

Studies on the Regulation of RNA

Synthesis in the Yeast, *Saccharomyces cerevisiae*

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by

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Abstract

The stringent control of RNA synthesis in the yeast Saccharomyces cerevisiae may be evoked either by starving for a required amino acid or by inhibiting protein synthesis. The response is non-coordinate in that the synthesis of ribosomal and messenger RNA is depressed whereas that of transfer RNA continues. If protein synthesis is blocked in starved cells then tRNA synthesis is stimulated.

In this thesis the relationship between the level of tRNA charging and the transcriptional and translational state of the yeast cell has been examined. When the cells are starved for an amino acid the corresponding tRNA species only becomes uncharged. This effect can be counteracted by the addition of protein synthesis inhibitors to the starved cells. In contrast, the same inhibitors provoked the discharge of tRNA in growing (non-starved) yeast. Similar results were obtained when protein synthesis was blocked using a temperature-sensitive mutant. These contrasting effects of translation inhibition on tRNA charging in starved and non-starved cells correlate with the changes that inhibition evoked in the transcriptional state of those cells. The data appear to indicate that tRNA synthesis is under autoregulatory control and that tRNA charging may also play an important role in the regulation of rRNA synthesis.

A study has also been made of the regulation of the synthesis of P1 double-stranded (ds) RNA, the genome of the yeast virus-like particle (VLP). When protein synthesis is prevented by starvation for a required amino acid or by addition of cycloheximide, the rate of P1 dsRNA synthesis is reduced markedly. During nitrogen starvation the synthesis of P1 dsRNA persists but is accompanied by the degradation of pre-existing molecules. This degradation appears to require the induction of new enzymes and it is likely that the breakdown products are used to enable the cell to complete its division cycle. However, not all of the copies of the VLP genome are degraded in this process, some are conserved and can replenish the amount of P1 dsRNA on return to growth conditions. These results are discussed with respect to possible models for dsRNA replication and the controls which appear to operate on this process are compared to those exerted on nuclear RNA synthesis.

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Abbreviations

dsRNA	double-stranded RNA
ssRNA	single-stranded RNA
RN'ase	ribonuclease
DN'ase	deoxyribonuclease
VLP	virus-like-particle
pGpp	guanosine 5'-monophosphate 3' diphosphate
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5' triphosphate 3' diphosphate
MS	magic spot
NTP	nucleoside triphosphate
EMS	ethyl methanesulphonate
CHI	cycloheximide
MDMP	2 -(4-methyl-2,6-dinitroanilino)-N-methyl proprionamide
PMSF	phenylmethylsulphonylfluoride
DTT	dithiothreitol
APE	acetyl-DL-phenylalanine β naphthylester
DMF	dimethyl formamide
TNS	sodium tri-isopropyl naphthalene sulphonate
PPO	diphenyloxazole
DEAE	diethylaminoethyl
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
YNB	yeast nitrogen base
OD	optical density
nm	nanometres
U.V.	ultra-violet
ts	temperature sensitive

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INTRODUCTION

The growth of all cells is dependent on precise co-ordination of the diverse array of biochemical reactions which constitute their metabolism. These processes are mediated by cellular enzymes, the catalytic units of growth, which are determined by the genomic composition of the cell. Balanced growth can be considered as the manifestation of the ability of an organism to regulate the expression of its genome. It follows, therefore, that the controls over transcription and translation are central to the regulation of growth itself. Hence the co-ordination of these functions is one of the basic homeostatic mechanisms of the cell. The means by which this control is achieved is most easily analysed when this steady state is disturbed. For example, the rate of protein synthesis may be altered by changing the composition of the growth medium. The manner in which the rate and pattern of RNA synthesis is altered in response to this perturbation may then be examined. The work presented in this thesis is the result of investigations performed using this strategy to study the regulation of RNA synthesis and the mechanisms underlying this process in the yeast, Saccharomyces cerevisiae.

Yeast is particularly suitable for this kind of study since it has all the technical advantages of a microorganism. The molecular genetics of yeast is very well defined and a large number of regulatory and other useful mutations have been characterised. The physiological environment of yeast cells can be freely manipulated. Yeast also appears to be a typical eukaryotic organism containing a nucleus, mitochondria, a golgi body and an endoplasmic reticulum, all of which are characteristic of the higher level of cellular organisation found in eukaryotes. In common with other eukaryotes yeast cells also possess three functionally distinct RNA polymerases. These facts suggest that Saccharomyces cerevisiae is a good model system for the study of the regulation of RNA synthesis in eukaryotic cells.

The factors affecting the regulation of RNA synthesis in living cells are considered in the following sections of this Introduction. These mechanisms have been studied most extensively in bacteria. The discussion will therefore concentrate on the work performed with yeast but will compare this to the situation both in other eukaryotic organisms and in bacteria.

I.1 The Co-ordination of RNA Synthesis with Growth and Protein Synthesis

Although the relationship between cell content and growth was initially studied in animal and plant tissues (Brachet, 1942; Cassperson, 1950) the problem has been most readily examined in microorganisms. Experiments with bacteria have shown a direct proportionality between the rate of growth and the amount of RNA in the cell (Schaechter, Maaløe and Kjeldgaard, 1958; Neidhardt and Magasanik, 1960). The amount of protein per cell at different growth rates was found to be constant with respect to the RNA content. Since the bulk of stable RNA in bacteria is ribosomal RNA (Friesen, 1966) the conclusion drawn from these experiments is that the rate of protein synthesis per ribosome, i.e. the ribosomal efficiency, is characteristic of the type of cell but not the rate at which it is growing (Maaløe and Kjeldgaard, 1966). However this is not the case in slowly growing bacteria. These cells have a substantial excess of ribosomes over that required to support the level of protein synthesis observed (Koch, 1970). This becomes evident when slowly growing batch or nutrient-limited chemostat cultures are suddenly enriched. The resulting stimulation of protein synthesis is too rapid to be explained by an increase in the rate of synthesis of rRNA (Harvey, 1970; Koch and Deppe, 1971). These results are accounted for by the presence of a pool of non-functional ribosomes, an increased rate of rRNA degradation and a slight decrease in the peptide chain elongation rate in slowly growing cells (see Neirlich, 1978). In general, however, for a wide range of growth rates, the level of protein synthesis in bacteria is related to the number of active ribosomes rather than the rate of polymerisation of peptides.

Similar studies have been performed with a number of eukaryotic micro-organisms including Saccharomyces cerevisiae (McMurrough and Rose, 1967), the filamentous fungus Neurospora crassa (Alberghina, Sturani and Gohlke, 1975) and the protozoan Tetrahymena pyriformis (Leick, 1967). The concentration of RNA per cell is found to be proportional to the growth rate but in contrast to bacteria, the rate of protein synthesis per amount of ribosomal RNA decreases as the cells grow more slowly. Thus in eukaryotic organisms ribosomal efficiency is dependent on growth rate (Waldron and Lacroute, 1975). This finding is supported by nutritional shift up experiments which show that protein synthesis increases immediately after the shift indicating that the number of ribosomes is not limiting (Ludwig, Oliver and McLaughlin, 1977). Benven and Gulløv (1979) have measured the actual peptide chain elongation rate in Saccharomyces cerevisiae and have found that this diminishes with decreasing growth rate. These workers have also postulated, based on theoretical calculations, that the proportion of ribosomes which are active in protein synthesis is also reduced at lower growth rates. Thus it appears that in eukaryotes the rate of translation is regulated both by the rate of peptide polymerisation as well as by the number of ribosomes actively engaged in this process. Nevertheless it is clear that in both prokaryotes and eukaryotes the controls over transcription and translation are closely interlinked.

Further evidence for co-ordinated regulation of these functions is provided by nutritional shift-down experiments. The restriction of RNA synthesis which occurs when cells are starved for a required amino acid has been called the 'stringent response' (Stent and Brenner, 1961). A detailed review of the stringent response, the analysis of which constitutes a large part of this thesis, is presented in later sections of this chapter.

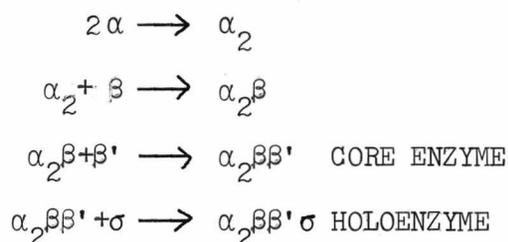
I.2. The Organisation and Structure of RNA Polymerases from Living Cells

The DNA dependent RNA polymerases catalyse the transcription of genetic information from DNA to RNA. It follows that the mechanisms involved in controlling transcription must all directly or indirectly affect the activity of the RNA polymerases. Thus, in discussing the regulation of RNA synthesis, it is necessary to consider some of the relevant properties of these important enzymes.

One of the major distinctions between eukaryotic and prokaryotic cells lies in the structure and organisation of the RNA polymerases. In bacteria there is only one enzyme responsible for the synthesis of RNA whereas three structurally different enzymes each with distinct functions have been identified in eukaryotic organisms. In general, however, it is found that RNA polymerases are large complex proteins having multi-subunit structures. A great deal is known about the bacterial enzymes and these will be considered first.

The Structure of Bacterial RNA Polymerases

Bacterial RNA polymerases are found to have four major polypeptide subunits denoted α , β , β' and σ (see review by Yura and Ishihama, 1979). Two active forms of polymerase occur which are known as the core enzyme and the holoenzyme. The core enzyme has the subunit composition, $\alpha_2\beta\beta'$. The σ subunit binds to this structure, relatively weakly, giving the holoenzyme. Reconstitution of polymerase activity from the separate subunits can be achieved (Ishihama and Ito, 1973; Palm et. al., 1975) and occurs in the following sequence:-



Temperature-sensitive mutants of Escherichia coli which have altered

subunits and which are blocked in polymerase assembly have been used to show that this same sequence occurs in vivo (Taketo and Ishihama, 1976, 1977).

A similar combination of genetic and biochemical approaches has been employed to elucidate functional roles for the different subunits. For example, the β subunit has been shown to be involved in both RNA chain initiation and elongation. This is known since mutations conferring resistance to antibiotics which inhibit these functions, e.g. rifampicin, streptolydigin, affect only the β subunit (Kawai, Ishihama and Yura, 1976). A nucleoside triphosphate binding site has also been found on the β subunit since this can be affinity labelled with UTP analogues (Armstrong, Sternbach and Eckstein, 1976). The β' subunit contains a DNA binding site as determined by mutant studies (Gross, Fields and Bautz, 1976) and by cross-linking with nucleic acid analogues (Frischauf and Scheit, 1973). It has also been reported to have a role in termination of transcription (see Yura and Ishihama, 1979 or Imai and Shigesada, 1978). No clear function has been assigned to the α subunit. It may simply perform a structural role in aligning the other subunits although it has been suggested to be involved with template recognition (Yura and Ishihama, 1979).

The two active forms of bacterial RNA polymerase show different specificity in their binding to DNA; RNA can be synthesised by the core enzyme alone but proper initiation from natural promoters on double-stranded DNA requires the σ -containing holoenzyme. The σ factor alone cannot bind to DNA (Zillig et. al., 1970, 1971). However the binding of σ factor to the core enzyme induces conformational changes in the latter (Wu, Yarborough and Wu, 1976) and an alteration in the DNA binding activity of the β' subunit (Fukuda and Ishihama, 1974). Thus the σ factor is implicated in the primary recognition of promoter DNA sequences. Genetic evidence using a temperature-sensitive mutant with an altered σ factor which has modified transcriptional specificity confirms this conclusion (Travers et. al., 1978).

In addition to the σ factor other protein factors with possible functions in regulating the activity of bacterial RNA polymerases have been found. A number of these factors are produced by the bacteriophages. Some of these phage-encoded proteins turn on the expression of other phage genes by altering the transcriptional specificity of the polymerase, e.g. in T4 infection of Escherichia coli (Rabussay and Geiduschek, 1977) and SPO 1 infection of Bacillus subtilis (Talkington and Pero, 1978). Others allow expression of phage genes by interacting with the polymerase to prevent the normal termination of transcription, e.g. in λ infection of Escherichia coli (Roberts, 1976).

Several different protein factors have been isolated from uninfected Escherichia coli which are proposed to influence the specificity of RNA polymerase. Similarly low molecular weight effector molecules, especially the guanosine polyphosphates, are known to affect polymerase specificity in vitro. It is clear that these observations indicate potential mechanisms for the control of transcription in bacterial cells. This possibility will be discussed more fully elsewhere in this chapter.

The Structure of Eukaryotic RNA Polymerases

In eukaryotic organisms the enzymes responsible for the transcription of genetic information are considerably more complex than those found in bacteria. Three distinct forms of RNA polymerase can be obtained by chromatographic resolution of extracts from eukaryotic cells. These separate forms, denoted I, II and III, have different catalytic properties in vitro, for example in their metal ion and template specificities and in their activity in varying salt concentrations (reviewed by Roeder, 1976).

The three classes of enzyme can also be distinguished by their sensitivity to the toxin α -amanitin. This inhibitor blocks the elongation step of transcription (Cochet-Meilhac and Chambon, 1974) by direct interaction with the polymerase (Mandel and Chambon, 1971). RNA polymerase II from animal cells is sensitive to low concentrations of the drug whereas animal polymerase I is insensitive. Polymerase III from animals has an intermediate sensitivity to

the drug (Seifart and Benecke, 1975; Hessenslop, Wells and Chambon, 1975). A similar pattern of sensitivities is shown by the enzymes isolated from insect cells except that polymerase III is also insensitive (Sklar and Roeder, 1975). In contrast, the yeast enzymes are more resistant to α -amanitin. Polymerase II is again the most susceptible but about twenty times the concentration used with the class II enzyme from animals is needed to give a similar level of inhibition. Yeast polymerase III is insensitive to α -amanitin but polymerase I is sensitive to very high concentrations of the drug (Schultz and Hall, 1976; Valenzuela et. al., 1976).

The differential effects of α -amanitin have been used to determine the specific functions of the different RNA polymerases. However the intact cells of many organisms are not freely permeable to the drug. In contrast, the endogenous RNA polymerase activities of isolated nuclei are instantaneously inhibited by α -amanitin and show the same sensitivities as the purified enzymes (Weinmann, Raskas and Roeder, 1975). These experiments have therefore been most fruitful using isolated nuclei. These studies indicate that polymerase I is responsible for the synthesis of the large molecular weight ribosomal RNA species. For example the synthesis of this RNA is fully resistant to α -amanitin concentrations that completely inhibit polymerases II and III in nuclei from mouse cells (Weinmann and Roeder, 1974) and Xenopus oocytes (Portner and Roeder, cited in Roeder, 1976). These data reinforce the earlier suggestion that the rRNA genes are transcribed by this enzyme since it is located in the nucleolus (Roeder and Rutter, 1970).

RNA polymerase II has been demonstrated to synthesise the heterogeneous nuclear RNA because the production of this class of RNA by isolated nuclei has the same sensitivity to α -amanitin as the purified enzyme (Weinmann, Raskas and Roeder, 1975; Zylber and Penman, 1971). The same conclusion was reached with intact cells using mutants resistant to α -amanitin which have an altered polymerase II (Chan, Whitmore and Siminovitch, 1972; Ingles et. al., 1976).

These observations suggested that messenger RNA is also synthesised by polymerase II. Direct evidence for this has been provided in isolated nuclei by examining the α -amanitin sensitivity of the synthesis of specific messages (Suzuki and Giza, 1976). Similar experiments have shown that the class III polymerases synthesise the low molecular RNA species, that is the 5S ribosomal RNA and transfer RNA (Weinmann and Roeder, 1974).

A different method that has been adopted to define the functions of the different polymerases is the analysis of transcriptional products from the isolated enzymes using purified templates. This approach is based on the assumption that the different polymerases have intrinsic capabilities for selective recognition and transcription of specific genes. Until recently these experiments have generally been unsuccessful using class I and class II polymerases which appeared to be no more capable of selective transcription of eukaryotic templates than were the bacterial RNA polymerases (see Roeder, 1976). Furthermore the activity of isolated polymerases on double-stranded DNA is poor. However preferential transcription of rRNA from total yeast DNA by the homologous class I enzyme has been reported (van Keulen, Planta & Retel, 1975; Hager et. al., 1976). The specific transcription of viral DNA templates by polymerase II purified from animal cells, in the presence of crude cell extracts, has been demonstrated (Weil et. al., 1979 a). These genes are actively transcribed by this enzyme during viral infection in vivo. A faithful and efficient cell-free transcription system for polymerase I has also recently been developed (Grummt, et. al. 1982). Activity is again dependent on the addition of crude cell free extracts which presumably supply additional components that are required for specific transcription.

The transcription of specific genes by cell free extracts has been most successful using type III polymerases. The faithful transcription of 5S rRNA and tRNA genes by polymerase III purified from a number of eukaryotes has been demonstrated (reviewed by Parker, Jaehnig and Roeder, 1978). Normally this occurs only when chromatin templates are employed. Specific transcription

TABLE I.1 - Comparison of subunit compositions of RNA polymerases from several eukaryotes.

The numbers refer to molecular weights $\times 10^{-3}$. Subunits enclosed in boxes have been shown to have identical molecular weights. In the case of yeast, Acanthamoeba, and cauliflower these polypeptides have been shown to be identical by two or more criteria (see text).

of 5S rRNA genes in purified DNA templates can be achieved, however, using cruder polymerase III preparations (Weil et. al., 1979b). These findings again imply a requirement for other functional transcription components which do not co-purify with the polymerase. These additional factors must interact with the DNA rather than the polymerase since the purified class III enzymes can faithfully transcribe chromatin templates.

The finding that the different eukaryotic RNA polymerases have distinct properties and functions is consistent with the observation that these enzymes also have distinct subunit structures. Detailed studies of the structure of eukaryotic polymerases have revealed that they are extremely complex multi-meric proteins. Table 11 compares the subunit structures of polymerases from several eukaryotic organisms. Polymerase I can be isolated chromatographically and electrophoretically in two distinct forms from a number of eukaryotes. The two forms normally differ by the presence or absence of the third largest subunit. In yeast the two types of polymerase I, denoted A and A*, differ also by another subunit (see table 12). It has been shown that these separate forms of yeast polymerase I are functionally distinct enzymes since the A* form is defective in both the DNA binding and elongation reactions of RNA synthesis (Cooper and Quincy, 1979).

RNA polymerase II has also been isolated in different forms from a variety of organisms. In all instances the distinguishing component is the subunit with the highest molecular weight (table 12). Two forms of yeast polymerase II can be isolated only when extreme precautions are taken to avoid protease action (Sentenac et. al., 1976). The enzyme normally prepared has ten subunits. When protease action is eliminated a form is isolated which contains a new polypeptide of very large molecular weight but which lacks two of the normal subunits. The two forms, BI and BII have similar α -amanitin sensitivities and enzymatic activities with purified DNA templates (Dezelee, 1977). It seems likely from this study of the yeast enzyme that the different forms of polymerase II from eukaryotes are proteolytic artefacts

I		II		III	
A	A*	BI	BII	C ₁	C ₂
		220	-		
185	185	180	180	160	160
137	137	150	150	128	128
				82	82
48	-			53	-
44	-	45	45		
41	41			41	41
36	36			37	37
			33.5	34	34
28	28	28	28	28	28
24	24	24	24	24	24
20	20	-	18	20	20
16	16				
14.5	14.5	14.5	14.5	14.5	14.5
12.3	12.3	12.5	12.5	11	11
		10	10		

Table I.2 - SUBUNIT STRUCTURES OF THE DIFFERENT FORMS
OF YEAST RNA POLYMERASE
THE FIGURES REFER TO MOLECULAR WEIGHTS $\times 10^{-3}$

arising from the method of purification itself. The alternative explanation, that the different forms of enzyme arise from a common precursor which is converted in vivo, is not discounted but is difficult to substantiate experimentally.

Two chromatographically distinct forms of RNA polymerase III have been detected in many eukaryotes. In yeast, one form contains 12 polypeptides while the other lacks the fourth largest of these (table 1.2). The existence of two types of class III enzyme could imply that the different forms are separately responsible for the transcription of the tRNA and 5S rRNA genes. However it is also possible that the alternative forms of enzyme are due to artefactual proteolysis or dissociation of subunits during the extraction procedure.

Electrophoretic analysis of the various types of polymerase has revealed yet more forms (Kedinger, Gissinger and Chambon, 1974; D'Alessio, Perna and Paule, 1979). This has been found to be due to heterogeneity in the charge associated with the individual subunits of the enzyme. It has been suggested that these charge differences are due to functional modifications of the polymerases (Roeder, 1976). The possibility of a regulatory role for such covalent modification is discussed in the next section of the Introduction.

The structural studies of eukaryotic RNA polymerase suggest that the three different classes of enzymes have common subunits. Comparison of the data shown in table 1.1 reveals that some of the subunits of all three types of eukaryotic RNA polymerase have the same molecular weights. Polymerases I and II from yeast have three subunits which are identical on the basis of molecular weight, isoelectric point and proteolytic fingerprint pattern (Buhler et. al., 1976a; Sentenac et. al., 1976). Detailed examination of the immunological cross-reactivity of the yeast polymerases has recently shown that these subunits are in fact common to all three types of enzyme (Buhler et. al., 1980 - see table 1.1). Polymerase I and III also have two other subunits which are common to both enzymes.

Similar conclusions have been reached about polymerases from other eukaryotes, e.g. cauliflower (Jendrisak, 1980), the free-living amoeba Acanthamoeba castellanii (D'Alessio, Perna & Paule, 1979) and other organisms (see review by Chambon, 1975) as shown in Table 1.1. Paule (1981) has pointed out that the common subunits from various organisms have similar characteristics. The largest common subunit is generally very basic and is present in a stoichiometry of two. The smallest is acidic and varies in stoichiometry between one and two.

The third common subunit is also acidic and is present in a single copy per polymerase molecule. The functional significance of common subunits is not known but may reflect a similarity in catalytic mechanism of the polymerases. Paule has noted that eukaryotic polymerases always contain two very large polypeptide subunits which are above a molecular weight of about 115,000 daltons (see Tables 1.1 and 1.2). He has suggested that these polypeptides together with the common subunits form the basic functional core of the eukaryotic polymerase analogous to the core enzyme of bacteria. The occurrence of common subunits may also allow a potential means for the co-ordinated regulation of the activity of the different polymerases.

I.3 The Regulation of RNA Synthesis by the Control of RNA Polymerase Activity

There are a large number of possible control mechanisms which can be postulated to regulate the synthesis of RNA. However any potential system of control over transcription must act by directly or indirectly affecting the activity of the enzymes which catalyse this process. Thus, changes in the overall level of RNA synthesis could be achieved by altering the actual concentration of polymerases within the cell. This form of control requires that the amount of RNA polymerase limits the rate of synthesis of RNA. The level of active polymerase could be varied by de novo synthesis of enzymes or by the activation of inactive precursors. Hence, in this model the control over the synthesis and assembly of polymerases is crucial to the regulation

of transcription.

The rate of RNA synthesis could alternatively be regulated by modulating the activity of existing polymerase molecules. This process could occur at the level of initiation or may effect the chain elongation rate. Either of these possibilities could be mediated through the action of regulatory effectors, by the covalent modification of polymerases, or by the level of substrates that are available. Control of initiation could lead to changes in the pattern of transcription as well as in the overall rate. This type of transcriptional control could also occur through alterations in the structure or conformation of the chromatin template. These modifications could be effected by factors associated with the chromatin itself. The regulation of the activity of these factors would, in turn, be critical to the control of RNA synthesis. This section of the Introduction will examine the experimental evidence that indicates which of these potential control mechanisms are relevant to the regulation of RNA synthesis in living cells.

Perhaps the most fundamental mode of control over RNA synthesis is through regulation of the number of active RNA polymerases. Since the major classes of RNA in eukaryotes are synthesised by distinct enzymes both the pattern and rate could be affected by such regulation. In a number of cases changes in the rates of synthesis of the different RNA species can be correlated with altered levels of enzymes. This result is most notable in differentiated cell types which respond to various stimuli with increased rates of protein synthesis or cell growth. For example marked increases in the levels of RNA polymerases I and III are observed in rapidly proliferating, as opposed to normal, mouse tissues (Schwartz et. al., 1974), and in regenerating rat liver (Yu, 1975). The same observation has been made for polymerase I in several other eukaryotic cells responding to growth promoting hormones (Van den Berg, et. al., 1974; Guilfoyle et. al., 1975; Babcock and Rich, 1973). In other instances, however, variations in the relative rates of synthesis of the major RNA species are not associated with altered polymerase levels

(Pong and Loomis, 1973; Detke and Paule, 1975; Borthwick and Smellie, 1975). It appears that in eukaryotes this type of transcriptional control is significant only in certain conditions. This conclusion is supported by experiments performed with yeast. The concentration of RNA polymerase I in yeast cells is found to be proportional to growth rate. Polymerase II levels are much less variable at different rates of growth (Sebastian, Mian and Halvorson, 1973). In contrast no difference in polymerase concentrations is detected in yeast cells and in other eukaryotes when protein synthesis is inhibited by a variety of methods (see next section).

In the bacterium, Escherichia coli, the cellular concentration of polymerase core enzyme, but not that of σ factor, increases in proportion to the growth rate (Matzura, Hansen and Zeuthen, 1973; Dablow 1973). Moreover when Escherichia coli cells are subjected to nutritional shift (up or down) the concentration of core enzyme is adjusted to the level characteristic of the post shift rate of growth (Iwakura and Ishihama, 1975). However the amount of core enzyme does not appear to limit the synthesis of RNA in these cells since a number of observations suggest that the RNA polymerase is synthesised in excess of that required. The rate of transcription increases immediately cells are shifted to a richer medium (Gausling, 1980) or when treated with chloramphenicol (Shen and Bremer, 1977a). The variation in polymerase concentration at different growth rates, measured by gel electrophoresis, is not sufficient to account for the changes in the rate of RNA synthesis observed (Travers, 1976a). Furthermore, it has been calculated that at fast growth rates there is about a two-fold excess of polymerase and this increases with decreasing growth rate (Nierlich, 1978).

The surplus of RNA polymerase found in growing bacterial cells over that actually engaged in RNA synthesis may be explained in several ways. These explanations generally involve regulation of the initiation of transcription since the rate of chain elongation is found to be fairly constant under most conditions in bacteria (Molin et.al., 1977; Shen and Bremer, 1977 a and b;

Mowbray and Nierlich, 1975). It is known that polymerases have an appreciable affinity for all regions of DNA as well as specific binding sites where successful initiation may occur. It appears that the rate limiting step in initiation may be the dissociation or displacement of these non-specific complexes (Chamberlin, 1976). Thus, at any given time, there would be a distribution of polymerases between these specific and non-specific sites giving equilibrium levels of functioning and non-functioning enzymes. It is possible that this distribution may be influenced in some way by the physiological state of the cell. This kind of argument has been developed by Maaløe (1966, 1979) who has put forward a 'passive' model for the regulation of transcription. The cellular environment is proposed to influence the overall pattern of transcription which indirectly affects the synthesis of the components of the protein synthesising system. According to this model, the maximum frequency at which each gene can be expressed is limited by the strength of the promoter with which it is associated. Genes with weak promoters have a lower frequency of initiation than genes having a strong promoter. Thus faster growth rates will favour a redistribution of polymerase to the genes with stronger promoters. These genes are proposed to include those encoding the protein synthesising system. In addition, an increased growth rate is usually associated with an enriched medium leading to the repression of many of the anabolic and catabolic enzymes. This repression will also favour the expression of genes whose increased function is essential for rapid growth.

Changes in the distribution of RNA polymerase resulting in an altered pattern of transcription could also be brought about by active mechanisms. Low molecular weight effector molecules, especially the adenine and guanine nucleotides are known to affect RNA synthesis by altering polymerase specificity (see other sections of this chapter). During amino acid starvation this process also involves protein factors (Ishihama and Saitoh, 1979; Aboud and Pastan, 1975) and tRNA (Aboud and Pastan, 1975). Evidence for a variety of other protein factors from bacteria with effects on transcription through

their action on the polymerase has also been reported (Muto, 1977; Block, 1976; Oostra, Geert and Gruber, 1980). In particular it has been found that a number of components of the translation machinery interact with the holoenzyme altering its specificity. These include f-met tRNA (Pongs and Ulbrich, 1976; Debenham, Pongs and Travers, 1980), IF-2 (Travers, Debenham and Pongs, 1980) and the elongation factors, EF-Tu (Travers, Kamen and Cashel, 1970), EF-Ts (Biebricher and Druminski, 1980) and EF-G (Travers et. al., 1981).

In the light of such data Travers has proposed a model of the bacterial polymerase as an allosteric protein able to exist in multiple conformations each of which favours the initiation of transcription at certain groups of promoters (Travers, 1976a; Travers, Kari and Mace, 1981). Thus the factors described here and others also serving as allosteric effectors might influence the pattern of transcription according to the physiological state of the cell. A similar, but more specific model for the control of polymerase activity has been proposed by Pribnow (1975). His scheme requires three distinct promoter elements: a recognition site which is characteristic of different classes of promoters, a binding site which is common to many or all promoters, and an initiation site. The first step in transcription is proposed to be the formation of a recognition site - holoenzyme complex which is influenced by the presence of regulatory subunits. The core moiety may then attach to the binding site and RNA synthesis may then commence at the initiation site. Thus in this model too, the various regulatory factors may direct the polymerase to produce different classes of transcript. Genetic and structural analysis of bacterial promoters (reviewed by Rosenberg and Court, 1979) has supported this model of initiation by RNA polymerase.

The low activity of eukaryotic polymerases on double-stranded DNA templates has encouraged an intensive search for stimulatory factors in eukaryotes analogous to these bacterial factors. The existence of such factors is indicated since the addition of crude cell extracts to purified eukaryotic

polymerases not only increases the overall rate of RNA synthesis but also facilitates the faithful transcription of specific genes (see above). There is evidence that these factors are similar in several different organisms since this effect occurs in heterologous cell-free transcription systems (Sprague, Larson and Morton, 1980; Wasylyk et. al., 1980). This is not the case for polymerase I, however (Grummt, Roth and Paule, 1982).

A number of proteins that stimulate transcription by polymerases from animal cells have been identified (Chuang and Chuang, 1975; Froehner and Bonner, 1973; Chesterton et. al., 1975; Revie and Dahmus, 1979; Sekimizu et. al., 1979). Some factors act on RNA initiation (Sugden and Keller, 1973; Lee and Dahmus, 1973) while others appear to affect the chain elongation rate (Seifart, Juhasz and Benecke, 1973; Stein, Hameister and Keding, 1973). Several other reports suggest that the modulation of rRNA synthesis in eukaryotes is brought about by an altered rate of chain elongation (Emerson, 1971; Barry and Gorski, 1971; Franze-Fernandez and Fontanive-Senguesa, 1975).

Stimulatory proteins have also been found in yeast. Di Mauro and co-workers (1972) have reported a factor which stimulates initiation by all three classes of polymerase. Another protein, P37, has been identified in yeast which greatly increases the specific activity of RNA polymerase II and can be isolated in complex with it (Sawadogo, Sentenac and Fromageot, 1980).

Covalent modification of RNA polymerases is another potential method for the control of polymerase activity. One modification which has been studied in eukaryotes is the phosphorylation of polymerase subunits. Several groups of workers have reported that protein kinases stimulate the activity of polymerases in cell-free systems (Jungmann, Heistand and Schweppe, 1974; Martelo and Hirsch, 1974; Dahmus, 1976). Furthermore the presence of phosphorylated subunits has been demonstrated in all three classes of polymerase from yeast (Buhler et. al., 1976b; Bell, Valenzuela and Rutter, 1976). The subunits were found to be phosphorylated in vitro using a protein kinase

which co-purified with the yeast polymerase I during part of the isolation procedure (Bell, Valenzuela and Rutter, 1977). However no significant effect of phosphorylation on the activity of the polymerases was observed using native yeast DNA templates. The activity of isolated polymerases with such templates is poor and this result does not necessarily reflect the situation within the cell. A possible role for the phosphorylation in the regulation of transcription cannot therefore be excluded. This would represent a means by which the synthesis of RNA could be related to the energetic state of eukaryotic cells, a form of control which is also apparent in bacteria (see below).

I.4. Other Factors Affecting the Regulation of RNA Synthesis

The Level of Substrates Available

The nucleoside triphosphates are substrates for RNA biosynthesis and substrate availability has been considered as a possible means of regulating the rate of RNA synthesis. Several investigations on the concentration of these compounds in cells growing at different rates and under differing conditions of physiological stress have been carried out.

In bacteria the general finding is that nucleoside triphosphate (NTP) levels are correlated with the growth rate (see Neirlich, 1978). The variations observed in NTP concentration, however, are not large enough to account for the range of rates of RNA accumulation that occur at different growth rates (Chapman and Atkinson, 1977). This conclusion is consistent with current views on the regulation of nucleotide biosynthesis in which the importance of product negative feedback is recognised (Chapman and Atkinson, 1977). Such a control mechanism is suited to the stabilisation of the level of these compounds and would be an inappropriate system for the regulation of RNA synthesis by precursor concentration. Furthermore bacterial RNA polymerase activity has been shown to be maximal at NTP concentrations below those normally observed in the cell (Anthony, Wu and Goldthwait, 1969; Rhodes and Chamberlin, 1974). Nevertheless the initiation reaction of RNA synthesis which requires ATP or GTP, has K_m values for these compounds of about ten times the

corresponding values for elongation (Anthony, Wu and Goldthwait, 1969). Thus, particularly in the case of GTP, the cellular concentrations may be in the range where they might influence the frequency of initiation. However during various types of nutritional shift-down experiments the inhibition of RNA accumulation could not be accounted for by depletion of the NTP pools (Nazar and Wong, 1972). Only during starvation for inorganic phosphate is there a relationship between the rate of RNA synthesis and NTP levels which might suggest that substrate limitation is a regulatory factor (Nazar, Tyfield and Wong, 1972).

In any discussion of this kind the part played by the guanine nucleotides which have a fundamental role in the regulation of RNA synthesis in bacteria must be considered. This important aspect is reviewed fully in a later section of this chapter. Travers and co-workers have suggested that the adenine nucleotides also have a similar role (Travers, Kari and Mace, 1981). The bacterial polymerase can be isolated by zonal sedimentation in two main structural forms which differ functionally in their promotor specificity (Travers, Buckland and Debenham, 1980). It is proposed that one of these forms is stabilised by the adenine nucleotides and the polymerase is said to be in the A state. The other form is in the G state and is proposed to be stabilised by the guanine nucleotides. These two types of polymerase can exist in a number of functionally different subforms depending on the specific nucleotide that is predominant and on the influence of other regulatory factors, for instance the components of the translation system (see above). The use of adenine nucleotide analogues which cannot be incorporated into RNA has shown that the action of these components is not due to their role as substrates (see Travers et. al., 1980).

This model relates the activity of the RNA polymerase and thus the synthesis of RNA with the energy metabolism of the cell. It is known that by perturbing the energy balance of cells, for example using strains with

temperature-sensitive mutations in fructose 1,6 diphosphate or adenylate kinase, results in the cessation of RNA synthesis (Bock and Neidhart, 1966 Frey, Newlin and Atherly, 1975). Since this effect occurs without the accumulation of ppGpp these findings are in agreement with the model of Travers et. al.. Furthermore measures of the energetic state of the cell, termed the 'energy charge' defined as
$$\frac{[ATP] + 0.5 [ADP]}{[ATP] + [ADP] + [AMP]}$$
 (Atkinson, 1969), give an indication of the degree of phosphorylation of nucleotides in general. That is, changes in ATP concentration generally cause changes in the levels of the other nucleotide triphosphates (Nazar and Wong, 1972; Nazar, Tyfield and Wong, 1972; Bagnara and Finch, 1973; Beck et. al., 1973). Hence the energetic state of the cell rather than the concentration of nucleotides would appear to be the more important factor in determining the level of RNA synthesis. However, the energy charge of bacterial cells remains fairly constant over a wide range of growth rates (Franzen and Binkley, 1961; Lowry et. al., 1971). In view of these facts Travers et. al. have suggested that changes in the pattern of gene expression are mediated by nucleotide induced changes in the template selectivity of RNA polymerase mainly during amino acid starvation or energy deprivation. During balanced growth this pattern is influenced by the interaction of the components of the translation system with the polymerase (Travers et. al., 1980).

In the majority of eukaryotes two ribonucleotide pools are thought to exist. Plageman (1972) has demonstrated the existence, in Novikoff hepatoma cells, of a cytoplasmic and a nuclear pool of which only the small nuclear pool serves as a functional one for RNA synthesis. Hence, although cellular nucleotide pools are relatively easy to determine these measurements may not necessarily correspond to the amount of nucleotides actually available for RNA synthesis. Moreover the determination of nuclear pools is a formidable task since prevention of nuclear leakage after cell lysis is difficult if not impossible. However it has been proposed that in most mammalian cells

while there is compartmentation of the nuclear and cytoplasmic nucleotide pools the two equilibrate very rapidly and that measurements of total cellular nucleotides do reflect their availability for polymerisation into RNA (Khym et. at., 1978).

By obtaining estimates of this kind in Ehrlich ascites cells, Grummt and Grummt (1976a) have suggested that rRNA synthesis may be controlled by the size of the purine nucleotide pools. The evidence for this is that during a amino acid starvation, when rRNA synthesis is reduced, shrinkage of these pools occurs. Also on adding adenosine or guanosine to starved cells the rate of rRNA transcription is fully restored although protein synthesis remains inhibited. These workers have also proposed that uncharged tRNA is an important component in this control mechanism. This proposal is based on the finding that animal ribosomes can degrade GTP to guanosine if the ribosomal A site is occupied by uncharged tRNA (Grummt and Speckbacher, 1975). In addition treatment of Ehrlich ascites cells and 3T6 fibroblasts with the histidine analogue histidinol also causes a reduction of the cellular ATP and GTP levels (Grummt and Grummt, 1976b). This compound acts as a competitive inhibitor of histidyl-tRNA synthetase thereby inducing high levels of uncharged his-tRNA and concomitant inhibition of RNA synthesis.

Contraction of the purine nucleotide pools has also been observed in Ehrlich ascites cells during histidinol treatment and amino acid starvation by other workers (Van Venrooj, Henshaw and Hirsch, 1972; Live and Kaminskas, 1975). However Dehlinger and co-workers have found that treatment of both Friend Leukemia cells and normal rat kidney cells with histidinol did not result in a decline in ATP or GTP pool size even though protein and RNA synthesis were both decreased (Dehlinger, Hamilton and Litt, 1977). These contrasting observations have been explained by considering the opposing influences on nucleotide levels. Ribosome-mediated hydrolysis of GTP would tend to decrease these levels while decreased utilisation of these nucleotides during inhibition of transcription would lead to an increase in the amounts of GTP and ATP.

Indeed these workers found that cycloheximide-induced reduction of RNA synthesis caused an increase in the GTP pool size in Friend leukemia cells (cited in Dehlinger, Hamilton and Litt, 1977).

In yeast a separate nuclear UTP pool has been proposed which is preferentially labelled and more rapidly saturated than the cytoplasmic pool when cells are incubated with radioactive uracil indicating that they are not in equilibrium (Gross and Pogo, 1976a). In contrast Peterson and co-workers have studied the kinetics of labelling of RNA and suggested that there is rapid equilibrium between the cytoplasmic and nuclear pools (Peterson, Nierlich and McLaughlin, 1978). Amino acid starvation of yeast cells has been found to have little effect on the total nucleoside triphosphate pools (Kudrna and Edlin, 1975). Inhibition of the uptake of labelled precursors into nucleotide pools by yeast sphaeroplasts has been reported (Shulman, Sripati and Warner, 1977) but this is not supported by others (Gross and Pogo, 1976a). The nuclear UTP pool is increased by approximately 50% during amino acid starvation but the addition of cycloheximide to starved cells has no effect on either the nuclear or total UTP pools (Kelker and Pogo, 1980). Thus no direct correlation between nucleotide pool size, either nuclear or cellular, and the level of RNA synthesis has been observed in yeast. A similar conclusion concerning total nucleotide pools has been reached in another eukaryotic microorganism, Neurospora crassa (Constantini, Zippel and Sturani, 1977).

The Activity of the DNA Template

Yeasts in common with most other eukaryotes possess multiple copies of the ribosomal RNA genes (Schweizer, Mackechnie and Halvorson, 1969) thereby conferring another possible level for the control of rRNA gene expression. Hybridisation data has shown that the rRNA genes are normally present in about 140 copies (Øyen, 1973; Finkelstein, Blamire and Marmur, 1972). Genetic analysis has located these genes in a single array on Chromosome XII (Petes, 1979 a & b). There is little evidence to suggest that changes in the rate of rRNA synthesis can be brought about by altering the number of transcriptionally

active rRNA genes.

Schweizer and Halvorson (1969) found that the amount of rDNA remained constant at different growth rates and concluded that variations in the rRNA synthesis cannot be due to differential amplifications of the rRNA cistrons. Nutritional shift up from a poor nitrogen source to medium containing ammonium ions again has no effect on the amount of rDNA per cell (Walmsley, 1982). The level of rDNA present in different strains of Saccharomyces cerevisiae has also been found to be fairly constant (Gimmler and Schweizer, 1972; Retel and Planta, 1968; de Kloet, 1970; Hollenberg, 1973). However strains with unusually high amounts occur (Harbitz and Øyen, 1974). Strains containing less than the normal complement of rDNA have also been constructed (Øyen, 1973; Kaback, Bhargava and Halvorson, 1973; Finkelstein, Blamire and Marmur, 1972). Kaback and Halvorson (1977, 1978) have shown that during repeated subculture of one such strain, which was initially deficient for 25% of rDNA, the number of rRNA genes increased to wild type levels. However no phenotypic differences between the original and the 'magnified' strains were detected and both had the same growth rate and RNA content per cell.

Ribosomal RNA gene magnification analogous to that found in yeast has been described in a number of other eukaryotes. In these organisms the phenomenon of gene amplification does not have a general role in the regulation of RNA synthesis (e.g. the fruitfly Drosophila melanogaster - Ritossa et. al., 1966) but rather is relevant to cells having unusual life cycles, e.g. the protozoan, Tetrahymena pyriformis (Elliot, 1974) or specialised functions e.g. in oocytes of the amphibian, Xenopus laevis (Brown and Dawid, 1968).

It appears that although in eukaryotes multiple copies of the rDNA genes are important in order to maintain sufficient levels of rRNA synthesis, alterations in gene dose do not have a role in the regulation of this process. It is possible, however, that such regulation could be effected through variation of the number of genes that are transcriptionally active, for instance, by altering the state of the promotor. This could be achieved by altering the

structure or conformation of the chromatin. In this connection the degree of DNA supercoiling (reviewed by Smith, 1981) and nucleosome structure (reviewed by Wiesbrod, 1982) have both been considered as possible means for the regulation of gene expression. Experiments using purified templates and polymerases (reviewed above) suggest that factors associated with chromatin do have a role in regulating the initiation of transcription. However, in yeast no profound structural rearrangements have been observed in transcriptionally active chromatin with respect to that which is inactive (Lohr and Ide, 1979; Lohr, 1981).

The work reviewed so far in this chapter has indicated that RNA synthesis in living cells is regulated in a complex manner through a variety of mechanisms. The interplay of factors controlling the synthesis of RNA during amino acid starvation of cells will now be considered.

I.5 The Stringent Response

The mechanisms by which living cells adjust their metabolism in response to the availability of amino acids is one of the most widely studied aspects of cellular regulation. In particular the dramatic reduction of RNA synthesis caused by the lack of a required amino acid, the stringent response, has been found to occur in a large variety of living organisms ranging from bacterial and eukaryotic microbes through to cells cultured from a number of higher organisms. Elucidation of the molecular basis of stringent control has been most readily performed in the bacterium, Escherichia coli, due to the abundance of suitable auxotrophic and regulatory mutants. This system will be discussed first.

The Stringent Response in Escherichia coli

Starvation of auxotrophic strains of Escherichia coli for required amino acids has been found to cause major readjustments of cellular activity in addition to the inhibition of RNA synthesis. These effects include reductions in the synthesis of nucleotides, glycolytic intermediates, carbohydrates, lipids, fatty acids, polyamines and peptidoglycans, restricted membrane

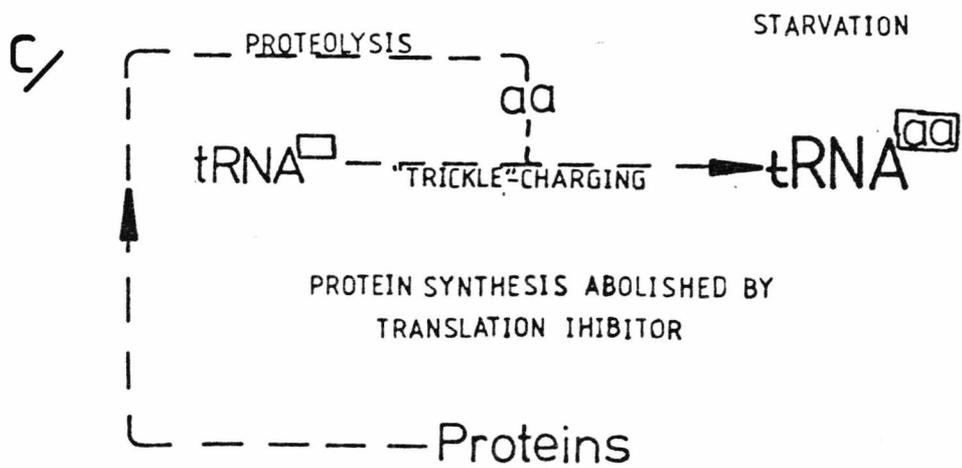
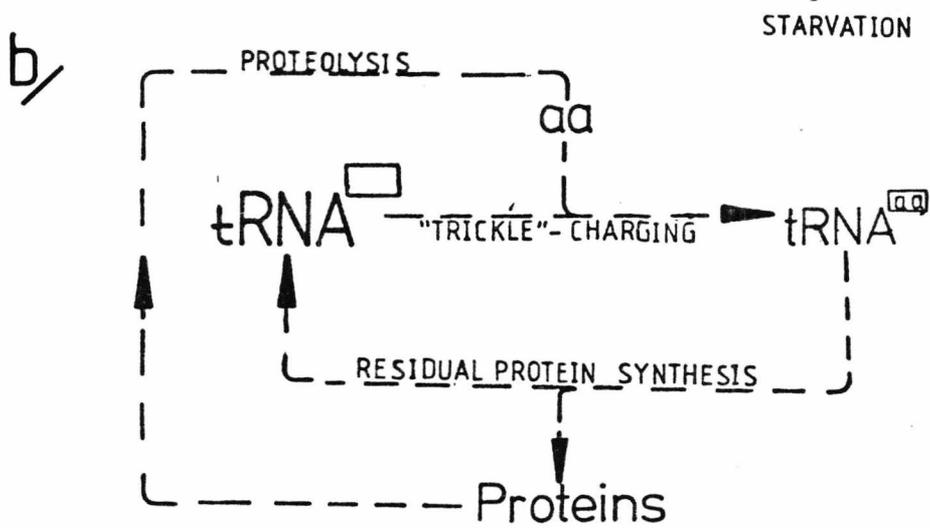
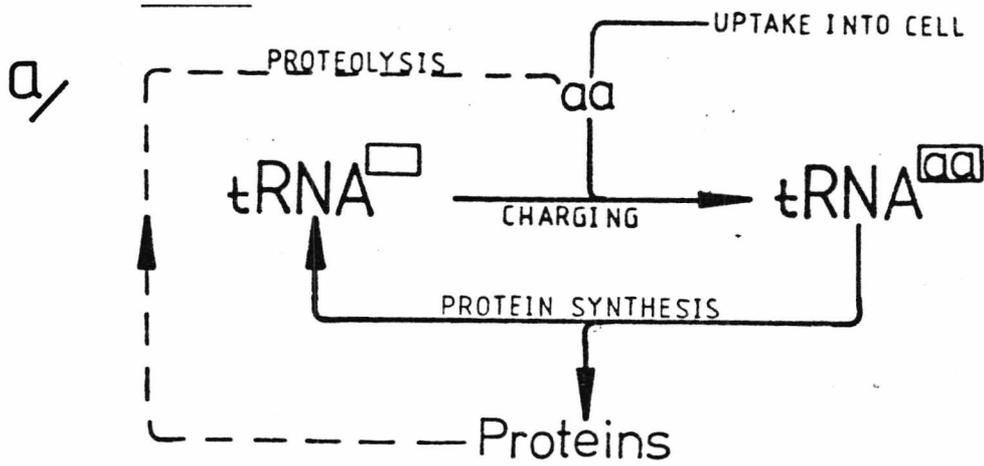
transport and an increased rate of protein turnover (see reviews by Cashel, 1975 and Gallant, 1979). Consideration of the variety of processes affected by deprivation of amino acids prompted a search for effector molecules which could mediate such a pleiotropic response. The key to the identification of these regulatory effectors was the discovery of mutant strains in which the amino acid dependence of RNA synthesis is relaxed (Borek, Rockenbach and Ryan, 1956). Although mapping at a single locus, the rel A gene (Alfoldi, Stent and Clowes, 1962), this mutation also relaxes the whole range of processes normally under stringent control (see review by Cashel and Gallant, 1974).

Amino acid starvation of normal strains was found to lead to the rapid accumulation of two low molecular weight compounds which do not appear in relaxed strains. These were first observed as spots on chromatograms of acid soluble extracts and were termed 'magic spots' I and II (Cashel and Gallant, 1969). Subsequently these substances were chemically identified as guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) (reviewed by Lipmann and Sy, 1976). The rel A - dependent synthesis of these compounds is triggered by starvation for a variety of amino acids and is not specific for any particular one (Cashel, 1969). The onset of ppGpp accumulation occurs within a few seconds of starvation preceding the effect on RNA synthesis which is not restricted until at least a minute later (Cashel, 1969; Gallant et. al., 1970). When amino acid — starved stringent strains are resupplied with amino acids the magic spot. (M.S.) nucleotides rapidly disappear and protein and RNA accumulation resume normally (Cashel, 1969; Lund and Kjeldgaard, 1972). All of these facts suggested that magic spot nucleotides were mediators of the physiological changes characteristic of the stringent response. Furthermore the rel A gene product appeared to be involved in adjusting the level of these nucleotides in response to the availability of amino acids. However other components were shown to be necessary for the formation of these compounds.

Fig I.1 'Trickle-charging' in amino acid-starved bacteria

- a) In normally growing auxotrophic cells the required amino acid is imported from the medium and is consumed during tRNA charging. Charged tRNA is then deacylated during protein synthesis.
- b) When auxotrophs are starved the sole supply of the required amino acid is through protein turnover and tRNA is 'trickle-charged' from this source. However, charged tRNA is consumed by residual protein synthesis and there is a net accumulation of uncharged tRNA.
- c) When residual protein synthesis is abolished by the addition of translation inhibitors to the starved cells, 'trickle-charging' results in high levels of charged tRNA.

FIG. I.1



The accumulation of ppGpp and concomitant restriction of RNA synthesis was found to be induced in mutants with temperature sensitive aminoacyl-tRNA synthetases held at the restrictive temperature even in the presence of all twenty amino acids (de Boer et. al., 1971; Neidhart, 1966). This result suggested a dependence of M.S. nucleotide formation on uncharged tRNA. In agreement with this idea was the finding that the addition of protein synthesis inhibitors, for example fusidic acid or chloramphenicol, to starved cells led to the resumption of a normal rate of RNA synthesis (Kurland and Maaløe, 1962) and also blocked the production of ppGpp (Cashel, 1969; Lund and Kjeldgaard, 1972). This may be explained in terms of the observed effects on tRNA charging which occur in cells undergoing these treatments (Lund and Kjeldgaard, 1972; Kurland and Maaløe, 1962; Kaplan, Atherly and Barret, 1973; Morris and de Moss, 1965; Ezekiel and Elkins, 1968). In starved cells the sole supply of the required amino acid is through intracellular protein turnover. Transfer RNA is 'trickle-charged' using these amino acids but the charged tRNA produced in this manner is continually consumed by residual protein synthesis (fig.I.1b). When this consumption is blocked directly by the action of inhibitors recharging occurs to near normal levels and RNA synthesis is stimulated (fig.I.1a).

The requirement for mRNA in the production of ppGpp was shown by the use of the drug rifampicin. This inhibitor blocks the formation of mRNA by interacting with the RNA polymerase (see above). Wong and Nazar (1970) showed that rifampicin also caused inhibition of ppGpp formation in amino acid-starved cells. This result was interpreted to show the direct involvement of mRNA in this process but could also be due to an indirect effect caused by the blockage of residual protein synthesis (Erlich, Laffler and Gallant, 1971).

The use of the protein synthesis inhibitor tetracycline led to the conclusion that magic spot production was also dependent on the ribosome itself. This drug, unlike fusidic acid or chloramphenicol, was still able to block ppGpp formation even in temperature-sensitive synthetase mutants which cannot trickle charge tRNA at restrictive temperature (Kaplan, Atherly and Barret,

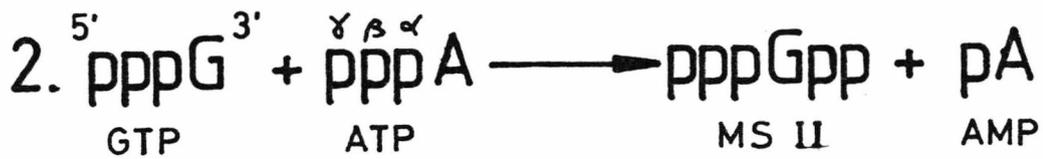
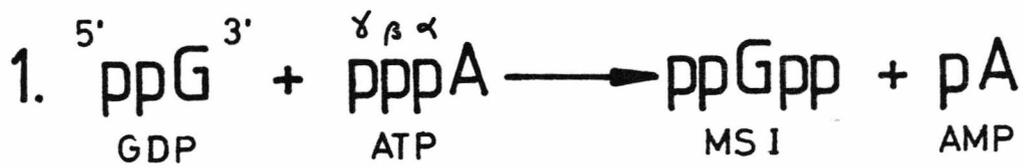


Fig. I.2— THE FORMATION OF 'MS' NUCLEOTIDES

1973). Thus tetracycline which inhibits the binding of both charged and uncharged tRNA to ribosomes (Levin, 1970), also acts as a direct inhibitor of ppGpp synthesis. In summary, it appeared that the conditions required for the synthesis of magic spot nucleotides include the restricted aminoacylation of any tRNA species, a functional mRNA-ribosome complex, the rel A gene product, and the target of tetracycline inhibition, presumably a functional ribosome-tRNA acceptor site. The hypothesis that emerged from these data was that ppGpp and pppGpp were synthesised by ribosomes in response to the codon specific binding of uncharged tRNA (Cashel, 1969; Lund and Kjeldgaard, 1972).

These ideas have been verified by a large number of in vitro experiments, which have been reviewed in detail by Block and Haseltine (1974). This biochemical evidence in favour of the model can be summarised as follows. The synthesis of ppGpp and pppGpp occurs by the transfer of the $\beta\gamma$ pyrophosphate group of ATP to the 3'-hydroxyl group of GDP or GTP (Sy and Lippmann, 1973) - see fig. I.2, and this reaction occurs at the ribosome (Cashel, 1969). In rel A⁺ strains the active component, 'stringent factor', can be dissociated from the ribosome by salt washing (Cochran and Byrne, 1974). This factor is active when reassociated with salt-washed ribosomes from rel A⁻ strains (Cochran and Byrne, 1974). The rel A gene codes for the stringent factor (Block and Haseltine, 1973). This factor has been purified (Block and Haseltine, 1975; Pederson and Kjeldgaard, 1977) and exhibits a low level of phosphotransferase activity when apart from the ribosomes (Sy et. al., 1973 a and b). Its activity is stimulated by the codon-specific binding of uncharged tRNA to the ribosomal A site (Pederson, Lund and Kjeldgaard, 1973; Haseltine and Block, 1973). Hence the working model of stringent control developed in E. coli is that during amino acid starvation ribosomes continue translation of the messenger RNA until a codon specifying the missing amino acid is encountered. An uncharged tRNA molecule then binds to this codon at the acceptor site of the ribosome resulting in the activation of the stringent factor and the synthesis of pppGpp and ppGpp - see fig. I.3.

FIGURE I.3 - The mechanism of stringent control in E. coli.

a) During normal growth the codon specific binding of aminoacyl-tRNA to the ribosomal A site occurs followed by peptide bond synthesis and translocation.

b) During amino acid-starvation the codon specific binding of uncharged tRNA to the ribosomal A site stimulates 'stringent factor' and the synthesis of MS nucleotides.

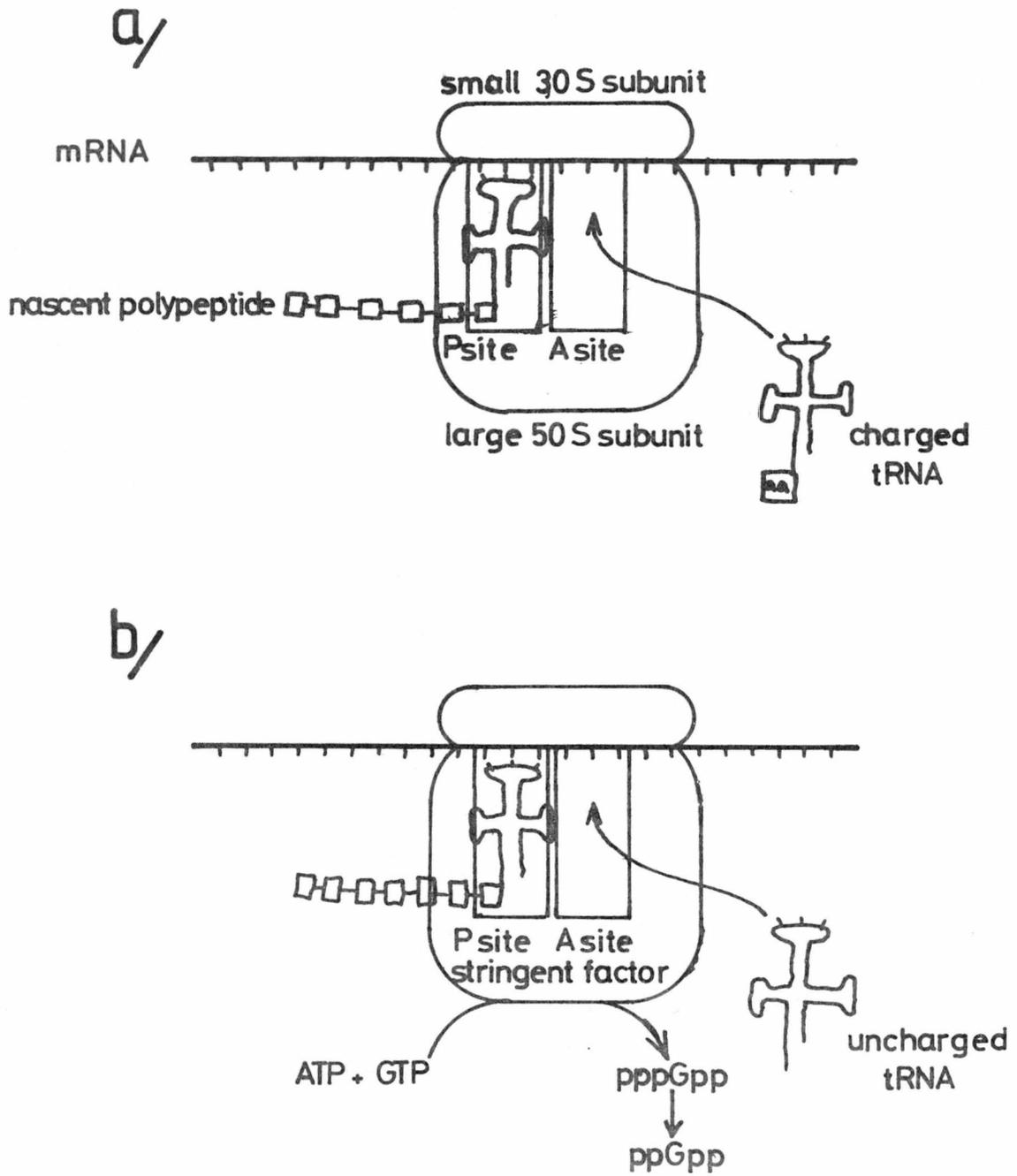


Fig.I.3

The metabolism of MS nucleotides has been investigated with the aid of other regulatory mutants of E. coli, designated spo T. These mutants do not accumulate pppGpp during the stringent response. The concentration of this nucleotide increases during the first two minutes of amino acid starvation but then drops to a barely detectable level (Chaloner-Larrson and Yamazaki, 1976). These mutants also accumulate levels of ppGpp two to four-fold higher than in normal strains when starved and have a slower rate of ppGpp decay when resupplied with amino acids (Weyer et. al., 1976). Kinetic studies of the formation of guanine nucleotides during the stringent response in these strains indicates that pppGpp is the precursor to ppGpp and that the major pathway of biosynthesis in these conditions is $GTP \rightarrow pppGpp \rightarrow ppGpp$ (Kari, Torok and Travers, 1977; de Boer et. al., 1977). These results together imply that the spo T gene product is responsible for ppGpp turnover and that this nucleotide inhibits the formation of pppGpp (see review by Gallant, 1979). An enzyme which degrades ppGpp to GDP has been identified (Sy, 1977; Heim Meyer and Richter, 1977; An et. al., 1979) and appears to be the spo T gene product. The activity of this enzyme has recently been shown to be inhibited by uncharged tRNA (Dietmar, 1980) indicating another role for this moiety in the mechanism of stringent control. The dephosphorylation of pppGpp to ppGpp can be carried out by at least five enzymes including EF-Tu and EF-G (Hamel and Cashel, 1973) but appears to be carried out in vivo by a specific enzyme encoded by the gpp gene (Somerville and Ahmed, 1979).

When E. coli cells are starved for a carbon/energy source the accumulation of ppGpp (but not pppGpp) is stimulated and there is an abrupt decrease in stable RNA synthesis. This response is observed in both rel A⁺ and rel A⁻ strains and thus represents rel A - independent synthesis of ppGpp, quite distinct from stringent control (Neidhart, 1963). Other evidence for rel A - independent synthesis of ppGpp has been found in relaxed strains since, as in normal strains, there is an inverse relationship between basal levels of this nucleotide and doubling time during normal exponential growth (Gallant &

Lazzarini,

1976; de Boer, Bakker and Gruber, 1977). Residual synthesis of ppGpp in the absence of decay will thus lead to its accumulation. The basal level of this nucleotide and its adjustment during downshifts are affected by the rel X gene, the product of which is yet to be identified (Pao and Gallant, 1978). A scheme summarising the current views of guanine nucleotide metabolism in E. coli is shown in fig.I.4. A third phosphorylated MS compound, ppGp, appears in this diagram and its role in stringent control is discussed below.

The specific regulatory effects of ppGpp in vitro have been investigated in some detail and are reviewed by Gallant (1979). There are three levels at which this compound appears to affect the metabolic processes of the cell. Firstly ppGpp directly inhibits the in vitro activity of a number of metabolic enzymes. Most of these effects correlate well with the metabolic adjustments observed during the stringent response in vivo. Thus there is little question that regulatory effects of ppGpp are central to the stringent adjustment of metabolism. Secondly ppGpp influences the transcriptional specificity of coupled transcription-translation systems reducing the total RNA synthesis by about a factor of two (see Gallant, 1979). When specific effects are studied the production of many transcripts is inhibited by this order of magnitude while some are more severely inhibited. Conversely stimulation of the production of some transcripts by ppGpp has been observed. However discrepancies are found concerning these studies and the correlation with in vivo effects is not very satisfactory. For example the synthesis of rRNA in these systems has been reported to be severely inhibited by some workers (Van Ooyen et. al., 1975; Reiness et. al., 1975; Travers, 1973) but only weakly inhibited by others (Haseltine, 1972; Atherly, 1974; Muto, 1975). Similarly the synthesis of Su^+tRNA^{tyr} is under stringent control in vivo (Primakoff and Berg, 1970) but there are contradictory reports on the effect of ppGpp on its synthesis in vitro (Debenham and Travers, 1977; Reiness et. al., 1975). These discrepancies could be due to differences in the assay conditions used, such as temperature or ionic strength. These factors have been found to be crucial in such experiments (Travers, 1976b). Thus it may be difficult to discriminate between

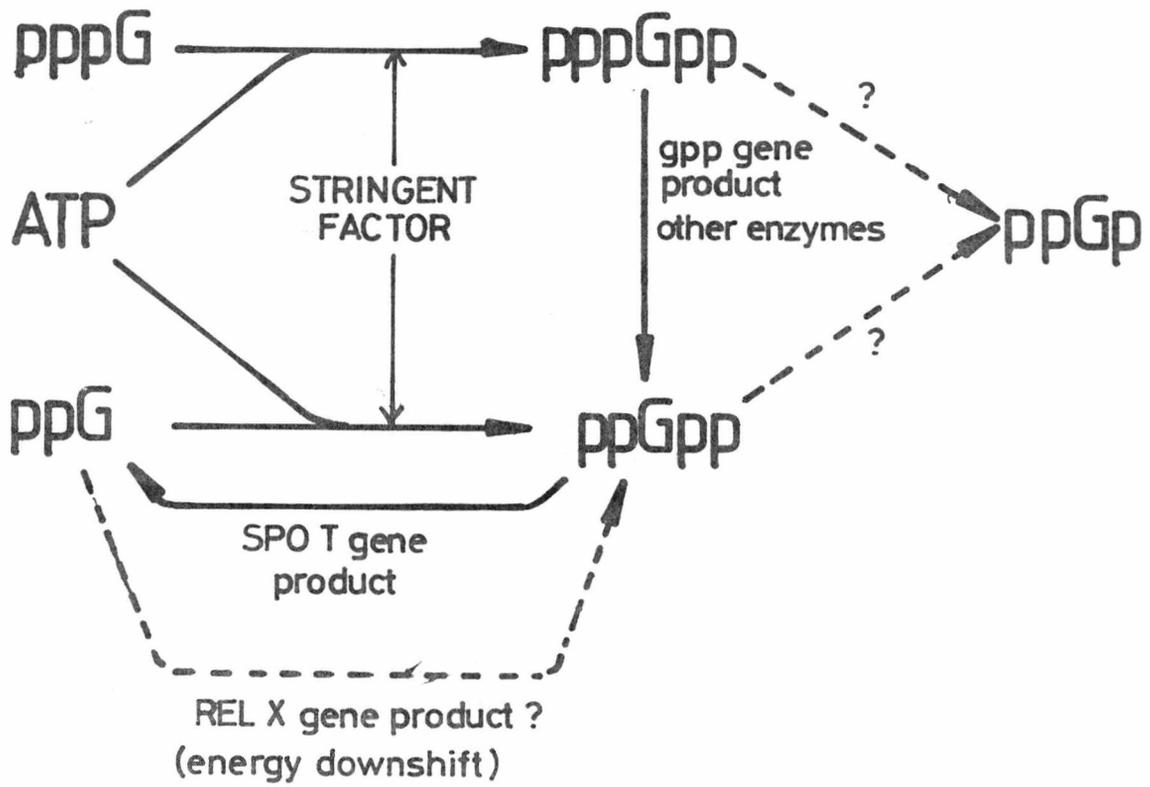


Fig.I.4 THE METABOLISM OF 'MAGIC SPOT' COMPOUNDS IN E.COLI

physiologically significant results and artefacts which are an intrinsic problem of the experimental system itself.

Additional factors may also be involved in modulating the promotor specificity of RNA polymerase during stringent control. One obvious candidate is the other magic spot compound, pppGpp. However this possibility has been left unexplored probably because the stringent response in spo T mutants is essentially normal. Another related nucleotide, guanine 5' disphosphate 3' monophosphate (ppGp), has recently been identified in E. coli cells undergoing the stringent response (Pao and Gallant, 1979). Experiments with permeabilised cells show a much better correlation between the effects of this nucleotide in vitro and the stringent response in vivo particularly in the case of rRNA and tRNA synthesis (Pao, Dennis and Gallant, 1980). It may well be that ppGp is the physiologically active nucleotide in the stringent control of RNA synthesis although in such a system it is difficult to exclude the possibility of further metabolism of this compound to give the actual regulatory moiety.

The third type of effect caused by ppGpp is the maintenance of translational accuracy. Residual protein synthesis during amino acid limitation proceeds at about the same rate in wild type cells and otherwise isogenic relaxed strains (Gallant et. al., 1970). However relaxed strains have a deficit in the levels of many enzymes (see review by Gallant, 1979). This deficit is found to be due to effects on both transcription and translation with that on the latter being greatest. For example, the large reduction in formation of active tryptophan synthetase is not accounted for by the much smaller deficit in the production of the tryptophan operon mRNA in fully derepressed rel A⁻ strains (Sokawa, Sokawa and Kaziro, 1974). Most of the reduction in the synthesis of active enzymes was shown to be due to an increased level of mistranslation (see review by Gallant, 1979). Mistranslation in rel A mutants is not confined to the codons which require the aminoacyl-tRNA species that is in short supply since phenotypic suppression of nonsense mutations has been detected (Gallant and Foley, 1979). The most straightforward interpretation of these data,

that mistranslation is a direct consequence of the mutant rel A gene product found on the ribosome does not apply. The effect was shown to be independent of the rel A genotype since it is alleviated by imposing a carbon/energy source downshift prior to amino acid starvation of rel A⁻ mutants. This raises the level of ppGpp in such cells by the rel A-independent pathway and also alleviates the mistranslation effect (Gallant and Foley, 1979). Thus ppGpp itself appears to maintain the accuracy of translation in cells undergoing amino acid starvation.

Gallant (1979) has put forward a model to account for this phenomenon. The binding of tRNA to the ribosome is considered to be a two stage reaction. In the first step, both correct and incorrect molecules may reversibly bind in an equilibrium manner. The second step is an irreversible reaction which alters the complex so as to remove ribosome-bound molecules from this equilibrium. A delay is proposed to occur between these steps which allows time for incorrect tRNA molecules to dissociate. Thus by increasing the length of this delay translational accuracy may also be enhanced. It is proposed that ppGpp works in this manner through delaying the GTP mediated steps in translation by acting as an analogue.

This model also accommodates the finding that a decrease in transcription occurs in rel A⁻ mutants as in the case of the derepressed tryptophan operon. This effect involves control of attenuation, that is when trp mRNA synthesis is terminated after transcription of a non-coding region (the leader sequence) at a site just before the coding region. This occurs when adequate levels of tryptophan prevail and the trp operon is repressed. When the supply of tryptophan is limited transcription of the structural locus is permitted and the operon is derepressed. This derepression is a direct consequence of the coupling of transcription and translation in bacteria. It has been shown to be due to conformational change in the secondary structure of the leader sequence which is permitted by the idling of ribosomes at a sequence rich in tryptophan specifying codons (Crawford and Stauffer, 1980). Gallant has proposed that rel A mutants fail to provide the proper signal for attenuation since in the

absence of ppGpp ribosome idling is reduced.

In conclusion the study of the stringent response and related phenomena in E. coli has shown that the guanine nucleotides are mediators of the metabolic changes that are made by the bacterial cell in response to changing environmental conditions. In particular the synthesis of magic spot nucleotides is related to the presence of the twenty amino acids. The biochemical mechanism whereby this is achieved involves a ribosome-associated enzyme the activity of which is dependent on a functional protein synthesising system and specifically requires uncharged tRNA. The ribosome may be considered to be a sensory organelle which can respond to the signal of uncharged tRNA by synthesising low molecular weight effectors, the magic spot nucleotides, which serve to regulate cellular metabolism as appropriate to the physiological environment.

The Stringent Response in Eukaryotic Organisms

The restriction of RNA synthesis in response to starvation for amino acids is a phenomenon which is common to many eukaryotic cells including yeast (Schmidt et. al., 1956; Ycas and Brawerman, 1957; Roth and Dampier, 1969; Wehr and Parks, 1969), Neurospora crassa (Buckel and Bock, 1973), the green algae, Ankistrodesmus braunii (Buckel and Bock, 1973) and Chlamydomonas reinhardtii (Heizmann and Howell, 1978; McMahon and Longstroth, 1972), the protozoan Tetrahymena pyriformis (Byfield and Scherbaum, 1967) and in several types of cultured mammalian cells e.g. Chinese Hamster Ovary cells (Fan, Fisher and Edlin, 1973), mouse fibroblast (Hershko et. al., 1971) and ascites cells (Grummt, Smith and Grummt, 1976), Ehrlich (Franze-Fernandez and Pogo, 1971) and Lanschutz ascites cells (Shields and Korner, 1970), lymphoma cells (Ellem, Fabrizio and Jackson, 1970) Friend leukaemia cells (Litt and Weiser, 1978), Changs Liver cells (Elliasson, Bauer and Hultin, 1967) and Hela cells (Vaughan et. al., 1967; Maden et. al., 1969).

As in bacteria the effect is elicited by uncharged tRNA rather than by a lack of amino acids per se. Thus, direct interference with the charging

reaction using the amino acid analogues, histidinol (Vaughen and Hansen, 1973; Warrington, Wratten and Hechtman, 1977; Dehlinger, Hamilton and Litt, 1977) or O-methyl threonine (Smulson, 1970), also inhibits RNA synthesis in most of the cell types mentioned above. This method of producing a 'functional amino acid deprivation' is often the most convenient way of studying the stringent response in higher eukaryotes where the number of genetically well-defined auxotrophic mutants is limited.

The addition of protein synthesis inhibitors to starved cells has been shown to stimulate RNA synthesis in some eukaryotes (Mauck and Green, 1974; Willis, Baseman and Amos, 1974; Smulson and Thomas, 1969; Pogo and Zbrzezna, 1978; Gross and Pogo, 1974; DeKloet, 1966; Foury and Goffeau, 1973; Hershko et. al., 1971). Hershko and colleagues (1971) have compared the amino acid controlled pleiotropic response of eukaryotes with the stringent response in bacteria. By analogy they have proposed a model to account for this phenomenon involving a hypothetical mediator with a similar role to that played by ppGpp in bacteria.

There have been many attempts to ascertain whether eukaryotes do synthesise such regulatory effectors (see reviews by Silverman and Atherly, 1979 and Oliver, 1981), but none have so far been unambiguously identified. In particular, studies of products found in vivo and evidence from in vitro experiments with isolated ribosomes either on their own or complemented with the E. coli stringent factor, indicate that eukaryotes do not generally produce the magic spot nucleotides. An exception to this occurs in C. reinhardtii where significant amounts of ppGpp were found (Heizmann and Howell, 1978). Evidence from in vitro studies together with the finding that ppGpp levels varied inversely with the rate of chloroplast rRNA synthesis suggested a chloroplast location for ppGpp production (Chua, Ogawa and Lippmann, 1974). Similarly yeast mitochondrial ribosomes were found to synthesise ppGpp in vitro (Richter, 1973) and also in vivo during heat shock (Pao, Paietta and Gallant, 1977). The production of ppGpp observed in this instance does not appear to

have any relevance to the RNA metabolism of the nucleus.

Other more unusual nucleotides have been found to accumulate in various organisms during nutritional shifts (see Silverman and Atherly, 1979; Oliver, 1981) but regulatory functions for these compounds have not been established. In yeast a low molecular weight compound has been found to accumulate during amino acid starvation (Lusby and McLaughlin, 1980). This substance is not a nucleotide but was found to be phosphorylated. Again the regulatory significance of this compound is unclear.

It is apparent that there are a number of other fundamental differences in the regulation of RNA synthesis in eukaryotic and prokaryotic cells. These differences have been most well defined in the yeast Saccharomyces cerevisiae since there are a large number of auxotrophic and other suitable mutants which are conditionally deficient in protein and RNA synthesis. Studies by various workers have shown that the synthesis of the different RNA species are differently affected by deprivation of amino acids. Gross and Pogo (1974) have found that the synthesis of mRNA is less inhibited than is stable RNA synthesis. Shulman and co-workers (1977) have confirmed this observation and have also examined the effect on the stable RNA species more closely. They found that rRNA synthesis was drastically reduced whereas the synthesis of tRNA was relatively unaffected by starvation. Similar results were obtained by Oliver and McLaughlin (1977). This finding that the regulation of the synthesis of the different RNA species is not co-ordinate during the stringent response has also been reported in other eukaryotes (Enger and Tobey, 1972; Tiollais et. al., 1971).

Non-coordinate control of RNA synthesis is also observed when protein synthesis inhibitors are added to amino acid starved cells. As previously described (see above) this treatment causes a stimulation in the overall rate of RNA accumulation in eukaryotes. Oliver and McLaughlin (1977) have found that in yeast this is due to stimulation of tRNA synthesis and that rRNA synthesis remains depressed. Furthermore this stimulation occurs only with

inhibitors, such as cycloheximide and trichodermin, which affect translation after the formation of the mRNA-ribosome complex. Inhibitors which act at the very early initiation steps of protein synthesis, e.g. edeine, MDMP, do not elicit this apparent reversal of the stringent response caused by the stimulation of tRNA synthesis. Similarly prestarvation for increasing periods before the addition of cycloheximide reduced the magnitude of the effect; thus the ability of this drug to stimulate the synthesis of tRNA is decreased by the disaggregation of polysomes which occurs during amino acid starvation (Van Venrooij, Henshaw and Hirsch, 1972; Elliasson, Bauer and Hultin, 1967; Bolcsfoldi, 1974).

These observations have been disputed by Shulman and colleagues (1977) who have reported that during cycloheximide treatment of starved cells the synthesis of rRNA and mRNA remains depressed but that tRNA synthesis returns only to the normal level. These results were obtained by pulse labelling sorbitol stabilised spheroplasts with ^3H -methylmethionine. In this procedure mature RNA species which are methylated during processing become radioactively labelled. Shulman and co-workers have suggested that the overall rate of RNA accumulation reported by others in these conditions is due to effects on the labelling of nucleotide pools which are avoided using their labelling protocol. This conclusion is based on the finding that amino acid starvation causes inhibition of the entry of labelled precursors into the nucleotide pools and that cycloheximide ameliorates this effect (Shulman, Sripati and Warner, 1977). This data and also that from a more exhaustive study of the behaviour of nucleotide pools during these treatments (Gross and Pogo, 1976a; Kelker and Pogo, 1980) was considered in another section of this chapter. The findings of Shulman and colleagues were not corroborated by these studies. Moreover their explanation does not account for the results of Oliver and McLaughlin since the stimulation of nucleotide pool labelling by cycloheximide would have an equal effect on the labelling of all RNA species.

A clue to the resolution of this discrepancy is provided by recent experiments performed by Kelker and Pogo (1980). They have found that D-sorbitol, the osmotic stabiliser used by Shulman et. al. cancelled the stimulatory effect of cycloheximide on RNA synthesis which is normally seen in amino acid starved cells or sphaeroplasts stabilised with magnesium ions. These workers reported that the addition of the drug did indeed stimulate the synthesis of tRNA, while the synthesis of the 35S rRNA precursor was only slightly increased. They also observed a stimulatory effect on the 5S species. The stimulation of tRNA synthesis by cycloheximide during amino acid starvation has also been observed in other eukaryotes (Mauck and Green, 1974; Willis, Baseman and Amos, 1974).

The addition of protein synthesis inhibitors to unstarved yeast cells causes a reduction in the rate of synthesis of the stable RNA species (Roth and Dampier, 1969; Shulman, Sripati and Warner, 1977). This is in marked contrast to the effect of translation inhibitors in bacteria but appears to be a response common to many eukaryotes (Willems, Penman and Penman, 1969; Ennis, 1966; Fiala and Davies, 1965; Warner et. al., 1966; Muramatsu et. al., 1970; Wanka and Schrauwen, 1971; Bolcsfoldi, 1974; Franze-Fernandez and Fontanive-Senguesa, 1973). Indeed this is another method commonly used to evoke the stringent response in higher organisms in the absence of auxotrophic strains. In yeast the response is very similar to that observed during stringency. Shulman and colleagues have reported that the synthesis of rRNA and mRNA is reduced while tRNA synthesis continues normally (Shulman, Sripati and Warner, 1977). Kelker and Pogo (1980) report that rRNA synthesis is depressed but that tRNA synthesis is only slightly reduced after a substantial lag. Thus, in yeast the stringent response would appear to be dependent on continued protein synthesis.

Gross and Pogo (1974, 1976 a and b) have proposed a model for the regulation of RNA synthesis during stringency which is based on the effect of blocking protein synthesis on the activities of the RNA polymerases. These workers have found that inhibition of protein synthesis had no effect on the total

amounts of the different species of polymerase that can be extracted from yeast cells. The same result was obtained whether translation was inhibited by amino acid starvation, by the action of cycloheximide or in a mutant defective in protein synthesis at the restrictive temperature. Similar results have been obtained using liver cells from cycloheximide treated rats (Benecke, Ferencz and Seifart, 1973) and in insect cells (Doenecke, Marmaras and Sekiris, 1973). However the level of RNA synthesis in nuclei isolated from these treated cells was found to be reduced in every case compared to those isolated from normal cells. It was inferred that these isolated nuclei reflect the distribution of polymerases within the cell since they were assumed to be incapable of reinitiating RNA synthesis and are only able to elongate RNA chains that have already been initiated in vivo. These findings imply that all these treatments limit the initiation of RNA polymerases. The inhibition of polymerase initiation during amino acid starvation has also been reported in mouse ascites cells (Grummt, Smith and Grummt, 1976). A similar experimental analysis was used to arrive at this conclusion except that the ability to synthesis rRNA by isolated nucleoli was monitored. Direct evidence was obtained to show that the rate of chain elongation was not affected.

Gross and Pogo postulated that the reduction in RNA chain initiation was due to the lack of a positive regulator which was probably an unstable polypeptide that cannot be synthesised in the absence of protein synthesis. The effect on initiation was found to vary in extent for the polymerase species and it was proposed that each type of enzyme must therefore have its own potential positive regulator. However the decrease in total RNA accumulation observed in intact cells which are blocked in translation occurs before polymerase initiation is inhibited. Moreover, the reduction in transcription by isolated nuclei is not sufficient to account for the larger decrease in total RNA accumulation seen in intact cells. Gross and Pogo have therefore suggested the existence of a second regulatory factor which acts as a repressor of polymerase activity by reducing the rate of chain elongation. The production of

this negative regulator is instantly stimulated by the inhibition of trans-lation possibly by the activation of an inactive precursor. Cycloheximide was proposed to prevent the activity of the repressor either by direct inter-action or by interfering with this activation reaction.

Stringent control in yeast was thus envisaged as a two step process. Immediately following amino acid starvation the negative regulator is activated which inhibits chain elongation by the polymerase reducing the rate of RNA synthesis. Initiation by the polymerase then becomes inhibited as the positive regulator begins to decay and cannot be replaced by protein synthesis. Phenotypic relaxation of RNA synthesis by cycloheximide occurs since the action of the repressor is prevented and although initiation is still inhibited the rate of the chain elongation is stimulated.

A number of reports suggest that the modulation of RNA synthesis can be brought about by altering the rate of chain propagation in eukaryotes (Barry and Gorski, 1971; Emerson, 1971; Franze-Fernandez and Fontanive-Senguesa, 1973 and 1975). In most of these instances this has been accounted for by postulating the absence of unstable proteins. Grummt and colleagues found that elongation in isolated nucleoli from starved mouse cells was stimulated by a factor contained in the cytoplasm of untreated cells (Grummt, Smith and Grummt, 1976). However the model proposed by Gross and Pogo is not consistent with their own more recent data which indicated that in nuclei from starved ascites tumour cells, cycloheximide causes a 'real augmentation in the number of polymerases engaged in rRNA transcription' rather than an increase in the rate of chain elongation (Pogo and Zbrzezna, 1978). Furthermore, this model predicts that cycloheximide would have the same effect in starved and non-starved cells which is clearly not the case.

Another model for stringent control in yeast which accommodates the dependence of RNA synthesis on continued protein synthesis was proposed by Shulman and Warner (1978 a). They suggested that the reduction in RNA synthetic rate was caused by the deficiency of new ribosomal proteins which would result

from the lack of protein synthesis. This deficit would be even more marked for the ribosomal proteins as a group compared to total protein since their messenger RNA's are subject to stringent control whereas the bulk of mRNA is not (Shulman and Warner, 1978b). This model could be tested directly since a mutant has been isolated which is temperature-sensitive for the synthesis of ribosomal proteins (Hartwell, McLaughlin and Warner, 1970). It was found that although the 35S rRNA precursor was not processed to the mature species in this mutant at the restrictive temperature its rate of synthesis was undiminished (Shulman and Warner, 1978a).

The importance of uncharged tRNA in stringent control in eukaryotes has been indicated by the use of amino acid analogues which competitively inhibit the aminoacyl-tRNA synthetases. In yeast this has been confirmed using mutants which have a temperature-sensitive isoleucyl-tRNA synthetase. The stringent response elicited in these mutants is similar to that seen in amino acid-starved wild-type strains (McLaughlin, Magee and Hartwell, 1969). However the stimulation of tRNA synthesis caused by cycloheximide in starved normal strains does not occur in a temperature-sensitive synthetase mutant (Oliver and McLaughlin, 1977). These workers have suggested the lack of relaxation is due to the inability of the mutant to trickle-charge tRNA at the restrictive temperature. They have therefore proposed that the cycloheximide stimulated synthesis of tRNA in starved wild-type cells is due to enhancement of trickle-charging (Oliver and McLaughlin, 1977). This is analogous to the trickle-charging observed in E. coli. Thus tRNA synthesis is suggested to be under a form of autoregulatory control such that the accumulation of charged tRNA results in the production of new tRNA molecules.

Another model in which RNA synthesis is dependent on the levels of tRNA aminoacylation has been proposed by Grummt and Grummt (1976) and has already been discussed in a previous section of this chapter. These workers found that ribosomes from Ehrlich ascites cells degrade GTP to guanosine when the acceptor site is occupied by uncharged tRNA (Grummt and Speckbacher, 1975). This finding is supported by the observation that treatment of these and

other eukaryotic cells with histidinol causes a reduction in the size of the cellular GTP and ATP pools (Grummt and Grummt, 1976; Vaughen and Hansen, 1973; Van Vendrooij, Henshaw and Hirsch, 1972; Live and Kaminskas, 1975). Thus, if in eukaryotes, RNA transcription is more sensitive to the concentration of these nucleotides than is chain elongation (as in prokaryotes) then the drop in the level of GTP caused by ribosome-mediated hydrolysis during amino acid starvation would result in an inhibition of RNA polymerase initiation. This has indeed been found to be the case in amino acid-starved ascites cells (see I.4a). However as has previously been discussed the contraction of purine nucleotide pools during starvation has been disputed by others (Dehlinger, Hamilton and Litt, 1977). Furthermore ribosome-mediated hydrolysis of GTP has not been observed in other eukaryotes. This model may not be applicable, therefore as a general control mechanism for the regulation of RNA synthesis in eukaryotes.

In summary it appears that while the stringent response in eukaryotes has some similarities to that found in prokaryotes, e.g. the involvement of uncharged tRNA, several fundamental differences are apparent. Firstly the regulation of the synthesis of RNA species in eukaryotes is distinctly less coordinated than in prokaryotes both during stringency and during phenotypic relaxation of the stringent response. Perhaps this finding is not surprising in view of the existence of three functionally distinct polymerases in eukaryotes. Secondly the addition of protein synthesis inhibitors to unstarved cells has opposite effects in eukaryotic and prokaryotic organisms. In considering these differences it becomes apparent that the mechanisms which exist to regulate RNA metabolism in eukaryotes and prokaryotes are likely to be equally distinct.

I.6 Cytoplasmic Synthesis of RNA

In addition to the three main nuclear encoded RNA species which are essential for growth, the yeast Saccharomyces cerevisiae also contains another major type of RNA. This class of RNA exists in double-stranded form and is

synthesised exclusively in the cytoplasm (see review by Bruenn, 1980). The presence of double-stranded RNA (dsRNA) is associated with the ability of the host cells to secrete a protein toxin that is active against other yeast strains but to which they themselves are immune. These toxin producing strains are lethal to sensitive strains and are therefore termed 'killer yeasts'. Two types of dsRNA are found: a larger species called P1 or L, and a smaller species called P2 or M which is never found in the absence P1. Killer strains contain both types of dsRNA, (are said to have the Kil-k genotype) and the killer/resistant phenotype is denoted K^+R^+ (see table I.3). Naturally occurring sensitive strains having the phenotype K^-R^- , do not contain P2 dsRNA [Kil-o] but the P1 species is usually present (Somers and Bevan, 1969). Neutral strains of S. cerevisiae which do not produce the killer toxin but which are resistant to its action (K^-R^+) also occur (Berry and Bevan, 1972). The correlation between killing ability and the presence of P2 dsRNA is confirmed by the observation that these characteristics co-segregate and are cytoplasmically inherited (Bevan, Herring and Mitchell, 1973). Furthermore a study of the in vitro translation products of denatured P2 dsRNA has provided convincing evidence that this species contains the gene for the protein toxin (Bostian et. al., 1980).

The two double-stranded RNA species are contained within protein capsids in a morphologically analogous structure to that found in many viruses (Herring and Bevan, 1974). However no infective cycle for these yeast 'virus-like-particles' (VLP's) has been demonstrated and transmission can occur only by cytoplasmic mixing during cytoduction or mating. Thus the killer associated genomes may be considered to be particle - associated plasmids or viruses showing only 'vertical' transmission.

Virus-like-particles are widespread among fungi and to date they have been discovered in over a hundred species from more than 60 genera (reviewed by Buck, 1980; Lemke, 1979). Several different morphological types of particle have been described. By far the most frequently occurring types are similar to that found in S. cerevisiae, being isometric, containing dsRNA, and being

GENOTYPE	PHENOTYPE	
KIL-k	K^+R^+	KILLER, has wild type killer M dsRNA, secretes toxin, and is immune to it.
KIL-o	K^-R^-	SENSITIVE, lacks M dsRNA, does not secrete toxin and is sensitive to it.
KIL-n	K^-R^+	NEUTRAL, has full sized M dsRNA, is defective in active toxin but has active immunity component.
KIL-s	K^-R^-	SUPPRESSIVE SENSITIVE, contains S dsRNA (a deletion derivative of the M species) which interferes with M dsRNA replication, thus causing its loss.
KIL-i	K^+R^-	SUICIDE strains, defective in immunity component but produces toxin. Can be maintained at pH 6.0.
KIL-sk	$K^{++}R^-$	SUPERKILLER, carries a mutated M dsRNA resulting in a higher copy number and a more stable toxin.
KIL-b	K^+R^+	M dsRNA mutant that bypasses the need for most <u>MAK</u> gene products (see fig. I.5)
KIL-sd	K^+R^+	M dsRNA mutant whose maintenance is conditional on the presence of a <u>ski</u> mutation.
KIL-d	K^+R^+	Mutant M dsRNA dependent on a diploid host.

TABLE I.3 - Summary of killer phenotypes and genotypes.

transmitted only by intracellular routes. However unlike those of S. cerevisiae many of these 'mycoviruses' do not have known functions. Notable exceptions are the killer systems of Ustilago maydis, where in killer strains up to seven species of dsRNA occur (Wood and Bozarth, 1973; Koltin and Day, 1976), and of Kluyveromyces lactis in which killers contain two linear dsDNA plasmids (Gunge et. al., 1981). Other examples include the fungal diseases transmitted by heterokaryosis and caused by dsRNA, for instance in Penicillium chrysogenum (Lemke, 1975) and Agaricus bisporus (Dieleman-van Zaayen, 1979).

The finding that VLP's of Sacchromyces cerevisiae are associated with a known phenotype together with the fact that this phenotype can be easily assayed have enabled extensive genetic analysis of the yeast killer system. The following section summarises the results of these studies.

i) The Genetics of the Yeast Killer System

The killer character of Saccharomyces cerevisiae has been found to be dependent on a number of chromosomal genes. These fall into two categories: those which affect the replication of P2 dsRNA and thus the maintenance of killing ability (MAK genes), and those which appear to be essential for processing or secretion of the toxin and therefore affect killer expression (KEX genes).

A mutation in a gene affecting the maintenance of P2 dsRNA (now known as mak 10) was first described by Somers and Bevan (1969). Subsequently a total of 28 such genes have been identified. These are, pet 18 (Fink and Styles, 1972; Wickner and Leibowitz, 1976), mak1, mak3 - mak27 (Somers and Bevan, 1969; Wickner, 1974a and 1978; Wickner and Leibowitz, 1976), and spe 2 (Cohn et. al., 1978a). Mutations in all these genes result in the loss of P2 dsRNA but only one, mak3, affects the maintenance of the P1 species. A mutation in this gene results in a significant decrease in the level of P1 but even in this case there is not complete loss of the plasmid (Leibowitz and Wickner, 1978). Mutants in the PET 18, MAK1 and MAK16 genes are all temperature sensitive for growth and several other mak mutations (mak 13, 15, 17, 20, 22, and 27) result in slow growth at any temperature. (Wickner,

1979). The pet 18 mutation also causes the loss of the mitochondrial genome (Leibowitz and Wickner, 1978).

Only one killer maintenance gene, SPE 2, has a known function; it is the structural gene coding for S-adenosylmethionine decarboxylase, which is involved in the synthesis of the polyamines, spermidine and spermine (Cohn et. al., 1978b). Mutants in the SPE 2 gene are also defective in sporulation (Cohn et. al., 1978b). Thus while the role of some MAK gene products in the replication of P2 dsRNA may be indirect, mutations in these genes have a greater effect on plasmid maintenance than on cell growth.

In contrast to the abundance of genes involved in the maintenance of P2 dsRNA, only two chromosomal genes affecting the expression of killing ability, KEX 1 and KEX 2, have been identified. Indeed Wickner and colleagues have isolated over 30 independent mutants of this class and all had alterations in one of these two genes (Wickner and Leibowitz, 1974b). Both are required for the expression of killing ability but not for resistance or for maintenance of the P2 plasmid.

Mutants in the KEX 1 gene show a normal pattern of secreted proteins except for the absence of the toxin polypeptide (Bussey, 1981). Mutants in the KEX 2 gene have a pleiotropic phenotype consistent with defective secretion. Strains which are α -mating and carry the kex 2 mutation cannot mate since they are unable to secrete an active α -factor pheromone or to respond to the α hormone. Spore maturation in homozygous diploids is also prevented by the kex 2 mutation (Leibowitz and Wickner, 1976). The extracellular proteins found in culture supernatants of kex 2 strains are abnormal since they are both larger and more basic. Some of the proteins secreted by wild type strains are not secreted by kex 2 mutants, including the killer toxin (Rogers et. al., 1979). These observations have led to the suggestion that the KEX 2 gene product is a protease required for the proper processing of exported proteins (Rogers et. al., 1979).

In addition to the MAK loci, another class of chromosomal genes which appear to affect the replication of P2 dsRNA have been found. These are called SKI genes since mutants in these loci were originally isolated as having a 'super-killer' phenotype. These mutants have an increased production of toxin activity due to an increase in the amount of P2 dsRNA (Toh-e, Guerry and Wickner, 1978). There are four SKI genes, SKI1-4 which are recessive in heterozygous diploids. These ski mutants have the ability to 'suppress' mak mutations such that ski,mak double mutants are able to replicate the P2 plasmid (Wickner, 1979; Toh-e and Wickner, 1980; Cohn et. al., 1978a). These results indicate a role for the SKI gene products in killer plasmid replication. Wickner (1979) has suggested that in addition to the MAK dependent pathway there is a second replication pathway for P2 dsRNA which is not normally used due to the influence of the SKI gene products. (Fig.I.5). In ski mutants however, both pathways may be used leading to an overproduction of the P2 plasmid. Other mutations, including those in a dominant chromosomal gene (KRB 1-killer replication bypass) and in a cytoplasmic locus (KIL-b - see table I.3) have also been described which have similar properties to the ski mutation (Wickner and Leibowitz, 1977).

Several other cytoplasmically located mutations affecting the killer character are known. Neutral strains [KIL-n], first described by Somers and Bevan (1969), have the K^-R^+ phenotype and are defective in toxin production. This trait is cytoplasmically inherited (Berry and Bevan, 1972) but dsRNA's isolated from these mutants co-electrophorese with the normal P1 and P2 species (Bevan and Mitchell, 1979). Thus neutral strains presumably carry point mutations or small deletions in the P2 plasmids.

Strains having large deletions in the P2 species giving a K^-R^- phenotype have also been described (Somers, 1973). These mutants are called suppressive sensitive strains [KIL-s] since they suppress the K^+R^+ phenotype in crosses such that most of the progeny are K^-R^- (Somers, 1973; Tzen, Somers and Mitchell, 1974; Vodkin, Katterman and Fink, 1974; Sweeney, Tate and Fink, 1976). Detailed analysis of the smaller dsRNA found in suppressive strains (S dsRNA) has shown

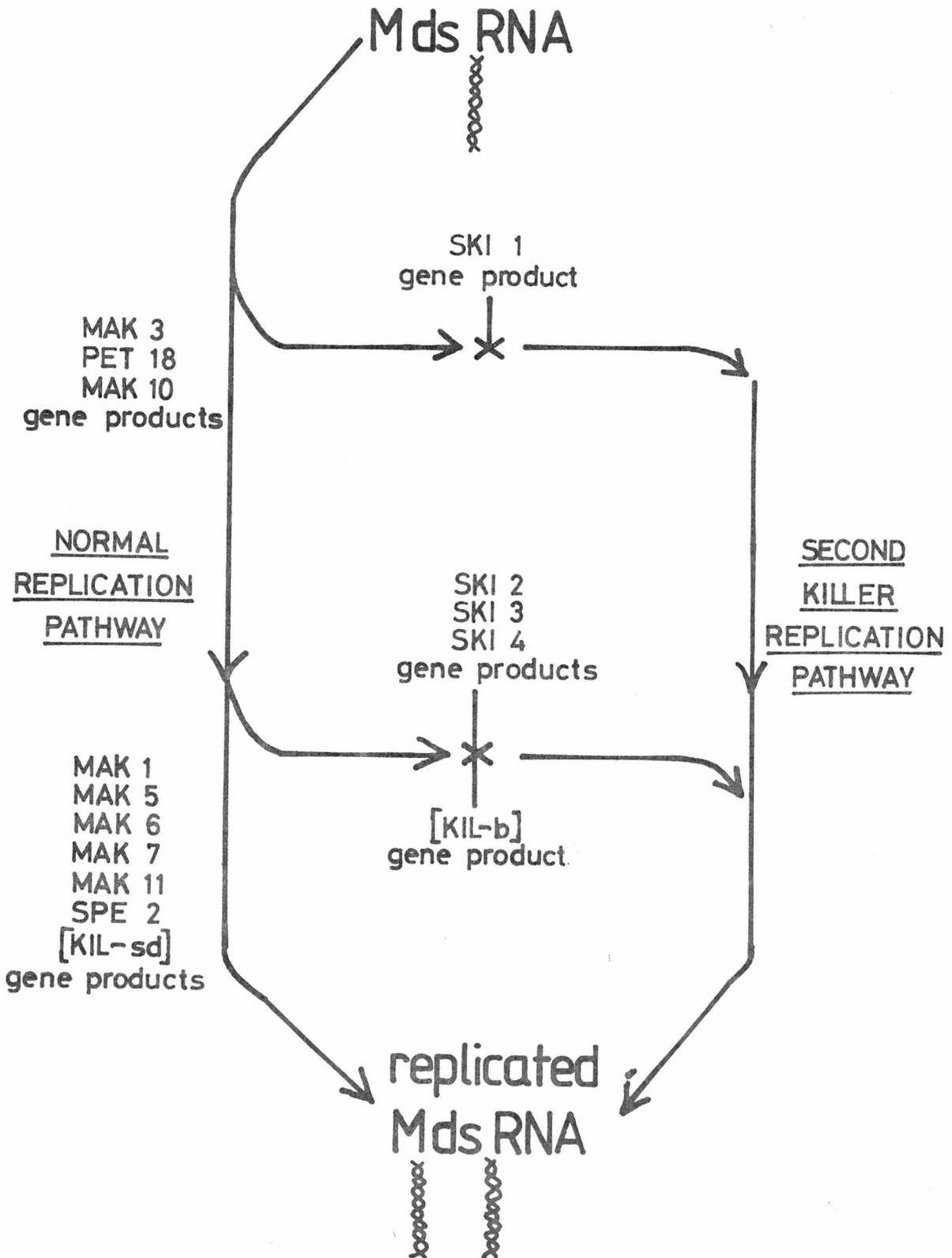


Fig. I.5 THE INTERACTION OF GENE PRODUCTS AFFECTING
M dsRNA REPLICATION (WICKNER, 1979)

that they arise from the P2 species by internal deletion and tandem duplication (Fried and Fink, 1978; Bruenn and Kane, 1978; Kane, Pietras and Bruenn, 1979). Suppressive strains provided the first good evidence that the killer toxin and resistance to it were encoded by P2 dsRNA . However they retain the sequences necessary for plasmid replication and appear to have a competitive advantage over the normal killerplasmid perhaps due to their smaller size (Bussey, 1981). The separate nature of P1 replication is again emphasised by the fact that the SdsRNA does not interfere with this process (Wickner, 1979).

A cytoplasmic superkiller mutation [KIL-sk] has been isolated (Vodkin et. al., 1974; Sweeney et. al., 1976) which has a higher level of P2 dsRNA and also produces a more stable toxin (Palfree and Bussey, 1979). Buck (1980) has proposed that, since the P2 dsRNA found in these mutants is unaltered in size, a point mutation on the coding strand resulting in a more stable toxin also leads to a complementary mutation on the non-coding strand which gives rise to the increased activity of a binding site involved in plasmid replication. Another mutation of the killer plasmid which appears to be in a gene required for its own replication has been isolated by Toh-e and Wickner (1979). This mutant contains a killer plasmid which can be maintained in ski⁻ strains but not in wild type yeast. It is therefore denoted [KIL-sd] for ski dependent. These workers have proposed that the mutation is in a P2 encoded gene which is required for P2 replication by the MAK dependent pathway but not by the second killer pathway (cited in Wickner, 1979). Finally Wickner (1976a) has described a mutant killer plasmid [KIL-d] which is only stably maintained and expressed in diploid cells. It is possible that in these mutants P2 replication is dependent on the overproduction of certain chromosomally coded gene products, a phenomenon which has been observed in diploid cells (Errede and Sherman, 1978).

ii) Biochemistry and Physiology of the Yeast Killer System

The two species of cytoplasmically inherited yeast virus-like-particles have been characterised by a large number of biochemical and physiological

studies. The nucleic acid content of VLP's was demonstrated to be double-stranded RNA by several criteria including mobility in polyacrylamide gels and CF-11 cellulose chromatography columns, staining with toluidine blue, resistance to RNase A, denaturation kinetics, equivalence of base ratios and negative response in DNA assays (reviewed by Bevan and Mitchell, 1979). The molecular weights have been independently determined by a variety of methods and are estimated to be $2.5-3.5 \times 10^6$ and $1.1-1.7 \times 10^6$ respectively (reviewed by Bruenn, 1980). The P1 and P2 molecules have little sequence homology by the criteria of hybridisation and T1 'fingerprint' analysis (Bruenn and Kane, 1978). Preliminary sequencing data has indicated that neither dsRNA is capped at the 5' end or polyadenylated at the 3' end (Bruenn and Keitz, 1976; Bruenn and Brennan, 1980).

The two dsRNA species are separately encapsidated since P1 and P2 containing particles can be separated on sucrose gradients (Bevan and Mitchell, 1979). However only one morphological type of VLP can be observed in killer strains. Electron microscopy of cell free extracts has shown that these are isometric being 40nm in diameter (Herring and Bevan, 1974; Adler, Wood and Bozarth, 1976; Oliver et. al., 1977). There appears to be only one major capsid protein in yeast virus-like-particles. It has been calculated that there are about 120 copies of this per particle (Bruenn, 1980). This polypeptide has been isolated from P1 VLP's and has a molecular weight of 88,000 as determined by various workers (reviewed by Bussey, 1981). The same protein has been shown to be present in P2 VLP's using the technique of partial proteolysis and peptide mapping on SDS-polyacrylamide gels (Bostian et al., 1980). This conclusion is supported by the finding that antibodies raised against particles obtained from sensitive strains cross react with both P1 and P2 VLP's obtained from a killer strain (Harris, 1978).

The common capsid protein is encoded by the P1 dsRNA species. This has been demonstrated since the major product when denatured P1 dsRNA is used as a message in a wheatgerm translation system is identical in size and has a

similar tryptic digest peptide map (Hopper et. al., 1977). These experiments establish a clear functional relationship between P1 and P2 VLP's with the P1 species acting as a 'helper virus' in maintaining the P2 dsRNA by providing a capsid protein. It has been calculated, assuming there is only one coding strand and that there are no overlapping genes, that the P1 dsRNA could also code for other proteins, for example an RNA polymerase (Holm et. al., 1978).

The major product of denatured P2 dsRNA in in vitro translation systems is a polypeptide which immunoprecipitates with antibodies raised against the killer toxin (Bostian et. al., 1980). This polypeptide has a molecular weight of 32,000 and is considerably larger than the toxin itself (molecular weight, 12,000). These results suggest that the toxin protein is formed by the post-translational processing of a larger precursor. This polypeptide could therefore include the immunity or replicase peptides in addition to the toxin itself. Alternatively these activities could be contained within the minor in vitro translation product which has a molecular weight of 30,000 (Bostian et. al., 1980).

The killer toxin has been purified 79,000 fold from culture supernatants to homogeneity (Palfree and Bussey, 1979). The active species is a monomer being 109 amino acid residues long which are mainly hydrophobic. The toxin is stable within a narrow pH range of between 4.2-4.6 which corresponds to its activity in vitro, and is secreted by killer strains during the exponential phase of growth. Growing sensitive strains treated with killer toxin show a rapid decrease in viability following a co-ordinate shut off of the synthesis of macromolecules (Bussey and Sherman, 1973). This biosynthetic shut off coincides with plasma membrane damage measured by loss of potassium ions and ATP (Skipper and Bussey, 1977). Membrane damaged cells shrink in volume but do not lyse or contain large pores as macromolecules are not lost from killed cells (Bussey, 1974).

The mechanism of toxin action has been elucidated biochemically, using radioactive toxin, and genetically, by isolating resistant mutants from sensitive cells (reviewed by Bussey, 1981). Killer resistant mutants fall

into three complementation groups, KRE 1-3. Mutants in the KRE 1 and KRE 2 genes have a lower binding of toxin to cells than the wild type, but sphaeroplasts from these cells are toxin sensitive. Mutants of KRE 3 bind toxin normally to the cell wall but are toxin resistant (Al-Aidroos and Bussey, 1978; Skipper, 1978). From these and other observations a two stage model for the action of killer toxin has been proposed - see fig. I.6 (Bussey, 1981). The toxin binds to the cell wall at a β -(1 \rightarrow 6) glucan receptor in an energy independent process that requires the KRE 1 and KRE 2 gene products. The toxin then interacts directly with a trans-membrane protein, the KRE 3 gene product, whose normal function is lost and it serves as a channel for potassium, ATP and small metabolites to leave the cell. A simpler model involves the toxin inserting into the membrane during the second stage to form a trans-membrane pore in its own right. In this scheme kre3 mutants would be defective in a component necessary for recognition or insertion of the toxin into the membrane. In either case the second step in toxin action is thought to be energy dependent.

The mechanism of immunity to these reactions in resistant cells is not known. A nuclear gene product is required for resistance expression. Mutants in this gene, rex 1, are not well characterised but are phenotypically K^+R^- (Wickner, 1974a). Such strains which produce the killer toxin but are no longer resistant to it are called 'suicide strains' and must be stored as heterozygous diploids or grown on medium buffered at high pH. Cytoplasmic 'suicides' which are defective in the immunity component [KIL-i] have also been described (Wickner, 1979).

Bussey (1981) has speculated that the immunity protein may be an integral membrane component which prevents the action of the toxin by blocking its recognition or by modification of the membrane receptor or of the toxin itself.

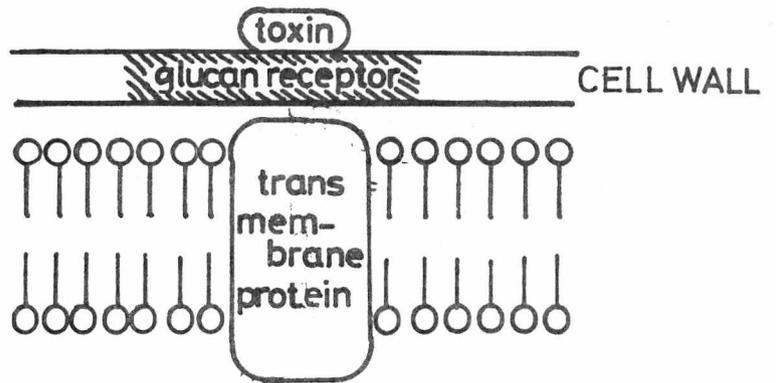
Despite extensive genetic analysis of the killer phenomenon in yeast very little is known about the biochemistry of the replication of these plasmids. Both P1 and P2 VLP's possess a single-stranded RNA polymerase activity (Herring and Bevan, 1977; Welsh and Leibowitz, 1978). This activity is

FIGURE I.6 - The mechanism of action of the yeast killer toxin

STAGE 1 The toxin binds to the cell wall at the β -(1 \rightarrow 6)glucan receptor

STAGE 2 Toxin interacts with a trans-membrane protein modifying it such as to form a channel for small metabolites.

Stage 1



Stage 2

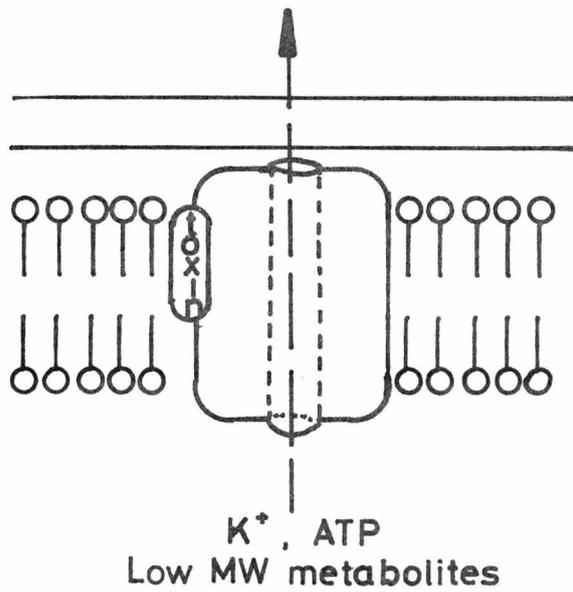


Fig.I.6

insensitive to DN'ase, α -amanitin and actinomycin D but sensitive to ethidium bromide. It requires all four ribonucleoside triphosphates and magnesium ions (Welsh and Leibowitz, 1978). The major product of the P1 enzyme is single-stranded RNA which appears to be entirely complementary to only one of the two P1 strands (Herring and Bevan, 1977; Hastie, Brennan and Bruenn, 1978; Brennan, Hastie and Bruenn, 1978). It is possible that these products represent the activity of a transcriptase or a replicase or both. P1 associated double-stranded RNA polymerase activity has also been reported (Bevan and Herring, 1976).(see Discussion for a further consideration of VLP replication).

Although many genes are known to affect P2 dsRNA replication none of these significantly affect replication of the P1 species. However a relationship between these two processes is evident from the observation that strains cured of P2 often have increased levels of P1 dsRNA (Sweeney, Tate and Fink, 1976; Mitchell, Herring and Bevan, 1976). This curing of killer character may be effected by treatment with heat (Wickner, 1974b), cycloheximide (Fink and Styles, 1972) or 5-fluorouracil (Mitchell, Herring and Bevan, 1976). The basis of this selective loss is not known but may be due to the lower copy number of P2. The virus-like-particles of yeast are non-infective and non-lytic and thus their replication must be integrated with the growth of the host cell. Selective loss of P2 by these curing agents may therefore represent a more efficient regulation of this process in the case of P1 dsRNA.

Despite the need for integration with the host the number of particles maintained per cell varies dramatically between different strains. Values ranging from 100 copies of P1 and 12 copies of P2 in killer strains (Wickner, 1976) to 24,000 (Oliver et. al., 1977) and 30,000 (Weber and Lindner, 1975) copies of P1 in sensitive strains have been reported. Nevertheless the number of particles maintained by cells of a particular strain appears to remain fairly constant during exponential growth (Oliver et. al., 1977). However there is a burst of synthesis when cells enter stationary phase which may be caused by the switch from fermentative to oxidative metabolism. This conclusion is consistent with the observation that cells grown oxidatively using

ethanol as a carbon source have elevated levels of P1 dsRNA compared to those grown on glucose (Oliver et. al., 1977). In conclusion while the genetic biochemical and physiological studies summarised above establish a clear relationship between the host cell metabolism and the replication of the dsRNA-containing VLP's further experiments are necessary to determine the nature of the controls which regulate the integration of these processes.

I.7 The Aims of the Investigation

In this Introduction the means by which the synthesis of RNA is regulated in living cells has been examined. One of the best understood of these control mechanisms is the stringent response of bacteria in which the importance of uncharged tRNA as a regulatory signal molecule has been established. The yeast Saccharomyces cerevisiae exhibits stringent control of RNA synthesis and the involvement of uncharged tRNA has also been implicated. This investigation therefore examines the role of uncharged tRNA in the regulation of RNA synthesis in this organism.

Saccharomyces cerevisiae has an additional property relevant to the study of transcription. In addition to the three major RNA species normally found in living cells this organism possesses another important class of RNA which is encapsidated within virus-like-particles. These double-stranded RNA containing VLP's are non-infective and non-lytic and so depend upon the growth of the host cell for their propagation. The manner in which transcription of this cytoplasmically located genome is regulated has therefore been investigated to determine the relationship between this process and the regulation of the RNA species encoded in the nucleus.

MATERIALS AND METHODSM1 Organisms

The following strains of Saccharomyces cerevisiae were used in the investigation:-

<u>Strain</u>	<u>Genotype</u>	<u>Source</u>
S7	a Prototrophic	P. Lhoas
S7A6	a ade ⁻	S. G. Oliver
S7A6L184	a ade ⁻ , leu ⁻	This work
X2180.1A	a <u>SUC2</u> , mal, <u>gal2</u> , <u>CUP1</u>	T. D. Petes
20B-12 parent (X2180.1C)	α <u>trp1</u> , <u>SUC2</u> , mal, <u>gal2</u> , <u>CUP1</u>	E. Jones
20B-12	α <u>trp1</u> , <u>pep 4.3</u> , <u>SUC2</u> , mal, <u>gal2</u> , <u>CUP1</u>	E. Jones
A3 pep.7	<u>his1</u> , <u>lys2</u> , <u>tyr1</u> , <u>trp1</u> , <u>pep4.3</u>	This work
A364A	a <u>ade1</u> , <u>ade2</u> , <u>ura1</u> , <u>his1</u> , <u>lys2</u> , <u>tyr1</u> , <u>gal1</u>	L. H. Hartwell

The following mutant derived from A364A:-

ts275	temperature sensitive for protein synthesis	C. S. McLaughlin
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M2 MediaM2.1 YEPD

1% w/v Yeast Extract (Oxoid)

2% w/v Peptone (Bacto)

2% w/v Glucose (Bacto)

Solid YEPD medium was prepared by adding 2% w/v Agar (Bacto)

M2.2 YNB-aa

0.67% w/v Yeast Nitrogen Base without Amino Acids (YNB-aa) (Difco)

2% w/v Glucose

The chemical composition per litre of YNB-aa at the above concentration is:-

Ammonium Sulphate 5g

Vitamins

Biotin 20µg

Calcium Pantothenate 2,000µg

Folic Acid 2µg

Inositol 10,000µg

Nicotinic Acid 400µg

p - Aminobenzoic Acid 200µg

Pyridoxine hydrochloride 400µg

Riboflavin 200µg

Thiamine hydrochloride 400µg

Trace Elements

Boric Acid 500µg

Copper Sulphate 40µg

Potassium iodide 100µg

Ferric chloride 200µg

Manganese sulphate 400µg

Sodium molybdate 200µg

Zinc sulphate 400µg

Salts

Potassium phosphate (monobasic)	0.85g
Potassium phosphate (dibasic)	0.15g
Magnesium sulphate	0.5g
Sodium chloride	0.1g
Calcium chloride	0.1g

M2.3 YNB-NH₄⁺

0.17% w/v YNB without amino acids and ammonium sulphate (Difco)

2% w/v Glucose

YNB-NH₄⁺ has the same chemical composition as YNB-aa but no ammonium sulphate

M2.4 Wickerhams Minimal Medium

Solid minimal medium was prepared according to Wickerham (1946):

Stock Salts (Solution A) per litre:-

(NH ₄) ₂ SO ₄	10g
KH ₂ PO ₄	8.75g
MgSO ₄ ·7H ₂ O	5g
NaCl	1g

Trace Elements (Solution B) per 100ml:-

H ₃ BO ₃	0.1g
CuSO ₄ ·5H ₂ O	0.1g
KI	0.1g
FeCl ₃ ·6H ₂ O	0.5g
ZnSO ₄ ·7H ₂ O	0.7g

Calcium Stock (Solution C)

CaCl ₂	100mg/ml
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Stock Vitamins (Solution D) per 100ml:-

Biotin	0.2mg
Thiamine	40.0mg
Pyridoxine	40.0mg
Inositol	200.0mg
Calcium Pantothenate	10.0mg

1 litre of solid medium contains:-

100mls	Solution A
10µls	Solution B
1ml	Solution C
1ml	Solution D
2% w/v	Glucose
2% w/v	Agar (Bacto)

M2.5 Sporulation Medium (Mortimer and Hawthorne, 1966)

	<u>Presporulation</u>	<u>Sporulation</u>
Yeast Extract (Oxoid) w/v	0.8%	0.1%
Peptone (Bacto) w/v	0.3%	-
Glucose w/v	10.0%	0.05%
CH ₃ COOK w/v	-	2.0%
Agar (Bacto) w/v	2.0%	2.0%

M2.6 Nutritional Supplements

Adenine, Uracil and all amino acids were added to minimal medium as required at 20µg/ml

M2.7 Sterilisation Procedures

YNB, nutritional supplements, vitamins and antibiotics were filter sterilised through 0.22µm pore size nitrocellulose filters (Millipore).

Glucose (50% w/v stock solution) was autoclaved at 15 lbs/sq. in. for 10 mins. All other media were sterilised by autoclaving at 15 lb/sq. in. for 15 mins.

M3 Growth and Maintenance of Organisms

All strains were kept at 5°C on YEPD agar. Inocula were prepared by transferring cells to 10mls of YEPD or YNB and incubating at 23°C or 30°C. Long term storage was achieved by suspending a colony from a YEPD agar plate in 1ml of YEP containing 50% glycerol which was then stored at -80°C.

Growth in liquid culture was performed using sterile conical flasks in a shaking water bath (Mickle) at 23°C. Growth on solid media was carried out in constant temperature incubators or rooms at 23°C, 30°C or 36°C as required.

M4 Buffers and Other Standard Solutions

Standard buffers were prepared as described by Gomori (1955).

M4.1 Buffer A (Andrulis and Arfin, 1979)

50mM CH₃COONa

0.15M NaCl

Adjusted to pH 4.5

M4.2 Buffer B (Andrulis and Arfin, 1979)

10mM CH₃COONa

10mM MgCl₂

1mM EDTA

15mM β-mercaptoethanol

Adjusted to pH 4.5

M4.3 Buffer C (Von der Haar, 1979)

30mM Potassium phosphate, pH 7.2

1mM EDTA

1mM Dithiothreitol (DTT)

0.01mM Phenylmethylsulphonylfluoride (PMSF)

M4.4 Buffer D (Von der Haar, 1979)

Buffer C containing:-

10%v/v glycerol

M4.5 5x E Buffer (Loening, 1967) per litre:-

Tris	21.7g	
NaH ₂ PO ₄ ·2H ₂ O	23.4g	pH7.7
EDTA	1.85g	

E Buffer

5x E buffer diluted to 1 x strength containing 2g/l SDS

M4.6 Buffer F (Faulhammer and Cramer, 1977)

100mM Potassium phosphate, pH7.3

5mM EDTA

1mM DTT

0.01M PMSF

5% v/v glycerol

M4.7 Buffer G (Hartwell and McLaughlin, 1968)

0.01M Tris maleate, pH7.6

0.5mM (CH₃COO)₂Mg

0.02M KCl

M4.8 Buffer H (Yang and Novelli, 1971)

0.3M Tris HCl, pH8.0

M4.9 Crushing Cocktail (Kirby, 1965)

50mM Tris HCl, pH7.5

0.1M NaCl

Where indicated crushing cocktail also contained 2% w/v TNS (Sodium tri-isopropyl-naphthalene sulphonate, Eastman-Kodak)

M4.10 Phenol-Cresol Mixture (Kirby, 1965)

Phenol 500g

m-Cresol 70ml

8-Hydroxyquinoline 0.5g

Distilled water 200ml

M4.11 Acetate - SDS (Oliver, 1974)

0.15M CH₃COONa, pH6.9

0.5% w/v sodium dodecyl sulphate

M4.12 Loading Mixture

5x E buffer diluted to 1 x strength containing

2% w/v SDS

15% w/v sucrose

M4.13 Stock Acrylamide (Loening, 1967) per 100ml :-

Acrylamide (Eastman-Kodak, Electrophoretic grade) 15g

Bis-acrylamide (Eastman-Kodak, Electrophoretic grade) 0.75g for 2.6%
or 0.375 for 12%

Stock acrylamide was stored in the dark at 5°C

M4.14 Saline Sodium Citrate

0.15M NaCl

0.015M Sodium Citrate

M4.15 STE Buffer (Franklin, 1966)

0.1M NaCl

1mM EDTA

0.05M Tris, pH6.85

M4.16 Scintillation Fluid

Water insoluble:

0.5% Diphenyloxazole (PPO) in Toluene

When counting gel slices, these were solubilised by adding 3% Protosol (New England Nuclear) and the vials allowed to stand for 24 hrs.

Water soluble:

Phase Combining System (PCS, Amersham)

M4.17 Synthetase Reaction Mixture (Hampel, Enger and Ritter, 1979)

At final strength reaction mixture contains:-

50mM Tris HCl, pH7.4

15mM MgCl₂

0.5mM EDTA (adjusted to pH7.5 with NaOH before adding to mixture)

5mM ATP (adjusted to pH7.0 with NaOH before adding to mixture)

0.35mM GTP (adjusted to pH7.0 with NaOH before adding to mixture)

5.8mM ³H-amino acid (approximately, according to specific activity)

0.135 mg/ml tRNA

Individual constituents with the exception of tRNA and labelled amino acids were kept as 20 x strength stock solutions. ATP and CTP were stored at -20°C in 200ul aliquots.

M4.18 Solutions for Folin-Lowry Assay (Lowry et. al., 1951)

Solution A	-	2% Na_2CO_3 in 0.1M NaOH
Solution B	-	1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
Solution C	-	2% NaKtartrate
Solution C*	-	50mls solution A with 0.5mls solution B and 0.5mls solution C
Solution D	-	5mls of Folin's Reagent (BDH) diluted to 15mls with distilled water.

M4.19 Radioisotopes

^{14}C -8-Adenine (62mCi/mmol)

^3H -2-Adenine (15Ci/mmol)

L - [4,5 - ^3H] Leucine (65 μCi /mmol)

L - [3,5 - ^3H] Tyrosine (50 μCi /mmol)

All radioisotopes were purchased from Amersham International Ltd.

M4.20 Protein Synthesis Inhibitors

Edeine was a gift from Dr. C. S. McLaughlin, University of California at Irvine, USA.

MDMP [2-(4-methyl-2, 6-dinitroanilino)-N-methyl propionamide] was a gift from Dr. R. Baxter, Shell Research, Sittingbourne, Kent, U.K.

Anisomycin was a gift from the Pfizer Corp., Groton, Connecticut, USA.

Cycloheximide was purchased from Sigma.

M5 Standard Techniques

M5.1 Monitoring Cell Growth

Cell growth in liquid culture was monitored in two ways:-

a) Electronic Particle Counting

An appropriate aliquot of culture was suitably diluted into 10mls of 0.9% w/v saline. This was subjected to sonication (15 seconds at full power in a MSE Ultrasonicator) to separate clumps and unseparated but divided pairs, before counting with a Coulter particle counter (Model F) fitted with a 70 μ probe.

b) Klett Meter

The optical density of the culture was determined using a Klett-Summerson Colourimeter with a no. 54 green filter.

M5.2 Cell Transfer

Cells were transferred from one medium to another in order to perform temperature shifts and also to effect starvation. This was carried out by rapidly filtering the cells onto 0.45 μ pore size filters (Millipore), briefly washing with the new medium, transferring the filter to a flask containing the new medium, prewarmed as necessary, and rapidly resuspending the cells by shaking.

M5.3 Incorporation of Radioactive Precursors into Acid Insoluble Material

This was performed by measuring acid precipitable radioactivity bound to filters as described by Bollum (1967) and modified by Johnston and Game (1978). Aliquots (0.2mls) of the radioactively labelled culture were taken and added to 20 μ l of ice-cold 50% Trichloroacetic acid (TCA). Extraction was allowed to proceed for at least 40 minutes. At the end of the experiment, the samples were transferred to glass fibre filters (4 cms x 2 cms, Whatman GF/C). The filters were attached to metal grids to allow simultaneous extraction of up to 25 samples per grid. The filters were allowed to dry so that they were no longer glistening but still damp and the grids then submerged for 5 minutes each in a series of 5 tanks containing respectively: 10% TCA, 5% TCA, 5% TCA, 5% TCA and methanol. After drying the filters were

transferred to vials containing 2mls of Toluene-PPO which were then counted in a liquid scintillation counter.

M5.4 Mutagenesis

a) EMS Mutagenesis

EMS mutagenesis was performed as described by Fink (1970). A mid-exponential phase culture was harvested by centrifugation, washed with distilled water and resuspended in 0.1M Sodium phosphate buffer pH8.0. Ethyl methanesulphonate (0.3mls) was added and the culture incubated at 23°C without shaking. After 90 minutes the cells were harvested by centrifugation and washed with sterile distilled water a total of three times, each time transferring to a clean sterile tube. The cells were finally resuspended in YNB-aa containing the appropriate nutritional supplements (e.g. leucine if selecting a leucine auxotroph) and incubated for at least one generation. An aliquot was then taken, diluted in sterile 0.9% w/v saline, and 0.1mls spread onto supplemented minimal agar plates such as to give 100 - 200 colonies per plate following incubation at 30°C. When colonies had appeared they were replica plated using the velvet transfer technique (Lederberg and Lederberg, 1952) onto non-supplemented selective minimal agar plates and again incubated at 30°C. Putative auxotrophic mutants were scored by absence of growth on the corresponding selective plate and then screened again by restreaking on supplemented and non-supplemented agar.

b) UV Mutagenesis

A mid-exponential phase culture was diluted to 10^5 cells per ml with sterile distilled water and 10ml aliquots placed into three sterile Petri dishes. With the lids removed these were irradiated for 15s, 45s, and 75s respectively using a Hanovia Bacteriocidal ultra-violet lamp (253nm emission) as the sole light source at a distance of 15 cms above the dishes. The irradiated cell suspensions were each added to an equal volume of supplemented 2 x YNB-aa and incubated in the dark. After 5 hrs, aliquots (0.1 mls) from the culture and also from 10-fold and 10^2 -fold dilutions were spread onto supplemented minimal agar, these were incubated at 30°C in order to identify

the optimum UV exposure and to determine the appropriate dilution. The irradiated cultures and corresponding dilutions were all stored at 5°C. When colonies had appeared the appropriate cell suspension was spread onto supplemented minimal agar plates which were incubated and screened for mutants as described for EMS mutagenesis.

M5.5 Disruption of Cells

Cells were harvested at exponential phase, washed with distilled water and resuspended in the appropriate buffer at a concentration of about 10^8 cells per ml. The suspension was vortexed with a half volume of acid-washed glass beads (0.45mm Braun-Melsungen) for four 30s bursts with 60s intervals of cooling on ice. This procedure gave an average cell breakage of 80%.

M5.6 Double Stranded RNA Extraction

Two methods were used to release dsRNA from the cells. In the first the cell suspension in TNS-containing crushing cocktail (5×10^8 cells in 5 mls) was subjected to 2 cycles of freezing and thawing prior to phenol-cresol extraction. This method causes the release of dsRNA together with tRNA (Bevan, Herring and Mitchell, 1973). In the second method the cell suspension in crushing cocktail without TNS (5×10^8 cells in 10 mls) was disrupted using glass beads. TNS was then added to give a final concentration of 2% (w/v).

In both cases the extraction was then carried out as described by Kirby (1965) and modified by Oliver (1974). All procedures were carried out at 5°C except where indicated. An equal volume of phenol-cresol was added and the mixture stirred vigorously for 3 minutes. After centrifugation at 10,000 rpm for 10 minutes the phenol layer was removed. To the aqueous layer 0.5 mls of 3M NaCl was added followed by 5 mls of fresh phenol-cresol. The mixture was stirred vigorously for 3 minutes and centrifuged as before. The aqueous layer was taken and 2 volumes of ethanol at -20°C added. The nucleic acids were then allowed to precipitate at -20°C for at least 90 minutes before harvesting by centrifugation at 12,000 rpm at -15°C. The precipitate

was resuspended in 3 mls of Acetate-SDS and 7 mls of ethanol (-20°C) added. The mixture was again held at -20°C for at least 90 minutes when the precipitate was harvested by centrifugation as before.

Samples prepared by the freeze/thaw method were then resuspended in an appropriate volume of loading mix. Samples prepared by bead crushing were resuspended in 2M LiCl and placed on watery ice overnight to precipitate rRNA (Baltimore, 1966). Following centrifugation at 12,000 rpm, 15 minutes the supernatant was ethanol precipitated and the precipitate harvested and resuspended in loading mix.

M5.7 Extraction of tRNA

Transfer RNA was prepared by phenol extraction of whole (i.e. unlysed) cells. This causes the release of tRNA virtually uncontaminated with other RNA species (Monier, Stephenson and Zamecnik, 1960; see also R 2.1). An equal volume of phenol-cresol was added to the cell suspension in buffer A (2×10^9 cells in 5 mls) and the extraction was carried out as described for dsRNA (M5.6).

M5.8 Separation of RNA Species

The different RNA species in nucleic acid extracts were separated using CF 11 cellulose column chromatography as described by Franklin (1966).

CF11 cellulose (Whatman) was suspended in STE buffer and repeated decantations were performed to remove fines. The resulting slurry was then poured into a 1.5 cm x 60 cm column.

This column was then washed with 2 column volumes of STE buffer containing 1% (v/v) β -mercaptoethanol and 0.01M EDTA followed by STE buffer containing 35% (v/v) ethanol until the OD_{260} of the effluent was equal to that of the fresh buffer (about 2 - 3 column volumes). The RNA sample, suspended in STE buffer with 35% v/v ethanol (0.1 column volumes), was then loaded on to the column. The different RNA species were then eluted in a stepwise manner in the following order: transfer RNA was eluted with STE buffer containing 35% v/v ethanol, ribosomal RNA was eluted with STE buffer containing 15% v/v ethanol, and double stranded RNA was eluted with STE

buffer without ethanol. Elution was monitored using a LKB Uvicord spectrophotometer at 280nm. The fractions corresponding to the absorbance peak eluted by each buffer were combined and RNA precipitated with 2 volumes of ethanol at -20°C .

M5.9 Determination of Purity and Concentration of Nucleic Acid Extracts

The optical density at wavelengths of 260nm and 280nm of nucleic acid solutions was determined using a Gilford spectrophotometer (Model 250). The concentration was then calculated using the formula $10D_{260} = 40\mu\text{g/ml}$ nucleic acid.

Contamination of nucleic acid solutions by protein was estimated from the $OD_{260} : OD_{280}$ ratio which should approximately be equal to 2 if the sample is protein free.

M5.10 Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (2.6% and 12%) were prepared according to the method of Loening(1967) which is outlined in figure M.1.

The 2.6% gels were prevented from falling out of the tubes by using nylon mesh supports fixed over the ends with polyethylene rings. The running buffer in all cases was 'E' buffer. Electrophoresis of 2.6% gels was carried out for 7 hrs with a constant current of 3.3mA per gel. The 12% gels were run at a constant current of 5mA per gel for 4 hrs. The current was supplied by a LKB power pack. All gels were pre-run with normal conditions for 30 minutes before loading. Nucleic acid samples in loading mix were layered onto each gel (not more than 200 μl for 2.6% gels, not more than 100 μl for 12% gels) using an automatic pipette.

After electrophoresis, gels were expelled from the tubes and the SDS removed by soaking in 1 x 'E' buffer without SDS for 30 minutes. The gels were then scanned at 260nm using the gel scanning attachment of the Gilford 250 spectrophotometer. For gels run with radioactively labelled samples, the radioactivity contained in the peaks was determined by freezing the gels on dry ice and cutting 1mm slices with a Mickle Gel Slicer. The slices were then solubilised by transferring to vials containing 5 mls of Toluene-PPO

Fig.M.1 The Preparation of Polyacrylamide Gels

The following mixture was prepared:

<u>12%</u>	<u>2.6%</u>
20 ml	5.0 ml Stock Acrylamide Solution
5 ml	5.8 ml 5 x "E" Buffer, pH 7.7
-	17.8 ml Distilled Water

↓
The mixture was degassed using a vacuum pump.

↓
25 μ l of N,N,N,N, tetramethyl ethylene diamine (Koch-Light) were added followed by 0.2 ml of freshly dissolved 10% w/v ammonium persulphate.

↓
A 20 ml syringe was used to rapidly transfer the gel mixture to perspex tubes (0.6 cm i.d. x 12.5 cm long) closed by rubber bungs, to give 7 cm long gels. Distilled water (0.1 ml) was then carefully layered onto the top of the gel mixture.

↓
The gels were allowed to polymerise at 25°C.

with 3% v/v protosol (New England Nuclear) which were allowed to stand for 24 hrs before counting in a liquid scintillation counter.

M5.11 Liquid Scintillation Counting

Samples labelled with single isotopes (^3H or ^{14}C) were counted in either a Beckman Liquid Scintillation Counter (using standard preset windows, Gain 300) or a LKB 1216 RackBeta II liquid scintillation counter (using manufacturers recommended windows). Double labelled samples (^3H and ^{14}C) were counted in the Beckman counter using an adjustable window set at 0-90 for ^3H , the standard preset ^{14}C with ^3H window and with increased gain (450). These settings gave the least spill over of counts from one channel into the other. When counting double labelled samples single isotope standards were routinely included to estimate such cross over between channels and the counts were corrected accordingly. All samples were counted for 5 minutes.

M5.12 Extraction and Purification of Amino-acyl tRNA Synthetases

The procedure adopted was developed during the course of the investigation and details are given in section R2.2. Standard techniques applied during this procedure are described below:

a) Preparation of Chromatographic Material

DEAE Cellulose (Whatman) was suspended and swollen in excess distilled water. This was decanted and the DEAE cellulose stirred in 0.2M NaOH for 30 minutes. It was then washed with distilled water to pH7 and stirred in 0.2M HCl for 30 minutes before again washing to neutrality. Finally it was resuspended in buffer 'F'.

CM Sephadex C50 (Pharmacia) was precycled in a similar manner; first stirred in 0.2M HCl, washed to pH7, stirred in 0.2M NaOH, washed again to neutrality and lastly equilibrated in buffer C.

b) DEAE Cellulose Chromatography

This was performed in batch as described by Faulhammer and Cramer (1977). About 0.2gms (wet weight) of DEAE cellulose was added per ml of the cell extract in buffer F and the mixture was stirred on ice for 30mins. The eluate was collected by centrifuging at 15,000 rpm for 10 minutes and

lifting off the supernatant (care was taken not to include any DEAE cellulose).

c) CM Sephadex C50 Chromatography

CM Sephadex C50 chromatography was carried out according to Von der Haar (1979) using two sizes of column. A small column, 0.75 cm x 15 cm (20 mls of gel), was used routinely and a larger one, 1.5 cm x 40 cm (250 mls of gel), for large scale preparations. After pouring, the column was washed with 2 column volumes of buffer C. The sample in buffer C (0.1 column volumes) was applied to the column which was then washed with buffer D (1 column volumes) followed by buffer D containing 50 mM KCl (1.5 column volumes). Tyrosyl synthetase activity was then eluted from the column using buffer D containing 0.5 M KCl (1.5 column volumes).

d) Ammonium Sulphate Precipitation

This procedure was used to precipitate proteins from crude cell extracts. An appropriate amount of solid ammonium sulphate was gradually added to the extracts with continuous stirring at 5°C to give the required percentage saturated solution. The mixture was kept on ice for a further 30 minutes with intermittent stirring. The precipitate was then harvested by centrifugation at 10,000 rpm for 15 minutes.

e) Polymin P Precipitation

Polymin P, a polyethylenediamine of average molecular weight of 6000, was used to precipitate nucleic acids and fine cell debris from crude cell extracts. A stock solution of 10X strength was prepared by dissolving 2.4 gm of Polymin P (Sigma) in water (25 mls). This solution was then dialysed against 5 l of water. After dialysis the solution was adjusted to pH 7.0 using hydrochloric acid. A suitable aliquot of this stock Polymin P solution was added to enzyme extracts at 5°C over a period of 5 minutes with continuous stirring. The precipitate was collected by centrifuging at 10,000 rpm for 15 minutes.

f) Streptomycin Precipitation

Precipitation of nucleic acids using streptomycin was performed by adding a suitable aliquot of a 10X strength solution to extracts at 5°C with stirring. The final concentration of streptomycin was 1.2% w/v. The

precipitate was harvested by centrifugation at 10,000 rpm for 15 minutes.

g) Alkaline Hydrolysis of RNA

To remove RNA from synthetase preparations the extracts were incubated at 37°C for 16hrs in 0.25M Tris HCl, pH 9.0. To limit the action of proteases the inhibitors phenylmethylsulphonylfluoride (0.01mM) and Diisopropylflourophosphate (50µM) were added.

h) Ribonuclease Digestion of RNA

Ribonuclease A (Sigma) was added to synthetase extracts at a final concentration of 1µg/ml to digest endogenous RNA. After incubation for 1hr at room temperature the extract was rendered nuclease free by differential ammonium sulphate precipitation.

M5.13 Assay Procedure for Aminoacyl-tRNA Synthetases

The procedure used to assay aminoacyl-tRNA synthetases was adapted from that described by Hampel, Enger and Ritter (1979). A solution of 2x final concentration reaction mixture was prepared immediately prior to use by diluting 20x strength stock solutions as explained in M4.17. The relative amounts of tRNA and radiolabelled amino acid added were adjusted such that the latter was present in molar excess. Aliquots (25µl) of this mixture were dispensed separately onto stretched "Cling Film" (Saran Wrap) so that each drop formed a tight bead and represents a replicate assay. An equal volume of synthetase preparation was added to each bead and the reaction allowed to proceed for 20 minutes at 25°C. For each group of assays a set of control assays without added tRNA were performed to monitor the endogenous activity of the enzyme extract and the results were corrected as appropriate for this value (see next chapter). The reaction was stopped by placing a glass fibre filter (Whatman GF/C), which had been previously been soaked in 10% w/v TCA and dried, onto each bead. After 2 minutes the filters were transferred to ice-cold 10% w/v TCA for at least 30 minutes. Un-esterified radiolabelled amino acids were then washed off the filters by transferring them to metal grids which were submerged for 10 minutes in each of a series of tanks containing respectively : 10% w/v TCA, 5% w/v TCA,

5% w/v TCA, 5% w/v TCA and methanol. The filters were then dried and the level of radioactivity determined by liquid scintillation counting.

M5.14 Assay for Proteinase Activity

Proteinase activity was assayed in three different ways:-

a) Rapid Screening of Colonies on Agar Plates (Jones, 1977)

Colonies on agar plates were tested for the ability to cleave N-acetyl-DL-phenylalanine β naphthyl ester (APE) which serves as a substrate for yeast proteinase C (Wolf and Fink, 1975). To 6 mls of molten agar (1%) at 50°C, 4 mls of dimethyl formamide (DMF) containing 5 mg of APE (Sigma) was added. After mixing this agar was poured over the colonies on solid medium. When the agar had solidified 10 mls of 0.1 M Tris HCl, pH7.3 containing 5 mg/ml Fast Garnet GBC (Sigma) was gently poured over the surface. After about ten minutes wild type colonies turned red. Proteinase deficient colonies turn red much more slowly.

b) Spectrophotometric Proteinase Assays

The activity of proteinases A, B and C in crude cell extracts was assayed spectrophotometrically.

i) Preparation of cell extracts

Cells were grown in YEPD (2 l) to stationary phase at 30°C and then harvested by centrifugation. After washing with distilled water the cells were resuspended in 10 mls of 0.1 M Tris HCl pH7.6 and broken using glass beads. Cell breakage was assessed by microscopic examination since the procedure normally used (M5.5) is not sufficient for lysis of concentrated suspensions of stationary phase cells. Using these conditions 60-80% breakage was achieved by 8-12 bursts of 30 seconds with intervals of cooling on ice. The extract was then centrifuged for 30 minutes at 18,000 rpm, the supernatant was collected and divided into two portions. The first portion was brought to 0.26% SDS by the addition of an appropriate amount of 20% SDS pH7.6 and incubated for 6 hrs at room temperature before assaying for proteinase B and C activities. The second portion was adjusted to pH5 using 3N acetic acid and assayed for proteinase A activity.

ii) Proteinase A Assay (Lenney et. al., 1974)

Proteinase A activity was assayed using haemoglobin as substrate. This was prepared by dissolving haemoglobin in water (2.5% w/v) and dialysing against two changes of water (5 l) at 5°C. The pH was brought to 1.8 using 1M HCl and the solution was stirred at 35°C for 1 hr before adjusting the pH to 3.2 with 1M NaOH. The concentration of the solution was then adjusted to 2% (w/v) with respect to haemoglobin. An aliquot (0.2 mls) of this solution was added to 0.1M Glycine Buffer, pH3.0 (0.2 mls) followed by 0.1 mls of cell extract. After incubation at 37°C for 30 minutes, 0.35 mls of water and 0.2 mls of 1M Perchloric acid were added. The mixture was then filtered using Whatman GF/C filters and the filtrate assayed for tyrosine containing peptides released into the supernatant using the Lowry assay (M5.15).

iii) Proteinase B Assay (Jones, 1977)

An aliquot (0.1 mls) of cell extract was added to 0.35 mls of 0.1 M Tris HCl pH7.6, 0.1 mls of 1% Triton x-100 and 20mg of Hide Blue Azure Powder (Sigma) at 37°C. After 20 minutes incubation 3.5 mls of ice water was added and the mixture centrifuged a 10,000 rpm for 5 minutes. The absorbance of the supernatant at 520nm was then determined.

iv) Proteinase C Assay (Jones, 1977)

0.1 ml of 6mM N-benzoyl-L-tyrosine-p-nitroanilide (Sigma) in DMF was added to 0.45 mls of 0.1M Tris HCl pH7.6 and 0.1 mls of cell extract at 37°C. After incubation for 30 minutes 1 ml of 1mM HgCl₂ was added followed by 0.5 ml of water and 0.2 mls of 20% SDS, pH7.6. After vortexing the absorbance at 410nm was determined.

For each of these spectrophotometric assays suitable controls were carried out to enable correction for absorbance due to the substrate and the cell extract.

c) Determination of 'in vivo' Protein Turnover

A culture was grown overnight to mid-exponential phase in YNB-aa containing 2μCi/ml ³H-Leucine. The culture was harvested and the cells resuspended in fresh YNB containing 200μg/ml non-labelled leucine.

At intervals after this switch aliquots (1 ml) were taken into ice-cold 50% w/v TCA (0.1 mls). After 1 hr the samples were filtered through nitrocellulose filters (poresize 0.45 μ) and the filtrates added to 4 mls of PCS scintillation fluid. The level of radioactivity was then determined by liquid scintillation counting.

M5.15 Folin - Lowry Assay for Proteins (Lowry et. al., 1951)

To the protein sample suitably diluted in 1 ml volume, 5 mls of solution C* was added and after mixing allowed to stand for 10 minutes. Solution D (0.5 mls) was then added, and after immediate mixing the sample was again allowed to stand for 30 minutes. The absorbance at 550nm was then determined. This value was then compared to those of standard protein solutions obtained by assaying a range of concentrations of Bovine Serum Albumen.

M5.16 Random Spore Analysis

The parent strains were mated by streaking each onto solid minimal medium such that the two streaks crossed and the cells were mixed. The diploid colonies which grew up were restreaked onto presporulation medium and after two days growth spread onto sporulation medium for about five days. The spores were liberated from their asci by resuspending a loop of the spore containing culture into 1M sorbitol to which Novozym 234 (5 mg /ml) was added. Following incubation for 20 minutes at room temperature the suspension was suitably diluted in sterile distilled water and aliquots (0.1 ml) spread onto suitably supplemented minimal agar such as to give 100-200 colonies per plate. After three days growth the colonies were replica plated onto minimal agar containing all supplements except that being scored using the velvet transfer technique (Lederberg and Lederberg, 1952).

RESULTS

R1 The Regulation of Double-Stranded RNA Synthesis

The maintenance of the double-stranded RNA containing virus like particle (VLP) and its relationship with the host cell metabolism was examined by subjecting growing cultures to specific changes in their nutritional environment. In the experiments described only P1 dsRNA was studied. This species occurs in larger amounts than P2 dsRNA and its presence can be readily followed using gel electrophoresis. The quantitation of P2 dsRNA in polyacrylamide gels is less reliable since it co-migrates with 25 S rRNA which may contaminate the preparation. Furthermore, P2 dsRNA is not found in all VLP containing strains and its maintenance appears to be dependent on the presence of the P1 species (see Introduction).

The strains used were derived from a prototroph (S7) containing elevated amounts of P1 as the sole dsRNA species (Oliver et al., 1977).

R1.1 The Effect of Nitrogen Starvation on the Maintenance of P1 dsRNA

The effect of nitrogen starvation on the synthesis and maintenance of P1 dsRNA was examined by means of double isotope labelling experiments using the adenine auxotroph S7A6 (M1). The cells were grown for about 5 generations in adenine supplemented YNB-aa containing ^{14}C -8-Adenine ($0.1\mu\text{Ci/ml}$) to a cell density of $0.5\text{--}1 \times 10^7$ cells/ml. The culture was divided into two and the cells from one portion were transferred (M5.2) to fresh YNB-aa whilst cells from the other portion were transferred to YNB-NH_4^+ . At the indicated times (see figure R.1) after the shift aliquots (50 mls) were removed from each culture and 'pulse'-labelled by adding ^3H -2-adenine ($1\mu\text{Ci/ml}$). Incorporation was stopped after 1 hour by adding iced deionised water and rapidly harvesting and washing the cells. Double-stranded RNA was then extracted (M5.6) and analysed by electrophoresis on 2.6% polyacrylamide gels. After scanning at 260nm, the regions of the gels containing dsRNA were sliced and the radioactivity counted.

FIGURE R.1 - Levels of radioactivity contained in P1 dsRNA gel peaks from normal and nitrogen starved cells.

S7A6 was grown for 5 generations in YNB-aa+ade containing ^{14}C -8-adenine ($0.1\mu\text{Ci/ml}$). This culture was divided in two and the cells rapidly collected by filtration. One filter was transferred into YNB-aa+ade and the other into YNB- NH_4^+ +ade. At the indicated time periods after the medium shift, aliquots (50ml) were labelled for 1 hr with ^3H -2-adenine ($1\mu\text{Ci/ml}$). Incorporation was stopped by pouring the cultures over iced deionised water. The dsRNA (extracted from the cells using the freeze thaw method -M5.6) was subjected to electrophoresis in 2.6% polyacrylamide gels (M5.10). The gels were sliced and the radioactivity contained in the P1 peaks determined (M5.11). These values are plotted in the figure and are shown together with the $^3\text{H}/^{14}\text{C}$ ratio for each peak. $\circ-\circ, ^{14}\text{C}$; $\bullet-\bullet, ^3\text{H}$.

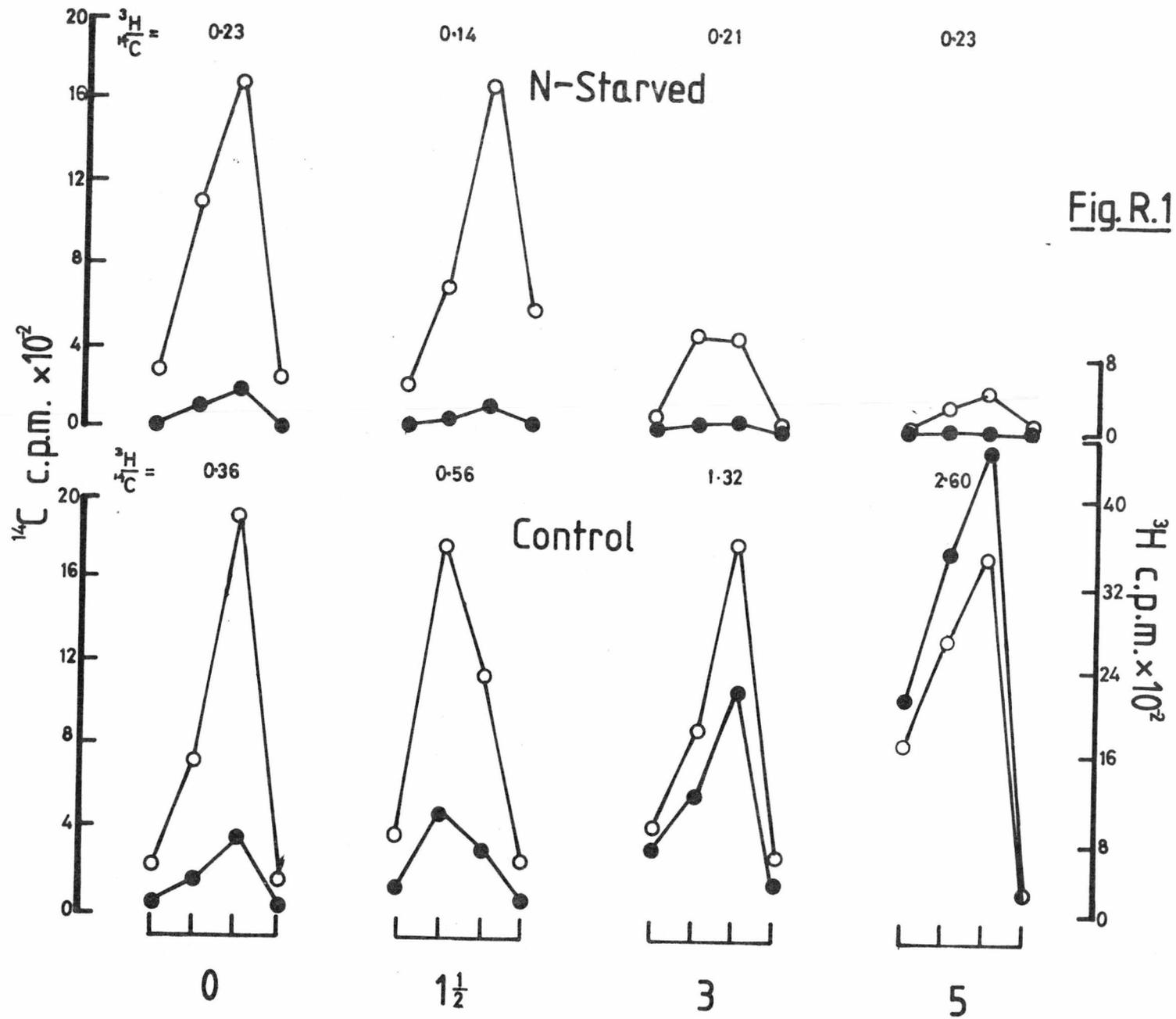


FIGURE R.2 - Degradation of P1 dsRNA in nitrogen — starved cells.

The total amount of ^{14}C recovered in the P1 dsRNA peaks produced in the experiment described in the legend to Fig. R.1 was determined. These amounts were normalised to a value of 1 for the 0-1 hr peaks. Thus, the figure demonstrates the proportion of pre-labelled P1 dsRNA remaining with time. o—o, control; ●—●, N stvd.

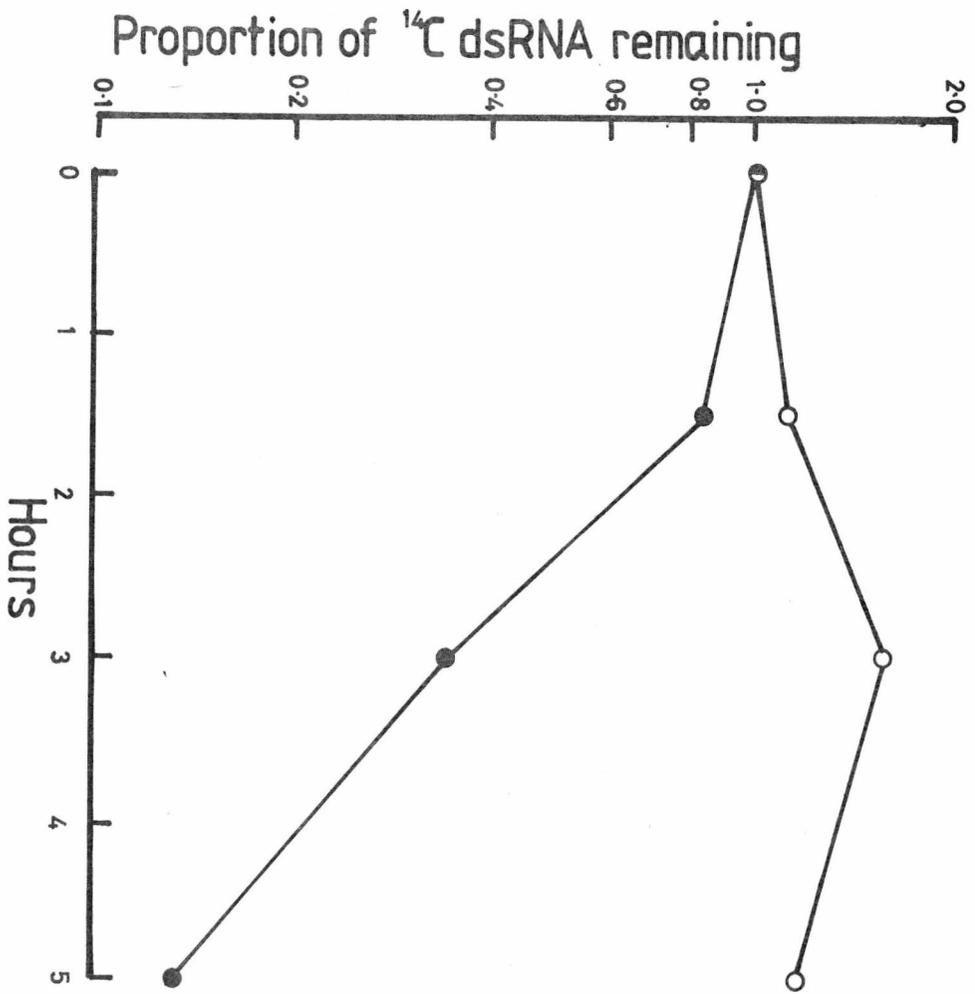


Fig. R.2

FIGURE R.3 - U.V. Absorbance scans of P1 dsRNA gel peaks from normal and nitrogen-starved cells.

The absorbance scans at 260nm of P1 dsRNA gel peaks obtained in the experiment described in the legend to figure R.1 are shown.

(left hand peaks = DNA ; right hand peaks = dsRNA - see fig R.18)

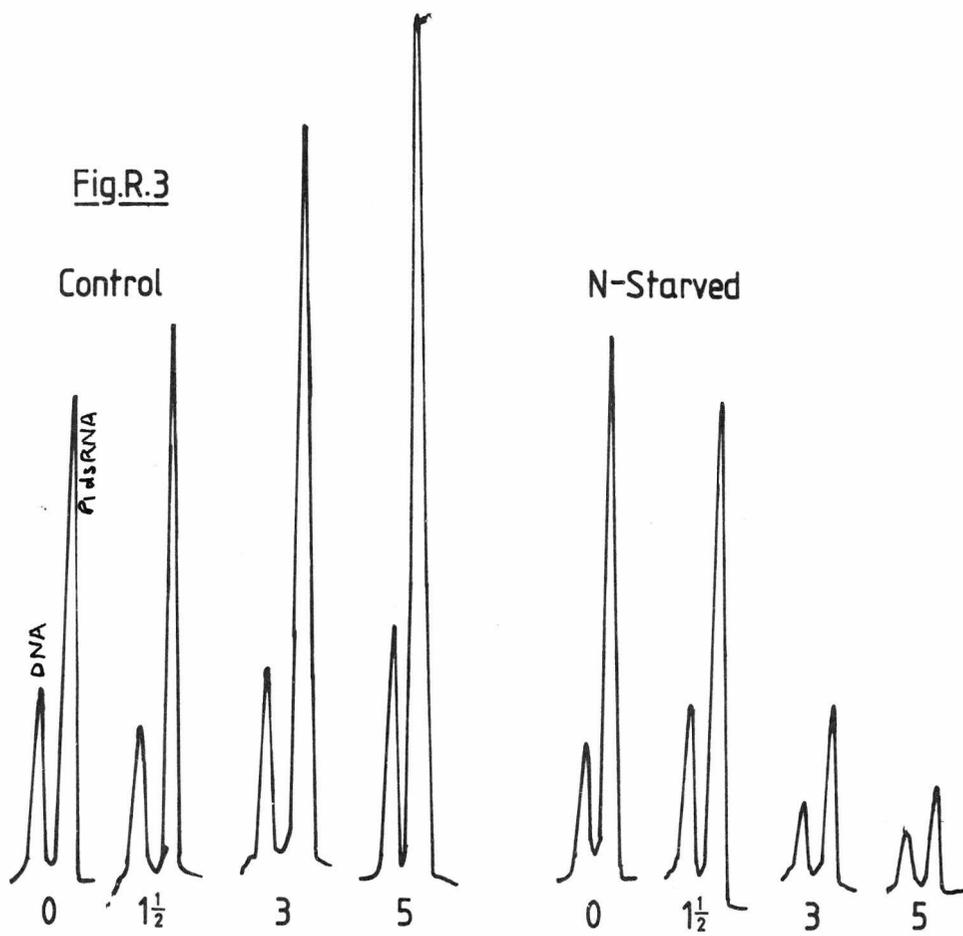


FIGURE R.4 - Synthesis of P1 dsRNA during nitrogen starvation.

The $^3\text{H}/^{14}\text{C}$ ratios from the P1 dsRNA peaks shown in figure R.1 were normalised to a value of 1 for the 0-1 hr sample. These values are plotted in the figure and the curves therefore describe the change in the rate of P1 dsRNA synthesis with time. ○—○, control; ●—●, N stvd.

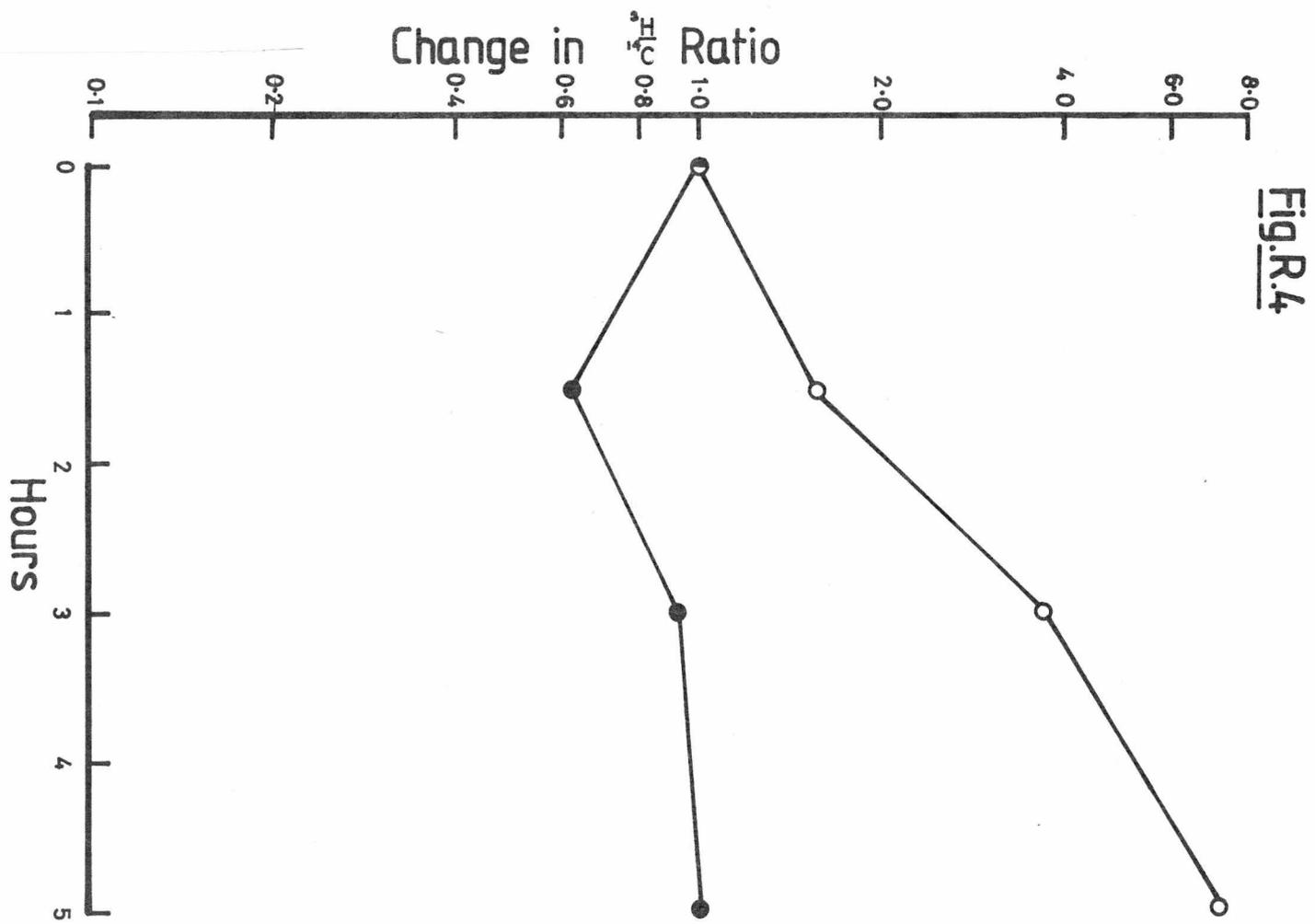


Fig.R.4

The radioactivity profiles of gels from one such experiment, using the freeze/thaw method of RNA extraction (see M5.6) are shown in figure R.1. The fate of dsRNA molecules existing at the beginning of the experiment can be followed by examining the total amount of ^{14}C in the gel peaks. In figure R.2 this value is plotted for each labelling period relative to the value for the 0-1 hr sample. It can be seen that little or no turnover of dsRNA occurs in control cells whereas during nitrogen starvation considerable degradation occurs. This loss of dsRNA from the culture can also be seen on the u.v.absorbance scans of the gels (figure R.3).

The ratio of ^3H to ^{14}C in the dsRNA peaks reflects the rate of synthesis of that molecule with respect to the number of templates remaining from the beginning of the experiment. In figure R.4 the change in this ratio relative to the 0-1 hr labelling period is plotted. It is clear that in control cells the $^3\text{H}/^{14}\text{C}$ ratio increases exponentially throughout the experiment. Since no appreciable turnover of dsRNA occurs in these cells (figure R.2) this is the pattern of synthesis to be expected if existing templates replicate to give molecules which themselves act as templates for further replication. In contrast the $^3\text{H}/^{14}\text{C}$ ratio in nitrogen starved cells remains fairly constant with time even though extensive degradation of P1 dsRNA is occurring. This pattern of synthesis can be explained most simply by assuming that pre-existing templates which survive degradation continue to replicate at the same rate throughout nitrogen starvation but newly formed dsRNA molecules do not themselves act as templates for further replication (see Discussion).

Similar experiments were also performed using the glass beads /LiCl procedure for RNA extraction (see Methods). The results obtained show exactly the same pattern of P1 dsRNA synthesis and degradation and are displayed in figures R5-9.

FIGURE R.5 - Levels of radioactivity contained in P1 dsRNA gel peaks from nitrogen-starved cells.

The experiment was performed as described in the legend to figure R.1 except that the cells were broken by the glass beads technique (M5.6).

○—○, ¹⁴C; ●—●, ³H

Fig.R.5

N-Starved

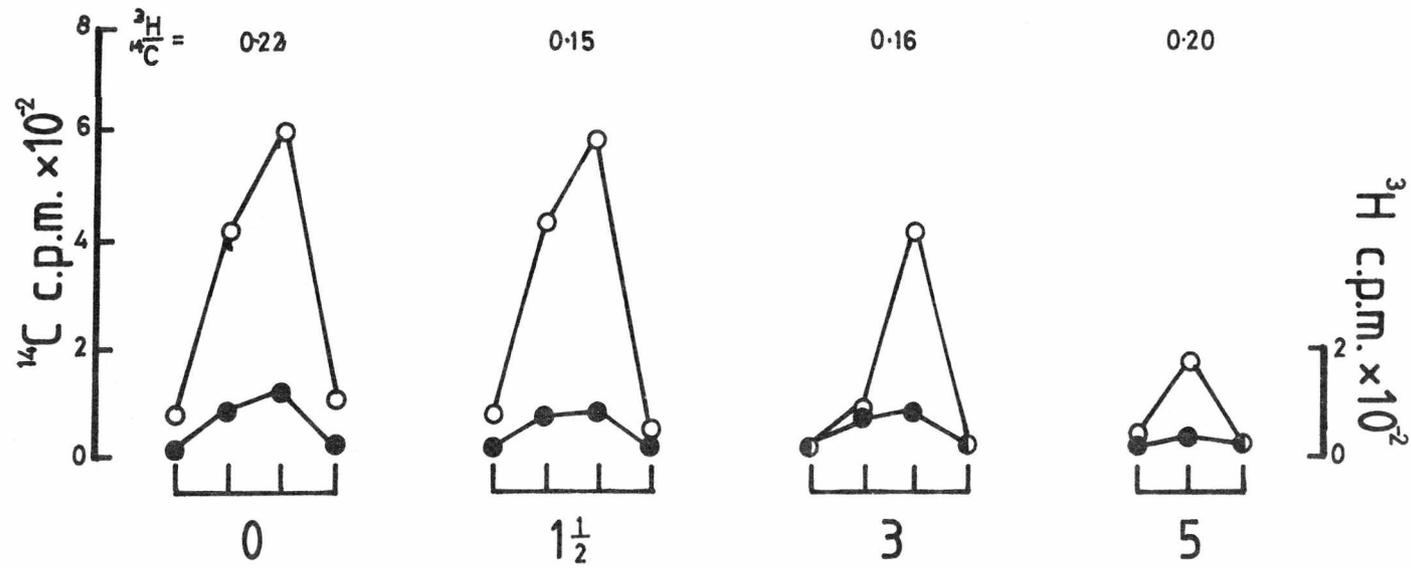


FIGURE R.6 - Levels of radioactivity contained in P1 dsRNA gels peaks from normally grown cells.

The values shown are from the same experiment as in figure R.5.

○—○, ¹⁴C ; ●—●, ³H

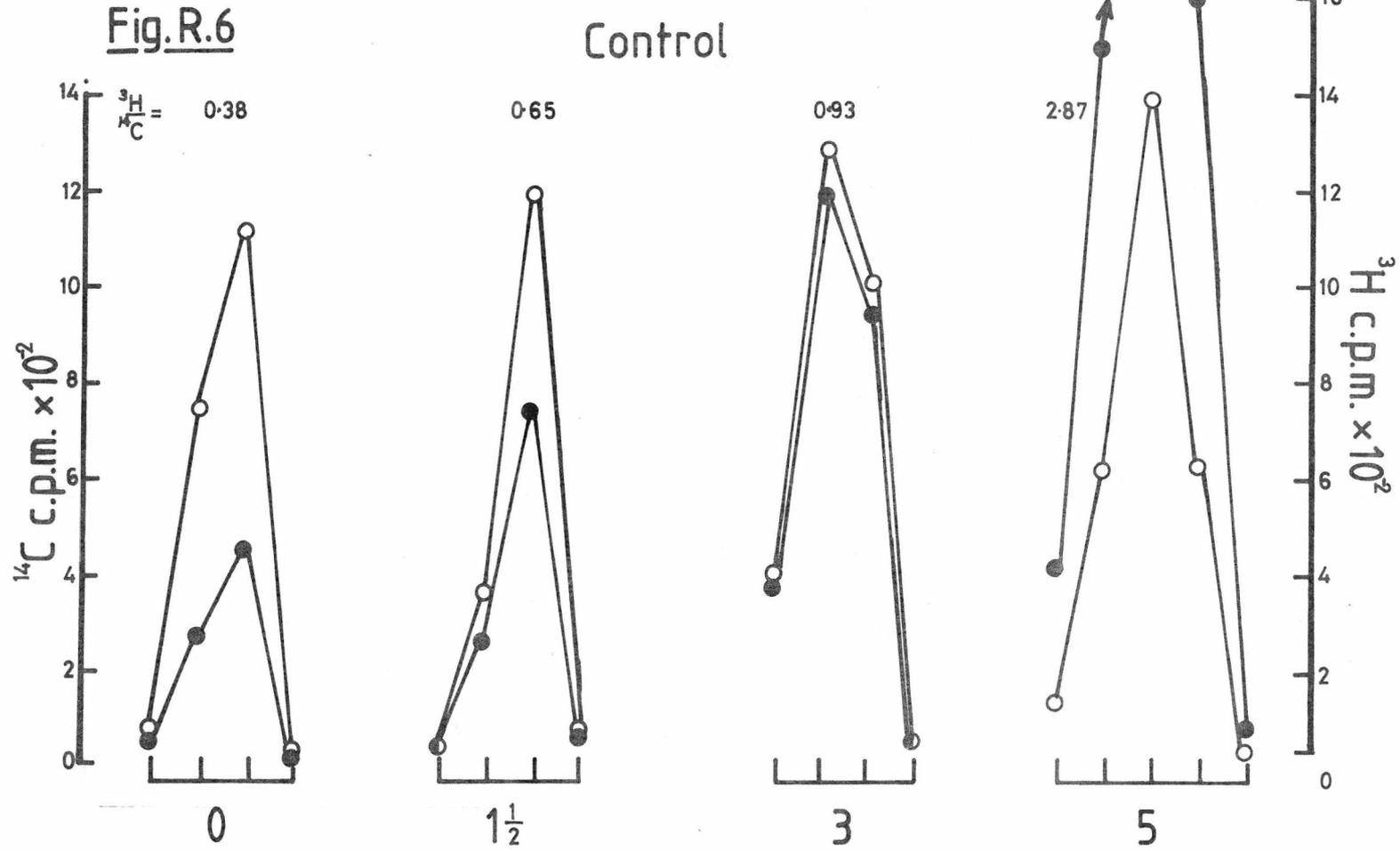


FIGURE R.7 - Degradation of P1 dsRNA in nitrogen-starved cells.

○—○, control; ●—●, N stvd.

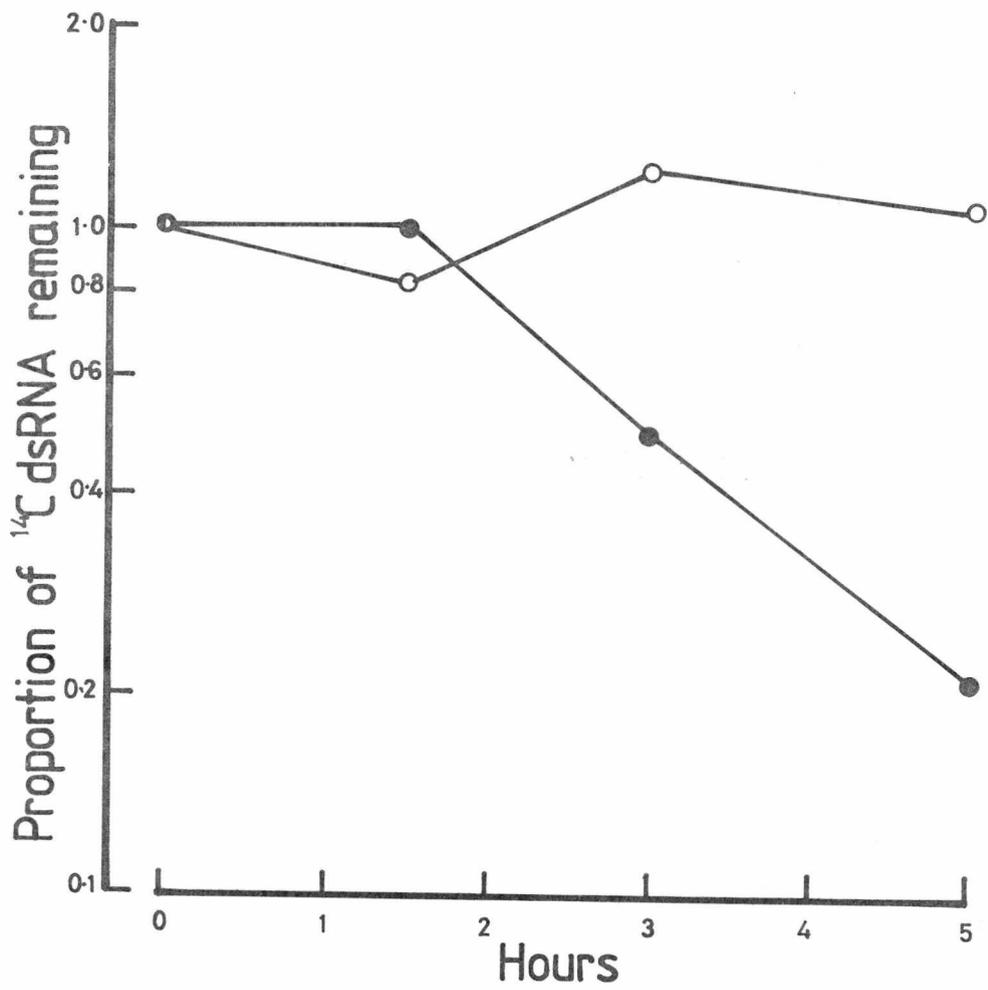
Fig.R.7

FIGURE R.8 - Absorbance scans of P1 dsRNA gel peaks.

(left hand peaks = DNA ; right hand peaks = dsANA - see fig. R.18)

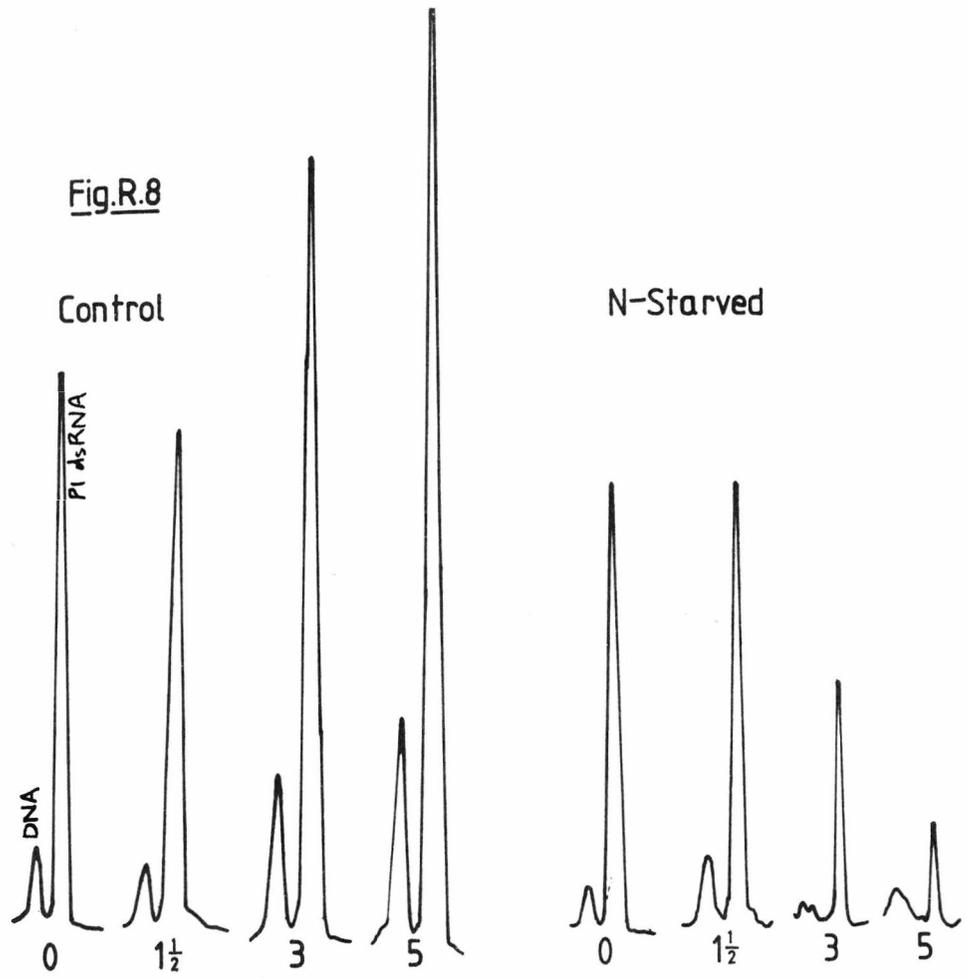


FIGURE R.9 - Synthesis of P1 dsRNA during nitrogen-starvation.

The $^3\text{H}/^{14}\text{C}$ ratios from the experiment shown in figure R.5 and R.6 were normalised to the 0-1 hr labelling period as explained in the legend to figure R.4. $\circ\text{---}\circ$, control; $\bullet\text{---}\bullet$, N stvd.

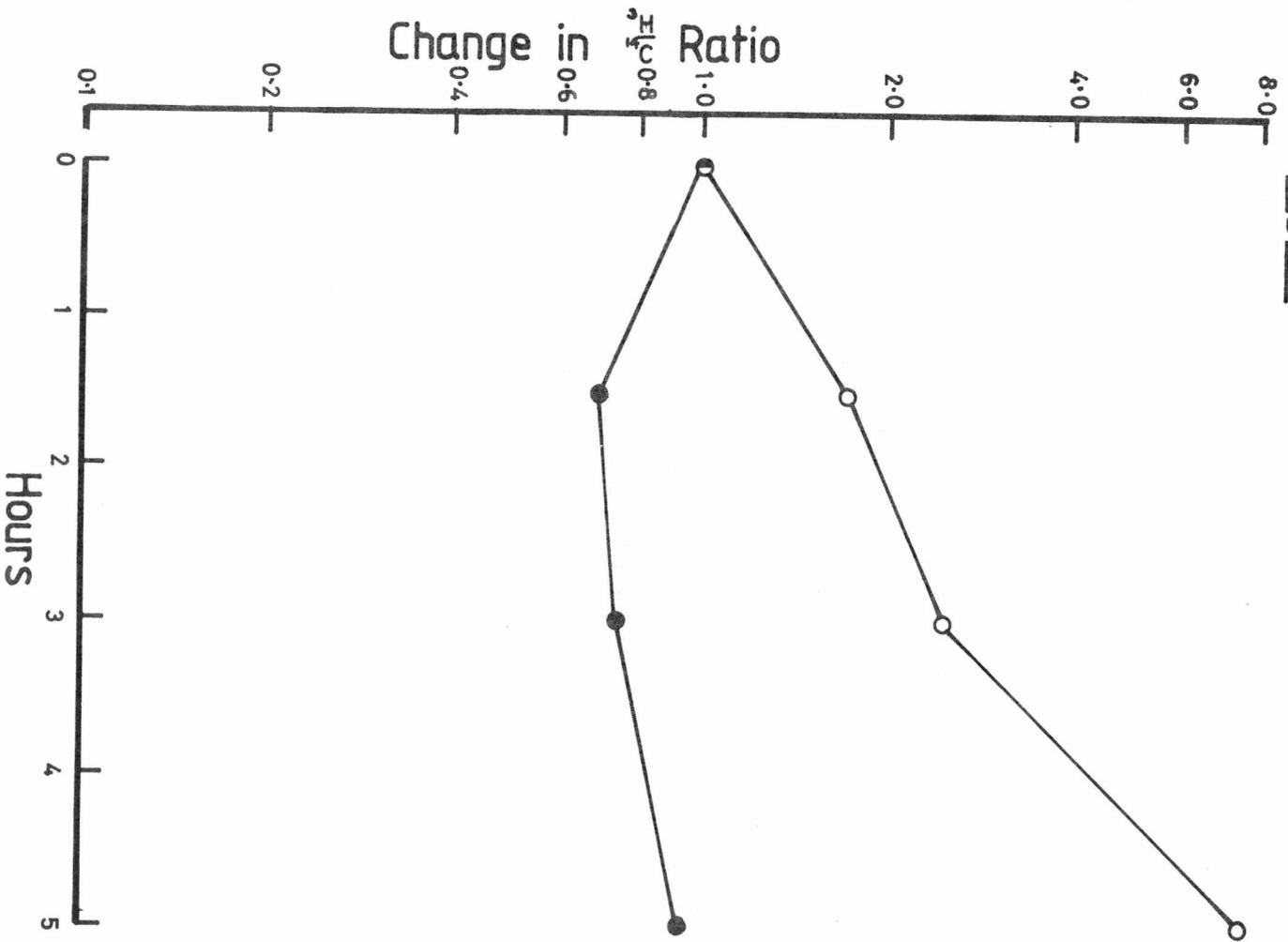


Fig.R.9

R1.2 The Effect of Prolonged Nitrogen Starvation on the Level of P1 dsRNA

The experiments described in section R1.1 show that during nitrogen starvation P1 dsRNA is degraded. To determine the extent of this process and to discover if cells can be completely cured of dsRNA, cultures were subjected to extended periods of starvation.

These experiments were performed by growing strain S7A6 in YNB-aa with adenine to a cell density of about 6×10^6 cells/ml. At the start of the experiment two equal aliquots (100 mls) of this culture were taken and the cells from the remainder were transferred to YNB-NH₄⁺ supplemented with adenine. Double-stranded RNA was extracted from one of the aliquots while the other was allowed to grow to stationary phase (12 h.) before extraction of the dsRNA. At the intervals shown in table R.1 two aliquots (both 100 mls) were taken from the nitrogen starved culture and dsRNA was again immediately extracted from one of these. To the other aliquot 10 mls of sterile 5% (NH₄)₂SO₄ was added and the culture allowed to grow to stationary phase (12 h.) before extraction of dsRNA. The RNA samples were then analysed on 2.6% polyacrylamide gels.

Table R.1 gives the results of one such experiment. The values shown correspond to measurements of the area under the P1 dsRNA peaks in each of the gels when scanned at 260nm. The amount of P1 dsRNA observed in cells after 26 h of nitrogen starvation is markedly reduced compared to that found in the unstarved culture (t=0). However even after starvation for 78 h some P1 still remained. Indeed the amount of P1 observed did not change during prolonged starvation but remained at the level found after 26 h. Thus it appears that some P1 dsRNA may be protected from degradation.

When cells were resupplied with nitrogen after differing periods of starvation the amount of P1 increased during subsequent growth to a similar level in each case. However this value was lower than that found in the stationary phase culture which had not been subjected to nitrogen starvation. This finding further demonstrates the interrelationship between P1 replication

SAMPLE	AREA UNDER P1 PEAK
0	236
Stationary Phase	832
N starved, 26 h	13
N added back, 26 h	106
N starved, 52 h	13
N added back, 52 h	98
N starved, 78 h	26
N added back, 78 h	150

TABLE R.1 - The effect of extended nitrogen starvation on P1 levels.

and the metabolism of the host cell since there is no preferential synthesis of dsRNA as cell growth is resumed.

R1.3 Amino Acid Starvation and the Synthesis of P1 dsRNA

In order to study the effect of amino acid starvation on the synthesis of P1 dsRNA it was necessary to isolate an amino acid auxotrophic mutant from S7A6. To obtain a rapid effect on protein synthesis starvation for leucine was chosen since this amino acid has the second smallest pool in S. cerevisiae (Watson, 1976) and is present in yeast protein at twice the concentration of tyrosine (Boulanger, quoted in Waldron, 1977), the amino acid with the smallest pool. Accordingly S7A6 was subjected to EMS mutagenesis (M5.4a) and by selecting for leucine requirement the mutant S7A6L184 was isolated.

The effect produced by leucine starvation on macromolecular synthesis in S7A6L184 is shown in figure R.10. There is a marked decrease in the incorporation of ^3H -tyrosine in the starved culture about 40 mins after the shift. Similarly the incorporation of ^{14}C -Adenine is sharply reduced at about 50 mins after starvation.

Strain S7A6L184 was then used to observe the effect of leucine starvation on P1 dsRNA synthesis by performing similar experiments to those carried out to study nitrogen starvation. In this case a shift from YNB-aa +leucine to YNB-aa -leucine replaced the YNB-aa to YNB-NH₄⁺ shift. Furthermore only the glass beads/LiCl RNA extraction procedure (M5.6) was used since consistent recoveries of dsRNA were not obtained from S7A6L184 with the freeze/thaw method.

Figure R.11 shows that no detectable turnover of ^{14}C label occurred in either control or leucine starved cells. Thus starvation for an amino acid does not lead to breakdown of dsRNA molecules. The synthesis of P1 dsRNA during starvation is shown in figure R.12. In unstarved cells the $^3\text{H}/^{14}\text{C}$ ratio again showed the exponential increase expected for the synthesis of a self-replicating molecule. In leucine starved cells, however, this ratio drops to 50% of the value seen at the 0-1 hr labelling period. Since no degradation is occurring this represents a genuine reduction in the rate of P1 dsRNA synthesis as a

FIGURE R.10 - Macromolecular synthesis in S7A6L184.

S7A6L184 was grown to 2.5×10^6 cells/ml in YNB-aa+ade+leu, the culture was divided into 4 portions and the cells harvested by filtration. One filter was transferred to YNB-aa +ade +leu containing ^3H -(3,5)-Tyrosine (0.2 $\mu\text{Ci/ml}$) and another to the same medium containing ^{14}C -8-adenine (0.5 $\mu\text{Ci/ml}$). The two remaining filters were transferred to the same two labelled media but without leucine. Aliquots (0.2mls) of these cultures were taken at the times indicated in the figure and the incorporation of radiolabel into TCA insoluble material was determined as explained in M5.3.

o—o, control; ●—●, stvd.

Fig.R.10

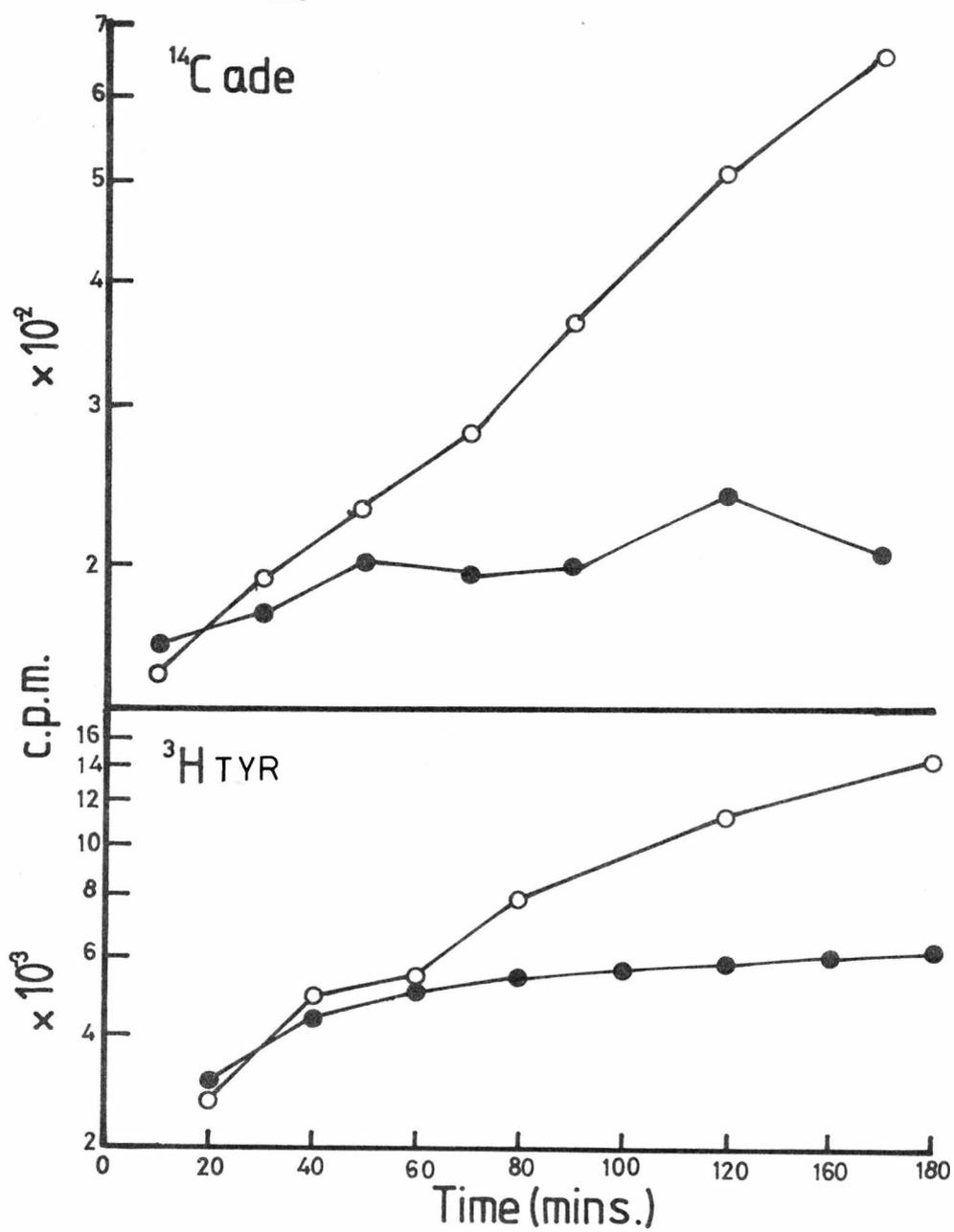


FIGURE R.11 - Maintenance of P1 dsRNA in leucine-starved cells.

The experimental protocol was as described in the legend to figure R.1. The only differences were that strain S7A6L184 was used, cell breakage was effected by vortexing with glass beads (M5.6), and starvation was effected by transferring cells from YNB-aa+ade+leu to YNB-aa+ade. The figure shows the total amount of ^{14}C recovered in the P1 dsRNA peaks normalised to a value of 1 for the 0-1 hr peaks, and thus shows the proportion of ^{14}C labelled P1 dsRNA remaining with time.

○—○, control; ●—●, leu stvd.

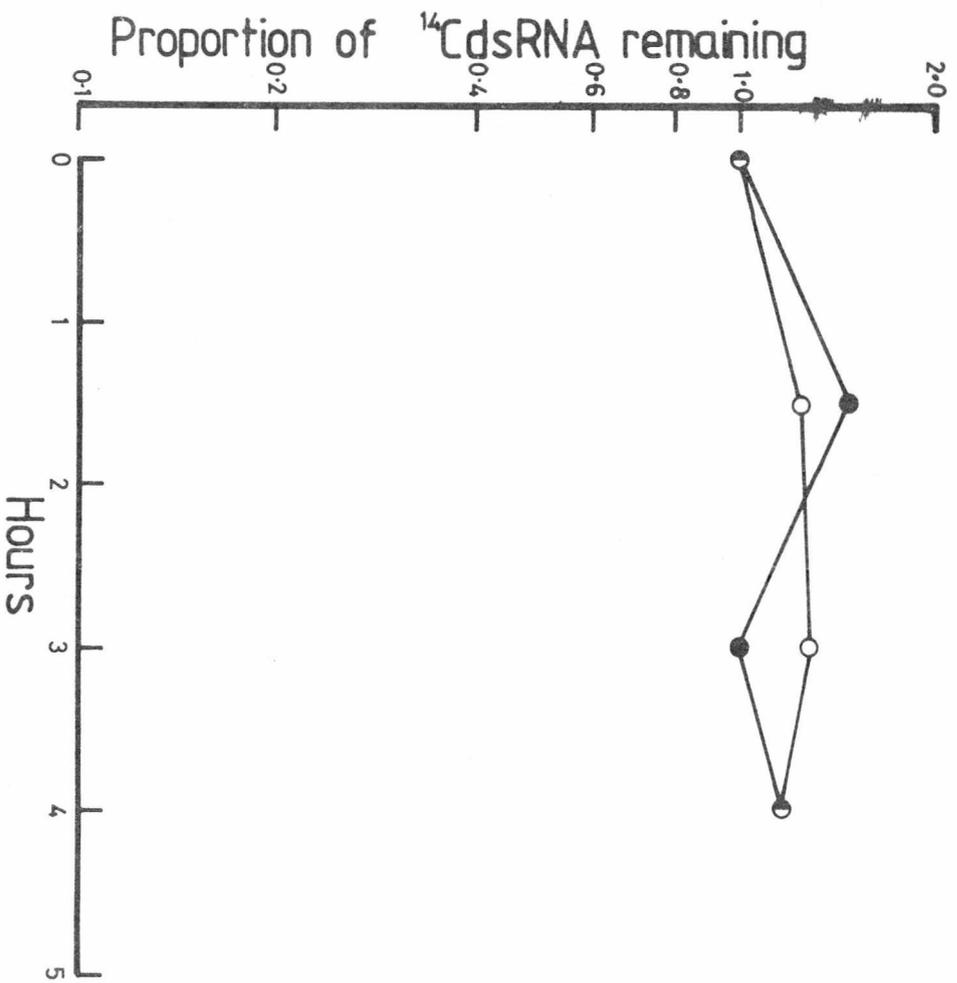


Fig.R.11

FIGURE R.12 - Synthesis of P1 dsRNA during leucine starvation.

The experiment was performed as outlined in the legend to figure R.11. The method of presentation of the data is exactly as described in the legend to figure R.4. The curves therefore show the change in the rate of P1 dsRNA synthesis with time. $\circ-\circ$, control; $\bullet-\bullet$, leu stvd.

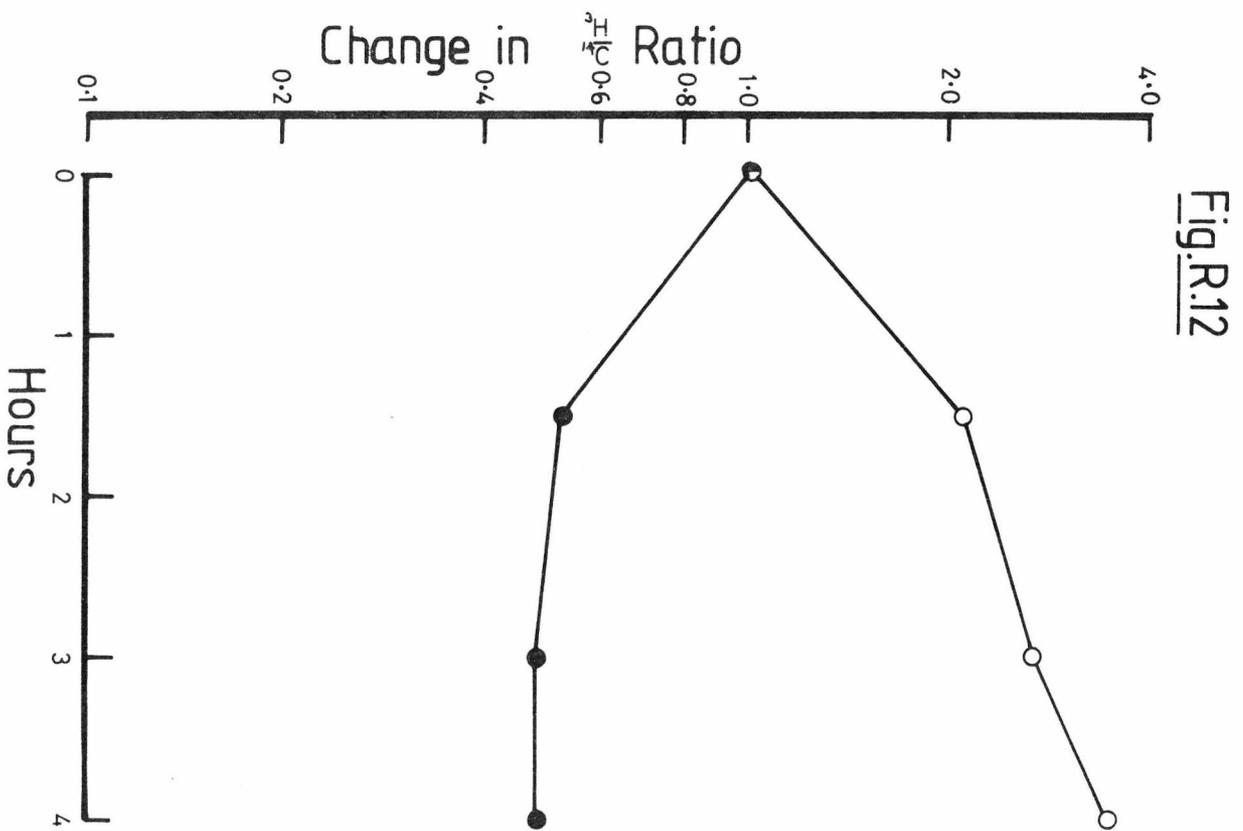


Fig. R.12

result of amino acid starvation.

R1.4 The Effect of Cycloheximide on the Level of P1 dsRNA

The results obtained in section R1.3 suggest that the synthesis of P1 dsRNA maybe sensitive to the protein synthetic rate. Alternatively this response could be due to the lack of amino acids per se or to the accumulation of uncharged tRNA. To investigate these ideas the effect of the translation inhibitor cycloheximide (CHI) on P1 dsRNA synthesis was examined. As before S7A6L184 was grown for about 5 generations in YNB-aa +leucine containing ^{14}C -8-adenine (0.1 $\mu\text{Ci/ml}$). The culture was then divided into four aliquots (50 mls) and the cells transferred into: (A) YNB-aa +leucine; (B) YNB-aa +leucine +CHI (20 $\mu\text{g/ml}$); (C) YNB-aa -leucine; (D) YNB-aa -leucine +CHI (20 $\mu\text{g/ml}$). All of these fresh media contained ^3H -2-adenine (1 $\mu\text{Ci/ml}$). After 2 hrs incorporation was stopped by adding ice, the cells were harvested and the dsRNA extracted and resolved on polyacrylamide gels as usual.

The radioactivity profiles of the P1 dsRNA peaks from one such experiment are shown in figure R.13. When cycloheximide was present in the unstarved culture the $^3\text{H}/^{14}\text{C}$ ratio drops to a third of that seen in untreated cells showing that the drug inhibits P1 dsRNA synthesis. However, little or no effect is seen when cycloheximide is added to leucine starved cells.

The effect of cycloheximide during nitrogen starvation was then determined. The experiment was performed in a similar manner to that outlined for leucine starvation except that strain S7A6 was used and the cells from the four portions of the culture were shifted into the following media all containing ^3H -2-adenine (1 $\mu\text{Ci/ml}$); (A) YNB-aa; (B) YNB-aa +CHI (20 $\mu\text{g/ml}$); (C) YNB-NH $_4^+$; (D) YNB-NH $_4^+$ +CHI (20 $\mu\text{g/ml}$). The results are shown in figure R.14. Again cycloheximide inhibited the synthesis of P1 dsRNA in control cells. In nitrogen starved cells the $^3\text{H}/^{14}\text{C}$ ratio was slightly reduced when the drug was present. In the experiment shown the ratio was reduced by about 20% and in others by as much as 36%. This is a true reduction in the P1 dsRNA synthetic rate since during the first 2 hrs of nitrogen starvation little or no degradation

FIGURE R.13 - The effect of cycloheximide on P1 dsRNA synthesis during leucine-starvation.

S7A6L184 was grown for 5 generations in YNB-aa+ade+leu containing ^{14}C -8-adenine (0.1 $\mu\text{Ci/ml}$). The culture was divided into four aliquots (50ml) and the cells from these transferred into YNB-aa+ade+leu; YNB-aa+ade+leu + 20 $\mu\text{g/ml}$ CHI; YNB-aa+ade -leu; YNB-aa+ade-leu + 20 $\mu\text{g/ml}$ CHI. All of these fresh media contained ^3H -2-adenine (1 $\mu\text{Ci/ml}$). Incorporation was allowed to proceed for 2 hr before the cells were harvested and dsRNA prepared and resolved on polyacrylamide gels. The figure shows the radioactivity profiles of the gels in the P1 dsRNA region.

○—○, ^{14}C ; ●—●, ^3H

Fig.R.13

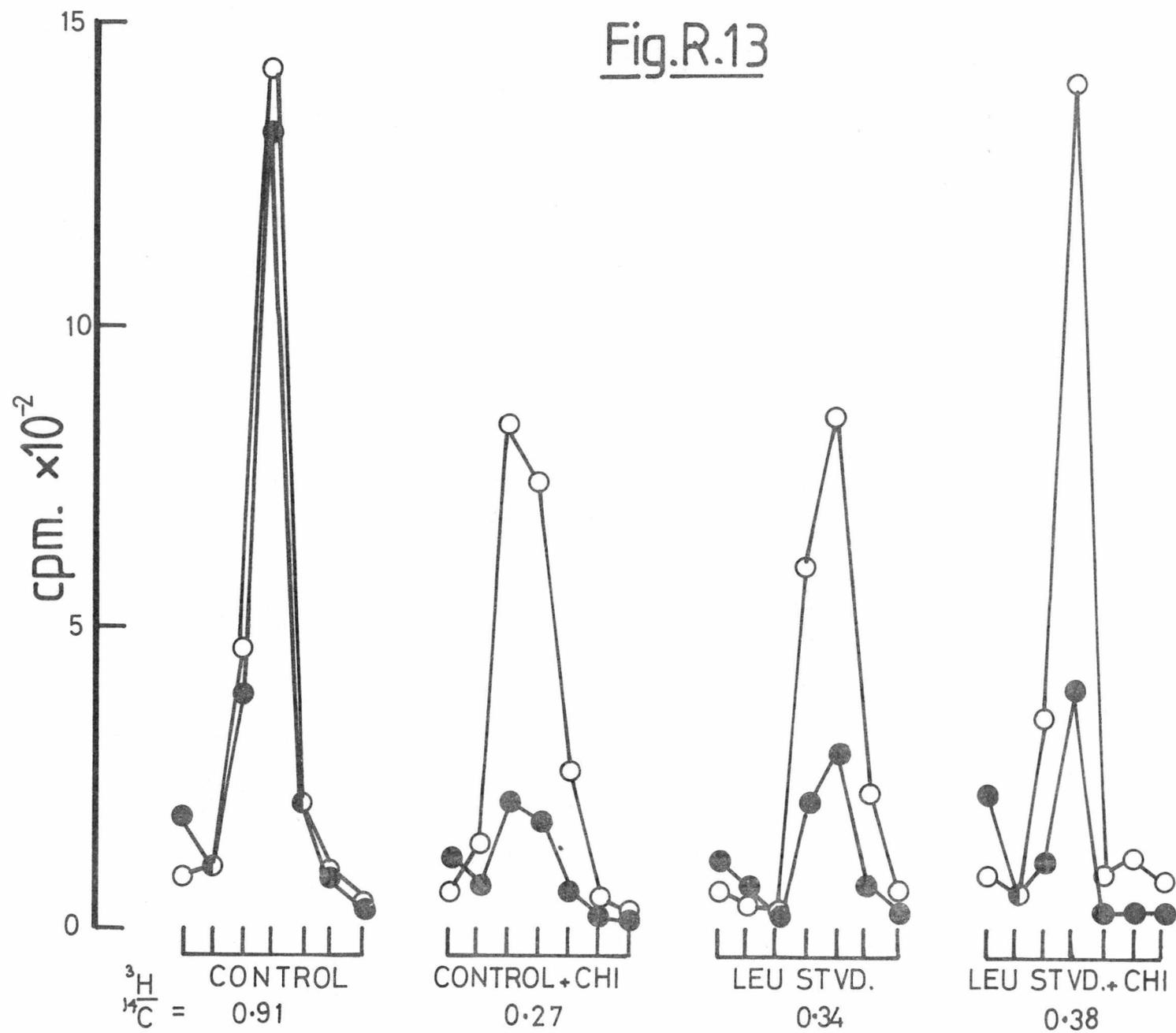


FIGURE R.14 - The effect of cycloheximide on P1 ds RNA synthesis during nitrogen-starvation.

S7A6 was grown for 5 generations in YNB-aa+ade containing ^{14}C -8-adenine ($0.1 \mu\text{Ci/ml}$). Cells from each of four aliquots (50 ml) of this culture were transferred into the following four media, which all contained ^3H -2-adenine ($1 \mu\text{Ci/ml}$): YNB-aa+ade; YNB-aa+ade+20 $\mu\text{g/ml}$ CHI; YNB-NH $_4^+$ +ade; YNB-NH $_4^+$ +ade+20 $\mu\text{g/ml}$ CHI. Incorporation was allowed to proceed for 2 hr before the cells were harvested and dsRNA extracted and run on polyacrylamide gels. The radioactivity profiles of the P1 dsRNA gel peaks are shown in the figure.

○—○, ^{14}C ; ●—●, ^3H

Fig.R.14

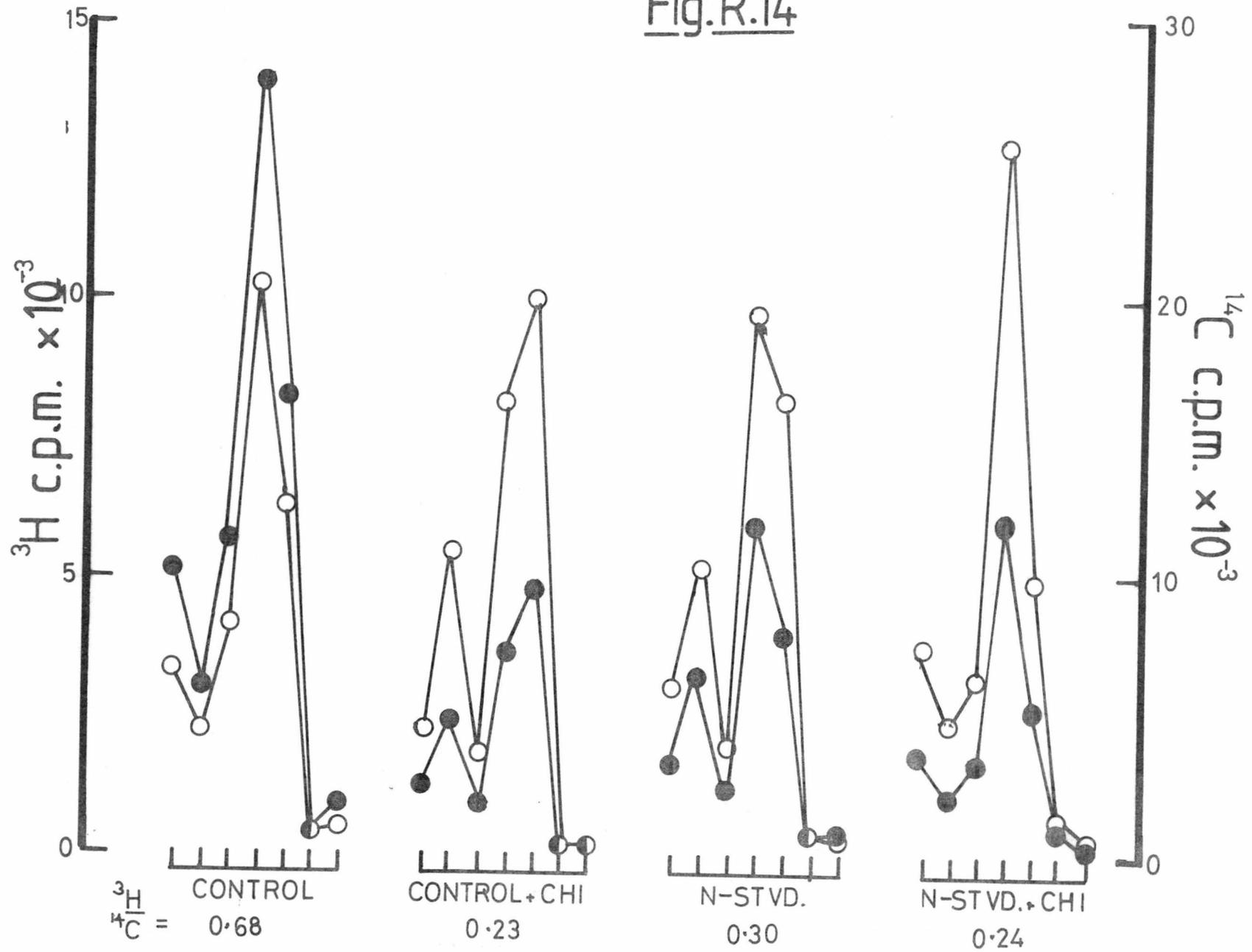


FIGURE R.15 - Effect of cycloheximide on the degradation of P1 dsRNA.

S7A6 was grown for 5 generations in YNB-aa+ade containing ^{14}C -8-adenine ($0.1 \mu\text{Ci/ml}$). This culture was divided in two and the cells transferred into YNB-NH_4^+ +ade and YNB-NH_4^+ +ade+CHI ($20 \mu\text{g/ml}$). At the times indicated, aliquots (50 ml) were removed from each of the two cultures. The cells were harvested and the amount of ^{14}C contained in P1 dsRNA determined. The figure gives the proportion of pre-labelled P1 dsRNA remaining with time.

o—o, N stvd.+CHI ; ●—●, N stvd.

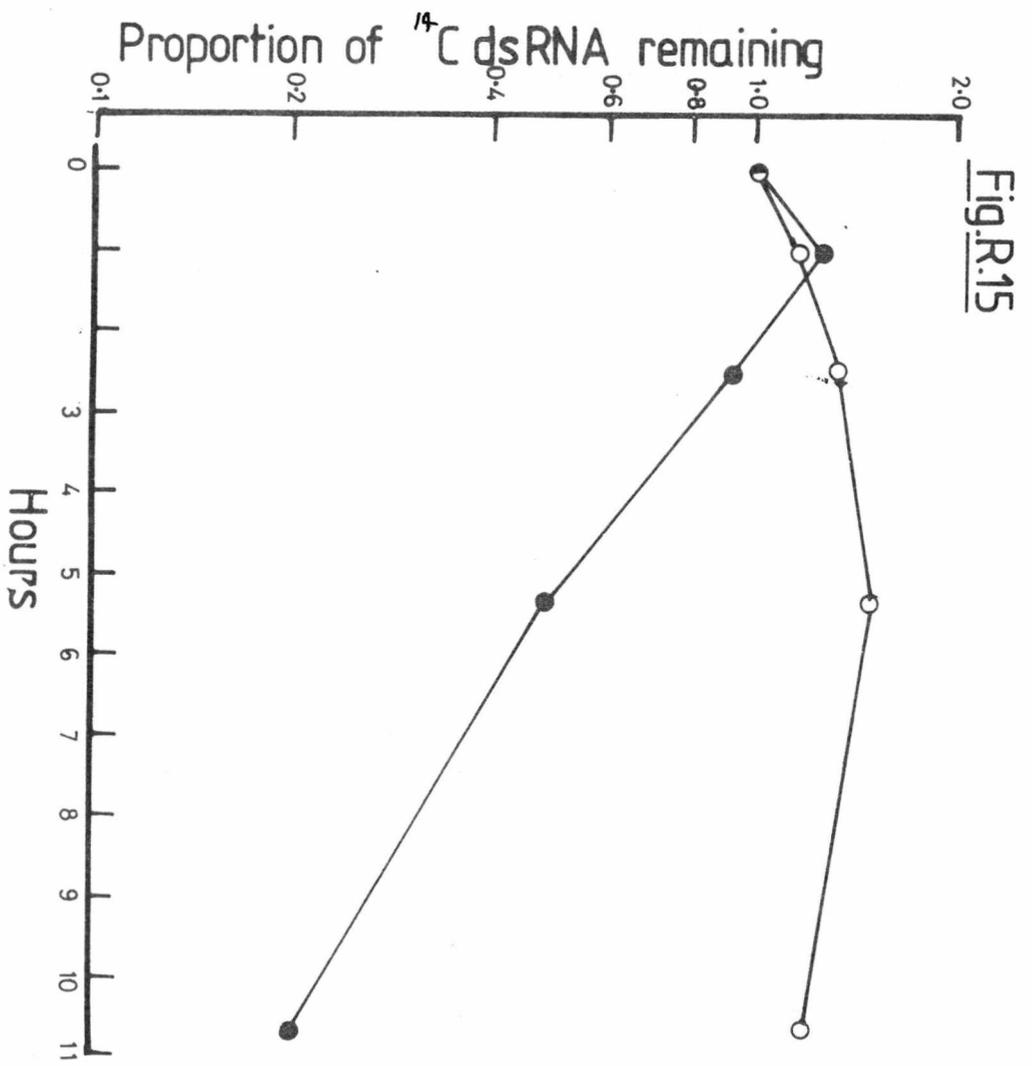


Fig. R.15

of ^{14}C labelled molecules is observed (see figures 2 and 6, where degradation occurs only after the 1.5 - 2.5 hr labelling period).

To verify this point further and also to determine the effect of cycloheximide on P1 dsRNA degradation the following experiment was performed. S7A6 was grown for about 5 generations in YNB-aa containing ^{14}C -8-adenine ($0.1\mu\text{Ci/ml}$). The culture was divided in two and the cells transferred into YNB-NH_4^+ and $\text{YNB-NH}_4^+ + \text{CHI}$ ($20\mu\text{g/ml}$). At the times indicated in figure R.15 aliquots (50mls) were taken and the amount of ^{14}C contained in P1 dsRNA determined by RNA extraction and gel electrophoresis. As can be seen in figure R.15 cycloheximide completely prevents the degradation of pre-existing dsRNA in nitrogen starved cells. Furthermore, as previously observed, in untreated nitrogen starved cells the level of ^{14}C starts to fall below the initial value only at the $2\frac{1}{2}$ time point.

R2 The Relationship Between RNA Synthesis and in vivo tRNA Charging Levels

In yeast, RNA synthesis is dependent on simultaneous protein synthesis (see I.5) and it has been proposed that uncharged tRNA has a role in the regulation of this phenomenon (Oliver and McLaughlin, 1977). In this section the effect of specific alterations in the rate of protein synthesis on the level of tRNA aminoacylation has been investigated.

The determination of in vivo tRNA charging levels was performed as shown in figure R.16. Transfer RNA was extracted from growing cultures by phenol extraction of whole cells (see M5.7 and R2.1) and the samples divided into two portions. One half was chemically deacylated by mild alkaline hydrolysis and used as a substrate for a preparation of aminoacyl-tRNA synthetases together with excess radio labelled amino acids. The level of trichloroacetic acid (TCA) insoluble radioactivity (i.e. counts esterified to tRNA) is equivalent to the total amino acid acceptor activity of the tRNA sample. The other half was treated with periodate. This reacts with uncharged tRNA oxidising the ribosyl hydroxyl groups on the terminal adenosine residues such that it can no longer esterify an amino acid (figure R.17). This sample was then treated in exactly the same way as the first half such that the level of radioactivity is now equivalent to the total acceptor activity minus the acceptor activity due to uncharged tRNA present in the original sample.

Preliminary experiments were performed to characterise and determine optimal conditions for each stage of the charging assay and the results of this investigation will be presented first.

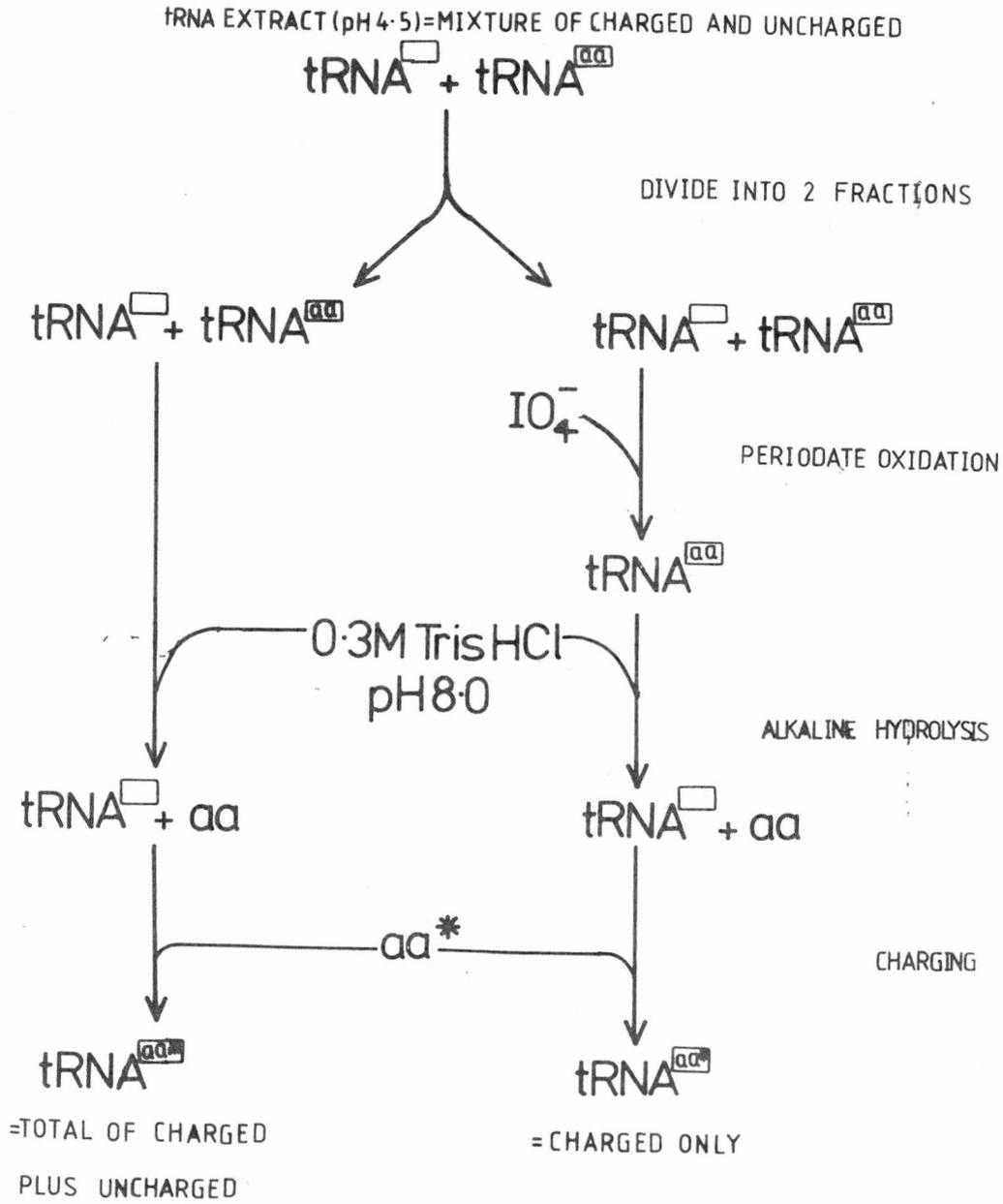
R2.1 Preparation of tRNA

Phenol extraction of whole cells has commonly been used as a convenient way of preparing yeast tRNA (Monier, Stephenson and Zamecnik, 1960). For the purpose of this investigation it was important to discover if this method releases all tRNA and is not preferential or selective against any of the tRNA species under examination. Furthermore the preparation should be uncontamination with other RNA species.



FIGURE R.16 - Protocol used for the determination of in vivo tRNA charging levels.

Fig.R.16



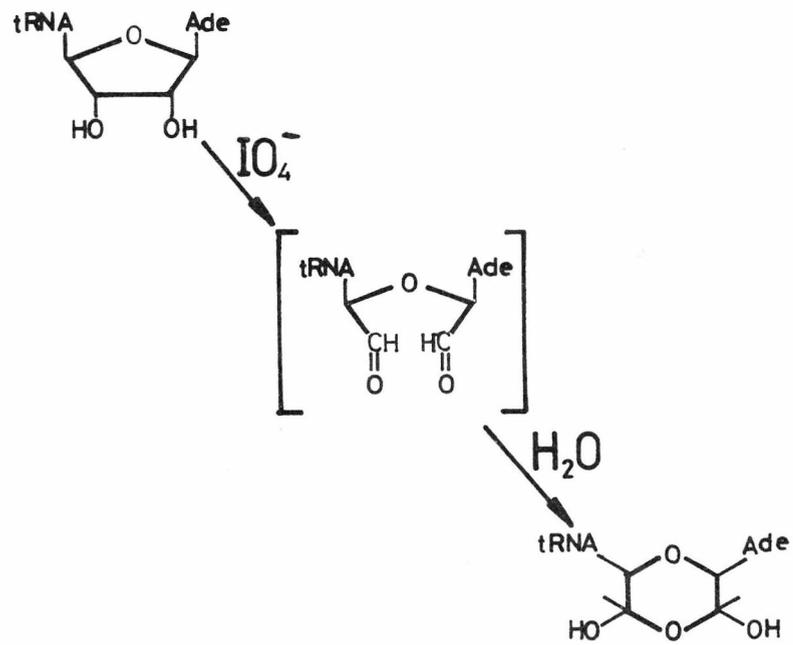


Fig.R.17 TERMINAL OXIDATION OF tRNA BY PERIODATE

Whole cells from a growing culture were harvested and washed before extraction with phenol (M5.7) giving sample A. These same cells were then disrupted with glass beads (M5.5a) and again phenol extracted as described in M5.6 to give sample B. The RNA samples from both extractions were compared by 12% and 2.6% polyacrylamide gel electrophoresis (M5.12). The 260nm absorbance scans of these gels are shown in figure R.18; as a reference, gels loaded with total yeast RNA prepared by phenol extraction of cells after breakage only were used. The small molecular weight RNA species in the total RNA sample are resolved as two peaks in 12% gels corresponding to 5S ribosomal RNA and tRNA. The 5.8S ribosomal RNA species is observed only after denaturation of the sample, eg with detergent. Samples obtained by phenol extraction before cell breakage show only one peak on 12% gels which correspond to the broad tRNA peak seen in 'normal' extractions. No tRNA peak could be detected in 12% gels of samples extracted after cell breakage. The 2.6% gel absorbance scan of sample B resembled very closely that of the total RNA sample. In sample A small amounts of dsRNA were found. This amounted to less than 2.5% of the total tRNA sample and this was considered to be an acceptably low level of contamination. However in order to confirm that this dsRNA had no effect on synthetase activity assays were performed using dsRNA purified by CF11 cellulose column chromatography (M5.10) as substrate at concentrations similar to that used with tRNA. No effect, either stimulatory or inhibitory was observed on synthetase activity (see R2.3).

R2.2 Purification of Aminoacyl-tRNA Synthetases.

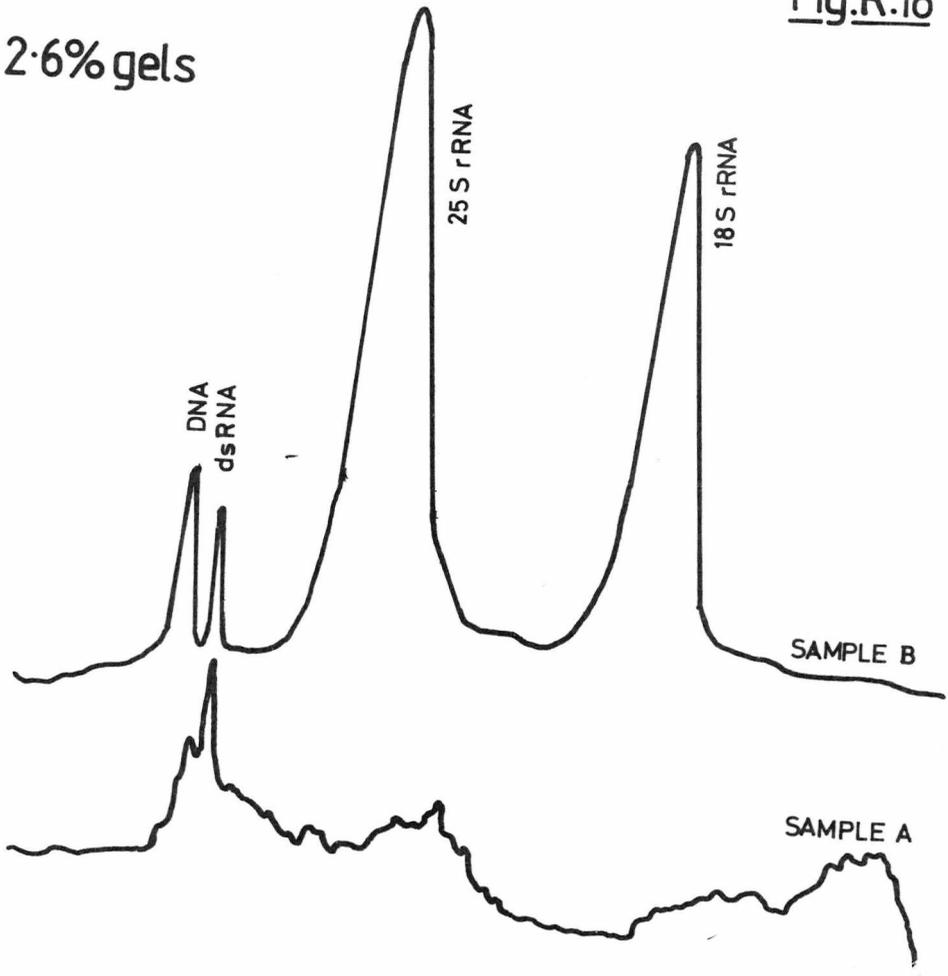
Active preparations of aminoacyl-tRNA synthetases are easily obtained from yeast. However difficulty is experienced in removing the high rate of endogenous activity, (i.e. activity occurring without the addition of exogenous tRNA), which is invariably present in these extracts. This endogenous activity is presumably due to co-purification of tRNA during synthetase extraction and a large number of methods for the removal of nucleic acids from solution were tried. Extraction was performed using anaerobically grown cells and also from petite mutant strains to avoid co-purification of mitochondrial tRNA. No

FIGURE R.18 - Electrophoretic analysis of different yeast RNA preparations.

Sample A was prepared by phenol extraction of unbroken cells (M5.7). Sample B was prepared by disruption of the same cells using glass beads (M5.5a) and phenol extraction as described in M5.6. These samples were electrophoresed in 2.6% and 12% polyacrylamide gels and the figure shows the absorbance scans at 260 nm. The 12% gels were scanned at the same sensitivity. The 2.6% gel containing sample A was scanned at 50 times the sensitivity of that of sample B.

Fig.R.18

2.6% gels



12% gels

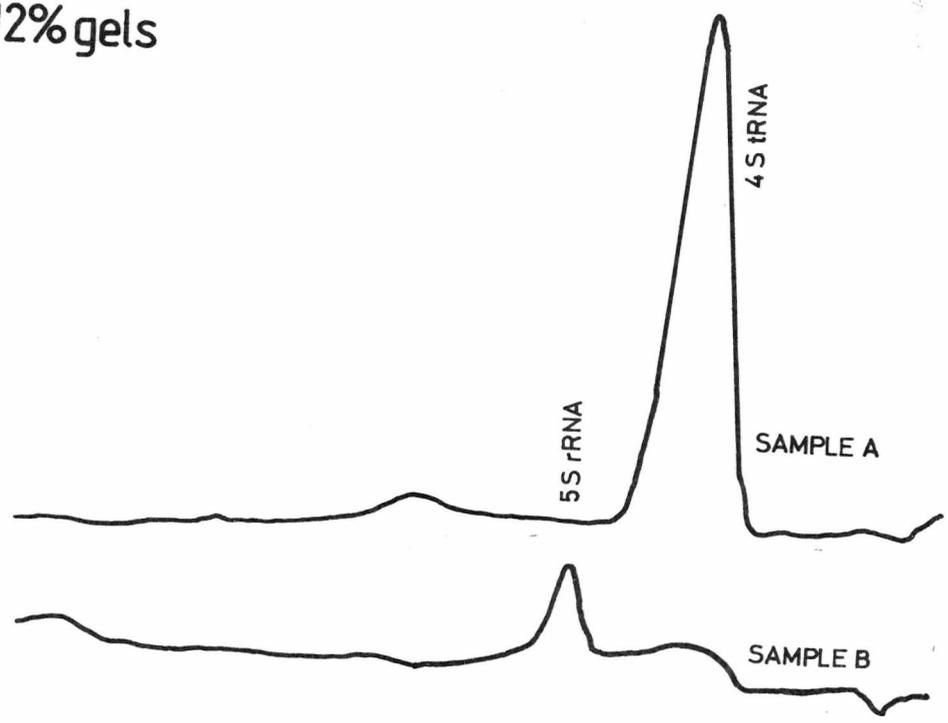


Table R.2 DETAILS OF THE PURIFICATION OF TYROSYL-tRNA SYNTHETASE

PURIFICATION STEP	PROTEIN		PROTEIN/RNA $A_{280}/260$	ACTIVITY		
	total (mg .)	conc. (mg /ml)		10^4 cpm/25 μ l	relative specific	stimulated by tRNA
BREAK CELLS (10^9 /ml)						
↓ 27,000g sup.						
↓ 229,000g sup.	396.0	4.4	0.62	2.07	0.47	1.64 X
↓ POLYMIN P						
↓ 40% $(NH_4)_2SO_4$ sup.						
↓ 70% $(NH_4)_2SO_4$ ppt. (no 229 000g spin)	47.2 (72.4)	2.1 (3.2)	0.87 (0.78)	1.51 (1.26)	0.72 (0.39)	3.13 X (1.60 X)
↓ DEAE Cellulose	28.8	1.6	1.16	1.25	0.78	6.60 X
↓ CM50 Sephadex	3.72	0.93	ND	1.98	2.11	15.8 X

difference in the exogenous tRNA stimulated activity was observed. Removal of tRNA by phenol extraction of whole cells (M5.7) prior to synthetase extraction was performed. This method proved impractical since it was not possible to remove all traces of phenol which severely inhibited synthetase activity. Digestion of endogenous tRNA in synthetase extracts by alkaline hydrolysis (M5.8) and with ribonuclease (M5.9) was attempted. Ribonuclease was then removed from the extract by ammonium sulphate precipitation (M5.1a). In both cases the overall loss of synthetase activity was too large for the method to be of use. Precipitation of tRNA using Streptomycin (M5.1f) and Polymin P (M5.1e) was also performed. Some improvement in the exogenous tRNA stimulated activity was noted in the case of Polymin P (Table R.2). Finally DEAE cellulose chromatography (M5.12b) was used and found to greatly improve the exogenous tRNA stimulated activity to levels acceptable for use in the charging assays.

Table R.2 gives purification data for one preparation of aminoacyl-tRNA synthetase. The procedure used was derived from Hartwell and McLaughlin (1968), Von der Haar (1979) and Faulhammer and Cramer (1977). Activity was assayed using ^3H -tyrosine by the method explained in section M5.15. The high speed centrifugation step greatly improved the effectiveness of the ammonium sulphate and Polymin P precipitation as judged by both specific activity and activity stimulated by the addition of exogenous tRNA. The greatest purifications were achieved by the DEAE cellulose and CM50 sephadex (M5.12) chromatography steps. Several strains of Saccharomyces cerevisiae were used for this purification procedure and no significant differences were found between them. From these observations two methods of purification were developed. A rapid purification procedure was used routinely to prepare synthetase for use in the tRNA charging assays and is outlined in the left hand side of figure R.19. Table R.3 shows the purification data for the extraction. After each centrifugation step a hard pellet and a softer, less compact pellet as well as the supernatant fraction were obtained. In both cases this soft pellet was found to be enriched for protein relative to the other fractions but with a low specific activity when assayed for synthetase. Thus in routine preparations

FIGURE R.19 - Procedures for the purification of aminoacyl-tRNA synthetases

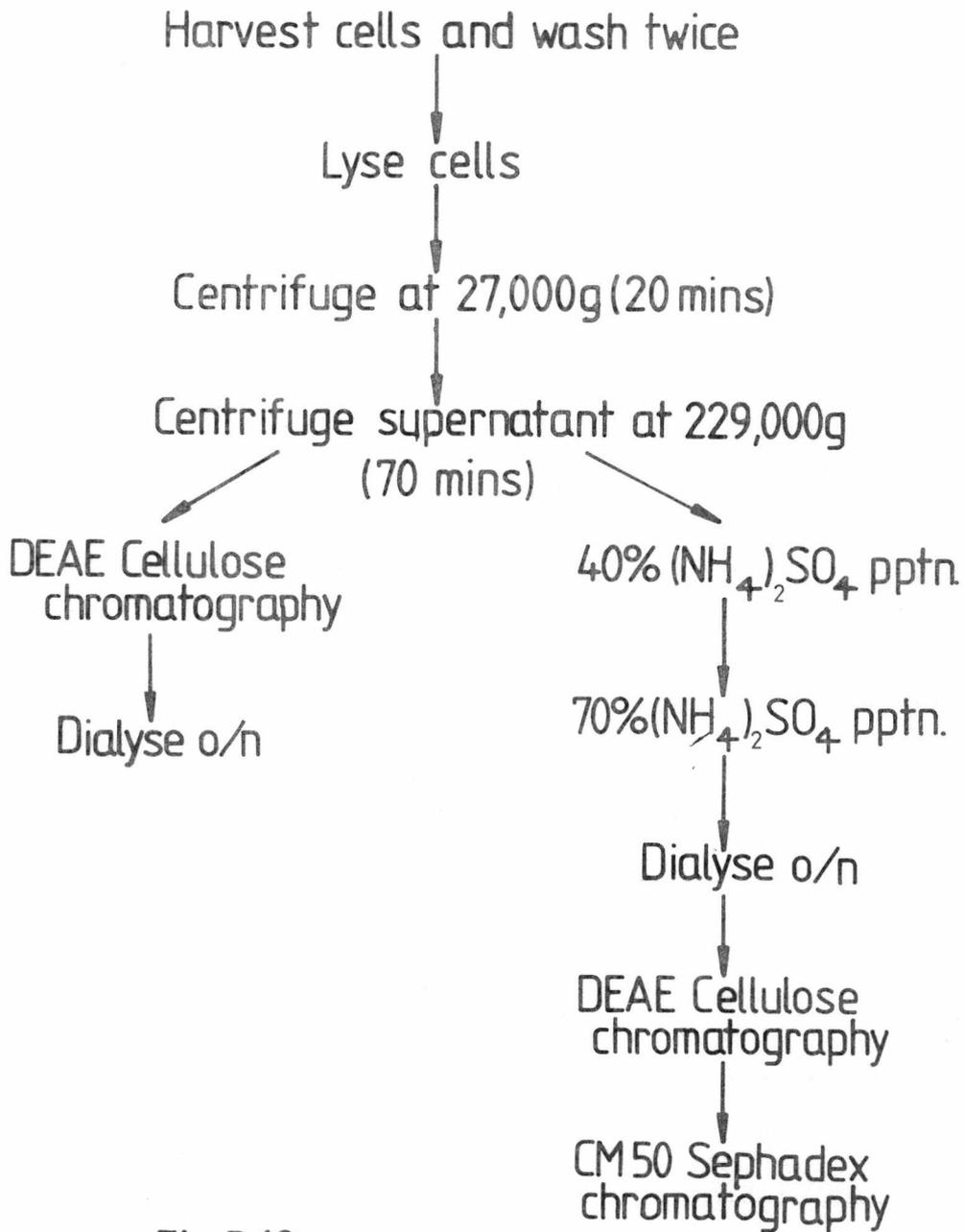


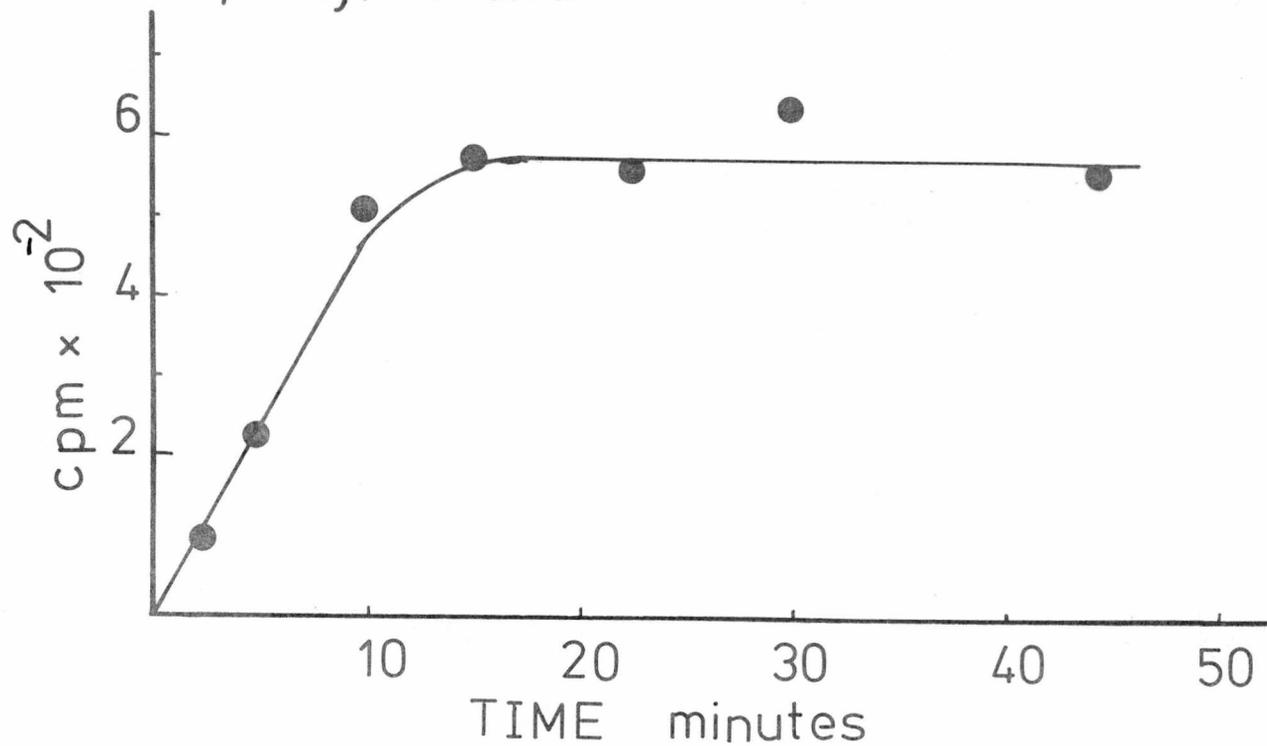
Fig.R.19

Table R.3 PURIFICATION DATA FOR THE PREPARATION OF TYR-tRNA SYNTHETASE

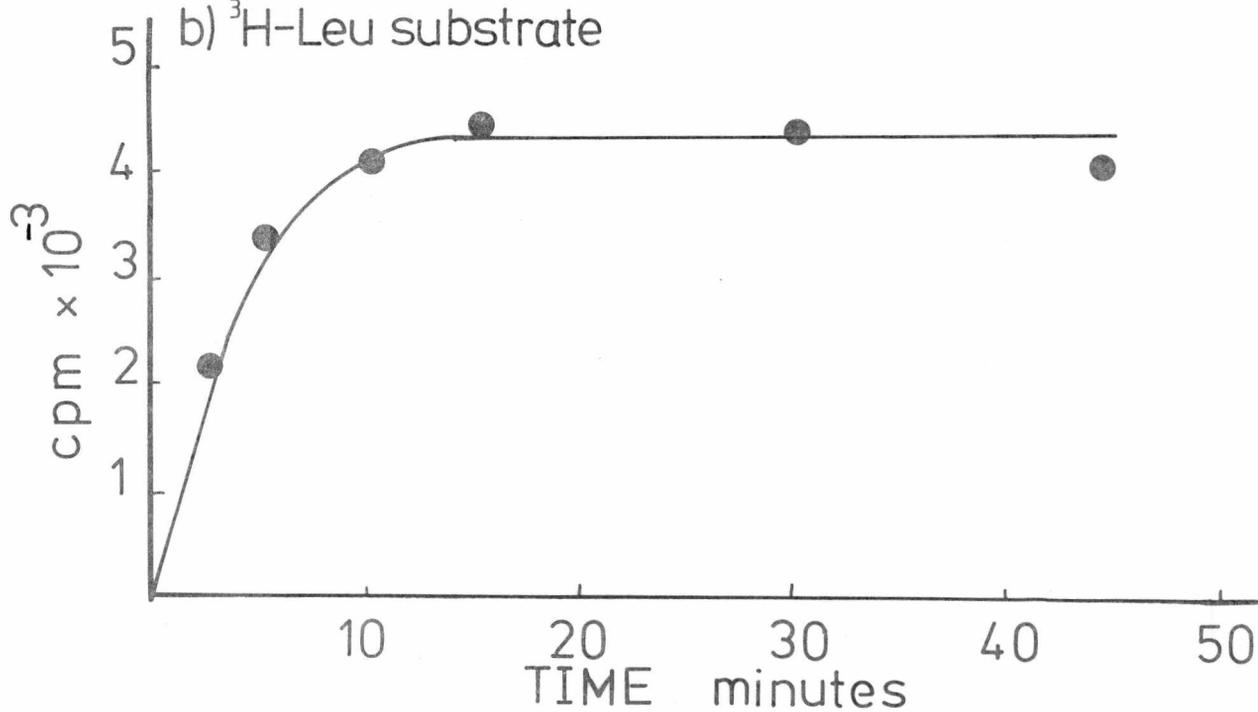
PURIFICATION STEP	PROTEIN		PROTEIN _{tRNA} $A_{280}/_{260}$	ACTIVITY		
	total (mgs)	conc (mg/ml)		10^3 cpm/ $_{25\mu l}$	relative specific	stimulated by tRNA
27,000g sup.	765	51	0.82	0.46	90.5	0.75 X
(27,000g soft pellet)	(130)	(65)	(0.88)	(0.37)	(56.9)	(1.19 X)
229,000g sup.	416	46	0.93	1.25	271.7	0.98 X
(229,000g pellet)	(34)	(34)	(0.66)	(0.34)	(109.8)	(0.87 X)
(229,000g soft pellet)	(45)	(90)	(0.78)	(0.58)	(64.4)	(1.30 X)
DEAE Cellulose + 50% glycerol, stored -80°C	320	20	1.18	0.95	475.0	14.70 X
DEAE Cellulose as above but no dialysis	14	14	1.05	0.27	192.8	0.92 X
DEAE Cellulose stored -80°C, no glycerol	42	42	1.18	0.82	195.2	1.91 X

Fig.R.20 Progress Curves for aa-tRNA synthetases

a) $^3\text{H-Tyr}$ substrate



b) $^3\text{H-Leu}$ substrate



these soft pellet fractions were discarded. In this rapid purification method the final dialysis step is important (see Table R.3) since at no other stage of the procedure are the synthetases separated from endogenous amino acids as they are by ammonium sulphate precipitation in the higher purification method. Stationary phase cultures of strain X2180.1A were normally used for the procedure and the cells were broken in buffer F at a concentration of about 10^{10} cells/ml. At this cell density breakage was achieved after vortexing for a total of 4-5 minutes in 30 second bursts with intervals of at least 1 minute on ice to allow for cooling. The concentration of protein in the final enzyme preparation was usually about 15-30 mgs/ml. The activity of final preparations was stimulated by the addition of exogenous tRNA about 20-30 fold in the case of tyrosyl-tRNA synthetase and about 50-60 fold with leucyl-tRNA synthetase (Table R.3). In tRNA charging level determinations control assays containing synthetase extract but without exogenous tRNA were always performed as a check for endogenous activity.

For better purification of synthetases the method shown on the right hand side of figure R.19 was used. In addition to the same steps involved in the rapid procedure this method included two ammonium sulphate precipitations and a final CM50 sephadex chromatography step.

Synthetase preparations were stored at -80°C in suitable aliquots such that the extract was frozen and thawed only once prior to use in charging assays. The addition of glycerol (50%) before freezing increased the specific activity, when subsequently thawed. (Table R.3).

R2.3 Characterisation of Aminoacyl-tRNA Synthetase Preparations

To measure the kinetics of the synthetase reaction a time course experiment was carried out by stopping the reaction in equal aliquots of the same assay mixture after various intervals and measuring the incorporation of radioactive amino acids into an acid-insoluble form. The progress curves obtained in this way are shown in figure R.20 for both the tyrosyl and leucyl-tRNA synthetases. It can be seen that for the concentration of tRNA and enzyme used routinely the kinetics are very similar for the two enzymes and in each case

	ACTIVITY (c.p.m.)	
	$^3\text{H-TYR}$	$^3\text{H-LEU}$
-RNA	4,495	1,071
tRNA _{phe.}	12,805	9,596
RNA _{phe.}	3,821	1,901
tRNA _{col.}	8,214	N.D.
rRNA _{col.}	3,064	2,393
dsRNA _{col.}	534	203
tRNA/dsRNA _{col.}	8,438	N.D.

TABLE R.4 Activity of tyrosyl and leucyl-tRNA synthetases using different RNA substrates.

The values shown are average values calculated from 3 replicate assays.

tRNA_{phe.} = tRNA prepared by phenol extraction of unlysed cells (M5.7)

RNA_{phe.} = RNA extracted from cells when disrupted after pretreated as above.

RNA_{col.} = RNA prepared by CF11 cellulose chromatography (M.5.8)

tRNA/dsRNA = tRNA sample containing 2.5% dsRNA (see text)

N.D. = Not determined

the reaction approaches completion after fifteen minutes. In subsequent charging assays the reaction was therefore allowed to proceed for 20 mins. The specificity of synthetase preparations obtained as described in R2.2 was determined. When rRNA or dsRNA purified by CF11 column chromatography (M5.10) were used as substrate no significant activity above that found without RNA was observed with either tyrosyl or leucyl-tRNA synthetases (Table R.4). These results show that the enzymic activity of the preparation is specific for tRNA.

After removal of tRNA by phenol extraction of whole cells, RNA was prepared from the same cells after disruption with beads by phenol extraction (M5.6). When this sample was used as substrate no significant activity above endogenous was observed using ^3H -tyrosine or ^3H -leucine (Table R.4). Since the activity of the preparation has been demonstrated to be tRNA specific this result implies that no leucyl or tyrosyl-tRNA was present in the sample. Thus phenol extraction of whole cells is not discriminatory against leucyl or tyrosyl-tRNA (see R2.1).

A slightly higher level of activity was stimulated by tRNA prepared by phenol extraction of whole cells than by the same concentration of tRNA purified by CF11 column chromatography (Table R.4). Furthermore when dsRNA obtained from the CF11 column was added to 'column prepared' tRNA at 2.5% (the level of contamination observed in whole cell phenol extracts) no change in acceptor activity was observed (Table R.4). Together these observations show that dsRNA has no stimulatory or inhibitory effect on synthetase activity and that some amino acid acceptor activity is lost during purification of tRNA by column chromatography.

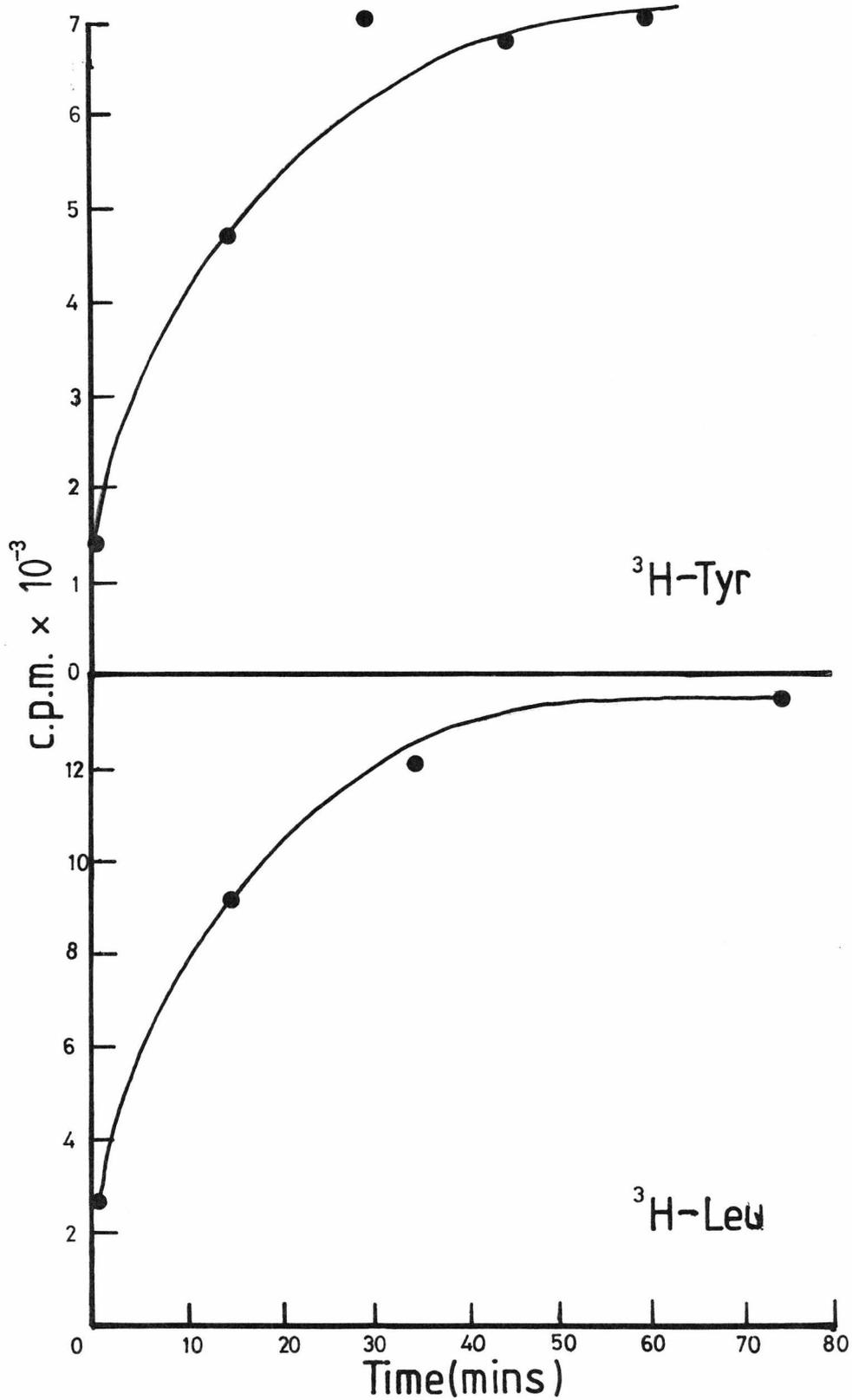
R2.4 Characterisation of the tRNA Charging Assay

1) Chemical Deacylation of tRNA

Chemical deacylation of tRNA was performed by mild alkaline hydrolysis in buffer H (0.3M TrisHCl pH8.0) as described by Yang and Novelli (1971).

FIGURE R.21 - Time course for alkaline hydrolysis of aminoacyl-tRNA

Fig.R.21



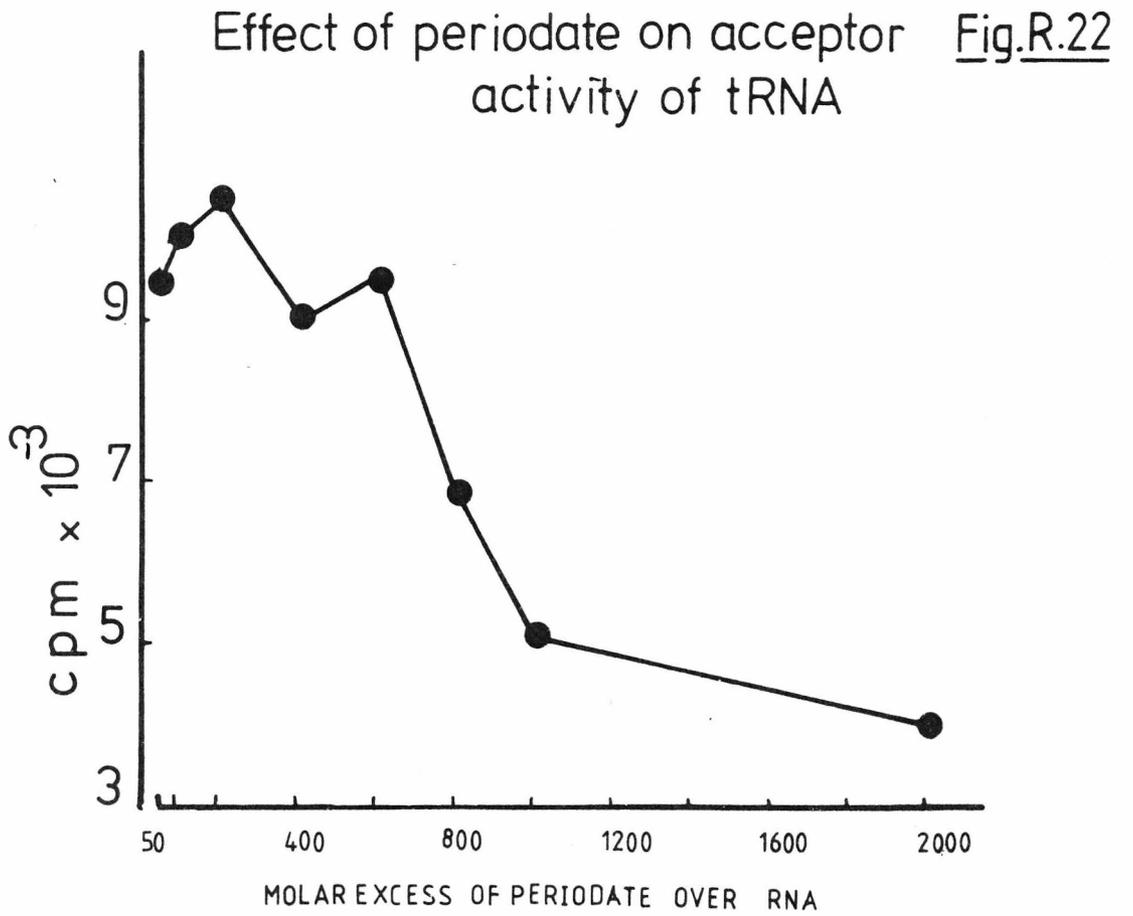
A time course for the reaction was carried out by taking aliquots at various intervals after resuspending ethanol precipitated tRNA (M5.6) in Buffer H and assaying with synthetase preparation. This experiment was performed for both tyrosyl and leucyl-tRNA and the results are shown in figure R.21. In each case the reaction had reached completion after 45 mins. Furthermore no loss in acceptor activity was observed up to 75 mins after addition of Buffer H. Alkaline hydrolysis was therefore allowed to proceed for 1 hr in subsequent charging assays.

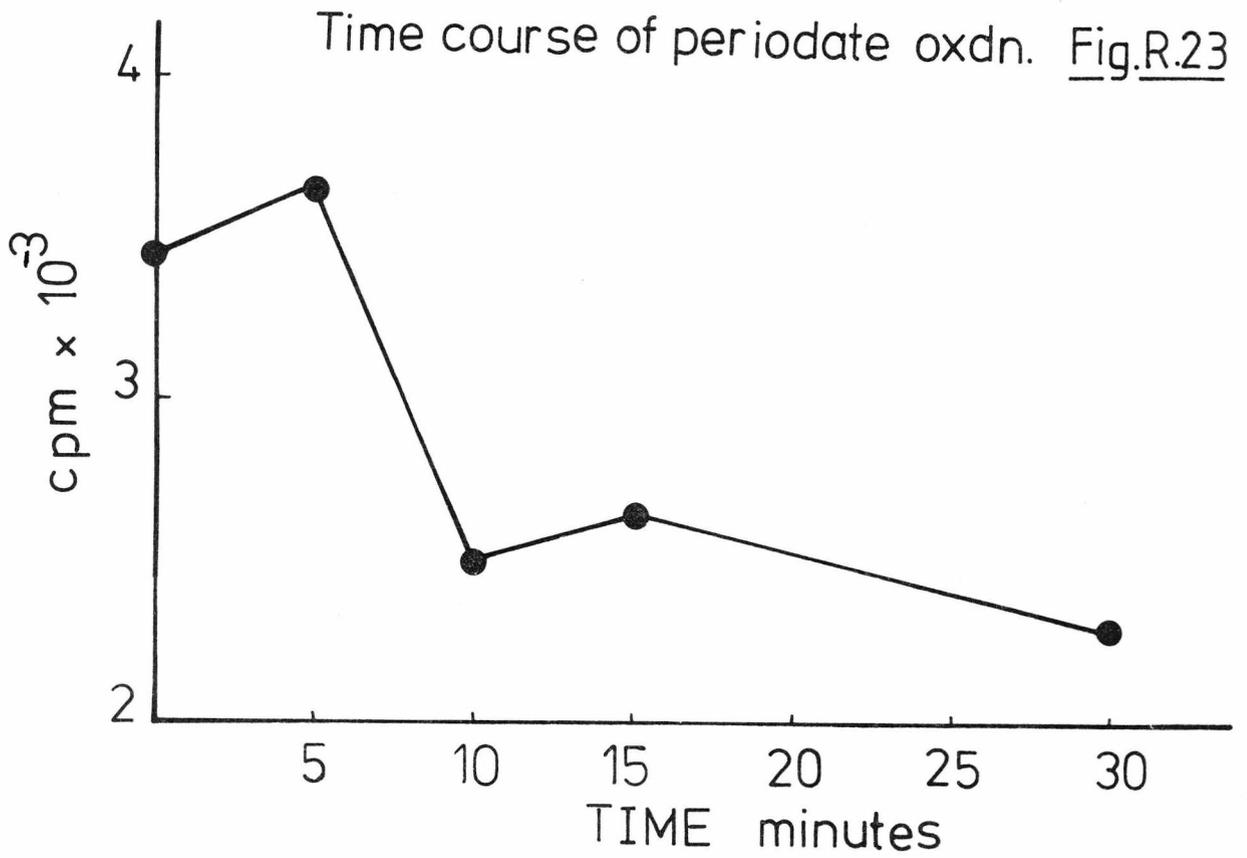
2) Periodate Oxidation of Uncharged tRNA

At low concentrations periodate preferentially oxidises terminal nucleoside residues of RNA molecules (see fig. R.17). However at higher concentrations, or at lower concentrations over extended periods of time, oxidative attack on ribosyl hydroxyl groups occurs at random points along the polynucleotide chain causing cleavage of the molecule (Schmidt, 1968).

In the case of transfer RNA this will result in a concomitant and progressive loss of amino acid acceptor activity. This loss of activity due to degradation will continue to completion whereas that due to terminal oxidation will cease when the supply of uncharged tRNA is exhausted. Thus using tRNA from growing cells (i.e. highly charged, see R2.5) the two processes can be distinguished by the extent to which amino acid acceptor activity is lost. Using this criterion the level of periodate required to fully deactivate uncharged yeast tRNA without causing degradation of the sample was determined.

The experiment was carried out by adding different concentrations of periodate to equivalent amounts of tRNA and incubating at 25°C in the dark. Periodate was normally prepared by dissolving in buffer B immediately prior to use. After 15 minutes oxidation was stopped by the addition of excess ethylene glycol (0.1 mls) and incubating for a further 10 minutes (Andrulis and Arfin, 1979). After ethanol precipitation and alkaline hydrolysis the tRNA samples were assayed with synthetase preparation using ^3H -tyrosine. Figure R.22 shows that concentrations between 200 to 600-fold molar excess of periodate over RNA gave acceptor activity of about 80% of that found in





untreated tRNA. Above this concentration range the acceptor activity declines sharply to less than 30% of the control value indicating that extensive degradation was occurring. Therefore in subsequent charging level assays a 400-fold molar excess of periodate over RNA was used.

A time course for the oxidation with this concentration of periodate was performed using tRNA extracted from amino acid starved cells (i.e. highly uncharged, see R2.5). Aliquots were taken from the reaction mixture at intervals, added to ethylene glycol (0.1 mls) and incubated for 10 mins and treated as before. The results show that the oxidation is complete after 5 mins from periodate addition (fig. R.23). Moreover, no further loss of acceptor activity can be seen up to 30 mins after the start of oxidation showing that at 400-fold molar excess no degradation of tRNA is occurring. Routinely, fifteen minutes were allowed for periodate treatment of RNA in charging level assays.

R2.5 Amino Acid Starvation and the Level of tRNA Charging

The effect of starvation for amino acid on the level of tRNA charging was determined using strain A364A (M.1). Cells were grown overnight to early exponential phase (2×10^6 cells/ml) in YNB-aa medium supplemented with histidine, tyrosine, lysine, adenine and uracil (complete YNB). Aliquots (200 ml) from this culture were transferred by filtration to the same volume of the following three media: complete YNB; complete YNB minus tyrosine; complete YNB minus lysine. After 2 hrs incubation in these new media the cells were harvested, tRNA extracted and the relative amounts of uncharged tRNA determined as explained in sections R2.1 - R2.4. In these experiments, and in all subsequent charging studies the acceptor activity of each sample was determined by performing 5 independent assays and calculating the mean value. All values were corrected for the endogenous activity of the synthetase preparation which was monitored in each experiment by performing incubations without exogenously added tRNA. The results of two such experiments are given in table R.5.

Experiment 1	% UNCHARGED tRNA	
	tyrosyl	leucyl
CONTROL	23.8	40.5
TYROSINE STARVED	90.1	17.3
LYSINE STARVED	5.0	7.3
Experiment 2		
CONTROL	8.1	43.2
TYROSINE STARVED	85.1	10.9
LYSINE STARVED	5.0	28.1

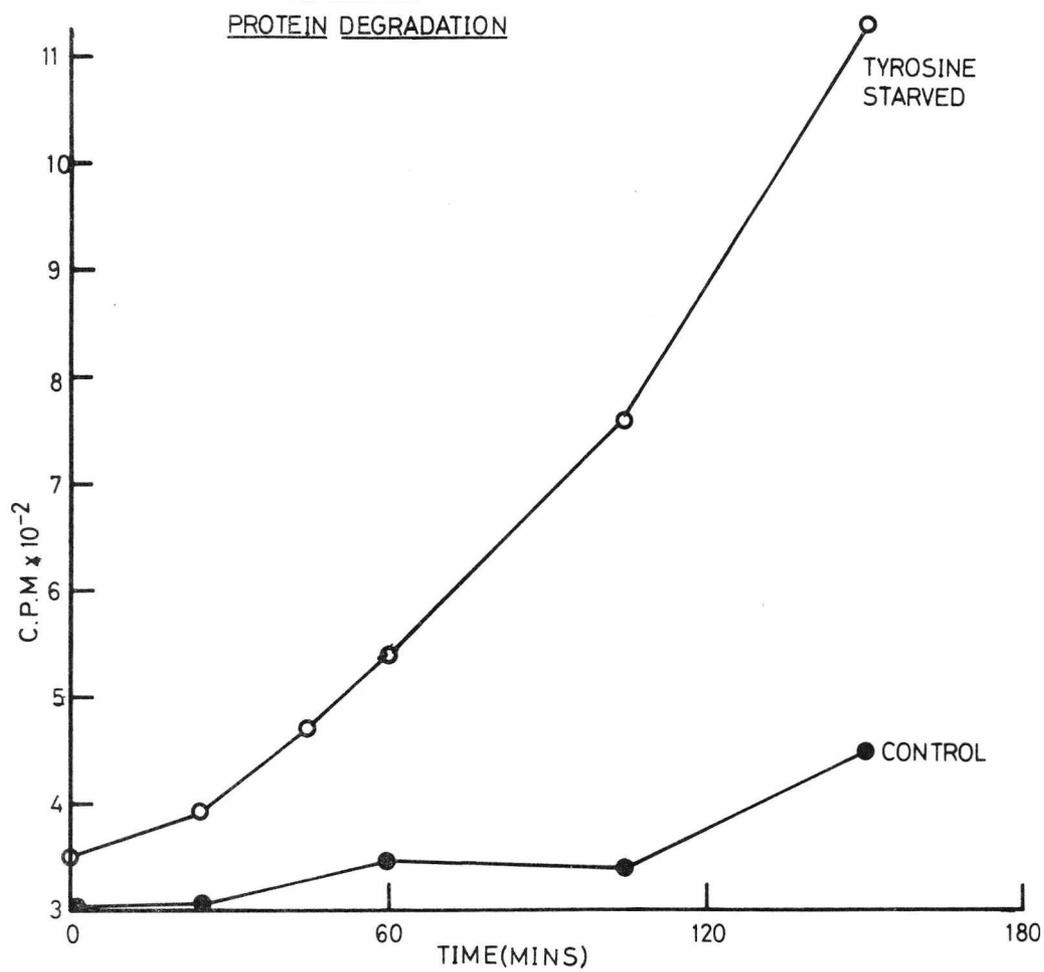
TABLE R.5 - The effect of amino acid starvation on tRNA charging

FIGURE R.24 - Proteolysis in amino acid-starved and normally growing cells.

A culture of A364A was grown overnight to mid-exponential phase in fully supplemented YNB-aa containing ^3H -(4,5)-Leucine (2 $\mu\text{Ci/ml}$) in order to uniformly label cell proteins. The cell culture was split and the cells from each half resuspended in fresh medium containing a high concentration (200 $\mu\text{g/ml}$) of nonlabelled leucine but either with or without tyrosine. At the times shown aliquots (1 ml) were taken into ice-cold 50% w/v TCA (0.1 ml) and left to precipitate for 1 hr. The samples were filtered through nitrocellulose filters and the filtrates added to 4 mls of PCS scintillation fluid. The level of radioactivity in each sample was then determined by liquid scintillation counting (see M.14c).

Fig.R.24

PROTEIN DEGRADATION



Starvation for tyrosine leads to the discharge of the tyrosyl-tRNA species. This effect is confined to the cognate species: during tyrosine starvation no discharge of leucyl-tRNA was observed and, similarly, lysine deprivation did not cause the deacylation of either tyrosyl-tRNA or leucyl-tRNA.

The removal of a required amino acid from the culture medium of an auxotrophic strain means that this amino acid can now only be supplied to the organism by the turnover of intracellular proteins. The level of protein turnover in tyrosine starved A364A cells was therefore examined using the procedure described in M5.14c. Figure R.24 shows that proteolysis is stimulated in amino acid starved yeast cells. Amino acids released in this way should be available to the synthetase reaction but the aminoacyl-tRNA molecules formed would presumably be discharged by residual protein synthesis (see figure I.) The next series of experiments examine whether it is possible to promote the 'trickle-charging' of the tRNA pool by blocking protein synthesis.

R2.6 The Effect of Translation Inhibitors on tRNA Charging in Starved Cells

These experiments were performed in a similar manner to that already described in R2.5. Strain A364A was grown overnight to early exponential phase and then a 200 ml portion of the culture was transferred into each of these four media: complete YNB; complete YNB plus a translation inhibitor; complete YNB minus tyrosine; complete YNB minus tyrosine plus a translation inhibitor. After 2 hrs the cells were harvested and the in vivo charging levels determined. The results of four such experiments, each using a different inhibitor, are shown in Table R.6.

For each of the four drugs used the level of charged tyrosyl-tRNA in tyrosine starved cells was equal to or approached that found in control (non-starved) organisms. Thus, in each case, the presence of the protein synthesis inhibitor counteracted the effect of starvation on tRNA charging.

In the experiments described above the inhibitor was added at the time of the transfer to starvation medium. Additional experiments were carried out in which cycloheximide was added after prestarvation for increasing

	%UNCHARGED TYROSYL-tRNA	
	WITHOUT INHIBITOR	WITH INHIBITOR
a) Cycloheximide		
Control	19.6	47.9
Tyrosine starved	53.8	19.2
b) Anisomycin		
Control	9.4	43.0
Tyrosine starved	53.8	1.9
c) MDMP		
Control	12.7	48.6
Tyrosine starved	58.8	14.6
d) Edeine		
Control	12.4	ND
Tyrosine starved	61.0	31.7

TABLE R.6 The effect of translation inhibitors on tRNA charging.

	% UNCHARGED TYROSYL-tRNA
CONTROL	3.4
TYR STARVED	66.0
TYR STARVED + CHI, 0h	15.9
TYR STARVED + CHI, 2h	38.1
TYR STARVED + CHI, 4h	33.0
TYR STARVED + CHI, 6h	13.3

TABLE R.7 - Trickle-charging caused by cycloheximide in cells prestarved for tyrosine.

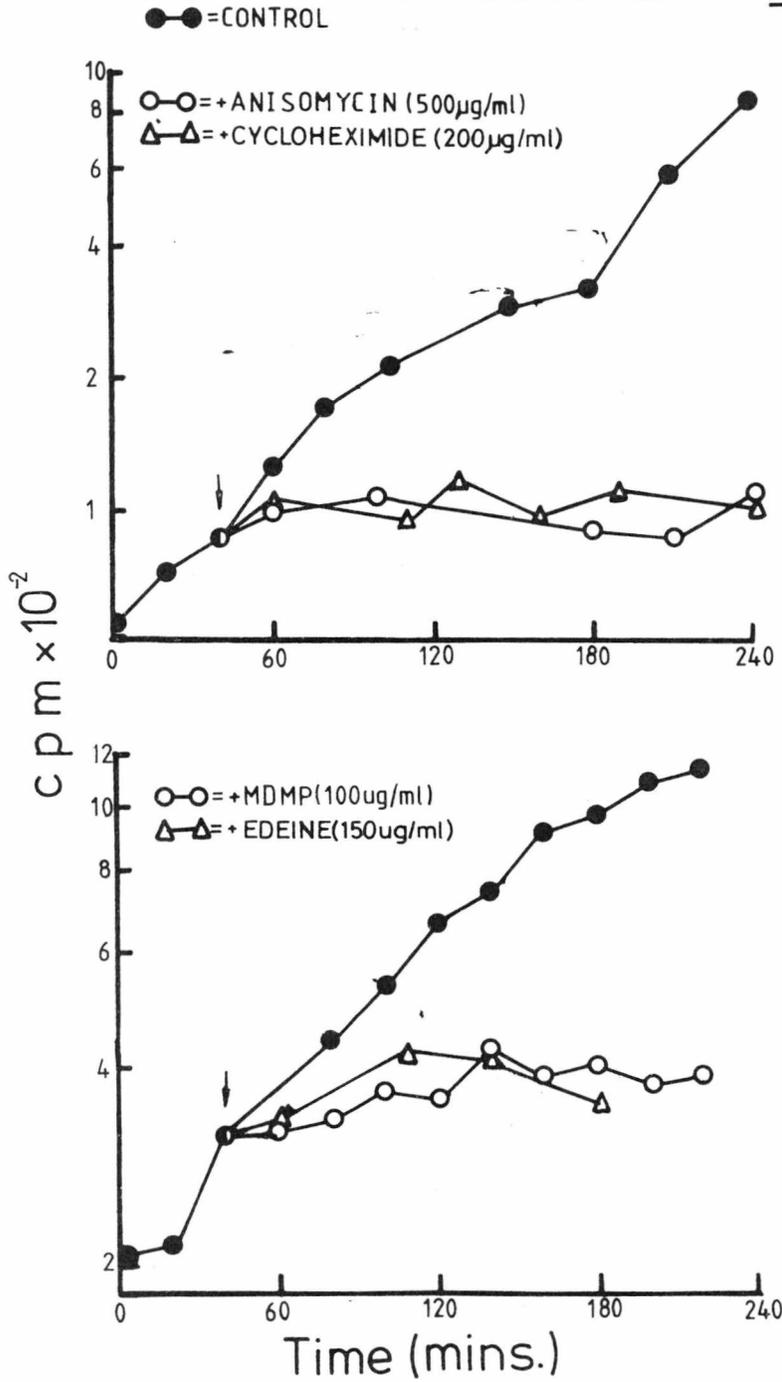
The drug was added following starvation for the indicated periods of time at a concentration of 200 ug/ml.

FIGURE R.25 - The effect of translation inhibitors on protein synthesis in A364A.

A364A was grown in fully supplemented YNB-aa to a cell density of 2×10^6 cells/ml and ^3H -(3,5)tyrosine (4 uCi/ml) was added. The culture was incubated for 1 hr. before aliquots (0.2 mls) were withdrawn into ice-cold 50% w/v TCA (20 ul) at the indicated times. After 40 minutes (indicated by the arrow) the culture was divided into three and translation inhibitors added, at the concentrations shown, to two of these portions. An equal volume of sterile distilled water was added to the third. Sampling of all three cultures was continued and the level of TCA-insoluble radioactivity in each sample was determined as described in M5.3.

EFFECT OF TRANSLATION INHIBITORS ON INCORPN.
OF 3H-TYR INTO T.CA INSOLUBLE MATERIAL

Fig.R.25



intervals of time. In similar experiments the extent to which the stringent response is apparently reversed by translation inhibitors has been found to be reduced by prestarvation (Oliver and McLaughlin, 1977). However as shown in Table R.7 prestarvation for tyrosine for up to 6 hrs before the addition of cycloheximide did not affect the final extent of trickle-charging of tyrosyl-tRNA that was observed.

Figure R.25 shows that the concentration of inhibitors used in these experiments were sufficient to arrest protein synthesis completely.

R2.7 Transfer RNA Charging in a Mutant Deficient in Proteinase Production

In section R2.6 it was found that the effect of amino acid starvation on tRNA charging could be counteracted by the addition of translation inhibitors. It was proposed that this is achieved by trickle-charging of tRNA using amino acids from protein turnover in an analogous manner to that which occurs in bacteria (see Introduction). In order to examine this explanation more fully, tRNA charging in a mutant deficient in proteinase production was studied.

In yeast seven intracellular proteinases have been identified but the specific functions of these enzymes have not yet been determined. Mutants have been isolated which are defective in the structural genes coding for the three major yeast proteinases: A, B and C (now known as carboxypeptidase Y) (Jones, 1977; Zubenko, Mitchell and Jones, 1979). These workers have also isolated a mutant pep 4,3, which is deficient in these proteinase activities presumably due to the lack of a processing event which is common to all three enzymes (Hemmings et, al., 1981). In order to examine the relationship between the aminoacylation of tRNA and protein turnover, tRNA charging in a pep 4,3 mutant was therefore studied. Before these experiments could be performed it was first necessary to obtain the pep 4,3 mutation in a strain which is also auxotrophic for tyrosine. This was achieved by crossing strain 20B-12 (pep4,3) with A364A (tyr 1) and sporulating the resultant diploids. Segregants were then screened for tyrosine auxotrophy by random spore analysis (M5.16). The tyrosine-requiring colonies obtained in this manner were then screened for

	A	CORRECTED FOR BLANK	Protein conc. (mg/ml)	ACTIVITY relative specific	percentage wild type
<u>PROTEASE A (550nm)</u>					
X2180 1A +Extract	0.537	0.432	26.3	16.4	100
- Extract	0.105				
20B-12 +Extract	0.179	0.059	21.7	2.7	16.5
-Extract	0.120				
A3pep7 +Extract	0.126	0.039	24.5	1.6	9.7
-Extract	0.087				
<u>PROTEASE B (520nm)</u>					
X2180 1A +Extract	0.291	0.217	63.0	3.2	100
-Extract	0.074				
20B-12 +Extract	0.062	0.033	48.0	0.69	21
-Extract	0.029				
A3pep7 +Extract	0.064	0.017	54.0	0.31	10
-Extract	0.047				
<u>PROTEASE C (410nm)</u>					
X2180 1A +Extract	0.325	0.304	63.0	4.8	100
-Extract	0.016				
20B-12 +Extract	0.033	0.028	48.0	0.58	12
-Extract	0.005				
A3pep7 +Extract	0.073	0.039	54.0	0.72	15
-Extract	0.034				

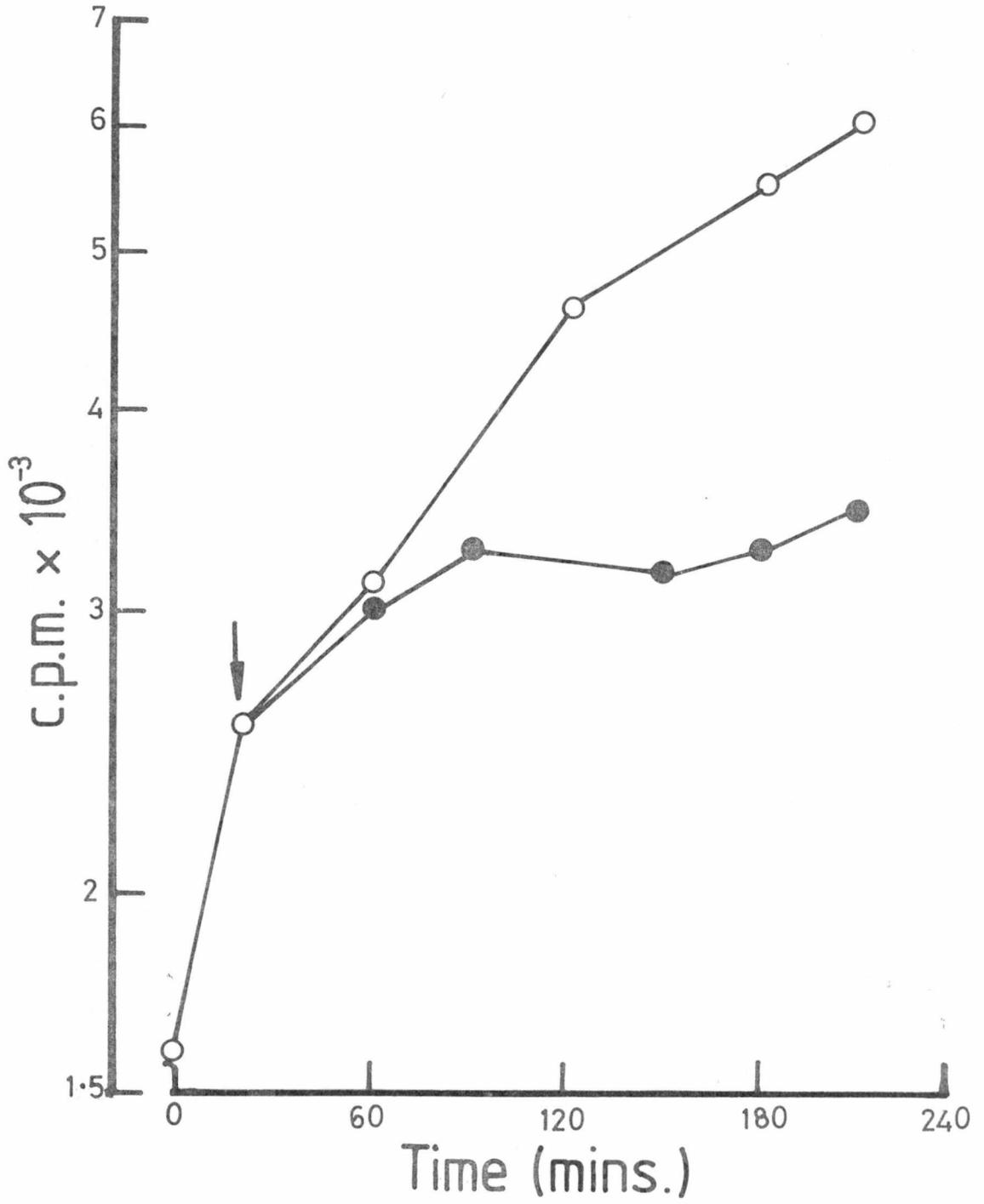
Table R.8 PROTEASE ACTIVITIES IN MUTANT AND WILD TYPE CELLS
 The **A** values shown are average values for three replicate assays.

FIGURE R.26 - The effect of tyrosine starvation on protein synthesis in

A3 pep.7.

A3 pep.7 was grown to 2×10^6 cells/ml in appropriately supplemented YNB-aa and ^3H -(4,5)-leucine ($4 \mu\text{Ci/ml}$) was added. After prelabelling the cells for 30 minutes ($t=0$) aliquots (0.2 ml) were taken into ice-cold 50% w/v TCA ($20 \mu\text{l}$) at the indicated times. One hour after the addition of radiolabel (indicated by the arrow) the culture was divided into two portions and the cells were harvested by filtration. The filters were transferred to fresh ^3H -(4,5)-leucine containing medium with or without added tyrosine. Sampling both cultures was continued and the level of TCA insoluble radioactivity in each sample was determined (see M5.3).

○—○, control; ●—●, tyr stvd.

Fig.R.26

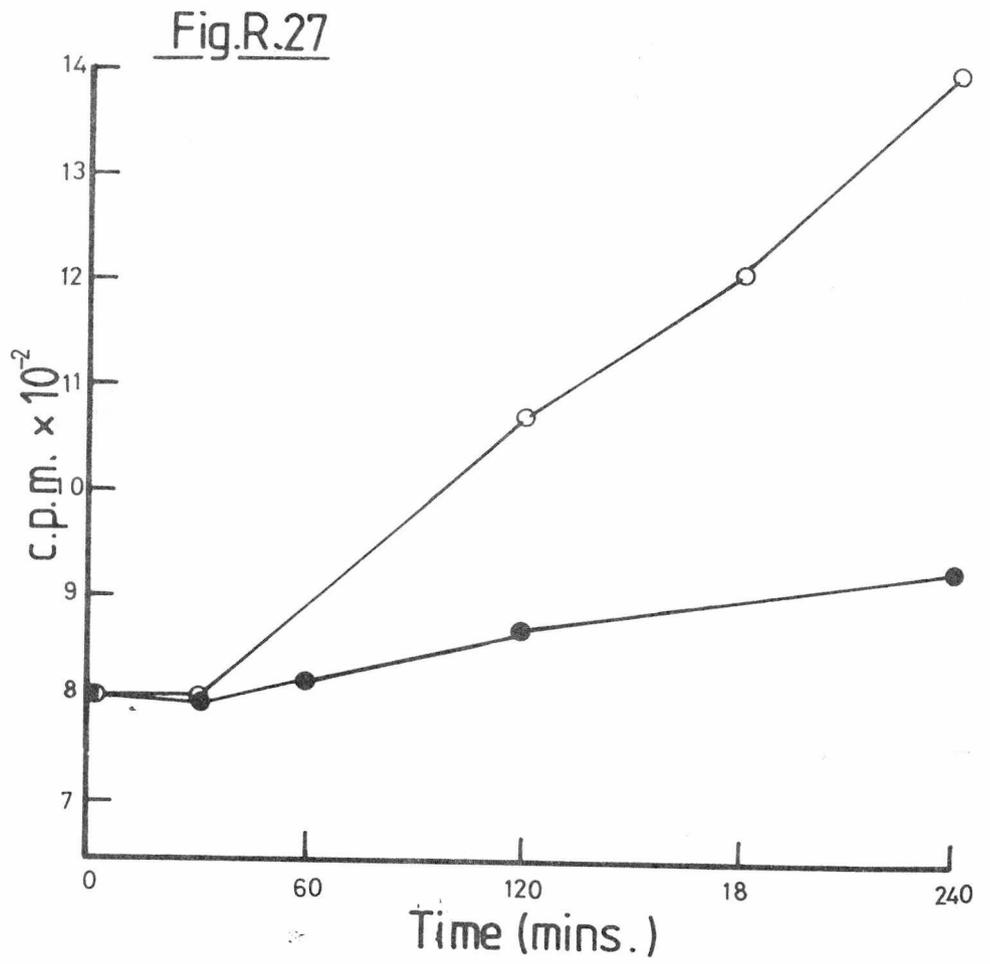
A3.pep7	% UNCHARGED TYROSYL-tRNA
CONTROL	23.8
CONTROL + CHI	49.9
TYR. STARVED	74.3
TYR. STVD + CHI	28.7

TABLE R.9 - tRNA charging in a protease deficient mutant

FIGURE R.27 - Proteolysis in A3 pep.7 during tyrosine starvation

A364A and A3 pep.7 were grown overnight to mid-exponential phase in appropriately supplemented YNB-aa containing ^3H -(4,5)-leucine (2 $\mu\text{Ci/ml}$). The cells were harvested by filtration and resuspended in fresh medium containing (200 $\mu\text{g/ml}$) non-labelled leucine but without tyrosine. Samples (1 ml) were taken from these cultures at the times shown in the figure and the level of TCA soluble radioactivity was determined by the method explained in the legend to figure R.24 and in M.14c.

○—○, A 364 A; ●—●, A3 pep.7



the production of proteinases A, B and C as described in M5.14. The results of these assays using one such tyrosine requiring segregant, A3pep.7 (M 1), are shown in Table R.8. It can be seen that in this strain the levels of proteinases A, B and C are reduced to similar values to those found in the original pep 4,3 mutant strain. The effect of tyrosine starvation on protein synthesis in strain A3pep.7 is displayed in figure R.26.

Strain A3pep.7 was then used in tRNA charging experiments which were carried out exactly as described in R2.6. Thus, the level of uncharged tRNA in starved and non-starved cells was determined both with and without the addition of cycloheximide. The results, which are presented in Table R.9, appear to indicate that the responses of tRNA charging to these treatments are the same in cells deficient in proteinases A, B and C as in normal cells.

Since the actual intracellular functional roles of these enzymes are not clear the effect of reduced levels on protein turnover during amino acid starvation was measured in A3pep.7. The experiment was carried out as described in M5.14 and the results are given in figure R.27. The level of proteinolysis in the pep 4,3 mutant during tyrosine starvation is reduced compared to that observed in A364A. However it is clear that some turnover of proteins does occur despite reduced levels of the three main proteinases. In fact the rate of proteinolysis in starved A3pep.7 cells is similar to that observed in non-starved A364A (c.f. fig. R.24). Thus it appears that this 'residual' protein turnover is sufficient to permit trickle-charging of tRNA during amino acid starvation.

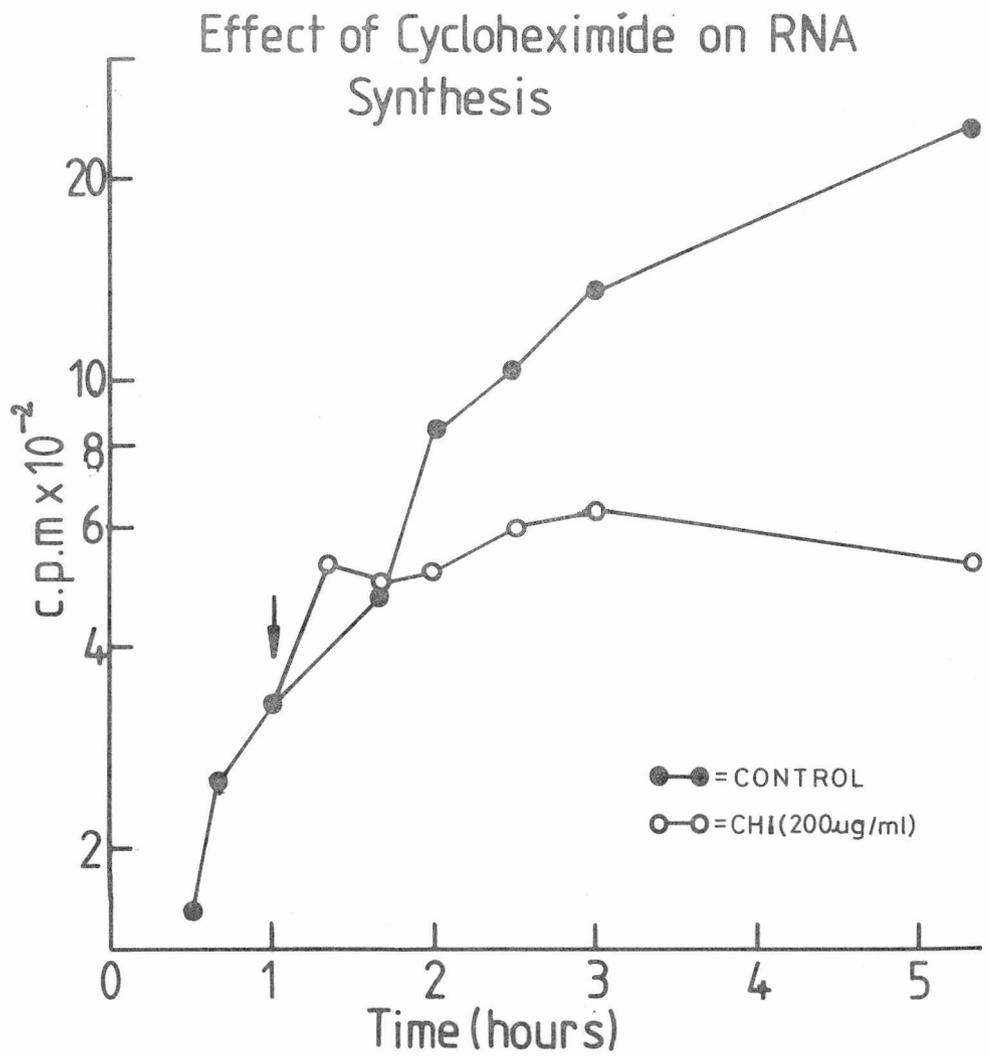
R2.8 The Effect of Translation Inhibitors on Charging Levels in Non-starved Cells

In contrast to their effect on starved cells the addition of protein synthesis inhibitors to growing yeast cells causes a reduction in the rate of accumulation of RNA (see Introduction, I.5). This effect is demonstrated in figure R.28. To determine the relationship between the level of tRNA aminoacylation and RNA synthesis it is therefore important to examine the effect of

FIGURE R.28 - The effect of cycloheximide on RNA synthesis in unstarved cells.

A364A was grown to early exponential phase in appropriately supplemented YNB-aa and ^{14}C -8-adenine ($0.5 \mu\text{Ci/ml}$) was added. After 30 minutes samples (0.2 mls) were taken into ice-cold 50% w/v TCA ($20 \mu\text{l}$). One hour after the addition of radiolabel (indicated by the arrow) the culture was divided into two and cycloheximide ($200 \mu\text{g/ml}$) was added to one half. Sampling was continued and the TCA precipitable radioactivity in each sample determined as explained in M5.3.

Fig.R.28



these inhibitors on charging in non-starved cells. The experimental procedure used is described in R2.6 and the results are given in Table R.6. The data presented demonstrate that, for all inhibitors tested, the block in protein synthesis appeared to produce a concomitant discharge of tRNA.

It was ~~was~~ then necessary to determine whether this effect was a direct result of the action of these inhibitors on protein synthesis or whether it was due to some secondary action of the drugs.

R2.9 The Effect of Translation Inhibitors on the Synthetase Reaction 'in vitro

Discharge of tRNA would clearly be promoted by these inhibitors if they interfered with the synthetase reaction itself. This possibility was examined by adding the drugs to the incubation mixture for the in vitro aminoacylation reaction. A suitable volume of reaction mixture was prepared and divided into five aliquots. Translation inhibitors were added to three of these to give final concentrations: cycloheximide, 200 $\mu\text{g/ml}$; MDMP; 100 $\mu\text{g/ml}$; Anisomycin, 500 $\mu\text{g/ml}$. An equal volume of 50mM Tris HCl, pH7.4 was added to the other two aliquots. An equivalent amount of alkaline-hydrolysed tRNA was then added to each of these inhibitor containing mixtures and to one of those without an inhibitor. These mixtures were then used to assay synthetase activity as explained in M5.13.

The results of these experiments, which are shown in Table R.10 demonstrate that little or no effect on the synthetase reaction is caused by any of the translation inhibitors tested. The figures shown are average values for five independent assays.

R2.10 Transfer RNA Charging in a Mutant Temperature-Sensitive for Protein Synthesis

A further check was made to ensure that tRNA discharge in unstarved cells occurs as a direct consequence of a block in protein synthesis. In these experiments a temperature-sensitive (ts) mutant, ts275 (Hartwell and McLaughlin, 1968), was used to arrest protein synthesis in a manner which is independent of drug action (see figure R.29). This strain has a mutation in

Sample	activity (C.P.M)	%control activity
No tRNA	414	3
tRNA	13,172	100
¹¹ +CHI 200 μg/ml	12,240	91
¹¹ +ANI 500 μg/ml	12,030	93
¹¹ +MDMP 100 μg/ml	13,583	103

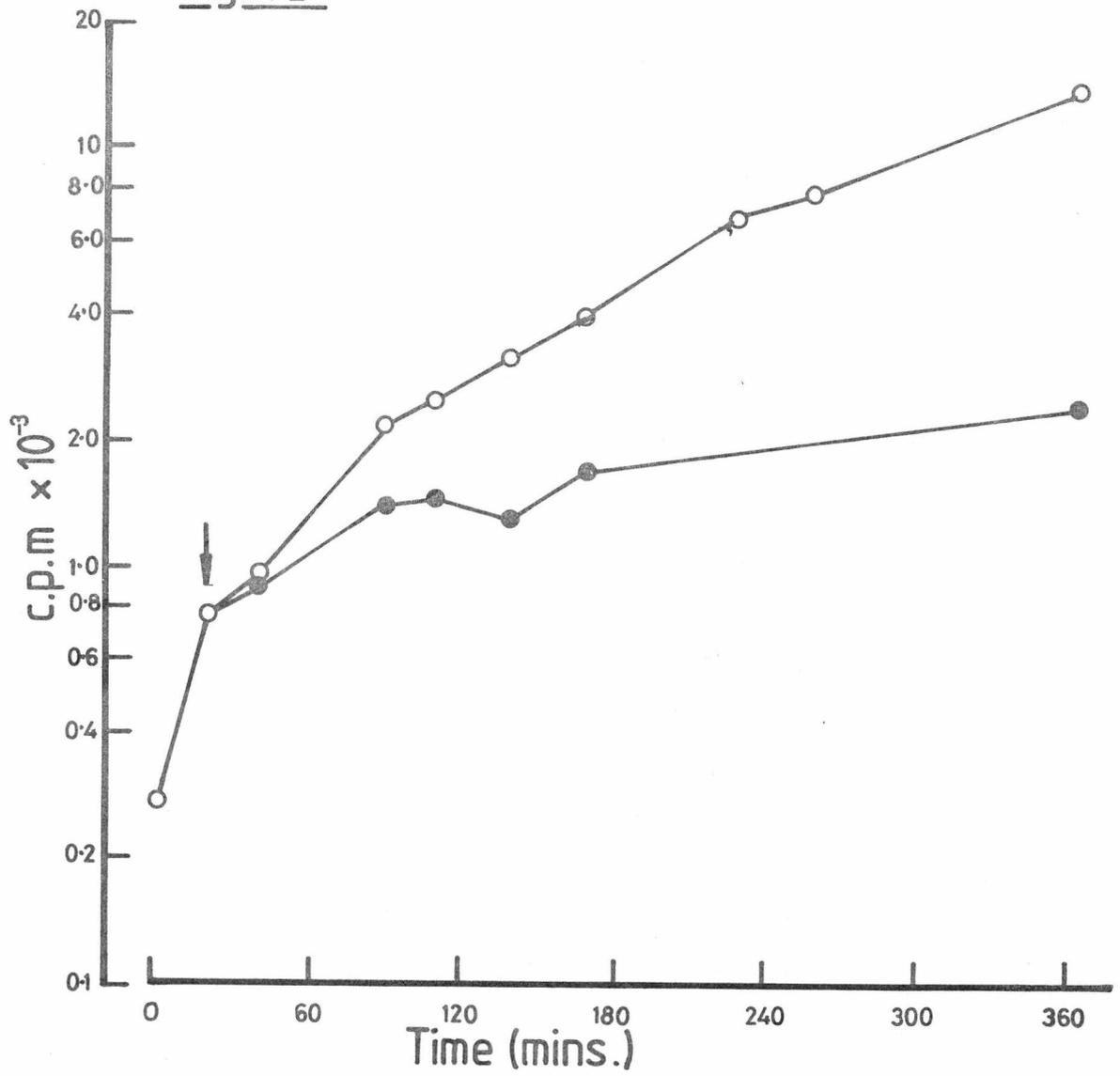
Table R.10 Translation inhibitors do not affect the synthetase reaction
in vitro

The effect of translation inhibitors on synthetase activity was determined by adding the drugs to the synthetase assay drops. A suitable volume of reaction mixture was prepared and divided into 5 aliquots. Translation inhibitors were added to three of these aliquots to give the final concentration shown and an equal volume of 50mM Tris HCl, pH 7.4 added to the other two. An equal amount of alkaline-hydrolysed tRNA was then added to each of these inhibitor containing mixtures and to one of those without an inhibitor. These mixtures were then used to assay synthetase activity as explained in Materials and Methods. The values shown in the table are average values for 5 replicate assays.

FIGURE R.29 - Protein synthesis in ts 275

ts 275 was grown to early exponential phase in supplemented YNB-aa at 23°C and ^3H -(3,5)-tyrosine (4 $\mu\text{Ci/ml}$) was added. After 30 minutes, samples (0.2 mls) were taken into ice-cold 50% w/v TCA (20 μl). At the time indicated by the arrow the culture was divided into two and one half was switched to 36°C for the remainder of the experiment. Sampling was continued and the TCA precipitable radioactivity in each sample determined as described in M5.3. ○—○, 23°C ; ●—●, 36°C

Fig.R.29



ts 275	%uncharged tyr-tRNA
Control 23°C	19.8
Control 37°C	64.3
Tyr. starved 23°C	67.4
Starved 37°C	21.9

TABLE R.11 - tRNA charging in a mutant temperature-sensitive for protein synthesis

the gene p_{rt} 3 resulting in a temperature-sensitive elongation factor II protein (McNeil and McLaughlin, 1973). Raising the incubation temperature of ts275 from the permissive value of 23°C to the restrictive value of 36°C is therefore analogous to treating wild-type cells with cycloheximide, since the translocation step of protein synthesis is blocked.

Experiments with the mutant were carried out exactly as already described except that a shift in temperature from 23°C to 36°C replaced the inhibitor treatment. Table R.11 demonstrates that discharge of tRNA occurred in non-starved ts275 cells when they were raised to 36°C. In tyrosine-starved cells, however, the level of charged tyrosyl-tRNA at the restrictive temperature approached that found in control cultures. This pattern of tRNA aminoacylation corresponds to that found in wild-type cells treated with inhibitors. Thus it appears that inhibition of protein synthesis in amino acid starved cells promotes trickle-charging of tRNA whilst the same block causes discharge of tRNA in non-starved cells.

DISCUSSION

The results reported in section R1 of chapter 3 show that the replication of P1 dsRNA is highly dependent on the metabolism of the host cell. In cultures growing logarithmically the synthesis of P1 was also found to be exponential (figures R.4, 9, 12). This is the expected pattern of synthesis for any cellular component during balanced growth. This would also be expected if existing templates replicate to give molecules which themselves act as templates for further replication. These findings are in agreement with the observations of Oliver et. al. (1977) who reported the exponential accumulation of P1 during balanced growth and of Newman et. al. (1981) who found exponential P1 synthesis throughout the cell cycle.

The suggestion that P1 dsRNA may be a self replicating species is supported by the demonstration of the required VLP associated enzymic activities. Herring and Bevan (1977) have identified a viral polymerase associated with P1 VLP's from stationary phase cells which produces single-stranded RNA. The activity has been characterised by Welsh and colleagues (Welsh, Leibowitz and Wickner, 1980) and was found to incorporate all four ribonucleotides triphosphates and requires magnesium ions. The single-stranded RNA product is entirely complementary to one of the two P1 strands (Herring and Bevan, 1977; Brennan and Bruenn, 1978; Brennan, Hastie and Bruenn, 1978) and is released from some of the enzymatically active virions (Welsh and Leibowitz, 1980). These data suggest that the single-stranded RNA polymerase is a viral transcriptase which catalyses the asymmetric synthesis of full length transcripts. This conclusion is supported by a number of other studies (Haylock and Bevan, 1981; Welsh and Leibowitz, 1980; Bruenn, Bobek, Brennan and Held, 1980.)

Bevan and Herring (1976) have also described an RNA polymerase activity that copurifies with VLP's from actively growing cells. An analysis of the products of this enzyme indicated that it probably catalysed the synthesis of double-stranded RNA from a single-stranded template.

It is possible that the viral transcriptase may also have a direct role in dsRNA replication and several models have been forwarded to describe this process. On the basis of the two types of polymerase activity, Herring and Bevan (1976,1977) have suggested that the replicative cycle of the yeast VLP is similar to that of reovirus, a dsRNA virus which infects animal cells. Thus, first a single-stranded copy of one of the two strands of the parental molecule is synthesised. This ssRNA may either act as a messenger for translation or be encapsidated together with a polymerase which will then synthesise the complimentary strand (fig D.1).

The study of a number of dsRNA 'mycoviruses', particularly that of Penicillium stoloniferum, has led Buck and Ratti (1975) to propose a model in which the VLP-associated RNA polymerase is able to bind equally to the ends of either strand of the dsRNA (see fig D.2). It therefore produces a single-stranded RNA molecule from either strand with equal probability. After one strand has been synthesised the polymerase may transfer from the 5' end of the template strand to the 3' end of the complementary strand. RNA synthesis on the complementary strand could then take place giving rise to particles containing two molecules of dsRNA. These particles could then be uncoated by the action of host cell proteinases. Single-stranded mRNA could then be synthesised from these non-encapsidated double-stranded templates, which in turn would be translated to give more viral coat proteins and polymerases. Finally the two dsRNA molecules would be separately re-encapsidated. The discovery of the viral polymerases described above make this model of replication less likely, however, in the case of yeast dsRNA-containing VLP's.

In considering these ideas it is clear that the ability of the host cell to synthesise proteins is an essential component of any model describing the replication of yeast dsRNA. The interdependence of these processes is reflected in the response of P1 synthesis to starvation for leucine as shown in section R1.3. The rate of P1 synthesis during the initial pulse labelling period after starvation was reduced to about 80% of the control value. This rate of synthesis continued to fall during amino acid starvation to less than 40%

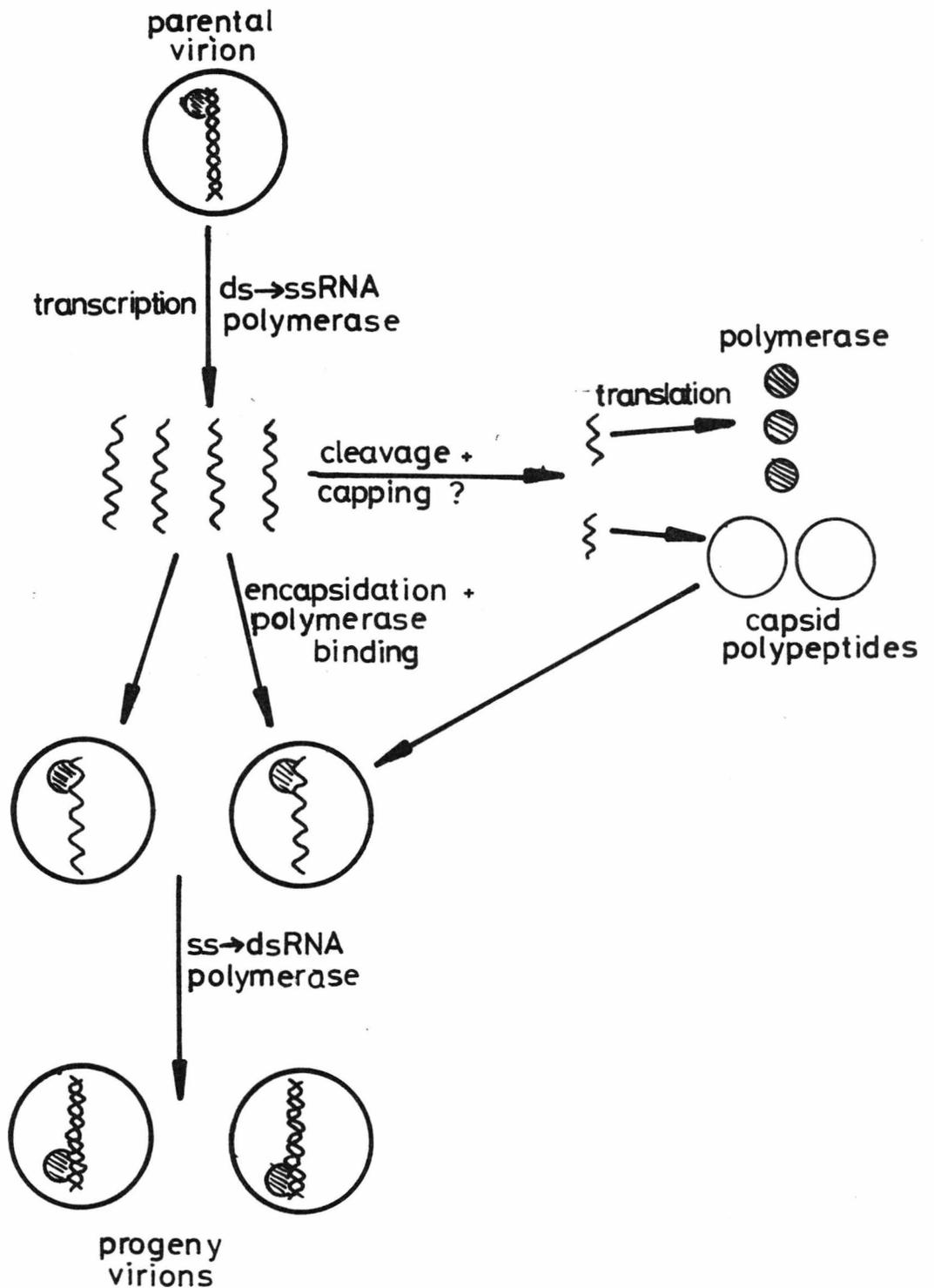


Fig.D.1 THE 'REOVIRUS' MODEL OF dsRNA REPLICATION

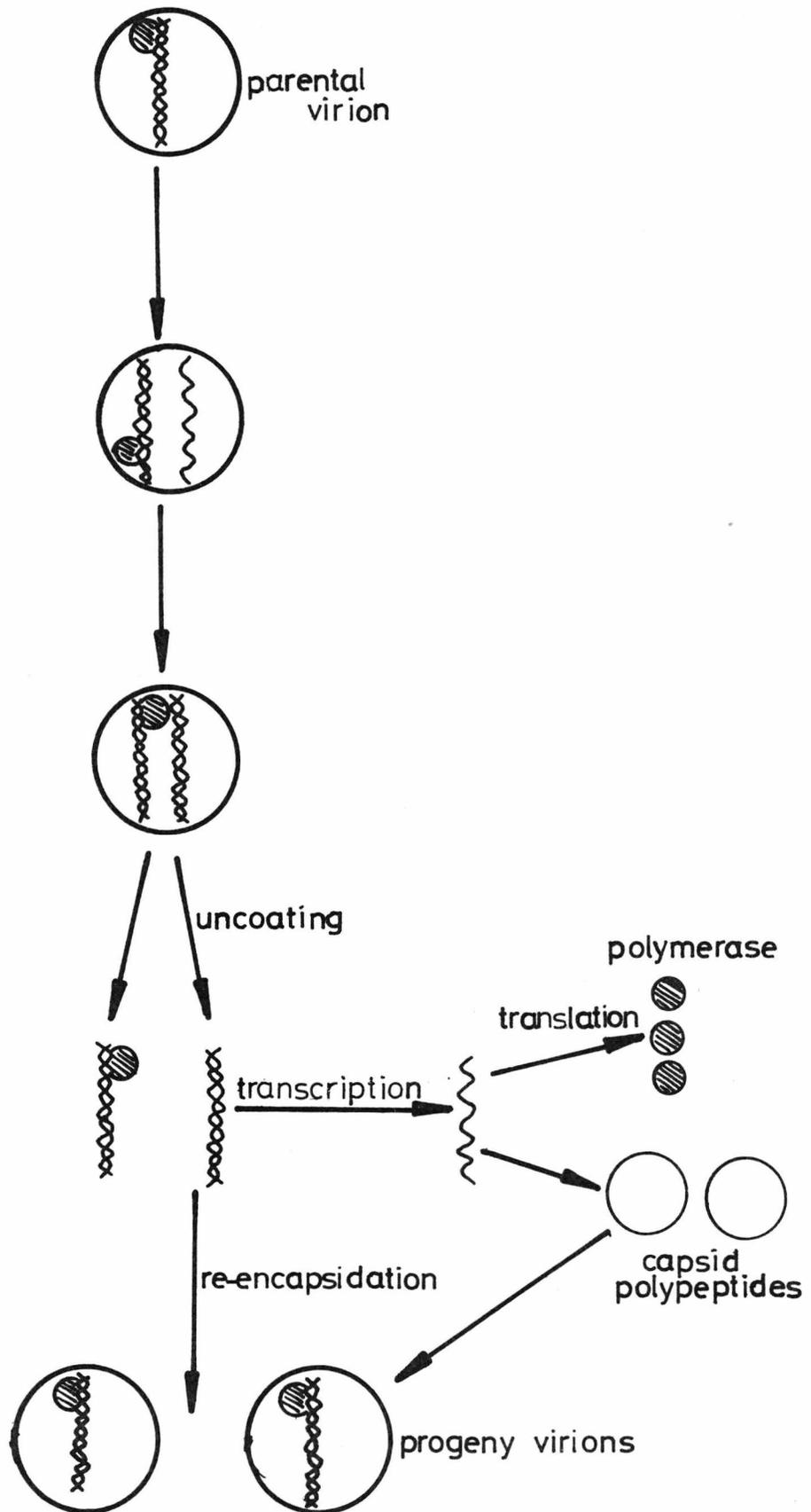


Fig.D.2 THE 'BUCK AND RATTI' MODEL OF dsRNA REPLICATION

of the initial control value (fig R.12). This represents a true decrease in the rate of P1 synthesis since no degradation of dsRNA was observed during amino acid starvation (fig R.11). Furthermore when protein synthesis in growing cells was inhibited by the addition of cycloheximide the P1 synthetic rate was markedly reduced (fig R.13). Finally, when amino acid starved cells were treated with this inhibitor no further effect on P1 synthesis was observed (fig R.13).

These observations show that there is a similarity between the response of P1 dsRNA synthesis during these conditions and that of rRNA synthesis. The results given in section R.2 of chapter 3 indicate a possible role for tRNA aminoacylation in the regulation of rRNA synthesis. The mechanism behind this control system could involve the direct action of uncharged tRNA or could be due to the action of a secondary effector molecule (s) (see later discussion). However the models of dsRNA replication discussed above require the action of viral polymerase within intact virions implying that these enzymes would be isolated from such a control mechanism. (This may not be true in the case of low molecular weight effectors to which VLP's may be permeable as they presumably are to nucleotides). That the viral polymerases have been isolated only in association with VLP's and that in vivo dsRNA is probably always encapsidated (Bostian, Sturgeon and Tipper, 1980) also support this conclusion. It is possible, however, that uncharged tRNA or other putative regulatory molecules may exert their effect on the viral polymerase(s) following translation but before encapsidation of these enzymes. Nevertheless, it appears more likely that continued protein synthesis is required for P1 dsRNA replication to provide the necessary viral capsid proteins and polymerases as they are removed into completed VLP's. Thus although the responses of dsRNA and rRNA synthesis are similar during amino acid starvation and inhibitor treatment it is probable that the controls operating on these processes are different. However, it may be noted that a relationship could exist between P1 synthesis and levels of uncharged tRNA if tRNA aminoacylation regulates the rate of

protein synthesis (see later discussion).

During starvation for nitrogen the synthesis of P1 dsRNA was constant with respect to those molecules remaining from the beginning of the experiment (figs R.4 and R.9). However, extensive degradation of P1 dsRNA was observed during these conditions (figs R.2 and R.7). The simplest explanation for a constant relative rate of P1 synthesis is that pre-existing templates which survive degradation continue to replicate at the same rate throughout nitrogen starvation but newly formed dsRNA molecules do not themselves act as templates for further replication. This conclusion may be justified in the following manner.

A constant relative rate of P1 synthesis cannot be explained by assuming that the rates of synthesis and degradation are equal. This cannot be so since the amount of P1 in nitrogen starved cultures actually decreases as measured by the absorbance at 260nm of the relevant gel peaks (see figs R.3 and R.8). Thus, during nitrogen starvation, the rate of degradation of P1 (loss of ^{14}C assuming degradation is not preferential) is faster than the rate of P1 synthesis (i.e. incorporation of ^3H) such that there is a net loss of P1 dsRNA (reduction in OD_{260}) from the culture. (That synthesis is occurring during starvation is demonstrated by the incorporation of ^3H label into dsRNA). However, a constant $^3\text{H}/^{14}\text{C}$ ratio would only be observed if the rate of P1 synthesis continually decreased at the same rate as P1 degradation (i.e. the observed decrease in the amount of ^{14}C -dsRNA is matched by an equal reduction in the incorporation of ^3H into dsRNA). Furthermore, this reduction in P1 synthetic rate must commence simultaneously with the onset of P1 degradation (since an increase in the $^3\text{H}/^{14}\text{C}$ ratio would otherwise be expected during the initial period of starvation when essentially little or no degradation is occurring). Thus in order to maintain a constant $^3\text{H}/^{14}\text{C}$ ratio throughout the period of the experiment, changes in the amount of ^{14}C -dsRNA caused by P1 degradation must be balanced by changing the incorporation of ^3H into dsRNA (P1 synthesis). This dependence of P1 synthetic rate on the number of templates existing before starvation (^{14}C) suggests that the majority of newly

formed dsRNA molecules do not act as templates for further replication.

The constant $^3\text{H}/^{14}\text{C}$ ratio observed throughout the experiment would, according to the argument presented above, indicate that pre-existing templates continue replication at about the same rate during nitrogen starvation. Comparison of the $^3\text{H}/^{14}\text{C}$ ratio for the initial labelling periods (see fig R.1 and R.5) show that, although this rate is constant throughout starvation, it is reduced to about 60% of the initial control value. This reduced rate of P1 synthesis would be expected since the overall rate of protein synthesis is decreased during nitrogen starvation and the production of viral capsid proteins and polymerases would also be reduced.

A decreased availability of viral proteins may also explain why newly formed P1 molecules do not normally act as templates during starvation. Thus, according to the model of Buck and Ratti (1975), the uncoating of VLP's containing newly replicated dsRNA will release both parental molecules to which the polymerase remains associated and daughter molecules which do not have a polymerase. Only a proportion of each will be re-encapsidated since the production of capsid proteins is limited. (Presumably those molecules which are not re-encapsidated are subject to degradation - see later discussion). Encapsidated parental dsRNA molecules may then continue replication as before but very few VLP's containing daughter molecules will also contain a polymerase since the production of these enzymes is also limited. Therefore very few of the daughter VLP's will be capable of replication.

Similar arguments can be made using the reovirus model of dsRNA replication. However, during the normal cycle of replication according to this model, only the ssRNA intermediate exists outside the protein capsid. Thus any replication intermediates or products which are coated without a polymerase molecule would remain as single-stranded RNA and would not be detected in the double labelling experiments since they would not migrate to the same region of the gels. If this were the case then very little ^3H labelled dsRNA would be observed during nitrogen starvation assuming, like reovirus, the replication of yeast dsRNA was completely conservative. (Similarly no

degradation of ^{14}C dsRNA could be observed if replication were fully conservative and VLP's were not uncoated during replication - see later discussion). It is possible however, that yeast dsRNA has a semi-conservative mode of replication (see Herring and Bevan, 1977; Bruenn et. al., 1980). The most direct study of the mode of yeast dsRNA replication, using density transfer experiments, indicated that this process is either fully conservative or is semi-conservative but carried out only by a proportion of the total dsRNA population (Newman et. al., 1981). Semi-conservative replication of other fungal dsRNA viruses has also been reported (Buck, 1978; Ratti and Buck, 1978).

The experiments described in section R1.2 and discussed above showed that during nitrogen starvation extensive degradation of P1 molecules was found to occur. It is known that when yeast cells are deprived of a primary nutrient such as nitrogen, cells that have already begun their cell cycle will complete that cycle in the absence of further growth (Johnston, Pringle and Hartwell, 1977a). It has also been suggested that the degradation of intracellular macromolecules could supply most of the intermediates required for this process (Johnston, Singer and McFarlane, 1977b). The observed loss of P1 dsRNA from nitrogen-starved cultures is consistent with this idea. It is possible that the very high levels of P1 which S7A6 contains provide a useful reserve of nucleotides to the starved cells and may even confer an advantage in such conditions. It has been observed (Clare, unpublished) that while strains containing less dsRNA can complete only 1-1.5 cell doublings from the onset of nitrogen starvation, S7A6 is able to undergo 2-3 cell doublings. However this observation was not conclusively verified since the extent of 'residual growth' during nitrogen starvation varied to some degree according to the normal growth rate of the strain being tested. Furthermore isogenic pairs of strains with similar growth rates and differing only in P1 content were not available.

The degradation of P1 dsRNA during nitrogen starvation was not observed until after a 'lag' period of about $2\frac{1}{2}$ hours (figs R.2 and R.7). Moreover,

degradation was prevented by the addition of the protein synthesis inhibitor, cycloheximide, to the culture at the time of starvation (fig R.15). These facts suggest that the starved cells must induce the de novo synthesis of nucleolytic enzymes before the degradation of P1 dsRNA may begin. The observation of Johnston et. al. (1977b), that a different complement of proteins is synthesised during nitrogen starvation is compatible with this suggestion. Furthermore McFarlane (1980) has also reported that RN'ase activity is stimulated when yeast cells are starved for nitrogen. This conclusion was reached by measuring the degradation of yeast total RNA by crude cell extracts and the activities involved were not well characterised. However, the starvation induced nuclease showed differing thermostability and specificity for homopolyribonucleotides compared to that found in growing cells.

Another inducible RN'ase has also been found in yeast. This enzyme is an exonuclease that produces 3' nucleotides and is induced by glucose starvation (Schulz-Harder and Hucherer, 1980) and at the transition from the exponential to stationary phase of growth (Swida et. al., 1981). Induction of this enzyme by glucose starvation is also inhibited by cycloheximide (Schulz-Harder and Hucherer, 1980).

The degradation of P1 dsRNA during nitrogen starvation probably represents the inducible activity of a double-stranded specific ribonuclease. However, this need not necessarily be the case. As pointed out previously, according to the reovirus model of replication, during the normal cycle of events only ssRNA intermediates are unencapsidated and thus accessible to nucleases. In this scheme no degradation of ^{14}C labelled dsRNA would occur unless replication was semi-conservative (see above). It appears more likely that nitrogen starvation induces the activity of host cell proteinases such that uncoating of dsRNA containing VLP's may occur, thus exposing double-stranded molecules to nuclease activity. This

alternative interpretation would also be predicted by the Buck and Ratti model of replication. Presumably substantial uncoating of dsRNA must occur during nitrogen starvation otherwise the minimum number of molecules surviving degradation would be equal to the number existing at the time of starvation. This is not the case since a decrease in absorbance at 260nm of the P1 peak as well as loss of the ¹⁴C label was observed.

Whatever the pattern of events leading to the degradation of dsRNA during nitrogen starvation it is clear that this process does not continue to completion since even after extensive starvation (e.g. 78 hours) some dsRNA still remained (see R 1.2). Thus it may be that the relative rates of synthesis and degradation eventually come to equilibrium in nitrogen starved cells. Alternatively it is possible that a certain proportion of the dsRNA population is protected from degradation. This may be because they are resistant to nuclease activity but could also be due to resistance of the VLP to uncoating by the action of proteinases. Furthermore this resistant VLP population retains the potential for replication since upon the readdition of nitrogen the level of dsRNA increased dramatically (see Table R.1). Thus during nitrogen starvation the replication of the VLP is subordinated to the needs of the host cell. The VLP genome is not sacrificed, however, and its ability to replicate once the host cell is returned to growth conditions is fully protected. In this respect the replication of the VLP appears well adapted to the fact that its survival is dependent on that of its host.

In the Introduction a possible role for uncharged tRNA in the regulation of RNA synthesis in yeast was discussed. Evidence for this suggestion was provided using amino acid analogues, temperature sensitive aminoacyl-tRNA synthetase mutants and by the observation of an apparent relaxation of the stringent response by translation inhibitors. In section R.2 of chapter 3 the relationship between tRNA aminoacylation and RNA synthesis was examined. When yeast cells are starved for a required amino acid RNA synthesis is restricted. The primary target of this stringent control is the synthesis of ribosomal RNA and that of transfer RNA continues relatively unaffected (see Introduction, I.5).

Table R.5 demonstrates that the tRNA species cognate to the missing amino acid became uncharged during starvation, while the charging of the other tRNA species continued normally. This finding is in agreement with results obtained with other eukaryotic cells (e.g. mouse ascites tumour cells - Ogilvie, Huschka and Kersten, 1979).

During starvation the required amino acid can only be supplied from the cells own resources. Figure R.24 shows that the degradation of intracellular proteins was stimulated under these conditions (see also Betz, 1976). Amino acids released by proteolysis are then available to the synthetase reaction and trickle-charging may therefore occur. Aminoacyl-tRNA molecules produced in this manner do not accumulate during amino acid starvation since they are discharged by residual protein synthesis. However, the results shown in Table R.6 demonstrate that high levels of charged tRNA of the cognate species occur when translation inhibitors are added to the starved cells. Thus in yeast, as in bacteria, the deacylated tRNA species may be trickle-charged by blocking residual protein synthesis (see fig. I.1). This interpretation of the data was also confirmed by arresting residual protein synthesis independently of inhibitor action using a temperature sensitive mutant (Table R.9).

The deacylation of a single species of tRNA may be promoted in yeast either by starvation for a single amino acid (Table R.5) or by raising a mutant with a temperature-sensitive aminoacyl-tRNA synthetase to its restrictive temperature (McLaughlin, Magee and Hartwell, 1969). Furthermore either

treatment evokes a stringent response (Oliver and McLaughlin, 1977). Therefore if the controls operating during stringency in yeast involve uncharged tRNA then it may be concluded that, as in bacteria, the lack of a single charged species is sufficient for the system to operate. However, in yeast, in contrast to bacteria, a stringent response is also evoked by the addition of translation inhibitors to growing cells (see Roth and Dampier, 1972; Shulman et. al., 1977; Kelker and Pogo, 1980; and fig. R.26). In section R2.7 it was found that the discharge of tRNA occurs in cells during this treatment. The effect was not due to any interference by the protein synthesis inhibitors with the charging reaction itself (Table R.8). The discharge of tRNA was also observed when a mutant which is temperature sensitive for protein synthesis was incubated at the restrictive temperature (Table R.9). Thus, all the conditions which cause an inhibition of rRNA synthesis in yeast also promote the discharge of tRNA. Moreover, the contrasting effect of translation inhibitors on RNA synthesis in starved and non-starved cells are paralleled by their opposing effects on tRNA charging. All of these data suggest that tRNA charging does indeed have a role in the regulation of rRNA synthesis.

The nature of this tRNA-mediated control mechanism remains unclear. That the response is triggered by the lack of a single charged tRNA species suggests the involvement of a codon-specific reaction. Perhaps, by analogy with E. coli, a secondary effector molecule is synthesised by the ribosome. As pointed out in the Introduction, to date no such low molecular weight regulators have been shown to exist in yeast. It is also possible that the codon-specific binding of uncharged tRNA to yeast ribosomes may lead to the degradation of GTP as has been found in Ehrlich ascites cells (Grummt and Speckbacher, 1975). However neither this phenomenon nor the contraction of the GTP pool has been observed in yeast. Another alternative is that the total level of deacylated tRNA (i.e. the accumulation of uncharged tRNA per se) is important rather than any putative reactions at the ribosome. Thus uncharged tRNA may, either directly or indirectly, interact with the polymerase(s) itself. It is

known that tRNA reversibly binds to both polymerases I and II inhibiting their activity in vitro (Sawadogo, 1981). Furthermore the inhibition of polymerase activity by tRNA was found to vary according to the nature and concentration of the template since it actually interferes with the binding of the template to the enzyme. No attempt was made to determine the levels of aminoacylation of the tRNA used in this investigation. However, since commercially prepared tRNA was used it is likely that it was not highly charged.

It should be remembered at this point, that starvation led to the deacylation only of the tRNA species corresponding to the missing amino acid. Examination of the data in Table R.5 reveals that other tRNA species if anything, became slightly more charged. This result would be expected assuming that charging continued normally but the utilisation of charged tRNA by protein synthesis was greatly reduced. An increase of about 60 - 70% uncharged tRNA of the cognate species would be counteracted by a decrease of only 3 - 3.5% uncharged tRNA for each of the other species. Nevertheless it could be that only certain tRNA species, or indeed certain tRNA isoacceptors, have a regulatory role. Another possibility is that any species of tRNA could have a regulatory function when the level of uncharging exceeds a certain threshold.

One further point relevant to this discussion is the finding that although translation inhibitors promote trickle-charging of the deacylated tRNA species, the synthesis of rRNA is still repressed. The effect of adding inhibitors on the non-cognate tRNA species in starved cells was not determined. If these tRNA species become uncharged, as occurs in inhibitor treated non-starved cells, then relaxation of rRNA synthesis would not be expected. If, on the other hand, they remain highly charged, as during amino acid starvation alone, then it is necessary to postulate another regulatory component in addition to uncharged tRNA, for example a short-lived polypeptide as proposed by Gross and Pogo (1974, 1976a, b). One way to distinguish between these alternatives would be to uncouple the discharge of tRNA from the cessation of protein synthesis (see later discussion).

The apparent relaxation of the stringent control on RNA synthesis which occurs on adding a translation inhibitor to amino acid-starved yeast cells is confined to the stimulation of tRNA synthesis (Oliver and McLaughlin, 1977). This stimulation is coincident with the trickle-charging of tRNA which is also promoted by the inhibitor. Furthermore no stimulation of tRNA synthesis by translation inhibitors is observed in a temperature sensitive aminoacyl-tRNA synthetase mutant at the restrictive temperature (Oliver and McLaughlin, 1977). These results are in agreement with the suggestion that the charging of tRNA may be part of an autoregulatory system controlling tRNA synthesis.

There is evidence to suggest that during normal growth yeast cells maintain an equilibrium level of tRNA charging (McLaughlin, Magee and Hartwell, 1969; Messenguy and Delforge, 1976). The published data and the results presented here suggest that the normal level of charging for the different tRNA species is in the range 60 - 90% charged. The stimulation of tRNA synthesis observed in starved cells on treatment with a protein synthesis inhibitor may reflect the action of a mechanism which maintains this charging equilibrium. The high levels of charged tRNA which occur under such conditions would thus be reduced by increased synthesis of new, uncharged, molecules in order to restore the equilibrium.

Autoregulatory control of tRNA synthesis involving tRNA aminoacylation has also been proposed to occur in other eukaryotes (Hamilton and Litt, 1976; Dehlinger, Hamilton and Litt, 1977). These workers found that drugs which inhibit tRNA charging in cultured Friend leukaemia cells, e.g. O-methyl threonine or histidinol, caused a decrease in the rate of tRNA synthesis whereas drugs that were presumed to stimulate tRNA charging, e.g. cycloheximide or pactamycin, had the opposite effect. Similar effects of these protein synthesis inhibitors on tRNA synthesis have been reported in other cultured eukaryotic cells (Bolcsfoldi, 1974; Westerberg, Bolcsfoldi and Eliasson, 1976).

Litt has also described another form of autoregulation of tRNA accumulation occurring in Friend leukaemia cells. It was found that in cells in which the level of a single charged tRNA species was low, as during amino acid starvation or treatment with amino acid analogues, the relative concentration of this tRNA species was correspondingly high (Litt and Weiser, 1978; Weiser and Litt, 1979; Litt and Howell-Litt, 1980). This phenomenon was apparently due to a decreased rate of degradation of the cognate tRNA species during amino acid deprivation and it was concluded that in Friend leukaemia cells the deacylated tRNA species is more stable than the corresponding charged form (Litt and Howell-Litt, 1980). Litt and colleagues therefore proposed that this charging-dependent mechanism regulates the concentrations of specific tRNA's according to the needs of the cells for their cognate amino acids in protein synthesis.

Other regulatory functions for tRNA involving aminoacylation have been proposed. For example the relation between tRNA charging and protein synthesis has been examined. Experiments to determine polysome profiles and peptide elongation rates during treatment with amino acid analogues, or amino acid starvation, of cultured human and mouse cells indicated that tRNA charging levels may govern the rate of translational initiation (Van Venrooij, Henshaw and Hirsch, 1972; Vaughan and Hansen, 1973; Warrington, Wratten and Hechtman, 1977). Van Venrooij and co-workers (1972) suggest that this effect on initiation is due to a decrease in the intracellular ATP pool which they observed in amino acid-starved Ehrlich ascites cells rather than uncharged tRNA (cf Grummt and Grummt, 1976). Vaughan and Hansen (1973) studying HeLa cells, have disputed this proposal as have other workers (Lofgren and Thompson, 1979). Furthermore, uncharged tRNA was found to block the formation of eukaryotic initiation complexes in vitro (Zasloff, 1973; Kyner, Zalos and Levin, 1973) although again this has recently been disputed (Austin et. al., 1982). However while it is still debateable whether tRNA charging levels do effect initiation of protein synthesis in eukaryotes it is apparent that the rate of peptide elongation is affected (see above references and Ogilvie, Huschka and

Kersten, 1979).

Another aspect of cellular regulation in which tRNA aminoacylation appears to have a role is in the 'general control' of amino acid synthesis of yeast. This 'general control' involves the derepression of enzymes from several amino acid biosynthetic pathways upon starvation for a single amino acid (Delforge, Messenguy and Wiame, 1975). Evidence using a t.s. aminoacyl-tRNA synthetase mutant indicated that this phenomenon occurs in response to the accumulation of uncharged tRNA rather than by the lack of an amino acid (Messenguy and Delforge, 1976).

In the preceding discussion it was proposed that the stimulation of tRNA synthesis observed in response to inhibitor treatment of amino acid-starved yeast cells occurred in order to restore equilibrium levels of charged tRNA. It is possible that the establishment of such an equilibrium is necessary to maintain the regulatory functions of tRNA such as those discussed above. The results described in section R.2 suggest that uncharged tRNA may have another regulatory function for which the maintenance of this equilibrium is important. As has been discussed, uncharged tRNA appears to be a component in a negatively acting control circuit which serves to control the synthesis of rRNA. An interesting consequence of this model is that tRNA synthesis may be regulated in such a manner as to maintain the balance between rRNA synthesis and cell growth. It has not yet been possible, however, to uncouple the discharge of tRNA from the cessation of protein synthesis. It may be that the rate of protein synthesis is the major controller of the rate of rRNA synthesis. Alternatively, protein synthesis and tRNA charging may together act as controllers. Further experiments are necessary to distinguish between these models. One method of achieving this aim would be to perform a detailed kinetic study of the events which occur when amino acids are added back to a starved culture.

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