PATTERNS OF POLYPEPTIDE SYNTHESIS DURING THE CELL CYCLE OF

SCHIZOSACCHAROMYCES POMBE.

by

Douglas P. Dickinson.

Thesis presented for the degree of Doctor of Philosophy of

the University of Edinburgh in the Faculty of Science.

1979

Regulation 2.4.15

    The work presented in this thesis has been entirely my own, and this thesis has been composed and written by myself.

CONTENTS

Abbreviations:

BSA	bovine serum albumin
butyl-PBD	2-(4'-tert-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole
cdc	cell division cycle
DD	DNA-division
DNase	deoxyribonuclease
EDTA	ethylene diamine tetra-acetic acid
EMM	Edinburgh minimal medium
β-ME	β-mercaptoethanol
PMSF	phenyl methyl sulphonyl fluoride
RNase	ribonuclease
SDS	sodium dodecyl (=lauryl) sulphate
TCA	trichloroacetic acid
TEMED	NNN'N'-tetramethylethylene diamine
Tris	tris (hydroxymethyl) methylamine
TX-100	Triton X-100 (iso-octylphenoxypolyethoxyethanol)

SUMMARY

The general patterns of polypeptide synthesis during
the cell cycle were investigated in the fission yeast
Schizosaccharomyces pombe. Evidence is presented to show
that the major cell polypeptides are normally synthesised
continuously, but that periodic synthesis can be induced
by perturbing the cells during synchronisation. Pulse
labelling of samples from a synchronous culture, followed
by separation of the labelled polypeptides using one-
dimensional SDS gel electrophoresis, was used to determine
the rate of synthesis of 96 polypeptides, including 36
ribosomal proteins, during the cell cycle. Synchronous
cultures prepared in minimal medium showed a perturbed pattern
of labelling, and three non-ribosomal proteins showed a
periodic pattern of synthesis. By supplementing the medium
with amino acids the perturbations were largely eliminated,
and all 96 polypeptides showed a continuous pattern of synthesis.
It is suggested that these periodic patterns are due to
oscillations induced in feedback loops caused by a shock,
perhaps nutritional, during the synchronisation procedure.

A search was conducted using two-dimensional gel electro-
phoresis for polypeptide synthesis dependent upon the occurence
of some preceeding cell cycle event. A survey of 716 poly-
peptides synthesised in cells of cell cycle mutants blocked at
different stages in the cell cycle failed to reveal any
significant differences between blocked, non-blocked, and
wild-type cells. It is concluded that dependent synthesis is

a very rare form of cell cycle control. It is proposed that post-translational control, with perhaps a few periodically synthesised control proteins, is responsible for cell cycle control.

An attempt was made using two-dimensional gel electrophoresis to identify the <u>wee</u> l gene product, which is involved in a size control over nuclear division. A strategy was developed for gene product searches, but a survey of nearly 800 polypeptides failed to identify the gene product.

Concepts:

The cell cycle of a growing cell is the period between
the formation of the cell by the division of its mother cell,
and the division of the cell itself to form two daughters.
The cell cycle of higher cells may be divided into interphase,
which typically occupies 95% of the cycle, and mitosis, when
the daughter chromosomes are separated. Interphase may be
further subdivided into G1, S, and G2, which leads into mitosis.
The nuclear DNA is replicated during the S phase, and in most
cells no nuclear DNA synthesis occurs during G1 or G2. Cell
division follows shortly after mitosis in the majority of cells,
but there are exceptions.

During the cell cycle the components of the cell must
be duplicated, and a major goal of research in cell biology
has been to elucidate the mechanisms underlying this duplication.
My aim in this thesis has been to examine the patterns of
doubling of individual proteins, and to investigate the cell
cycle controls.

Thirty years ago, no visible changes had been observed
in cells going through interphase, and it was believed that
the cell doubled itself during this time by a process of continuous
uniform growth. The only well defined cell cycle event in which
cell components were doubled discontinuously was mitosis, which
is essentially a physical separation of the interphase products.

This view was changed dramatically with the discovery in
the early fifties that DNA was synthesised discontinuously,
raising the possibility that other cell components, especially

the proteins, were synthesised in the same periodic manner.

Over the past twenty-five years, periodic changes during the

cell cycle have been observed in a considerable number of

cell components: the cell cycle may therefore be considered

as the passage of a cell through an orderly sequence of periodic

biochemical and morphological changes. These changes may be

properly regarded as processes of morphogenesis and differentiation,

directly analogous to those of a developing organism, such as a

growing embryo, or an outgrowing spore. All appear to show

ordered patterns of chemical synthesis and morphogenesis,

which are repeated in consecutive generations, and the cell

cycle may be seen as a miniature developmental system. Ten

years ago, there was a growing body of evidence to suggest that

development involved the expression of sequences of genes.

Application of this concept to the cell cycle provided us with

a theme which dominated cell cycle work: a programmed sequence of

gene expression during the cell cycle generates periodic changes

in the proportions of molecular components of the cell. These

changes are in turn responsible, at least in part, for the

structural changes observed during the cell cycle.

Periodic changes during the cell cycle:

(a) Structural:

Most of the known structural changes during the cell cycle are directly associated with the replication of the genetic material. The most conspicuous occur during mitosis, when the chromosomes condense, the mitotic apparatus is assembled, and the daughter chromosomes are physically separated. At the same time the nucleoli, and in many cases the nuclear membrane disappear, to be reformed at the end of mitosis.

There are few such dramatic changes during interphase. Mazia has suggested that changes in chromosome structure may occur throughout interphase, constituting a 'chromosome cycle' (1). Decondensation of the chromosomes may continue during G1, and condensation begin after S and continue through G2. Unfortunately the evidence for this cycle is at present limited (2,3,4). Changes during the cell cycle in the distribution and density of nuclear pores have been observed (5), but their significance is unknown.

The centrioles are an integral part of the mitotic apparatus in animal, and some primitive plant cells. They replicate by the formation of a new centriole near (but not touching) one end of a mature centriole and at right angles to it (6). Interestingly, this process occurs out of phase with mitosis. Replication begins at the start of S phase, and is complete by the end of G2, when the cell contains two adjacent pairs of centrioles, each comprised of one new and one old centriole. At mitosis each daughter cell receives one pair, which sebsequently

separate during G1 into two mature centrioles (7).

The oral apparatus in ciliates is also replicated during interphase. In _Tetrahymena_, for example, this occurs half a cycle before its use as a mouth in a daughter cell (8).

Other, more subtle changes may occur throughout the cell cycle, but present techniques may have insufficient resolution to detect them. One difficulty lies in identifying spatial and temporal changes in thin sections of cells.

(b) _Molecular_:

There is a considerable body of evidence from a wide range of prokaryotic and eukaryotic cells for periodic changes in the composition of the cell, primarily as a result of periodic synthesis (reviewed in 2,9). This evidence has been derived from three sources: (i) assay of specific substances, or specific fractions of substances; (ii) assay of enzyme activity; (iii) more recently, electrophoretic separation of crude protein extracts into many individual components. Our models of the cell cycle have been based mainly on the evidence from the first two methods. As electrophoretic separation has been used extensively in this thesis, a review of work using this technique is deferred until the concluding discussion.

(i) The chromosomes show extensive periodic behaviour, and as might be anticipated, this is paralleled by periodic synthesis of some of their components. The periodic synthesis of DNA has been known for over twenty years (e.g. 10, 11). Recent work on eukaryotic DNA has revealed an extensive heterogeneity at the molecular level, such as satelite and repetitive DNA,

which is reflected in a non-uniformity in the pattern of DNA

replication (reviewed in 12). In mammalian cells, for example,

guanine-cytosine rich sequences are preferentially replicated

at the start of the S phase, and adenine-thymine rich sequences

at the end. The histones are an essential component of the

chromosomes (reviewed in 13). Basic proteins, they form complexes

with themselves and with DNA, and are partly responsible for

the 'packaging' of the DNA. There is good evidence to show

that the histones accumulate in the nucleus during S phase (14,15),

and that this is almost certainly due to periodic synthesis of

the histones (15,16), probably from mRNA transcribed during

S phase (17,18).

Changes in the levels of phosphorylation of the histones

correlate with changes in chromatin organisation (19, reviewed in 20).

In mammalian cells, H1 and H3 are phosphorylated during prophase,

metaphase and anaphase; dephosphorylation of these molecules

occurs as the cells leave anaphase. Histone H1 is phosphorylated

at different sites during interphase. It has been suggested

that interphase phosphorylation is correlated with a change

in organisation at the molecular level; H2a phosphorylation

with heterochromatin condensation; and H1 and H3 phosphorylation

with condensation to chromosomes. Cause and effect relationships,

however, remain to be determined; it is not known if these

phosphorylations bring about changes in the chromatin, or if

these changes bring about the phosphorylation of the histones.

The acidic chromatin proteins also show periodic synthesis,

although the periodicity is generally less well defined than

that of the histones. For example, a series of non-histone, DNA binding proteins in mouse cells are synthesised during S phase (21). The synthesis of another protein stops at the end of G1. In HeLa cells, an increased rate of synthesis of tightly bound acidic chromosomal proteins occurs during late G1, though some synthesis occurs throughout the cycle (22).

Periodic synthesis has been identified directly in very few specific cytoplasmic molecules. In human lymphoid (23) and mouse myeloma cells (24), the immunoglobulins have been reported to be synthesised from late G1 until nearly the end of S. However, Liberti and Baglioni found continuous synthesis of immunoglobulins in mouse myeloma cells (25). Finally, chitin, a macromolecular component of the bud scar in budding yeast is synthesised about halfway through the cycle (26). (ii) The largest body of evidence in favour of periodic synthesis comes from measurements of enzyme activity in synchronous cultures. Mitchison, in his monograph on the cell cycle (2), lists 103 cases where enzymes have been assayed in synchronous cultures in a large variety of cells. The results ranged from no periodic enzymes detected (rat HTC cells) to all being periodic (Saccharomyces cerevisiae); but in the majority of cells roughly 70% of the enzymes were found to be periodic. There is little consistency in the pattern of increase of an individual enzyme from one cell type to another. For example, alkaline phosphatase shows one step per cycle in Chlorella, two steps per cycle in Saccharomyces cerevisiae, a peak pattern in Human Henle cells, and continuous synthesis in the fission

yeast, _Schizosaccharomyces_ _pombe_ (see refs. in 2). A few
enzymes associated with DNA replication and metabolism, such
as thymidine kinase, or DNA polymerase, always appear to be
periodic and their synthesis is usually coincident with S phase,
although there is insufficient evidence to exclude the possibility
that this is a fortuitous result.

It is generally assumed that a step is produced by periodic
synthesis of a stable enzyme, and a peak by the periodic
synthesis of an enzyme which is unstable, or in some way
inactivated. However, a step could equally well be produced
by the continuous production of an unstable enzyme with a
doubling of the rate of synthesis at the time of the step (27).
This doubling would change the enzyme concentration from one
steady state to another, higher one. Alternatively, a step
could result from periodic activation of a continuously accumulated
inactive protein.

Perturbations and periodicity:

The existence of periodic changes in the composition of
a cell would be expected for two main reasons. One, already
given, is that the cell can be regarded as a developmental
system. The other is that the periodic changes in chromosome
structure and behaviour can be considered to stem from the
doubling in DNA during the S phase. It is therefore conceivable
that other morphological changes are in some way the result,
at least in part, of similar periodic changes in the proportions
of other molecules. However, the most striking fact about
the structure of a growing cell is how few changes during
the cell cycle are detectable by light or electron microscopy.
To some extent this might be expected for the prokaryotes, as
they have a comparatively simple structure. But it is surprising
to find a similar situation in the vastly more complex eukaryotes,
where many discrete components have to be replicated. Indeed,
it is difficult, and often impossible, to distinguish a G1 cell
from a G2 cell, except on the basis of size and in a few
cases, chromatin differences. In addition, most of the work
on the bulk properties of cells, such as dry mass or total
protein, show patterns of continuous increase (reviewed in 2,9).
Total RNA also shows a similar pattern, apart from a block
on its synthesis at mitosis, presumably due to chromosome
condensation (2). These patterns suggest that growth occurs
without extensive changes in composition.

It is therefore paradoxical that whilst the overall growth
of a cell appears to be a fairly smooth, continuous process,

periodic changes in the levels of enzyme activity are so common.
Continuous synthesis would seem a much more logical way of
increasing the components of a growing cell without upsetting
its delicate balance, particularly when some of these are
components of major pathways. It is possible that many enzymes
are present in excess, so a sharp increase has no effect.
Alternatively, periodic synthesis may be linked with structural
changes which are too fine for us yet to resolve. Periodic
synthesis may be forced upon the cell by some nature of the
control mechanisms, or it may be the result of a cellular
clock whose timing is essential for progress through the cell
cycle.

Whilst we have as yet no way of answering these questions,
we can examine more closely the evidence which gives rise to
them. Most of this evidence for periodicity comes from the
measurement of enzyme activity in synchronous cultures, on
the assumption that the events observed are equivalent to
those in a single cell in asynchronous cultures. Some reports
have differed in the patterns found for an enzyme in a particular
organism (reviewed in 12). Early work in Chlorella on ribulose
1,5-diphosphate carboxylase (28), and in mouse L cells on
thymidine kinase (29,30), showed that the pattern of enzyme
activity obtained might be dependent upon how the cells were
synchronised; raising the possibility that a synchronisation
procedure could perturb the cells, and produce an artefactual
deviation from the true pattern of enzyme activity. In recent
years, evidence has emerged which suggests that at least some

cases of periodicity are due to perturbation of the cells by
the synchronisation procedure, rather than being true cell
cycle events.

The effects of synchronising cells has been extensively
studied in S. pombe by Mitchison and workers (reviewed in 31).
Synchronous cultures were prepared by selection synchrony,
which involved concentrating the cells, centrifuging them
whilst concentrated through increasing concentrations of a
sugar, selecting the small cells, and inoculation into fresh
medium. Particular attention was paid to asynchronous control
cultures, which were designed to reproduce many of the features
of the synchrony technique which might have caused perturbations.
The cells were treated in the same way, except that the gradient
was mixed after centrifuging to reconstitute the asynchronous
population. This was then inoculated into fresh medium at
the same density as the synchronous culture. If unperturbed,
an enzyme activity would rise exponentially in such a control.

Out of ninteen enzymes investigated in S. pombe, only
one, thymidine kinase, showed a periodic pattern in synchronous
cultures and a continuous increase in asynchronous controls.
Three enzymes (alcohol dehydrogenase, homoserine dehydrogenase,
maltase), showed persistent step-like patterns in controls
(although the steps were often not accurate doublings, nor always
a generation apart), as well as in synchronous cultures, and
which in some cases were repeated over three successive cycles.
A further four enzymes (arginase, ornithine transaminase, leucine
aminopeptidase, glutamine synthetase) showed a large

initial perturbation, from which the cells recovered after about an hour, and thereafter showed a continuous increase in both synchronous cultures and controls. The remaining enzymes showed a continuous pattern of increase.

Only one of these nineteen enzymes, thymidine monophosphate kinase, can be interpreted as a periodic enzyme, the remaining periodicities probably being due to perturbations. It is possible that alcohol dehydrogenase, homoserine dehydrogenase, or maltase are periodic in an unperturbed cell, but it seems more likely that their pattern is an extreme case of the perturbation seen in arginase, for example.

The exact cause of these perturbations is unclear, although it seems likely that there are several contrubutory factors. Mitchison, et.al., found that the size of the perturbations was considerably reduced, but not eliminated, by inoculating the cells back into the medium they were growing in before being placed on the gradient, rather than inoculating into fresh medium (31). Folkes, et. al., obtained a similar result in Candida yeast (32). Using isopycnic gradients for selection they initially found step patterns of activity for various enzymes. These steps became continuous increases when the cultural procedure was refined, and a sudden change into fresh medium was avoided. Starvation whilst the cells are concentrated may also be a major cause of perturbations.

Two main conclusions can be drawn from these results. Firstly, synchronisation can perturb cells, and induce artefactual periodicities in enzyme activity. Secondly, in the absence

of controls, these perturbations can be mistaken for cell
cycle events, and the proportion of periodic enzymes in the
cell seriously overestimated. It is therefore important to
test for perturbations, both by running suitable controls,
where possible; and using different synchronisation techniques,
on the assumption that they produce different perturbations.
This approach is complemented by the minimisation, and elimination
of perturbations by refining the techniques employed.

A great deal of our evidence for periodic synthesis comes
from S. cerevisiae, where 32 out of 33 enzymes studied so
far have shown step or peak patterns (reviewed in 2. 31).
Most of these results have come from synchronous cultures
prepared by gradient selection, and although it is unlikely that
all these steps are the result of perturbations, it is important
to re-examine them. Halvorson and workers have used age
fractionation of chilled asynchronous cultures as an alternative
method of investigating cell cycle patterns (33). Assuming any
changes are stopped by the chilling, (and in some cases cyclo-
heximide was added), the cells should not be perturbed.
This method has the disadvantage of only permitting study
over one cycle, and results from fractions representing older
cells may show variation as the fractionation is less consistent
with older cells. Six enzymes were found to show a step pattern,
and some of these confirmed earlier results. One continuous
enzyme, RNA polymerase, was found (34).

Another major body of evidence on patterns of synthesis
comes from Chlorella, where many periodic increases in enzyme

activity have been found (reviewed in 35). Much of this work has been done with synchronous cultures synchronised by alternate light/dark cycles. As Chlorella is a photosynthetic organism, darkness is equivalent to starvation, and light might induce some enzymes: a change from dark to light will inevitably cause major metabolic shifts. Recent work has shown that many periodic changes disappear when cells are studied under conditions designed to minimise perturbations (36).

Two further assumptions are generally made in interpreting changes in enzyme activity in terms of the cell cycle: that enzyme activity is equivalent to enzyme synthesis; and that changes in enzyme synthesis are a direct result of changes in gene expression. Both these assumptions are difficult to test rigorously. In principle, it should be possible to measure the specific activity of an enzyme during the cell cycle, but this has very rarely been done. A simpler test is to follow enzyme activity after the addition of a protein synthesis inhibitor, and this has revealed an interesting situation in S. pombe (37). With alkaline phosphatase, there seems to be a delay of about thirty minutes between the synthesis of enzyme protein and its final activation. It is possible that a gene might be transcribed continuously, but the mRNA translated only periodically. Unfortunately, inhibitors of RNA synthesis tend to have multiple effects, making interpretation of results difficult, and transcriptional control hard to test for (38).

One possibility which has received very little attention is that apparent periodic patterns in enzyme activity might

be produced _in vitro_ during the course of an enzyme assay
as a result of, for example, variations in protease activity
during the cell cycle. During an assay, samples from different
stages of the cycle might be degraded to different extents,
resulting in artefactual variations in activity.

Undoubtedly, some periodic synthesis does occur during
the cell cycle; however, in the absence of controls for many
of the reports of periodicity, the contribution of periodic
synthesis to total protein accumulation remains unclear.

Continuous synthesis;

The alternative to periodic synthesis is continuous synthesis of a substance. This may be a smooth exponential rise, or a linear pattern with a constant rate until some point in the cell cycle, when a fairly sharp doubling in rate occurs. A differential measure such as a pulse of radioactive tracer, is generally necessary to distinguish these possibilities. Few measurements of enzyme activity have been sufficiently accurate for this distinction to be made, but a linear pattern with a rate change has been found for three enzymes in S.pombe (37), and one enzyme in Chlorella (39). A similar pattern has been found for total dry mass (40), RNA synthesis (41) and CO_2 production in S. pombe (42). Creanor has measured CO_2 production in synchronous cultures of this organism made by selection. When grown in minimal medium, a linear pattern was observed which was not found in asynchronous controls. The rate change still occurred in synchronous cultures of DNA synthesis was inhibited by deoxyadenosine, or if nuclear division was blocked with mitomycin C. The change was not found in synchronous cultures produced by induction synchrony, where DNA synthesis and division were synchronised but 'growth' was not. Curiously, the rate change was not found in cells growing in complex media. In contrast to S. pombe, an exponential increase in RNA has been found in S.cerevisiae (43).

Models of the Cell Cycle

(a) Periodic Synthesis:

Two theories have been put forward to explain the periodicity
in synthesis of enzymes. The first, which can be called
'oscillatory repression', is concerned with enzymes which are
controlled by end product repression (44, 45). It was developed
primarily for prokaryotes, but can be applied equally well to
eukaryotes. A system where an enzyme product can repress the
synthesis of that enzyme has negative feedback: if the end
product pool is high, the enzyme will be repressed; if it is
low, the enzyme will be produced. With the right choice of
constants, stable oscillations can be generated. There is no
intrinsic reason why these oscillations should bear any relation-
ship to the frequency of the cell cycle. In order to meet
this objection, it has been suggested that the oscillations
are entrained by an event which is dependent upon the cycle,
such as by a pulse of mRNA produced at the time of gene re-
plication (46).

This theory elegantly explains why sucrase and alkaline
phosphatase in Bacillus subtilis (44), and ribulose 1,5-diphosphate
carboxylase in Chlorella (28), are synthesised continuously
when repressed, and periodically when derepressed. However,
some enzymes, the α- and β-glucosidases in budding yeast, for
instance, show steps at the same time in the cycle both with
and without induction (47). Arginine and ornithine carboxyl-
ases in budding yeast also show steps in activity at the same
time both with and without induction by arginine, and there

are no major cyclic changes in the arginine pool (48).

Some of these results may be explained by postulating an additional control by a general catabolite repressor. However, there is also some doubt about the theoretical side of this model, as it is difficult to devise oscillating feedback systems with biologically realistic parameters (49). Even if oscillatory repression is a control mechanism, it seems unlikely that it is the control for all step enzymes.

The second theory is the 'linear reading' model, developed to explain periodicity in enzymes in budding yeast (reviewed in 50). It suggests that genes are transcribed in the same order as their linear sequence on the chromosomes, perhaps by the movement of an RNA polymerase along the chromosome. As a result a gene would only be transcribable, and therefore inducible, for a short time in the cell cycle.

There are several experimental results to support this hypothesis. The budding yeasts Saccharomyces dobzhanskii and S. fragilis have antigenically distinct β-glucosidase enzymes. Both enzymes show a single step in activity per cycle, and are subject to the same regulatory controls. When these yeasts are hybridised, two enzyme steps per cycle are found, suggesting the non-allelic genes are transcribed at different points in the cell cycle (51). The oscillatory repression model would predict a single step.

Tauro and Halvorson have studied the multiple M genes for α-glucosidase in budding yeast (52). The homozygote M_1M_1 and the heterozygote M_1m_1 both showed a single step at the

same point in the cycle, indicating that an increase in gene

dosage at one locus does not affect the timing of the steps.

In contrast, the introduction of other non-allelic genes

produce extra steps, one for each non-allelic gene. Tauro,

et. al., have compared the timings of steps of nine enzymes

with their gene position in budding yeast (53). Four enzymes

located on the same chromosome gave results in good agreement

with the linear reading model. The remaining five enzymes

were too widely distributed over the chromosomes for any con-

clusions to be drawn from their timing.

There is similar good evidence for linear reading of three

enzymes in the normal cell cycle of B. subtilis (54), and for

five enzymes during synchronous spore germination (55).

As with the oscillatory repression theory, linear reading

is unable to accommodate many important exceptions. Firstly,

it is unable to explain the significant proportion of continuously

synthesised enzymes (2). Secondly, there is good evidence to

show that enzymes can be induced at all stages of the cycle

in bacteria (reviewed in 2), fission yeast (56), and Chlorella

(58, 59), which would not be permitted in a straightforward

linear reading model.

Both models therefore fail to provide a comprehensive

explanation for the patterns of enzyme synthesis found in cells.

As yet, there are too many gaps in our knowledge to be able to

judge their significance to cell cycle control, and it is

possible that they may be both partially correct. It is equally

possible that other mechanisms remain to be discovered.

(b) <u>Sequences of Events</u>:

Whatever the molecular basis for generating periodic synthesis, some mechanisms must exist to maintain the order and timing of these, and other, periodic events in the cell cycle at both the molecular and structural levels. A number of possible relationships could exist between observable events in a temporal sequence (reviewed in 2, 60). The events could lie in a 'dependent sequence', where a direct causal connection exists between successive events such that each one cannot occur unless the preceeding one has occurred. Alternatively, the events could be independent and not have direct causal connections, a master timing mechanism initiating them in sequence. This can be called an 'independent, single timer sequence'. The linear reading model would be such a system. The oscillatory repression theory can be considered as a permutation of this mechanism, where each event is independent, with its own timer.

These basic elements can be combined in a variety of ways, introducing the problem of maintaining the correct phase relationships between separate sequences. Timers could run independently, or alternatively could all start at one time. Separate sequences could converge at a series of 'checkpoints', where one sequence could not proceed past the point till another sequence had reached some critical stage.

The controls which regulate cell cycle events can be investigated by disturbing the progress of the cell through the cell cycle. Blocking an event in a dependent sequence would prevent all subsequent events, and perturbing the sequence

might alter the timing. If each event had its own timer, blocking one event would not necessarily affect the others, whilst a perturbation might alter both their order and their timing. In principle, this sort of approach permits any control model to be tested.

Three main techniques are available for probing cell cycle controls: specific chemical inhibitors of events; changes in physiological conditions, such as nutrients, or temperature; and temperature sensitive mutants which are affected in progress through the cell cycle at the restrictive temperature.

Early work in this field established that DNA synthesis, mitosis and cell division form part of a dependent sequence. Blocking DNA synthesis or mitosis prevents the other events occuring. Growth however, appears to be unaffected. Total protein and RNA synthesis increase as normal in HeLa cells blocked at DNA synthesis by amethopterin, fluorodeoxyuridine (61) or thymidine (62, 63); and in S. pombe blocked at the same stage by hydroxyurea (64). Volume increase is unaffected in mouse L5178 Y cells treated with fluorodeoxyuridine or hydroxyurea (65). Similar results have been obtained for inhibition of mitosis by heat shock in Terrahymena (66), and by colchicine arrest in mouse L 5178 Y cells (65), although in the latter interpretation was complicated by the normal suppression of RNA synthesis at mitosis.

There is some limited evidence to suggest that in the reverse situation, where growth is blocked, DNA synthesis and division can still proceed. If cells of S. pombe are deprived

of a nitrogen source growth is much reduced, but the cells go
on to divide once or twice, producing abnormally small progeny (67).

Mitchison has suggested that these observations can be
explained if the cell cycle is comprised of two dissociable
sequences of events which normally run together, but can be
made to run separately (2, 60). One of them contains DNA
synthesis, mitosis and cell division, and may be called the
'DNA-division cycle' (DD-cycle); the other contains the main
events of growth, such as enzyme patterns and rate changes,
and may be called the 'growth cycle'.

The evidence for the growth cycle has become fairly limited
with the discovery that many of the previously reported periodic
patterns of enzyme activity were due to perturbations. However,
the rate change in CO_2 production in S. pombe appears to be
a valid marker of progress through the 'growth cycle' (42),
lending credence to the original proposal. The rate change is
seen in synchronous cultures prepared by selection synchrony,
even after treatment with hydroxyurea, to inhibit DNA synthesis
or mitomycin C to block nuclear division. In contrast, no
rate change was seen in synchronous cultures prepared by
induction with deoxyadenosine.

The introduction of temperature-sensitive cell division
cycle mutants (cdc mutants) has enabled the earlier studies
on the 'DD-cycle' to be both confirmed and considerably extended
(reviewed in 68). Mutants have been isolated in E. coli (reviewed
in 69), budding (70) and fission yeasts (71), Chlamydomonas
(72), Tetrahymena (73), and mammalian cells (74). At the
restrictive temperature these mutants are blocked at some

critical stage in the DD-cycle after passing a point in the
cell cycle called the transition point. Cells prior to the
transition point become blocked; those after this time proceed
through division, and their daughters become blocked. Those
events which are subsequent to, and dependent upon the blocked
stage, do not occur. The availability of cdc mutants con-
siderably extends the range of cell cycle events that can
be blocked by chemical inhibitors, and they permit delicate
tests for causality to be made. In the majority of cases the
molecular basis of the lesion is unknown, but recently Nasmyth
has identified a cdc mutant in S. pombe which is blocked in
DNA synthesis as having a temperature-sensitive DNA ligase (75).

The existence of a dependent sequence of events, in which
gene products complete their function at particular points in
the cell cycle, raises the possibility that the dependency
lies at the level of gene expression: the products of one
group of genes might have to complete their function before
another group of genes are expressed. There is some limited
evidence available to support this model. The synthesis of
the histones is temporally coincident with DNA replication,
suggesting a close co-ordination between them (14-16). Inhibition
of DNA synthesis causes a marked decrease in histone synthesis (16),
accompanied by the disappearance of histone mRNA (17). Ex-
pression of the histone genes would therefore seem to be de-
pendent upon DNA synthesis. Other proteins involved in DNA
replication have been shown to exhibit step increases around
the time of S phase, but the controls are unknown.

(c) <u>Division</u>:

Cells growing in a constant environment tend to have a relatively constant size at division. It is therefore likely that there is a homeostatic mechanism for regulating cell size, which co-ordinates the DNA division cycle with growth. This is further substantiated by experiments where DD-cycle events are blocked. The cells continue to grow and produce abnormally large cells (e.g. 79). When the block is released, the succeeding cycles are shorter than normal. This produces smaller cells each time, until the cells reach their correct size, when the correct generation time resumes. Fantes has shown that this shortening of succeeding cycles occurs for larger cells of <u>S</u>. <u>pombe</u> in normal growth, where there is some spread in size at division (76).

The concept that emerges from this work is that the primary trigger for mitosis and cell division comes from a mechanism that measures cell size. Fantes and Nurse (77) have shown that cell size in <u>S</u>. <u>pombe</u> alters when the growth rate is changed by using different nutrients. In general, cell size was diminished as the growth rate was slowed and cycle time increased. In a nutritional shift-down, when cells were transferred from rich to poor medium, the cells were accelerated through G2 and into nuclear division, and size at division started to fall shortly after the change. After a shift-up, there was a rapid inhibition of nuclear division followed a little later by a plateau in cell numbers, and a sharp increase in size at division. These results suggest that entry into mitosis

is governed by a size control, and that the size control is modulated by the nutritional conditions.

Mutants with lesions in the size control over nuclear division have been identified in S. pombe (78, 79). These wee mutants have the same generation time as the wild-type, but divide at half its size. Mutants, some temperature sensitive, have been found in two independent genes, wee 1, and wee 2. Nurse has shown that shifting a ts wee 1 mutant to the restrictive temperature causes an acceleration of cells through G2 and into division, accompanied by a rapid decrease in the size of dividing cells (78). This result is analogous to those from the nutritional shift-down experiments. Significantly, the size of wee cells is insensitive to nutritional changes, and it seems likely that the wee gene products are also essential for the nutritional control over division.

The wee 1 gene product would seem to inhibit mitosis until the cell has grown to the requisite size at division. Inactivation of the gene product allows division to occur at a much smaller size. The wee 2 locus has been found to map in the middle of the cdc 2 gene. Temperature sensitive cdc 2 mutants are blocked in nuclear division at the restrictive temperature, and the wee 2/cdc 2 gene product is therefore required for both the control of division, and for division itself (reviewed in 67).

The molecular basis of the size control is unknown. One simple model would be for the wee 1 gene product to bind to the wee 2/cdc 2 gene product, thereby inactivating it, and

blocking division. The binding would be modulated by the nutritional state of the cell. At the correct size the block would be released, for instance by dilution out of the wee 1 product (67).

Outline of thesis

I have outlined some of the main areas of cell cycle

research, and highlighted four questions of major importance:

(i) What is the contribution of periodic synthesis to total

protein accumulation? The answer to this question is

fundamental to our understanding of the mechanisms of growth.

(ii) What is the effect of perturbing the cells upon the

pattern of growth? (iii) Is the synthesis of some proteins

dependent upon completion of some cell cycle event? This has

bearing on our models of cell cycle control and gene expression.

(iv) What is the molecular basis of the size control over

division? These questions have one technical problem in

common; their solution requires the direct determination

of the relative proportions of a substantial fraction of the

cells proteins under a variety of different conditions.

One approach to this problem is to use gel electrophoresis.

This technique resolves proteins into fractions according to

some physical parameter, for example, molecular weight, or

iso-electric point. For maximum resolution these fractions

should be as small as possible, and ideally only one protein.

The advent in recent years of sophisticated gel electrophoresis

techniques enables changes in hundreds, or even thousands, of

proteins to be measured directly, and obviates the need to

measure enzyme activities. The discontinuous one-dimensional

SDS gels devised by Laemmli are capable of resolving several

hundred proteins in a sample according to their molecular

weight (80). A number of samples are readily compared by

using slab gels. The recent introduction by O'Farrell and workers of high resolution two-dimensional gel electrophoresis techniques capable of resolving several thousand proteins -perhaps all of a cells proteins- offers to provide a comprehensive picture of patterns of synthesis (81,82). Needless to say, with this increase in sophistication there is an increase in attendant problems: the vast amount of information on such gels causes problems in handling the data; the gels are more prone to artefacts; and they are more expensive and time consuming to use.

The most sensitive way to measure changes in the pattern of synthesis of a protein is to measure changes in the rate of synthesis using radio-isotope pulse labelling. In principle, separation of pulse labelled samples by gel electrophoresis followed by autoradiography is the best approach now available to investigating general patterns of growth.

In this thesis I have used these techniques to study the cell cycle of the fission yeast Schizosaccharomyces pombe. S. pombe is an excellent organism for such a study. A considerable amount is already known about its cell cycle, including the most sophisticated cell cycle control model yet developed in a eukaryote. Several approaches to cell cycle analysis can be used with S. pombe. Temporal patterns of events can be determined from synchronous cultures, and good, relatively unperturbed synchronous cultures can be prepared by size selection using the elutriator rotor (83). In addition, asynchronous cultures can be removed from the rotor after loading, providing

a control for some of the perturbations induced during the
selection procedure. Secondly, there is a series of
temperature sensitive cdc mutants: these mutants are affected
in cell cycle progress at the restrictive temperature, and
arrest at some characteristic stage in the cell cycle (71).
Thirdly, another class of mutants are altered in the regulation
of the cell cycle. These wee mutants have lesions in the size
control over entry into mitosis, and divide at a smaller
size (78,79).

Chapter 2: MATERIALS AND GENERAL METHODS

MATERIALS

Most chemical reagents were supplied by BDH ('Analar' grade), and most biochemicals by Sigma, with the following notes and exceptions:

1) Radiochemicals were obtained from Amersham Radiochemical Centre. [^{35}S]-Sulphate was supplied as an aqueous solution pH 6-8, carrier free, at either 2 mCi/ ml or ca. 30 mCi/ ml. L-[^{35}S]-Methionine was supplied as a sterilised aqueous solution, ca. 8 mCi/ ml, >5000 mCi/ mmol, containing 0.2% β-ME.

2) The butyl-PBD was supplied by Fisons.

3) The DNase I and RNase A were supplied by Worthington.

4) The ampholytes used were from LKB (supplied under the name 'Ampholine'), pH ranges 5-7, 6-8 and 3.5-10, as specified in the text.

5) The following were supplied by BDH:

    SDS (specially pure grade)

    TX-100 (scintillation grade)

    urea ('Aristar' grade)

    acrylamide (reagent grade). Recrystallised from chloroform to give silvery-white plates.

    bis-acrylamide (reagent grade). Recrystallised from acetone to give pure white fine needles.

    glycine (electrophoretically pure grade).

    Folin-Ciocalteu phenol reagent

    TEMED

6) The tris was supplied by Sigma. For the SDS slab gel buffer 'Trizma' pre-set pH 8.7 crystals were used, as it was found

that most pH electrodes gave inaccurate readings at the high
concentrations of tris used, resulting in a considerable loss
in resolution by the gels.

MEDIA

The EMM 3 medium (sulphur free) contained per litre:

Carbon source	glucose	20 g
Nitrogen source	NH_4Cl	5 g
Phosphate source	Na_2HPO_4 (anhyd.)	1.8 g
Buffer	KH pthalate	3 g
Vitamins	inositol	10 mg
	nicotinic acid	10 mg
	calcium pantothenate	1 mg
	biotin	0.01 mg

The vitamins were kept as a 1000X strength stock solution at $4^{o}C$. A few drops of preservative (1 pt dichloroethane, 2 pts chlorobutane, 1 pt chlorobenzene) were added, and boiled off before use.

Salts	$MgCl_2 \cdot 6H_2O$	1.07 g
	$CaCl_2 \cdot 6H_2O$	0.02 g
	KCl	1.0 g

Stored as a 20X strength solution.

Trace	H_3BO_3	0.5 mg
	$MnCl_2 \cdot 4H_2O$	0.46 mg
	$Zn(CH_3COO) \cdot 2H_2O$	0.305 mg
	$FeCl_3 \cdot 6H_2O$	0.2 mg
	H_2MoO_4	0.145 mg
	$CuCl_2 \cdot 2H_2O$	0.026 mg
	citric acid	1.0 mg
	KI	0.01 mg

Stored as a 100X strength solution. Supplemented with Na_2SO_4 (anhyd.) as specified in the text.

CELLS

The fission yeast <u>Schizosaccharomyces pombe</u> is a cylindrical cell with rounded ends (ca. 10μm long x 3.5μm in diameter), that grows mainly in length during the cell cycle. It has most of the typical features of a eukaryotic cell, except that its mitosis is somewhat different from that of higher eukaryotes. Nuclear division takes place at 0.75 of the cycle. At 0.85 of the cycle, a cell plate, or septum, appears across the middle of the cell, which cleaves at the end of the cycle to form the new ends of the daughter cells (84). There is therefore an unusually large interval between mitosis and cell separation. The S period is positioned right at the end of the cycle. Earlier work suggested a G1 period of 0.2 of the cycle, and an S period of 0.1, but recent evidence from autoradiographs after pulse-labelling of DNA molecules indicates a shorter G1 of ca. 0.1 of the cycle and a longer S period of 0.2. With these short G1 and S periods, the cell spends most of the cycle (ca. 0.7) in G2 (67).

1) Construction of Strains:

Where necessary, the required strains were constructed from existing strains by standard genetical methods for <u>S</u>. <u>pombe</u>, as described by Gutz, et. al. (85). Strains were crossed to the opposite mating-type strain (i.e. 972h$^-$, or 975h$^+$) on malt extract agar plates. The resulting ascospores were them streaked out on yeast extract agar plates, the tetrads separated by micromanipulation, and clones of each spore grown up. The required strain was identified by phenotype, and test-crossing

on replica plates to both 972h$^-$ and 975h$^+$.

2) Maintainance of Stocks:

Stocks of strains were kept on yeast extract slopes, stored
at 4°C. Before use, strains were re-isolated from stock by
streaking out onto yeast extract-magdala red plates. Pre-
cultures were made by innoculating 20 mls of EMM 3 with a
healthy, (i.e., pale pink), clone, and growing to stationary
phase. These cultures were discarded after 1-2 weeks, when
the viability began to decline. Stocks were replaced every
three months with a fresh slope prepared from a healthy clone,
derived by re-isolation form the stock being replaced (84).

3) Media:

The yeast extract and malt extract agar plates have been
described by Gutz, et. al. (85). The basic formula used for
liquid media was EMM 3 (78) modified to contain no sulphur (see
materials). This was supplemented by up to 40 mg/l Na$_2$SO$_4$,
and in some cases amino acids, as specified in the text.
Flasks were innoculated with stationary phase pre-culture cells,
and grown overnight at 25° or 35°C, as specified, to an optical
density of about 0.1 - 0.3 at the start of an experiment.

4) Cell Density:

A rough estimate of cell number/ ml was obtained by the
measurement of optical density at 595nm using a Unicam SP 600
spectrophotometer, taking a conversion factor of 1.5x10^6 cells/
ml/0.1 O.D. units (84). An accurate value was obtained by
fixing samples in a filtered 0.22% formaldehyde-1% saline
solution and counting cells with a Coulter counter (Model B).

Cell plate index was determined by counting the proportion of cells with cell plates in a sample using a PZO microscope set up for dark field illumination.

LABELLING

Choice of Label:

Proteins in S. pombe may be labelled by $[^3H]$- or $[^{14}C]$-amino acids, $[^{35}S]$-methionine and cysteine, or by $[^{35}S]$-sulphate (which is converted into $[^{35}S]$-methionine and cysteine in the cell). To give a good exposure of an autoradiograph in 1-6 weeks, a gel needs to be loaded with around $0.5-5 \times 10^6$ cpm of radioactive protein. Limitations on the amount of protein that can be loaded onto gels without a loss of resolution require specific activities of about 10^4 cpm/μg for SDS gel electrophoresis, and $0.4-4 \times 10^5$ cpm/μg for two-dimensional gels. Any radio-label employed must therefore be incorporated into protein to at least this extent in the time of labelling used (20 minutes for rate determinations, 1 hour for 'dependent protein' searches, and overnight for wee 1 protein searches). It would be advantageous to be able to use the same radio-isotope, if not the same label, for each type of experiment. Otherwise, the relative proportions of proteins on gels would appear to vary, and a protein which appeared as a major component on gels when labelled with, for example L-$[^{35}S]$-methionine, might be absent when labelled with, say, $[^3H]$-tryptophan.

At the activities required, cost becomes an important consideration. In the presence of alternatives, the $[^3H]$- and $[^{14}C]$-labelled amino acids can all be excluded for this reason; they are either taken up too slowly, neccessitating large amounts of label to attain a suitable specific activity; or the final cost of label in a sample itself is too high.

$[^{35}S]$-Sulphate was used for the long-term labelling of cells
(1hour or more) for three reasons: firstly, it is taken up
rapidly; secondly, it is converted in the cell into $[^{35}S]$-
methionine and $[^{35}S]$-cysteine. This is important in the search
for the _wee_ 1 protein, as even an abundant protein would not
be detected on gels if it did not contain the amino acid used
for labelling. The chance of a protein not containing both
amino acids is much smaller. Thirdly, $[^{35}S]$-sulphate is
inexpensive, so relatively large amounts may be used, affording
short exposure times for autoradiographs.

1) Growth in Sulphate Limitation:

Preliminary experiments showed that overnight labelling
with 1 mCi $[^{35}S]$-sulphate of a 25 ml culture of wild-type
$(972h^-)$ S. pombe, growing in EMM 3 containing 40 mg/l sulphate
at $25^{\circ}C$, would give a specific activity of ca. 20 000 cpm/μg
protein. This is at least a factor of four too low for use
with two-dimensional gel electrophoresis, and to raise the
specific activity the amount of sulphate in the medium was
reduced. Figure 1 shows the growth of S. pombe into stationary
phase at a variety of sulphate concentrations, followed by
measurement of the optical density at 595nm. This method is
effectively a crude measure of cell mass/ ml, and masks an
important effect on cell division. Figure 2 shows the increase
in cell number during the growth of S. pombe into stationary
phase at 2.5 mg/l sulphate. At about $5.6x10^6$ cells/ml, the
cells were accelerated into their final division, presumably
by a nutritional effect upon the size control over nuclear
division (77). The final stationary phase cells were as a

Figure 1

The growth of 972 h⁻ into stationary phase due to
sulphate limitation was followed by measurement of
$O.D._{595}$. Cells were grown at $25°C$ in EMM 3 containing
different amounts of sulphate.

● 0 mg/ l,   ○ 0.5 mg/ l,   ▲ 1.0 mg/ l,   △ 2.5 mg/ l,
□ 5 mg/ l,   ■ 40 mg/ l.

The innoculum used was washed once by pelleting
by centrifugation and resuspension in sterile sulphate-
free EMM 3. Some growth occurred in the 0 mg/ l sulphate
culture due to carry-over from the innoculum and to
sulphate present as an impurity in the reagents used.
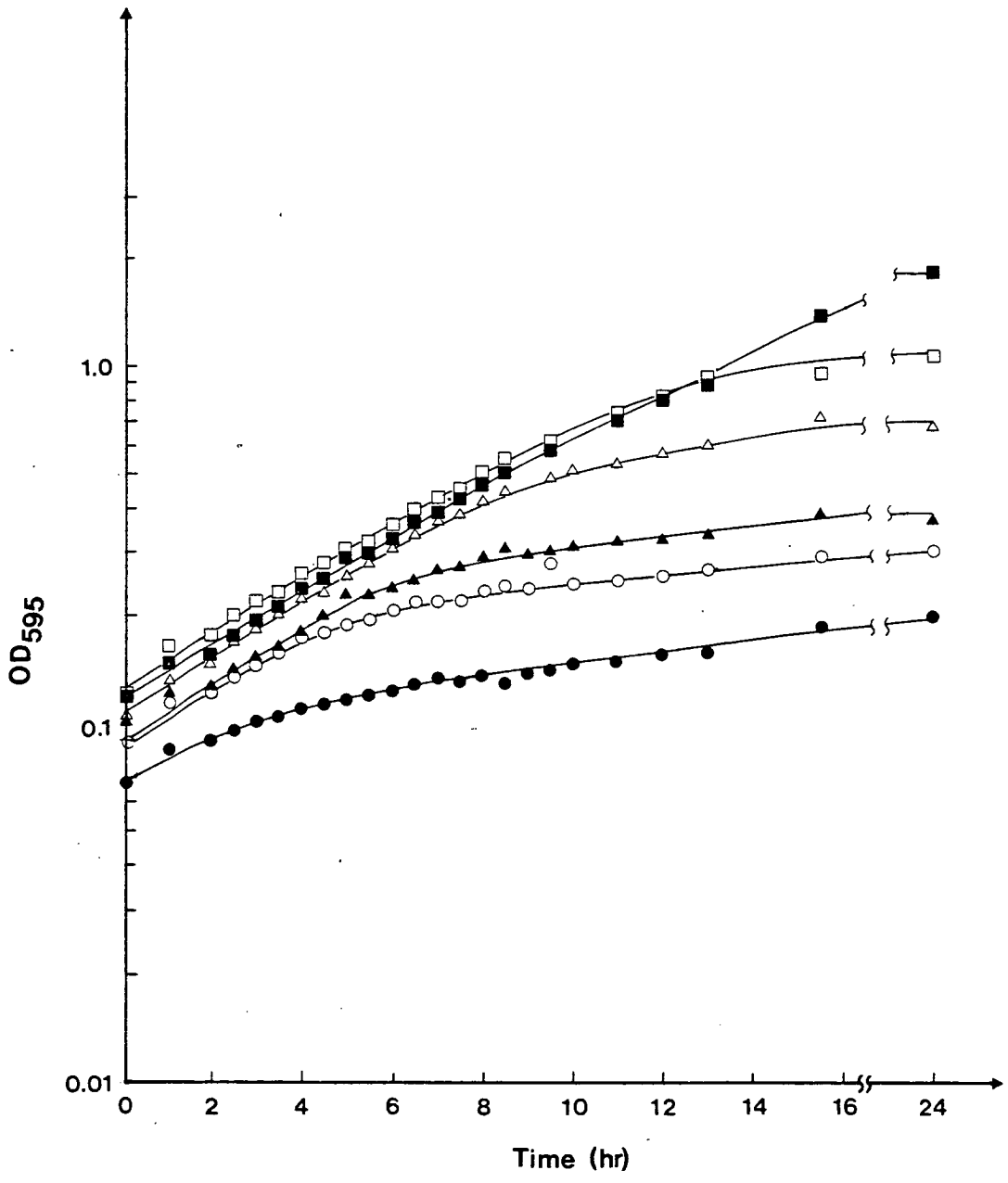
Figure 1

Figure 2

The growth of 972 h⁻ into stationary phase in EMM 3 containing 2.5 mg/ l sulphate at 25°C was followed by measurement of cell number.

● 2.5 mg/ l sulphate, ○ 40 mg/ l sulphate control.
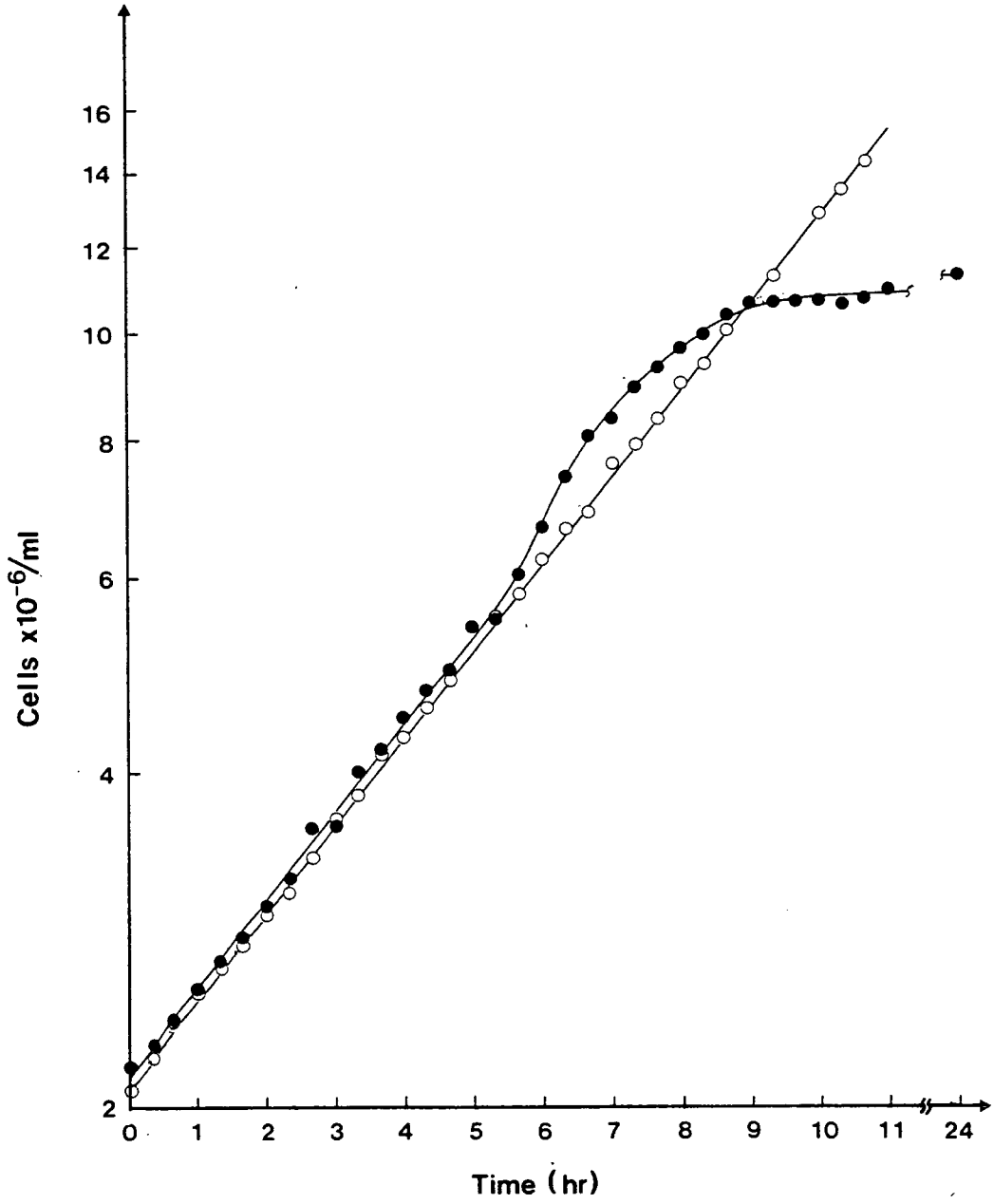
Figure 2

Figure 2

result smaller than exponential phase cells. If such effects
at the end of the exponential phase of growth are to be avoided,
cells cannot be used within two cycle from stationary phase;
in this case, not more than ca. $3x 10^6$ cells per ml. A sulphate
concentration of 10 mg/ 1 was therefore chosen for labelling
cells with $[^{35}S]$-sulphate: This would give a specific activity
of ca. 80 000 cpm/ µg, sufficient for gels; and the cells can
be used at up to ca. $12x 10^6$ cell/ ml (Fig. 3), which gives
a sufficient range for experimental purposes. Interestingly,
these cells did not show such a sharp acceleration into
stationary phase as those growing in 2.5 mg/ 1 sulphate. This
choice of sulphate concentration is further supported by the
fact that this concentration was successfully used for many
years in the EMM 2 medium (84).

The kinetics of uptake and incorporation of $[^{35}S]$-sulphate
by S. pombe growing in EMM 3 containing 10 mg/ 1 sulphate at
$25^{o}C$ are shown in Figure 4. Incorporation was not linear,
and diverged from uptake. These results suggest that the
sulphate pool is expanding during growth, and therefore whilst
$[^{35}S]$-sulphate is adequate for long term labelling, it cannot
be used for the determination of rates of synthesis. Approx-
imately 1% of sulphate label is incorporated into protein for
each 0.1 O.D.$_{595}$ unit increase in cell density.

2) $[^{35}S]$-Methionine labelling:

To measure rates of synthesis of polypeptides in synch-
ronous cultures, not less than ca. $5x 10^5$cpm must be incorporated
during 20 minutes into 2.5 ml of culture containing typically,

Figure 3

The growth of 972 h⁻ into stationary phase in
EMM 3 containing 10 mg/ 1 sulphate was followed by
measurement of cell number.

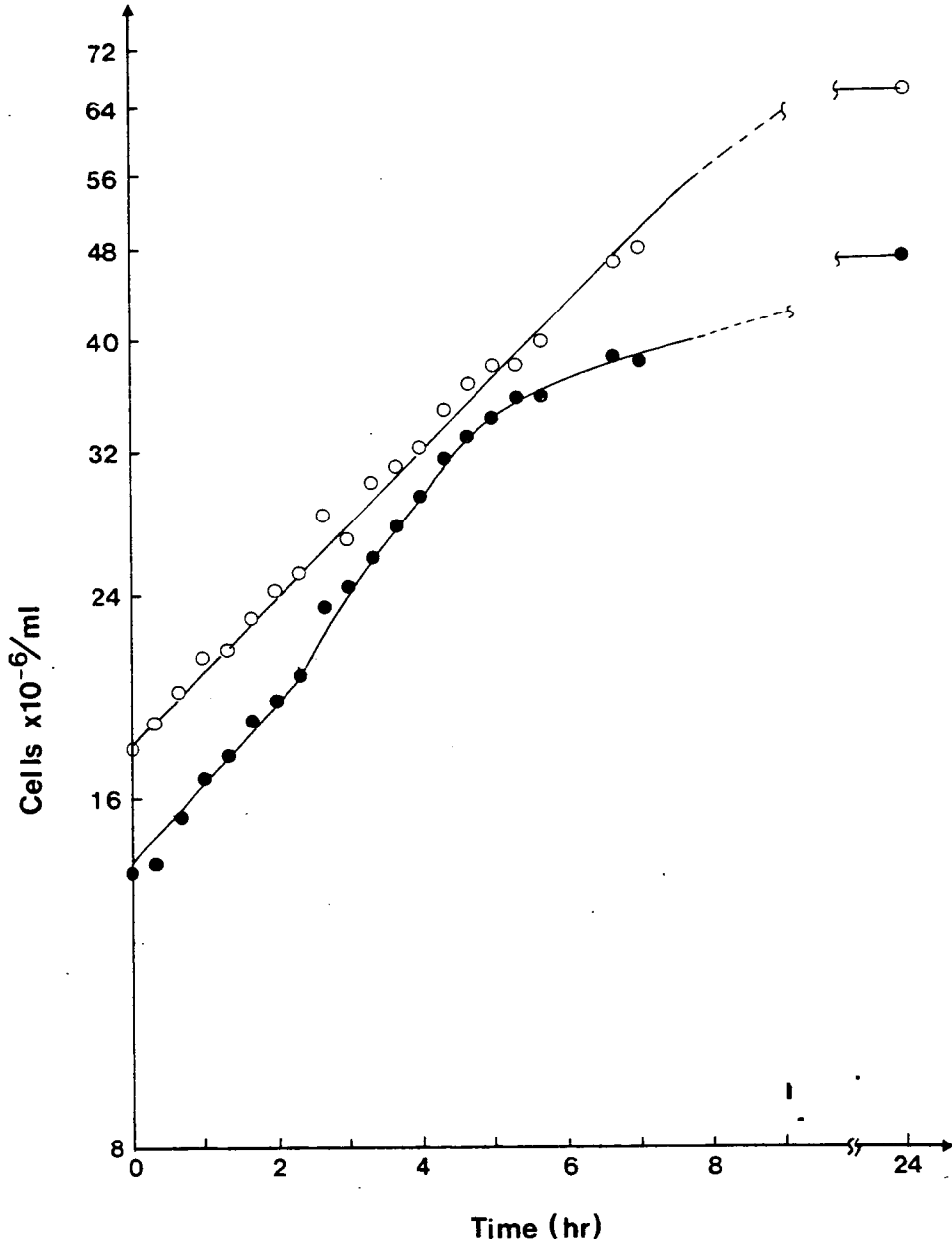● 10 mg/ 1 sulphate, ○ 40 mg/ 1 sulphate control.

Figure 3

Figure 4

Kinetics of $[^{35}S]$-sulphate uptake and incorporation. A 100 ml culture of 972 h$^-$ at an O.D.$_{595}$ of 0.14 growing in EMM 3 medium containing 10 mg/ l sulphate at 25°C was pulse-labelled with 100 µCi of $[^{35}S]$-sulphate and uptake (O) and incorporation (●) followed.
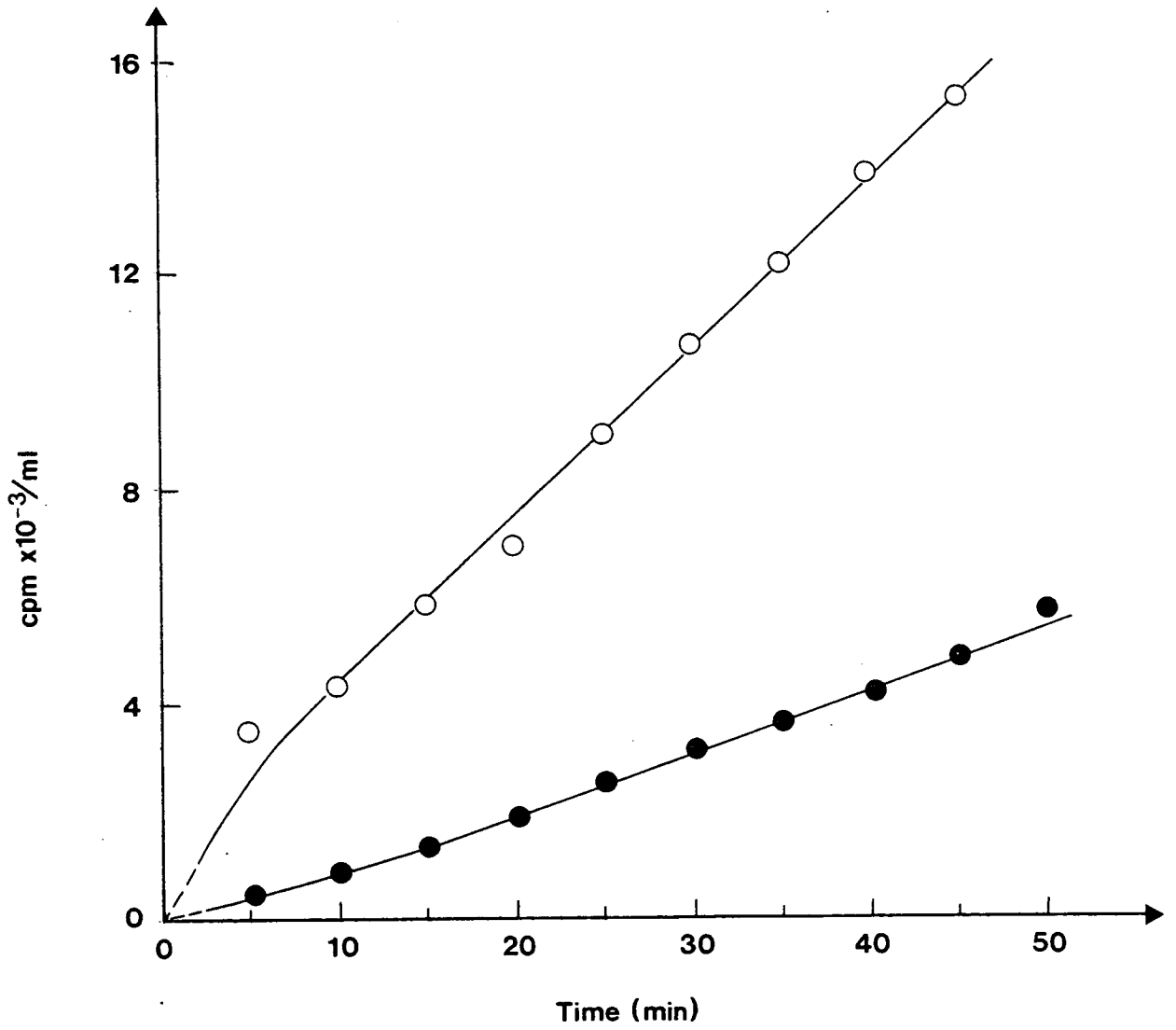
Figure 4

51

1.5 x10^6 cells/ml, at 35°C. Preliminary experiments showed
that 30-40 µCi of [^{35}S]-methionine per pulse would be adequate,
using EMM 3 +40 mg/l sulphate. Figure 5 shows the kinetics
of uptake and incorporation of L-[^{35}S]-methionine in asynchronous
culture. The rate of incorporation became linear after about
two minutes, and then paralleled uptake. These kinetics,
and the small pool size, make methionine an ideal label for
rate determinations. Approximately 2% of the label is incorpor-
ated during a 20 minute pulse.

3) <u>Measurement in Cells</u>:

The uptake of [^{35}S]- labelled sulphate of methionine by
filtering samples of cells onto 2.5 cm Whatman GF-C discs,
and washing with a solution of carrier (10 mg/l sulphate, or
2 mg/ml L-methionine). Incorporation was measured by adding
samples of cells to 2.5 ml of ice-cold 10% TCA containing
carrier (10 mg/ml sulphate, or 2 mg/ml L-methionine). Samples
were allowed to fix on ice for not less than 15 minutes, then
filtered onto GF-C discs and washed well with ice-cold 10% TCA.
In both cases, the discs were dried and counted in a Packard
model 2425 scintillation counter using a 0.5% Butyl-BPD solution
in sulphur-free toluene as scinctillant (counting efficency
83% for ^{35}S).

4) <u>Measurement in Protein</u>:

The counts incorporated in protein extracts were determined
by adding a sample (5-50 µl) to 25 µl of a 10 mg/ml solution
of BSA, then adding 0.5 ml of ice-cold 25% TCA. samples were
then allowed to fix on ice for not less than 15 minutes, then

Figure 5

Kinetics of L-[^{35}S]-methionine uptake and incorporation. A 15 ml culture of 972 h$^-$ at an O.D.$_{595}$ of 0.10 growing in EMM 3 containing 40 mg/ l of sulphate at 35°C was pulse-labelled with 30 µCi L-[^{35}S]-methionine and uptake (○) and incorporation (●) followed.
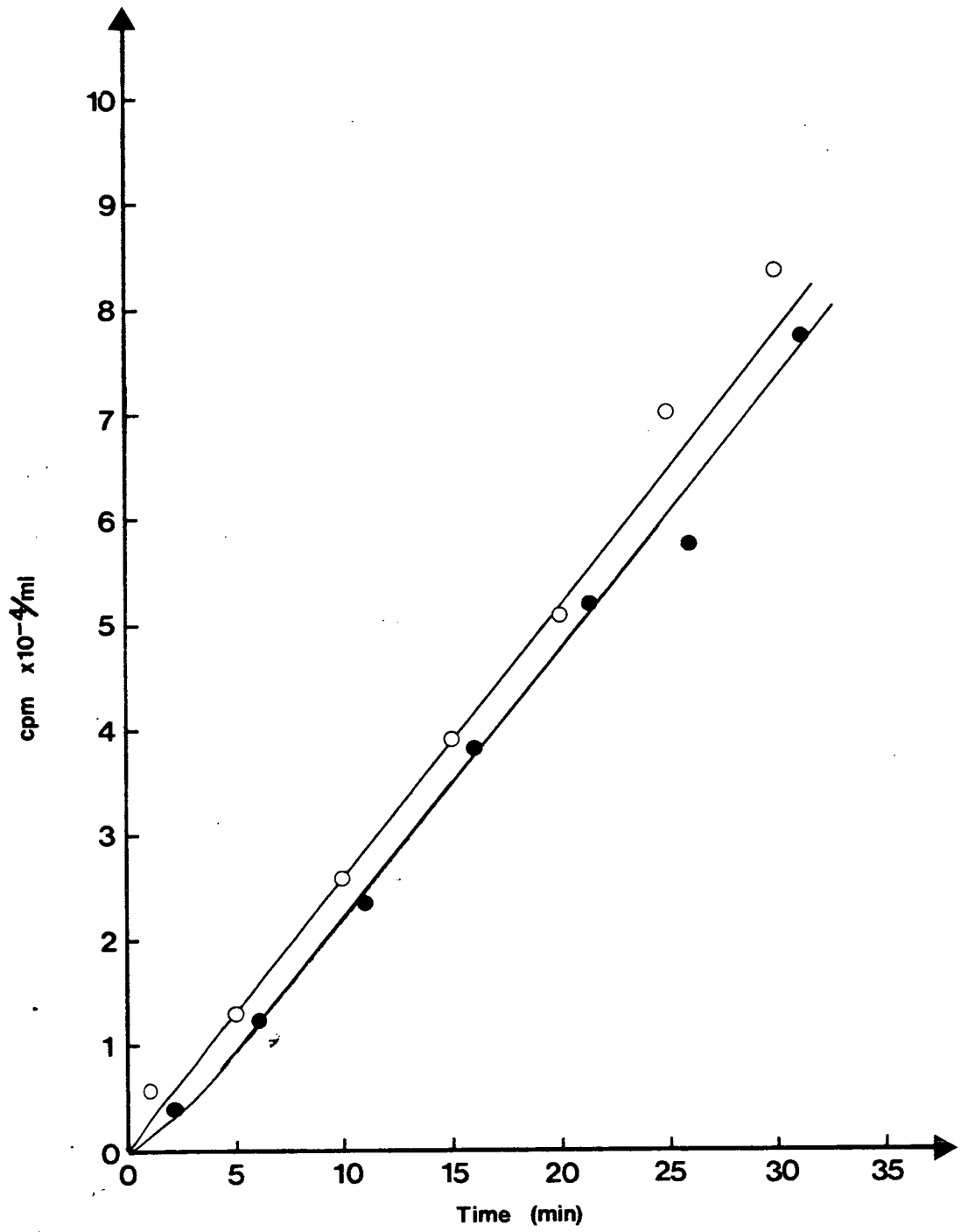
Figure 5

filtered onto GF-C discs and washed well with ice-cold 10%

TCA. The filters were dried and counted, as above.

SOLUBILISATION

The aim of a solubilisation technique is reproducibly to

take as many polypeptides as possible up into solution.

However, the vigour of the method has to be tempered by the

need to avoid the introduction of artefacts; for example,

guanidinium thiocyanate, although a good solubilising agent,

introduces artefactual spots on two-dimensional gels (86).

Most important, the final solution of solubilised proteins has

to be compatible with the gel system employed. Aimes and

Nikaido (86) have extensively studied the solubilisation of

membrane proteins for gel electrophoresis, and their results

can be generalised to total cell protein. They found that

non-ionic detergents solubilised membrane proteins inefficiently,

but that the ionic detergent, dodecyl sulphate, was an efficient

and fairly non-discriminating solubilising agent, especially

at higher temperatures. Heating samples at $100^{o}C$ for 3 minutes

at an SDS: protein ratio of 2.6 gave optimum solubilisation.

The presence of β-mercaptoethanol during the solubilisation was

not essential (although, of course, it was required before

the gels were loaded). The resulting samples were compatible

with SDS gel electrophoresis, and by adding a non-ionic detergent

(NP-40), could be made compatible with iso-electric focusing,

due to the formation of mixed micelles.

In addition, modification of the proteins by the action

of the cells own proteins, particularly proteases, has to be

prevented, or at least minimised. Yeast cells contain several

different proteolytic enzymes, of which three (proteases A,

B, and C) have been studied in detail (reviewed in 87). These proteases are remarkably stable: protease A is quite stable at pH 5-6 at temperatures up to $50^{o}C$, and may be stable in 6 M urea at some pH values; protease B can survive treatment at $60^{o}C$, and remains active temporarily in 5.5 M urea; protease C loses little activity after incubation for 30 minutes at $50^{o}C$ at pH 6, and is quite resistant to 6 M urea at $25^{o}C$. This resistance to denaturation creates a special hazard during solubilisation. The majority of cellular proteins are rendered more susceptible to proteolysis when denatured; thus even a temporary retention of activity by a protease can cause severe degradation during solubilisation by detergents. Indeed, in some cases denaturing conditions can actually activate a protease.

Pringle (87) lists two approaches to preventing the formation of proteolytic artefacts:

1. The use of protease inhibitors. PMSF and do-isopropyl fluorophosphate are both potent inhibitors of some, but not all yeast proteases.

2. Abrupt denaturation to denature the proteases as rapidly as possible, thereby reducing the time available for degradation. Boiling samples with 1% SDS, 1% β-ME was found by Pringle to provent further proteolysis, and subsequent incubation of samples at room temperature gave no further changes.

These guide-lines were combined with the solubilisation methods of Aimes and Nikaido (86) and the requirements of two-dimensional gel electrophoresis (81), in the solubilisation schemes used in this work.

1) <u>Solubilisation for One-dimensional Electrophoresis</u>:

All samples were kept on ice until boiled, and all solutions used were ice-cold. The cells were pelleted by centrifugation, and washed by resuspension in 2 ml Cell Suspension Buffer (10mM Tris-HCl, pH 7.4; 5mM $MgCl_2$) and repelleting. They were then resuspended in 250 µl Cell Suspension Buffer, and 5 µl of 0.1 M PMSF in ethanol added. 250 µl of ice-cold 40 mesh glass beads were added, and the cells broken by vortexing with a 'whirlimixer' (Fisons) for 15 second intervals, then allowing to cool on ice for not less than 45 seconds until the majority of cell walls (i.e. 85-95%) were ruptured (3-5 Minutes total), as shown by phase contrast microscopy. The resulting homogenate was removed from the beads, the beads washed twice with 250 µl of Cell Suspension Buffer, and the washes combined with the homogenate. 20 µl of 10% SDS was added, the the mixture boiled on a water bath for 3 minutes, then freeze-dried. The resulting solid material was then resuspended/dissolved in 50 µl 2% SDS, and boiled for 4 minutes. A 5 µl sample was taken for protein assay by the Lowry metho (88), and 2.5 µl of β-ME added to the remainder, which was then boiled for a further one minute to ensure complete breakage of disulphide bridges. 7 µl of a 0.5% solution of bromophenol blue in 70% glycerol was then added, and the samples immediately loaded onto SDS slab gels(80). The yield of protein from this procedure is 88% $\pm$ S.E. 6%. Without SDS solubilisation, the yield from a homogenate after centrifugation is 23 $\pm$ 4%.

2) Solubilisation for Two-dimensional Electrophoresis:

The main modifications required to the one-dimensional
technique involved the need to enzymatically degrade the
nucleic acids in the sample, and to make the final sample
compatible with iso-electric focusing (81). All solutions
used were ice-cold, and samples were kept on ice until boiled.
Cells were pelleted by centrifugation, washed by resuspension
in Cell Suspension Buffer (12 ml for 25 ml cultures, 2 ml
for 2.5 ml Cultures), and repelleted. The cells were resuspended
in 250 μl Cell Suspension Buffer, and 200 μl ice-cold acid
washed 40 mesh glass beads, 5 μl of 0.1 M PMSF (ethanol solution)
30 μl of 2% SDS, 10% β-ME added. Cells were broken by vortexing,
as described above, and then 40 μl of 10x DNase-RNase (1mg/ml
DNase I; 0.5mg/ml RNase A; 50mM $MgCl_2$; 0.5M Tris-HCl, pH 7.0)
added. The mixture was allowed to stand on ice for 10-15
minutes, then 5 μl of 0.1 M PMSF solution added and the mixture
heated in a boiling water bath for 4 minutes. 20 μl of 2%
SDS, 10%β-ME was added to the hot mixture, which was then
allowed to cool for 5 minutes. A further 30 μl of 10xDNase-
RNase was added, and the mixture allowed to stand at room temperature
for 5 minutes. The homogenate was removed from the beads,
which were washed twice with 150 μl of Cell Suspension Buffer,
and the washes combined with the homogenate. The cell debris
was removed by centrifuging for 5 minutes in a Beckmann micro-
centrifuge, and the clear supernatant freeze-dried. The solid
material was resuspended in Lysis Buffer (10M urea; 4% TX-100;
5% β-ME; 2% pH3.5-10 Ampholytes) (50 μl or 100 μl, depending
on the amount of cells used), mixed for 2 minutes, then Triton

X-100 added to 10% v/v, and mixed for a further 10 minutes. The solution was then centrifuged for 3 minutes, and the supernatant applied to gels. Samples were stored at $-20^{o}C$ when not required, and discarded after six weeks.

GEL ELECTROPHORESIS

The two-dimensional gel system used was essentially that devised by O'Farrell (81), with a few minor modifications. This technique involves separation of the proteins in a sample in the first dimension according to their iso-electric point, by equilibrium iso-electric focusing on tube gels; then separation in the second dimension according to their molecular weight in SDS slab gels, as originally devised by Laemmli (80). This latter system was also used for the one-dimensional separations in this work.

1) Iso-Electric Focusing

a) Gel Preparation:

The iso-electric focusing gels were made in alcoholic-KOH cleaned, siliconised glass tubes, 13.5 cms long, I.D. 0.2 cm, O.D. 0.7 cm. The gels were made essentially as described by O'Farrell, but with the following modifications: The final gel composition was 4.0% acrylamide, cross-linked 60: 1 with 0.067% bis-acrylamide (this composition gave excellent mechanical stability), and TX-100 was used instead of NP-40 (they are qualitatively and quantitatively very similar in action). No TEMED was added to the gel mixture, the ampholytes serving as the initiators of polymerisation. The gels were poured to a depth of 12 cms. For the wee protein search, the gels contained 2% of pH 3.5-10 ampholytes; for the 'dependent protein' search, the gels contained 0.8% pH 5-7, 0.8% pH 6-8, and 0.4% pH 3.5-10 ampholytes.

b) Electrophoresis:

After polymerising for 90 minutes, the tops of the gels were washed with 10 µl of lysis buffer, then covered with a further 10 µl of lysis buffer. The tubes were then filled to the top with 0.1 M sodium hydroxide, and pre-focused by electrophoresis for two hours at constant current from a starting voltage of 200 V. 0.1 M sodium hydroxide was used as the negative(upper)electrode solution, and 0.01 M ortho-phosphoric acid as the positive(lower)electrode solution. After this time the upper electrode tank was emptied, the liquid above the gels removed, and the gel covered with 10 µl of IF gel Overlay Buffer (8M urea; 4% v/v TX-100; 5% v/v β-ME; 2% pH3.5-10 Ampholytes). The sample (up to 25µl) was loaded under this layer using a micro-syringe. The tubes were then filled to the top with 0.1 M NaOH. Fresh 0.1 M NaOH was poured into the upper tank, and the gels electrophoresed overnight (15-16 hours) at 1000 V.

C) Gel Equilibration and Storage:

After electrophoresis, the gels were removed from the tubes, and equilibrated with Equilibration Buffer (10% v/v glycerol; 5% v/v β-ME; 2.3% SDS; 0.0625M Tris-HCl, pH6.3) for 1½-2 hours. Then were then either loaded onto SDS slab gels, or placed on aluminium boats, which were then wrapped in 'Cling-film' and placed at -20°C. Gels could be stored in this way for several days before they began to become dehydrated.

2) SDS Gel Electrophoresis

Electrophoresis was performed as described by O'Farrell. In the studies on periodicity and dependency, 16% gels cross-linked 160:1 with 0.1% bis-acrylamide were used, with a 4% 36.5:1 cross-linked stacking gel. These 16% gels were found to give good resolution over a wide molecular weight range (Fig. 6), and dried down without cracking. In the wee 1 protein search 12% acrylamide, 0.33% bis-acrylamide; and 9.4% acrylamide, 0.26% bis-acrylamide gels were used to cover the molecular weight range (Fig. 7). The slab gels were 1.5mm thick, 15.5cms wide, and 14cms deep, with a 3cm deep stacking gel. If the gel was to be used for one-dimensional electrophoresis, sample wells 1.5cm deep were made in the stack gel by inserting a 14-tooth 'comb' into the stacking gel prior to polymerisation. For two-dimensional electrophoresis, the iso-electric focusing gels were placed on the top of the stacking gel, and 'glued' in place with a molten solution of 0.05% bromophenol blue, 1% agarose in Running Buffer (0.025M Tris; 0.192M glycine; 0.1% SDS). Gels were electrophoresed at 35 mA each at constant current, until the dye-front was 0.5cms from the end (ca. 3hrs.).

3) Slab Gel Treatment

Slab gels were either shaken with water for an hour, and then dried down directly onto Whatman 17 chromatography paper using a Bio-Rad gel dryer; or stained before drying down using Coomassie Brilliant Blue R. Gels were soaked for several hours, with shaking, in a 0.125% solution of Coomassie Brilliant

Figure 6

SDS-gel molecular weight markers (Sigma), comprised of enzymes of known molecular weight, and chemically cross-linked BSA; actin, spectrin (supplied by Dr. S. Thomson); and myosin (supplied by Dr. R. Burns) were solubilised and electrophoresed in a 16% acrylamide, 0.1% bis-acrylamide SDS gel.
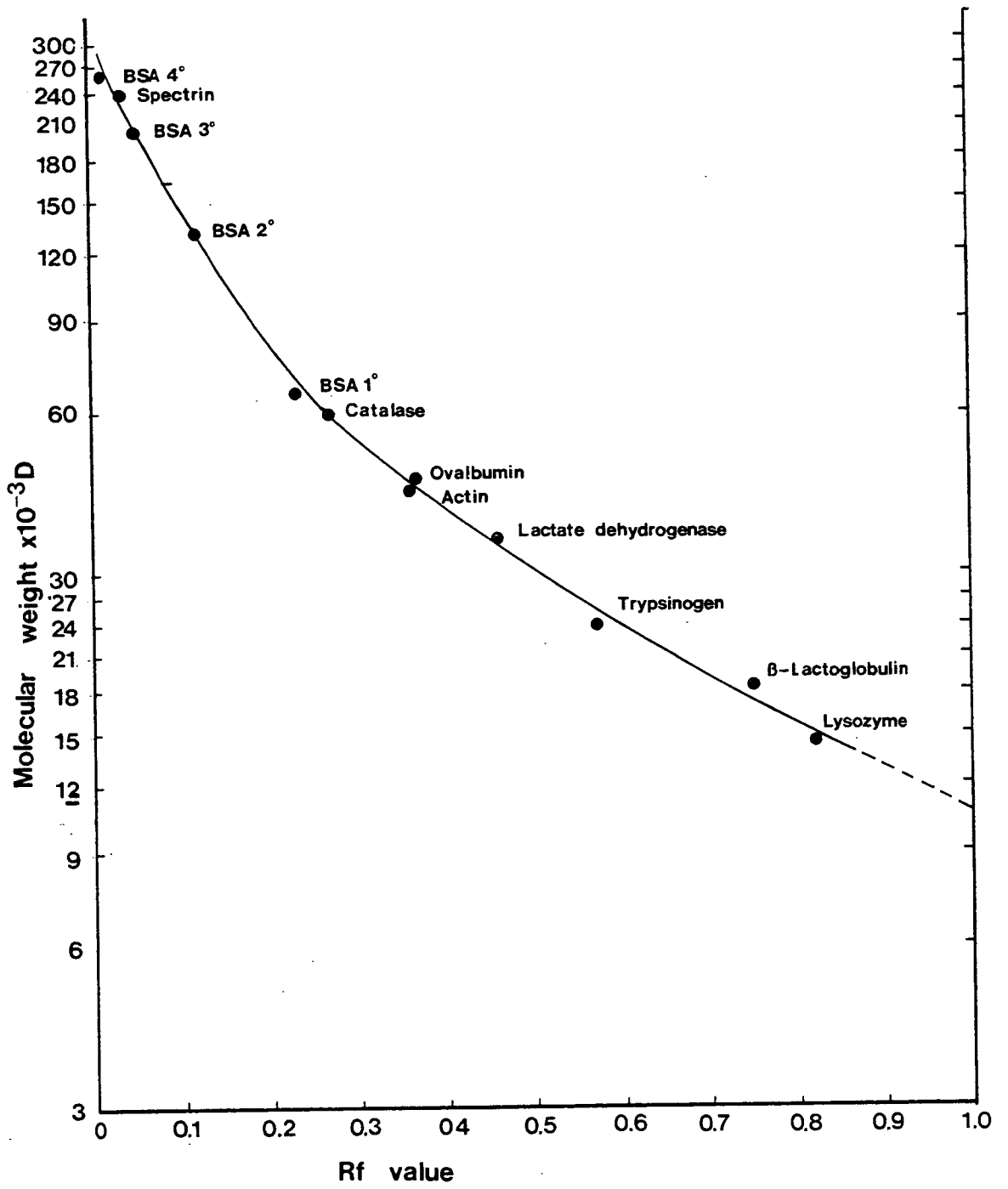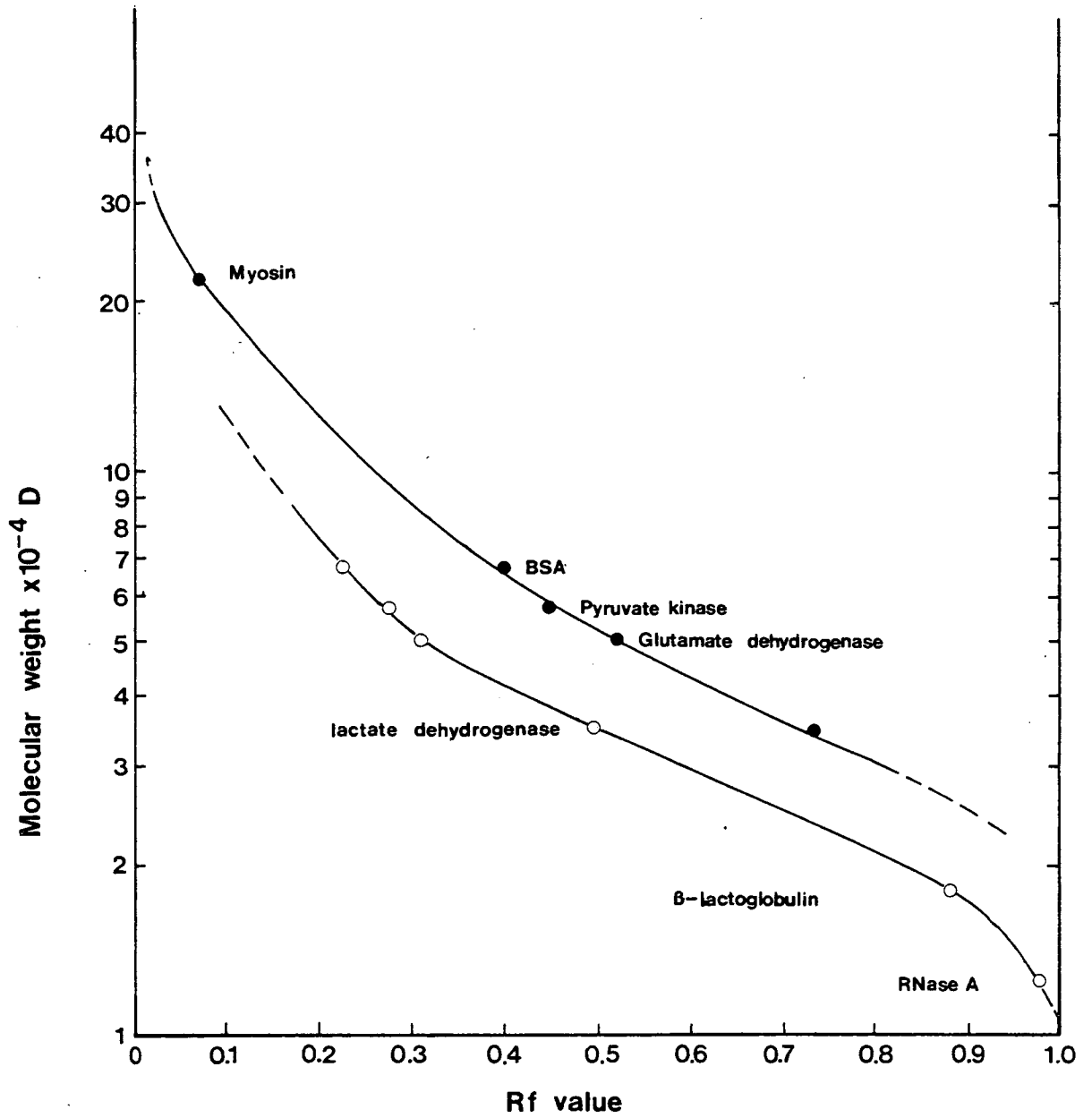
Figure 6

Figure 7

Blue R in 25% iso-propanol, 10% acetic acid, then destained
by repeated changes of 10% acetic acid.

RIBOSOME PREPARATION

To prepare unlabelled ribosomes, 0.5-1.0 l of 972h⁻ cells at 2-3 x 10^6 cells/ml growing in EMM 3, 40mg/l sulphate at 35°C. were collected by filtration, and resuspended in 5ml ice-cold NMT buffer (0.1M NaCl, 0.03 M MgCl$_2$, 0.01 M Tris-HCl, pH 7.4), to which 500 µl of 2.5% TX-100 and 50 µl of 0.1 M PMSF in ethanol were added. 5ml of ice-cold, acid washed 40 mesh glass beads were added, and the cells vortexed until more than 90% were disrupted, (ca. 5 minutes). The homogenate was removed from the beads, the beads washed twice with 2.5ml of ice-cold NMT buffer, and the washes combined with the homogenate.

To prepare labelled ribosomes, 25ml of cells were labelled with 100 µCi L-[^{35}S]-methionine for 3 hours. The cells were then collected by centrifugation, and washed by resuspension and repelleting from 2.5ml of ice-cold NMT buffer. The cells were then resuspended in 2.5ml of ice-cold NMT buffer, to which 250µl of 2.5% TX-100 and 25µl of 0.1 M PMSF in ethanol were added. 2.5ml of ice-cold acid-washed 40 mesh glass beads were added, and the cells vortexed as above. The homogenate was removed from the beads, which were washed twice with 2.5ml of ice-cold NMT buffer, and the washes combined with the homogenate.

Both labelled and unlabelled homogenates were then treated in the same way; essentially as described by Coddington and Fluri (89). They were centrifuged at 10 000 rpm for 10 minutes at 4°C in an MSE Prepspin 50 in a 10 x 10ml rotor, to remove

debris; then the supernatants were centrifuged at 50 000 rpm

for 90 minutes at 4°C in the same rotor. This gave a crude

ribosomal pellet which was either solublised for SDS gel electro-

phoresis, or further purified. To purify the ribosomes further,

the pellets were resuspended in 5ml of 5 mM $MgCl_2$, 0.2 $KHCO_3$,

pH 8.0, and centrifuged at 50 000 rpm for 90 minutes, as above.

The pellets were solublised by resuspension in 50 µl of NMT

buffer, adding 10 µl of 10% SDS, 3µl β-ME, and heating in a

boiling water bath for 4 minutes. To test that the pellets

contained ribosomes, 250 µl of unlabelled homogenate, and

a ribosomal pellet resuspended in 75 µl were each mixed with

saturated Na_2EDTA (25 & 10 µl respectively) phenol-cresol

(500 & 150 µl respectively) for 2 minutes. The aqueous layers

were then loaded onto 0.75% agarose E-buffer (36mM Tris:

31 mM $Na_2H_2PO_4$ 1mM Na_2EDTA) tube gels, and electrophoresed

for 80 minutes at 50 V constant voltage (90). The gels were

then stained in 1 µg/ml ethidium bromide, and photographed

under UV illumination. Figure 8 shows the RNA fractions

obtained in these gels (at these loadings, the DNA is below

the level of detection). The ribosomal pellets were clearly

enriched for rRNA. Figure 9 shows a Coomassie Brilliant

Blue stained SDS gel which had been loaded with total cell

extract, and a ribosomal fraction. The distribution of proteins

in the ribosomal fraction was radically different from the

total cell protein pattern. As expected, the ribosomal fraction

was considerable enriched in lower (i.e. less than 45 000 M)
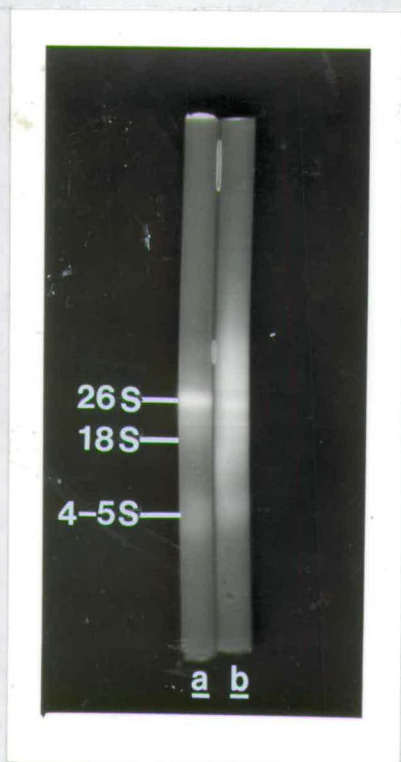
molecular weight major cell proteins.

Figure 8

a) RNA profile of total cell extract.
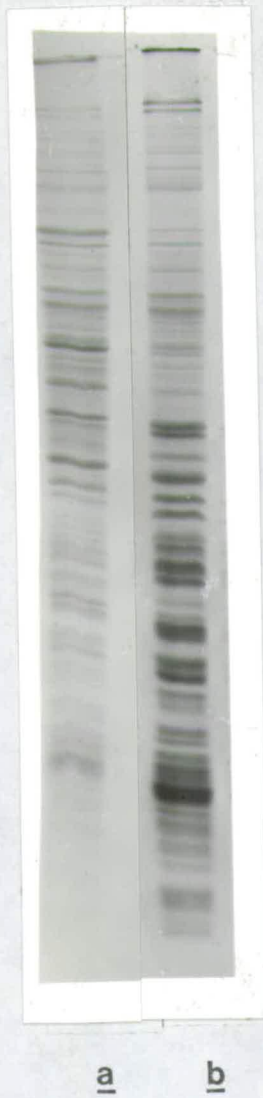
b) RNA profile of ribosome pellet.

a   b

Figure 9

Samples were fractionated on 16% acrylamide gels
and stained with Comassie Brilliant Blue R.

a) Polypeptide components of total cell extract.

b) Polypeptide components of ribosome fraction.

Chapter 3: <u>PATTERNS OF POLYPEPTIDE SYNTHESIS, AND THE EFFECTS</u>

<u>OF PERTURBATIONS</u>

INTRODUCTION

Synchronous cultures of <u>Schizosaccharomyces</u> <u>pombe</u> have

been used for more than ten years in studies of the patterns

of change in enzyme activity during the cell cycle. Bulk

synchronous cultures of <u>S</u>. <u>pombe</u> can be prepared by selecting

small cells on tube gradients (84), or the zonal rotor (91).

Unfortunately these techniques tend to produce perturbed

cultures (31). Recently, a method has been developed for

producing small volumes of relatively unperturbed synchronous

cultures of <u>S</u>. <u>pombe</u> by size selection using the Beckmann

elutriator rotor (83). Although these cultures are too small

for most enzyme activity measurements, they are ideal for the

determinations of rates of synthesis by pulse labelling with

radio-isotopes. The elutriator rotor technique also has

the advantage of permitting asynchronous control cultures to

be prepared from the remaining cells, allowing tests to be

made for at least some perturbations.

In this section, I shall describe the results of using

this technique, in conjunction with one-dimensional SDS gel

electrophoresis (80), to study the rates of synthesis of

96 proteins, in both perturbed and unperturbed cultures.

One-dimensional, gel electrophoresis was used, instead of

two-dimensional methods, because its resolving power is quite

adequate for detecting periodic protein synthesis, and it is

not susceptible to as many artefacts as two-dimensional electro-

phoresis, which might otherwise be interpreted as spurious

perturbations. In addition, it permits the study of the

pattern of synthesis of the ribosomal proteins, which represent

about 25% of the total proteins in S. pombe. These proteins

are not resolved in two-dimensional gels.

METHOD

Wild-type (972h⁻) Schizosaccharomyces pombe was used in all experiments, and all cultures were grown at 35°C, at which the generation time was 2hr 20min. A basic medium of EMM 3 (40 mg/l sulphate, unless otherwise stated), was used, supplemented with various L-amino acids, as described for each experiment. The kinetics of uptake and incorporation of L-[^{35}S]-methionine by S. pombe growing in these different media were determined by taking 15ml of cells from a one litre asynchronous culture at an O.D.$_{595}$ of 0.2 - 0.3 pulse-labelling with 40 µCi L-[^{35}S]-methionine, and sampling as described in General Methods, at the intervals of time shown. Synchronous and asynchronous cultures of S. pombe were prepared from the remaining 985ml of cells using the Beckmann elutriator rotor, as described (83). Twenty minutes after removal of the cells from the rotor, 2.5ml samples were taken at intervals of 20 minutes, and the cell proteins pulse-labelled for twenty minutes by adding the cells to L-[^{35}S]-methionine. Generally, 3 µCi of label was used per pulse, unless the proteins were to be run on gels. In this case, 40 µCi of label was used; and after sampling for incorporation at the end of the pulse, the samples were rapidly frozen in an acetone/dry-ice freezing mixture, and stored at -20°C, till required. During these pulses, concommitant samples were taken from the main cultures for cell number and cell plate determinations (see General Methods).

Frozen pulse-labelled samples were thawed on ice, the

cells pelleted by centrifugation, and washed by resuspension and repelleting from 10ml of ice-cold Cell Suspension Buffer. The cells were then broken, the proteins solublised for SDS gel electrophoresis as described in General Methods, and the yield of protein was determined by the Lowry method. The minimum yield was ascertained, and all the other samples were loaded at this mass onto 16% (160:1 cross-linked) Laemmli SDS slab gels. L-[^{35}S]-methionine labelled ribosomal proteins were loaded onto one slot. After electrophoresis, the gels were stained in Coomassie Brilliant Blue R, dried, and auto-radiographed for up to 26 days.

The autoradiographs were scanned by eye, as no mechanical means of sufficient resolution was available. The intensity of a band was estimated by comparison with a calibration strip, made by exposing a film to a strip of pieces of acrylamide gel containing [^{35}S]-sulphate. The activity/unit area of the pieces increased by a factor of two between successive pieces.

RESULTS

a) Choice of Medium

   L-[^{35}S]-methionine was chosen to label the cell protein

for reasons already discussed (see 'General Methods' section).

However, as shown in Figure 10, an asynchronous culture was

perturbed when the cells were grown in unsupplemented EMM 3

medium containing 40 mg/l sulphate. Creanor found similar

results using L-[^{3}H]-tryptophan to label total protein;

presumably due to fluctuations in the tryptophan pool, as the

perturbations disappeared when the pool was expanded by including

10 mg/l of tryptophan in the medium (92). In contrast, in-

creasing the size of the methionine pool by the addition of

methionine to the medium was found to increase the size of

the perturbations (Fig. 11). Moreover, the kinetics of labelling

became non-linear at 5 mg/l methionine (Fig. 11, inset),

preventing the determination of rates of synthesis from in-

corporation data, and thereby precluding higher methionine

concentrations.

   Perturbations in methionine labelling of total protein

in S. cerevisiae are reduced by supplementing the medium with

all the amino acids, with the exception of cysteine (93). A

similar result was found in S. pombe (Fig. 12), where no repeated

periodic patterns were seen over four hours in an asynchronous

control prepared in EMM 3 containing 2 mg/l of each amino acid,

except methionine and cysteine (compare Fig. 10). However,

the culture was still perturbed, and showed fluctuations in

rate which persisted for about 2½ hours, before the rate became

Figure 10

An asynchronous control culture was prepared from 972 h⁻ growing in EMM 3 + 40 mg/ 1 sulphate at 35°C. Every 20 minutes a 2.5 ml sample was pulse-labelled for 20 minutes with 3.7µCi L-[S^{35}]-methionine to determine the rates of uptake and incorporation of label. The experimental parameters are plotted per unit volume of culture on an arbitrary log scale. The real values of the experimental parameters per ml of culture equivalent to one log unit are given within the brackets in the symbol key.

○ Rate of uptake ($4x\ 10^4$), ● Rate of incorporation ($4x\ 10^4$), □ Cell number ($2x\ 10^6$).

Inset: Kinetics of uptake (○) and incorporation (●). 15 ml of asynchronous culture at an O.D.$_{595}$ of 0.10 was pulse-labelled with 30 µCi of label.
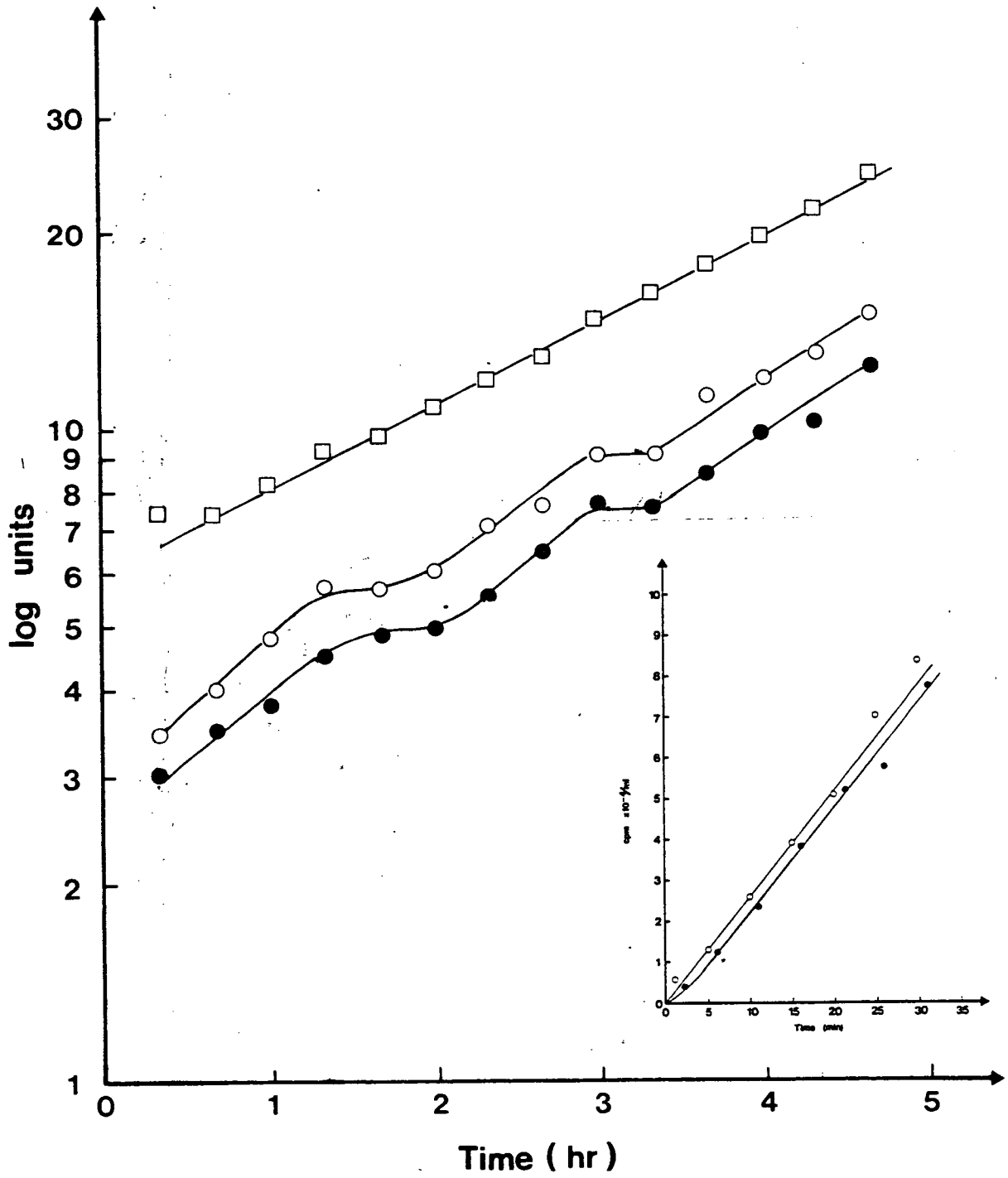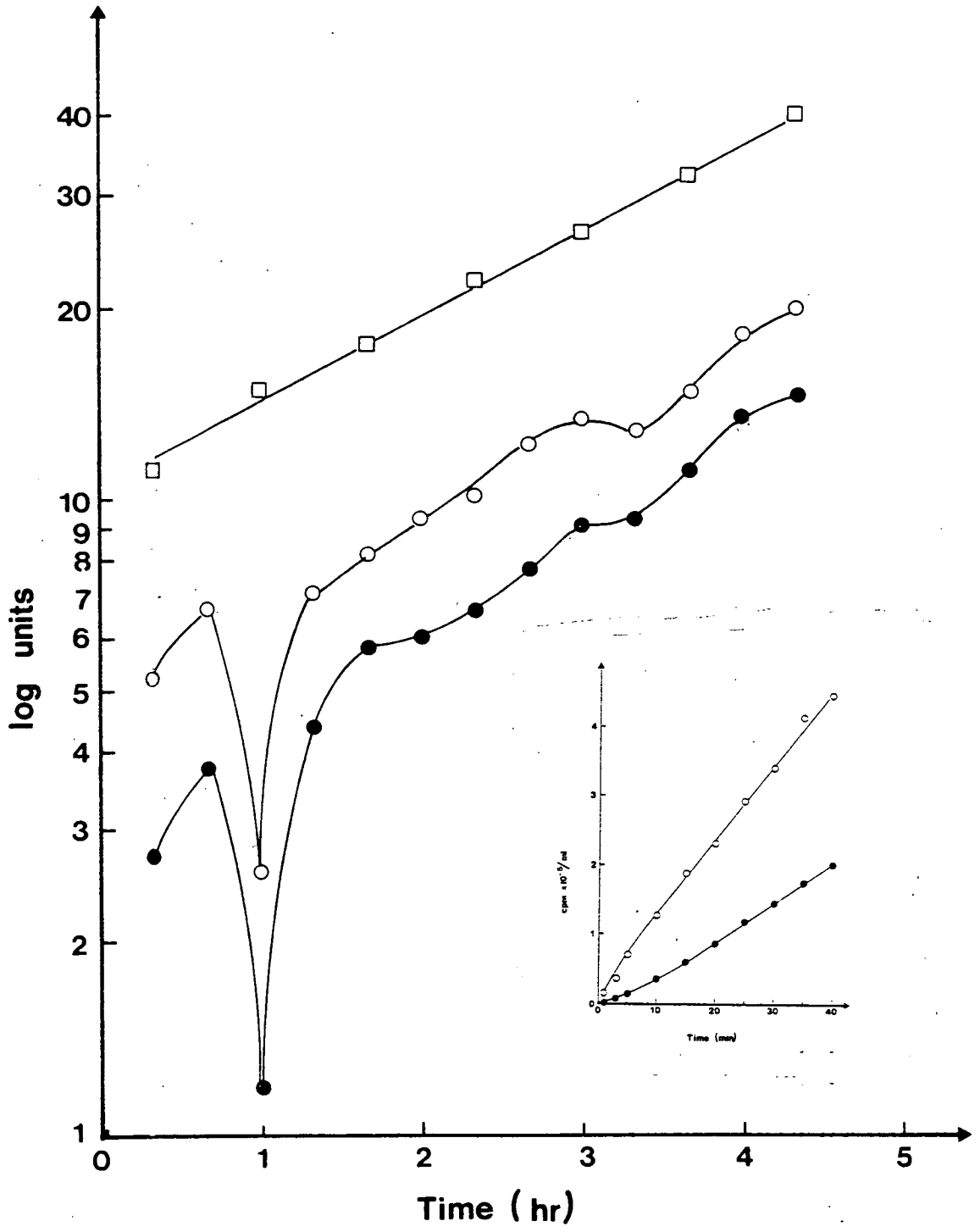
Figure 10

Figure 11

An asynchronous control culture was prepared from 972 h⁻ growing in EMM 3 + 40 mg/ l sulphate + 5 mg/ l L-methionine at 35°C. Every twenty minutes a 2.5 ml sample was pulse-labelled for 20 minutes with 3.7 µCi L-[^{35}S]-methionine to determine the rates of uptake and incorporation of label. An explanation of the arbitrary log scale is given in the legend of Figure 10.

○ Rate of uptake (1.6x 10^4), ● Rate of incorporation (1.6x 10^4), □ Cell number (1x 10^5).

Inset: Kinetics of uptake (◎) and incorporation (●). 15 ml an asynchronous culture at an O.D.$_{595}$ of 0.18 was pulse-labelled with 37 µCi of label.

Figure 11

<u>Figure 12</u>

An asynchronous control culture was prepared

from 972 h‾ growing in EMM 3 + 40 mg/ 1 sulphate +

2 mg/ 1 of each amino acid except methionine and cysteine

at 35°C. Every 20 minutes a 2.5 ml sample was pulse-

labelled for 20 minutes with 3.7 µCi L-$[^{35}S]$-methionine

to determine the rates of uptake and incorporation.

An explanation of the arbitrary log scale is given in

the legend of Figure 10.

○ Rate of uptake $(4x\ 10^{4})$, ● Rate of incorporation

$(4x\ 10^{4})$, □ Cell number $(4x\ 10^{3})$.
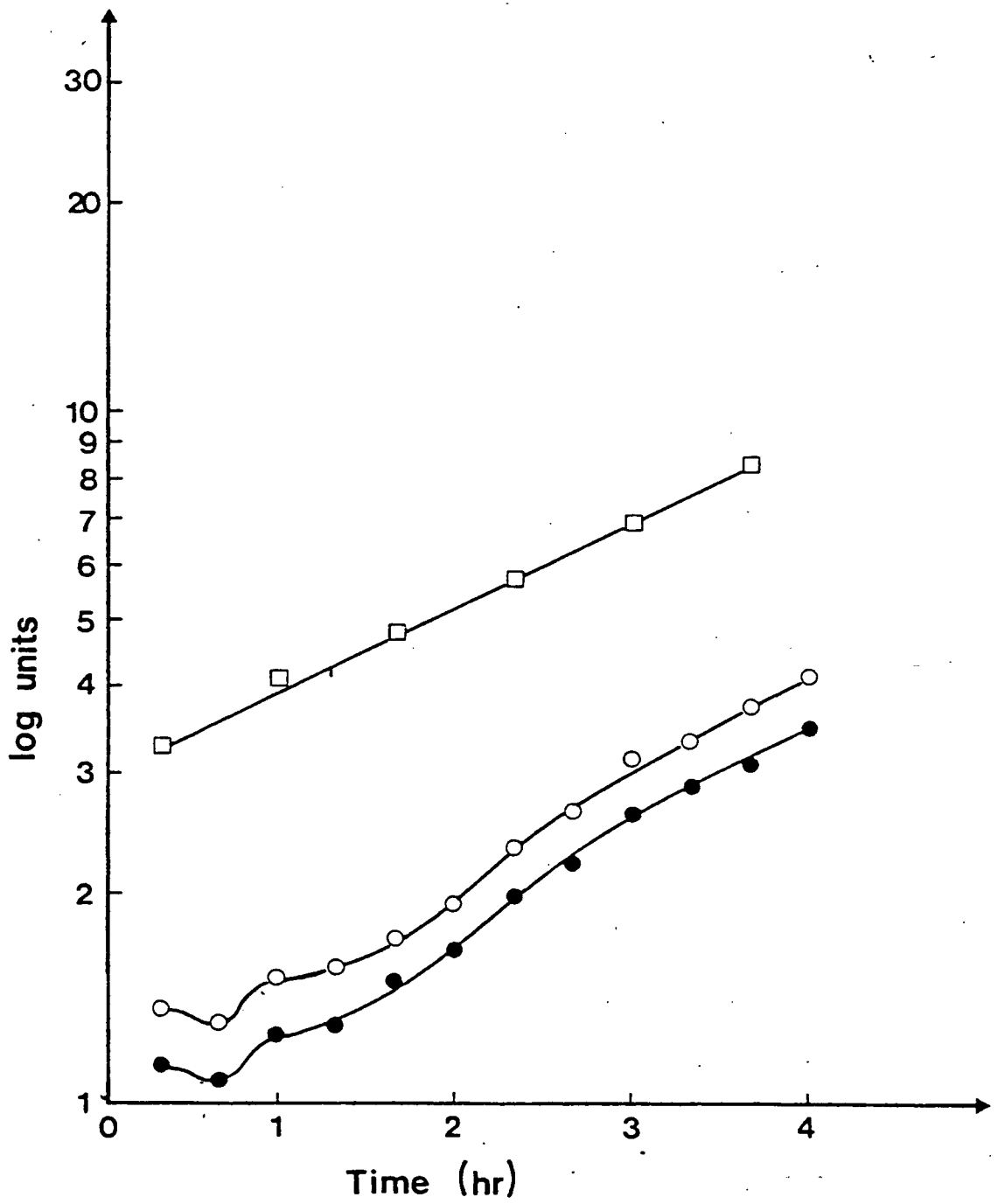
Figure 12

exponential. The addition of methionine in the presence of amino acids still increased the size of the perturbation (Fig. 13).

Cysteine is involved in the biosynthesis of methionine from homoserine, and cysteine was therefore added to the growth medium to investigate its effects upon the perturbations in methionine labelling. In the presence of the other amino acids (except methionine), cysteine was found to reduce·the size of the perturbations considerably. At 10 mg/l of each amino acid, 50 mg/l cysteine the perturbations were more-or-less eliminated (Fig. 14, control), and these supplements to EMM 3 medium were used to produce relatively unperturbed synchronous cultures. The kinetics of uptake and incorporation of $[^{35}S]$-methionine in this medium are shown in Figure 15.

It should be noted that in all these cultures, the pattern of uptake closely followed that of incorporation.

b) <u>Labelling in Synchronous Cultures</u>

The patterns of rate of total protein labelling in a synchronous culture and its accompanying asynchronous control prepared in EMM 3 containing amino acids and cysteine are shown in Figure 14. The asynchronous culture showed an initial perturbation, and thereafter an approximately exponential, i.e., nearly unperturbed pattern of labelling. The minor fluctuations in rate bore no relationship to the generation time of the cells. The synchronous culture showed an initial plateau in rate of about 40 minutes (0.3 of a cycle), presumably due to a perturbation. Thereafter the rate rose until 0.68 in the cell cycle, when a small inflexion occured. This covered

Figure 13

  An asynchronous control culture was prepared from
972 h⁻ growing in EMM 3 + 40 mg/ l sulphate + 2 mg/ l
L-methionine + 2 mg/ l of each amino acid except cysteine
at 35°C. Every 20 minutes a 2.5 ml sample was pulse-
labelled for 20 minutes with 3.7 μCi L-[^{35}S]-methionine
to determine the rates of uptake and incorporation of
label. An explanation of the arbitrary log scale is
given in the legend of Figure 10.

  ○ Rate of uptake (2×10^4), ● Rate of incorporation
(2×10^4), □ Cell number (2×10^5).
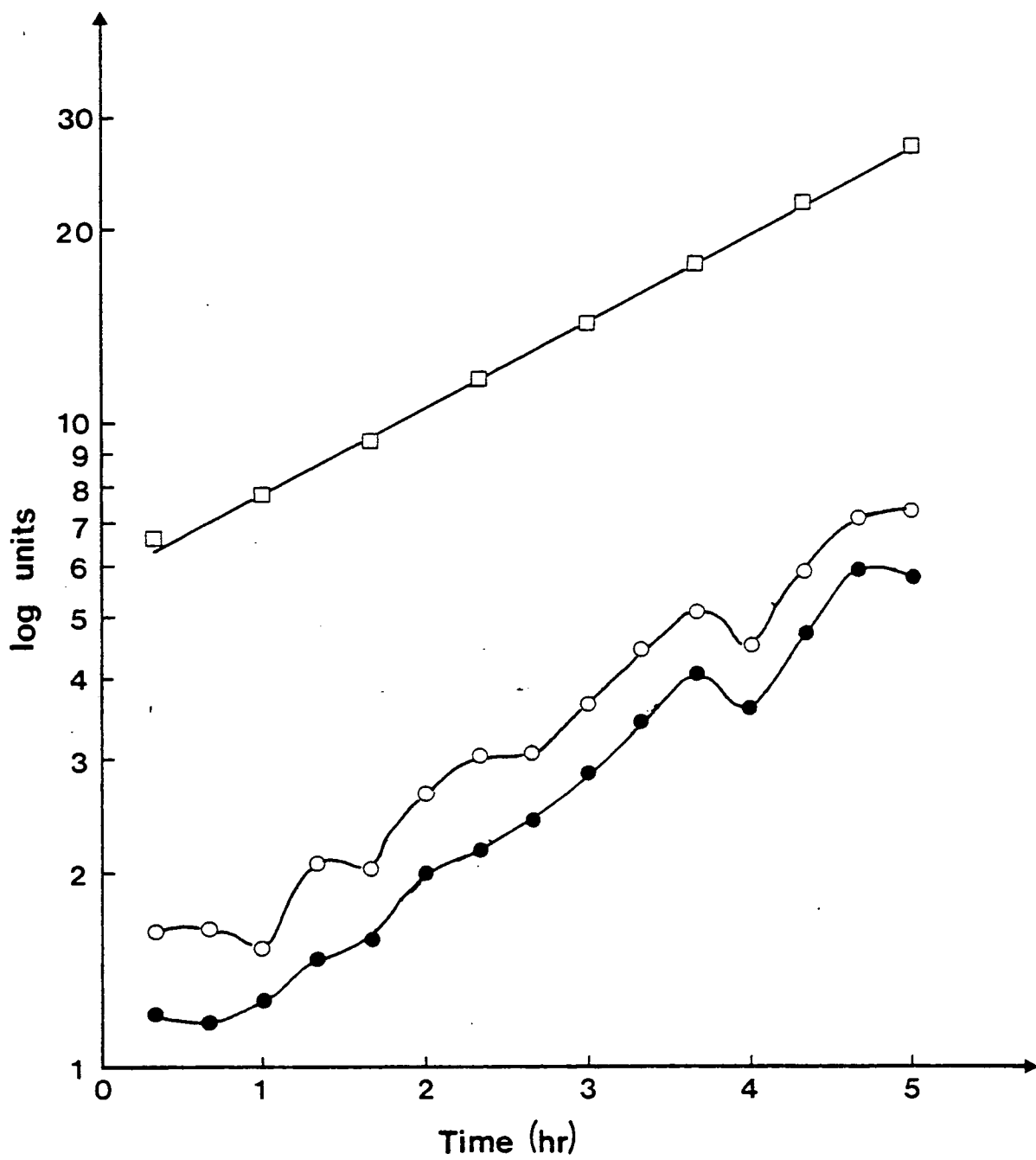
Figure   13

<u>Figure 14</u>

Synchronous and asynchronous control cultures were prepared from 972 h⁻ growing in EMM 3 + 40 mg/ 1 sulphate + 10 mg/ 1 each amino acid + 50 mg/ 1 cysteine, but no methionine, at 35°C. Every 20 minutes a 2.5 ml sample was pulse labelled for 20 minutes with 37 μCi L-[^{35}S]-methionine to determine the rate of incorporation of label. An explanation of the arbitrary log scale is given in the legend to Figure 10.

O Asynchronous control culture incorporation (1.2×10^5).

☐ Asynchronous control culture cell number (4×10^5).

● Synchronous culture incorporation (2.67×10^4).

■ Synchronous culture cell number (1×10^5).

▲ Cell plate index

↓ Indicate the mid-point of cell number rise.
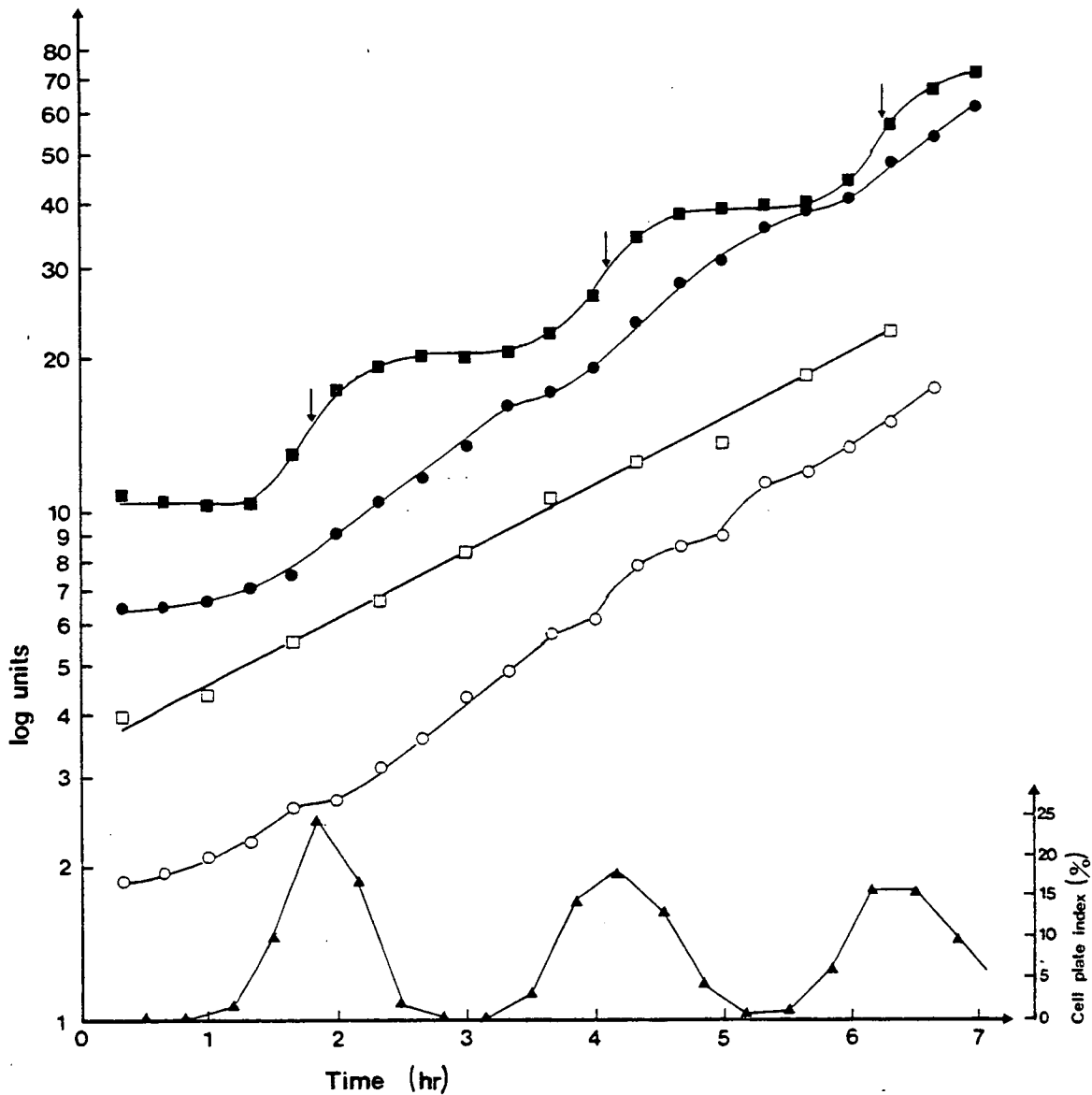
Figure 14

Figure 15

Kinetics of uptake (O), and incorporation (●)
of L-[^{35}S]-methionine by 972 h$^-$ growing in EMM 3 +
40 mg/ l sulphate + 10 mg/ l each amino acid + 50 mg/ l
cysteine, but no methionine at 35°C were determined by
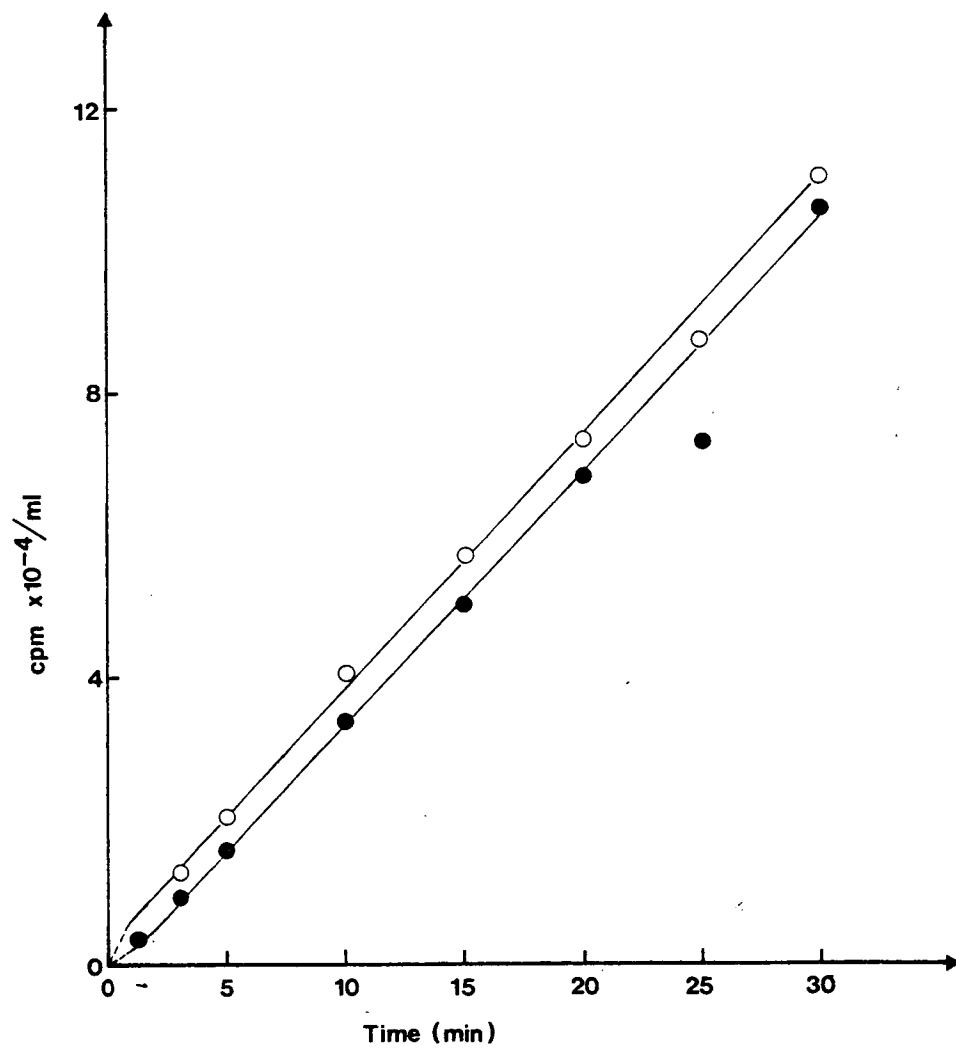pulsing 15 ml of asynchronous culture with 37 μCi of
label.

Figure 15

a period of about 25 minutes, after which the rate began to rise again at 0.87 in the cycle. This pattern was repeated in the succeeding cycle. In both cultures the doubling in rate occured slightly faster than the doubling in cell number, presumably due to ageing of the medium causing changes in the sizes of amino acid pools in the cells.

The pattern of labelling in a perturbed synchronous culture prepared in EMM 3 with 40 mg/l sulphate, but no supplements, is shown in Figure 16. The rate of incorporation dropped sharply by about 25% shortly after cell division then rose rapidly until 0.56–0.06 in the cell cycle (compare the perturbation in Fig. 11). At 0.56 in the cycle a short inflexion occured, similar to that seen in the presence of amino acids (Fig. 14). At 0.76±0.02 in the cycle the tate then began to rise again. The timing of this inflexion is about the same as that of the inflexion seen in unperturbed conditions.

One possible cause of perturbations is starvation whilst the cells are in the rotor. To test the effects of starvation upon the cells, a synchronous culture was prepared from cells growing in EMM 3, but with only 10 mg/l sulphate, and no amino acids. In such a culture, cells are likely to be limited for sulphate whilst in the rotor, and hence starved. As Figure 17 shows, the overall pattern during the first two cycles is similar to that seen in Figure 16, for a culture prepared in normal EMM 3. A short inflexion occured between 0.74 and 0.84 in the first cycle, after which the rate rose
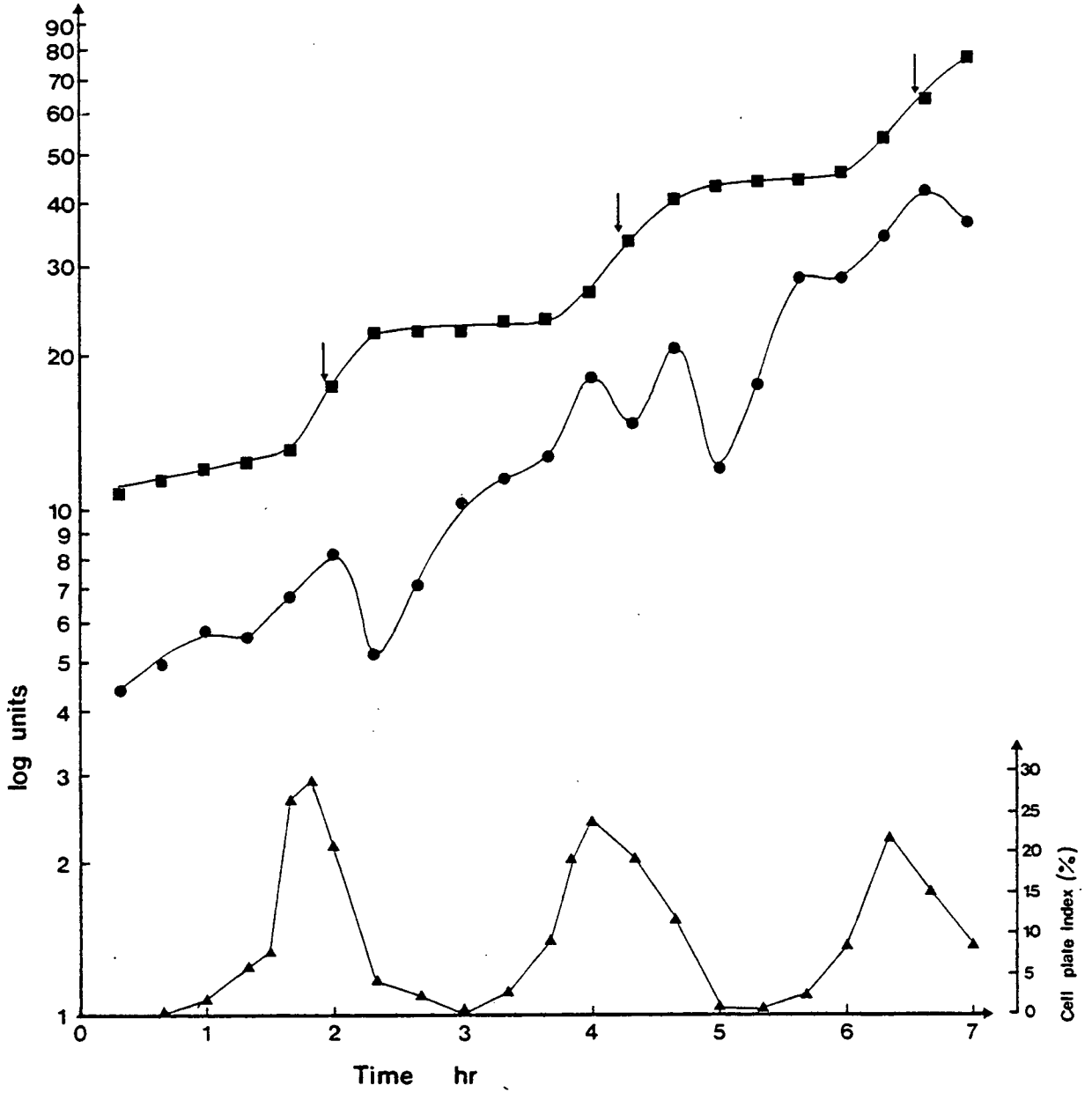
Figure 16

A synchronous culture was prepared from 972 h⁻

growing in EMM 3 + 40 mg/ l sulphate and no amino

acid supplements at 35°C. Every 20 minutes a 2.5 ml

sample was pulse-labelled for 20 minutes with 37 µCi

of L-[^{35}S]-methionine, to determine the rate of

incorporation of label.

● Rate of incorporation (7.5x 10^4).

■ Cell number (2x 10^5).

▲ Cell plate index


↓ Indicate the mid-point in cell number rise.

Figure 16

<u>Figure 17</u>

A synchronous culture was prepared from 972 h$^-$ growing in EMM 3 + 10 mg/ l sulphate and no amino acid supplements at 35°C. Every 20 minutes a 2.5 ml sample was pulse-labelled for 20 minutes with 2 µCi L-[^{35}]-methionine, to determine the rate of incorporation of label.

● Rate of incorporation (5x 10^3).

■ Cell number (2x 10^5).

▲ Cell plate index

↓ Indicate the mid-point in cell number rise.
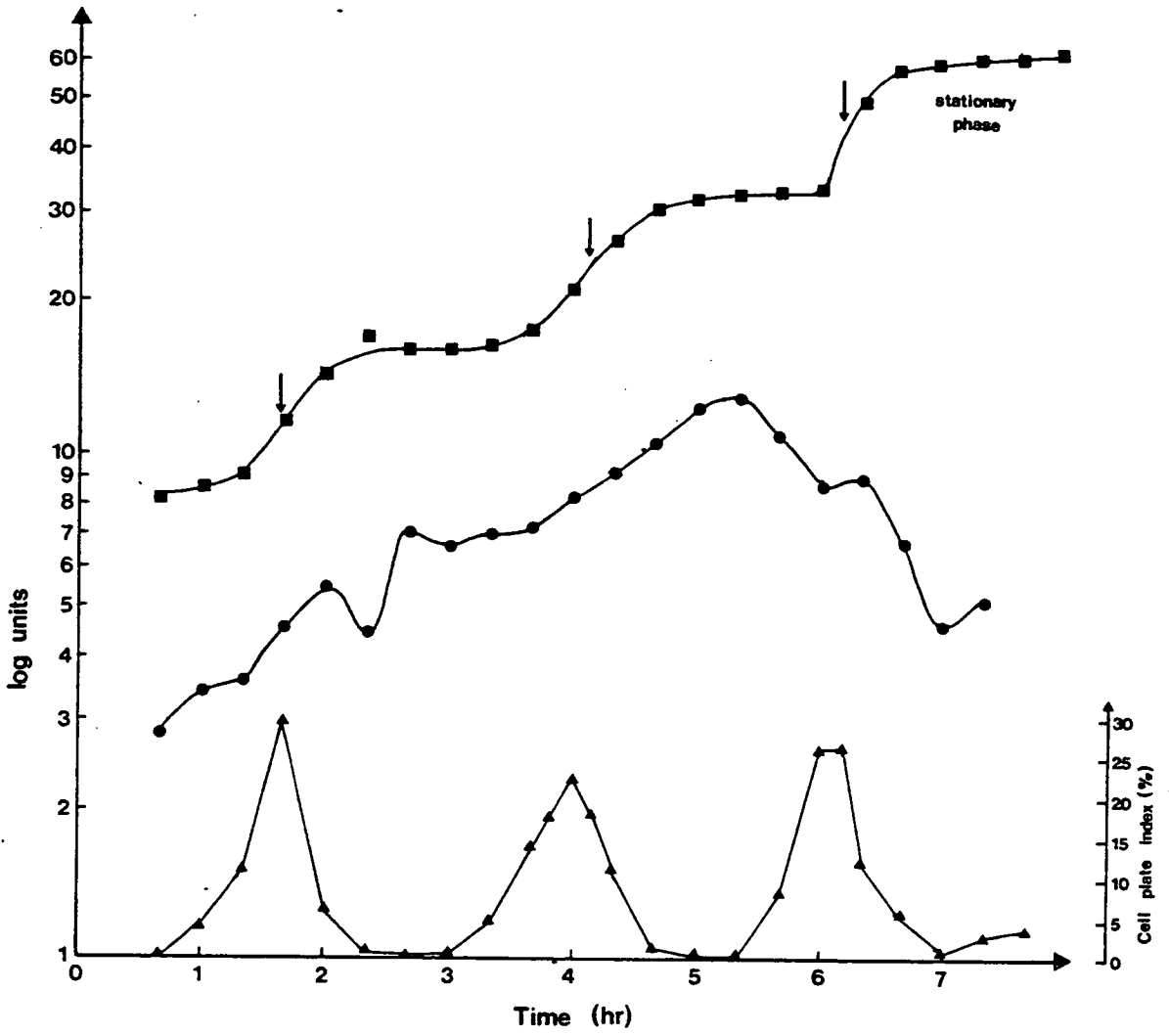
Figure 17

rapidly until just after cell division, when it fell sharply
by about 20%. The rate then rose rapidly until 0.5 in the
cell cycle, when a plateau occured which lasted for 0.3 of
the cycle. At 0.8 in the cycle the rate then began to rise,
and continued to increase until 0.5 in the next cycle, when
it began to decline steadily. The cells divided for the last
time at the end of this cycle, which was slightly shorter than
normal (120 minutes), to form small, stationary phase cells.

c) Gel Electrophoresis

Total protein extracts were prepared from the pulse-
labelled cells used in the experiments described in Figures 14
and 16. The component polypeptides in these extracts were
then separated according to their molecular weight by SDS gel
electrophoresis and bands detected on the gels by staining and
autoradiography. Typically, 96 bands in the molecular weight
range 12 000-270 000 Daltons were resolved on autoradiographs
of these gels. Of these, some 36 major bands co-migrated with
the bands of a ribosomal protein extract.

As these gels were loaded at constant protein, the intensity
of each band on an autoradiograph is a measure of the specific
rate of labelling of that band. Since the accumulation of
protein in S. pombe is approximately exponential, the bands
of protein whose rate of labelling increases exponentially
will therefore have a constant intensity on these gels
(Diag. 1 a). In contrast, a protein whose pattern of labelling
is a linear increase with a rate change will show a sharp
increase in intensity at the rate change (Diag. 1·b). A

a
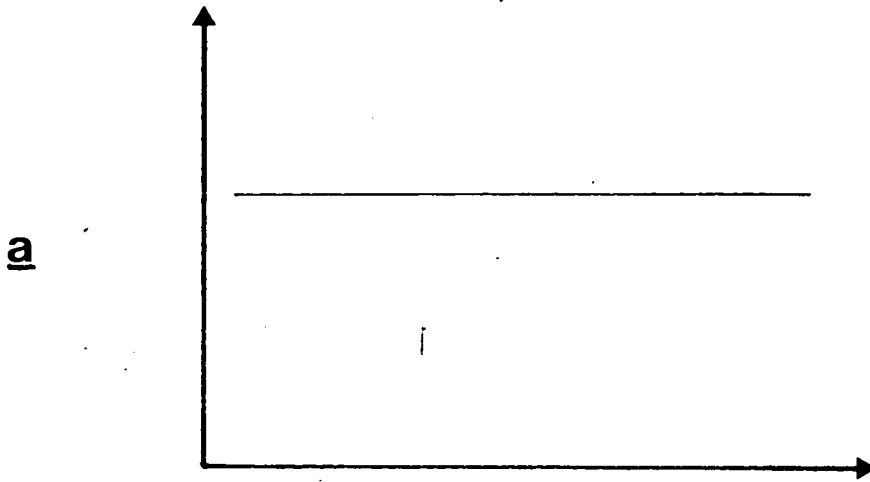
Exponential labelling.

b

Linear increase with a rate change.

c
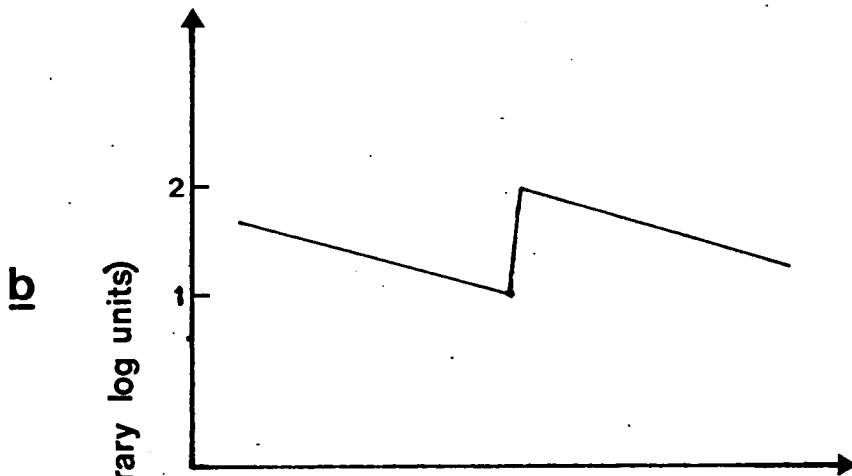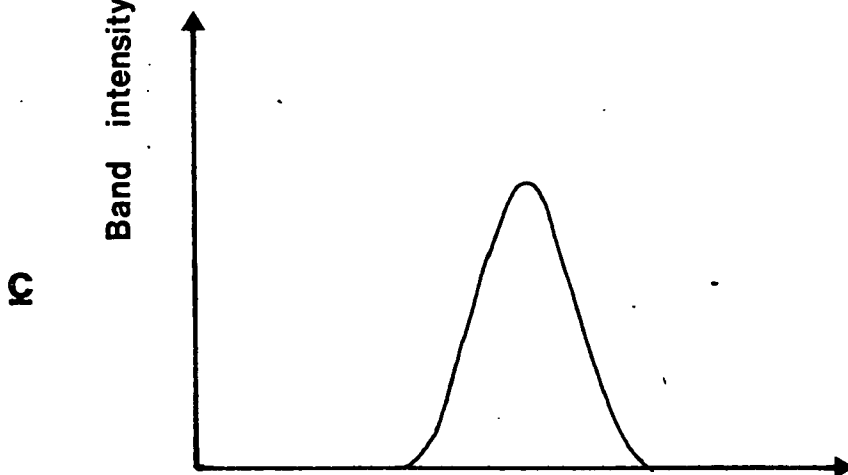
Periodic labelling

Diagram 1

Patterns of polypeptide labelling.

protein which is labelled periodically would be absent from the gels altogether until labelling began (Diag. 1 c). Each band on autoradiographs of gels was examined for the pattern of labelling it corresponded to.

(i) Unperturbed Cultures:

Autoradiographs of SDS slab gels of samples from the synchronous and asynchronous cultures of Figure 14, which were prepared in EMM 3 with amino acid and cysteine supplements, are shown in Figures 18 and 19 respectively. No periodic changes were detected in either the synchronous or the asynchronous culture. Further, no sharp increases or decreases in the intensity of bands in consecutive samples consistent with a rate change were found. The relative intensities of bands in a sample remained the same, and samples from different times in the cycle, and different cycles, were indistinguishable. These results are consistent with all the polypeptides being labelled at an exponentially increasing rate.

(ii) Perturbed Culture:

Figure 20 shows autoradiographs of SDS slab gels of samples from the perturbed synchronous culture in Figure 16, which was prepared in unsupplemented EMM3 (40 mg/l sulphate). The intensity of 93 bands followed the pattern of total protein labelling, reaching a minimum at times corresponding to cell division, but the relative intensities of these 93 bands in different samples remained the same, suggesting that the pattern of labelling underlying the perturbation is an

Figure 18

The synchronous culture samples labelled in the experiment described in Figure 14 were solubilised for one-dimensional SDS-gel electrophoresis and separated by this method.

● Indicates a band that co-migrates with a ribosomal protein (see Fig. 19).

◁ Indicates a band that can show periodicity (see Fig. 20).

↓ Indicates the mid-point in cell number rise.
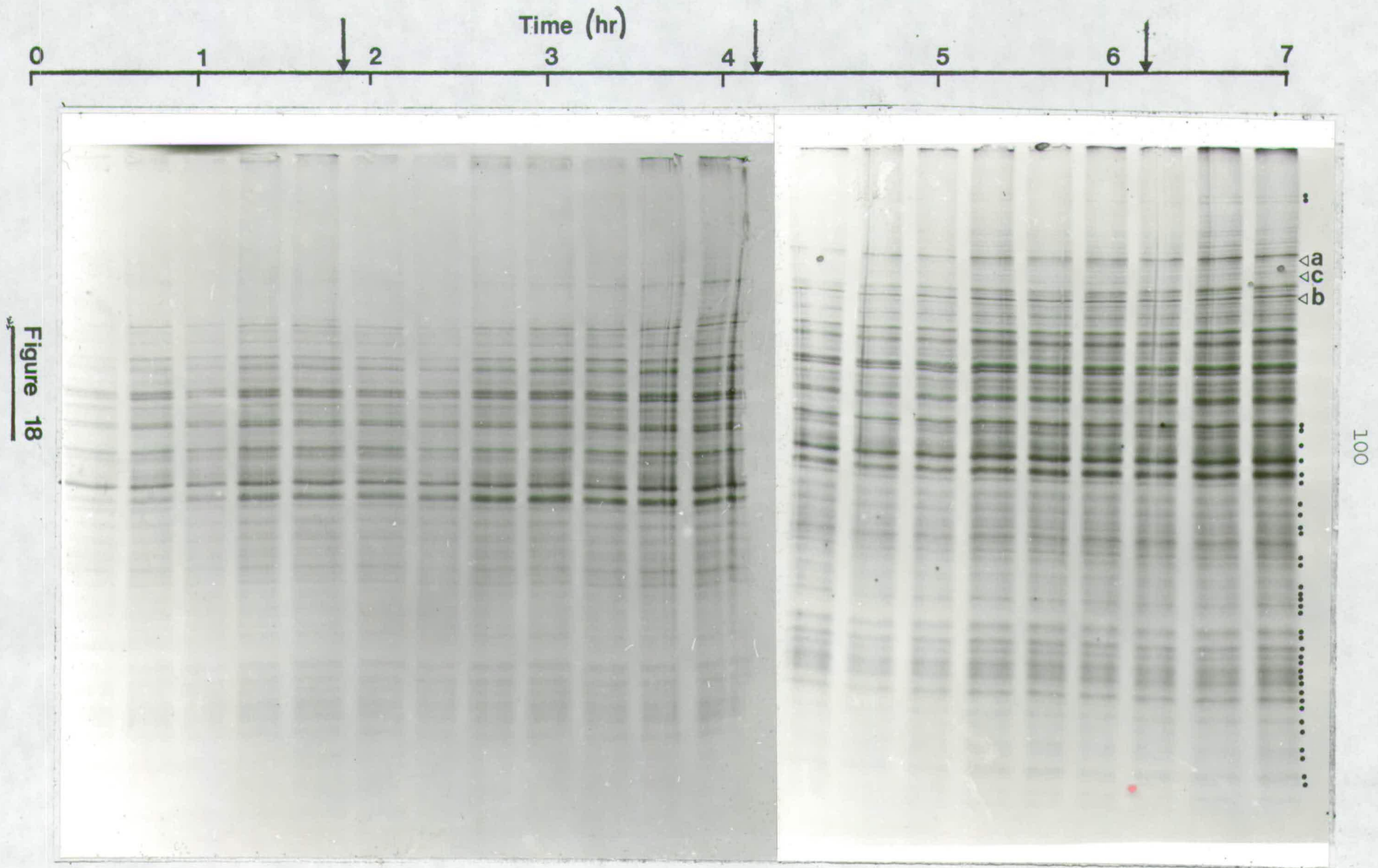
Figure 18

Figure 19

The asynchronous control culture samples labelled
in the experiment described in Figure 14 were solubil-
ized for one-dimensional SDS-gel electrophoresis and
separated by this method.

● Indicates a band that co-migrates with a ribosomal
protein (ribosomal markers are on the right).

◁ Indicates a band that can show periodicity (see
Fig. 20).

↓ Indicates the mid-point in cell number rise in the
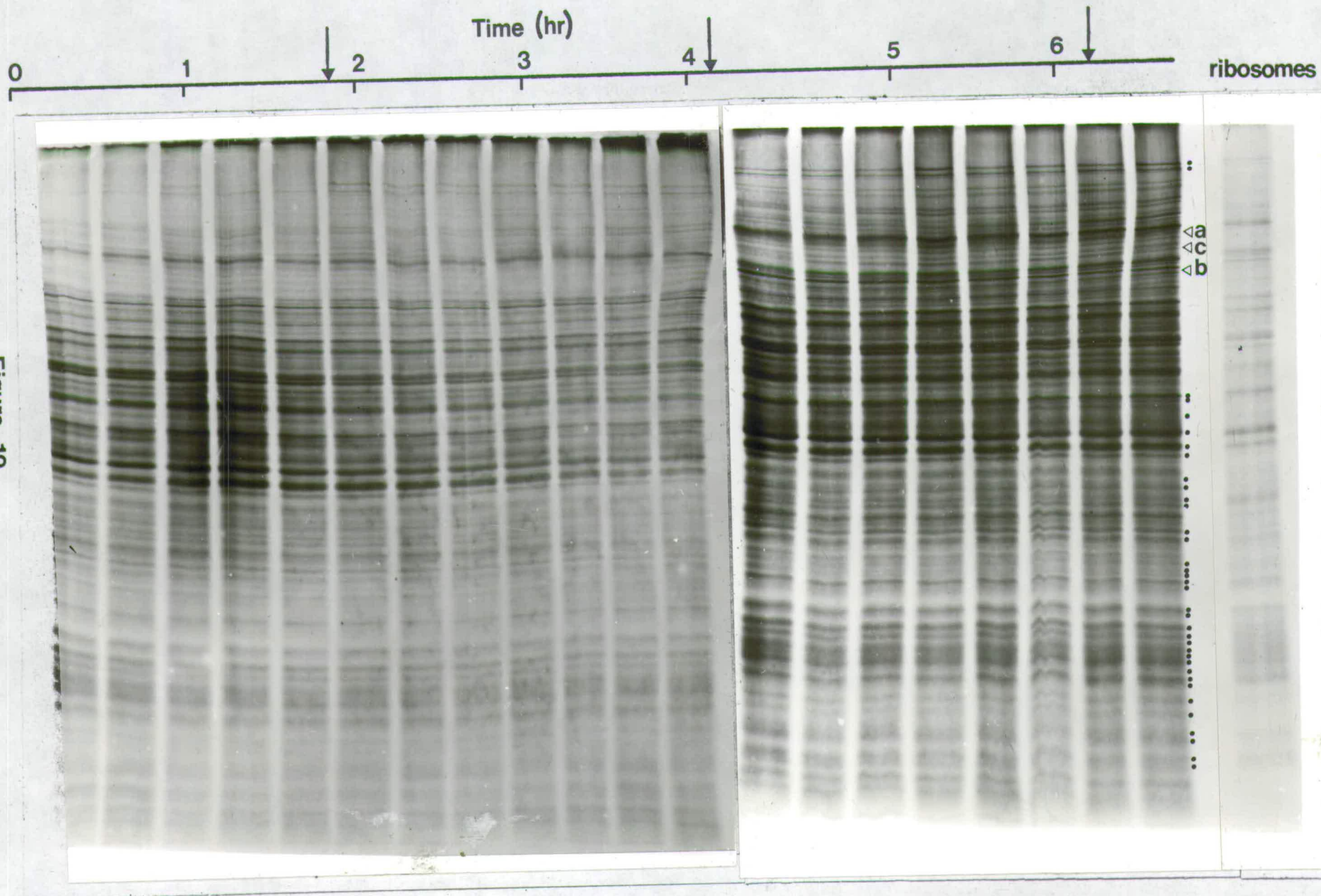accompanying synchronous culture.

Time (hr)

0   1   2   3   4   5   6   ribosomes

◁a
◁c
◁b

Figure 19

102

Figure 20

The synchronous culture samples labelled in the
experiment described in Figure 16 were solubilised for
one-dimensional SDS-gel electrophoresis, and separated
by this method.

● Indicates a band that co-migrates with a ribosomal
protein (ribosomal markers are on the right).

◁ Indicates a band that shows periodicity.

↓ Indicates the mid-point in cell number rise.

Time (hr)

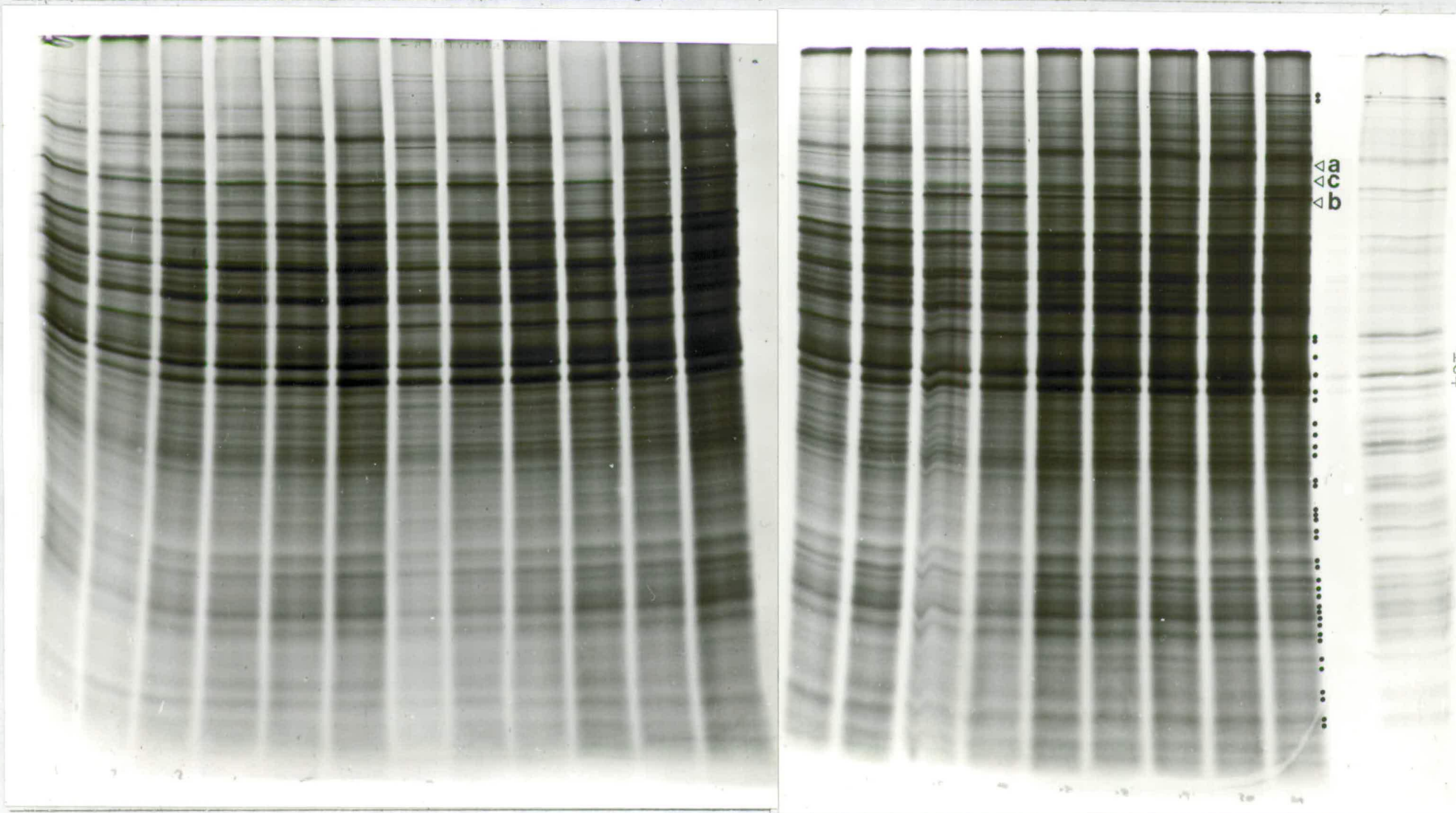0   1   2   3   4   5   6   7

ribosomes

a
c
b

Figure 20

104

exponential one, as in the unperturbed culture. In contrast
to the unperturbed culture, three proteins showed periodic
changes. All three proteins were labelled throughout the
cycle (Fig. 21), but the rate of labelling of proteins A and B
rose substantially at the time of cell division, when the
other proteins were being labelled at their minimum rate.
The pattern of labelling of protein C paralleled that of
total protein, in that its rate had a minimum at cell division,
and which them rose during the rest of the cell cycle. How-
ever, its proportion relative to the majority of the proteins
changed considerably, being very low just after cell division,
and rising considerably to a maximum around 0.7 in the cycle.
All three proteins were detectable on the unperturbed culture
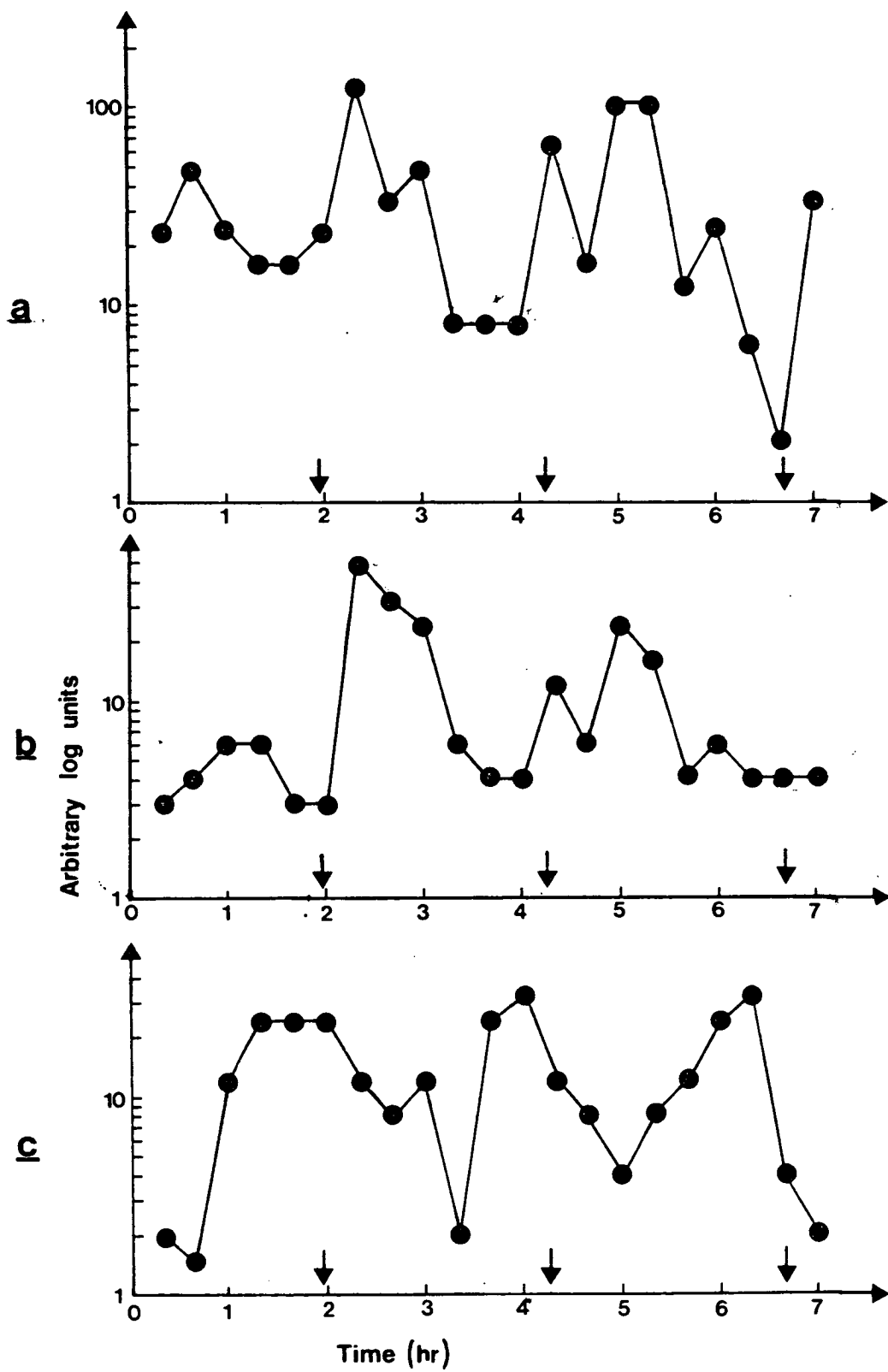gels, were they were labelled non-periodically, at a relatively
low level.

Figure 21

DISCUSSION

Synchronous and asynchronous cultures of wild-type S. pombe, perpared using the elutriator rotor in normal EMM 3 medium, show marked perturbations in their pattern of labelling with L-[^{35}S]-methionine. Creanor also found perturbations when L-[^{3}H]-tryptophan was used to label cells in media without tryptophan (92). It seems unlikely that these variatiohs in the rate of labelling correspond directly to variations in the rate of synthesis, since the patterns of perturbation for tryptophan and methionine are to some extent different. The apparent variations are more likely to be artefacts due to changes in the specific activity of the label in the cell, caused by changes in the amino acid pools. This is supported by the observation that changing the average size of the amino acid pool alters the size of the perturbation. Thus increasing the pool reduced the perturbation for tryptophan, whilst for methionine the perturbations are increased. The patterns of labelling for methionine are strongly reminiscent of damped oscillations, with the amount of damping decreasing as the pool size is increased by the addition of carrier methionine. If a suitable feedback loop exists, an initial change in the pool size caused by the synchronisation procedure could give sustained oscillations in the pool size. Altering the size of the pool, or the activity of the enzymes involved, by the addition of carrier, would affect the parameters of the feed-back system, and hence the size of the oscillations. Whatever the molecular basis for the oscillations, the most likely

cause of the initial shock is starvation of the cells whilst

in the rotor. Starvation would cause an imbalance in many

of the cells processes, and would explain the multiple effects

of synchronisation upon the cell. Reduction of the perturbations

in labelling by supplementing the medium with amino acids is

consistent with this hypothesis.

If pool effects are taken into account, the rate of labelling

of a protein corresponds to its rate of synthesis. However,

this statement needs qualifying when the rate of synthesis

is measured from bands on gels. Firstly, two-dimensional gel

electrophoresis has shown that each band on a one-dimensional

gel is often composed of several polypeptides (see next chapter).

A major band on a one-dimensional gel may thus be composed of

one, or a few rapidly labelled proteins; or of several proteins

labelled at an intermediate rate. The rate change measured

from a band is therefore the average of the rate changes of

each protein. The minimum change detectable by eye is probably

about a factor of two, so a linear increase with a rate doubling

may go undetected if the timings of the rate changes of the

polypeptides in a band occur out of phase. Secondly, the

intensity of a band may change as a result of changes in the

formation or loss of a polypeptide by proteolysis, rather

than by direct changes in the rate of synthesis on the ribosomes.

The general pattern of total protein synthesis in S. pombe

seems to be by an approximately exponential increase in the

rate of synthesis; that is, total protein accumulates

exponentially. This result is in agreement with the results

of Stebbing (94) and of Mitchison and Wilbur (95). The significance of the inflexion in the increase in rate is uncertain, even though such a repeated pattern is not observed in the asynchronous culture, as such cultures are only partial controls. A perturbed asynchronous control strongly implies that the synchronous culture is also perturbed, but the converse, i.e., the absence of perturbations in a control, does not necessarily mean that the synchronous culture is unperturbed. In addition, the control is qualitative, not quantitative, and the magnitude and timing of the perturbations in a control culture need bear no direct relationship to those of the synchronous culture. The inflexion in methionine labelling seen here could therefore simply be a cyclic continuation of the initial perturbation, and it does resemble some of the perturbations seen in other asynchronous controls (Fig. 10). One piece of evidence in favour of it being a true cell cycle event is that when tryptophan is used to label S. pombe in medium containing tryptophan, probably involving different perturbations, a pattern similar to that of methionine is found, and the timing of the inflexion is about the same (92). The inflexion is also seen at about the same time in a perturbed synchronous culture prepared in unsupplemented EMM 3, and could therefore be a genuine cell cycle event upon which the perturbations are superimposed. However, it could equally well be a part of these perturbations. The pattern of labelling in sulphate limited media is similarly open to both interpretations. The culture is perturbed, and the inflexion

seen in the first cycle is increased to a plateau in the second,
which may therefore be an increased perturbation due to
starvation. Alternatively, the inflexion, the plateau, and
the subsequent shut off in synthesis may all represent the
operation of some control co-ordinating nutrition, growth,
and the DD-cycle. The increase in rate of synthesis may be
geared to the nutrient supply such that the increase stops
at some point in G2, determined by the available nutrients.
With an adequate supply this point is at 0.66 in the cell
cycle (mean of inflexions in figs. 14, 16 and 1st. cycle of 17),
just before nuclear division. Under poor nutrient conditions,
this point is brought forward (2nd. cycle in 17) to about
0.5 in the cell cycle. Before entering stationary phase,
synthesis is shut off at this point. With an adequate supply
of nutrient, the increase in rate would normally recommence
at 0.81 in the cycle.

If the inflexion is a genuine cell cycle event, instead
of a persistent perturbation, it could be caused in a variety
of ways. It occurs at about the time of nuclear division
in the cell cycle, and the simplest explanation is that the
cells cease to produce, or at least to utilise, new messenger
RNA at this time . An inhibition of RNA synthesis has been
found in many higher eukaryotic cells, and some lower ones (2).
This mechanism would also be valid if the inflexion was due
to a perturbation. However, it is not consistent with the
results of Fraser and Moreno (41), who showed that RNA synthesis
is not shut off in S. pombe. Alternatively, the step could

be due to an underlying pattern of gene expression. An inflexion would be the result if the majority of the cells proteins are synthesised periodically, with their times being out of phase, but falling into two broad groups on either side of the plateau. It would also be the result if many of the proteins are synthesised linearly with a rate doubling, if the timings are spread about the time of the plateau. Within the limitations discussed below, this work finds no evidence for either of these patterns of gene expression.

No patterns of periodic synthesis were found in 96 bands of polypeptides from a relatively unperturbed synchronous culture. These bands represent a high proportion of the major cell proteins, and therefore periodic synthesis does not make a significant contribution to the pattern of total protein accumulation. Further, no evidence was found for a significant proportion of proteins synthesised linearly, with a rate change. This could be due to the averaging of rates in a band, but several of the 36 major bands which co-migrate with ribosomal proteins probably contain only 1-3 major polypeptides. A rate change might have been seen in these bands, if it existed. Thise results are consistent with the majority of the cells major proteins accumulating exponentially during the cell cycle similar to total protein. However, the existance of a substantial minority of major proteins being synthesised in a linear pattern with a rate change cannot be entirely eliminated.

The comparative ease by which perturbations may be eliminated

from synchronous cultures perpared using the elutriator rotor suggests that the initial shock to the cells is quite minor. Even so, in the absence of amino acid supplements, it is sufficient to induce a sustained periodic pattern of labelling in three bands (i.e., 3% of the total resolved). The simplest explanation is that these proteins are synthesised periodically in such a culture, but the possibility that the changes result from periodic proteolytic cleavage cannot be eliminated. However, whichever explanation is true, this result shows that periodic changes in the protein composition of a cell can be induced by even mild shocks, and these changes resemble 'cell cycle events'.

Chapter 4: <u>A SEARCH FOR DEPENDENT PROTEINS</u>

INTRODUCTION

Cells of temperature-sensitive cdc mutants that are before their transition point in the cell cycle become arrested in cell cycle progress when shifted to the restrictive temperature(68). Subsequent DD-cycle events whose occurrence is dependent upon the blocked stage do not occur. Diagram 2 shows the main events in the DD cycle of S. pombe, and their dependency relationships (71). Thus, shifting a mutant with a temperature sensitive lesion in nuclear division to the restrictive temperature blocks DNA synthesis and cell plate formation, as well as nuclear division. Growth, however, continues unaffected, and such cells have a markedly different qualitative and quantitative structural composition to wild-type cells. It is conceivable that this difference is a reflection of change in the pattern of gene expression brought about by the block in cell cycle progress. To take the above example, the synthesis of at least some proteins required for DNA synthesis and cell plate formation might be dependent upon the completion of some stages of nuclear division, and would not occur in cells blocked at this stage. The histones appear to be an example of such proteins, and their synthesis has been reported to be dependent upon DNA synthesis in a variety of cells (see chapter 1).

To test the contribution of dependent proteins to cell cycle control in S. pombe, the synthesis of a large number of polypeptides was examined in wild-type and cdc cells at the permissive and non-permissive temperatures (25° and 35°re-
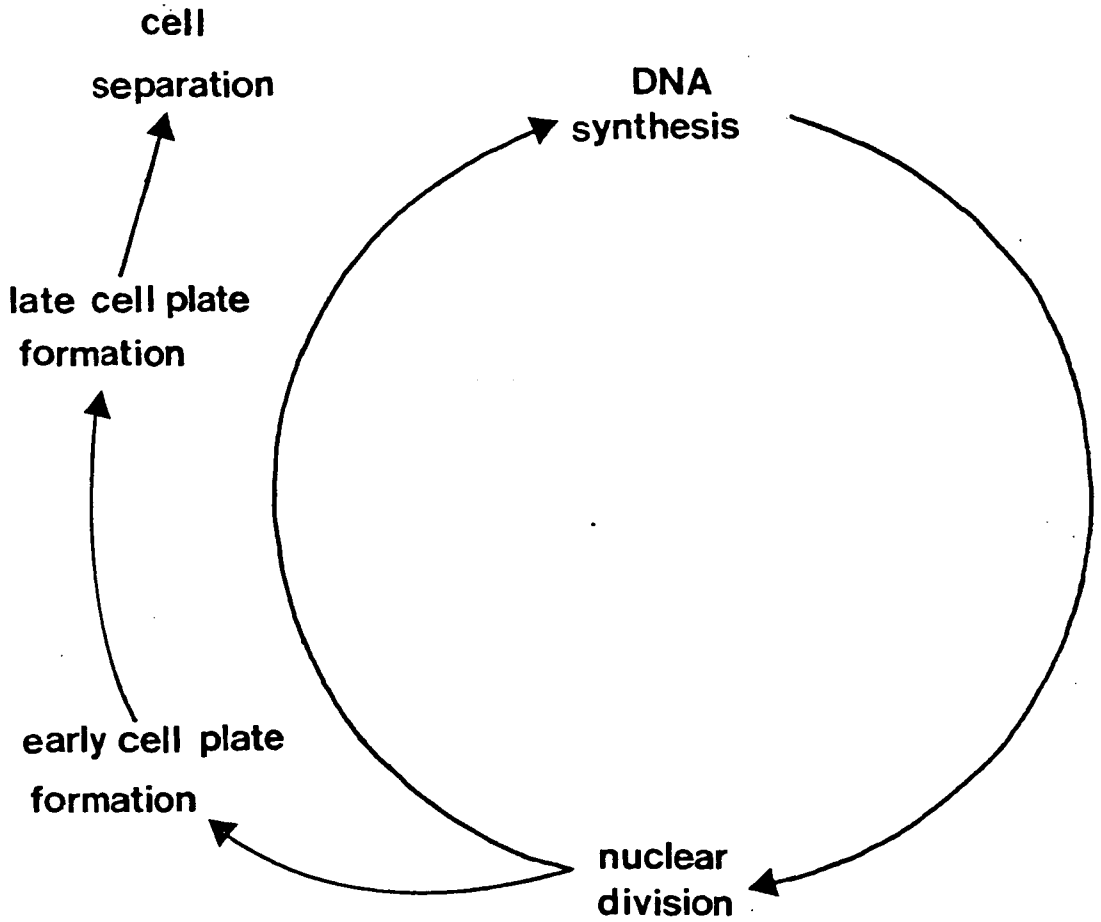
Diagram 2

The main DD-cycle events in S. pombe.

spectively) by labelling cells with $[^{35}S]$-sulphate and
separating the proteins on two-dimensional gels. Any protein
whose synthesis was dependent upon progress through any stage
of the cell cycle would be absent from gels of proteinswhich
were labelled in cells which had not completed this stage.

METHOD

a) Choice of Strains

Strains were chosen with genetic lesions in the three main DD cycle events and having transition points coincident with these events: nuclear division, cdc 2-33 h⁻; DNA synthesis, cdc 10-129 h⁻; and cell plate formation, cdc 3-6 h⁻ (71). Strain cdc 3-6 h⁻ is a late cell plate mutant which accumulates disorganised cell plate material. Some proteins involved in cell plate formation might as a result be over-produced, thereby offering an alternative means of detecting proteins involved in a cell cycle event.
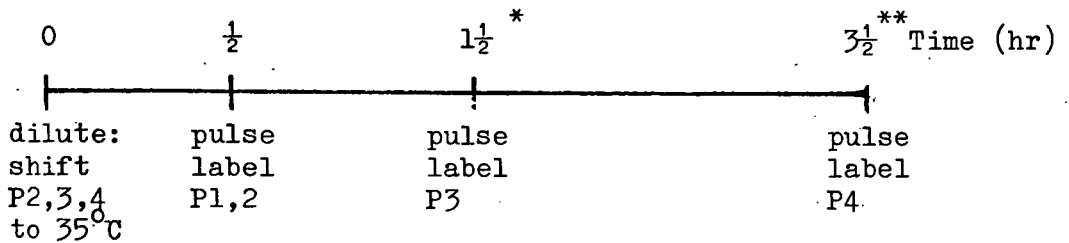
b) Labelling Protocol

Cells were grown overnight at 25°C to an O. D.$_{595}$ of 0.15-0.2 in 100mls of EMM 3 containing 10 mg/l of sulphate, instead of the usual 40 mg/l. Before use the cells were diluted with fresh EMM 3 (10 mg/l sulphate), pre-warmed to 25°C, as shown in Table 1.

Pulse number	Volume of cells	Volume of EMM 3 added	Activity of isotope used
P1	25ml	0ml	3mCi
P2	25	12	3
P3	13	12	2
P4	11	16	2

TABLE I·

Culture P1 was kept at 25°C, and cultures P2,3 and 4

were shifted to 35°C. At the times after the shift indicated
in Diagram 3, 2.5ml samples were taken from the appropriate
culture and added to the amount of carrier free $[^{35}S]$-sulphate
shown in Table 1. After labelling for 1 hour, the cells
were frozen in an acetone-dry ice freezing mixture and stored
at -20°C till required.

0	½	1½ *	3½ ** Time (hr)
dilute:	pulse	pulse	pulse
shift	label	label	label
P2,3,4	P1,2	P3	P4
to 35°C			

*   2½ hr in the case of cdc 10-129 h⁻

** 4½ hr in the case of cdc 10-129 h⁻

Diagram 3

c) Gel electrophoresis

Cells were thawed on ice, the proteins solublised for
two-dimensional gel electrophoresis, and then separated by
this technique, as described in General Methods. Iso-
electric focusing gels containing 0.2% pH 3.5-10, 0.8% pH 5-7,
and 0.8% pH6-8 Ampholytes were used, giving a pH gradient of
roughly pH3.5 to 6.5 as measured in degassed water. The gels

were loaded at constant activity ($1\text{-}6 \times 10^6$ cpm, depending on yield). In the second dimension, 16% SDS acrylamide gels, cross-linked with 0.133% bis acrylamide were used, covering the molecular weight range 12 000 - 270 000 D. After electrophoresis, the slab gels were soaked in water for about an hour, then dried down and autoradiographed for 3 - 4 weeks.

To analyse the autroadiographs, a map was drawn to show the spots resolved on autoradiographs of wild-type gels. This was then cross-checked against some of the gels of mutants. The map was then divided up into 1 cm^2 squares, and then the spots on each autoradiograph were examined, square by square.

RESULTS AND DISCUSSION

a) Labelling conditions

The time required for a significant decrease to occur in the synthesis of a dependent protein after shifting an asynchronous culture of a ts cdc mutant to the non-permissive temperature is dependent upon the transition point of the mutant, and the relative timing of the cell cycle events involved. The time required for a large decrease in a proteins synthesis can thus give information about the position of its synthesis in a dependent sequence of events. Samples of an asynchronous culture of cells were therefore labelled with a long (1 hour) pulse at intervals after shifting the cells to $35^{\circ}C$. The mass doubling time is abbut 2 hr 20 min at this temperature, so the cells in the last pulse had been blocked for at least one cycle. The cells were grown in sulphate limited EMM 3, containing only 10 mg/l of sulphate, to increase the specific activity of the labelled protein. Stationary phase occurs at an O.D.$_{595}$ of ca. 2.0 (ca. 30×10^6 cells/ ml) in this medium. Stationary phase with the normal 40 mg/ l sulphate (i.e. glucose limited) occurs at about 70×10^6 cells/ ml. When an asynchronous culture of a cdc mutant growing in normal EMM 3 is shifted to $35^{\circ}C$ the cell plate index drops to zero, and the cells become elongated. However, it was found that if cells of cdc 2-33 h⁻ growing in sulphate limited EMM 3 were shifted to $35^{\circ}C$ at an O.D.$_{595}$ of ca. 0.4 units, the cell plate index did not reach zero, and the cells did not elongate to the normal extent. Division continued at a low level in the culture, even after 4 hrs. In contrast, control cultures which were shifted at

the lower O.D. $_{595}$ of 0.16, or shifted at an O.D. $_{595}$ of 0.4

but growing in 40 mg/l sulphate, behaved as normal. The cdc

mutation was therefore incompletely expressed at the restrictive

temperature when the cells were growing in very low levels of

sulphate, caused by depletion of the medium due to cell growth.

To avoid this nutrient effect upon the expression of the mutation

the culture was split into fractions before shifting to 35°C,

and each fraction diluted with fresh EMM 3 (10 mg/l) such

that they would all be at a density of ca. 0.2 O.D. units

when labelled. Cells shifted to 35°C under these conditions

behaved in the same way as cdc cells growing in EMM 3 containing

40 mg/l sulphate.

b) Autoradiographs

Figures 22 to 25 show autoradiographs of two-dimensional

gels of pulse labelled samples of each strain, labelled at

25°C (pulse P1), and at different times after shifting to

35°C (pulses P2,3, and 4). A total of 716 different spots

were identified from these gels by comparing their relative

positions. However, not all of these spots were seen on any

one autoradiograph. On a typical gel about 500 spots (ca. 71%)

were positively identified, a further 75 or so (ca. 10%)

were of uncertain identification, and the remainder (ca. 19%)

were not resolved. Many spots were only resolved on a pro-

portion of the gels due to different areas of each gel being

resolved to different extents. The reasons for this variation

are unknown. The main limitation to resolution is 'streaking',

i.e., the polypeptide does not form a spot, but a long line

Figures 22a-d

Autoradiographs of two-dimensional gels of total protein extracts of cells of 972 h⁻ grown in EMM 3 + 10 mg/ l sulphate and labelled for one hour at the temperatures and times given below:

a) 0 hr, 25°C.

b) 0.5 hr after diluting and shifting from 25°C to 35°C.

c) 1.5 hr after diluting and shifting from 25°C to 35°C.

d) 3.5 hr after diluting and shifting from 25°C to 35°C.

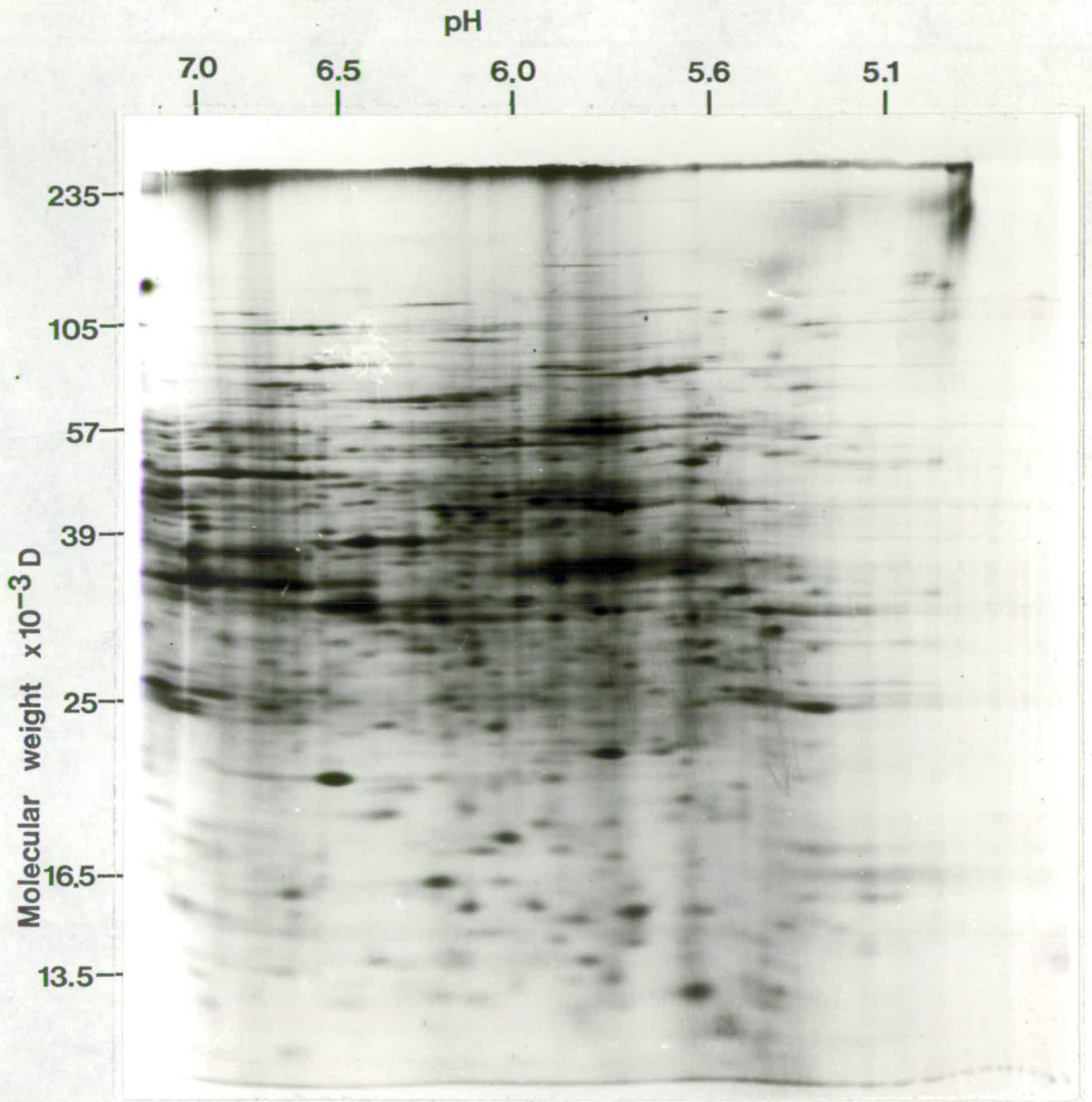Molecular weights were taken from Figure 6, and pH determined by the method of O'Farrell (81).
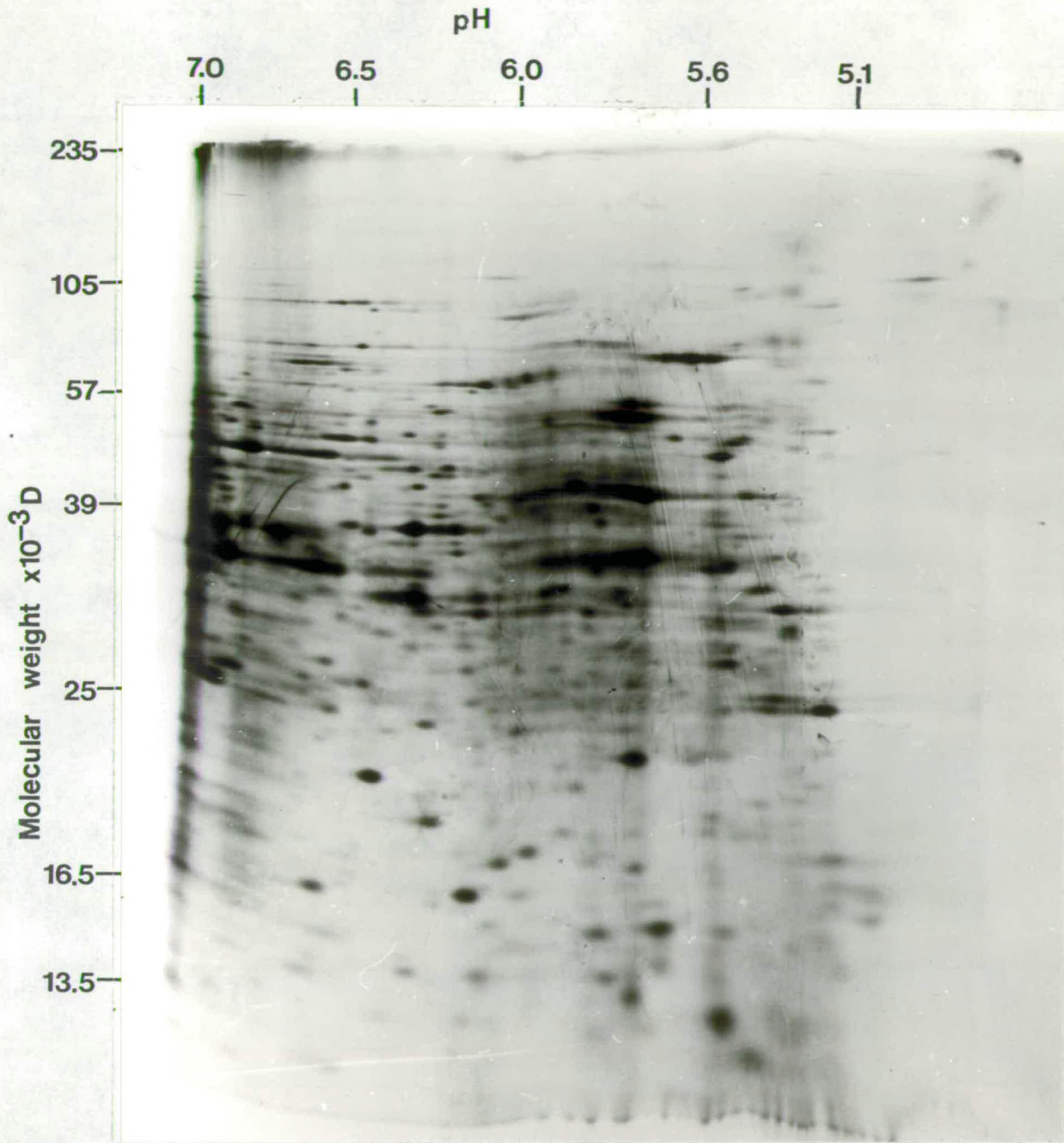
Figure 22a

Figure 22b

Figure 22c

Figure 22 d

Figures 23a-d

Autoradiographs of two-dimensional gels of total
protein extracts of cells of cdc 2-33 h⁻ (nuclear
division mutant) grown in EMM 3 + 10 mg/ l sulphate and
labelled for one hour at the temperatures and times
given below:

a) 0 hr, 25°C.

b) 0.5 hr after diluting and shifting from 25°C to 35°C.

c) 1.5 hr after diluting and shifting from 25°C to 35°C.

d) 3.5 hr after diluting and shifting from 25°C to 35°C.

Molecular weights were taken from Figure 6, and
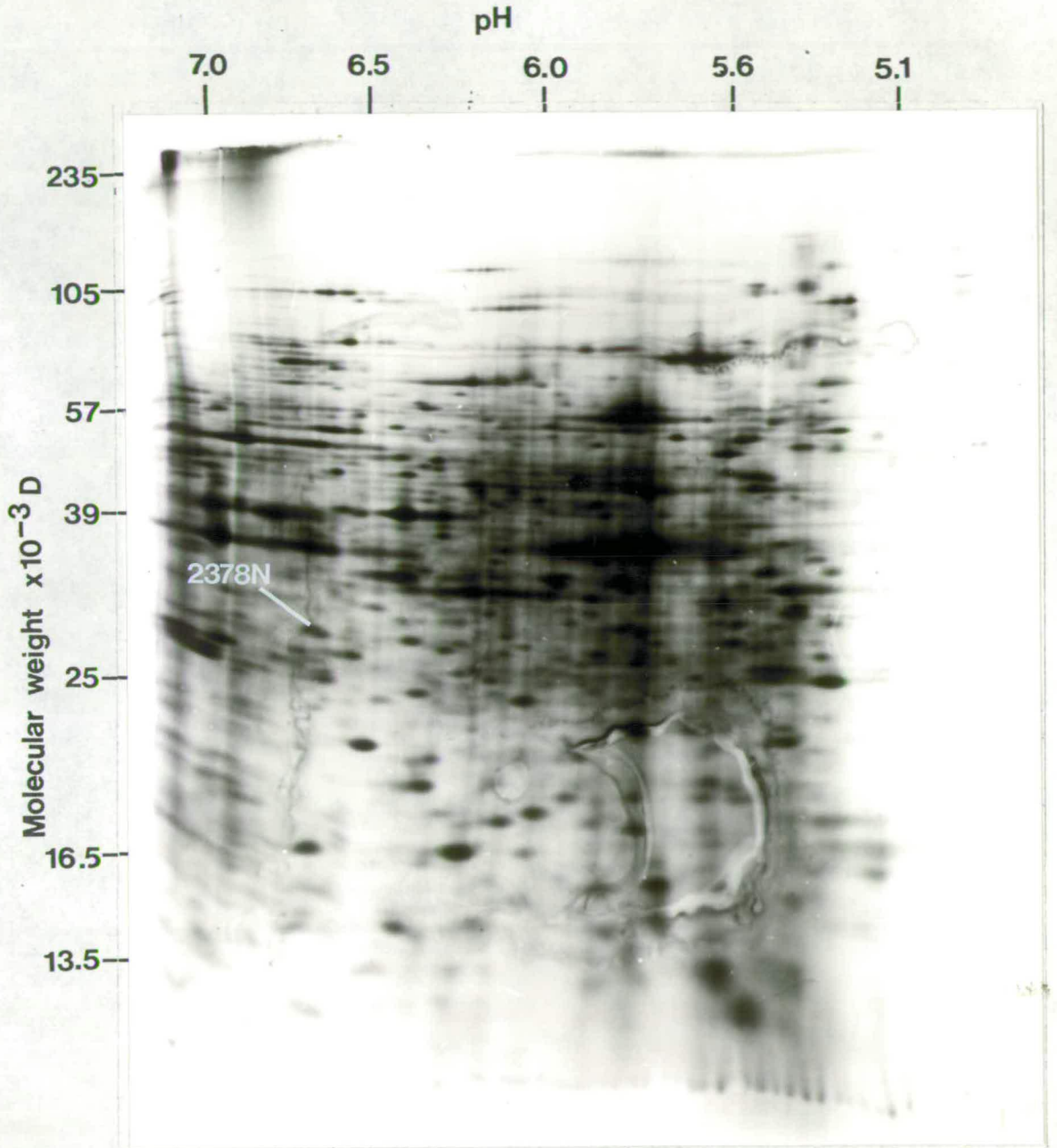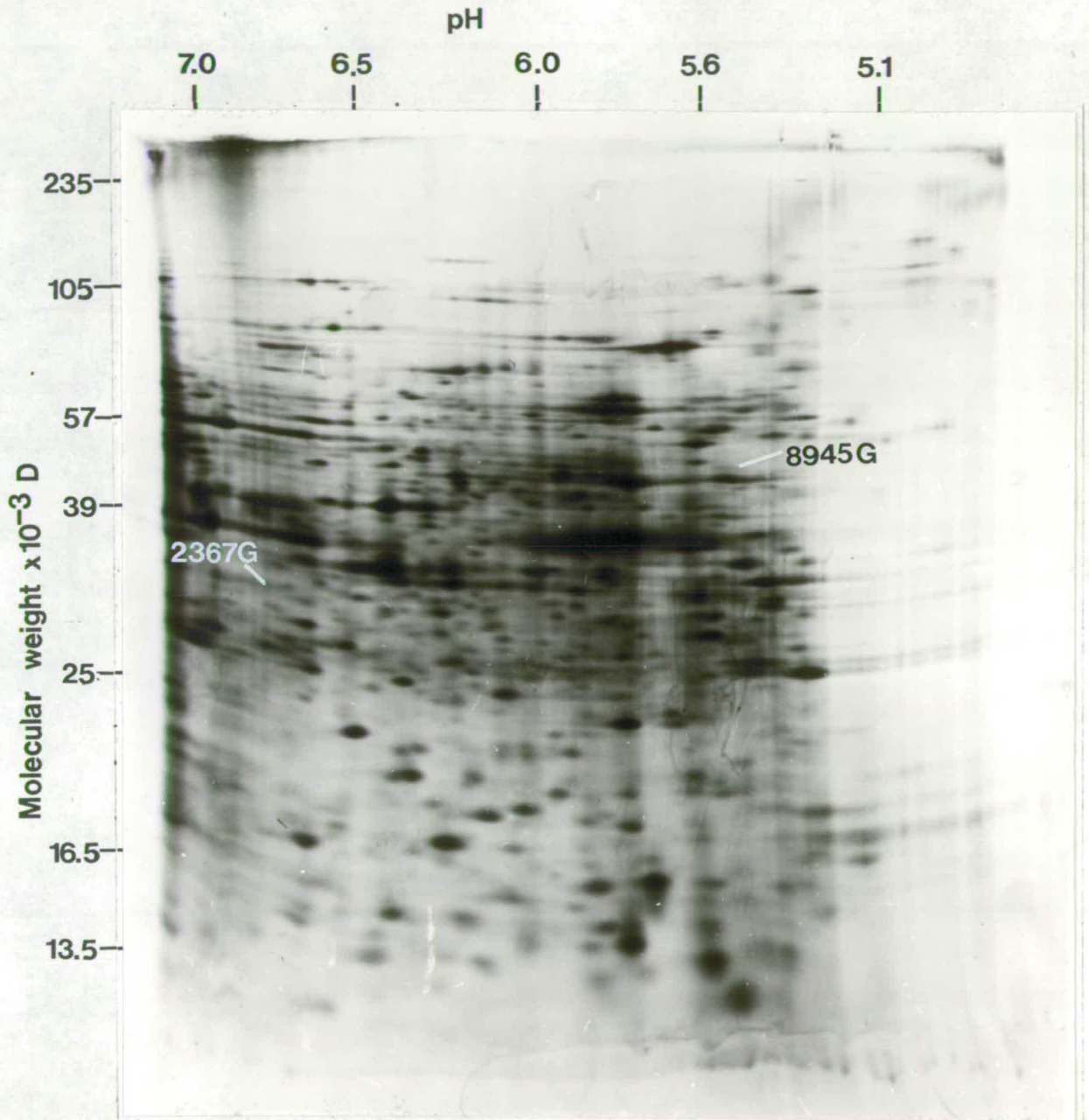pH determined by the method of O'Farrell (81).

Figure 23 a

Figure 23b

Figure 23c

Figure 23d

Figures 24a-d

Autoradiographs of two-dimensional gels of total protein extracts of cells of cdc 10-129 h⁻ (DNA synthesis mutant) grown in EMM 3 + 10 mg/ l sulphate and labelled for one hour at the temperatures and times given below:

a) 0 hr, 25°C.

b) 0.5 hr after diluting and shifting from 25°C to 35°C.

c) 2.5 hr after diluting and shifting from 25°C to 35°C.

d) 4.5 hr after diluting and shifting from 25°C to 35°C.

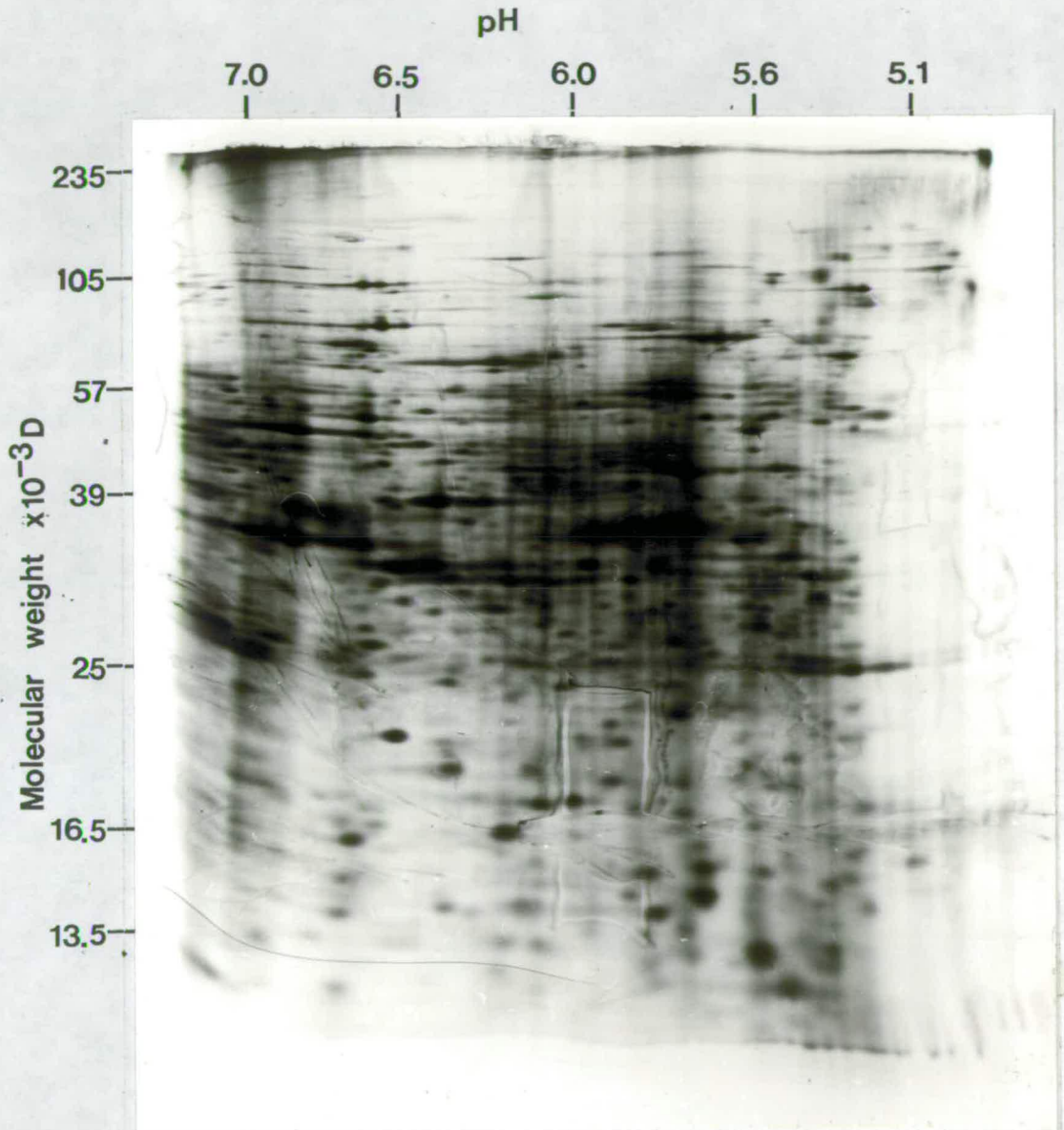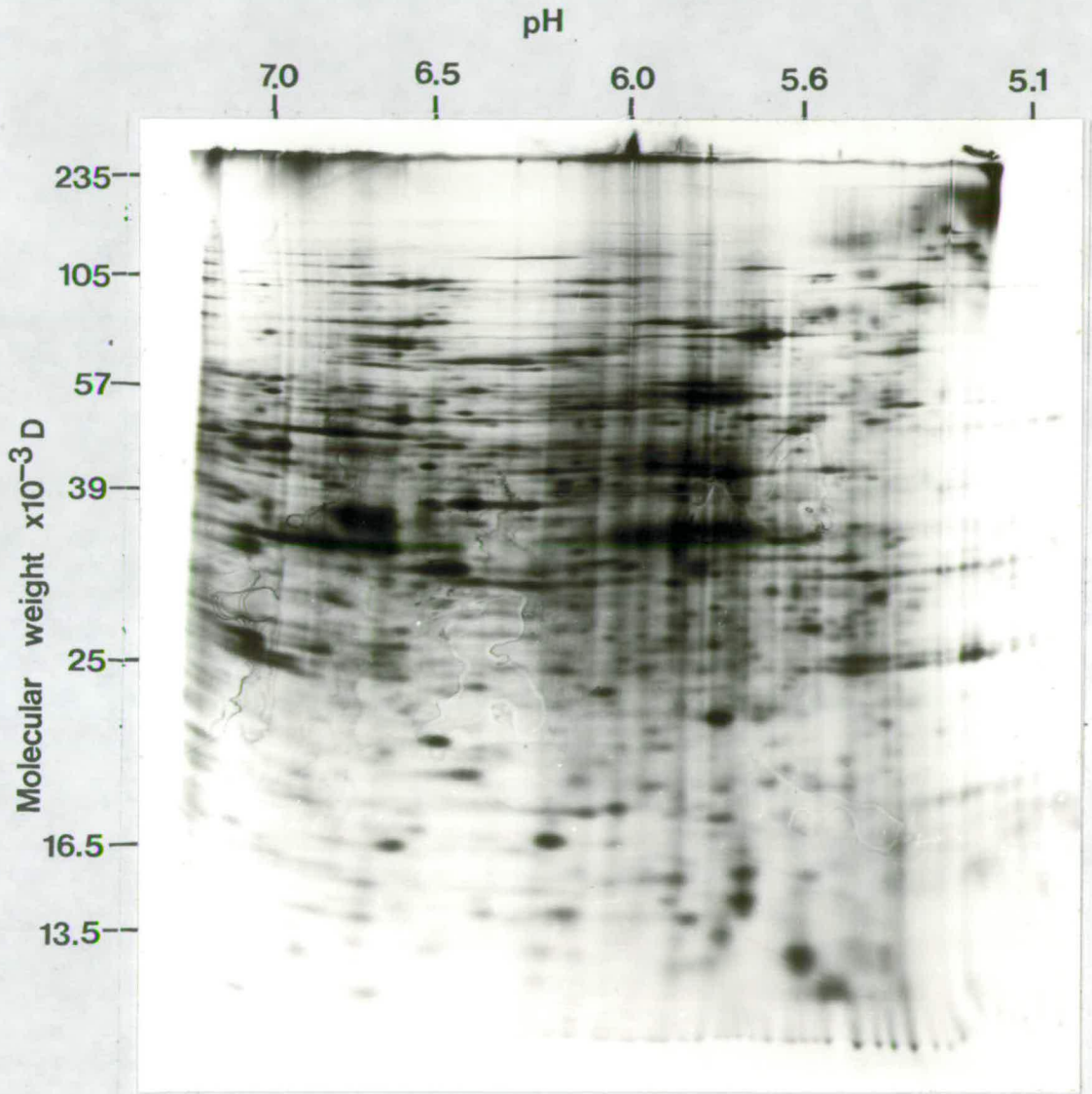Molecular weights were taken from Figure 6, and pH determined by the method of O'Farrell (81).

pH

7.0 6.5 6.0 5.6 5.1

Molecular weight x10^{-3}D

235—
105—
57—
39—
25—
16.5—
13.5—

Figure 24 a

Figure 24 b

Figure 24c

Figure 24 d

Figures 25a-d

Autoradiographs of two-dimensional gels of total protein extracts of cells of <u>cdc</u> 3-6 h⁻ (cell plate mutant) grown in EMM 3 + 10 mg/ l sulphate and labelled for one hour at the temperatures and times given below:

a) 0 hr, 25°C.

b) 0.5 hr after diluting and shifting from 25°C to 35°C.

c) 1.5 hr after diluting and shifting from 25°C to 35°C.

d) 3.5 hr after diluting and shifting from 25°C to 35°C.

Molecular weights were taken from Figure 6, and pH determined by the method of O'Farrell (81).
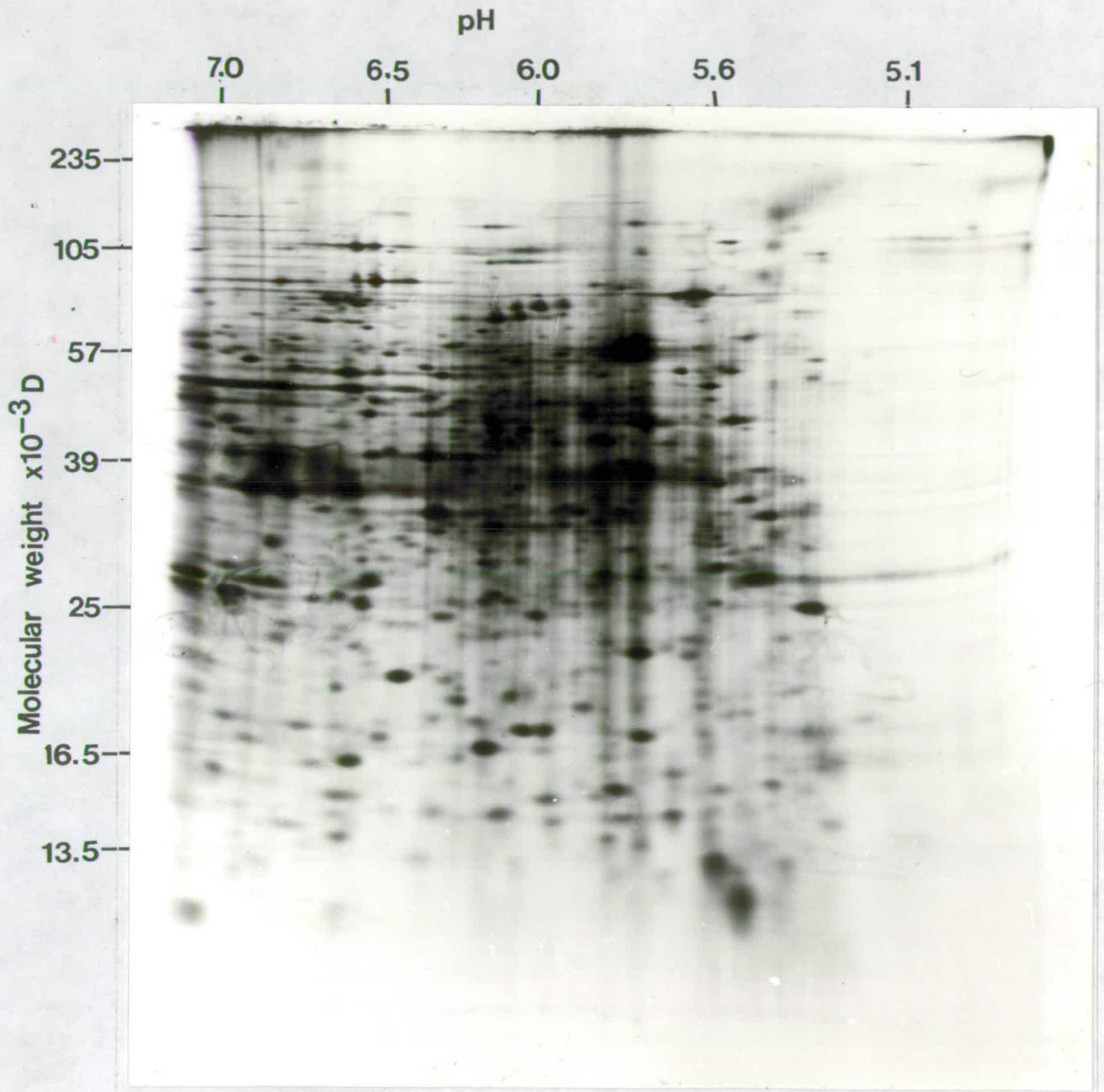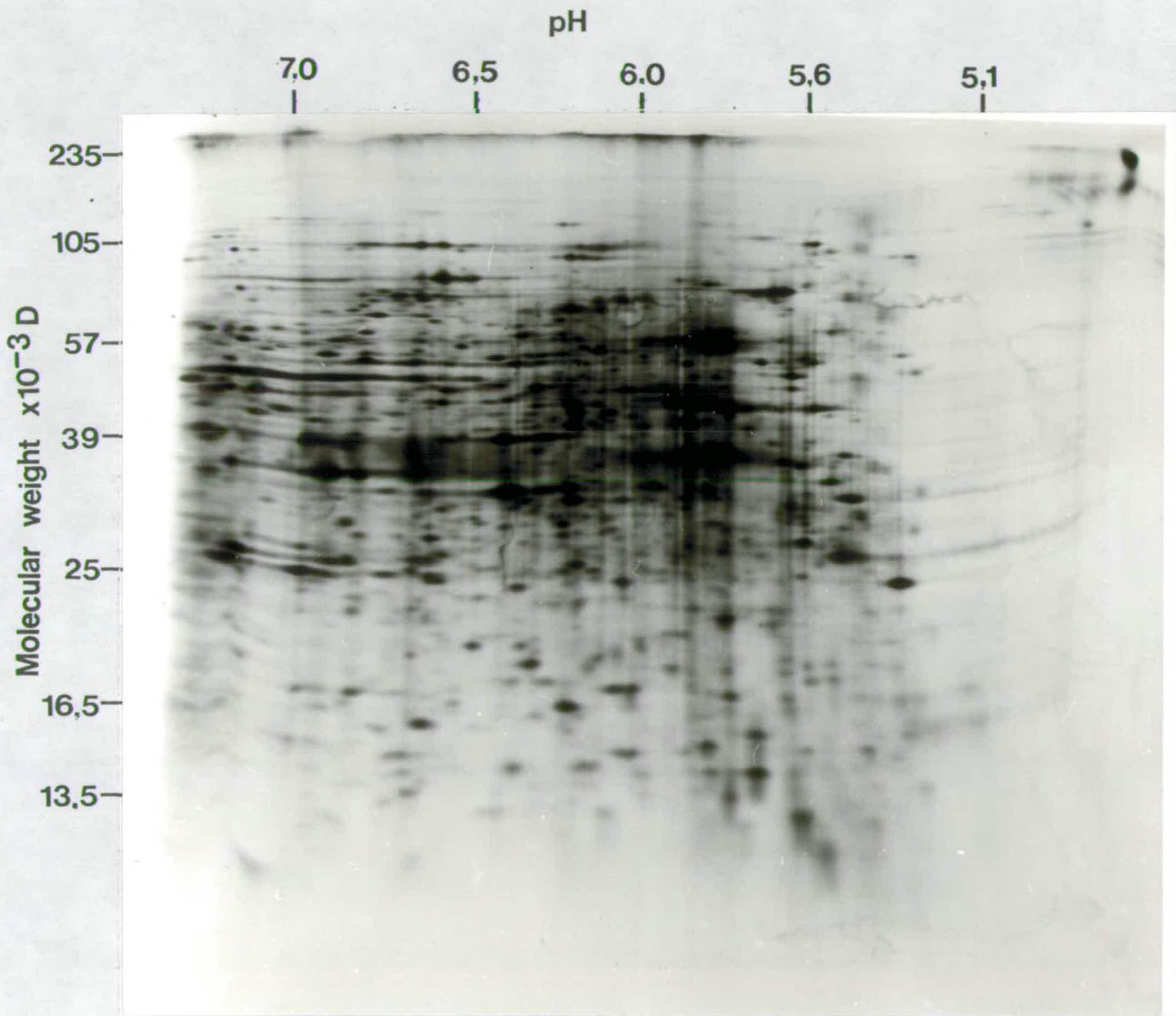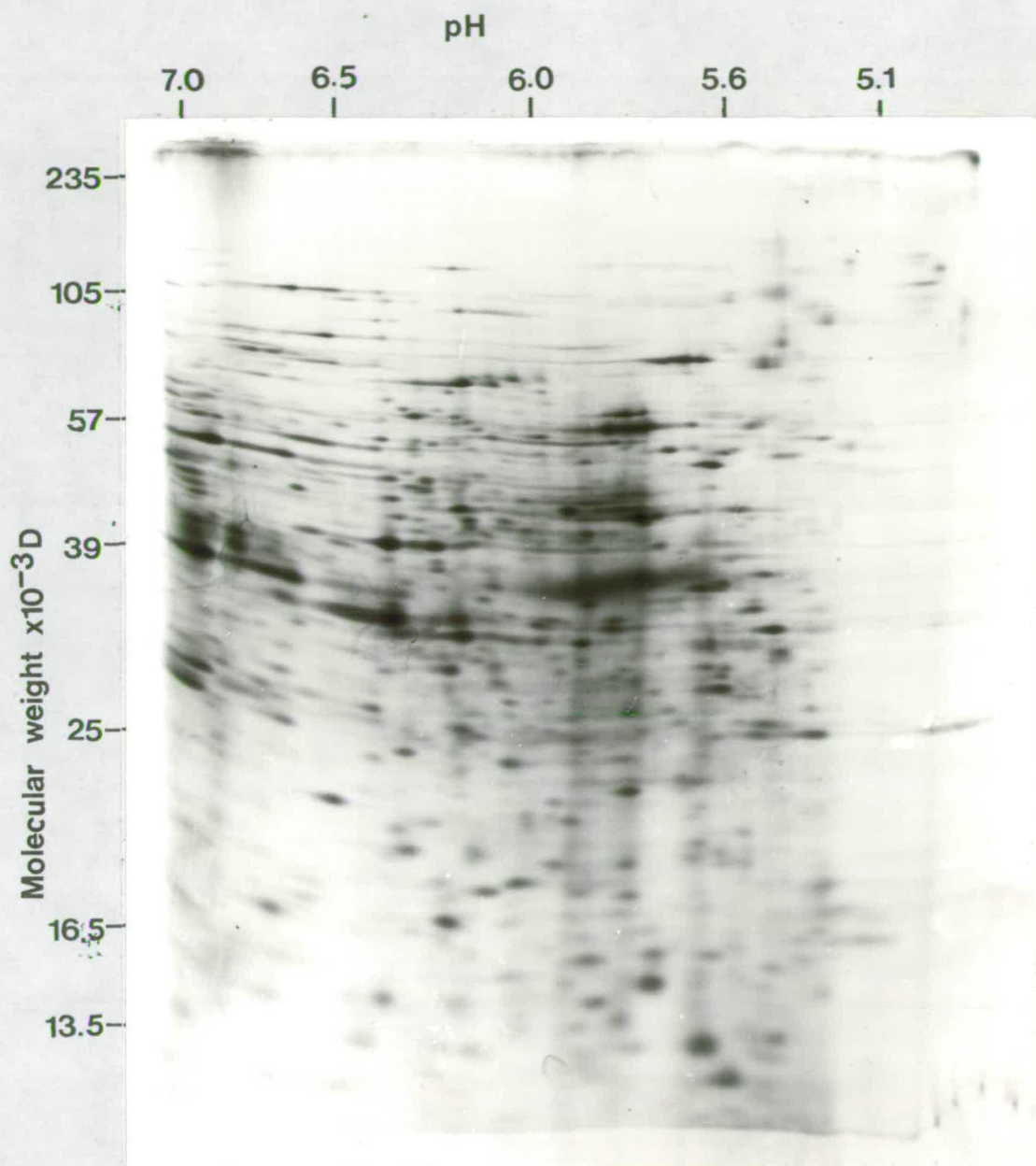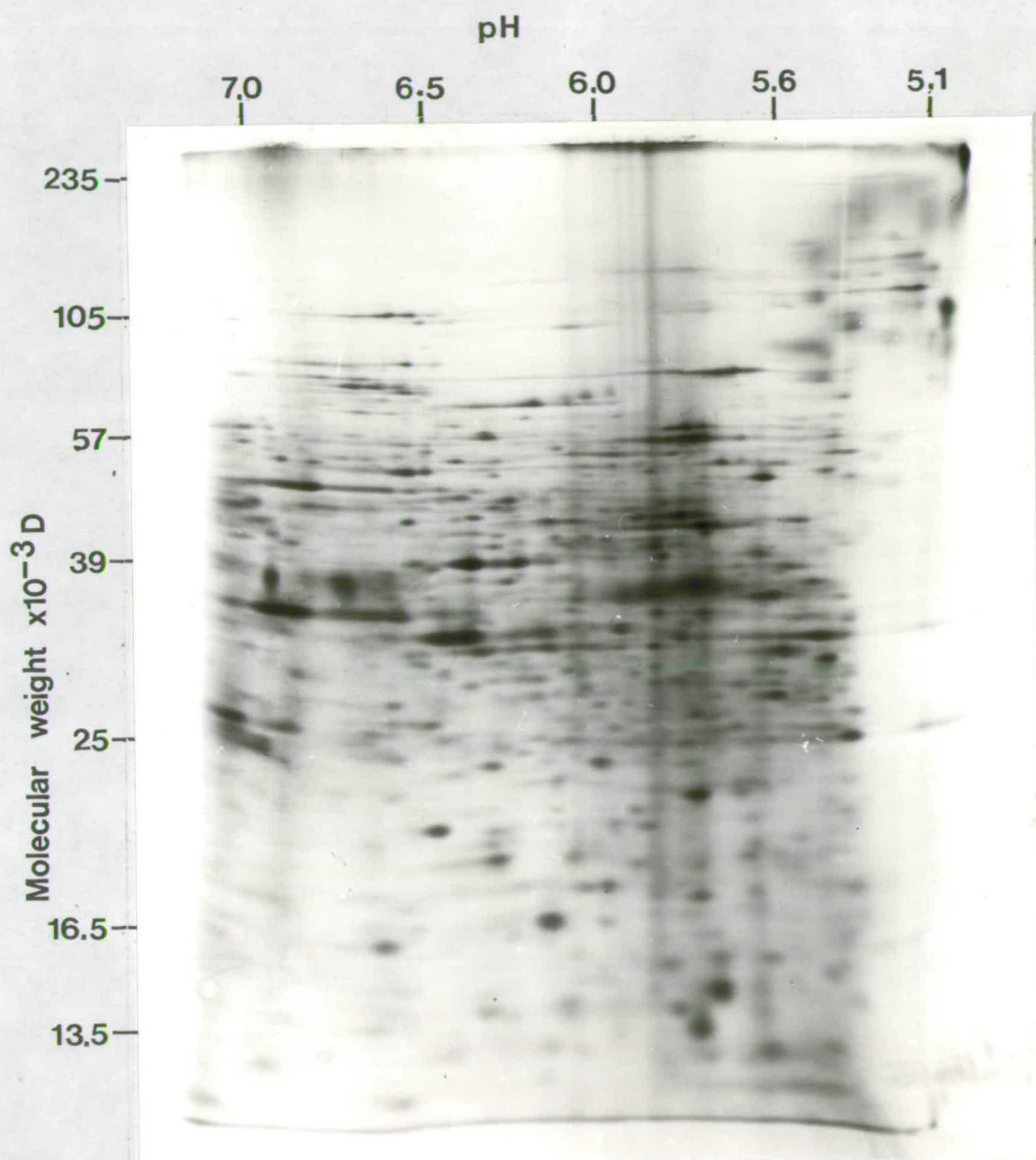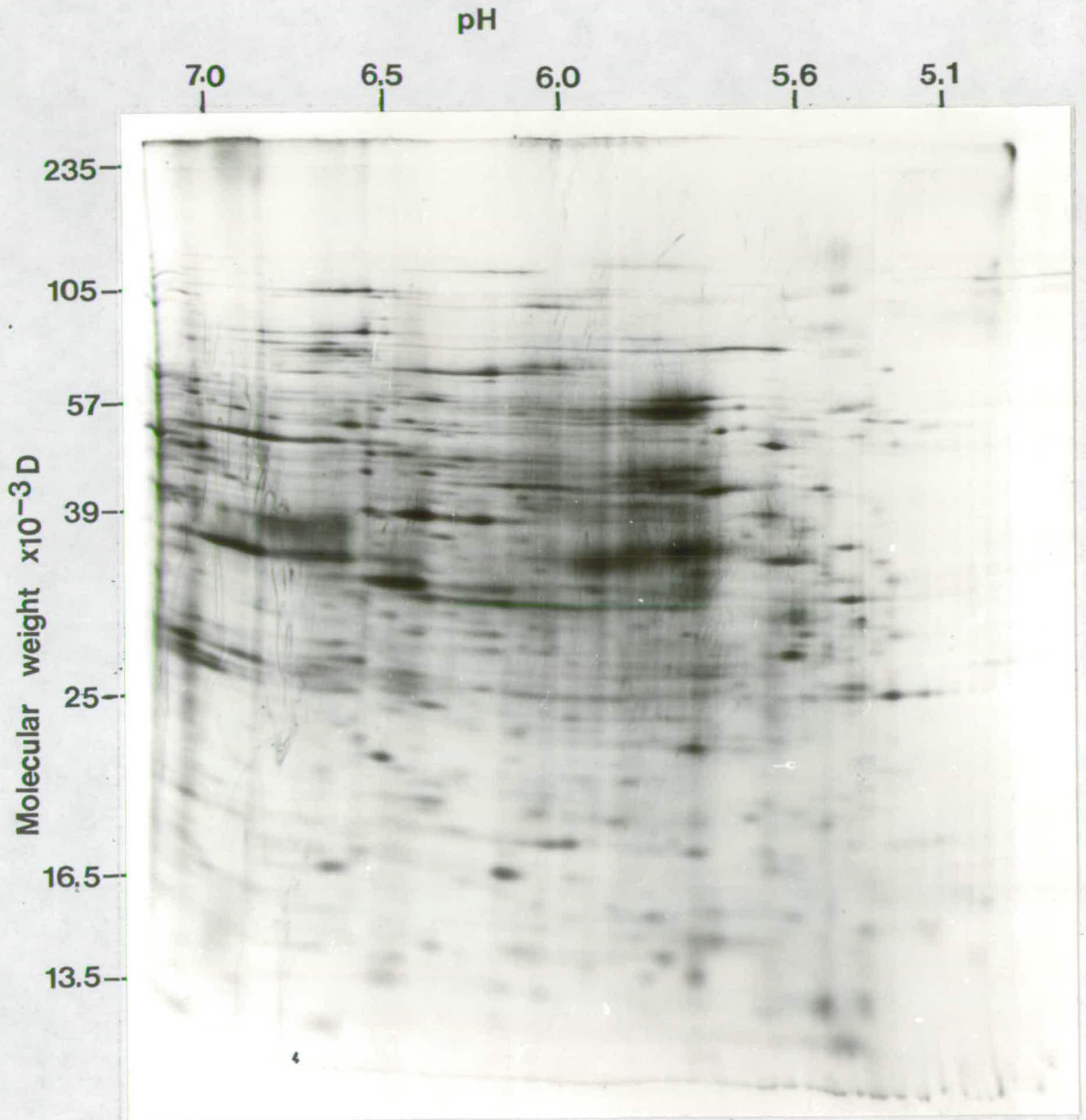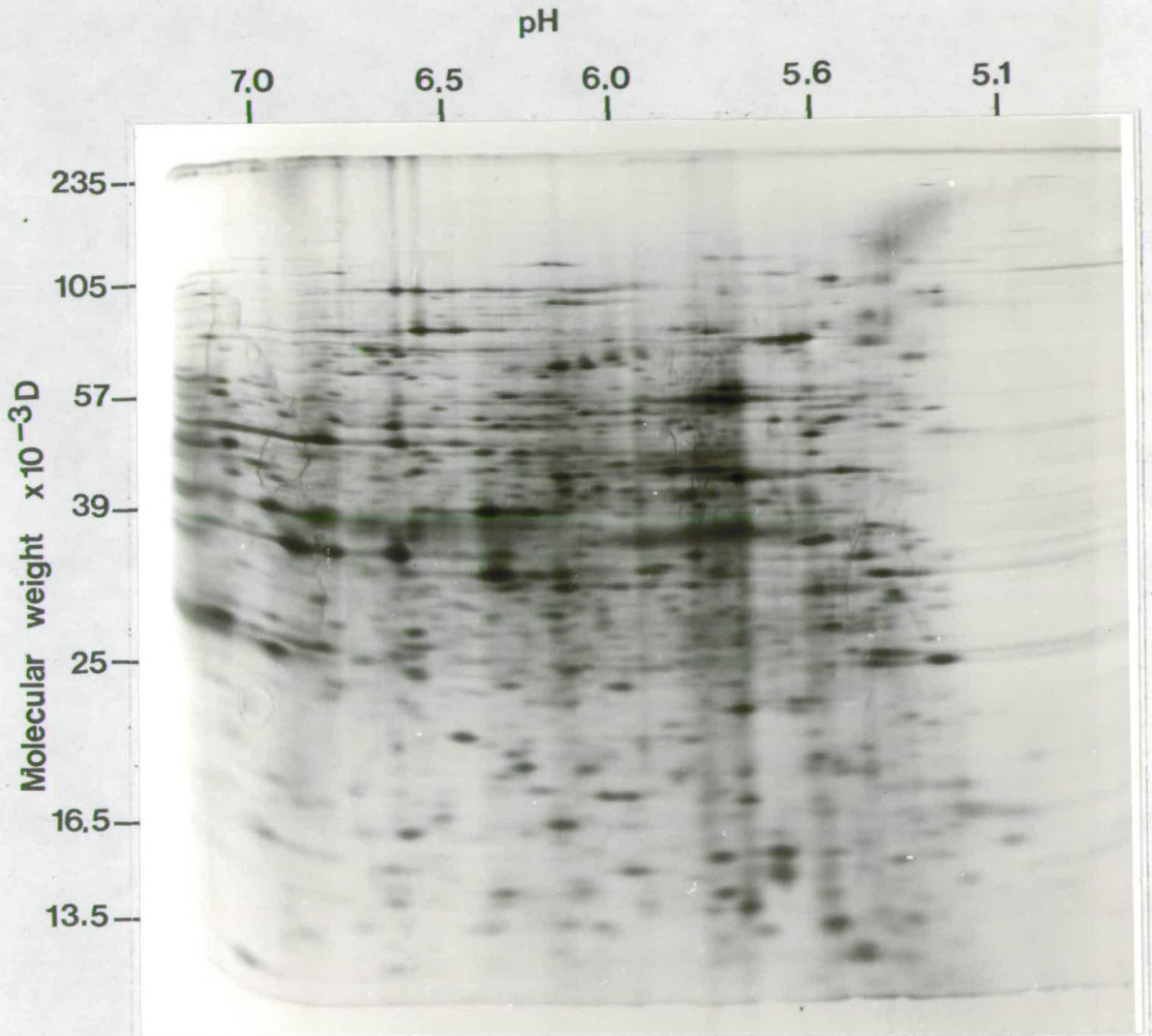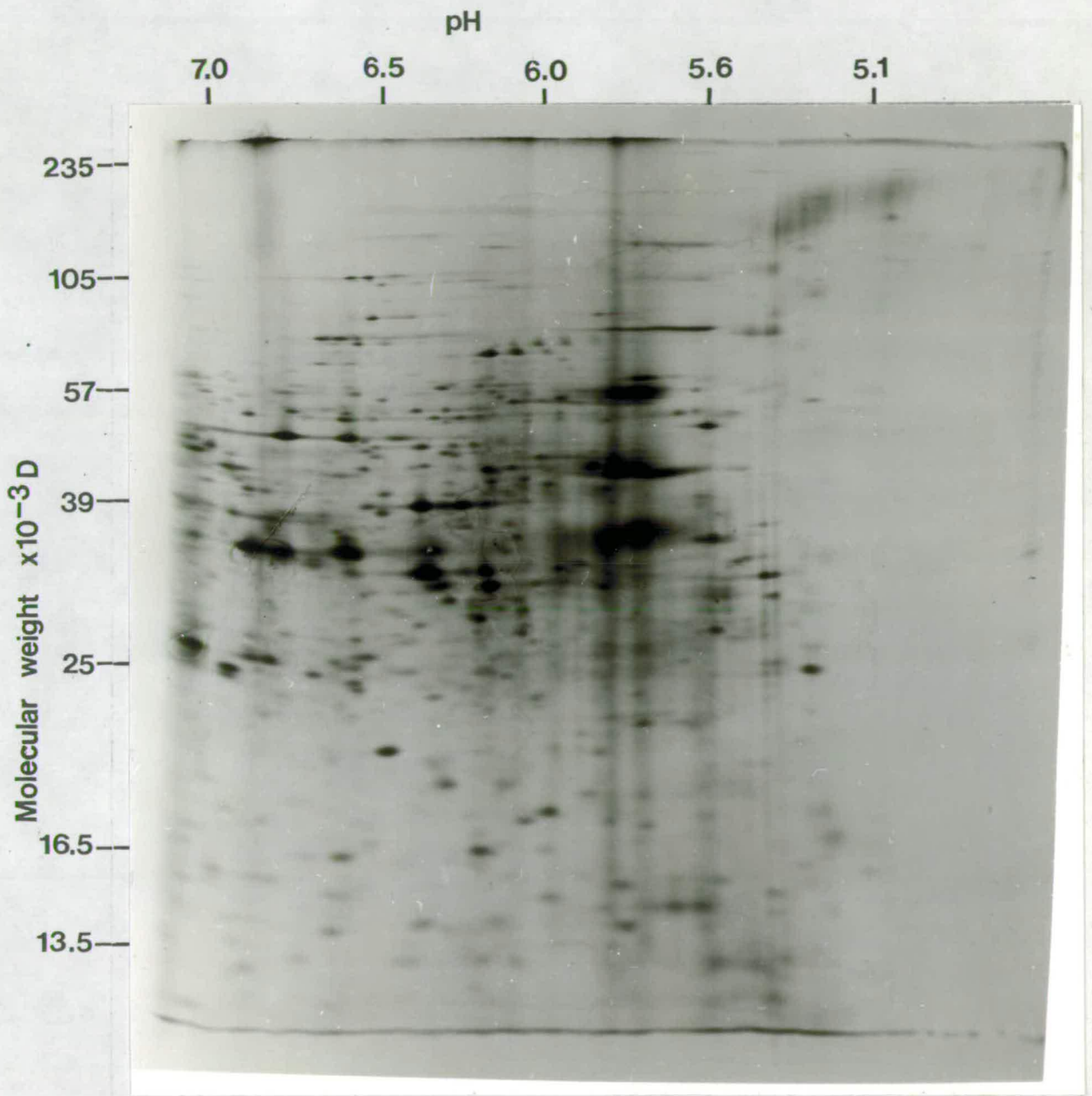
Figure 25 a
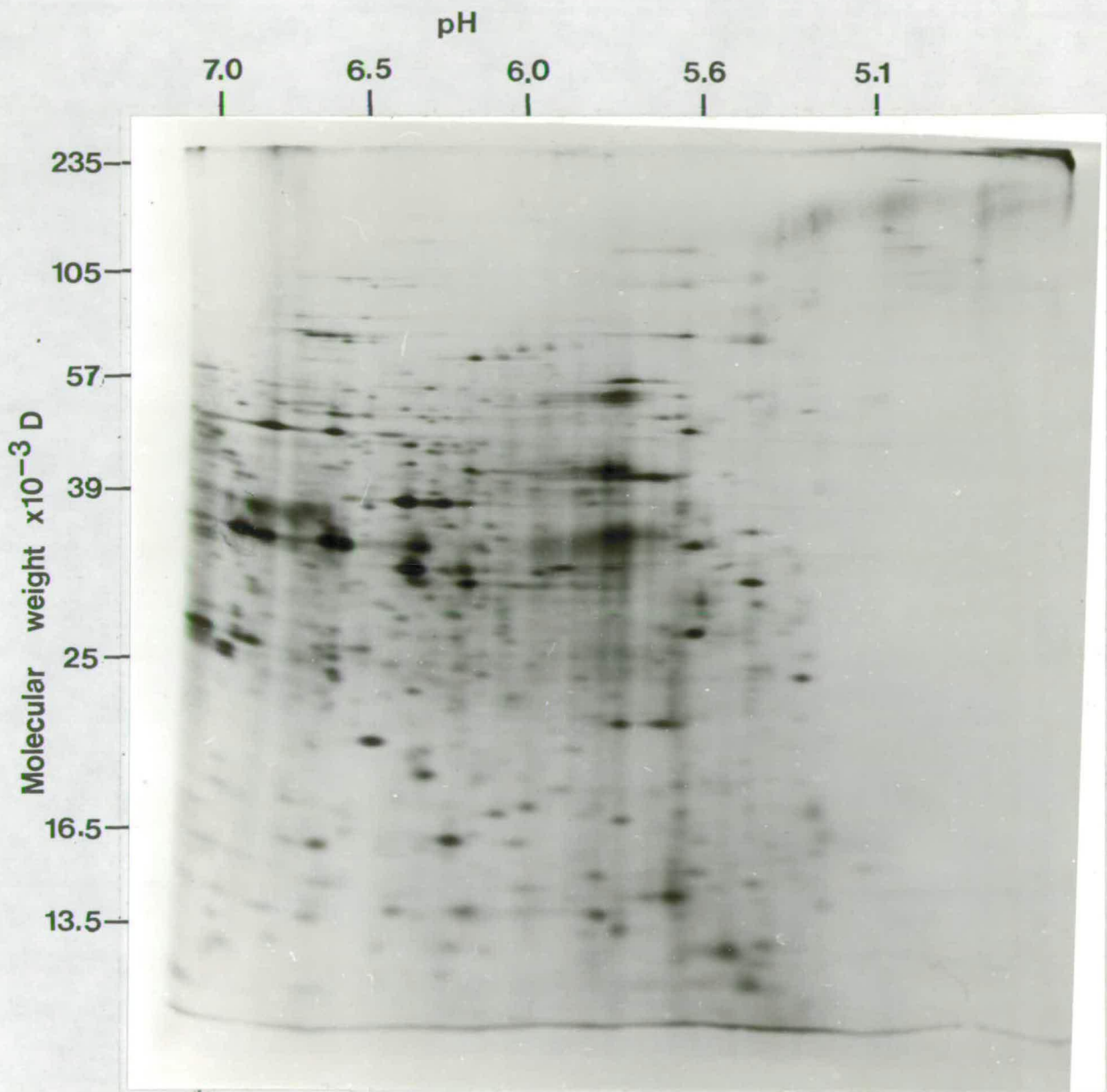
Figure 25 b
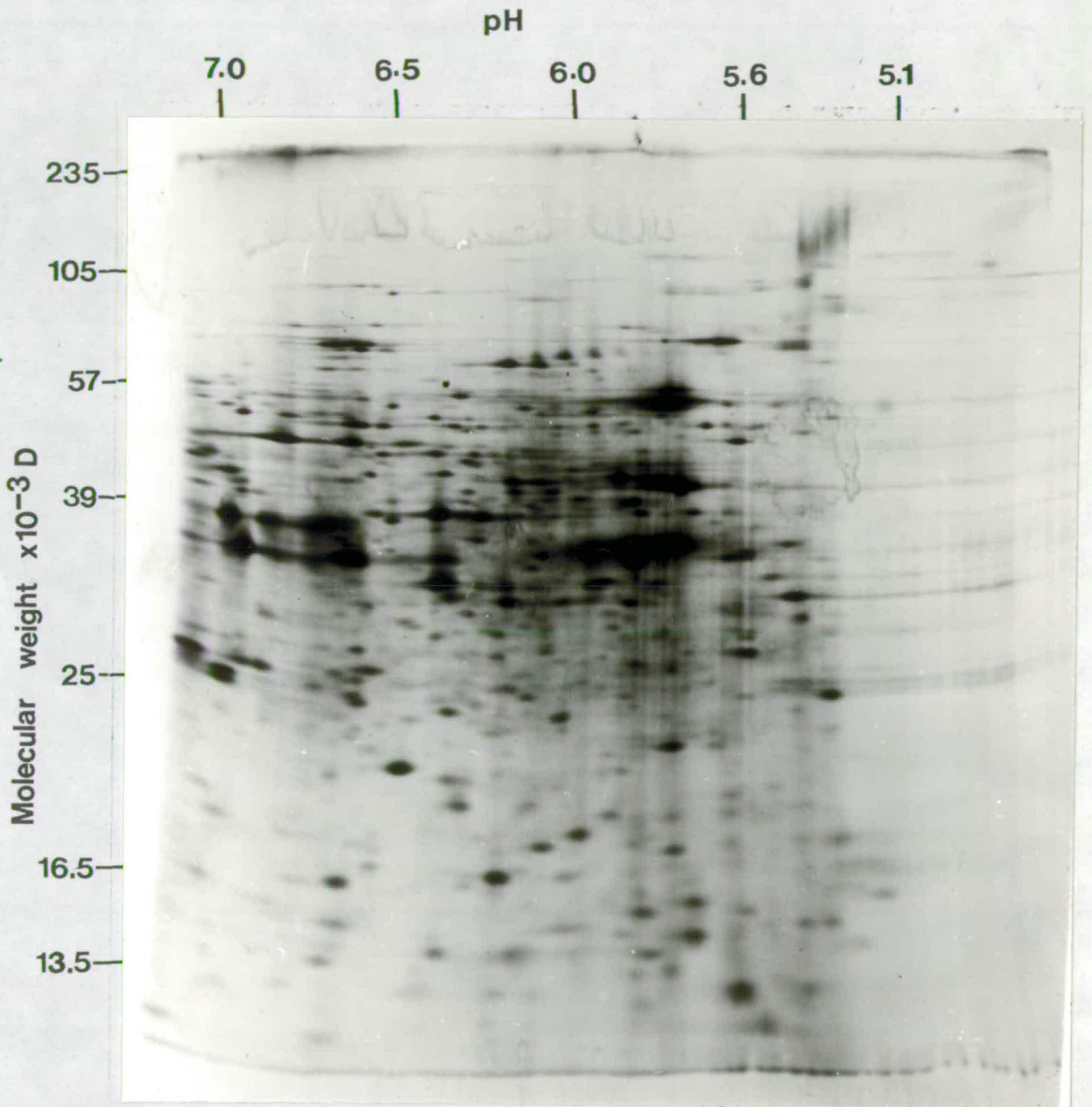
Figure 25 c
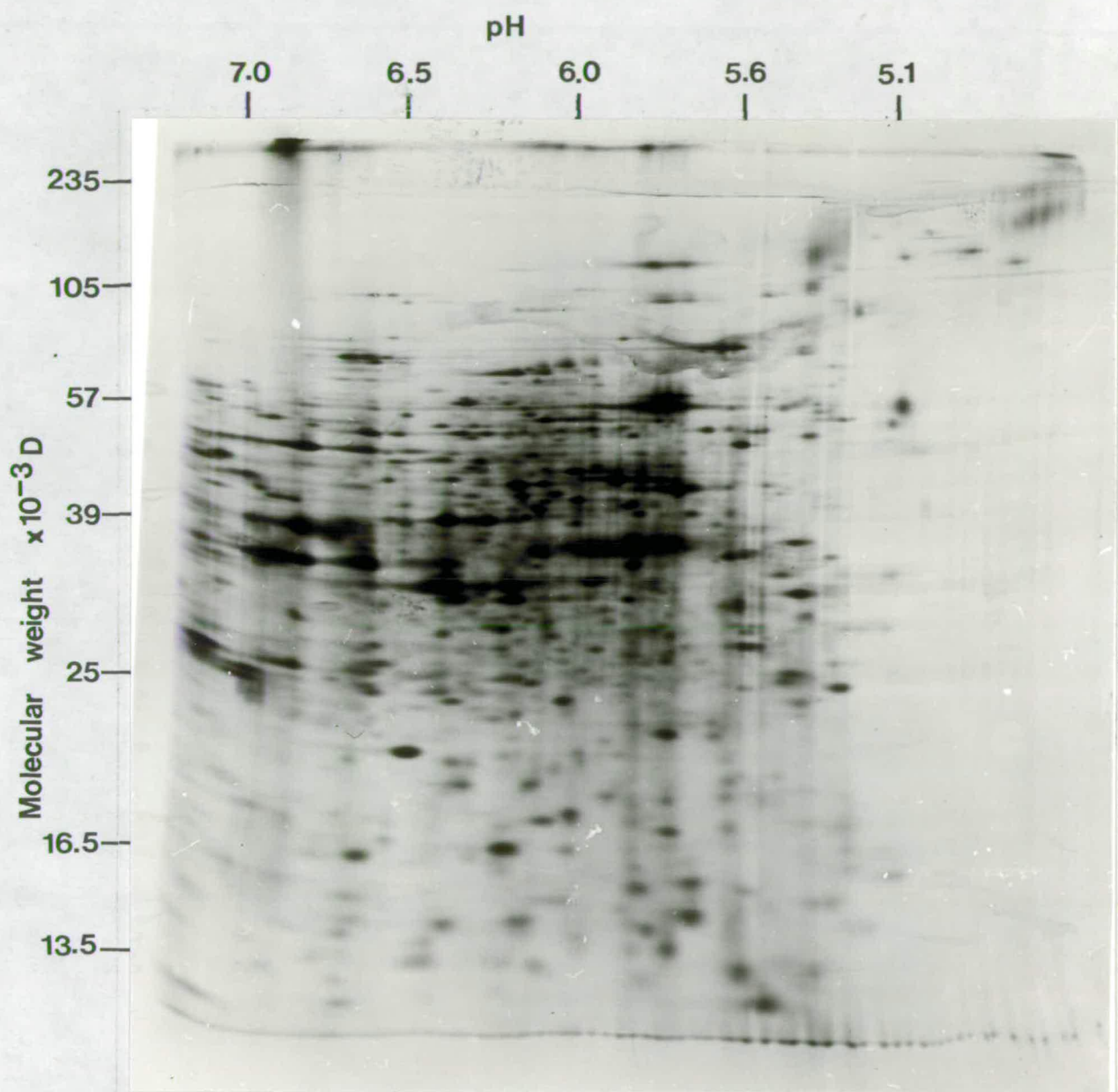
Figure 25 d

which can obscure adjacent spots. This occurs rarely in the SDS dimension, but is very common in the iso-electric focusing dimension, particularly at the extremes of the pH gradient. In addition, minor components are often only detectable on very sharply defined gels. Table 2 shows the distribution of the spots resolved on the gels. Some 206 (28.8%) of the spots were at least partially resolved on all 16 gels, and 517 (72.2%) were resolved on 12 or more gels.

The polypeptide patterns on these gels all appeared very similar. No 'overproduced' spots were seen on the cdc 3-6 h⁻ gels. To study the patterns in detail, a map was drawn from the wild-type autoradiographs (Diag. 4). This map was then divided into 1 cm^2 squares, and the spots in each square examined individually on each autoradiograph, yielding data on nearly 11 500 spots. A dependent protein would have been absent from at least gel P4 in both cdc 2-33 h⁻ and cdc 10-129 h⁻. Only 57 of the spots satisfied this minimum condition: 3 of major abundance; 10 of intermediate abundance; and 44 minor components. None of these spots showed a pattern that corresponded to a dependent protein. Of the major components, spot 2378 N was identified on only one gel (Fig. 22c), and was most probably an artifact. The other two major components, 2367 G and 8945 G were generally very badly streaked (e.g. Fig. 22d), and hence a failure to detect them on some gels was due to resolution, rather than a definite absence. All the intermediate abundance spots were poorly resolved, either due to streaking, or to variations at the most basic end of the pH gradient.

Number of gels	Number of spots	% of total spots
1	2	0.28
2	5	0.70
3	7	1.0
4	10	1.4
5	16	2.2
6	12	1.7
7	5	0.70
8	24	3.4
9	36	5.0
10	43	6.0
11	39	5.5
12	62	8.7
13	86	12.0
14	78	10.9
15	85	11.9
16	206	28.8

Table 2

The number of spots resolved on a given total number of gels is shown.
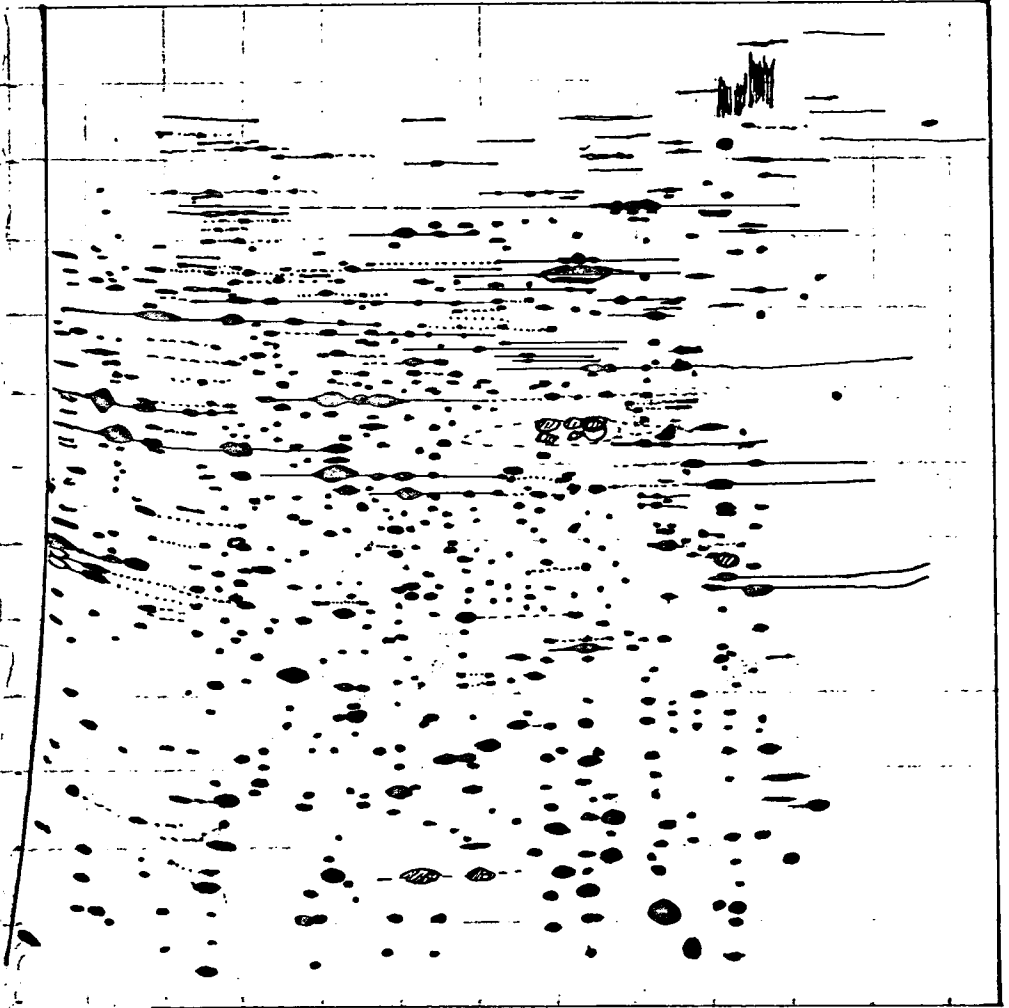
Diagram 4

Drawing of spots observed on autoradiographs shown in Figures 22-25.

Most of the minor components were barely detectable, even on very good gels. In addition many were in areas of very high spot density, and were easily obscured during the long exposure needed to detect them by autoradiographic spreading of adjacent spots. The absence of these 57 spots from the P4 gels of cdc 2 and cdc 10 was therefore much more likely to have been due to their low level of resolution in this gel system, rather than to their being dependent proteins. It is possible that perhaps 2 or 3 of the minor components are dependent proteins, but that this fact was obscured by their low resolution. Nonetheless, such proteins would constitute a very small proportion of the total protein, both in terms of number (less than 0.4%), and mass (ca. 0.0005%, as estimated by comparison to a calibration strip; equivalent to ca. 1000 molecules/ cell).

Chapter 5: A SEARCH FOR THE wee 1 GENE PRODUCT

INTRODUCTION

The <u>wee</u> mutants isolated in <u>S</u>. <u>pombe</u> have lesions in the control co-ordinating growth with cell size (77,78,79), and an identification of the molecular basis of this control would be of great importance to our understanding of the control of cell proliferation. Two types of <u>wee</u> 1 mutant suggest that the <u>wee</u> 1 gene product is a protein. Firstly, several temperature sensitive <u>wee</u> 1 mutants have been isolated. Temperature sensitivity is usually the result of instability in the structure of a protein caused by an amino acid substitution. Temperature sensitivity in other molecules, such as tRNAs, are much rarer. Secondly, a <u>wee</u> 1 mutant exists which is suppressible by nonsense suppressors (96). These suppressors are altered tRNAs which substitute an amino acid against a stop codon on an mRNA. This <u>wee</u> 1 mutant is therefore probably a nonsense mutant which produces only a polypeptide fragment of the <u>wee</u> 1 gene product.

The identification of a spot on a gel corresponding to the <u>wee</u> 1 gene product would be valuable, not only as an 'assay' for the protein, but also studies of its level during the cell cycle would enable models of cell cycle control to be devised on a sounder biochemical basis than is possible at present.

The high resolving power of two-dimensional gel electrophoresis, which can detect single charge changes in missense mutants (81), allows searches for individual gene products to be made in crude cell extracts. In principle the spot on a gel corresponding to the wild-type polypeptide might be shifted in the iso-electric focusing dimension due to a charge change

in a missense mutation; or, absent altogether, due to a nonsense

mutation, or proteolytic cleavage of the defective protein.

This approach was used to search for the <u>wee</u> 1 gene product by

comparing $[^{35}S]$-labelled wild-type protein extracts with those

of <u>wee</u> 1 strains, including a nonsense mutant.

METHOD

The missense mutants used in this study were <u>wee</u> 1.1 h⁻, <u>wee</u> 1.6 h⁻, and <u>wee</u> 1.6 h⁻. A nonsense mutant, <u>wee</u> 1.112 h⁻, was also used. Cells were grown overnight at 25°C in 25 ml of EMM 3 containing 10 mg/ 1 of sulphate, and 1 mCi of $[^{35}S]$-sulphate. The cells were collected at an O.D.$_{595}$ of 0.1-0.4, and the proteins solublised for two-dimensional gel electrophoresis, as described in General Methods. Two approaches were used to maximise the number of proteins resolved. Firstly, the iso-electric focusing gels contained 2% of pH 3.5-10 Ampholytes only. This reduced the resolution of the middle range to some extent, but considerably extended the basic end of the gradient to cover the range pH 3.5-8.5. Secondly, two gels were used to increase the resolution in the second dimension. A 12% acrylamide, 0.33% bis-acrylamide gel was used to cover the molecular weight range 12 000-25 000 Daltons, and a 9.4% acrylamide, 0.26% bis-acrylamide gel to cover the range 24 000-270 000 Daltons.

After electrophoresis the gels were soaked in water for about one hour, then dried down and autoradiographed for 4-6 weeks. To compare the autoradiographs a map was drawn of the spots seen, and divided into 1 cm^2 squares. The spots on each gel were then compared, square by square.

RESULTS AND DISCUSSION

Five 972 h⁻ wild-type controls were compared with two
wee 1.1 h⁻, one wee 1.6 h⁻, one wee 1.3 h⁻, and three wee 1.112 h⁻
experiments. Figures 26 and 27 show autoradiographs of 972 h⁻
and wee 1.112 h⁻ samples, and diagram 5 shows a drawing
constructed from these, and other gels. A total of 794 spots
were identified on the autoradiographs, which is about 10%
higher than the figure obtained for a single 16% gel. Of these,
some 484 (61.0%) were at least partially resolved on all but
the poorer gels. None of these spots could therefore be
identified with the wee 1 gene product on the basis of the
criteria discussed above. The remaining spots were resolved
with a lower frequency, but all except 103 spots, mostly minor
components, were resolved on at least one wee 1.112 h⁻ preparation.
None of these spots showed a consistent absence from the gels of
other wee strains, and their absence was most probably due to
variability in their resolution. The possibility that one of
the remaining 103 spots is the wee 1 gene product cannot be
excluded, as they were only poorly resolved on the majority of
the gels. This survey therefore failed to identify the wee 1
gene product.

There are several possible reasons for failure to detect
single protein changes in a sample, the most trivial being the
absence of methionine or cysteine in the protein. The properties
of the protein, such as extremes of charge or size, might exclude
it from the range studied. Indeed, a majority of the cells
proteins are not resolved on these gels. It is also possible

Figure 26

An asynchronous culture of 972 h⁻ growing in 25 ml of EMM 3 + 10 mg/ 1 sulphate at 25°C was labelled overnight with 1 mCi [^{35}S]-sulphate, and samples of a total protein extract separated by two-dimensional gel electrophoresis on 12% and 9.4% acrylamide gels. Only the bottom half of the 12% gel is shown, including a small region of overlap with the bottom half of the 9.4% gel.
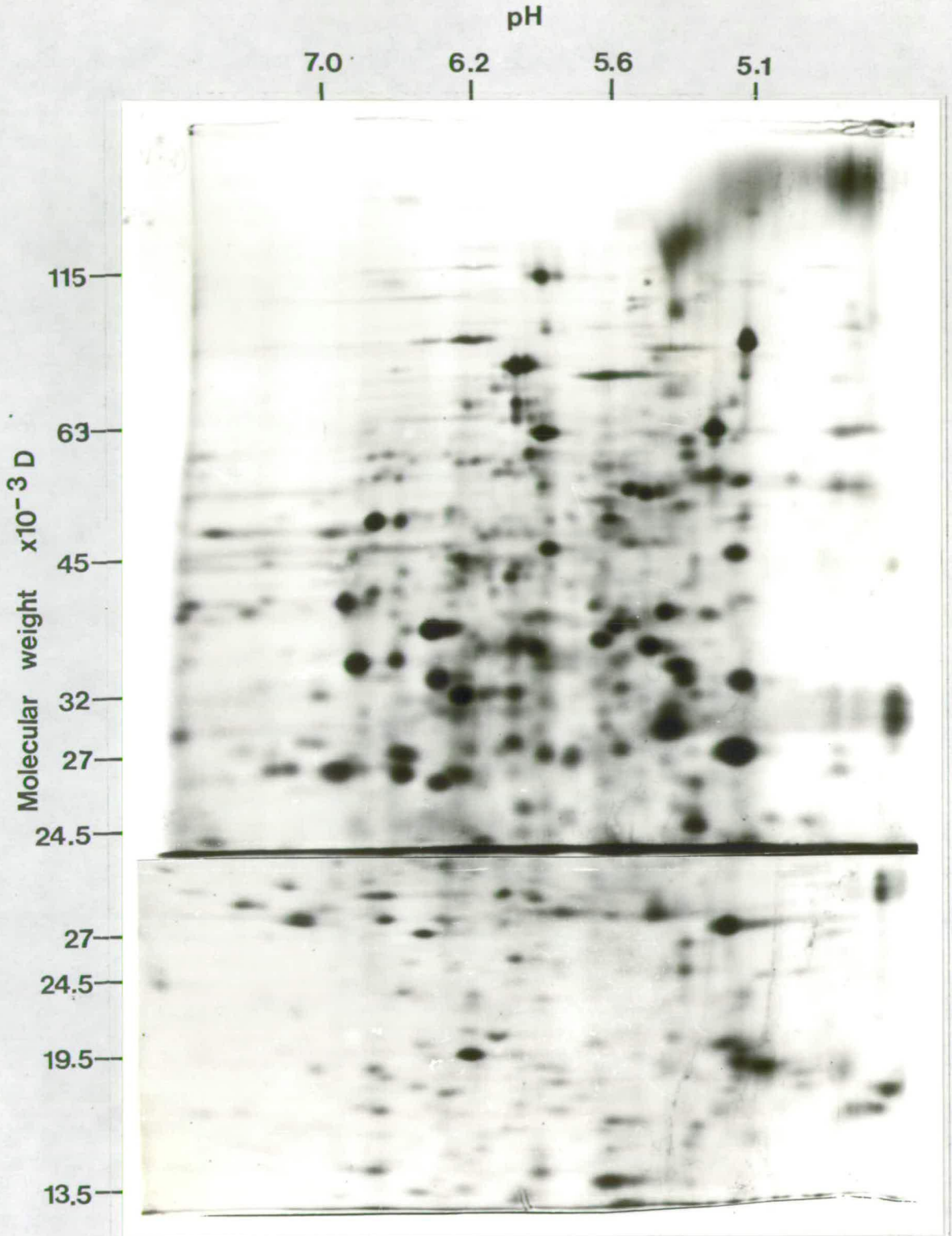
Figure 26

Figure 27

An asynchronous culture of wee 1.112 h⁻ growing in
25 ml of EMM 3 + 10 mg/ l sulphate at 25°C was labelled
overnight with 1 mCi [^{35}S]-sulphate, and samples of a total
protein extract separated by two-dimensional gel electro-
phoresis on 12% and 9.4% acrylamide gels. Only the bottom
half of the 12% gel is shown, including a small region of
overlap with the bottom of the 9.4% gel.
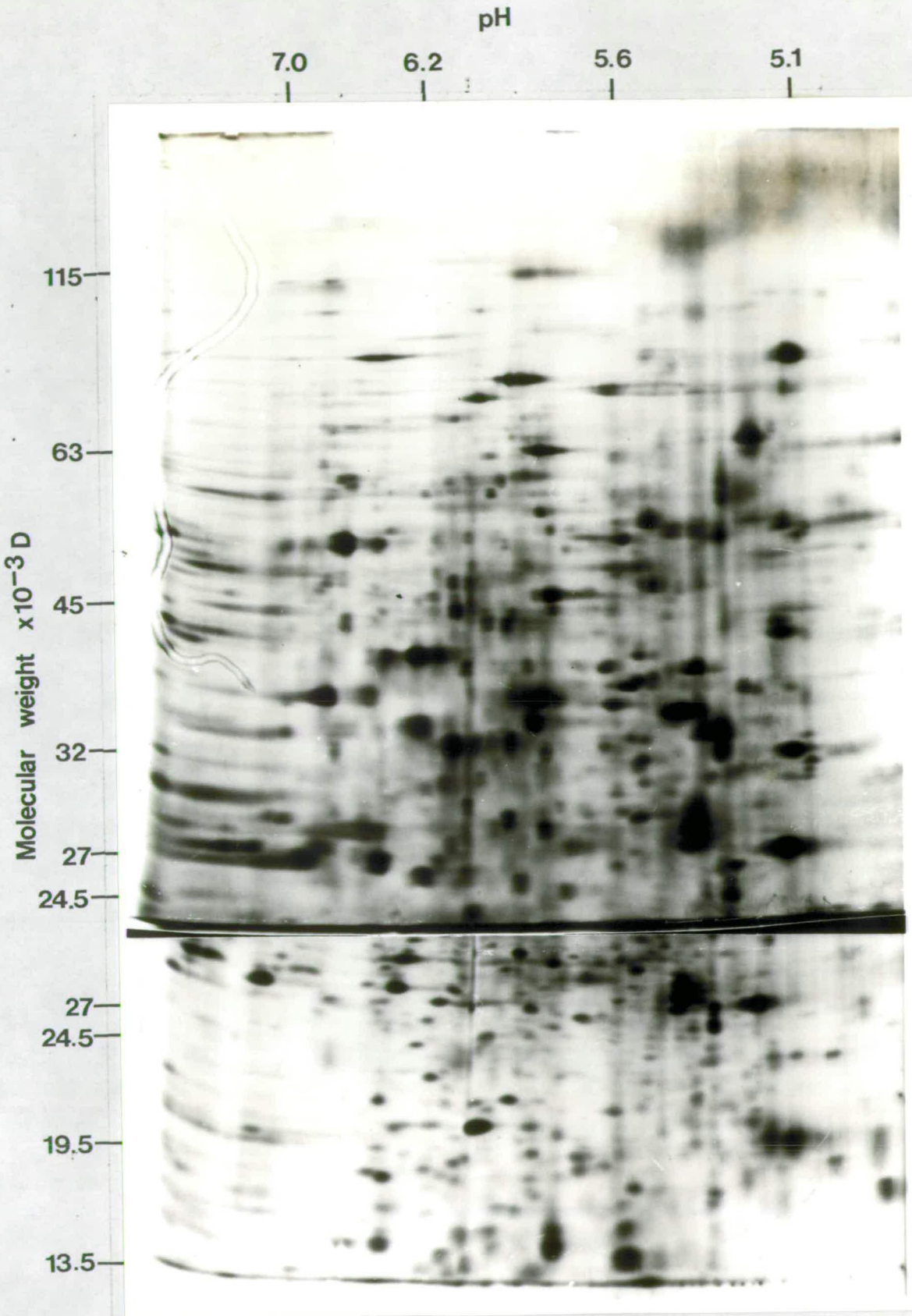
Figure 27
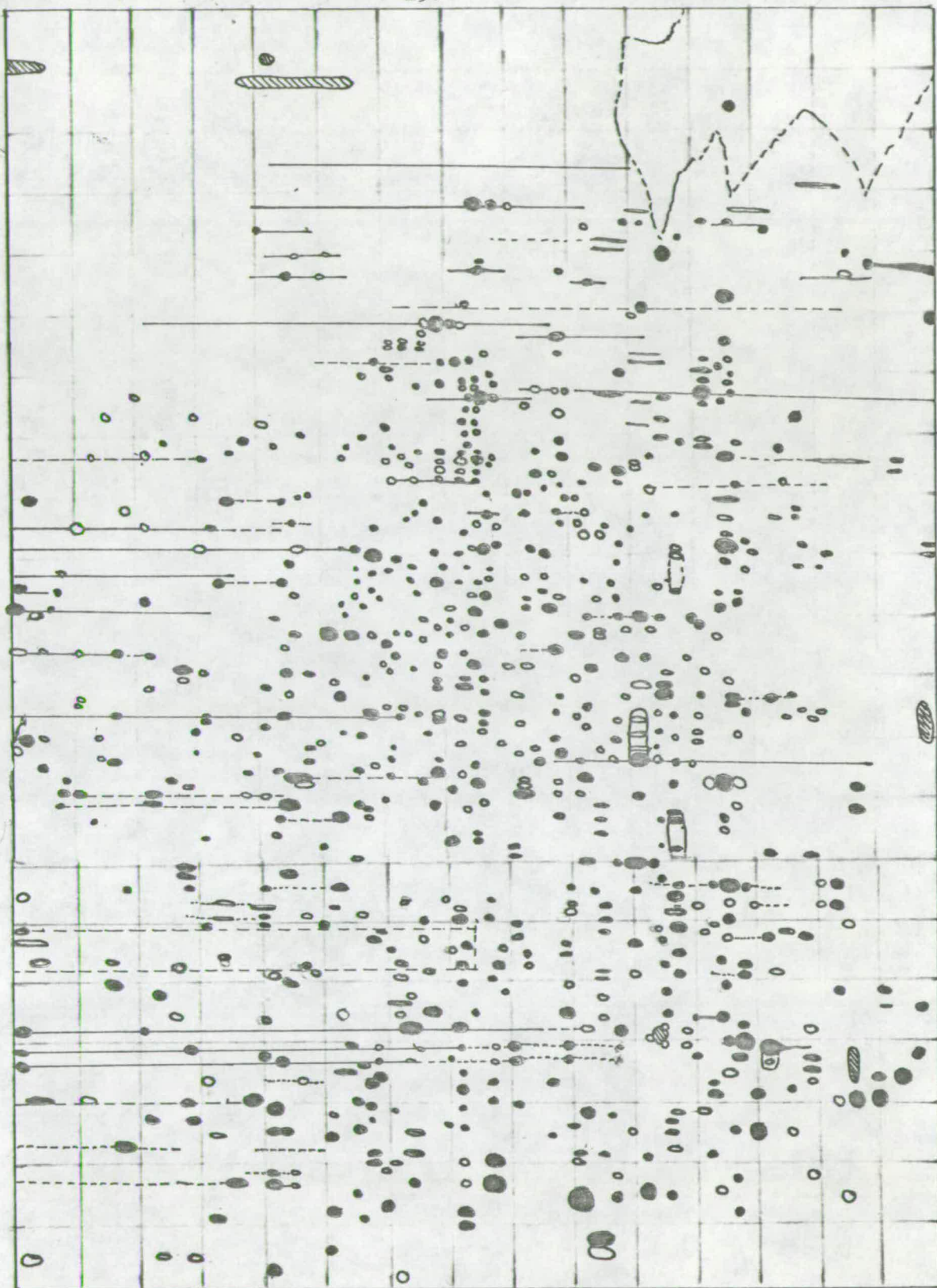
Diagram 5

Drawing of spots observed on autoradiographs shown in Figures 27,28, and others.

that the _wee_ 1 gene product is one of the 794 spots seen on
these gels, but the mutants screened did not have a sufficiently
different protein structure to the wild-type protein to be
detectable by this technique. Recently, Milman and workers
used a similar approach to examine hypoxanthine phosphoribosyl
transferase (HPRT) mutants in HeLa cells (97). They began by
identifying the HPRT protein spot on gels by comparison with the
purified enzyme. This spot was absent in 24 HPRT mutants, and
one missense mutant showed a new spot with the same molecular
weight as the wild-type protein, but a slightly higher pI.
This shift was probably due to a charge change caused by the
mutation. These results therefore confirm the principle behind
the approach used here. Clearly, these experiments were
something of a gamble, but the potential results would have been
of great value.

Chapter 6:   CONCLUDING DISCUSSION

Periodic Events in the Cell Cycle:

Prior to the discovery, nearly thirty years ago, that the synthesis of DNA is periodic, it was believed that in macro-molecular terms cell growth during the cell cycle was a relatively smooth, continuous process. Beginning in the mid-sixties, measurements of changes in enzyme activity during the cell cycle suggested the contrary, and that ordered patterns of periodic protein synthesis occured during the cell cycle. Further work, however, indicated that at least some of the observed periodic patterns were due to perturbations, and also that enzyme activity could not always be directly equated with enzyme synthesis. The general patterns of gene expression during the cell cycle therefore remained uncertain until the recent development of sophisticated gel electrophoresis techniques which provided a means of studying the patterns of synthesis of large numbers of polypeptides directly. In addition, the need has been recognised to minimise the effects of the synchronisation procedure, and to test for perturbations. The results from this approach suggest that our original view of cell growth was correct: the majority of proteins in a wide range of organisms all appear to be synthesised continuously during the cell cycle, and periodic protein synthesis during the cell cycle is the exception.

Lutkenhaus and workers have examined the patterns of synthesis of 750 polypeptides during the cell cycle of the prokaryote E. coli, using two-dimensional gel electrophoresis (98). They found all of these proteins to be synthesised continuously, and found no evidence for periodic synthesis. The E. coli

genome can code for roughly 2300 mRNA species, but only about

1500 of these are present, on average, at more than one molecule

per 35 cells (99). Assuming that these code for the normal

protein complement of a cell, and that rarer mRNAs make no

significant contribution to the total, them at least half of

the cells polypeptides are synthesised continuously. In terms

of mass, this proportion represents almost the entire bulk of

the protein.

Several eukaryotic cells have been examined, and in most

cases they show general agreement with this result. In this

thesis, I have described evidence to show that 56 non-ribosomal,

and 38 ribosomal proteins in the fission yeast S. pombe are

synthesised continuously, and possible exponentially. This

work confirms, and considerably extends the early work in this

yeast by Wain, who demonstrated continuous synthesis in 14

bands on disc gels of a pH 8.1 soluble protein fraction (100).

Clearly, it would be advantageous to repeat this work using

two-dimensional gel electrophoresis. Unfortunately, time

did not permit this in the present study.

Elliott and McLaughlin used two-dimensional gel electro-

phoresis to show that 550 polypeptides are synthesised con-

tinuously during the cell cycle of S. cervisiae, and that at

least 110 of these are synthesised exponentially (43). Similarly,

Elliott et. al., showed that the synthesis of 49 ribosomal

proteins is exponential (101). Yeast probably contains 3000-

4000 different mRNA species (102), so, as in E. coli, a sub-

stantial proportion of the cells proteins, both in terms of

number and mass, are synthesised continuously. Using one-dimensional gel electrophoresis, Ernst found no evidence for periodic synthesis of proteins during the cell cycle of Physarum(103).

The algae, however, would appear to provide an exception to the general pattern of continuous synthesis of individual proteins seen in these lower eukaryotes. However, since a light-dark growth regime was used to synchronise the cells, it is not clear if the observed periodicities were real cell cycle events.

It is possible that many of the reported periodicities were due to the induction or repression of synthesis by the illumination conditions. Howell et. al. examined the patterns of synthesis of 100 polypeptides in synchronous cultures of Chlamydomonas reinhardtii (104). They found significant variations in the rate of synthesis of thirty polypeptides, but many of these polypeptides also showed extensive variations in their rate of labelling in control asynchronous cultures shifted from light to dark. Although some of these variations were not directly correlated with those seen in the synchronous culture, the possibility remains that many of the observed periodicities were due to perturbations induced by the synchronisation method. Similarly, recent results from Chlorella indicate that most of the previously observed periodicities may have been due to the growth conditions employed (36). In this study on S. pombe, I have demonstrated that a relatively mild synchronisation procedure can produce periodic patterns of labelling of some polypeptides due to quite subtle nutritional effects.

The situation in higher eukaryotic cells is not clear. Milcarek and Zahn used pulse labelling and two-dimensional gel electrophoresis to examine the patterns of synthesis of 90 abundant cytoplasmic proteins in HeLa cells (105). The cells were synchronised by three different methods: a double thymidine block; colcemid arrest; and mitotic wash-off. Only six proteins were found to show more than a four-fold variation in their rate of labelling during the cell cycle; four at a greater rate during M, and two at a lower rate during G2. None showed synthesis during only a restricted period of the cell cycle. In contrast, Al-Bader et. al. found evidence for extensive variations in the levels of proteins during the HeLa cell cycle (106). Using a double thymidine block, to synchronise the cells either alone, or followed by colcemid arrest, they found that out of 150 proteins examined on Coomassie stained two-dimensional gels, 35 were absent during S phase, but present during G2. This result would suggest that many major proteins are synthesised periodically during G2, and are then subsequently rapidly removed, possibly by proteolysis. Clearly, it will be important to resolve this contradiction.

Apart from the technical difficulties in interpreting gel data, such as variability in resolution, or 'streaking', several other factors need to be considered. Firstly, gel electrophoresis studies are restricted to that fraction of the cells proteins soluble in SDS. This fraction is further limited by two-dimensional electrophoresis to these proteins with an isoelectric point,in the 3-8 pH range. This would

exclude the very basic or acidic proteins, such as some nuclear proteins. Some of the nuclear proteins might be expected to show periodicity associated with chromosome replication, as has been found for histones.

Secondly, the gel studies described above were generally restricted to the more abundant proteins. Most of these are probably major metabolic enzymes, and it would seem that the best way to maintain balanced growth would be for them to be synthesised continuously. The minor components, such as regulatory proteins, which also might be expected to show periodic synthesis, have not been studied. However, there is a statistical argument against the least abundant proteins being synthesised periodically. The low probability of the appropriate collisions occuring between a rare molecule and its target, such as a rare mRNA and a ribosome, would result in large fluctuations in the timing of translation, making the control of timing periodic synthesis difficult.

Thirdly, proteins can undergo a wide range of post-translational modifications, such as methylation of arginine or lysine (reviewed in 107), and many enzymes have been shown to be regulated by covalent modification of amino acid residues, (reviewed in 108), particularly phosphorylation (reviewed in 109). Some enzymes are also activated by proteolytic cleavage (e.g.110). The polypeptide population of a cell may therefore be much more diverse than the number of mRNA species would suggest. Many of the differences between such molecules, for example methylation, would not be detectable by gel electrophoresis.

Some of the modifications, for example, a single charge change

due to the acetylation of an amino group, or the proteolytic

removal of a short terminal sequence of neutral amino acids,

would affect the migration of a protein in a gel electro-

phoresis system but would produce only a small net change

which would go undetected.

Finally, the method of pulse-labelling a polypeptide

only measures the rate of its accumulation. The rate of

accumulation equals the rate of synthesis only if the loss of

the polypeptide, for instance by proteolysis, is negligible

during the time of the pulse, and is constant. These assumptions

are undoubtedly true for a great many proteins, but there may

be exceptions. It is important to note that in the studies

discussed above, a change in the rate of accumulation during

the cell cycle of at least a factor of two would have been

required for its detection.

Many of the reported periodic patterns of change in enzyme

activity were undoubtedly due to perturbations induced by the

synchronisation technique. In this thesis, I have demonstrated

that periodic labelling of some polypeptides in perturbed

synchronous cultures of S. pombe can be induced during

synchronisation. Assuming that these polypeptides did not

represent transient proteolytic fragments, them at least some

of the observed periodic patterns caused by perturbations are

due to periodic synthesis. However, it seems improbable that

all the cases of periodic changes in activity seen during the

cell cycle were due to perturbations. S. cerevisiae is an

extreme case, where 32 out of 33 enzymes show periodic variations

in their activity (2,31). It seems highly unlikely that all

of these periodic patterns are due to perturbations. But this

is clearly paradoxical: a substantial proportion of polypeptides

in S. cerevisiae appear to be synthesised continuously, yet

at least several percent of a randomly chosen group of enzymes

would seem to show periodic changes in activity. Even if all

the observed periodicities were due to perturbations, it remains

to be explained how such patterns could be produced, when four

cases of periodic enzyme activity were found using the same

synchronisation technique that Elliott and McLauglin used to

show a high proportion of continuous protein synthesis (43).

Any explanation put forward must also explain how step-wise

doublings in activity can occur. This requires the activity

of an enzyme to remain constant (and therefore the specific

activity must decrease at a controlled rate), until some time

in the cell cycle, when the total activity is doubled.

The paradox would be resolved if many of the observed

periodic patterns in activity, whether genuine cell cycle

events or the results of perturbations, were due to post-

translational changes, rather than changes in the patterns of

synthesis at the ribosome. Support for this hypothesis is

provided by the results from a study of DNA-polymerase activity

during the cell cycle of S. cerevisiae, by Golombek, et. al.(111).

They found that DNA-polymerase activity varied periodically

during the cell cycle, and that this oscillation process was not

due to a periodic synthesis and degradation, nor to a change

in the molecular size of the enzyme, as measured by sedimentation

studies. One possibility was that the activity was being

regulated by reversible covalent modifications.

Although only twenty amino acids are used as the building

blocks during protein synthesis, extensive post-translational

chemical modification occur, and about 140 amino acid derivatives

have been identified as constituents of different proteins

in various organisms (reviewed in 112). These derivatives

arise from a diverse range of modifications, including phosphoryl-

ation, acetylation, methylation, glycosylation, ADP-ribosylation,

and oxidation of thiol groups. Some of these modifications

have been shown to be very important in the control of enzyme

activity. One of the best characterised systems is the regulation

of glycogen metabolism in mammalian tissues by the phosphoryl-

ation and dephosphorylation of the enzymes involved (reviewed

in 108). Glycogen synthetase, which polymerises UDP-glucose

to glycogen, is converted into a glucose-6-phosphate dependent

b form by phosphorylation brought about by a 3',5'-cyclic AMP

dependent synthetase kinase. A synthetase phosphatase can

reverse the reaction to give the glucose-6-phosphate

independent a form. Degradation of glycogen to glucose-1-

phosphate is brought about by phosphorylase a, produced by

phosphorylation of the inactive phosphorylase b by a

phosphorylase kinase. The kinase is itself activated by

phosphorylation by a cyclic AMP dependent kinase. The histones

also undergo extensive phosphorylation and dephosphorylation,

although the significance of these modifications remains un-

known (reviewed in 20).

Recently, Wolosuik and Buchanan have shown that fructose-1,
6-bisphosphatase, a key metabolic enzyme in chloroplasts, is
activated by a photochemical reduction brought about by the
ferredoxin-thioredoxin system, and deactivated in the dark by
oxidation with glutathione (113).

In addition to the modification of the constituent amino
acids, proteolytic cleavage of the polypeptide chain has been
shown to be important in enzyme activation or deactivation
in several systems. Examples of this type of modification
are provided by the activation of trypsinogen, and the activation
cascade involved in blood clotting. Cabib and workers have
studied the synthesis of chitin in S. cerevisiae, and have
shown a similar mechanism to be involved. Chitin is a
polysaccharide involved in bud scar formation, and its synthesis
is restricted to a localised part of the wall and a specific
time in the cell cycle. The enzyme involved, chitin synthetase,
was found to be present in the cell membrane in an inactive
form. The most probable mechanism of activation of the enzyme
involves both spatial and temporal control by the fusion of
vesicles produced by the Golgi bodies with the cell membrane
at the appropriate time and place. These vesicles contain
a protease, and on fusion with the membrane the chitin synthetase
zymogen is cleaved, and thereby activated (reviewed in 114).
However, Elliott and McLaughlin have shown that periodic proteolysis
is not a general feature of the cell cycle in S. cerevisiae (115),
so chitin synthetase may be an exception.

There is now a great deal of evidence to show that the

vast majority of enzymes are composed of subunits, and that
physical interactions between these subunits are important
in the regulation of enzyme activity (reviewed in 116). Such
interactions could be extensively involved in generating periodic
changes in the activity of continuously synthesised enzymes,
and would go completely undetected by gel electrophoresis.
For example, acetyl-CoA carboxylase from a variety of mammalian
sources exists in an inactive form of molecular weight ca. 410 000.
The monomer is itself composed of subunits, only one of which
contains biotin. In the presence of phosphate, citrate,
isocitrate, malonate, high protein, or slightly acid pH, the
monomers polymerise into enzymically active filamentous structures.
Similarly, bovine glutamate dehydrogenase undergoes a con-
centration dependent polymerisation influenced by purine nucleo-
tides in the presence of the co-enzyme.

A further example of a protein-protein interaction affecting
enzyme activity is afforded by E. coli RNA polymerase (reviewed
in 117). The core enzyme is composed of four subunits: twoα,
one β,and oneβ'. The core enzyme will polymerise RNA using a
T4   DNA template, but the transcription is found to be
innaccurate. A further subunit, sigma factor which co-purifies
with the enzyme, is found to stimulate the rate of T4 transcript-
ion and confers accuracy of transcription of T4 and T7 DNA
templates upon the enzyme.

The step-wise doublings observed in the activation of some
enzymes could be produced by the periodic activation of a
continuously accumulated ,inactive form of the enzyme. This

mechanism closely parallels that already observed in chitin synthetase activation. Alternatively, a step could be produced by a rate change in the synthesis of an unstable enzyme. The concentration of the enzyme would change from one steady-state value to another, higher one (27).

In conclusion, periodic protein synthesis during the cell cycle would appear to be very rare. Despite this, a few enzymes may still show periodic changes in activity, whether as the result of a perturbation, or as genuine cell cycle events. Numerous mechanisms exist which could generate such changes in continuously synthesised enzymes, and a substantial proportion of these mechanisms would go undetected by gel electrophoresis.

Control of the Cell Cycle.

The rarity of periodic protein synthesis during the cell cycle suggests that dependent sequences of gene expression are also rare. This is confirmed by evidence from gel analysis of the proteins synthesised in mutants blocked in cell cycle progress. In this thesis, I have shown that the synthesis of up to 716 proteins in S. pombe is independent of cell cycle progress. Elliott used a similar approach, and found no evidence for dependent synthesis in S. cerevisiae (118). Howell et. al. showed that the patterns of polypeptide labelling remained unchanged when a temperature sensitive mutant of Chlamydomonas reinhardtii was shifted to the restrictive temperature, when it became blocked in division (104). Landy-Otsuka and Sheffler used two-dimensional gel electrophoresis to study the polypeptides synthesised in a ts cdc mutant of Chinese hamster fibroblasts that is blocked in G1 at the restrictive temperature (119). No differences that could be ascribed to a dependency upon cell cycle progress were found.

Dependent protein synthesis would therefore appear to be a very rare form of control, although once again the histones would seem to be an exception, and their synthesis has been reported to be dependent upon DNA synthesis (16,17). The unusual properties of histone synthesis suggest that it might be better regarded as a special case, rather than as a model system for a postulated general pattern of periodic or dependent synthesis.

Dependent sequences of periodic cell cycle events therefore remain to be explained. These events involve extensive

changes in the structures of the cell. Nuclear division

is the most obvious example of this, but DNA replication

requires the deposition of chromatin proteins which are synthesised

in the cytoplasm upon the DNA, and the incorporation of the new

chromatin into the nuclear structure. Dramatic changes in the

structure of Tetrahymena macronuclei can be brought about by

treating the cells with actinomycin when large nucleoli form

and accumulate around the inside surface of the nucleus.

However, Herlan et. al. found no difference in the polypeptide

composition of isolated normal and abnormal nuclei when compared

by one dimensional gel electrophoresis, suggesting that the

structural changes were predominantly organisational, rather

than due to changes in composition (120). By analogy, I would

suggest that the changes seen during the cell cycle are primarily

the result of changes in the organisation of existing components,

rather than the result of periodic synthesis of new ones.

The behaviour of the microtubules during the cell cycle

lends further credence to this hypothesis. The microtubules

are long, filamentous structures composed of tubulin protein

subunits, and are frequently found as components of biological

machines involved in motility, such as sperm flagella, and the

mitotic spindle (reviewed in 121). Immunofluorescent staining

of cells with tubulin antibodies has revealed that the cytoplasm

of interphase mammalian cells contains a network of microtubules,

which is thought to contribute, at least in part, to the maintain-

ance of cell shape (122). This network disappears during

mitosis, when the mitotic spindle is formed, and reappears

after mitosis, when the spindle disappears. There are therefore two alternating cycles of microtubule assembly and disassembly during the cell cycle, occuring at different locations within the cell. It seems unlikely that each cycle is due to periodic synthesis of specific tubulins (121). Tubulin synthesis has been shown to increase continuously during the cell cycle of mammalian cells, and to remain constant during oogenesis in sea urchins and Drosophila. In both systems, the mitotic spindles appear and disappear at times when the apparent pools of tubulin are constant. Moreover, spindle formation and function will proceed for about one hour when protein synthesis is inhibited by 80% with puromycin. This also tends to exclude a role for the synthesis of microtubule associated proteins (MAP's) in the initiation of spindle activity.

The most plausible explanation for the microtubule cycles is that the cytoplasmic and spindle microtubules are assembled from pre-existing pools of monomer, and that it is the control of assembly which is important, rather than the synthesis of particular proteins. This control could be exerted by low molecular weight effectors produced in the cytoplasm, and as such could be relatively independent of protein synthesis.

Calcium ion concentration has been implicated as a factor regulating tubule assembly in vivo (121). Recently, a protein has been identified in mouse 3T3 cells that exhibits a Ca^{2+} dependent inhibition of microtubule assembly in vitro (123). Antibodies raised against this protein, indicate that it is located in the cytoplasm during interphase, but at mitosis

antibodies are found at the spindle poles of cells. These
results suggest a protein mediated control of microtubule dis-
assembly by Ca^{2+} concentration. Other factors undoubtedly
contribute to the control of microtubules. For example, two
MAP's have been found to be efficient substrates for c AMP-
stimulated phosphorylation _in vivo_, and their function, what-
ever it might be, could be regulated by this post-translational
modification (121). One MAP has been found which initiates
tubulin assembly and increases tubule length stiochiometrically.
This protein is partitioned in the cytoplasm of interphase
cells, but in the spindle of dividing cells (124). There are
therefore several known possible mechanisms by which microtubule
assembly and disassembly could be controlled.

The location of the sites of assembly could be determined
by the position and orientation of microtubule organising
centres (MTOC's). These structures serve as sites from which
tubules are 'spun-out', and are themselves replicated periodically
during the cell cycle. In budding yeast the spindle plaque,
a MTOC, is replicated at the time of the 'start' function, and
has been implicated in growth control (125,126). These MTOC's
could be turned on at different times in the cycle, for example,
by the accumulation of a certain amount of some material.
This is reminiscent of the 'division-protein' model of Zeuthen,
formulated to account for division delay in Tetrahymena(127).
He suggested that a protein accumulated during the cycle,
which was assembled into a structure essential for division
and that this structure became temperature stable at some

point in the cycle. However, there is no evidence to identify the MTOC's with this hypothetical protein structure.

The microtubule cycles therefore represent organisational changes in existing components, leading to changes in their location and function. Primarily, these changes are probably brought about by post-translational controls rather than by controls at the level of gene expression. It is therefore conceivable that other cell cycle events, such as nuclear division, are also due to organisational changes controlled at the level of the cytoplasm. Control in this way would also allow a greater flexibility and speed of response to changing conditions, as a response to a change in nutrient, for instance, could occur directly, without the need for repression or derepression of synthesis .

The timing of these events could be controlled by varying levels of cytoplasmic factors. By analogy with the 'oscillatory repression' model of gene control, oscillations in the level of some material, for example cAMP, might activate a continuously synthesised control protein, which in turn causes a change in cellular structure, or iniates a periodic event. Dependent sequences of events could be generated by coupled oscillators, or by different events being initiated by different levels of some component. Of course, these mechanisms are not mutually exclusive. Also these mechanisms, and the results described here and elsewhere, do not exclude the possibility that ultimately the cell cycle is controlled by a few, rare, periodically synthesised dependent proteins. There is now a great deal of

evidence to suggest that development in Drosophila larvae is controlled by a program of genes which produce a 'binary code' that designates the structure a particular group of cells will form (reviewed in 128). Changing one gene can cause completely different structures to develop. A similar mechanism may be operating during the cell cycle, and the turning on or off of a single gene might initate, for example, nuclear division, or DNA synthesis. If these mechanisms are correct, then an entirely new field in cell cycle research is opened up. What are the control molecules? How are the structural changes brought about? Microtubule assembly could prove to be an important model system in answering these questions.

1. Mazia, D. (1963). Synthetic activities leading to mitosis.
    J. cell. comp. Physiol. 62, Suppl. 1 : 123-140.

2. Mitchison, J.M. (1971). The Biology of the Cell Cycle.
    Cambridge University Press.

3. Moser, G.C., Müller, H. & Robbins, E. (1975). Differential
    nuclear fluorescence during the cell cycle. Expl. Cell Res.
    91 : 73-78.

4. Hittelman, W.N. & Rao, P.N. (1976). Premature chromosome
    condensation. Expl. Cell Res. 100 : 219-222.

5. Scott, R.E., Carter, R.L. & Kidwell, W.R. (1971). Structural
    changes in membranes of synchronised cells demonstrated by
    freeze-cleavage. Nature, New Biology, Lond. 233 : 219-220.

6. Petelka, D. (1969). Centriole Replication. In: Handbook
    of Molecular Cytology, pp 1199-1218. Ed. by A. Lima-de-Faria.
    Amsterdam & London, North Holland Publishing Co..

7. Robbins, E., Jentzsch, G. & Micali, A. (1968). The centriole
    cycle in synchronised cells. J. Cell Biol. 36 : 329-339.

8. Williams, N.E. (1964). Structural development in synchronously
    dividing Tetrahymena pyriformis. In: Synchrony in Cell
    Division and Growth, pp 159-175. Ed. by E. Zeuthen.
    Interscience Publishers.

9. Prescott, D.M. (1976). Reproduction of Eukaryotic Cells.
    New York & London, Academic Press.

10. Swift, H.H. (1950). The desoxyribose nucleic acid content
    of animal nuclei. Physiol. Zoöl. 23 : 169-198.

11. Howard, A. & Pelc, S.R. (1953). Synthesis of desoxy-
ribonucleic acid in normal and irradiated cells and its
relation to chromosome breakage. Heredity, Lond. Suppl.
6 : 261-273.

12. Bostock, C.J. & Sumner, A.T. (1978). The Eukaryotic
Chromosome. Amsterdam & London, North Holland Publishing Co..

13. Elgin, S.C.R. & Weintraub, H. (1975). Chromosomal Proteins
and Chromatin Structure. A. Rev. Biochem. 44 : 725-774.

14. Prescott, D.M. (1966). The synthesis of total macromolecular
protein, histone, and DNA during the cell cycle in Euplotes
eurystomus. J. Cell Biol. 31 : 1-9.

15. Robbins, E. & Borun, T.W. (1967). The cytoplasmic synthesis
of histones in HeLa cells, and its temporal relationship
to DNA replication. Proc. natn. Acad. Sci. U.S.A. 57 : 409-416.

16. Moll, R. & Wintersberger, E. (1976). Synthesis of yeast
histones in the cell cycle. Proc. natn. Acad. Sci. U.S.A.
73 : 1863-1867.

17. Borun, T.W., Scharff, M.D. & Robbins, E. (1968). Rapidly
labelled, polysome associated RNA having the properties of
histone message. Proc. natn. Acad. Sci. U.S.A. 58 : 1977-1983.

18. Wilkes, R.R., Birnie, G.D. & Old, R.W. (1978). Histone gene
expression during the cell cycle studied by in situ
hybridisation. Expl. Cell Res. 115 : 441-444.

19. Bradbury, E.M., Inglis, R.J. & Matthews, H.R. (1974). Control
of cell division by very lysine rich histone F1 phosphorylation.
Nature, Lond. 247 : 257-261.

20. Isenberg, I. (1979). Histones. A. Rev. Biochem. In press.

21. Salas, J. & Green, H. (1971). Proteins binding to DNA and their relation to growth in cultured mammalian cells. Nature, New Biology, Lond. 229 : 165-169.

22. Stein, G.S. & Borun, T.W. (1972). The synthesis of acidic chromosomal protein during the cell cycle of HeLa S-3 cells. I. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. J. Cell Biol. 52 : 292-307.

23. Buell, D.N. & Fakey, J.L. (1969). Limited periods of gene expression in immunoglobulin synthesising cells. Science, N.Y. 164 : 1524-1525.

24. Byars, N. & Kidson, C. (1970). Programmed synthesis and export of immunoglobulin by synchronised myeloma cells. Nature, Lond. 226 : 648-650.

25. Liberti, P. & Baglioni, C. (1973). Synthesis of immunoglobulin and nuclear protein in synchronised mouse myeloma cells. J. cell. Physiol. 82 : 113-120.

26. Cabib, E. & Farkas, V. (1971). The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. Proc. natn. Acad. Sci. U.S.A. 68 : 2052-2056.

27. Donachie, W.D. (1965). Control of enzyme steps during the bacterial cell cycle. Nature, Lond. 205 : 1084-1086.

28. Molloy, G.R. & Schmidt, R.R. (1970). Studies on the regulation of ribulose-1,5-diphosphate carboxylase during the cell cycle of the eukaryote Chlorella. Biochem. biophys. Res. Commun. 40 : 1125-1133.

29. Littlefield, J.W. (1966). The periodic synthesis of thymidine kinase in mouse fibroblasts. Biochim. biophys. Acta. 114 : 398–403.

30. Mittermeyer, C., Bosselmann, R. & Bremerskov, V. (1968). Initiation of DNA synthesis in a system of synchronised L-cells: rhythmicity of thymidine kinase activity. Europ. J. Biochem. 4 : 487–489.

31. Mitchison, J.M. (1977). Enzyme synthesis during the cell cycle. In: Cell Differentiation in Micro-organisms, Plants and Animals, pp 377–401. Eds. L. Nover & K. Mothes. VEB Gustav Fischer Verlag.

32. Folkes, B.F., Bishop, R., Box, V., Hinde, R.W., Sims, A.P. & Walls, D. Quoted in reference 31.

33. Sebastian, J., Carter, B.L.A. & Halvorson, H.O. (1971). Use of yeast populations fractionated by zonal centrifugation to study the cell cycle. J. Bact. 108 : 1045–1050.

34. Carter, B.L.A. & Dawes, I.W. (1975). Synthesis of two DNA-dependent RNA polymerases in yeast. Expl. Cell Res. 92 : 253–258.

35. John, P.C.L., McCullough, W., Atkinson Jr., A.W., Forde, B.G. & Gunning, B.E.S. (1973). The cell cycle in Chlorella. In: The Cell Cycle in Development and Differentiation, pp 61–76. Eds. M. Balls & F.S. Billett. Cambridge University Press.

36. John, P.C.L.. Personal communication.

37. Mitchison, J.M. & Creanor, J. (1969). Linear synthesis of sucrase and phosphatases during the cell cycle of Schizosaccharomyces pombe. J. Cell Sci. 5 : 373-391.

38. Mitchison, J.M., Creanor, J. & Sartirana, M.L. (1973). Enzyme synthesis, RNA synthesis and the effects of Actinomycin D in protoplasts of the fission yeast Schizosaccharomyces pombe. In: Yeast, Mould and Plant Protoplasts, pp 229-245. Ed. by J.R. Villanueva, I. Garcia-Acha, S. Gascon and F. Uruburu. New York and London, Academic Press.

39. Molloy, G.R., Sitz, T.O. & Schmidt, R.R. (1973). Evidence for continuous availability of the phosphoribosylglycinamide synthetase structural gene for transcription during the cell cycle of the eukaryote Chlorella. J. biol. Chem. 248 : 1970-1975.

40. Mitchison, J.M. (1963). Pattern of synthesis of RNA and other cell components during the cell cycle of Schizosaccharomyce pombe. J. cell. comp. Physiol. 62, Suppl. 1 : 1-13.

41. Fraser, R.S.S. & Moreno, F. (1976). Rate of synthesis of poly A mRNA and rRNA during the cell cycle of Schizosaccharomyces pombe. J. Cell Sci. 21 : 497-521.

42. Creanor, J. (1978). Carbon dioxide evolution during the cell cycle of the fission yeast Schizosaccharomyces pombe. J. Cell Sci. 33 : 385-397.

43. Elliott, S.G. & McLauglin, C.S. (1978). Rate of macromolecular synthesis through the cell cycle of the yeast Saccharomyces cerevisiae. Proc. natn. Acad. Sci. U.S.A. 75 : 4384-4388.

44. Masters, M. & Donachie, W.D. (1966). -Repression and the control of cyclic enzyme synthesis. Nature, Lond. 209 : 476-479.

45. Donachie, W.D. & Masters, M. (1969). Temporal control of gene expression in bacteria. In: The Cell Cycle: Gene-Enzyme Interactions, pp 37-76. Ed. by G.M. Padilla, G.L. Whitson & I.L. Cameron. New York and London, Academic Press.

46. Goodwin, B.C. (1966). An entrainment model for the timing of enzyme synthesis in bacteria. Nature, Lond. 209 : 479-481.

47. Halvorson, H.O., Boch, R.M., Tauro, P., Epstein, R. & La Berge, M. (1966). Periodic enzyme synthesis in synchronous cultures of yeast. In: Cell Synchrony, pp 102-116. Ed. by I.L. Cameron & G.M. Padilla. New York & London, Academic Press.

48. Carter, B.L.A., Sebastian, J. & Halvorson, H.O. (1971). The regulation of synthesis of arginine catabolising enzymes during the cell cycle in Saccharomyces cerevisiae. Adv. Enzyme Regulation, 9 : 253-263.

49. Walter, C. (1969). Oscillations in controlled biochemical systems. Biophys. J. 9 : 863-872.

50. Halvorson, H.O., Carter, B.L.A. & Tauro, P. (1971). Synthesis of enzymes during the cell cycle. Adv. micro. Physiol. 6 : 47-106.

51. Gorman, J. Tauro, P., La Berge, M. & Halvorson, H.O. (1964). Timing of enzyme synthesis during synchronous division in yeast. Biochem. biophys. Res. Comm. 15 : 43-49.

52. Tauro, P. & Halvorson, H.O. (1966). Effect of gene position on the timing of enzyme synthesis in synchronous cultures of yeast. J. Bact. 92 : 652-661.

53. Tauro, P., Halvorson, H.O. & Epstein, R.L. (1968).
    Time of gene expression in relation to centromere distance
    during the cell cycle of Saccharomyces cerevisiae.
    Proc. natn. Acad. Sci. U.S.A. 59 : 277-284.

54. Masters, M. & Pardee, A.B. (1965). Sequence of enzyme synthesis
    and gene replication during the cell cycle of Bacillus subtilis.
    Proc. natn. Acad. Sci. U.S.A. 54 : 64-70.

55. Kennett, R.A. & Sueoka, N. (1971). Gene expression during
    outgrowth of Bacillus subtilis spores. The relationship between
    gene order on the chromosome and temporal sequence of enzyme
    synthesis. J. molec. Biol. 60 : 31-44.

56. Benitez, T., Nurse, P. & Mitchison, J.M. (1979). Enzyme
    potential in the fission yeast Schizosaccharomyces pombe.
    In press.

57. Carter, B.L.A., Sebastian, J. & Halvorson, H.O. (1971).
    Regulation of enzyme synthesis in synchronous cultures of
    yeast. Bact. Proc. 71 : 156.

58. Baechtel, F.S., Hopkins, H.A. & Schmidt, R.R. (1970).
    Continuous inducibility of isocitrate lyase [E.C. 4.1.3.1]
    during the cell cycle of the eukaryote Chlorella.
    Biochim. biophys. Acta 217 : 216-219.

59. Turner, K.J., Gronostajski, R.M. & Schmidt, R.R. (1978).
    Regulation of initial rate of nicotinamide adenine dinucleotide
    phosphate-specific glutamate dehydrogenase during the cell
    cycle of synchronous Chlorella. J. Bact. 134 : 1013-1019.

60. Mitchison, J.M. (1974). Sequences, pathways and timers in
    the cell cycle. In: Cell Cycle Controls, pp 125-142. Ed. by
    G.M. Padilla, I.L. Cameron and A. Zimmermann. New York &
    London, Academic Press.

61. Rueckert, R.R. & Mueller, G.C. (1960). Studies on unbalanced
     growth in tissue culture. I. Induction and consequences of
     thymidine deficiency. Cancer Res. 20 : 1584-1591.

62. Kim, J.H., Kim, S.H. & Eidinoff, M.L. (1965). Cell viability
     and nucleic acid metabolism after exposure of HeLa cells to
     excess thymidine and deoxyadenosine. Biochem. Pharmac.
     14 : 1821-1829.

63. Studzinski, G.P. & Lambert, W.C. (1969). Thymidine as a
     synchronising agent. I. Nucleic acid and protein formation in
     synchronous HeLa cultures treated with excess thymidine.
     J. cell. Physiol. 73 : 109-118.

64. Mitchison, J.M. & Creanor, J. (1971). Induction synchrony in
     the fission yeast Schizosaccharomyces pombe. Expl. Cell Res.
     69 : 368-374.

65. Rosenberg, H.M. & Gregg, E.C. (1969). Kinetics of cell volume
     changes of murine lymphoma cells subjected to different agents.
     Biophys. J. 9 : 592-606.

66. Andersen, H.A., Rasmussen, L. & Zeuthen, E. (1975). Cell
     division and DNA replication in synchronous Tetrahymena
     cultures. In: Current Topics in Microbiology and Immunology,
     72 : 1-20.

67. Mitchison, J.M. (1977). The timing of cell cycle events.
     In: Mitosis: Facts and Questions, pp 1-19. Ed. by M. Little,
     N. Poweletz, C. Petzelt, H. Ponstingl, D. Schroeter & H.-P
     Zimmermann. Berlin and Heidelberg, Springer-Verlag.

68. Hartwell, L.H. (1978). Cell division from a genetic
     perspective. J. Cell Biol. 77 : 627-637.

69. Slater, M.L. & Schaechter, M. (1974). Control of cell division
    in bacteria. Bact. Rev. 38 : 199-221.

70. Hartwell, L.H. (1974). Saccharomyces cerevisiae: cell cycle.
    Bact. Rev. 38 : 164-198.

71. Nurse, P., Thuriaux, P. & Nasmyth, K. (1976). Genetic control
    of cell division in the fission yeast Schizosaccharomyces
    pombe. Molec. gen. Genet. 146 : 167-178.

72. Howell, S.H. (1974). An analysis of cell cycle controls in
    temperature sensitive mutants of Chlamydomonas reinhardtii.
    In: Cell Cycle Controls, Ed. by G.M. Padilla, I.L. Cameron
    & A. Zimmermann. New York, Academic Press.

73. Frankel, J., Jenkins, L.M., Doerder, F.P. & Nelsen, E.M. (1976).
    Mutations affecting cell division in Tetrahymena pyriformis.
    I. Genetic analysis. Genetics 83 : 489-506.

74. Roscoe, D.H., Robinson, H. & Carvwell, A.W. (1973). DNA
    synthesis and mitosis in a temperature sensitive Chinese
    hamster cell line. J. cell. Physiol. 82 : 333-338.

75. Nasmyth, K.A. (1977). Temperature-sensitive lethal mutants in
    the structural gene for DNA ligase in the yeast Schizosaccharomyce
    pombe. Cell 12 : 1109-1120.

76. Fantes, P.A. (1977). Control of cell size and cycle time in
    Schizosaccharomyces pombe. J. Cell Sci. 24 : 51-67.

77. Fantes, P. & Nurse, P. (1977). Control of cell size at division
    in fission yeast by a growth-modulated size control over nuclear
    division. Expl. Cell Res. 107 : 377-386.

78. Nurse, P. (1975). Genetic control of cell size at cell
    division in yeast. Nature, Lond. 256 : 547-551.

79. Thuriaux, P., Nurse, P. & Carter, B. (1978). Mutants altered
     in the control co-ordinating cell division with cell growth
     in the fission yeast Schizosaccharomyces pombe.
     Molec. gen. Genet. 161 : 215-220.

80. Laemmli, U.K. (1970). Cleavage of structural proteins during
     tha assembly of the head of bacteriophage T4. Nature, Lond.
     227 : 680-685.

81. O'Farrell, P.H. (1975). High resolution two-dimensional
     electrophoresis of proteins. J. biol. Chem. 250 : 4007-4021.

82. O'Farrell, P.Z., Goodman, H.M. & O'Farrell, P.H. (1977).
     High resolution two-dimensional electrophoresis of basic as
     well as acidic proteins. Cell 12 : 1133-1142.

83. Creanor, J. & Mitchison, J.M. (1979). Reduction of perturbations
     in leucine incorporation in synchronous cultures of
     Shizosaccharomyces pombe made by elutriation. (1979).
     J. Gen. Microbiol. 112 : 385-388.

84. Mitchison, J.M. (1970). Physiological and cytological methods
     for Schizosaccharomyces pombe. In: Methods in Cell Physiology,
     vol IV, pp 131-165. Ed. by D.M. Prescott. New York,
     Academic Press.

85. Gutz, H., Heslot, H., Leupold, U. & Loprieno, N. (1974).
     Schizosaccharomyces pombe. In: Handbook of Genetics, 1
     pp 395-446. Ed. by R.C. King. New York, Plenum Press.

86. Ames, G.F.-L. & Nikaido, K. (1976). Two-dimensional gel
     electrophoresis of membrane proteins. Biochemistry 15 :
     616-623.

87. Pringle, J.R. (1975). Methods for avoiding proteolytic
     artefacts in studies of enzymes and other proteins from
     yeast. In: Methods in Cell Biology, 12 : 149-180.
     Ed. by D.M. Prescott. New York & London, Academic Press.

88. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J.
     (1951). Protein measurement with the folin phenol
     reagent. J. biol. Chem. 193 : 265-275.

89. Coddington, A. & Fluri, R. (1977). Characterisation of
     the ribosomal proteins from Schizosaccharomyces pombe by
     two-dimensional polyacrylamide gel electrophoresis.
     Molec. gen. Genet. 158 : 93-100.

90. Loening, U.E. (1969). The determination of the molecular
     weight of ribonucleic acid by polyacrylamide gel electro-
     phoresis. The effects of changes in conformation.
     Biochem. J. 113 : 131-138.

91. Mitchison, J.M. & Carter, B.L.A. (1975). Cell cycle
     analysis. In: Methods in Cell Biology, 11 : 201-219.
     Ed. by D.M. Prescott. New York & London, Academic Press.

92. Creanor, J. Personal communication.

93. Elliott, S.G. Personal communication.

94. Stebbing, N. (1971). Growth and change in pool and
     macromolecular components of Schizosaccharomyces pombe
     during the cell cycle. J. Cell Sci. 9 : 701-717.

95. Mitchison, J.M. & Wilbur, K.M. (1962). The incorporation
     of protein and carbohydrate precursors during the cell cycle
     of fission yeast. Expl. Cell Res. 16 : 49-58.

96. Nurse, P. Personal communication.

97. Milman, G., Lee, E., Gangas, G.S. & McLauglin, J.R. (1976). Analysis of HeLa cell hypoxanthine phosphoribosyl transferase mutants and revertants by two-dimensional gel electrophoresis: evidence for silent gene activation. Proc. natl. Acad. Sci. U.S.A. 73 : 4589-4593.

98. Lutkenhaus, J.F., Moore, B.A., Masters, M. & Donachie, W.D. (1979). In press.

99. Hahn, W.E., Pettijohn, D.E. & Van Ness, J. (1977). One strand equivalent of the Escherichia coli genome is transcribed: complexity and abundance class of mRNA. Science N.Y. 197 : 582-585.

100. Wain, B. (1971). Synthesis of soluble protein during the cell cycle of the fission yeast Schizosaccharomyces pombe. Expl. Cell Res. 69 : 49-56.

101. Elliott, S.G., Warner, J.R. & McLaughlin, C.S. (1979). The synthesis of ribosomal proteins during the cell cycle of the yeast Saccharomyces cerevisiae. In press.

102. Hereford, L.M. & Rosbach, M. (1977). Number and distribution of polyadenylated RNA sequences in yeast. Cell 10 : 453-462.

103. Ernst, G. Quoted in Sauer, H.W. (1978). Regulation of gene expression in the cell cycle of Physarum. In: Cell Cycle Regulation, pp 149-166. New York & London. Academic Press.

104. Howell, S.H., Posakony, J.W. & Hill, K.R. (1977). The cell cycle program of polypeptide labelling in Clamydomonas reinhardtii. J. Cell Biol. 72 : 223-241.

105. Milcarek, C. & Zahn, K. (1978). The synthesis of ninety proteins, including actin through the HeLa cell cycle. J. Cell Biol. 79 : 833-838.

106. Al-Bader, A.A., Orengo, A. & Rao, P.N. (1978). G2 phase specific proteins of HeLa cells. Proc. natl. Acad. Sci. U.S.A. 75 : 6064-6068.

107. Cantoni, G.L. (1975). Biological methylation: selected aspects. In: A. Rev. Biochem. 44 : 435-451.

108. Holtzer, H. & Duntze, W. (1971). Metabolic regulation by chemical modifications of enzymes. In: A. Rev. Biochem. 40 : 345-374.

109. Rubin, C.S. & Rosen, O.M. (1975). Protein phosphorylation. In: A. Rev. Biochem. 44 : 831-887.

110. Ulane, R.E. & Cabib, E. (1976). The activating system of chitin synthetase from Saccharomyces cerevisiae: purification and properties of the activating factor. J. biol. Chem. 251 : 3367-3374.

111. Golombek, J., Wolf, W. & Wintersberger, E. (1974). DNA synthesis and DNA polymerase activity in synchronised yeast cells. Molec. gen. Genet. 132 : 137-145.

112. Uy, R. & Wold, F. (1977). Post-translational covalent modification of proteins. Science N.Y. 198 : 890-896.

113. Wolosiuk, R.A. & Buchanan, B.B. (1977). Thioredoxin and glutathione regulate photosynthesis in chloroplasts. Nature Lond. 266 : 565-567.

114. Cabib, E., Ulane, R. & Bowers, B. (1974). A molecular model for morphogenesis. In: <u>Current Topics in Cell Regulation</u>, <u>8</u> : 1-32. Ed. by B.L. Hoecker & E.R. Staalman. New York & London, Academic Press.

115. Elliott, S.G. & McLauglin, C.S. (1979). Synthesis and modification of proteins during the cell cycle of the yeast <u>Saccharomyces cerevisiae</u>. <u>J. Bact.</u> <u>137</u> : 1185-1190.

116. Frieden, C. (1971). Protein-protein interaction and enzyme activity. In: <u>A. Rev. Biochem.</u> <u>40</u> : 653-696.

117. Burgess, R.R. (1971). RNA polymerase. <u>A. Rev. Biochem.</u> <u>40</u> : 711-740.

118. Elliott, S.G. Personal communication.

119. Landy-Otsuka, F. & Scheffler, I.E. (1978). Induction of ornithine decarboxylase activity in a temperature sensitive cell cycle mutant of Chinese hamster cells. <u>Proc. natl. Acad. Sci. U.S.A.</u> <u>75</u> : 5001-5005.

120. Herlan, G., Quevado, R. & Wunderlich, F. (1978). Structural transformation of the nuclear matrix <u>in situ</u>. <u>Expl. Cell Res.</u> <u>115</u> : 103-110.

121. Snyder, J.A. & McIntosh, J.R. (1976). Biochemistry and physiology of microtubules. <u>A. Rev. Biochem.</u> <u>45</u> : 699-720.

122. Brinkley, B.R., Fuller, G.M. & Highfield, D.P. (1975). Cytoplasmic microtubules in normal and transformed cells in culture: analysis by tubulin antibody immunofluorescence. <u>Proc. natl. Acad. Sci. U.S.A.</u> <u>72</u> : 4981-4985.

123. Marcum, J.M., Dedman, J.R., Brinkley, B.R. & Mecus, A.R. (1978). Control of microtubule assembly-disassembly by calcium dependent regulator protein. Proc. natl. Acad. Sci. U.S.A. 75 : 3771-3775.

124. Lockwood, A.H. (1978). Tubulin assembly protein: immunochemical and immunofluorescent studies on its formation and distribution in microtubules and cultured cells. Cell 13 : 613-627.

125. Byers, B. & Goetsch, L. (1975). Behaviour of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J. Bact. 124 : 511-523.

126. Hyams, B. & Bonsy, G.G. (1978). Nucleation of micro-tubules in vitro by isolated spindle pole bodies of the yeast Saccharomyces cerevisiae. J. Cell Biol. 78 : 401-414.

127. Zeuthen, E. & Williams, N.E. (1969). Division-limiting morphogenetic processes in Tetrahymena. In: Nucleic Acid Metabolism, Cell Differentiation and Cancer Growth, pp 203-216. Ed. by E.V. Cowdry & S. Seno. Oxford, Pergammon Press.

128. Lawrence, P.A. (1976). Insect development. Symposia of the Royal Entomological Society of London, 8.

Acknowledgements

I would like to express my thanks to all those who have advised, helped and supported me during the three years I have worked on this thesis.

Firstly, my supervisor, Professor J.M. Mitchison, who first introduced me to much of the cell cycle, and whose advice (and occasional gentle prodding!) has been invaluable.

Doctors J. Creanor, P. Fantes, and P. Nurse have been a constant source of information (and chemicals and glassware) at all stages of this work, and their help has been of inestimable value.

I have at some stage sought advice from almost everyone in the Department of Zoology, and I would like to extend a collective 'Thank-you' to 'The Department', not only for the help I have always been freely given, but for making my time here so pleasant.

My thanks must go to Ms. M. Downie for her excellent typing in the face of insurmountable odds (my writing).

Finally, my thanks must go to Miss J. Barwick, who has provided me with food, transport, and moral support, all of which are warmly appreciated.