ROLE OF CYCLIC AMP IN THE REGULATION OF
BIOSYNTHESIS OF SOME ENZYMES IN YEAST

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by
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To

my parents and my wife.
SUMMARY

10 mM cyclic AMP causes a slow lifting of catabolite repression of α-glucosidase (but not invertase), in the presence of 2% glucose, in yeast protoplasts but not in whole yeast. α-Glucosidase is partially protected in vivo from loss under deadaptation conditions by maltose. Protection in vitro by cyclic AMP or maltose is shown, against chemical inhibitors or heat.

The concentration of intracellular cyclic AMP was found to be low under high glucose conditions and to parallel the appearance of cytochrome P-450 (but not its loss). In agreement with this, the cytochrome P-450 concentration is high in high glucose media (5-20%): and cytochrome a+a₃ formation is repressed as expected. Maximal levels of cytochrome P-450 are reached at the end of the growth phase (40 hours of culture) in high glucose media. Rapid disappearance of the enzyme occurs after this, but the cyclic AMP level did not rise, despite the rise in cytochrome a+a₃ level. Cytochrome P-450 is produced rapidly during early phase of growth, up to 17 hours in 1% and 2.5% glucose media (none is produced in 0.1% glucose medium where cyclic AMP levels are high). The cytochrome P-450 subsequently disappears from the yeast while the appearance of cytochrome a+a₃ can now be observed.

Cyclic AMP added to the yeast protoplast can prevent the accumulation of cytochrome P-450 in 20% glucose medium, where it is otherwise formed. Conversely, induction of cytochrome P-450 by cyclic GMP has been demonstrated in yeast protoplasts. Sodium phenobarbital, at 0.2%, causes the maximal accumulation of cytochrome P-450 in yeast growing in 0.5% glucose medium. Also, various antimitochondrial drugs had been studied in relation to the accumulation of cytochrome P-450.
The optimal conditions of cytochrome P-450 production in yeast have been determined in continuous culture.

The opposing behaviour of cyclic AMP and cyclic GMP in the regulation of these yeast enzymes is discussed.
ACKNOWLEDGEMENTS

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CHAPTER I

General Introduction
I GENERAL INTRODUCTION

1.1 It was shown by Reese (1975) that a bacterial species grown in a cellulose medium produced a cellulose hydrolyzing enzyme. The micro-organism clearly adapted to the substrate in the medium by producing a substrate metabolising enzyme and the presence of substrate 'switched on' the appropriate mechanism for its production. This process is now recognised as induction. The phenomenon of enzyme induction is of great ecological importance to micro-organisms since with foodstuffs in the environment, enzymes able to break down these foodstuffs will be produced. The other way by which micro-organisms can adapt and hence survive in the environment, is by the selection of mutants in a genetic population which can utilize these foodstuffs, by having the ability to produce the necessary enzymes.

1.2 Basal enzyme and induction ratio

Monod (1956) showed that enzyme induction involved the de novo synthesis of protein, or group of proteins, in response to the addition of a specific molecule. Enzyme formation from amino acids occurs, rather than from some inactive peptide or protein precursor existing up to addition of the inducer of the culture.

The introduction of a highly sensitive assay for β-galactosidase (Lederberg, 1950) activity has shown that non-induced cells did contain very low levels of β-galactosidase activity. There is considerable evidence that basal and induced enzymes are biochemically identical and are made by transcription of the identical structural genes. Clearly slow or occasional transcriptions of these genes does occur, which is accelerated by the switch on effect of the inducer.
However, the demonstration of the presence of a basal level of enzyme activity in non-induced cells raised a number of inter-related problems. The first was whether the basal level of activity could be due to the presence of constitutive mutant cells which produced a high level of enzyme even in the absence of substrate. Although such mutants are present in cultures of non-induced cells (Ryan, 1952) quantitative analysis by Holmes, et al (1961) indicated that their numbers are insufficient to account for the basal level of enzyme. The non-induced cells themselves produced a basal level of enzyme. This enzyme was related but it raised the question as to whether exogenous substrate could in some way affect the genetic material of the cell so that it can produce an enzyme of altered structure and efficiency as a catalyst.

This has been examined in some detail only for the β-galactosidase enzyme of *E.coli* and the extracellular penicillinase, or β-lactamase enzyme produced by strains of *B.cereus* and *B.licheniformis*. Evidence for all other systems is far less complete and usually rests upon the currently accepted concepts of the mechanisms controlling synthesis of inducible enzyme as demonstrated by the β-galactosidase system. The enzyme and antigenic properties of β-galactosidase in a variety of ionic environments proved the identity of the molecular species of this enzyme (Cohen-Bazire and Monod, 1951). When such a comparison was made, no differences were found between the basal and induced β-galactosidases or the enzyme produced by a constitutive mutant. Similarly, Manson et al (1954) were unable to demonstrate any differences between the basal and induced penicillinases for *B.cereus* and *B.licheniformis* nor were differences found for enzymes produced by a constitutive mutant (Kogut, et al, 1956).
Therefore, the available evidence for the β-galactosidase and penicillinase enzymes indicates that the phenomenon of induced enzyme synthesis is one of regulation of the quantity of enzyme formed.

The differences between the basal and the induced level is measured by the inducibility of the system and this quantity is usually measured in terms of the induction ratio which is defined by

\[ \text{IR} = \frac{\text{induced enzyme level}}{\text{basal enzyme level}}. \]

1.3 Gratuitous induction

A gratuitous inducer can be defined as an inducer which is insensitive to the action of the enzyme they induce. Certain substrates related structurally to the natural inducer expected in a system may be much better inducers, whether they serve as a productive substrate of the inducible enzyme or not.

In yeast, methyl-β-glucosidase is an excellent inducer of β-glucosidase but is not a substrate of the enzyme (Halvorson, 1960). The effect of the inducer cannot therefore depend on the concept of substrate breakdown or even on affinity, as induction is achieved at a much lower concentration of inducer than is needed to half-saturate the active site of the enzyme.

1.4 Kinetics of induction

Benzer (1953) provided evidence that cultures of *E. coli* synthesized β-galactosidase enzyme at approximately the same rate under conditions approximating to those of gratuity. He also showed that the increase in
rate of synthesis of the enzyme followed after only a very short lag period, after the addition of inducer. On removal of the inducer, synthesis of enzyme ceased rapidly. It may be noted that when the inducer served as the source of carbon and energy for growth Benzer (1953) found substantial heterogeneity among the cells of the population. Subsequently, Rotman (1961); Rotman et al. (1963) introduced the use of fluorogenic galacto-ide substrates to measure the enzymic activity of individual cells before and after induction and demonstrated the participation of all cells in the process. Similarly, Collin (1964) demonstrated the synthesis of penicillinase enzyme to approximately the same extent by individual cells of B. licheniformis. In the yeast cell, β-glucosidase is shown to have its biosynthesis switched on at maximal rate immediately by the addition of this inducer (Duerksen and Halvorson, 1959). The level of the enzyme rises rapidly in the culture differential rate is constant of that inducible enzyme.

This differential rate of synthesis is expressed as percentage of enzyme activity in the culture (per ml of culture), ignoring the low basal level of inducible enzyme and the low culture density at the time of addition of inducer. Some authors also use the term differential to refer to a change in induced enzyme level with respect to time, that is referring to the rate of enzyme biosynthesis.

1.5 Sequential induction

There are two distinct processes by which a single inducer may evoke the induced synthesis of a series of enzymes, comprising a complete metabolic pathway. The successive steps occurring in the oxidation of the indole ring of tryptophan to β-keto-adipic acid has been shown in
Pseudomonas species during the development of the ability to metabolize aromatic acids. The first is the process of sequential induction, which was discovered independently by Karlsson and Barker (1948). Whenever induced enzyme synthesis is elicited by an inducer which is a substrate of the enzyme formed, the latter will convert the inducer substrate into the corresponding product. This may serve as an endogenous inducer, i.e. as substrate of a second enzyme. Switch on of biosynthesis of the required enzyme occurs when the appropriate concentration of potential substrate is reached to effect overall the efficient degradation of the substrate.

1.6 Coordinate induction

My emphasis so far has been on describing the regulation of a single enzyme protein in response to the addition of inducer or repressor to the culture, because in many respects this is a typical situation. The switch-on mechanism of inducers may cause the appearance of two or more enzymes in constant molar ratio usually equal. For example, in the Lac system of E.coli three proteins, β-galactosidase itself, the galactoside permease and β-galactoside transacetylase, whose function is unknown, are produced by the inducers of β-galactosidase. In practice it appears from mapping data that the structural genes for the three components of the Lac operon system are contiguous on the genome and are controlled by one gene and one operator. Thus in this case, addition of inducer leads to the coordinate expression of all three enzymes and the group of enzymes is said to constitute an operon. Experiments on the nature of the messenger RNA molecule made by the genes of the Lac operon suggest strongly that the whole region is transcribed as a single messenger molecule and that this is then translated to give the separate polypeptide chains which
correspond to the three structural genes. This well known model is widely accepted and certainly applies to many bacterial systems. With negative control the operon is normally switched off by the action of a protein repressor molecule acting at the operator. Positive control, however, has been suggested more recently for the operon that controls the biosynthesis of enzymes in *E. coli* responsible for the pathway of arabinose degradation.

Most of the well characterised operons in bacteria are each under the negative control of a protein regulator molecule (repressor) which switches off the operator by combining with the DNA at this regulatory point. The repressor protein is synthesized in the usual way on a messenger RNA template made by a distinct regulatory gene. The mode of action of this gene does not require it to be closely linked to the operon it represses, whereas the operon itself consists of the contiguous segments of DNA forming in linear sequence, one region called the promoter (next to the operator region) and then the appropriately linked structural genes that show coordinate induction. It is the combination of inducer with this protein repressor that moves the latter from its DNA binding site on the operator, presumably by eliciting a conformational change that lowers its affinity for this site. The promoter region is responsible for the initiation of transcription of messenger RNA by RNA polymerase (see Fig. 1.1).

1.7 Catabolite repression of inducible enzyme

There is an additional phenomenon of regulation of enzyme synthesis in response to the presence in the medium of readily available sources of catabolic intermediates, superimposed on the regulatory phenomenon of induced
Fig. 1.1 Basic "Jacob-Monod" hypothesis for negative control of induced biosynthesis of some enzymes in bacteria (E.coli)

The inducer removes the repressor protein from the operator gene causing switch-on of structural genes of the operon, coding via messenger RNA for the biosynthesis of the corresponding enzymes subject to coordinate induction by that inducer.

Regulator gene

Operator

Structural genes
1, 2, 3 of the operon

Gene DNA specifies messenger RNA

m. RNA

Switch off

m. RNA1

m. RNA2

m. RNA3

Messenger RNA specifies enzyme biosynthesis

Switch on by INDUCER

m. RNA

Repressor protein

Enzyme1

Enzyme2

Enzyme3
enzyme synthesis and end product repression. This phenomenon was well known as 'the glucose effect'. For many years it had been recognised that the levels of various enzymes found in bacterial cells could be markedly affected by the addition of carbohydrates and particularly glucose to the medium. In some cases the effect of glucose or other carbohydrates was indirect and due to changes in pH of the medium resulting from acids produced by incomplete oxidation of the sugar. However, in other cases glucose inhibited the formation of enzymes by some mechanism other than by serving as a source of acids (Gale, 1943). Many of the enzymes present in markedly reduced levels during growth in medium supplemented with glucose are inducible enzymes. However, it is not a general rule that synthesis of all inducible enzymes is repressed in the presence of glucose. Indeed in some cases where the inducible enzyme is not one acting on a carbohydrate substrate the provision of glucose, or an equivalent source of energy, is an absolute requirement for enzyme formation (e.g. Magasanik, 1957). Glucose is not the only carbon source able to repress enzyme formation (Mandelstam, 1957; Perlman et al, 1969) but it is one of the most potent inhibitors of enzyme formation with the greatest effects. The basis of this glucose effect was first formulated by Neidhardt and Magasanik (1956, 1957) and elaborated in detail by Magasanik (1961).

The enzymes subject to end product repression play key roles in a number of divergent anabolic pathways which serve to balance most efficiently the flow of common metabolic intermediates and energy into production of all the materials required for metabolism and growth of the cells. Conversely, enzymes subject to the glucose effect participate in a variety
of convergent catabolic pathways, controlling the flow of common intermedi­
mediary metabolite and energy into the endogenous intracellular pools. Neidhardt and Magasanik (1956, 1957) therefore suggested that just as the divergent anabolic pathways were regulated by end product repression so were the convergent catabolic pathways.

1.8 Transcription of genes factors and inhibitors

Initiation of transcription demands the selection by RNA polymerase of specific binding site on DNA. Recently RNA polymerase holoenzyme of E.coli has been fractionated by chromatography on phosphocellulose into apoenzyme and another protein (eluted) namedSigma factor (Burgess et al, 1969). This factor transiently associates with RNA polymerase and confers on it an ability to discriminate between promoter sites of operons, so that positive control is exerted over messenger RNA synthesis. No doubt a variety of such Sigma factors are involved in regulation of microbial enzyme biosynthesis. Many Sigma factors are thought to be involved in induction of enzymes of a bacterial cell. Another factor rho, directs the termination of the action of RNA polymerase after completion of appropriate operons (Roberts, 1969).

Most studies on inhibition of transcription have used Actinomycin-D (combines with DNA) and use of this inhibitor is usually assumed to block transcription in all organisms. Growing E.coli does not take up this compound but EDTA treated cells can (Leive, 1968). Recently rifampicin had been shown to be a competitive inhibitor of RNA polymerase for its promoter binding site.

1.9 Translation of Messenger RNA

A large number of inhibitors have been found to inhibit overall
translation in microbial organisms, by affecting one or more steps e.g. transfer (of amino acids from its transfer RNA complex), chain elongation or translocation of ribosomes along the messenger RNA. Studies have been reported on tetracyclines (Cerna et al, 1969) pactamycin (Cohen et al, 1969) streptomycin (Ozaki et al, 1969) all of which mainly inhibited transfer chain elongation, mediated by peptidyl transferases. Other inhibitors are puromycin, chloramphenicol and sparsomycin (Monro et al, 1969). Cycloheximide, a translation inhibitor, is often used to inhibit cytoplasmic protein synthesis in yeasts.

All of these compounds inhibit protein synthesis in animals or animals systems. Thus cycloheximide inhibits several animal systems, so does puromycin. Chloramphenicol is of particular interest. Although bacteria are much more sensitive to its effects at low concentrations, this antibiotic inhibits mainly mitochondrial protein synthesis and induced protein synthesis.

1.10 Control of Lac operon in E.coli

A number of recent studies have indicated that cyclic AMP plays a major role in the synthesis of inducible enzymes. Many of these studies have been concerned primarily with the effects of cyclic AMP in relieving an inhibition of induced enzyme synthesis caused by catabolite (glucose) repression. Some studies have been made of the effects of cyclic AMP on induced enzyme synthesis. Addition of an exogenous source of cyclic AMP enhances the differential rate of β-galactosidase synthesis in E.coli (Ullman and Monod, 1968). However it does not affect the duration of the lag period before onset of rapid enzyme synthesis (de Crombrugghe et al, 1969, Pastan and Perlman, 1968).
In agreement with this work, cyclic AMP degrading enzyme (Monard et al, 1969) and adenyl cyclase (Tao and Lipmann, 1969) and (Ike, 1964) have been isolated from E.coli. Furthermore glucose exhaustion has been shown to derepress adenyl cyclase activity in intact E.coli (Peterkofsky and Gozdor, 1973). A mutant strain with a marked deficiency of adenyl cyclase enzyme activity shows very poor growth on a number of media for which an essential prerequisite for growth is the induced synthesis of enzyme acting on the carbon source. Addition of cyclic AMP markedly stimulates induced β-galactosidase synthesis and growth on the various test media (Pastan and Perlman, 1970, Perlman et al, 1969). Conversely a mutant strain lacking enzyme degrading cyclic AMP shows little effect of varying the carbon source upon β-galactosidase formation (Monard et al, 1969). More general studies extended to this work show that glucose repression of many enzymes of E.coli could be decreased by cyclic AMP and furthermore that this applied to β-galactosidase biosynthesis in many bacterial species (de Crombrugghe et al, 1969, Carpenter and Sells, 1973).

1.11 Level of Control of β-galactosidase by Cyclic AMP

Cyclic AMP appears to stimulate transcription of messenger RNA from the Lac operon (Jacquet and Kepes, 1969, Varmus et al, 1970(a) 1970 (b), Chambers and Zubay, 1969) observed a very marked stimulation by cyclic AMP of the in vitro induced synthesis of β-galactosidase in a coupled system able to transcribe m.RNA and to synthesis protein. The site of action of cyclic AMP appears to be the promoter of the operon. Either point mutation within this region or deletion of most of the promoter, markedly reduces the sensitivity to stimulation by cyclic AMP (Pastan and Perlman, 1968, Perlman et al, 1969, Silverstone et al, 1969). On the other hand mutants
affected in the operator 0 and regulator I genes show similar responses
to those of the wild type strain (Pastan and Perlman, 1968, Perlman and
Rickenberg (1974) claimed that catabolite repression of the proteins
coded by the Lac operon as well as the reversal of catabolite repression
by cyclic AMP, occurred at the level of translation. However it may be
explained on the basis of the use of presumptive inhibitors of transcription
which blocked transcription only particularly under the conditions of the
experiments. Ramirez et al (1972) and Dahl et al (1971) suggested that
cyclic AMP exerts its effect predominantly at the level of transcription.
An effect of cyclic AMP on transcription, but not on translation, has been
shown in other bacterial systems, Chao (1974) reported that glucose and
methyl glucoside and deoxyglucose and pyridoxal phosphate repress
maltodextrin phosphorylase synthesis in E. coli but this repression is
reversed by 2mM-cyclic AMP. Therefore action of cyclic AMP appears to be
at the transcription level. Haggery et al (1975) reported that the
addition of glucose but not glycerol to the E. coli lowered the initiation
frequency of Lac mRNA within 10 seconds. Interestingly, intracellular
cyclic AMP level responded almost identically to glucose or glycerol
addition.

1.12 Cyclic AMP Receptor Protein

Cyclic AMP seems unlikely to react directly on the DNA of the promoter
and the protein which mediates its action has been identified by the
existence of the second class of mutants which has normal levels of cyclic
AMP but nevertheless fails to induce many operons. Cells of this Crp-
mutant genotype contain little lactose mRNA even when induced with IPTG.
Two approaches have been used to identify the protein inactivated in these mutants. Emmer et al (1970) have compared the ability to bind cyclic AMP of extracts from wild type and mutant cells. Most of the cyclic AMP binding activity of wild type cells present in the supernatant can be bound to phosphocellulose columns, and elutes from them when the ionic strength is increased. One Crp\textsuperscript{−} mutant lacks this protein activity, another possesses a protein which has a much lower affinity for cyclic AMP.

By using a DNA dependent cell free system for synthesising \(\beta\)-galactosidase, Zubay et al (1970) have shown that extracts from Crp\textsuperscript{−} mutants have only about 5\% of the activity of wild type extracts. Cyclic AMP stimulates transcription of lactose m.\(\text{RNA}\) in the presence of the proteins extracted from wild types cells but cannot do so when the proteins are derived from mutant cells. By using this system they were able to purify the cyclic AMP binding protein using its activity in stimulating transcription of the lactose operon. Riggs et al (1971) and Anderson et al (1971) have reported the Crp factor (cyclic AMP receptor protein) is a dimer of sub-units of 22,300 daltons and has an isoelectric point of just above pH 9.

The promoter region was diagrammatically divided into the two sites. One is in a cyclic AMP protein recognition site at the left and an RNA polymerase binding site at the right. Both these sites must be occupied by the proteins presumably so the Crp protein interacts with polymerase before transcription can be initiated (see fig. 1.2).
Fig. 1.2 Regulation of transcription of the lac operon of E. coli

The genes marked 1, 2, 3, α, p and probably are contiguous in this organism. Additional control (positive) by cyclic AMP plus its receptor protein (CRP) is of particular interest.
1.13.1 Some advance with other enzyme systems

(a) Catabolite repression and induction of glucosidase with respect
to the utilisation of maltose, maltotriose and glucose by brewery
yeasts.

During sugar fermentation it is known that the presence of glucose
in the medium suppresses the ability of yeast to utilise maltose and that
both glucose and maltose suppress the use of maltotriose, Hough et al (1971).
The phenomena lead to the following order of sugar utilisation (a) glucose,
fructose (b) maltose (c) maltotriose. This is because glucose, fructose and
sucrose are the direct substrates of hexokinase and the other enzymes of
glycolysis.

Maltose and maltotriose are only used at an appreciable rate by the
yeast after preliminary growth on one of these two sugars. The adaptation
depends on two enzymes, a specific maltose permease for the active transport
of the substrate and an α-glucosidase which hydrolyse the endogenous maltose
to glucose. The transport of maltotriose is also achieved by means of
specific permease (Harris and Thompson, 1960). It is the intervention of
specialised proteins, their stereospecificity and their specialised
functions that distinguished the metabolic enzymes implicated in the
utilisation of carbohydrates. The permeases are all genetically controlled
as their activity can be altered specifically following a mutation. In
yeast the synthesis of α-glucosidase is controlled by six non-linked genes.
Halvorson et al (1963) have shown the gene controlling α-glucosidase
synthesis are structural genes. Halvorson et al (1963) indicated that in
the dominate state the system is inducible and that in the diploid
heterozygote the inducibility is a dominant character. The inducible and
and repressible maltose and maltotriose permease and α-glucosidase are the cause of the sequential utilisation of wort sugar by brewery yeasts. The rapid metabolism of glucose leads to an accumulation of catabolites which act as the regulating agents. The regulation of α-glucosidase has been shown to be involved with cyclic AMP level. Cheung (1966) has shown that the presence of cyclic AMP in S. carlsbergensis. Van Wijk and Konijn (1971) and Schlenderer and Dellweg (1974) have shown that the yeast grown on glucose contains six times less cyclic AMP than yeast grown on galactosidase. Sy and Richter (1972) showed in S. fragilis that the intracellular content of cyclic AMP and adenyl cyclase varied during growth on 0.15% glucose with a maximum concentration at the end of growth which corresponded to the depression of respiratory and glyoxylic shunt enzymes. Vauler Plaat and Van Solinger (1974) reported that trehalase was under control of a system regulated by cyclic AMP of Baker's yeast during the lag phase of growth. An increase in the intracellular concentration of cyclic AMP was followed by 6-8 fold increase in trehalase activity. The cryptic trehalase was found to be activated by cyclic AMP to the same degree in vivo. Montene court et al (1973) reported that the levels of cyclic AMP were correlated with the sensitivity of invertase synthesis to glucose repression. Schlanderer and Dellweg (1974) reported that in Schizosaccharomyces pombe an inverse proportionality between glucose concentration and intracellular cyclic AMP was observed in cells grown in glycerol or maltose. Wheeler et al (1974) found that adenyl cyclase (the cyclic AMP-forming enzyme) in S. cerevisiae is inhibited by fluoride, but this was contradicted by Londesborough and Nurminen in 1972.
1.13.2 Induction and breakdown of cytochrome P-450

(a) hepatic system.

Cytochrome P-450 is an inducible enzyme, its formation in animal tissues being induced by phenobarbital (Remmer, et al, 1976; Garner and McLean, 1969; Kuntzman et al, 1968) and by 3-methylcholanthrene and other polycyclic hydrocarbons (Conney and Gilman, 1969; Sladek and Mannering, 1966; Long, 1969; Jeffcoate and Taylor, 1969; Nebert, 1969). The increase is a synthetic increase rather than due to a decrease in its breakdown (Garner and McLennan, 1969; Grein et al, 1970). It requires protein synthesis (Orrenius, et al, 1965; Marshall and McLean, 1968, 1969) and leads to the formation of specific apoproteins of P-450 depending on the inducing substrate (Sladek and Mannering, 1966; Long, 1969). This is of importance insofar as the dependence of induction on protein synthesis might have been due to more indirect causes. Thus induction of δ-ALA-synthetase is required for haem synthesis (Baron and Tephley, 1969a,b., 1970) and the labelled δ-ALA is found in P-450 (Levin et al, 1970). Haematin present acts as a repressor of the induction of microsomal protein synthesis by drugs (Marver et al, 1968). An inducible enzyme must also undergo rapid breakdown in the absence of an inducer. This has been shown for P-450 (Schmid et al, 1966; Robinson et al, 1966, Robinson, 1969).

(b) reaction mechanism of cytochrome P-450

The endoplasmic reticulum consists of a bimolecular layer of lipid covered on both sides with protein. The protein forms a strong lipoprotein complex (outer layer). The outer layer contains the readily solubilized components of microsomal electron transport. NADH cytochrome
bj reductase, cytochromes bj, NADPH P-450 reductase and NADH reductase. The inner strong complex contains P-450 held by lipoprotein in a specific configuration. The function of this organisation being the metabolic control of the oxidation and reduction of (a) water soluble metabolites and (b) lipid soluble metabolites.

The hydroxylation reactions effected by cytochrome P-450 are termed mixed function oxidation or mono-oxygenations. The molecular basis of P-450 activity is as shown (see Fig. 1.3).

Lu et al (1969) postulated that NADPH enters the reaction twice, once as reductant of ferric P-450 and then again transforming the initial oxygen adduct of ferrous P-450 into an active hydroperoxocomplex, and the bound substrate molecule is oxidised after transformation into a ferryl form.

(c) Other systems of cytochrome P-450 in mammals (Mitochondrial systems)

Cytochrome P-450 has been identified as participating in steroid hydroxylation reactions in the adrenal cortex (McKerns, 1968) the corpus luteum of the ovary (McIntosh et al, 1975) and in the testis (Manson, et al, 1973) and placenta (Meigo and Ryan, 1968). This enzyme has been identified in both the mitochondria and microsomes (McIntosh et al, 1973 Meigo and Ryan, 1968). The hydroxylase system in these systems is as below (see Fig. 1.4).

The mitochondrial P-450 is associated with unique iron-sulphur protein, and a flavoprotein serves as necessary electron transport components. (Simpson et al, 1969) for the transfer of reducing equivalents from NADPH to cytochrome P-450. It should be noted that the one electron transfer iron sulphur protein (adrenodoxin) interacts at two steps (Kimura and Suzuki, 1965) during the cyclic function of cytochrome P-450.
Fig. 1.3 Hydroxylation reactions of cytochrome P-450 in liver microsomes
Fig. 1.4. The electron transport system functional in adrenal cortex mitochondria of cytochrome P-450 catalysed reaction (Estabrook, et al, 1975)
(d) Other systems of cytochrome P-450

In insect microsomes cytochrome P-450 acts as a terminal oxidase of a mixed function oxidase system concerned with xenobiotic metabolism (Temple, 1971). A cytochrome of the P-450 type has been shown to exist in the microsomal fraction derived from Arum Spadices (Yahiel, et al, 1974). Cauliflower microsomes were found to contain cytochrome P-450 exhibiting the same characteristic as mammalian microsomes but cytochrome P-450 in Mung Bean could not be detected (Rich and Bendell, 1975).

In higher plants P-450 has been implicated in the biosynthesis of one of the plant hormones, the gibberellins (Murphy and West, 1969), whereas in other higher plants such as pea and bean, in which cytochrome P-450 may be detected in the cotyledons, no function has yet been ascribed (Moore, 1967). In the ergot producing fungi, Claviceps purpurea, cytochrome P-450 is involved in the production of the ergot alkaloids (Wilson and Orrenius, 1971).

(e) Cytochromes P-450 of microorganism

In microbial systems only Pseudomonas putida has aroused much interest to date. When this organism is grown on D-(+) camphor, induction of cytochrome P-450 can occur (P-448 in fact, Peterson and Griffin, 1972). This type of cytochrome was found as one component of the inducible methylene hydroxylase system that catalyses the hydroxylation of camphor to its exo 5 alcohol which is followed by further degradative steps (Hedegaard and Gunsalus, 1965; Katagiri, et al, 1968; Gunsalus et al, 1967). The early enzymes of the camphor oxidation metabolic pathways are induced by a variety of bicyclic monoterpenes. There is considerable interest in the use of the relatively low-molecular weight cytochrome P-450 as a model compound,
and relatively large scale isolation and purification of it has been achieved (Peterson, 1971) and it had been crystallized in a stable form as its substrate couple with D-camphor (Yu and Gunsalus, 1970). The purified P-450 was a single polypeptide chain and has a molecular weight in the range $4.4 \times 10^4$ to $4.6 \times 10^4$ (Yu, et al, 1974). Dus et al (1975) found that the substrate specificity of P-450 from P. putida and the cytochrome P-450 from rabbit liver microsomes was different but they are structurally similar.

Though the cytochrome P-450 from P.putida has many similarities to the hepatic system it is strictly not a mixed function oxidase, which by definition utilises molecular oxygen and NADPH whereas the camphor hydroxylase uses NADH. In addition to cofactors and P-450 the camphor hydroxylase system requires two other proteins, putidaredoxin and putidaredoxin reductase (Tyson et al, 1972) which performs functions of electron transport from NADH to cytochrome P-450 similar to cytochrome $b_5$ and $b_5$ reductase in liver systems (see Fig. 1.5).

Cytochrome P-450 has been detected in several other microbial species under some conditions of growth. The role of this enzyme is still unknown. Some interest has been shown in the cytochrome P-450 concerning tetradecane metabolism by C.tropicalis (Lebeault, et al, 1971). Thus cytochrome P-450 was isolated from C.tropicalis grown on tetradecane with high oxygenation and stirring rate (Lebeault, et al, 1971; Gallo et al, 1971). A soluble enzyme system containing cytochrome P-450 as one component was subsequently isolated and shown to be capable of oxidizing n-alkenes to the corresponding primary alcohol. This enzyme was also able to oxidise fatty acids to their $\omega$-hydroxylation derivative, being specially active in the oxidation of lauric acid. This oxygen-
Fig. 1.5 Hydroxylase System in P. Putida
dependent reaction needs NADPH.

A species of Torulopsis, when grown on a medium containing glucose, has been reported to hydroxylate added fatty acids at the terminal or penultimate carbon atom depending on chain length and degree of unsaturation (Tulloch et al, 1962). Stearic and oleic acids are hydroxylated mainly at the penultimate carbon atom, giving 17-L hydroxy acids as the product. Further studies with this yeast system suggest the involvement of cytochrome P-450 e.g. it required O\textsubscript{2} and NADPH and was inhibited by carbon monoxide but not cyanide (Heinz et al, 1970).

(f) Cytochrome P-450 in S.cerevisiae

Cytochrome P-450 was discovered in baker's yeast grown anaerobically in 10% glucose supplemented with oleic acid and ergosterol (Lindermayer and Smith, 1964). When yeast was grown aerobically in high concentrations of glucose, the P-450 was present (Ishidate et al, 1969a). In deadaption studies, cytochrome P-450 decreased sharply over 4 hours under aerobic conditions. Ishidate et al (1969b) suggested that P-450 was associated with biogenesis of functional mitochondria. An inverse relationship has been shown between the presence of mitochondria and the presence of cytochrome P-450 in yeast. High concentrations of glucose or of chloramphenicol repressed the mitochondria formation and also prevented the loss of cytochrome P-450.

The cytochrome a+a\textsubscript{3} is needed for aerobic respiration which is parallel to the uptake of oxygen. As it was demonstrated that the oxygen uptake presumed to be due to cytochrome P-450 was Antimycin insensitive, it was suggested that its location was non-mitochondrial. This Antimycin -
Insensitive respiration was proportional to the cytochrome P-450 content of the yeast under a variety of conditions in wild type yeast and RD mutants. These RD mutants accumulate much larger amounts of cytochrome P-450 than the wild type under semi anaerobic conditions. This level also drops sharply on aeration even though no cytochrome a formation can be detected here. In their subsequent work (Kawaguchi et al, 1973) most of the respiratory activity of semi-anaerobically grown yeast was found in the soluble and large particulate fraction (25,000 times g for 20 min.) rather than in small particulate fractions: it showed similar properties in the use of NADPH (and to some extent NADH) in an Antimycin insensitive respiration, sensitive to inhibition by carbon monoxide. Both of these particulate systems were considered to be analogous to the microsomal fraction of liver.

The drug specificity of cytochrome P-450 in yeast is not fully elucidated. 4-hydroxylation of biphenyl is catalysed by yeast microsomal fraction (Wiseman et al, 1975b). Demethylation of N-ethylmorphine and aminopyrine was studied as reported for the cytochrome P-450 from Candida tropicilis (Lebeault et al, 1971). Studies on the biphenyl-4-hydroxylation system have shown its dependence on NADPH and almost equally on NADH.

Isolation of the soluble form of yeast cytochrome P-450 has been reported (Yoshida and Kumoaka, 1975). The spectral characteristics were reported to be identical to the cytochrome P-450 of hepatic microsomes. The enzymes achieved the demethylation of aminopyrine and may be able to hydroxylate aniline. The reactions are NADPH dependent.
CHAPTER II

Materials and Methods
2. MATERIALS AND METHODS

2.1 Materials

A pure strain of brewer's yeast, Saccharomyces cerevisiae, No.240 was obtained from the National Collection of Yeast Culture, Nutfield, Surrey. Mycological peptone and powdered yeast extract were obtained from Oxoid Co. Ltd. Snail gut enzyme was obtained from Micro-Bio Ltd., London. Cyclic guanosine 3',5' monophosphate, cyclic adenosine 3',5' monophosphate, pure α-glucosidase, pure β-galactosidase, chloramphenicol, rotonone, tetracycline, 2-4 dinitrophenol, erythromycin, Actinomycin D cycloheximide, 3,5 dinitrosalicyclic acid and ergosterol, were obtained from the Sigma Chemical Corp. (London) Ltd.

Glucose (Analar Grade), sodium phenobarbital, adenosine 5' monophosphoric acid, adenosine 2',3' monophosphoric acid, adenosine 5 triphosphoric acid, guanosine 5 monophosphoric acid and 2-nitrophenyl-β-D galactopyranoside were obtained from BDH Chemicals Ltd.

4 nitrophenyl-α-D. glucopyranoside was obtained from Koch Light Laboratories Ltd. Imipramine was a gift obtained from Biorex Laboratories Ltd.

A perid kit was purchased from Boehringer Mannheim Corporation. Cyclic AMP assay kit was purchased from Amersham Radiochemical Centre.

2.2 Methods

The yeast was maintained on agar slopes and 100 ml of culture medium was inoculated using a wire loop. (Prior to inoculation the culture medium was autoclaved for 15 min. at 151bs/in^2). The media consisted of 1-20% glucose (autoclaved separately)

0.5% sodium chloride
1% yeast extract
2% peptone

The cultures were incubated at 30°C in a gently shaking water bath for various times as indicated.

2.2.1 Preparation of protoplast (Eddy and Williamson, 1957)

The cells were harvested by decantation followed by filtration. They were then incubated for 30 min. at 30°C with the following media:

0.02M 2-mercaptoethanol
0.04M EDTA
0.2M Sodium phosphate buffer pH 5.8.

The concentration of yeast cells was 0.2g wet weight cells per ml of medium. The cells were resuspended in 5 ml sodium phosphate buffer (pH 5.8) and 0.33 ml of snail gut enzyme per g. wet weight of cells was added and the incubation maintained for 50-60 min. at 30°C. The suspension was then centrifuged for 5 min. at 1,000 g to sediment the protoplasts from the cell wall debris. The sediment containing the protoplasts was resuspended in 0.66M phosphate buffer (pH 6.8) which prevented lysis of the protoplasts.

2.2.2 Disruption of yeast (Wiseman et al, 1975b)

Vibro mill disruption. 3g wet weight of yeast was added to a 10 ml stainless steel container of the vibro mill and 5 grams of glass beads (diameter 0.15 mm) was added. The mixture was then violently shaken at maximum setting of the vibro mill (E. Buhler, Tubingen, West Germany) with cooling for half a minute. More beads were then added and shaking continued for a further 4½ min. The beads were then decanted off and washed in 0.1M phosphate buffer pH 7.4. The solution was then centrifuged
for 10 min. at 1,000 g and the supernatant was used for obtaining the microsomal fraction. Centrifugation was performed at 5,000 g for 20 min. and the pellet was discarded. The supernatant was then centrifuged at 18,000 g for 60 min. and the pellet was collected and resuspended in 0.1M phosphate buffer (pH 7.4) at 0°C.

2.2.3 Spectrophotometric determination

An SP1800 Unicam spectrophotometer was used for scanning absorption between the wavelengths 390 nm and 490 nm. The scanning speed was 40 mm per second, slit width 1 mm and usually the sensitivity was set at 0-0.5 (depending on the preparation). Two cuvettes each filled with 3 ml of yeast suspension or microsomal fraction were scanned to provide a base line, then each of them was reduced by the addition of a few grains of sodium dithionite. Carbon monoxide was bubbled steadily through the solution in the test cuvette for 30 sec. and the difference in spectrum was recorded against the reference cell.

This method had been used for liver microsomes and the P420-P490 and P450-P490 differences used to determine the concentration of P420 and P450 respectively, using the extinction coefficient, based on protoporphyrin content, 91 cm⁻¹ mM⁻¹ (Omura and Sato, 1962). This is indeed valid for purified liver microsomes, but when yeast suspensions were used the base line showed significant drift with wavelength. Therefore, although the results can also be expressed as P450-P490 values (for the purpose of comparison with literature) or as symmetrical peak heights, the P450 values used by us were obtained from subtraction of CO-difference spectra from the base line, and the extinction coefficients for liver were assumed to be applicable (see Fig. 2.1a).
Fig. 2.1a
YEAST GROWN IN 1% OR 20% GLUCOSE

YEAST GROWN IN 0.1% GLUCOSE
2.2.4 Absolute spectra for cytochrome a + a₃

Estimation of cytochrome a + a₃ was made from absolute spectra at 605 nm of whole yeast, read as is usual against filter paper or filter paper pieces in glycerol to balance the light scattering of yeast cells at the necessary concentration. Another method is to read against the empty cell turned sideways to allow the light to traverse the opalescent sides. The extinction of cytochrome a + a₃ was determined by the difference between absorbance measured at 605 nm and 630 nm (see Fig. 2.1a).

2.2.5 Disruption of the yeast for α-glucosidase assay (Wiseman & Jones, 1971)

10 ml of yeast suspension (about 1 mg/ml) was suspended in 0.75 M KH₂PO₄ buffer (pH 6.5) and ultrasonicated for the relatively long time of 10 min. with cooling at 0°C. The Dawe Soniprobe was used at max. 4.4 amperes.

Estimation of dry weight of yeast: The yeast dry weight standard curve was produced in the range of 0.2-1.0 mg/ml dry weight against an optical density at the wavelength 600 nm (see Fig. 2.1). The dry weight of yeast was determined after heating at 100°C overnight and cooling.

2.2.6 Assay of α-glucosidase using the PNPG method (Wiseman & Jones, 1971)

Yeast α-glucosidase catalyses the hydrolysis of glucosides to glucose plus an alcohol

\[
\begin{align*}
\text{O-H} & \quad \text{O-H} + \text{R-OH} \\
\text{D-glucose} + \text{alcohol}
\end{align*}
\]
Fig. 2.1 Yeast dry weight against extinction
In preliminary experiments 4-nitrophenyl-\(-D\) glucosidase was used as the substrate:

\[ \text{PNPG} \quad \text{glucose} \quad \text{p-nitrophenol (PNP)} \]

The reaction is followed by measuring the production of p-nitrophenol which has a yellow colour in alkaline solution.

0.15ml of yeast disruptate suspension was incubated for 5 min. at 30°C with 1 ml of 30 mM PNPG in buffer and 3.50 ml of 0.1M KH$_2$PO$_4$ buffer pH 6.5. The reaction was then stopped by the addition of 1 ml of IN NaOH and after centrifugation at 1,000 r.p.m. the optical density of the solution was measured at 400 nm.

A standard curve was prepared using different solution concentrations of PNP (0.04M in IN NaOH) and made up to 5 ml, incubated with IN NaOH and the extinction was read at 400 nm as before. This allowed the results to be expressed in terms of µmoles/ml PNP by use of the standard curve (Fig. 2.2).

2.2.7 Assay of invertase from the whole yeast (Wiseman & Jones, 1971)

Dinitrosalicylate (DNS) method: It is known that reducing sugars from a reduced coloured compound (amines) with aromatic nitro compounds, such as picramic acid from picric acid, the intensity of which can be related to sugar concentration. Wiseman and Woodward (1975) have developed
Fig. 2.2 Standard curve of PNP/ml against extinction at 400 nm.
an assay based on this method. The method employs a reagent consisting of 5g of NaOH plus 150g DNS and 24g of NaOH plus 150g potassium sodium tartrate, dissolved in 500 ml distilled water.

Assay procedure: 1.0 ml of yeast disruptate was incubated with 1.0 ml, 0.3M sucrose plus 2.5 ml, 0.5M sodium acetate buffer (pH 4.7) at 25°C for 10 min. The reaction was stopped by adding 2.5 ml DNS reagent. The colour was developed by keeping at 50°C for 5 min. The solution was then cooled and centrifuged to clarify and the absorption read at 540 nm.

The standard curve (Fig.2.3) was using 1 ml of glucose and fructose mixed solution (concentration range 5-50mM) 2.5 ml 0.5M sodium acetate buffer (pH 4.7) and 1 ml 0.3M sucrose (incubated at 25°C for 10 min.). The reaction was stopped by adding 2.5 ml DNS reagent and heating 5 min. at 50°C. The blank was prepared by adding 1 ml of H₂O instead of glucose and fructose mixed solution.

2.2.8 Assay of "pure" β-galactosidase (Ullman et al, 1965)

Reagents:
Tris acetate buffer. (1) 0.01M Tris-acetate containing 0.01M sodium-phosphate buffer.
(2) 0.3M sodium phosphate containing 0.003M MgCl₂ at pH 7.5.

Enzyme:
Stock solution of 1.0 mg/ml in 0.1M Na₂HPO₄ and KH₂PO₄ at pH 7.5.

Enzyme assay as below at 37°C.

<table>
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<th>Control</th>
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<td>Sodium phosphate buffer</td>
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</tr>
<tr>
<td>ONPG(O-nitrophenyl β-D-galactoside)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Fig. 2.3 Standard calibration line for invertase estimation (DNS method)
The optical density was read at 400 nm after 5 min.

2.2.9 Assay of 'pure' α-glucosidase

Reagents: phosphate buffer 0.1M KH$_2$PO$_4$ buffer (pH 6.5)
Enzyme stock solution of 1.0 mg/ml in phosphate buffer
Enzyme incubation medium (at 30°C)

<table>
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<tr>
<td>H$_2$O</td>
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<tr>
<td>Enzyme</td>
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<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>3.0 ml</strong></td>
<td><strong>3.0 ml</strong></td>
</tr>
</tbody>
</table>

The optical density was read at 400 after 5 min.

2.2.10 Perid kit assay of glucose (from Boehringer Mannheim)

The method was followed by Woodward et al (1974). The glucose standard curve (Fig. 2.4) was produced in the range 10-50 g of glucose in 5.0 ml of Perid reagent. Colour development was carried out at room temperature (18°C) for 25 min. and extinction was measured in the EEL portable colorimeter (OB3 filter 600-650 nm range).

Also glucose extinction in the medium was measured by the DNS method as before (12-75 mg range) see Fig. 2.5.
Fig. 2.4  Standard curve for Perid Kit's Assay

Optical density

μg of glucose

10 20 30 40 50

0.1 0.2 0.3 0.4 0.5 0.6 0.7
Fig. 2.5 Standard curve (DNS method) glucose concentration against optical density.
2.2.11 Alcohol estimation in the medium

An alcohol distillate from cell free culture fluid was obtained by conventional distillation at 77°C. The alcohol recovery using this assembly was determined by distilling a known dilution of alcohol (0 to 10% v/v) in a final volume of 5 ml and estimating the alcohol in the distillate. The standard curve was plotted, specific gravity against different concentrations of alcohol in the water mixture (Fig. 2.6).

2.2.12 Measurement of cyclic AMP in yeast

Extraction of nucleotides from the cells: Yeast cells 0.2g, wet weight were resuspended and kept 5 min. in 2 ml 10% TCA. The cells were then centrifuged at speed 4 (bench centrifuge) for 10 min. and re-extracted with 1 ml 10% TCA, and centrifuged again. To the combined supernatant fractions one tenth of the volume of IN HCl and 2 volumes of ether were added. The mixture was shaken in the cold for 10 min. and the ether phase discarded. The water phase was reextracted 5 times with ether and then evaporated to dryness under a steam of nitrogen at 55°C.

The dried samples were dissolved in 10 ml of 100 mM Tris-HCl buffer (pH 7.4) 50 µl were taken out for cyclic AMP assay.

2.2.13 Assay of cyclic AMP by using protein binding method using Amersham Radiochemical Centre cyclic AMP assay kit

a) General description of the kit:-

The kit provides a rapid, simple and specific method for the determination of cyclic AMP in the range 0.2 to 16 picomoles per incubation tube.

b) The assay is based on the competition between unlabelled cyclic AMP and a fixed quantity of tritium labelled compounds for binding to a protein which has a high specificity and affinity for cyclic AMP. The amount of labelled protein cyclic AMP complex is inversely related to the amount of
Fig. 2.6 Standard curve for alcohol estimation by using specific gravity method
unlabelled cyclic AMP present. Measurement of the protein bound radioactivity enables the amount of unlabelled cyclic AMP in the sample to be calculated.

Separation of the protein bound cyclic AMP from the unbound nucleotide is achieved by absorption of the free nucleotide on to coated charcoal followed by centrifugation. An aliquot of the supernatant is counted. The concentration of unlabelled cyclic AMP in the sample is then determined from a linear standard curve.

c) Contents of the kit:-

The kit contains the following reagents, all of which contain Tris/EDTA buffer and are in freeze dried form.

1) Tris/EDTA buffer
2) Binding protein, purified from bovine muscle
3) (8-3^H)Adenosine 3'5' cyclic phosphate 180 pmole containing approximately 5 Ci.
4) Adenosine -3'5' cyclic phosphate standard 1600 pmole
5) Charcoal absorbant.

d) Assay procedure:

(i) 150 ml of the buffer reagent was pipetted into assay tubes for the determination of the blank counts per minute for the assay.

(ii) the buffer reagent (50 ml) was pipetted into the assay tubes for determination of binding in absence of unlabelled cyclic AMP. The levels of standard cyclic AMP were ranged between 1-14 pmole. The samples were Vortex mixed for about 5 sec. The samples were placed in a cold room at 2°-4°C and left for 2 hr. 100 μl of the charcoal suspension was added to all tubes and Vortex mixed for 10-12 seconds. Then all the tests were centrifuged to sediment the charcoal. This
operation should be done immediately. Without disturbing the sediment, 200 l samples from each test were removed after centrifuging each batch and placed in the scintillation vials for counting.

Cyclic AMP assay protocol (see chart)

<table>
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<tr>
<th>Tube No.</th>
<th>Blank 1</th>
<th>2</th>
<th>Zero 3</th>
<th>4</th>
<th>Standards 5 6 7 8 9 10 11 12 13 14</th>
<th>Unknowns 15 16 17 18</th>
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<td>150</td>
<td>50</td>
<td>50</td>
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<td></td>
<td>1pmol cyclic AMP</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
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<td>50</td>
<td>50</td>
<td>100</td>
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<td>100</td>
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<tr>
<td></td>
<td>Unknown 2</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

All volumes are in microlitres

Scintillant contained:-

Diphenyloxazole (PPO) 5 gram and 1.4 di (2-4 methyl 1-5 phenyloxolyl benzene (POPOP) 0.3 gram in 1 litre of toluene plus 2 litres of triton x-100, mixed well and 10 ml of scintillant were used for each vial.

Counting times were used for 10 minutes. All the tests were done in duplicate.

e) Calculation of cyclic AMP in protein-bound complex:-

(i) The cpm of cyclic AMP after substraction of the blank cpm gives the result in cpm bound in the absence of unlabelled cyclic AMP (Co). Then after substraction of the blank cpm from each result from the cpm bound in the presence of standard or unknown unlabelled cyclic AMP the
(Cx). Co/Cx for each level of cyclic AMP and the test was calculated. CO/Cx values were plotted against pmoles of inactive cyclic AMP. A straight line was obtained with the intercepts of 1.0 on the ordinate (see Fig. 2.7).

2.2.14 Adenyl cyclase assay (Albano et al, 1973)

0.1g of yeast disruptate (by vibro mill) was suspended 10 ml Tris-HCl in buffer (0.1M pH 7.4). The incubation medium contained the following:

\[
\begin{align*}
2 \text{mM ATP} & \quad 200 \mu l \\
3 \text{mM MgCl}2 & \\
10 \text{mM KCl} & \\
6 \text{mM Theophyline} & \\
50 \text{mM Tris HCl pH 7.4} & \\
10 \text{mM NaF} & 100 \mu l \\
50 \mu g \text{ Yeast disruptate} & 50 \mu l \\
\text{Total incubation medium} & 350 \mu l
\end{align*}
\]

The incubations were done for 10 min, 20 min, and 40 min. at 30°C. The reaction was stopped by boiling for 3 mins. Then deep freezing -4°C overnight and thawing before centrifuging (speed 4 bench centrifuge). The supernatant (50µl) was collected for cyclic AMP assay (see Fig.2.8).

2.2.15 Preparation of RD mutant (Millbank, 1962)

The preparation of RD mutant was done as follows:- The yeastNCYC 240 was transferred with a platinum wire from an agar slant to 100 ml flasks containing 100 ml of 1% glucose, 0.5% NaCl, 2% peptone and 1% yeast extract plus 0.01% acriflavin. The cultures were incubated at 30°C for 24 hrs. The streaks from each culture were made on an agar slant.
Fig. 2.7 Measurement of cyclic AMP by protein binding method

![Graph showing cyclic AMP levels in p moles/incubation tube]
Fig. 2.8 Assay of adenyl cyclase incubated for different time.

P Moles Cyclic AMP released/mg of yeast

Minutes
The slants were incubated at the same temperature as the flasks. Mutant colonies are smaller, less opaque.

2.2.16 Continuous culture assembly:-(see fig. 2.9) 

a) General description

The fermenter is designed as a versatile laboratory tool for the batch culture as well as continuous culture of a wide range of microbes. The unit comprises of a glass vessel of total volume 1400 ml (working volume approximately 400-1100 ml) with a polypropylene head plate and backing flange. Head plate penetrations are made via nylon screw-in connectors of " or ø" inner diameter. Culture temperature is regulated by a solid-state controller using a thermometer sensor. Aeration is provided by a diaphragm pump, with front panel indication and needle valve control of the air flow rate. An encapsulated magnetically-coupled follower acts as the impeller. A 1/10 H.P. universally-wound motor derives the impeller at speeds regulated by a thyristor controller, with front-panel adjustment.

The system is designed for sterilisation of the vessel and all ancillaries in most bench-top autoclaves.

b) Operation of the fermenter

(1) Power supply:- a 240v 50HZ earthed power supply is required.

(2) pO2 controller:- the solid state meter/controller is suitable for use with any galvanic pO2 probe. The controller provides a single switched output suitable for controlling the motor speed in the fermenter.

Before calibration the whole system was autoclaved, in order to stabilise the electrode (e.g. to remove any protective coating of the lead

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Fig. 2.9  Continuous Culture Assembly

A  Controller
   T° control
   RPM control
   Airflow indication

B  Controller
   pO₂ control
   pH control
   RPM, T° indication

C  Fermentor
   pH probe
   Temperature probe
   O₂ electrode probe
   Magnetic stirrer

D  Peristaltic Pump

E  Feed Medium

F  Titrant Pump
anode). The electrode reading was adjusted to zero by placing it in saturated sulphite solution. The electrode was removed from the sulphite solution, rinsed the previously-immersed section well with distilled water, and then placed in air saturated water. The span control was adjusted to 99.9%. The temperature was at 30°C.

(3) pH controller: the solid state controller incorporates a high impedance amplification stage. In its standard form the controller provides a single switch mains output, suitable for activation of a single pump. The controller will thus actuate the titrant pump when the pH falls below the set point control valve.

The range of the pH meter is 0-10, it should be standardised at two valves reasonably far apart (e.g. pH 4 and pH 9) before used.

(4) Continuous culture pump system

The system consists of a double-track peristaltic pump with slip-out pumping elements for ease of sterilisation and a medium addition pipe entering the vessel. The culture removal pipe uses the inverted weir device which lowers and maintains the culture level when the pump is running. The shroud ring ensures the removal of culture from the body of liquid and not from the surface.

Dilution rate is determined by the rate of medium addition, pumping elements of different sizes are used, that for the medium addition being smaller in diameter.

The pump allows regulation of pumping rate 0 and 100% of the system capacity determined by the diameter of the pumping element and maximum
rotor speed. The system is stabilised against mains voltage changes and the effects of temperature to ensure long-term constancy of pumping rate. This is also assisted by the rotor which kinks the pumping tube without appreciable stretching, to reduce changes in the volume per revolution caused by tube fatigue.
CHAPTER III

Regulation of α-glucosidase biosynthesis
by cyclic AMP level in yeast
REGULATION OF α-GLUCOSIDASE BIOSYNTHESIS BY CYCLIC AMP LEVEL IN YEAST

Cyclic AMP has been shown to control a variety of functions in prokaryotes as well as in eukaryotes (Pastan and Perlman, 1970). This control includes catabolite repression in *E. coli*, differentiation in slime mould and various hormonal responses in mammalian systems. Yeast (*S. carlbergensis*) has been shown to contain a lower concentration of cyclic AMP when grown under conditions of catabolite repression in 2% glucose. During subsequent adaption of this yeast to maltose utilization the intracellular concentration of cyclic AMP rose from 0.04M to 0.32M over 40 minutes (Van Wijk and Konijn, 1971). Sy and Richter, (1972) reported that the cyclic AMP content of *S. fragilis* was dependent on growth conditions and the carbon source. This yeast when grown in 1.5% glucose or 2% lactate for 12 hours contains 3 times as much cyclic AMP as grown in 10% glucose for 12 hours.

The removal of glucose repression of β-galactosidase by cyclic AMP addition has been achieved in *E. coli*, in non-growing Tris-EDTA treated cells at 1mM-cyclic AMP (Perlman and Pastan, 1968) and in normally permeable, growing cells with at least 5mM-cyclic AMP (Ullmann and Monod, 1968). It has been shown that a similar effect occurs using 10mM-cyclic AMP for removal of glucose repression of α-glucosidase in yeast protoplast. (Wiseman and Lim, 1974).

3.1 Materials and methods

(NCYC No:240) was grown at 30°C in 1% glucose 2% w/v peptone 1% yeast extract 0.5% NaCl. Late exponential phase cells were harvested by
centrifugation and washed three times with 0.66M potassium phosphate buffer containing respectively 0.1% glucose, 0.1% glucose + 4% maltose, 1% glucose 4% maltose, 2% glucose w/v + maltose, 3% glucose w/v + 4% maltose, 10% glucose + 4% maltose, 3% glucose and 10% glucose. Suspensions of yeast cells were used at approximately 500 μg ml dry weight of yeast. No weight changes occurred under these non growth conditions. Samples (in triplicate) were taken at hourly intervals disrupted by ultrasonication and then α-glucosidase was assayed by the PNPG method (Wiseman and Jones, 1971).

In the protoplast experiment, yeast NCYC No:240 was grown in a medium containing 1% glucose, 1% yeast extract, 2% peptone and 0.5% NaCl for 24 hours at 30°C. Cells were harvested and we prepared protoplasts (100 mg wet weight as previously described). The yeast protoplasts were resuspended in 10 ml of 0.66M phosphate buffer pH 6.8. 1 ml samples of this suspension were added to 10 ml of the induction medium at 30°C containing either 0.1% glucose + 4% maltose or 2% glucose + 4% maltose + 10mM cyclic AMP. One such sample of each was taken hourly and disrupted by ultrasonication. The assay of α-glucosidase was achieved by using the PNPG technique. In control experiments 0.1% glucose + 4% maltose was used with and without 10mM cyclic AMP.

In the sodium fluoride experiments, the yeast (1g wet weight batches) were transferred to 100 ml of fresh medium with 2% glucose + 4% maltose, and grown for up to 24 hours with or without sodium fluoride, followed by sampling of individual batches as described. Other yeast batches were suspended in non-growth medium containing only 2% glucose + 4% maltose in
0.66M phosphate buffer pH 6.8.

3.2 Results:

3.2.1 Effect of maltose protection of α-glucosidase in yeast disruptate.

The thermostability of α-glucosidase in ultrasonic disruptates was investigated by monitoring the recovery of enzyme activity after heating at 50°C for up to 30 minutes, alone and with various additives such as maltose. Also the disruptate of 15 mg of yeast was used along with 0.5 ml of 100mM KI or 100mM p-chloromercurobenzoate (PCMB) alone and in the presence of maltose (100mM 3.8%). Fig 3.1 showed that PCMB caused rapid progressive loss of α-glucosidase activity down to 20% recovery in 20 minutes, 100mM maltose increased this recovery to 50%. Similar findings were reported by Halvorson and Ellias (1958). Fig 3.2 shows the 8% recovery with KI after 20 minutes; this was increased to 38% by 100mM maltose.

3.2.2 Effect of cyclic AMP protection of α-glucosidase in thermal stability study:

Cyclic AMP can remove catabolite repression of yeast α-glucosidase by true induction involving enzyme synthesis. Investigations were carried out to see whether it could also cause stabilisation of the enzyme in vitro and possibly in vivo. Fig 3.4 shows the significant stabilisation in vitro against heat at 50°C, unlike other nucleotides tested (Fig 3.8 5'AMP, 3'-AMP, ATP and adenosine 2', 3' cyclic phosphate). Fig 3.84 shows that α-glucosidase was protected at high cyclic AMP concentration of 10 and 1mM but not at the physiological concentration of 1mM. Thus
Fig. 3.1  $\alpha$-Glucosidase stability of yeast ultrasonic disruptate in 100mM Maltose and 100mM PCMB.

A: Control.
B: 100mM Maltose + 100mM PCMB
C: 100mM PCMB.
Fig. 3.2 $\alpha$-Glucosidase stability of yeast ultrasonic disruptate with 4% maltose and 0.1 mM KI at 30°C incubation

% of Remaining Activity

Incubation Time (mins)

A: 4% Maltose + 0.1 mM KI
B: 4% Maltose + 10 mM KI
C: 0.1 mM KI
D: 10 mM KI
Fig. 3.3 α-Glucosidase Heat Stability with 10 mM Nucleotides

- 10 mM Nucleotides without heating
- 10 mM 2′:3′-AMP
- 10 mM 3′-AMP
- 10 mM ATP
- Control

% of remaining activity

Time (mins)
Fig. 3.4  Heat stability of α-Glucosidase with 10 mM cyclic AMP or other nucleotides

A: 10 mM cyclic AMP
B: 10 mM 5'-AMP
C: Control
Fig. 3.5
Heat Stability of α-Glucosidase at 50°C with:

A: 0.1m Maltose + 1mM Cyclic AMP.

B: Control.

% of Remaining Activity

Time (mins)

100
90
80
70
60
50
40
30
20
10
0

5 10 15 20 25 30
after 15 minutes heating at 50°C, the recovery of α-glucosidase activity in ultrasonic disruptates of yeast is increased to 110% by 10mM maltose and to 160% by 100mM maltose. (Fig 3.5)

### 3.2.3 Effect of substrate protection of α-glucosidase during deadaptation

Fig. 3.6 shows the gradual loss of this enzyme that occurs with 2% w/v glucose and is especially marked at higher glucose concentrations. Some protection of the enzymes by the presence of the 4% w/v maltose in the suspending buffer solution was evident. The basal activity of α-glucosidase drops maximally from about 15 to 5 μmole/min/g of dry weight of yeast.

There is an increase of 100% with 10mM cyclic AMP and of 33% with 1mM cyclic AMP (1mM maltose of 1μM cyclic AMP shows no protective effect nor does 10mM maltose and 3μM cyclic AMP). The protective effect of 100mM maltose is not increased by 1mM cyclic AMP. Figs. 3.7 and 3.8 show similar protective effects with cyclic AMP as obtained with a purified preparation of α-glucosidase (baker's yeast) and β-galactosidase (E.coli).

### 3.2.4 Effect of cyclic AMP in reversal of catabolite repression:

Fig.3.9 shows the usual induction in four hours of α-glucosidase by 4% maltose in the presence of 0.1% glucose. 10mM cyclic AMP caused no additional induction.

Fig. 3.10 shows the usual lack of induction of α-glucosidase by 4% maltose in the presence of 2% glucose. It is clear that 10mM cyclic AMP causes a slow induction of α-glucosidase that is able to occur despite
Fig. 3.6  Induction of $\alpha$-glucosidase in yeast and protection against deadaptation by maltose

Concentrations are given as % (w/v)

$\bullet$ 0.1% glucose + 4% maltose.,
$\circ$ 1% glucose + 4% maltose.,
$\triangle$ 0.1% glucose only.,
$\blacktriangle$ 3% glucose + 4% maltose.,
$\square$ 2% glucose + 4% maltose.,
$\blacksquare$ 3% glucose only.,
$\blacktriangledown$ 10% glucose + 4% maltose.,
$\blacktriangledown$ 10% glucose only.
Fig. 3.7  Heat stability of β-Galactosidase (pure) in E. Coli at 50°C

% of remaining activity

- 10 mM Cyclic AMP
- 10 mM 5' AMP
- Control

Time (mins)
Fig. 3.8 Heat stability of $\alpha$-Glucosidase (pure) from yeast at 50°C

- $\times$ 10 mM cyclic AMP
- $\triangle$ Control
- $\bullet$ 5 AMP (10 mM)
Fig. 3.9  α-Glucosidase induction in Yeast protoplasts

- 0.1% Glucose + 4% Maltose + 10mM Cyclic AMP.
- 0.1% Glucose + 4% Maltose.
Kinetics of $\alpha$-glucosidase induction in baker's yeast protoplasts. The induction of $\alpha$-glucosidase, the preparation of a lysate and the assay of $\alpha$-glucosidase activity were carried out. The induction medium (0.7 M MgSO$_4$ as osmotic stabilizer) was supplied with 0.25% glucose ($\times$), 1% glucose (□) or 1% glucose + 0.3 mM cAMP (○).

(Haarasilta and Oura 1973)

**Fig. 3.10** Slow reversal of 2% glucose repression of $\alpha$-Glucosidase in yeast protoplasts by 10 mM cyclic AMP

- 2% Glucose + 4% Maltose + 10 mM CAMP

- 2% Glucose + 4% Maltose
the catabolite repression by the 2% glucose present.

Fig. 3.11 shows results obtained using the highest concentration of cyclic AMP with yeast protoplasts sampled at intervals of time. The derepression of α-glucosidase synthesis increased with cyclic AMP concentration up to 4mM, and is demonstrated at its maximum (after 10 hours) with yeast protoplasts. Cyclic AMP has no effect on whole yeast in this connection.

3.2.5 Effect of cyclic AMP on the synthesis of α-glucosidase:-

Fig. 3.12 shows protoplasts obtained from yeast in 1% glucose and transferred to the 0.66M phosphate buffer containing 2% glucose + 4% maltose and 10mM cyclic AMP only. 80µg/ml Actinomycin-D was added at zero time and after one hour to the buffer when Actinomycin-D was added at zero time α-glucosidase synthesis was completely inhibited. When Actinomycin was added after one hour α-glucosidase synthesis rose steadily which can be compared to the control enzyme levels which also rose steadily, when no Actinomycin was added.

3.2.6 Effect of lifting of glucose repression of α-glucosidase by sodium fluoride:-

Fig. 3.13 shows the small effect of 0.04% sodium fluoride and the much greater effect of 1% sodium fluoride in lifting the glucose repression of α-glucosidase synthesis observed in the control experiment in non growth medium. Some loss of α-glucosidase is seen in the control medium due to the essentially deadaptation condition prevailing.
Fig. 3.11 Removal of glucose repression of $\alpha$-glucosidase synthesis in yeast protoplasts by added cyclic AMP, sampled after 10 hours

Protoplasts were suspended in medium containing 0.66 M-potassium phosphate buffer, pH 6.8, with 2% glucose - 4% maltose plus cyclic AMP in the range 0.5 to 10mM as indicated
Fig. 3.12  Reversal of 2% glucose repression of \( \alpha \)-glucosidase in yeast protoplasts by 10 mM cyclic AMP with the addition of actinomycin-D at 0 or 1 hour.
Fig. 3.13 Removal of glucose repression of α-glucosidase synthesis in yeast by sodium fluoride at 0.04% or 1% concentration

- non-growth medium
- growth medium

The controls are growth medium (containing 2% glucose + 4% maltose) and non-growth medium (2% glucose + 4% maltose in 0.66 M-potassium phosphate buffer, pH6.8). There is no glucose repression in induction medium (0.1% glucose-4% maltose)
3.3 Discussion

Catabolite repression involves the inhibition of formation of certain enzymes (i.e. repression) by the catabolite products of a readily utilisable carbon source. The repressed enzyme can be inducible or constitutive but in most cases, inducible enzymes are involved. The classical example of catabolite repression is the repression of β-galactosidase in *E. coli* by growth on glucose. In yeast, there also exists a similar system which can be repressed: this is α-glucosidase and occurs when growth is in a high glucose medium (Halvorson, 1956).

α-Glucosidase is induced in Brewer's yeast by maltose in the presence of low glucose concentration, but catabolite repression occurs at about 2% glucose concentration in the suspending medium. Loss of induced α-glucosidase occurs on aerobic incubation, with shaking for a few hours at high glucose concentration in buffer solution (Robertson and Halvorson, 1957). It has been shown that maltose, the substrate for α-glucosidase in yeast, protects the enzyme from loss under these conditions of deadaptation. The same effect is also demonstrated against thermal denaturation and inhibition by various chemicals *in vitro*.

The rapid destruction of the induced enzyme can be achieved by incubation of induced cells in the buffer solution, i.e. without growth being possible in the presence of inducer. When cells are incubated with glucose in buffer solution, the enzyme itself may be rapidly destroyed (Halvorson, 1967). The basic character of the inactivation of both the glucoside permeation and the α-glucosidase mechanism during deadaptation is unknown, clearly protection of α-glucosidase activity by maltose is
often found \textit{in vitro}. The conformational protection against loss by aerobic shaking in the presence of high glucose concentration is clearly evident. Many enzymes are protected, against thermal denaturation or attack by irreversible inhibitors, by their substrate. The mechanism is well known, and when the adjoining protein chains are separated by unfolding during denaturation the activity is lost in practically all cases. The fact that some affinity for substrate has sometimes been found after denaturation although the activity has been completely lost, would suggest that the individual combining groups are still present although the pattern of the active centre has been disrupted. This pattern may be reformed in certain cases where the denaturation can be reversed, and the activity then returns. Such a picture is a consistent with a fact that combination with the substrate often protects the enzyme from denaturation since it would tend to hold the chains together.

Maltose has been shown to affect transcription of coded mRNA during synthesis of $\alpha$-glucosidase (Van Wijk \textit{et al}, 1969). The addition of both 2\% glucose + 4\% maltose did not reverse the catabolite repression. This is in agreement with the deadaptation of $\alpha$-glucosidase that occurs during incubation in 2\% glucose or 2\% glucose + 4\% maltose. It has been found that 10$\mu$M cyclic AMP added to brewer's yeast protoplasts could indeed remove glucose repression of $\alpha$-glucosidase synthesis in the presence of 2\% glucose + 4\% maltose. This finding was in agreement with reports that cyclic AMP addition could remove the glucose repression of $\beta$-galactosidase synthesis in \textit{E.coli} (Pastan and Perlman, 1970). In whole yeast, there is no removal of catabolite repression. Presumably cyclic AMP is a molecule which is unable to enter the cell, without prior removal of the
rigid cell wall.

The range of cyclic AMP concentration (5-10mM) to reverse catabolite repression of α-glucosidase synthesis in the protoplasts is far beyond the physiological level. This may be explained by the fact that cyclic AMP has difficulty in entering into the cell.

Unlike other workers (Haarasilta and Oura, 1973), it has been shown that a greater effect on the synthesis of α-glucosidase is achieved at higher concentrations of cyclic AMP. They studied the acidic maltose (pH 4.7) found in baker's yeast (but not in our brewer's yeast) which is probably surface located. In protoplasts they observed lifting of glucose repression at 1% glucose with little maltose (0.65%) and 0.3mM cyclic AMP. Much less effect was observed at 2mM cyclic AMP. This quite different sensitivity to external cyclic AMP requires explanation. Clearly this surface located acidic α-glucosidase has a promoter of greater sensitivity to cyclic AMP, or the transport of cyclic AMP into protoplasts is less efficient in this system. This investigation showed that 10mM cyclic AMP in 2% glucose + 4% maltose medium did not give the maximal induction whereas 0.1% glucose + 4% maltose did. It is clear that 10mM cyclic AMP slowly enters into the cell. 10mM cyclic AMP caused no additional induction in 0.1% glucose and 4% maltose during incubation for four hours. It is possible that cyclic AMP had already reached the maximal physiological level during adaptation in the maltose medium. No effect for the same concentration (10mM) of ATP, 5'AMP and Adenosine 2' 3' cyclic phosphate was found which agreed with Haarasilta & Oura (1973). Surprisingly they found that 3' 5' cyclic GMP showed the same derepression effect, which
is different from other systems. Fang and Butow (1970) found that cyclic AMP (1-2mM) and several other nucleotides including ATP caused the partial reversal (within 8 hours) of mitochondrial repression by 5% or 10% glucose in protoplasts of *S. cerevisiae*.

The restoration rate of α-glucosidase in yeast when incubated with 2% glucose + 4% maltose with or without 10mM cyclic AMP for 6 hours was 1.3/3; this ratio compared to Haarasilta's finding of a 2/3 ratio. In comparison to the maximal induction medium (which is 0.25% glucose) the medium containing 1% glucose and 0.3mM cyclic AMP (Haarasilta and Oura, 1973), the restoration rate was 2/5.5 in ratio in 6 hours. It was found that maximal induction (0.1% glucose and 4% maltose) gave a restoration rate of 1.3/3.2 in ratio compared to the medium containing (2% glucose + 4% maltose). The efficiency of cyclic AMP action in these results is \((1-1.3/3) \times 100\% = 57\%\). In Haarasilta's finding, it was \((1-2/3) \times 100\% = 34\%\). However the efficiency of maximal induction, \(1-\frac{1.3}{3.2}\) = 51.4%, \(1-\frac{2}{5.5}\) = 63.7% (Haarasilta and Oura, 1973) is lower.

The Stimulation by cyclic AMP appears after 1 hour of exposure of the cells to cyclic AMP (10mM). Cyclic AMP might regulate maltose induced α-glucosidase synthesis at the level of transcription of DNA into m.RNA or at the level of translation of m.RNA into protein. To investigate the site of cyclic AMP action, α-glucosidase was induced with 2% glucose + 4% maltose and cyclic AMP for 1 hour and then 80μg/ml Actinomycin-D was added to inhibit DNA-dependent RNA synthesis. There was no inhibition of α-glucosidase synthesis when 80μg/ml Actinomycin-D was added at zero time to the medium containing 10mm cyclic AMP + 2% glucose + 4% maltose, the amount of α-glucosidase activity observed did not increase after 1 hour.
Thus cyclic AMP does not stimulate production of α-glucosidase in the presence of Actinomycin-D. The results also show that cyclic AMP does not regulate the synthesis of the enzyme in the absence of α-glucosidase coded m.RNA production. The m.RNA had been transcripted for α-glucosidase synthesis within one hour when incubated in cyclic AMP + 2% glucose and 4% maltose medium, and when 80μg Actinomycin-D was added, after 1 hour no effect was observed. No m.RNA coded for α-glucosidase synthesis was made when Actinomycin-D was added at zero time.

Actinomycin-D inhibits transcription of DNA to m.RNA. Growing E.coli did not take up this compound, but tris EDTA treated cells can (Leive, 1965) and so can the yeast protoplast (Van Wijk et al, 1969).

Stimulation of adenyl cyclases isolated from mammalian sources by low concentration of fluoride (40%) has been documented (Jost, Rickenberg 1971). A relatively small stimulation (40%) has more recently been reported for another eukaryotic system, the cytoplasmic membrane - located adenyl cyclase in baker's yeast, using a low concentration of sodium fluoride (10mM i.e. 0.04%) (Londesborough, and Nurininen 1972). It was demonstrated that sodium fluoride, at relatively high concentration can lift the glucose repression in growing or non-growing brewer's yeast. This effect in intact yeast is considered to be correlated with the direct action of cyclic AMP.

Nevertheless, the repression is lifted only slowly so that the α-glucosidase level is at about 90% of the non-repressed system in induction medium (4% maltose + 0.1% glucose) after 12 hours although there is only 67% after 4 hours (but note basal level of about 50% of maximum level).
Fig. 3.13, showing the dotted lines referring to growth medium, look similar, but must be interpreted differently (Wiseman, 1974), because of growth occurring in the medium with 0.04% but not with 1% sodium fluoride. Doubling of yeast weight had occurred within 24 hours with 0.04% sodium fluoride and the control without fluoride. Under these conditions some production of enzyme had occurred in all three cases, of about 10 enzyme units in the control, 20 enzyme units in 0.04% sodium fluoride but only 15 enzyme units in 1% sodium fluoride, when the weight of yeast remained unchanged as in non-growth medium. Inhibition of yeast growth by a particular concentration of required metals, such as Mg$^{++}$ or chloride ion may have occurred in this particular growth medium. Inorganic fluoride at 10mM concentration or above, inhibits many enzymes in all organisms and tissues (Wiseman, 1970), and it may be responsible for bacteria (de Robertis et al., 1973) or animal cell (de Asun and Rozengurt, 1974) growth inhibition via cyclic AMP. On the contrary fluoride (5mM) has been reported to cause 80% inhibition (Jost and Rickenberg, 1971) of a soluble adenyl cyclase isolated from Escherichia coli after disruption by grinding with alumina (Tao and Lipmann, 1963) despite its usual growth inhibiting ability with this and other bacteria. Fivefold stimulation by fluoride (20mM) has been reported however for adenyl cyclase from Streptococcus SPP (Khandalwal and Hamilton, 1971) presumably of membrane location.

The action of sodium fluoride appears to be mediated by the cyclic AMP produced by allosteric stimulation of a membrane located adenyl cyclase. (Londesborough and Nurminen, 1972). There is no need to postulate that the fluoride ion enters the yeast cell. Animal cells may be similarly
susceptible to fluoride which may mimic the effect of certain hormones such as glucagon and adrenaline. Conversely it was found that insulin does not cause the onset of catabolite repression of α-glucosidase synthesis in growing yeast, contrary to a report that a membrane bound adenyl cyclase in Neurospora crassa was inhibited by insulin (Fiawia and Torres, 1973) as is rat liver plasma membrane adenyl cyclase (Khandelal and Hamilton, 1971).
CHAPTER IV

Regulation of yeast invertase.
Regulation of yeast invertase

B-fructofuranosidase (EC 3.2.1.26, trivial name invertase) is located externally to the protoplast either in the cell wall or in the space between the protoplast and the wall in yeast (Metzenberg, 1963; Lampen et al, 1967). Glucose had been found to play an important role as a repressor of synthesis of invertase. It was shown that the activity of invertase in yeast was dependent on the growth condition (R. Davies, 1953; A. Davies, 1956; Suomalainen and Oura, 1957; Dodyk and Rothstein, 1964). Davies (1953) shows that when yeast cell was incubated in 0.2M K$_2$HPO$_4$ citrate buffer (pH 4.5) containing 10% sucrose the invertase increased ten-fold after 2½ hours. It is well known that sucrose can protect invertase against thermal denaturation. The basal level of the invertase in yeast and effect of cyclic AMP, sodium fluoride and sucrose on its biosynthesis was determined.

4.1 Materials and methods

The yeast was grown on a shaking water bath at 30°C in 100 ml medium containing 1% glucose or 2% glucose or 20% glucose plus 2% peptone, 1% yeast extract and 0.5% NaCl. Cells were harvested by filtration (24 hrs) and washed with distilled water. Then about 1 gram wet weight of yeast was used to prepare protoplasts. The protoplasts were then transferred to 10ml 0.66M citrate phosphate buffer (pH 4.7) containing 2% glucose plus 4% maltose or 10 mm cyclic AMP or 1% NaF. Invertase was assayed by Perid method (Woodward, et al, 1974).
4.2 Results

4.2.1. Effects on enzyme production during growth of cells on different carbon sources has shown a marked different value in the total level of invertase. The amount of enzyme produced on 4% sucrose or 0.1% glucose in growth medium was shown to be at a maximal level of 500 μmoles/min/g of yeast (wet weight) in 24 hours. In 20% or 2% glucose growth medium this was 70-180 μmole/min/g of yeast respectively after 24 hours, as shown in Fig. 4.1.

4.2.2. Study of invertase deadaptation when incubated in 0.66 M citrate phosphate buffer containing 2% glucose with and without 4% sucrose:-

The invertase level decreased to 65% of the original activity when incubated in 0.66M citrate phosphate buffer (pH 4.7) with 2% glucose for 4 hours. But with 4% sucrose, the invertase level was maintained at 100% in 4 hours. (see Fig.4.2).

4.2.3. Effect of 4% maltose (in non-growth medium) for the production of invertase in yeast protoplasts. When yeast protoplasts were incubated in the 0.66M citrate phosphate buffer pH 4.7 at 30°C with shaking, the invertase level decreased to 75% in 4 hours. Even the presence of 4% maltose did not prevent a loss of enzyme. There was no invertase excreted into the medium. The invertase was assayed by the Perid method, as the medium contained 4% maltose only (see Fig.4.3).
Fig. 4.1  Invertase activity (growth in different medium)

A with 4% sucrose
B with 0.1% glucose
C with 2% glucose
D with 20% glucose
Fig. 4.2  Deadaptation of yeast invertase when incubated in 0.66 M citrate phosphate buffer containing 2% glucose with and without 4% sucrose.

- 2% glucose + 4% sucrose
- 2% glucose

% of remaining activity of invertase

Incubating hours
Fig. 4.3  Yeast protoplasts were incubated in the 0.66 M citrate phosphate buffer with and without 4% maltose
4.2.4. Effect of 10mM cyclic AMP or 1% sodium fluoride added to the protoplasts in the medium containing 2% glucose in 0.66 M citrate phosphate buffer.

When yeast protoplasts were transferred to the medium containing 10mM cyclic AMP and 2% glucose in the 0.66 M citrate phosphate buffer, invertase was shown to have decreased to 55% in 4 hours. There is no induction or protection of the enzyme (see Fig. 4.4).

4.3 Discussion

Enzyme adaptation due to aeration in non-growth medium has been shown (Wiseman and Lim, 1974). Yeast invertase shows the same effect when incubated at 30°C with shaking in 0.66 M phosphate buffer containing 2% glucose. The activity of invertase was down to 65% in 4 hours, but with 4% sucrose, invertase was shown to be 100% protected. Glucose repression of invertase synthesis in yeast had been studied (Dodyk and Rothsbern, 1964). They showed that high glucose concentration inhibited the synthesis of invertase. In 0.1% glucose or 4% sucrose growth medium the invertase was derepressed, enzyme level rose up to 500 μmole/min/g of yeast.

Invertase has a different regulatory system to α-glucosidase. Cyclic AMP does not show any effect on reversing the catabolite repression of invertase biosynthesis. It was demonstrated that invertase biosynthesis does not have a simple relationship with the cyclic AMP level in relation to sensitivity to repression. Lampen (1973) reported that cyclic AMP level did not parallel the level of invertase. This is in agreement with the finding that addition of exogeneous cyclic AMP did not lift the catabolite repression. Moreover, maltose
Fig. 4.4 10mM cyclic AMP or 1% NaF were added to the yeast protoplasts in the medium containing 2% glucose in 0.66 M citrate phosphate buffer.

- × × with 10mM cyclic AMP
- ○ ○ with 1% NaF
- - - Control

% of activity of invertase remaining

Incubating hours
-grown yeast produced a high level of cyclic AMP, but invertase did not increase when incubated with 4% maltose. Similarly, incubation with 1% NaF did not raise the level of invertase. Nevertheless, NaF can stimulate the formation of cyclic AMP by increasing adenyl cyclase activity (Londesborough and Nurininen, 1972). In summary it is clear that cyclic AMP does not affect the rate of invertase production by yeast protoplast.
CHAPTER V

Regulation of cytochrome P-450 biosynthesis in yeast
5. Regulation of cytochrome P-450 biosynthesis in yeast

Yeasts will synthesize a cytochrome P-450, with similar spectral characteristics (peak at 450 nm in reduced form in complex with carbon monoxide), to the drug metabolising enzyme from liver (Parke, 1972), under well defined conditions of growth all involving mitochondrial repression (Ishidate et al, 1969). These conditions include anaerobic growth, and aerobic growth in high glucose media or in low glucose media containing inhibitors (Ishidate et al, 1969), such as chloramphenicol or erythromycin, that prevent the biosynthesis of mitochondrial enzymes especially cytochrome a + a₃. Similar behaviour is found with respiratory deficient mutants. This enzyme is at a particularly high level in 0.1% glucose medium where no cytochrome P-450 is produced even in the early phase of growth. In 20% glucose a late rise in the level of cytochrome a + a₃ corresponds to the fall in cytochrome P-450 after 40 hours of culture. It is not clear, however, why the levels fall rapidly to zero by 70 hours. The glucose concentration also falls rapidly at this time. It has reached about 8% residual glucose by 48 hours and presumably some enzymes may be destroyed rapidly during this stationary phase of culture.

5.1 Materials and Methods

Saccharomyces cerevisiae (NCYC No.240) was grown from loop at 30°C in shake culture (50 revs/min, stroke 2") in a medium containing defined quantities of glucose (in the range 0.1 to 20%). The glucose was sterilized separately to avoid the production of breakdown pigments that coated the yeast cells and prevented the detection of the cytochrome P-450 in the whole yeast. The media contained also, 1%
yeast extract, 0.5% NaCl and 2% peptone. Separate experiments were terminated at intervals between 17 and 70 hours. The yeast growth data was derived from samples weighed after filtration (wet weight). Cytochrome P-450 was estimated on well shaken whole yeast (0.1g wet weight/ml), by automatic scanning difference spectrum at 450nm with carbon monoxide added to the one cuvette, and sodium thionite added to both cuvettes to achieve full reduction of the cytochrome P-450. Details of this estimation and the estimation of cytochrome a + a₃ by absolute spectral determination have been described in Chapter 2 (page 32).

To confirm that the cytochrome P-450 in the whole yeast is the same cytochrome P-450 existing in microsomal fraction, experiments were carried out on disruption and later centrifugation. Microsome P-450 levels parallel the level of whole yeast, although the microsomal P-450 yield is only a quarter of the whole yeast's levels.

It was found that the medium took on a dark colour after autoclaving more than 15 minutes if glucose was present and P-450 could not be detected. It was likely that the glucose in the medium is degraded, to form masking pigments, due to the high temperature required for sterilisation. Separate sterilization of the glucose was always done therefore (see Fig 5.1).

Cyclic AMP measurements were done using the cyclic AMP assay kit obtained from Amersham Radiochemical Centre. Adenyl cyclase assay were performed by the method of Albano et al (1973) (see Chapter 2, page 45).
Fig. 5.1  Sterilization time of medium (with 20% glucose or with glucose autoclaved separately) against yield of cytochrome P450

A  Sterlized separately with 20% glucose
B  Sterlized together with 20% glucose
5.2.1 Results:— (yeast weights are expressed as wet weight)

Fig. 5.2 shows the growth curves for yeast in media containing particular concentrations of glucose, as indicated. Much faster growth of yeast was observed at high glucose concentrations. These curves should be considered in relation to the change in cytochrome P-450 levels shown in Fig. 5.3. Rapid production of this enzyme, in early growth phase, occurs only at 1% and 2.5% glucose, while none is formed in 0.1% glucose medium. This appearance of cytochrome P-450 is delayed in 5% glucose or above, and rapidly rising levels are observed only towards the end of the logarithmic growth phase. The accumulation of cytochrome P-450 would not be correlated with the rise in alcohol content of the media in late growth phases.

Consideration of Fig. 5.4 indicates the inverse correlation of cytochrome a + a₃ to cytochrome P-450. Clearly, the level of cytochrome P-450 drops in the 1% glucose medium in correspondence with the rise in the cytochrome a + a₃ level.

It has been shown that cytochrome P-450 is present transiently even in medium containing as little as 1% glucose, in the early growth phase 17-40 hours of culture (Wiseman et al, 1975a). Several intermediate glucose concentrations between 1% and 20% glucose have now been done. The change to high-glucose behaviour, characterised by high levels of cytochrome P-450 in late logarithmic phase of growth, is observed to begin at 3% or 4% glucose concentration in the medium. The subsequent loss of cytochrome P-450 in 1% glucose medium can be correlated with the rise in concentration of cytochrome a + a₃.

91
Fig. 5.2 Growth curves for yeast in 1% to 20% glucose medium (as indicated)
Fig. 5.3 Changes in concentration of cytochrome P450 in yeast during growth in 0.1% to 20% glucose medium (as indicated)

Original % glucose in medium as shown

Time after inoculation of yeast (Hours)
Fig. 5.4 Changes in concentration of cytochrome a + a₃ in yeast during growth in 0.1%, 1% and 20% glucose medium (as indicated)

Cytochrome a₈ + a₃ as optical density at 607 nm of yeast

original % of glucose in medium as shown

Time after inoculation of yeast (h)

94
5.2.2 Effect of cyclic AMP level due to glucose concentration of media

Yeast grown in different concentration of glucose shows different levels of cyclic AMP. Cells were removed at 17 hours, 40 hours and 70 hours. Fig. 5.5 shows that the intracellular amount of cyclic AMP increased considerably during growth when cells were cultured in 0.1% glucose. This increase was almost linear for 40 hours. Under these conditions the maximal level of cyclic AMP was reached with growth at stationary phase (40 hours). Comparison of the results in 1% glucose and 20% glucose show that cyclic AMP level was 2 to 3 times lower than with growth in 0.1% glucose.

These results are almost reciprocal to the formation of cytochrome P-450 in all cases. The level of cyclic AMP has an inverse relationship with the biosynthesis of cytochrome P-450. However, yeast grown in 20% glucose shows the same level of cyclic AMP in 40 hours and 70 hours, cytochrome P-450 decreased to zero at 70 hours. Some other mechanism is involved therefore in the loss of the enzyme, presumably by degradation.

5.2.3 Effect on adenyl cyclase under glucose repression

The specific activity of adenyl cyclase was measured in a disruptate of yeast grown in 0.1% or 20% glucose. The activity was significantly repressed (55%) in 20% glucose growth medium in 30 hours of growth compared to 0.1% glucose growth medium in 24 hours of growth.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>p.mole cyclic AMP released/mg of yeast wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% glucose</td>
<td>1.4</td>
</tr>
<tr>
<td>0.1% glucose</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Adenylcyclase assay (one min.)
Fig. 5.5 Levels of cyclic AMP growth in different concentration of glucose
Addition of 20% glucose or 0.1% glucose to the assay system (yeast originally grown in 0.1% glucose) caused a significant reduction (50%) in production of cyclic AMP with 20% glucose but not with 0.1% glucose (see Fig. 5.6).

5.2.4 Cytochrome P-450 in anaerobically grown yeast cells:-

Yeast was grown semi-anaerobically with the medium containing 0.1% glucose, 2% peptone, 1% yeast extract and 0.5% NaCl. The 100 ml medium was flushed with nitrogen. The yeast was kept at 30°C in a shaking water bath for 24 hours. Cytochrome P-450 was assayed and found to be 1.2 n moles/g of yeast. Cytochrome a + a₃ was completely repressed.

5.2.5 Cytochrome P-450 in RD mutants of yeast:-

The RD mutants of yeast were prepared as described before. The RD mutants were inoculated into the medium (100 ml) containing 0.1% glucose, 1% yeast extract, 2% peptone and 0.5% NaCl at 30°C for 24 hours. The yeast was assayed for P-450. The activity of cytochrome P-450 was 2.3 n mole/g of yeast. With an RD mutant grown in 20%, P-450 was shown to be 5.5 n mole/g of yeast. Cytochrome a + a₃ was absent.

5.2.6 Effect of antibiotics in causing increase of P-450 production:-

The minimum inhibitory concentration of each antibiotic has been determined by growing with non-fermentable substrate 3% glycerol, 1% yeast extract, 2% peptone and 0.5% NaCl plus the antibiotics to be as follows:-

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>M.I.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol 3%</td>
<td>Chloramphenicol</td>
</tr>
</tbody>
</table>
Fig. 5.6 Adenyl cyclase assay

Yeast were grown in 0.1% glucose for 24 hours then disrupted by vibro mill

A 20% glucose were added to the incubation medium for the assay

B 0.1% glucose were added to the incubation medium for the assay
Glycerol 3% Imipramine 100µg/ml
Glycerol 3% Tetracycline 1000µg/ml
Glycerol 3% Rotonone 1000µg/ml
Glycerol 3% Erythromycin 1000µg/ml
Glycerol 3% 2:4-dinitrophenol 50µg/ml
Glycerol 3% Sodium Azide 50µg/ml

With the minimum inhibitory concentration of antibiotics determined, the yeast was grown in fermentable medium containing 0.1% glucose, 1% yeast extract, 2% peptone and 0.5% NaCl for 24 hours. The results are shown in table 1. Chloramphenicol gives a good yield of P-450. Cytochrome a + a₃ was repressed (see table 5.1).

With yeast cells grown in 0.1% glucose and then transferred to 20% non-growth medium, Fig. 5.6A shows that P-450 was increased to 1 n mole/g of yeast at 1 hour, then dropped to zero at 4 hours.

When yeast cells were grown on 0.1% glucose for 24 hours then transferred to 20% glucose (non-growth medium) incubated anaerobically for 24 hours, P-450 was increased to 1.25 n moles/g of yeast and cytochrome a + a₃ was repressed.

In deadadaptation experiment, yeast cells were grown in 20% glucose for 30 hours in 30°C. The harvested yeast (about 1g wet weight) was transferred to 100 ml non-growth medium of 0.1M phosphate buffer pH 7.4 containing 0.1% glucose. The yeast cells were incubated in a shaking water bath for several hours at 30°C. The minimal inhibitory concentration of antibiotics, such as chloramphenicol, tetracycline, erythromycin, imipramine, were added to the suspension containing 0.1%
Fig. 5.6a  Yeast growth in 0.1% glucose for 24 hours then transferred to 20% glucose (Non-growth) for 4 hours.
<table>
<thead>
<tr>
<th>Glucose concentration</th>
<th>Antibiotics</th>
<th>Cytchrome P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>Rotonone 1000μg/ml</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Tetracycline 1000μg/ml</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Erythromycin 1000μg/ml</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Imipramine 100μg/ml</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol 2 mg/ml</td>
<td>3.5</td>
</tr>
<tr>
<td>20%</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>0.1%</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Rotonone 1000μg/ml</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>Tetracycline 1000μg/ml</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Erythromycin 1000μg/ml</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Sodium Azide 50μg/ml</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2,4-Dinitrophenol 50μg/ml</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Imipramine 100μg/ml</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol 2mg/ml</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Fig. 5.8 shows the degradation of P-450 in 6 hours when suspended in the solution containing only 0.1% glucose. The P-450 was decreased after 6 hours, whereas when suspended in antibiotics it shows a greater activity of P-450 (see Figs. 5.8, 5.9 and 5.10).

Fig. 5.11 shows that when yeast cells are incubated in 0.1% glucose plus 1.0mM EDTA this stabilises the P-450 at 6 hours. Fig. 5.12 shows that when yeast cells are incubated in 0.1% glucose with and without aeration, cytochrome P-450 was restored to the same activity in non-aerated conditions. P-450 was destroyed under aeration conditions. Fig. 5.13 shows that when yeast was grown in 1% glucose for 40 hours then transferred to non-growth medium containing 12mM chloramphenicol with 0.1% glucose, the P-450 rose from 1mmole/g of wet weight of yeast to 2.75 in 4.5 hours and then dropped to zero in 6 hours. The control dropped to zero at 1 hour. The yeast was transferred from 0.1% glucose (yeast grown for 24 hours) to 0.1% glucose with 2mM chloramphenicol, cytochrome P-450 rose from 0 to 1.15 at 2 hours, and dropped to 0 in 4 hours.

5.2.7 Effect of P-450 appearance during adaptation

With yeast either grown in 0.1% glucose for 24 hours or grown in 1% glucose for 70 hours, P-450 was undetectable. When the yeast was transferred to the medium containing 20% glucose, P-450 appeared in 17 hours at a level of 3mmole/g of wet weight of yeast, and then declined to 1.5 in 40 hours. No P-450 was found when 1 mg/ml cycloheximide was added to the same medium. 80 mg/ml Actinomycin D added to the same medium could not inhibit the biosynthesis of cytochrome P-450. But when the yeast cells grown in 20% glucose were then transferred to 20% non-growth medium with or without 1μg/ml
Fig. 5.7  Yeast growth for 40 hours in 1% glucose medium then transferred to non-growth medium containing 12mM chloramphenicol with 0.1% glucose

A  Incubating with 12mM chloramphenicol plus 0.1% glucose non-growth medium

B  Control

C  Yeast cells were transferred from 0.1% glucose growth medium to 0.1% glucose plus 12mM chloramphenicol medium (non-growth) no P450 was found in control

Cytochrome P450 n moles/g of yeast (wet wt)

Incubation time (Hours)
Fig. 5.8 Deadaptation study of P450 when incubated in 0.1% glucose with and without 12mM chloramphenicol
Fig. 5.9 Yeast grown in 20% glucose for 30 hours then transferred to 0.1% glucose + 1000 µg/ml erthromycin

Cytochrome P450 n moles/g of yeast (wet wt)

Erthromycin

No erthromycin

Incubation time (Hours)
Fig. 5.10  Yeast grown in 20% glucose for 30 hours then transferred to 0.1% glucose plus
A  100 μg/ml imipramine or
B  1000 μg/ml tetracycline
C  Control
Fig. 5.11 Yeast grown in 20% glucose for 30 hours then transferred to 0.1% glucose + 1mM EDTA.
Fig. 5.12 Yeast grown in 20% glucose then transferred to 0.1% glucose (non-growth)

a. Anaerobic condition
b. Aerated the medium
cycloheximide, P-450 was found to be protected by the 20% glucose (see Fig. 5.14).

5.2.8 Effect of potential inhibitor of cytochrome P-450

Carbon monoxide is the best known inhibitor of cytochrome P-450. Carbon monoxide pregassing of the medium containing 20% glucose did not inhibit the growth of the yeast. However, since the cytochrome P-450 with carbon monoxide binding is reversible by oxygen, it is possible that only during the initial stages of growth was the cytochrome P-450 inhibited. Since the cells were harvested after three days it seemed likely that by this time the cytochrome P-450 may have been uninhibited.

5.2.9 Effect of alkanols on the growth of yeast

Hexanol and butanol at 1% in the culture medium totally inhibited growth in a 1% glucose medium and severely inhibited growth in 20% glucose. Though yeast cells can grow in 0.1% butanol in 20% glucose medium, no P-450 was present.

Fig. 5.15 shows ethanol formation in different times with growth in 20% glucose. The maximal production of ethanol was at 5% glucose concentration which had dropped from 20% to 5% during 60 hours of growth. In growing yeast with 5% ethanol plus 0.1% or 20% glucose, P-450 was not found in 0.1% glucose and there was no increase of P-450 in 20% glucose, though yeast can grow remarkably well (see Fig. 5.16). Various concentrations of other sugars were also tried for the biosynthesis of P-450.
Fig. 5.13  Yeast grown in 0.1% glucose for 24 hours as transferred to 20% glucose growth medium.

Fig. 5.14  Yeast grown in 20% glucose as transferred to 20% glucose (non-growth medium) with 10 \( \mu \text{g/ml} \) cycloheximide.
Fig. 5.15 Glucose consumption in yeast growth (against time) and formation of ethanol (against time)
Fig. 5.16 Yeast grown in 20% glucose or 20% glucose plus 5% ethanol
Fig. 5.17 shows that with yeast growth in sucrose, P-450 was found in the same pattern as yeast growing in glucose. In maltose the pattern was slightly different, P-450 was absent with growth in 4% maltose, but was found in 20% maltose. Cytochrome a + a₃ was found with 4% maltose but not 20% maltose.

5.2.10 Potential inducers for cytochrome P-450 are 3MC (methylcholanthrene) and phenobarbital. Effect of phenobarbital concentration on the induction of P-450 in yeast:

Fig. 5.18 shows the phenobarbital concentration-dependence of cytochrome P-450 production in 24 hours of growth. None of this enzyme was found at 0.05% phenobarbital in the growth medium, while at 0.15% phenobarbital, a concentration of 2nmole cytochrome P-450/g wet weight of yeast was reached. But the cyclic AMP level did not change compared with growth in 0.5% glucose. 3MC and ergosterol does not give any induction.

Fig. 5.19 shows that the concentration of cytochrome P-450 in the yeast drops during the stationary phase of growth in 0.5% glucose, with 0.2% phenobarbital present. This behaviour is similar to that reported for 1% glucose medium where transient accumulation of the enzyme is followed by a rapid decline to 30%, at 40 hours, of the maximum level reached at 17 hours. This pattern is quite unlike that in 20% glucose where there is relatively little cytochrome P-450 present after 17 hours but high levels are reached at 30 hours followed by a decline when growth ceases, to zero at 70 hours.

With yeast grown in 20% glucose for 30 hours or 40 hours in the presence of 0.2% phenobarbital, it did not increase the existing high level of cytochrome P-450.
Fig. 5.17 Yeast grown in different concentrations of sucrose

![Graph showing yeast growth in different sucrose concentrations over time. The x-axis represents growing time in hours (10-70), and the y-axis represents P450 n moles/g of yeast (wet wt). Curves for 2%, 8%, 10%, and 20% sucrose concentrations are shown, with peak growth around 40 hours for each concentration.]
Fig. 5.18 Effect of phenobarbital concentration on accumulation of cytochrome P-450 in yeast in 0.5% glucose medium in 24 hours.
Fig. 5.19  Time course of cytochrome P-450 accumulation and loss in yeast in the presence of 0.2% phenobarbital in 0.5% glucose medium •—•
Yeast growth curve in 0.5% glucose plus 0.2 phenobarbital ▲▲
Addition of 10 μg/ml cycloheximides in 17 hours ■—■
In deadaptation experiments the cytochrome P-450 was lost in 4 to 5 hours with or without the presence of 0.2\% phenobarbital (see fig. 5.20) and this rate of loss was not affected by the addition of 0.1\% glucose. Fig. 5.19 shows the addition of cycloheximide at 17 hours of yeast culture was associated with a halved recovery of cytochrome P-450 at 30 hours. Also the growth of the yeast between 17 hours and 30 hours (only a 25\% weight increase normally occurs as growth ceases early with only 0.5\% glucose present) is prevented by the addition of the cycloheximide (10 $\mu$g/ml).

5.3 Discussion:-

The glucose repression of mitochondrial cytochrome formation is known to be associated with the lower intracellular levels of adenosine 3' 5' cyclic AMP in high glucose media.

Fang and Butow (1970) reported that when yeast protoplasts prepared from anaerobically grown cultures were transferred to a low glucose medium they showed respiratory adaptation. In a medium containing 5\% or 10\% glucose, derepression was inhibited but could be overcome by addition of cyclic AMP. Cytochrome P-450 was inversely related to the formation of mitochondria in the yeast system. Under these conditions, the biosynthesis of cytochrome P-450 may be associated with the loss of cyclic AMP during incubation in high glucose media.

The activity of adenyl cyclase has been observed to be affected by the concentration of glucose in the growth medium of S. fragilis (Sy and Richter, 1972). Almost no adenyl cyclase activity could be detected
Fig. 5.20  Yeast grown in 20% glucose for 30 hours then transferred to
a  0.1% glucose (non-growth medium)
b  0.1% glucose + 0.15% phenobarbital
with cells grown in 10% glucose, and cyclic AMP levels were low under these conditions (Sy and Richter, 1972). It is likely that glucose may cause both repression and inhibition of adenyl cyclase as shown in our in vitro and in vivo studies.

Rapid destruction of cytochrome P-450 can occur when the glucose level falls. The mechanisms of these effects is entirely unknown.

The transient appearance of cytochrome P-450 in 1% glucose medium is associated with the only slow rise of cytochrome a + a₃ as the growth of the yeast begins during the first 17 hours. The much faster growth rate of yeast occurs with low levels of intracellular cyclic AMP (in 20% glucose rather than in low glucose media). Inhibition of the growth of \textit{E. coli} by exogenous cyclic AMP has been reported (de Roberts \textit{et al}, 1973).

The inverse relationship between cytochrome P-450 content and formation of mitochondria with cells grown in 20% glucose and transferred to 0.1% glucose, which permits formation of mitochondria, is associated with a large decrease in cytochrome P-450 content. When aerated in the presence of a high concentration of glucose (20%) where the mitochondria formation is also repressed, the decrease in P-450 was prevented, in contrast to the case of 0.1% glucose. The effect of 0.1% glucose was enhanced when the cells suspension was kept standing instead of shaking. This may be explained by the fact that in the presence of oxygen, unsaturated fatty acids of phospholipids undergo peroxidation (Hochstein \textit{et al}, 1968), a process which destroys the architecture of microsomal membranes and leads to the formation of malonyldialdehyde. This process could be blocked by EDTA (Levin \textit{et al}, 1973), by inhibiting lipid peroxidation. But in the
adaptation study, with the addition of 1mM EDTA plus 0.1% glucose, this did not restore the level of cytochrome P-450. This leads to the explanation that the degradation of cytochrome P-450 during adaptation may not be only due to lipid peroxidation of microsomal membrane. Yu and Coon (1974) showed that cytochrome P-450 in Pseudomonas can be reconverted from cytochrome P-420 by treatment with sulfhydryl compounds such as cysteine, β-mercaptoethanol or thioglycolic acid. Hence the loss of cytochrome P-450 during deadaptation may be due to the oxidation of a sulfhydryl group in this enzyme.

Cytochrome P-450 accumulates in yeast cells when grown anaerobically. Cytochrome a + a₃ was repressed when cytochrome P-450 was derepressed. Ishidate et al. (1969b) reported that with yeast grown under semi-anaerobic conditions mitochondria are absent in yeast cells and fermentation supplies the energy demands of the cells. However, the mitochondria do not disappear but exist as pro-mitochondria which are unable to support oxidative phosphorylation. Clark-Walker (1972) reported that pro-mitochondria occur in yeast grown under condition of high glucose concentration in the nutrient media or under semi-anaerobic conditions. Here the yeast is unable to synthesize the inner mitochondria membrane, due to deficiency of unsaturated fatty acids during growth anaerobically or in high glucose concentration media.

In the study of an RD mutant, wild type yeast grown in 0.1% glucose forms cytochrome a + a₃, but the RD mutant does not produce any cytochrome a + a₃. The latter showed a small amount of cytochrome P-450 present when grown even in 0.1% glucose. The inverse relationship between cytochrome P-450 content and formation of mitochondria was shown under these conditions,
which repress the adaptive formation of mitochondria.

Many antibiotics have been used to investigate the process of aerobic adaptation and of derepression in glucose-grown yeast. Various antibiotics inhibit protein synthesis in bacteria and they prevent the formation of mitochondrial cytochromes in yeast cells in a similar manner to glucose repression.

Chloramphenicol, tetracycline, erythromycin, imipramine, rotonone and 2-4 dinitrophenol are specific inhibitors of mitochondria protein synthesis, and they inhibit the synthesis of mitochondrial enzymes by reacting with the ribosome (Wilkie, 1972, Rousseau and Halvorson, 1973).

The yeast is unable to divide in the presence of inhibitor in the non-fermentable medium containing glycerol. Yeast can grow at the minimum concentration of the inhibiting drug in the fermentable medium. The inhibition of the mitochondrial system is selective, a significantly greater concentration than that required to inhibit growth on glycerol medium will be necessary to inhibit cell growth on fermentable medium. In fact, specific inhibitors e.g. rotonone, an extremely toxic insecticide, blocks electron transfer from NAD to cytochrome b, and hence prevents the process of synthesis of cytochrome \( a + a_3 \). Clark-Walker and Linnane (1967) showed that cells grown in 1% glucose are permeable to chloramphenicol. Cytochrome \( a + a_3 \) levels decrease in the presence of chloramphenicol with increase in cytochrome P-450 level. Tetracycline, erythromycin and imipramine showed the same effect as chloramphenicol.

The role of cytochrome P-450 in yeast grown on glucose is unknown.
Alexander et al (1974) reported that the conversion of lanosterol to zymosterol, by demethylation, in yeast disruptates could be inhibited (57%) by carbon monoxide but not by inhibitors of mitochondrial cytochromes such as cyanide or antimycin-A. This is suggestive of a role for cytochrome P-450 in this conversion, which is in the pathway of ergosterol synthesis in yeast, and may explain its accumulation in the yeast under conditions of glucose repression of the structural completion of the mitochondrion. The accumulation of ergosterol, or its precursors, under condition of glucose repression may be beneficial for the rapid restoration of functional mitochondrial membranes after glucose depletion, under aerobic conditions. But yeast growth in glucose medium supplemented with ergosterol did not lower the level of cytochrome P-450 by 30 hours of growth.

An alternative explanation is that the ergosterol is required for proliferation of the membranes of the endoplasmic reticulum that contain the cytochrome P-450, such as with the observed proliferation of these membranes upon induction of cytochrome P-450 in liver by phenobarbital. An increased cytochrome P-450 content of the yeast (Gibbons and Mitropoulos, 1973) may therefore encourage this proliferation of the endoplasmic reticulum, so allowing an increasing amount of cytochrome P-450 to be present in the cell in the form of membrane-bound enzyme. It is of interest in this connection that the cytochrome P-450 of liver is involved also in the biosynthesis of cholesterol.

The role therefore, in liver, of cytochrome P-450 is in the metabolism of drugs and perhaps the removal of toxic lipid hydroperoxides. The latter role is more likely in yeast growth, although accumulation of cytochrome
P-450 only in the absence of functioning mitochondria (when cyclic AMP level is low) may reflect the absence then of toxic radicals produced as a by-product of oxidative phosphorylation.

The molecular basis for the phenomenon of microsomal enzyme induction is not fully elucidated. Administration of phenobarbital to rats increases the rate of synthesis of certain microsomal drug metabolising enzymes in a selective manner and promotes proliferation of smooth endoplasmic reticulum in the liver.

Conney and Gilman (1963) suggested that the increased enzyme activity by phenobarbital induction was inhibited by Actinomycin-D and implied that the mechanism of this system is de novo protein synthesis. Venkatesan and Steele (1975) reported that phenobarbital could produce a 30% increase in the amount of ribosomes and mRNA in cells. In rat liver Ambike et al, (1970) reported that a small increase occurs in cytochrome P-450 level caused by phenobarbital in Claviceps purpurea, associated with alkaloid metabolism. In a deadaptation study, phenobarbital did not show any protection in P-450 degradation. This suggested that phenobarbital is not decreasing the rate of degradation of the cytochrome P-450 but is acting as a genuine inducer of biosynthesis as suggested for liver (Holtzman, 1969).

Rapid degradation of cytochrome P-450 occurs as growth ceases, as indicated in fig. 5.18, between 30 and 40 hours of culture. This degradation plus the inhibition of its biosynthesis therefore is responsible for the halved recovery at 30 hours. This dual effect explanation is confirmed by the fact that yeast is still able to grow with cycloheximide.
(1μg/ml) added at zero time, and then no cytochrome P-450 is produced.

Kawaguchi et al, (1973) concluded that cytochrome P-450 is not involved in ergosterol synthesis, nor in the desaturation required for the formation of the unsaturated fatty acid moiety in yeast phospholipids, unless saturated fatty acids could replace these. This conclusion is contrary to studies on a cell-free system from Saccharomyces cerevisiae that can convert lanosterol to zymosterol (Alexander et al., 1974). The system in the pathway of ergosterol biosynthesis, involves a demethylation reaction, and it was inhibited by carbon monoxide. This suggests the involvement therefore of cytochrome P-450 in this pathway.

It is possible that carbon monoxide is incapable of penetrating to the site of cytochrome P-450 binding in membranes, in the whole yeast. Kawaguchi et al (1973) however, considered such ω-hydroxylations to be unlikely in their yeast because of the presence of the cytochrome P-450 in semi-anaerobically grown yeast and its disappearance upon aerobic adaptation. An alternative view would be that the hydroxylation of oleic acid might be required under such conditions of mitochondrial repression. In support of this, 3% Tween 80 (but not ergosterol) was found to increase the level of cytochrome P-450 in brewer's yeast at late phases of growth (Wiseman, Lim and McCullough unpublished work). This would be a case of substrate induction which would suggest that cytochrome P-450 catalyses the hydroxylation of oleic acid prior to incorporation into membranes possibly of the endoplasmic reticulum or more likely of the mitochondrion.
CHAPTER VI

Regulation of cytochrome P-450 by cyclic AMP and cyclic GMP in the yeast protoplasts
6. Regulation of cytochrome P-450 by cyclic AMP and cyclic GMP in the yeast protoplast

It is well known that cyclic AMP is required for the synthesis of inducible enzymes in bacteria (Perlman and Pastan, 1968). Glucose represses inducible enzyme synthesis both by lowering the intracellular concentration of AMP (Makman and Sutherland, 1965) and by inhibiting the uptake of inducers (Adhyas and Echols, 1966). Added cyclic AMP is known to remove the catabolite repression of β-galactosidase biosynthesis by glucose in Escherichia coli (Ullman and Monod, 1968). In yeast, several enzymes responsible for the hydrolysis of disaccharides and the development of active mitochondria are known to be under catabolite repression.

There is only a little information concerning the function of cyclic AMP in yeast. Van Wijk and Konijn (1971) as well as Sy and Richter (1972) have shown some evidence that cyclic AMP may exert a regulatory role in the mechanism of catabolite repression of yeast.

6.1 Materials and Methods

Saccharomyces cerevisiae (NCYC No. 240) was grown in shake cultures in a medium containing 0.1% glucose, 1% yeast extract, 2% peptone, and 0.5% NaCl for 24 hours at 30°C. Cells were harvested by centrifugation and washed three times with 0.1% citrate-KH$_2$PO$_4$ buffer pH 5.8. The yeast (500 mg wet weight) was resuspended in this buffer (5 ml) with the additives 0.14M 2-mercaptoethanol and 0.04M EDTA and preincubated for 30 min., followed by resuspension after washing, in the same buffer (0.5 ml) without the additives and incubating at 30°C for 45 mins with 0.2 ml of snail gut enzyme
(33 mg/g wet weight of yeast). Yeast protoplasts were collected by centrifugation and resuspended in 10 ml of the growth medium now containing 20% glucose, and incubated at 30°C with one of 10 mM cyclic AMP or 5 mM cyclic AMP plus 5 mM cyclic GMP or other nucleotides, or 80 g/ml Actinomycin D (also added in one experiment after 6 hours) or 1 g/ml cycloheximide, or with no additives. Separate experiments were terminated at intervals up to 17 hours as indicated. No significant loss of protoplasts occurred under these conditions.

Cytochrome P-450 was estimated on a well shaken suspension of protoplasts at 450 nm, by automatic scanning difference spectrum with CO (with sodium dithionite in both cuvettes to complete the reduction of the cytochrome P-450). In the other experiments, the yeast was grown in the 20% glucose medium (as above except for glucose concentration). Protoplasts were prepared and suspended in the growth medium and incubated at 30°C with one of cyclic AMP, 5'AMP, 2'/3'AMP, GMP, 5GMP, GTP, 2/3 GMP or ATP (each at 10 mM concentrations) or with no additives as indicated.

In the study of cyclic GMP induction of cytochrome P-450, yeast cells were grown in 0.5% glucose for 24 hours. Protoplasts were prepared and resuspended in the growth medium (with 0.5% glucose) and incubated at 30°C with 5 mM cyclic GMP, or 5 mM cyclic GMP plus 5 mM cyclic AMP or 5 mM cyclic AMP or 5 mM AMP.

Yeast was grown in 20% glucose for 30 hours and protoplasts were prepared and transferred to (a) non-growth medium (0.1 M phosphate buffer) with 10 mM nucleotides. Protoplasts were incubated also for 8 hours in (b) 20% glucose in 0.1 M phosphate buffer with 10 mM nucleotides.
6.2 Results

6.2.1 Effect of cyclic AMP - a proposed negative effect on transcription in the biosynthesis of cytochrome P-450 in yeast (Wiseman and Lim, in press).

Cytochrome P-450 in Brewers' yeast is formed *de novo* under growth conditions of mitochondrial repression, which is at high glucose concentration in aerated media, where the intracellular level of cyclic AMP is low. In agreement with this, a cytochrome P-450 was formed in yeast protoplasts when Actinomycin D (80μg/ml), cycloheximide (10μg/ml) or cyclic AMP (10mM) is added upon transfer from 0.1% glucose to 20% glucose medium, where this enzyme is otherwise formed after 4 hours (see Fig. 6.1).

The following nucleotides (at 10mM) mimic the effect of cyclic AMP in the protoplast system; 5'AMP, 2'/3' AMP, and slightly by ATP which may not enter the protoplasts easily.

6.2.2 Effect of cyclic GMP - a proposed positive effect on transcription in the biosynthesis of cytochrome P-450 in yeast (Wiseman and Lim, in press).

Cyclic GMP, on yeast protoplasts in 0.5% glucose medium to induce the appearance of cytochrome P-450 1.1 nmoles/g wet weight of yeast in 17 hours (mean of 4 determinations). This too is prevented by Actinomycin D (80μg/ml) added at zero time. This induction effect is specific for cyclic GMP and is not shown by 5'GMP, 2/3' GMP or GTP. Moreover, an equal concentration of cyclic AMP added to the cyclic GMP causes a 50% reduction of the yield of the cytochrome P-450. Fig. 6.3 - suggesting true competition between these cyclic nucleotides probably at the level
Fig. 6.1 Hours of incubation of yeast protoplasts after transfer from 0.1% to 20% glucose medium

Control

Actinomycin D added at 6 hours

Zero time addition of cyclic AMP or 5'-AMP or 2'/3' AMP or actinomycin D or cycloheximide

Fig. 6.2 Hours of incubation of yeast protoplasts in 20% glucose medium

Control or plus ATP

With cyclic AMP or 5'-AMP or 2'/3' AMP
of transcription by competition for a binding protein (or enzyme) as an \textit{E. coli} (but with reversal of normal roles).

Protoplasts with high cytochrome P-450 content prepared from yeast grown in 20\% glucose medium, loses 88\% of this enzyme within 17 hours upon subsequent incubation with cyclic AMP (also 5' AMP 2'/ AMP but not ATP) in the growth medium. Only 50\% is lost in the absence of these nucleotides or in the presence of ATP (see Fig. 6.2, 6.2a). All of the enzyme is lost within 5 hours during incubation in 0.1M phosphate buffer instead of growth medium, with or without the cyclic AMP, (Figs. 6.4 and 6.5).

In order to study the control level at which cyclic AMP acts in the synthesis of cytochrome P-450, cyclic AMP was added after 6 hours, in the 20\% glucose medium cytochrome P-450 was not effected when assayed at 17 hours (Fig. 6.3).

6.3 \textbf{Discussion}

It is well established that yeast growing anaerobically or in high concentrations of glucose have a low rate of cyanide sensitive respiration and are deficient in cytochrome of the mitochondrial electron transport chain. This repressed condition can be reversed by growth on a low glucose medium or by simply aerating cells under non-growth conditions (Tustanoff and Bartley, 1964). Interestingly, cytochrome P-450 level was found to parallel this repressed condition in yeast. The presence of 10\% glucose in the medium severely inhibited derepression. When the medium is supplemented with 1.2mM cyclic AMP, protoplasts achieve a rate of cyanide-sensitive respiration comparable to the 0.1\%
Fig. 6.2a  Hours of incubation of yeast protoplasts (grown in 20% glucose) in 20% glucose medium
Fig. 6.3  Hours of incubation of yeast protoplasts after transfer from 0.1% to 20% glucose medium

- Control
- Added 10mM cyclic AMP at 6 hours
- Zero time addition of 5mM cyclic GMP
- Zero time addition of 5mM cyclic GMP + 5mM cyclic AMP
Fig. 6.4  Hours of incubation of yeast protoplasts in 0.1 M phosphate buffer (pH 7.4) with the nucleotides.
Fig. 6.5  Hours of incubation of yeast protoplasts in 20% glucose (non growth) with nucleotides
This reversal of glucose repression, however, is not specific for exogeneously added cyclic AMP. Although cyclic AMP consistently enhances adaptation, other nucleotides effectively lift the catabolite repression of mitochondrial cytochrome appearance and therefore respiration in a yeast protoplast system by added nucleotides. In vivo, however, this role seems to be confined to cyclic AMP and as with the effect the other nucleotides may be confined in the yeast mitochondria so that it is cyclic AMP that exerts the controlling function of β-galactosidase and tryptophanase in *E. coli*, growing in minimal medium on glucose or glycerol. Its action was considered to be on transcription in competition with cyclic GMP for binding protein (Artman and Werthamer, 1974). This is in agreement with our results when 5mM cyclic GMP acts on yeast protoplasts in 0.5% glucose medium inducing the appearance of cytochrome P-450 1.1nmoles/g of yeast in 17 hours. This is prevented by Actinomycin D (80µg/ml) added at zero time.

The induction is only specific for cyclic GMP. Other nucleotides do not show induction. Moreover, an equal concentration of cyclic AMP added to the cyclic GMP causes a halving of the yield of the cytochrome P-450 so we can suggest that the action occurs at a site (presumably transcription site) with equal affinity for these two nucleotides (Wiseman and Lim, in press).

All of the cytochrome P-450 is lost within 5 hours during incubation in 0.1M phosphate buffer instead of growth medium, with or without the cyclic AMP. Biosynthesis of the enzyme is presumably halted even in growth medium in the presence of cyclic AMP, and the
loss that occurs is due to the turnover mechanisms (observed in stationary phase of growth at glucose concentration below 8%). Once again the cyclic AMP effect is halved by the simultaneous presence of an equal concentration of cyclic GMP. Other negative type effects of cyclic AMP have been reported. The production of sex pill is inhibited in \textit{E.coli} K-12 carrying derepressed sex factors (Harwood and Heynell, 1975). The authors commented that this need not be a direct effect on transcription of genes involved in pilus production, but could be an indirect effect of cyclic AMP. Also it suppresses the induction of antibody synthesis in male spleen cell cultures (Bosling - Schnelder, 1975). A recent report (Klaipongan \textit{et al}, 1975) has concluded that cyclic AMP promotes a coordinated inhibition of hepatic anabolic pathways, including protein synthesis, under conditions of enhanced gluconeogenesis. Studies in a cell free system suggests a membrane associated effect in translation. Effects on translation in the cytochrome P-450 system cannot be ruled out therefore in view of the general doubt as to whether translational effects have been demonstrated for cyclic AMP in microbial and animal systems. Nevertheless, we are able to note the possibility that some promoters may have the reverse requirements for transcription from start or stop, compared with the classical \textit{lac} operon of \textit{E.coli}. (Wiseman and Lim, in press).
CHAPTER VII

Studies of cytochrome P-450 in yeast in continuous culture
7. Studies of P-450 of yeast in continuous culture

7.1 General introduction

Single-stage fermentation is the simplest type of cultivator in which the medium is thoroughly mixed to attain maximum homogeneity. Fresh sterile medium flows into the cultivator at a defined and constant rate. The volume in the cultivator is kept at a constant level. The practically exhausted medium together with a fraction of the grown cells leaves the fermenter at the same rate. The composition of medium with respect to cell density, substrate concentration, pH, dissolved oxygen concentration temperature is identical at all points in the cultivator and hence in the out flowing culture fluid.

Single stage homogenous continuous cultivation may be worked as a chemostat or turbidostat.

7.1.1 The turbidostat

By this method, the organisms are allowed to grow in a medium in which all the nutrients are present in excess, and therefore the growth rate is maximal (i.e. $\mu_{\text{max}}$) for the defined conditions. The inflow rate, to the culture vessel, is controlled in such a way that the turbidity of the culture is kept constant and the steady state is kept constant and the steady state is attained when the dilution rate ($D$) (proportional to flow rate) is constant. In this situation $\mu_{\text{max}} = D$. Automatic methods of operation, in which a photocell (measuring the turbidity of overflow) is linked back to regulate the rate of inflow, have been developed (Northrop, 1960) but the control is difficult to maintain due to 'clumping out' of cells in the culture.
The turbidostat is not a self regulating system, since changes in the growth rate at a fixed dilution rate produced a rapid change in biomass. Therefore, in the absence of control the system is unstable.

7.1.2 The *chemostat*

Steady state conditions in continuous culture can be achieved when the concentration of one of the nutrients in the medium is reduced to a growth-limiting value (Herbert *et al*, 1956). This system is called the chemostat.

The chemostat has the property of being self regulating since if the inflow of medium is set at a suitable constant value the system adjusts itself to the steady state (for the defined conditions), in which the microbial concentration, the substrate concentration in the vessel and the physiological properties of the cells remain constant (N.B. dx/dt and ds/dt = 0). Cells in the culture can be maintained in active growth over a wide range of growth by setting the flow rate (dilution rate) to the desired value.

7.2 Principles of continuous culture of microorganisms

The theoretical aspects of continuous cultivation were worked out by Herbert, Elsworth and Telling (1956). They formulated the basic mathematical derivations for growth kinetics of microbial cells in a homogenous continuous culture.

The specific growth rate: $\mu$

$$\mu = \frac{1}{(X \frac{dx}{dt})}$$ is determined by the substrate
concentration and may be described by the expression

\[ \mu = \mu_{\text{max}} \cdot \frac{S}{K_s + S} \]

where \( \mu_{\text{max}} \) is the maximum value of \( \mu \) at saturation concentration of substrate.

\( S \) is the substrate concentration.

\( K_s \) is a saturation constant numerically equal to the substrate concentration at which \( \mu = \mu_{\text{max}}/2 \)

There is a simple relationship between growth and utilization of substrate such that the growth rate is a constant fraction of the rate of substrate utilization. Thus

\[ \frac{dx}{dt} = -Y \frac{ds}{dt} \]

Where \( x \) is the concentration of organisms (weight of organisms per unit of volume) and

\( Y \) is the yield coefficient

Thus for any finite period of growth

\[ Y = \frac{\text{weight of micro-organisms formed}}{\text{weight of substrate used}} = -\frac{dx}{ds} \]

In the more usual type of apparatus the reactor consists of some form of culture vessel in which the organisms can be grown under suitable conditions. Sterile growth medium is fed into the vessel at a flow rate \( F \) and culture emerges at the same rate, a constant level device keeping the volume of the culture, \( V \) constant. Efficient stirring is necessary so that the entering medium approaches the ideal condition of instant mixing and uniform distribution throughout the vessel, the so called 'completely-mixed' vessel.
The period that a particle remains in the culture vessel, referred to as the residence time, will be determined not by the absolute values of flow rate and culture volume, but by their ratio, f/v. The wash-out rate (i.e. the rate at which organisms initially present in the vessel would be washed out if growth ceased with the flow of medium continuous) is therefore \( \frac{dx}{dt} = Dx \) where \( x \) is the concentration of organisms in the vessel.

In the growth vessel, the net rate of change of concentration of organisms is expressed by the balance equation:

\[
\text{increase} = \text{growth} - \text{output}
\]

\[
\frac{dx}{dt} = \mu x - Dx \tag{1}
\]

when \( \mu = D \) \( \frac{dx}{dt} = 0 \)

\( x \) is constant and independent of time, thus gives steady state

If \( \mu > D \) \( \frac{dx}{dt} > 0 \)

Therefore the concentration of organisms will increase. If \( \mu < D \) \( \frac{dx}{dt} < 0 \)

The concentration of organisms will decrease. Eventually to zero corresponding to washing out of the culture vessel.

In the growth vessel, substrate enters at a concentration \( S_R \) and is consumed by the organisms, and flows out at concentration \( S \). The substrate net change concentration is as follows:

Increase = input-output-consumption

\[= \text{input} - \text{output} - \text{growth} \]

Yield coefficient

Therefore \( \frac{ds}{dt} = DS_R - D_S - \frac{\mu x}{Y} \quad \text{-------- (2)} \)
From equation (1)
\[ \frac{dx}{dt} = x \left( \mu_{\text{max}} \left( \frac{S}{K_s + S} \right) - D \right) \] ............(1')

since \( \mu = \mu_{\text{max}} \frac{S}{K_s + S} \)

From equation (2)
\[ \frac{ds}{dt} = D (S_r - S) - \mu_{\text{max}} \frac{S}{K_s + S} \frac{X}{Y} \]
\[ = D (S_r - S) - \mu_{\text{max}} \frac{X}{Y} \left( \frac{S}{K_s + S} \right) \] (2')

In steady state
\[ \frac{ds}{dt} = 0; \frac{dx}{dt} = 0 \]

From (2')
\[ X \left( \mu_{\text{max}} \left( \frac{S}{K_s + S} \right) - D \right) = 0 \]

\[ \mu_{\text{max}} \left( \frac{S}{K_s + S} \right) = D \] ............(4)

\[ S = K_s \left( \frac{D}{\mu_{\text{max}} - D} \right) \] ............(5)

From (1')
\[ \frac{ds}{dt} = D (S_r - S) - \mu_{\text{max}} \frac{X}{Y} \left( \frac{S}{K_s + S} \right) = 0 \] ............(6)

Substitute (4) to (6)
\[ \mu_{\text{max}} \frac{S}{K_s + S} (S_r - S) - \mu_{\text{max}} \frac{X}{Y} \left( \frac{S}{K_s + S} \right) = 0 \]
\[ \mu_{\text{max}} \frac{S}{K_s + S} \left[ (S_r - S) - \frac{X}{Y} \right] = 0 \]
\[ X = Y (S_r - S) = Y \left[ (S_r - K_s) \left( \frac{D}{\mu_{\text{max}} - D} \right) \right] \]
With these equations, and knowing the values of the constants $\mu_{\text{max}}$, $K_s$ and $Y$ for a given organism and growth medium, the steady state concentrations of organisms and substrate in the culture vessel can be predicted for any value of the dilution rate and concentration of inflowing substrate. The critical dilution rate $D_c$ is important from the equation (see Fig. 7.1a).

$$D_c = \mu_{\text{max}} \left( \frac{S_R}{K_s + S_R} \right)$$

when $S_R \ll K_s$

\[ \therefore \quad D_c = \mu_{\text{max}} \]

\[ D_c > D \]

\[ \frac{dc}{dt} < 0 \quad \text{The organism will be washed out of the culture vessel.} \]

An important factor in the growth of aerobic bacteria is the rate of supply and dissolution of oxygen. Methods of aeration and means of increasing the oxygen transfer rate (from gas to liquid) have been reviewed by Solomons (1961).

The amount of available oxygen in solution depends on the rate of transfer of oxygen from gas to liquid (Elsworth et al., 1957; Pirt, 1957).

$$\frac{dc}{dt} = \phi \ (C_s - C)$$

where $\phi$ : absolute rate constant

$C_s$: Sat. concentration of $O_2$

$C$ : Concentration of dissolved $O_2$

$\phi$ depends upon the degree of agitation, viscosity and composition of the solution.

The oxygen demand of the cells is proportional to the dilution rate.
Fig. 7.1a Steady-state relationships in a continuous culture (theoretical).

Steady-state substrate concentration, \( s \) (g/l)

Output of bacteria, \( D_s \) (g/l/hr)

Steady-state bacterial concentration, \( x \) (g/l)

Substrate concentration

Bacterial concentration

Dilution rate, \( D \) (hr\(^{-1}\))
and the substrate concentration in the inflowing medium (Pirt, 1957).

7.3 Materials and methods:-

Yeast (NCYC No. 240) was grown in 0.1% glucose, 2% peptone, 1% yeast extract and 0.5% NaCl medium for 24 hours and was then transferred to the fermenter containing 20% glucose, 2% peptone, 1% yeast extract and 0.5% NaCl. The working volume of the fermenter was 800 ml which was agitated by a magnetic stirrer, incorporated into the fermenter (see fig. 7.6). The rotating speed was in the range of 225-725 r.p.m. and the aeration rate was in the range of 400-800 ml/min. The antifoam was rubbed onto the top of the fermenter wall. The dilution rate was varied, using a peristaltic pump. Cultures were considered to be in a steady state after 4 days at a dilution rate of 0.08 hour⁻¹. At least three samples were taken at each steady-state value and the measurements of each sample were done in triplicate. Assay of cyclic AMP and cytochrome P-450 were done as described previously (see chapter 5, page 88).

7.4 Results:-

The effect of variables on some cellular properties in the chemostat. The effect of changes in the pH, temperature substrate concentration and air rate were studied in turn at a fixed dilution rate in glucose limited cultures.

7.4.1. Substrate concentration and the steady state cell mass; and cytochrome P-450 for glucose limited cultures is shown in fig. 7.1.

In the region studied the steady state values of cytochrome P-450
Fig. 7.6 Flow diagram of continuous fermentation
Fig. 7.1 The relationship between growth, cyclic AMP level, cytochrome P450, and glucose concentration in continuous culture. The dissolved oxygen was at 16% (dilution rate at 0.08 hr⁻¹).
were directly in proportion to the glucose concentration (reciprocal therefore to cyclic AMP) is of cytochrome P-450. Levels in 20% glucose were the maximum level but 0.1% glucose did not give any P-450.

7.4.2 pH

In these experiments the pH of the culture was maintained at the desired value by means of automatic control.

The glucose limited cultures showed the same models of response to the change in the pH. The cytochrome P-450 and the cellular mass were decreased remarkably at the pH below the pH of 3 or above pH 7. At pH 4 to 5 optimal production of cytochrome P-450 was found (see Fig. 7.2).

7.4.3 Temperature

Temperature is known to have marked influence upon the growth of yeast. It can be seen that the cytochrome P-450 existed and was found at lower temperatures in the glucose limited system. The optimal temperature was 30°C for the production of P-450. Above this temperature the activity of P-450 decreased (see Fig. 7.3). Growth above 30°C gave a low rate of growing.

7.4.4 Dissolved oxygen

The dissolved oxygen concentration governed the production of cytochrome P-450 during growth in high glucose concentration medium. The presence of 16%-30% oxygen, gives a maximal production of P-450 (4 n mole/g of yeast) whereas in 80% oxygen P-450 levels dropped to 2 n moles/g of yeast. The control of dissolved oxygen in the medium was controlled by
Fig. 7.2  Yeast growth at different values of pH (20% glucose) in continuous culture showing different yield of cytochrome P450
Fig. 7.3 Yeast growth at different temperature in continuous culture with different yield of cytochrome P450
automatic on-off stirring and therefore by the rate of impeller rotation. The inflow rate was fixed all the time (see fig. 7.4).

7.4.5 Dilution rate:

The effect of dilution rate under conditions of glucose limitation on cytochrome P-450 is shown in fig. 7.5. Cytochrome P-450 activity varied little between $D = 0.10/hr^{-1}$ and $D = 0.06/hr^{-1}$. Cytochrome P-450 levelled off to 4 mmole/g of yeast at the dilution rate $D = 0.10/hr^{-1}$. The level of P-450 was higher at $D = 0.06/hr^{-1}$.

Cyclic AMP content in the different growth condition: it was very interesting to observe the cyclic AMP content to be found in continuous cultures under different growth conditions i.e. different glucose concentration and dissolved oxygen. In 0.1% glucose there was a maximal level of cyclic AMP. The higher the glucose concentration in the medium the lower the cyclic AMP found. This is in agreement with the previous results found in shake cultures. For low and high dissolved oxygen, cyclic AMP content remained at the same level, though cytochrome P-450 levels varied under these different conditions.

7.4.6 Discussion:

Cytochrome $a + a_3$ has been noted to display an inverse relationship to glucose concentration. (Tustanoff and Bartley, 1964). When yeast cells (S. cerevisiae) were grown anaerobically on glucose and then exposed to air, the rate of development of respiration and cytochrome oxidase activity were directly dependent on the concentration glucose during adaptation. This suggested that glucose inhibited protein and lipoprotein synthesis.
Fig. 7.4  Yeast growth at different % of dissolved oxygen in continuous culture with different yield of cytochrome P450
Fig. 7.5 Cytochrome P450 levels in 20% glucose against dilution rate in continuous culture
and led to a diminished activity of lipoprotein bound enzymes in mitochondria. Ishidate et al (1969) reported that cytochrome P-450 levels decreased concomitantly with the development of the mitochondria respiratory system. Similarly, in continuous culture, cytochrome P-450 formation was glucose concentration dependent. Cyclic AMP levels in yeast are inversely related to glucose concentration. When the yeast was under catabolite repression, cyclic AMP levels dropped.

Hence it is suggested that cyclic AMP prevents the formation of cytochrome P-450. Fang and Butow (1971) reported that cyclic AMP and other nucleotides could derepress mitochondria development and hence reverse the catabolite repression of the synthesis of mitochondria enzymes in protoplasts of *S.cerevisiae* that have been anaerobically grown and are adapting to aerobiosis in the presence of glucose. Furthermore Van Wijk and Konijn (1971) have shown that the intracellular level of cyclic AMP in cells of *S.carbsbergensis* grown on glucose is low, but increases during adaptation to growth on maltose. In agreement with the above finding, it was found that cyclic AMP level was high at low glucose concentration (0.1% glucose) in continuous culture.

The regulation of the respiratory structures of micro-organisms by the concentration of oxygen during growth had been studied (Smith, 1961). In *E.coli*, cytochrome was repressed when oxygen was added to the medium, (Wimpeny et al, 1963). *E.coli* cytochrome a₂ was repressed during growth in continuous culture with limited oxygen. It has been suggested that it is likely that both the apoprotein and the prosthetic group are suppressed by abundant oxygen (Lascelles, 1964). *Saccharomyces* sp. show particularly complex responses to dissolved oxygen tension when grown on glucose because
of the phenomenon of repression of respiration.

In yeast, oxygen may be incorporated into unsaturated fatty acids and sterols (Goldfine and Block, 1963) which are important lipid components of mitochondria membranes. As reported by Ishidate et al (1969), cellular content of cytochrome P-450 can vary under different environmental conditions and is found in very small quantity in aerobic cells containing mitochondria. The decrease of cytochrome P-450 on aeration is strongly inhibited by the addition of chloramphenicol to suppress the formation of mitochondria. It has been suggested that cytochrome P-450 has a role in cellular metabolism and that it may be involved in a system operating in the oxygen-dependent transformation of lipid. Some of the active oxygen may be attributed to the formation of hydrogen peroxide which cannot be removed in the absence of catalase but in some anaerobes oxygen may be inhibited through the autoxidation of cytochrome. An excess of oxygen has been shown to retard steps in porphyrin biosynthesis. Thus δ-aminolaevulinic acid synthetase is repressed by oxygen in *Rhodospirillum spheroides* (Lascelles 1960). Hyperbaric oxygen toxicity has been attributed to oxidation of thiol groups, enzyme inactivation, lipid peroxidation and free radical accumulation. In this investigation it was found that P-450 was degraded when yeast was grown in higher dissolved oxygen concentration. The loss of cytochrome P-450 in high dissolved oxygen is not associated with an increase in the level of cyclic AMP however. This is also true when cytochrome P-450 is lost in stationary phase of culture.

Increased dilution rate will change the growth rate. The growth of
micro-organisms is regulated by the limiting substrate, and this growth rate is raised when limiting substrate is increased. The increase of growth of the micro-organism is thought to be due to the increased RNA content; the RNA (ribosomal and messenger) regulate protein synthesis and this regulates growth rate of the micro-organism. Enzymes usually involved in metabolism are repressed by the growth-limiting substrate and are regulated by the concentration of that substrate. At low dilution rates catabolite repression is minimal. However, above certain dilution rates the growth rate has increased to a point where metabolic intermediates are being formed at a rate sufficiently high to create significant catabolite repression. Surprisingly, the cytochrome P-450 level did not vary when the dilution was increased. Temperature might be expected to influence the yeast in a continuous culture through altered growth rate. Other metabolic functions of the yeast cells such as biosynthesis of carbohydrate, lipid, and RNA may also be sensitive to temperature (Rose, 1969). The optimal temperature for cytochrome P-450 production is between 25-30°C. pH often causes quite a sharp change in cell metabolism. In anaerobically grown culture of K.aerogenes pH 6.5 the metabolic products are mostly ethanol and butanodiol together with small amounts of acetic acid (Harrison and Pirt, 1967). Cytochrome P-450 did not appear when the medium pH was 7, perhaps because some other metabolic products cause inhibition of cytochrome P-450 biosynthesis.
CHAPTER VIII

Concluding remarks
8.1 Introduction

Van Wijk et al (1969) reported that the ability of maltose to induce α-glucosidase was controlled by the concentration of glucose present. 1% glucose caused catabolite repression, but 0.1% glucose was able to derepress α-glucosidase synthesis. It is possible that derepression involves the initiation of translation of some existing messenger RNA for α-glucosidase. Glucose at 1% prevented both transcription and translation, each to different extents. Van Wijk and Konijn (1971) showed lowering of cyclic AMP in catabolite repression by 2% glucose in *S. carlsbergensis*. Intracellular levels of cyclic AMP were lowered six-fold. No mention was made of the effect of addition of cyclic AMP to yeast or yeast protoplasts under conditions of glucose repression. Cyclic AMP will easily enter only damaged *E. coli* and yeast too is usually impermeable to negative charged molecular species. No lifting of catabolite repression was observed by us with 10mM cyclic AMP or 10mM dibutyryl cyclic AMP in the presence of 2% glucose + 4% maltose in a non-growing yeast culture. Removal of glucose repression of α-glucosidase synthesis was however achieved in the yeast protoplast.

It is believed that cyclic AMP stimulates the synthesis of α-glucosidase mRNA by increasing the frequency of initiation of α-glucosidase coded mRNA chains at the promoter site. A cyclic AMP binding protein has been isolated from yeast cells (Sy and Richter, 1971). This is an important discovery and tends to uphold the hypothesis that a cyclic AMP receptor protein exists in yeast cells and plays a part in
derepression of yeast by forming the complex between receptor protein and cyclic AMP and α-glucosidase DNA.

Anaerobically grown yeast shows a good yield of cytochrome P-450. The cytochrome P-450 may be involved in the respiratory adaptation of repressed yeast. Evidence for this theory received strong support from the fact that in respiratory adaptation the decline of cytochrome P-450 is paralleled by the increase of cytochrome α + α<sub>3</sub>. On this hypothesis, the P-450 may be degraded or converted to some other cytochrome perhaps during the biogenesis of functional mitochondria.

The nature and function of the haematin compounds are not known and on aeration of the cells they are replaced by the normal cytochrome of the aerobic cell, α + α<sub>3</sub>, b, and c. The synthesis of the mitochondrion appears to be dependent on the presence of oxygen. Formation of mitochondria is induced on aeration of the anaerobically grown cells with the induction being accompanied by the synthesis of the cytochrome characteristic of the aerobic cells (many inducible enzyme systems are repressed by glucose and its metabolic products). In addition to oxygen another parameter which exerts a controlling influence on the development of the respiratory activity of yeast is the glucose concentration available to the cell. Anaerobically grown yeast cells aerated in the presence of low concentrations of glucose rapidly develop ability to respire, whereas high concentrations of glucose restrict this development. Cells cultured aerobically also develop limited respiratory activity in the presence of high concentrations of glucose but pursue essentially aerobic metabolic pathways when grown on low concentrations of glucose.
or on galactose, independently of the galactose concentrations (Strittmatter, 1957). Linnane et al (1972) reported that the number and the manner of formation of the mitochondria from the oxygen-induced vesicles of *S. cerevisiae* are controlled by the concentrations of glucose available to the cell. Glucose maintained at concentrations in excess of 5% repress the oxygen-induced formation of mitochondria and the synthesis is partially repressed in a medium of 0.66% glucose with the glucose repression appearing to function separately at two different levels; on the development of electron transparent vesicles to dense granules and on the stage of vesicle development which gives rise to the mitochondria (high glucose concentration 5% arrest both the synthesis of mitochondria and the development of the vesicles). Cells aerated in intermediate glucose concentration (2%) form mitochondria largely from the more electron dense vesicles. Low glucose levels (0.6%) permit the cell to elaborate mitochondria via the vesicles in all stages of their development.

The relationship between the accumulation of cytochrome P-450 in brewer's yeast and the growth phase of the yeast, grown aerobically in glucose, containing media in the range 0.1% to 20% glucose had been studied (Wiseman et al, 1975). No cytochrome P-450 is produced in 0.1% glucose medium. Early accumulation of the enzyme was observed in low glucose (1%, 2%) and late accumulation was observed in high-glucose media (5% upwards) and with intermediary behaviour in 3% or 4% glucose media. There is an inverse correlation of cytochrome P-450 level with that of cytochrome a + a^3. It was thought unlikely that cytochrome P-450 is converted to cytochrome a + a^3 upon aeration, and concomitant
mitochondriogenesis. Ishidate (1969) noted that high concentrations of glucose or of chloramphenicol prevented the development of mitochondrial respiration and also prevented the loss of the cytochrome P-450. It was possible to demonstrate an uptake of oxygen in the presence of Antimycin-D, which was therefore non-mitochondrial.

8.2 The possible role of cytochrome P-450 in peroxidation

However an interesting proposition as to the significance of the cytochrome P-450 induction involves peroxidation (Hrycay and O'Brien, 1972). In anaerobic fermentation there will be a marked decrease in the metabolism of oxygen by the yeast although this is not due to total absence of oxygen metabolism. This was demonstrated by the experiments involving shaking and non-shaking in which it was shown that shaken cultures grew faster than unshaken when both media contained 20% glucose. Presumably this difference was due to the greater aeration of the shaken yeast. Thus although oxygen is still metabolised, it is a very low level compared with the derepressed state. This also means that the amount of free superoxide radical declined in the repressed yeast and that the content of fatty acid hydroperoxide formed by reaction of radicals with unsaturated fatty acids declines. Since the metabolism of this superoxide and fatty acid hydroperoxide causes cytochrome P-450 to be converted to cytochrome P-420 (Hrycay and O'Brien, 1971), this has therefore detoxified the superoxide radical or the fatty acid hydroperoxide and is itself then degraded by scission of its heme prosthetic group with decline in the concentration of free superoxide radical. It has been pointed out (Greim, 1970) that fasting decreases the degradation of
cytochrome P-450. In rats, fasting decreases the oxidative phosphorylation in mitochondria and hence decreases the formation of free superoxide radicals and hence may explain why fasting potentiates the phenobarbital induction of cytochrome P-450. Also in mammalian systems (Gilbert, 1969) antioxidants act as inducer of cytochrome P-450 possibly to decrease the amount of free radicals in this way.

8.3 Cytochrome P-450 and cyclic AMP

The stimulation of this enzyme by various chemicals, including glucose, and mitochondrial inhibitors, all points to different modes of achieving mitochondrial repression in the yeast cell. Glucose acts by a mechanism very much like the catabolite repression by other metabolites also observed in bacteria (Perlman and Pastan, 1971). The drug phenobarbital seems to act by classical enzyme induction (that is by an increased rate of de novo synthesis of enzyme protein after removal of a cytoplasmic repressor), since cycloheximide prevented the formation of the cytochrome P-450.

Cyclic AMP level mediates the cytochrome P-450 level: glucose is thought to exert its effect upon the system by lowering the concentration of cyclic AMP in the cell. Cyclic AMP can lift the glucose repression and therefore induce the formation of functional mitochondria (Fang and Butow, 1971), also decreasing the level of cytochrome P-450 of yeast protoplasts in our work.

Added cyclic AMP and some other nucleotides enter the protoplast and prevent the accumulation of cytochrome P-450 and increases its rate of disappearance on transfer to low glucose medium. On the contrary, cyclic GMP can cause its accumulation in 0.5% glucose medium, where its
otherwise not formed.

This evidence suggested that the transcription of the gene responsible for cytochrome P-450 synthesis in yeast is controlled in the opposite fashion to the Lac operon of E.coli.

It appears that, in higher vertebrates, evolution has developed a complicated set of hormonal controls that regulate cell processes. Some hormones operate indirectly by way of a two messenger system. The first messengers travel from the site of synthesis to their specific target cells where they stimulate the formation of a second messenger which at the present time is believed to be cyclic AMP. The level of cyclic AMP is controlled by two important enzymes, one is adenyl cyclase and the other a specific cyclic AMP diesterase. The profound metabolic importance of cyclic AMP resides in the fact that adenyl cyclase activity responds to a wide variety of hormones in intact cells. The hormones react with specific hormones receptor sites (HRS) in the target membrane which are in close proximity to the adenyl cyclase site and activated the enzyme protein by allosteric interaction. The concept of the first messenger (hormone) and the second messenger (cyclic AMP) system is as below.
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