The role of cyclic AMP in the growth and proliferation of Saccharomyces cerevisiae

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The role of cyclic AMP in the growth and proliferation of *Saccharomyces cerevisiae*

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SUMMARY

The role of cyclic AMP (cAMP) in the control of growth and proliferation was investigated in *Saccharomyces cerevisiae*. After modification of an assay method for cAMP, it was found that the concentration of intracellular cAMP (i-cAMP) decreased throughout the growth of an asynchronous culture, and that the concentration of i-cAMP was in excess during exponential phase. It was also found that the concentration of i-cAMP was similar in cells that had been grown on rich, minimal, fermentable or non-fermentable carbon sources. Centrifugal elutriation experiments showed that there were no cell cycle-specific fluctuations in the i-cAMP concentration, but fluctuations were observed. The presence of extracellular e-cAMP (e-cAMP) was also observed, although it was subsequently found that the assay method used was not suitable. HPLC analysis of the growth medium was not conclusive either as a peak that eluted close to where cAMP eluted was found not to be cAMP. It is concluded form this work that feedback inhibition of cAMP synthesis is not operable during exponential growth of *S. cerevisiae*, i-cAMP is not an indicator of catabolite repression and the concentration of i-cAMP does not fluctuate during the cell cycle.

LIST OF ABBREVIATIONS

A	absorbance
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine triphosphate
cAMP	cyclic AMP
cdc	cell division cycle
cCMP	cytosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
cUMP	uridine 3',5'-cyclic monophosphate
cTMP	thymidine 3',5'-cyclic monophosphate
c.p.m.	counts per minute
cyclic AMP	adenosine 3',5'-cyclic monophosphate
DAG	diacylglycerol
DMG	dimethyl glutarate
e-cAMP	extracellular cAMP
EDTA	ethylenediaminetetraacetic acid
FM	fructose minimal
fmol	femtomoles (x 10^{-15} moles)
GDP	guanosine diphosphate
GlyM	glycerol minimal
GM	glucose minimal
GMP	guanosine monophosphate
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IBMX	3-butyl-1-methylxanthine
i-cAMP	intracellular cAMP
IP_3	inositol-1,4,5-trisphosphate

kDa	kilodaltons
M	minimal (medium)
mM	millimolar (x 10^{-3} M)
mRNA	messenger RNA
n.d	not determined
nM	nanomolar (x 10^{-9} M)
nm	nanometres (x 10^{-9} m)
nmol	nanomoles (x 10^{-9} moles)
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP	phosphatidylinositol 4-phosphate
P_i	inorganic phosphate
PKA	protein kinase A
PM	picomolar (x 10^{-12} M)
pmol	picomoles (x 10^{-12} moles)
PMSF	phenylmethylsulfonyl fluoride
protein kinase A	cAMP-dependent protein kinase
r.p.m	revolutions per minute
TCA	trichloroacetic acid
ts	temperature sensitive
YEP	yeast extract peptone
YEPD	yeast extract peptone dextrose (glucose)
YEPF	yeast extract peptone fructose
YEPG	yeast extract peptone glycerol
YEPP	yeast extract peptone pyruvate

LIST OF MUTANTS OF THE cAMP PATHWAY.

Gene	Function of encoded protein
BCY1	Regulatory subunit of cAMP-dependent protein kinase
CAP	Stabilizing protein of adenylyl cyclase
CDC25	GTP-GDP exchange factor for Ras
CDC35	Structural gene for adenylyl cyclase
CYR1	Structural gene for adenylyl cyclase
CYR2	Allelic to *CDC25*, thought to encode catalytic subunit of the cAMP-dependent protein kinase
DIS2S1	Type 2A phosphoprotein phosphatase
IRA1	GTPase activating protein (of Ras2)
IRA2	GTPase activating protein (of Ras1)
PDE1	Phosphodiesterase, high K_m isoenzyme.
PDE2	Phosphodiesterase, low K_m isoenzyme.
PPD1	Allelic to *IRA1*, thought to encode phosphoprotein phosphatase.
PPH1	Type 2A phosphoprotein phosphatases
PPH21	Type 2A phosphoprotein phosphatase
PPH22	Type 2A phosphoprotein phosphatase

PPH3	Type 2A phosphoprotein phosphatase
RAS1	G protein that activates adenylyl cyclase. *RAS1* mRNA is preferentially expressed on non-fermentable carbon sources.
RAS2	G protein that activates adenylyl cyclase. *RAS2* mRNA is produced in larger amounts when cells are grown on glucose.
SIT4	Type 2A phosphoprotein phosphatases, allelic to *PPH1*
SRA1	Cyclic AMP-dependent protein kinase
SRA5	Phosphodiesterase, low K_m isoenzyme.
SRV2	Stabilizing protein of adenylyl cyclase
TPK1	cAMP-dependent protein kinase catalytic subunit
TPK2	cAMP-dependent protein kinase catalytic subunit
TPK3	cAMP-dependent protein kinase catalytic subunit

CHAPTER 1.

INTRODUCTION.

1.1. Cyclic nucleotides.

Cyclic nucleotides are reportedly ubiquitous. There are many pathways in which cyclic nucleotides are reported to be involved: these include the coordination of cellular growth and proliferation. Only two of the cyclic nucleotides, adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) have been of significant interest; however, their precise roles and the way in which they achieve them are still uncertain. Cytosine 3',5'-cyclic monophosphate (cCMP) has also been detected, along with its cyclase and phosphodiesterase (see Kuo *et al.*, 1978), and although there is only one report available, it is also involved in proliferation.

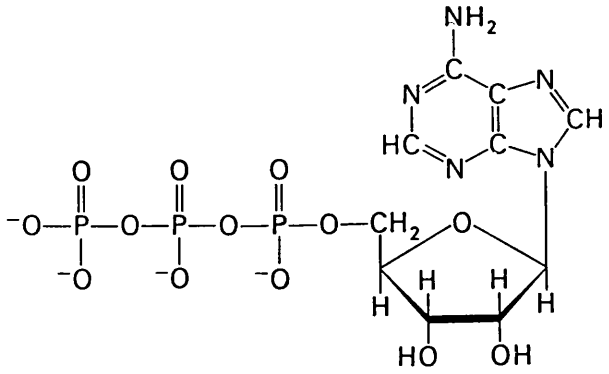
Cyclic AMP is composed of a purine ring linked to a ribose sugar moiety, which is itself attached to a phosphate group by 3'-5' bonds (Figure 1). It is synthesized from adenosine triphosphate (ATP) by adenylyl cyclase, which is usually situated in the plasma membrane, and is degraded to 5'-AMP by a cyclic nucleotide-dependent phosphodiesterase. Cyclic AMP exerts its effects through a cAMP-dependent protein kinase.

1.2. The role of cAMP.

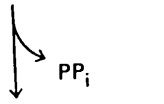
1.2.1. Intracellular cAMP.

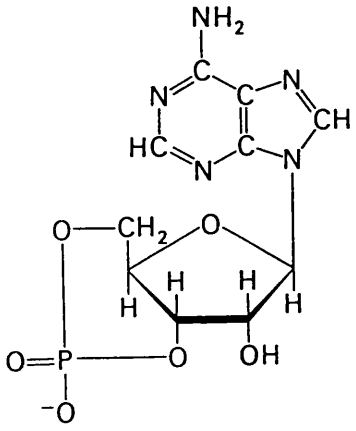
Despite the reported ubiquity of intracellular cAMP (i-cAMP), it would be incorrect to say that the cAMP regulatory system has been entirely conserved throughout evolution (Pall, 1981; Gancedo *et al.*, 1985). First, there is an apparent fundamental difference in the role of i-cAMP between prokaryotes and eukaryotes and between members of each category. Secondly, in prokaryotes the intracellular receptor for cAMP is a transcription regulation protein and in eukaryotes it is the cAMP-dependent protein kinase (PKA). Prokaryotes tend

Figure 1. The enzyme-catalysed synthesis and degradation of cAMP. Cyclic AMP is synthesized from ATP *via* adenylyl cyclase and causes the release of pyrophosphate. Cyclic AMP is degraded to 5'-AMP by a hydrolysis reaction *via* phosphodiesterase.

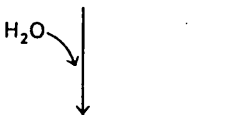


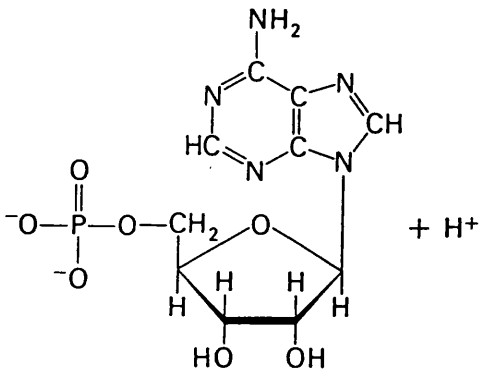
ATP





Cyclic AMP





AMP

to be affected by glucose availability and membrane depolarization; in lower eukaryotes, i-cAMP concentrations reflect glucose availability, membrane depolarization and stimulation of membrane receptors, as in higher eukaryotes; both higher and lower eukaryotes have a PKA that affects processes such as transcription, cell proliferation, meiosis, translation, membrane permeability, degradation of reserves, hormone secretion and muscular relaxation.

Much of the attention drawn to prokaryotes concerning the role of cAMP has been towards catabolite repression in *Escherichia coli*. Catabolite repression is the process whereby a cell will preferably metabolize one sugar, for example glucose, rather than another, such as lactose. Cyclic AMP and a cAMP receptor protein (CRP) are required to interact for induction of carbon catabolite operons such as *lac*, *gal*, *trp* and *ara*, which are required for growth on alternative carbon sources. When *E. coli* is growing on a medium containing glucose, the synthesis of cAMP and the transcription of the above operons are suppressed. The activity of adenylyl cyclase is high if the components of the sugar transport pathway are phosphorylated, and this is the case in the absence of transportable sugars such as glucose and fructose. In *E. coli*, the presence of cAMP is indicative of a low energy status. In fact, cAMP and CRP are also required for induction of carbon starvation proteins (Schultz *et al.*, 1988).

Cyclic AMP in fungi is involved in processes related to growth, proliferation and multicellular development such as the utilization of exogenous and endogenous carbon sources, conidiation, dimorphism, phototropism, regulation of hierarchical hyphal growth, control of hyphal branching and spore germination (for references see Dumbrava and Pall, 1987; Robson *et al.*, 1991). Cyclic AMP was also the first identified second messenger in hormone action. With respect to normal or non-tumour cells, cAMP can stimulate, inhibit or have no effect on proliferation. The effect of cAMP on cell growth appears to depend upon the differentiation state of the cell (for review see Dumont *et al.*, 1989). Positive and negative regulatory elements that are affected by cAMP

have been found in the promoters of mammalian genes. It is believed that PKA or a protein that has been phosphorylated by PKA enters the nucleus and phosphorylates chromosomal proteins that regulate the transcription of genes.

There appears to be no paradigm for the role of cAMP in proliferation for both eukaryotes and prokaryotes. Despite this, and the two different modes of control of cAMP between them, there are reports of similarities. For example, the amino acid sequence of the regulatory subunit of PKA from bovine cardiac muscle and the CRP protein from *E. coli* show approximately 40% homology, with the cAMP-binding domain showing conservation. Also, in *E. coli*, slime moulds, fungi and mammals, adenylyl cyclase is membrane-bound and requires interaction of an effector with the membrane-bound adenylyl cyclase, and also may transmit the signal *via* a coupler (Peterkofsky, 1976).

From this brief account of the role of cAMP, it is clear that cAMP is required for essential programmes in the life cycle of a cell, enabling an organism to switch life style in order to exploit its environment or to coordinate with other cells to form a multicellular organism.

1.2.2. Extracellular cAMP.

The escape of cAMP from intact cells has been detected in cultures of both prokaryotic and eukaryotic cells (for references see Fehr *et al.*, 1990). Many reports suggest that cAMP is extruded from cells *via* an energy-dependent process. However, as yet, no carrier has been found; there is also no universal reason why cAMP should be extruded from any cell. The efflux of cAMP does not, however, have a consistent role in the regulation of i-cAMP concentrations; it may be part of a feedback loop. It has been reported that the amino acid sequence of adenylyl cyclase in bovine brain is topologically similar to several channels and transporters, possibly resulting in adenylyl cyclase transporting the cAMP out of the cell (Fehr *et al.*, 1990).

The role that extracellular cAMP (e-cAMP) plays in *Dictyostelium discoideum* is clearly understood to be an intercellular signal. On starvation of *D. discoideum*, a programme of development is initiated resulting in differentiation from the free-living amoeba form to the multicellular slug form, eventually resulting in dispersal of spores. Extracellular cAMP is an essential requirement for differentiation of *D. discoideum* and is released from a cell under these conditions. Cyclic AMP serves as a chemoattractant and regulates the expression of genes essential for development and differentiation (for review see Saxe *et al.*, 1988). One class of developmentally regulated genes require oscillations in e-cAMP and others require either oscillatory or constant levels of e-cAMP for induction (Kessin, 1988). *D. discoideum* also possesses an extracellular phosphodiesterase that degrades e-cAMP in order to control concentrations of e-cAMP and therefore control its development and differentiation.

Extracellular cAMP has been found in the growth medium of cultured fibroblasts and avian erythrocytes. The release of cAMP from avian erythrocytes was found to be a significant mechanism for control of i-cAMP concentrations; however, the release of cAMP from cultured fibroblasts was not (for references see Fehr *et al.*, 1990). There is efflux of cAMP from WI-38 fibroblasts also and the amount that is released from these cells is reported to be approximately 18% of cAMP turnover, therefore these cells also depend on e-cAMP as a significant factor in controlling the concentration of i-cAMP (Barber and Butcher, 1983). Cyclic AMP has also been found in the growth medium of pig aortic smooth muscle cells when inhibitors of phosphodiesterase activity were used, implying that extrusion of cAMP from these cells was a consequence of the i-cAMP concentration becoming too high due to lack of phosphodiesterase activity. It has also been reported that e-cAMP may function as an intercellular signal in mammalian cells (Boxer *et al.*, 1980). Other putative roles for e-cAMP may be its modification into another active compound

or it may be coupled with the counterflow of an ion. It has been suggested that e-cAMP is rapidly degraded in the medium into adenosine monophosphate (AMP), inosine, adenine and hypoxanthine. It has been reported that the function of e-cAMP in bacteria, such as *E. coli*, is a function of i-cAMP concentration (Epstein *et al.*, 1975), similar to mammalian cells.

In some filamentous fungi, basal levels of e-cAMP are required for normal mycelial development (Robson *et al.*, 1991) and increases in e-cAMP cause decreases in hyphal extension rates and increases in the degree of branching.

The loss of cAMP from any cell type to the medium would be extremely biologically inefficient if it was not re-utilized. Alternatively, it is thought that extrusion of cAMP may be a biological mistake that cannot be corrected, usually resulting from abnormalities and pathological conditions (Hamet *et al.*, 1984).

1.3. The life and cell division cycles of *S. cerevisiae*.

S. cerevisiae cells can exist in either haploid (*n* DNA) or diploid (2*n* DNA) forms (Figure 2). Haploid cells of *S. cerevisiae* are one of two mating types, **a** or α. Only an α and an **a** cell can mate to form an α/**a** diploid. Both haploids and diploids undergo budding and cell division, have the same mitotic cycle and can enter G_0 upon starvation; only diploids can undergo meiosis and sporulation. The purpose of the cell division cycle (cell cycle) is to produce two cells genetically identical to the mother cell. The cell cycle of *S. cerevisiae*, which is typical of eukaryotes, comprises four major phases G_1, S, G_2 and M (Figure 3). G_1, initially just thought to be a preparation step for DNA synthesis, contains a major regulatory step in the cell cycle of *S. cerevisiae*; in S phase the entire DNA content of the cell is replicated; G_2 is the phase in which chromosomes are prepared for separation; separation of chromosomes, or nuclear division, occurs in M phase; M phase is followed by cell separation.

Figure 2. The life and cell division cycles of *S. cerevisiae*. Reproduced from Russell (1986).

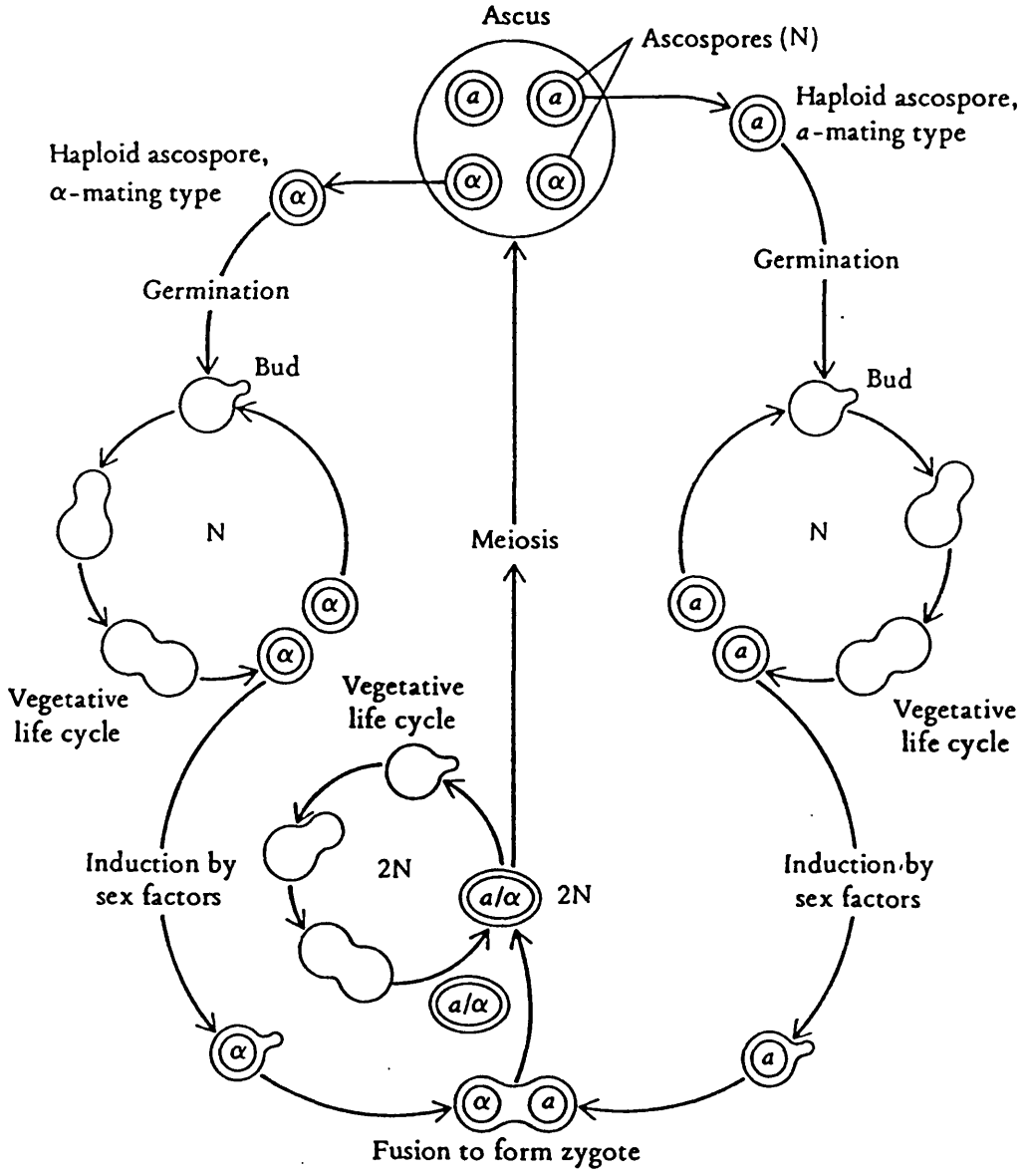
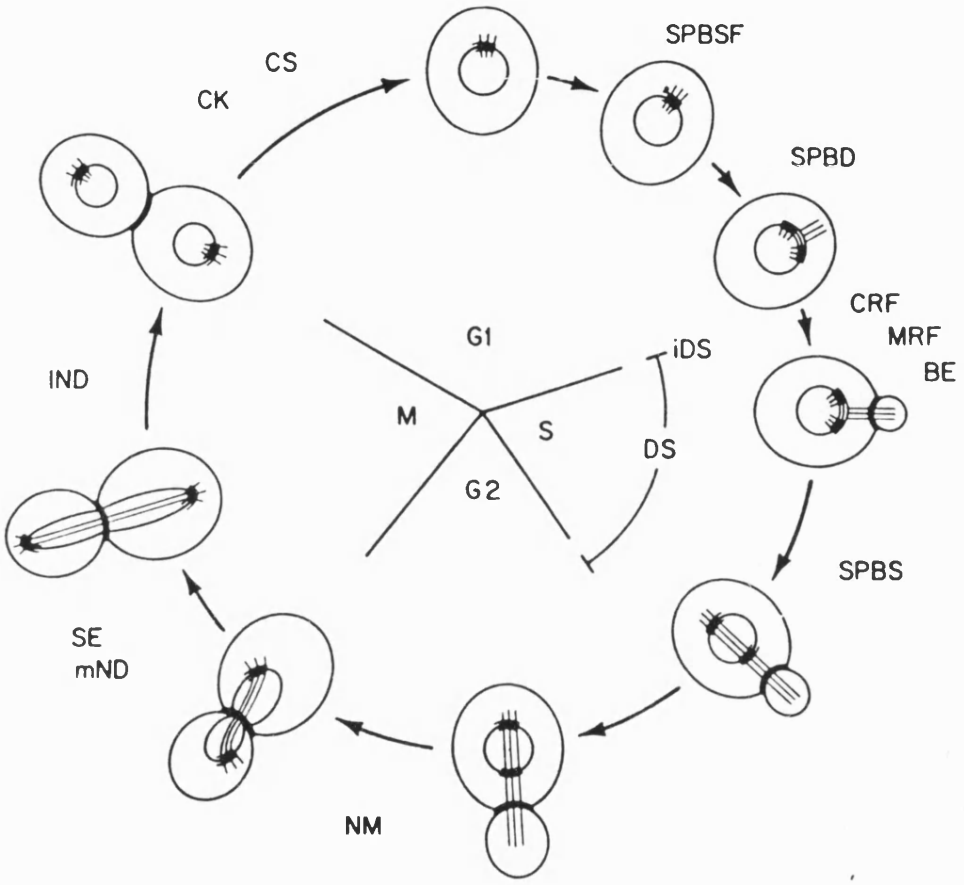

Ascus
Ascospores (N)
Haploid ascospore, a-mating type
Haploid ascospore, α-mating type
Germination
Germination
Bud
Bud
N
N
Meiosis
Vegetative life cycle
Vegetative life cycle
Vegetative life cycle
Induction by sex factors
Induction by sex factors
2N
2N
a/α
a/α
Fusion to form zygote

Figure 3. Major landmark events of the *S. cerevisiae* cell cycle. Abbreviations: SPBSF, spindle pole body satallite formation; SPBD, spindle pole body duplication; CRF, chitin ring formation (shown as a heavy line between the mother and daughter cells); MRF, microfilament ring formation; BE, bud emergence; iDS, initiation of chromosomal DNA synthesis; DS, chromosomal DNA synthesis; SPBS, spindle pole body separation; NM, nuclear migration; mND, medial stage of nuclear migration; SE, spindle elongation; lND, late stage of nuclear migration; CK, cytokinesis; CS, cell separation. Reproduced from Pringle and Hartwell (1981).


SPBSF
CS
CK
SPBD
CRF
MRF
BE
G1
iDS
M
S
DS
G2
IND
SPBS
SE
mND
NM

So far, over one hundred mutations have been isolated that affect the cell cycle. The largest class of cell cycle mutants are known as cell division cycle (*cdc*) mutants and these have a stage-specific defect in the cycle. Conditional lethal *cdc* mutants at the restrictive condition uniformly arrest and accumulate at a particular stage in the cell cycle, known as the terminal phenotype. *CDC* genes help to mediate the control of the cell cycle and represent the various dependent, independent and interdependent pathways of the nucleus and cytoplasm that are necessary for a normal cell cycle. All of these pathways converge at G_1.

A *S. cerevisiae* cell begins the cell cycle as an unbudded mother cell or a newborn daughter cell. The first visibly detectable event is the emergence of a bud. This grows continually through nuclear division and cytokinesis until cell separation, when it becomes the daughter cell. At the beginning of the cell cycle in G_1, there is a major decision making process called Start (Hartwell, 1974). At Start, a cell is unbudded, responsive to mating pheromone and has a spindle pole body satellite that has not yet replicated (Pringle and Hartwell, 1981). Start is the point at which the cell assesses its environmental status. It would be biologically efficient for the cell if it knew whether it should initiate and complete a new cycle or to follow an alternative developmental pathway. The pathway that a cell can follow is dependent on whether it is haploid or diploid and its environment: if a cell is haploid it will either enter into G_0, a non-proliferative stage, or undergo conjugation and mating if mating pheromone is present; and if a cell is diploid it can undergo meiosis and sporulation. The coordination of cell division and growth at Start is achieved by the necessity to attain a critical cell size, the efficient monitoring of nutrient availability and the ability to detect the presence of mating pheromone. There are two classes of Start mutants, I and II. On arrest, class I mutants resemble mating pheromone-arrested cells that shmoo and include mutants such as *cdc28*, *cdc36*, *cdc37* and *cdc39*. On arrest, Class II mutants such as *cdc25*, *cdc33* and *cdc35* are

unbudded and resemble nutritionally-arrested cells. The reason for these controls is presumably so that a cell will not waste valuable resources by initiating a new cycle if there are insufficient nutrients available to complete it. Therefore it is clear that cells of *S. cerevisiae* are capable of coordinating growth and proliferation and this occurs in G_1.

According to Johnston *et al.* (1977), growth is the rate limiting step for continuation of the cell cycle of *S. cerevisiae*, as daughter cells can be distinguished from mother cells by the fact that they must undergo further growth until they can initiate a new cell cycle (Hartwell and Unger, 1977). The size of a cell is a function of its growth rate. A low growth rate supports a small cell size and a high growth rate supports a large cell size. Initiation of sporulation is also subject to size control (Calvert and Dawes, 1984). Size control is partly controlled by the *WHI1* gene (Sudbery *et al.*, 1980). *whi1-1* mutants initiate the cell cycle at half the volume of wild type cells and deletion of *WHI1* results in a larger than normal cell size (Nash *et al.*, 1988); overexpression of *WHI1* results in a smaller cell size (Nash *et al.*, 1988). These observations indicate that the Whi1 protein is an activator of Start and the *whi1-1* mutation results in a hyperactive Whi1 protein (Nash *et al.*, 1988). Whi1 is modulated by nutritional conditions and manipulation of *WHI1* can affect the length of G_1, but not the total length of the cell cycle. As a consequence of bypassing Start and possibly activating *CDC28*, *whi1-1* cells are immune to mating pheromones. It is possible that Whi1 and α factor may be antagonists (Nash *et al.*, 1988). Whi1 has been found to have sequence homology with a class of proteins called cyclins, which are inducers of mitosis (see below). The *Whi1-1* mutation has been found to be co-dominant and subsequent to the original discovery of the mutant (Sudbery *et al.*, 1980), it has been referred to as *WHI1-1*.

Nutritional control of the cell cycle is mediated partly by the cAMP pathway, which is involved in sensing sugars (to be discussed later), and

CDC33, which is involved in nitrogen signalling. *CDC33* encodes a mRNA Cap-binding protein (Brenner *et al.*, 1988). The role of *CDC33* is to activate PKA in order to repress genes that encourage G_1 arrest (Brenner *et al.*, 1988). The *cdc33-1* mutation leads to preferential synthesis of certain proteins that promote G_1 arrest.

WHI2 also plays an important part in the monitoring of the nutritional status of the cell (Sudbery *et al.*, 1980; Saul *et al.*, 1985; Rahman *et al.*, 1988; Mountain and Sudbery, 1990a). Whereas the *whi1-1* mutation results in a small cell size during the logarithmic phase of growth, the *whi2* mutation results in a small critical cell size in stationary phase (Sudbery *et al.*, 1980). *whi2* cells become committed to a new cell cycle as they are not capable of recognizing a lack of carbon source on which to grow; therefore *whi2* cells arrest at all stages of the cell cycle when nutrients eventually run out (Saul *et al.*, 1985). As *whi2* cells have a higher specific growth rate and are smaller during growth on non-fermentable carbon sources compared with fermentable carbon sources (Rahman *et al.*, 1988), and overexpression of the wild type gene prevented growth on glycerol (Mountain and Sudbery, 1990b), it was proposed that the function of *WHI2* is to repress functions that are only expressed in the absence of glucose. It appears that *WHI2* expression and cell size are dependent on growth rate rather than catabolite repression/derepression (Mountain and Sudbery, 1990b).

One of the most obvious biochemical aspects of G_1 is its regulation by a protein kinase cascade. For example *CDC7* and *DBF2*, which are required for passage through this part of the cell cycle, encode protein kinases (Jazwinski, 1988; Johnston *et al.*, 1990). The Start gene, *CDC28*, isolated by Reed (1980) encodes a 34 kDa serine/threonine protein kinase and its activity is required for the traverse of Start. Cdc28 complexes with and phosphorylates a 40 kDa protein, which is necessary for the activation of Cdc28. $p34^{CDC28}$ was thought to function only at the G_1-S transition. Subsequently it has been found that it has a role similar to that in other organisms was demonstrated (Reed and

Wittenberg, 1990; Surana *et al.*, 1991). In other organisms the function of p34 is to regulate the G_2-M transition. It has also been found that $p34^{CDC28}$ is phosphorylated in S phase on the Ser19 residue (Amon *et al.*, 1992; Sorger and Murray, 1992), similar to other p34 proteins. Although, mutations that affect the phosphorylation of Ser19 and an adjacent threonine do not accelerate mitosis or disrupt feedback controls that link cell division with completion of DNA synthesis.

Although the activity of the Cdc28-p40 complex in *S. cerevisiae* is regulated during the cell cycle, the concentration of the complex does not alter (Wittenberg *et al.*, 1990). The activity of Cdc28 is directly dependent on cyclins. Cyclins are proteins that ensure passage through particular transition points in the cell cycle and have been subdivided into two groups: mitotic and G_1 cyclins.

Mitotic cyclins accumulate in G_2 and are degraded at mitosis (Hunt, 1989; Pines, 1991). Recently, genes have been found in *S. cerevisiae* that encode proteins which are homologous to mitotic cyclins: *SCB1* (Ghiara *et al.*, 1991), *CLB1*, *CLB2*, *CLB3* and *CLB4* (Surana *et al.*, 1991). The level of *SCB1* mRNA fluctuates during the cell cycle and maximum accumulation occurs in G_2 (Ghiara *et al.*, 1991). *CLB1* and *CLB2* are only expressed in late S, G_2 and M phases, neither are essential but one must be functional (Surana *et al.*, 1991).

G_1 cyclins are required at Start for entry into S phase (Hadwiger *et al.*, 1989; Richardson *et al.*, 1989; Wittenberg *et al.*, 1990), after which time they are no longer required (Cross, 1990); they also associate with $p34^{CDC28}$. Three such cyclins have been isolated from *S. cerevisiae*, Cln1, Cln2 and Cln3 and they are homologous to higher eukaryotic cyclin A types (Richardson *et al.*, 1989). The *CLN* gene family is required for the G_1-S transition and is involved in the timing of Start. Elimination of all three cyclins is lethal. The mRNA of *CLN1* and *CLN2* is cell cycle-dependent, whereas the mRNA of *CLN3* is not. *CLN3* was originally described as *WHI1* (Sudbery *et al.*, 1980) and also *DAF1*

(Cross, 1988). Cyclin gene expression can be controlled by either mating pheromones, as the mRNAs of *CLN1* and *CLN2* decrease on addition of mating pheromone (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990), by nutrient limitation which decreases protein synthesis, or by modification of cyclin by Cdc28 (Wittenberg *et al.*, 1990). Cln3 is also affected by mating pheromones *via* two other proteins, Far1 and Fus1 (Cross and Tinkelenberg, 1991). Recent evidence (Lew *et al.*, 1992) shows that different cyclins act differently in mother and daughter cells. In daughter cells, transcription of *CLN1* and *CLN2* is induced in a size-dependent manner, presumably so that daughters can only enter the cycle if the level of cyclin transcription is sufficient. *CLN3* is not required in daughter cells, but is crucial for the traverse of Start in mother cells in which it is constitutively expressed.

Regulation of the cell cycle by cyclins and Cdc28 is extremely complex and there is a seemingly endless supply of proteins that participate in this pathway. For example it is possible that Swi4 and Swi6, which are transcription factors responsible for the Start-dependent transcription of the HO endonuclease, are involved in a positive feedback loop (Ogas *et al.*, 1991). This loop may be responsible for activation of Cln1 and Cln2 transcription in order to activate the Cdc28-Cln complex. As both Swi4 and Swi6 contain potential sites for Cdc28 phosphorylation, Cdc28-Cln may activate the activity of Swi4 and Swi6 (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). Cln3 is also dependent on Swi4 and Swi6 proteins.

1.4. The cyclic AMP pathway in *S. cerevisiae* and its control.

1.4.1. The cAMP pathway.

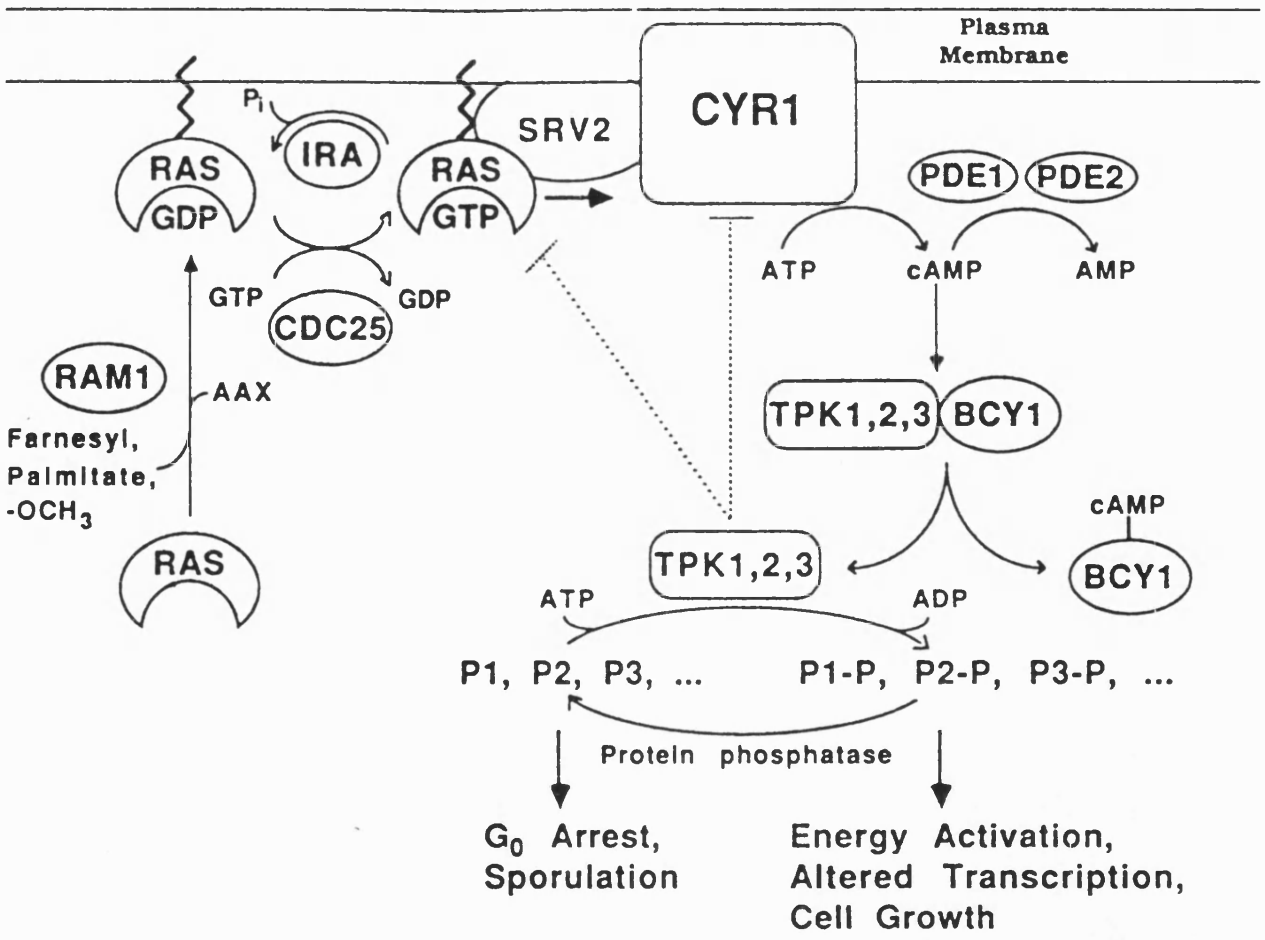
An entire cAMP pathway has been found in *S. cerevisiae*, comprising adenylyl cyclase (Londesborough and Nurminen, 1972), both low K_m and high K_m phosphodiesterases (Londesborough, 1975, 1974, respecively) and a PKA (Hixson and Krebs, 1980). With the isolation of a considerable number of

mutants of this pathway, an intricate control system has emerged. The latest model of this pathway is summarized in Figure 4. There are many more proteins that have been implicated in the cAMP pathway than are shown here, but only those of importance and interest to this work are discussed below.

The most upstream function of the cAMP pathway is encoded by *CDC25*. A temperature-sensitive mutant of this gene was first isolated by Pringle and Hartwell (1981) and at the non-permissive temperature for growth, this mutant arrests as an unbudded cell in G_0, showing that a functional *CDC25* is essential for traverse of Start. *CDC25* was cloned and sequenced by Camonis *et al.* (1986) and encodes a 180 kDa polypeptide, which is tightly linked to the yeast plasma membrane (Garreau *et al.*, 1990). The biochemical role of Cdc25 is thought to be a GTP-GDP exchange factor for the guanine nucleotide-binding proteins (G proteins) encoded by the *RAS* genes (Robinson *et al.*, 1987; Daniel *et al.*, 1987). It has been found that the C-terminus of Cdc25 was required for stabilizing the Ras-adenylyl cyclase complex (Daniel *et al.*, 1987) and the C-terminal ß-domain is able to mediate the glucose-induced activation of Ras (Munder *et al.*, 1988; Van Aelst *et al.*, 1990). Mutations in *CDC25* therefore result in a lack of response to glucose. Cdc25 is also thought to be involved in the mitosis-meiosis decision (Tripp and Piñón, 1986; Munder *et al.*, 1988) as *cdc25* mutants sporulate in rich medium (Shilo *et al.*, 1978). It has been found that the N-terminus of Cdc25 is required for growth on non-fermentable carbon sources resulting in defective sporulation if altered (Munder *et al.*, 1988; Van Aelst *et al.*, 1990) and the C-terminus is essential for sporulation to proceed.

In the absence of glucose, the glucose receptor is thought to interact with the C-terminal domain (Ramos *et al.*, 1989b) of Cdc25 causing transduction of a signal to adenylyl cyclase *via* GTP-GDP exchange on Ras, resulting in synthesis of cAMP. The C-terminal of Cdc25 then interacts with another G protein, Ira1, which stimulates the GTPase activity of the Ras proteins by causing the conversion of the active GTP-bound Ras protein to the inactive GDP-bound

Figure 4. The cAMP pathway of *S. cerevisiae*. Reproduced from Broach (1991). Each component of the pathway is represented by its gene designation; P1, P2 and P3 represent targets of PKA; solid lines with arrows represent the direction in which a reaction occurs; broken lines with bars represent the control that the catalytic subunit of PKA has over particular components.


Plasma
Membrane
CYR1
SRV2
RAS
GDP
IRA
P_i
RAS
GTP
PDE1
PDE2
ATP
cAMP
AMP
GTP
GDP
CDC25
RAM1
AAX
Farnesyl,
Palmitate,
$-OCH_3$
TPK1,2,3
BCY1
cAMP
RAS
TPK1,2,3
BCY1
ATP
ADP
P1, P2, P3, ...
P1-P, P2-P, P3-P, ...
Protein phosphatase
G_0 Arrest,
Sporulation
Energy Activation,
Altered Transcription,
Cell Growth

form (Tamanoi *et al.*, 1984). In the presence of glucose, the glucose receptor (as yet unknown) directly activates Ras independently of Cdc25 and Ira1 (Ramos *et al.*, 1989b).

It is thought that Cdc25 could be a transmembrane protein sensing nutrients (Munder and Küntzel, 1989) or it may rely on other glucose sensors for example hexose transporters. A functional *CDC25* is also required for the pheromone response (Perlman *et al.*, 1989). *cdc35*, *cyr1*, *bcy1*, *ras1 ras2 bcy1-1*, *ras2 bcy1-1* and *RAS2*^{val19} mutants all respond to pheromone, but *cdc25* mutants do not and transient intracellular alkalinization, which is the first biochemical event that is detectable after the addition of pheromone (Perlman *et al.*, 1989), does not occur in these cells. *CDC25* disruption strains can be made fertile by the overexpression of the gene encoding the catalytic subunit of PKA, *TPK1*, on a multicopy plasmid.

There have been reports that *cdc25* mutations can result in a decrease in the concentration of i-cAMP (*cdc25-5*; Camonis *et al.*, 1986; Broek *et al.*, 1987) or no change (*cdc25-1*; Martegani *et al.*, 1986). Petitjean *et al.* (1990) characterized the *cdc25-1* and *cdc25-5* missense mutations and concluded that both of the mutations occur within the C-terminus. Apart from the possibility that changes in i-cAMP concentration are due to differences in the genetic backgrounds (Van Aelst *et al.*, 1991), the observation that *cdc25-5* is a temperature sensitive mutation, in which adenylyl cyclase is inactivated at the restrictive temperature only, and *cdc25-1* which has a constantly inactivated adenylyl cyclase, may be relevant. Both of these mutations can be suppressed by e-cAMP (Boutelet *et al.*, 1985; Martegani *et al.*, 1986).

IRA1 was first isolated by Tanaka *et al.* (1989) and encodes a 350 kDa polypeptide; another gene *IRA2* has since been isolated (Tanaka *et al.*, 1990b). Both Ira1 and Ira2 additively regulate the GTPase activity of Ras, although according to Tanaka *et al.* (1991), it is mainly Ira1 that is responsible for stimulating the GTPase activity of Ras2, and Ira2 for Ras1 (Tanaka *et al.*,

1989). Both Ira1 and Ira2 have similarities to the human GAP (GTPase activating protein). *IRA1* was originally isolated as *PPD1*, which was supposedly deficient in phosphoprotein phosphatase (Matsumoto *et al.*, 1985b). A functional *IRA1* is required for regulating the concentration of cAMP *via* Ras and adenylyl cyclase in response to nutrient limitation. Usually, Ras1 and Ras2 are found in the GDP-bound form, however, in *ira*⁻ mutants they occur in the GTP-bound form (Tanaka *et al.*, 1990a), i.e. the form that enables stimulation of adenylyl cyclase (Broek *et al.*, 1985; Toda *et al.*, 1985), *ira1* mutants also have an elevated i-cAMP concentration as there is no downregulation of Ras GTPase activity. Disruption of *IRA1* results in sensitivity to nitrogen starvation and heat shock, it can also lead to suppression of *cdc25* mutations, but not *ras1* *ras2* or *cyr1*.

There are two classes of G protein: the hormone-linked trimeric G proteins, which transduce signals from the cell surface to effectors on or near the plasma membrane, and the single subunit G proteins that are localized in the plasma membrane and are involved in cell division signalling. Ras proteins belong to the single subunit class and in *S. cerevisiae* are encoded by *RAS1* and *RAS2* (for review see Tamanoi, 1988). *RAS1* and *RAS2* show a high degree of sequence homology with human *ras* (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984), which if mutated, can induce cancer. Unlike *S. cerevisiae*, however, *S. pombe*, *D. discoideum*, *Drosophila melanogaster* and human *ras* do not regulate adenylyl cyclase (Gibbs and Marshall, 1989), although cells of *S. cerevisiae* that are deficient in both *RAS* genes can be revived by a human *ras* gene substitution.

Both Ras1 and Ras2 possess a highly distinctive Cys-Ala-Ala-X tail. This consensus pattern of amino acids is found in proteins involved in signal transduction. Without this sequence, Ras proteins would not stably associate with membranes (Deschenes and Broach, 1987). Ras proteins are synthesized in the cytosol and are processed before attachment to the plasma membrane

(Fujiyama and Tamanoi, 1986). Mutations in the genes encoding proteins that are responsible for correct processing of Ras, for example *RAM1*, *RAM2* and *DPR1* (Fujiyama *et al.*, 1987), result in temperature sensitivity for growth and defective mating. Neither *RAS1* nor *RAS2* are essential, but one functional *RAS* gene is needed for growth and viability of *S. cerevisiae* (Kataoka *et al.*, 1984; Tatchell *et al.*, 1984) and this can be demonstrated by incubation of *ras1*$^{-}$ *ras2*^{ts} cells at the non-permissive temperature, which will fail to form buds (DeVendittis *et al.*, 1986). If diploid, *ras2* mutants will sporulate on rich media, but are still able to accumulate storage carbohydrates (Toda *et al.*, 1985). Cells with an activated Ras2, where the amino acid at position 19 is substituted for a valine (*RAS2*^{val19}), fail to accumulate storage carbohydrates, are sensitive to heat shock and nutrient starvation, and diploids are defective in sporulation (Tatchell *et al.*, 1984). Therefore cells with oncogenic Ras fail to respond to deprivation and cells with disrupted *RAS* genes arrest in rich media, but are able to enter G_0. This phenomenon is thought to be related to the concentration of i-cAMP as cells with disrupted *RAS* genes have a lowered concentration of i-cAMP and cells with activated *RAS2* have an elevated concentration of i-cAMP.

Although both Ras1 and Ras2 can activate adenylyl cyclase, it is thought that the major role of Ras2 is to perform this function (Toda *et al.*, 1985), whereas the major role of Ras1 is to negatively control glucose-induced inositol phospholipid turnover (Kaibuchi *et al.*, 1986). This work has since been repeated and the original results have been disputed (S. Henry, R. Irvine and P. Hankins, personal communication). However, functional differences between Ras1 and Ras2 have been found. When cells are grown on glucose, *RAS2* mRNA is produced in larger amounts than *RAS1* mRNA; on non-fermentable carbon sources, *RAS1* mRNA is preferentially expressed (Breviario *et al.*, 1986).

In fact, critical size attainment may be affected by the *CDC25* and *RAS2* gene products implying that the cAMP pathway can regulate size control (Baroni *et al.*, 1989). At the permissive temperature, *cdc25-1* mutants have a smaller critical size for traverse of Start and $RAS2^{val19}$ mutants have an increased critical size. This increase in critical cell size was apparent in all types of growth medium, however, the change in critical cell size for *cdc25-1* was only apparent in rich medium.

Adenylyl cyclase was first isolated by Londesborough and Nurminen (1972). It is dependent on either Mg^{2+} or Mn^{2+} ions (Londesborough and Nurminen, 1972; Casperson *et al.*, 1983). It was assumed that adenylyl cyclase was tightly anchored to the plasma membrane (Heidemann *et al.*, 1987). Recently, however, adenylyl cyclase activity was found not only in membrane preparations, but also in cytosolic fractions. It was then proposed that adenylyl cyclase is only peripherally attached to the plasma membrane (Mitts *et al.*, 1990). The ability of adenylyl cyclase to bind to the membrane may be significant to its regulation as its binding to the membrane was inhibited by the addition of cAMP and in mutants with a constitutively active PKA (Mitts *et al.*, 1990). The component that binds adenylyl cyclase to the membrane is still unknown, although Ira1 has been implicated (Mitts *et al.*, 1991). Other work supporting this idea has shown that in wild type cells, adenylyl cyclase is found in the membrane fraction, whereas in *ras1 ras2 bcy1* mutants, adenylyl cyclase is found in the soluble fraction (Engelberg *et al.*, 1990). Overexpression of *CDC25* relocalizes adenylyl cyclase to the membrane, thus revealing a possible link between *CDC25* and adenylyl cyclase in the absence of *RAS*.

The structural gene for adenylyl cyclase, *CYR1* (Matsumoto *et al.*, 1982a, 1984) or *CDC35* (Boutelet *et al.*, 1985), has been cloned (Masson *et al.*, 1984; Casperson *et al.*, 1985) and sequenced (Kataoka *et al.*, 1985; Masson *et al.*, 1986). $cdc35^{ts}$ mutants have a similar phenotype to $cdc25^{ts}$ mutants (Camonis *et al.*, 1986). The catalytic domain of adenylyl cyclase is located at the

N-terminus (Kataoka *et al.*, 1985) and a small region adjacent to this is involved in interacting with Ras proteins (Uno *et al.*, 1987). This region has been found to contain leucine-rich repeat sequences (Kataoka *et al.*, 1985; Field *et al.*, 1990b) and loss of these repeats results in a loss of binding to Ras and therefore lack of activity (Colicelli *et al.*, 1990). A Ras-responsive adenylyl cyclase has been isolated as a 200 kDa complex, although other researchers have isolated 220 kDa (Kataoka *et al.*, 1985), 450 kDa (Varimo and Londesborough, 1976) and 594 kDa (Heidemann *et al.*, 1987) complexes. The larger sizes are probably due to the association of adenylyl cyclase with other proteins such as Ira and Cap.

Cap was first isolated by Field *et al.* (1988) as a 70 kDa polypeptide and without it adenylyl cyclase is not stimulated by Ras (Field *et al.*, 1990a). Cells that do not have a functional Cap protein are viable and resistant to heat shock, but are sensitive to nitrogen starvation, unable to grow on rich medium and have a swollen cell morphology. The N-terminal domain of Cap is required for response to Ras and the C-terminal domain is required for cell morphology and responses to nutrient extremes (Gerst *et al.*, 1991). Cap may interact with other proteins involved in nitrogen starvation. *CAP* is allelic to *SRV2* (Fedor-Chaiken *et al.*, 1990). *srv2-2* null mutations are lethal on their own, but can alleviate the lack of response to stress in $RAS2^{val19}$ cells. The product of *SRV2* may be responsible for the integrity of membranes as an unusually high amount of debris is observed in the growth medium of *srv2-2* mutants. Srv2 may affect adenylyl cyclase either by acting as an intermediate or as a stabilizer.

Cyclic AMP exerts its effects through a cAMP-dependent protein kinase (PKA). Specific cellular proteins have their activities controlled by phosphorylation *via* PKA and dephosphorylation events *via* a phosphoprotein phosphatase. The target enzymes of PKA are involved in numerous pathways: carbohydrate and phospholipid metabolism, including trehalase (Uno *et al.*, 1983), phosphofructokinase and fructose 1,6-bisphosphatase (Purwin *et al.*,

1982) (the first two enzymes are activated by PKA and the last enzyme is inactivated by PKA); the regulation of transcription factors (free catalytic subunits have been located in the nucleus) (Buechlet *et al.*, 1991); the control of strategic points in both glycolytic and gluconeogenic pathways; and regulation of proteins involved in the production of cAMP, for example Cyr1 (Kataoka *et al.*, 1985), Cdc25 (Camonis *et al.*, 1986), Ras2 (Resnick and Racker, 1988) and Ira1 (Tanaka *et al.*, 1989). In *S. cerevisiae*, PKA comprises two catalytic and two regulatory subunits. In the absence of cAMP, the regulatory and the catalytic subunits are bound *via* interaction between a negatively charged residue on the catalytic subunit and a positively charged residue on the regulatory subunit. On binding to each of the regulatory subunits, cAMP causes the release of the catalytic subunit by stabilizing an altered conformation in which the positively charged residue is displaced (Levin and Zoller, 1990). The family of catalytic subunits of PKA encoded by *SRA1*, *TPK1*, *TPK2* and *TPK3*, have been cloned and sequenced (Cannon and Tatchell, 1987; Toda *et al.*, 1987b). At first it was thought that *CYR2* encoded the catalytic subunit of PKA (Matsumoto *et al.*, 1985a), however, *CYR2* has now been identified to be *CDC25* (I. Uno, personal communication). Expression of any of the *TPK* genes on multicopy plasmids results in the suppression of *ras1 ras2* and *cyr1* mutations. Haploid spores lacking all three Tpk proteins are able to germinate, but grow rather slowly. The non-lethal nature of *tpk* mutations may be due to the presence of other protein kinases, for example Sch9 (Toda *et al.*, 1988) and Yak1 (Garrett and Broach, 1989). Neither Sch9 nor Yak1 is essential, but Sch9 can suppress *tpk1 tpk2 tpk3*, *ras1 ras2* and *cyr1* mutations, and if *YAK1* is expressed on a multicopy plasmid, it can also suppress all of these mutations (Toda *et al.*, 1988; Garrett and Broach, 1989).

Matsumoto *et al.* (1982a) were first to isolate mutants defective in the regulatory subunit of PKA, which is encoded by *BCY1* or *SRA1* (Cannon and Tatchell, 1987; Toda *et al.*, 1987a). *BCY1* was cloned and sequenced by Toda

et al. (1987a). Due to the lack of a regulatory subunit, *bcy1* strains have an unregulated PKA and thus do not respond to nutrient limitation by arresting in G_1, fail to accumulate storage carbohydrates and do not survive heat shock (Matsumoto *et al.*, 1985a). *bcy1* mutants also do not require functional Ras proteins for growth and proliferation. Cells without the regulatory subunit, but possessing an attenuated catalytic subunit (*tpk*^{w}) are responsive to nutrient limitation and can sporulate, are resistant to heat shock and accumulate glycogen (even when other components of the cAMP pathway are absent).

Dephosphorylation of proteins is carried out by phosphoprotein phosphatase. *PPD1* was first isolated by Matsumoto *et al.* (1985b) and was thought to encode such an enzyme, but it has since been found to be allelic to *IRA1*. The serine/threonine phosphatase superfamily includes types 1, 2A and 2B; types 1 and 2A have been implicated in cell cycle control (Cyert and Thorner, 1989). *S. cerevisiae* possesses type 2A phosphoprotein phosphatases and these are encoded by *PPH1* (*SIT4*) (Arndt *et al.*, 1989), *PPH3*, which is related to the mammalian PP2A enzyme, *PPH21* and *PPH22*, which are homologues of the mammalian PP2A enzyme (Sneddon *et al.*, 1990; Ronne *et al.*, 1991), and *DIS2S1*, which was isolated as a homologue of the *S. pombe* *dis2*$^+$ gene (Ohkura *et al.*, 1989). Pph1/Sit4 performs an important function in the G_1 phase of the cell cycle. A *sit4* mutation results in cells that are unable to grow on non-fermentable carbon sources (Arndt *et al.*, 1989) similar to *bcy1* mutants. It therefore appears that as a consequence of increased phosphorylation, a cell loses the ability of growth on non-fermentable carbon sources. A *pph3* mutation does not affect growth and is therefore thought not to be essential, however, disruption of spores containing both *pph21 pph22* mutations is lethal (Sneddon *et al.*, 1990). Other researchers have found that disruption of spores containing both *pph21 pph22* mutations results in a few viable, but very small colonies (Ronne *et al.*, 1991), and disruption of spores lacking all three of these genes results in no growth. Therefore there is an

essential role for phosphoprotein phosphatase activity in *S. cerevisiae*. As yet, no *pph21 pph22* double mutants have been isolated which would allow the study of the cell cycle and the role of phosphoprotein phosphatases, but it has been found that *pph* mutants have a lengthened G_1 (Sneddon *et al.*, 1990).

Phosphodiesterase, which is responsible for the breakdown of cAMP, has two isoenzymes, with low and high K_m values. These are thought to be responsible for the total phosphodiesterase activity in *S. cerevisiae*. The low K_m enzyme is active in actively growing cells especially with high glucose concentrations and the high K_m enzyme is active in stationary phase cells. The low K_m enzyme was first isolated by Londesborough (1975) and the gene encoding this enzyme, *PDE2* (or *SRA5*; Wilson and Tatchell, 1988), was cloned and sequenced by Sass *et al.* (1986). It has a K_m of 0.15 μM between pH 6-9. The low K_m enzyme is loosely bound to microsomal particles, whereas the high K_m enzyme is cytosolic in location. The high K_m enzyme was first isolated by Londesborough (1974) and the gene encoding this enzyme, *PDE1*, was cloned and sequenced by Nikawa *et al.* (1987b). The high K_m enzyme has a K_m of 0.1 mM at pH 7.5 and is strongly pH-dependent.

The phenotypic effects of the *RAS2*^{val19} mutation can be suppressed by both *PDE1* and *PDE2* when expressed on a high copy number plasmid. Neither *PDE1* nor *PDE2* appear to be essential and unexpectedly, the concentration of i-cAMP in *pde1 pde2* cells is only two to three times higher than normal (Nikawa *et al.*, 1987b), however, disruption of both phosphodiesterases suppresses the lethality of *ras1 ras2* mutations. The phenotype of *pde1 pde2* mutants resembles that of *RAS2*^{val19}: cells do not endure starvation, are sensitive to heat shock and do not accumulate storage carbohydrates.

1.4.2. Control of cAMP concentration in *S. cerevisiae*.

The intracellular concentration of cAMP in *S. cerevisiae* is under tight feedback control. This control is capable of affecting the generation of cAMP, the rate of

loss or both, can operate over a 10,000-fold range of i-cAMP concentration and requires an active PKA. Cdc25 and Ras are also required as they are the targets of PKA. *CDC25*, *CYR1* and *IRA1* have multiple consensus sites for PKA phosphorylation in their N-terminal domains (Nikawa *et al.*, 1987a; Tanaka *et al.*, 1989) and they are also necessary for feedback to occur. No such sites have been found on *PDE1* or *PDE2* (Sass *et al.*, 1986; Nikawa *et al.*, 1987a). Ras proteins are also reported to be involved in this feedback control: *RAS2*^{val19} mutants are able to override the control that PKA has, as the concentration of i-cAMP is modestly elevated in these cells. The concentration of i-cAMP in *tpk*^{w} *ras1 ras2* mutants is reportedly below the limit of detection (Nikawa *et al.*, 1987a), demonstrating the stringent control that Ras has over adenylyl cyclase. A low concentration of i-cAMP is observed in cells with an unregulated PKA, for example *bcy1* mutants, and a high concentration of i-cAMP is observed in cells with an attenuated PKA, for example *tpk*^{w} mutants. Attenuation of *TPK* genes increases cAMP concentrations more than disruption of both phosphodiesterase genes (Nikawa *et al.*, 1987b).

It has been discovered also that Ras1 and Ras2 are phosphorylated. In fact, Resnick and Racker (1988) found that bacterially-produced Ras2 is phosphorylated by purified PKA and is 40-60% less effective than unphosphorylated Ras in stimulating adenylyl cyclase. Ras purified from yeast is also phosphorylated by PKA (Cobitz *et al.*, 1989) and the phosphorylated forms of Ras1 and Ras2 are predominantly found in the membrane, where they are able to activate adenylyl cyclase when unphosphorylated. Sreenath *et al.* (1988) reported that Ras2 was phosphorylated by PKA and another protein kinase that was independent of the concentration of cAMP or active with a very low concentration i-cAMP. It is possible that Sch9 or Yak1 is this protein kinase.

1.5. The role of cAMP in *S. cerevisiae*.

1.5.1. Cyclic AMP and sensory mechanisms involved in nutrient detection.

On addition of glucose to derepressed (respiring) cells, germinating ascospores or stationary cells of *S. cerevisiae*, there is a transient increase in the concentration of i-cAMP (Van der Plaat and Van Solingen, 1974). This signal indicates that glucose, the preferred sugar for metabolism, is present and the increase in i-cAMP results in a phosphorylation cascade. Reports by Thevelein *et al.* (1987a, b) showed that the increase in i-cAMP concentration is partly caused by intracellular acidification and the optimum pH of adenylyl cyclase is pH 6. They also concluded that intracellular acidification occurred downstream of glucose phosphorylation and either at or upstream of Cdc25 (Van Aelst *et al.*, 1991). This decrease in pH was thought to be due to the first three steps in the glycolysis pathway in which H^+ ions are generated (Ramos *et al.*, 1989a). The phosphorylation of sugars in these reactions depletes ATP availability, but the H^+ ions produced stimulate the plasma membrane H^+-ATPase and restore the pH and ATP levels. Cyclic AMP is reported to be involved in the regulation of the H^+-ATPase and acts as a positive effector: the addition of exogenous cAMP to arrested *cdc35* or *cdc25* cells restores the H^+-ATPase activity (Ulaszewski *et al.*, 1989). It has been confirmed that Cdc25 is also involved in mediating the activity of the H^+-ATPase but not Cyr1, Ras1 or Ras2 (Mazón *et al.*, 1989).

Mbonyi *et al.* (1988) proposed that the presence of either Ras1 or Ras2 is required for the transmission of the glucose-induced cAMP signal. Cells with an oncogenic *RAS2*^{val19} did not show a glucose-induced cAMP signal and were found to inhibit transmission of the signal. Possible reasons for this could stem from the fact that the *RAS2*^{val19} protein does not respond to PKA.

The transient nature of the glucose-induced cAMP signal can be explained in terms of a temporary override of feedback inhibition on lowering of pH. This would explain the transient signal observed in *bcy1* mutants. Further evidence for the involvement of PKA-mediated feedback inhibition in the

transient nature of the cAMP signal came from Mbonyi *et al.* (1990). They showed that cells having a reduced PKA activity, carrying the *tpk1*^{w1}, *tpk2*^{w1} or *tpk3*^{w1} mutations, are defective in glucose repression and show hyperaccumulation of cAMP. Strains that only have one *TPK* gene still show the transient glucose-induced cAMP signal. Tpk2 caused greatest feedback inhibition and Tpk3 caused the least.

It was Munder and Küntzel (1989) that first showed *CDC25* to be involved in the glucose-induced cAMP signal. However, it was Van Aelst *et al.* (1990) who correctly concluded that the C-terminal was responsible for mediation of the glucose-induced cAMP signal and the glucose-induced cAMP signal is unnecessary for growth on glucose. The N-terminus of Cdc25 has been shown to possess multiple sites for PKA phosphorylation, which could be used in feedback control of the cAMP signal (Camonis *et al.*, 1986).

Thevelein *et al.* (1987b), however, have subsequently found that since glucose did not cause a transient increase in the concentration of i-cAMP when cells were growing on glucose, and the cAMP signal was still present when the pH drop was abolished, another factor must be involved in the glucose-induced cAMP signal, i.e. a glucose-repressible protein. Van Aelst *et al.* (1991a) showed that in glucose grown cells where *CDC25* is overexpressed, there is a cAMP signal. Using the *cat1* mutation that prevents derepression, they concluded that Cdc25 was not the glucose repressible protein, but acts as a positive stimulator of derepression. Although the regulatory components of the glucose-induced cAMP pathway have been identified and assigned roles, the identity of the glucose repressible protein is still unanswered.

1.5.2. Catabolite repression and carbohydrate metabolism.

Growth of *S. cerevisiae* on glucose results in repression of transcription of a large number of genes necessary for alternative carbohydrate metabolism and respiration. As *S. cerevisiae* can utilize similar sugars to those of *E. coli*, it was

thought that the same catabolite repression system may be present in yeast. Early work by Singh *et al.* (1980) showed that exogenously added cAMP, which was thought to be taken up by the cells, could not relieve catabolite repression of the *N*-acetylglucosamine pathway. They concluded that catabolite repression does occur in *S. cerevisiae*, but is not under cAMP control. However, Matsumoto *et al.* (1982b) showed that cells of *S. cerevisiae* were not permeable to cAMP and required mutation before any uptake of cAMP could occur, thus placing the results of Singh *et al.* (1980) in doubt. By using the *cam1*, *cam2* and *cam3* mutations, which conferred permeability to cAMP, they also showed that cyclic AMP did not relieve catabolite repression of galactokinase. Eraso and Gancedo (1984) also demonstrated that catabolite repression in *S. cerevisiae* was not mediated by cAMP. They measured higher i-cAMP concentrations in cells that were grown on glucose medium compared with cells grown on ethanol medium. The demonstration that the addition of glucose causes an increase in the i-cAMP concentration (Van der Plaat and Van Solingen, 1974; Mazón *et al.*, 1982; Purwin *et al.*, 1982) also supports the theory that catabolite repression in *S. cerevisiae* is not connected with low concentrations of i-cAMP as in *E. coli*. However, there is controversy concerning whether i-cAMP are higher on glucose and other fermentable carbon sources than on non-fermentable carbon sources (Eraso and Gancedo, 1984; Olempska-Beer and Freese, 1987; Mbonyi *et al.*, 1990). Also there have been reports demonstrating that exogenous cAMP can derepress the synthesis of various mitochondrial enzymes and release cells from catabolite repression (Mahler and Lin, 1978) and low i-cAMP concentrations are associated with catabolite repression in other yeasts (Van Wijk and Konijn, 1971). So far, it appears that cAMP is either not involved in catabolite repression or it mediates an opposite effect to that seen in *E. coli*.

It has been well documented that cAMP is a key organizer in carbohydrate metabolism (François *et al.*, 1987). PKA phosphorylates various enzymes that

are involved in carbohydrate metabolism: trehalase and 6-phosphofructose-2-kinase are activated by phosphorylation by PKA; fructose-1,6-bisphosphatase and trehalase-6-phosphate synthase are inactivated by PKA. By using mutants of the cAMP pathway, it has been shown also that glycogen metabolism is regulated by the cAMP pathway and this occurs at the transcriptional level: a *RAS2*^{val19} mutant, which has increased levels of cAMP, has reduced accumulation of *GAC1* mRNA (*GAC1* encodes glycogen synthase) (François *et al.*, 1992). However, it is probable that it is not increased i-cAMP concentrations but a regulated PKA which is a prerequisite for regulation of glycogen metabolism.

Nitrogen sources are also able to cause drastic activation or inactivation of enzymes that are regulated by PKA, however, nitrogen sources have no effect on cAMP concentrations in vegetative *S. cerevisiae* cells (see Thevelein, 1991).

1.5.3. cAMP and conjugation, meiosis and G_0 cell cycle arrest.

Another signal transduction system in yeast is mediated by mating pheromones. However, there are two conflicting ideas about the role of cAMP in conjugation. Liao and Thorner (1980, 1981) found that α factor inhibited adenylyl cyclase and exogenously added cAMP to pheromone-arrested cells shortened this arrest. However, this theory has since been disputed many times (Matsumoto *et al.*, 1982a; Casperson *et al.*, 1983; Sy and Tamai, 1986). In fact, Matsumoto *et al.* (1985a) reported that cAMP acts as a positive effector in conjugation as a *cyr1* mutant failed to conjugate, but a *bcy1* mutant did not.

Data that support a role for cAMP in conjugation include the responses of various mutants of the pathway to pheromone. Addition of pheromone to *ras1 ras2 bcy1*, *cdc35*^{ts}, *bcy1* and *cdc25*^{ts} mutants results in reduced shmoo formation. *cdc25*^{ts} mutants were most severely affected.

Factors that are associated with the cAMP pathway and are thought to be important in the decision to sporulate are: a functional PKA that can regulate

entry into meiosis; cAMP, which is known to be essential for particular sporulation events, for example completion of the spore wall (Matsumoto *et al.*, 1985b); the N-terminal domain of adenylyl cyclase, which is negatively controlled by interaction with a factor in response to a signal for sporulation (Uno *et al.*, 1990); and the initiation of *IME1* transcription, which is usually inhibited by the presence of Ras2 (Smith and Mitchell, 1989). However, there is also conflicting evidence about a role for cAMP in this developmental pathway. According to Matsumoto *et al.* (1983a, 1985a), sporulation is dependent on inhibition of cAMP production and inactivation of PKA, as *bcy1* mutants do not sporulate in sporulation medium and a *cyr1* diploid sporulates in nutrient rich medium.

Olempska-Beer and Freese (1987) found that cAMP mutants that are derepressed for sporulation in nitrogen rich, gluconeogenic medium sporulate because they can utilize the carbon source at a rate that permits sporulation but not proliferation. They found that during sporulation, cAMP concentrations decreased by not more than 20% and not at all in the presence of 3-butyl-1-methylxanthine (IBMX; an inhibitor of phosphodiesterase; the sporulation efficiency was 76 or 100% in the presence or absence of IBMX, respectively). Cells also appeared to sporulate more efficiently if the concentration of i-cAMP levels was increased. Salmon *et al.* (1989) agreed with Olempska-Beer and Freese (1987) in that a cAMP decrease is not a requirement for sporulation to occur. Sporulation requires the appropriate nutritional conditions and Ras2 plays a part in controlling meiosis through its regulation of adenylyl cyclase (Kao *et al.*, 1990).

A multicopy plasmid carrying *IME1* relieves the sporulation defects of *bcy1* and *RAS2*^{val19} cells (Matsuura *et al.*, 1990). *IME1* is a positive regulator of meiosis (Kassir *et al.*, 1988) and its transcriptional regulation occurs *via* an intermediary protein that is probably phosphorylated by PKA (Matsuura *et al.*, 1990). This can be demonstrated by the findings that the level of *IME1*

transcription is affected by *cdc25*, *cyr1* and *bcy1* mutations, and addition of cAMP to *cyr1-2* diploids suppresses *IME1* transcription and prevents sporulation (Matsuura *et al.*, 1990).

If cells are starved of cAMP they will arrest at Start; therefore cAMP is required for traverse of Start and to initiate a new cell cycle. When cells cease to divide due to decreased nutrient supply, they also arrest at Start, which is analogous to the G_0 state in mammalian cells. Cells at this stage are still metabolically active, but become thermotolerant, acquire the ability to withstand nutrient deprivation and synthesize a set of G_0 proteins, including a subset of heat shock proteins (Boorstein and Craig, 1990). The transition to this status is thought to be regulated by cAMP *via* PKA activity as *RAS2*^{val19} (Matsumoto *et al.*, 1985a) and *bcy1* (Matsumoto *et al.*, 1983) mutants are unable to arrest in G_0, and they do not synthesize these particular proteins. Other evidence linking cAMP to the G_0 and heat shock is that synthesis of heat shock proteins is suppressed by the addition of cAMP (Shin *et al.*, 1987).

Other genes that participate in the heat shock response and are affected by cAMP and PKA include *SSA3* and *UBI4*. *SSA3* encodes a member of the *HSP70* gene family of heat shock proteins, which is activated when the concentration of i-cAMP is lowered (Boorstein and Craig, 1990). The yeast polyubiquitin gene, *UBI4*, is essential for resistance to high temperatures, starvation and other stresses; *UBI4* is also activated by decreases in the concentration of i-cAMP (Tanaka *et al.*, 1988).

Cyclic AMP is obviously not the sole regulator of all these pathways. Cameron *et al.* (1988) have suggested that there might be a cAMP-independent pathway for controlling responses to nutrient deprivation. By demonstrating that a *cyr1*$^-$ *bcy1*$^-$ *tpk*^{w} mutant is viable and responsive to nutrient conditions, they concluded that modulation of cAMP concentration was not an absolute requirement for regulation of glycogen accumulation, sporulation and thermotolerance.

1.6. Extracellular cAMP and *S. cerevisiae*.

Watson and Berry (1977a) were the first to measure extracellular cAMP from medium in which *S. cerevisiae* cells had been growing after release from sporulation. However, it was Olempska-Beer and Freese (1987) and Eraso and Gancedo (1984) that measured cAMP in the growth medium of vegetatively growing cells. They concluded that *S. cerevisiae* requires an optimal concentration of cAMP for growth and excretes the rest that may otherwise have a detrimental effect on the cell. A report from Jakubowski and Goldman (1988) stating that diploid yeasts cooperate during sporulation and meiotic development using purine and pyrimidine nucleotides and nucleosides as mediators, may demonstrate a role for cAMP outside the cell during exponential growth.

1.7. Other signal transduction systems in *S. cerevisiae* involving the cyclic AMP pathway.

It is thought that inositol-1,4,5-triphosphate (IP_3) is rapidly turned over in proliferating yeast cells (Steiner and Lester, 1972) and cells that are in G_0 and are deprived of a carbon source, induce this turnover in response to glucose addition (Kaibuchi *et al.*, 1986). It has been reported that when cells are rescued from G_0 by the addition of glucose, it results in the incorporation of P_i into phosphatidic acid, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Kaibuchi *et al.*, 1986). Glucose-induced P_i incorporation was reported to be greater in *ras1*, *ras1 ras2 bcy1* or *RAS2*^{val19} mutants. However, this work has since been repeated and the original results have been disputed (S. Henry, R. Irvine and P. Hankins, personal communications). However, it has been found that PKA-mediated protein phosphorylation enhanced PI kinase and PIP kinase (Kato *et al.*, 1989) and incorporation of P_i into PIP, PIP_2 and PI has also been recorded. The incorporation was reduced in *ras2* mutants and increased in *bcy1* mutants.

Incorporation was the same in wild type, *ras1*, *ras2* and *bcy1* mutants. The activities of PI and PIP kinases was severely reduced in *cyr1-2* and *ras2* mutants but was high in *ras1 ras2 bcy1* mutants. Other experiments have shown that an antibody to PIP_2 inserted into yeast inhibited growth and PIP_2 turnover to IP_3, and diacylglycerol (DAG) was found to be essential for mitosis (Uno *et al.*, 1988). Cyclic AMP added to a *cyr1* mutant resulted in an increase in the rate of PI synthesis.

Further evidence to suggest that cAMP has a connection with the phosphoinositide signal transduction pathway is that multicopy plasmids expressing the *PFY* gene, which encodes profilin and binds phosphoinositides, can suppress morphological and nutritional defects of *cap* cells (adenylyl cyclase is not bound to the membrane in these cells) (Vojtek *et al.*, 1991). This may be due to a restoration in the integrity of the plasma membrane or this may indicate that Cap (and cAMP) also has a role in the phosphoinositide signal transduction pathway.

Evidence to provide a link between the cAMP and Ca^{2+} pathways is not in great supply. It has been reported that the activation or inactivation of calcium influx depends on the addition or removal of glucose (Eilam and Othman, 1990), the concentration of which cAMP is an indicator. Also, as decreases in Ca^{2+} concentrations result in decreases in cAMP concentrations, there is thought to be some degree of cross-talk between the two pathways (Iida *et al.*, 1990).

Cyclic GMP has been found also in *S. cerevisiae* (Thevelein and Beullens, 1985; Eckstein, 1988). The concentration of cGMP is reported to be approximately 10- to 50-fold lower than the concentration of cAMP (Thevelein and Beullens, 1985; Eckstein, 1988). Its concentration is reported to be affected by the concentration of glucose (Eckstein, 1988), but not by nitrogen sources.

1.8. Cyclic AMP and cell cycle analysis.

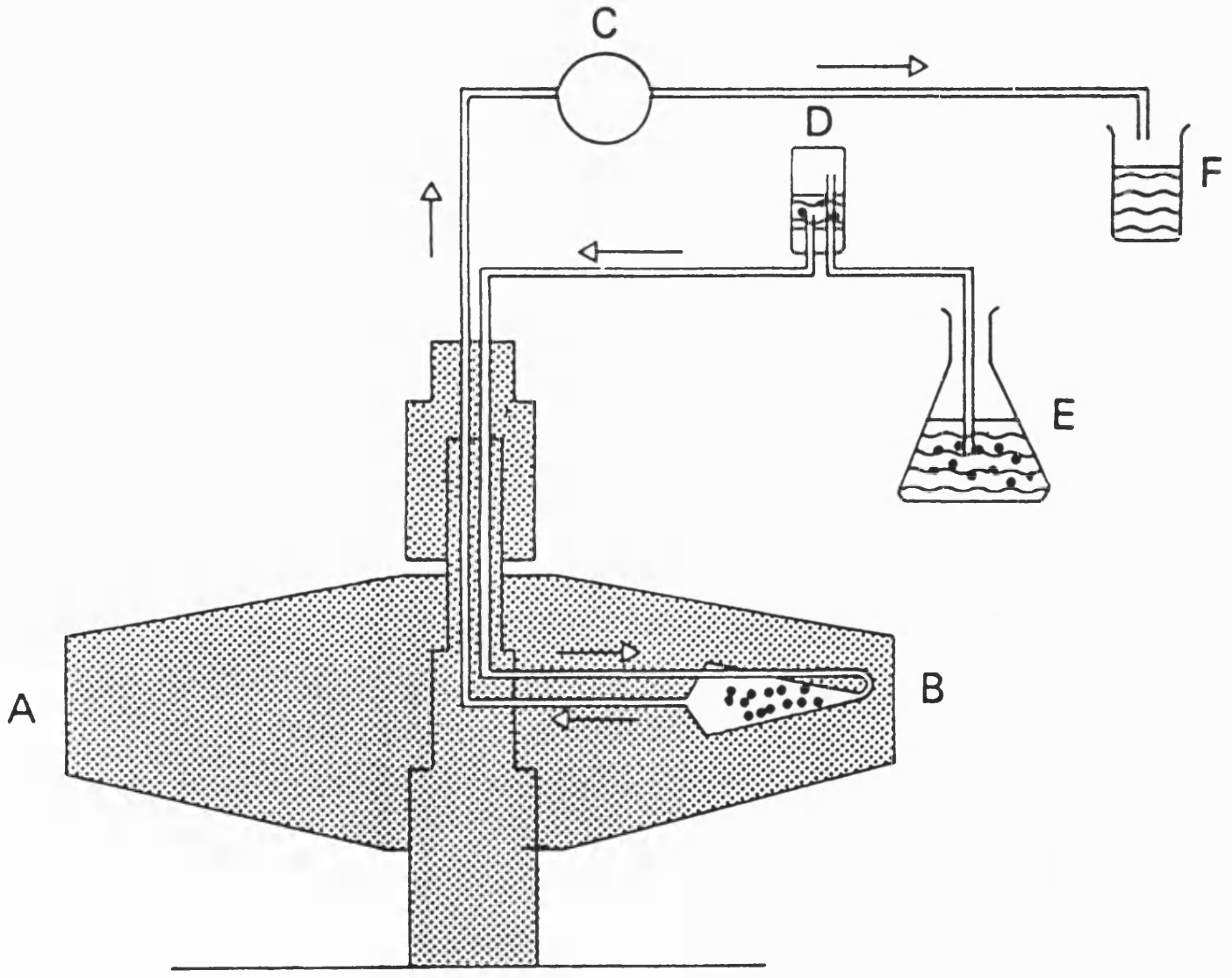
Research concerning cAMP in *S. cerevisiae* has been largely directed towards genetic evidence, with the isolation of genes and their sequences being determined rather than activities and concentrations of key molecules being measured throughout the cell cycle. It is known that cAMP plays a role in the cell cycle, but the question of when exactly cAMP is required and what happens to cAMP throughout the cell cycle is still to be answered. Do the levels of i-cAMP directly regulate the onset of proliferation or is it a conditional control, with the actual control exerted elsewhere?

Cell cycle-dependent i-cAMP fluctuations have been observed in other organisms (Boynton and Whitfield, 1983), such as diatoms, actinomycetes and *Tetrahymena pyriformis* (Dickinson *et al.*, 1976). However, these and a previous study of cAMP fluctuations in *S. cerevisiae* (Watson and Berry, 1977b), who found cell cycle-dependent fluctuations in the concentration of i-cAMP, used inadequate synchronization procedures.

Proteins have been found in *S. cerevisiae* whose concentrations fluctuate in relation to specific stages of the cell cycle. Many of the genes that are involved in DNA synthesis are expressed under cell cycle control (for a review see Johnston and Lowndes, 1992). As cAMP is involved in the regulation of the cell cycle, it may be that the i-cAMP concentration fluctuates similarly.

In order to study cell cycle stage-specific fluctuations in the concentration of i-cAMP, methods that select for a particular fraction of cells were used: synchronous cultures that are produced by selection methods are preferred to those created by induction methods as they do not interfere with the metabolic state of the cell and do not cause serious perturbations. *cdc* mutants that arrest at points of interest in the cell cycle were used in order to map the concentration of i-cAMP throughout the cell cycle and to reinforce observations obtained when using centrifugal elutriation (Figure 5; Creanor and Mitchison, 1979; Van Doorn *et al.*, 1988b), which is the preferred method for synchronization.

Figure 5. Diagram of the centrifugal elutriation rotor. A, rotor; B, rotor cell; C, peristaltic pump; D, bubble trap; E, culture flask; F, effluent flask. Reproduced from Creanor and Mitchison (1979). Briefly, 4 l of an early exponentially growing culture of *S. cerevisiae* (E) is loaded into a Beckman JE10X elutriator rotor (A, B) in a Beckman JE-6M centrifuge, maintained at 25°C. Cells should be loaded into the rotor cell as quickly as possible and when the rotor cell has filled, and a stable cell front has formed, the rotor speed and the flow rate should be lowered. The small unbudded cells that are expelled from the chamber are used as the synchronous culture (F). The growth medium in which the cells have been growing is continually recycled into the rotor. The remaining cells are removed, and after dilution with GM medium, should be used as an asynchronous control culture.


C
D
F
E
A
B

Centrifugal elutriation is the preferred method for synchronization as cells are kept in their growth medium at their growth temperature and the 'selection' of the smallest unbudded cells, which comprise the synchronous culture, occurs by selection rather than induction. Related methods such as velocity sedimentation in zonal rotors, velocity separation in tubes and continuous flow centrifugation, can cause perturbations because cells are taken out of their growth medium, are bathed in sucrose or lactose and may not be kept at a constant growth temperature, and may not give sufficient yields. The disadvantages with centrifugal elutriation are that the equipment is expensive and yields may be lower than by using velocity sedimentation from a zonal rotor.

Cell cycle stage-specific fluctuations in the activities of some enzymes have been seen (Van Doorn *et al.*, 1988a,b). However, these fluctuations were found to disappear when the concentration of protein in some of the samples, in particular those at both ends of the gradient, were taken into account. It was found that there were differences in the specific activities of the enzymes when the extracts were incubated in unusually low or high concentrations of protein (De Koning *et al.*, 1991). This should not be a problem for cAMP determinations as the concentration of i-cAMP is not dependent on the protein concentration of the cell harvest.

1.9. Aims of research.

Cyclic AMP is present in living tissues at extremely low concentrations, therefore a sensitive and reliable assay method has to be used. The most common and reliable method to achieve this is an immunoassay that employs a specific binding protein, which is competed for by *bona fide* cAMP. The most widely used method of cAMP binding protein isolation is that of Brown *et al.* (1971, 1974); the binding protein is also commercially available as the BDH cAMP binding protein; the cAMP assay kit that can be obtained from Amersham International plc also uses a similar binding protein. The binding

protein that is used in the assay protocol is isolated from bovine adrenal glands and is not affected by the addition of adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), 3',5'-cyclic thymidine (cTMP) and 2',3'-cAMP at a concentration of 30 nmol/assay tube; cyclic inosine monophosphate (IMP), cGMP, cCMP and cyclic uridine monophosphate (cUMP) cross-react slightly, although the binding protein has 10-1000 times less affinity for cGMP. The probability that these nucleotides are present in *S. cerevisiae* at such concentrations is very small. Only cGMP has been detected (Thevelein and Beullens, 1985; Eckstein, 1988) and its concentration is reported to be 10- to 50-fold lower than the i-cAMP concentration, thus not sufficient to interfere with the binding protein.

Once the assay method for cAMP had been developed for use in *S. cerevisiae*, experiments were performed that would enable examination, and perhaps clarification, of the role that cAMP has in the control of proliferation, growth and catabolite repression. For this, i-cAMP concentrations were obtained comparing time and various types of growth medium, including rich and minimal, and fermentative and non-fermentative carbon sources.

The possible existence of cell cycle stage-specific fluctuations in the i-cAMP concentration was also examined by using centrifugal elutriation and *cdc* mutants. Observations from these experiments would show whether the concentration of i-cAMP fluctuates during particular stages of the cell cycle and if these fluctuations could control traverse of Start.

Although e-cAMP has been previously detected in the growth medium of *S. cerevisiae* (Eraso and Gancedo, 1984; Olempska-Beer and Freese, 1987), its role has not been extensively considered. Experiments were also undertaken in order to test the possibility that e-cAMP functions as a control mechanism for excess i-cAMP and whether its presence affects the rate of proliferation.

CHAPTER 2.

METHODS AND MATERIALS.

2.1. List of strains.

Saccharomyces cerevisiae.

D1: *MATa/MATα ade1/ADE1 ade2/ADE2 ura1/URA1 his7/HIS7 lys2/LYS2 tyr1/TYR1 gal1/GAL1 MET3/met3 LEU2/leu2*

A364A: *MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1*

GR57: *MATa cdc5-1 his6 ura1 leu2*

JL138: *MATa cdc28-D1 ade1 ade2 ura1 his7 lys2 tyr1 gal1*

DJ23-3C: *MATα his3 leu2 ura3 trp1 ade8 pde1::LEU2 pde2::URA3*

SP1: *MATa his3 leu2 ura3 trp1 ade8 can1*

S7-7A: *MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*

S18-1D: *MATα his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1*

GR2: *MATa his6 ura1*

GR6X-7A: *MATα ade2 ura1*

GRD-7A: *MATa/MATα his6/HIS6 ura1/ura1 ade2/ADE2*

2.2. Maintenance of strains.

All chemicals were analytical grade and purchased from BDH unless otherwise stated.

2.2.1. Storage of strains.

Short-term storage of strains involved maintenance on agar plates made of suitable media for up to 1 month (see section 2.2.2). Strains could be kept on agar slopes of suitable media for up to 6 months. In both cases storage was at 4°C. Strains were stored long-term on glycerol slopes (per litre: 0.63 g K_2HPO_4, 0.18 g KH_2PO_4, 45 mg sodium citrate, 9 mg $MgSO_4$, 90 mg

$(NH_4)_2SO_4$, 20 ml glycerol and distilled water to 50 ml), made according to the following method. A fresh culture of the strain to be stored was grown overnight in suitable growth medium to a cell density of 1-2 x 10^6 cells/ml. 1 ml of culture was added to a small vial containing 1 ml of glycerol mixture. The vials were quickly frozen by immersion in liquid nitrogen. For use, vials were rapidly thawed at 37°C. Strains were checked frequently to test for strain-specific auxotrophic markers.

2.2.2. Media.

Yeast strains were grown on two basic types of medium: rich [YEP plus carbon source, per litre: 10 g yeast extract (Difco), 20 g Bacto-peptone (Difco), 20 g D-glucose or other carbon source, 0.1 g adenine (Sigma), 0.1 g uracil (Sigma), 0.1 g supplements for auxotrophic markers, 20 g agar (Difco; for solid plates)] and minimal [M plus carbon source, per litre: 5 g $(NH_4)_2SO_4$, 1.67 g yeast nitrogen base (w/o amino acids; Difco), 20 g D-glucose or other carbon source, 0.1 g supplements for auxotrophic markers, 20 g agar (for solid media plates)]. Cultures were grown in conical flasks containing suitable medium and the volume of the medium was 40% of the total volume of the flask. Cultures were shaken in a shaking water bath at a speed of 120 rev/min at 25°C unless otherwise stated.

2.3. Cell counting and sizing.

2.3.1. Determination of cell numbers using a particle counter.

In experiments performed at the University of Wales, College of Cardiff: 250 μl of culture were taken and added to 5 ml of a 30% solution of Isoton II (Coulter Electronics Ltd) in filtered distilled water with 50 ml of formaldehyde added per litre. Solutions could be kept in this solution for at least 2 days without shrinkage of cells. Prior to cell counting a suitable amount of the cell solution was added to 20 ml of Isoton II. Cell numbers were obtained by the use of a

Coulter Counter model ZM. An orifice with a diameter of 100 μm was used. In experiments performed at the University of Bath: cells were counted by essentially the same method, except that cells were counted with a Particle Data Electrozone and an LTSIII counter and analyser, and a 70 μm orifice was used.

2.3.2. Determination of cell numbers using a haemocytometer.

Samples were diluted in the same concentration of Isoton II and formaldehyde as in section 2.4.1., but cells were counted under a microscope using and haemocytometer chamber and a x40 objective. The total number of small squares counted per sample was 40. The total number of cells from the 40 squares was multiplied by 1 x 10^5 to give the final concentration of cells per ml.

2.3.3. Fluorescent staining of cell nuclei.

A stock solution of 4,6-diamidino-2-phenylindole (DAPI; Sigma) was made up to a concentration of 1 mg/ml in distilled water and kept at -20°C. At all times it was protected from the light. 1 ml of cell culture to be stained was pipetted into a microfuge tube and spun briefly at 13,000 r.p.m. The supernatant was discarded and 100 μl of 95% ethanol were added to the cell pellet. Cells were fixed in ethanol for 5 min, after which DAPI stock solution was added to a concentration of 1 μg/ml. Cells could be left in this solution for a few hours at room temperature in darkness. 200 cells were recorded and examined for DNA distributions (unbudded cells, budded cells with a single nucleus, cells with migrating nuclei and binucleate cells).

2.4. Quantification of cellular protein.

2.4.1. Sample preparation.

Samples prepared by the Braun homogenization were added directly to the assay, diluted appropriately. Proteins precipitated by treatment with 8%

trichloroacetic acid (TCA) were resolubilized by boiling in 0.2 M NaOH for 10 min according to Matsumoto *et al.* (1983b).

2.4.2. Assay for protein.

Protein was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin (BSA; Sigma) at a concentration of 1 mg/ml as the standard. A standard curve was constructed using concentrations of BSA ranging from 10-200 μg/ml. Reagent A contained 2% (w/v) Na_2CO_3 in 0.1 M NaOH; reagent B contained 1% sodium potassium tartrate in 0.5% $CuSO_4$; reagent C contained 1 ml of reagent B plus 49 ml of reagent A; reagent D contained Folin and Ciocalteu's phenol reagent diluted 1:1 with distilled water.

Briefly, 200 μl of sample or standard were mixed with 1 ml of reagent C. This was incubated for 10 min at room temperature and then 100 μl reagent D were added. Samples were incubated for a further 30 min at room temperature and then the absorbance at 750 nm was measured.

2.5. Construction of synchronous cultures by centrifugal elutriation and age fractionation.

This method has been previously described by Creanor and Mitchison (1979) and White *et al.* (1986). Briefly, 4 l of an early exponentially growing culture (A_{600nm} 0.3) of *S. cerevisiae* was loaded into a Beckman JE10X elutriator rotor in a Beckman JE-6M centrifuge, maintained at 25°C. Cells were loaded into the rotor cell as quickly as possible and when the rotor cell had filled, and a stable cell front had formed, the rotor speed and the flow rate were lowered. The growth medium in which the cells had been growing was continually recycled into the rotor.

The small unbudded cells that were expelled from the chamber were used as the synchronous culture. The remaining cells were removed and after dilution with GM medium used as an asynchronous control culture. Both the

synchronous and asynchronous cultures were transferred to conical flasks and reincubated at 25°C. Samples were taken at specific time intervals for cell numbers, i-cAMP and e-cAMP.

For age fractionation, the cells were loaded at a rotor speed of 4500 r.p.m and at a flow rate of 150 ml/min. These speeds were lowered to 2800 r.p.m. and 100 ml/min for removal of fractions. The flow rate was increased by approximately 3 ml increments until all cells had been washed out of the rotor cell. Samples for cell numbers, DAPI staining, i-cAMP and e-cAMP were taken immediately after elution.

2.6. Temperature shift-up experiments.

A 600 ml culture of *S. cerevisiae* was grown in suitable medium overnight at 25°C to an approximate A_{600nm} 0.1. This was then split into two parts, one was kept at 25°C (the permissive temperature) and the other was transferred to the restrictive temperature of 36.5°C. Samples were taken immediately and at specific time intervals for cell numbers, budding index and i-cAMP measurements.

2.7. Cyclic AMP assay.

2.7.1. Introduction.

The method chosen was based on that of Brown *et al.* (1971, 1974). The assay is based on the competition between labelled and unlabelled (standard or sample) cAMP for binding sites on the regulatory subunit isolated from bovine adrenal glands. After a suitable period of incubation charcoal is added to remove all unbound material. In order to determine the amount of cAMP present in a particular sample, the resulting supernatant after the addition of charcoal is counted by liquid scintillation: the amount of cAMP present is inversely related to the c.p.m. To obtain an actual concentration of cAMP, the c.p.m. value of the blank control is subtracted from the sample value, then this value is divided

by the total control value. This value is then inverted and read against the standard curve, which is constructed in a similar manner.

2.7.2. Preparation of assay constituents.

Preparation of the binding protein and buffers used in the assay were the same as Brown *et al.* (1971, 1974) with modifications.

Binding protein. This was extracted from fresh bovine adrenal glands from which traces of medulla and fat had been removed. The remaining cortices were homogenized with approximately 1.5 vol ice-cold homogenate buffer (0.25 M sucrose, 50 mM Tris-HCl pH 7.4, 25 mM potassium chloride and 5 mM magnesium chloride). The homogenate was then spun at 2000 *g* for 5 min, and the supernatant was spun at 5000 *g* for 15 min. The supernatant from this was stored as 1 ml aliquots at -20°C. Any subsequent dilution of the binding protein was by using assay buffer (50 mM Tris-HCl pH 7.4, 8 mM theophylline and 6 mM 2-mercaptoethanol).

The optimal concentration of binding protein for use in the assay was determined to ensure maximal sensitivity. (This was the concentration at which approximately 50% of the radioactive cAMP was bound.)

Charcoal suspension. The charcoal suspension was made up to 10% in assay buffer and then spun for 1 min at 10,000 *g*. The supernatant was discarded and the pellet was then made up to 10% in assay buffer containing 2% BSA (crystallized, lyophilized and globulin free; Sigma). Aliquots were stored at -20°C and were not reused after defrosting. During the assay the charcoal suspension was kept on ice.

[8-^{3}H]cAMP. For each assay, 0.5 μl of stock [8-^{3}H]cAMP (Amersham) was added to 1 ml of assay buffer in lieu of a stock [8-^{3}H]cAMP solution. From this solution, 50 μl were added to each assay tube.

2.7.3. Assay protocol.

50 μl of either a known standard or an unknown sample were added to 150 μl of assay buffer and 50 μl of [8-^{3}H]cAMP. Two control tubes (in duplicate) were set up: total control, which had assay buffer added instead of charcoal, and blank control, which had assay buffer added instead of binding protein. At time zero, 100 μl of binding protein were added, the assay tubes were mixed and then incubated on ice. After 100 min, 100 μl of charcoal suspension were added to eight tubes at a time. The tubes were mixed twice over a 2 min incubation period on ice. Then the tubes were centrifuged in a microfuge for 1 min at 13,000 *g*. 150 μl were then taken from the supernatant and mixed with 3 ml of Optiphase Safe (LKB).

2.7.4. Sample preparation for cAMP assays.

For i-cAMP assays, per sample, 30 ml of cell culture were harvested by rapid filtration using 0.45 μm pore size cellulose acetate filters (Sartorius) and washed with distilled water. It was found that potential contamination by e-cAMP was very slight and was only 4.926 $\pm$ 1.25 fmol (obtained from 10 samples). After collection, the cells were mixed with 500 μl of 8% TCA in a 1.5 ml microfuge tube and incubated on ice for 90 min. This mixture was freeze-thawed three times before spinning in a microfuge for 3 min at 13,000 r.p.m. The sediment was used in protein assays (see 2.4.1.). The supernatant was transferred to a 10 ml Quickfit tube and washed five times with 2 vol of water-saturated diethylether. The washed supernatant was transferred to a 2 ml microfuge tube, freeze-dried and reconstituted in 200 μl of assay buffer.

For e-cAMP assays, the filtrate from a cell culture was used. This was collected before washing with distilled water.

2.7.5. Evaluation of the assay for cAMP.

Enzymatic degradation of *bona fide* and sample cAMP by phosphodiesterase would verify whether it was cAMP that was being assayed.

A suitable dilution of phosphodiesterase (3',5'-cyclic nucleotide 5' nucleotidohydrolase; Sigma) that is capable of degrading approximately 80% of cAMP present, in buffer G (0.1 M glycyl glycine pH 7.5, 0.15 mM $MgSO_4$) was added to 1 ml of 100 pmol/ml. *Bona fide* cAMP (Sigma) or sample extract were also in buffer G. Tubes were incubated along with control tubes containing no phosphodiesterase at 25°C for a few hours. Every hour a 100 μl sample were taken from each tube and boiled for 3 min to destroy any remaining phosphodiesterase activity. Each sample was assayed for cAMP, using standards that were made in buffer G.

Control experiments using various dilutions of known amounts of cAMP were also performed to establish the validity of the cAMP assay.

2.8. Purification and assay of yeast phosphodiesterases.

2.8.1. Isolation of both yeast phosphodiesterases.

A 1 l culture of *S. cerevisiae* was grown in appropriate medium and harvested by centrifugation for 1 min at 10,000 g. After discarding the supernatant 5 ml of breakage buffer [50 mM Tris-HCl, pH 7.4 (Sigma), 1.0 mM ethylene diamine tetraacetic acid (EDTA; Sigma), 1.0 mM 2-mercaptoethanol (Sigma), 0.5 mM phenylmethylsulphonyl fluoride (PMSF; Sigma)] were added to the cell pellet. Cells were broken by Braun homogenization with 425-600 μm diameter glass beads (Sigma). Broken cells were spun for 10 min at 1000 *g* and the supernatant was spun for 30 min at 20,000 *g*. The supernatant was applied to a 20 cm DEAE-cellulose (Merck) column equilibrated in breakage buffer. Samples were eluted with buffer P and 0.5 M NaCl at a flow rate of 1 ml/min and with two linear gradients (50% of 0.5 M NaCl was reached after 124 min and 100% after 164 min). Each fraction had a volume of 4 ml.

2.8.2. Assay for phosphodiesterase.

To 800 μl of sample, 10 μl of 0.02 M cAMP, 10 μl [8-^{3}H]cAMP and 100 μl buffer P (46 mM $MgCl_2$, 0.37 M Tris-HCl, pH 8.0, 34.5 mM 2-mercaptoethanol and 23 mg/ml BSA) were added. This mixture was incubated at 30°C for 30 min before boiling for 2.5 min to destroy phosphodiesterase activity. After the mixture had cooled, 100 μl (1 mg/ml) of King Cobra venom (*Ophiophagus hannah*; Sigma) in 0.1 M Tris-HCl pH 7.5 were added. Tubes were re-incubated at 30°C for 10 min. To stop the reaction, 1 ml of 1:1 AG1-X8 (200-400 mesh; Sigma) anion exchange resin-water solution was added, mixed thoroughly and then the tubes were spun in a microfuge. 500 μl supernatant were mixed with 3 ml of Optiphase Safe and counted in a liquid scintillation counter. As a background control, duplicate tubes were set up containing buffer P instead of sample. As a total count control, duplicate tubes were set up containing buffer P instead of sample and 1 ml of distilled water was added instead of the resin-water mixture.

2.9. Identification of extracellular phosphodiesterase.

A culture of *S. cerevisiae* was grown to an A_{600nm} 0.2 in GM medium. 1 ml samples of the culture were taken at various times and harvested by filtration through sterile Acrodisc filters (0.45 μm pore size). Each sample was added to 100 μl of 10 μM cAMP in 10x buffer G pH 8 and incubated at 30°C for 120 min. A control was set up by using 1 ml of fresh sterile glucose medium and adding this to the cAMP in 10x buffer G. After specific times, samples were taken for cAMP measurements.

2.10. Construction of a pH range in glucose minimal medium.

2,2-dimethylglutaric acid (DMG; Aldrich) was used as a buffer in order to construct a pH range in GM medium between pH 3-7.5. DMG was added to a

concentration of 0.1 M in 50 ml GM medium. This was adjusted with 1 M NaOH to the desired pH and then diluted to 100 ml. Cells were inoculated into flasks containing medium at specific pH values and also a control culture containing no DMG. At specific times, samples were taken for extracellular pH, e-cAMP measurements and A_{600nm} values.

2.11. Identification of e-cAMP using high performance liquid chromatography.

2.11.1. Sample preparation.

Culture of *S. cerevisiae* was grown to an approximate A_{600nm} 1.0 in either GM or YEPD medium made up with milli-Q water. Cells were filtered through 0.45 μm cellulose acetate filters and the filtrate was used for HPLC chromatography. Samples were diluted in an appropriate amount of 7 mM KH_2PO_4 (HPLC grade; Sigma) before applying to the column.

2.11.2. Nucleotide separation using high performance liquid chromatography.

Nucleotides were separated by injecting a 20 μl sample and eluting with 7 mM KH_2PO_4 (HPLC grade; Sigma), adjusted to pH 4.0 with H_3PO_4, for 15 min. A strong anion exchange column (Partisil 10-SAX, 25 cm x 4.6 mm) was used in conjunction with a guard column (Partisil 10-SAX, 2.5 cm length). The column was regenerated by elution with 0.5 M KH_2PO_4 pH 3.3 for 10 min and 7 mM KH_2PO_4 for 15 min. The column was washed with 20 μl of HPLC grade methanol (Aldrich) after every chromatogram to remove organic impurities.

2.12. Rapid transformation of *Escherichia coli*.

For this, strain DH5α was used (Sambrook *et al.*, 1989). This method is described in Sambrook *et al.* (1989). The following modifications were used after step 10. 1 ml of prewarmed LB broth was added to the cells and tubes

were then placed at 37°C for 2 h to allow expression. Aliquots were then spread onto selective plates.

2.13. Extraction of DNA from *E. coli.*

Plasmid DNA was extracted from DH5α transformed in 2.12. This method is also described in Sambrook *et al.* (1989).

2.14. Lithium acetate transformation of *S. cerevisiae.*

A culture was grown to stationary phase in YEPD medium before inoculation into 50 ml of fresh YEPD; this was grown at 30°C until an A_{600nm} 1.5-2.0 was reached. Cells were harvested by centrifugation in a bench centrifuge for 10 min at 4,000 r.p.m. The pellet was first washed with 10 ml of TE buffer, pH 7.5 and re-centrifuged before being resuspended in 5 ml of 0.1 M lithium acetate in TE and incubated with agitation for 1 h at 30°C. Cells were centrifuged as before and the pellet was resuspended in an equal volume of 0.1 M lithium acetate in TE. To 300 μl of competent cells, 1-10 μg of DNA was added, isolated by the method in 2.13. Tubes were then left at 30°C for 10 min. 1 ml of a 50% solution of polyethylene glycol (PEG 4000) was then added and tubes were inverted gently three times. Tubes were then incubated for 1 h at 30°C. After this incubation, cells were heated for 5 min to 42°C and then centrifuged in a microfuge by pulsing gently six times. The pellet was washed with 1 ml of water and centrifuged as before. The pellet was resuspended in 0.5 ml of YEPD for 15 min at 30°C. Cells were plated onto selective plates of GM medium. Transformants appeared after 2-3 days.

CHAPTER 3.

RESULTS.

3.1. The cAMP assay.

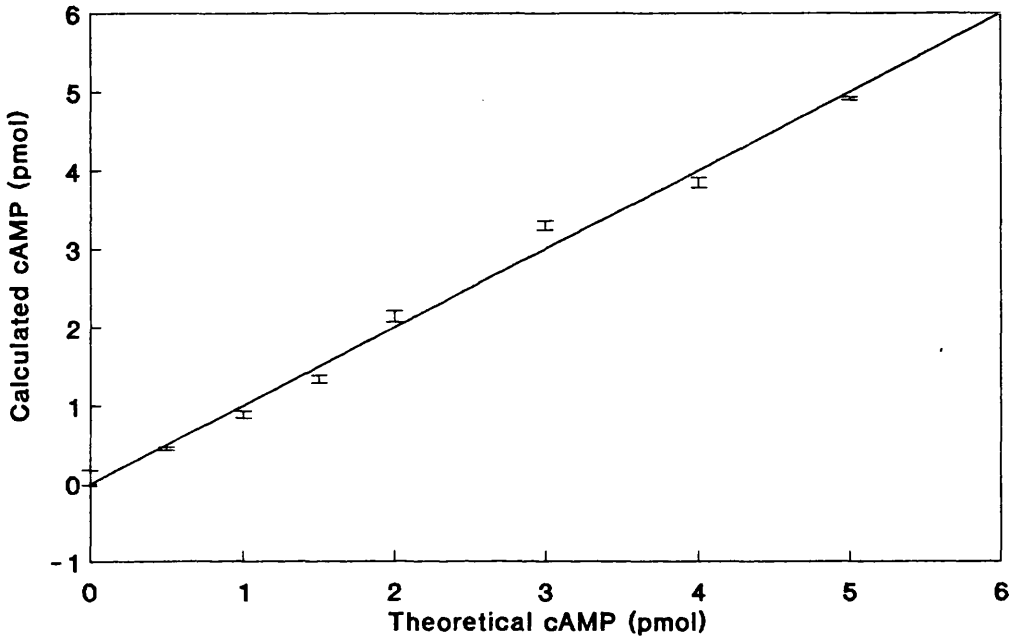
3.1.1. Introduction.

In order to evaluate the cAMP assay method of Brown *et al.* (1971, 1974), various checks had to be made to ensure that values represented true concentrations of i-cAMP in *S. cerevisiae*; this series of checks included rigorous examination of the reproducibility of the standard curve and ensuring that the concentrations of cAMP obtained from the standard curve were not artefactual.

3.1.2. Standard curve.

The binding protein that was to be used in the assay had to be tested in order to determine whether a sensitive and reproducible standard curve could be obtained. Not all of the bovine adrenal glands that were obtained performed satisfactorily; adrenal glands obtained from two abattoirs in Avon gave substandard binding protein, which resulted in a non-existent standard curve; whereas binding protein that had been isolated from adrenal glands obtained from an abattoir in South Glamorgan (coincidentally the same as that used by Amersham), gave sensitive and reproducible standard curves. A typical standard curve from this work is shown in Figure 6. The standard deviation from the mean values obtained gave percentage errors of 3.9 $\pm$ 3.0% (obtained using 28 samples from four standard curves n = 46). The percentage error decreased with increasing concentration of cAMP and the scintillation counter had an error of 1.6 $\pm$ 1.5%. By assay, the percentage recovery of cAMP after freeze-drying alone was 79.5 $\pm$ 8.1%, and after freeze-drying and ether extraction was 70.1 $\pm$ 6.2%; by weight, 89.4 $\pm$ 2.8% of cAMP was recovered after ether extraction.

Figure 6. A typical standard curve for the assay of cAMP according to the method of Brown *et al.* (1971, 1974). Duplicate tubes were used for all standards, except 0 pmol, which was in quadruplicate. The error bars represent the range of cAMP that was measured from all tubes. The assay for cAMP was performed as described in section 2.7.


Calculated cAMP (pmol)
6
5
4
3
2
1
0
-1
0
1
2
3
4
5
6
Theoretical cAMP (pmol)

3.1.3. Charcoal absorption capacity.

By adding increasing concentrations of cAMP to blank control tubes (see Materials and methods), the cAMP-absorbing capacity of the charcoal could be determined. The absorption capacity of the charcoal was found to be at least 60 pmol/50 μl (Table I), far greater than any concentration of cAMP that was present in assays. Thus, it can be concluded that the amount of charcoal that was used in the assay, had the capacity to absorb any unbound cAMP.

3.1.4. Dilution of sample cAMP.

To determine whether the concentration of cAMP measured in assays was accurate and that there were no interfering substances present in the extracted sample, serial dilutions of samples were made (Table II).

Results show that the concentrations of cAMP measured closely reflected the successive dilutions of samples and the assay was accurately measuring cAMP and there were no artefacts. There were slight differences between the final concentrations of successive dilutions of the same sample, resulting in an average variation of 12 $\pm$ 6%.

3.1.5. Phosphodiesterase degradation of cAMP.

The final test of whether the assay for cAMP was really measuring cAMP and not artefacts, involved the use of the phosphodiesterase enzyme, which degrades cAMP to 5'-AMP. A known amount of phosphodiesterase was added to tubes containing a known amount of cAMP. By previously assaying the sample and obtaining the specific activity of phosphodiesterase, it was possible to calculate the amount of enzyme that needed to be added in order to degrade approximately 80% of the cAMP. Table III shows that phosphodiesterase degraded both *bona fide* and sample cAMP, with all six samples showing degradation. The *bona fide* cAMP samples had a lower percentage degradation

Table I. The absorption capacity of charcoal used in the cAMP assay.

Amount of cAMP added (pmol)	% c.p.m.[a]
0 (total)	100.0
0 (blank)	1.5
10	1.6
20	1.4
40	1.3
60	1.3

[a]This value is the percentage of c.p.m. of [^{3}-H]cAMP that the charcoal did not bind. The results suggest that the charcoal bound as much [^{3}-H]cAMP in the tubes that had the largest amount of [^{3}-H]cAMP than in the blank control tubes. The data shown here represent the mean values of one experiment.

Table II. The effect of dilution upon cAMP measurements.

	Dilution				
Sample	None	1/2	1/4	1/8	1/16
1	2.1	2.2	2.3	2.4	2.5
2	1.0	1.1	1.2	-	-
3	0.9	1.3	1.2	-	-
4	2.7	3.0	-	-	-
5	2.0	2.0	-	-	-
6	2.1	2.2	2.7	-	-
7	1.76	-	1.8	-	-

The data presented here represent the amount of cAMP measured when multiplied by the dilution factor. Samples for cAMP assays were obtained from cultures of D1 cells grown at 25°C in GM medium at various A_{600nm} and serially diluted.

- represents not determined.

Table III. Phosphodiesterase degradation of bona fide and sample cAMP.

Sample	cAMP/50 μl assayed at time (h)			Percentage degraded
	0	4	4[a]	
A[b]	4.6	2.6	4.3	43.5[d]
B[b]	5.2	1.9	-	63[d]
C[b]	5.0	2.0	-	60[d]
D[c]	4.2	0.6	4.1	85
E[c]	5.3	0.7	4.5	88
F[c]	5.1	0.8	4.7	85

The amount of cAMP present in the assay tubes was comparable to the K_m of the low K_m phosphodiesterase at the beginning of the experiment.

[a]This is material that has been incubated for 4 h but has had no phosphodiesterase added.

[b]*Bona fide* cAMP.

[c]Sample cAMP.

[d]Percentage degradation was lower than the predicted 80% as the incubation reaction with phosphodiesterase was designed to occur over a longer period of time.

- represents not determined.

as these experiments were designed to degrade 80% over a longer period of time.

3.2. Intracellular cAMP concentrations in batch cultures.

3.2.1. Introduction.

Prior to determining whether there were cell cycle stage-specific fluctuations in the concentration of i-cAMP, it was necessary to examine the behaviour of i-cAMP concentrations with particular reference to time, the proliferation rate and growth of cells, and glucose concentration.

3.2.2. Intracellular concentrations of cAMP from cells grown on glucose.

Figure 7 shows that in an exponentially growing asynchronous culture, as the cells were growing and proliferating from lag to exponential phase through to stationary phase utilizing glucose as the carbon source throughout, the i-cAMP concentration fell with time. Throughout both lag and exponential phases the concentration of i-cAMP fell continually. Throughout stationary phase the concentration of i-cAMP remained constant. Figure 7 also shows that although the proliferation rate remained constant throughout exponential phase, the i-cAMP concentration fell.

3.2.3. Intracellular concentrations of cAMP in different strains.

In order to determine whether it would be possible to compare results from various experiments, it was necessary to compare the i-cAMP concentrations within a family of closely related strains, according to the volume and the ploidy of the cell, and between various unrelated strains. GR2 and GR6X-7A are haploids and are closely related to GRD7A, which is a diploid resulting from the mating of the two haploids. D1, JL138, GR57 and A364A are unrelated strains. The results of this comparison are shown on Table IV.

Figure 7. The relationship between the concentration of i-cAMP and cell density with time from a wild type strain of *S. cerevisiae*. Cells from a stationary culture of D1, which had been growing in GM medium at 25°C, were inoculated into fresh GM medium at t = 0, grown at 25°C in a shaking water bath and sampled at the time intervals indicated. Cell numbers and i-cAMP concentrations were measured as described in sections 2.3 and 2.7. All points represent the average of two values, the error bars represent the range of i-cAMP concentration obtained. Closed circles, cells/ml; open circles, i-cAMP/cell.

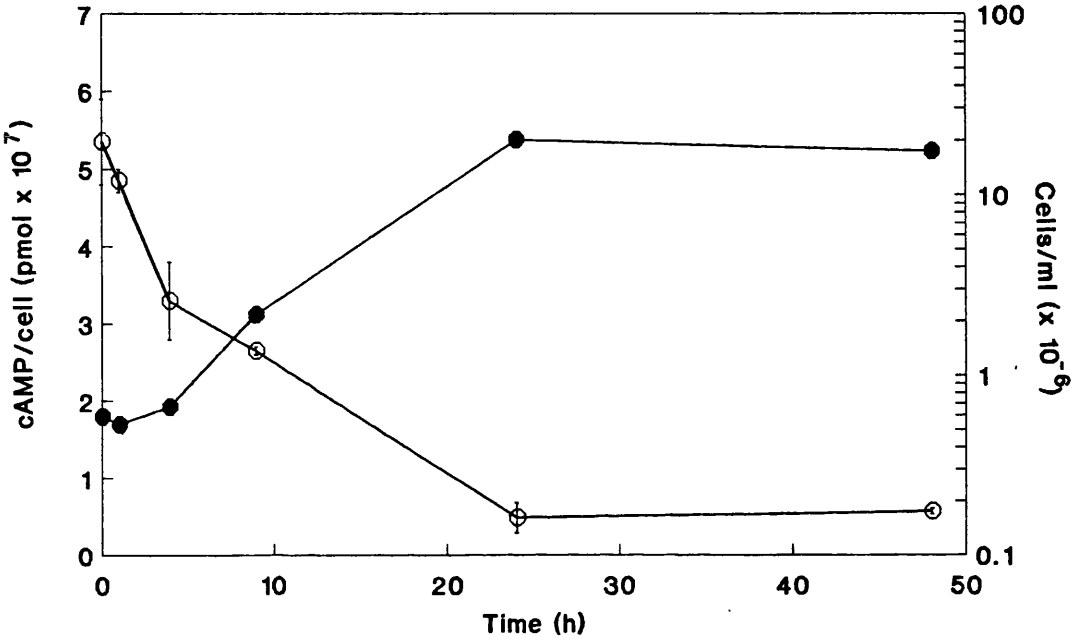

cAMP/cell (pmol x 10^7)
Cells/ml (x 10^-6)
Time (h)
0
1
2
3
4
5
6
7
0.1
1
10
100
0
10
20
30
40
50

Table IV. Comparison of i-cAMP concentrations between related and unrelated strains.

Strain	Cells/ml (x 10^5)	i-cAMP (pmol/ml)	Modal cell volume (μm^3)	[cAMP] (μM)
GR2	9.64	0.29 ± 0.03	77	3.9
GR6X-7A	13.2	0.30 ± 0.06	84	2.8
GRD7A	10.6	0.30 ± 0.01	149	1.9
JL138	10.7	0.17 ± 0.02	54	2.9
GR57	9.72	0.35 ± 0.04	76	4.7
A364A	13.8	0.20 ± 0.06	-	-
D1	12.7	0.26 ± 0.05	58	3.5

The data represented here were obtained from two (GR2, JL138, A364A and D1), three (GR6X-7A and GRD7A) or four (GR57) separately grown cultures. Cultures were grown in YEPD at 25°C and harvested as close to an A_{600nm} of 0.1 as possible. The micromolar concentration was calculated by multiplying the number of cells/l by the cell volume, and then dividing that value into the concentration of i-cAMP/l culture.

- represents not determined.

Referring to the related strains, when the concentration of i-cAMP was expressed per ml of culture harvested, concentrations between strains were very similar. When the concentration of i-cAMP was expressed as a function of volume, however, differences were apparent: the i-cAMP concentration was inversely related to the cell volume. This indicated that between related strains, the i-cAMP concentration was independent of cell volume, but not cell density.

When comparing the i-cAMP concentration per ml of culture harvested between all strains, a wider range (0.17-0.30) was observed. This represented a 23% variance compared with 2% between the related strains. The i-cAMP concentrations, when expressed as a molar concentration, were similar to those of the related strains, and varied within a similar range to the cAMP/ml values.

To summarize, the data in Table IV demonstrate that differences between unrelated strains exist, but differences are within a 23% variance band and are therefore not considered to be high enough to be significant. Also, when the i-cAMP concentration was expressed as a micromolar concentration, it was independent of the volume of the cell. The data do not indicate that a universal, absolute i-cAMP concentration was present.

3.3. Comparisons of i-cAMP concentrations from cells grown on rich or minimal media, and on different carbon sources.

There have been various reports stating that i-cAMP concentrations varied depending on the carbon source used and the concentration of the carbon source present in the growth medium (see Chapter 1). In an attempt to clarify the inconsistencies between these reports, a series of experiments was performed that enabled analysis of i-cAMP concentrations in rich and minimal media, with either fermentable or non-fermentable carbon sources.

3.3.1. Comparisons of i-cAMP concentrations on rich and minimal media.

In order to determine whether there were significant differences in the i-cAMP concentration depending on whether cells were grown on rich or minimal media, a wild type strain was grown on rich and minimal glucose-based media.

The i-cAMP concentrations (Table V) obtained from one experiment were subjected regression analysis and a pooled *t*-test (this tested the constants and the gradients of the regression co-efficients), and it was found that there was no significance difference between i-cAMP concentrations from rich or minimal media at $P = 0.001$. The value of P gives an indication as to how significant a value is, and refers to the probability of observing a particular (t) value.

When i-cAMP concentrations from cultures grown on rich and minimal media, with both fermentative and non-fermentative carbon sources (Figure 8), were subjected to regression analysis and a pooled *t*-test, there was a significant difference between the constants and the gradients at $P = 0.01$. However, as the analysis may have been affected by differences between the different carbon sources, it was still possible that there were no significant differences between i-cAMP concentrations obtained on rich or minimal media.

3.3.2. Comparisons of i-cAMP concentrations on various carbon sources.

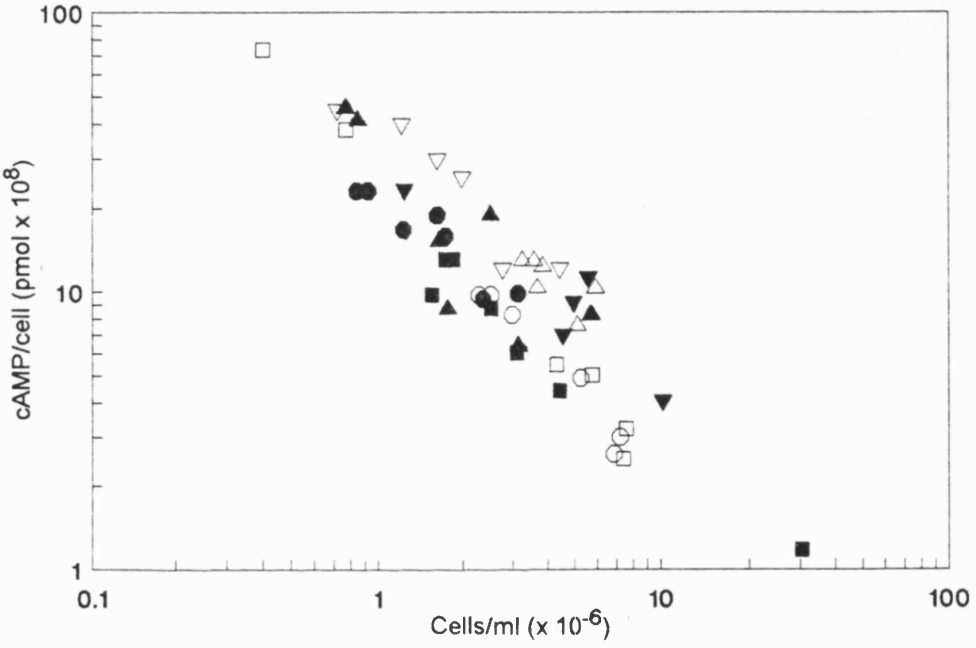
Cyclic AMP is not believed to be associated with catabolite repression in yeast (Eraso and Gancedo, 1984; Matsumoto *et al.*, 1982b), but evidence to prove this is inconclusive. Therefore in order to determine whether i-cAMP concentrations are dependent upon the carbon source present in the growth medium of the cells, experiments were performed to test possible differences in i-cAMP concentrations. Cells were grown in rich and minimal media, with various carbon sources (including glucose, fructose, glycerol and pyruvate).

In order to determine any relationships between fermentable and non-fermentable carbon sources (Figure 8), four experiments were combined as it

Table V. Comparison of rich and minimal medium-grown cells.

Medium	A_{600nm}	i-cAMP (pmol/10 ml)
YEPD	0.22	0.75
	0.18	0.75
	0.59	0.75
	0.50	1.00
	0.86	1.45
	0.98	1.75
	1.80	1.10
	1.78	1.90
GM	0.27	0.50
	0.29	0.70
	0.51	0.80
	0.78	1.25
	1.29	1.60
	1.70	1.50
	2.04	2.20

Figure 8. The relationship between the i-cAMP concentration of cells grown on fermentable and non-fermentable carbon sources, and cell density from a wild type strain of *S. cerevisiae*. Flasks of rich (YEP) or minimal (M) media containing 2% (w/v) of glucose, fructose, glycerol or pyruvate, were inoculated from an exponentially growing culture of strain D1 that had been growing at 25°C in GM medium. The cells were then incubated at 25°C in a shaking water bath. Samples were taken for cell numbers and i-cAMP concentration when the cells had reached early to mid-exponential phase. YEPD, closed circles; GM, open circles; YEPF, closed squares; FM, open squares; YEPG, closed triangles (pointed side up); GlyM, open triangles; YEPP, inverted closed triangles (pointed side down); PM, open inverted triangles. The data plotted here are from four separate experiments.


100
10
1
cAMP/cell (pmol x 10^8)
0.1
1
10
100
Cells/ml (x 10^-6)

was difficult to harvest cultures at the same cell density due to the different generation times that the wild type strain had in different carbon sources.

Cells were grown in a particular medium and harvested in exponential phase. Samples were taken for i-cAMP analysis and cells/ml. The data obtained were plotted (Figure 8). By studying Figure 8, it appeared that there were two separate relationships; one represented cells grown on fermentable carbon sources (lower band of co-ordinates) and the other represented the cells grown on non-fermentable carbon sources (upper band of co-ordinates).

The regression coefficients of both lines were then compared in order to statistically verify whether there was a significant difference between the two relationships. As it was found that there may be significant differences between cells that are grown on rich or minimal medium, the data was split into rich carbon sources and minimal carbon sources. The data were subjected to regression analysis and a pooled *t*-test.

It was found that there were no significant differences between i-cAMP concentrations from rich fermentable and rich non-fermentable carbon sources at $P = 0.01$. However, it was found that there was a significant difference between i-cAMP concentrations from minimal fermentable or minimal non-fermentable carbon sources at $P = 0.001$.

3.4. The relationship between i-cAMP concentrations and cell density.

From the results obtained so far (Figures 7 and 8), it was evident that there was a relationship between cell density and the i-cAMP concentration. Figure 8 shows that there was a very significant relationship between i-cAMP concentration and cell density ($P = 0.000$), and the regression had an R^2-adjusted value of 80.7% (this value indicated how well the data fits the regression co-efficient). Figures 7 and 8 also show that cell density and i-cAMP concentration per cell have an inverse relationship, the higher the i-cAMP concentration the lower the cell density and *vice versa*.

Table VI shows the regression analyses of i-cAMP concentration and A_{600nm}. A_{600nm} reflects cell mass, including cell number and volume, unlike cells per ml, which only reflects the number of cells per ml of culture. All but one of the experiments showed a very significant relationship between A_{600nm} and i-cAMP concentration in both rich and minimal grown cells. However, despite a significant relationship between the tested variables, as shown by *P*, the closeness of fit of the regressions for GR57, D1 and JL138 was poor. The low R^2-adjusted values suggest that there could be some other factor or factors affecting i-cAMP concentrations.

Throughout the experiments described in this section it was assumed that there was no interstrain difference in possible relationships between A_{600nm} or number of cells per ml and i-cAMP per ml of culture; even if there were interstrain differences, different strains will still show similar patterns.

3.5. Shift-up experiments using cell division cycle (*cdc*) mutants.

3.5.1. Introduction.

cdc mutants have a defect in stage-specific functions of cell cycle control and under restrictive conditions, for example an increase in growth temperature from 25°C to 36.5°C, show a stage-specific arrest. The stage at which the original defect occurs is usually different from the stage at which the cell arrests. *cdc* mutants may be useful in attempting to map where any changes in concentrations of cAMP occur in the cell cycle.

Strains used here were: A364A, a *CDC*$^+$ haploid; GR57, a haploid containing the *cdc5-1* mutation, the wild type gene encoding an as yet unknown protein that is essential for sporulation and for transfer of mitochondria during mating; and JL138, a haploid containing the *cdc28-D1* mutation encoding a 34 kDa protein that has kinase activity, associates with cyclins (Wittenberg *et al.*, 1990) and aids the control of Start (Reed, 1980). A functional *CDC28* gene is a prerequisite for progression through Start and entering the cell cycle.

Table VI. Regression analyses between A_{600nm} and i-cAMP per ml of culture.

Strain	Medium	*P* value	R^2-adjusted (%)	*n*
GR57	YEPD	0.029	24.5	18
JL138	YEPD	0.011	44.2	17
DJ23-3C	GM	0.004	86.6	6
D1	YEPD + GM	0.000	64.2	16
DJ23-3C	YEPD	0.002	78.9	8

These strains were chosen because they arrest at points in the cell cycle that were useful for studying cell cycle stage-specific fluctuations of cAMP. Also, they have the execution point of their gene function close to the terminal phenotype and therefore their terminal phenotype represents the part of the cell cycle in which they are defective. *cdc28-D1* arrests at the beginning of the cycle before Start, whereas *cdc5-1* arrests at the end of the cycle near cytokinesis or a late stage of nuclear reorganization.

3.5.2. A364A temperature shift-up.

Strain A364A was used as it does not arrest at 36.5°C and has only a slight increase in cell numbers due to the effect of temperature on the growth rate, and thus any changes in cAMP concentrations at this temperature would be used as a control. Figure 9 shows the concentration of i-cAMP, percentage buds and cell density for a shift-up experiment of A364A. The consistency of the percentage buds throughout the time-course of the experiment demonstrated the ability of A364A to maintain similar growth characteristics at 36.5°C as at 25°C, however, the cell density in the 36°C culture increased slightly. The concentration of i-cAMP at 25°C before shift-up fell as expected and continued to do so in the culture remaining at 25°C. One hour after shift-up, the i-cAMP concentration in the 36.5°C culture was approximately twice that in the culture at 25°C. Two hours after shift-up, the concentration of i-cAMP in the 36.5°C culture began to decrease and resembled that in the 25°C culture.

3.5.3. *cdc28-D1* temperature shift-up.

cdc28-D1 arrested cells are unbudded uninucleate growing cells that shmoo, similar to when cells are subjected to mating pheromones. Figure 10 shows that when JL138 was transferred to 36°C, arrest occurred quickly; both the cell density and percentage buds fell quickly and dramatically in the 36°C culture. Figure 10 also shows that before shift-up and in the culture that remained at

Figure 9. Temperature shift-up experiment of A364A (*CDC*+). Flasks were inoculated with an exponentially growing culture of A364A and grown at 25°C in a shaking water bath until a cell density of approximately 1 x 10^6 cells/ml was reached. Samples were taken for budded index, cell density and i-cAMP concentration. At t = 0, the culture was split into two and one-half was transferred to a shaking water bath at 36.5°C. Each point represents the average value of two measurements, the error bars represent the range of i-cAMP concentration obtained. This figure is representative of three separate cultures performed on two separate occasions. Closed circles, 25°C; open circles 36.5°C; arrow, shift-up time.

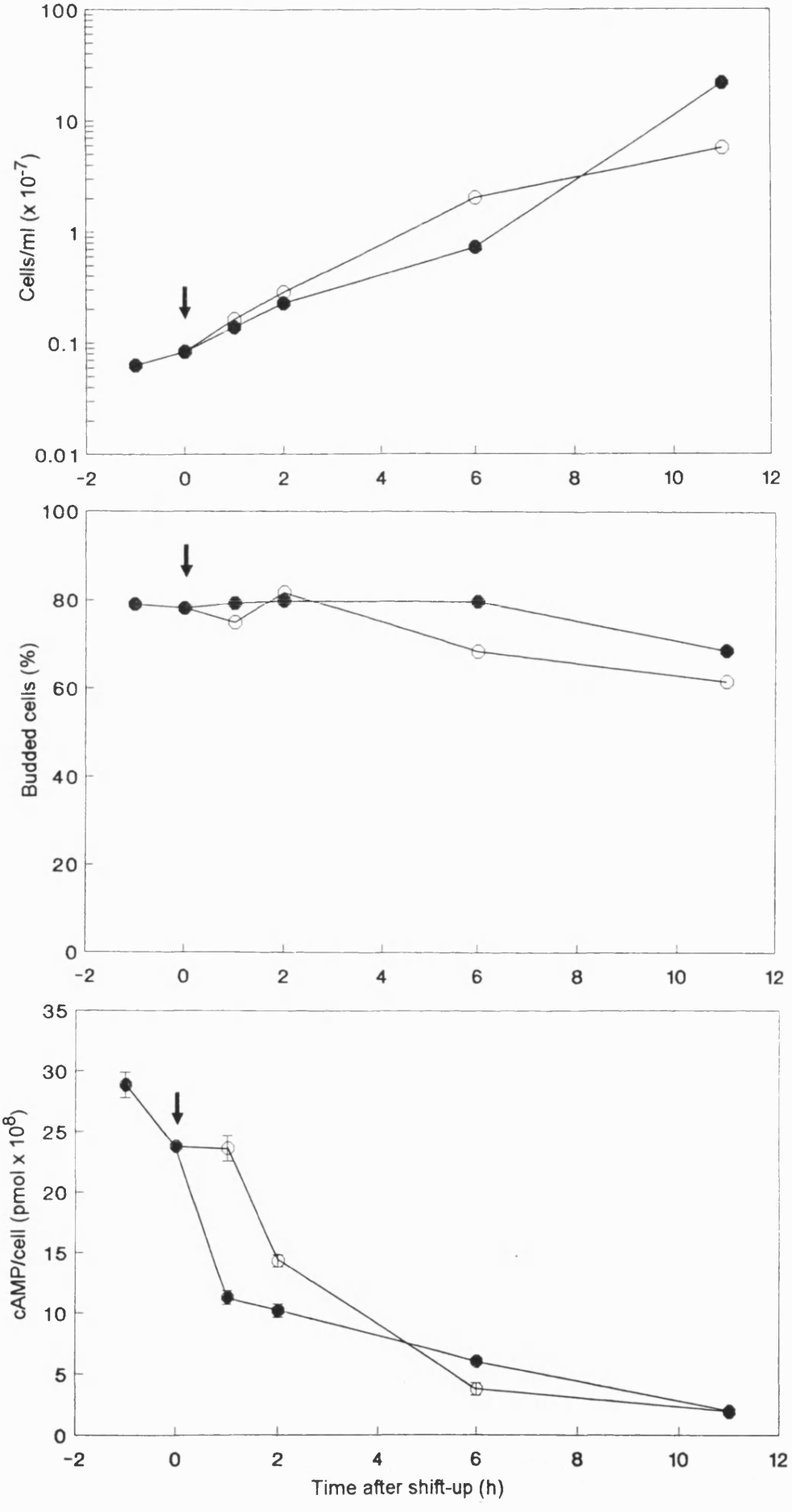
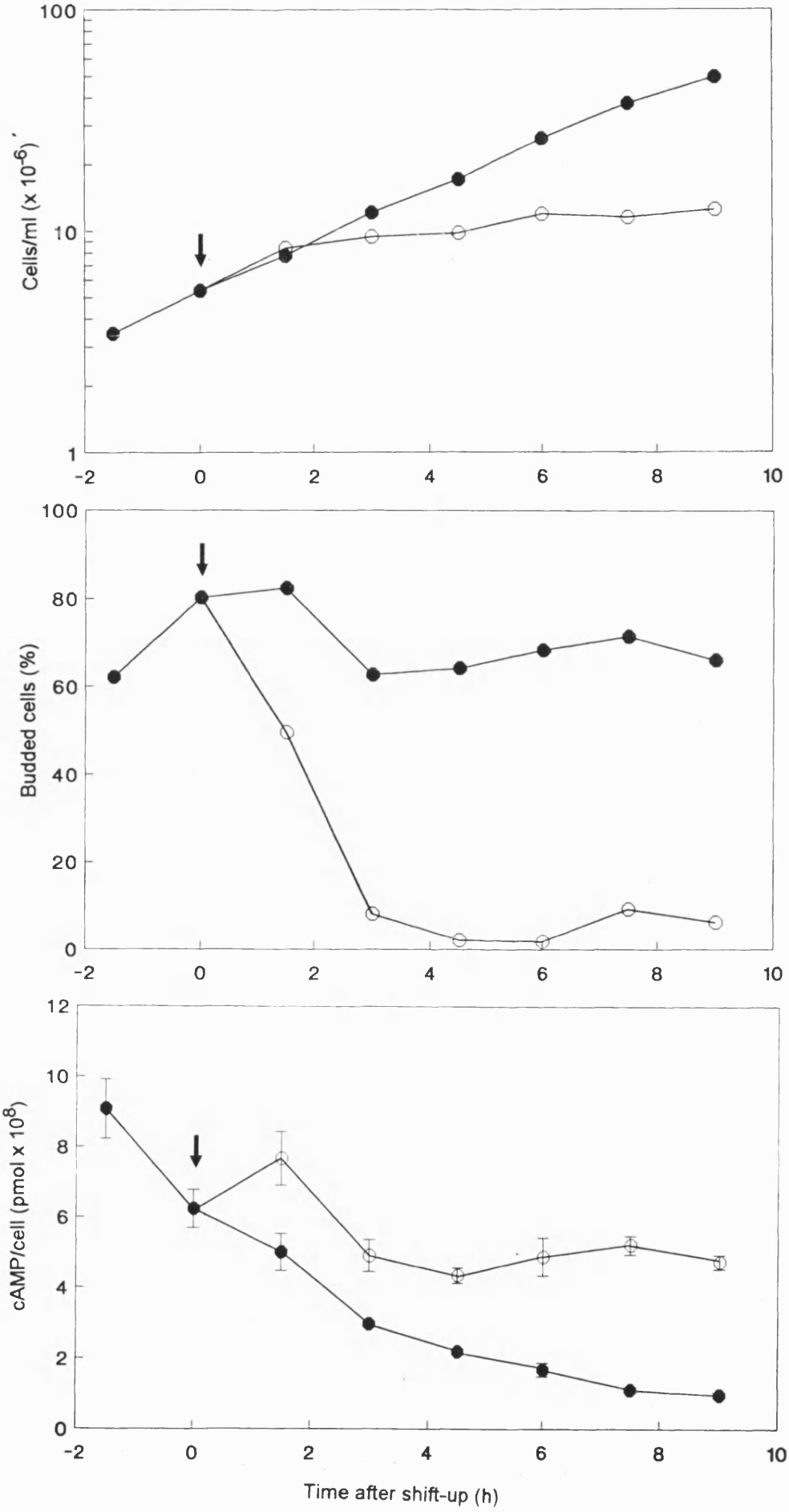

Cells/ml (x 10^-7)
100
10
1
0.1
0.01
-2
0
2
4
6
8
10
12
Budded cells (%)
100
80
60
40
20
0
cAMP/cell (pmol x 10^8)
35
30
25
20
15
10
5
0
Time after shift-up (h)

Figure 10. Temperature shift-up experiment of JL138 (*cdc28-D1*). Flasks were inoculated with an exponenially growing culture of JL138 and grown at 25°C in a shaking water bath until a cell density of approximately 3 x 10^6 cells/ml was reached. Samples were taken for budded index, cell density and i-cAMP concentration. At t = 0, the culture was split into two and one-half was transferred to a shaking water bath at 36.5°C. Each point represents the average value of two measurements, the error bars represent the range of i-cAMP concentration obtained. Closed circles, 25°C; open circles 36.5°C; arrow, shift-up time. This figure is representative of four separate cultures performed on two separate occasions.


Cells/ml (x 10^{-6})
100
10
1
-2
0
2
4
6
8
10
Budded cells (%)
100
80
60
40
20
0
cAMP/cell (pmol x 10^{8})
12
10
8
6
4
2
0
Time after shift-up (h)

25°C, the trends in i-cAMP concentration were similar to those in the wild type, with an increase in the concentration of i-cAMP occurring within the initial 1.5 h. The concentration of i-cAMP in the 36.5°C culture eventually began to fall throughout the time-course of the experiment, but remained at a higher concentration than that of the 25°C culture. Thus, although the cells had ceased to proliferate, cAMP was still being produced or not being degraded. The final concentration of i-cAMP was 5-fold higher in the 36.5°C culture than in the 25°C culture.

3.5.4. *cdc5-1* temperature shift-up.

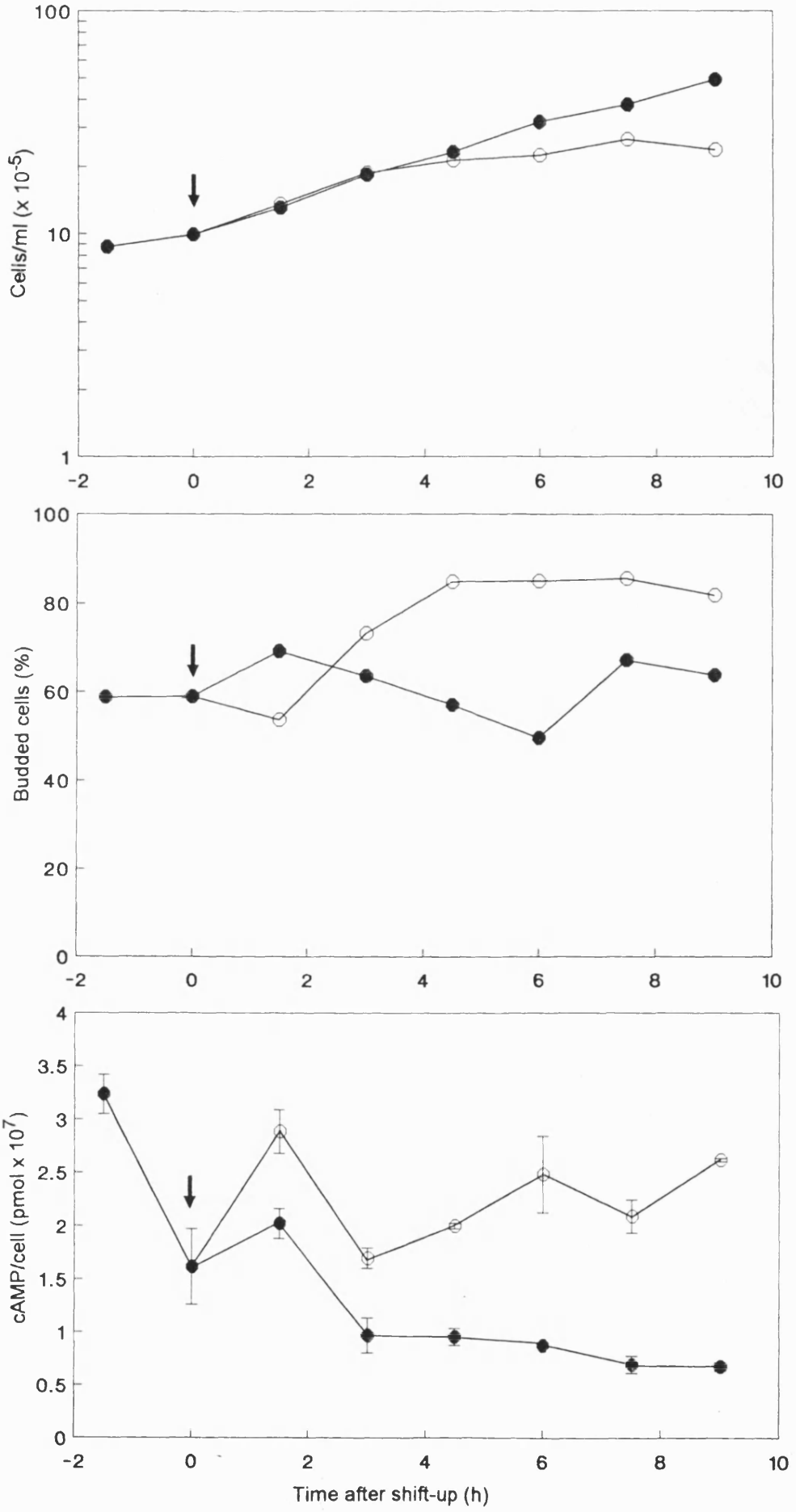
Results similar to those of *cdc28-D1* were found when performing a temperature shift-up on *cdc5-1* cells. Figure 11 shows that after transfer to 36.5°C, the cells began to arrest. This arrest was not as immediate as that observed for JL138, however, the cell density did not increase as much as in the 25°C culture and the percentage buds rose; GR57 arrests with a dumbbell morphology and therefore percentage buds should be higher after arrest. Although the culture at 25°C showed a more erratic trend for percentage buds, the percentage buds for the 36.5°C culture remained higher throughout. Figure 11 also shows that after shift-up, the concentration of i-cAMP increased and remained at a high concentration, 5-fold higher, compared with the concentration from the culture grown at 25°C. However, because GR57 has a longer generation time than JL138, *cdc5-1* cells took longer to arrest.

3.6. Centrifugal elutriation and age fractionation experiments.

3.6.1 Introduction.

In a synchronous culture it should be possible to determine whether there are stage-specific cell cycle fluctuations in the concentration of i-cAMP. For this, a wild type strain (D1) was used. To investigate how the enzyme responsible for the degradation of cAMP behaved during the cell cycle, a double

Figure 11. Temperature shift-up experiment of GR57 (*cdc5-1*). Flasks were inoculated with an exponenially growing culture of GR57 and grown at 25°C in a shaking water bath until a cell density of approximately 1 x 10^6 cells/ml was reached. Samples were taken for budded index, cell density and i-cAMP concentration. At t = 0, the culture was split into two and one-half was transferred to a shaking water bath at 36.5°C. Each point represents the average value of two measurements, the error bars represent the range of i-cAMP concentration obtained. Closed circles, 25°C; open circles 36.5°C; arrow, shift-up time. This figure is representative of four separate cultures performed on two separate occasions.


100
10
1
Cells/ml (x 10^-5)
-2
0
2
4
6
8
10
100
80
60
40
20
0
Budded cells (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
cAMP/cell (pmol x 10^7)
Time after shift-up (h)

phosphodiesterase mutant (DJ23-3C; *pde1 pde2*) was used. If cell cycle stage-specific fluctuations occurred after elutriation of the double phosphodiesterase mutant then any changes in the i-cAMP concentration would have been due to another activity or mechanism, thus demonstrating its presence. Figure 12 shows the phosphodiesterase activities of D1, SP1 and DJ23-3C.

Age fractionation, which utilizes the elutriator rotor to construct a size gradient, was also employed to produce synchronous fractions, but instead of just collecting the smallest unbudded cells, successive fractions of the size gradient were harvested.

3.6.2. Centrifugal elutriation using a wild type strain.

Four separate elutriations were performed on wild type strain D1. This strain is ideal for elutriation purposes as at cell separation the mother and daughter cells readily separate. Studying the plots of cell density and percentage buds, it can be seen that there was good first cycle synchrony, but this had deteriorated by the second cycle (Figure 13). As the time for cell separation to occur was long, daughters became delayed with respect to mothers cell cycle, resulting in a breakdown of synchrony; daughter cells require a longer period of time to traverse Start as they have to grow more. After an initial peak in the concentration of intracellular cAMP in the first three samples, the concentration of cAMP per cell assumed a straight line relationship with time. This relationship, except the initial peak, could also be seen in the asynchronous culture. This inferred that there were no obvious stage-specific fluctuations in the concentration of cAMP.

The second elutriation experiment (Figure 14) showed a slightly improved degree of synchrony compared with the first experiment, with the percentage buds reaching approximately 95% and in the second cycle the percentage buds reached 80%, although after cell separation the percentage buds remained at approximately 45%, which was too high. However, by looking at

Figure 12. The phosphodiesterase activities of D1, SP1 and DJ23-3C. The two separate peaks of activity shown are the low and the high K_m isoenzymes, respectively. 1 l cultures of D1, SP1 or DJ23-3C were grown in YEPD at 25°C in a shaking water bath and harvested in exponential phase. Upper panel: D1, 75 mg. Middle panel: D1, 150 mg. Lower panel: SP1, 60 mg (closed squares) and 40 mg (open squares); DJ23-3C, 60 mg (closed triangles) and 40 mg (open triangles).

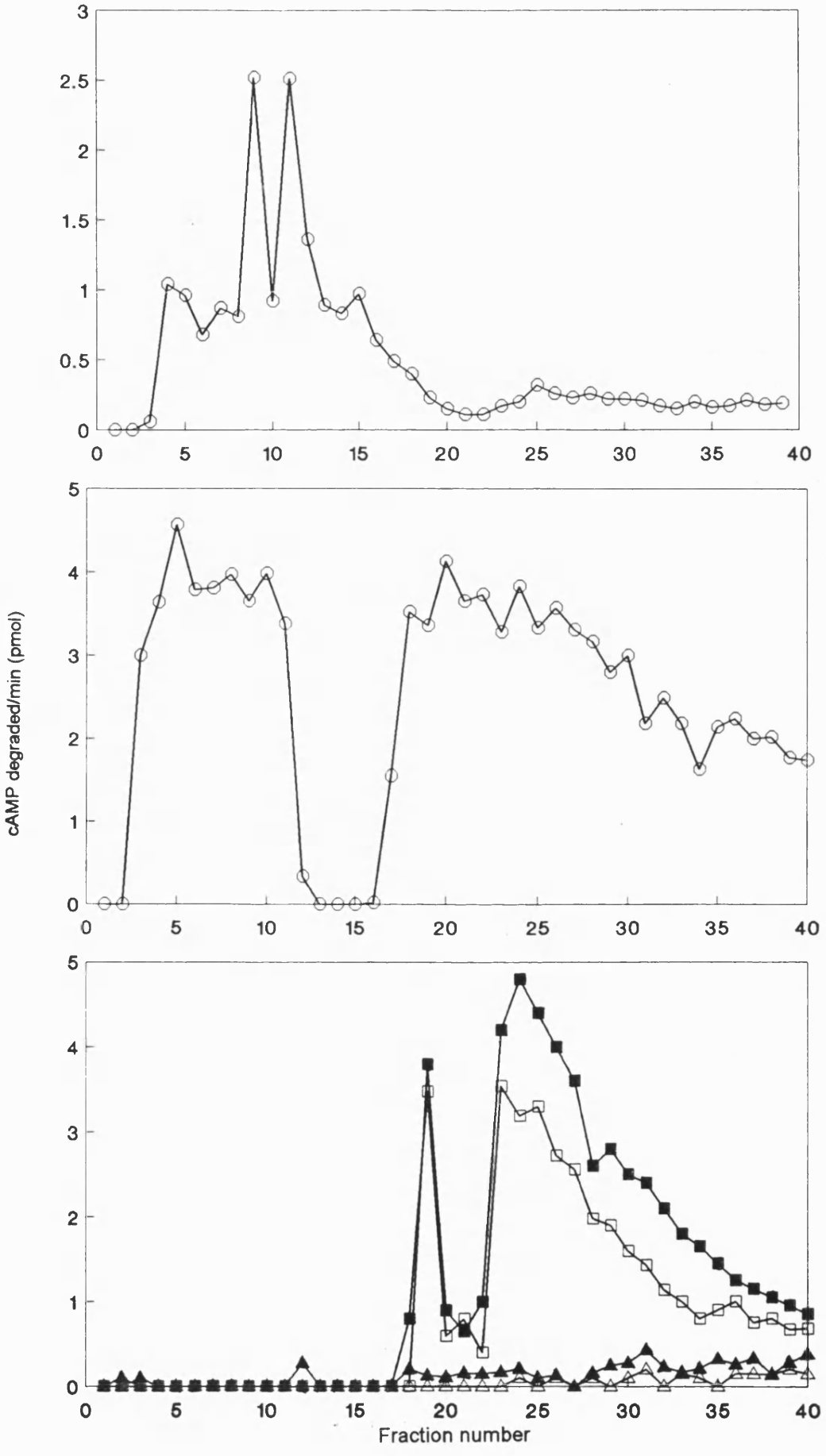

cAMP degraded/min (pmol)
Fraction number

Figure 13. Centrifugal elutriation experiment of a wild type strain of *S. cerevisiae*. Strain D1 was grown at 25°C in GM medium and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 2170 r.p.m. at a flow rate of 68 ml/min and unloaded at 2000 r.p.m. at a flow rate of 56 ml/min. Experimental details are given in section 2.5.

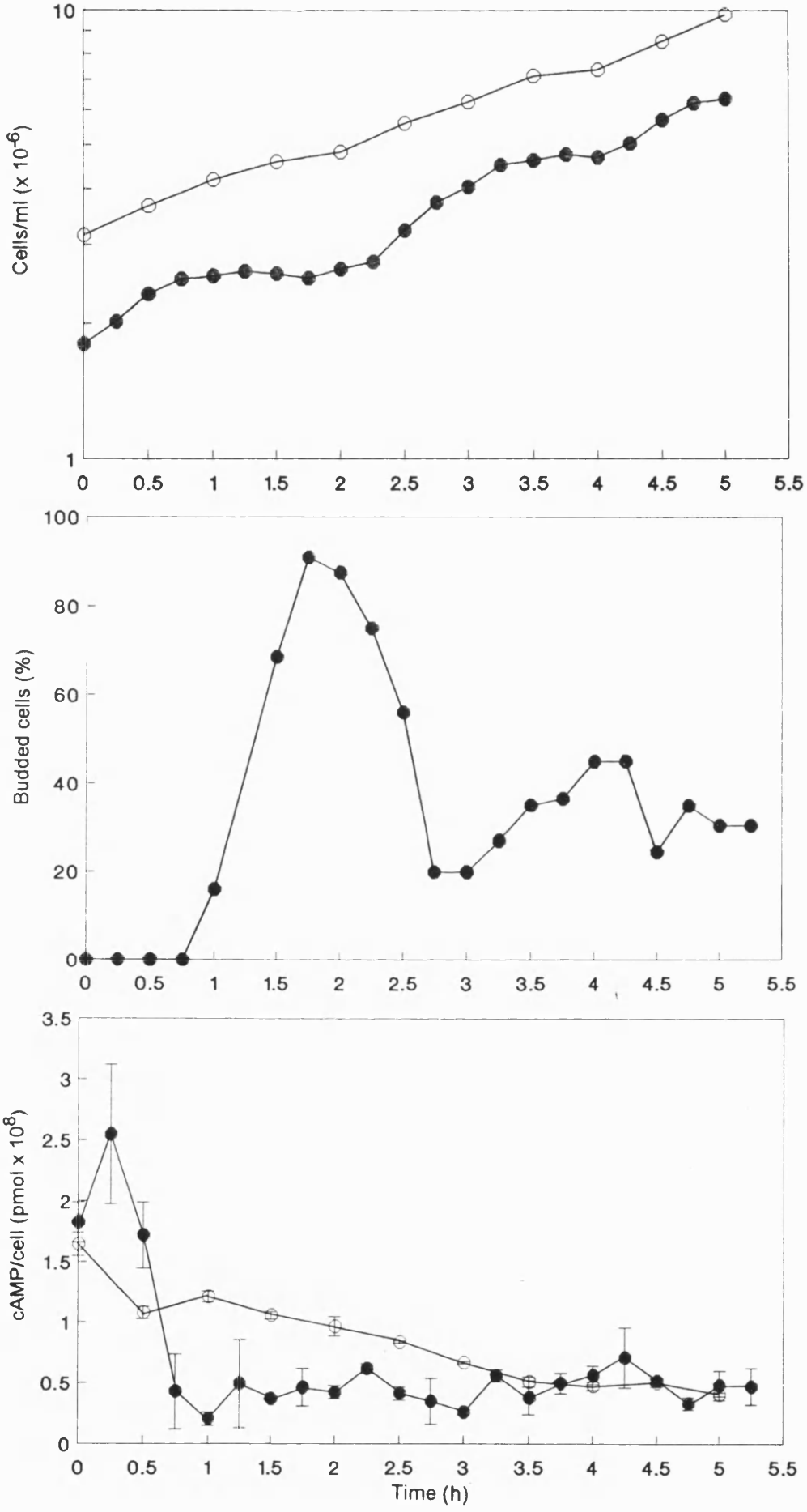
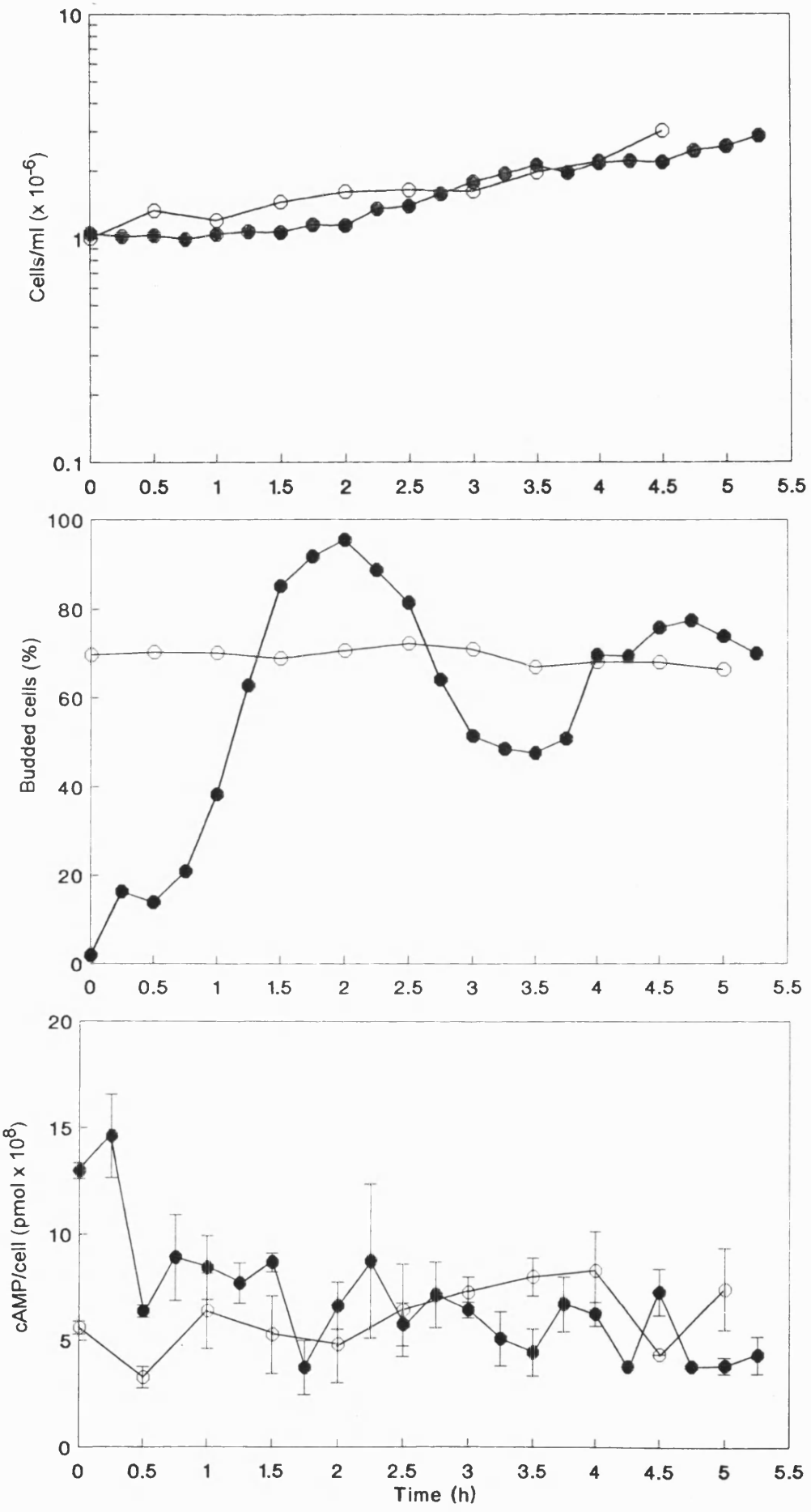

Cells/ml (x 10^-6)
10
1
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Budded cells (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
cAMP/cell (pmol x 10^8)
3.5
3
2.5
2
1.5
1
0.5
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Time (h)

Figure 14. Centrifugal elutriation experiment of a wild type strain of *S. cerevisiae*. Strain D1 was grown at 25°C in GM medium and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 2200 r.p.m. at a flow rate of 70 ml/min and unloaded at 2000 r.p.m. at a flow rate of 70 ml/min. Experimental details are given in section 2.5.


Cells/ml (x 10-6)
10
1
0.1
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Budded cells (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
cAMP/cell (pmol x 10^8)
20
15
10
5
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Time (h)

the cell density plot, it is apparent that the synchronous curve is only slightly different from the asynchronous curve. Slight fluctuations were observed although they were not stage-specific. Unexpectedly, the concentration of cAMP per cell from the asynchronous culture did not follow an expected decline, but rose and fell before the end of sampling.

The third elutriation experiment (Figure 15) gave the best synchrony obtained so far, with a recognizable second cycle. With respect to concentrations of cAMP, the asynchronous culture behaved typically, whilst the synchronous culture had a high concentration of cAMP during the first cell cycle that fell at cell separation and then rose slightly for the second cycle, the rise not being of the same magnitude as the concentration before cell separation.

The fourth elutriation experiment also gave good cell cycle synchrony, but only for one cycle; despite this the results (Figure 16) were in sharp contrast to the previous experiment. For both the synchronous and asynchronous cultures there were no obvious stage-specific fluctuations with both the synchronous and the asynchronous fluctuating haphazardly.

3.6.3. Centrifugal elutriation using a phosphodiesterase mutant.

Problems were encountered when attempting to produce a size gradient in the rotor cell with strains DJ23-3C and SP1, due to the glutinous quality of the cells. Usually at cell separation, the mother and daughter cells fall apart, as demonstrated by D1. Both DJ23-3C and SP1 formed clumps in the rotor cell that resulted in a depletion of newborn cells and unbudded mothers at the top of the rotor cell. Mild sonication slightly improved the situation, but the yield of unbudded cells was still too low. In order to obtain a synchronous culture of sufficient volume, a larger fraction of the size gradient was taken although contamination by budded cells occurred. Due to the inevitable contamination, synchrony broke down rapidly and even a good synchronous first cycle was

Figure 15. Centrifugal elutriation experiment of a wild type strain of *S. cerevisiae*. Strain D1 was grown at 25°C in GM medium and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 2200 r.p.m. at a flow rate of 68 ml/min and unloaded at 2000 r.p.m. at a flow rate of 68 ml/min. Experimental details are given in section 2.5.

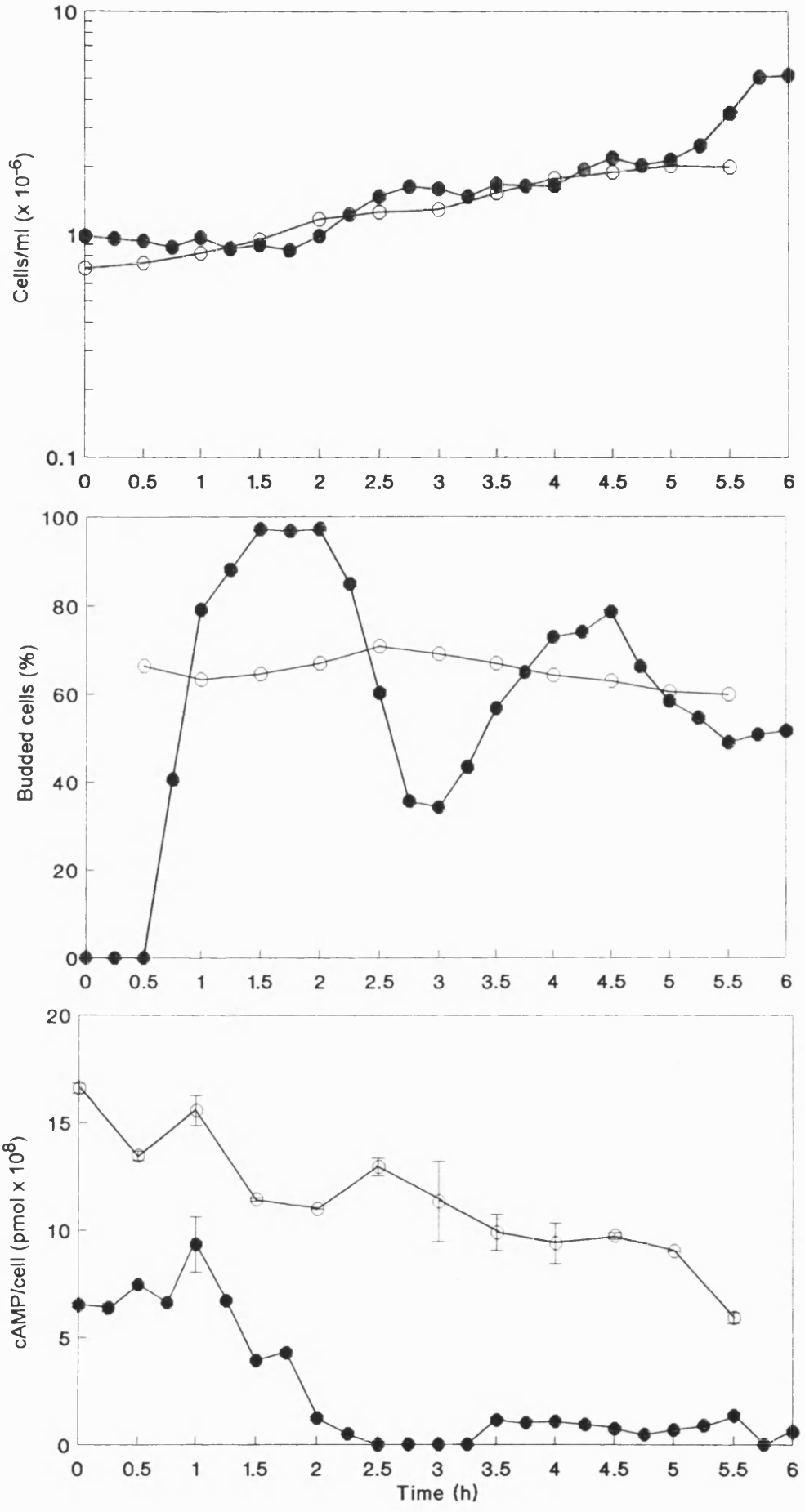
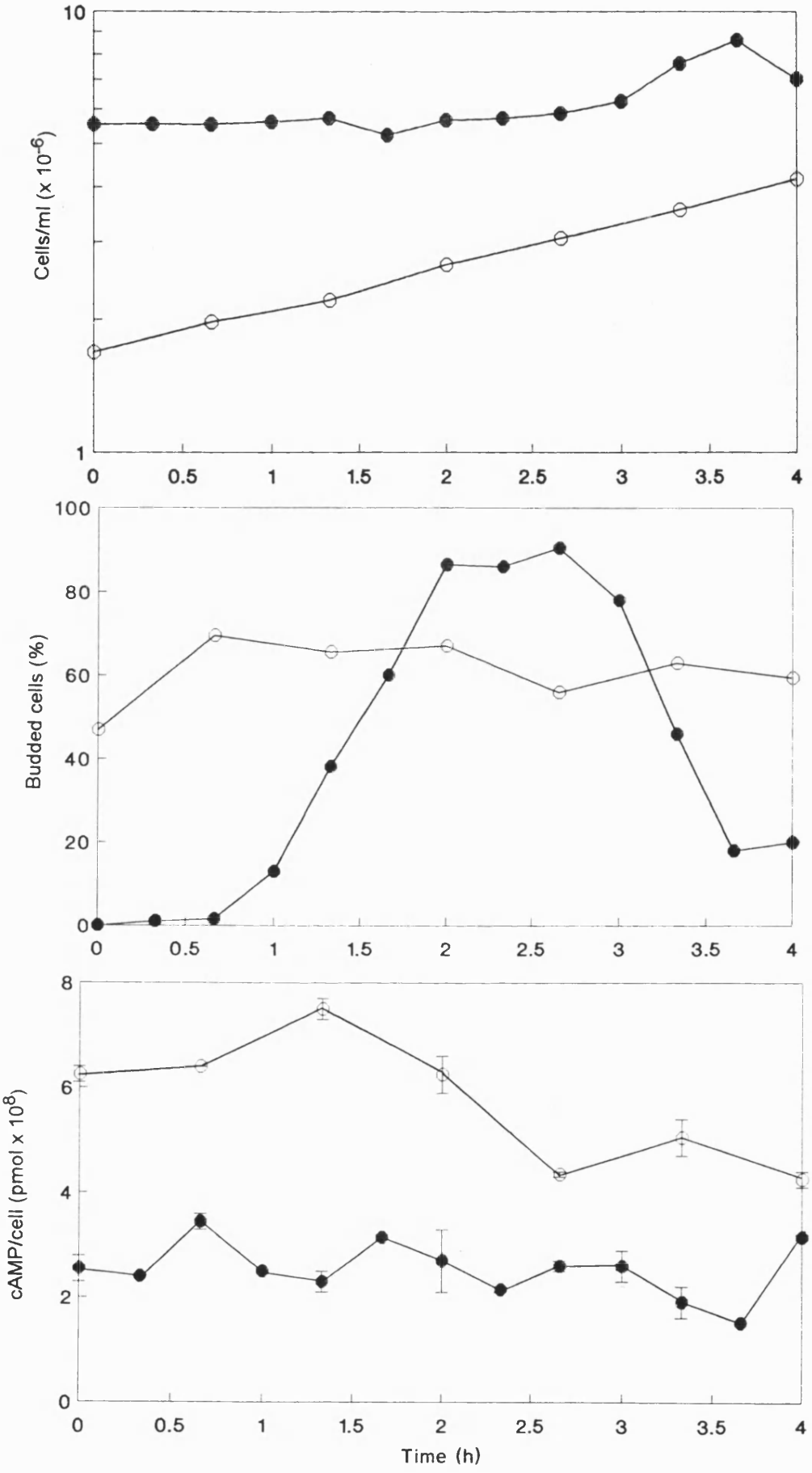

Cells/ml (x 10^-6)
10
1
0.1
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Budded cells (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
cAMP/cell (pmol x 10^8)
20
15
10
5
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Time (h)

Figure 16. Centrifugal elutriation experiment of a wild type strain of *S. cerevisiae*. Strain D1 was grown at 25°C in GM medium and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 4500 r.p.m. at a flow rate of 150 ml/min and unloaded at 3300 r.p.m. at a flow rate of 100 ml/min. Experimental details are given in section 2.5.


Cells/ml (x 10^-6)
10
1
0
0.5
1
1.5
2
2.5
3
3.5
4
Budded cells (%)
100
80
60
40
20
0
cAMP/cell (pmol x 10^8)
8
6
4
2
0
Time (h)

difficult to obtain. Despite these problems, two elutriations with DJ23-3C were analysed.

The first of these experiments is shown in Figure 17. The beginnings of good synchrony, apparent during the first cycle, rapidly broke down to asynchrony for the second cycle. Despite the poor synchrony, a pattern of increasing and decreasing i-cAMP concentration in tandem with the percentage buds emerged. If this pattern was true, then it would be in sharp contrast to D1, in which concentrations of cAMP remained at a high concentration and then dropped, and higher concentrations of cAMP occurred at G_1, not at the end of the cycle. However, the asynchronous control culture showed no fluctuations and fell steadily with time as expected.

Results from the second elutriation (Figure 18) were less promising although the synchrony in the first cycle was similar to the first experiment. Concentrations of cAMP per cell in the asynchronous culture were as expected, but the synchronous culture showed disorderly fluctuations of intracellular cAMP concentrations, unrelated to any stage in the cell cycle. However, synchrony became rather poor so stages were difficult to distinguish. It is notable that despite being unable to distinguish cell cycle stage-specific fluctuations, the concentration of cAMP in the asynchronous culture fell steadily during the course of the experiment whereas the concentration of cAMP from the synchronous culture did not. This may have been partly due to the synchronous culture not dividing synchronously.

3.6.4. Age fractionation of SP1.

Because of the difficulty in the construction of synchronous cultures for either SP1 or DJ23-3C, age fractionation was used.

The beginning of the cell cycle was well fractionated after age fractionation of SP1 (Figure 19). However, due to the original problem of adherent cells, unbudded cells were collected in the latter half of the gradient

Figure 17. Centrifugal elutriation experiment of a double phosphodiesterase mutant. DJ23-3C was grown at 25°C in GM medium plus 2% YEPD and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 4500 r.p.m. at a flow rate of 175 ml/min and unloaded at 3500 r.p.m. at a flow rate of 125 ml/min. Experimental details are given in section 2.5.

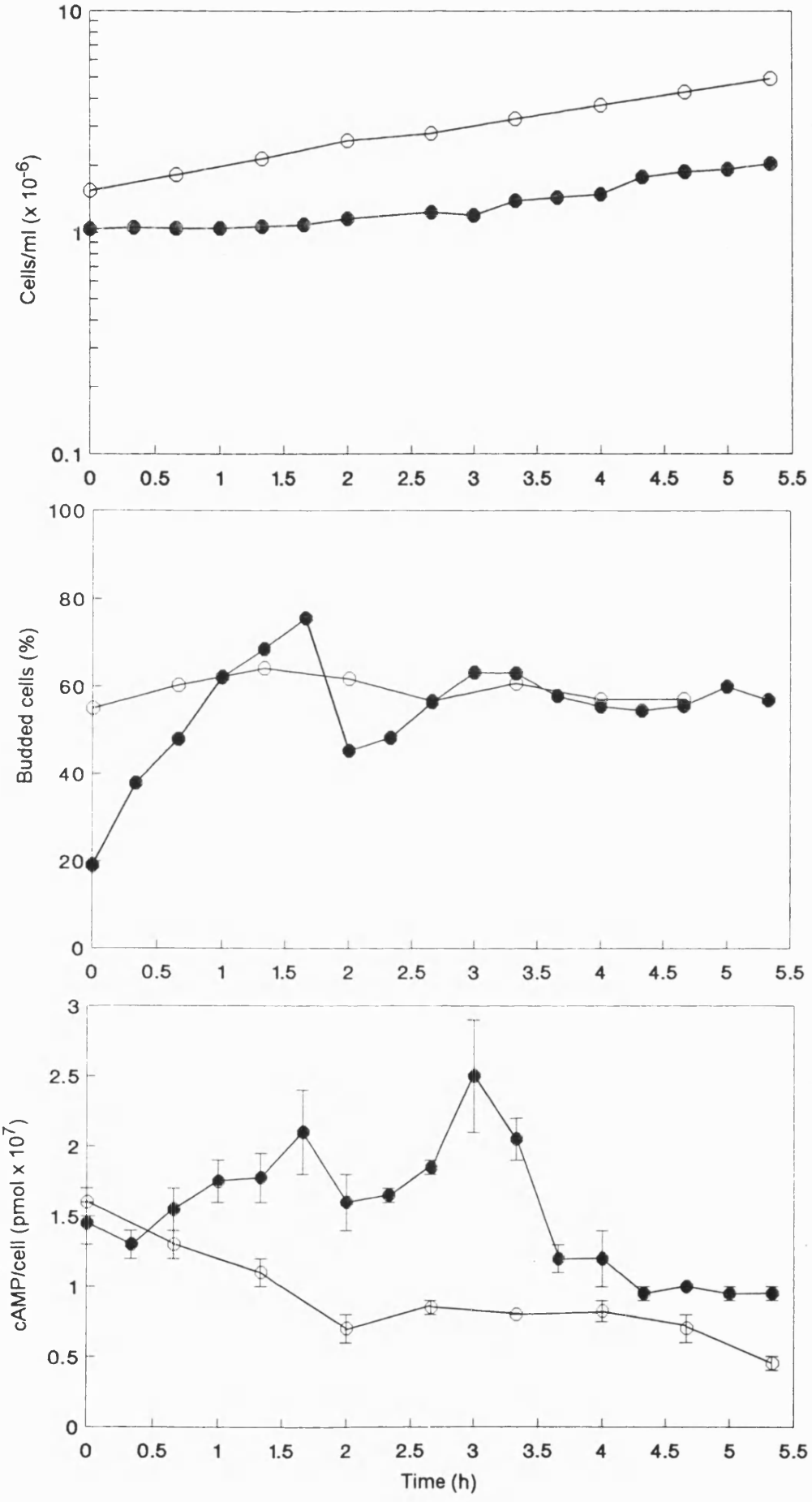

Cells/ml (x 10^-6)
10
1
0.1
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Budded cells (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
cAMP/cell (pmol x 10^7)
3
2.5
2
1.5
1
0.5
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Time (h)

Figure 18. Centrifugal elutriation experiment of a double phosphodiesterase mutant. DJ23-3C was grown at 25°C in GM medium plus 2% YEPD and used to produce the synchronous (closed circles) and asynchronous (open circles) cultures. The cells were loaded at 4500 r.p.m. at a flow rate of 175 ml/min and unloaded at 3500 r.p.m. at a flow rate of 125 ml/min. Experimental details are given in section 2.5.

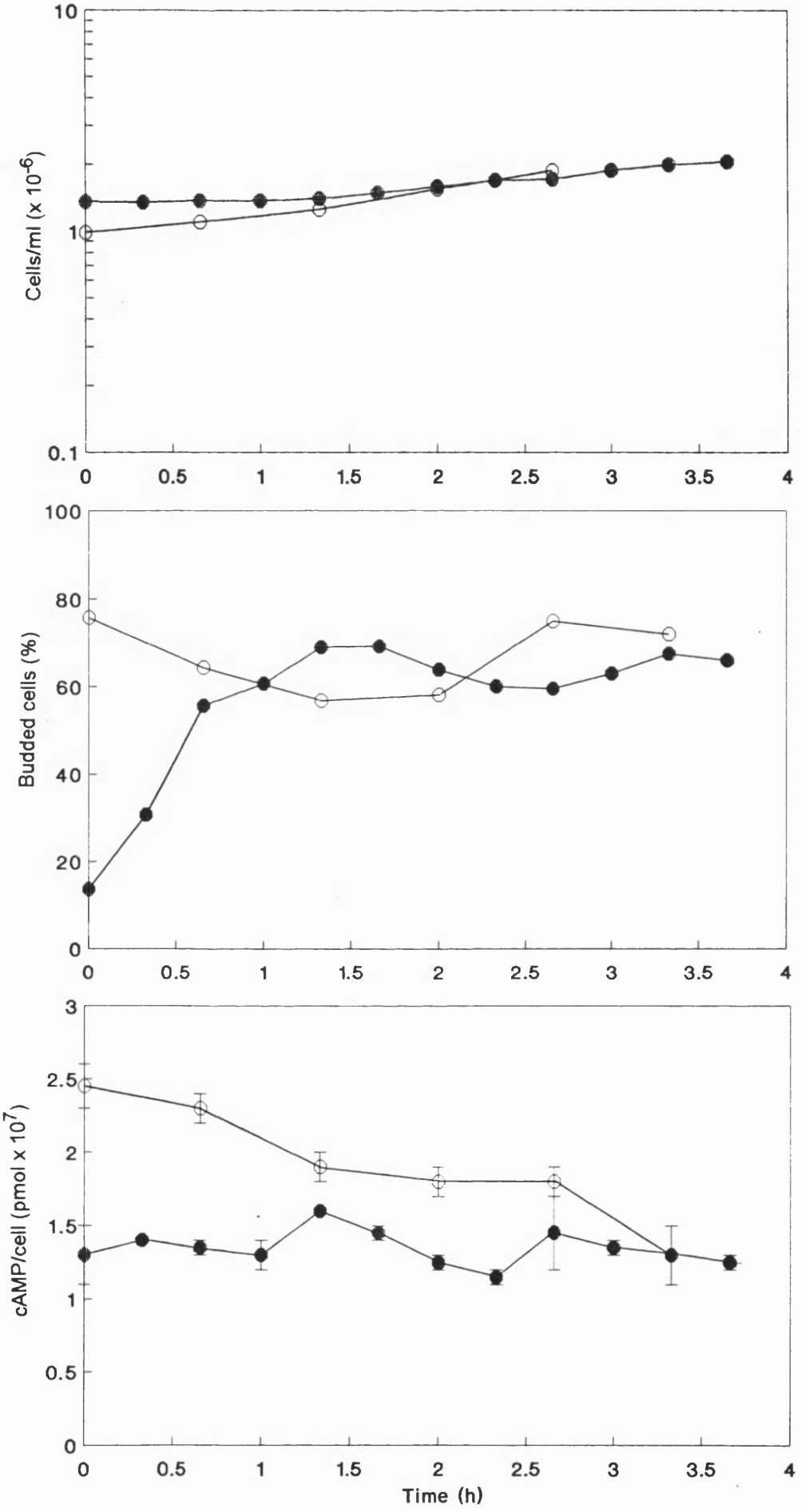
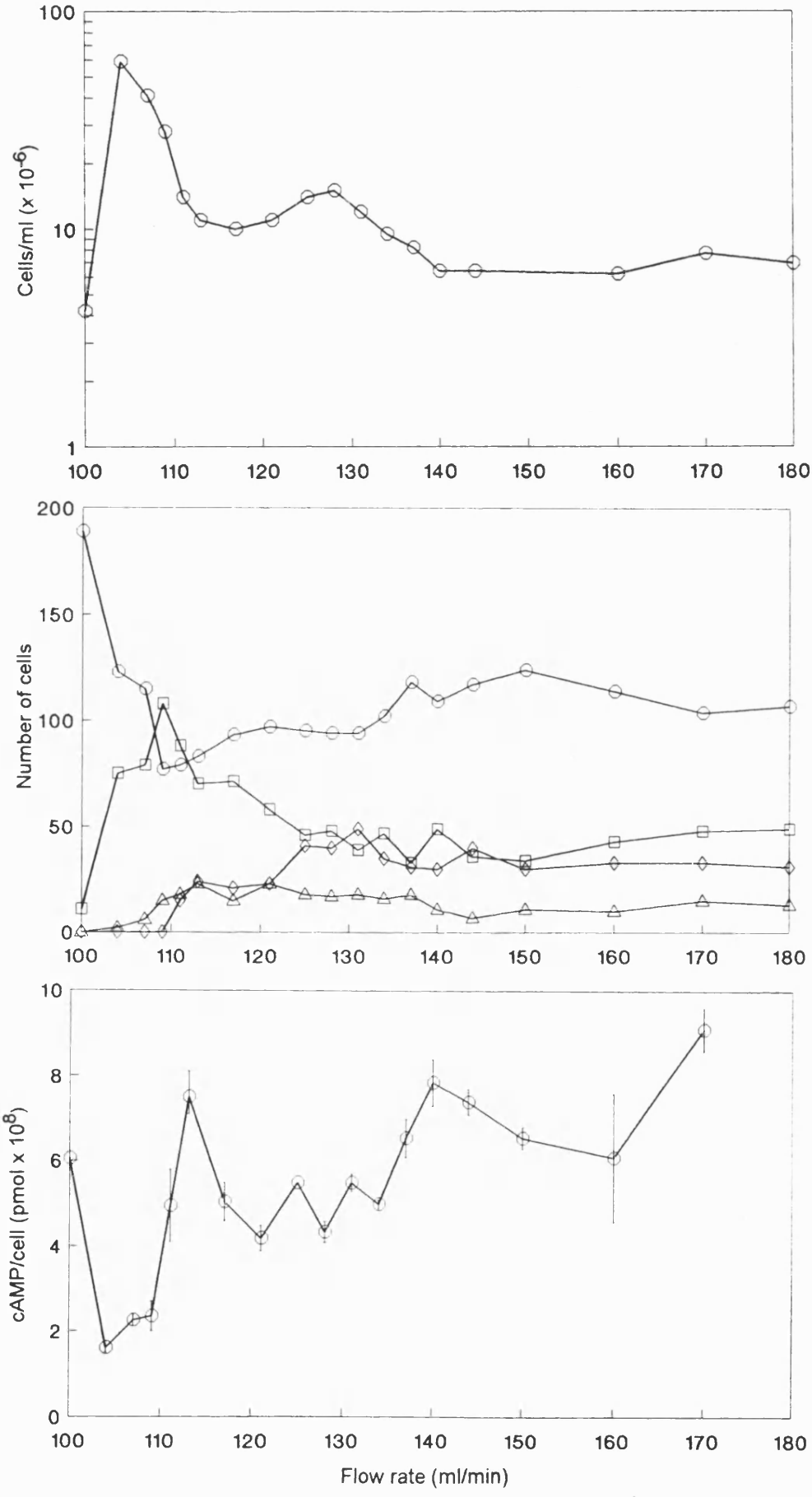

Cells/ml (x 10^-6)
10
1
0.1
0
0.5
1
1.5
2
2.5
3
3.5
4
Budded cells (%)
100
80
60
40
20
0
cAMP/cell (pmol x 10^7)
3
2.5
2
1.5
1
0.5
0
Time (h)

Figure 19. Age fractionation of a wild type stain of *S. cerevisiae*. SP1 was grown at 25°C in GM medium and prepared as described in section 2.5. Cells at different stages of the cell cycle could be distinguished by DAPI staining: unbudded, open circles; budded, open squares; undergoing nuclear division, open triangles; near to cell separation, open diamonds.


Cells/ml (x 10^-6)
100
10
1
100 110 120 130 140 150 160 170 180
Number of cells
200
150
100
50
0
100 110 120 130 140 150 160 170 180
cAMP/cell (pmol x 10^8)
10
8
6
4
2
0
100 110 120 130 140 150 160 170 180
Flow rate (ml/min)

thus distorting the amount of G_2 and M cells. SP1 showed an erratic pattern of i-cAMP concentration, with an increase in cAMP concentration concomitant with the appearance of budded cells, although the cell possesses both phosphodiesterases. This may indicate that the i-cAMP concentration can fluctuate during the cell cycle, although whether there are stage-specific fluctuations cannot be determined from these data as the remainder of the cell cycle was not well fractionated.

3.6.5. Age fractionation of DJ23-3C.

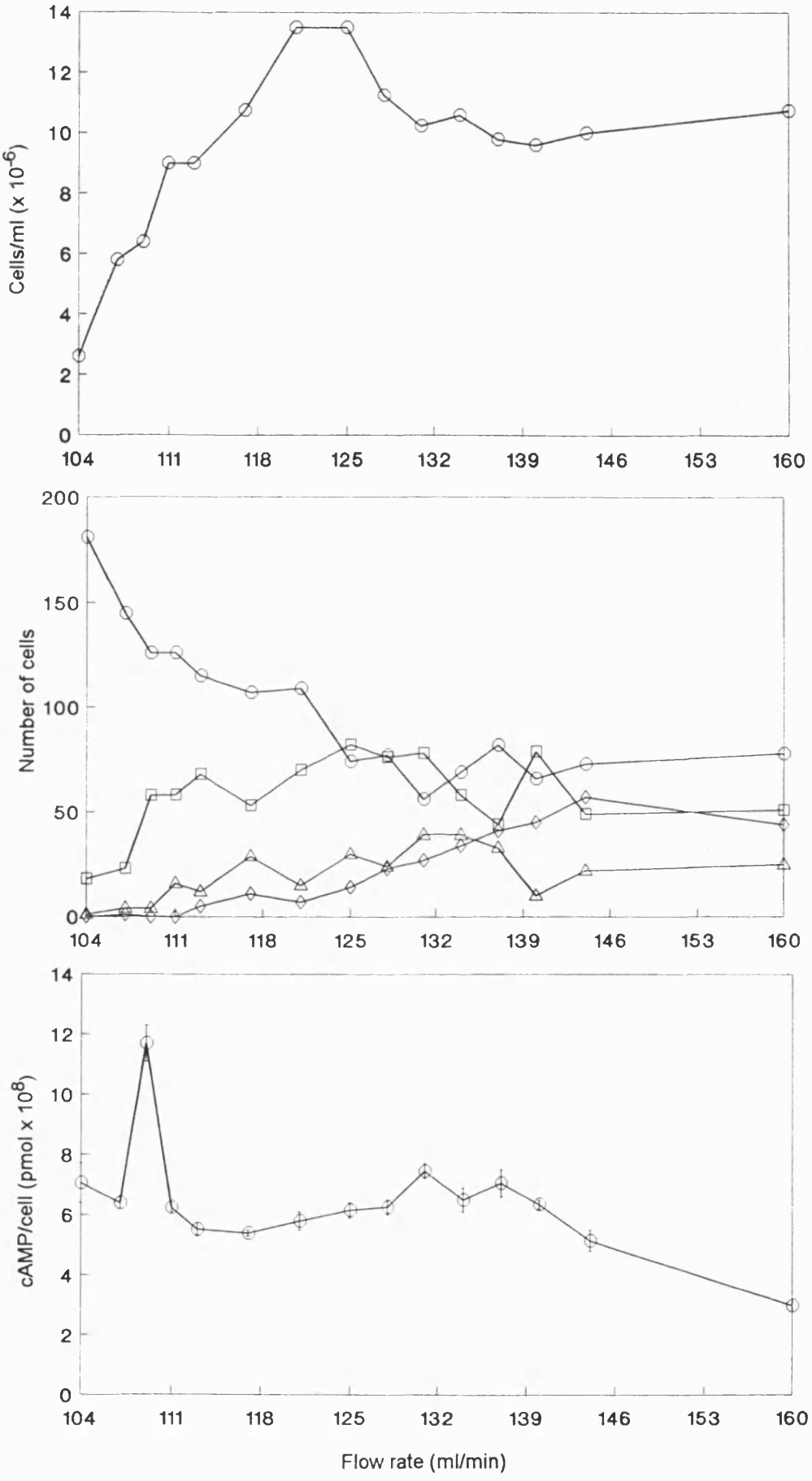
The concentration of intracellular cAMP from an age fractionation of DJ23-3C (Figure 20) shows an erratic fluctuation in the third sample where the cell cycle was well separated, but there was no change in the composition of cells that were present in that sample. In contrast to SP1 (Figure 19), there were no fluctuations in the concentration of i-cAMP and once again, the beginning of the cell cycle was well fractionated. The smooth line representing the i-cAMP concentration pointed towards the idea that phosphodiesterase may be responsible for any fluctuations in the concentration of i-cAMP.

3.7. Extracellular cAMP.

3.7.1 Introduction.

As described in Chapter 1, extracellular nucleotides are known to be involved in cellular responses and differentiation. For this reason, and the possibility that if cAMP fluctuates then it may involve extrusion into the medium, the existence of extracellular cAMP (e-cAMP) in growth media of *S. cerevisiae* was investigated. If the concentration of cAMP does fluctuate then there are a number of possibilities of how this could be achieved: first, cAMP could be degraded by phosphodiesterase; secondly, feedback inhibition could affect the rate of cAMP synthesis and loss; and thirdly, cAMP could be extruded into the medium. If cAMP is present in the growth medium then why it is there and

Figure 20. Age fractionation of a double phosphodiesterase mutant. DJ23-3C was grown at 25°C in GM medium plus 2% YEPD and prepared as described in section 2.5. Cells at different stages of the cell cycle could be distinguished by DAPI staining: unbudded, open circles; budded, open squares; undergoing nuclear division, open triangles; near to cell separation, open diamonds.


Cells/ml (x 10^-6)
0
2
4
6
8
10
12
14
104
111
118
125
132
139
146
153
160
Number of cells
0
50
100
150
200
cAMP/cell (pmol x 10^8)
Flow rate (ml/min)

whether it has a positive or negative effect on proliferation should be determined.

3.7.2. The presence of cAMP in the growth medium of *S. cerevisiae*.

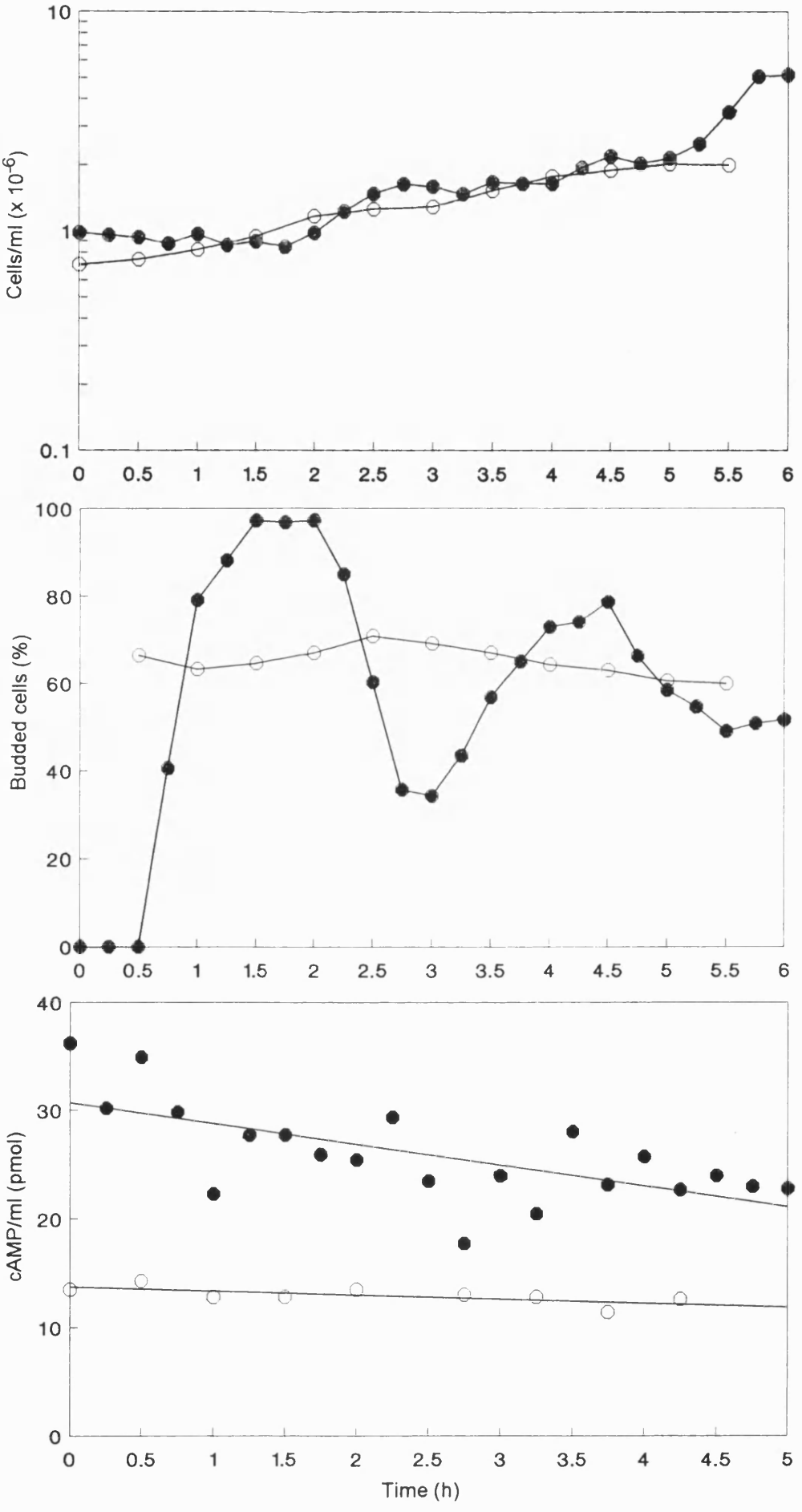
Figure 21 shows that in both the asynchronous and synchronous cultures e-cAMP was assayed to be present. There was between 20-100 times more cAMP outside a cell than inside per ml of culture analysed. However, expressed in relation to volume, i-cAMP and e-cAMP are of the order μM and nM, respectively.

Due to the large quantities of e-cAMP assayed (Figure 21), any stage-specific fluctuations of cAMP would be masked, therefore it was impossible to determine whether the presence of e-cAMP was due to putative stage-specific extrusion. The concentration of e-cAMP from the synchronous culture was higher than that of the asynchronous culture. This could be due to the fact that the synchronous culture was diluted into medium in which cells had been growing overnight and had been possibly releasing cAMP. For both the synchronous and asynchronous cultures, the concentration of e-cAMP fell very slightly over the 5 h of the experiment.

3.7.3. Effect of extracellular cAMP on proliferation.

To determine whether the presence or absence of e-cAMP enhanced proliferation, various concentrations of cAMP were added to the medium in which cells were growing. A physiological concentration of cAMP (5 pmol/ml), as measured by the assay, was added to seven flasks out of a total of 13, which contained stationary phase cells that had been transferred to fresh medium to a cell density of 10^5 cells/ml. Cells were then grown for a further 6 h. The number of cells per ml was determined for each culture and a Mann-Whitney test was performed on the data in order to determine whether there was a significant difference between cells that had been growing in medium with or

Figure 21. Extracellular cAMP from a wild type strain of *S. cerevisiae*. The medium of the synchronous (closed circles) and asynchronous (open circles) cultures from the third elutriation experiment was taken for e-cAMP determination. The upper panel represents cells/ml; the middle panel represents budded index; the lower panel represents e-cAMP/ml culture. For experimental details see section 2.7.


Cells/ml (x 10^-6)
10
1
0.1
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Budded cells (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
cAMP/ml (pmol)
40
30
20
10
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5
Time (h)

without exogenously added cAMP. After 6 h there was a significant difference between the two types of medium ($P = 0.001$). The cells that had been grown in medium to which cAMP had been added had lower cell densities compared with the cells that had not been grown in medium to which cAMP had been added.

In a separate experiment, cells were diluted to 10^0 and 10^1 cells per ml and cAMP was again added at 5 pmol/ml (four flasks out of a total of eight had cAMP added to them). There was no significant difference between the two sets of cultures, showing that exogenously added cAMP had no effect on proliferation. However, for this experiment large dilution errors could obviously be obtained therefore diminishing the value of these results.

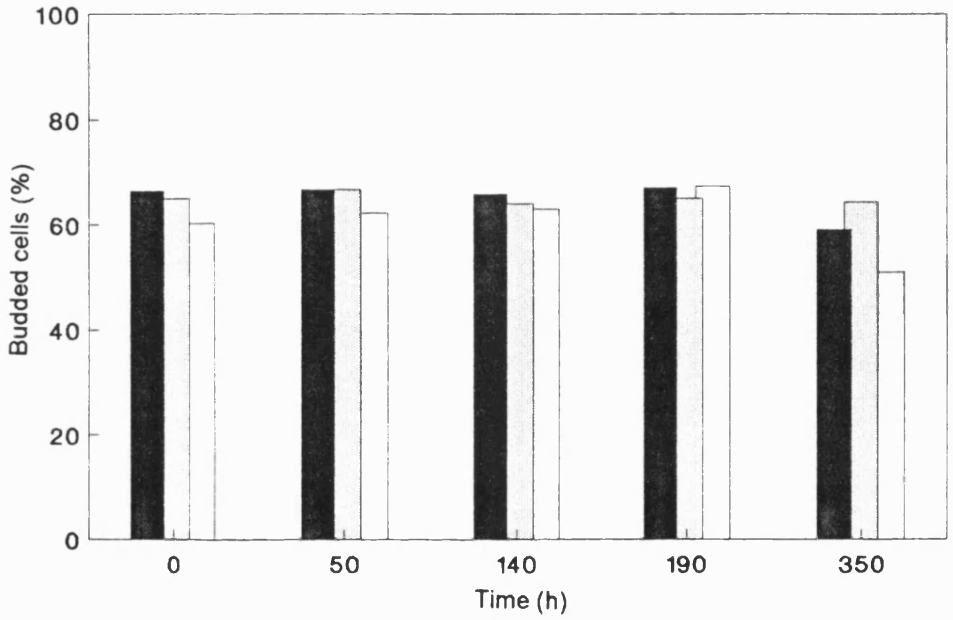
Another experiment that employed exponentially growing cells and had various concentrations of cAMP added to cultures also showed no effect on the percentage bud value (Figure 22). Any change in the percentage budded cells would indicate a change in the proliferation rate; an increase in this value would indicate an increase in the proliferation rate and a decrease would indicate a decrease in the proliferation rate.

The sequestering of cAMP from a growing culture would also enable the examination of any effects that e-cAMP had on proliferation or growth. However, a substance that would remove e-cAMP in sufficient amounts, for example charcoal or binding protein, but would not interfere with the yeast cells, could not be found.

3.7.4. The uptake of cAMP by *S. cerevisiae*.

It may be possible that cells of *S. cerevisiae* take up cAMP from the medium in which they are growing as has been previously reported (Singh *et al.*, 1980; Matsumoto *et al.*, 1982b). In order to attempt to demonstrate this, [8-^{3}H]cAMP was added to an exponentially growing culture of a wild type strain. At t = 0 and subsequent time points, samples were harvested as usual for cAMP assays

Figure 22. Effect of exogenous cAMP on the proliferation of a wild type strain of *S. cerevisiae*. Cells from an exponentially growing culture of D1 were inoculated into fresh GM medium with (50 pmol/ml, stippled bars; 5 nmol/ml, open bars) or without (0 pmol, filled bars) exogenous cAMP. Cells were grown at 25°C in a shaking water bath. Samples for percentage buds were taken at the times indicated.


100
80
60
40
20
0
Budded cells (%)
0
50
140
190
350
Time (h)

and the soluble fraction after TCA precipitation was counted for the presence of [8-^{3}H]cAMP. It can be seen from Figure 23 that the c.p.m., and therefore the amount of [8-^{3}H]cAMP, increased with time before reaching an equilibrium. This result may demonstrate that *S. cerevisiae* can take up cAMP from the growth medium.

3.7.5. Extracellular cAMP from asynchronous batch cultures.

Extracellular cAMP was also found in asynchronous batch cultures of a wild type and a double phosphodiesterase mutant (Figure 24). In contrast to the results from section 3.7.2., the concentration of e-cAMP increased with time; the increase in e-cAMP concentration was found to be more characteristic of the relationship between e-cAMP concentration and time (data obtained from five separate cultures). There was no significant difference in e-cAMP concentrations between cells grown on GM or YEPD, but a 6 h lag period existed before the appearance of e-cAMP when cells were grown on YEPD. On approach to stationary phase, DJ23-3C cells in both GM and YEPD continued to have increased e-cAMP concentrations, whereas in D1 the concentration of e-cAMP fell. Even though the cell density of DJ23-3C did not increase in GM medium, the pattern of increasing e-cAMP concentration was still apparent.

3.7.6. Construction of pH gradients using dimethylglutarate.

As the concentration of e-cAMP assayed by the method of Brown *et al.* (1971, 1974) was higher than that due to extrusion alone, and e-cAMP appeared to increase with time and decreasing pH (the range of pH for GM and YEPD medium uninoculated to stationary phase was pH 4.8 to 2.7 and pH 6.1 to 5.9, respectively), a relationship between e-cAMP and the pH of the growth medium was examined. Dimethylglutarate (DMG) was chosen to buffer GM medium at specific pH values and Table VII shows that DMG was capable of maintaining a specific pH for at least 6 h.

Figure 23. The uptake of cAMP from GM medium by a wild type strain of *S. cerevisiae*. [8-^{3}H]cAMP was added at t = 0 to an exponentially growing culture of strain D1 that had been growing in GM medium at 25°C in a shaking water bath. Samples were taken for determination of [8-^{3}H]cAMP concentration as described in section 2.7., except that after the TCA precipitation stage, the supernatant was mixed with Optiphase Safe scintillation fluid for determination of [8-^{3}H]cAMP c.p.m. The error bars represent the range of [8-^{3}H]cAMP c.p.m. obtained. This figure is representative of two separate experiments.

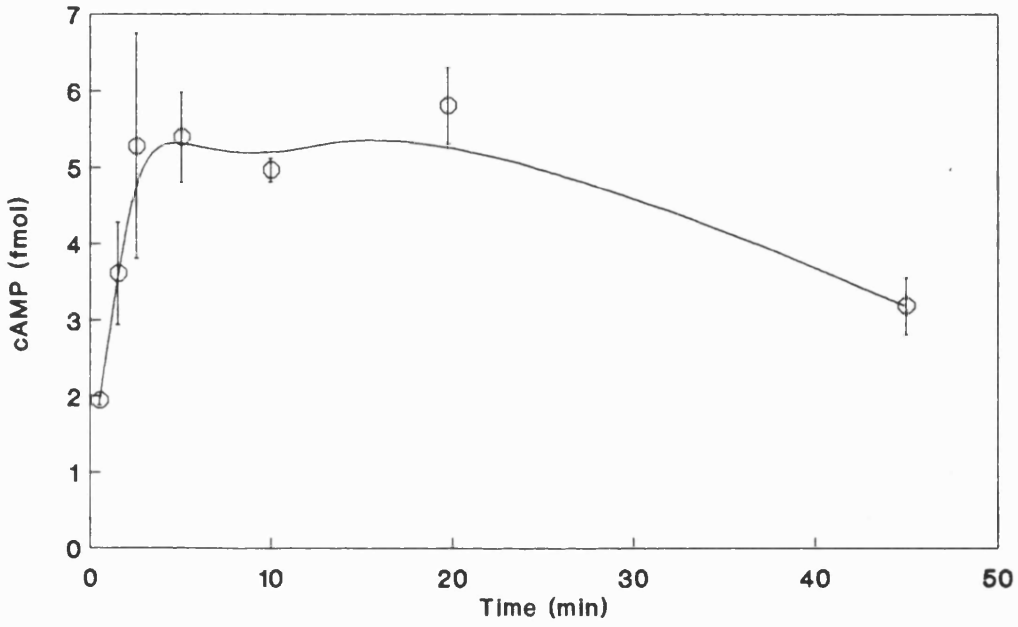

cAMP (fmol)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
Time (min)

Figure 24. The relationship between e-cAMP and time for wild type and double phosphodiesterase mutant strains of *S. cerevisiae*. D1 (cicles) and DJ23-3C (squares) were grown on YEPD (closed symbols) and GM (open symbols) at 25°C in a shaking water bath. Samples were taken for i-cAMP concentration and cell numbers at the times indicated.

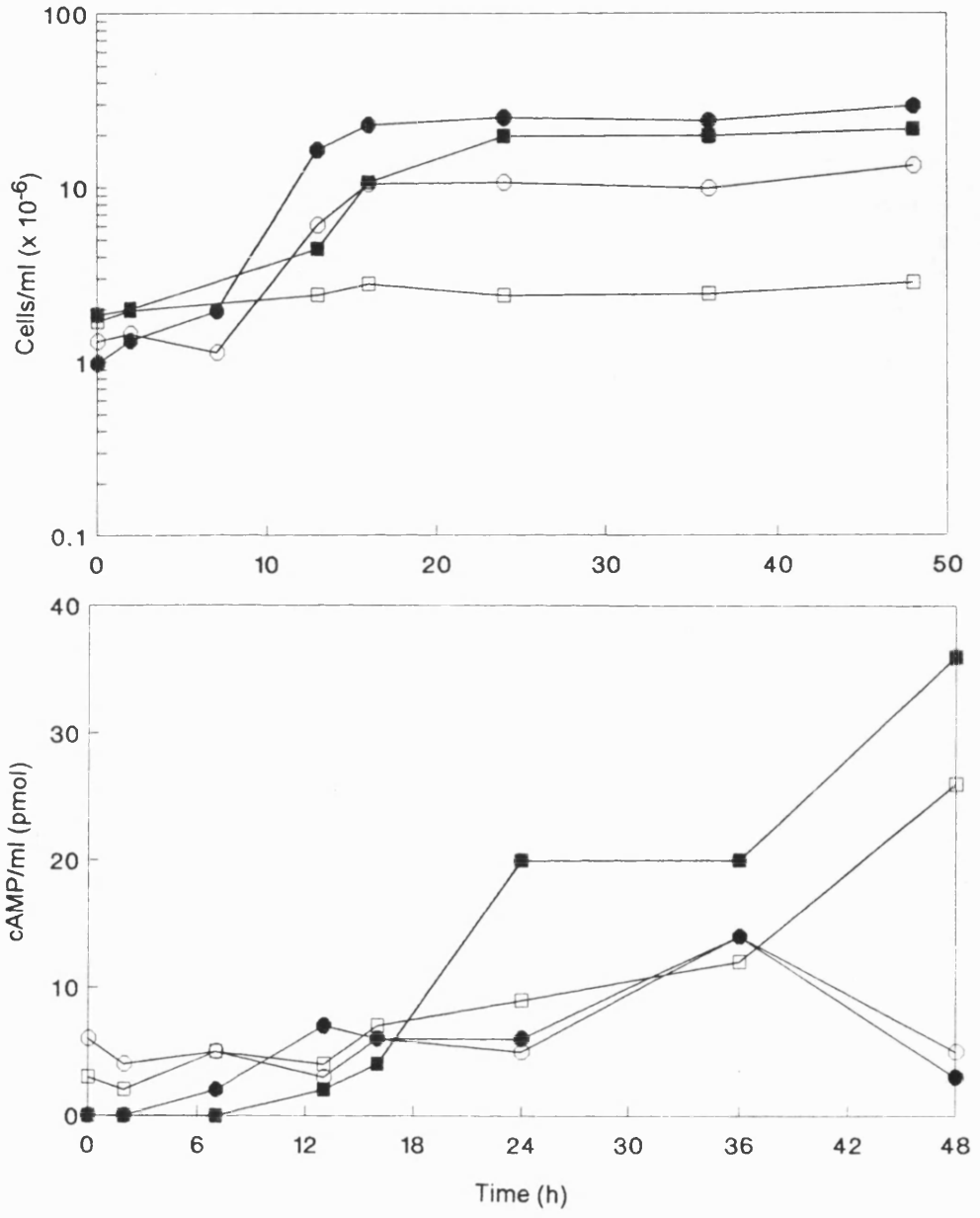

100
10
1
0.1
Cells/ml (x 10-6)
0
10
20
30
40
50
40
30
20
10
0
cAMP/ml (pmol)
0
6
12
18
24
30
36
42
48
Time (h)

Table VII. The effect of DMG-buffered GM medium on growth rate.

Sample	% increase in A_{600nm}	pH at		
		0 h	1 h	6 h
Control	506	4.21	3.64	4.38
A	173	3.38	3.31	3.37
B	195	4.13	4.07	4.16
C	475	5.09	5.03	5.15
D	449	5.97	5.85	6.02
E	409	6.51	6.39	6.63
F	262	7.22	7.04	7.73

Cells of a wild type diploid strain that had been growing in DMG-buffered medium at various pH values had different growth rates. The highest proliferation rates were found in the control culture and cultures grown at pH 5, 6 and 6.5. It was found that e-cAMP concentration was inversely proportional to the pH of the medium in which cells grew (Figure 25). However, when DMG was incorporated into the standard curve, no such standard curve could be obtained, indicating that DMG or the pH interfered with the assay for cAMP. To test which of these two factors interfered with the standard curve various concentrations of cAMP were added to DMG-buffered GM medium at different pH values. Table VIII shows that medium of a low pH interferes with the cAMP assay and values measured were either off the standard curve or non-responsive to any increase in added cAMP. Cyclic AMP measurements from pH 6-7 DMG-buffered medium resembled expected cAMP measurements best, i.e. DMG-buffered medium at particular pH values plus 0 pmol cAMP was approximately 0 pmol and when 0.5 pmol was added the value corresponded to approximately 0.5 pmol. An amount of cAMP was measured from pH 6 and pH 7 DMG-buffered GM medium that cells had been growing in and this may indicate the presence of cAMP in the medium. pH values measured from non-GM-buffered growth medium from 'normal' experiments were approximately pH 6-7, and therefore no interference should be present under 'normal' circumstances.

3.7.7. Degradation of extracellular cAMP by *bona fide* phosphodiesterase.

As the results shown in section 3.7.6. pointed towards the possibility that the cAMP assay method of Brown *et al.* (1971, 1974) was not measuring e-cAMP, *bona fide* cAMP and sample e-cAMP were incubated in the presence of phosphodiesterase. This would degrade any e-cAMP present and thus, if the assay method was measuring e-cAMP, a decrease in the concentration of e-cAMP obtained from the standard curve would occur.

Figure 25. The relationship between e-cAMP concentration of a wild type strain and pH of the culture. Aliquots of an exponentially growing culture of D1, which had been growing at 25°C, were inoculated into fresh GM medium and buffered with DMG to the pH values shown. The data shown were obtained after 6 h. The error bars represents the range of e-cAMP concentrations obtained.

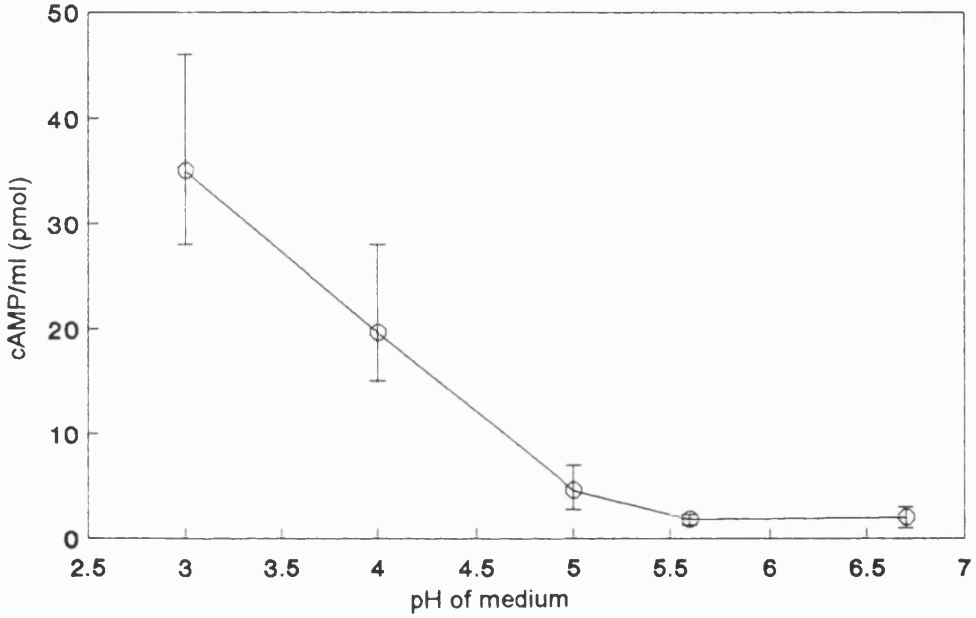

50
40
30
20
10
0
cAMP/ml (pmol)
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
pH of medium

Table VIII. The effect of DMG-buffered GM media at various pH values on e-cAMP concentrations.

Medium pH	[e-cAMP][a] (pmol/50 μl)	[e-cAMP][b] (pmol/50 μl)	
		+ 0 pmol[c]	+ 0.5 pmol[d]
pH 3	NA	NA	NA
pH 4	3.38	4.92	4.58
pH 5	0.46	0.61	1.0
pH 6	0.10	0.03	0.48
pH 7	0.19	0.02	0.48

NA, not available due to values being off the standard curve.

[a]The concentration of e-cAMP in GM medium in which cells had been growing overnight.

[b]The concentration of e-cAMP in 'blank' GM medium.

[c]'Blank' GM medium plus normal constituents of cAMP assay tubes.

[d]'Blank' GM medium plus 50 μl containing 0.5 pmol cAMP and only 100 μl of assay buffer.

Only one of the nine attempts (Table IX) gave any signs of e-cAMP degradation by phosphodiesterase. The principal problem was in obtaining sufficient e-cAMP for it to be detectably degraded because the concentration of cAMP assayed was low and close to 0 pmol, according to the assay. The medium was concentrated, but only *bona fide* cAMP was degraded. This indicated that either something in the medium was preventing phosphodiesterase activity or that the assay method of Brown *et al.* (1971, 1974) identification of e-cAMP was giving misleading cAMP measurements and was therefore probably unusable.

3.7.8. Identification of extracellular cAMP using HPLC.

HPLC was used as an alternative method to determine whether e-cAMP was present in the growth medium of *S. cerevisiae*.

Figure 26 shows a series of chromatograms of 259nm-absorbing substances from the growth medium of a wild type strain of *S. cerevisiae* over a period of time. Chromatograms A-E show the concentration and appearance with time of those nucleotides that had a retention time of between 0 and 12 min, when a wild type strain of *S. cerevisiae* was grown in GM medium. Cyclic AMP had a retention time of 9.2 min (marked by an arrow).

At t = 0, cAMP was already present in the medium. Also there was no visible change in the concentration of e-cAMP or any other nucleotides until 23 h had elapsed. At this point, the range of nucleotides present in the medium increased; the concentration of e-cAMP at this point was approximately halved. The concentration of e-cAMP after 48 h was approximately the same compared with that after 23 h.

Due to the low sensitivity used for this set of chromatograms the amount of cAMP recorded in cAMP assays would not have been seen in this set of chromatograms. However, by adding a known amount of cAMP to the samples

Table IX. Percentage degradation of cAMP by phosphodiesterase.

Experiment	Percentage degradation		
	bona fide cAMP	medium[a] + *bona fide* cAMP	medium[a]
1	88.0	51.8	-[b]
2	97.6	97.4	-[b]
3	98.7	76.4	100[c]
4	99.8	97.7	0
5	98.3	98.2	-[b]
6	100.0	100.0	-[b]
7	90.7	92.0	-[b]
8	91.2	87.3	-[b]
9	93.9	0.0	0[d]

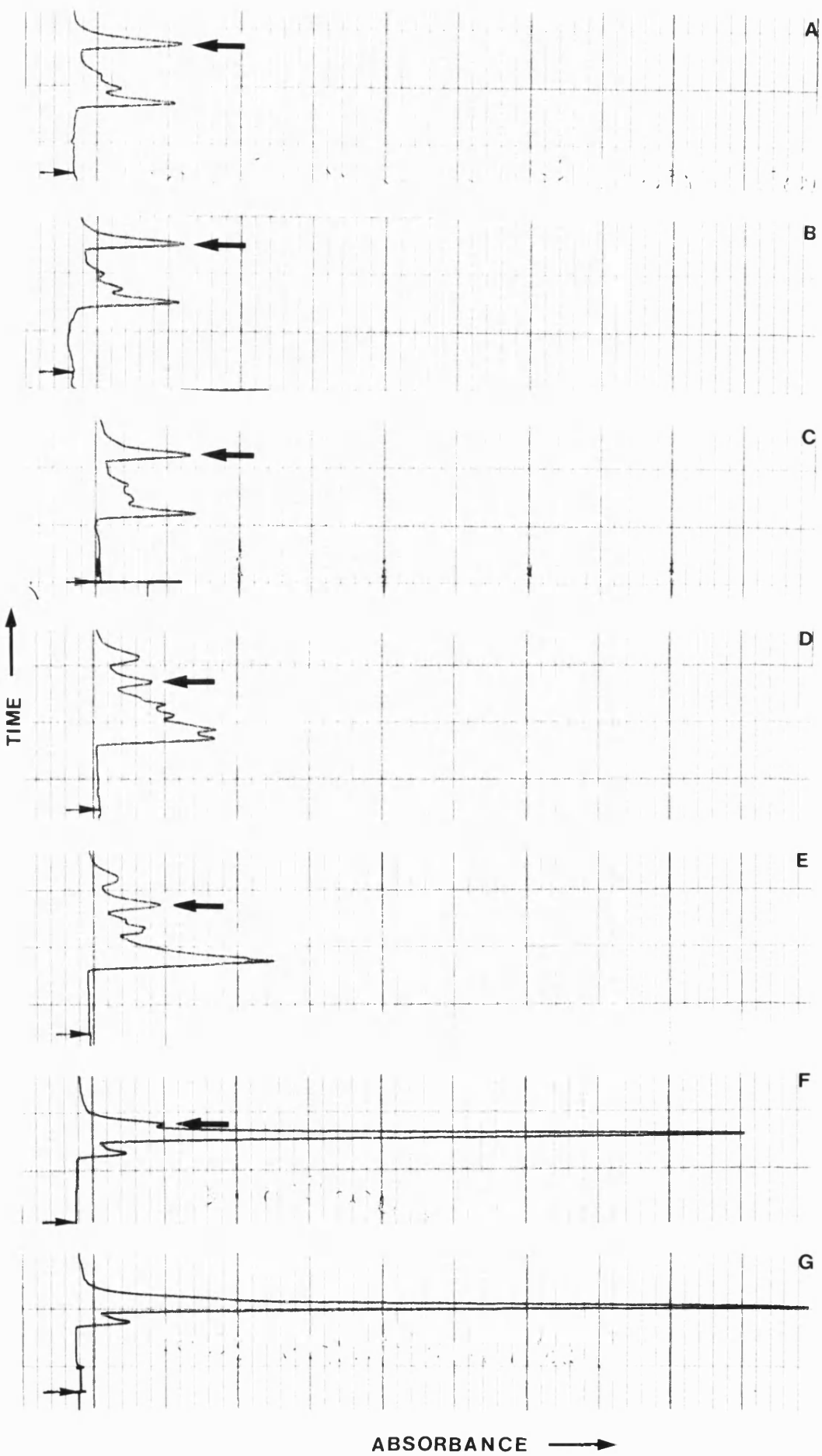
[a]'medium' represents GM medium in which cells had been growing.

[b]The concentration of cAMP measured was higher in the original sample where enzyme had been added than when the enzyme had been omitted.

[c]This was the only experiment to show degradation.

[d]GM medium had been concentrated 10-fold for this experiment.

Figure 26. HPLC chromatograph of 259nm-absorbing substances with time from a wild type strain of *S. cerevisiae*. A flask containing GM medium was inoculated with cells from an exponentially growing culture of D1 that had been growing at 25°C. Cells were then grown overnight in a shaking water bath. Samples were taken after inoculation into fresh GM medium for analysis at the times indicated. All GM medium was filtered before being loaded onto the column. A, 'blank' medium, 0 h; B, 3 h; C, 6 h ;D, 23 h ; E, 48 h; F, 'blank' GM medium plus 1.3 nmoles *bona fide* cAMP; G, same as F. Flow rate was 1.5 ml/min; sensitivity was 0.2; path length was 10 mm; chart recorder was set at 15 cm/h, except for F and G when it was 30 cm/h. Sample volume was 20 μl. The small arrow represents t = 0, the large arrow represents the time at which *bona fide* cAMP elutes (except for chromoatogram F, where it represents a putative peak of e-cAMP).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(chromatograms F and G), the concentration of cAMP present in peaks could be determined and this was found to be approximately 500 pmol.

Figure 27 shows a second set of chromatograms that also demonstrated the presence and release of cAMP into the medium. By spiking a sample with *bona fide* cAMP in order to identify the cAMP peak (chromatogram B), it was apparent that cAMP was present in blank GM medium (chromatogram A). Chromatograms C and D both show the pattern of nucleotides that were released into the growth medium. By spiking these samples with *bona fide* cAMP (chromatogram E), any cAMP that may have been extruded into the growth medium could be identified. A peak corresponding to the cAMP spike was identified in medium in which the cells had been growing and by comparing the area of the spike, the amount of cAMP in blank and used GM medium could be determined. For this batch of GM medium there was 20 pmol/20 μl in blank medium and 60 pmol/20 μl in medium in which cells had been growing overnight.

Figure 28 shows a more sensitive measurement of putative e-cAMP. Unlike the other attempts, no e-cAMP was found in blank GM medium. However, for each sample run a spike of *bona fide* cAMP was included to ensure correct identification of e-cAMP. Chromatogram A shows a spike of *bona fide* cAMP (32 pmol). Chromatogram B shows the elution profile of GM medium in which wild type yeast (A_{600nm} 1.06) had been growing, and it appears that there was a peak that eluted at the same time as *bona fide* cAMP. However, when spiked with *bona fide* cAMP, this peak eluted just after the spike. Therefore it appeared that cAMP was not present in the medium in which wild type yeast had been growing.

Next, a strain that lacked any phosphodiesterase activity was tested (A_{600nm} 0.9). As the strain could not degrade cAMP, it was possible that excess cAMP may be extruded into the medium. Chromatogram D shows that not only was the elution pattern of this strain different from the wild type, but also, on

Figure 27. HPLC chromatograph of 259nm-absorbing substances from a wild type strain of *S. cerevisiae*. A flask containing GM medium was inoculated with cells from an exponentially growing culture of D1 that had been growing at 25°C. Cells were then grown overnight in a shaking water bath. A, 1/50 'blank' GM medium; B, 1/50 'blank' GM medium plus 2.26 nmoles *bona fide* cAMP; C, 1/50 GM medium from a culture of D1; D, same as C; E, same as C plus 0.69 nmoles cAMP. All GM medium was filtered before being loaded onto the column. The flow rate was 1 ml/min. UV absorbance was measured at a sensitivity of 0.02; path length was 10 mm. Peaks were recorded onto a chart recorder with a running speed of 15 cm/h. The sample volume was 2 ml. The small arrow represents t = 0, the large arrow represents the time at which *bona fide* cAMP elutes.

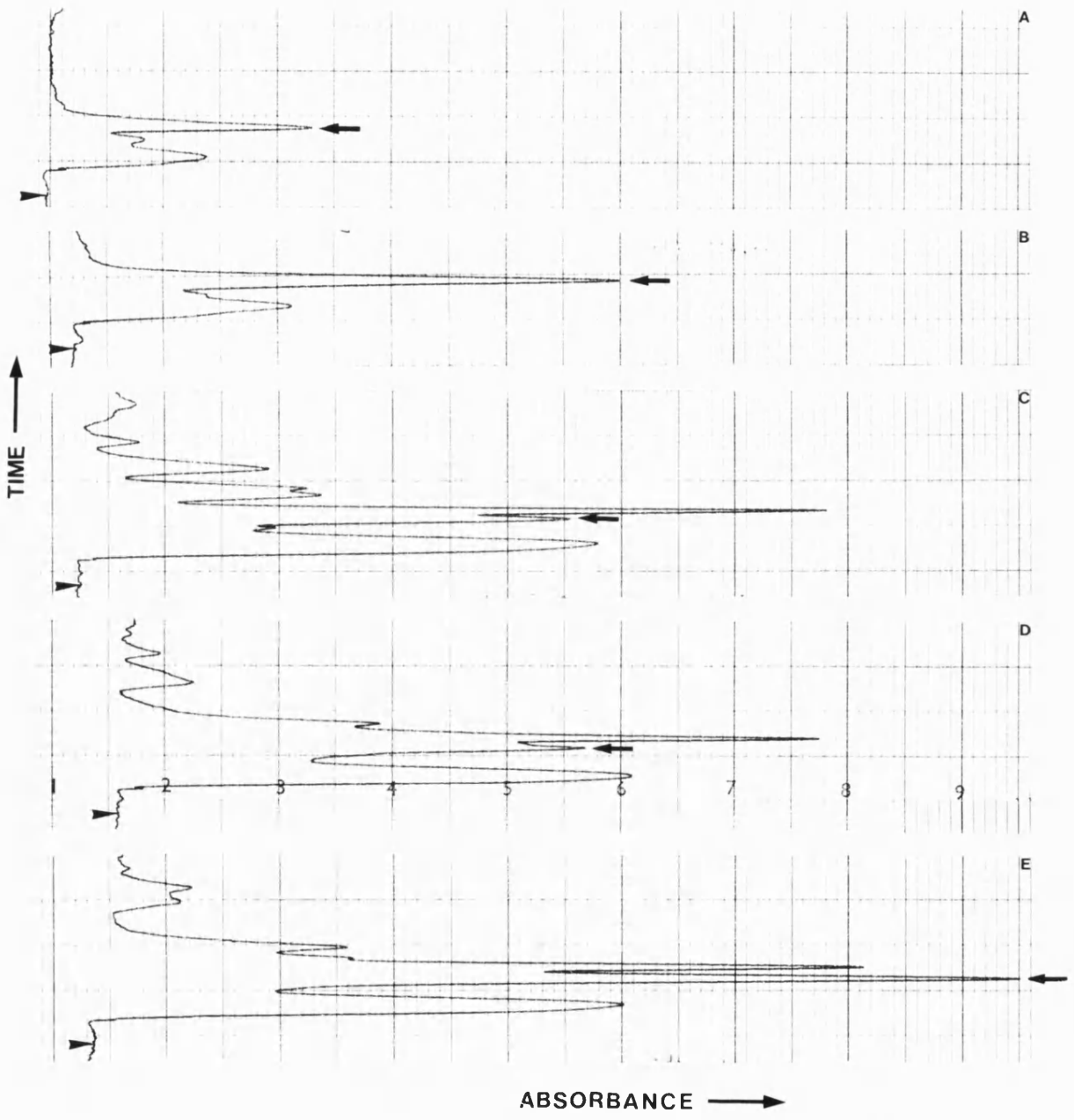
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Figure 28. HPLC chromatograph of 259nm-absorbing substances from the growth medium of two unrelated wild types (D1 and S7-7A) double phosphodiesterase mutant (DJ23-3C) and triple PKA catalytic subunit mutant (S18-1D) strains of *S. cerevisiae*. S18-1D was also transformed with the low K_m phosphodiesterase or the *TPK1* PKA catalytic subunit gene (pPDE2 and pTPK1, respectively). All strains had been grown in GM medium at 25°C in a shaking water bath to the A_{600nm} indicated. All GM medium was filtered before being loaded onto the column. The flow rate was 1.5 ml/min. UV absorbance was measured at a sensitivity of 0.002; path length was 5 mm. Peaks were recorded using a chart recorder with a running speed of 15 cm/h. The samples were diluted by the addition of 0.5 vol of *bona fide* cAMP or 'blank' elution buffer. The sample volume was 20 μl. The small arrow represents t = 0, the large arrow represents the time at which *bona fide* cAMP elutes.

A, 'blank' GM medium plus 32 pmol *bona fide* cAMP

B, D1 A_{600nm}1.06

C, D1 A_{600nm}1.06 plus 32 pmol *bona fide* cAMP

D, DJ23-3C A_{600nm}0.803

E, DJ23-3C A_{600nm}0.803 plus 32 pmol *bona fide* cAMP

F, S18-1D A_{600nm}1.05

G, S18-1D A_{600nm}1.05 plus 32 pmol *bona fide* cAMP

H, S7-7A A_{600nm}1.05

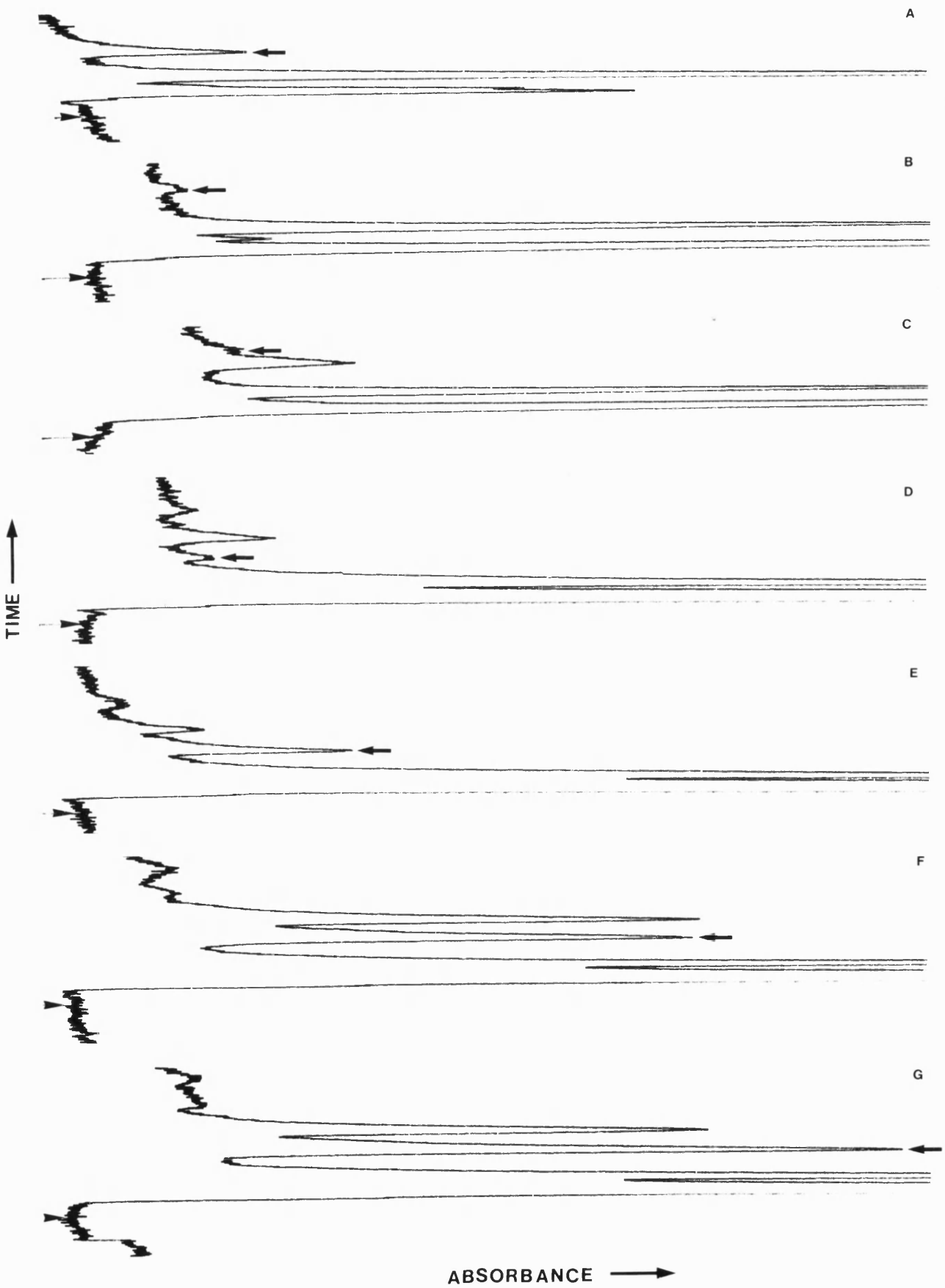
I, S7-7A A_{600nm}1.05 plus 32 pmol *bona fide* cAMP

J, S7-7A(pPDE2) A_{600nm}0.902

K, S7-7A(pPDE2) A_{600nm}0.902 plus 32 pmol *bona fide* cAMP

L, S7-7A(pTPK1) A_{600nm}0.704

M, S7-7A(pTPK1) A_{600nm}0.704 plus 32 pmol *bona fide* cAMP.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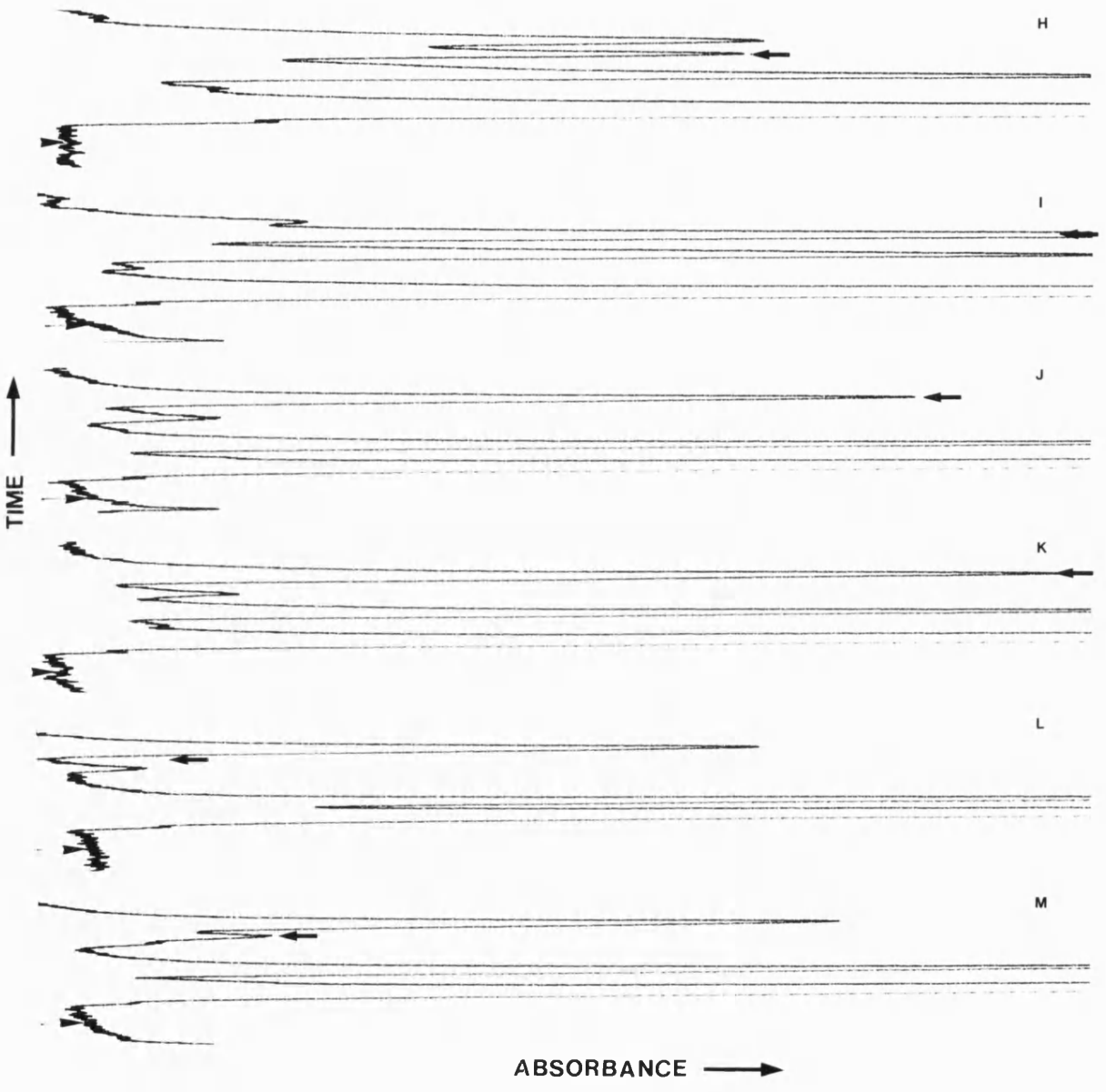
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checking with *bona fide* cAMP (chromatogram E), it appeared that e-cAMP was present. The concentration of e-cAMP was approximately 15 pmol/20 μl medium).

Chromatograms F and G show the elution profiles of a strain that contained attenuated catalytic subunits of Tpk1. This attenuation results in an dramatic increase in i-cAMP concentrations and if similar to the phosphodiesterase mutant, e-cAMP may be present. Again the elution profile of this strain was different to any others and had a considerably increased concentration of 259nm-absorbing compounds. When spiked with cAMP, a peak of cAMP was identified and corresponded to approximately 90 pmol/20 μl medium.

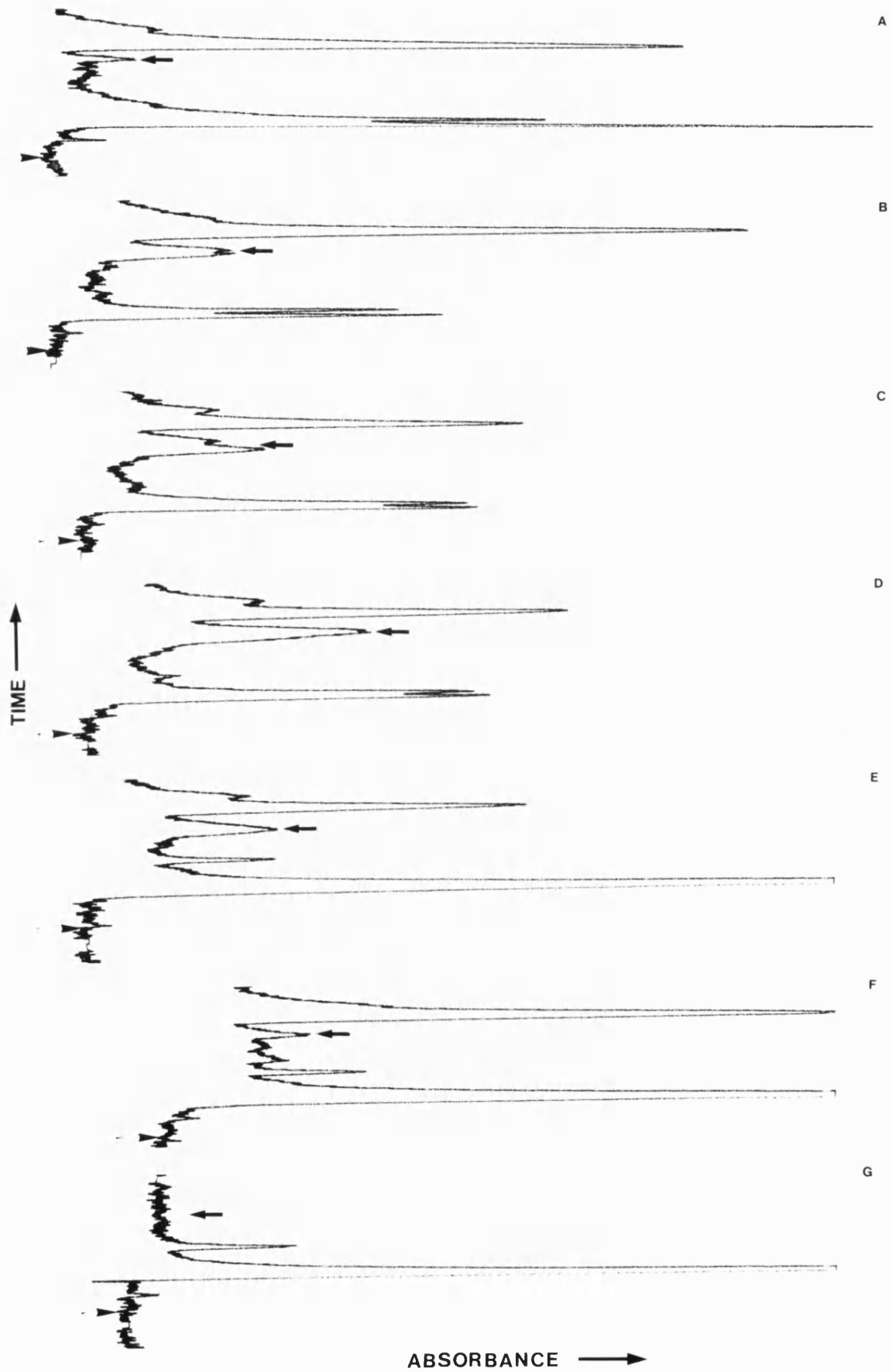
Another test to determine whether the genotype and/or status of the i-cAMP-generating pathway of a strain had any effects of the presence of e-cAMP involved another wild type strain, S7-7A (this is related to S18-1D). The elution profile of this strain was very similar to S18-1D and indicated that the excess e-cAMP observed in that strain was probably not due to its phenotype but just a characteristic of this particular family of strains. When *bona fide* cAMP was added to S7-7A, the profile changed dramatically and the last peak decreased whist the previous one increased. This was a exceptional result, but it could be possible that cAMP was the second from last peak.

When S7-7A was transformed with a plasmid harbouring the *PDE2* gene (Sass *et al.*, 1986), a different elution profile was observed. By spiking with *bona fide* cAMP it was thought that the last peak probably corresponded to cAMP. However, when S7-7A was transformed with a plasmid carrying the *TPK2* gene (Toda *et al.*, 1987b), cAMP appeared to elute between the last and the second from last peak. Thus indicating that there was no cAMP present in the medium in which this strain had been growing. This may also mean that the cAMP observed in the previous chromatogram may not have been cAMP either.

Due to the inconsistencies observed in the above experiments, i.e. cAMP may or may not be present in blank medium, cAMP is or is not extruded from cells, and the unexplainable changes in elution profile, it was necessary to determine whether the peaks of cAMP observed were e-cAMP and not another compound that co-eluted. For this, phosphodiesterase, which would degrade any e-cAMP, was added to samples and the appearance of the nucleotide that corresponded to e-cAMP was monitored. Figure 29 shows that as usual, there was a peak co-eluting or eluting very close to *bona fide* cAMP (chromatograms A, B and C). However, when phosphodiesterase was added to samples, no degradation of cAMP was observed. This lack of degradation was found not to be due to inactivity of phosphodiesterase, as chromatograms F and G indicated a disappearance of cAMP. From these data it appeared that the peaks observed were not e-cAMP, but something that co-eluted, possibly 2'-3' cAMP, which is a breakdown product of RNA and can be found in the medium.

Concentrations of e-cAMP were also investigated on YEPD-grown cells (data not shown). However, as there was an increased number of nucleotides in blank YEPD than blank GM medium, the identification of e-cAMP was difficult to determine.

Figure 29. HPLC chromatogram of phosphodiesterase degradation of *bona fide* and putative e-cAMP. Cells of strain D1 were grown in GM medium at 25°C in a shaking water bath until a sufficient A_{600nm} had been reached. All GM medium was filtered before being loaded onto the column. The flow rate was 1.5 ml/min. UV absorbance was measured at a sensitivity of 0.002; path length was 5 mm. Peaks were recorded using a chart recorder with a running speed of 15 cm/h. The samples were diluted by the addition of 0.5 vol of *bona fide* cAMP or 'blank' elution buffer. The sample volume was 20 μl. The small arrow represents t = 0, the large arrow represents the time at which *bona fide* cAMP elutes. A, blank GM medium; B, blank GM medium plus *bona fide* cAMP; C, D1 GM medium; D, D1 GM medium plus 32 pmol *bona fide* cAMP; E, D1 GM medium plus phosphodiesterase; F, blank GM medium plus phosphodiesterase and 32 pmol *bona fide* cAMP; G, water plus phosphodiesterase and 32 pmol *bona fide* cAMP.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CHAPTER 4.

DISCUSSION.

4.1. The cAMP assay.

The control experiments, which included checking the standard curve, serial dilution and phosphodiesterase degradation of *bona fide* and sample cAMP, clearly demonstrated that the assay method of Brown *et al.* (1971, 1974) was suitable for this work. Although the assay was extremely sensitive, and therefore showed minor differences in cAMP concentrations between experiments, the degree of reproducibility demonstrated that the assay was reliable.

This work expressed i-cAMP concentrations on a per cell basis; others have expressed i-cAMP concentration in relation to the amount of protein (pmol cAMP per mg protein), as a molar concentration (μM) or as a concentration in relation to dry or wet weight (nmol per g wet weight).

Although comparisons of i-cAMP concentrations were difficult due to the variety of ways in which they have been expressed, Table X shows that i-cAMP concentrations from this work compare well with the i-cAMP concentrations obtained by other researchers. The slight differences observed between values obtained from this work and those obtained from other researchers can be explained. First, there may be strain differences that cause different i-cAMP concentrations; this is known already (Van Aelst *et al.*, 1991) and has been demonstrated here (see section 3.2.3.). Secondly, not only is there a variety of ways to express the concentration of i-cAMP, but there is a variety of extraction and assay methods that can be used, possibly resulting in slight concentration differences. Thirdly, most of the researchers that have measured i-cAMP concentrations have tended to use cells that have been harvested from high cell density cultures, whereas in this work cells have been harvested from low density cultures in which the cells are in early to mid exponential phase.

Table X. Comparisons of cAMP concentrations.

Reference	Range of [i-cAMP]	This work
François *et al.* (1987)	0.8-2.0 nmol/g dry wt	0.17-0.4[a]
Eraso and Gancedo (1984)	0.71-1.13 μM	0.3-0.4[b]
Matsumoto *et al.* (1982a, 1984)	1.6-2.0 pmol/mg protein	1.1-12.2[c,d]
Tanaka *et al.* (1990b)	$\approx$5 pmol/mg protein	1.1-12.2[c,d]
Boutelet *et al.* (1985)	$\approx$5 pmol/mg protein at 0.8-1.2 x 10^7 cells/ml	1.4-2.8
Fedor-Chaiken *et al.* (1990)	10 pmol/mg protein at 1 x 10^7 cells/ml	1.4-2.8
Camonis *et al.* (1986)	0.7 pmol/2 x 10^7 cells	0.13-0.48
Watson and Berry (1977b)	35-55 pmol/5 x 10^7 cells at $\approx$2 x 10^7 cells/ml	$\approx$0.4
Eilam *et al.* (1990)	0.02-0.04 pmol/10^6 cells at 1 x 10^8 cells/ml	[e]

[a]François *et al.* (1987) expressed i-cAMP as a function of dry weight. Using their dry weight and optical density curve, a dry weight value was obtained for samples prepared in this work.

[b]The molar concentration could be calculated using an i-cAMP concentration obtained in this work that corresponded to the cell density at which Eraso and Gancedo (1984) harvested the cells and the assumption that 1 g wet weight yeast contains 0.74 ml yeast cell sap.

[c]The range of i-cAMP concentration was calculated using TCA-precipitated protein and obtaining protein concentrations by micro Lowry assays.

[d]As authors did not state the cell density at which they harvested the cells, the range of i-cAMP concentration was obtained by using i-cAMP concentration values from experiments over a wide range of cell density.

[e]Not determined, estimated to be between 1 and 3 pmol; authors harvested at a cell density that was not often obtained in this work.

Fourthly, most of the values obtained from other researchers reflect the i-cAMP concentration at only one time point or when the cells have been grown in medium containing buffers or depolarizing agents, therefore comparisons between patterns of i-cAMP concentrations or i-cAMP in similar growth medium were difficult to obtain.

François *et al.* (1987) performed experiments that can be directly compared with this work. They measured i-cAMP concentrations over a long period of time and a wide cell density range. They also used TCA in order to extract the i-cAMP and the Amersham cAMP assay kit in order to measure i-cAMP. Their i-cAMP concentrations were 3-4.5 times higher than those found here, but considering the approximate calculation, concentrations were reasonably comparable.

Eraso and Gancedo (1984) also used the Amersham kit to assay i-cAMP and TCA in order to extract cAMP from the cell harvest. They also harvested cells at a low cell density and therefore their i-cAMP concentrations could also be compared with those obtained here. In order to calculate the concentration of i-cAMP, Eraso and Gancedo (1984) used the value of 0.6 ml yeast cell sap per gram of wet weight yeast (Conway and Downey, 1950). However, Conway and Downey had in fact concluded that 1 g of wet weight yeast corresponds to 0.74 ml yeast cell sap per gram wet weight. Therefore the i-cAMP concentrations of Eraso and Gancedo (1984) should in fact be lower, 0.57-0.92 μM. The corresponding i-cAMP concentration obtained here was lower than found by Eraso and Gancedo, but was still comparable.

Many researchers (Van Aelst *et al.*, 1991; Thevelein *et al.*, 1987a,b) harvested their cells at higher cell densities than those measured here and therefore could not be directly compared.

Another group that have measured cAMP was Matsumoto *et al.* (1982b, 1984). Using either the method of Brown *et al.* (1971, 1974) or the Amersham cAMP kit, their i-cAMP concentrations were similar to those measured in this

work. However, the authors did not state the cell density at which the cells were harvested, and as the i-cAMP concentration varies depending on the cell density of the culture (François *et al.*, 1987; this work), values obtained in this work could not be compared with their values.

There have been many researchers that have measured i-cAMP concentrations and expressed them as pmol/mg protein (Boutelet *et al.*, 1985; Toda *et al.*, 1985; Nikawa *et al.*, 1987a,b; Fedor-Chaiken *et al.*, 1990; Tanaka *et al.*, 1990b). All of the ranges in i-cAMP concentration obtained in the papers cited above corresponded to values that have been measured here, although Nikawa *et al.* (1987a,b) used the acetylation assay method, Camonis *et al.* (1986) used the cAMP- succinyl-TME-^{125}I kit and Fedor-Chaiken *et al.* (1990) used the New England Nuclear [^{125}I]cAMP kit for measuring i-cAMP concentrations. The acetylation method is reportedly able to measure i-cAMP in the order of femtomoles per assay tube.

Whereas all of the researchers cited so far have measured i-cAMP concentrations that corresponded to those reported here, Eilam *et al.* (1990) and Watson and Berry (1977b) have reported concentrations of i-cAMP that are much lower and much higher, respectively, than any other values measured (despite Eilam *et al.* (1990) using the Amersham cAMP assay kit).

4.2. Intracellular cAMP concentrations in asynchronous batch cultures.

The majority of the experiments presented here involved the use of asynchronous batch cultures. The results of these experiments all showed a decrease in the concentration of i-cAMP with time. The i-cAMP concentration per ml of culture harvested increased with time, but not in proportion to the increase in cell numbers, therefore the i-cAMP concentration per cell decreased.

François *et al.* (1987) have suggested a theory as to why the i-cAMP concentration decreases with time. They proposed that the i-cAMP concentration mirrors glucose concentration and as glucose is metabolized and

its concentration falls, the i-cAMP concentration falls. As discussed in Chapter 1, the concentration of i-cAMP responds to nutritional conditions and when glucose is present, cAMP is synthesized. Therefore as the glucose concentration falls, so does the concentration of i-cAMP.

However, the results also showed that as the glucose was being metabolized and its concentration was decreasing along with the i-cAMP concentration, the cells were proliferating at the same rate, for example when the cells were in exponential phase. This means that although the i-cAMP concentration fell, there was no effect on the rate of proliferation. It could be inferred that the i-cAMP concentration in asynchronous cultures was much higher than any threshold concentration that is required for entry into the mitotic cycle and the traverse of Start, and the cell produced i-cAMP in excess of this amount. When the cells had stopped proliferating, the i-cAMP remained at a low but constant level, indicating that either the degradation or the synthesis of cAMP had not stopped altogether. However, this lower concentration of i-cAMP was not necessarily the putative threshold required for traverse of Start.

The concentration of i-cAMP is thought to act as a switch mechanism, probably in conjunction with other factors, governing when a cell can traverse Start. Data presented here suggest that this putative switch mechanism is gradual in the transition from the ability to the inability to traverse Start.

There is evidence to suggest that the i-cAMP concentration is controlled by feedback inhibition (Nikawa *et al.*, 1987a), although as seen here, the i-cAMP concentration was not kept at a minimum level during the exponential phase of growth and therefore no feedback control appeared to be acting. Feedback inhibition (discussed in section 1.4.2.) is supposed to be able to control i-cAMP concentrations tightly over a 10,000-fold range. From the data obtained here, however, it appears that this is not the case and perhaps feedback inhibition only occurs when there are drastic changes in the i-cAMP concentration. Instead it appears that the amount of i-cAMP synthesized is far

greater than that required for growth and proliferation; the apparent excess of i-cAMP may be related to the higher concentration of glucose present in the medium at the beginning of the experiment. It is possible that the i-cAMP concentration is related to the concentration of available carbon source and does not function solely as a signal, as it would not be economical to produce a signal molecule in excess, but only when required.

Other results presented here show that there was a strong relationship between cell density and i-cAMP per ml of culture harvested. A linear relationship existed and this can be seen in Figures 7 and 8, and Table IV. This is what would be expected if the i-cAMP concentration were dependent on the concentration of the carbon source. As the carbon source is utilized and the cells grow and increase in number, the i-cAMP concentration would be expected to decrease in proportion to the increase in cell number.

It was also found that the concentration of i-cAMP was not kept constant when related to changes in volume, possibly inferring that as long as there is i-cAMP, its actual concentration is not crucial for proliferation. There were also no significant differences in the i-cAMP concentration between different strains, which also displayed different volumes. The actual concentration of i-cAMP may not be important as it only takes one molecule of cAMP to activate PKA, which can then phosphorylate many proteins, thus producing a signal. Baroni *et al.* (1989) suggested that the critical size difference between *RAS2*^{val19} and *cdc25-1* mutants (*RAS2*^{val19} cells have to achieve a larger critical size before traverse of Start than *cdc25-1* cells) was due to the activity of adenylyl cyclase and cAMP concentrations. It would be agreeable if the control of cell size was linked to the cAMP pathway and this is still conceivable, however, the data obtained here do not provide evidence for or support any control that cAMP may have over cell size.

Although statistical analysis showed that there were significant differences in the i-cAMP concentrations between cells grown on rich and

minimal media or fermentable or non-fermentable carbon sources, the difference was not too gross, for example more than 2-fold. In *E. coli*, the difference in i-cAMP concentrations between glucose and non-fermentable carbon sources is at least 4-fold: the shift from glucose- to succinate-containing medium results in a 10-fold increase and glucose- to glycerol-containing medium results in a 7.5-fold increase in i-cAMP concentration. It appeared that the i-cAMP concentrations were on a similar scale and therefore i-cAMP is not an indicator of catabolite repression, as in *E. coli*.

There have been many reports concerning the i-cAMP concentration with respect to catabolite repression (see section 1.5.2). Eraso and Gancedo (1984) concluded that catabolite repression was not associated with low i-cAMP concentrations. They found that the i-cAMP concentration was higher in cells that had been growing on glucose rather than ethanol. However, as they only tested one non-fermentable carbon source (ethanol), a definite conclusion from their data cannot be made. There are other reports that have compared i-cAMP concentrations between different carbon sources (Olempska-Beer and Freese, 1987; Mbonyi *et al.*, 1990). Olempska-Beer and Freese (1987) found that the i-cAMP concentrations of cells grown on either glucose or acetate are similar, thus supporting similar results found here. Also, Mbonyi *et al.* (1990) found that although non-fermentable carbon sources do not result in the production of a cAMP signal, similar concentrations of i-cAMP exist in cells that have been grown on fermentable or non-fermentable carbon sources. Results from this work appear to agree with those of Olempska-Beer and Freese (1987) and Mbonyi *et al.* (1990) in that i-cAMP concentrations are similar when cells are grown on fermentable or non-fermentable carbon sources.

Although the i-cAMP concentrations were similar when cells were grown on fermentable or non-fermentable media, it is interesting to note that the proliferation rates were not. The proliferation rate of cells grown on glycerol was lower than that of cells grown on glucose, and also the cells were smaller.

These data not only add to the theory that the i-cAMP concentration is size-independent, but also suggest that the i-cAMP concentration is not related to the growth rate and therefore unlike Whi2, whose transcription is sensitive to growth rate (Mountain and Sudbery, 1990b).

Theories (Thevelein, 1991) relating to the RAS-adenylyl cyclase pathway not being operative during growth on non-fermentable carbon sources and the RAS pathway is only triggered by fermentable sugars, and that this pathway is not operative on glucose and cAMP cannot be a signal for traverse of Start, are in contrast to results obtained in this work and by others, or there is confusion between the glucose-induced i-cAMP signal (a putative switch from non-fermentable to fermentable carbon sources) and i-cAMP synthesis that is present all of the time. The findings that show the requirement of Ras for growth on non-fermentable carbon sources (*ras2* mutants cannot grow on such carbon sources) and the domain of *cdc25* that allows growth on non-fermentable carbon sources, together with data obtained here, demonstrate that cAMP is synthesized on non-fermentable sources and this must occur via Cdc25, Ras and adenylyl cyclase.

4.3. Cell cycle stage-specific fluctuations in i-cAMP from *S. cerevisiae*.

It is well known that cAMP has an important role in the regulation of growth and also in the control of the cell cycle of *S. cerevisiae*. A series of experiments was performed in order to define this role more clearly and to determine whether the i-cAMP concentration fluctuates at specific stages in the cell cycle.

One method of determining whether there are cell cycle stage-specific fluctuations in the concentration of i-cAMP is to select cell cycle mutants that arrest at particular points in the cell cycle and study their patterns of i-cAMP concentration.

For this, a CDC^+ control strain was compared with two other cell cycle mutants, one of which arrested at Start and the other arrested at the end of the

cell cycle. On shifting to the higher temperature all three strains showed a transient increase in the concentration of i-cAMP. The reason for this increase and decrease in i-cAMP levels around shift-up could be explained by a number of reasons. First, adenylyl cyclase activity approximately doubles for every 10°C increase in temperature (Londesborough and Varimo, 1979) and the high K_m phosphodiesterase is independent of increases in temperature (Londesborough and Lukkari, 1980); nothing is known of the effect of temperature on the low K_m enzyme as yet. Secondly, feedback inhibition may shut down the synthesis of cAMP, although it appeared that feedback inhibition was not operative during normal growth on glucose, a temperature increase may stimulate feedback inhibition. Thirdly, cells at 36.5°C are proliferating faster and therefore use up glucose faster and may assume a lower level of i-cAMP sooner than slower proliferating cells at 25°C; i-cAMP concentrations at 36.5°C were soon similar to those in the 25°C cultures.

The control strain, A364A, showed a typical decreasing level of i-cAMP throughout the time-course of the experiment. However, *cdc28-D1* had a constant i-cAMP concentration after the transient increase and *cdc5-1* cells showed an increase. A constant i-cAMP concentration may mean that synthesis of cAMP equals degradation or that there is no synthesis or degradation. An increasing i-cAMP concentration may mean that synthesis is greater than degradation.

It may be significant that *cdc5-1* cells have a continuously increasing level of i-cAMP, as opposed to the constant levels of *cdc28-D1*. Neither of the mutations affect cAMP production or degradation directly. It is possible that the concentration of i-cAMP increases throughout the cell cycle and at cell separation, and it falls in preparation for Start, where a cell's environmental status is assessed (see section 1.3). A decrease at this stage of the cell cycle could function as a gate through Start: if there were nutrients present, then cAMP would be synthesized and the cell would traverse Start. The results from

the temperature shift-up of *cdc28-D1*, showing that there is a decrease in the i-cAMP compared with *cdc5-1*, may indicate that there is a lowering of i-cAMP concentration before Start. Table XI shows the ratio of intracellular cAMP at 36.5°C to 25°C, after a number of generations. After each generation of A364A, the ratio of cAMP remained close to a value of 1. However, for both *cdc5-1* and *cdc28-D1*, this ratio increased with successive generations. However, the ratio after two generations of arrest for *cdc5-1* is much higher than that of *cdc28-D1* therefore it seems possible that this increase may be due to its position or timing of arrest in the cell cycle.

To reinforce any theory concerning cell cycle stage-specific fluctuations of i-cAMP concentrations, a technique that will produce true synchronous cultures and not affect the activities of enzymes that may effect the i-cAMP concentration should be used. For this, centrifugal elutriation was chosen as the best available method.

Although good synchrony was obtained for all centrifugal elutriation experiments of the wild type strain, there was no consistent pattern of i-cAMP cell cycle stage-specific fluctuations. There was, however, a high level of i-cAMP measured at the beginning of the first three elutriations, particularly the first two experiments. This may mean that cAMP is high in cells that have just separated from the mother cell or those at Start. The first two elutriations showed decreases on commencement of budding, the third showed a decrease on cell separation and a rise on commencement of budding and the fourth showed no fluctuations at all. (Differences in magnitude between the two high plateaux observed in the results from the third elutriation experiment may have been due to two factors: first, the concentration of cAMP decreases over a period of time in relation to glucose, but only approximately 2-fold; secondly, because cell separation occurs over a period of time, daughter cells become delayed in respect to the cell cycle of their mother.

Table XI. The ratio of i-cAMP concentrations between cells grown at 36.5°C and 25°C after successive generations.

Strain	Number of generations		
	1	2	3
A364A	1.2	1.15	0.89
JL138	1.6	2.36	3.93
GR57	1.84	5.5	n.d.

n.d. represents not determined.

The difficulty in interpreting the information from the centrifugal elutriation experiments is that 100% synchrony was impossible to achieve and so trends in i-cAMP levels were difficult to identify as no two experiments were the same. Even with a good first cycle synchrony, which was achieved in all elutriations, results from each elutriation were different from the next. The possibilities that could cause such differences could be (i) the elutriation procedure is interfering with the cells (ii) extraction procedures for cAMP are affected differently in the cell cycle and (iii) there are no cell cycle stage-specific fluctuations in the i-cAMP concentration. It is doubtful that the first possibility affected the results, as the asynchronous cultures, which have been in the rotor for longer, did not show any serious perturbations with reference to the cell density, budding index or concentration of i-cAMP throughout the time-course of the experiment. Also, the asynchronous batch cultures showed reproducibility and consistency between experiments. The second possibility that the extraction procedure for cAMP is affected stems from the observation that in the laboratory of M. Wigler, cAMP cannot be detected after extraction with TCA (J.M. Thevelein, personal communication). It is thought (J.M. Thevelein, personal communication) that the inability to detect cAMP in the third elutriation experiment may have been due to the TCA extraction method. However, i-cAMP was observed at this same stage in other experiments and similar fluctuations to those seen in the third elutriation experiment have been observed (J.M. Thevelein, personal communication). The third possibility that there are no cell cycle stage-specific fluctuations, is supported by unpublished observations (J.W.M. Oehlen, personal communication to J.M. Thevelein); although this group tend to use the technique of age fractionation, which does not produce well defined stages throughout the whole of the cell cycle. Watson and Berry (1977b) also measured i-cAMP concentrations, and although the technique that they used was incomparable with centrifugal elutriation, they observed an increase at bud

initiation and a decrease at nuclear separation and division. Also, there were increases before decreasing finally at cell separation. The results of the third elutriation experiment (only) also showed increases or high concentrations of i-cAMP during bud initiation and decreases at cell separation.

One assumes that any reduction in the concentration of i-cAMP is due to phosphodiesterase activity, but if none is present then either feedback inhibition or extrusion into the medium is responsible. Elutriation using a strain that was deficient in both phosphodiesterases did not make it easier to define any cell cycle stage-specific fluctuations of i-cAMP. In the first experiment, the i-cAMP concentration appeared to mirror the budded index and as the cells separated, the i-cAMP concentration decreased, as in the third experiment with the wild type. For the second experiment, when the synchronous culture was compared with the asynchronous control it appeared that the level of cAMP fluctuated but not in relation to the cell cycle. Obviously phosphodiesterase has a role in controlling i-cAMP concentrations, however, the concentration of i-cAMP did not appear to fluctuate 'wildly' in the phosphodiesterase mutant, possibly meaning that there may be another factor controlling i-cAMP concentrations. The synchrony, however, was very poor for both experiments.

Age fractionation was also used to separate stages of the cell cycle. Interpretation of the results was extremely difficult as the cells were aggregating, forming clusters in the rotor and therefore a good separation could not be obtained. However, further evidence demonstrating that phosphodiesterase is not the only controlling element of i-cAMP concentrations was obtained, as the i-cAMP concentration did not fluctuate to a great extent overall compared with the i-cAMP concentration of the wild type. It is possible that extrusion of cAMP into the growth medium is an alternative method for control of i-cAMP concentrations (see sections 1.1.2 and 4.4).

From the centrifugal elutriation and age fractionation experiments it appears that there were fluctuations in the i-cAMP concentration, but these were

not specific to any stage of the cell cycle. However, the results from the *cdc* mutants experiments matched those of the third elutriation experiment, when the concentration of i-cAMP built up during the cell cycle and decreased after cell separation and before Start.

Despite the lack of evidence for stage-specific fluctuations in the i-cAMP concentration, it does not, however, necessarily follow that i-cAMP has no role in the regulation of the cell cycle. Intracellular cAMP is required for activation of PKA in order to control various processes such as carbohydrate and phospholipid metabolism, transcriptional regulation, and control of strategic points in the glycolytic and gluconeogenic pathways, but i-cAMP may not be tightly regulated for this purpose. The theory that i-cAMP has a role as a signalling molecule during the cell cycle has been disputed (Thevelein, 1991) on the basis of the glucose-repressible cAMP signal and feedback inhibition information. Much of the work in this area has largely been directed towards studying 'moments in time' with mutants, rather than studying wild type strains (and mutants) over a long period of time. First, it has been demonstrated here that feedback inhibition does not appear to be operating efficiently, as the levels of i-cAMP were more than the minimum concentration required for proliferation. Secondly, the glucose-repressible cAMP signal has been confused with the normal regulation of the cAMP concentration. As this signal is reportedly glucose-repressible, its role has been confined to the respirative-gluconeogenic switch and according to Thevelein (1991) cannot operate as a trigger for traverse of Start. But their results are only concerned with the signal and in no way can be related to normal cAMP synthesis or control of proliferation. Thevelein concluded by stating that a role for i-cAMP is to provide a basal level for control of proliferation, however, they experimented with the addition of glucose to cells that were already growing on glucose, and not cells that are growing on glucose and are subject to normal cell cycle regulation and cAMP synthesis.

4.4. Extracellular cAMP.

Results from centrifugal elutriation experiments, especially the third elutriation run (section 3.3.2.) showed possible stage-specific fluctuations in the level of i-cAMP. This result and those from the *cdc* mutants, may indicate that the i-cAMP concentration is high at end of the cycle and low at the beginning in preparation for Start. This led to the possibility that cells may actively release cAMP, perhaps at cell separation in preparation for Start. Disposal of cAMP, released at any stage of the cell cycle (specific or non-specific), may be part of the assessment of nutritional status. This could occur by phosphodiesterase, feedback inhibition or extrusion. In fact, it has been reported that cAMP is present in the medium of proliferating *S. cerevisiae* cells (Eraso and Gancedo, 1984; Olempska-Beer and Freese, 1987) and sporulating cells (Watson and Berry, 1977a). Extracellular cAMP is continually excreted during vegetative growth during guanine starvation (Olempska-Beer and Freese, 1987) and it is thought that cells need an optimum i-cAMP concentration for growth and excrete the excess, which may otherwise be harmful for growth (Olempska-Beer and Freese, 1987). Both reports showed similar e-cAMP concentrations to those found in this work: approximately 2 nM (Eraso and Gancedo, 1984) and 0-40 nM (Olempska-Beer and Freese, 1987). Watson and Berry found that e-cAMP was present in concentrations of the order of 50-800 nM.

Cyclic AMP was found in the media of synchronous and asynchronous cultures from the third elutriation experiment and in asynchronous batch cultures of a wild type and double phosphodiesterase mutant. A role for e-cAMP could not be assigned, however, as exogenously added cAMP did not appear to affect the proliferation of *S. cerevisiae*. Although, due to the design of both of the experiments, only large changes would have been detected. The role of e-cAMP could be to enable communication between cells, as found with other

nucleotides during sporulation of *S. cerevisiae* (Jakubowski and Goldman, 1988).

It also appeared that cells of *S. cerevisiae* possibly take up cAMP from the medium in which they are growing. This has been previously reported (Singh *et al.*, 1980; Matsumoto *et al.*, 1982b). Although uptake experiments were performed in a similar way to those of Matsumoto *et al.* (1982b) and Singh *et al.* (1980), the results may represent other phenomena. Cyclic AMP could have bound to a putative cAMP receptor protein on the wall of the cell and remained bound after washing, but then become unbound after TCA precipitation. It is possible that cAMP diffused into the intermembrane space and after TCA precipitation was mixed with the cytoplasmic solute. Therefore this observed uptake is not definite, but it may be useful to a cell if cAMP is extruded at some stage of the cell cycle.

The concentration of e-cAMP usually increased with time, perhaps increasing as cAMP was being extruded into the medium at cell separation. However, it was calculated that the e-cAMP concentration measured was higher than could possibly be present due to extrusion by cells at the end of the cell cycle. Perhaps the e-cAMP concentration was a function of the pH of the medium as the e-cAMP concentration increased with the decreasing pH of the medium with time. However, it was subsequently shown that the pH of the growth medium was found to interfere with the cAMP assay. This implied that values measured by this assay method were incorrect and unreliable, although control experiments had been performed that showed the concentration of e-cAMP correlated with the dilution factor, and addition of growth medium actually depressed the concentration of e-cAMP, as measured by the assay. It also meant that the e-cAMP concentrations measured by Eraso and Gancedo (1984), Olempska-Beer and Freese (1987) and Watson and Berry (1977a) were incorrect, as they used essentially the same method to assay e-cAMP.

Due to the unreliability of the e-cAMP concentrations measured using the assay method of Brown *et al.* (1971, 1974), phosphodiesterase was added to used growth medium in order to see if e-cAMP could be identified by degradation. Only one experiment out of nine showed any degradation. This result indicated that no e-cAMP was present in the medium. The lack of observed phosphodiesterase activity may have been due to a constituent of the growth medium that interfered with its activity, although it was subsequently found that phosphodiesterase was able to function in GM medium.

It was then necessary to employ an alternative method of measuring the e-cAMP concentration. A technique was required that would not only ensure the accurate identification of cAMP, but also that there was minimal interference. The technique of HPLC was chosen, although, if by coincidence the range of concentration of e-cAMP measured by the Brown *et al.* (1971, 1974) method was correct (approximately 1-10 pmol/ml), a peak of e-cAMP would be too small and difficult to determine. It is, however, possible that the concentration of e-cAMP is higher (or lower) than that determined by the assay method for cAMP.

Bona fide cAMP was used in order to determine the elution time of cAMP on the chromatograph. A peak was observed that eluted at the same time as the *bona fide* cAMP and the concentration of this peak varied between 15 and 500 pmol/20 μl, much higher than the concentration of e-cAMP measured by assay was between 1 and 10 pmol/ml. Not only were there large differences between the e-cAMP concentration measured between individual HPLC experiments, but sometimes there was e-cAMP and sometimes there was not. This may have been due to differences in batches of yeast nitrogen base, which is a constituent of GM medium, or it may be due to insufficient separation of nucleotides resulting in a false peak of cAMP. Yeast nitrogen base does in fact contain compounds that absorb at around 259 nm, for example folic acid, inositol, niacin, *p*-aminobenzoic acid, pyridoxine hydrochloride, riboflavin and

thiamine hydrochloride. Where these particular substances elute in relation to cAMP is not known.

The possibility that the e-cAMP observed may be another substance was tested by adding phosphodiesterase to growth medium. It was found that the enzyme could degrade cAMP present in the growth medium, but no degradation was observed of the putative e-cAMP. It therefore seems probable that the e-cAMP observed may be a compound that co-elutes with cAMP, thus giving misleading results. A possible candidate is 2',3' cAMP, which is a breakdown product of RNA and may be present in the medium due to break up of dead cells. Although the peak identified was probably not e-cAMP, it is still possible that cAMP is present in the growth medium of *S. cerevisiae*, but it is masked by other compounds.

4.5. Overall conclusions.

The assay method of Brown *et al.* (1971, 1974) and the modifications developed during this work show that cAMP can be reliably measured from cell extracts of *S. cerevisiae*. Results obtained showed that the i-cAMP concentration decreased during exponential growth on glucose and this concentration was in excess of a putative threshold level required for traverse of Start. Feedback inhibition also appeared not to exist. No gross differences were found between the i-cAMP concentrations from cells grown on either rich or minimal media and non-fermentable or fermentable carbon sources.

The results from the centrifugal elutriation experiments were not conclusive, but it would appear that no cell cycle stage-specific fluctuations in the i-cAMP concentration exist, although fluctuations were seen to occur. It appeared that there may be another factor controlling i-cAMP concentrations. The presence of e-cAMP is still questionable as the experiments performed could not conclusively state whether e-cAMP was present. However, it is now known that e-cAMP should not be measured by the use of an immunoassay, in

particular that of Brown *et al.* (1971, 1974), unless interfering compounds have been completely removed.

4.6. Further work.

Although no differences could be found between cells grown on fermentable and non-fermentable carbon sources, useful information may be obtained if the i-cAMP concentration from cells that are growing on these carbon sources was monitored throughout the lag, exponential and stationary phases. Differences in concentration may be found due to the different metabolism of these carbon sources.

In order to complete the evidence for or against cell cycle stage-specific fluctuations in the i-cAMP concentration, it would be desirable to assay the activity of adenylyl cyclase or PKA. This could be achieved only if the yield obtained from elutriation is increased.

The presence of e-cAMP is yet to be confirmed, however, it has been subsequently found in the literature that a phenylboronate-agarose column can be used to remove interfering compounds from the growth medium (Fehr *et al.*, 1990). The detection of cAMP may be additionally improved after removing all ribonucleotides from the medium by adding barium and zinc sulfates and then separating the growth medium on a reversed phase column (Perrett, 1986). These modifications should be used to provide a more accurate method for determination of e-cAMP.

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