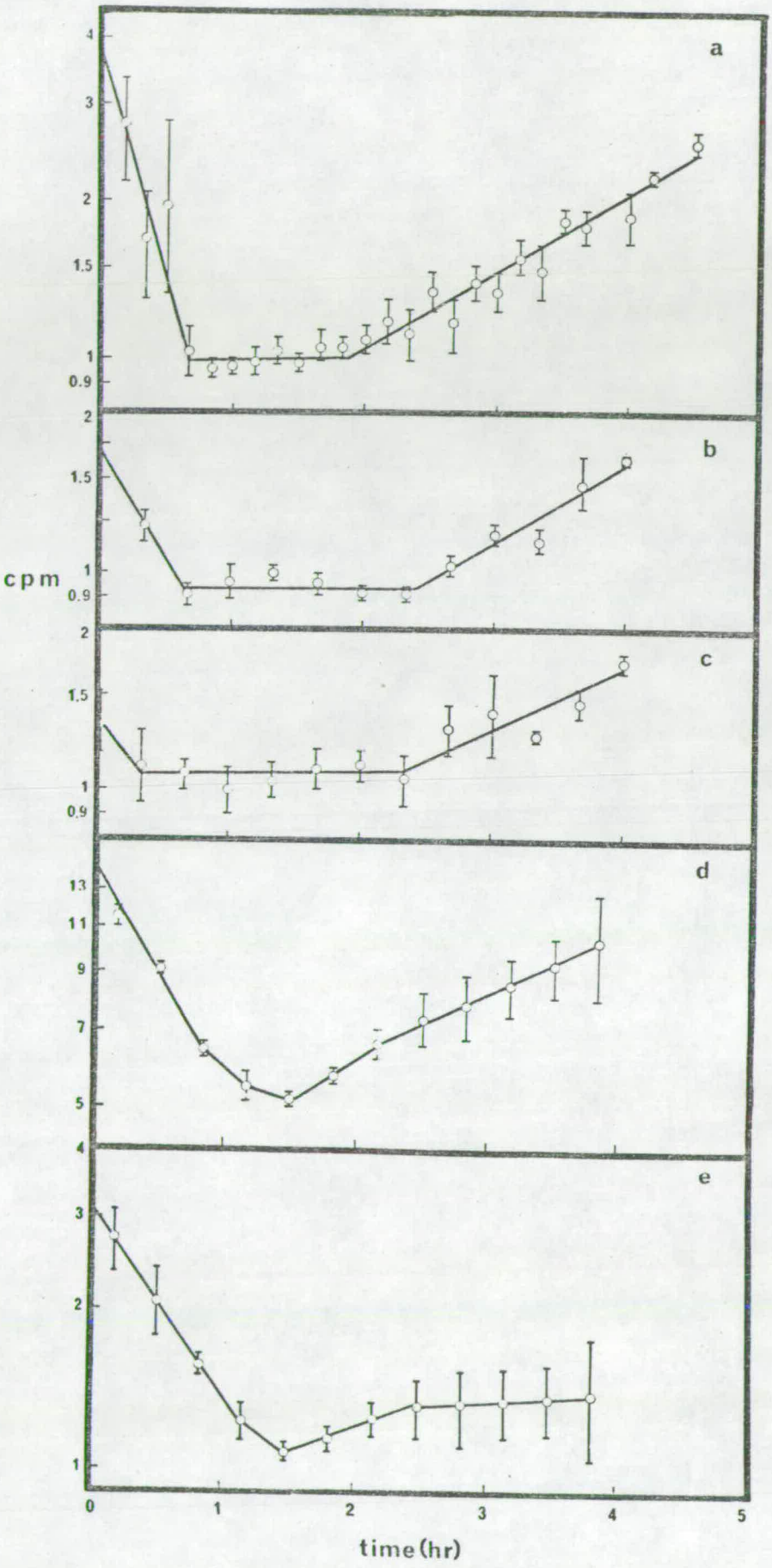
Table 7/1: Ratios of ^{3}H-uridine incorporated into ribosomal RNA fractions during 1-PE treatment. Cultures labelled at 1 µCi/ml for 19 min. RNA separated electrophoretically on 2.2% polyacrylamide gels. Data presented as ratios of counts in ribosomal RNA peaks determined as in Chapter 3.

	pre-r-	25-S	18-S
control	0.458	1.0	0.425
45 min	0.555	1.0	0.544
180 min	0.593	1.0	0.725

proportion of the nuclear label also appears to accumulate in the smaller species of r-RNA in HeLa cells grown under "Step-down" conditions (Bramwell & Handmaker, 1975). These results suggest that two things are possibly happening: First, since both 25-S and 18-S r-RNA are derived from the ribosomal precursor RNA molecule, there can be no differential synthesis occurring. Instead, the 25-S species seems to be degraded more rapidly than 18-S. This degradation could also increase the relative radioactivity in the precursor peak. Second, a decrease in the stability of the 25-S r-RNA sufficient to produce a 70% relative increase in the label found in the 18-S fraction would also cause a similar proportional increase in the radioactivity in the precursor peak unless its breakdown were also speeded up. A similar selective degradation of selected RNA species can be induced in Novikoff hepatoma cells by 2-PE (Plagemann, 1968,b), and in this case it was suggested that protected sequences may be bound to membranes. Such protection may well be acting in both HeLa and S. pombe, since the smaller ribosomal subunit is thought to attach to both messenger RNA and to membranes before joining the larger subunit (McLaughlin, 1974).

Figure 7/6.

The effect of 0.2% 1-phenylethanol upon the rates of ^{3}H-uridine uptake and incorporation in exponentially growing cultures of *S. pombe*. Rates of uridine incorporation during (a) 19 min pulses, labelling with 1µCi/ml at 28.7 nM to 5 µCi/ml at 820 µM; (b) 19 min pulses, labelling with 20 µCi/ml at 2 mM; (c) 1 min pulses, labelling with 10 µCi/ml at 287 nM; rates of uridine uptake during (d) 19 min pulses labelling with 2 µCi/ml at 8.2 µM, and (e) rate of soluble pool filling, obtained by subtracting (a) from (d). Data from at least 2 of each type of experiment were pooled, presented as means ± 95% confidence limits, and expressed as relative cpm.


a
b
c
d
e
cpm
time (hr)
0
1
2
3
4
5

EFFECT ON RATES OF ^{3}H-URIDINE USE IN LOG PHASE CULTURES

In order to help clarify the relationships between the rates of RNA synthesis and uridine uptake on the one hand, and DNA synthesis and cell division on the other, a series of pulse labelling experiments were carried out. The rates of ^{3}H-uridine uptake, incorporation and soluble pool filling were followed in log phase cultures to which 0.2% 1-PE was added. The initial decrease in the rate of incorporation lasted 20-40 min and was followed by a plateau of about 100 min. Thereafter the rate of uridine incorporation began to increase with a mean doubling time of 4-4.5 hr.

The initial drop in rates showed wide variations not only between cultures, as reflected in the very wide 95% confidence limits of the initial points in Fig. 7/5a, but also between counts on replicate filters taken from the initial samples of any given culture. Much of this scatter was almost certainly due to the insolubility of 1-PE, so that in initial samples the drug was probably not evenly dispersed. This, plus several other factors, including slight differences in 1-PE concentration, density of the culture and experimental errors produced plateaux in the rate of incorporation which ranged from 1.5 hr to just over 2 hr in individual experiments. In all cases where DNA was measured however, this plateau continued only as long as there was no increase in the amount of DNA in the culture. Once DNA synthesis resumed, the rate of uridine incorporation began to increase in parallel. This same pattern was also found when cells were labelled either in the presence of 2 mM uridine or by 1 min pulses, although in these last two cases the initial rate decreases were noticeably less (Fig. 7/6b,c). Since 2 mM uridine in the labelling medium should make the rate of incorporation

independent of any active transport mechanisms (See Chapter 3), this result suggests that, as does 2-PE in budding yeast (Burns, 1968), 1-PE partially inhibits RNA synthesis as well as uridine uptake.

Both the rate of ^{3}H-uridine uptake and that of radioactive pool filling per cell decreased for the first 70-80 min of 1-PE treatment (Fig. 7/6d,e). Thereafter they both began immediately to increase. By 130 min of treatment, the rate of uptake had stabilized at an exponential rate of increase with a mean doubling time of 4-4.5 hr. The cellular rate of pool filling increased for only an hour. It then remained constant until the end of the experiment. Thus, the rate of uridine uptake again appears to increase following a decrease in both the cellular rate of pool filling (Fig. 7/6e) and the size of the radioactive uridine pool (Fig. 7/3).

EFFECTS OF 1-PE ON SELECTION SYNCHRONIZED CULTURES

Bostock (1970,b) reported that if S. pombe cultures maintained in 0.2% 2-PE for several generations were selection synchronized, the S period occurred 2-2.5 hr after cell division. Cultures, were therefore grown over night in the presence of 0.2% 1-PE, then selection synchronized and resuspended in fresh 1-PE containing EMM2. The DNA content and the rates of ^{3}H-uridine uptake and incorporation were followed for up to 2.5 generations. Cell numbers and the CPI were also monitored. Under these conditions the cultures usually had a mean generation time of about 3.5 hr. DNA appeared to be made over a period of 1-1.5 hr with the midpoint of the increase about 0.29 of a cell cycle after cell division (Fig. 7/7). The DNA data show a lot of scatter and do not cover an entire cell cycle because of thedifficulty in getting a culture of 1-PE grown cells which was at the same time large enough to take sufficient

Figure 7/7.

Rates of ^{3}H-uridine uptake and incorporation in selection synchronized cultures of cells growing in 0.2% 1-phenylethanol. Data from 2 of each type of experiment were normalized, pooled and presented as means ± 95% confidence limits.

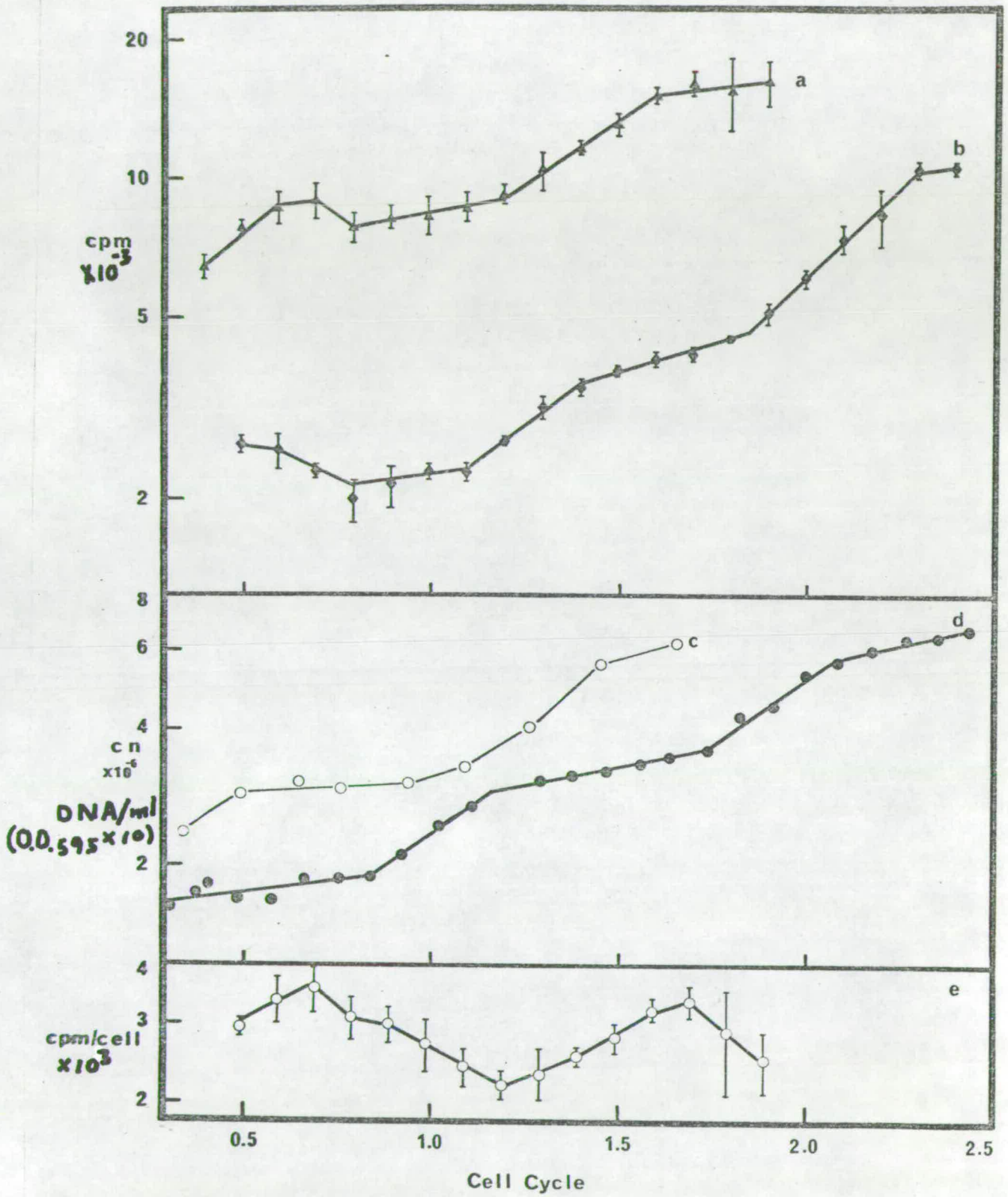
(a) rate of uptake from 19 min pulses, 1 µCi/ml at 4.1 µM,

(b) rate of incorporation during 19 min pulses, 2 µCi/ml at 8.2. µM;

(d) cell number increase from a typical experiment;

(e) rate of soluble pool filling per cell $(\frac{a-b}{c})$;

(c) DNA/ml as OD_{595} of the diphenylamine reaction.


20
10
5
2
cpm
x10⁻³
a
b
8
6
4
2
cn
x10⁻⁶
DNA/ml
(O.D.595 x 10)
c
d
4
3
2
cpm/cell
x10³
e
0.5
1.0
1.5
2.0
2.5
Cell Cycle

samples for a DNA assay and synchronous enough to relate the data to cell cycle changes. The rates of ^{3}H-uridine uptake and incorporation suffered from some of the same problems, since the longer cell cycle of PE grown cells meant that much more asynchrony arose during a cell cycle than in untreated synchronous cultures. In spite of this difficulty, the rates of both uptake and incorporation double sharply in the middle of the cell cycle. Furthermore, the rate of uptake clearly doubles after the rate of incorporation. From the beginning of the experiment until 0.6 of the first cell cycle, the rate of uridine uptake increases. It then decreases slightly before remaining nearly constant until the end of that cell cycle. At 1.2 cell cycle uridine uptake increases sharply to nearly double its rate. There is a similar, but more marked decrease in the rate of uridine incorporation between 0.6 and 0.8 cell cycle. But if a previous rate increase occurred, it did so before 0.5 of the first cell cycle.

From the timing of the initial rise in the rate of uptake, it must be the tail end of the rate doubling which, by comparison with the one in the next cycle, started about 0.2 cell cycle. The decrease in rates was not thoroughly investigated but it could have been produced by increased cell death around the time of cell plate formation. At this time there is an increase in the number of cells which have a much higher refractive index than normal cells in the drops of culture used to count the cell plates. According to Creanor (pers. comm.) these cells are dead. In an exponentially growing culture, the percentage of these dead cells rose from under 0.5% before 1-PE was added to a peak of 4.5% at 170 min after starting drug treatment. Thereafter the percentage decreased to a fairly constant level of 2%. Decreases in rates of uptake and incorporation are probably not seen in the later cell cycles because

the increasing asynchrony of the cultures obscures them.

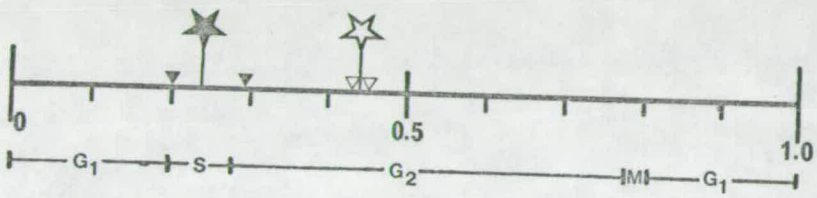
The results from these cultures are very similar to those reported in Chapter 4 for selection synchronized cultures of untreated cells. In this present data, however, the doubling in the rate of RNA synthesis is clearly associated at least temporally with changes in the DNA content of the culture rather than with cell division. Such a distinction was not possible in Chapter 4. Likewise, although the rate of uridine uptake doubles 0.2 of a cell cycle after the similar rate doubling in RNA synthesis, the same basic relationship appears to remain between them as in the untreated cultures.

DISCUSSION

The initial experiments involving growth of cells in medium containing 1-PE show that *S. pombe* responds to this compound as much as it responds to its isomer, 2-PE (Bostock, 1970,b). Both sensitivity to various inhibitor concentrations (Fig. 7/1) and the response of several growth parameters to 0.2% 1-PE (Fig. 7/2) show this similarity. The 2-isomer does, however, appear to prolong the generation time more than the 1-isomer. Thus, at a final concentration of 0.2%, 1-PE extends the cell cycle of this yeast by 1-1.5 hr whereas 2-PE extends it 1.5-2 hr. Cells are very sensitive to both isomers, however, and increasing the PE concentration in the medium from either 0.1% to 0.2% or from 0.2% to 0.3% roughly doubles the generation time of the culture. The basis for the increased generation time caused by 2-PE has been shown to be an insertion of a prolonged G_1 period into an otherwise unaltered DNA-division cycle (Bostock, 1970,b). The 2-isomer of PE is known to inhibit the initiation, but not the continuation of DNA synthesis in bacteria (Treick & Konetzka, 1964), mammalian cells (Bruchovsky, Maks &

Figure 7/8.

Cell cycle map of the mid-points of the doublings in rates of ^{3}H-uridine uptake and incorporation of synchronous cultures growing in 0.2% 1-phenylethanol. Uptake, open symbols; incorporation, solid symbols; individual experiments, triangles; normalized pooled data, stars. Relative contributions to the cycle: (G_1) 0.39; (S) 0.08; (G_2) 0.50; (M) 0.03.


0
0.5
1.0
G1
S
G2
M
G1

Till, 1967) and fission yeast (Bostock, 1970,b). The 1-isomer may well do the same in S. pombe. In selection synchronized cultures of 1-PE treated cells, the beginning of the S period is delayed, but by about 1.0 hr only (Fig. 7/7). This delay produces an altered DNA-division cycle in which the S, G_2 and M periods are not altered in actual time, but, because of the added G_1, the relative contributions of G_1, S, G_2 and M to the cycle are altered from 0.2, 0.1, 0.65 and 0.05 to 0.4, 0.08, 0.5 and 0.2 respectively (Fig. 7/8). The 1-1.5 hr plateau in the pattern of DNA accumulation following the addition of 1-PE to a log phase culture (Fig. 7/2a) is very likely also to be caused by the insertion of such a G_1 into the cell cycle. Thus, those cells about to initiate DNA synthesis when the compound is added, postpone entry into S for 1-1.5 hr and the DNA content of these cultures remains constant for the initial part of drug treatment.

When S. pombe was treated with 1-PE, a rapid decrease in the rate of ^{3}H-uridine uptake was noted (Fig. 7/6). But after 1-1.5 hr of drug treatment the rate of uptake recovered and increased at a rate comparable to that of culture growth. This decrease in the rate of uptake was also reflected in the kinetics of precursor use during the initial hours of 1-PE treatment (Figs. 7/3 & 7/4). These kinetic experiments suggest that the decreases in the rates of ^{3}H-uridine incorporation and pool filling are the direct result of the reduced rate of precursor uptake, since the length of 1-PE treatment appears to have little effect on the percentage of the label taken up by the cells which was finally incorporated into RNA (Fig. 7/5). Bruchovsky and Till (1967) demonstrated a similar decrease, followed by recovery, in the rates of uridine and leucine uptake by mouse L cells treated with 2-PE. This latter, 2-PE-induced reduction in the rate of uridine uptake is also reflected in a

lower rate of uridine incorporation into RNA (Higgins, Shaw, Tillman & Leach, 1969).

Unlike the rate of ^{3}H-uridine uptake, the rate of uridine incorporation in S. pombe decreases for only the first 30-60 min of 1-PE treatment (Figs. 7/2,7/6). After this initial decrease, the rate of incorporation again parallels the DNA content of the culture and appears to reflect the rate of RNA synthesis. In doing so, both DNA content and rate of RNA synthesis remain constant until about 2 hr after the 1-PE was added, after which they increase with mean doubling times of about 4 hr. Support for the claim that the rate of ^{3}H-uridine incorporation reflects the rate of RNA synthesis after 60 min of 1-PE treatment comes from several sources. First, RNase digestion of cells labelled after 30, 90 or 240 min of 1-PE treatment reduced the incorporated radioactivity to the background level. Second, the rate of ^{3}H-uridine incorporation in the presence of 2 mM uridine (Fig. 7/6b) has the same pattern of decrease, plateau, slow increase, even though precursor uptake under these conditions occurs primarily by diffusion (Chapter 3). And third, after the first hour of 1-PE treatment, the cellular rate of ^{3}H-uridine pool filling remains fairly constant (Fig. 7/6e). From a low point of 1.1 after 90 min of treatment it increases for an hour and then becomes roughly constant at about 1.4. Such variation in the rate of pool filling does not appear to affect the pattern of incorporation. From these three points it appears that in the presence of 0.2% 1-PE, ^{3}H-uridine is incorporated specifically into RNA and, at least after the first hour of treatment, changes in the rate of ^{3}H-uridine uptake and cellular rate of pool filling do not noticeably alter the rate of incorporation. However, 1-PE does appear to affect the rate of RNA

synthesis directly, since the rate of ^{3}H-uridine incorporation decreases during the first 40 min of treatment, even in the presence of 2 mM uridine (Fig. 7/6b). Part of this initial decrease in the rate of incorporation might be caused by a more rapid turnover of the 25-S ribosomal RNA (Table 7/1). This possibility is supported by the smaller initial decrease in the rate of rapidly labelled RNA synthesis (Fig. 7/6c) than in that of stable RNA synthesis (Fig. 7/6a,b).

In budding yeast, 2-PE has been shown to act at two levels: one solely at the cell membrane and the other at both the membrane and some internal site (Burns, 1968). In these cells, metabolite uptake shows a linear response to changes in inhibitor concentration while DNA and RNA synthesis show sigmoid responses. Since the 1- and 2-isomers of PE have similar chemical and physical properties (Weast et al., 1964) and appear to affect growth and DNA synthesis in S. pombe in the same way, the two isomers probably affect many biological systems in the same way. The similarity between the separate effects of 1-PE upon uridine uptake and incorporation demonstrated above and those of 2-PE upon glucose uptake and RNA synthesis in S. cerevisiae (Burns, 1968) seem to bear this out. In other cell types, 2-PE also appears to produce similar groupings of effects associated with membranes (Knutsen, 1966; Nunn & Tropp, 1972; Crifo et al., 1973) and with membranes plus internal non-membrane sites (Bruchovsky, Mak & Till, 1967; Plagemann, 1968,a,b). From his work with rat hepatoma cells, Plagemann (1968,a) suggested that the inhibitor acts on a regulatory site, since it induces treated log phase cells to behave like untreated cells entering stationary phase. In support of this suggestion, Wright, Ceri and Lewis (1973) demonstrated that 2-PE can induce changes in the morphology

of hamster cells similar to those caused by dibuteryl cyclic AMP, a known growth regulating agent.

By growing cultures overnight in the presence of 1-PE before labelling them, the cells should be equilibrated with their drug-containing environment. The effects of changes in the rate of uptake and possible changes in the specific activities of radioactive pools should no longer pose the problems they did in the experiments described above. When such cultures are selection synchronized, the rate of RNA synthesis doubles at the time of DNA synthesis with a mid-point at about 0.24 of a cycle (Fig. 7/7). This pattern, along with the similar patterns of DNA synthesis and the rate of RNA synthesis following 1-PE addition to log phase cultures, further supports the gene dosage control model described in previous Chapters. The rate of ^{3}H-uridine uptake also doubles sharply in mid-cycle, but does so 0.2 of a cell cycle after the rate of RNA synthesis doubles. This produces an oscillating pattern of increase and decrease in the rate of filling the cellular pool of ^{3}H-uridine (Fig. 7/7d). As described in Chapter 4, a decrease in the rate of pool filling could cause the pool to empty and when the pool emptied sufficiently, the rate of precursor uptake could be induced to increase. Further support for such a feedback control of ^{3}H-uridine uptake can be found in the pattern of the cellular rate of uridine uptake following the addition of 1-PE to log phase cultures (Fig. 7/6e). After an initial 1.5 hr decrease, this rate increases for about an hour and then becomes constant. During this same 1.5 hr, the rate of uridine uptake recovers and begins to increase. And when the rate of uptake becomes equilibrated at its new rate of exponential increase the rate of pool filling per cell becomes constant.

Therefore, after the initial adjustments to the presence of 1-PE in the culture, S. pombe appears to continue to control its rate of RNA synthesis and ^{3}H-uridine uptake by the same mechanisms that it uses in untreated cultures. Furthermore, the isomers 1- and 2-PE appear to have very similar effects on cultures of S. pombe, although the 2-isomer may produce slightly longer generation times than 1-PE.

CHAPTER VIII

STEP-DOWN

In the 1960's several workers reported that selective synthesis of "messenger" RNA could be induced by rapidly transferring exponentially growing cells from a rich medium to a minimal medium in which the cells had a much slower growth-rate. Such step-down procedures were developed for bacteria (Hayashi & Spiegelman, 1961) and for yeast (Gross and Mitchison, 1962; Kitazume, Ycas & Vincent, 1962). For a short period immediately after stepping a culture down, ribosomal RNA did not appear to be made. The RNA that was made by S. pombe during the first 15-30 min after step-down was shown to be unstable and to have base ratios closer to DNA than to r- or t-RNA (Gross & Mitchison, 1962). Because of these characteristics and the heterodisperse sedimentation properties this RNA had on sucrose gradients, it was considered to be messenger RNA (Mitchison & Gross, 1965). In an autoradiographic study (Mitchison et al., 1969), the rates of uptake and incorporation of a number of bases were examined in stepped-down cultures of S. pombe. Cells were related to their position in the cell cycle according to their length. Two important observations were made about the step-down RNA: about half the silver grains were found over the cytoplasm after a 10 min pulse, and the base ratios vary during the cell cycle. It was concluded that step-down RNA may be a mixture of different types of m- and Hd- RNAs.

Since stepping-down a culture appears to inhibit r-RNA synthesis, the step-down situation might provide a method of examining the rate of synthesis of heterogeneous nuclear and other types of heterodisperse RNA during the cell cycle with little ribosomal contamination. A series of experiments was therefore carried out to see what sort of RNA was labelled with ^{3}H-uridine following step-down.

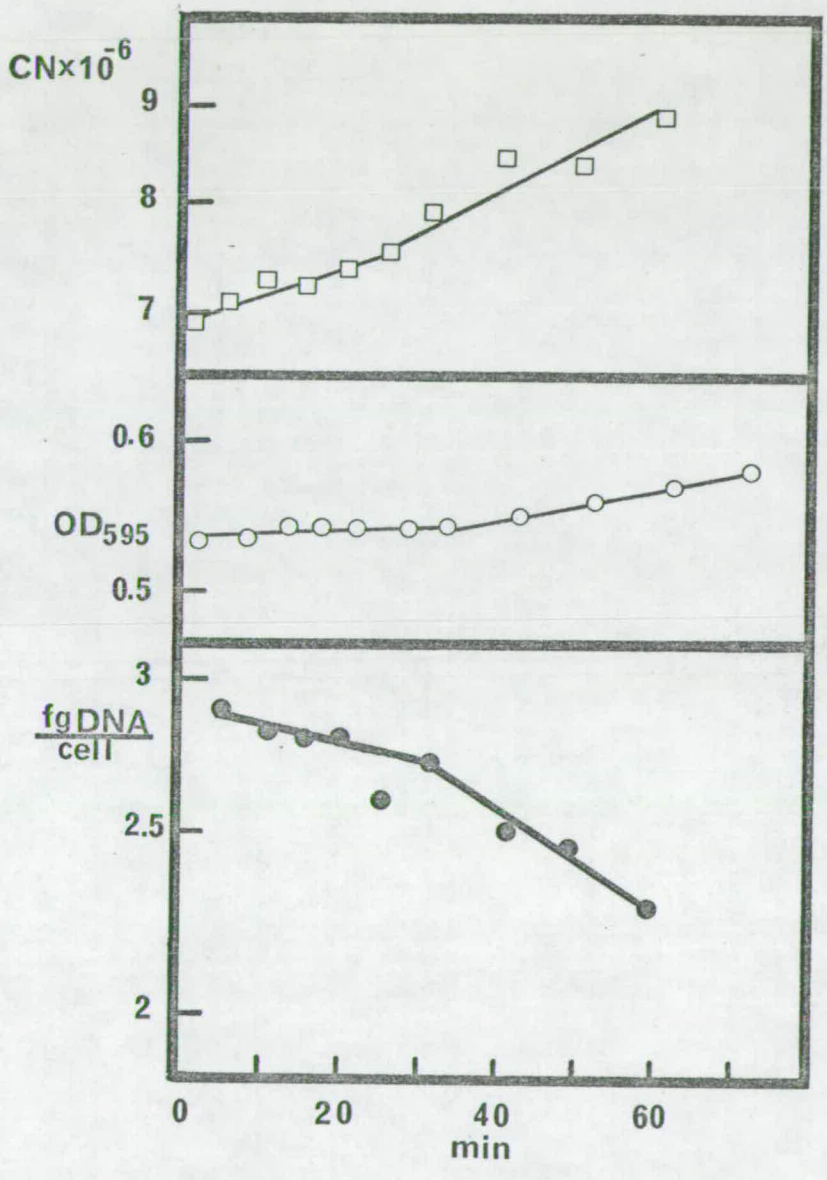
Figure 8/1.

The effects of step-down upon growth and the amount of DNA per cell. Cultures were stepped-down at 0 min.

(a) cell number x 10^{-6};

(b) cellular dry mass (OD_{595});

(c) fg DNA/ml.


CN×10⁻⁶
9
8
7
0.6
OD595
0.5
3
fg DNA / cell
2.5
2
0
20
40
60
min

EFFECT ON GROWTH AND DNA SYNTHESIS

An exponentially growing culture of S. pombe was rapidly transferred from 2% malt extract broth (MEB) to low phosphate minimal medium (EMM1) which contained 4.75% ethanol (Gross & Mitchison, 1962). As expected, the cell mass (OD_{595}) of the culture remained constant for the first 30 min after step-down; thereafter it increased with a doubling time of 14-16 hr (Fig. 8/1). During the first hour after step-down, cell numbers were found to increase at an appreciably greater rate than did cell mass. Microscopic observations also gave the general impression that cells got smaller following step-down. The DNA content per cell decreased during this same period and the rate of decrease was greater when, 30 min after step-down, cell mass began to increase. Although DNA was followed for only 60 min, it presumably continued to decrease to a level near the 1C value, because of a very long (presumed) G_1 being inserted into the cell cycle. Likewise, the size of the cells must reach a stable mean size some time after 60 min. Thereafter cell numbers and OD_{595} should increase in parallel.

KINETICS OF ^{3}H-URIDINE USE

The kinetics of ^{3}H-uridine uptake and incorporation after step-down (Fig. 8/2a) are very different from those in normal culture. Although linear for the first 10 min after step-down, the rate of ^{3}H-uridine uptake then decreases until it is nearly the same as the rate of incorporation. In untreated cultures, uptake remains linear and the incorporation increases until it is parallel to uptake (Fig. 8/2b). After step-down, the uridine pool fills within the first 15-20 min of labelling and the slow increase in the ^{3}H-uridine pool label after 20 min most likely reflects growth of the culture. These results are again in marked

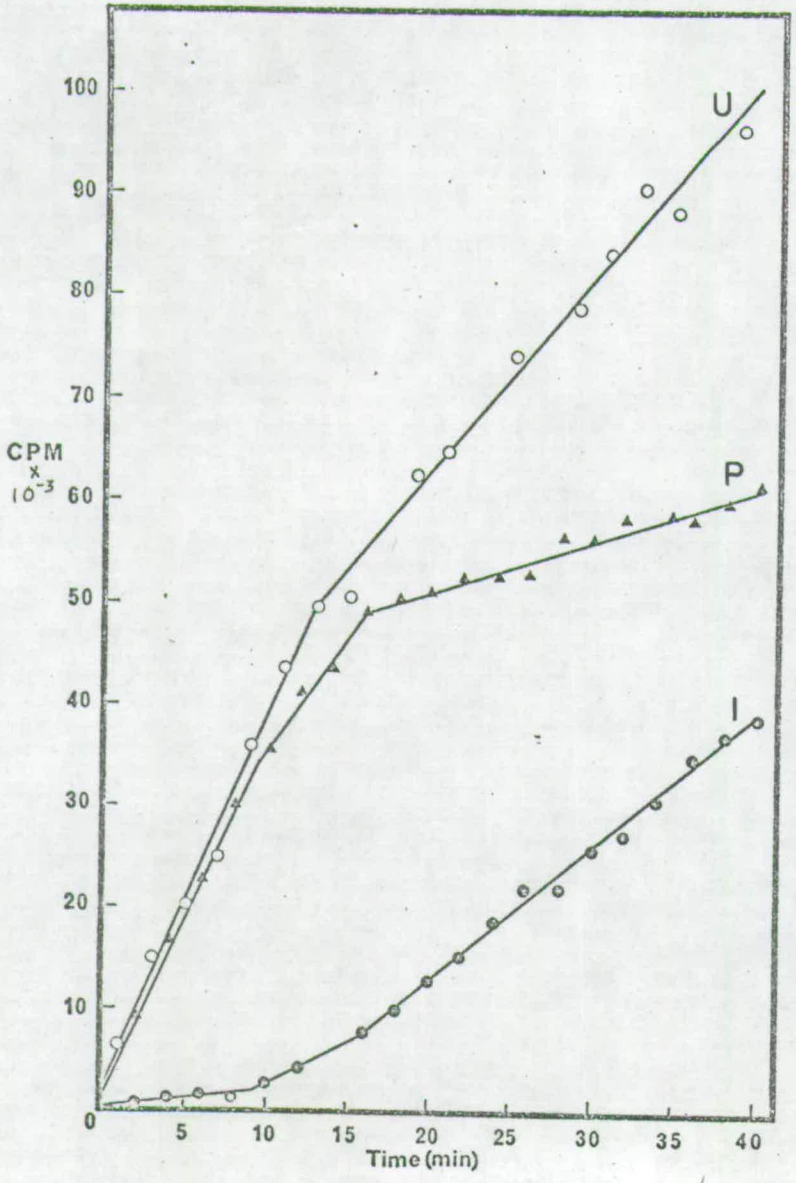
Figure 8/2.

The effects of step-down upon the kinetics of ^{3}H-uridine use in log phase cultures of S. pombe.

(a) Step-down culture labelled with 20 µCi/ml ^{3}H-uridine at a final concentration of 8.2 µM.

(b) control culture and the same data as presented in Fig. 3/1a.

(u) uptake; (i) incorporation; (p) soluble pool filling.


CPM
x
10^-3
U
P
I
Time (min)
0
10
20
30
40
50
60
70
80
90
100
5
10
15
20
25
30
35
40

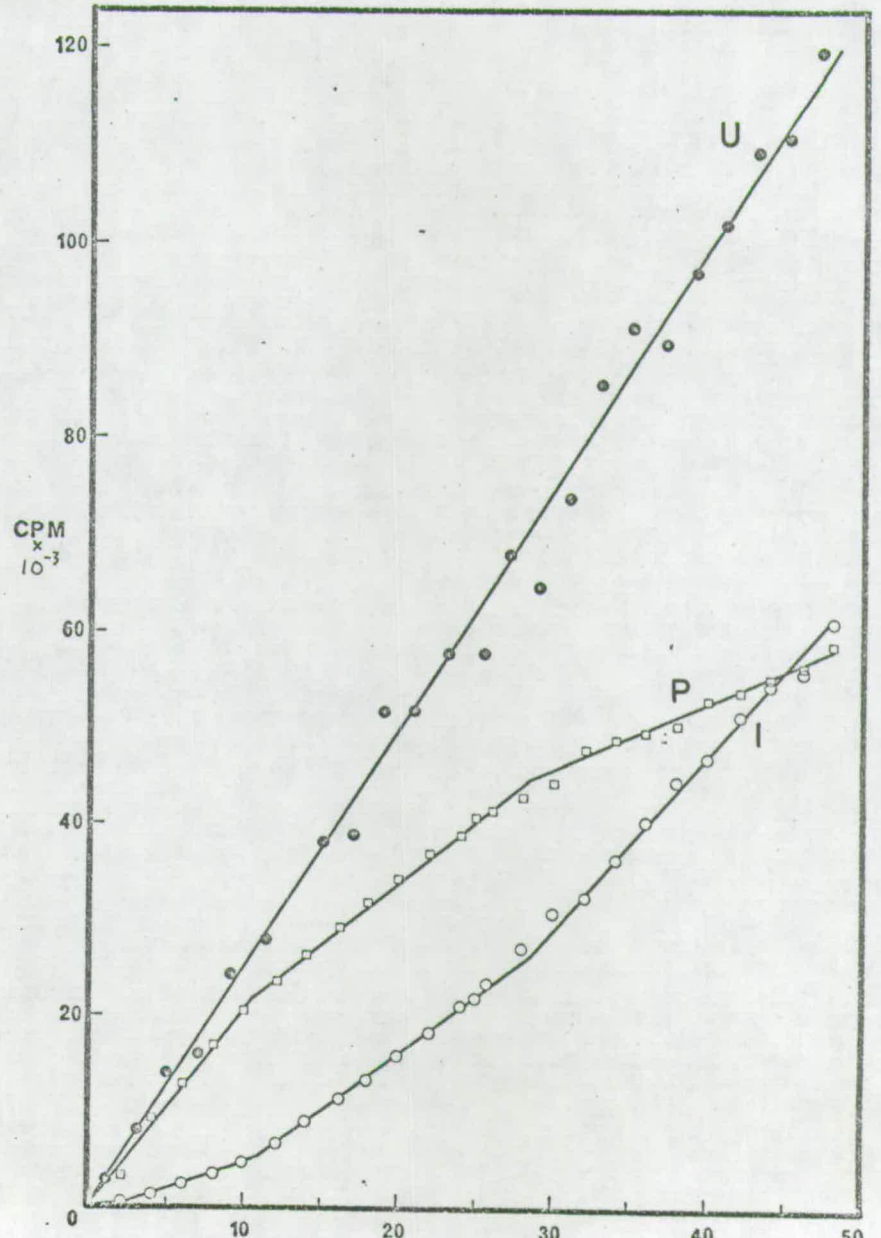

CPM
x
10^-3
U
P
I
0
20
40
60
80
100
120
10
20
30
40
50

Figure 8/3.

The effect of step-down upon the percentage of ^{3}H-uridine taken up by cells which was incorporated into TCA-insoluble material. Labelling same as for Fig. 8/2. (c) control; (sd) step-down.

contrast to those from the untreated cultures, in which the cells require at least 30 min to fill their pools. If uridine incorporation is rate limiting, a feedback control mechanism like the one proposed in Chapter 4 could well control the rate of uridine uptake during step-down. This assumption gains support from the low level of uridine incorporation after step-down (Fig. 8/2). Further support comes from a comparison of the percentage of the label taken up which is also incorporated (Fig. 8/3). Clearly, RNA synthesis during the first 10 min after step-down remains at a very low level compared to that in the control culture. Thereafter, the two curves are parallel. So, as reported earlier (Gross & Mitchison, 1962; Mitchison & Gross, 1965), RNA synthesis is inhibited during the plateau in OD_{595} following step-down. Furthermore, this reduced use of uridine probably allows the uridine pool to fill much faster than usual.

RNA LABELLING PATTERNS

Mitchison and Gross (1965) puzzled over sucrose gradients and base ratios which showed that the RNA made following step-down was neither r- nor t-RNA, but did little to explain what it was. Mitchison *et al.* (1969) suggested it could be a mixture of m-RNA and unstable, heterodisperse RNAs of various classes. A partial answer to this question was obtained by pulse labelling a culture with ^{3}H-uridine (20 µCi/ml) during the second 5 mins following step-down, and separating the RNA extracted from these cells by electrophoresis on 2.4% polyacrylamide gels. The only distinct peak of label in the step-down-RNA (SD-RNA) corresponds to the heavier of the two ribosomal precursor peaks seen in the control RNA (Fig. 8/4). This is presumably the 38-S precursor reported by Taber and Vincent (1969). Except for this peak, all of the

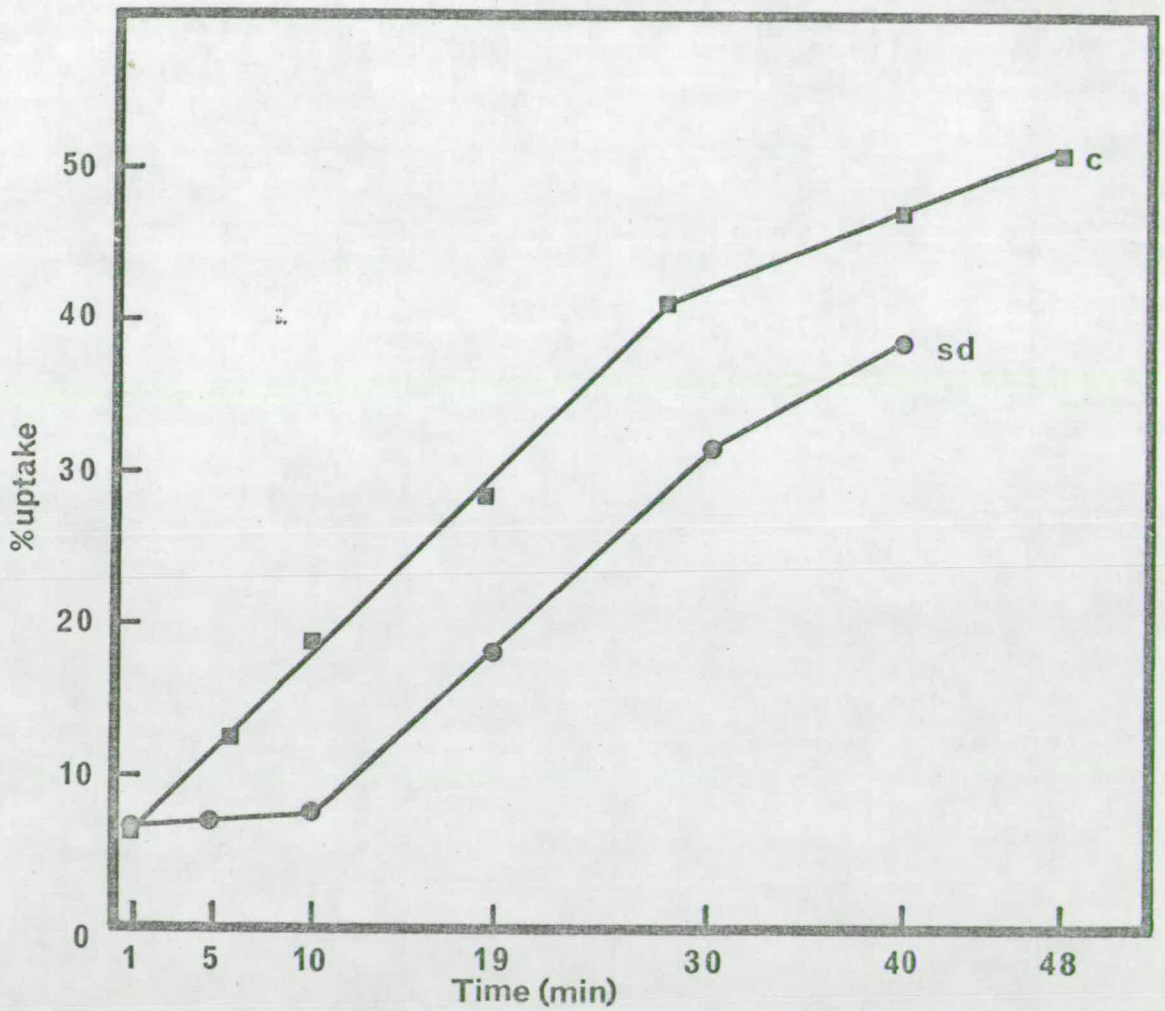
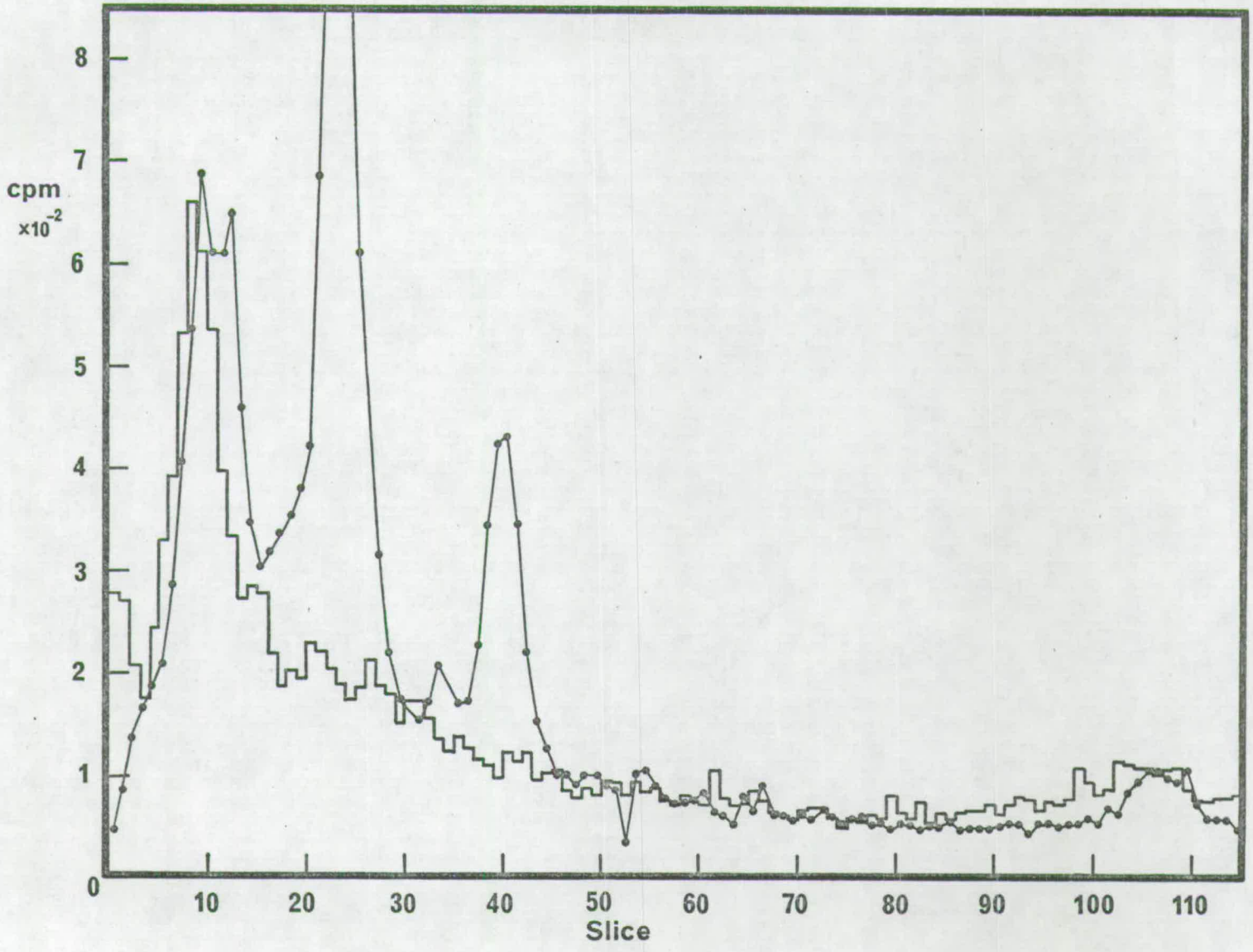

50
40
30
20
10
0
%uptake
c
sd
1
5
10
19
30
40
48
Time (min)

Figure 8/4.

The effect of step-down upon the distribution of ^{3}H-uridine in various fractions of RNA separated by polyacrylamide gel electrophoresis. Cultures labelled for 5 min at 8 µCi/ml. RNA fractionated on 2.4% gel run for 2.5 hr at 8V/cm. Step-down (histogram); control (-●-●-).


cpm
x10^-2
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
90
100
110
Slice

SD-RNA has about the same size distribution as the RNA below the peaks of the control sample. This suggests that during step-down, cells transcribe but do not process ribosomal gene products. As initial transcripts accumulate further synthesis of them may be inhibited by feed-back. Only at the very bottom of the gel is the situation uncertain. The control RNA contains a distinct peak of 4- and 5-S RNA, but the SD-RNA has only a broad region of slightly increased counts in the corresponding part of the gel. This could reflect either loss of definition because of a poor gel or a real heterodispersity of RNA, perhaps caused by inhibition of processing in this region as well as in the ribosomal region.

To put these observations on a more quantitative basis, the percentage of the total label found in various RNA fractions was calculated for both SD- and control-RNA (Table 8/1). The minor difference in the relative amounts of ribosomal precursor and the complete lack of mature ribosomal species in the SD-RNA confirm that ribosomal RNA processing, but not its transcription, is inhibited. The relative increases in all the SD-RNA fractions over the same fractions in the control RNA can be accounted for by the accumulation of 18- and 25-S r-RNA in the control. If the SD-RNA data are adjusted to account for 28.4% stable r-RNA, then there is a 3.5% loss in pre-r-RNA and the other species differ 1.5% or less from the control. These differences are well within the limits of experimental error.

SYNCHRONOUS CULTURES

The step-down was the only condition which, when examined in a selection synchronized culture, did not produce a discrete step-like doubling in rate of RNA synthesis at the time of DNA synthesis.

Table 8/1. Percentage Total Label in Various Gel Fractions

Slices	Content	Control	Step-down	Change (C-SD)
1 - 115	Total	100%	100%	-
5 - 17	Pre-r	15.9	17.3 (12.4)	1.4 (-3.5)
18- 32	25S	21.5	- (21.5)	-21.5 (0)
36- 46	18-S	6.9	- (6.9)	- 6.9 (0)
1 - 46	Hd- [1]	34.6	48.6 (34.8)	14.0 (0.2)
47- 101	sHd- [2]	16.2	26.1 (18.7)	9.9 (1.5)
102-112	4 & 5-S	4.1	6.2 (4.4)	2.1 (0.4)

1 Hd-RNA: refers to counts below the bases of the peaks and at least as large as the leading edge of the 18-S r-RNA peak in the control as described in Chapter 3.

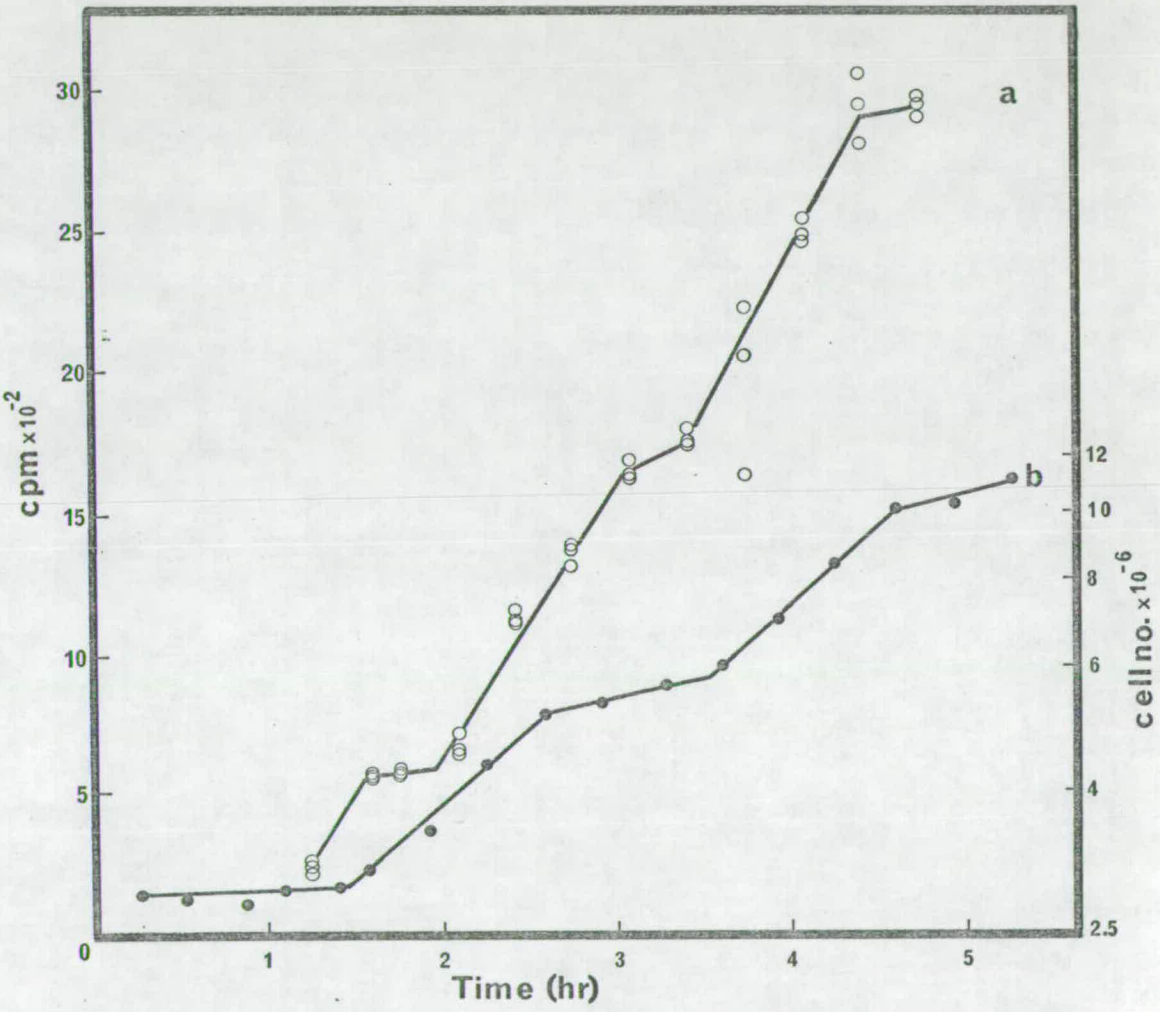
2 sHd-RNA: refers to counts intermediate in size between 18-S and Hd-RNA, on the one hand, and 5-S RNA on the other as described in Chapter 3. Numbers in () refer to a step-down culture corrected for the 28.4% stable r-RNA accumulated in the control culture.

Figure 8/5

The rate at which ^{3}H-uridine is incorporated by selection synchronized cells stepped-down at various times during the cell cycle. Cells were labelled for 5 min with 5 µCi/ml at 1.5 µM.

(a) rate of incorporation; as 10^{-2} x cpm/ml culture;

(b) cell number x 10^{-6}.


30
25
20
15
10
5
0
cpm x10^-2
a
b
12
10
8
6
4
2.5
cell no. x10^-6
1
2
3
4
5
Time (hr)

At various times during the cell cycle, samples from a slection synchronized culture in MEB were stepped down. During the second 5 min of step-down, these samples were pulsed with 5 µCi/ml ^{3}H-uridine. The rate of incorporation increases linearly, but short plateaux occur approximately once per cell cycle, (Fig. 8/5). These plateaux do not, however, occur at the same time in each successive cycle: 0.95 in the first cycle, 1.61 in the second cycle, and 2.23 in the third. Such irregular spacing corresponds to no known cell cycle event. Similar data has, however, been obtained for the uptake and incorporation of ^{14}C-adenine and ^{14}C-uracil by S. pombe following step-down (Mitchison, unpublished). Furthermore, RNA is synthesized at an extremely low level after step-down. A control culture of 3×10^{6} cells/ml incorporates 5×10^{3} cpm when pulsed for 5 min with 1 µCi/ml ^{3}H-uridine in the presence of 8.2 µM uridine. After step-down, on the other hand, a similar culture incorporates only 5×10^{2} cpm in 5 min when labelled with 10 µCi/ml in the presence of 0.03 µM uridine.

DISCUSSION

We now have some idea of the size distribution of the RNAs made, but very little idea about their function. Until the functions of a major proportion of these RNAs are known, the cell cycle data presented here will probably remain uninterpretable. From earlier work (Gross & Mitchison, 1962; Mitchison & Gross, 1965; Mitchison et al., 1969) we know SD-RNA is rapidly turned over, hybridizes with a large part of the genome, and has a base composition more akin to DNA than to r- or t-RNA. It also has no appreciable amounts of any single RNA species, apart from 38-S ribosomal precursor, and it is made in much smaller quantities

than RNA in control cultures. One point of interest is that, although cells stop processing RNA after step-down, there is no appreciable accumulation of Hn-RNA similar to that seen in mammalian cells. Taber and Vincent (1969) reported a similar accumulation of 38-S RNA in S. pombe when they inhibited the processing of ribosomal RNA with cycloheximide. They showed that this 38-S RNA is probably the primary transcription product of the ribosomal genes in this yeast.

It is much more difficult to assess the extent of inhibition of synthesis of non-ribosomal gene products than it is to do the same for the ribosomal RNA. The reduction in the rate of RNA synthesis by several orders of magnitude indicates that many genes other than just the ribosomal ones are no longer transcribed after step-down. Furthermore, when HeLa cells undergo a stepdown from growth medium to a balanced salt solution, they decrease their rates of precursor uptake into their amino acid and nucleotide pools and the rate of uridine phosphorylation. At the same time, they increase their RNA polymerase activity (Bramwell & Handmaker, 1975). Thus, labelling experiments with step-down cells do not appear to yield results which are directly comparable with those from control cultures.

Much more work needs to be done to properly characterize the types of RNA made after step-down. Even then, because of the massive perturbations the step-down procedure is likely to produce in cells, the results may have little importance to cell cycle events in normal cultures. This line of investigation was, therefore, abandonned in favour of the work presented in earlier Chapters.

CHAPTER IX

GENERAL DISCUSSION

In the previous Chapters I have presented data on the uptake and incorporation of ^{3}H-uridine by Schizosaccharomyces pombe in both log phase and in synchronous cultures. In each of these chapters I have discussed the relationships between the various results presented in that Chapter and tried to establish the pattern of the rate of RNA synthesis under the conditions peculiar to that set of experiments. In doing so, I have attempted to account for sources of error or possible misinterpretation and have tried to explain contradictions between these results and those published by others.

In this Chapter I, therefore, wish to correlate the data from all of the previous Chapters and to describe any general cell cycle patterns which emerge. In doing so I shall discuss possible mechanisms by which S. pombe may control its rates of RNA synthesis and precursor uptake.

CONTROL OF RNA SYNTHESIS

One of the common correlations that runs throughout the experiments presented above is that RNA synthesis occurs at a rate proportional to the DNA content of the culture. This holds regardless of whether the rates of synthesis are measured with 1 or 19 min pulses of ^{3}H-uridine or ^{3}H-adenine. In those instances in which the rate of precursor incorporation did not parallel the DNA content of the culture, the data suggests that, due to changes in the specific activity of the precursor pools, the rate of incorporation did not accurately reflect the rate of RNA synthesis.

The correlation between DNA content and the rate of RNA synthesis

also appears to hold for a wide variety of other cell types. In several species of Bacillus, the rates of both r-RNA and t-RNA increase in proportion to DNA content and these rate increases occur in both log phase cultures and in cultures of germinating spores (Donnellan et al. 1965; Dubnau et al., 1965). Bluegreen algae maintain constant ratios of ribosomal and transfer RNAs to DNA over a range of different growth rates (Mann & Carr, 1974). These reports from prokaryotes suggest that changes in the rates of DNA and RNA synthesis are linked. But they are all based upon the average ratios of RNA to DNA in log phase cultures. As such, they are indicative of a general RNA/DNA control. They do not, however, specify any control which regulates the rate of synthesis within one cell cycle. In moving from prokaryotes to protozoa, several early reports suggest that the rate of cytoplasmic RNA synthesis in Paramecium aurelia increases sharply at the beginning of macronuclear DNA synthesis (Woodward, Gelber & Swift, 1961; Kimball & Perdue, 1962). But the rate of synthesis in these cells appears to continue to increase exponentially until the end of the cell cycle so its exact relationship to DNA content remains unclear.

In sea urchin embryos, on the other hand, there seems to be a direct relationship between the amount of DNA and the rate of RNA synthesis as shown by the work of von Ledebur-Villiger (1972). She measured the rate of RNA synthesis in blastulae derived from parthenogenetically activated eggs and found it to be half the rate found in normal embryos from fertilized eggs. A variety of mammalian cell types also increase their rates of RNA synthesis in proportion to their DNA content. Zetterberg and Killander (1965) demonstrated that RNA accumulates continuously throughout the cell cycle of mouse fibroblasts. But the rate

of accumulation appears to double about the middle of the S period. Direct measurement of the rate of r-RNA synthesis in plasmacytoma cells also showed a rapid increase during the S period, with relatively constant rates in both G_1 and G_2 (Eikhom, Abraham & Dowben, 1975). In these cells as in many others which condense their chromosomes during mitosis, the rate of synthesis decreased markedly during cell division (See Mitchison, 1971, p. 116 for 16 further references). In prokaryotes and simple eukaryotes, such as S. pombe, the chromosomes do not appear to condense, and the rate of RNA synthesis does not decrease during division.

In HeLa cells not only does the rate of total RNA synthesis double at the time of DNA synthesis (Pfeiffer & Tolmach, 1968), but the rates of synthesis of a number of different RNA fractions do so as well (Pfeiffer, 1968). To explain his data Pfeiffer proposed that the rate of synthesis is regulated by the number of active cistrons available for replication. Under constant culture conditions the number of such genes should double during the S period. Furthermore, major changes in the growth rate would then simply involve changes in the number of genes which are active. Thus,the condensation of chromosomes during mitosis noted above would be considered to be a temporary deactivation of the entire genome.

Such a gene dosage control of the rate of transcription was first proposed for S. pombe based upon the observations of stable RNA synthesis presented in Chapter 4 (Wain & Staatz, 1973), and this suggestion has been supported by data in the later Chapters of this thesis. In selection synchronized cultures, the rates of both stable- and rapidly labelled RNA synthesis double within about 0.1 of a cell cycle after the

time of cell division (Figs. 4/1, 7/7). And as cells adapt to growth in l-PE, the rate of RNA synthesis starts to increase only when DNA synthesis begins to recover (Fig. 7/2, 7/6). In these experiments other culture parameters, such as rate of precursor uptake and number of cells/ml, do not consistently change their patterns in parallel with such changes in DNA content as does the rate of RNA synthesis. They therefore are less likely to be the basis for the control of the rate of RNA synthesis.

On the other hand, when log phase cultures are treated with AdR, the rate of RNA synthesis becomes constant anywhere from 20 to 120 min after DNA synthesis is inhibited (Figs. 6/1 & 6/2). The length and variability of this delay suggest that the rate of RNA synthesis may become constant because of a general decrease in the rate of growth, rather than as the result of a gene dosage control. But the inhibition of DNA synthesis by AdR can also take anywhere up to 120 min to be inhibited. And this inhibition is thought to involve alterations of the nucleoside triphosphate pools (Reichard et al., 1960). Alterations in the rate of RNA synthesis might also be affected by these pool changes, as suggested in Figure 6/1. For this reason, AdR may not be the best inhibitor to use in studying alterations in the rate of RNA synthesis in log phase cultures. A compound which reacts more directly might give a more clear cut answer.

Recently a study of the rates of synthesis of polyadenylated messenger RNA and ribosomal RNA during the cell cycle has been carried out in S. pombe by Fraser and Moreno (1976). They found that the rates of synthesis of both of these RNA species double during the S period. They then went on to show that when DNA synthesis is inhibited in log phase cultures by adding hydroxyurea, the rate of total RNA synthesis

becomes constant about 20 min later. They concluded that ribosomal and messenger RNAs are synthesized at rates proportional to the number of genes available for transcription.

The transcription of sea urchin chromatin *in vitro* occurs at a much higher rate on template material which is capable of DNA synthesis than on material in which DNA synthesis has been inhibited before isolation (Flickinger & Humphreys, 1974). This suggests that gene activation is equally as important as DNA content for controlling the rate of RNA synthesis. The importance of gene activation is also illustrated by the autoradiographic studies of nucleolar formation in *Drosophila* carried out by Krider and Plant (1972). They demonstrated that regardless of the number of nucleolar organizers present in a cell, only two were ever active at any one time. Furthermore, if a cell only possessed one organizing centre it still made about as much r-RNA as normal cells with two centres. Their conclusions mirror those of Pfeiffer (1968) above: r-RNA synthesis is controlled at two separate levels. An on-off control sets the general level of synthesis by activating the required genes, while a modulator adjusts the rate of synthesis at that level.

If such a two tier control regulates the rate of RNA synthesis, then the modulatory control appears to act by gene dosage in *S. pombe*. The gene activation control might then be affected by such things as nutrient supply, hormones, c-AMP and at least some inhibitors. The activities of such agents would then be expected to alter the amounts or types of gene product synthesized. But unless they completely inhibited synthesis, they should not alter the overall pattern of rate changes in relation to changes in the DNA content. Evidence for gene activation control of RNA synthesis in *S. pombe* comes from several sources. First, when spores are first suspended in nutrient medium, they begin to make

protein and RNA almost immediately, although DNA synthesis only starts after 2.5 hr of incubation (Padilla, Creanor & Fraser, 1974). And second, the synthesis of RNA in log phase cultures can be stimulated in glucose-starved cells by the addition of c-AMP to the medium (Foury & Goffeau, 1973, 1975). This stimulation appears to be independent of a similar, but greater, simultaneous stimulation of the rate of uridine uptake. But the type of RNA synthesized and the cell cycle patterns have yet to be examined, so it is not known whether the normal rate pattern is altered by c-AMP.

The general sensitivity of the on-off component of the control of RNA synthesis to compounds such as c-AMP and hormones which act at the cell membrane suggests that at least one component of this control is either located in or closely associated with this membrane. Certainly, the hydrophobic inhibitor, 1-PE, does not appear to alter the relationship between DNA content and the rate of RNA synthesis, although it clearly alters the time of DNA synthesis within the cell cycle (Fig. 7/7). Plagemann (1968,a) also suggested that 2-PE acts on a regulatory site in mammalian cells, since 2-PE treated log phase cultures behaved like untreated cultures entering stationary phase. In fission yeast, more work appears to be needed on the exact nature and location of this part of the overall control of RNA synthesis. But from what information there is available, it appears to be a part of larger control of the general level of metabolism.

CONTROL OF PRECURSOR UPTAKE

In the previous Chapters, the rate of precursor uptake by S. pombe has often, but not always paralleled the rate of its incorporation. Under the relatively constant conditions in log phase and in selection

synchronized cultures, the uptake increases in rate at the same time as, or shortly after the incorporation rate increases (Figs. 3/17, 4/4 & 4/5). Only when inhibitors were used to alter the size of the intracellular pool of precursors did this relationship break down. With the addition of AdR, the pool expanded (Figs. 5/1 & 6/1), while with 1-PE it contracted (Fig. 7/5). Thus, models for the control of precursor uptake rate must be able to account for both partial linkage to, and partial independence from the rate of incorporation.

In synchronous cultures of E. coli B/r the rate of potassium uptake doubles sharply once per cell cycle (Kubitschek, Freedman & Silver, 1971). The time of this rate doubling depends upon the degree to which the cells have been perturbed during synchronization. In relatively undisturbed cultures the rate doubles at the time of cell division. In more perturbed cultures they double in the second half of the cell cycle, but before division. Superficially, the rate of ^{3}H-uridine uptake by S. pombe behaves similarly. In selection synchronized cultures, uridine uptake doubles just after cell division (Fig. 4/4). But if selection synchronized cultures are perturbed either by the presence of 1-PE (Fig. 7/7) or by a pulse treatment of AdR (Fig. 5/1) the rate of uridine uptake doubles in the second half of the cell cycle. Kubitschek et al., (1971) advanced three possible explanations for their results:

(1) assuming that only active uptake sites are made, the number of uptake sites may double at a given point in the cell cycle;

(2) new uptake sites may be made continuously throughout the cell cycle but remain inactive until they are switched on at a given point in the cycle;

(3) active uptake sites may be synthesized throughout the cell cycle and their activity then regulated by a feed-back mechanism.

In all three of these models the trigger for the increase in the number of fully active sites must be dissociable from cell division. But only the third model specifies the nature of the trigger, and in doing so it requires an extra control system which must itself change abruptly during the cycle.

Changes in the rate of ^{3}H-uridine uptake during the cell cycle of S. pombe might also be explained in terms of one of these models. But each model has distinctive and testable features which should allow us to choose between them. For example, the third model states that the activities of the individual uptake sites vary continuously during the cell cycle. In Chinese hamster ovary (CHO) cells synchronized by isoleucine deprivation Hopwood, Dewey and Hejny (1975) demonstrated a maximum in the rate of thymidine uptake during the period of DNA synthesis. As the third model predicts, the rate increase that provides this maximum appears to be due to increases in both the number of functional uptake sites and the activity of individual sites. But this pattern is not universally observed. When Sander and Pardee (1972) examined the rates of uptake of thymidine, -aminobutyric acid (AIB) and uridine in CHO cells synchronized by mitotic wash off, they found that increases in the rates of uptake did not involve changes in the K_m of any of the transport reactions. Thus, in these latter cultures the activities of individual permease molecules apparently remain constant and the increases in V_{max} are produced by increasing the number of active molecules. In Novikoff rat hepatoma cells, increases in the rates of uptake of uridine, thymidine, hypoxanthine, choline and 2-deoxyglucose were also shown to occur without changes in the K_m's of the respective transport reactions (Plagemann et al., 1975). And similar results have been obtained from S. pombe by Foury and Goffeau (1975). When they examined c-AMP stimulated

increases in the rates of uptake of 6 amino acids, 3 nucleosides including uridine, AIB, sucrose and 2-deoxy-D-glucose, they found that the K_m of each uptake reaction remained unchanged from the control level. Their work effectively eliminates the third model of Kubitschek et al. (1971) as a likely method by which S. pombe controls its rates of active metabolite uptake.

In theory, it should be relatively simple to distinguish between the induction or derepression of existing uptake sites, as proposed in the second model, and the synthesis of fully active, new sites, as in the first model. All that is necessary is to correlate the amount of permease protein in cells at different stages of the cell cycle with the rates of uptake at the same stages of the cycle. In practice, the task may prove very difficult. The fraction of protein involved directly in permease activity in most cells is likely to be minute, and thus all but impossible to detect. However, in a variety of cells which make measurable quantities of single enzyme proteins, changes in enzyme activities are reflected by comparable changes in the amounts of enzyme proteins (Harland, Jackson & Yeoman, 1973; McCullough & John, 1972; Watson, 1970). Furthermore, in several types of mammalian cells, cycloheximide appears to rapidly inhibit increases in the rate of metabolite uptake (Sander & Pardee, 1972). And in suspension cultures of Novikoff rat hepatoma cells, Plagemann et al. (1975) demonstrated that maintenance of existing uptake activity is sensitive to inhibitors of either RNA or protein synthesis. They interpreted their results as an indication that transport sites in these cells are continually turning over. Thus, more by analogy than by direct evidence, it appears that increased rates of uptake may, indeed, reflect synthesis of permease molecules. But

supporting evidence for this being the case in actively metabolizing fission yeast has yet to be obtained.

As mentioned above, only the third possible model of Kubitschek et al. (1971) specifies a control element which would trigger the rapid increase in uptake activity. In discussing the step-like increases in the rates of uptake of several amino acids, Carter and Halvorson (1973,a) point out that increases in cell volume, cell mass and the total cellular content of RNA are unlikely to supply the basis for regulation of such periodic events since they all increase continuously throughout the cell cycle of budding yeast (Gorman, Tauro, Laberge & Halvorson, 1964; Mitchison, 1958; Scopes & Williamson, 1964). Carter and Halvorson (1973,a) suggest that the discontinuous increases in uptake activity are analogous to those of step enzymes, some 30 of which they previously described in budding yeast (Halvorson, Carter & Tauro, 1971). Differences in the timing between steps in the rates of uptake of different amino acids would then be attributed to differences in the time of replication of the various permease genes during the S period (Gorman et al. 1964).

A second possible method of controlling uptake rates could be through their energy supply. The energy for active transport reactions can be supplied directly from high energy compounds like ATP, as is the amino acid transport in frog oocytes (Merriam & Pollack, 1969). But it can also be supplied by harnessing the free energy of an electrochemical gradient of some other ion or molecule (Stein, 1967). The gradient of this second species would itself, however, have to be maintained by an energy producing reaction. Such energy coupling appears to supply the energy requirements for the transport of many amino acids

by the budding yeast, Saccharomyces carlsbergensis (Eddy & Nowacki, 1971). They found that in cells treated with antimycin and 2-deoxy-glucose to inhibit energy metabolism, the uptake of amino acids was coupled to the uptake of hydrogen ions and the concomitant loss of potassium ions. The stimulation, by cycloheximide, of uridine uptake in metabolically starved cultures of S. pombe and of S. cerevisiae (Foury & Goffeau, 1973) suggests that the energy for this transport reaction may also be derived only indirectly from ATP. And further experiments with c-AMP and the ATPase inhibitor, Dio-9, suggest that active uridine transport is coupled to proton transport (Foury & Goffeau, 1975). Edmunds and Cirillo (1974) suggest that fluctuations in such coupled electrochemical gradients could produce not only cell cycle fluctuations, but may be involved in the generation of ultraradian and circadian cycles as well.

By far the most commonly described form of control over metabolic processes found in the literature is a negative feedback loop. Such feedback controls have been proposed as parts of models for gene regulation in both prokaryotes (Jacob & Monod, 1961) and in eukaryotes (Britten & Cohen, 1969) and demonstrated for a number of enzyme activities.

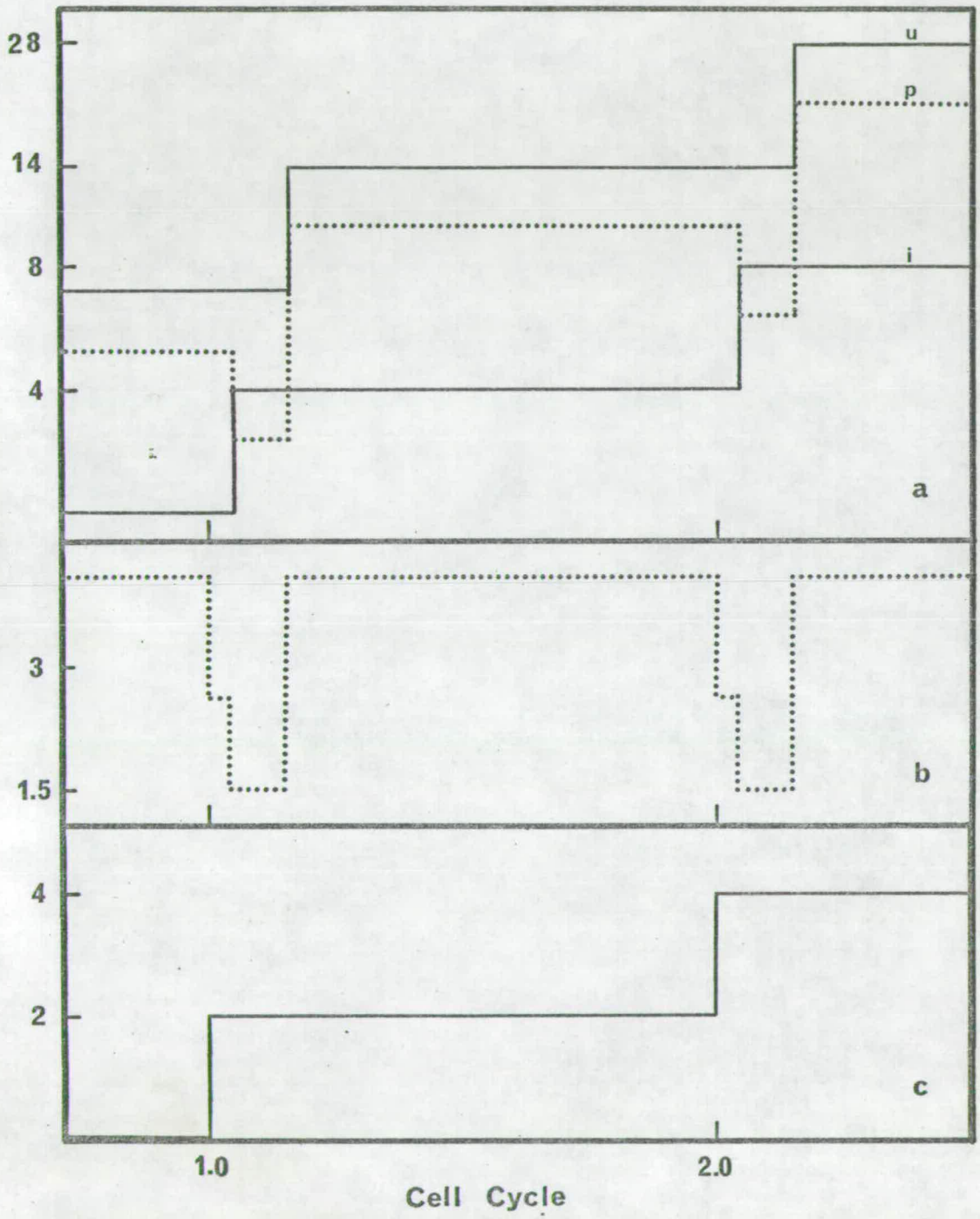
A well-known, analogous mechanical-hydrodynamic system has been in use for some considerable time (Harington, 1596). There is no a priori reason why permeases and kinases should not also be regulated by feedback. Indeed, feedback controls have been implicated in the regulation of the rates of valine uptake by sea urchin embryos (Mitchison & Cummins, 1966) and of several other amino acids by budding yeast (Carter & Halvorson, 1973,a; Crabeel & Grenson, 1970). The uptake and phosphorylation of thymidine by Tetrahymena (Stone et al., 1965) and by mouse L cells (Adams, 1969) have also been shown to be inhibited by feedback from the TTP pools at the end of DNA synthesis. In syn-

chronous cultures of Chlorella fusca the rate of uracil uptake closely follows its rate of use for either RNA synthesis or for energy (Knutsen, 1972), which suggests that a similar control may be in operation there. In a study involving a number of uracil and uridine uptake deficient mutants of S. cerevisiae, Grenson (1969) demonstrated that when the use of either metabolite was decreased, the rate of uptake was diminished by feedback inhibition of internal pyrimidines on the appropriate permease. Cummins and Mitchison (1967) demonstrated that adenine uptake by this organism was at least partially regulated by the accumulation of the precursor and its metabolites in the soluble pool. This feedback control is reflected in the timing of the step-like doubling in the rate of ^{3}H-adenine uptake in selection synchronized cultures (Fig. 4/5). The mid-point of the increase in the rate of adenine uptake occurs about 0.1 of a cell cycle after that of adenine incorporation. The rate of ^{3}H-uridine uptake also doubles sharply 0.1 of a cell cycle after its corresponding rate of incorporation doubles (Fig. 4/4). This similarity suggests that both of these uptake rates may be under similar types of control. The rates of ^{3}H-uridine uptake and incorporation in selection synchronized cultures of PE grown cells also double sharply at a point in the cell cycle. In these cultures, however, the difference between the mid-points of the rate doublings is 0.2 cell cycle. In both PE-treated and untreated selection synchronized cultures the rate of ^{3}H-uridine pool filling per cell increases and decreases in a cyclic manner (Figs. 7/7 & 4/4). The minimum rate of filling occurs between the time the rate of incorporation starts to double and when the uptake rate begins to increase.

By taking the above rate changes into account a feedback model for

Figure 9/1.

Feed-back control model for the control of the rate of uridine uptake (a) rates/ml culture; (u) uptake; (P) Pool filling, (i) incorporation; (b) rate of soluble pool filling/ cell; (c) cell numbers/ml.


28
14
8
4
u
p
i
a
3
1.5
b
4
2
c
1.0
2.0
Cell Cycle

the control of uridine uptake can be constructed (Fig. 9/1) which is analogous to that proposed for the control of adenine uptake in S. pombe (Cummins & Mitchison, 1967). The timing of the various events in this model are the same as that in selection synchronized cultures of non-treated cells (Figs. 4/4 & 4/10). The rates of RNA synthesis and uridine uptake, therefore, double at 1.04 and 1.15 respectively, while cells divide at 1.0 and 2.0 cell cycle. As in the adenine model mentioned earlier, the link between changes in the rates of uridine uptake and incorporation appears to be at least one component of the soluble uridine pool. When the rate of incorporation increases, the cellular rate of pool filling decreases and the amount of uridine in the pool decreases. At a critical level the reduced content of the pool triggers a compensatory increase in the rate of uridine utpake. The model predicts that the rate of pool filling per cell should remain constant except from the time the rate of incorporation begins to double in rate until uptake has completely doubled in rate. During this interval the cellular rate of pool filling should decrease in two steps of 0.4 and 0.6 cell cycle respectively. In practice (Fig. 4/4d) this rate does decrease at the same time as the rate of incorporation begins to increase, but scatter in the experimental data is too great to establish the details of the pattern of decrease. The cellular rate of uridine pool filling does decrease in two steps in selection synchronized cultures of PE-grown cells (Fig. 7/6). The slow decreases and increases in the cellular rates of pool filling are most likely caused by the asynchrony of the cultures. But they may represent a part of the real control system not accounted for by the model system.

Data obtained from log phase cultures to which PE was added supports

this feedback model. When the drug is added to the culture it causes a very marked decrease in the rate of uridine uptake for the first 1-1.5 hr of treatment. The cellular rate of pool filling and the rate of incorporation also decrease initially although the rate of incorporation becomes constant after about 40 min of treatment. The rate of pool filling per cell and presumably the ^{3}H-uridine content of the pool reach their minima about 40 min after the rate of incorporation becomes constant. The rate of ^{3}H-uridine uptake then begins to increase more or less exponentially. The cellular rate of pool filling, on the other hand, increases for about an hour and then remains constant for the remainder of the experiment.

The results from the AdR treated cultures also generally agree with the model. The rate of ^{3}H-uridine incorporation increases after the inhibitor has been removed. This causes a decrease in the cellular rate of uridine pool filling, which in turn stimulates the rate of uridine uptake to increase. The AdR results do, however, differ from the predicted response in several ways. During the third hour of treatment, the cellular rate of uridine pool filling increases and during the synchronous cell divisions after AdR removal this rate decreases again. As discussed in Chapter 6, these changes appear to be produced by the AdR treatment but they imply that either the size of the uridine pool or its steady state rate of filling can be regulated independently of the feedback control. But if either this feedback control or any other control regulates the rate of uridine uptake in S. pombe, the system must still allow for some regulation from other sources. For example, c-AMP has a demonstrated stimulatory effect on the rate of uridine uptake in both fission yeast and in budding yeast (Foury & Goffeau, 1973). The rate of

uridine uptake in S. pombe is also stimulated to increase by the presence of AdR in the culture medium (Figs. 5/1 & 6/1). This latter increase continues after the rate of uridine incorporation has become relatively constant. Similar increases in the rates of RNA precursor uptake can be induced in a variety of cell types (e.g. Hutchison & Porter, 1972) but in many instances they may be caused by a concomitant stimulation of RNA synthesis (e.g. Luck & Hamilton, 1972). In AdR treated cultures of S. pombe, after the first cell division following the removal of the inhibitor, the cells also decrease their rates of uridine uptake at a time when the rate of uridine use is increasing exponentially (Figs. 5/1 & 6/1). Membrane affective agents such as phenylethanol in S. pombe and many other organisms (See Chapter 7) and certain colicins in bacteria (Luria, 1975) can also produce marked decreases in the rates of uptake of many compounds. Decreases in the overall rate of cell growth as caused by step-down (Fig. 8/2), also see: Bramwell & Handmaker, 1975) and other forms of nutrient depletion (e.g. Lien & Knutsen, 1973) are also known to decrease the rates of metabolite uptake. Thus, by analogy with the two-level model of the control of RNA synthesis (Krider & Plaut, 1972; Pfeiffer, 1968) cells probably have one mechanism which sets the overall level of uptake. A second one then modulates the pattern of uptake at that level. If such a bi- or multi-level control does indeed regulate the rate of uridine uptake in S. pombe, then I have only been examining the modulating control level. The existence and nature of any higher level controls might be the topic of further investigations.

EFFECTS OF GRADIENT SELECTION

Up to this point I have been discussing situations in which the rates of ^{3}H-uridine uptake and incorporation have been relatively unperturbed. I would now like to consider instances in which either one or both of these rates appears to be disturbed.

There has been a growing body of evidence from many laboratories that the different methods of synchronization produce different cell cycle patterns in the same or similar types of cells. For example, if CHO cells are treated with hydroxyurea they are prevented from initiation of DNA synthesis, but if they are treated with a high concentration of thymidine many cells make a portion of their DNA before being arrested (Meyn et al., 1973). And in the ciliate, Tetrahymena, temperature shocks, hypoxia and deprivation of nutrients all cause rapid and reversible inhibition of the initiation of protein synthesis (Klemperer & Rose, 1974). The selection procedure of Wolfe (1973,a,b), on the other hand, does not appear to cause such changes.

In work on S. pombe, the gradient selection procedure of Mitchison and Vincent (1965) has been used extensively to provide synchronous cultures. In this method cells are subjected to a temperature shift from 32° to about 22-25°, accompanied by manifold concentration. These are followed by the dual stresses of centrifugation and increasing osmotic pressure as they move down the sucrose gradient. And finally, they are suddenly returned to 32° upon dilution in fresh medium. It might be expected that such treatment would perturb cellular metabolism in some way, since at least in a more severe form each of these treatments can do so individually. In his thesis, Polanshek (1973) was able to synchronize log phase cultures of S. pombe by shifting them from 32°

to 41° for 15-30 min and then returning them to 32°. But perturbations of cell growth have also been shown to occur when cells experience temperature shifts much closer to the range of those experienced during the gradient synchronizing procedure. The original heat shock technique for synchronizing Tetrahymena involved a series of shifts between 28° or 29.5° and 33° (Scherbaum & Zeuthen, 1954). And recently Nurse (1975) demonstrated that if strain 972^{h-} of S. pombe is shifted from growth at 25° to 35°, the length of cells undergoing division increases significantly. The increase in cell size involved parallel increases in the RNA and protein content of the cells. In several papers Harvey (1932, 1936) demonstrated the ability of centrifugation to differentially sediment subcellular fractions within the cytoplasm of sea urchin eggs. Upon prolonged or more vigorous centrifugation parts of the cytoplasm can actually be pulled from the main body of the cell. But at the much lower levels of force used in the gradient selection method the actual centrifugation appears to have very little effect (Mitchison, pers. comm.). The effects of concentration and resuspension can be considered to be at least partially linked, since upon concentration cells may find themselves running out of a particular nutrient which is resupplied to them upon resuspension. Nutrient starvation followed by refeeding has been used to synchronize a variety of cell types (Tobey & Chrissman, 1972; Rudnick & Cameron, 1972). A single burst of synchronous cell division can also be produced by reinoculating late log phase S. pombe (Mitchison & Creanor, 1971,a). To produce such a synchronous division, however, how close cells are to stationary phase appears to be critical. But some disturbance of metabolism may still occur after even short periods of metabolite limitation.

Finally, Duffus & Mitchell (1970) demonstrated the disturbing effects that high osmotic pressure can have on S. pombe. They found that cells grown in 1 M glucose or sorbitol had longer generation times than cells in ordinary EMM2. The increase appeared to be caused by an extension of the G_1 phase with little alteration to the other phases of the cycle. After centrifugation, cells undergoing gradient selection are removed from the middle region of the sucrose gradient where they were suspended in 0.7-0.9 M sucrose.

Whether or not any one of the above possible perturbations of cellular metabolism occurs, there is a visible gradient centrifugation effect on nucleic acid metabolism. If, after cells are separated on a gradient, the gradient is thoroughly mixed before cells are reinoculated, the rates of both uptake and incorporation of ^{3}H-adenine are depressed for about 30 min after reinoculation (Fraser & Moreno, 1976). A similar effect can also be seen in the rates of uptake and incorporation of ^{3}H-uridine in the first cell cycle of synchronous cultures (Figs. 4/1 & 4/4). In each of these cases the rate increases more rapidly before the first cell division than between the first and the second divisions. The effect is even more marked in the rate of ^{3}H-adenine incorporation in synchronous cultures (Fig. 4/1). This rate appears to increase almost exponentially until the end of the first synchronous division. The rates of uptake and incorporation, thus, appear to increase their synchrony as they pass from the first to the second cell cycle after synchronization. During the same time, however, the cell number curve and the CPI are losing their synchrony. The adenine incorporation pattern for the first cell cycle in Fig. 4/1 is very similar to that for rates of ^{14}C-incorporation in synchronous cultures given by Mitchison et al. (1969).

These authors used the pattern as support for conclusions drawn from autoradiographic evidence that the rate of RNA synthesis increased exponentially during the cell cycle. If their sampling had been continued during the second cell cycle, however, they most likely would have found a different pattern.

Perhaps the clearest evidence that the selection technique perturbs uridine metabolism comes from its use in conjunction with AdR. In a selection synchronized culture treated with AdR, the cellular rate of ^{3}H-uridine pool filling increases for the first 80 min of treatment and then becomes constant (Fig. 5/1). In log phase cultures, the addition of AdR does not markedly alter the rate from the untreated control value (Fig. 6/1). Later changes in the rate of pool filling per cell are very similar in both types of AdR treated cultures. The selection procedure thus appears to produce an expansion of the uridine pool during the first 80 min following resuspension.

A third possible set of side-effects of the gradient selection procedure can be seen in synchronized cultures of cells grown in PE. The rates of ^{3}H-uridine uptake and incorporation in these cultures decreases sharply just before the first synchronous division (Fig. 7/6). About the same time in the samples taken to count cell plates there is an increase in the number of cells which have a much higher refractive index than normal cells. Thus, in dark field illumination they appear very bright compared to the rest of the culture. Such highly refractive cells are thought to be dead (Creanor, pers. comm.). The percentage of such "dead" cells rose to 4.5% just before the first division, then decreased slowly to 2% 3 hr later. From the coincidence of timing the decreases in the rates appear to be caused by a decrease in the number

of metabolizing cells. Certainly, under the microscope PE treated cells do not look healthy. They have bulbous ends and many of them are either bent or branched. It is quite possible that the side-effects of the synchronizing procedure could be fatal to such "unhappy" cells.

From the above considerations it appears likely that the gradient selection technique of Mitchison and Vincent (1965) disturbs the normal patterns of RNA precursor uptake and incorporation in S. pombe. This disturbance takes the form of a temporary depression of the rates of uptake and incorporation following resuspension from the gradient. The method also seems to decrease the viability of cells already stressed by being maintained in l-PE-containing medium. And if, upon resuspension, cells are treated with AdR, the combined action of this compound and the synchronizing technique appear to cause an expansion of the cellular uridine pool. Because these alterations are produced in one small part of total cellular metabolism, disturbances in other parts may also be expected. Therefore, where possible in the work reported above, patterns of change were followed for at least two cell cycles after selection synchronization. In the second cycle temporary perturbations, such as those in adenine uptake and incorporation (Fraser & Moreno, 1975), should no longer affect the observed patterns. However, long range perturbations cannot be ruled out.

CELL DIVISION AND CHANGES IN THE PHYSIOLOGICAL STATE

When S. pombe is recovering from an imposed physiological stress, cell division often appears to play an important role in the recovery process. For example, cells do not appear to recover from the gradient selection procedure until the onset of the first synchronous division.

This is true for the rates of RNA synthesis and precursor uptake in both AdR treated and in untreated synchronous cultures (Figs. 4/4, 4/5 & 5/1). Furthermore, cultures recovering from AdR treatment are suddenly "locked in" to an apparently normal relationship between the timing of DNA synthesis and cell division only after the first induced round of synchronous cell division (Figs. 5/1 & 6/1). Similarly, if a culture of PE-grown cells is selection synchronized and then resuspended in fresh minimal medium without l-PE, the first synchronous cell division occurs after 3.5 hr, but the second after only 2.5 hr (Staatz, unpubl.). Thus, although culture conditions permit a 2 to 2.5 hr generation time, the cells apparently need to divide before they can adjust their growth rates. A cell size control such as proposed by Nurse (1975) seems unlikely to explain these changes in growth rates since l-PE does not appear to affect cell size. But since the size estimates were made subjectively while counting cell plates, a change in size at division cannot be ruled out. However, Bostock (1968) reports that 2-PE also does not seem to alter the size at which S. pombe divides. The cells which exhibited this prolonged generation time were the small cells of a log phase population and must have entered G_1 in the presence of l-PE just before being selected off the gradient. These results suggest that either at cell division or shortly thereafter, early in G_1, S. pombe may set the level of its overall rate of growth for the coming cell cycle. If circumstances change during that cycle it would modulate its growth and metabolism about the set level. But it would have to wait until the next cell division to alter the basic metabolic level. As such, the control would be analogous to the two level control of nucleolar transcription advanced by Krider and Plaut (1972). Many types of mammalian cells also appear to collect or be

delayed in G_1 when subjected to stress (Froelich & Rachmeler, 1974; Gautschi, Schindler & Hürni, 1971; Tobey & Chrissman, 1972). Terminal differentiation of mammary epithelial cells also appears to occur only when several hormones act together on cells in mid-G_1 (Vonderhaar & Topper, 1974). It also appears that upon depleting the medium of any one of a wide range of nutrients, budding yeast enter stationary phase in early G_1 (Pringle, pers. comm.). Furthermore, the α mating factor in this yeast also arrests cells of the a mating type at the same point in the cell cycle (Bücking-Throm, Duntze, Hartwell & Manney, 1973). From these results and those from several cell cycle mutants, Hartwell, Culotti, Pringle and Reid (1974) have proposed that early in the cycle there is a point at which the cell must commit itself to a further division cycle or to differentiate. They call this commitment point "start". Once the cell has passed through "start" it is unable to alter its developmental commitment until it divides. The above results from S. pombe support the possibility that the fission yeast cell cycle also contains such a "start" region. Further support comes from studies with the inhibitors of DNA synthesis, mitomycin C and hydroxyurea. When DNA synthesis and cell division are inhibited in synchronous cultures, step like increases in enzyme activity continue to occur as they do in the untreated control cultures (Robinson, 1972). Thus, once the cells go through "start" their metabolic patterns may remain set until altered. Bostock (1968) examined the effect of cells entering stationary phase due to phosphate depletion. He found that upon exhausting the phosphate supply in the medium, EMM1, S. pombe entered stationary phase in G_1, as would be consistent with the "start" hypothesis. But, if cultures were allowed to enter stationary phase

in the phosphate rich medium, EMM2, they remained in G_2. Thus, the G_1 block does not seem to be as absolute in fission yeast as it appears to be in budding yeast. It would be interesting to examine the effects on S. pombe of limitation of other major metabolites, such as nitrogen and carbon. Along this line, preliminary experiments suggest that in sulphate limited growth S. pombe enters stationary phase as G_1 cells (Wain, pers. comm.). Since cells store both phosphate and sulphate there may be a correlation between the presence of metabolite storage pools and the ability to stop in G_1.

From the above considerations, I have concluded that during undisturbed growth the rate of RNA synthesis in S. pombe appears to be under gene dosage control and that the rate of uridine uptake is controlled by feedback from the internal uridine pool. However, much of the evidence was obtained when steady growth was disturbed by either AdR or 1-PE, which drugs were also found to have other effects. Therefore the evidence cannot be conclusive. The same control systems may continue to operate but the data presented in this thesis do not allow us to determine whether or not they do so. As cells adapt to these inhibitors, sets of genes may be switched off or on independently of changes in gene number.

One method of examining the problem would be to compare the specific activities of various types of RNA labelled under a given set of conditions. Differences in such specific activities would indicate gene dosage-indpendent regulation. Changes in the size and specific activity of the ^{3}H-uridine pool could also be directly measured and correlated with changes in rate of incorporation, especially during treatment with inhibitors. Another method would be a genetic approach, using mutants with lesions in the regulation of RNA synthesis may likewise provide information about how often cells rely solely upon gene dosage regulation of the rate of RNA synthesis.

SUMMARY

The uptake and incorporation of ^{3}H-uridine by the fission yeast, Schizosaccharomyces pombe has been examined under a variety of conditions in both log phase and in synchronous cultures.

The active uptake of uridine appears to occur by a single step reaction with a K_m of about 3.15×10^{-5} M at moderate precursor concentrations. If the concentration of uridine in the medium exceeds approximately 2 mM, the kinetics of uptake change markedly and uridine entry into the cell seems to be chiefly by diffusion. The intracellular uridine pool is not expandable, and the rate of uptake does not appear to limit the rate at which ^{3}H-uridine is incorporated into RNA. In synchronous cultures, the rate of uridine uptake parallels the rate of incorporation, but changes in the uptake rate occur approximately 0.1 of a cell cycle after those of the incorporation rate. The specific activity of the ^{3}H-uridine pool in these cultures remains relatively constant. Inhibitors which alter the relationship between the rates of uptake and RNA synthesis have been shown to alter the size of the uridine pool.

The incorporation of ^{3}H-uridine into the acid-precipitable fraction of the cells has been used to measure RNA synthesis in both synchronous and log phase cultures. The relationship between pulse length and the distribution of ^{3}H-uridine label in the various species of RNA was examined by gel electrophoresis. After 1 min of labelling all the radioactivity is in the ribosomal precursor-, heterodisperse- and 4-5 S RNA fractions, whereas following pulses of 10 min or longer the label is distributed in essentially the same pattern as during steady state labelling.

In synchronous cultures the rate of RNA synthesis doubles at the time of DNA synthesis. When cultures are treated with the inhibitors of DNA synthesis, 2'-deoxyadenosine and 1-phenylethanol, they go through a period of adjustment during which the rate of RNA synthesis no longer appears to proceed at a rate directly proportional to the DNA content of the cell. Once the cells have adjusted to the inhibitors, however, the relationship between DNA and RNA synthesis is re-established.

A step down in culture conditions was used in an attempt to identify a nonribosomal species of RNA which might be preferentially labelled. Under such conditions the various species of RNA appear to be transcribed in approximately the same proportions as in control cultures. However, the total rate of synthesis is depressed and the processing of ribosomal precursor RNA is inhibited.

The results are discussed and mechanisms by which the cells may control their rates of RNA synthesis and uridine uptake are considered. Some possible perturbing effects the gradient selection procedure might have upon the cell cycle, and the role of cell division or a G_o in the recovery from such perturbations, are contemplated.

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APPENDIX I

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RATES OF SYNTHESIS OF RIBOSOMAL PROTEIN AND TOTAL RIBONUCLEIC ACID THROUGH THE CELL CYCLE OF THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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SUMMARY

The rates of synthesis of ribosomal proteins through the cell cycle of the fission yeast *Schizosaccharomyces pombe* have been examined by spec. act. estimations of isolated 80S ribosomes pulse-labelled with ^{35}S-sulphate. The spec. act. have minimum values at the beginning (0.0) and maxima between 0.6 and 0.9 of the cell cycle. This pattern in spec. act. is also shown by isolated 80S ribosomes pulse-labelled with ^{3}H-uridine during synchronous cultures and is in marked contrast to the small, random variations in the spec. act. of isolated 80S ribosomes from control, asynchronous cultures pulse-labelled with ^{35}S-sulphate or ^{3}H-uridine.

A detailed examination of the rates of synthesis of total RNA through the cell cycle measured by the rates of incorporation of ^{3}H-uridine and ^{3}H-adenine shows a step in the rates of incorporation at the time of DNA synthesis. This step has further been shown to be independent both of the uridine concentration, over a range from 0.03 μM to 820 μM, and of pre-filling the adenine pool. This step thus appears to be independent of variations in rates of uptake of both purines and pyrimidines, or fluctuations in the pool size of the precursors and may be explained as a gene-dosage effect.

The step in the pattern of synthesis of total RNA has been shown to yield a cyclic pattern in the spec. act. of the total RNA through the cell cycle. This pattern is similar to that of the spec. act. of RNA and of protein recovered from ribosomes. The variation exhibited by the ribosomal proteins is believed to be a consequence of the step in the pattern of RNA synthesis, with a concomitant fluctuation in the pool of ribosomal proteins synthesised continuously through the cell cycle.

In the fission yeast, *Schizosaccharomyces pombe*, both total protein and also individual groups of soluble proteins similar in charge and molecular weight are synthesized continuously during the cell cycle [22]. As in other cells, however, a number of enzymes in *S. pombe* show discontinuous rises in their activities [19], possibly due to discontinuous synthesis of the enzymes involved.

In contrast to the small amounts of individual enzymes and to the non-specific association between the different soluble proteins, ribosomal proteins constitute a significant proportion of the total protein in *S. pombe* and have a specific association in the ribosomes. There are about 70 ribosomal proteins in *Saccharomyces cerevisiae* [24] and it can be assumed that there is a similar number in *S. pombe*. Warner [24] has shown that the 50 proteins on the large ribosomal subunit may be separated by one-dimensional gel electrophoresis to permit the detection of at least 2 single proteins as individual bands.

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However, in view of the complexity of the rest of the electrophoretic pattern and of the number of proteins involved, it was decided to examine the synthesis of ribosomal proteins in toto during the cell cycle of *S. pombe*. This was carried out by pulse-labelling synchronous cultures of *S. pombe* with radioactive sulphate and using a spec. act. measurement (cpm/μg RNA) as an estimate of the rate of synthesis of the proteins recovered with 80S ribosomes.

The close relationship between the synthesis of ribosomal RNA and of ribosomal protein [1, 25] suggests that ribosomal protein might be synthesised in a pattern similar to that of ribosomal RNA. Mitchison et al. [11] have reported that the synthesis of total RNA during the cell cycle of *S. pombe* is continuous and approximately exponential. A similar pattern (apart from mitosis) has been demonstrated in a number of eukaryotes [4, 16]. In other eukaryotes, however, although RNA synthesis is continuous, a sharp step in the rate of total RNA synthesis occurs at the time of DNA synthesis [6, 15]. In *Physarum* [14] and in mouse leukaemic cells [7] cyclic patterns were observed which were consistent with neither a smooth increase nor a step-like patterns of rates of RNA synthesis through the cell cycle. In his review, Mitchison [9] suggested that the observed pattern of RNA synthesis may vary with both medium and cell type. This view is supported by the experiments of Stambrook & Sisken [20], who were able to convert a step-like pattern in rates of total RNA synthesis in mammalian cells into a smooth, continuous pattern by raising the uridine concentration in the medium.

In view of these variations in the pattern of RNA synthesis, we have re-examined the rates of total RNA synthesis during the cell cycle of *S. pombe* using both purine and pyrimidine precursors. In addition, we have examined the rates of uridine incorporation into 80S ribosomes.

METHODS

Cell culture and synchronization

Schizosaccharomyces pombe (N.C.Y.C. 132) was maintained in Edinburgh Minimal Medium (EMM2), [8]. Synchronous cultures were prepared using the sucrose density gradient technique of Mitchison & Vincent [13], and monitored for cell number and cell plate index as previously described [22]. Asynchronous cultures were prepared by suspending concentrated cells in 5 ml 25 % (w/v) sucrose solution (equivalent of halfway down at 10 to 40 % sucrose gradient) for 5 min and then inoculating into fresh EMM2 at 32°C at a cell density of $2–3 \times 10^6$ cells/ml. The cultures were usually grown for 6 h to include 2 cell cycles in the synchronous cultures, with pulse-labelling experiments starting 30 to 40 min after re-inoculation from the sucrose suspension.

Radioisotopes

Radioactive tracers from the Radiochemical Centre, Amersham, were used at the following spec. act. and activities/ml of culture: ^{35}S as sulphate (carrier-free) 2 μCi/ml; ^{3}H-5-uridine (29 Ci/mmol) and ^{3}H-8-adenine (20 Ci/mmol), 1 μCi/ml and ^{14}C-2-uridine (56 mCi/ml) and ^{14}C-8-adenine (27.7 mCi/ml), 2 μCi/ml of cell culture.

Isolation of labelled ribosomes

Fifty ml aliquots of the culture were added to the isotope (^{35}S and ^{3}H-uridine) in a 250 ml flask and shaken at 32°C. Na_2SO_4 is present in EMM2 at a conc. of 10 μg/ml, and uridine was added to yield final conc. of 7 ng/ml or 1 μg/ml. Consecutive pulses were 21 min apart and each was stopped after 20 min by addition of 50 ml of 1 % Na_2SO_4 solution containing 100 μg/ml uridine at 0°C. The flask was placed on ice for 5 min and the cells were then collected on a 2.1 cm Whatman GF/A glass fibre filter (pre-washed with 10 ml of the 1 % Na_2SO_4-uridine solution), washed with a further 10 ml of the Na_2SO_4-uridine solution and finally with 10 ml TKM buffer (10 mM Tris, 10 mM Mg-acetate, 50 mM KCl, pH adjusted to 7.4 with 1 N HCl). The GF/A filter, frozen and stored at −20°C until completion of the experiment, was covered with 5 ml TKM buffer, refrozen and crushed by passage through an Eaton press [3]. The broken cells and GF/A filter were transferred to a capped tube and stored at −20°C until all samples had been crushed. The samples were allowed to thaw at 0°C, 0.25 ml of 20 % Triton X-100 were added to each tube and the contents were thoroughly mixed and then centrifuged for 10 min at 1 000 g at 0°C. The supernatants were clarified by further centrifugation (10 min at 25 000 g, 0°C) and were then layered on to 1 to 2 ml of 34 % sucrose (1 M) in TKM buffer, centrifuged for 120 min at 125 000 g at 0°C. The

transparent ribosomal pellets were drained, resuspended in 100 μl 0.1 % Triton X-100 in TKM buffer, layered on to a 5 ml gradient of 10 to 34 % sucrose, in TKM buffer and centrifuged for 60 min at 173 000 g. The gradients were analysed by upward displacement with 40 % sucrose through a 2 mm light-path flow cell in an ISCO UV analyser. The region of each gradient corresponding to the 80S ribosome peak was collected as 20 three-drop fractions of about 50 μl each, which were then diluted with 100 μl TKM buffer. After pre-soaking GF/A filters in 5 % trichloroacetic acid (TCA) containing 1 % Na_2SO_4 and 100 μg uridine/ml and drying overnight, 50 μl of each diluted fraction was spotted on to one of the pretreated filters, dried and washed thrice with 5 % TCA containing 1 % Na_2SO_4 and 100 μg uridine/ml (5 min each) once each with ethanol buffered with acetate (5 min), with diethyl ether/96 % ethanol (3/1 mixture, 5 min) and finally with diethyl ether. After drying overnight the filters were counted for 10 min in scintillation vials with 6 ml of scintillator (5 g butyl-PBD (Ciba Ltd) per litre in toluene). The optical density of the remaining 100 μl of each fraction was measured at 260 nm in a Beckman DB spectrophotometer. The isotope crossover and spec. act. (cpm of 50 μl/OD_{260nm}) were calculated for each fraction. Spec. act. measurements were used to take into account differential losses from the sample during preparation. The mean and standard error of the 7 to 10 fractions at the 80S peak were calculated and this value was used as the estimate of the spec. act. of the ribosomes synthesized at that particular time in the cell cycle. The final selection of 7 to 10 from the 20 fractions taken from each gradient was necessary in order to eliminate a "tail" of non-spec. radioact. associated with the ^{35}S-labelled ribosome preparations which interfered with the 40S and 60S regions of the gradients. These sub-units comprised from 1 to 5 % of the total free ribosomes.

High-voltage electrophoresis

Ribosomes labelled with $Na_2{}^{35}SO_4$ were isolated in larger yields, dialysed to remove the sucrose and evaporated to dryness under vacuum. The dry ribosomes were dissolved in 6 N HCl, sealed in vacuo and hydrolysed at 105°C for 24 h. High voltage paper electrophoresis at pH 2.1 [23] showed that more than 60 % of the ^{35}S was present as methionine and cysteine, and that the remainder was SO_4 and oxidation products of methionine. This indicated that the ^{35}S label of the ribosomes was incorporated into amino acid residues of ribosomal proteins.

Total RNA specific activities

Total RNA estimations were made using a modification of the assay described by Hutchison & Munro [5]. Five ml of culture at a density of 1 to 8×10^6 cells/ml were frozen in a capped 15 ml centrifuge tube and stored at −20°C. The sample was thawed at 0°C in the presence of 5 ml of 10 % perchloric acid (PCA), extracted at 0°C for 30 min and centrifuged (10 min at 1 500 g, 0°C). The pellet was resuspended in 5 ml of 5 % PCA, left for 10 min at 0°C and recentrifuged as before. The pellet was resuspended in 1.0 ml of 5 % PCA and transferred to a water bath at 70°C for 20 min, with vigorous stirring every 90 sec. At the end of the hydrolysis the tube was cooled to 0°C and centrifuged for 10 min at 1 500 g at 0°C. The supernatant was scanned in a UV spectrophotometer (Unicam SP800) and the $OD_{\lambda max\,264nm}$ was used to estimate the conc. of total RNA using $E^{1.0}_{0.1\,\%,\,264} = 25$. An aliquot of the supernatant, either 50 or 100 μl, was then counted for radioactivity and the cpm used to calculate the spec. act. (cpm/μg RNA) of the total RNA.

Rates of total RNA and protein synthesis

Five ml aliquots of cells were shaken at 32°C for 19 min in the presence of ^{3}H-uridine and $Na_2{}^{35}SO_4$ or ^{14}C-uridine and ^{3}H-adenine. The pulse was stopped by adding 5 ml of ice-cold 10 % TCA (containing 100 μg of the appropriate non-radioactive precursors per ml) to the flask, which was then placed on ice to allow for the extraction of small molecules. Triplicate 1.0 ml samples were collected on 2.1 cm Whatman GF/A glass fibre filter discs which had been prewashed with 5 ml of 5 % TCA and precursors. The filtered cells were washed thrice with 10 ml 5 % TCA and precursors, twice with 10 ml water and once with 5 ml 96 % ethanol, and then placed in scintillation vials and dried in vacuo for 1 h before being counted as above. The rate of total RNA synthesis was followed for 1.5 to 4 generations. The 5 ml aliquots of culture were pulse-labelled at 10 min intervals beginning 40 min after making the synchronous cultures. To study the effect of external uridine concentrations upon the rate pattern, uridine concentrations in the labelling medium were varied from 7 ng/ml to 200 μg/ml. Adenine was used at 1.5 μg/ml except during expts with a prefilled pool in which cells were maintained in 20 μg adenine/ml starting 4 h before synchronizing the cells.

RESULTS

The ribosomal protein synthesis rate during the cell cycle was examined in *S. pombe* by pulse-labelling the cells with $Na_2{}^{35}SO_4$. The synthesis rate of sulphur-containing proteins associated with RNA in 80S ribosomes was measured as the spec. act. of ribosomes purified by sucrose gradient centrifugation, expressed as cpm of 50 μl/OD_{260}. Fig. 1*a* shows the pattern of spec. act. from 4 synchronous cultures. The 4 cultures all show a minimum value for the spec. act. at the time of the cell plate index peak and with maxima between 0.6 and 0.9 of a cell cycle. By comparison with asynchronous control cul-

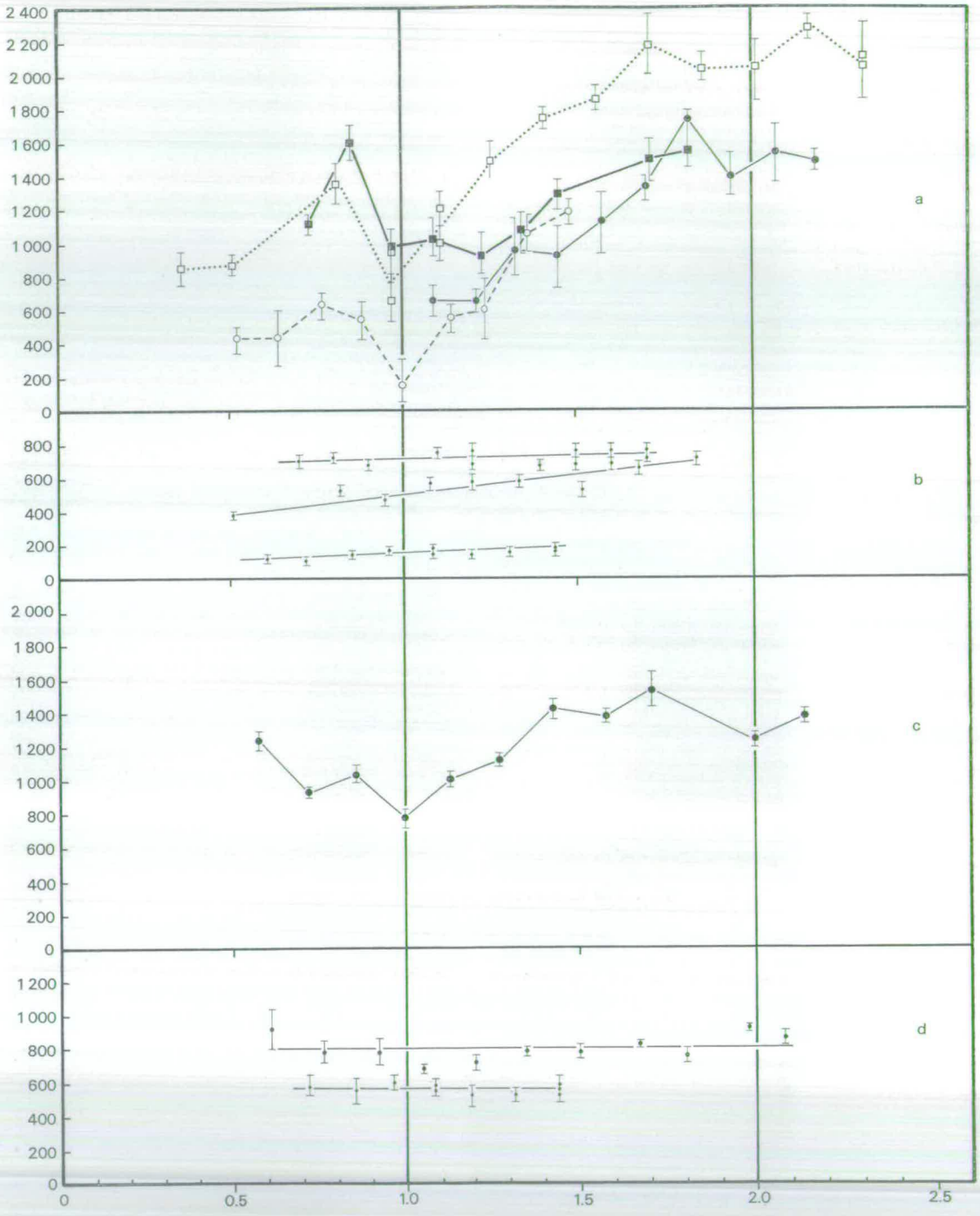


Fig. 1. Abscissa: cell cycle time; *ordinate:* cpm/OD_{260}.

Spec. act. of protein and RNA from isolated 80S ribosomes. Data given as means and S.E., with no adjustments for differences in cell numbers between repeat experiments. (*a*) 4 synchronous cultures labelled with $Na_2{}^{35}SO_4$; (*b*) 3 asynchronous control cultures labelled with $Na_2{}^{35}SO_4$; (*c*) 1 synchronous culture labelled with ^{3}H-5-uridine; (*d*) 2 asynchronous control cultures labelled with ^{3}H-5-uridine.

tures (fig. 1*b*) it is clear that the spec. act. of ribosomal proteins pulse-labelled with ^{35}S vary during the cell cycle and that this variation is much greater than the random variation shown by the asynchronous control cultures.

There is a close similarity in pattern between the spec. act. of 80S ribosomes from synchronous cultures pulse-labelled with ^{3}H-uridine (fig. 1*c*) and those of ^{35}S-labelled ribosomes (fig. 1*a*). The random variation in the asynchronous ^{3}H-uridine-labelled cultures (fig. 1*d*) is again much less than the pattern shown in the synchronous cultures. We have no explanation for the overall tendency of the spec. act. of 80S ribosomes pulse-labelled during synchronous cultures to rise during the course of the experiment (fig. 1*a*, *c*) but it appears that as the expts progressed an increasing percentage of newly made ribosomes was isolated. This is shown in only one of the asynchronous, ^{35}S-labelled experiments (fig. 1*b*).

From their earlier work, Mitchison et al. [11] had concluded that the rate of total RNA synthesis during the cell cycle of *S. pombe* was continuous and approximately exponential. The uridine incorporation into the acid-insoluble fraction of *S. pombe* cells was investigated in order to confirm their findings. Fig. 2*a* shows the pattern of rates of total RNA synthesis measured by the amount of incorporation during 19 min pulses of ^{3}H-uridine or ^{14}C-uridine throughout the cell cycle of *S. pombe*. The data from each of 6 expts using uridine at conc. of 7 ng/ml, 1 μg/ml and 200 μg/ml in EMM2 have been normalised to show a common mean level of incorporation during the plateau between the first and second peaks of cell plate index. This plateau was chosen as the baseline for normalisation because all expts, irrespective of their duration, included the whole of this plateau. The data from all 6 expts were then pooled and the means and 95% confidence limits

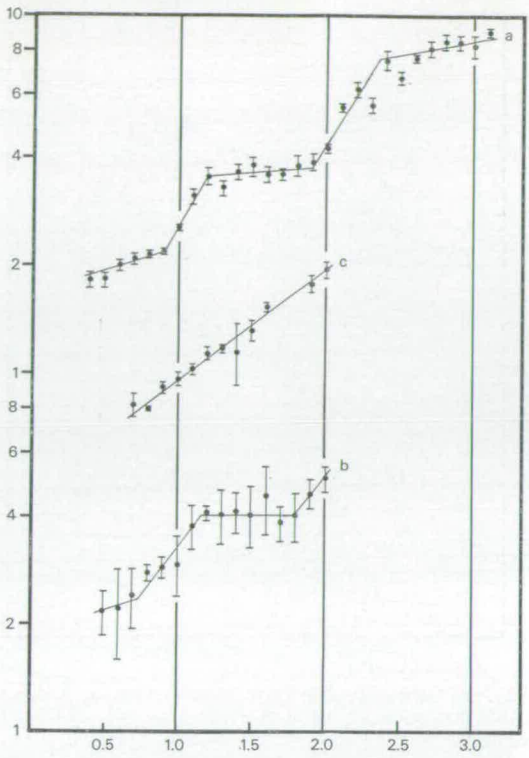


Fig. 2. Abscissa: cell cycle time; *ordinate:* radioactivity in relative units.

Rates of uridine, adenine and sulphate incorporation into the acid-insoluble fraction of whole cells during the cell cycle. Cells were pulse-labelled for 19 min. The mean value of the plateau between the first and second peaks in the cell plate index of each expt was normalized at 4.0. The data from repeat expts were then pooled and the means and 95% confidence limits were calculated for all points within each 0.1 cell cycle. (*a*) from 6 synchronous cultures labelled with ^{14}C-uridine or ^{3}H-uridine, with uridine concentrations in the labelling medium ranging from 7 ng/ml to 200 μg/ml in separate expts; (*b*) from 2 synchronous cultures labelled with ^{3}H-adenine in the presence of an additional 1.5 μg adenine/ml of culture; (*c*) from 2 synchronous cultures labelled with $Na_2{}^{35}SO_4$ in the presence of 10 μg Na_2SO_4/ml medium.

calculated for all points in each tenth of the cell cycle. The pattern of increase in the rate of uridine incorporation into the TCA-precipitable fraction of *S. pombe* (fig. 2*a*) is not exponential but has a sharp step in the rate coincident with the rise in cell number.

In contrast, the pattern of rates of $Na_2{}^{35}SO_4$ incorporation into the TCA-precipitable frac-

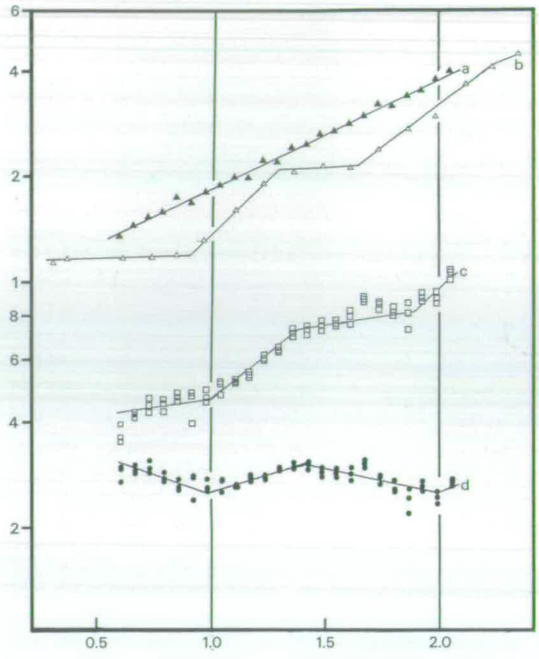


Fig. 3. Abscissa: cell cycle time; *ordinate:* (*a*) μg RNA/5 ml culture; (*b*) cpm $\times 10^{-3}$; (*c*) cpm/μg RNA; (*d*) cell number $\times 10^{-5}$.

Specific activity of extracted total RNA during the cell cycle. (*a*) total RNA extracted from 5 ml of cells, measured as the OD_{264} of a hot PCA digest of acid-washed cells; (*b*) radioactivity counted in 50 μl of the PCA digest; (*c*) spec. act. of the PCA digest; (*d*) cell number.

tion of *S. pombe* was exponential throughout the cell cycle (fig. 2*c*). The $^{35}SO_4$ was used as a measure of total protein synthesis and although ribosomal proteins are a large group within the total cell proteins in *S. pombe*, the contribution of a step in their synthesis rate to that of total protein synthesis is likely to be obscured by overlapping patterns of synthesis of many other proteins.

Two approaches were used to provide at least partial confirmation that the incorporation of uridine pulses was a true measure of the rate of RNA synthesis. First, unlike Stambrook & Sisken [21], the pattern of adenine incorporation during the cell cycle of *S. pombe* (fig. 2*b*) was found to be similar to that of uridine (fig. 2*a*). (The 100-fold RNA excess over DNA in *S. pombe* [12] permits the use of adenine as a precursor for the estimation of RNA synthesis). The second approach involved the extraction of total RNA after pulse-labelling cells with ^{3}H-uridine. The increase in cell number, in total RNA, in the rate of uridine incorporation into RNA and the spec. act. of the extracted RNA, derived from the values for total RNA and incorporated counts, are shown in fig. 3. It is clear that the pattern of uridine incorporation into extracted total RNA (fig. 3*b*) is similar to that of uridine incorporation into the acid-insoluble fraction of *S. pombe* cells (fig. 2*a*). Although the total RNA conc., measured by OD_{264} of the hot PCA digest, appears to increase exponentially (fig. 3*a*), this is probably due to the imprecision in the method which prevents the detection of the difference between an exponential curve and a series of straight lines with a doubling in their slopes at the time of each successive cell division. By dividing the radioisotope incorporation (fig. 3*b*) by the total RNA conc. (fig. 3*a*) one obtains the spec. act. of the extracted RNA (fig. 3*c*) which shows a pattern of cyclic variation through the cell cycle which is similar to the pattern shown in fig. 1*c* for ribosomal RNA. This correlation would be expected in view of the high proportion of ribosomal RNA in total RNA.

The rate of uridine incorporation into the acid precipitable fraction of *S. pombe* cells thus appears to be a reasonable estimate of the rate of RNA synthesis. Changes in the rates of synthesis estimated in this way bear a close correlation with the variation in the spec. act. of ribosomal RNA (fig. 1*c*) and ribosomal protein (fig. 1*a*), but since these methods are all based upon incorporation of labelled exogenous precursors given only during a relatively short pulse-time, they could be explained by changes in the spec.

act. of the internal pools on the immediate synthetic pathway, e.g. the nucleoside triphosphate pools. Changes in these pools could be caused by (1) changes in the rate of uptake at a point during the cycle; (2) changes in the size of the pool proportional to the amount of precursor in the medium (expandable pool); (3) changes in the size of the pool at some point in the cell cycle; (4) changes in the ratio between the contribution of the external and internal supplies of precursor to the synthesis at a point in the cell cycle.

Stambrook & Sisken [20] have demonstrated that the pattern of uridine incorporation into synchronised Chinese hamster lung cells can be altered by changing the uridine concentration in the medium. Indeed, by presenting the cells with a vast excess of precursor during the pulse-labelling, one would expect to flood all uptake mechanisms and obtain a measurement of the true rate of synthesis. They suggest that in their cells the rate of RNA synthesis can be limited or controlled by the rate of entry of uridine into the cells. The patterns of increase in the rates of uridine incorporation at two uridine concentrations (0.03 μM and 820 μM) were found to be identical in *S. pombe* (fig. 4*a*, *b*). This is a greater range than that used by Stambrook & Sisken [20] (0.25–200 μM). Synchrony induced by high uridine concentration, in a manner analogous to thymidine block, was described for *Tetrahymena* by Villadsen & Zeuthen [26]. Since there was no significant departure from the asynchronous level of 7% cell plate index of asynchronous cultures of *S. pombe* grown in the presence of 820 μM uridine for 5 h, the possibility of uridine-induced synchrony in *S. pombe* can therefore be disregarded. The data presented in fig. 4*a*, *b* suggest that, unlike the Chinese hamster cells used by Stambrook & Sisken [20], the rate of RNA synthesis in *S. pombe* is probably not limited or controlled by the rate of entry of uridine.

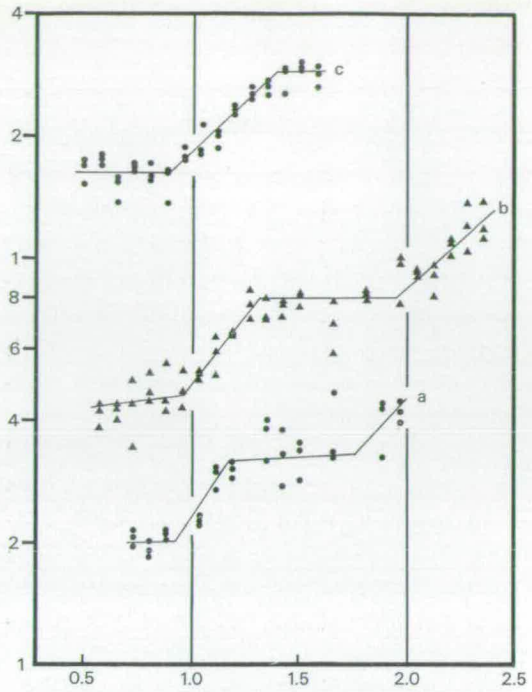


Fig. 4. Abscissa: cell cycle time; *ordinate:* (*a*, *c*) cpm $\times 10^{-2}$; (*b*) cpm $\times 10^{-3}$.

Effects of precursor concentration upon the pattern of the rates of total RNA synthesis during the cell cycle. (*a*) ^{3}H-uridine at a final conc. of 0.03 μM; (*b*) ^{3}H-uridine at a final concentration of 820 μM; (*c*) ^{14}C-adenine incorporation after pre-filling the adenine pool by growing the cells in the continuous presence of 20 μg adenine/ml of culture from 4 h before synchronizing the cells.

The role of pool size variation in controlling the rate of RNA synthesis may be studied by pre-filling the expandable adenine pool by growing an asynchronous population of *S. pombe* in the presence of 20 μg adenine/ml for more than one generation prior to synchronisation or to any pulse-labelling experiments [2]. Pre-filling the adenine pool did not, in fact, affect the step-like pattern of increase in rate of adenine incorporation during the cell cycle (fig. 4*c*). Variations in uptake and pool size of RNA precursors are,

thus, probably not factors controlling or limiting the rate of labelled adenine incorporation.

DISCUSSION

The synthesis of ribosomal proteins recovered on 80S ribosomes (fig. 1*a*) parallels the synthesis of ribosomal RNA (fig. 1*c*) and total RNA (fig. 3*c*). These results are supported by the experiments with *Saccharomyces cerevisiae* by Warner & Udem [25] in which temperature-sensitive mutants affecting ribosomal RNA synthesis also affected ribosomal protein synthesis. Craig & Perry [1] showed that synthesis of ribosomal proteins by L cells could continue when ribosomal RNA synthesis was inhibited by actinomycin D, but that such proteins were not incorporated into ribosomes formed after release of the actinomycin inhibition.

The similarity in the patterns of synthesis shown by ribosomal proteins and ribosomal RNA (fig. 1*a*, *c*) suggested that it was unlikely that there could be either gross variation in the patterns of synthesis of major individual ribosomal proteins or that there would be large pools of these proteins. However, some minor ribosomal proteins could have very different patterns of synthesis without noticeably affecting the pattern of total ribosomal protein synthesis. Recent work by Shulman et al. [19] has shown that both total and several individual ribosomal proteins are synthesised at nearly equivalent rates to one another at four points in the cell cycle of *Saccharomyces cerevisiae* and that there is no evidence of discontinuous synthesis of these proteins. Our results are in agreement with their work. They are more limited in being restricted to total ribosomal protein, but they have the advantage of a large number of samples over more than one cell cycle. They have, therefore, revealed discontinuities of rate within the pattern of continuous synthesis. The work of Schleif [17] suggests that there are insignificantly small pools of ribosomal protein in *Escherichia coli* although Sells & Davis [18] have shown that one class of 50S sub-unit ribosomal proteins may take 11 min to pass through a pool, a significant proportion of the generation time of *E. coli.*

However, the pattern of rates of uridine incorporation into fission yeast cells (figs 2, 4) and into RNA (fig. 3*b*) imposes severe controls on the synthesis of ribosomal proteins if the possibility of ribosomal protein pools is excluded. The doubling in the rate of RNA synthesis shown by the step in the incorporation of uridine (figs 2, 4) accompanied by a doubling in the rate of ribosomal protein synthesis in the cytoplasm would imply some control of protein synthesis closely linked to the mechanism controlling the doubling in the rate of RNA synthesis. Alternatively, there may be pools of ribosomal proteins with a rapid turnover and which in synchronous cultures would have a maximum level at the beginning of the rise in the rate of uridine incorporation (0.8 of the cell cycle) and a minimum level, or possibly zero, at the start of the plateau in the rate of uridine incorporation (0.2 of the cell cycle). Consequently, RNA synthesised during the rise in the rate of uridine incorporation could be associated with pre-existing ribosomal proteins without requiring similar changes in the rates of synthesis of the ribosomal proteins. A continuous pattern for ribosomal protein synthesis would not require close temporal links between RNA synthesis in the nucleus and ribosomal synthesis in the cytoplasm. One consequence is that any attempts to measure pools of ribosomal proteins should be concentrated on specific parts of the cell cycle where the pools might be expected to be at a maximum (in *S. pombe* about 0.8 of the cell cycle.)

Although the findings of Mitchison et al.

[11] appear to be in contrast to the experiments presented in this paper, in fact, the bulk of their work was based on autoradiographic, single-cell estimations covering only one cell cycle with all the limitations of such analyses, especially the greater errors in the points at the ends of the cycle. Since the increase in rate of incorporation occurs between 0.8 of one cycle and 0.2 of the next cycle (figs 2, 3, 4), single-cycle estimations could only include parts of two increases, with a plateau in between. Such a pattern would be difficult to detect and would require the examination of many points in the cell cycle. The data of Mitchison et al. [11] obtained from bulk measurements, at 5 points in the cell cycle of a different strain of *S. pombe* (fig. 4 of [11]) may be interpreted as showing this pattern: a rise, a plateau and a final rise in the rate of adenine incorporation). We feel that our data are not inconsistent with the data of Mitchison et al. [11], although our more detailed information supports a different conclusion.

We were unable to find the effects of external uridine concentrations on the incorporation pattern described by Stambrook & Sisken [20] for mammalian cells, although their maximum uridine conc. was increased by a factor of 4, to 820 μM (fig. 4*b*). The similarity of the pattern of adenine incorporation in cells with a pre-filled adenine pool (fig. 4*c*) to that of uridine at various conc. (figs 4*a*, *b*) is an indication that the rates of uptake and pool size fluctuations are unlikely to be factors controlling the rate of incorporation of precursors into RNA. However, changes in the specific activity of the nucleoside triphosphate pools due to changes in the contribution from non-labelled internal pathways cannot be excluded.

An alternative explanation for the stepwise pattern of increase in the rate of RNA synthesis is a gene dosage effect, since the timing of the step coincides with the DNA synthesis of *S. pombe* [10]. A causal relationship between the steps in the rate of RNA synthesis and the timing of DNA synthesis has been suggested by Klevecz & Stubblefield [6] and Pfeiffer & Tolmach [15].

Experiments are currently in progress to examine the relationship between DNA synthesis and the changes in rate of RNA synthesis, rates of uridine uptake and changes in the spec. act. of the internal nucleotide pools. We hope to report on them in a subsequent paper.

We would like to express our thanks to Professor J. M. Mitchison for his advice, criticism and encouragement and to our colleagues for their interest and discussion. We are grateful for the skilled technical assistance of Miss Yvonne Pryde and R. Pitcairn, and we thank J. J. Holmes for producing fig. 1, and the secretarial staff for their unflagging efforts in the production of this manuscript. We are grateful to Professor Hartwell for a pre-print of the paper by Shulmann, Hartwell & Warner.

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APPENDIX II

ESTIMATION OF DNA BY THE DIPHENYLAMINE METHOD*

Samples of 1-5 x 10^8 cells were collected by filtration on Millipore membrane filters. The pad of cells was washed on the filter with 10 ml of 0.1% NaCl, and filters were then placed in plastic tubes (LP3, Luckham), and frozen before processing. Samples could be stored frozen for at least a week without any effect on estimated values of DNA/cell.

Processing of the samples included the following operations in the order given:

1. Incubation of samples in 1.5 ml of ice-cold 5% perchloric acid (PCA), with frequent mixing, for 30 minutes. The filter was removed from each tube at the end of this period.
2. Samples were spun for 10 minutes at 2500 RPM, 4°C (MSE 4L centrifuge), and the supernatant was discarded. The pellets were washed with another 1.5 ml PCA, samples were respun, the supernatant was again discarded.
3. The pellet was resuspended in 0.3 ml PCA, tubes were capped and incubated for 20 minutes at 70°C, with frequent mixing. At the end of this extraction the samples were again centrifuged, and 0.25 ml of the supernatant was placed in a clean tube.
4. To the 0.25 ml fraction was added 0.4 ml of diphenylamine reagent, consisting of 0.8 g diphenylamine (Sigma), 20 ml glacial acetic acid, and 0.05 ml of 16 mg/ml acetaldehyde in water. 0.25 ml aliquots of deoxyadenosine standards (2,4,6,8,10,12 µg/ml in 5% PCA) were treated in the same way.

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* From Polanshek (1973).

5. Standards and samples were incubated 12-16 hours at 32°C. All tubes were then centrifuged before reading optical densities at 595 nm and 720 nm in an Hitachi-Perkin spectrophotometer. Readings at 720 nm were subtracted from those at 595 nm to give a calibration curve going through the origin (Giles & Myers, 1965). Equivalents of deoxyadenosine/sample were estimated from the calibration curve, and were converted to values of µg DNA/ml using the equivalence, .1 µg deoxyadenosine/ml = 2.605 µg DNA/ml.

APPENDIX III

POLYACRYLAMIDE GELS FOR RNA FRACTIONATION

From: Loening (1967 & Pers. Comm.)

Stock Acrylamide Solution:

* recrystallized acrylamide	15 g
+ recrystallized bisacrylamide	0.75 g for 2.0-5% gels or 0.375 g for 5-8% gels
water to 100 ml final volume	

* recrystallized from chloroform
\+ recrystallized from acetone

Stock Electrophoresis Buffer (5 x E buffer):

Tris	36 mM
NaH_2PO_4	30 mM
Na_2-EDTA	1 mM
pH 7.6 to 7.8 at room temperature	

Running Buffer (E-Buffer) is a 5 x dilution of 5 x E with 0.2% SDS (low UV absorbtion SDS, e.g. BDH Specially Purified).

To make gels, 5 ml of stock acrylamide solution is mixed according to the Table below to give the desired stiffness of gel:

Volume	Gel Concentration[1]							
	2.0%	2.2%	2.4%	2.5%	2.6%	3.0%	5.0%	7.5%
5xE, (ml).	7.5	6.8	6.25	6.0	5.8	5.0	3.0	2.0
H_2O, (ml).	24.7	22	19.7	18.7	17.3	14.7	6.7	2.7
total vol. (ml).	37.2	33.8	30.95	29.7	28.1	24.7	14.7	9.7

[1] Gel Concentration refers to the final concentration of acrylamide in the gel.

The solution is degassed under vacuum for 15 sec. 25 µl of N,N,N,N-tetramethylethylenediamine (Temed) and 250 µl of freshly dissolved 10% ammonium persulphate are added and mixed. The solution is immediately pipetted into gel tubes (0.9 cm x 11 cm diameter Perspex), carefully overlayered with water and allowed to set for 2 hr. Gels were pre-run ½ to 1 hr in E buffer at 5 mA per gel, 8 V per cm length. RNA was then loaded in ½-strength running buffer containing 12-16% sucrose and the gels run for 2½ to 6 hr depending upon pore size and the degree of separation desired.

ABSTRACT OF THESIS

Name of Candidate William D. Staatz

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Degree Ph.D. *Date* 1976

Title of Thesis Some Aspects of RNA Synthesis During the Cell Cycle of the Fission Yeast, Schizosaccharomyces pombe.

Uridine uptake by Schizosaccharomyces pombe is by a single step reaction with a K_m of 3.15×10^{-5}M. If the uridine concentration in the medium exceeds 2mM, uridine uptake appears to be chiefly by diffusion. The rate of uptake does not appear to limit the rate at which ^{3}H-uridine is incorporated into RNA. In synchronous cultures, the rate of uridine uptake parallels the rate of its incorporation, but changes in the uptake rate occur 0.1 cell cycle after those in incorporation rate. The Specific activity of the ^{3}H-uridine pool in such cultures remains constant. Inhibitors which alter the relationship between the rates of uptake and RNA synthesis alter the size of the uridine pool.

The relationship between pulse length and the distribution of ^{3}H-uridine in various species of RNA was examined electrophoretically. After 1 min of labelling, all radioactivity was in the ribosomal precursor, heterodisperse and 4-5 S RNA fractions. After pulses of 10 min or longer, label is distributed in essentially a steady-state labelling pattern.

In synchronous cultures, the rate of RNA synthesis doubles at the time of DNA synthesis. When cultures are treated with the inhibitors of DNA synthesis, 2'-deoxyadenosine and 1-phenylethanol, they go through a period of adjustment during which the rate of RNA synthesis no longer is proportional to the DNA content of the cell. After cells have adjusted, however, the relationship between DNA and RNA synthesis is restored.

Following a step-down in culture conditions the various RNA species are transcribed in approximately the same proportions as in control cultures. But the total rate of RNA synthesis is depressed and the processing of ribosomal precursor RNA is inhibited.

The results are discussed and possible control mechanisms are considered.

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