

"DIFFERENTIAL PROTEIN SYNTHESIS DURING
NITROGEN STARVATION OF THE FISSION
YEAST, *Schizosaccharomyces pombe*"

THESIS

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by

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ABSTRACT

In an effort to highlight potential "cell division cycle proteins" in the fission yeast, *Schizosaccharomyces pombe*, cultures growing exponentially in defined medium were starved of nitrogen. This treatment causes a semi-synchronous burst of accelerated cell division at a reduced cell size and can thus be envisaged as an uncoupling of the growth cycle from the DNA-division cycle. ³⁵S-methionine labelling during the period of accelerated division reveals the differential synthesis of two polypeptides of molecular weights 46kd and 27kd.

In order to distinguish between the alternative hypotheses that these proteins (denoted as p46 and p27) are a) "division proteins", or b) "stress proteins" (synthesized as a direct result of nitrogen starvation), use was made of the temperature-sensitive mutant of *S.pombe*, wee 1.50, which, at the restrictive temperature, divides at half the size of wild type cells. This mutant does NOT accelerate into division upon nitrogen starvation at the restrictive temperature for reasons satisfactorily explained by current models of cell cycle control in *S.pombe*. ³⁵S-methionine labelling during the period following nitrogen starvation does, however, reveal the same differential synthesis of proteins p46 and p27 as seen in wild type.

Heat shock analysis reveals the existence of two heat shock proteins in *S.pombe* of similar sizes to p46 and p27. It was therefore postulated that nitrogen starvation in this yeast induces a subset of the total array of heat shock proteins. The hypothesis is supported by the observation that nitrogen-starved cells acquire thermotolerance at a time only shortly after the transient appearance of proteins p46 and p27.

A heat shock protein in *Saccharomyces cerevisiae*, HSP 48, has recently been identified as the glycolytic enzyme ENOLASE. Furthermore, it has been proposed that this protein may be solely responsible for the acquisition of thermotolerance in pre-heated cells. Using an anti-HSP 48 antiserum the possibility that, in *S.pombe*, protein p46 is enolase was tested by immunoblotting techniques. While the antiserum reacted specifically to a single polypeptide of *S.pombe* of approximate

molecular weight 50kd, the amount of this antibody-binding protein was not found to increase during nitrogen starvation. On this basis, it was concluded that protein p46 is not enolase. The implied divergence in the heat shock responses of budding and fission yeasts is emphasized further by the suggestion, by similar immunoblotting techniques, that, in *S.pombe*, enolase is not a heat shock protein at all.

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1. INTRODUCTION

Introductory Remarks

This research thesis is based on an observation made by Walker and Kramhoft (1984) concerning the transient appearance of two proteins during the division cycle which follows nitrogen-starvation of the fission yeast *Schizosaccharomyces pombe*. It has been the object of the research to elucidate the characteristics of these proteins.

A full introduction to the work necessitates a brief synopsis of two as - yet - unrelated areas of yeast molecular biology. An understanding of the rationale behind the original investigations requires acquaintance with studies that have attempted to identify "cell division cycle proteins" in yeast and other cell types (Section 1.1). While initial experiments (Section 3.1) require a specific knowledge of current ideas on cell cycle control in *S.pombe* (Section 1.2), subsequent experiments are based on ideas not at all related to cell division cycle phenomena, but rather to "stress responses" in yeast. This sharp division in the work not only requires a brief review of the heat shock response (Section 1.3), but also explains the subdivision of the Results Section into two chapters (Sections 3.1 and 3.2, respectively). The Introduction concludes with a summary of the Aims of this Research (Section 1.4) and, while results are specifically discussed in each chapter, the thesis concludes with a General Discussion of findings.

1.1 The Search for "Division Proteins"

In recent years, there has emerged the concept of the cell division cycle as a programmed series of stage-specific events whose execution is effected by the activity of a series of cell division cycle (CDC) genes. Although the cell division cycles of a variety of organisms have been genetically characterized, it is, nevertheless, the monumental genetical studies on the cell division cycle of the budding yeast *Saccharomyces cerevisiae* by Leland Hartwell in Seattle to which the emergence of this concept can be most closely attributed. From

these studies by Hartwell and others (eg. Pringle & Hartwell, 1982), a series of 32 temperature sensitive cell cycle mutants in *S.cerevisiae* (Mortimer & Schild, 1985) and 29 in *S.pombe* (Kohli, 1986) have been isolated. Each mutation has been mapped to different chromosomal loci in both budding (Mortimer & Schild, 1985) and fission yeast (Gygas & Thuriaux, 1984) and each, at the restrictive temperature (usually 36°C), causes the homogeneous arrest of a population of mutant cells at a specific stage of the cell division cycle. Through the construction of double mutants it has been possible to assess the functional interrelationships between *cdc* mutants and thus to create functional sequence maps of the cell division cycle. In short, it has been possible to describe the cell division cycle in terms of a series of mutually dependent gene-controlled events.

Despite these impressive advances in the genetic characterization of the cell division cycle, attempts to elucidate the nature and function of cell division cycle gene products have proven less fruitful. Nevertheless, with the advent of recombinant DNA technology and its application to the area of cell division cycle genetics, the situation is improving and, to date, a number of cloned gene products have been identified. For example:

<i>S.pombe cdc 22</i>	- Nucleoside diphosphokinase (Dickinson, 1981)
<i>S.pombe cdc 17</i>	- DNA ligase (Nasmyth, 1977)
<i>S.cerevisiae cdc 28</i> & <i>S.pombe cdc 2</i>	- cAMP dependent protein kinase (Beach <i>et al</i> , 1982)
<i>S.cerevisiae cdc 31</i>	- Homologous with calmodulin - involved in spindle pole body duplication (Byers, 1986)
<i>S.cerevisiae ras</i>	- GTP binding protein which regulates adenylate cyclase (Matsumoto, 1986).

One of the most exciting developments emanating from this approach is the finding that the CDC 28 gene of *S.cerevisiae*, and its functional counterpart, CDC 2, in the fission yeast *S.pombe* (Beach *et al.*, 1982) probably code for phosphorylated protein kinases (Lorincz & Reed, 1984; Hindley & Phear, 1985). Both of these genes are crucially involved in cell division cycle control.

Of the other approaches which have been adopted in the search for "cell division cycle proteins", most have rested on the expectation that if genetic studies have been able to describe the cell division cycle as a dependent series of gene-controlled events then at least some of these genes should exert their control through sequential synthesis of their respective products (rather than by sequential activation of products that are present continuously). Thus, many studies in *S.cerevisiae* and in other cell types have attempted to identify proteins whose synthesis is modulated periodically during the cell cycle. Such studies involve synchronization of cell populations, pulse-labelling of proteins at different stages of the cell cycle, followed by resolution of total protein extracts on two-dimensional gels. Individual spots on the resultant autoradiograms are then compared for changes in intensity. In one such study in *S.cerevisiae* (Elliott & McLaughlin, '78) out of almost 200 spots examined, each appeared to be synthesized at a constant rate throughout the cell cycle and only two proteins showed labelling patterns suggestive of periodic degradation or modification. In HeLa cells, Bravo and Celis (1980) also found that the vast majority of proteins were synthesized at constant rate during the cell cycle; among the few variable polypeptide markers, α and β tubulin were found to increase in mitosis. In *S.pombe*, a slightly different approach was adopted by Dickinson (1981) who compared the two-dimensional protein synthetic patterns of various *cdc* mutants and wild type cells. In this case, examinations of over 700 polypeptides failed to reveal any qualitative differences at all.

Despite these generally unsuccessful studies, Lorincz *et al.* (1982) have reported the identification by similar techniques of eight proteins in *S.cerevisiae* whose rates of synthesis are cell cycle modulated and a further nine which may be regulated in the cell

cycle by periodic synthesis, modification or degradation.

Of the relatively few proteins, then, whose synthesis has been shown to be cell cycle modulated, even fewer have been identified. The few examples are: histones, tubulins and certain enzymes of DNA synthesis like nucleoside diphosphokinase (Henley, 1986).

Moreover, the *a priori* assumption that cell cycle modulated proteins are likely to be the products of cell division cycle genes is called into question by the work of Byers & Sowder (1980) who have shown that a large range of hybrid cells containing temperature-sensitive *cdc* nuclei in wild type cytoplasms retain the capacity to complete several division cycles at the restrictive temperature. It is possible, however, that cyclically modulated proteins may be the products of as-yet-undiscovered *cdc* genes since, as has been argued by Pringle & Hartwell (1982), probably only a fraction of the total number of such genes has been found.

Against this background, it is now pertinent to discuss the work carried out by Walker and Kramhoft (1984) which forms the basis of this research thesis. Arguing that the inability to reveal cell cycle periodicity in protein-synthetic patterns is due to the failure to distinguish between those proteins relating to cell division and those relating to continuous cell growth, Walker and Kramhoft (1984) sought to uncouple the "growth cycle" from the "DNA-division cycle".

Using the fission yeast, *Schizosaccharomyces pombe*, this objective was achieved by a simple nitrogen starvation protocol. It has been known for many years that cells of the yeast *S.cerevisiae* may, upon completion of the "START" event, proceed through the DNA-division cycle with little need for net growth (Johnston *et al*, 1977), and the same is true for *S.pombe*. However, unlike budding yeast, nitrogen starvation of fission yeast not only uncouples growth from division, but it also results in an accelerated burst of semi-synchronous division (for reasons explained in Section 3.1.1). The system is therefore an ideal one in which to look for the production of potential cell division cycle proteins since the means by which growth is uncoupled from division also serves to bring dividing cells into

synchrony. Thus, while a general decline in the synthesis of presumed "growth proteins" is to be expected in this system, the synthesis of potential "division proteins" should become highlighted.

When protein synthetic patterns were followed at 10 min intervals throughout the period of accelerated cell division following nitrogen-starvation of fission yeast, Walker and Kramhoft (1984) observed the sudden appearance of two polypeptides of approximate molecular weights 46 kd and 27 kd after about 70 min (the period of maximum cell number increase). The result is shown in Fig. 1.1 and is reprinted with permission.

It has been the object of this research to investigate the nature of these proteins in *S.pombe* and they will be denoted throughout this thesis as proteins p46 and p27. The temperature-sensitive cell size mutant of *S.pombe*, wee 1.50 (Nurse, 1975) has been of great value in this work and it will be necessary to explain current ideas on cell cycle control in *S.pombe* in order to understand the contrasting behaviour of wild type and wee 1.50 cells during experiments described in Section 3.1.

1.2 Cell Cycle Control in *S.pombe*

1.2.1. The Cell Cycle of *S.pombe*

Fig. 1.2 shows a photomicrograph of actively dividing cells of *S.pombe*. The cells are cylindrical with rounded ends (ca. 10 μm long and 3.5 μm in diameter) and grow mainly in length during the cell cycle (May & Mitchison, 1986). Nuclear division (M phase) take place at 0.75 of the cycle. At 0.85 of the cycle a cell plate, or septum, appears. This is visible as a bright line under phase-contrast microscopy and is located centrally across the width of the cell. (Fig. 1.2, arrows). The cell plate lasts until the end of the cycle when the daughter cells separate physically. The pre-synthetic

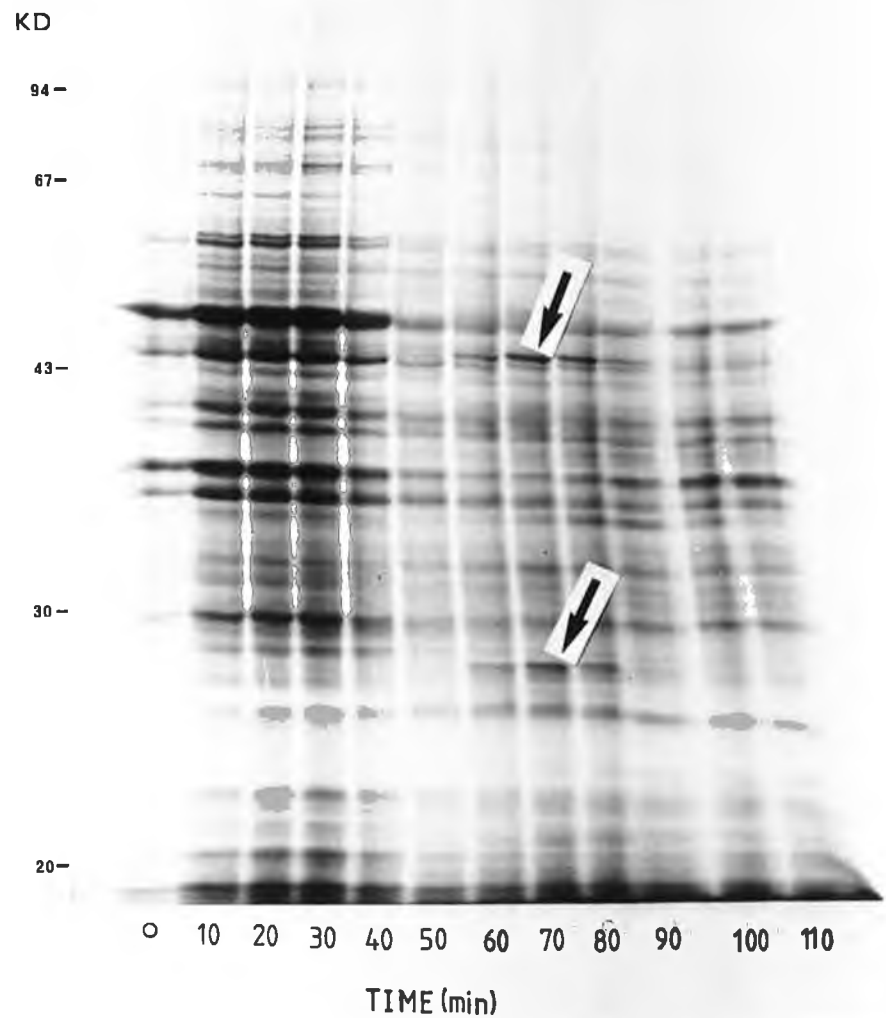


FIG. 1.1 Differential Protein Synthesis during Nitrogen Starvation of Fission Yeast

Cytoplasmic protein synthesis followed at 10 min intervals during nitrogen starvation at 32°C of the fission yeast *Schizosaccharomyces pombe*, reveals the differential synthesis of two polypeptides (arrow) at approx. 60-90 min after the initiation of starvation.

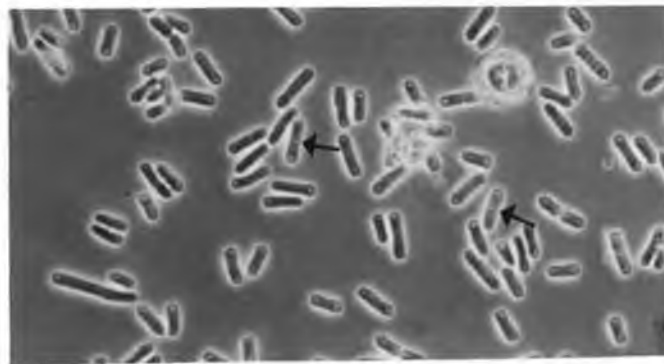


FIG. 1.2 Actively Dividing Cells of *S. pombe*

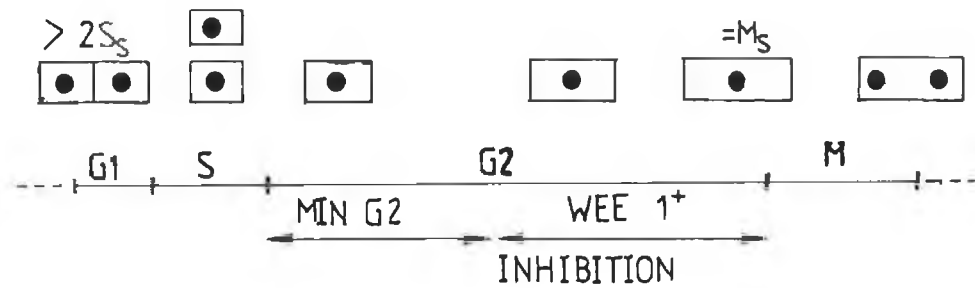
Photomicrograph of cells of *S. pombe* in exponential growth in EMM2. Under phase-contrast microscopy the cell plate (arrows) is visible as a bright line located centrally across the width of the cell.

G1 phase is very short (ca 0.1 of the cycle) and DNA synthesis (S-phase) occurs right at the end of the cycle. Thus, cells spend most of the cycle (ca 0.7) in the post-synthetic G2 phase. The cell cycle of *S.pombe* has been reviewed in depth by Mitchison (1970).

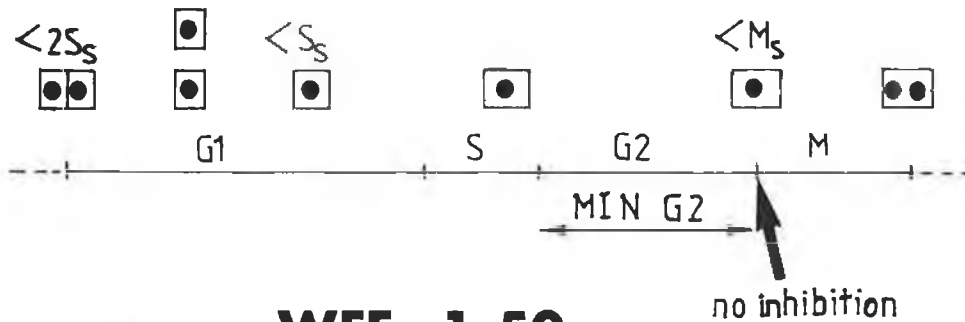
1.2.2. A Model for Cell Cycle Control

From the analysis of size mutants and from nutritional-shift experiments (Fantes, 1984; Nurse, 1975; Fantes & Nurse, 1977 & 1978; Nurse & Thuriaux, 1977) the following model of cell cycle control in *S.pombe* has emerged (Fig. 1.3). There are two size-related controls acting over two separate events in the cycle. The first (S_g) acts at S phase requiring that cells reach a certain minimal size before DNA synthesis can be initiated. In wild type cells under normal conditions of growth this size control is unexpressed, or "cryptic", because by the time cell plate formation takes place the unseparated daughter cells are already greater than the critical size required for initiation of DNA synthesis. The second size control (M_g) regulates the size at which cells enter mitosis. The size at which the mitotic control is set depends on the temperature of incubation and on the nutritional status of growing cells such that at high (but still favourable) temperatures and in rich medium, the critical size for entry into mitosis is greater than under sub-optimal growth conditions. Thus, the observed variation of cell size at nuclear division with growth rate which is a feature of most organisms, both procaryotic and eucaryotic, is achieved in fission yeast by a thermally and nutritionally regulable size control over mitosis.

Much of the evidence for this model of cell cycle control in *S.pombe* comes from the study of cell size mutants which are altered in the co-ordination of growth and division. The most notable of these is the temperature-sensitive single gene mutant, *wee 1.50*. At the restrictive temperature this mutant grows at much the same rate as the wild type but divides at half the wild type size. Although the molecular machinery which comprises the mitotic sizing mechanism in wild type cells of *S.pombe* is poorly understood, the WEE 1+



WILD TYPE



WEE 1-50

S_s — size (constant) reqd. for initiation of DNA synthesis

M_s — size (variable) reqd. for initiation of mitosis

FIG. 1.3 A Model for Cell Cycle Control in *S. pombe*

See text (Section 1.2.2) for explanation.

gene product is thought to constitute a negative regulatory element which acts by inhibiting mitosis until a critical cell size is achieved. In the absence of a functional WEE 1⁺ gene product, the G2 phase becomes compressed to a minimum time and mitosis is initiated at a reduced cell size. As a consequence of this, however, wee 1.50 cells at cell plate formation and cytokinesis are smaller than the size required for initiation of DNA synthesis. The G1 phase of wee 1.50 cells at the restrictive temperature thus becomes expanded and the S phase is delayed.

Knowledge of this model of cell cycle control in *S.pombe* is required to explain the contrasting behaviour of wild type and wee 1.50 cells during the nutritional shift-down and temperature shift-up experiments described in Section 3.1. The nature of the results obtained in this Section, however, is such as to suggest a "stress" role rather than a "division" role for proteins p46 and p27. It will therefore be of value to offer a brief review of stress responses in yeast and other organisms, with particular emphasis on the heat shock response.

1.3 The Response of Yeast to Heat Shock and Other Stresses

1.3.1. The Induction of Heat Shock Proteins

Since the now classical observation by Ritossa (1962) that shifting larvae from 20°C to 37°C results in a distinctly altered pattern of puffs in polytene chromosomes of *Drosophila*, the so-called heat shock response has been documented for a wide variety of organisms, both procaryotic and eucaryotic. In fact, the response has been observed in every species examined from bacteria to man (Craig, 1985; Schlesinger *et al*, 1982). The highly conservative nature of the response provides both reason and justification for the intensity with which it has been investigated in recent years.

The heat shock response describes the reaction of cells to a sudden increase in temperature, whereupon the synthesis of a group of proteins, termed heat shock proteins (HSPs) becomes enhanced. Another factor in the response is the concomitant repression of synthesis of a large number of proteins which were actively synthesized before the temperature shift. (These have been called "heat stroke proteins") (Ludwig *et al.* 1982). The exact kinetics of induction of HSPs and repression of pre-shift proteins depend on the exact conditions of heat shock. For example, in the budding yeast *S.cerevisiae*, it has been demonstrated that, following a temperature shift from 23°C to 36°C, maximum changes in synthetic rates are attained 20-30 min after the shift and pre-shift synthetic patterns are regained after 60-90 min (McAlister *et al.*, 1979). This example raises several important points about the heat shock response. Firstly, it serves to emphasize the transient nature of the response. Secondly, the magnitude of the response, at least in terms of the induction of HSPs, appears to be unaffected by returning cells to 23°C after 20-30 min (McAlister & Finkelstein, 1980). Thus, it is not temperature *per se* which induces the response, but rather the shock imposed by the sudden change in temperature. Thirdly, since yeast grows well at both 23°C and 36°C, the example demonstrates that the heat shock response can be engaged by alteration of temperature even within the normal growth range.

Although, by definition, heat shock is the one universal stimulus which induces synthesis of HSPs, many other stimuli are known to induce the synthesis of either all of the HSPs or a subset of them. An extensive list of inducers in a number of species has been published (Ashburner & Bonner, 1979) and these include: recovery from anoxia, treatment with heavy-metal ions, arsenite, amino acid analogues, hydrogen peroxide, ethanol, uncouplers of oxidative phosphorylation and many more. Therefore, the response might be more appropriately referred to as a stress response. However, the induction of HSP synthesis by non-thermal means does not give as consistent and universal a response as temperature shift. For

example, ethanol has been shown to induce HSPs in yeast (Plesset *et al*, 1982), and Chinese hamster fibroblasts (Li & Werb, 1982) but not in *Drosophila* or HeLa cells. Also, in view of the results presented in this thesis, there is no report citing nitrogen stress as an inducer of HSP synthesis.

1.3.2. The Structure of Heat Shock Proteins

The heat shock response in *Drosophila* is the most thoroughly characterized. In *D.melanogaster*, there are seven major heat-induced proteins designated: HSP 83, HSP 70, HSP 68, HSP 27, HSP 26, HSP 23, and HSP 22, the proteins being named according to their apparent molecular weights on SDS-polyacrylamide gels. These seven proteins have been placed into three families based upon structural homologies. HSP 83 is encoded by a single gene and is the sole member of the first group. The second group is composed of HSP 70 and HSP 68. The *D.melanogaster* genome usually contains five closely related genes at two distinct loci which encode HSP 70. The third group includes the four related small HSPs: HSP 27, HSP 26, HSP 23 and HSP 22.

The heat shock proteins show a remarkable conservation throughout evolution. Nearly all organisms induce the synthesis of proteins in the size ranges 80-90 kd, 65-75 kd and 15-30 kd. Thus, by a variety of criteria, including sequence analysis, tryptic peptide mapping and cross-reactivity with antibodies, homologies have been established between the *Drosophila* HSP 83 protein and HSPs of similar sizes derived from *E.coli*, yeast, frog, rodent, chicken and human cells (Kelley & Schlesinger, 1982). The most abundant HSP of *Drosophila*, HSP 70, also has homologous counterparts in a wide variety of species. In the case of *E.coli*, the HSP 70 homologue has been identified as the dnaK gene product (Bardwell & Craig, 1984). Similarly, not only do the small HSPs of *Drosophila* show extensive amino acid sequence homology to each other as found by Ingolia & Craig (1982), but they also show the same homologies to the small HSPs synthesized by nematodes (Rusznak *et al*. 1983) and frogs

(Bienz, 1984). In addition, these small HSPs all show sequence homology to the bovine α - crystallin chain (Ingolia & Craig, 1982), a highly polymeric protein which, in the form of large aggregates, perform a major structural role in determining the properties of the eye lens. How the formation of cytoplasmic aggregates of the small HSPs might be valuable to stressed cells is, however, not quite clear.

1.3.3. The Function of Heat Shock Proteins

While HSPs are well characterized at the level of expression and structure, their functions remain relatively obscure. Given the stressful conditions which trigger their induction, it is a reasonable *a priori* assumption that HSPs must somehow protect cells from the otherwise lethal effects of heat and a variety of potentially toxic chemicals. This concept has been borne out by the observed correlations between the synthesis of HSPs and the acquisition of resistance to what would normally be lethal heat-shocks. Thus, McAlister and Finkelstein (1980) have shown that a rapid shift of yeast cells from 23°C to 36°C results in protection from death due to extreme heat treatment (5 min at 52°C). The acquired thermotolerance was shown to be dependent on protein synthesis through the use of cycloheximide and mutants temperature sensitive for RNA transport from the nucleus to the cytoplasm. Moreover, the kinetics of acquisition of thermotolerance were such as to reflect the kinetics of induction of HSP synthesis. Similar observations have been made for a variety of organisms including *Drosophila* and mammalian cells.

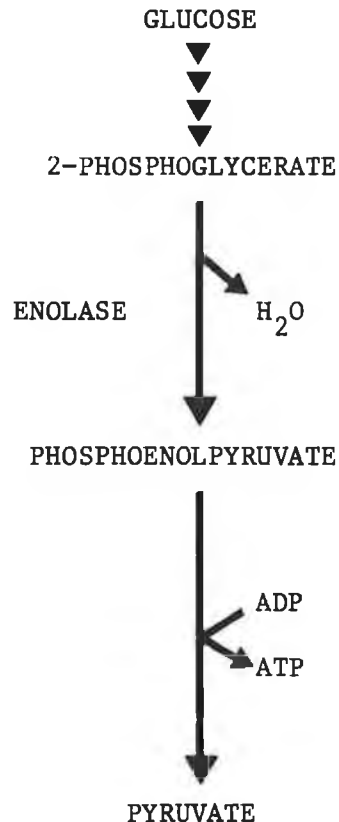
The idea that HSPs are responsible for thermotolerance is further substantiated by observations that stimuli other than heat which result in the synthesis of HSPs also confer thermotolerance (Plesset *et al*, 1982; Li & Werb, 1982). The reciprocal effect - the induction of resistance to chemical stresses by heat treatment - has also been shown (Velazquez & Lindquist, 1984).

The question remains, however, as to which HSPs are involved in conferring thermotolerance. That not all HSPs are so required has

been shown by a number of studies with cloned heat shock genes. Finkelstein and Strausberg (1983) have reported that the over expression of the HSP 90 gene, introduced into yeast cells on a multicopy plasmid vector, does not alter the sensitivity to lethal heat shock. Conversely, using site-directed mutagenesis techniques with cloned genes of the HSP 70 family, Craig and Jacobsen (1984) have shown that the lack of functional HSP 70 does not effect the ability of pre-heated yeast cells to withstand lethal heat shock. In *Dictyostelium*, a mutant has been isolated which fails to become thermally resistant following mild heat shock (Loomis & Wheeler, 1982). Analysis of the mutant shows that while it continues to synthesize moderate amounts of HSP 70, it fails to synthesize any small HSPs at all. These observations argue against the role of HSP 90 and HSP 70 in thermoprotection and suggest that this function may be with the small HSPs. An argument against this, however, is provided by the very recent demonstration by Petko and Lindquist (1986) that site-directed mutations in the unique HSP 26 gene of *S.cerevisiae* have no effect whatsoever on the acquisition of thermotolerance in pre-treated cells.

Some compelling work in the area of HSP function has been performed by Iida & Yahara and their results will be alluded to frequently in this report. Firstly, these workers have found that, in *S.cerevisiae*, six out of a total of thirteen HSPs (including HSP 48) are durably induced when cells enter the resting state of the cell cycle, or G_0 (Iida & Yahara, 1984a). This observation has also been shown to apply to certain higher eucaryotic cells including chick embryonic fibroblasts and mouse T lymphocytes. Iida and Yahara (1984a) found that G_0 yeast cells were much more resistant to lethal heat shock than growing cells, probably because of the accumulation of some or all of the above six HSPs. In addition, Iida and Yahara (1984b) have isolated a heat-shock-resistant mutant (hsr 1) which is defective in a gene designated HSR 1 and which constitutively synthesizes six proteins, including two isoforms of HSP 48. The hsr 1 mutant reveals unusual properties involved in growth control in addition to the selected property that it is ca. 1000-fold more resistant to lethal shock than the parental HSR 1+ strain. Because HSP 48 is the

only protein that is commonly induced in both growing hsr 1 cells and pre-heated or G₀-arrested HSR 1 cells, all of which are highly thermotolerant, Iida & Yahara have proposed that HSP 48 is solely responsible for the acquisition of thermotolerance in *S.cerevisiae*. They have additionally shown that, by a variety of criteria, HSP 48 is an isoprotein of the glycolytic enzyme, ENOLASE (Iida & Yahara, 1985), which catalyses the following reaction:



This finding is so surprising that it has been suggested that some function other than enolase activity may be ascribed to HSP 48 in its thermoprotective role, although it is not clear what the details of such a role might be.

Conceivable mechanisms for the repair of (reversible) damage caused by heat and other forms of stress can, however, be conjectured. Localization studies with monoclonal antibodies have detected a concentration of HSP 70 in nucleoli of heat-shocked cells of *Drosophila* (Velazquez & Lindquist, 1984). Artificial constructs linking the HSP 70 gene to a strong constitutive promoter have shown that although nucleoli are visibly altered by heat shock, they recover their morphology much faster in strains where HSP 70 is overproduced (Pelham;

1985 and references therein). These and related experiments suggest that HSP 70 might aid reassembly of damaged pre-ribosomal ribonuclear proteins (RNPs).

HSP 90 has been found in a complex with newly synthesized Rous sarcoma virus *src* protein in infected chick cells (Brugge *et al*, 1981; Opperman *et al*, 1981). As the *src* transforming protein is normally associated with membranes, it has been suggested that HSP 90 recognizes the hydrophobic nature of the protein and keeps it in solution until it reaches the membrane. It is possible then, although there is no direct evidence for it, that HSP 90 might also recognize the hydrophobic surfaces of denatured proteins after heat shock and, by binding to them, prevent them from forming an insoluble and potentially damaging precipitate.

Although neither HSP 70 or HSP 90 appear to be essential in the acquisition of thermotolerance, these examples at least serve to bring us closer to plausible molecular roles for heat shock proteins.

1.4 Aims of this Research.

Having thus briefly reviewed the areas upon which this thesis impinges, it remains only to emphasize that the work represents a piece of investigative science into the nature of two proteins. Both the experimental motive which revealed their appearance, and the circumstances under which they appear, strongly suggest that these proteins may have an interesting and potentially fundamental biological function. It has been the purpose of this research to assess that function in whatever way that might turn out to be possible.

2. MATERIALS & METHODS

2.1 Organisms, Media and General Culture Conditions

2.1.1 Organisms

The following haploid strains of *Schizosaccharomyces pombe* were used:

- a) The wild type strain, 972h⁻, and
- b) The single gene mutant strain, wee 1.50h⁻.

Wee 1.50h⁻ was derived from the wild type strain as described by Nurse (1975). Both strains were received from Dr. P.Fantes, Univ. of Edinburgh, Edinburgh, Scotland.

The wild type haploid strain of *Saccharomyces cerevisiae*, X2180, was also used. This strain was received from Prof. P.Whittaker, St. Patrick's College, Maynooth.

2.1.2 Media

The following media were used:

- a) YEPG
- b) EMM2 (Edinburgh Minimal Medium No. 2) (Mitchison, 1970 - as modified by Fantes & Nurse, 1977).

The compositions of these media are described in Tables 2.1 and 2.2, respectively. YEPG was sterilized at 15 psi for 15 min; EMM2 was sterilized at 10 psi for 10 min with the exception of vitamins which were filter sterilized and added to the medium last.

TABLE 2.1: Composition of YEPG

<u>INGREDIENTS</u>	<u>Amount per litre distilled water</u>
Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Agar (Technical, No. 3 - Oxoid)*	20 g *For solid medium only.

TABLE 2.2: Composition of EMM2

<u>Ingredients</u>	<u>Amount per litre dH₂O</u>
Glucose	10 g
NH ₄ Cl	5 g
Na ₂ HPO ₄	1.42 g
Potassium phthallate	3.06 g
MgCl ₂ ·6H ₂ O	500 mg
CaCl ₂	10 mg
KCl	1 g
Na ₂ SO ₄	10 mg
H ₃ BO ₃	0.50 mg
MnSO ₄ · H ₂ O	0.40 mg
ZnSO ₄ · 7H ₂ O	0.40 mg
FeCl ₃ · 6H ₂ O	0.20 mg
H ₂ MoO ₄ · H ₂ O	0.16 mg
KI	0.10 mg
CuSO ₄ · 5H ₂ O	0.04 mg
Citric Acid	1.00 mg
Inositol	10 mg
Nicot ⁱ nic acid	10 mg
Pantothenate (Ca ²⁺)	1 mg
Bioti ⁿ n	0.01 mg

2.1.3 General Culture Conditions

Stocks of cells were maintained on YEPG slopes, subcultured monthly and stored at 0 - 5°C. Viability staining (with 0.01% methylene blue in aqueous 2% sodium citrate) indicated no loss of cell viability under this subculture regime.

Experimental cultures were routinely established as follows: Cells were inoculated from a slope into fresh, liquid EMM2 and grown overnight in a shaking water-bath (100 revs/min approx.). The following day, log-phase cells from this culture were used to inoculate experimental flasks to cell densities of 1×10^6 cells/ml (unless otherwise indicated). Experimental treatments were initiated at cell densities of 5×10^6 cells/ml after 2 - 3 generations in exponential growth.

2.2 Cell Number Determinations

Cell number determinations were achieved by repeated counts (2 - 4 readings of 150 - 300 cells) in a haemocytometer (Improved Neubauer model) under phase - contrast microscopy (Nikon, Type 104).

2.3 Cell Morphology Examinations

Phase-contrast microscopy reveals the cell plate as a bright line centrally placed across the width of the cell and thus allows easy estimation of the percentage of cells with cell plates, or cell plate index (CPI).

Photomicrographs were taken with a camera fitted to the body of the microscope. The film used was Kodak EPT 135 - 136.

2.4 Nitrogen Starvation Protocol

Exponentially growing cultures (5×10^6 cells/ml) were filtered onto Millipore membranes (Type HAWG, pore size $0.45 \mu\text{m}$) and immediately resuspended in equal volumes of pre-warmed nitrogen-free EMM2 (made by simple omission of NH_4Cl from the recipe).

2.5 Temperature Shift Protocols

2.5.1 Temperature Shift of wee 1.50 cells ($25^\circ\text{C} \rightarrow 36^\circ\text{C}$)

The shift between permissive and restrictive temperature conditions of wee 1.50 cells was performed increasing the temperature of growing cultures GRADUALLY from 25°C to 36°C over a ten minute period.

2.5.2 Heat Shock

Temperature shifts designed to achieve heat shock were performed by the RAPID change of cultures between water-baths pre-set to different temperatures (either 25°C and 36°C, or 32°C and 41°C).

2.5.3 Lethal Heat Shock and the Analysis of Thermotolerance

Resistance to lethal heat-shock was assessed as follows:

1 ml aliquots of exponentially growing cultures were pipetted aseptically into 100 ml flasks (pre-heated to 52°C) and incubated at 52°C for 5 min . The use of such small volumes in such relatively large flasks was designed to maximize heat transfer and thus minimize the time required for temperature change of the culture aliquot. Immediately after heat shock, 0.1 ml samples were extracted and serially diluted in 5 - 10 ml volumes of sterile distilled water warmed to pre-shock temperatures. 0.1 ml aliquots of appropriately diluted cultures were then plated, in duplicate, onto EMM2 agar plates and incubated at pre-shock temperatures. Dilutions were calculated to yield 100 - 300 colony-forming-units per plate. Unless otherwise indicated, unshocked control samples were similarly diluted and plated and estimates of percentage survival were based on the total number of colonies on these control plates.

2.6 Protein Synthesis

2.6.1 Radiolabelling Procedures

Upon treatment (eg. nitrogen-starvation, heat shock) experimental cultures were divided into aliquots (usually 30 ml) and placed in 100 ml conical flasks. ³⁵S-methionine was added sequentially to each flask to give a net radioactive concentration in the range 1 - 3 µCi/ml. At the end of each labelling period (usually 15 min .) culture aliquots were filtered under suction onto Millipore membranes (Type HAWG, pore size 0.45 µm) and washed twice with 50 ml ice-cold buffer

(10mM Tris - HCl, pH 6.8). Filtered cells were stored at -20°C before homogenization and electrophoresis.

2.6.2 Incorporation Studies.

The incorporation of radiolabel into acid-precipitable material was assessed as follows:

After labelling and before filtering culture aliquots, duplicate 50µl samples were spotted onto Whatman DE81 filters and dried immediately in a 75°C oven to prevent further incorporation. These filters were washed twice in ice-cold 5% w/v trichloroacetic acid (TCA) for 15 min , and once in boiling 5% TCA for 15 min . After drying, filters were placed in vials containing 10 ml scintillation fluid (3g/l PPO, 0.1g/l POPOP, 30% v/v methanol and 70% v/v toluene) and counted on the ¹⁴C channel of a Beckman LS7500 liquid scintillation counter.

Where incorporation data are expressed per unit weight of protein, protein assays were performed as described in Section 2.7.

2.6.3 Preparation of Samples for Electrophoresis.

All solutions used were ice-cold and, between all manipulations, samples were kept on ice.

Stored cells were washed off filters with 1ml Homogenization Buffer (HB, 10mM Tris - HCl, pH 6.8), pelleted by centrifugation and re-suspended in 250 µl HB . Unless other wise indicated in the text, the following protease inhibitors were added:

pepstatin A, chymostatin, antipain (10 µl of a 2 mg/ml solution of each in dimethylsulphoxide (DMSO) and phenylmethylsulphonyl fluoride (PMSF, 5 µl of a 30 mg/ml solution in DMSO). Cell suspensions were transferred to microfuge tubes and 0.75 g 40 mesh glass beads added. Cells were broken by agitation on a vortex mixer (Vortex-Genie , Scientific Industries Inc., USA) for 1 min

intervals with intermittent cooling on ice. The procedure was repeated (5-10 times) until the majority of cell walls (ie. greater than 90%) were ruptured as estimated by phase contrast microscopy. After breakage, homogenates were removed from the beads, cell debris were pelleted, and protein supernatants transferred to fresh microfuge tubes and freeze dried. The solid material was resuspended in 50 μ l electrophoresis sample buffer (0.0625 M Tris -HCL at pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and treated in a boiling water-bath for 4 min. Prior to their application to the gels, prepared samples were centrifuged (13,000 rpm for 10 min) to remove any contaminating particulate matter. Also in order to allow the equalization of cpm/lane, duplicate 5 μ l aliquots were spotted onto Whatman DE81 filters and radioactivity counted according to the procedure described in 2.6.2.

2.6.4 SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

A vertical electrophoresis system (LKB) was employed and the method of Laemmli (1970) was followed. Electrophoresis was performed in 10% polyacrylamide resolving gels (pH 8.8) and 3% polyacrylamide stacking gels (pH 6.8). The running buffer consisted of 28.8 g/l glycine, 6.0 g/l Tris, and 1.0 g/l SDS in aqueous solution. Gels were run at 40 mA for 4 hr at 15°C. Low molecular weight protein markers (from Pharmacia) were used throughout.

Gels were fixed and stained simultaneously for 15 min at 36°C with a solution of 0.25% w/v Coomassie Brilliant Blue R-250 in a mixture of methanol, water, and glacial acetic acid (5:5:1). De-staining was performed overnight at 36°C with gentle shaking in an aqueous solution of 7% glacial acetic acid and 0.7% glycerol.

2.6.5 Autoradiography

Autoradiographic enhancement of de-stained gels was achieved by in-

cubation in 22.2% PPO in DMSO for 3-4 hours at 36°C after two preliminary 30 min incubations in DMSO alone. PPO was precipitated in the gels by placing them under running water for a minimum of 1 hr. Gels were then placed on a sheet of filter paper (Whatman No. 1), covered with a layer of cling-film and dried under suction in a Pharmacia Gel Slab Drier (GSD-4) for at least 4 hr at 70°C. Dried gels were exposed to Kodak X-ray film. Films were developed (in Kodak LX 24), fixed (in Kodak FX-40), dried and photographed with a Nikon F3 camera onto Kodak Technical film (Pan 2415, 135 mm).

2.7 Immunoblotting Analysis

2.7.1 Preparation of Samples

Although culture aliquots were not radiolabelled, cell samples for immunoblotting analysis were obtained as described in 2.6.1. Cells were homogenized as described in 2.6.3. Protein supernatants were assayed by the Bradford method (Bradford, 1976) (using the Bio-Rad Protein Assay kit) and were generally found to be in the range 2-4 mg/ml. Supernatants were not freeze-dried, but rather appropriately diluted (see Section 3.2.3) with Electrophoresis Sample Buffer and boiled for 4 min. Equal amounts of protein were applied to each lane of the gel and gels were run as described in 2.6.4.

2.7.2 Electroblotting

After SDS-polyacrylamide gel electrophoresis, gels were placed on nitrocellulose sheets (0.45 μ m pore size in roll form, Biorad) cut to appropriate size and pre-soaked in blotting buffer (see below). The combination was sandwiched between two scouring pads (Scotch Brite) backed on each side by close-fitting plastic grids. This assembly was placed in a Trans-Blot Transfer Cell (Bio-Rad) filled with blotting buffer (a 1:4 dilution v/v with high-grade methanol of the Tris-glycine electrophoresis running buffer described in 2.6.4). Electroblotting

was performed at 100 mA for a minimum of 12 hr at room temperature with the nitrocellulose sheet facing the positive electrode.

2.7.3 Staining of Molecular Weight Markers

After electroblotting, nitrocellulose sheets were reversibly stained with amido black (0.1% in distilled water) for 1-2 min in order to allow the positions of molecular weight markers to be recorded. The stain was then easily removed by frequent washes in distilled water.

2.7.4 Immunological Detection of Protein

After de-staining, nitrocellulose blots were soaked in Blocking Solution (BS: 10 mM Tris, 150 mM NaCl, and 0.5% v/v Tween 20 in aqueous solution, pH 7.5) for 2 hr in order to saturate additional protein binding sites. Blots were then incubated for 3 hr at 37°C with rabbit anti-yeast HSP 48 antiserum (received from Dr. H. Iida, Dana-Farber Cancer Institute, Boston, USA) diluted 1:1000 in BS. Non-specifically bound primary antibody was removed by washing overnight with frequent changes of BS. Blots were next incubated at 37°C for 2 hr with a 1:1000 dilution in BS of alkaline phosphatase conjugated goat anti-rabbit IgG (obtained from Sigma) before a second overnight washing with frequent changes of BS. Prior to substrate addition, the pH of nitrocellulose sheets was increased by several washes with 10 mM Tris-HCl buffer, pH 8.9. Per 10 ml, the development mixture consisted of:

- a) 1 ml of a 0.1% solution of nitro-blue tetrazolium in 10 mM Tris HCl, pH 8.9
- b) 0.4 ml of a 5 mg/ml solution of p-nitrophenyl phosphate (toluidine salt) in dimethylformamide
- c) 40 µl of an aqueous 1M solution of $MgCl_2$ and
- d) 8.86 ml 0.5 M Tris-HCl buffer, pH 8.9.

The development reaction was stopped by the addition of water. Nitrocellulose blots were finally dried between filter papers sandwiched together by glass plates and placed in a 75°C oven for 30 min.

3. RESULTS & DISCUSSION

3.1 CHAPTER 1: GENE EXPRESSION & CELL DIVISION IN *S.pombe*

Introductory Remarks

As explained in Section 1.1, Walker and Kramhoft (1984) have shown that upon nitrogen-starvation of actively dividing cells of *S.pombe* there ensues an accelerated burst of division at a reduced cell size and that two proteins, p46 and p27, are observed to appear transiently during the period of rapid cell number increase.

The strategy adopted initially in these investigations rested on an attempt to distinguish between two hypotheses:

Proteins p46 and p27, transiently appearing during the period of accelerated division that follows nitrogen-starvation of the fission yeast, *Schizosaccharomyces pombe*, are either:

- A) Associated with the acceleration into cell division, being essential for, or facilitating in some way, the division process;
- or
- B) Synthesized as a direct response to nitrogen starvation.

The temperature-sensitive cell size mutant of *S.pombe*, wee 1.50, (Nurse, 1975) has proven most useful in testing these alternative hypotheses because its behaviour is such as to furnish examples of:

- a) acceleration into division without nitrogen-starvation (following a shift from permissive to restrictive temperature conditions - Section 3.1.2) and
- b) nitrogen starvation without acceleration into division (starvation at the restrictive temperature - Section 3.1.3).

Before describing the results obtained with this mutant, the results of wild type studies will be presented.

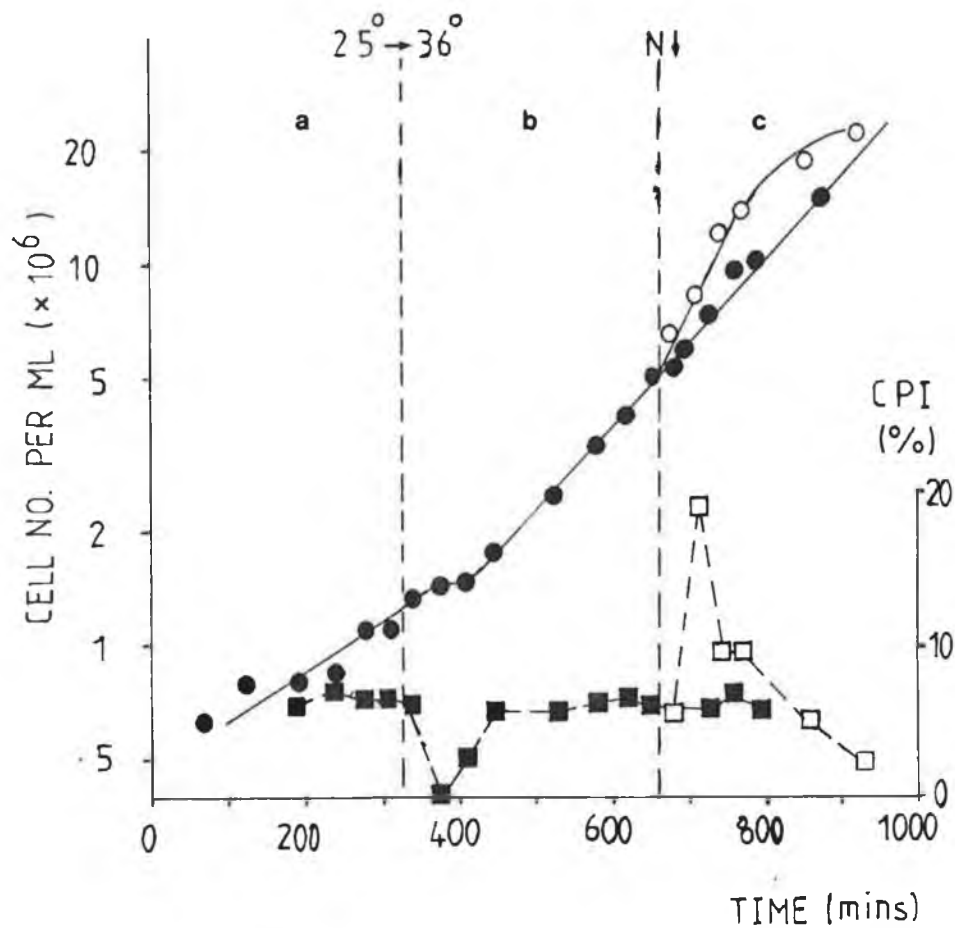
3.1.1. Nitrogen Starvation of Wild Type *S.pombe* Cells

3.1.1.1 Kinetics of Cell Division during Nitrogen Starvation

Using the wild type haploid strain of *S.pombe*, 972h⁻, the work of Walker and Kramhoft (1984) was repeated under slightly elevated temperature conditions: 36°C rather than 32°C. The higher temperature was necessary for purposes of comparison with nitrogen-starvation experiments at the restrictive temperature (36°C) of *wee* 1.50 cells. For both these experiments cells were grown at 25°C for 2-3 generations before the shift to nitrogen-free medium. This procedure was followed because it is known that, under prolonged exposure to restrictive conditions, cultures of *wee* 1.50 accumulate abnormal cells and spontaneous diploids (Fantès & Nurse, 1978). In an effort to minimize heat shock (and therefore concomitant heat-shock protein synthesis) the temperature change was effected gradually over a 10 minute period.

Exponentially growing cells of strain 972h⁻ were inoculated into 400 ml EMM2 at a cell density of 0.5×10^6 /ml and propagated with shaking at 25°C (Fig. 3.1a). After approximately one cell doubling the temperature was increased gradually to 36°C. Cell number and cell plate indices were followed at regular intervals and are shown in Fig. 3.1a,b. Although protein synthesis was not followed during the period following temperature shift of wild type cells, it will be useful to explain the kinetics of cell number increase on the basis of the cell cycle control model outlined in Section 1.2.2.

Upon shift from 25°C to 36°C there ensues a temporary inhibition of cell division. This inhibition is manifested not only by a cessation of cell number increase, but also by a fall in the cell plate index (CPI) from 5-7% (the typical value for an asynchronous exponential culture) to zero. After the inhibition, cell division resumes at the increased steady rate (and larger cell size) typical of the higher temperature.



WILD TYPE

FIG. 3.1 Growth of *S. pombe* wild type strain 972h⁻ in EMM2 following temperature-shift and nitrogen starvation

- A) Exponential growth at 25°C;
 - B) Upon temperature-shift to 36°C, cells stop dividing (cell plate index drops to zero), cell elongation occurs, and then exponential growth resumes at the rate typical of the higher temperature (Nurse, 1975)
 - C) Upon nitrogen starvation, cells are accelerated into semi-synchronous division at a reduced cell size (see Fig. 3.2) as manifested by the kinetics of cell number increase (o-o) and by the "peaking" of the cell plate index (□--□).
- , ■ values for an unstarved control culture.

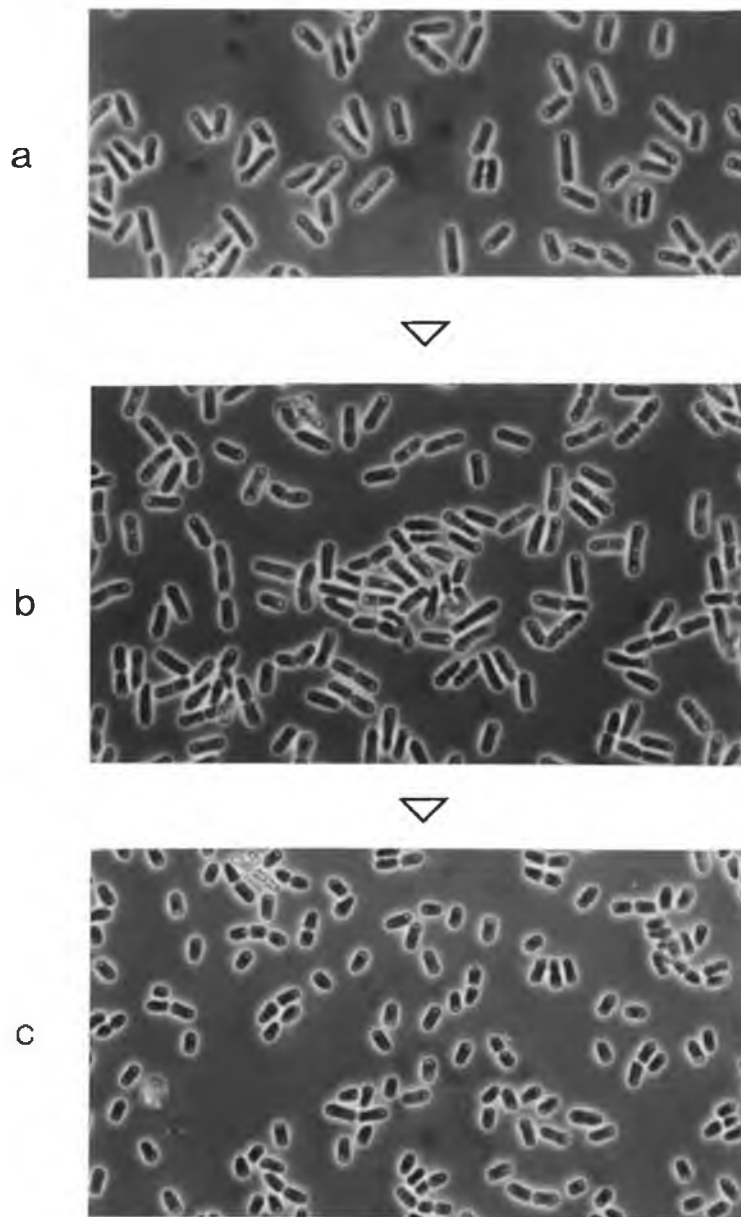


FIG. 3.2 Semi-synchronous, accelerated division at a reduced cell size following nitrogen starvation of wild type *S.pombe* cells

- a) cells growing exponentially at 36°C; cell plate index = 5-7%
- b) Semi-synchronous, accelerated division following nitrogen starvation; notice the high percentage of cells with cell plates.
- c) small cells produced after accelerated division in nitrogen-free medium.

This behaviour can be explained as follows: Upon shift to 36°C, the mitotic size control is re-set to a higher value. All those G2 phase cells which would, at 25°C, have been just ready to enter mitosis now find that they must continue growing to a larger size before doing so. The result is therefore a delay in the otherwise continuous entry of cells into nuclear and cell divisions.

At a cell density of 5×10^6 /ml, the culture was starved for nitrogen as described in 2.4. Aliquots (10 X 30 ml) were immediately withdrawn from this culture: 8 aliquots for protein radio-labelling purposes (see below) and 2 for cell counting purposes. To one of these last two aliquots (the unstarved control) solid NH_4Cl (0.15 g) was added to give the final concentration of ammonium ions typical of EMM2. Cell number and cell plate indices were followed in both starved and unstarved cultures throughout the radio-labelling procedure and the results are shown in Fig. 3.1c.

The acceleration of cells into division upon nitrogen-starvation is manifested by a rapid rise in the cell proliferation rate. Moreover, the concomitant sharp rise and fall of the cell plate index belies the semi-synchronous nature of the accelerated division. The process is illustrated photomicrographically in Fig. 3.2 (a-c) and this figure serves to emphasize the reduction in cell size at division of nitrogen-starved cells.

Fantes and Nurse (1977) have reported the acceleration of wild type cells into division at a reduced cell size upon nutritional shift-down from medium with glutamate as nitrogen source (which supports fast growth) to medium with proline as nitrogen source (which supports slow growth). They have explained this observation as follows: a substantial fraction of G2 phase cells which are not yet large enough to enter mitosis under the initial conditions are nevertheless large enough to do so under the poor growth conditions in which the nutritionally regulated mitotic size control becomes re-set to a lower value. The result is a semi-synchronous burst of accelerated division. Since nitrogen starvation is qualitatively similar to this nutritional shift-down, its

effect may be understood in the same terms.

3.1.1.2 Kinetics of Radiolabel Incorporation

Radio-labelling during the period of nitrogen-starvation was accomplished as follows: After an initial 10 min delay, ^{35}S -methionine was added in sequential fashion, at 12.5 min intervals, to each of the eight culture aliquots to give a net radioactive concentration in the range 1-3 $\mu\text{Ci/ml}$. Incorporation of radiolabel was ceased by filtering and washing the cells as described in 2.6.1.

Before discussing the results of electrophoresis it is appropriate to explain two points concerning the radiolabelling protocol adopted in this and subsequent nitrogen-starvation experiments. These are:

- a) The 10 minute delay between the initiation of starvation conditions and the first addition of radiolabel, and
- b) The use of variable radioactive concentrations of ^{35}S -methionine.

A preliminary nitrogen-starvation experiment was performed in which the incorporation of radiolabel into TCA-precipitable material over the initial 2-hour period of starvation was found to be highly variable. The kinetics of incorporation were assessed as follows: Upon re-suspension of cells in nitrogen-free medium, aliquots (8 X 30 ml) were withdrawn and radiolabelled sequentially at 15 min intervals. The net radioactive concentration of ^{35}S -methionine was uniform at 2 $\mu\text{Ci/ml}$. At the end of each labelling period, duplicate 50 μl samples of culture aliquots were spotted onto DE81 filters and dried immediately at 75°C to prevent further incorporation. These filters were then treated with TCA as described in Section 2.6.2 and counted in a liquid scintillation counter. The results are illustrated graphically in Fig. 3.3. Note that results are expressed per unit volume of culture rather than per unit weight of protein since there is little increase in cell protein during nitrogen starvation.

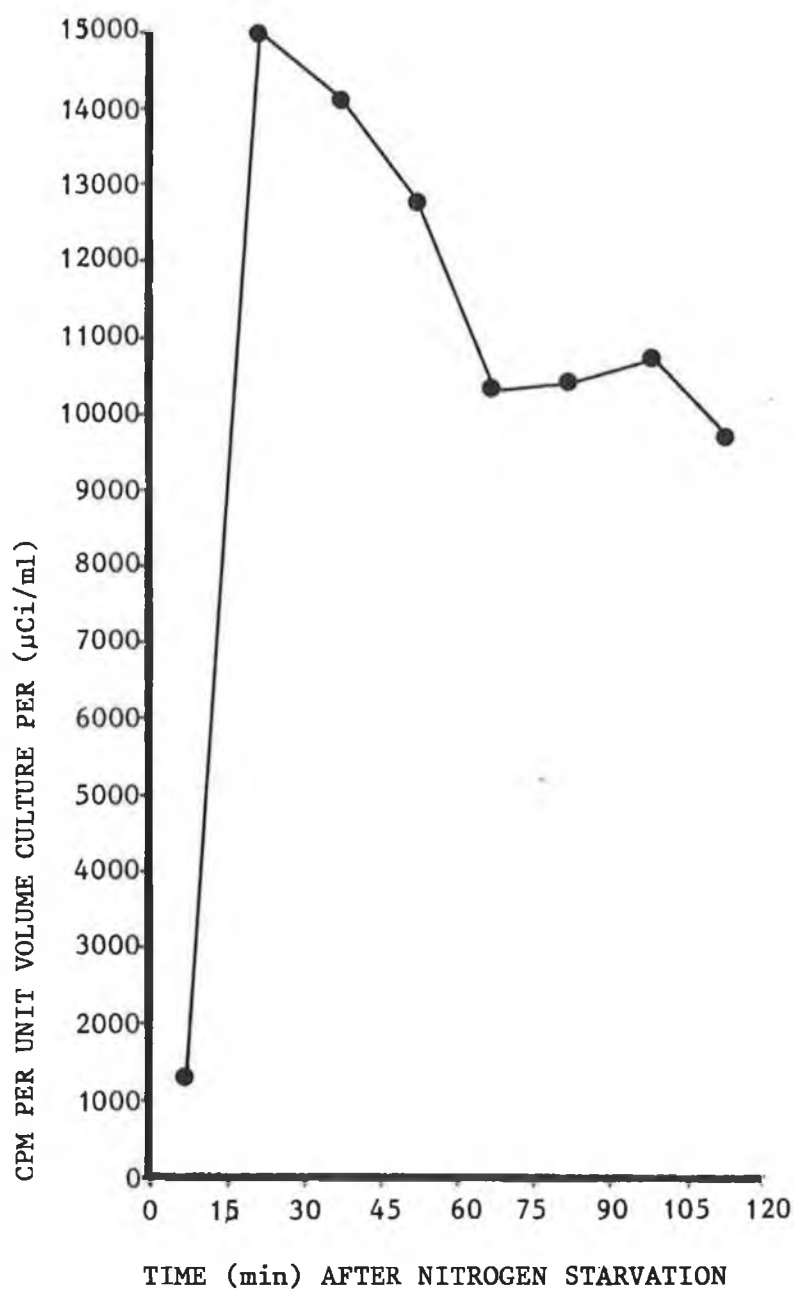


FIG. 3.3 Kinetics of incorporation of ^{35}S -methionine into TCA-precipitable material during nitrogen starvation of wild type *S.pombe* cells

Incorporation values are expressed as count per minute (cpm) per unit (50 μl) volume of culture per unit concentration ($\mu\text{Ci/ml}$) of radiolabel in the medium. With respect to the horizontal axis, values are positioned at the mid-point of each 15 min radiolabelling period.

It can be seen from Fig. 3.3 that there is a large increase in radio-label incorporation into acid-precipitable material within the first 15-30 min following nitrogen-starvation. The low initial rate of incorporation may reflect the fact that wild type cells of *S.pombe* are prototrophic, requiring no exogenous amino acids for growth. The subsequent increase suggests the induction of a general amino acid permease as has been shown to occur in *S.cerevisiae* (Grenson *et al*, 1970). Thereafter, the general trend in radiolabel incorporation is downward and this may reflect a decrease in permease activity or, alternatively, as in *S.cerevisiae* (Woodward & Cirillo, 1977), the mobilization of transaminase activity in starved cells resulting in metabolism of transported ^{35}S -methionine rather than its incorporation into protein.

The relationship between the radioactive concentration of ^{35}S -methionine in the medium and the eventual activity in acid-precipitable material in the cells was also determined. Following nitrogen-starvation, 4 X 10 ml aliquots were, after 15 min, labelled simultaneously with increasing concentrations of ^{35}S -methionine (1-4 $\mu\text{Ci/ml}$) for 15 min. Samples (50 μl) were treated as before with TCA and prepared for liquid scintillation counting. The results are shown in Fig. 3.4. It can be seen very clearly that there is a linear, or direct proportionality relationship between the radioactive concentration of label in the medium and the resultant activity of acid-precipitable fractions.

This result (although strictly applying only to the 15-30 min period of starvation) combined with the observed variability of ^{35}S -methionine incorporation during nitrogen starvation suggested that it might be possible to achieve a more uniform specific activity of radio-labelled protein during the 2 hr following starvation by appropriately varying the concentration of ^{35}S -methionine in each culture aliquot. In general, the strategy was to ignore the first 10 min period of starvation when incorporation was low, for the next 30-40 min to label at a concentration of 1.5 $\mu\text{Ci/ml}$, and for the following hour at twice this concentration. In this way, it was possible to achieve protein samples for electrophoresis within a small enough range of

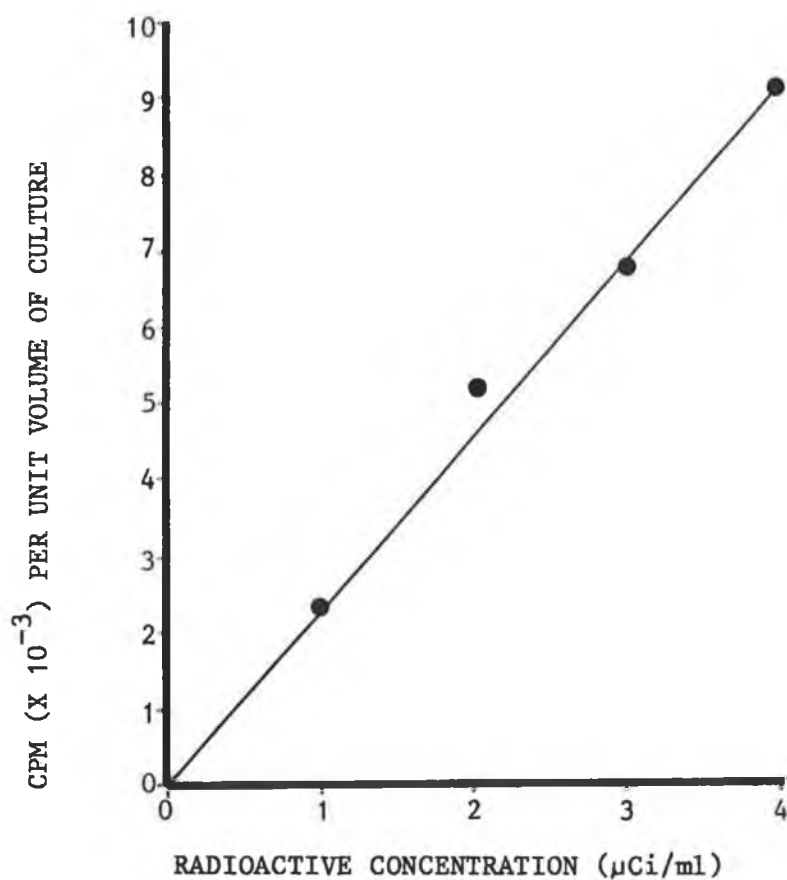


FIG. 3.4 Incorporation of ^{35}S -methionine as a function of concentration

The figure show the linear relationship, during the 15-30 min period following nitrogen starvation of wild type cells, between the radioactive concentration of ^{35}S -methionine in the medium and the resultant activity in TCA-precipitable material.

specific activities as to minimize variation in gel loading volumes.

3.1.1.3 Protein Synthesis during Nitrogen Starvation

Using this modified labelling procedure, protein-synthetic patterns during nitrogen-starvation of wild type cells of *S.pombe* were investigated and are shown in Fig. 3.5. The transient appearance of two polypeptide bands (indicated by arrows in lanes 5 & 6) can clearly be seen at 60-85 min after nitrogen-starvation. These bands describe polypeptides of apparent molecular weights 47 kd and 27 kd. Unlike the lower band, the upper band is faint and poorly resolved. This may be due to the omission of protease inhibitors during sample preparation for electrophoresis (see below). Nevertheless this result must be construed as a successful repetition of the original experiment by Walker and Kramhoft (1984).

Concerning the role of proteases, it is known that these enzymes play an integral role in the normal cellular metabolism of a wide range of organisms, including yeast (see Wolf, 1982 for review). Furthermore, it has been shown that nitrogen-starvation of *S.cerevisiae* actively stimulates protein turnover (Sumrada & Cooper, 1978), possibly due to the selective activation or derepression of a range of proteases. Therefore, it is perhaps doubly negligent to prepare cell homogenates from nitrogen-starved cultures in the absence of any form of protease inhibitor. Consequently, the nitrogen-starvation experiment of wild type cells was repeated by incubating cultures at the optimal growth temperature of 32°C and radiolabelling protein at 15 min intervals. Samples were homogenized in the presence of the following known protease inhibitors: pepstatin A (80 µg/ml), chymostatin (80 µg/ml), antipain (80 µg/ml), and PMSF (0.6 mg/ml) - see Section 2.6.3. Fig. 3.6 shows the resultant autoradiogram. When compared with Fig. 3.5, it is evident that the effect of the protease inhibitors is a general improvement in the resolution of all bands. Moreover, the effect on proteins p46 and p27 is dramatic; both bands again make a transient appearance 60-90 min after starvation, but in Fig. 3.6, their synthesis is seen with much greater

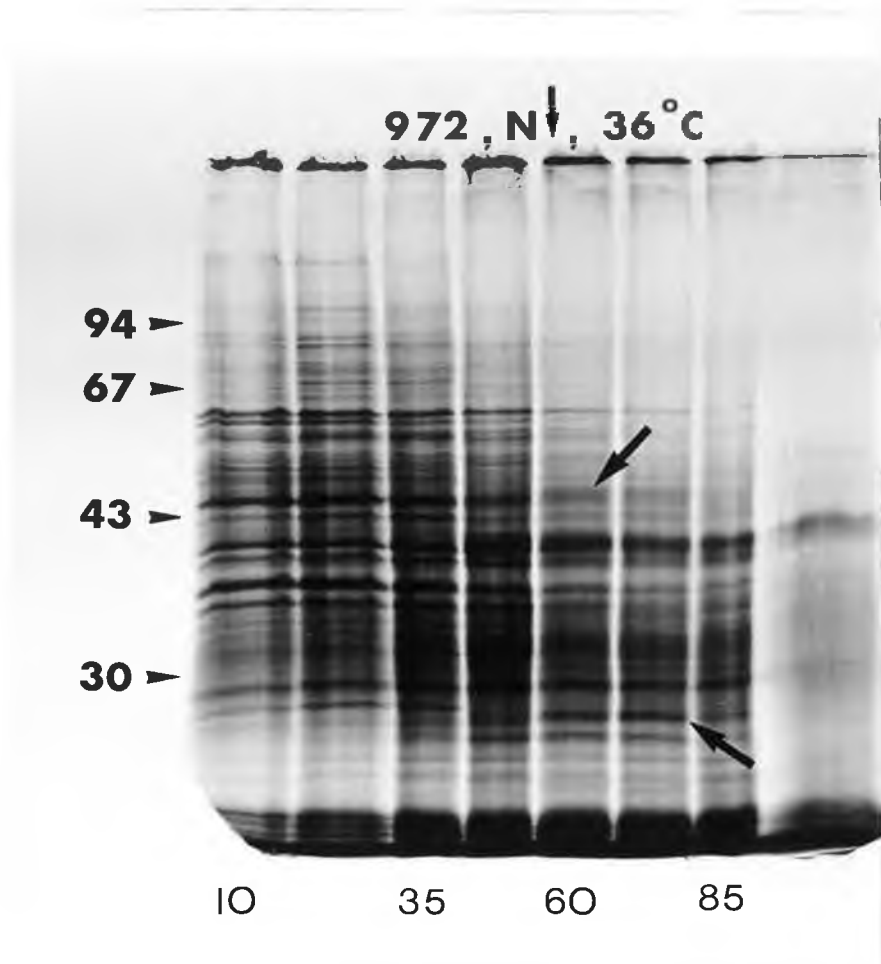


FIG. 3.5 Protein Synthesis during Nitrogen Starvation of Wild Type *S.pombe* cells

Cytoplasmic protein synthesis followed at 12.5 min intervals during the accelerated division that follows nitrogen starvation ($N\downarrow$) at 36°C of wild type cells (strain 972) reveals, at approx. 60-90 min, the transient appearance of polypeptides p46 and p27 (arrows) just as in Fig. 1.1. Protein samples were prepared in the absence of protease inhibitors (see text).

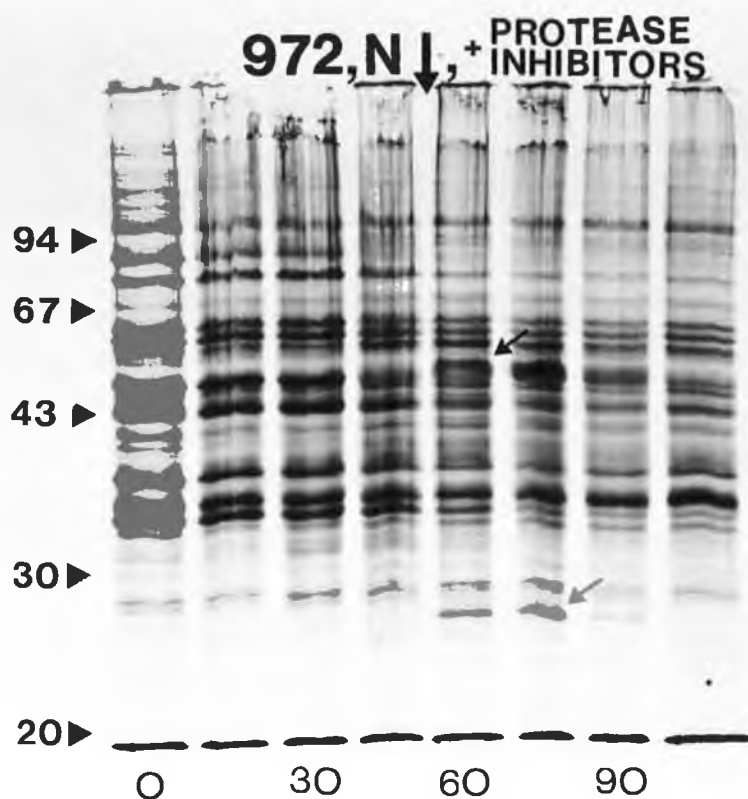


FIG. 3.6 The Effect of Protease Inhibitors

The use of protease inhibitors during the preparation of protein samples for electrophoresis dramatically increases the intensity of polypeptides p46 and p27 (arrows).

intensity and therefore seems all the more remarkable. Even without the facility for densitometric scanning, it appears visually that proteins p46 and p27 form two of the three most abundant polypeptide species present (the third lying around the 35 kd region).

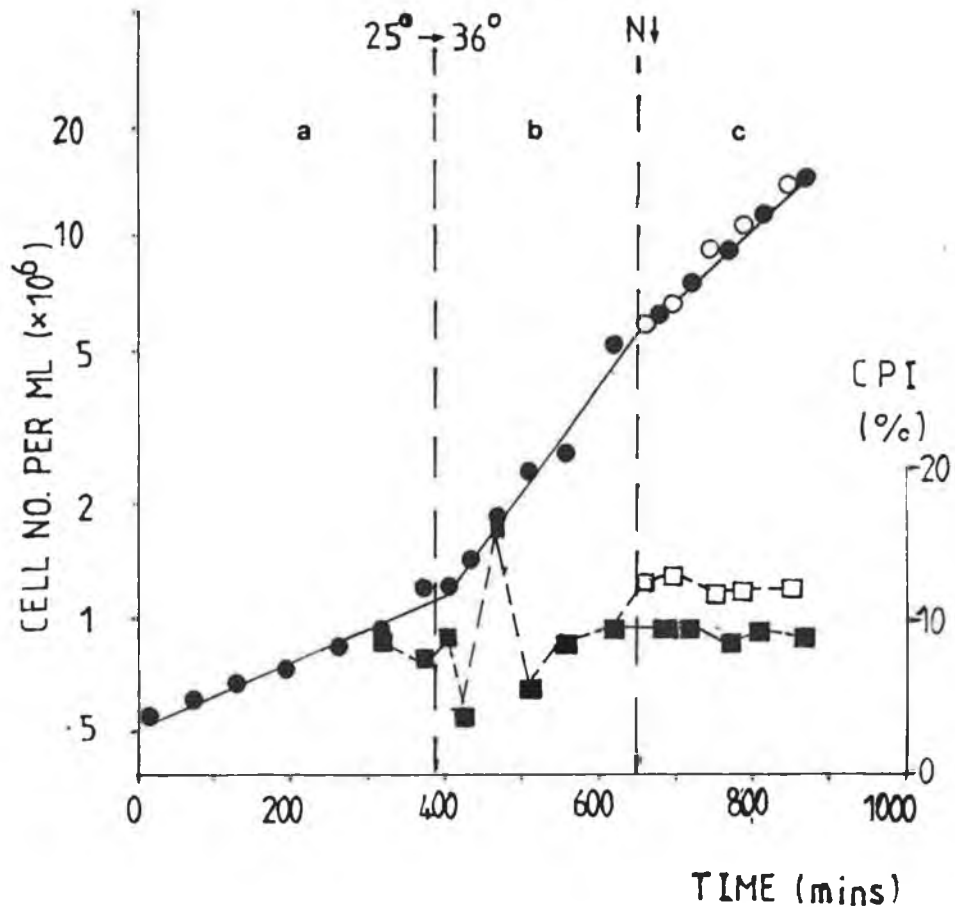
In all subsequent electrophoresis procedures, therefore, protease-inhibitors were routinely used during cell homogenization (except in the experiments described in Sections 3.1.2 & 3.1.3).

3.1.2. Protein Synthesis during Temperature Shift of wee 1.50 Cells

As outlined in Section 3.1, the first of two alternative hypotheses states that proteins p46 and p27 are associated in some way with the accelerated division process that follows nitrogen starvation. This hypothesis would be corroborated by demonstrating the similar appearance of proteins p46 and p27 during an acceleration of cells into division that is not dependent on nitrogen starvation. An example of such a system is furnished by the behaviour of wee 1.50 cells shifted from the permissive temperature (25°C) to the restrictive temperature (36°C) (Nurse, 1975).

Exponentially growing cells of strain wee 1.50h⁻ were inoculated into EMM2 at a cell density of 0.5×10^6 /ml and grown at 25°C. (Fig. 3.7a). After one generation, the temperature was gradually increased to 36°C. The kinetics of cell number increase following this shift are shown in Fig. 3.7b. The rapid increase in cell proliferation rate and the peaking of the cell plate index both illustrate the kinetic similarities between this accelerated division of wee 1.50 cells and that achieved by nitrogen-starvation of wild type cells. Fig. 3.8 also shows how the reduction of cell size at division following temperature-shift of wee 1.50 cells leads to morphological similarities between these cells and nitrogen-starved cells of the wild type (cf. Fig. 3.2c).

This behaviour of wee 1.50 cells can be explained on the basis of the model for cell cycle control in *S.pombe* outlined in Section 1.2.2.



WEE 1.50

FIG. 3.7 Growth of *S. pombe* mutant strain wee 1.50h⁻ in EMM2 following temperature shift and nitrogen starvation

- a) Exponential Growth at 25°C
- b) Upon temperature shift, cells are accelerated into semi-synchronous division at a reduced cell size (see Fig. 3.8) as manifested by the (transient) increase in growth rate (●) and by the "peaking" of the cell plate index (Nurse, 1975)
- c) Upon nitrogen starvation, no increase in growth rate (○) relative to an unstarved control (●) is observed, and neither does the cell plate index (□) "peak", although it does rise to a slightly higher level than in the unstarved control (■).

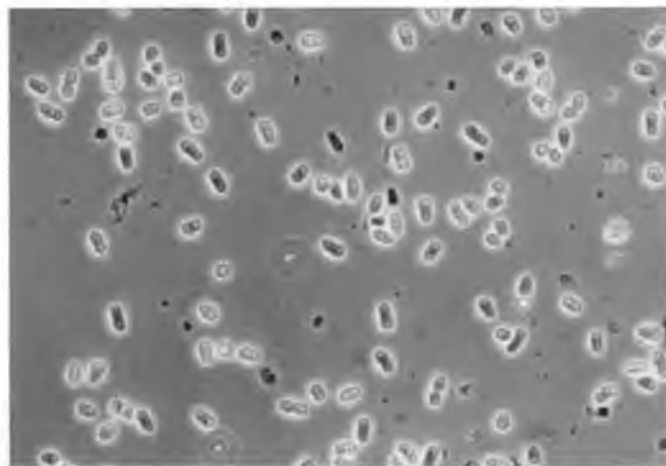


FIG. 3.8 Morphology of *S. pombe* wee 1.50 cells following temperature shift from 25°C to 36°C

Photomicrograph showing the reduced size of cells of strain wee 1.50h⁻ after the accelerated division that follows a temperature shift from permissive (25°C) to restrictive (36°C) conditions.

That is, upon shift to 36°C, the WEE 1⁺ inhibitor of mitosis becomes inoperative such that all those cells which have traversed the minimum G2 phase of the cycle (Fig. 1.3) are now able to initiate mitosis. The result is therefore a semi-synchronous burst of accelerated cell division at a reduced cell size.

The radio-labelling protocol adopted in this experiment was essentially similar to that already described for the nitrogen-starvation experiment of wild type. After labelling, samples were prepared for electrophoresis and autoradiography as before. The result is shown in Fig. 3.9. There is no evidence from the figure of any differential protein synthesis at all during temperature shift of wee 1.50 cells, and certainly no indication of the transient appearance of any polypeptides in the 46 kd and 27 kd ranges. This finding seems to argue against the "division" role of proteins p47 and p27 since the temperature shift of wee 1.50 cells results in a kinetically and morphologically similar response to nitrogen-starvation of wild type cells. However, as Fig. 3.9 is a poor-quality autoradiogram, this is a tentative conclusion. The main reason for the poor resolution stems from the low rate of incorporation of radio-label during the first hour after temperature shift - a finding also reported for *S.cerevisiae* by Miller *et al* (1979). This necessitated overloading the first few lanes with protein (in order to equalize cpm/lane) and this, in turn, gave rise to "spreading" of proteins during electrophoresis. In addition, it should be noted that, unlike nitrogen starvation, temperature shifting wee 1.50 cells does not uncouple growth from division. Therefore, the appearance or non-appearance of tentative "division" proteins may well be masked by the relatively more abundantly synthesized "growth" proteins.

3.1.3 Protein Synthesis during Nitrogen Starvation of wee 1.50 cells

The second hypothesis outlined in Section 3.1 postulates that proteins p46 and p27 are formed as a direct response to nitrogen starvation. This hypothesis would be supported by demonstrating the appearance of p46 and p27 under nitrogen starvation conditions which have no

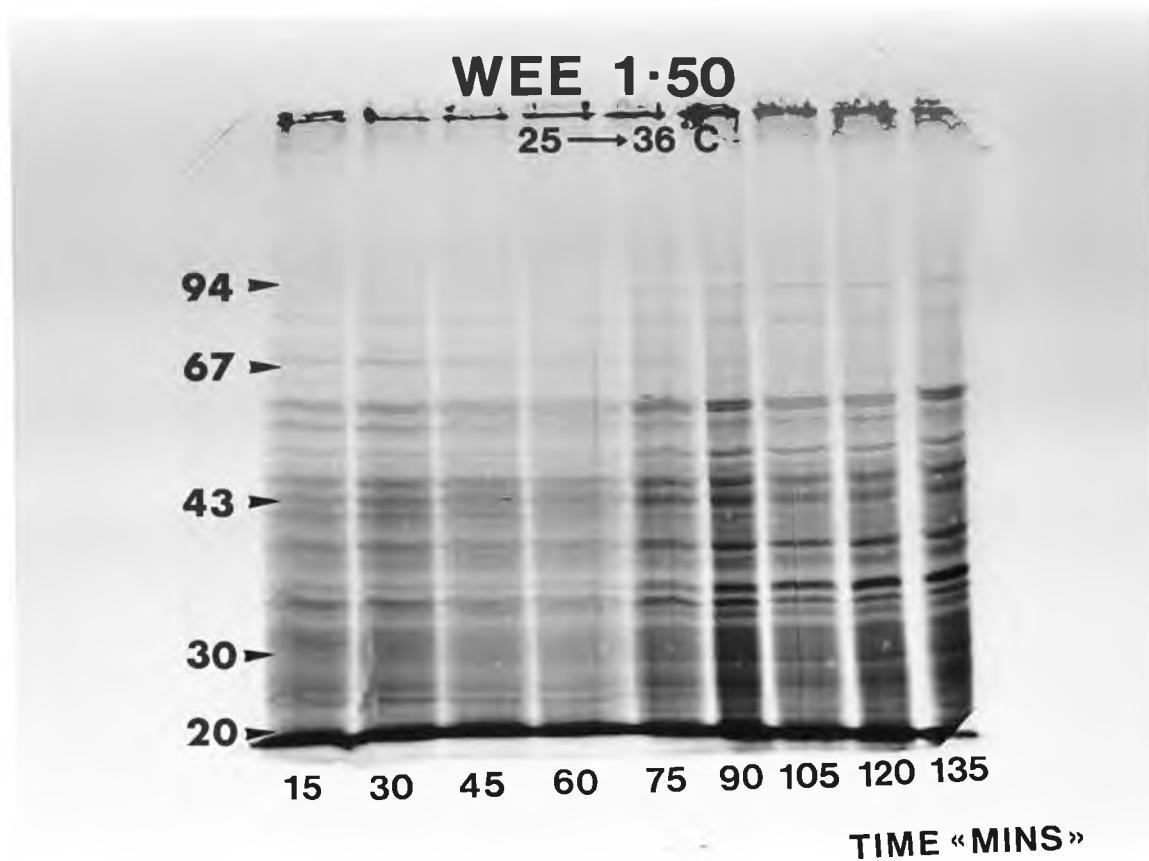


FIG. 3.9 Protein Synthesis during Temperature-Shift of wee 1.50 Cells

Cytoplasmic protein synthesis followed at 15 min intervals during the accelerated division that follows temperature-shift from 25°C to 36°C of wee 1.50 cells fails to reveal differential banding patterns.

effect on the rate of cell proliferation. An example of such a system is furnished by the behaviour of wee 1.50 cells during nitrogen-starvation at the restrictive temperature. The kinetics of cell number increase following such a shift are shown in Fig. 3.7c and it can clearly be seen that there is no acceleration of cells into division relative to an unstarved control. Correspondingly, there is no peaking of the cell plate index (although this does rise to a slightly higher steady level).

Fantes and Nurse (1978) have similarly reported that, unlike wild type cells, cells of strain wee 1.50 are NOT accelerated into division upon nutritional shift-down from glutamate to proline. Both these observations can be explained in terms of cell cycle control models as follows: In wee 1.50 cells, the size control over entry into mitosis is broken down and the rate of cell proliferation is determined by a non-adjustable size control over the initiation of DNA synthesis followed by a constant time ($S + \text{min. } G_2$) until the onset of nuclear and cell division (Fig. 1.2.). This mode of cell cycle control is unlike that operating in wild type cells and more like that known to operate in bacteria, the budding yeast *S.cerevisiae* and many other cells. It does not predict any acceleration of cells into division upon nutritional shift-down for reasons clearly outlined by Fantes & Nurse (1979).

Protein synthesis during nitrogen-starvation of wee 1.50 cells at 36°C was examined exactly as described for wild type cells (Section 3.1.1). The incorporation of radiolabel during starvation was found to follow a similar pattern to wild type. The absolute level of activity at all stages of starvation was, however, approximately half the value for wild type. This finding may be indicative of the smaller cell surface area available for the transport of ³⁵S-methionine.

The results of electrophoresis and autoradiography are shown in Fig. 3.10. As in Fig. 3.5, the transient appearance of two polypeptides (apparent molecular weights of 46 kd and 26 kd) is indicated (lane 7). Their appearance occurs maximally 80-95 min after starvation, somewhat later than in wild type cells. Nevertheless,

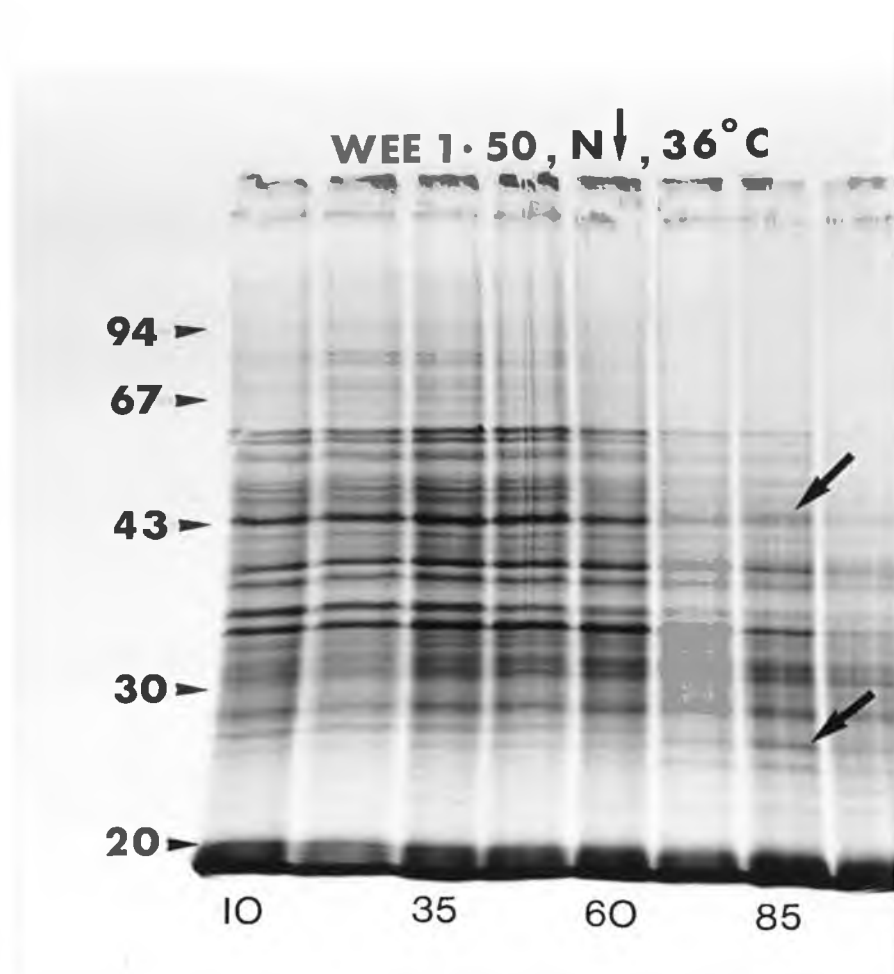


FIG. 3.10 Protein Synthesis during Nitrogen Starvation of wee 1.50 cells

Cytoplasmic protein synthesis followed at 12.5 min intervals during the UNaccelerated division that follows nitrogen-starvation ($N\downarrow$) at the restrictive temperature (36°C) of wee 1.50 cells reveals, at approx 70–100 min, the transient appearance of polypeptides p46 and p27 (arrows) in a manner very similar to wild type (Figs. 3.5 and 3.6). (Protein samples were prepared in the absence of protease inhibitors).

this protein synthetic pattern is strikingly similar to that observed in wild type and provides strong support for the hypothesis that proteins p46 and p27 are synthesized as a direct response to nitrogen-starvation. Conversely, the hypothesis that these proteins are required for, or facilitate in some way, the accelerated division process becomes severely weakened.

3.2 CHAPTER 2: DOES NITROGEN STARVATION INDUCE A HEAT SHOCK TYPE RESPONSE IN FISSION YEAST?

Introductory Remarks

Given the results from Chapter 1, it seemed that proteins p46 and p27 might constitute a reaction by cells to nitrogen stress and research was therefore directed towards the elucidation of this idea. In particular, it was thought that if proteins p46 and p27 were synthesized in response to nitrogen stress, might they not also be synthesized in response to other forms of stress such as heat shock? This concept is not, in essence, a novel one since it is known that a variety of stressful treatments, including recovery from anoxia, ethanol, inhibitors of oxidative phosphorylation and a number of other chemicals all induce synthesis of heat shock proteins in a wide range of organisms (Ashburner & Bonner, 1979). Thus, the hypothesis was formulated that, in fission yeast, nitrogen stress induces the synthesis of a subset of the total array of heat shock proteins. The methods by which this hypothesis was tested, and the results obtained, will now be discussed.

3.2.1. The Heat Shock Response of *S.pombe*

A rudimentary analysis of the heat shock response in fission yeast was carried out as follows: A culture of wild type *S.pombe* strain 972h⁻, growing exponentially in EMM2 at 32°C was split into two aliquots, one remaining at 32°C and the other shifted to 41°C. ³⁵S-methionine was immediately added to each culture at a net concentration of 3 µCi/ml. After 30 min, both cultures were harvested, homogenized and soluble proteins subjected to electrophoresis and autoradiography.

Fig. 3.11 show that protein synthetic patterns at the two temperatures are markedly different. While the repression of synthesis of

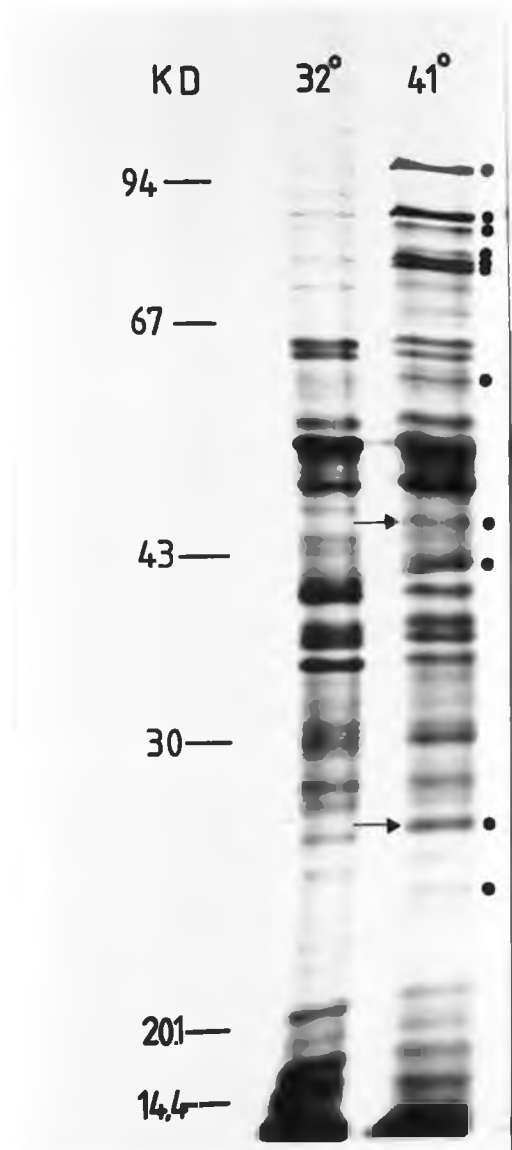


FIG. 3.11 Heat Shock Protein Synthesis in *S. pombe*

³⁵S-methionine radiolabelling before and after temperature shift from 32°C to 41°C reveals the synthesis of a variety of heat shock proteins (●). Two of these HSPs (indicated by arrows) are of approximately the same molecular weight as "nitrogen stress proteins" p46 and p27.

many proteins in normally cycling cells before the temperature shift is obvious, even more dramatic is the synthesis of a number of new proteins after the shift. The more abundant of these heat-shock proteins (HSPs) may be ascribed the following apparent molecular weights: 95 kd, 85 kd, 83 kd, 79 kd, 77 kd, 76 kd, 60 kd, 46 kd, 42 kd, 26 kd, and 23 kd. As outlined in Section 1.2, the HSPs of almost all organisms fall into the following size ranges: 80-90 kd, 65-75 kd, and 15-30 kd. It follows that the majority of HSPs shown in Fig. 3.1.1 fall outside these ranges. Nevertheless, more accurate determination of molecular weight might alter this general picture; thus, HSPs 79, 77, and 76 might easily be placed into either the 80-90 kd group or the 65-75 kd group. However, HSPs 95, 60, 46 and 42 fall very far outside the given ranges and this probably reflects a genuine deviation from the norm in the heat shock response of *S.pombe*. The budding yeast, *S.cerevisiae*, has also been shown to exhibit similar deviations from the norm and heat-shock proteins of M.W. 100 kd (McAlister & Finkelstein, 1980; McAlister *et al*, 1979), 49 kd, 48 kd and 46 kd (Iida & Yahara, 1984a) have been reported.

In context of this research, the most important information to be gained from Fig. 3.1.1 is the existence of HSPs of molecular weights 46 kd and 26 kd. These weights are very similar to those of proteins p46 and p27 induced during nitrogen starvation of the same yeast (Section 3.1). While the similarity of molecular weights between proteins on one-dimensional gels falls far short of a test for identity, this observation nevertheless provides circumstantial evidence for the working hypothesis that nitrogen-starvation in fission yeast induces a subset of heat-shock proteins.

As explained in Section 1.3, a correlation between the induction of HSPs and the acquisition of resistance to lethal heat shock has been observed in many species. Moreover, Iida and Yahara (1984b) have suggested that, in *S.cerevisiae*, sole responsibility for this thermo-protection lies with HSP 48. Given the similarity in molecular weight between HSP 48 of *S.cerevisiae* and protein p46 of *S.pombe*, and given the generally conservative nature of the heat-shock response, the

above hypothesis was tested by examining thermotolerance levels of cells of the fission yeast during nitrogen starvation.

3.2.2. Are Nitrogen-Stress Proteins also Heat-Shock Proteins?

Functional Studies

To test the hypothesis that nitrogen starvation induces a subset of HSPs in fission yeast it was necessary, before examining thermotolerance levels during nitrogen starvation, to establish the correlation between HSP synthesis and thermotolerance in this organism. Since the correlation is well documented for *S.cerevisiae* (McAlister & Finkelstein, 1980), this yeast was also included in the analysis and the results obtained with it will be presented first.

3.2.2.1 Thermotolerance & HSP synthesis in *S.cerevisiae*

A culture of *S.cerevisiae* strain X2180 was grown exponentially in EMM2 at 25°C. At a cell density of 5×10^6 /ml, 1 ml of culture was withdrawn and subjected to a 5 min incubation at 52°C, while the remainder of the culture was placed at 36°C. During the following 90 min period, 1 ml samples of the culture were exposed to the same heat shock conditions at 15 min intervals. Samples were then diluted and plated out in duplicate onto EMM2 agar plates as described in Section 2.5.3. Unshocked control samples were included at all points in this experiment. Fig. 3.12 shows a plot of percentage survival versus time at 36°C.

It is clear from Fig. 3.12 that while pre-treatment at 36°C undoubtedly enhances survival after heat shock at 52°C, thermotolerance is acquired only slowly. By 90 min after the shift to 36°C, thermotolerance is still apparently increasing although the levels of survival achieved are only around 15%. These results contrast qualitatively with those of McAlister and Finkelstein (1980) who have reported the attainment of maximal levels of survival of approximately 50% 60-120 min after a similar temperature shift of *S.cerevisiae*,

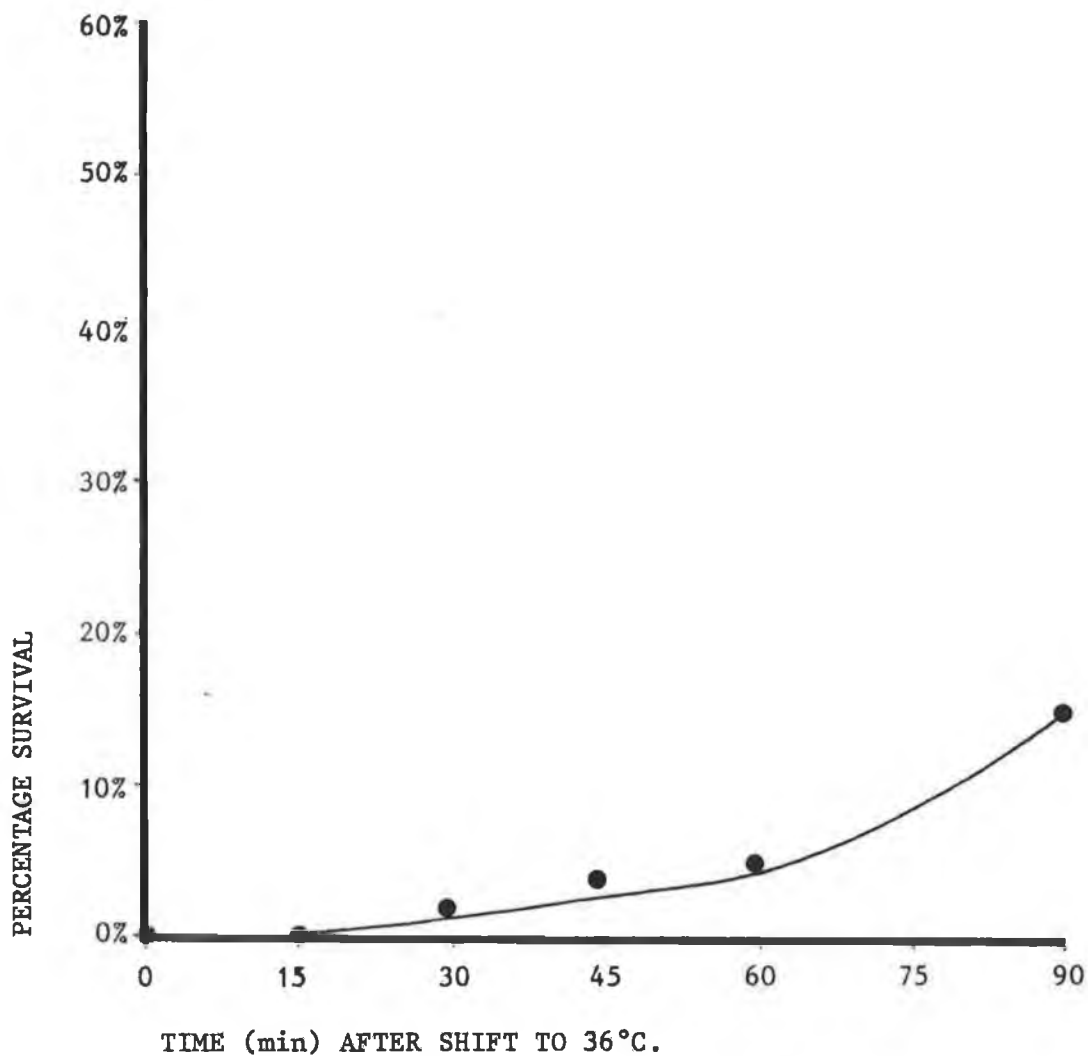


FIG. 3.12 Acquisition of Thermotolerance following Heat Shock
(25°C → 36°C) of *S. cerevisiae*

A culture of *S. cerevisiae* X2180 growing exponentially in EMM2 at 25°C was shifted to 36°C at time $t = 0$.

At 15 min intervals aliquots were tested for the ability of cells to withstand a lethal heat shock (52°C for 5 min).

strain A364A. These differences may reflect strain specific variation and/or their slightly altered pre-treatment regimes (where the initial incubation temperature was 23°C rather than 25°C).

To affirm the correlation between HSP synthesis and the acquisition of thermotolerance, protein synthesis patterns following temperature shift of X2180 cells from 25°C to 36°C were examined.

A culture of strain X2180 growing exponentially at 25°C in EMM2 was split into 5 X 15 ml aliquots. To one of these ³⁵S-methionine was added to a net concentration of 3 µCi/ml. After 15 min, this aliquot was harvested and washed while the others were placed at 36°C. At 15 min intervals, radiolabel was sequentially added to each of these.

The kinetics of ³⁵S-methionine incorporation into acid-precipitable material during this heat-shock experiment are shown in Fig. 3.13. Since, unlike nitrogen starvation, the temperature shift does not inhibit growth, incorporation values are expressed as cpm per µg protein (per unit concentration of added radiolabel). It can be seen from Fig. 3.13 that the temperature shift inhibits ³⁵S-methionine incorporation slightly during the first 30 min and more severely during the second 30 min. These results are similar to those already described (Section 3.1.2) for the temperature shift of wee 1.50 cells of *S.pombe*, and are not unlike those reported by Miller *et al* (1979) for *S.cerevisiae*, strain M25, following shift from 22°C to 37°C.

Protein synthetic patterns during the temperature shift are illustrated in Fig. 3.14. The resolution of bands in this gel was particularly good and the induction of six HSPs is clear. The calculated apparent molecular weights of these HSPs are: 90 kd, 84 kd, 80 kd, 79 kd, 75 kd and 68 kd. With the exception of HSP 80, these compare favourably with the list of HSPs published by Iida and Yahara (1984a) for *S.cerevisiae*, strain A364A. Surprisingly, no HSPs of molecular weights less than 68 kd are observed in Fig. 3.14. Given the apparent absence of the 20.1 kd molecular weight marker (Section 2.6.4) during Coomassie Blue staining of this gel prior to autoradiography, it is possible that any HSPs in the 20-25 kd range might have remained un-

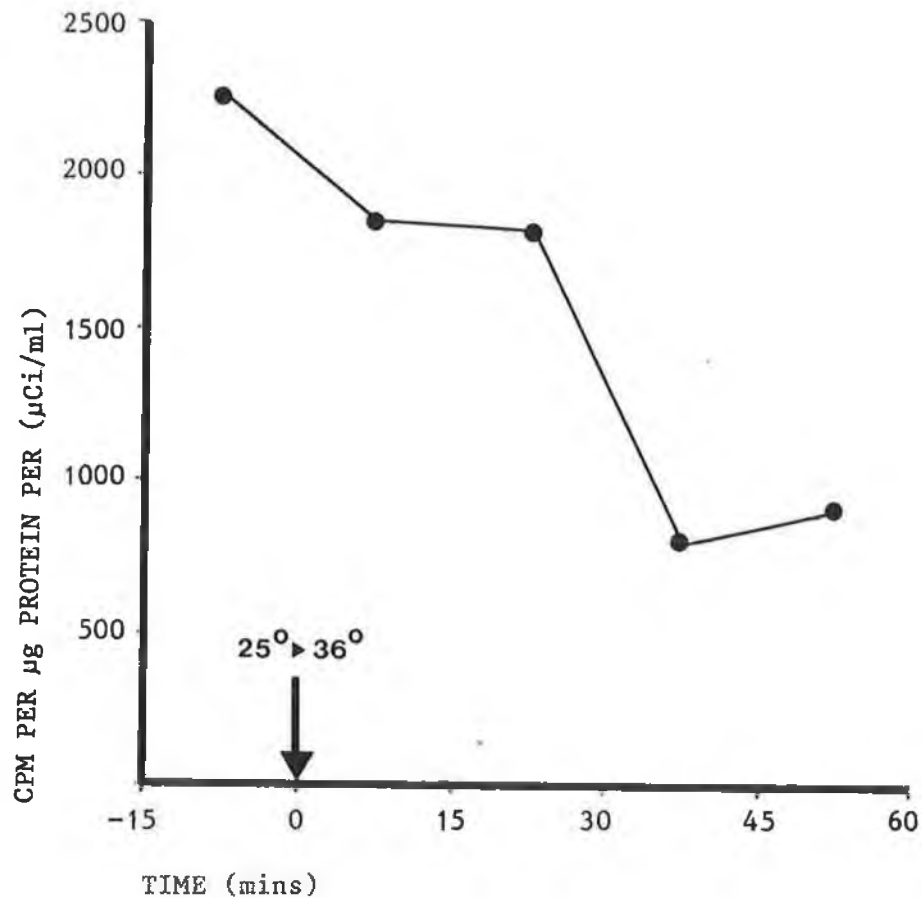


FIG. 3.13 Kinetics of Incorporation of ^{35}S -methionine into TCA-precipitable material during heat shock ($25^\circ\text{C} \rightarrow 36^\circ\text{C}$) of *S.cerevisiae*

Incorporation values are expressed as counts per minute (cpm) per μg of protein per unit concentration ($\mu\text{Ci/ml}$) of ^{35}S -methionine. With respect to the horizontal axis, values are positioned at the mid-point of each 15 min radiolabelling period. Arrow shows the time ($t = 0$) of shift to 36°C .

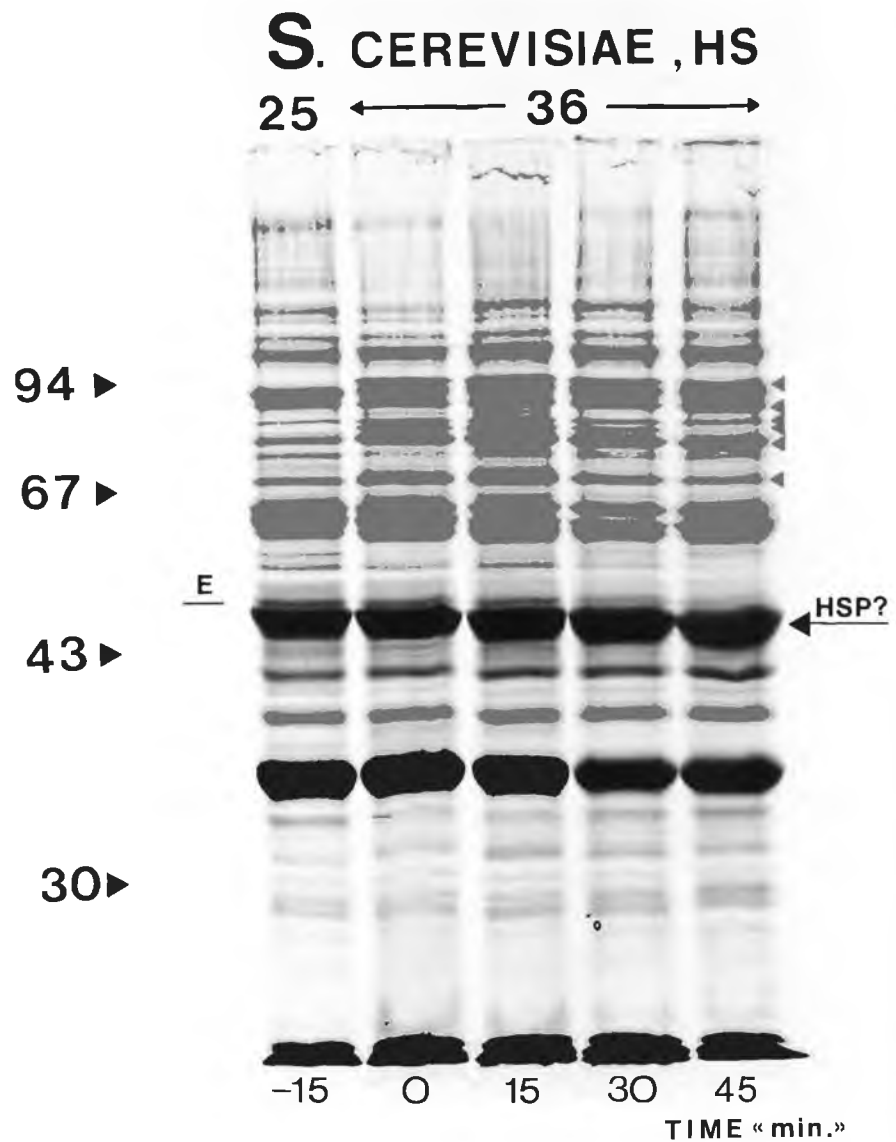


FIG. 3.14 The Temporal Pattern of Heat Shock Protein Synthesis in *S. cerevisiae*

^{35}S -methionine radiolabelling before and after temperature shift from 25°C to 36°C reveals the synthesis of HSPs (arrowheads). Maximal rates of synthesis of these HSPs are jointly achieved during the 15-30 min period (lane 3) following the shift. Pre-shift synthesis patterns are largely re-established by 45-60 min (lane 5).

[See Section 3.2.4.1. for discussion of symbols E and HSP?]

resolved from the bromophenol blue dye front. Nevertheless, the complete absence of HSPs in the 40-50 kd range is in direct conflict with the results of Iida and Yahara (1984a) and remain unexplained (but see Section 3.2.4).

Fig. 3.14 demonstrates well the transient nature of the heat-shock response. Without the facility for densitometry available, it is nevertheless clear that maximal synthetic rates for all of the observed HSPs are achieved 15-30 min after the shift to 36°C (lane 3), and that pre-shift synthetic patterns are largely re-established after 60 min (lane 5). These results are in good agreement with others (eg. McAlister & Finkelstein, 1980). However, the results imply a poor correlation between the cellular levels of HSPs and the acquired levels of thermotolerance since, by 60 min after temperature shift, thermotolerance levels are still low, but continuing to rise (Fig. 3.12).

3.2.2.2 Thermotolerance & HSP synthesis in *S.pombe*

Thermotolerance in pre-heated cells of *S.pombe*, strain 972h⁻, was assessed in a manner analogous to that described for *S.cerevisiae*. The standard lethal heat-shock regime (52°C for 5 min) that has been documented for *S.cerevisiae* by McAlister & Finkelstein (1980) was found, in a preliminary experiment, to be equally effective for *S.pombe*.

The acquisition of thermotolerance in cells of *S.pombe* following a temperature shift from 25°C to 36°C is shown in Fig. 3.15. Unlike the smooth, slow increase in thermotolerance observed with X2180 cells (Fig. 3.12), the response of *S.pombe* cells is more sudden and of a greater magnitude. For example, 15-30 min after the shift to 36°C percentage survival jumps from 1% to 29%, continuing thereafter to increase to levels of 60% at 60-75 min. By 90-105 min, levels of thermotolerance have begun to drop to about 40%.

Although no data on the temporal pattern of HSP induction after a 25°C

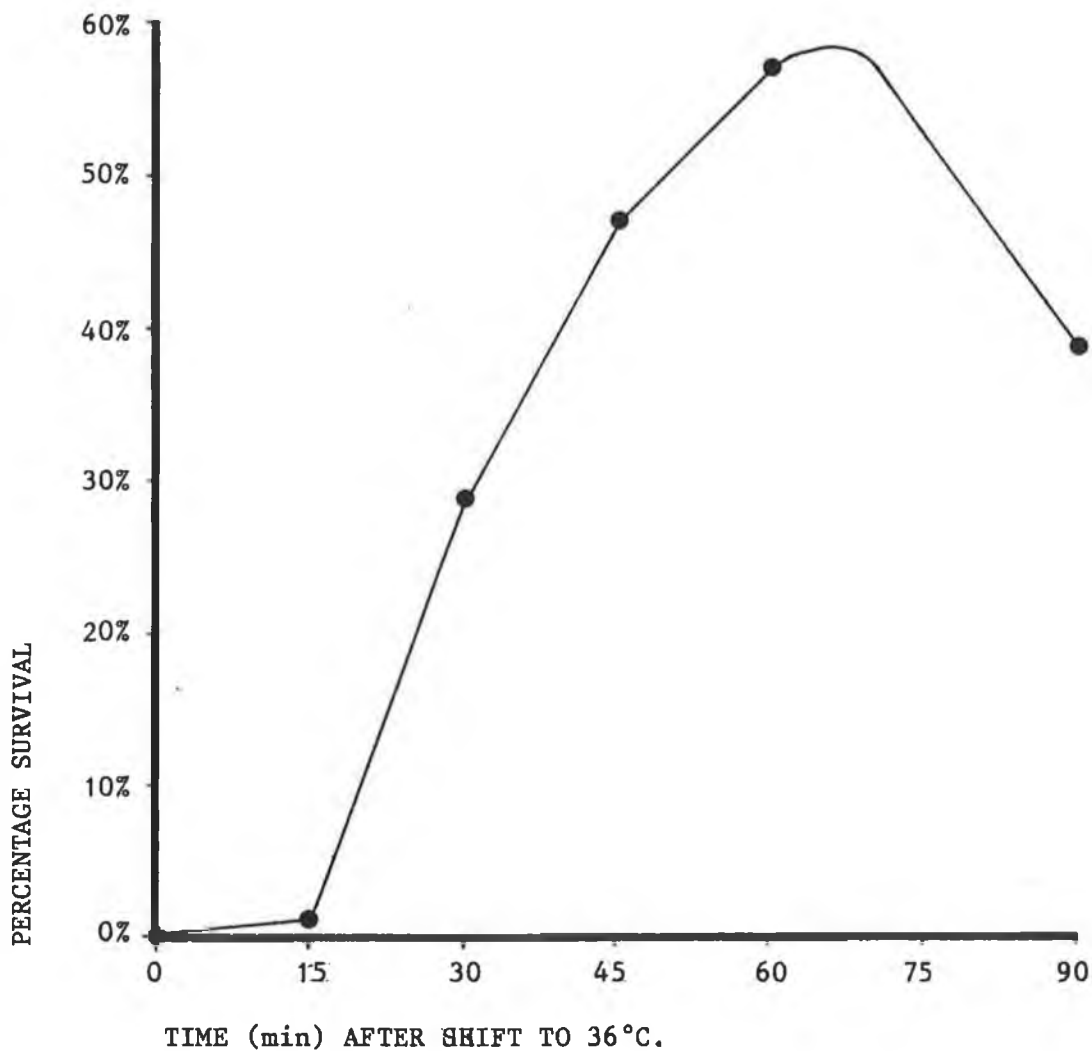


FIG. 3.15 Acquisition of Thermotolerance following Heat Shock (25°C → 36°C) of *S.pombe*

A culture of *S.pombe* 972 growing exponentially in EMM2 at 25°C was shifted to 36°C at time $t = 0$. At 15 min intervals aliquots were tested for the ability of cells to withstand a lethal heat shock (52°C for 5 min).

to 36°C heat shock are available, HSP induction following such a shift was confirmed by a procedure identical to that already described in Section 3.2.1 for the shift from 32°C to 41°C. Note that the acquisition of thermotolerance under this pre-treatment regime was not examined since 41°C, although a sub-lethal temperature, is nevertheless, outside the growth range for *S.pombe*.

3.2.2.3 Thermotolerance during Nitrogen Starvation of *S.pombe*

Having thus established an imperfect but satisfactory correlation between the induction of HSPs and the acquisition of thermotolerance in fission yeast, cells were studied for similar properties during nitrogen starvation.

A culture of 972h⁻ growing exponentially in EMM2 at 36°C was divided into two; one culture was starved for nitrogen and the other acted as an unstarved control. At 15 min intervals cells from both cultures were tested for their ability to withstand exposure at 52°C for 5 min. Unshocked control samples were not plated out and therefore estimates of percentage viability are based on the growth curves of the two cultures (the starved culture undergoing accelerated division as described in Section 3.1.1).

The results, shown in Fig. 3.16, reveal that for up to 90 min after nitrogen starvation the lethality of exposure to 52°C is complete for both starved and unstarved cells. However, in starved cells, thermotolerance levels begin to increase after 90-105 min, reaching levels of approx 60% after 3 hr and 75% after 4 hr. It is most significant that the rise in thermotolerance begins between 90 and 105 min after nitrogen starvation since the appearance of proteins p46 and p27 (Figs. 3.5 and 3.6) is observed only shortly before this period at 60-90 min. On the other hand, the appearance of these proteins is transient; their synthesis is no longer apparent after 105 min. However, the acquired thermotolerance of nitrogen-starved cells is of relatively long duration (as compared for instance, to that acquired by cells after shift from 25°C to 36°C (Fig. 3.15)). It is

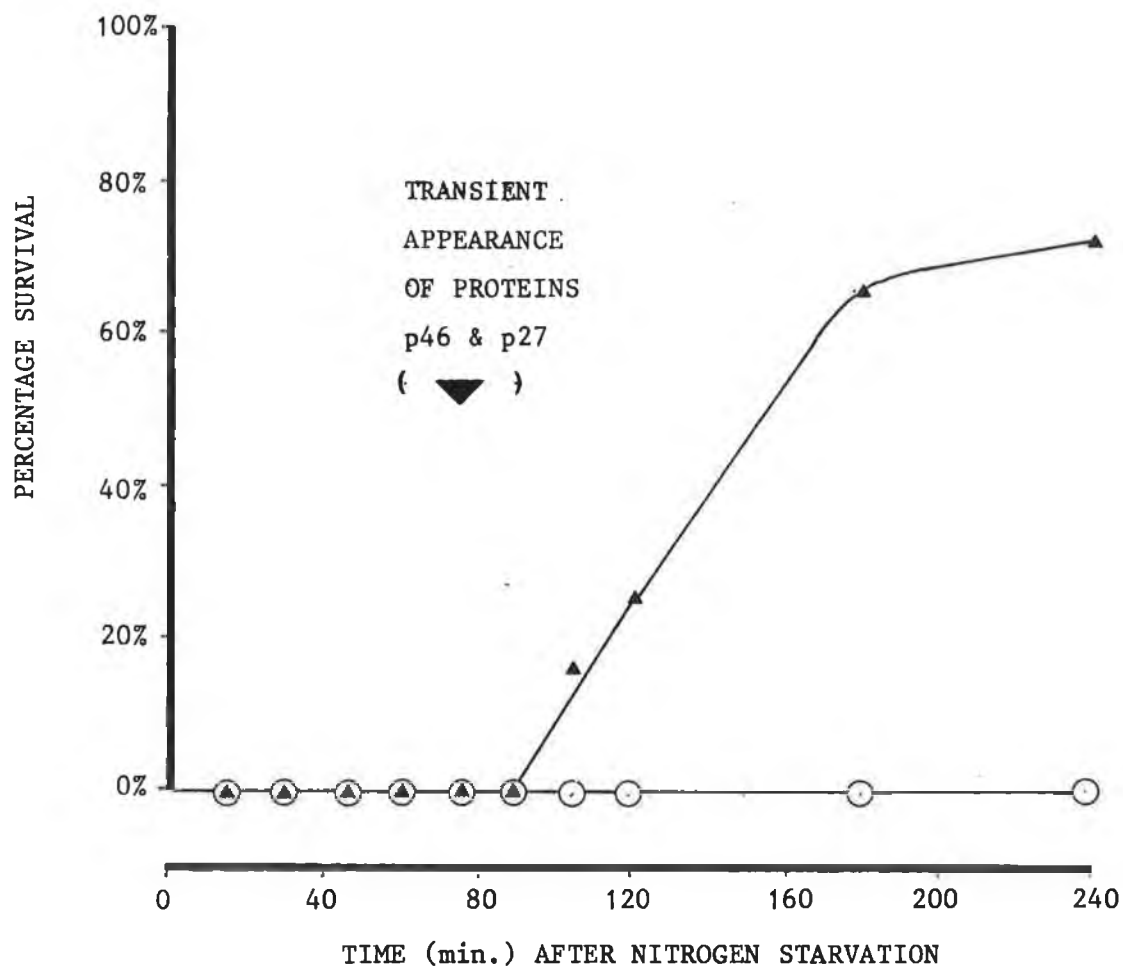


FIG. 3.16 Acquisition of Thermotolerance during Nitrogen Starvation of *S. pombe*

A culture of *S. pombe* 972 growing exponentially in EMM2 at 32°C was shifted to nitrogen-free medium at time $t = 0$. At the times shown (▲) aliquots were tested for the ability of cells to withstand a lethal heat shock (52°C for 5 min). The results are compared with those obtained for an unstarved control (o). Arrowhead indicates the transient appearance of proteins p46 and p27.

possible, although purely conjectural, that there is a delay between synthesis and execution of function for proteins p46 and p27, and that they are very stable proteins. Whatever reasons are speculated, these results provide corroborative evidence for the hypothesis that nitrogen-starvation in fission yeast induces a subset of HSPs.

3.2.3. Are Nitrogen-Stress Proteins also Heat-Shock Proteins? - Immunological Studies

If the connection between the appearance of proteins p46 and p27 and the subsequent increase in thermotolerance of nitrogen-starved cells is accepted, then the logical implication of this is that either one or both of these proteins is responsible for thermoprotection. As discussed in Section 1.3, Iida and Yahara have proposed that, in *S.cerevisiae*, thermoprotection is conferred solely through the action of HSP 48 (Iida & Yahara, 1984b). Furthermore, they have purified this protein, produced an anti-HSP 48 polyclonal rabbit antiserum and shown that the antiserum reacts to the yeast glycolytic enzyme, ENOLASE (Iida & Yahara, 1985). By a variety of other criteria they have compounded this relationship. Given the results quoted in this thesis concerning protein p46, ie.: i) the similarity of molecular weights between p46 of *S.pombe* and HSP 48 of *S.cerevisiae* and ii) the acquisition of thermotolerance in nitrogen-starved cells, and given the conservative nature of the heat shock response generally, it seemed reasonable to ask if protein p46 might not also be enolase. Since Dr. Iida responded favourably to a request for some of the anti-HSP 48 antiserum it became possible to test this hypothesis by immune blotting procedures.

3.2.3.1 Does Anti-HSP 48 Antiserum Bind *S.pombe* Proteins?

The following experiments were based on the assumption that an antibody directed against budding yeast enolase would specifically react to the equivalent enzyme of fission yeast, especially if this enzyme

were also a heat-shock protein. Although no specific justification for this assumption can be furnished, and while no literature on the comparative size and properties of the enzyme in the two yeasts is available, support for the assumption would be achieved by demonstrating specific binding of anti-HSP 48 antibody to a single polypeptide species on SDS-polyacrylamide gels of total soluble fission yeast protein. It might also be expected that the molecular weight of this polypeptide would be around 48 kd. To test this proposal and to assess the sensitivity of binding, the following experiment was performed: A cytoplasmic protein extract of cells of strain 972h⁻ growing logarithmically in EMM2 at 32°C was prepared by the usual procedures. Serial ten-fold dilutions of this sample were prepared for electrophoresis and run on consecutive lanes of an SDS-polyacrylamide gel. In a separate lane, 10 µl of a 10 µg/µl solution of purified baker's yeast enolase (from Sigma) in Electrophoresis Sample Buffer (Section 2.6.3) was run. After electrophoresis, polypeptides were blotted onto nitrocellulose paper and incubated firstly with anti-HSP 48 antiserum, then with goat anti-rabbit IgG conjugated to the enzyme alkaline phosphatase (from Sigma) and finally with enzyme substrate. While this procedure is described in detail in Section 2.7.4, it will be useful to note here that the length of washing times between incubations was only 2 hrs.

Several points arise from the results shown in Fig. 3.17. Firstly, the immune blot shows a high degree of non-specific background reaction - to such an extent that there is even extensive coloration of the nitrocellulose paper between lanes. For this reason, washing times between antibody/antibody/substrate incubations were increased to overnight periods. The effectiveness of this alteration in procedure was evidenced by the fact that, in subsequent experiments, not only was no background reaction observed, but no further binding to molecular weight markers (Fig. 3.17, lane 1) occurred in spite of the fact that the quantity of markers loaded could not be reduced. (This is because the reversible amido black staining by which the position of marker proteins is visualized and recorded, prior to antibody incubation (Section 2.7.4), is a relatively insensitive procedure).

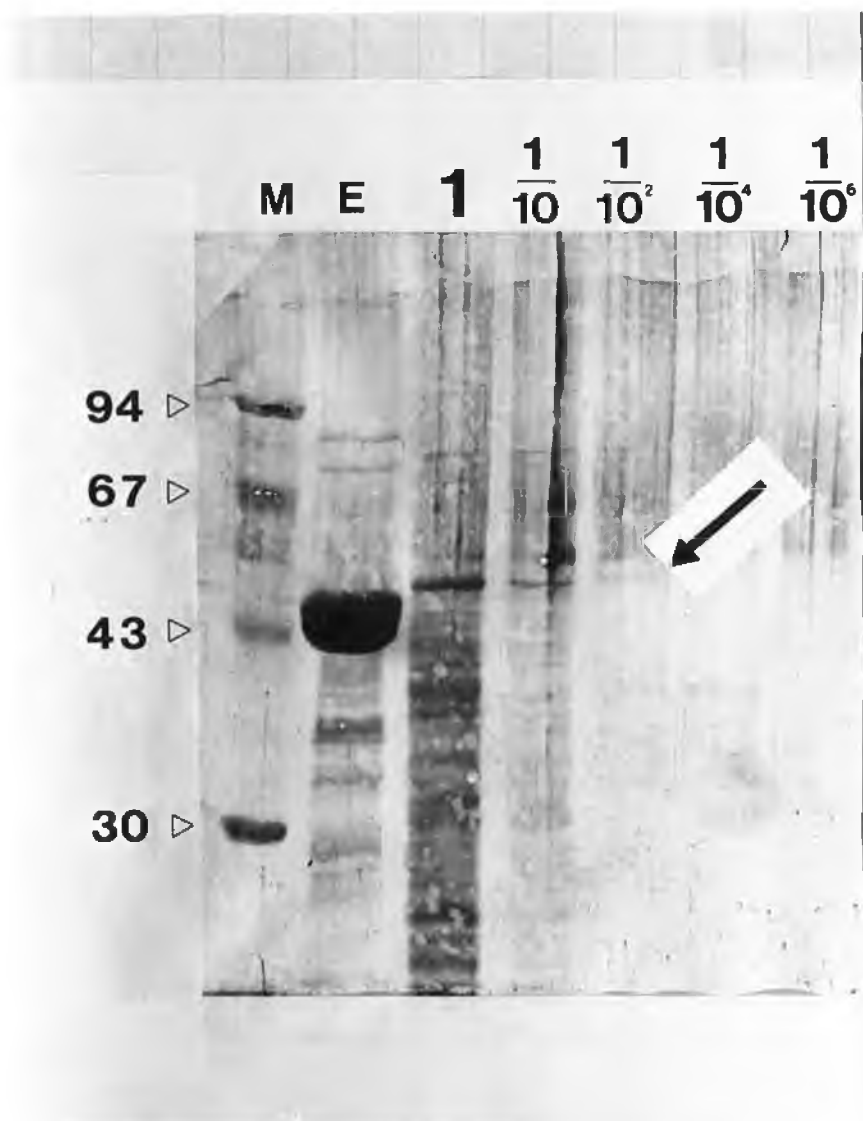


FIG. 3.17 Anti-HSP 48 Antibody Binding to *S. pombe* Proteins

The figure shows an immune blot of total cytoplasmic protein extracts from cells of *S. pombe* growing exponentially in EMM2. The nitrocellulose paper was incubated with a polyclonal anti-serum raised against the HSP 48/enolase protein of *S. cerevisiae*.

- | | |
|----------|--|
| Lane 1 | Non-specific antibody binding to Pharmacia low |
| (M) | molecular wt. markers |
| Lane 2 | Specific binding to purified baker's yeast enolase from Sigma |
| (E) | (Catalogue No. E-6126) |
| Lane 3-7 | Specific and non-specific binding to serially diluted samples |
| | of a total cytoplasmic protein extract of <i>S. pombe</i> . Arrows |
| | show specific binding to only one polypeptide in the hundred- |
| | fold diluted sample. |

Secondly, the anti-HSP 48 antibody reacts strongly to purified baker's yeast enolase (Fig. 3.17, lane 2). This lane was obviously overloaded (100 μ g protein was added), and in subsequent experiments much less enzyme was used (typically 0.1 μ g).

Finally, although antibody reaction to many polypeptide species in the undiluted protein extract of fission yeast has occurred (Fig. 3.17, lane 3) the reaction is by far the strongest to a polypeptide running slightly behind purified enolase in lane 2. The apparent molecular weight of this polypeptide is 49 kd. Furthermore, a hundred-fold dilution of the protein sample (lane 5) shows specific antibody reaction to this band alone. Higher dilutions (lanes 6 and 7) show no specific reaction at all. Since the total amount of protein added to lane 3 (undiluted sample) was 26 μ g, the calculated limit of sensitivity of the anti-HSP 48 antiserum (diluted 1:1000 in Blocking Solution - Section 2.7.4) to total cytoplasmic protein of *S.pombe* is therefore approximately 0.26 μ g.

These results are consistent with the idea that anti-HSP 48 antiserum directed against the HSP 48/enolase protein of *S.cerevisiae* should specifically react with the equivalent protein of *S.pombe*. Furthermore, the antibody-binding protein of *S.pombe* is in the expected molecular weight range of around 48 kd, and therefore also in the same weight range as the "nitrogen stress protein" of *S.pombe*, p46 (which in spite of its title, has variously been measured on different gels at 45-50 kd).

3.2.3.2 Is Protein p46 the anti-HSP 48 Antibody Binding Protein?

It was decided to test the hypothesis that p46 is enolase by using the anti-HSP 48 antiserum to detect differential levels of the antibody-binding protein during nitrogen-starvation of fission yeast. Samples for electrophoresis and immune blotting were collected in exactly the same way as described for nitrogen-starvation experiments in Section 3.1. While radiolabelling procedures affirmed the induction of proteins p46 and p27 during nitrogen starvation in a manner exactly analogous to that illustrated in Fig. 3.6, the results

of immune blotting are shown in Fig. 3.18.

The specificity of anti-HSP 48 antibody for a single polypeptide species of total fission yeast protein is clearly evident in this experiment. Once again, the polypeptide runs with an apparent molecular weight 1-2 kd greater than the 48 kd baker's yeast enolase marker. However, levels of this antibody-binding polypeptide do NOT increase during nitrogen starvation in the manner observed autoradiographically for p46; on the contrary, a small decrease is observed. The direct conclusion, therefore, is that the antibody-binding protein is not p46, and by implication, that p46 is not enolase. This conclusion depends, however, on the quantitative transfer of protein onto nitrocellulose during electroblotting of SDS-polyacrylamide gels. In order to demonstrate this, immune blotting procedures with anti-HSP 48 antiserum were applied to protein extracts of heat-shocked cells of *S.cerevisiae* in which HSP 48 is known to be induced. At the same time, heat-shocked cells of *S.pombe* were similarly studied to see if enolase is a heat shock protein in this yeast.

3.2.4 The Role of Enolase as a Heat Shock Protein in Budding and Fission Yeast

3.2.4.1 Enolase IS a Heat Shock Protein in Budding Yeast

Heat shocked cell samples of *S.cerevisiae* X2180 were collected following a shift from 25°C to 36°C as described in Section 3.2.2.1. Protein samples were electrophoresed, blotted and incubated with anti-HSP 48 antiserum as described above (Section 3.2.2.1). The results are shown in Fig. 3.19A.

There are two points for discussion. Firstly, in unshocked X2180 cells (lane 2), there appear to be two polypeptides which bind the anti-HSP 48 antibody since the stained area consists of a doublet. This observation is not surprising since, in *S.cerevisiae*, there are known to be two non-tandemly repeated enolase structural genes per

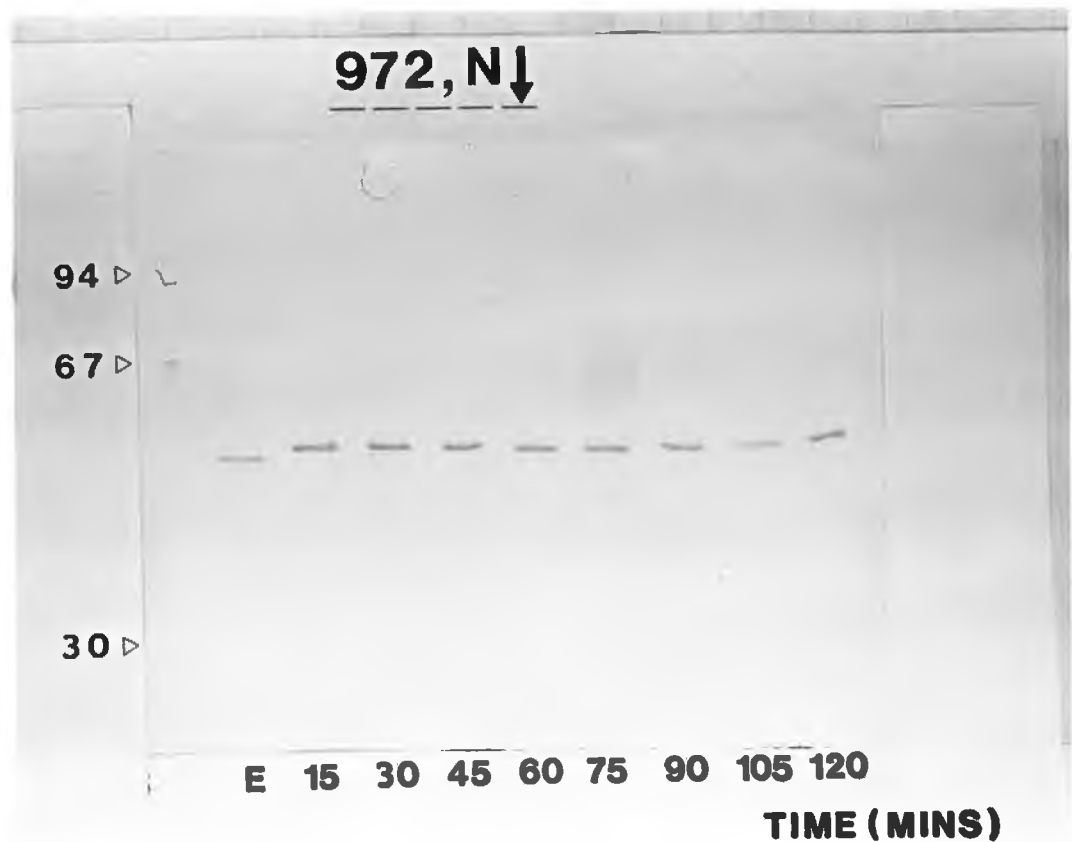


FIG. 3.18 Anti-HSP 48 Antibody Binding to a Single Polypeptide Species during Nitrogen Starvation of Fission Yeast

Cytoplasmic protein extracts from cell samples taken from a nitrogen-starved culture of *S.pombe* 972 at the times indicated were separated on SDS-polyacrylamide gels, blotted to nitrocellulose, and incubated with anti-HSP 48 antiserum. The antiserum reacts specifically with a single polypeptide species running slightly behind the baker's yeast enolase marker from Sigma. Since the quantity of this polypeptide does not appear to increase during nitrogen starvation, it is tentatively concluded that "nitrogen stress protein" p46 is not the antibody-binding protein.

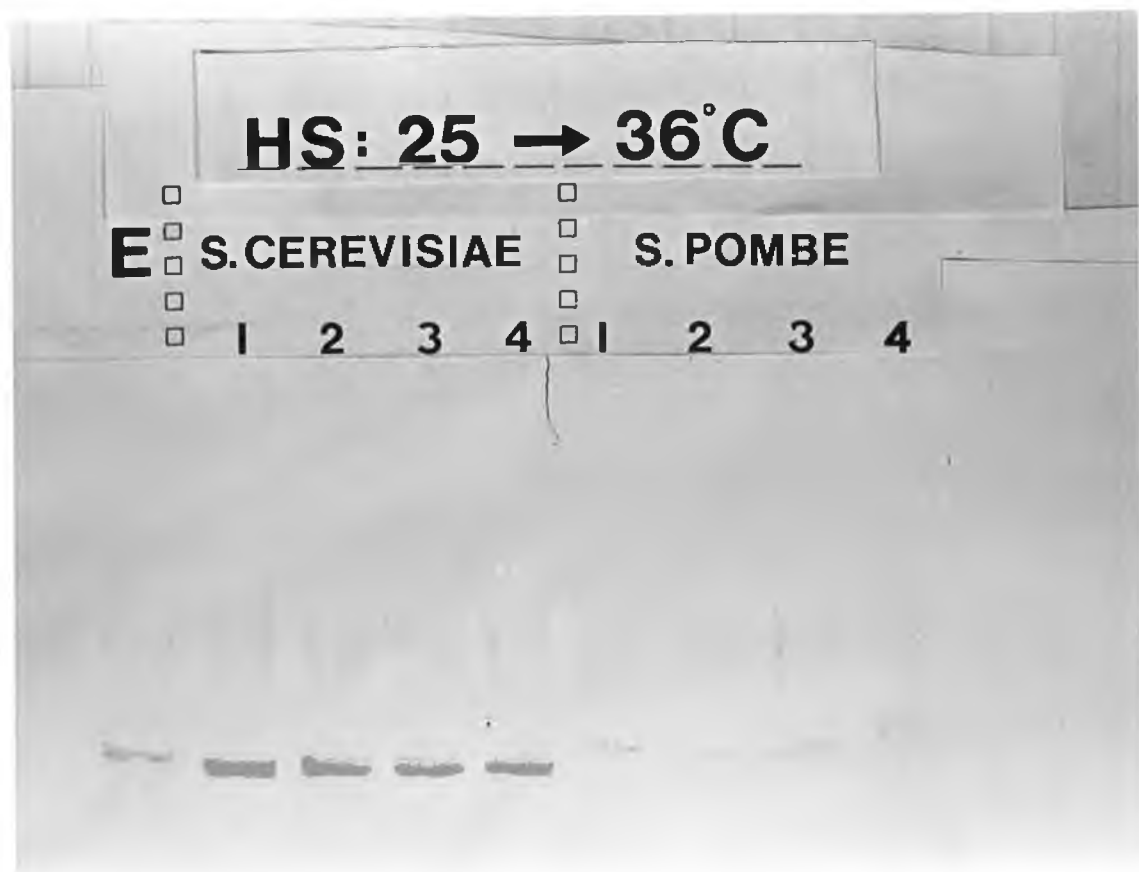


FIG. 3.19 Anti-HSP 48 Antibody Binding during Heat Shock of Budding and Fission Yeast

Cytoplasmic protein extracts from cells of budding & fission yeasts before & after heat shock (HS: 25°C → 36°C) were electrophoresed, blotted onto nitrocellulose and incubated with anti-HSP 48 antiserum.

- A. *S. cerevisiae* X2180. Lane 1: -15 to 0 min (before heat shock)
 Lane 2: 0 - 15 min (after heat shock)
 Lane 3: 15 - 30 min
 Lane 4: 30 - 45 min

- B. *S. pombe* 972 Lanes 1-4: As above

E:- Baker's yeast enolase marker from Sigma

haploid genome, designated ENO 1 and ENO 2 (Holland *et al*, 1981). Moreover, both of these genes are expressed in glucose-grown cells and it has been shown that, in SDS-PAGE, enolase 2 polypeptide, the product of ENO 2, migrates slightly faster than enolase 1 polypeptide, the product of ENO 1 (McAlister & Holland, 1982). Secondly, and more importantly in the context of this work, the intensity of coloration increases upon heat shock (lanes 3-5). While the increase in intensity may not appear startling, the effect is, in fact, all the more marked since only one of the two enolase polypeptides in unshocked cells appears to increase. This finding is consistent with the report by Iida & Yahara (1985) that only one of the two yeast enolase polypeptides is heat-shock inducible.

These results support the argument that protein transfer onto nitrocellulose during electroblotting of SDS-polyacrylamide gels is at least, a semi-quantitative process. The conclusion of the previous section, that protein p46 of *S.pombe* is not enolase is therefore substantiated.

The demonstration by immune blotting procedures that enolase is a heat-shock protein in *S.cerevisiae* is at odds with the autoradiographic analysis of the same heat shock procedure described in Section 3.2.2.1 where no HSPs of molecular weights less than 68 kd were observed (Fig 3.14). While it could be argued that this discrepancy reflects the greater sensitivity of the antibody assay, this argument is dubious because it is known that enolase messenger RNA is one of the most abundant mRNAs in vegetative yeast cells (Holland & Holland, 1978) and that, together with the other glycolytic enzymes, enolase comprises 25-65% of the soluble cellular protein (Holland *et al*, 1982). It is clear from the immune blot shown in Fig. 3.19A that the HSP 48/enolase polypeptide of X2180 cells runs slightly faster than the baker's yeast enolase marker obtained from Sigma and shown in lane 1 of the figure. Since the same enolase marker polypeptide was also run in the gel shown in Fig. 3.14, it is possible to tentatively identify the X2180 enolase polypeptide with the very intense band (indicated on the autoradiogram by an arrow) that runs just ahead of the Sigma enolase marker. If this identification is accurate,

it is not surprising that the X2180 enolase polypeptide fails to be recognized as a heat shock protein since its expression is so abundant even before heat shock. Nevertheless, it is possible to test the foregoing proposal by an under-exposure of the same gel shown in Fig. 3.14. From the result (shown in Fig. 3.20) it is indeed verified that the intense band tentatively identified as the X2180 enolase polypeptide is heat shock inducible. Thus, synthesis of this polypeptide is now seen to increase after heat shock (lanes 1 and 2) unlike, for instance, synthesis of another very abundant polypeptide lying at about 35 kd which is seen to decrease.

Therefore, by both immune blotting procedures and autoradiographic analysis, the status of enolase as a heat shock protein in *S.cerevisiae* is affirmed.

3.2.4.2 Is Enolase a Heat Shock Protein in Fission Yeast?

Heat-shocked (25°C → 36°C) cells of *S.pombe* 972 were collected and total soluble proteins analysed with anti-HSP 48 antiserum exactly as described in the previous section for X2180 cells. The results are shown in Fig. 3.19B alongside those already discussed for *S.cerevisiae* (Fig. 3.19A).

There are again two points to be made. Firstly, since the same amounts of total protein were added to each lane in both Fig. 3.19A and B, the fainter antibody reaction to *S.pombe* protein may reflect the lesser specificity of anti-HSP 48 antibody for *S.pombe* antigen. (This is to be expected since the antiserum was specifically raised against *S.cerevisiae* protein). Secondly, despite the faintness of the reaction, it is clear that there is no increase in the levels of the antibody-binding protein upon heat shock of *S.pombe* cells. The implication is, therefore, that enolase is not a heat shock protein in fission yeast.

While the antibody-binding protein of X2180 cells was observed during SDS-PAGE to run slightly faster than the Sigma enolase marker, it

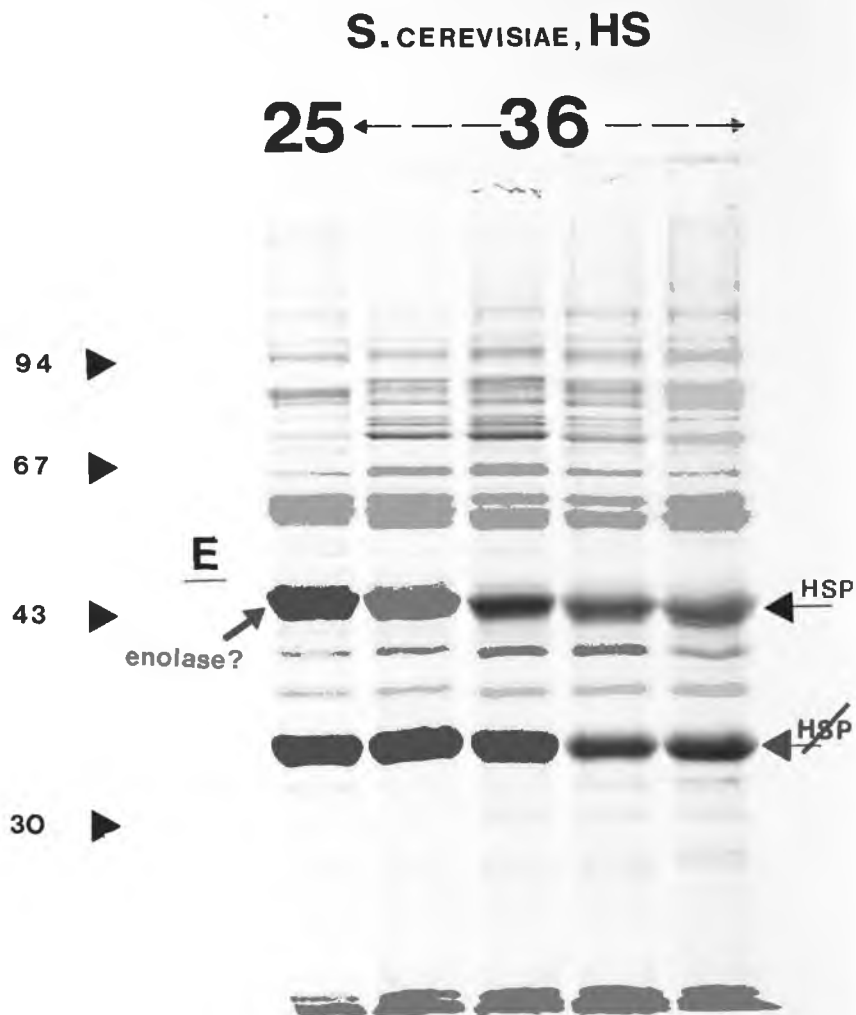


FIG. 3.20 *S. cerevisiae*: Heat Shock Protein Synthesis Re-Examined

The figure shows a decreased exposure of the gel presented in Fig. 3.14. The position of the Sigma enolase marker is denoted by an "E". Running slightly faster than this is an abundant polypeptide tentatively identifiable as the enolase monomer of *S. cerevisiae* X2180. Unlike the intense band lying at about 35 kd, this polypeptide is clearly heat shock inducible.

can be seen from Fig. 3.19B, and more clearly from Fig. 3.18, that the equivalent protein of 972 cells runs slightly slower than this marker. If, as in *S.cerevisiae*, it is assumed that enolase is also a very abundant protein in *S.pombe*, then it should be possible, by a similar line of reasoning to that outlined in the previous section, to test the hypothesis that enolase is not a heat shock protein in fission yeast by autoradiographic analysis.

Heat-shocked samples of 972 cells were prepared by shifting a culture growing exponentially at 32°C to 41°C for 15 min and then returning the culture to 32°C. Aliquots were radiolabelled with ³⁵S-methionine for 15 min before, during and after heat shock. Samples were prepared for SDS-PAGE and autoradiography as already described. In a separate lane of the gel the Sigma enolase marker was run. The results are shown in Fig. 3.21.

As already discussed in Section 3.2.1, the shift from 32°C to 41°C causes the rapid synthesis of a number of HSPs in the molecular weight range 60-95 kd. Most interesting in this context, however, is the positioning of an extremely abundant polypeptide (indicated by an arrow in Fig. 3.21) just behind the Sigma enolase marker (indicated by the letter "E"). On the basis both of its abundance and its electrophoretic mobility relative to this marker, the polypeptide may be tentatively identified as the *S.pombe* enolase polypeptide. If this identification is correct, then enolase in *S.pombe* is clearly not a heat shock protein since synthesis of the indicated polypeptide in Fig. 3.21 is strongly repressed by the shift to 41°C (lanes 1 and 2).

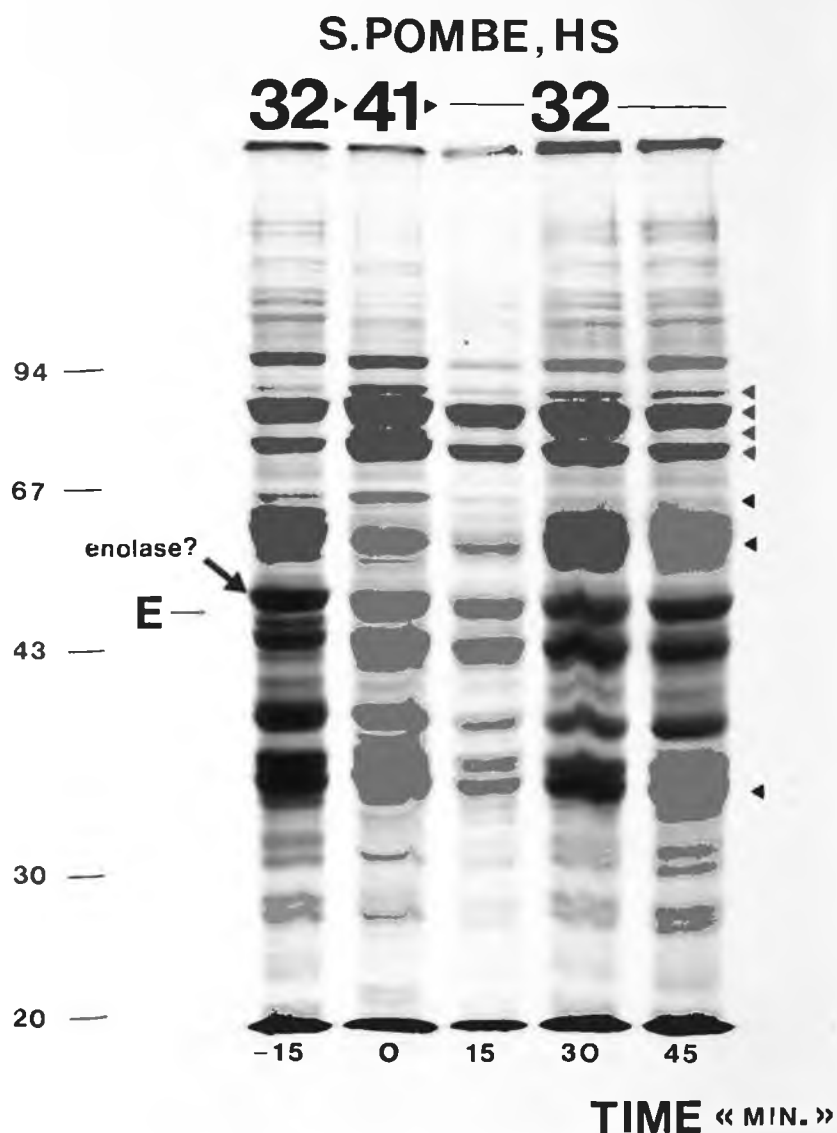


FIG. 3.21 *S. pombe*: Heat Shock Protein Synthesis Re-Examined

The figure shows patterns of polypeptide synthesis before (lane 1, 15 min period before shift), during (lane 2, 15 min at 41°C), and after (lane 3-5, consecutive 15 min intervals after return to 32°C) heat shock. A number of HSPs are indicated by arrowheads. Running slightly slower than the sigma enolase marker (E) is an abundant polypeptide tentatively identifiable as the enolase monomer of *S. pombe* 972. This polypeptide is clearly not heat shock inducible; rather, its synthesis is repressed by the temperature shift.

4. GENERAL DISCUSSION

The experiment upon which this research is based and of which it is a logical extension, was conceived as a means of "uncovering" potential cell division cycle proteins in the fission yeast, *Schizosaccharomyces pombe*. (Section 1.1). Insofar as two proteins, p46 and p27, were indeed "uncovered" (Fig. 1.1) the experiment may be regarded as successful. Nevertheless, in view of the many thoroughly documented responses of protein synthesis to stress in a wide variety of organisms (Section 1.3), it was a clear possibility that proteins p46 and p27 might represent a response of cells to the conditions of the experiment, ie. nitrogen starvation, rather than play the "division" role with which it was hoped they were endowed.

On the basis of the properties of the wee 1.50 mutant of *S.pombe*, it was possible to address this ambiguity through the use of systems designed to separate stimulus and response - nitrogen starvation and accelerated division (Section 3.1). Accelerated division without nitrogen starvation was achieved by temperature shifting wee 1.50 cells from permissive to restrictive conditions (Section 3.1.2). While this experiment furnished poor results (Fig. 3.9), it is ironic, in the light of the subsequent direction this research took, that the alternative stimulus to accelerated division in this system was, in fact, a heat shock. If proteins p46 and p27 are indeed heat shock proteins, then their synthesis perhaps ought to have been observed in this system and so compounded, at least temporarily, the proposition that they are division proteins. It might be noted, however, that their synthesis as HSPs would probably have been immediate and so followed a much different time course than that perceived during nitrogen starvation. Although precautions were taken to avoid heat shock by a gradual increase in temperature, all of this must, for the present, be assigned to the realm of conjecture since, as emphasized above, the results of this experiment were poor. Any elaboration of the research described in this thesis might benefit from a repetition of the experiment.

The implementation of nitrogen starvation conditions without consequent acceleration into division was achieved by starving wee 1.50 cells at the restrictive temperature (Section 3.1.3).

The result of this experiment (Fig. 3.10) was quite unambiguous and clearly pointed to a direct association of the synthesis of proteins p46 and p27 with the starvation process. Therefore, proteins that had tentatively been labelled as "cell division cycle proteins" were now, somewhat less tentatively, labelled "nitrogen stress proteins"

The research, thus re-directed, provoked the testing of a subsequent hypothesis that nitrogen starvation induces a subset of the heat shock proteins. This gained immediate momentum from the observation that, upon heat shock, *S.pombe* cells synthesize two proteins of similar molecular weights to proteins p46 and p27. (Fig. 3.11). Support of a functional and more substantial nature derived from the further observation that nitrogen-starved cells acquired thermotolerance characteristics at a time only shortly after the transient appearance of proteins p46 and p27. (Fig. 3.16)

In the light of these results, and those of Iida and Yahara (1984b, 1985) that, in *S.cerevisiae*, a heat shock protein of molecular weight 48 kd is also the glycolytic enzyme enolase, and that this HSP alone may be responsible for thermoprotection, it was decided to study the heat shock response in fission yeast further. Given the suspected properties of either one or both of proteins p46 and p27, and given the extremely conservative nature of the heat shock response, it seemed entirely conceivable that p46 might be the functionally equivalent protein of *S.pombe*. If the functional equivalent, then this also implies the structural and immunochemical equivalent. In other words, it seemed conceivable that p46 was enolase. The availability of rabbit anti-HSP 48 antiserum permitted an immunological test of this idea.

Why should it be expected that an antibody raised against the enolase enzyme of *S.cerevisiae* should cross-react with the enolase enzyme of *S.pombe*? Wold (1971) has reviewed the comparative properties of enolase enzymes from a variety of organisms, from bacteria to mammals. A number of structural properties are common. The enzymes exist *in vivo* as dimers with identical or near-identical subunit molecular weights in the range 41-50 kd. The existence of multiple electrophoretic forms of the active dimers is common and, while it was once thought that these were an artefact of the purification

procedures (Westhead and McLain, 1964), it is now known that in yeast the three active isozymes reflect the random assortment of monomers encoded by two non-tandemly repeated but homologous structural genes (McAlister and Holland, 1982). All the enzymes studied require magnesium not only as part of the catalytic apparatus, but also as a structural component lending stability to the dimeric complex. An anti-rabbit enolase antibody from chicken was found to cross-react with enolase from dogs, cows, mice, frogs and turtles. Despite the fact that an anti-yeast enolase antibody from rabbit failed to cross-react with a variety of fish and mammalian enolases, this general picture lends credence to the view that enolases from two different yeasts are likely to be immunochemically cross-reactive.

In view of the expectations then, it was not surprising to find that the anti-HSP 48 antibody reacted with a single polypeptide species of *S.pombe* of approximate molecular weight 50 kd. (Figs. 3.17 and 3.18). and it is assumed that this polypeptide is the enolase monomer of *S.pombe*. The antibody analysis of nitrogen-starved cells (Fig. 3.18) indicated, however, that enolase is not preferentially synthesized during nitrogen starvation, and the implication is therefore that protein p46 is not enolase.

In the context of a hypothesis that nitrogen starvation of fission yeast induces a subset of heat shock proteins, this study with anti-HSP 48 antiserum represents only a specific test. Had the result been positive, it might have corroborated the hypothesis, but being negative, it does not refute it. In other words, the conclusion that protein p46 is not enolase does not exclude the possibility that either one or both of proteins p46 and p27 are heat shock proteins conferring thermotolerance upon cells. In view of the well-known conservative nature of the heat shock response, however, it is surprising that the *S.pombe* protein which is immunologically related to the HSP 48/enolase protein of *S.cerevisiae* does not appear to share its functional properties. Thus, while the HSP 48/enolase protein may be crucially involved in the acquisition of thermotolerance in *S.cerevisiae*, the role of the glycolytic enzyme in this respect may, if it exists at all, be less important in *S.pombe*.

This last conclusion finds further support in the final section of the Results (Section 3.2.4.2.). While, both by immunoblotting procedures and by autoradiographic analysis, the status of enolase as a heat shock protein in *S.cerevisiae* was affirmed (Figs. 3.19A & 3.20) similar procedures and similar arguments denied the same status to the enolase protein of *S.pombe* (Figs. 3.19B & 3.21). If, as discussed above, it seemed surprising at first to find that protein p46 was not enolase, it seems all the less so in view of the proposition that, in *S.pombe*, enolase is not a heat shock protein at all. Moreover, if the argument upon which this proposition is partially based, is correct, namely, that enolase is as abundant a protein in normally cycling cells of *S.pombe* as it is known to be in *S.cerevisiae* (Holland and Holland, 1978; Holland *et al*, 1982), then it is once again not surprising that protein p46 is not enolase since its pattern of synthesis during nitrogen starvation is not consistent with that of a protein initially present in abundance.

Perhaps the most important point to be made however, is that these findings point to a major difference in the heat shock responses of two closely related organisms and therefore warn against over-emphasis of the conservative aspects of that response.

The main theme of this thesis has been the investigation of proteins p46 and p27. One of the questions that remains to be asked is this: Are these proteins induced as a result of nitrogen starvation or as a result of the consequent premature entry of cells into stationary phase? The question is particularly relevant since it has been noticed, although not previously discussed, that upon reaching stationary phase under normal conditions of batch culture cells become thermotolerant (an observation which concurs with those of Schenberg-Frascino and Moustacchi, 1972). Moreover, Iida and Yahara (1984a) have shown that, upon entry into the G₀ phase of the cell cycle in *S.cerevisiae* and in other eucaryotic cells, the synthesis of several heat shock proteins is induced. While it has not been ascertained whether or not nitrogen - starved cells of *S.pombe* enter a G₀ phase, it can be said that the synthesis of putative HSPs p46 and p27 is transient, whereas a particular feature of the G₀ synthesis

is its durability. In addition, G_0 HSP synthesis was not observed to include any low molecular weight HSPs (around 25kd). While any further research would be directed towards this line of investigation, the possibility that proteins p46 and p27 are G_0 -cell-cycle-specific heat shock proteins provides a fitting thought with which to conclude a thesis that has separately touched on both cell cycle and heat shock phenomena.

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