

AN ABSTRACT OF THE THESIS OF

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(Name of student) (Degree)

in Microbiology presented on July 28, 1972
(Major) (Date)

Title: CELL DIVISION AND DNA SYNTHESIS IN SACCHAROMYCES
CEREVISIAE FOLLOWING NUTRITIONAL SHIFT-UP
Redacted for privacy

Abstract approved: L. W. Parks
L. W. Parks

Nutritional shift-up experiments have revealed many important macromolecular interactions in bacteria. It has been shown that RNA synthesis can be dissociated from protein and DNA synthesis. The rates of protein synthesis were found to be, at a given temperature, strictly dependent on the numbers of mature ribosomes present. DNA synthesis remained unaffected by the increased rates of RNA and protein synthesis for some time after shift-up. The rate of cell division was not coupled to the new rates of RNA, DNA, and protein synthesis for one pre-shift generation time after the shift.

Recent experiments with yeast, however, have indicated that the rates of DNA synthesis and cell division are tightly coupled to the rates of RNA and protein synthesis after nutritional shift-up. Our experiments have demonstrated that these results are in error. The rates of cell division are maintained in yeast for one generation time

after shift. Also a rate maintenance phenomenon is observed with respect to DNA synthesis. These results indicate that the times of genome replication and the time for cell division to occur are constant between generation times of 120 - 300 minutes. Under our experimental conditions mitochondrial DNA is preferentially synthesized during the first 20 - 30 minutes after shift-up. There is some indication that this preferential synthesis is due to partial respiratory adaptation.

Cell Division and DNA Synthesis in Saccharomyces cerevisiae
Following Nutritional Shift-up

by

Gunnard Kenneth Jacobson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1973

APPROVED:

Redacted for privacy

Professor of Microbiology

in charge of major

Redacted for privacy

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Date thesis is presented July 28, 1972

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ACKNOWLEDGEMENTS

I would like to thank Dr. L. W. Parks who was a constant source of help and encouragement during the course of these investigations. Recognition is also due to my co-workers, Dr. Ed Thompson, Rich Bailey, Ron Trewyn, and Ken Nakamura, whose presence in the lab made working there an interesting experience. The excellent technical assistance of Mrs. Verlyn Stromberg who performed some of the experiments presented here is also acknowledged. I would especially like to thank my wife, Claudia, who was able to tolerate my presence during the preparation of this thesis, for her services as typist and proofreader.

To my father, Kenneth B. Jacobson, Sr., who first aroused my interest in the sciences, and mother, Mary Jane Jacobson, who encouraged it.

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CELL DIVISION AND DNA SYNTHESIS IN SACCHAROMYCES
CEREVISIAE FOLLOWING NUTRITIONAL SHIFT-UP

INTRODUCTION

Over the last 15 years the research of many workers has been directed toward understanding the control mechanisms of macromolecular synthesis. These complex interrelationships which govern the syntheses of RNA, DNA, and protein have proved to be efficient means of self-regulation. Several of these important macromolecular interactions were first elucidated in bacterial systems. By shifting a culture in balanced growth to a richer growth medium, it was shown that the synthesis of RNA could be dissociated from protein and DNA synthesis. The rates of protein synthesis were found to be, at a given temperature, strictly dependent on the rates of RNA synthesis and the subsequent numbers of mature ribosomes present. DNA synthesis was found to remain unaffected by the increased rates of RNA and protein synthesis for some time after shift-up. The cell divisional rate was found to remain uncoupled from the new rates of RNA, DNA, and protein synthesis for one pre-shift generation time after shift.

Recent experiments with yeast, however, have indicated that the rates of DNA synthesis and cell division are tightly coupled to the rates of RNA and protein synthesis after nutritional shift-up.

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INTRODUCTION

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Recent experiments with yeast, however, have indicated that the rates of DNA synthesis and cell division are tightly coupled to the rates of RNA and protein synthesis after nutritional shift-up.

The research presented here was undertaken to confirm the possibility of these new modes of macromolecular regulation. Also, since yeast contain two complements of DNA, mitochondrial and nuclear, it was of interest to examine the possible role of mitochondrial DNA on the transition between steady states during nutritional shift-up.

REVIEW OF THE LITERATURE

During the last 15 years, much emphasis has been put on the effects of nutritional shift-up in bacterial systems; much of this research has been extensively reviewed (Maaløe and Kjeldgaard, 1966). In the beginning, Schaechter, Maaløe, and Kjeldgaard (1958) demonstrated that cell size and chemical composition of bacteria can vary with changing growth medium, and that these variations are dependent on the culture's generation time at a given temperature. Temperature variation will affect the generation time but not cellular composition in a given medium. They state, therefore, that ". . . irrespective of temperature, a given medium defines a steady state condition of growth to which corresponds a certain average cell size and a certain chemical composition." When cells in a steady state condition of growth in poor culture medium are shifted to a rich broth, characteristic modifications in the synthetic rates of RNA, DNA, and protein occur which bring the culture to the steady state characteristic of the new medium.

The most dramatic change occurs in the rate of RNA synthesis which immediately adopts an accelerated rate of synthesis in excess of the definitive rate characteristic of the new medium (Kjeldgaard, 1961). It has been demonstrated that this elevated rate of synthesis begins within five seconds after shift and that the full rate is obtained

in 90 seconds (Koch, 1965). After several minutes at this accelerated rate, the RNA/mass ratio reaches a value characteristic of the post-shift medium, whereupon the rate of RNA synthesis drops to the characteristic rate. It has been shown that the majority of the RNA synthesized after shift-up is ribosomal RNA (r-RNA) and that the number of mature ribosomes present increase to the characteristic post-shift level during the period of excessive RNA synthesis (Kjeldgaard, 1961). In contrast, the increase in the rate of protein and DNA synthesis lags behind that of RNA synthesis. This suggests that the control of RNA synthesis is not dependent on the amounts of protein and DNA present or on their rates of synthesis. One can draw an identical conclusion from the work of Fraenkel and Neidhardt (1961) which demonstrated that when chloramphenicol (CAP) was added to cultures in minimal medium, RNA synthesis was accelerated while protein synthesis was inhibited. Earlier work had demonstrated that RNA synthesis ceased when a required amino acid is removed from the medium, but resumed, even though protein synthesis was inhibited, when the amino acid was returned to the medium with 10 $\mu\text{g}/\text{ml}$ CAP (Pardee and Prestidge, 1956). In 1958, Aronson and Spiegelman found that high levels of CAP (400 $\mu\text{g}/\text{ml}$) relieved the necessity of an external supply of the required amino acid so that RNA synthesis could continue in its absence. These data led Kurland and Maaløe (1962) to propose a model involving transfer

RNAs (t-RNA) and amino acids as a means of control of RNA synthesis. They hypothesize that uncharged t-RNAs are repressors and the individual amino acids are inducers. When cells are shifted to rich broth, the internal concentration of amino acids increases dramatically, all t-RNAs are charged, repression of RNA synthesis is therefore removed, and maximal rates of RNA synthesis ensue. Conversely, if a required amino acid is removed, its respective uncharged t-RNA accumulates and represses transcription either by blocking the DNA template or by inhibiting a polymerase.

The fact that the t-RNA/DNA ratio remains constant during the transition between steady states suggests that the t-RNA control mechanism is different from r-RNA and may be regulated in a manner similar to DNA (Maaløe and Kjeldgaard, 1966). Gros et al. (1965) have shown that the amount of messenger RNA (m-RNA) present is proportional to the number of ribosomes present. This proportionality suggests that m-RNA may never be produced in excess during a shift-up and that the r-RNA/m-RNA ratio remains constant.

A most important observation was that the rate of protein synthesis after shift-up is maintained at the pre-shift level several minutes past the time at which RNA synthesis accelerates (Kjeldgaard, 1961). MacCarthy (1962) has shown that the rate of protein synthesis depends on the number of ribosomes present by first starving cultures for Mg^{++} and then adding the ion back. After a lag, the numbers of

ribosomal particles increased logarithmically; and the rate of incipient protein synthesis closely paralleled the number of mature ribosomes present. When Kjeldgaard (1961) reported that the RNA synthesized immediately after shift-up is primarily r-RNA, he suggested that the synthesis of ribosomal protein is also accelerated during this time. Support already existed for this proposal. Neidhardt and Magasanik (1960) demonstrated that labeled leucine is incorporated predominately into particulate proteins after shift-up.

Like protein synthesis, DNA synthesis also was shown to be maintained at the pre-shift rate; but unlike the rate of protein synthesis, it is maintained for quite a while longer after shift-up. When the rate characteristic of the new medium is attained, the definitive RNA/DNA and protein/DNA ratios characteristic of the new medium are also attained (Maaløe and Kjeldgaard, 1966).

The manner of DNA synthesis and chromosome replication per se in bacteria has elicited much research. The classic experiments of Meselson and Stahl (1958) revealed that the DNA molecule is replicated semi-conservatively; and that in slowly growing bacteria, a second round of replication is not initiated until the first is completed. Lark, Repko, and Hoffman (1963) have shown in tritium pulsed and density labeled experiments that radioactivity moved into hybrid DNA peaks only after one replication time of growth. This supported Meselson and Stahl's evidence for semi-conservative replication and

indicated that the replication of the genome is ordered and proceeds unidirectionally from an unique origin. This view has also been supported by genetic evidence (Yoshikawa and Sueoka, 1963). The autoradiograms of Cairns (1963) also show that the act of replication proceeds from one end of the molecule to the other, and that the bacterial genome is a large structure, which can have one or more replication points per genome.

Maaløe (1961) has suggested that, for a given temperature, each of these replication points proceeds at a constant rate to completion; the rates of synthesis are independent of the cellular growth rate. There are two predictions of Maaløe's theory that have been closely studied. The first of these is that when the cellular growth rate is slower than the DNA replication rate, there exists a gap of DNA non-synthesis between subsequent rounds of replication (Maaløe and Hanawalt, 1961). Lark (1966) has shown in Escherichia coli 15T⁻ growing in proline or acetate that a definite gap does exist between rounds of replication. This gap was not measurable in cells with generation times of less than 120 minutes. Her autoradiograms suggest that in slowly growing cells (generation times greater than 120 minutes) events triggering initiation of a round of replication occur in the first half of the cell cycle and that DNA synthesis occupies the second. Her data also suggest that the rate of DNA chain elongation is slower in these cells, presumably due to shortages of nutrients.

Helmstetter (1967) has bound labeled Escherichia coli B/r to membrane filters, inverted the filters, and eluted the cells with medium. Parental cells adhere to the membrane so that only new-born cells are eluted. Thus, when pulse labeling experiments were done, the amount of radioactivity in the eluted cells was a function of cell age. With this technique, Helmstetter was able to show that the rate of DNA synthesis in glucose grown cells doubles during mid-cycle, thus supporting the similar data of Maaløe and Clark (1967) rather than the data of Abbo and Pardee (1960) which had indicated that the rate increases logarithmically during the cycle. Also evident was the gap of DNA non-synthesis in cells slowly growing in acetate first reported by Lark (1966). However, there is an important discrepancy between Lark's and Helmstetter's work in that Helmstetter finds the period of non-synthesis in the second half of the cell cycle. Kubitscheck, Bendigkeit, and Loken (1967), using pulse labeled cells growing in a chemostat, have plotted radioactivity/cell against cell volume. New-born cells are smaller than cells near division, and they find no incorporation into these cells. They agree with Lark: DNA is synthesized continually in glucose grown cells and that in slowly growing (acetate) cells, DNA synthesis occurs in the latter part of the division cycle and ceases before division.

A second prediction of this theory is that, since the rate of DNA synthesis at the replication point is independent of cellular growth

rate, increased rates of synthesis may be due to multiple replicating points on the chromosome. The first evidence of multiple forked replication in vegetative cells was demonstrated by Yoshikawa, O'Sullivan, and Sueoka (1964) in Bacillus subtilis. They looked at recombination frequency ratios of a marker known to be near the origin of replication relative to one near the terminus. In slowly growing cells this ratio is two; however, in rapidly growing cells with a DNA doubling time of 20 minutes, this ratio was found to approach four. This, they hypothesized, meant that there was multiple forked replication of the genome. Helmstetter and Cooper (1968) then conclusively demonstrated using membrane elution techniques that the rates of DNA synthesis in rapidly growing cells followed patterns indicative of multiple replication forks and that a constant time was required for each point to traverse the chromosome.

Such prolific outputs of scientific data invariably lead to numerous hypothetical models in search of support. The two most prominent models on the control of DNA replication have been those of Lark and Helmstetter and Cooper (Lark, 1966; Cooper and Helmstetter, 1968). Lark's model is proposed from data on DNA content/cell and from autoradiographic data. He found in broth grown cells pulsed with ^3H -thymidine that no unlabeled cells appeared in the culture until four generations had passed. Lark suggested that, instead of multiple replication forks, there were four complete genomes

replicating at the time of the pulse. Essentially, the model of Lark is one of multiple chromosomes which are replicated either in unison or alternately, depending on the growth rate. In all but slowly growing cells, initiation occurs just after division; and in very slow growers there is only one chromosome per cell which is replicated in the latter half of the division cycle.

Earlier work of Maaløe and Hanawalt (1961), which demonstrated that the total DNA of a culture increased 40% then ceased to be synthesized when a culture was deprived of essential amino acids, led to the idea that protein synthesis was necessary for the initiation of new rounds of replication, but not for completion for those rounds already in progress. Lark states that, in order for replication to occur, a proteinaceous membrane binding site for the 5' end of the chromosome must be synthesized. In slowly growing cells (grown in acetate or proline), the rate of synthesis of this protein is not fast enough to support the required number of binding sites necessary for multiple chromosomes per cell.

To satisfy the experimental data in glucose grown cells, he proposed that the length of time required to replicate a DNA molecule decreases with decreasing generation time. Glucose grown cells contain two simultaneously replicating chromosomes, and succinate grown cells contain two chromosomes which are replicated alternately. This was proposed when data from cells labeled for long

periods of time suggested that both cultures contained the same number of replicating units, but short pulsed experiments indicated active synthesis in only half the units in succinate grown cells. Also proposed by Lark for glucose and succinate grown cells is that just before division each cell contains four chromosomes, and that a template strand just completed at the end of the present cycle pairs and segregates with a template replicated in the previous cycle (Lark et al., 1967).

A year after Lark published his model of chromosome replication, Cooper and Helmstetter (1968) outlined a new model relating chromosome replication and the division cycle of bacteria. Their work was done with E. coli B/r at generation times ranging from 20 to 60 minutes, and they state that this relationship can be described by two parameters. These parameters, C and D, are defined as the time required to replicate a chromosome (C), and as the elapsed time from the completion of a round of replication to division (D). In this relationship cell division always follows an initiation event by C + D minutes, and it is based on the idea of Maaløe and Kjeldgaard (1966) that once a round is initiated, it proceeds to completion at a constant rate, and on their own experimental observations (Helmstetter and Cooper, 1968; Cooper and Helmstetter (1968)). The central theme of their model is that rapidly growing bacteria contain multiple forked replicating chromosomes which are initiated before and terminate

after cell division. In short, their model states that the time at which an initiation begins depends on C, D, and the growth rate. Small changes in these parameters have great effects on the age at which a cell initiates new rounds of replication. The main predictions of the model for the growth rates studies are:

1. Cells with generation times longer than C minutes have periods of DNA non-synthesis.
2. Cells with generation times less than C minutes exhibit multiple replication forks during part of the ir division cycle.
3. Cells with generation times equal to C minutes exhibit no multiple forks, no period of DNA non-synthesis, and the rate of DNA synthesis doubles at mid-cycle.
4. With decreasing generation times, rounds of replication are initiated earlier and earlier in the cell cycle. At very fast rates of growth, rounds are initiated in the previous cycle.

This model also is based on the rates of thymidine incorporation; a decrease in the rate was taken to reflect the advent of a replication point with the end of a chromosome, and an increase was taken to reflect the initiation of new synthesis. Their data correspond to theoretical predictions based on the model, and was shown to be "insensitive" to the actual values of C and D. They have shown that the values for C, D, and (C + D) were constant over the growth rates studied. The prediction that rounds of replication can begin and end in mid-cycle has ample support (Schaechter et al., 1962; Clark and Maaløe, 1967; and Koch, 1966). The model, however, is not strictly

adhered to in slowly growing cells. It predicts a gap in DNA synthesis at the beginning and end of the cell cycle, but the actual data show that replication begins at or somewhat before the beginning of the cell cycle and that C increases with increasing generation times above 100 minutes so that the fraction of the cell cycle devoted to DNA synthesis is relatively constant.

The model of Helmstetter and Cooper was developed in its entirety and published in 1968 (Helmstetter et al., 1968). They incorporated the idea of the existence of an initiator protein required for a round of replication to commence. A unit of this protein accumulates in I minutes and is consumed in the process of DNA synthesis initiation. At slower growth rates, I was found to equal $C + D$ minutes. A cell divisional event, therefore, occurs $I + C + D$ minutes after the initiation of I synthesis. With this model they explain the rate maintenance phenomenon observed during nutritional shift-up. Since C and D are constants, new replication points are not expressed as a divisional event until D minutes after their termination. Those cells in their C and D periods at the time of shift divide in the first $C + D$ minutes (one pre-shift generation time) after shift. After $C + D$ minutes in the new medium, the cells start dividing at the new rate, a result of new replication points being inserted into the genome by a decreased rate of I synthesis.

Since the earlier experiments depicting a rate maintenance

phenomenon of DNA synthesis utilized colorimetric assays for DNA determination, they were not sensitive enough to distinguish between an abrupt attainment of the definitive post-shift rate of DNA synthesis and a gradual one (Maaløe and Kjeldgaard, 1966; Cooper, 1969). This gradual increase in rate could occur if there is no change in the rate of cell division and the cell ends one round of replication and, by pre-mature insertion of replication points due to increased rates of I synthesis, initiates a second before it divides. Cooper (1969), using radiotracer techniques, has shown this to be the case.

In deciding which of the two models is more correct, one must remember that Lark and Helmstetter and Cooper used different strains of E. coli. Lark's strain 15T⁻ is not suitable for membrane elution techniques and cannot be tested by Helmstetter's procedure. The possibility exists that the regulation of replication may be different in 15T⁻. However, the model of Helmstetter and Cooper has gained more widespread acceptance due to several anomalies existing in the model of Lark. First, Lark's model predicts that rounds of replication begin immediately after division. It has been shown that initiation of replication can begin in mid-cycle. Second, according to Lark, C decreases with decreasing generation times even in cells growing at rates of less than 60 minutes/doubling. It has been shown that C is relatively constant in cells with doubling times of less than 60 minutes. Third, the model of Lark predicts an irregular

segregation of the four chromosomes present in glucose and succinate grown cells. In Helmstetter and Cooper's model, each bacterium has only two chromosomes which segregate. Finally, the data of Lark for such glucose and succinate grown cells can be accounted for in the other model by older cells containing two complete non-replicating chromosomes.

It must be pointed out again that for both models much of the data for one is equally compatible with the other and that the possibility of different mechanisms of replication may exist in the two strains. However, this author prefers the model of Helmstetter et al. (1968), and the data presented in this thesis will be discussed in its terms.

In 1969, Wehr and Parks published data on the effects of nutritional shift-up in the eucaryotic system. Some of the data obtained with the simple ascomycete Saccharomyces cerevisiae were strikingly similar to those obtained in bacteria. The data for RNA synthesis after shift showed that a rate of synthesis in excess of the definitive post-shift rate was assumed immediately after shift. This elevated rate was held for approximately 70 minutes before it dropped to the definitive rate of the post-shift medium. The rate of protein synthesis was unchanged for approximately 30 minutes after shift, then it also accelerated to a rate approaching that characteristic of the new medium. There was an apparent partial synchrony between RNA

and protein synthesis at 70 minutes post-shift. This was the time RNA synthesis slowed to its new rate, and this event apparently caused a momentary decrease in the rate of protein synthesis. Between 30 and 70 minutes after the shift-up the rate of protein synthesis was slightly in excess of the rate characteristic of the new medium. After the partial phasing with RNA synthesis at time 70 minutes, the rate of protein synthesis dropped to a more characteristic rate. Aside from the decrease in the rate of protein synthesis, and its initial elevated rate of synthesis, these data are in excellent agreement with the data from bacterial systems. However, the rate of synthesis of DNA after the shift was found to be maintained or to slightly decrease for only 40 minutes before the new rate of synthesis ensued. After approximately 100 minutes, a 30 minute transient decrease in the rate of DNA synthesis concomitant with a decrease in the rate of cell division was observed. It was found that cell growth rate was not maintained after shift. The growth rate increased to the definitive rate immediately after shift, and aside from the slight transient decrease in rate at 100 minutes post-shift, this rate was maintained for the duration of the experiment. These data are not in accordance with the observations from bacterial systems.

The study of DNA constituents in yeast is still in its infancy. At least three fractions of DNA are known to exist, the α or nuclear fraction (n-DNA), the β or mitochondrial fraction (m-DNA), and a

γ fraction. The G + C content for whole cell DNA in the genus Saccharomyces has been reported to be 39% with a 6% spread within the genus (Bicknell and Douglas, 1970).

It has been shown that the nucleus of a haploid yeast contains 17 chromosomes (Mortimer and Hawthorn, 1969). Petes and Fangman (1972) studied the sedimentation properties of yeast nuclear DNA. They found the DNA isolated ranged in size from 6×10^7 to 1.4×10^9 daltons with a number average molecular weight of 6.2×10^8 daltons. This information plus information on the amount of DNA per nucleus (between 8.4×10^9 and 12.0×10^9 daltons) have led these authors to propose that each yeast chromosome contains only one DNA duplex. Early autoradiographic studies by Sarkar and Poddar (1965) demonstrated that the nuclear fraction was stable during log growth. Since then, the nuclear fraction has been shown to have a density of 1.698 g/cm^3 in CsCl gradients (Moustacchi and Williamson, 1966). Recent studies by Christiansen et al. (1971) have shown that 5 - 16% of the nuclear DNA fraction contains repetitive sequences. This high percentage cannot be explained by the 140 copies of r-RNA genes in the genome which only account for 2.4% of the total. Although their intracellular origins are unknown, homogeneous circular DNA molecules have been isolated with the same density as n-DNA (Guerineau et al., 1971). These molecules are 2.2μ long, or multiples of 2.2μ , and account for only between 1 and 5% of the total cell DNA. These

closed circular molecules are closely associated with membranes; however, it is not certain yet whether or not these molecules represent extraction artifacts.

Mitochondrial DNA from many different organisms exhibit quite different properties than the nuclear DNA. Sinclair et al. (1967a) have shown the m-DNA of chick, rat, and beef tissue to be quite similar. They are circular with a contour length of 5 μ and a molecular weight of 10^7 daltons. Nass (1966) reported that while n-DNA is linear, m-DNA is circular, with two to six molecules per mitochondrion which appear to be membrane bound in at least one point. Frog m-DNA behaves as a closed, circular, double stranded molecule with a constant number of superhelices per unit length of DNA. Circular m-DNAs have been reported in yeasts (Shapiro et al., 1968), but the percentage of covalently closed circular molecules was small. But, it was shown that the linear yeast m-DNA had cohesive ends that could form hydrogen-bonded circles. The length of yeast m-DNA was 4.0 to 4.5 μ although linear oligomers of integral multiples of the basic unit exist. Other researchers have found no evidence for the existence of circular m-DNAs in yeast (Sinclair et al., 1967b).

The initial studies on m-DNA demonstrated that this fraction has a lighter buoyant density than the nuclear fraction (1.684 g/cm³ vs. 1.698 g/cm³) and that it undergoes renaturation more easily than DNA from the nucleus, thus suggesting a greater homogeneity of base

composition (Corneo et al., 1966). Depending on growth conditions, this fraction accounts for 5 - 15% of the total DNA (Moustacchi and Williamson, 1966) and is very AT rich (Tewari et al., 1966). Tewari et al. (1966) demonstrated that there were great differences between m-DNA and n-DNA with respect to their absorption spectra at various pHs, thermal transition temperatures, buoyant densities, and base composition. They found values of 35% and 21% for the G + C content of yeast nuclear and mitochondrial DNA respectively. Mitochondrial DNA exists as a double helix which exhibits a density increase in CsCl gradients of 0.015 g/cm^3 after heating at 100° C for 10 minutes (Sinclair, et al., 1967b). Recent ORD and CD studies on purified yeast m-DNA suggest that both alternating poly (dAT:dAT) and non-alternating poly (dA:dT) structures are responsible for the peculiar properties of m-DNA (Bernardi and Timasheff, 1970). These peculiarities include G + C contents calculated from buoyant densities in disagreement with empirical analyses, higher phosphate buffer elution molarities from hydroxyapatite columns than n-DNA, and apparent higher buoyant densities in $\text{Ag}^+ \text{Cs}_2\text{SO}_4$ gradients than DNAs of higher G + C content. Tewari et al., (1966) suggested that the preferential interaction of m-DNA with acridine dyes is a result of its AT rich nature.

Although lesions resulting in petite phenotype can originate in either the nuclear or mitochondrial genomes, the preferential

interaction of m-DNA with intercalating agents such as acridine and ethidium bromide make these dyes ideal mutagens for the induction of cytoplasmic petites. Petite mutants are those in which respiratory capability is lost. Neutral petites are cytoplasmic petites which give rise to wild-type progeny when crossed with a wild-type mate. Suppressible petites are petites which give rise to petite progeny when crossed with a wild-type mate. Mounolou, Jacob, and Slonimski (1966) have shown that while a wild-type m-DNA had a buoyant density of 1.687 g/cm^3 , the cytoplasmic neutral petite tested had a lesser density at 1.683 g/cm^3 and the cytoplasmic suppressible petite tested had a greater density at 1.695 g/cm^3 . Bernardi et al. (1970) also reported that irreversible loss of respiration in cytoplasmic petites was accompanied by altered m-DNA buoyant densities and base composition. A large heterogeneity of base composition existed in both petite and grande (wild-type) m-DNAs and their melting curves were dissimilar. The variable decrease in the G + C content in the petites tested, suggested that the respiratory factor, ρ , responsible for cytoplasmic petite mutation, was lodged in the mitochondrial genome. Slonimski, Perodin, and Croft (1968) have studied the induction of cytoplasmic petites with ethidium bromide, and have shown that the conversion of grandes to petites follows first order kinetics. Nass (1970) demonstrated with mammalian cells treated with $1 \mu\text{g/ml}$ ethidium bromide that the synthesis and integrity of

closed circular m-DNA was destroyed while n-DNA remained unaffected. This led to enlarged mitochondria with few or incomplete cristae, the formation of which was proportional to incubation time in the presence of the dye. Recent evidence indicates that brief exposure to ethidium bromide results in a novel species of m-DNA which is characterized by a lighter buoyant density and a greater heterogeneity than the wild-type m-DNA. This species of reduced size is stable after 20 generations of growth (Perlman and Mahler, 1971). It has been demonstrated that as incubation time in 10 $\mu\text{g/ml}$ ethidium bromide increased, the molecular weight of m-DNA decreased until mitochondrial DNA-less mutants were obtained (Goldring et al., 1970; Goldring, Grossman, and Marmur, 1971). Fukuhara and Kujawa (1970) demonstrated that acriflavin and ethidium bromide inhibited in vivo m-DNA synthesis and inhibited transcription of the mitochondrial genome.

The γ fraction of yeast DNA is still quite unintelligible. It appears as a shoulder on the heavy side of the nuclear peak and has a density of 1.704 g/cm^3 (Moustacchi and Williamson, 1966). This fraction was more prominent in older cultures than in younger ones and it was suggested that this fraction is nucleolar in origin and represents DNA in the act of being transcribed.

Williamson and Scopes (1960) using synchronized cultures of S. cerevisiae showed that the appearance of buds was closely followed

by a doubling of cellular DNA. Williamson (1964) demonstrated that DNA synthesis was initiated coincident with bud outgrowth which occurred just after completion of division. He later demonstrated that DNA synthesis occupied the first 25% of the division cycle (Williamson, 1965). Tonino and Rozijn (1966) reported that although yeasts do not form condensed chromosomes during cell division, a well defined nucleus is present. They reported similar data for Saccharomyces carlsbergensis (Rozijn and Tonino, 1964). Ramirez and Miller (1962) demonstrated that nuclear division occurred in the isthmus between the mother and daughter cell. The actual division occurred in either the mother cell or bud, with a subsequent nuclear migration to the cell lacking a nucleus. The cell cycle of the fission yeast Schizosaccharomyces pombe is somewhat different; the growth cycle is broken down into four steps: a G_1 , pre-DNA synthetic phase; S, DNA synthetic phase; G_2 , post-DNA synthetic phase; and M, division phase (Duffus and Mitchell, 1970). Eckstein, Paduch, and Hilz (1967) showed that DNA polymerase activity was cyclic in synchronous cultures of S. cerevisiae, and Wintersberger and Wintersberger (1970a, b) have isolated three DNA polymerases--two nuclear and one mitochondrial.

There are conflicting reports in the literature with respect to the time of synthesis of mitochondrial DNA in the growth cycle. Smith et al. (1968) have presented evidence that for cells derepressed

for mitochondrial function, m-DNA was synthesized periodically just prior to n-DNA synthesis in synchronized cultures of Saccharomyces lactis. Similar data was obtained in Tetrahymena and Physarum. In all three cases, n-DNA was synthesized in the early part of the cycle as in S. cerevisiae. They suggest that a nuclear coded initiator protein is necessary for m-DNA synthesis. Williamson and Moustacchi (1971), however, showed in synchronous cultures of S. cerevisiae that n-DNA is synthesized discontinuously and m-DNA continually throughout the cell cycle. This implies that the initiation of new rounds of m-DNA synthesis occurs randomly, rather than at a specific initiation site on the genome. There is no doubt, however, that a considerable degree of independent control from n-DNA exists with respect to m-DNA synthesis. The synthesis of m-DNA can be dissociated from nuclear DNA. Rabinowitz et al. (1969) have shown that there is preferential synthesis of m-DNA during respiratory adaptation. The specific activity of the mitochondrial fraction was shown to increase from 6 to 30 times over n-DNA when an anaerobic culture was pulsed with oxygen. Uptake of label was maximal during the first 30 minutes after shift. It was shown that this preferential synthesis of m-DNA was inhibited by 5% glucose in the medium. Grossman, Goldring, and Marmur (1969) demonstrated preferential m-DNA synthesis under conditions of protein synthesis inhibition. These conditions could be induced by cyclohexamide (CH), amino acid

acid starvation, and ts mutants. Oddly, m-DNA synthesis continued in the presence of either CH or CAP. This suggests that either an initiator protein for m-DNA synthesis does not exist (since m-DNA synthesis continues in the absence of both cytoplasmic and mitochondrial protein synthesis) or that there is a large enough pool of the protein to initiate one or more rounds of m-DNA synthesis.

MATERIALS AND METHODS

Organisms

In the course of these investigations, two haploid strains and one diploid strain of Saccharomyces cerevisiae were used. Strain 5015-D was obtained from Dr. H. L. Roman, Department of Genetics, University of Washington, and has the haploid genotype: a, me-2, ad-2, ur, tr-1. From this grande, a mitochondrial DNA-less petite, EB-5, was derived. In addition to adenine, uracil, tyryptophan, and methionine, this organism required thiamine and lysine. The initial attempts to produce a mitochondrial DNA-less petite were performed with a diploid. This strain was a cross between 5015-D and 5011-D. 5011-D is a sister strain of 5015-D, an a mating type with the same nutritional requirements.

Growth Conditions

Minimal medium in all experiments was Wickerham's minimal medium plus required nutritional supplements (Wickerham, 1946). Added amino acids were present at a concentration of 5 mg/l, nucleic acid precursors at 10 mg/l, and thiamine at 0.4 mg/l. For the remainder of this discussion Wickerham's minimal medium plus the required added nutrients for a particular organism will be referred to as WM. Tryptone culture broth (TCB) contains 5 g yeast extract,

10 g tryptone, and 20 g glucose per liter. In order to maximize the yields of mitochondrial DNA for the CsCl analyses of 5015-D and two petite strains, cells were grown to stationary phase in 25 ml of WM containing galactose rather than glucose. This was used in only one experiment as growth of petites in galactose was poor. Stationary glucose grown cells were used for the remaining petite strains tested.

The petite strain used in the remainder of this work grows extremely slowly in WM with the four supplements of the parental strain. Consequently, feeding experiments were performed that revealed that lysine in combination with thiamine could restore normal growth (defined as a generation time similar to the parent--300 minutes in WM). For the double labeled shift-up experiments, it was desirable to limit the amounts of exogenous cold adenine in the medium; with TCB, this was impossible. An artificial medium was prepared, using WM as a base that, in addition to all nucleic acid precursors and vitamins, contained 10 g/l vitamin-free casamino acids. This medium, designated as Ultra+, gave 5015-D and EB-5 approximately the same generation times as TCB. Table 1 lists the components of the media used. In all experiments growth temperature was 30° C. Thermal shock to the culture at the time of shift was prevented by pre-warming the post-shift medium and filter apparatus to 30° C.

Table 1. Composition of growth media.

Component	Concentration in Grams/Liter			
	Ultra+	WM (5015-D)	WM (EB-5)	TCB
Glucose	20	20	20	20
Yeast Extract	-	-	-	5
Tryptone	-	-	-	10
H ₃ BO ₃	1 x 10 ⁻⁵	1 x 10 ⁻⁵	1 x 10 ⁻⁵	-
CuSO ₄ · 5H ₂ O	1 x 10 ⁻⁵	1 x 10 ⁻⁵	1 x 10 ⁻⁵	-
KI	1 x 10 ⁻⁵	1 x 10 ⁻⁵	1 x 10 ⁻⁵	-
FeCl ₃ · 6H ₂ O	5 x 10 ⁻⁵	5 x 10 ⁻⁵	5 x 10 ⁻⁵	-
ZnSO ₄ · 7H ₂ O	7 x 10 ⁻⁵	7 x 10 ⁻⁵	7 x 10 ⁻⁵	-
KH ₂ PO ₄	0.875	0.875	0.875	-
KH ₂ P ₂ O ₄	0.125	0.125	0.125	-
MgSO ₄ · 7H ₂ O	0.5	0.5	0.5	-
NaCl	0.1	0.1	0.1	-
CaCl ₂ · 2H ₂ O	0.1	0.1	0.1	-
(NH ₄) ₂ SO ₄	1.0	1.0	1.0	-
Biotin	2 x 10 ⁻⁶	2 x 10 ⁻⁶	2 x 10 ⁻⁶	-
Adenine Sulfate	0.01	0.01	0.01	-
Uracil	0.01	0.01	0.01	-
Methionine	0.005	0.005	0.005	-
Tryptophan	0.005	0.005	0.005	-
Lysine	-	-	0.005	-

Table 1. Composition of growth media (continued).

Component	Concentration in Grams/Liter			
	Ultra+	WM (5015-D)	WM (EB-5)	TCB
Casamino Acids	10.0	-	-	-
Guanine	0.001	-	-	-
Cytosine	0.005	-	-	-
Thymine HCl	4×10^{-4}	-	-	-
Thiamine	4×10^{-4}	-	4×10^{-4}	-
Pyridoxine HCl	4×10^{-4}	-	-	-
Inositol	0.002	-	-	-
Ca pantothenate	4×10^{-4}	-	-	-
p-aminobenzoic Acid	2×10^{-4}	-	-	-
Riboflavin	2×10^{-4}	-	-	-
Nicotinic Acid	4×10^{-4}	-	-	-

Manner of Shift-up

For a shift-up experiment, cells were grown in WM overnight at 30° C before shift to TCB. Shifting of cultures was performed by filtration on Millipore filters (type HA, pore size 0.45 μ) and by placing the collected cells and filter into pre-warmed (30° C) TCB. Filters had been washed thoroughly, firstly in distilled water for 3 - 4 hours, secondly, 500 ml distilled water was passed through each filter prior to sterilization, and finally on the filter holder with 250 ml pre-warmed (30° C) distilled water just prior to shift-up. The washing was done to remove glycerol and detergents in the filter that keep it pliable.

Cell growth was monitored with a Model F Coulter Counter equipped with a 100 μ aperture, aperture setting 16, attenuation 0.707, and threshold setting of 10.4. Cells were diluted 1:50 to 1:200 in 0.9% saline containing 0.2% formalin. Since 5015-D forms cell aggregates rather than separating after division, cell suspensions were sonicated 45 seconds prior to counting with a Model W-140-C Branson Cell Disruptor set at a power output of 100 watts. As the diploid clumped to a lesser degree, it was sonicated only 10 seconds. During the adenine uptake studies, cell mass increase was monitored by change in A_{520} on a Zeiss PMQ II Spectrophotometer.

When a double label shift-up was performed, cell growth was

monitored on a Klett-Summerson Colorimeter. Procedures were identical in these experiments except that cells were grown overnight in the presence of 5 - 10 $\mu\text{Ci/ml}$ ^3H -adenine; and at 45 minutes prior to shift-up, 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine was added. At the time of shift the cells were washed on the filter with 50 ml pre-warmed WM plus 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine. The filter was placed in pre-warmed Ultra+ containing 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine, and samples were taken at timed intervals. In one experiment, cells were shifted to Ultra+ medium containing 5% glucose rather than 2%.

DNA Determination

DNA was assayed by the 3,5-diaminobenzoic acid fluorometric procedure of Kissane and Robins (1958). Duplicate 5 ml cell samples were added to 0.5 ml ice cold 55% trichloroacetic acid (TCA) in chilled centrifuge tubes. Samples were stored under refrigeration until use. The cells were collected by a 10 minute centrifugation, washed with 5% ice cold TCA, re-collected by centrifugation, and the TCA extracted by two washings with ice cold 0.1 N potassium acetate in 95% ethanol. The pH of the pooled second washings was determined and if acidic, a third wash was performed. The pH was generally between 8.0 and 8.5. At this point two hot (60°C) 95% ethanolic extractions were performed, each of 30 minute duration. In all cases, the supernatant after centrifugation was carefully pipetted off,

leaving about 0.5 ml covering the pellet. The pellet following the last hot alcohol extraction was evaporated to dryness under vacuum overnight and 200 μ l diaminobenzoic acid reagent was added. The reagent was mixed well and care was taken to insure that the entire inside of the centrifuge tube was covered. This was done to insure that DNA which had precipitated on the glass walls of the tube during processing came in contact with the reagent. The tubes were tightly capped and incubated, with mixing at 15 and 30 minutes, at 60^o C for 30 minutes. The fluorescence was diluted with 5.0 ml 0.6 N perchloric acid, the cell debris collected by centrifugation, and 3 ml samples were read on an Aminco Fluoro-microphotometer with a primary wavelength of 405 nm and a secondary wavelength of 520 nm. Values for DNA/ml were read from a standard curve using calf thymus DNA.

Petite Induction

Mitochondrial DNA-less petites were induced by a modified ethidium bromide technique of Goldring et al. (1970). To an exponential culture of 5015-D in WM, 15 μ g/ml ethidium bromide was added. At regular intervals 1 ml aliquot fractions were diluted in 0.1 M phosphate buffer, pH 6.6, and plated on tryptone culture agar. Petites were scored by the triphenyltetrazolium chloride overlay procedure of Ogur, St. John, and Nagai (1957).

Assay for Mitochondrial DNA

Suspected petites were assayed for the presence of mitochondrial DNA by CsCl gradients. Cells were grown to stationary phase in WM plus 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine and then spheroplasted by the method of Goldring et al. (1970). Cells were collected by centrifugation; washed once with distilled water; incubated 30 minutes at 23 $^{\circ}\text{C}$ with 0.5 M sodium thioglycollate in 0.1 M TRIS buffer, pH 8.8; and converted to spheroplasts by the action of 0.05 ml Glusulase in 2.0 ml 1 M sorbitol for one hour at 30 $^{\circ}\text{C}$. The spheroplasts were washed once with cold 1 M sorbitol and transferred to nitrocellulose ultracentrifuge tubes where the method of Grossman, Goldring, and Marmur et al. (1969) for preparative ultracentrifugation was used. The spheroplasts were lysed by 0.6 ml 1.3% sodium lauryl sulfate in 0.1 X SSC (SSC is 0.015 M sodium citrate in 0.15 M NaCl) and 0.2 ml nuclease-free 2.0 mg/ml Pronase was added. The Pronase was heat treated by the method of Hotta and Bassel (1965) to destroy DNase present in the crude preparation. After enzymatic digestion for one hour at 23 $^{\circ}\text{C}$, 4.0 ml of saturated CsCl solution were added and the gradients overlaid with mineral oil. Samples were centrifuged 60 - 66 hours at 18 $^{\circ}\text{C}$ and 31,000 rpm in Beckman L2 or L2-65 Preparative Ultracentrifuges. Eight drop fractions were collected, adjusted to 0.5 M NaOH, and incubated overnight at 37 $^{\circ}\text{C}$. After

alkaline RNA digestion, a carrier of 100 μg bovine serum albumin was added to each tube and was then precipitated with 0.6 ml 20% TCA. The samples were chilled and collected on nitrocellulose membrane filters (Schleicher and Schuell, type B-6), dried, and counted on a Model 3000 Packard Tri-Carb Liquid Scintillation Spectrometer. A toluene based cocktail containing 8.04 g/l PPO (2,5 diphenyloxazole) and 0.2 g/l POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene) was used.

Adenine Uptake

Adenine uptake into the pools was measured by adding 0.2 μCi /ml of ^{14}C -adenine to a balanced culture of 5015-D growing in WM. At frequent intervals duplicate 1.0 ml aliquot samples were pipetted into 1 ml ice cold 10% TCA. These samples were refrigerated overnight to allow low molecular weight components to diffuse out of the cells. The cells were collected on nitrocellulose membrane filters and washed with 15 ml ice cold 5% TCA. The filters were dried and counted by standard liquid scintillation techniques. For determination of label uptake into RNA and DNA, duplicate 1.0 ml samples were pipetted both into ice cold 2 M NaOH and ice cold 10% TCA. The TCA samples were treated as above; the base samples were allowed to incubate overnight at room temperature. They were then chilled, neutralized with cold 2 M HCl, 100 μg bovine serum albumin

was added as a carrier, and they were then brought up to 5% TCA. All samples were then collected on nitrocellulose membrane filters, dried, and counted by standard liquid scintillation techniques. As all small molecular weight components, such as nucleotides, etc., were extracted from the cells in the TCA treatment, these samples represented total radioactivity incorporated into nucleic acids. The base treated cells represented label in DNA. The label in RNA was the difference between the two.

Materials

Unless otherwise indicated all chemicals used were of the highest available purity and were used as obtained from commercial sources. 3,5-diaminobenzoic acid dihydrochloride was obtained from Aldrich Chemical Co. The reagent was prepared by dissolving 0.4 g per milliliter of glass distilled water.

RESULTS

Effects of Shift-up in 5015-D

The effects of shift-up from a medium supporting a doubling time of 0.2 doublings/hour (WM) to one supporting 0.6 doublings/hour (TCB) is shown in Figure 1. This typical experiment shows that during the first 30 minutes after shift-up there is an increase in the division rate from 0.20 to 0.48 doublings/hour. This approximate doubling of growth rate results in an increase in total cell number amounting to an average 7% over an unshifted culture after an equivalent 30 minutes growth. At 30 minutes post-shift, cellular division ceases for an apparent 5 - 10 minutes before resuming a rate approximating that characteristic of the pre-shift medium. This rate is maintained for an approximate pre-shift generation time, whereupon the culture's growth rate abruptly shifts into the definitive rate characteristic of TCB grown cells. The actual cell concentration at the time of shift in this experiment was 5.52×10^6 cells/ml; one would expect a shift-up in growth rate to occur at a concentration of 11.04×10^6 cells/ml. The experimental value at this time was 10.4×10^6 -- a value within 6% of the predicted value. This is considered to be within experimental error. Also, the culture's generation rate shifts up 20 minutes earlier than predicted. This is a 7% reduction of the expected time of shift and is presumably due to the 7% increase in

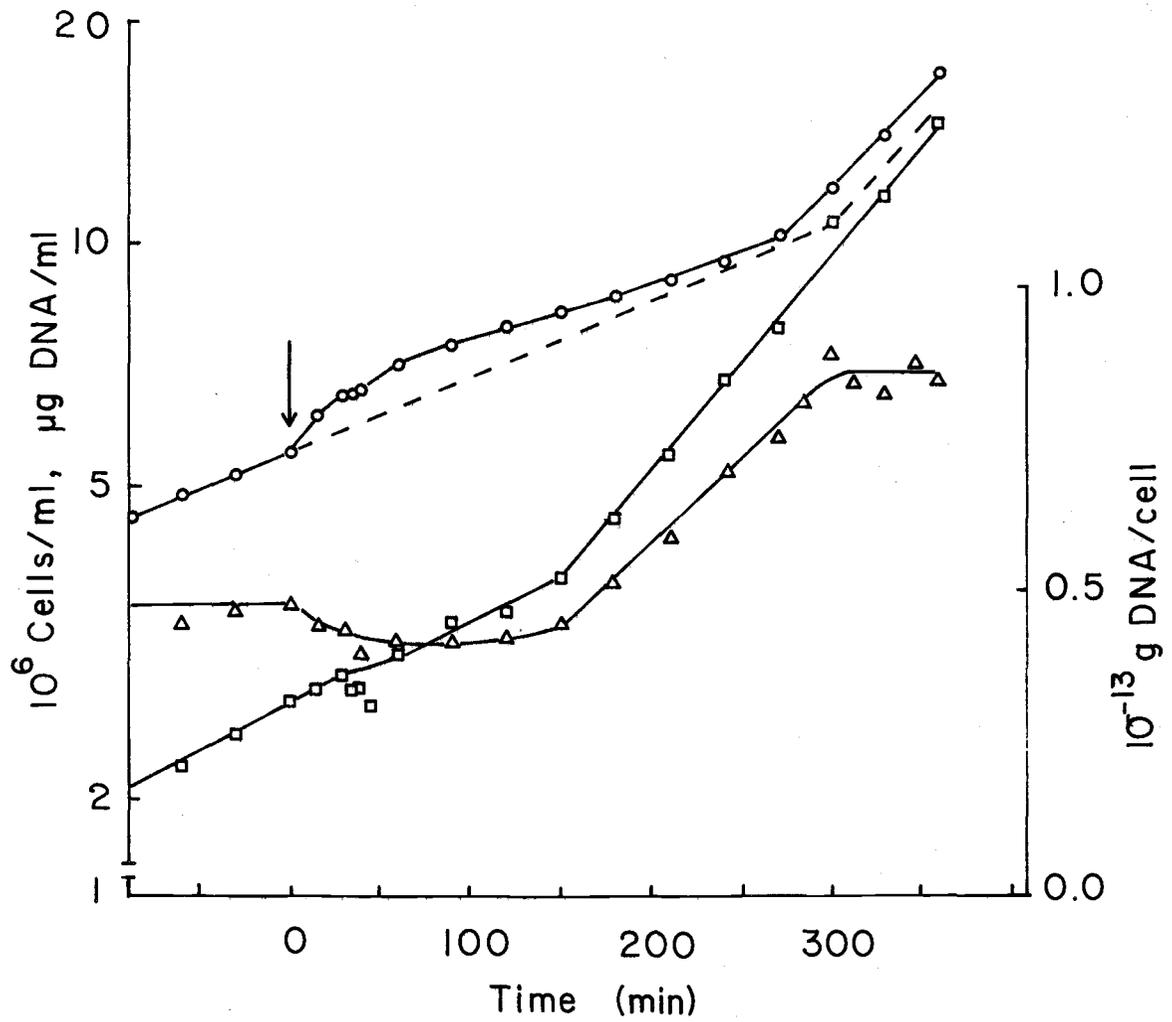


Figure 1. Shift-up of 5015-D. Symbols: (○) cell number; (□) DNA/ml; (△) DNA/cell. The dashed line represents a theoretical curve for the expected behavior of a culture in which C and D remain constant after shift and are equal to 300 minutes. The arrow denotes time of shift.

cell population during the first 30 minutes after shift-up.

The pre-shift rate of DNA synthesis is maintained 150 minutes following nutritional enrichment before the rate of synthesis characteristic of the new medium is assumed. The scatter of points between 30 and 45 minutes after time 0 are within 10% of the 30 minute value and are assumed to be within experimental error of each other. These points seem to indicate a decrease in the rate of DNA synthesis concomitant with the decrease in cell division at this time.

DNA per cell is constant before the shift. The characteristic value in minimal medium of 0.50×10^{-13} g/cell drops slightly following shift-up as the cellular growth rate is higher than the DNA synthetic rate. After the apparent partial synchrony between DNA synthesis and cell growth, the DNA/cell ratio climbs rapidly until at one pre-shift generation time after shift, the proper DNA/mass ratio for the new medium has been established and is maintained at the level characteristic of TCB grown cells, approximately $.75 \times 10^{-13}$ g/cell.

If the values of C and D for a given growth medium are held constant during the transition of steady states in a shift-up, one would expect a rate maintenance phenomenon (Helmstetter et al., 1968). The dashed line in Figure 1 represents a theoretical curve for the expected behavior of such a culture growing in WM with a value of C + D equal to 300 minutes.

Petite Induction

Although it was not observed in all experiments, several shift-up experiments with 5015-D indicated an apparent increase in DNA synthesis during the first 30 minutes after time 0; and, as it was of interest to ascertain the role of the mitochondrial DNA fraction during the transition of steady states (i. e. partial respiratory adaptation to meet increased energy needs), a mitochondrial DNA-less mutant was obtained. The initial study of petite induction was performed with a diploid strain as it was hoped that shift-up experiments with synchronous cultures could be performed. However, the diploid was abandoned when difficulty was encountered in obtaining a true mitochondrial DNA-less mutant. Data for the kinetics of petite induction in the diploid, a cross between 5015-D and 5011-D, a mutant with the same markers and originating from the same parental strain, are shown since such detailed studies were not performed in the haploid 5015-D. As the intercalating agent, ethidium bromide, is specific for cytoplasmic DNA (Nass, 1970; Fukuhara and Kujawa, 1970), there is no reason to suppose that the kinetics of induction would be different. Figure 2 shows a culture of 5015-D x 5011-D growing in WM to which is added 10 μ g/ml ethidium bromide. It can be seen that there is a transient reduction in the growth rate for the first 30 minutes after the cells are in contact with the dye. The cells then resume a

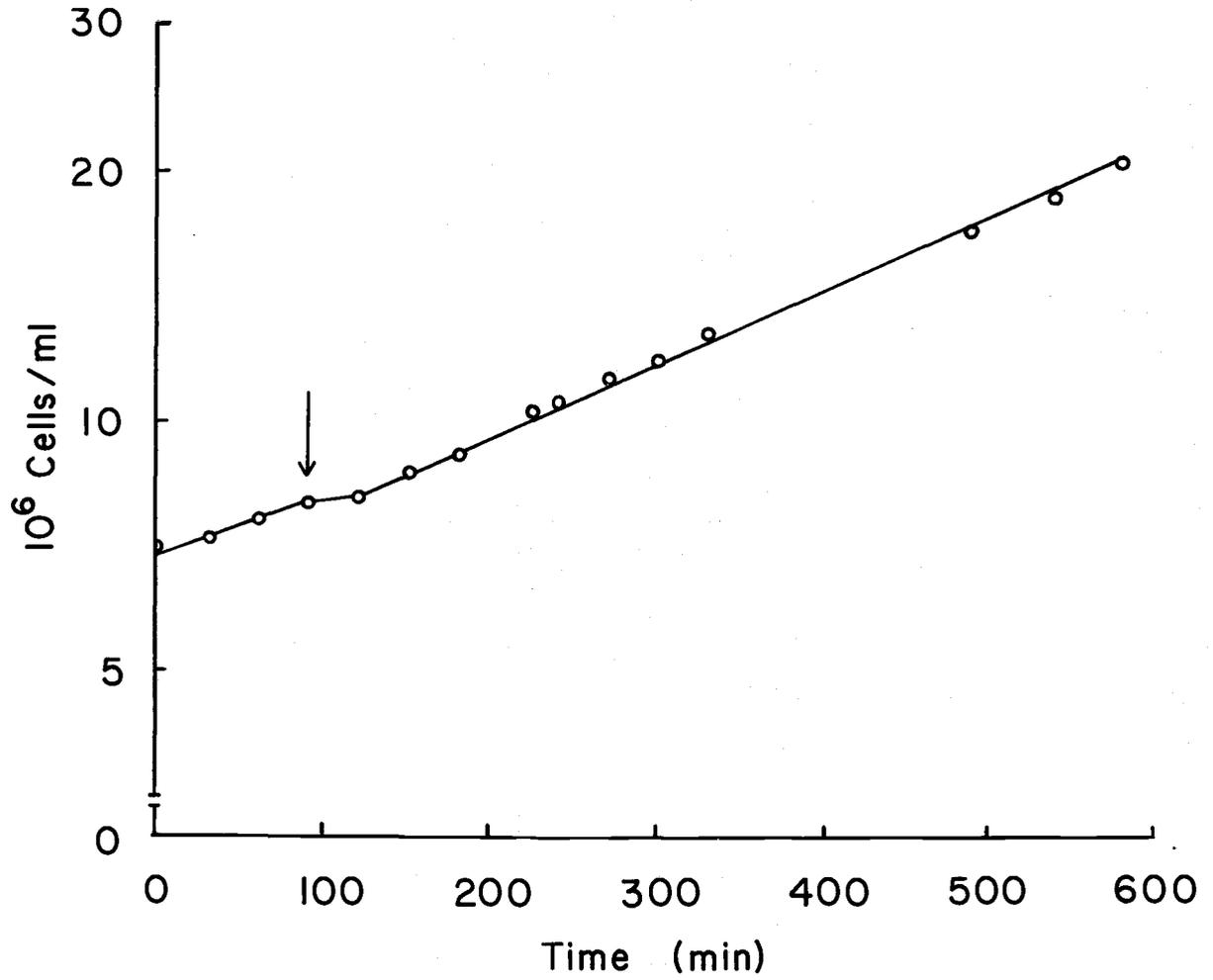


Figure 2. Induction of petites with ethidium bromide in 5015-D X 5011-D. The arrow marks addition of 15 $\mu\text{g/ml}$ ethidium bromide.

generation time of 335 minutes, apparently unaffected by the dye even after more than one generation after addition.

Figure 3 depicts the seven hour rise in the percentage of petites after dye addition from a normal spontaneous frequency of approximately 2.5% to a maximum of 90%. When one plots the log of the percent petites against time as in Figure 4, one obtains a straight line until maximum conversion is reached. This is indicative of a first order reaction. This is in agreement with the data of Slonimski, Perodin, and Croft (1968). The log of percent petites scored by triphenyltetrazolium chloride overlays was plotted as it eliminated plating irregularities due to the aggregates of yeast cells. The diploid doesn't clump as much as the haploid parentals, but a 10 second sonication is required to produce a maximum cell count as assayed by a Coulter Counter. Such a 10 second sonication could not be performed aseptically; consequently, cell samples were mixed thoroughly in the dilution blanks on a vortex mixer. It was hoped that this would give good enough separation so that each colony resulted from a single cell. Petite colonies are white under triphenyltetrazolium chloride, while grandes are pink. Many sectored colonies were observed which were not scored. There was still a larger discrepancy between plate count and Coulter count values than can be accounted for by the non-scored sectored colonies, suggesting that considerable clumping was still present.

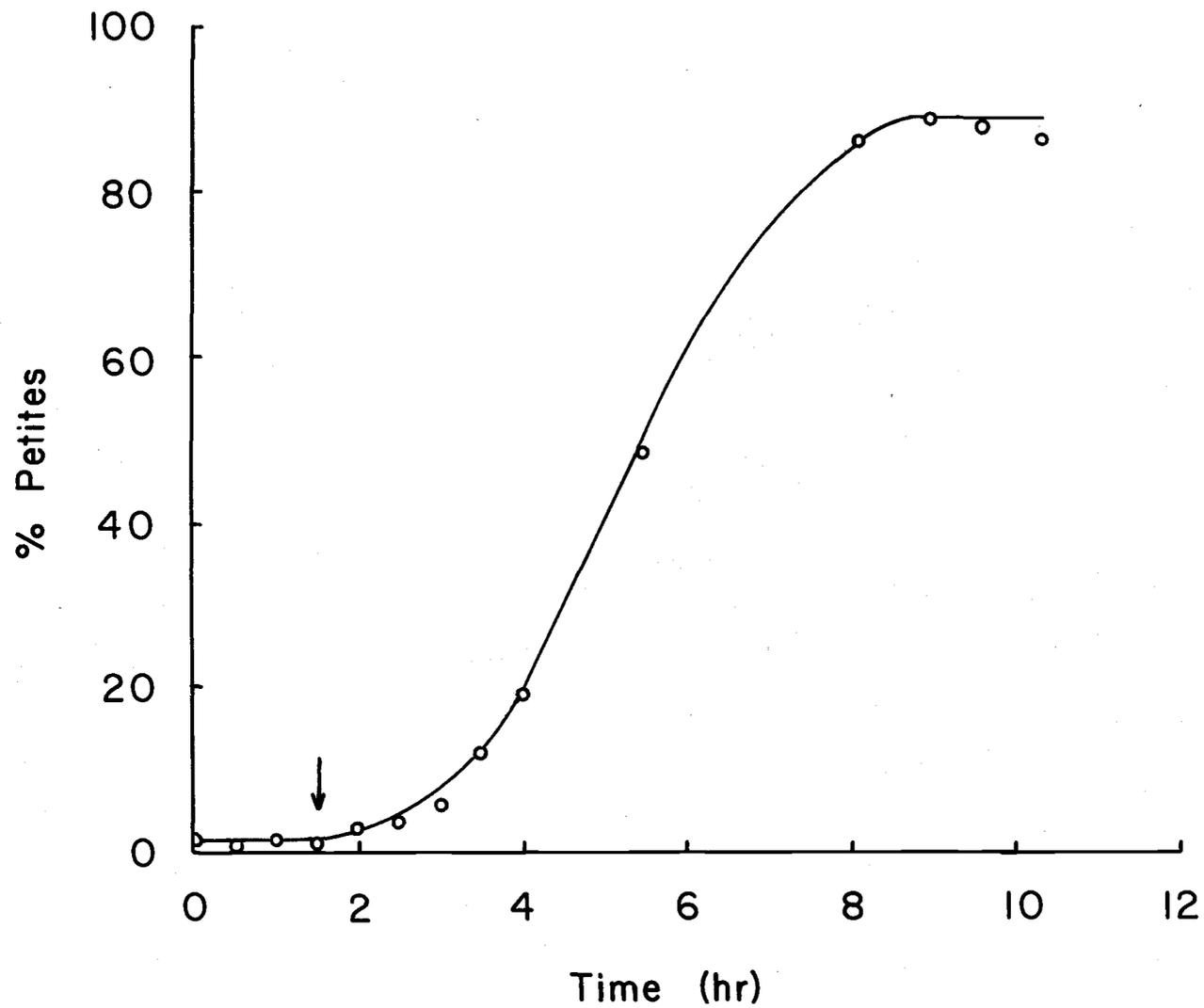


Figure 3. Increase in percentage of petites in a culture of 5015-D X 5011-D. The arrow denotes time of addition of 15 $\mu\text{g/ml}$ ethidium bromide.

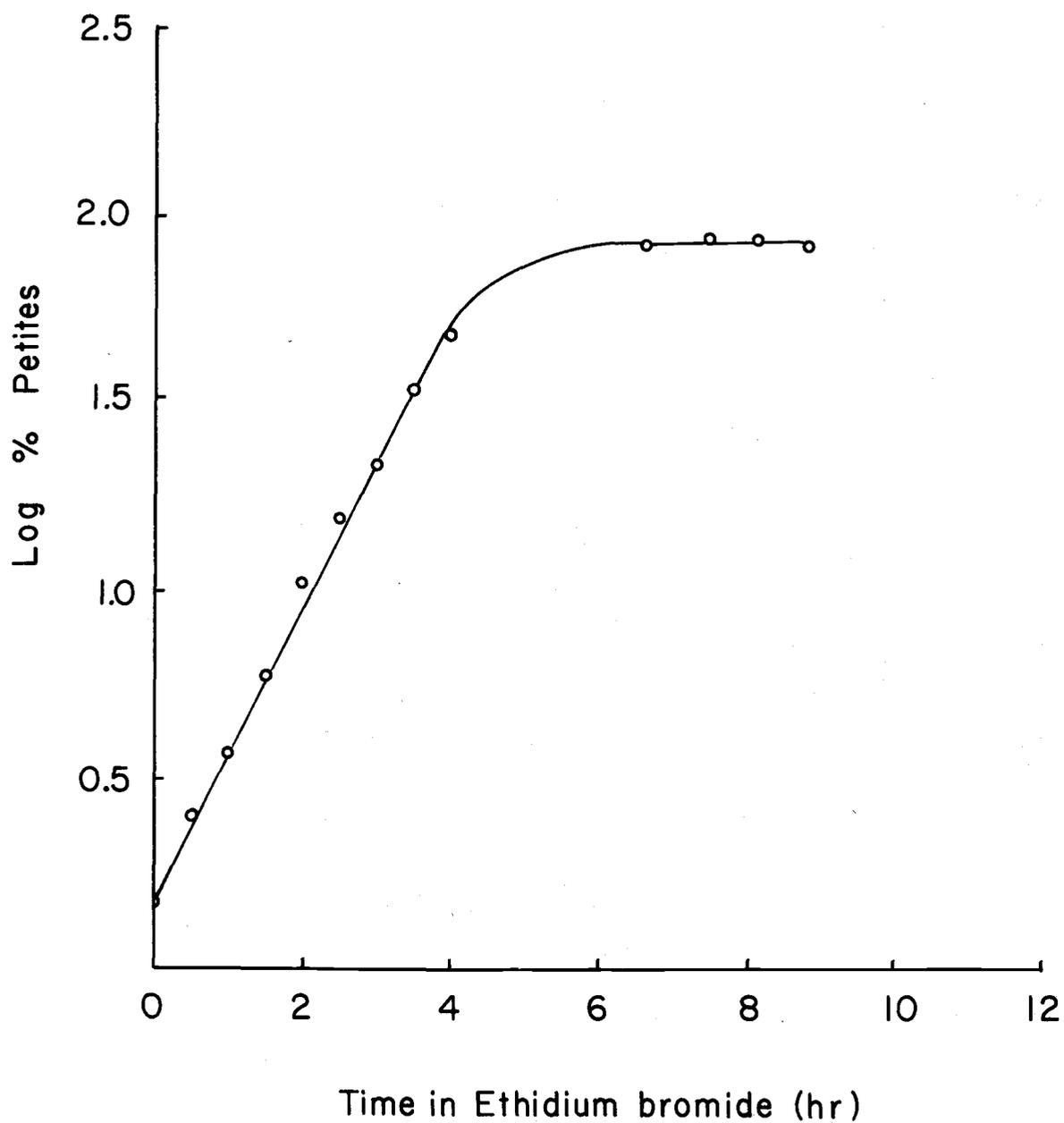


Figure 4. Kinetics of petite induction. At time 0, 15 $\mu\text{g/ml}$ ethidium was added to a culture in balanced growth.

Since 10 $\mu\text{g/ml}$ ethidium bromide failed to induce a mitochondrial DNA-less mutant in the diploid, the haploid 5015-D was incubated in WM containing 15 $\mu\text{g/ml}$ of the dye. EB-5 was isolated after 24 hours in the presence of the dye. Mitochondrial DNA was assayed by CsCl density gradient centrifugation. To maximize the amount of DNA in the positive control, 5015-D, the organism was grown to stationary phase in WM + galactose rather than glucose. This sugar does not inhibit mitochondrial function (Tustanoff and Bartley, 1964).

Figure 5 depicts a gradient pattern of 5015-D. As determined by refractive indices, the density of the nuclear peak is 1.702 g/cm^3 , the mitochondrial peak was 1.678 g/cm^3 . Figure 6 depicts a gradient pattern of the mutant EB-5; density of the nuclear peak is 1.703 g/cm^3 .

Shift-up of EB-5

A shift-up experiment identical in procedure to that using 5015-D was performed using EB-5. Figure 7 shows a 10 hour culture of EB-5 shifted from a medium supporting a growth rate of 0.175 doublings/hour (WM) to one supporting 0.455 doublings/hour. Aside from a 15 minute lag in the short burst of cell division after shift, there is no difference between the wild-type (Figure 1) and the mitochondrial DNA-less mutant. The 15 minute lag in the 30 minute burst of cell division was consistently observed, and also resulted in an

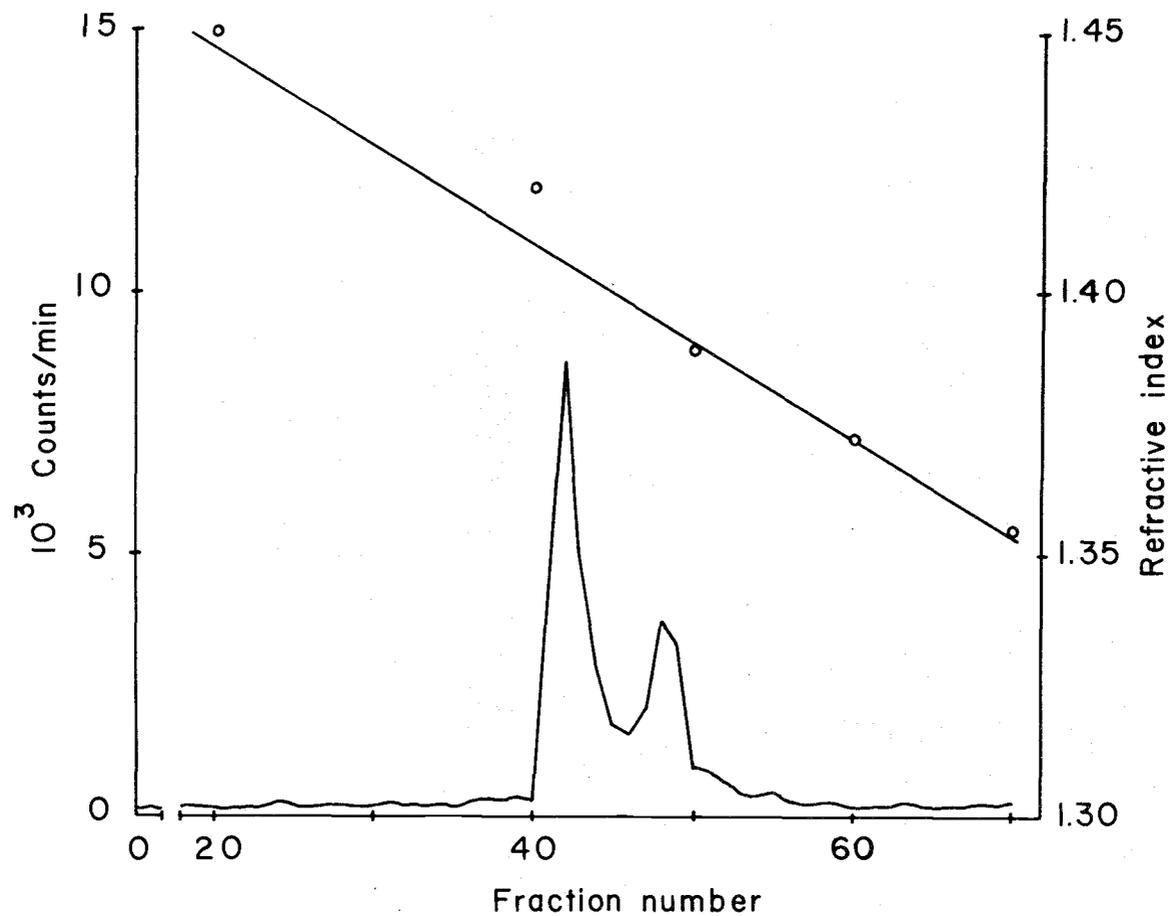


Figure 5. CsCl gradient pattern of 5015-D. Symbols: (○) Refractive index, the solid line represents ¹⁴C counts per minute. The densities of the nuclear and mitochondrial peaks were 1.702 g/cm³ and 1.678 g/cm³ respectively.

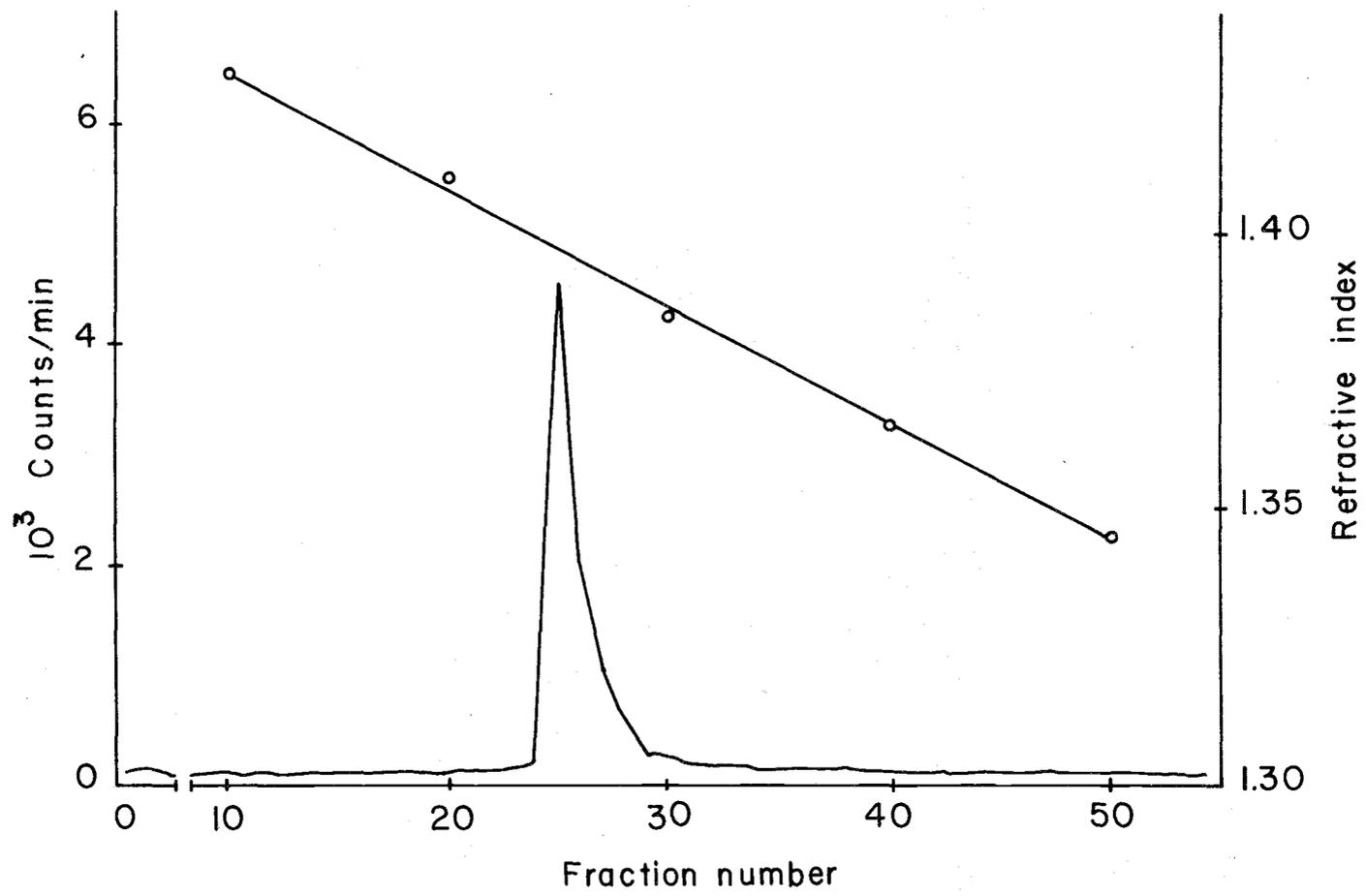


Figure 6. CsCl gradient pattern of EB-5. Symbols: (○) Refractive index, the solid line represents ¹⁴C counts per minute. Density of the peak was 1.703 g/cm³.

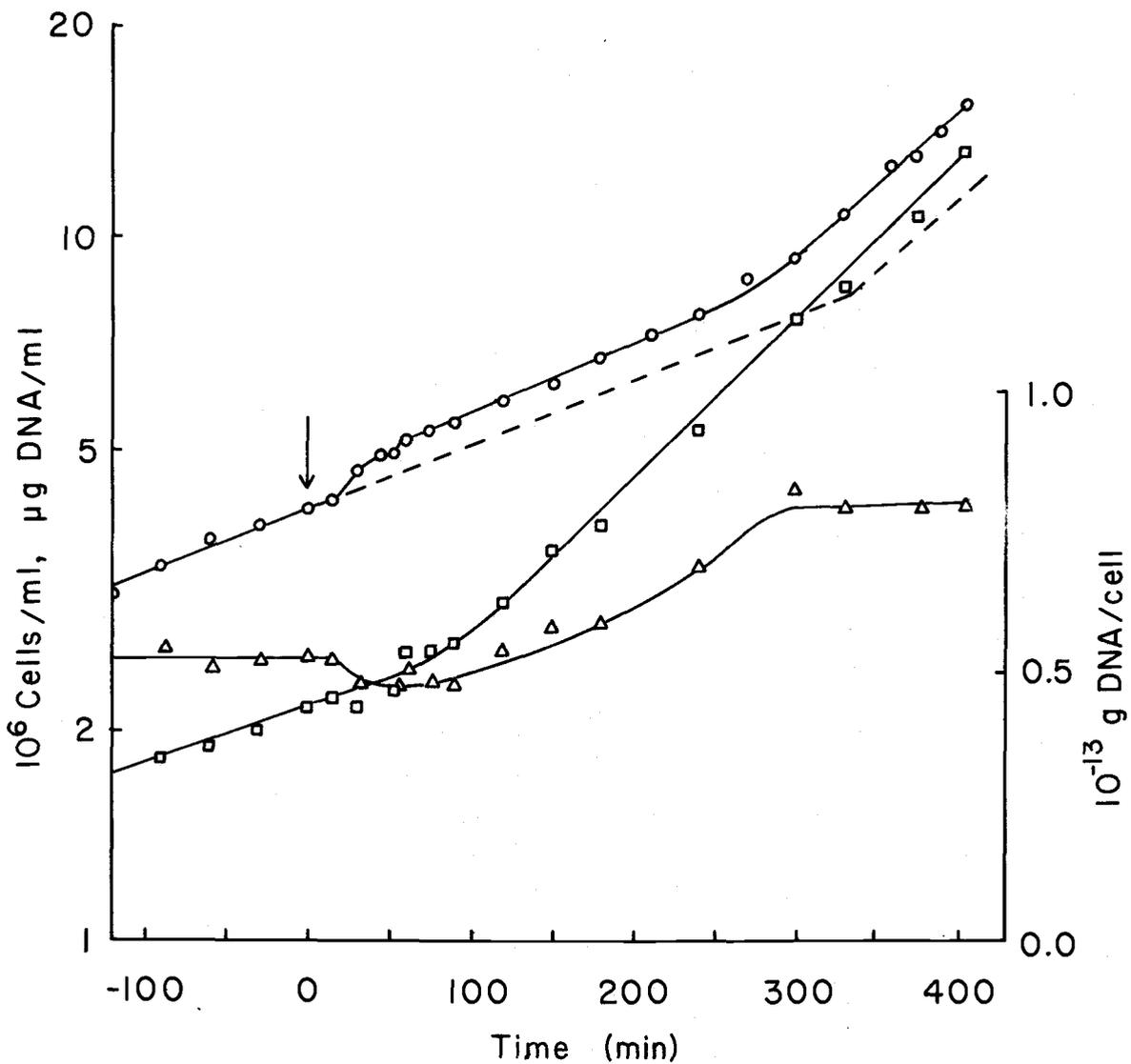


Figure 7. Shift-up with EB-5. Symbols: (○) cells/ml, (□) DNA/ml, (△) DNA/cell. The dashed line represents a theoretical curve for the expected behavior of a culture in which C and D remain constant after shift and are equal to 340 minutes. The arrow denotes time of shift.

average 7% increase over an unshifted culture after an equivalent 30 minutes growth. After an apparent decline in cell divisional rate between 45 and 60 minutes after time 0, the pre-shift growth rate was maintained for approximately one generation time whereupon the new growth rate was adopted. The culture's doubling time was 340 minutes in the pre-shift medium and adaptation of the new growth rate of 130 minutes occurred at 300 minutes post-shift. These results with the petite can be explained with the same argument used with 5015-D.

The rate of DNA synthesis is approximately maintained for 90 minutes following shift-up. At this time the rate of synthesis begins to increase, but the definitive new rate doesn't start until 120 minutes after shift. There is also some suggestion of a decline in the rate of synthesis between 15 and 50 minutes after time 0.

The amount of DNA/cell is constant at a value characteristic of WM before shift. Some 15 minutes after shift, this characteristic value of 0.50×10^{-13} g/cell drops in a manner similar to the wild-type and then rises until the proper DNA/mass ratio has been obtained. At a value of 0.79×10^{-13} g/cell, which is reached coincidentally with the increase in cell divisional rates, the new steady state characteristic of TCB is reached.

The values of DNA/cell in the petite and wild-type are 0.79 and 0.75×10^{-13} g/cell respectively. The 5 - 15% difference in DNA

between cells with mitochondrial DNA and those without is probably not detectable by this assay and the small cell samples employed.

Uptake of ^{14}C -adenine and Pool Equilibration

To ascertain if there was preferential incorporation of label into m-DNA or n-DNA after shift, a culture of 5015-D was shifted from WM + 20 $\mu\text{Ci/ml}$ ^3H -adenine to Ultra+ containing 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine. Advent of ^{14}C into the two peaks was monitored for 120 minutes by means of peak separation on CsCl gradients and liquid scintillation. The level ^{14}C incorporated into DNA did not significantly rise above background for 120 minutes. Consequently, it was felt that pool equilibration problems were being encountered. Using increased cell volumes to increase the sample size were ruled out because of the expense of the large quantities of radio-isotopes required and by the fact that large culture volumes could not be handled rapidly during the shift-up procedure.

It was, therefore, necessary to study the incorporation of adenine into the pools. It was hoped that the pools could be pre-equilibrated with ^{14}C prior to shift, and that counts going into the nuclear and mitochondrial peaks would, therefore, be significantly above background at the time of shift.

Figure 8 shows the result of adding 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine to a culture of 5015-D growing in WM. Increase in cell mass was

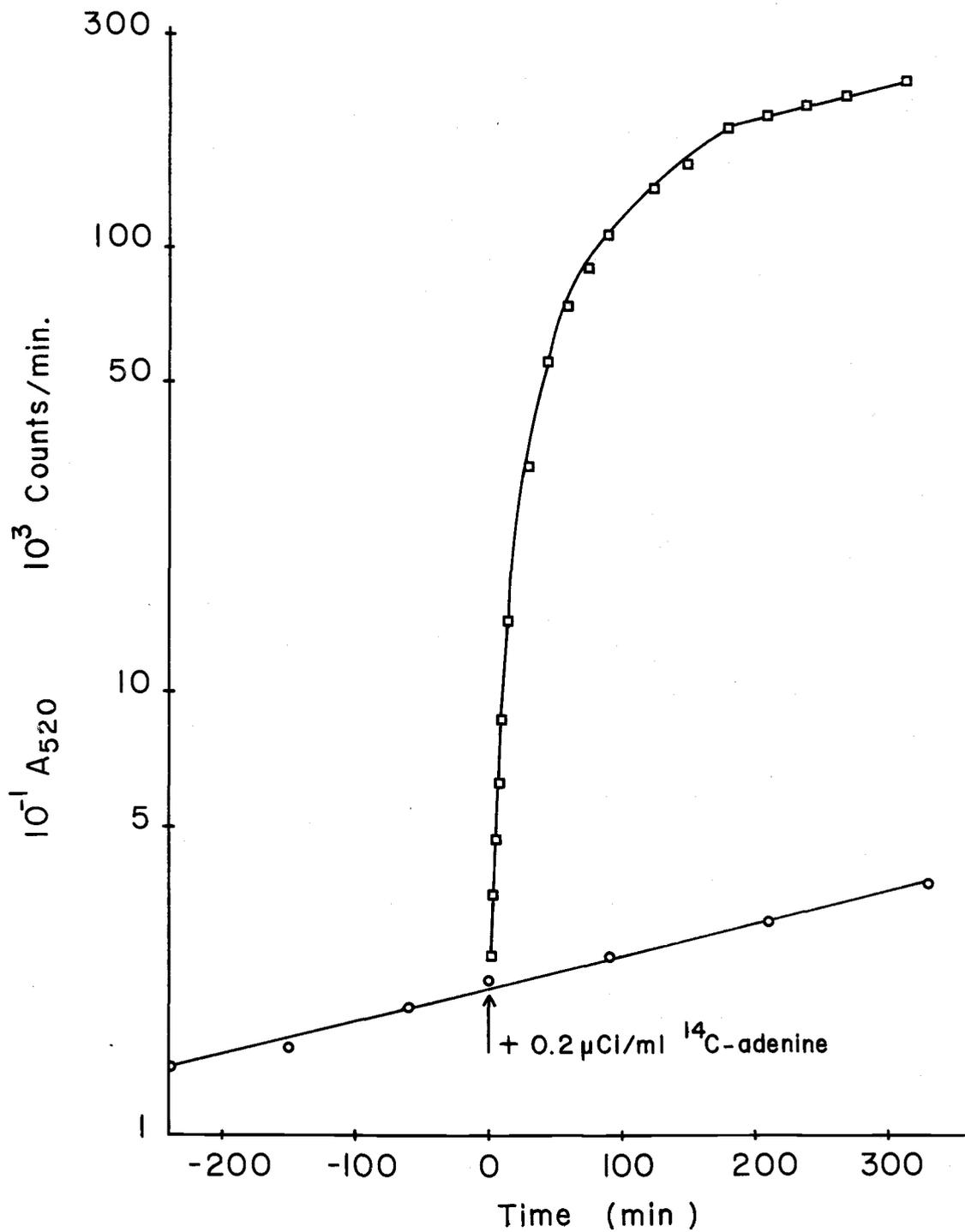


Figure 8. Uptake of ^{14}C -adenine into nucleic acid of 5015-D. Symbols: (○) cells/ml, (□) ^{14}C counts/min. The arrow denotes time of label addition.

monitored by absorption at 520 nm on a Zeiss PMQ II Spectrophotometer. There is a rapid uptake of label into nucleic acid. The uptake is linear for the first 10 minutes after which the rate of uptake decreases. In this experiment equilibration of the pools occurred at 180 minutes. In the series of experiments performed, pool equilibration required 1/3 to 1/2 of a generation time. The equilibration time was taken to be that time at which label was being incorporated into nucleic acids with a doubling time equal to the generation time.

The log of the specific activity and log of total counts incorporated are shown in Figure 9. Specific activity here is defined as $\frac{\text{counts/minute}}{A_{520}}$. It can be seen that this value becomes constant after equilibration while the log of total counts incorporated continues to increase. The insert shows that the incorporation of label is linear for the first 10 minutes, indicative of first order kinetics.

To determine the ratio of label segregation into RNA and DNA, a second experiment was performed. This was important since it was reported that yeast will incorporate on the order of 100 times more label into RNA than DNA during respiratory adaptation (Rabinowitz et al., 1969). This could considerably affect the length of pre-incubation time required to raise the DNA counts significantly above background for the shift-up experiment. A ten hour culture of 5015-D growing in WM was labeled with 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine. Duplicate samples were taken for both DNA and RNA label

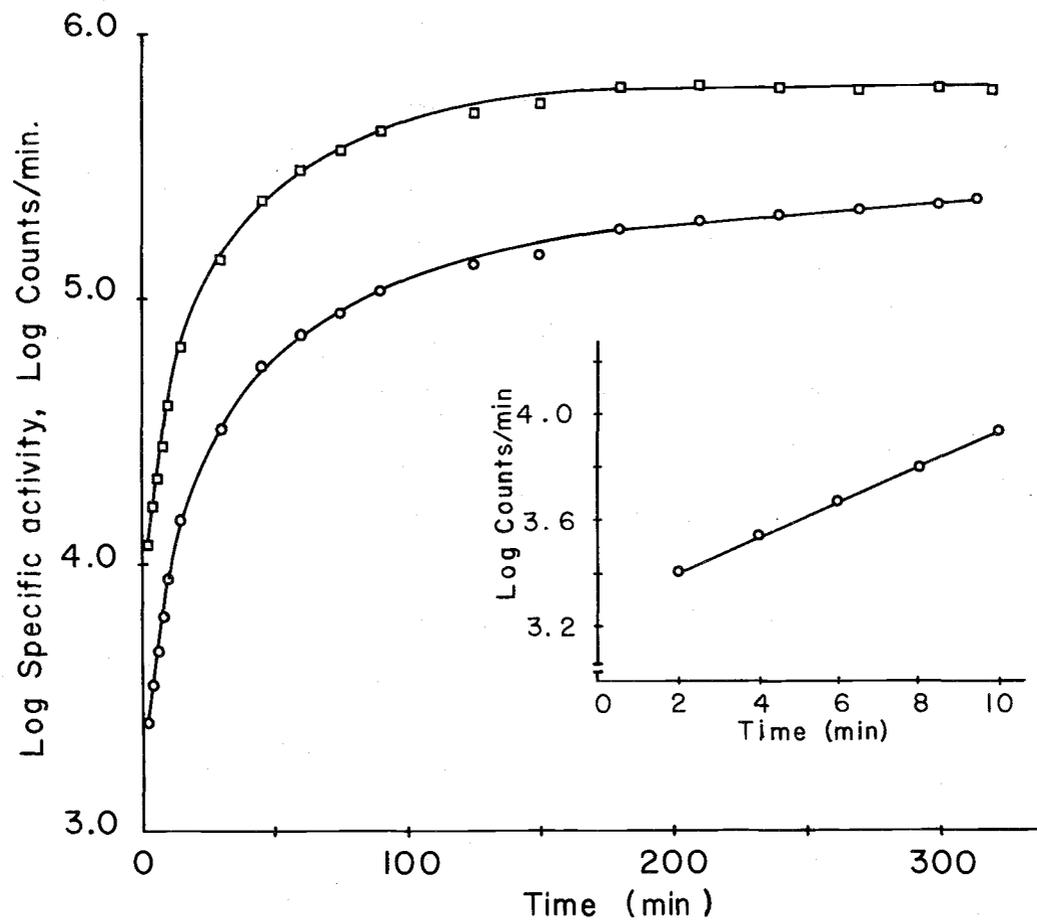


Figure 9. Kinetics of total uptake in 5015-D. Symbols: (○) log total counts per minute, (□) log specific activity.

determinations. Figure 10 shows the growth of the culture and uptake into RNA and DNA. One can see that the amount and rate of uptake is greater into RNA than DNA. Figure 11 shows the logs of the specific activities of RNA counts and DNA counts. Equilibration had not taken place by the end of the experiment. When one plots the ratios of counts in RNA to total counts and counts in DNA to total, the results in Figure 12 are obtained. The ratio of RNA total rises for 40 minutes and the ratio of DNA total decreases for 40 minutes; after this time they are stable. The significance of these data will be discussed later.

Double Label Shift-ups with 5015-D and EB-5

A 120 ml, 10 hour culture of 5015-D in WM plus 10 $\mu\text{Ci/ml}$ ^3H -adenine was equilibrated with respect to ^{14}C in the dATP pools by the addition of 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine 45 minutes prior to shift-up. At the time of shift, cells were rapidly collected on a filter and washed with 50 ml pre-warmed WM + 0.2 $\mu\text{Ci/ml}$ ^{14}C -adenine. At times 0, 10, 20, 30, and 60 minutes after shift, 20 ml samples were removed by volumetric pipet. Samples were prepared and analyzed for n-DNA and m-DNA by standard procedures. Figure 13 shows the growth of the culture before and after shift-up. The amount of tritium incorporated into each peak was taken to represent a measure of total DNA, thus the ratio $^{14}\text{C}/^3\text{H}$ would be a measure of the DNA newly

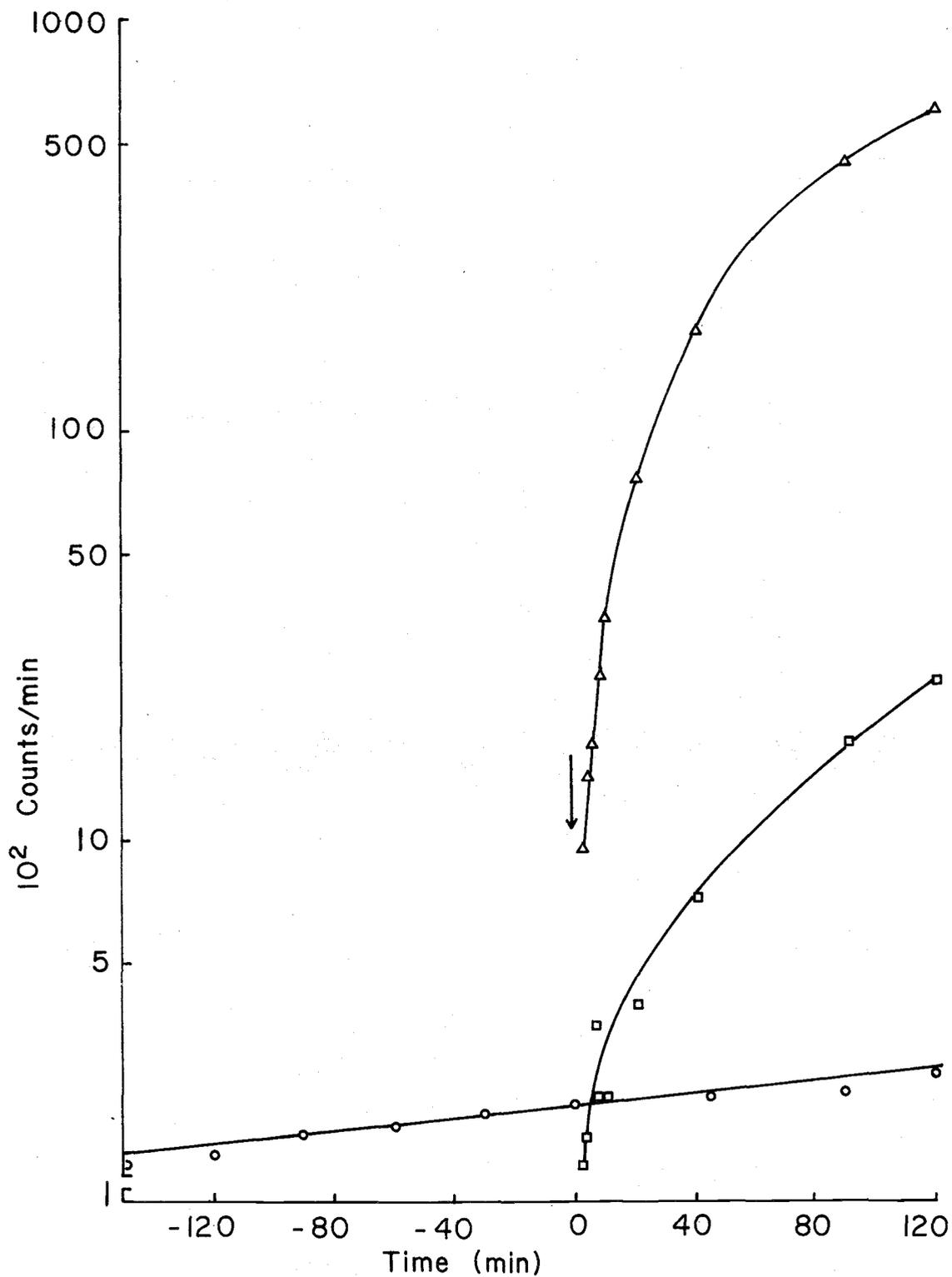


Figure 10. Cell growth and uptake of label into RNA and DNA of 5015-D. Symbols: (○) cells/ml, (□) counts into DNA, (△) counts into RNA. Arrow marks time of label addition.

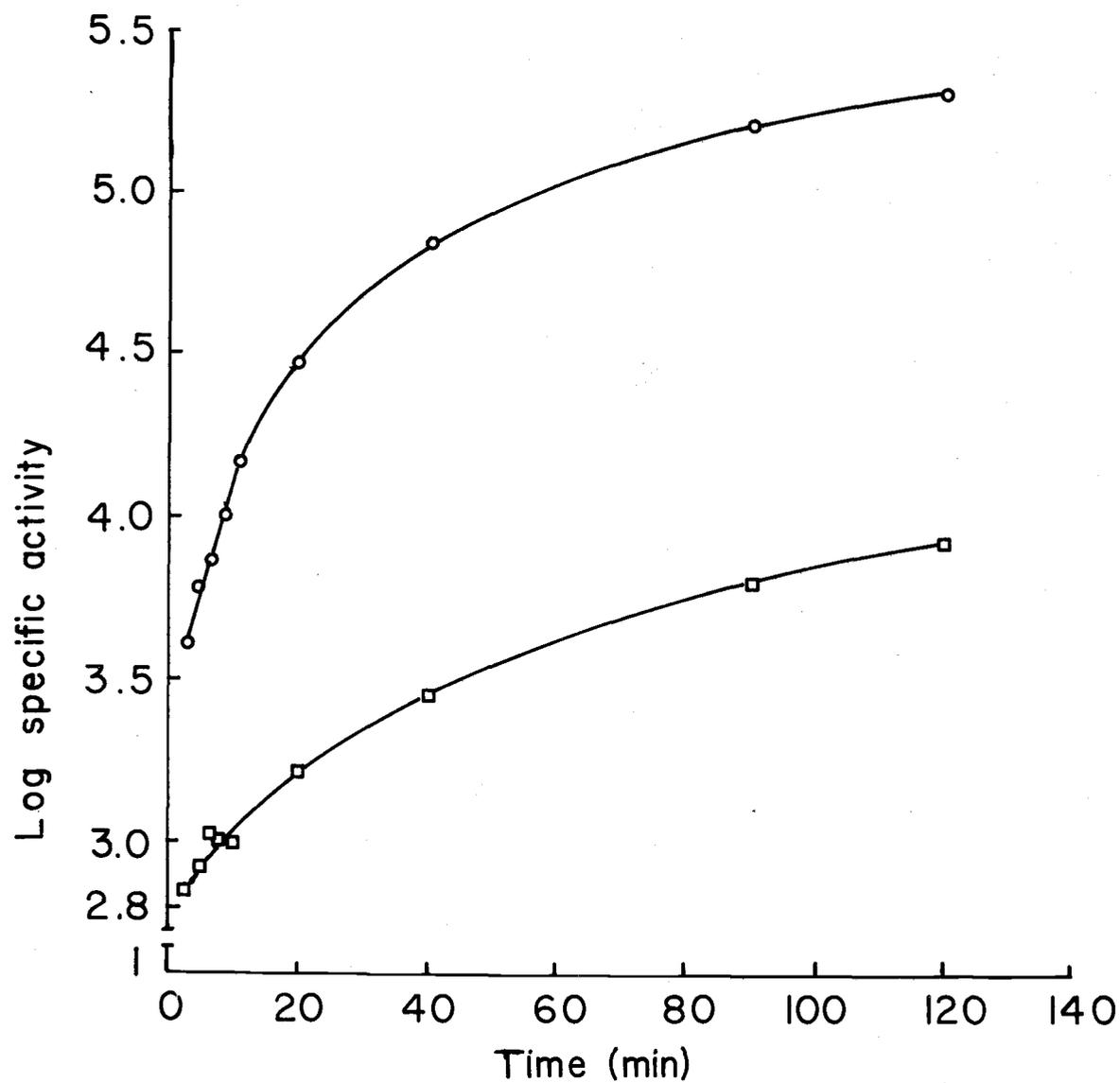


Figure 11. Kinetics of DNA and RNA label incorporation in 5015-D. Symbols: (○) RNA, (□) DNA. ^{14}C -adenine was added at time 0.

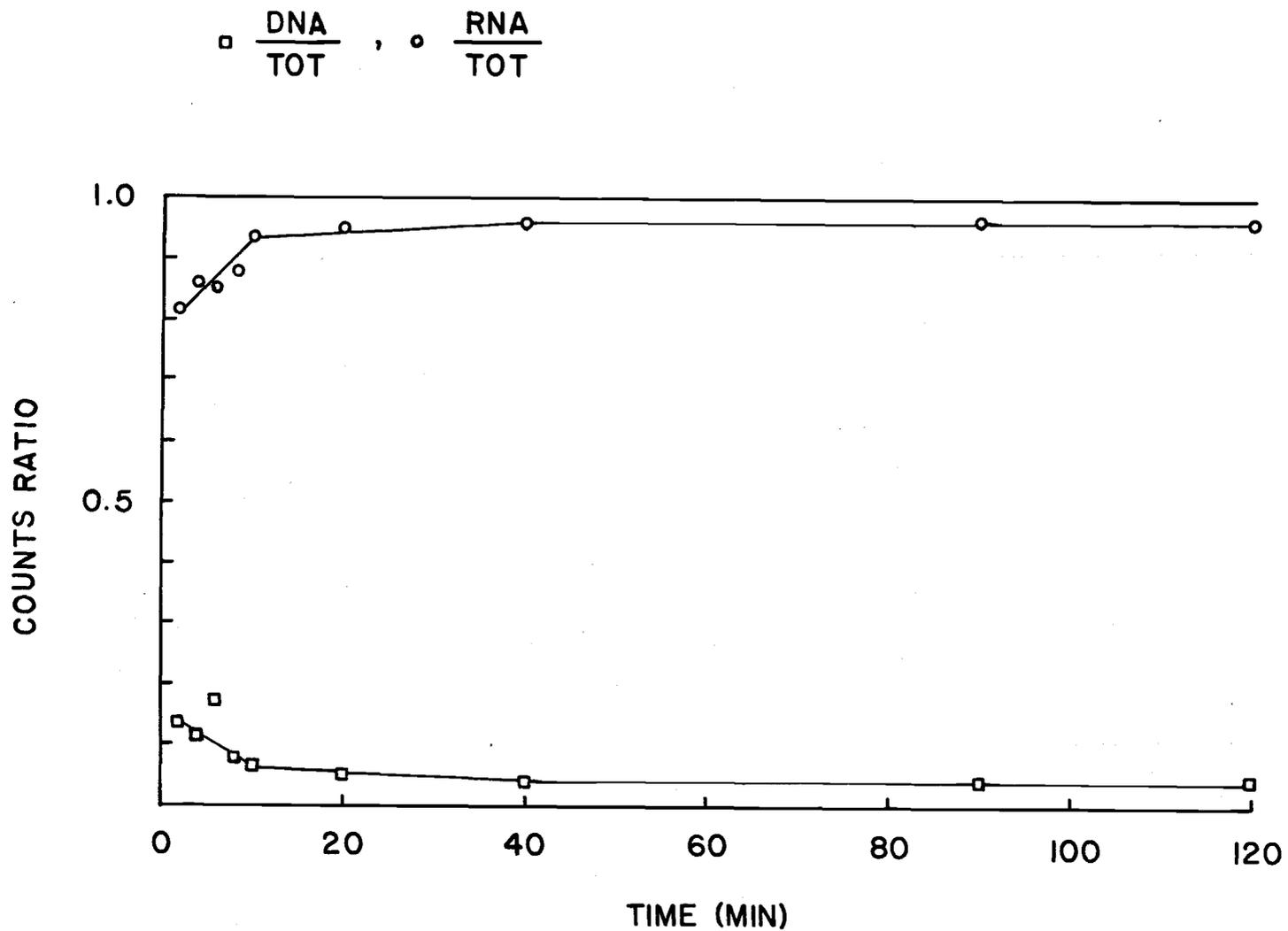


Figure 12. Segregation of label into RNA and DNA in 5015-D. Symbols: (○) radioactivity into RNA/total radioactivity, (□) radioactivity into DNA/total radioactivity. Time 0 marks time of isotope addition.

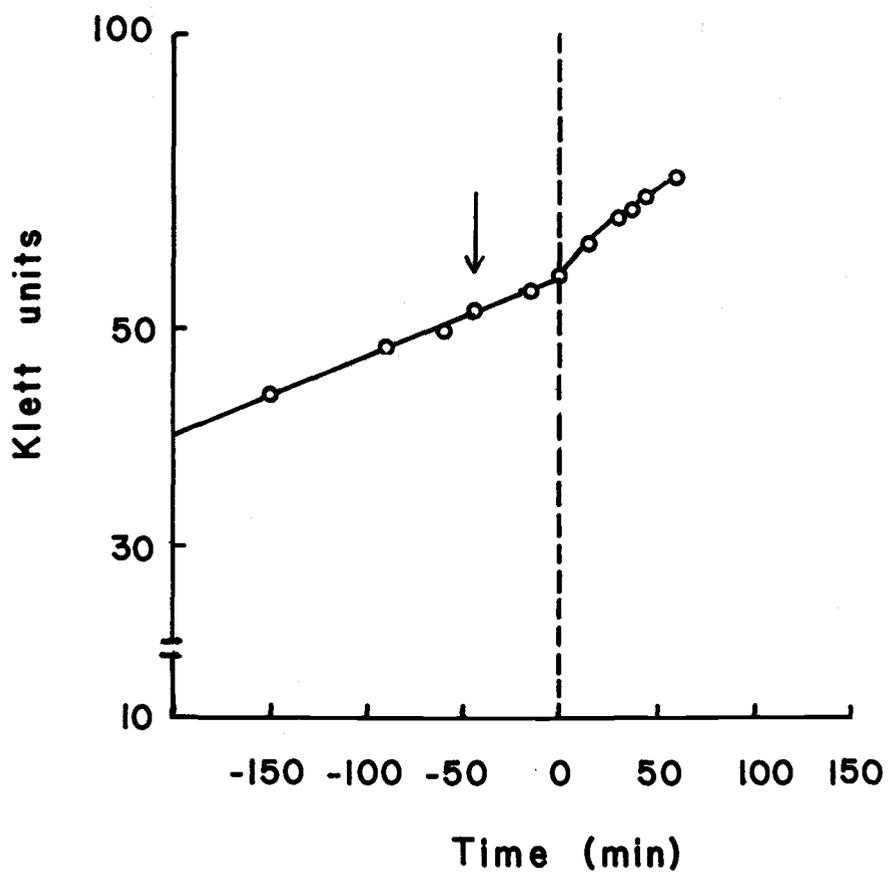


Figure 13. Growth of 5015-D during double label shift-up. The arrow denotes time of ^{14}C -adenine addition, dotted line denotes time of shift-up.

synthesized after shift, and would represent its specific activity. From Figure 14 it can be seen that the specific activity of the nuclear fraction is constant during the first 30 minutes after time 0, while the mitochondrial ratio increases by 50% over the same time interval. The ratio of the nuclear peak begins to rise after 30 minutes and has increased 50% by 60 minutes after time 0.

A control experiment, Figure 15, shows that the ratios of both the mitochondrial and nuclear fraction were still increasing at the time of shift-up. Figure 16 depicts growth of this culture during the experiment.

A repetition of this experiment gave similar results; however, the specific activity of the nuclear fraction was stationary for only the first 20 minutes after time 0. At 20 minutes post-shift, the mitochondrial fraction had increased 47.5% over that of the nuclear fraction. If the ratios from Figure 14 are calculated as ^{14}C in each respective peak to total ^3H (the sum of the tritium in the nuclear and mitochondrial peaks) one would obtain specific activities of the nuclear or mitochondrial peak relative to the total DNA of the cell. This is presented in Figure 17. The data are not as graphic, but they still indicate that the rate of m-DNA synthesis is larger than that of n-DNA synthesis.

A similar experiment was performed with EB-5 and the ratios of the single nuclear peak calculated. The data are the result of a

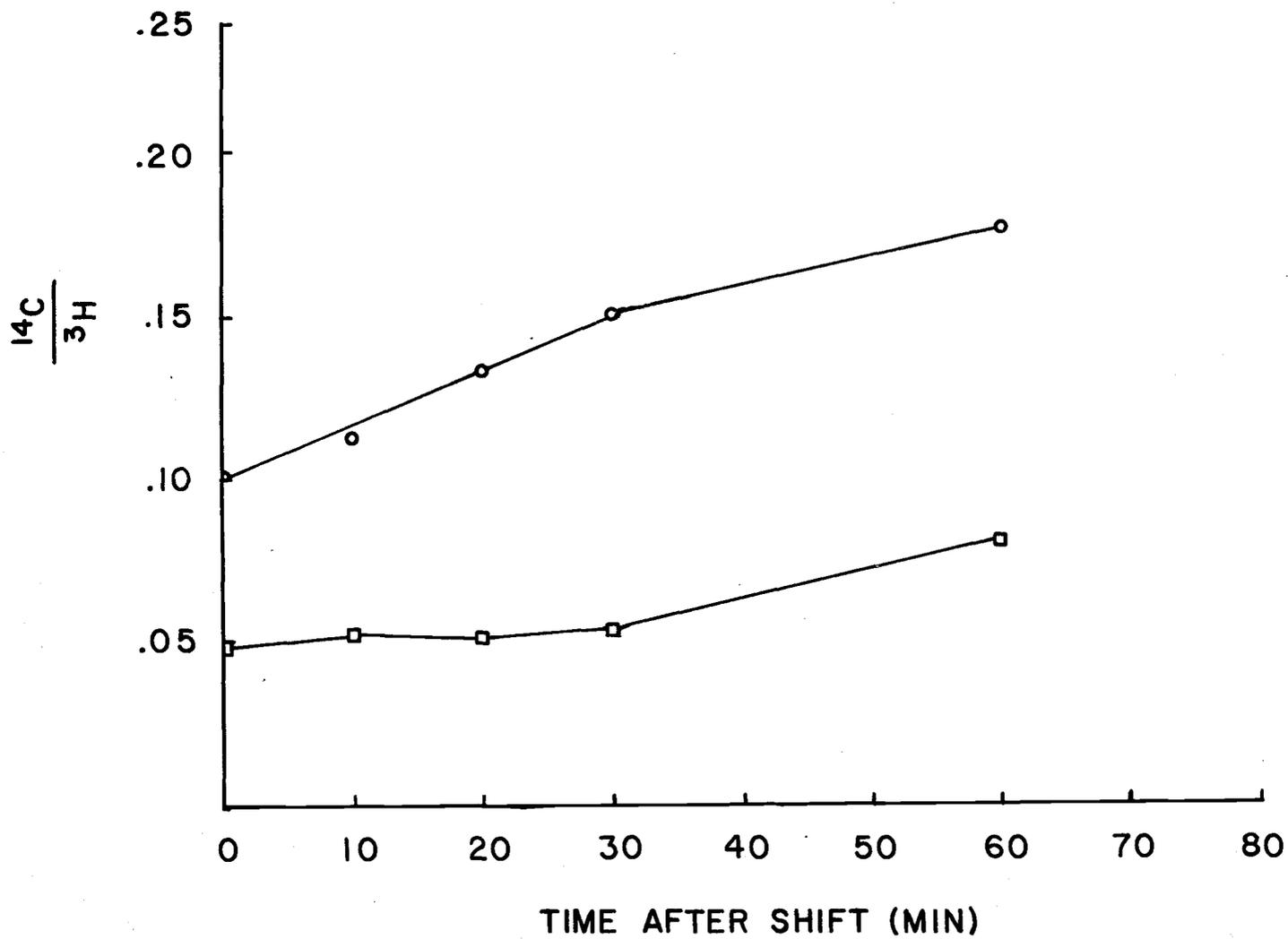


Figure 14. Specific activities of nuclear and mitochondrial DNA of 5015-D after shift-up. Symbols: (○) mitochondrial ratio, (□) nuclear ratio. Cells were shifted at time 0.

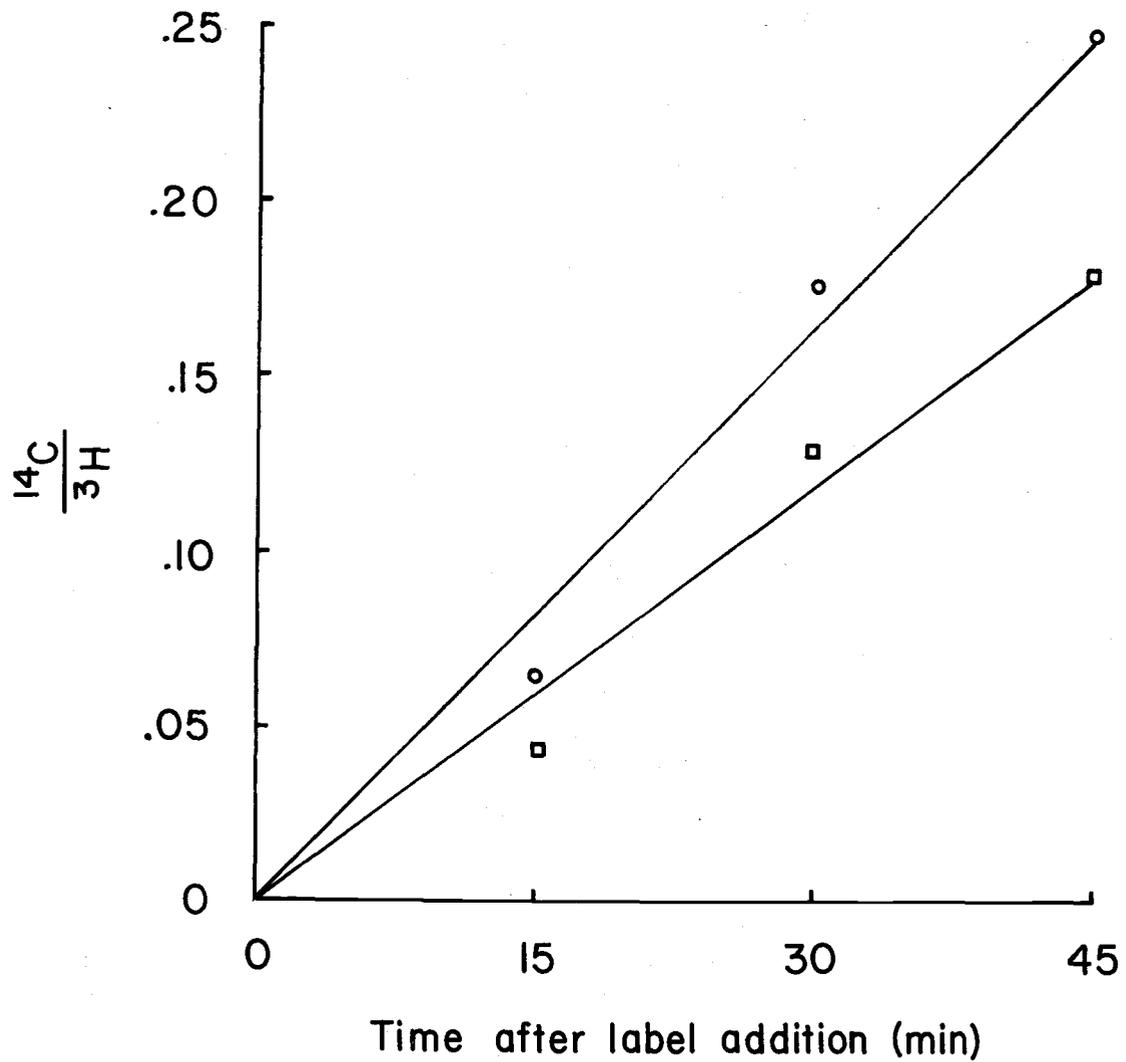


Figure 15. Pre-shift control of 5015-D. Symbols: (○) mitochondrial ratio, (□) nuclear ratio. Label was added at time 0.

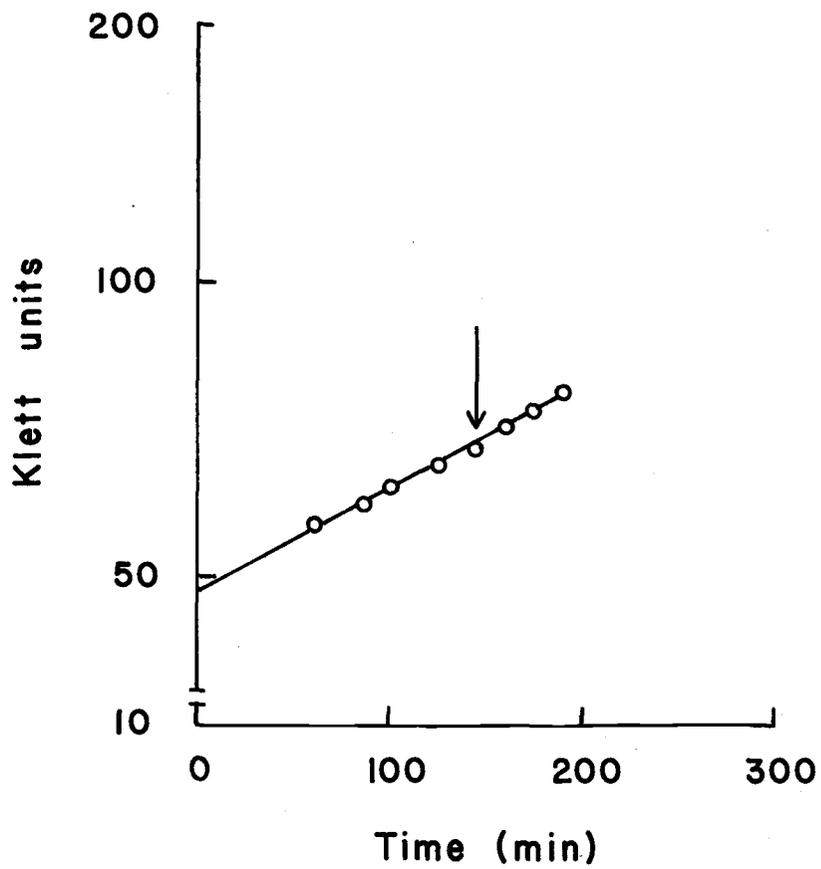


Figure 16. Growth of 5015-D during pre-shift control experiment. Arrow denotes time of ^{14}C -adenine addition.

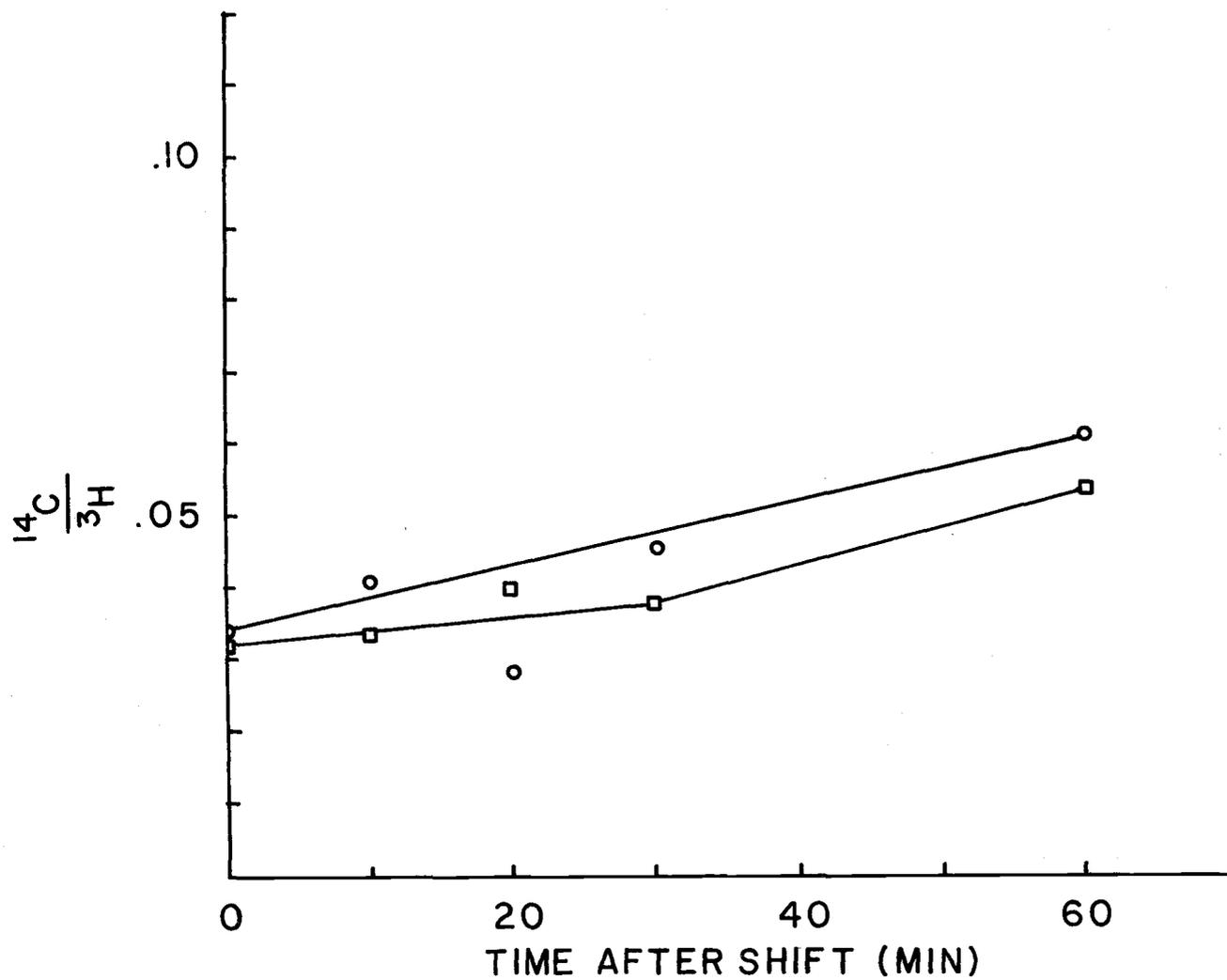


Figure 17. Ratios of label in nuclear and mitochondrial peaks to total radioactivity in 5015-D after shift-up. Symbols: (\circ) mitochondrial ratio, (\square) nuclear ratio. Cells were shifted at time 0.

single experiment and are not unequivocal. Figure 18 depicts cell growth before and after shift-up. Figure 19 shows that, apparently, the nuclear fraction is synthesized during the first 30 minutes after time 0 and is not affected by the shift in environments.

Double Label Shift-up Under Conditions of Respiratory Adaptation Inhibition

It was of interest to see the effects of respiratory adaptation inhibition on a shifted culture of 5015-D. High levels of glucose are known to repress mitochondrial function (Slonimski, 1956) and to inhibit preferential synthesis of m-DNA during respiratory adaptation (Rabinowitz et al., 1969). Figure 20 depicts the effects of shift-up into Ultra+ containing 5% glucose. There is an obvious effect on the growth of the organism; cell increase is inhibited for the first 15 minutes after shift. Growth recommences after 15 minutes and continues at a rate less than the 290 minute pre-shift generation time. Figure 21 shows the effects on the $^{14}\text{C}/^3\text{H}$ ratios. In the first 30 minutes after shift, the ratio for the mitochondrial peak increased only 6.8%. The nuclear fraction was affected, too, having increased only 10.2% after 60 minutes, whereas, in the unrepressed cultures it had increased an average of 50% by this time.

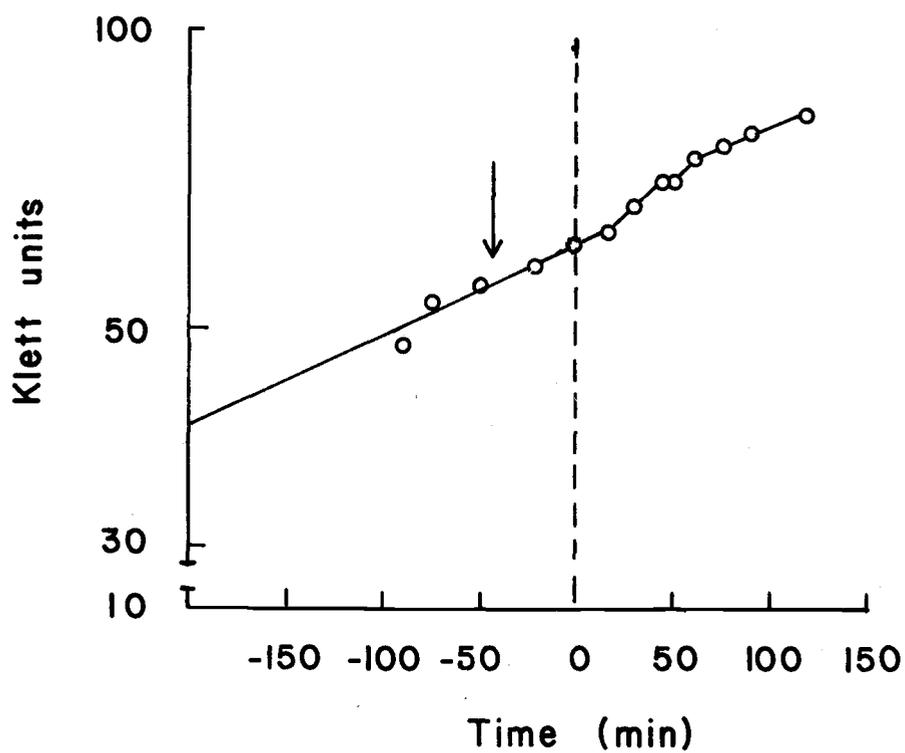


Figure 18. Growth of EB-5 during double label shift-up. The arrow denotes time of ^{14}C -adenine addition, dotted line denotes time of shift.

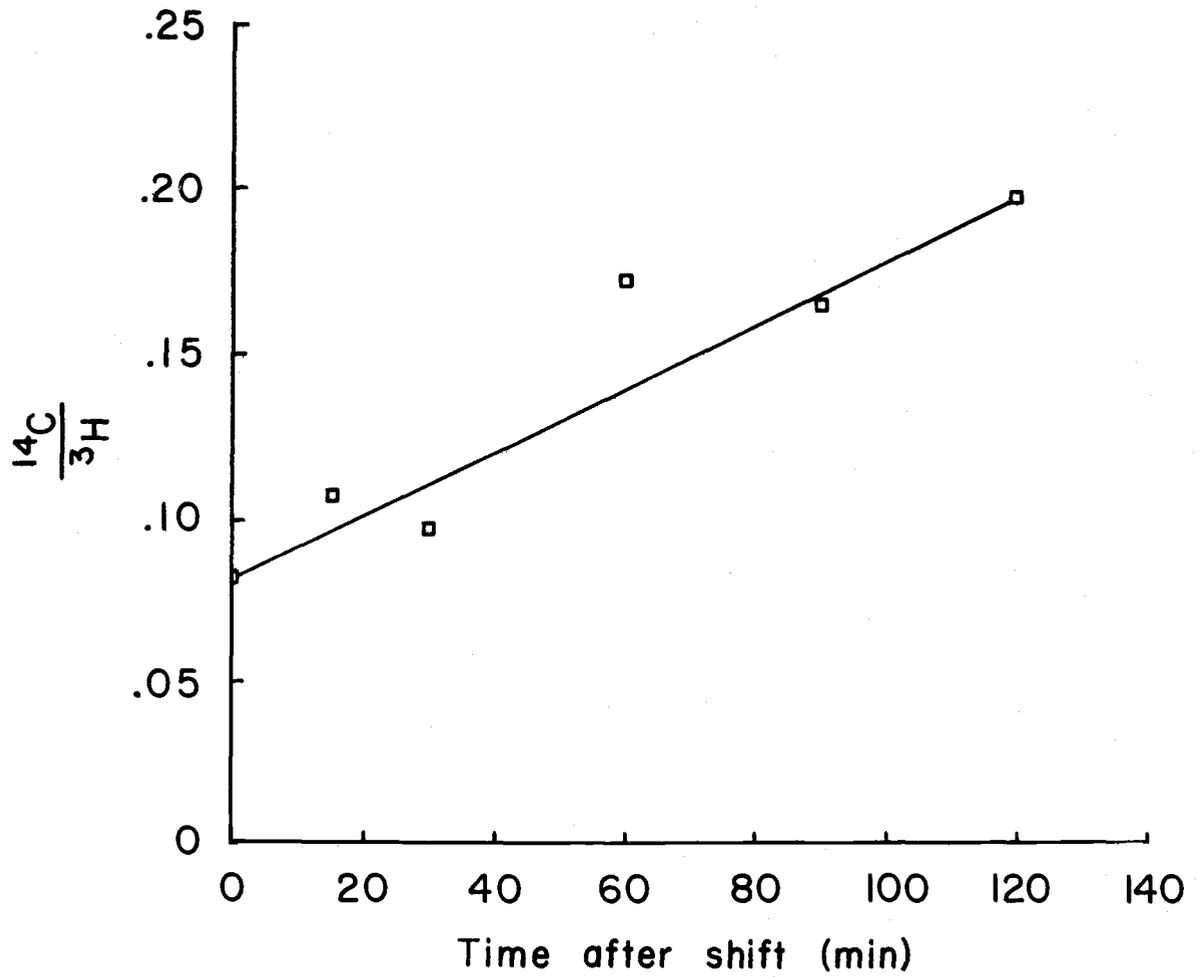


Figure 19. Specific activity of nuclear DNA in EB-5 after shift-up. The cells were shifted at time 0.

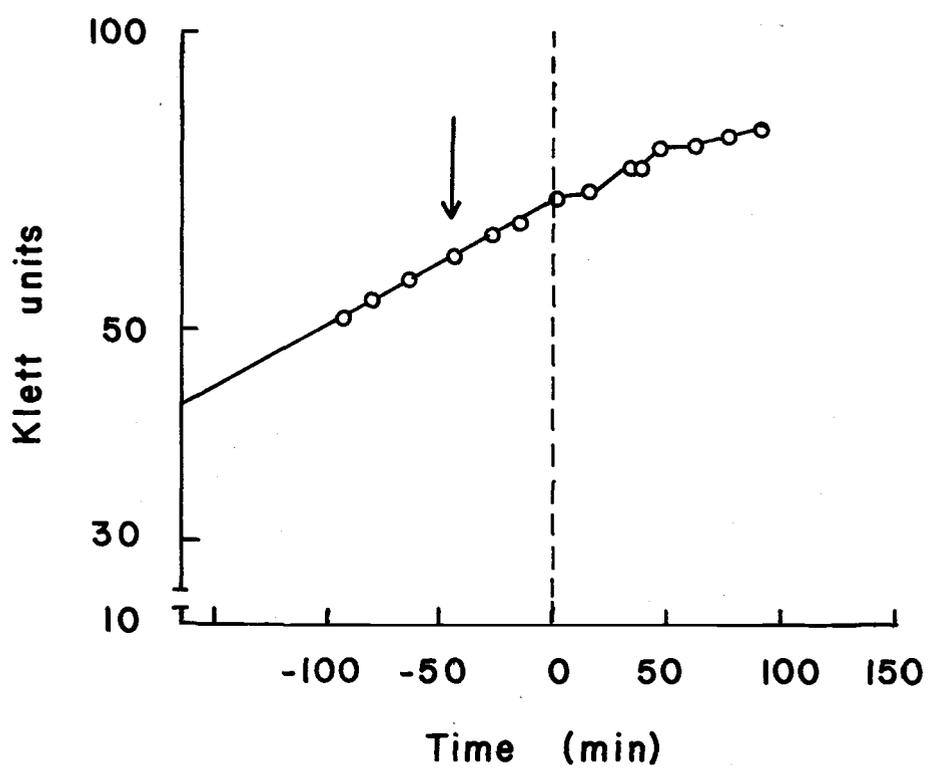


Figure 20. Growth of 5015-D after shift to conditions of respiratory adaptation inhibition. The arrow marks time of ^{14}C -adenine addition, the dotted line marks time of shift-up.

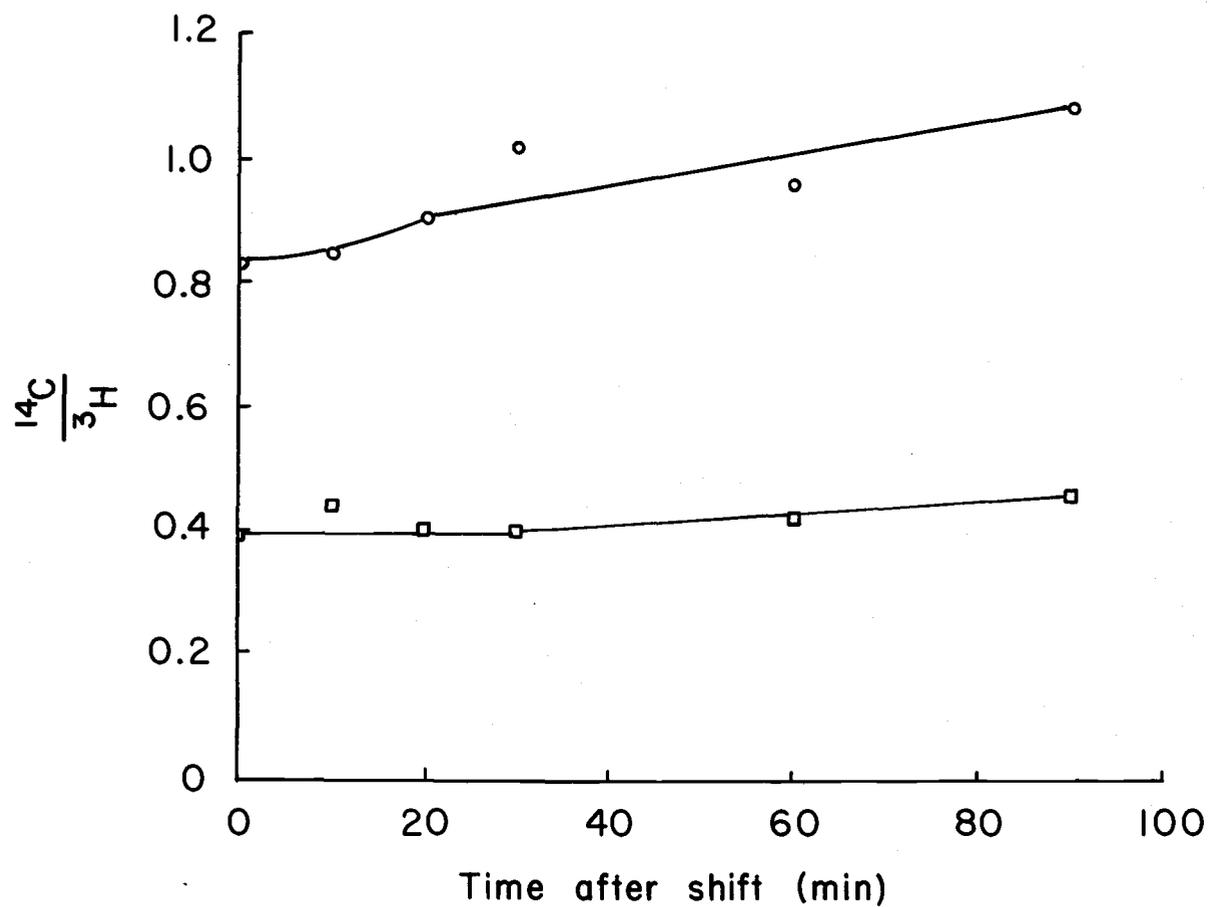


Figure 21. Specific activities of nuclear and mitochondrial DNA of 5015-D after shift-up to 5% glucose. Symbols: (○) mitochondrial, (□) nuclear. Cells were shifted at time 0.

DISCUSSION

The results of the traditional shift-up experiments presented here in conjunction with the results of previous workers, demonstrate that the eucaryotic system, in particular the yeast system, behaves in the manner of simpler procaryotes. Post-shift synthetic patterns of RNA, DNA, and protein in yeast all mimic those of the genetically less complex bacteria. At slow growth rates in glucose the generation time is equal to $C + D = I$ minutes (Helmstetter et al., 1968); and it would be expected that if such a culture were shifted to a richer medium, the rate of cell increase would be maintained for one generation following the shift. This was shown to be so in yeast. The slight variations in rate maintenance can be explained by slight variations in the C and D periods. It is suggested that the burst of cell division immediately after shift-up is due to a slight decrease in the D period, in that a certain population of the culture which has completed chromosome replication is late in its D period and is accelerated through division by the increased availability of nutrients. This initial 30 minute elevated rate of cell division explains why the culture assumes its post-shift growth rate 20 - 30 minutes earlier than expected.

The definitive post-shift rate of DNA synthesis in 5015-D is reached about 150 minutes after time 0. This is a reflection of the

time needed to synthesize a component of I under post-shift conditions. The proposal is based on the fact that a balanced culture contains a heterogeneous mixture of yeast populations in all stages of the growth cycle. Consider for a moment that population of cells which will be the last to adapt to the new rate of DNA synthesis. These would be those cells that have just initiated DNA synthesis at 30 minutes after time 0. These cells will have just consumed a unit of initiator and would require the I minutes of the new medium to accumulate another unit of initiator. Since, according to the model of Helmstetter et al. (1968), $I = C + D$ minutes, I is, therefore, equal to the generation time of 5015-D in TCB. Wehr and Parks (1969) demonstrated that the rate of protein synthesis is maintained about 30 - 40 minutes after shift-up. Thus, the DNA synthetic rate of those cells which consumed a unit of I at this time, and consequently initiated the synthesis of a new unit of I, will not reflect the insertion of new rounds of replication at the new rate until $120 + 30 - 40$ minutes or 150 minutes post-shift.

That there is little difference between the post-shift cell divisional and DNA synthetic patterns of the grande and petite strains suggests that there is little if any control of the adaptation process by the mitochondrial genome.

The similar patterns of post-shift events between bacteria and yeast allow one to draw similar conclusions about macromolecular

synthesis regulation in the two systems. It has been proposed in bacteria that the flooding of amino acid pools at the time of shift results in the charging of all t-RNA species. This derepresses RNA synthesis and results in rates of synthesis in excess of the definitive rate. This effect is almost instantaneous. This, and the fact that RNA synthesis in bacteria can be stimulated in the absence of protein synthesis, indicates that the control mechanism for RNA does not involve significant changes in the levels of the enzymes involved (Maaløe and Kjeldgaard, 1966). RNA synthesis can be dissociated from the syntheses of protein and DNA and during this dissociation, in the first minutes after shift, r-RNA is primarily synthesized; the r-RNA/DNA ratio climbs to the value of the new steady state. The fact that the t-RNA/DNA ratio remains constant suggests that the t-RNA control mechanism is different from r-RNA and may be controlled in a manner similar to DNA (Maaløe and Kjeldgaard, 1966). Gros et al. (1965) have shown in bacteria that the amount of m-RNA present is proportional to the number of ribosomes. This proportionality suggests that m-RNA may never be produced in excess. During a shift-up, then, the r-RNA/m-RNA ratio would be expected to remain constant. Wehr (Ph. D. Thesis, 1970) stated that pulse labeled experiments in S. cerevisiae under conditions of shift-up revealed that label was incorporated into all three RNA fractions rather than r-RNA alone, but he felt that his preliminary experiments

could not yet be considered conclusive.

Protein synthesis after shift-up in yeast follows the bacterial pattern; however, a transient decline in the rate of synthesis concomitant with the drop in the excessive rate of RNA synthesis to the definitive level occurs (Wehr and Parks, 1969). This apparent phasing is only a minor difference between the yeast and bacterial systems. Since the rate of protein synthesis is dependent on the number of mature ribosomes present (McCarthy, 1962), and consequently on their rates of formation, this transient effect on the rate of protein synthesis may merely be a reflection of the drop in rate of ribosome formation. This author is in agreement with Wehr and Parks that this is probably not a synchronous event, as Williamson (1964) has demonstrated that protein is produced continuously in synchronous cultures.

A rate maintenance phenomenon exists in both systems with regards to DNA synthesis. This is a reflection of the fact that DNA synthesis has a delayed dependence on protein synthesis. During this delay, new rounds of replication are inserted prematurely with respect to rounds of replication in unshifted cultures. Evidence found in bacteria by Cooper (1969) supports the hypothesis that units of initiator protein are accelerated to completion after shift-up and result in premature insertion of new rounds of replication into replicating DNA. The maintenance phenomenon in DNA synthesis in

yeast suggests similar control.

Finally, the cell divisional rate is maintained for one generation after shift in both yeast and bacteria. This suggests that the C and D periods in yeast are constant during the transition between the two steady states of growth.

The time required for the equilibration of the adenine pool was $1/3$ to $1/2$ a generation time. This indicates a relatively large adenine pool; evidence to support this has been reported by Wehr (Ph. D. Thesis, 1970), who demonstrated that there is a 10% increase in DNA synthesis after shifting 5015-D to adenine starvation medium. This large pool explains the reason for the failure of the initial double label shift-up experiments in which label was added at time 0. Figure 9 shows that the rate of incorporation of label into nucleic acid is linear for the first 10 minutes. This indicates that during this time, label is entering and leaving the pool at the same rate with no net accumulation. In other words, the ratio of unlabeled adenine in the pools is essentially 0 for the first 10 minutes. After this time label starts to accumulate in the pools and the ratio of unlabeled adenine/labeled adenine in the pools starts to approach the ratio in the medium asymptotically. When this ratio is reached, label is leaving and entering the pools at the same rate, and this rate is equal to the rate of nucleic acid synthesis, which is equal to the generation rate of the organism. The fact that the ratio of RNA radioactivity/

total radioactivity is increasing while the ratio of DNA radioactivity/total radioactivity is decreasing indicates that label is being incorporated into RNA very much faster and earlier than into DNA. In fact, when the first sample was taken two minutes after addition of label to the medium, there was nine times the amount of label in RNA as in DNA. The increase in the RNA/total ratio and decrease in the DNA/total ratio was most marked during the first ten minutes after addition of ^{14}C -adenine. During the next 30 minutes, the changes were only slight and the ratios became stabilized after 40 minutes. I suggest that these data do not mean that RNA is synthesized preferentially to DNA for the first 40 minutes after addition, but rather, there is much more RNA than DNA and the apparent preferential incorporation of label into RNA is due to pool effects. There are two adenine nucleic acid precursor pools in the cell, an ATP pool for RNA synthesis and a dATP pool for DNA synthesis. It is suggested that 40 minutes is required for these two pools to become "equilibrated" with respect to each other; that is, for label to be leaving both pools at a fairly rapid rate.

It is for this reason that 45 minutes was chosen as the time for pre-incubation with ^{14}C -adenine prior to a double label shift-up. The cells were washed free of ^3H -adenine with WM and shifted into Ultra+ medium, each containing identical amounts of label as the pre-shift medium. Therefore, I was confident that the process of

^{14}C -adenine pool equilibration had not been interrupted by the shifting process.

The amount of ^3H incorporated into each peak was taken to be a constant. Some dilution in the ^3H counts relative to the rising ^{14}C counts was expected due to depletion of ^3H in the pool. The large pool size would somewhat alleviate this problem, but it was not considered important since the tritium counts per peak were very much higher than the ^{14}C counts and, therefore, would not change the ratios much. Also important was the fact that there is some loss in the transfer of spheroplasts from 30 ml Corex centrifuge tubes (in which samples were collected and spheroplasts prepared) to the 5 ml cellulose nitrate ultracentrifuge tubes. It was, therefore, difficult to ascertain whether or not there was a loss of ^3H counts from the DNA peaks due to dilution with unlabeled and ^{14}C -adenine during the course of the experiment. This less than quantitative transfer of spheroplasts was also ignored as one would not expect to transfer one DNA fraction preferentially over the other in whole spheroplasts.

The results of the double label shift-up suggest that under the conditions of these experiments there is preferential incorporation of label into the mitochondrial DNA of the grande yeast during the first 20 - 30 minutes following shift. No correction was made for the different adenine contents of the two DNA fractions, since the increasing m-DNA ratio and static n-DNA ratio after shift indicate that doing

so would not alter the interpretation of the data. This preferential uptake of label could be the result of two occurrences. First, it is obvious from the increased rates of RNA and protein synthesis after shift-up that there are higher energy demands on the cell. As the 2% glucose used in TCB does not fully repress mitochondrial function (Criddle and Schatz, 1969), partial respiratory adaptation could be occurring. Since Rabinowitz et al. (1969) have demonstrated that preferential synthesis of m-DNA occurs during this event, it is possible that this could be occurring in the first 20 to 30 minutes after shift. If this were the case, it could explain the origin of the energy that supports the initial 30 minute burst of cell synthesis in 5015-D. Second, Criddle and Schatz (1969) have shown that the number of pro-mitochondria per yeast cell varies with cultural conditions. The increase in m-DNA over n-DNA may reflect the number of pro-mitochondria in the cell increasing in adaptation to the new medium. These two hypotheses were examined by shifting a culture of 5015-D to Ultra+ containing 5% glucose. This high concentration of the sugar is known to repress respiratory adaptation (Rabinowitz et al., 1969). The results of such an experiment show profound effects on the growth of the organism--not only are the rates of m-DNA and n-DNA affected, but so is the cell divisional rate. A shift-up of EB-5 (incapable of respiratory adaptation) into Ultra+ containing 2% glucose shows that the specific activity of n-DNA increases from the time of shift and

does not demonstrate a stable specific activity for the first 20 - 30 minutes as does the wild-type. This suggests that emphasis in DNA synthesis during the absence of respiratory adaptation or pro-mitochondrial replication shifts to nuclear DNA. Such a shift in emphasis was not observed in 5015-D shifted to 5% glucose. The rate of incorporation into m-DNA was definitely reduced in the 5% glucose shift giving some evidence that partial respiratory adaptation may be occurring. It does not rule out the possibility, however, that replication of the pro-mitochondria are under catabolite repression. The fact that nuclear DNA synthesis was inhibited, too, adds difficulty in interpreting the data. The effects of 5% glucose on the shifted culture are apparently not limited to the inhibition of respiratory adaptation.

SUMMARY

The data presented in this work indicate that yeasts undergoing nutritional shift-up behave in a manner similar to bacteria. The synthetic patterns of RNA, DNA, and protein synthesis are all similar. There is a rate maintenance phenomenon associated with cell division and DNA synthesis. It is proposed that the mechanisms of control of macromolecular biosynthesis operating in bacteria are also in effect in yeast. The control of DNA synthesis in yeast appears to be similar to that of bacteria. It is not known, however, if the replication of the 17 chromosomes in yeast are controlled individually or coordinately. It is conceivable that the 17 chromosomes could be replicated sequentially throughout the replication cycle or that they are all replicated together.

It has been shown that there is preferential synthesis of mitochondrial DNA during the first 20 - 30 minutes following a shift-up. It is hypothesized that this synthesis could be due to either partial respiratory adaptation or to an increase in the number of pro-mitochondria per cell. The data from shift-ups of EB-5 to Ultra+ containing 2% glucose and 5015-D to 5% glucose do not rule out either possibility. The fact that 5% glucose also affects nuclear DNA synthesis makes matters harder to interpret, but the fact that the mitochondrial $^{14}\text{C}/^3\text{H}$ ratios are suppressed the most suggests that

partial respiratory adaptation may be the cause of preferential incorporation. This presupposes that the replication of pro-mitochondria are not regulated by catabolite repression.

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