THE RELATIONSHIP OF GLYCOLYTIC/GLUCONEOGENIC INTERMEDIATES IN BREWING YEAST (SACCHAROMYCES UVARUM) FERMENTATIONS TO GROWTH

by

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CERTIFICATION OF SUPERVISORS

In terms of paragraph eight of "General regulations for the degree of Ph.D." we, as supervisors of the candidate, D.S. Ryder, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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CONTENTS

ABSTRACT

The objective of this study has been to understand the metabolic inter-relationship between yeast growth, regulation of glycolytic/gluconeogenic flux and accumulation of glycosyl donors for polysaccharide synthesis in brewing yeast (*Saccharomyces uvarum*) fermentations.

Loss of fermenting power of a brewing yeast population may be created by a condition that inhibits growth by limiting amino acid formation and protein synthesis. In commercial strains of *S. uvarum* this loss may be transitory, or, if not corrected, may ultimately lead to yeast degeneration. The potential industrial impact is realised for fermentation systems which may limit yeast growth, eg. continuous systems, use of pressure and, particularly, systems utilizing immobilised cells.

In order to chronicle the associated metabolic events occurring under conditions which promote cellular proliferation and under conditions which terminate growth in the exponential growth phase, a specific analytical strategy was adopted. It was considered at the outset of this work that the multivalent nature of the glycolytic/gluconeogenic controlling allosteric enzymes, often complicated by numerous intracellular modulators, prevented reproducing *in vitro*, a relatable *in vivo* enzymatic situation. To gain an accurate overall picture of intracellular glycolytic/gluconeogenic control, it was, therefore, decided to measure the levels of pertinent cellular intermediates and effectors. To this end, the development of isotachophoresis and bioluminescence as measurement techniques provided the specificity and sensitivity (picomole range) required to examine a wide

range of intracellular nucleotides, organic acids and hexose and triose phosphates.

Experiments indicated that growth-limiting conditions are associated with high ATP, GTP, citrate and phosphoenolpyruvate levels and low levels of AMP, fructose-1,6-bisphosphate and pyruvate in agreement with the proposed regulatory mechanisms of phosphofructokinase I (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40), phosphoenolpyruvate carboxykinase (EC 4.1.1.49), fructose-1,6-bisphosphatase (EC 3.1.3.11) and aldolase (EC 4.1.2.13).

The aggregate effect of these changes is in favour of inhibiting phosphofructokinase I and pyruvate kinase activity and activating phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, thus orientating the glycolytic pathway in a gluconeogenic direction. Regulation at the phosphofructokinase I/fructose-1,6-bisphosphatase junction created a build-up of fructose-6-phosphate and subsequently glucose-6-phosphate which, by feed-back, cooperatively inhibited glucose utilization.

Measurements showed that the end-point of gluconeogenesis in yeast and/or inhibition of phosphofructokinase I is not glucose-6-phosphate in yeast as has been previously reported. Under growth-limiting conditions, the glycogenic pathway was utilized as far as the glycogen substrate, uridine-5'-diphosphoglucose (UDPG), creating an augmented UDPG pool. Concomitantly, a build-up of the cell wall precursor, uridine-5'-diphosphate-N-acetylglucosamine, occurred. This indicated that control of glutamine fructose-6-phosphate transaminase (EC 5.3.1.19) by UDP acetylglucosamine, was incompletely suppressed, at least as far as the yeast strains utilized were concerned.

Under non-proliferating conditions, the augmented UDPG pool was not channelled

to glycogen. Moreover, under these conditions the level of glycogen decreased to a base level or to a level where formation and consumption are virtually equal. Thus, glycogen synthesis and degradation in brewing strains of S. uvarum appears to be a highly regulated, physiological process, dependent not only on the availability of UDPG and glycogen as respective substrates. It is proposed that allosteric and covalent regulation of the interconvertible phosphorylated and dephosphorylated forms of glycogen synthetase (EC 2.4.1.11) and phosphorylase (EC 2.4.1.1) is of major significance. In this regard, the intracellular levels of the adenine nucleotides and glucose-6-phosphate would appear to play a particularly important role. A regulation model is proposed of glycogen synthesis and degradation when exponential growth is inhibited in S. uvarum. The physiological meaning of the increased UDPG concentration under the described conditions is unclear. It may be that the maintenance of a UDPG pool is a more efficient mechanism for the yeast cell to tenuously support viability and integrity even though, under the test procedures, the cell is ultimately unable to ferment.

CHAPTER 1

INTRODUCTION

1.1 INDUSTRIAL SIGNIFICANCE

Inadequate yeast growth is important for the brewing industry since it results in pitching yeasts with undesirable characteristics such as poor attenuations, altered beer flavour notes and inconsistent fermentation times.

The principle that the rate of yeast fermentation is a function of the rate and extent of cellular growth is well established. In 1953, Slonimski commented on the decrease and even transitory loss of fermentation capacity per unit yeast in non-growing conditions. Subsequent to this work, Harris and Millin (1963) and Masschelein et al. (1960, 1965) studying brewing yeast fermentations concluded that each growth period is characterised by a maximal fermentation capacity for glucose and maltose per unit yeast, the extent of which is specific for each yeast strain and that this diminishes rapidly during the stationary phase to a maintenance level. Moreover, during these studies, it was determined that fermentation capacity is subject to cyclic variations depending upon the physiological state of the yeast cell and that one aspect of yeast degeneration may be a sequential lowering of maximum fermentation capacity levels during the brewing yeast generation cycle.

Kirsop (1978) more recently reported the results of investigations on the wort compositional aspects of yeast growth and fermentation capacity with respect to studies conducted at varying α-amino nitrogen,

oxygen and lipid concentrations. This work demonstrated the importance of nutrient interaction for improved yeast growth and performance.

Ploss and Eschenbecher (1981) studied the effects of brewing yeast storage and treatment conditions on fermentation capacity levels and demonstrated that conditions preventing yeast growth inhibited yeast performance.

Thus, there have been increasing demands for fundamental information towards a complete understanding of the metabolic inter-relationship between the rate of glycolytic flux and the dependency on yeast growth.

1.2 THE GLYCOLYTIC PATHWAY AND TRICARBOXYLIC ACID CYCLE IN YEAST METABOLISM

1.2.1 A Perspective

Yeasts are able to adopt either a fermentative or an oxidative sugar metabolism according to the aerobic nature of the environment and the nature and concentration of the carbon source in the substrate.

The reaction sequences and associated catalyzing enzymes for complete glucose oxidation are shown in Figure 1.1 and Table 1.1. In the glycolytic pathway, glucose is metabolised to three-carbon and two-carbon compounds, the oxidation is continued in the tricarboxylic acid cycle which produces more carbon dioxide and reducing power. The reduced respiratory nucleotides are then oxidised in the electron transport chain, via the flavin, ubiquinone and cytochrome systems, where the electrons from hydrogen are transferred to molecular oxygen.

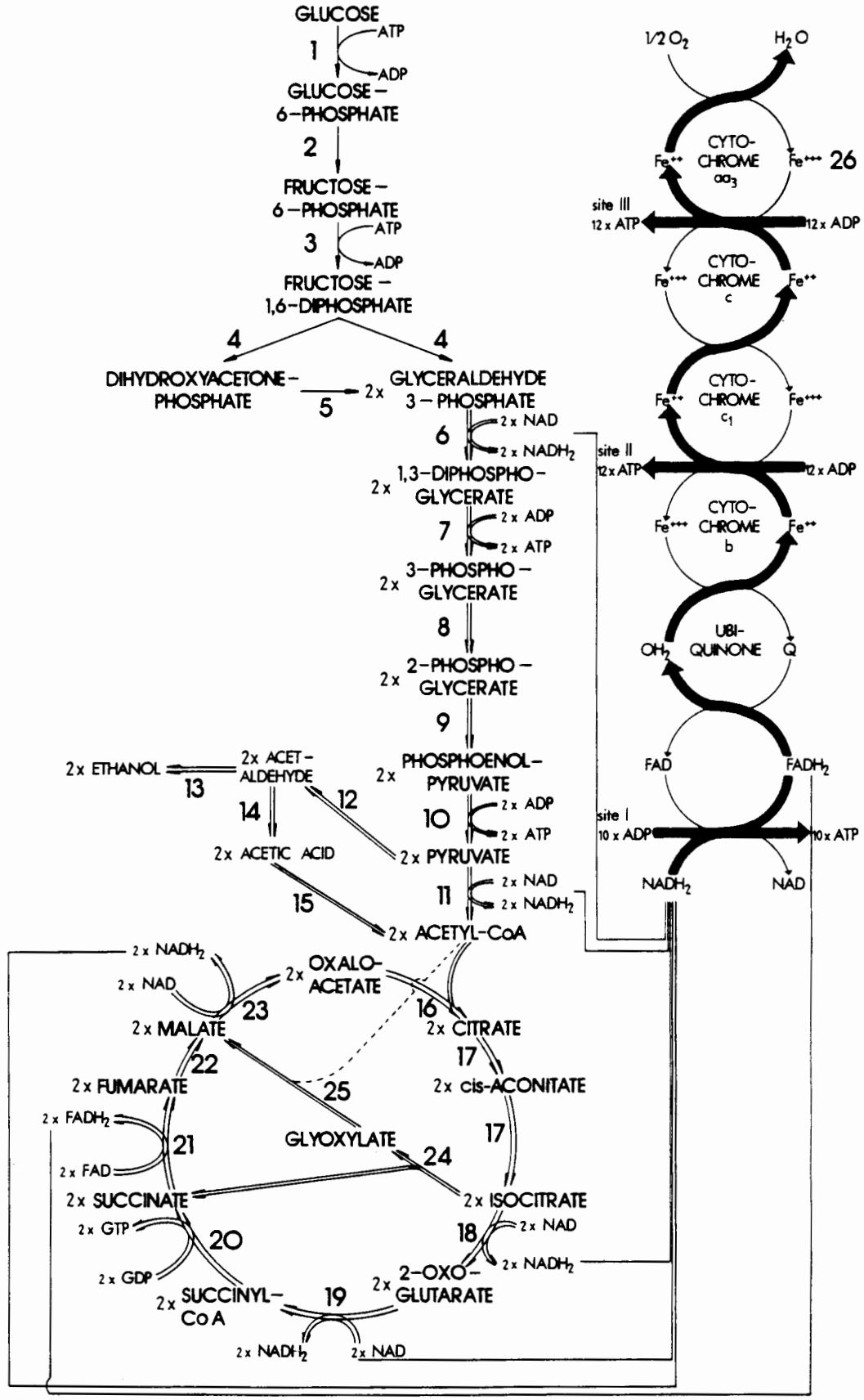


FIGURE 1.1: Reactions of complete glucose oxidation via the Embden-Meyerhof-Parnas pathway, the tricarboxylic acid cycle and the electron transport chain. Branch reactions to and from ethanol are indicated.

TABLE 1.1: Enzymes catalysing change: ref. Figure 1.1.

GLYCOLYSIS

1.	Hexokinase	(EC 2.7.1.1)
1.	Glucokinase	(EC 2.7.1.2)
2.	Phosphoglucose isomerase	(EC 5.3.1.9)
3.	Phosphofructokinase	(EC 2.7.1.11)
4.	Fructose bisphosphate aldolase	(EC 4.1.2.13)
5.	Triose-phosphate isomerase	(EC 5.3.1.1)
6.	Glyceraldehyde-3P dehydrogenase	(EC 1.2.1.12)
7.	Phosphoglycerate kinase	(EC 2.7.2.3)
8.	Phosphoglycerate mutase	(EC 2.7.5.3)
9.	Enolase	(EC 4.2.1.11)
10.	Pyruvate kinase	(EC 2.7.1.40)
11.	Pyruvate dehydrogenase	(EC 1.2.4.1)
12.	Pyruvate decarboxylase	(EC 4.1.1.1)
13.	Alcohol dehydrogenase	(EC 1.1.1.1)
14.	Aldehyde dehydrogenase	(EC 1.2.1.5)
15.	Acetyl-CoA synthetase	(EC 6.2.1.1)

TRICARBOXYLIC ACID CYCLE

16.	Citrate synthase	(EC 4.1.3.7)
17.	Aconitate hydratase	(EC 4.2.1.3)
18.	Isocitrate dehydrogenase	(EC 1.1.1.42)
19.	2-Oxoglutarate (α-ketoglutarate) dehydrogenase	(EC 1.2.4.2)
20.	Succinyl-CoA synthetase	(EC 6.2.1.4)
21.	Succinic dehydrogenase	(EC 1.3.99.1)
22.	Fumarase	(EC 4.2.1.2)
23.	Malate dehydrogenase	(EC 1.1.1.37)

TABLE 1.1: (Cont'd)

GLYOXYLATE CYCLE

24. Isocitrate lyase ——————————— (EC 4.1.3.1)

25. Malate synthase ——————————— (EC 1.1.3.2)

16-17 and 21-23 as in TCA Cycle

ELECTRON TRANSPORT CHAIN mentioned only

26. Cytochrome C oxidase ——————————— (EC 1.9.3.1)

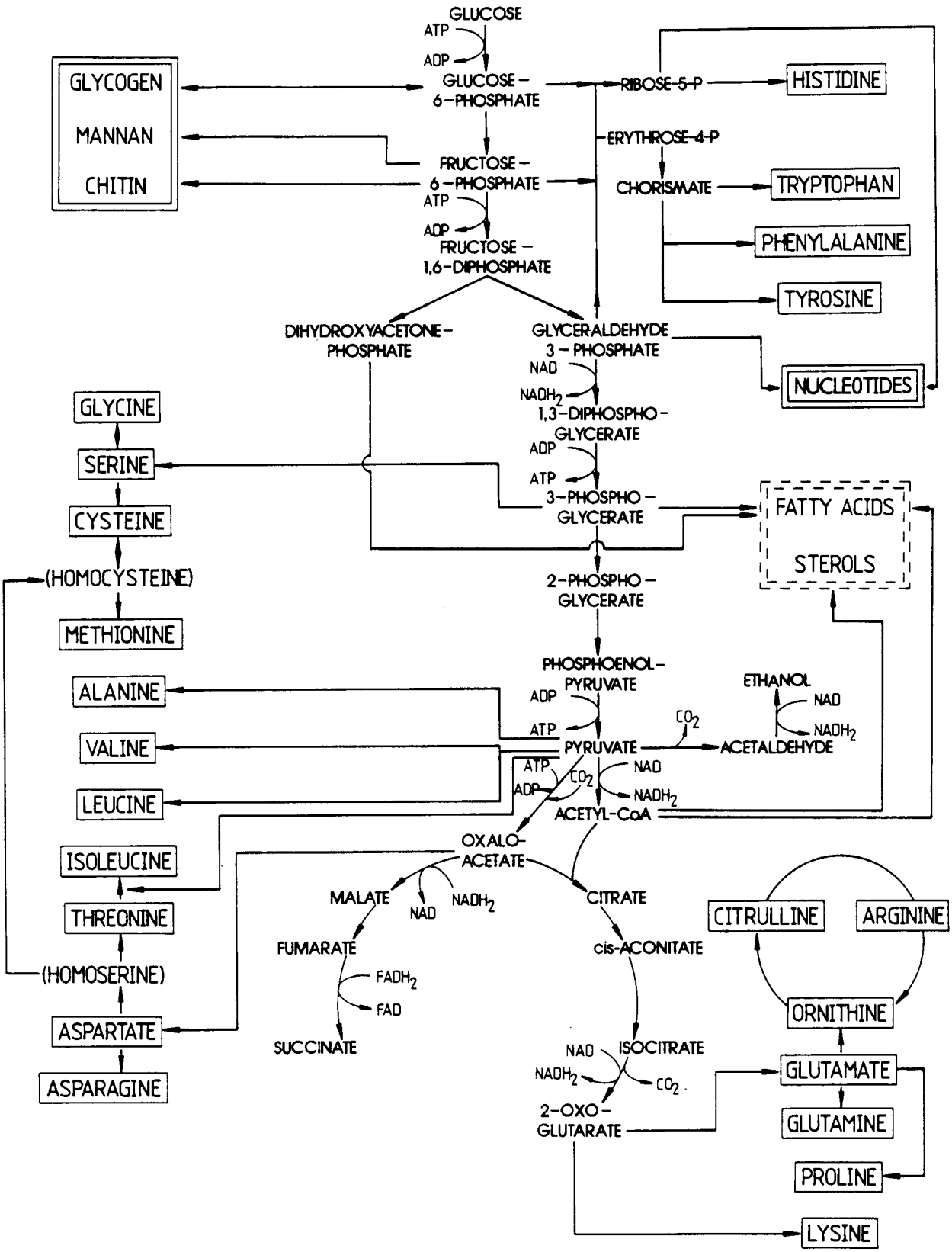


FIGURE 1.2: Intermediates of glucose metabolism in anaerobic fermentation showing the relationship to amino acid synthesis and other major biosynthetic pathways (incomplete tricarboxylic acid cycle explained in text).

As the growing yeast cell uses the intermediates of carbon catabolism as precursors for anabolic reactions, in polysaccharide, amino acid, lipid and nucleotide biosynthesis (Figure 1.2), the inter-relationship between glycolysis, the tricarboxylic acid cycle, and growth, is evident.

The continued oxidation of acetyl-CoA by the tricarboxylic acid cycle requires oxaloacetate. As the biosynthetic processes associated with the tricarboxylic acid cycle are proceeding concomitantly to the oxidation of pyruvate, there is a continual utilization of 2-oxoglutarate and the C4-dicarboxylic acids such that the regeneration of oxaloacetate is disturbed. In order to aid replenishment of oxaloacetate the yeast cell provides anaplerotic pathways (Kornberg, 1966). These pathways produce oxaloacetate from $C_1 + C_3$ reactions or from C_2 units (acetyl-CoA). In the first case, a Wood-Werkman type condensation of CO_2 with pyruvate or phosphoenolpyruvate (Cannata and Stoppani, 1963) to form oxaloacetate occurs as follows:

Pyruvate + ATP + CO_2

$\rightleftharpoons$

pyruvate carboxylase (EC 6.4.1.1)

Oxaloacetate + ADP + Pi

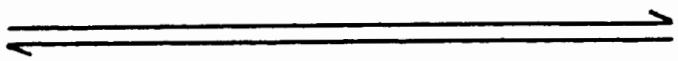
Phosphoenolpyruvate + ADP + CO_2

$\rightleftharpoons$

phosphoenolpyruvate carboxykinase (EC 4.1.1.49)

Oxaloacetate + ATP

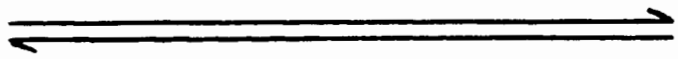
Phosphoenolpyruvate + Pi + CO_2 + H^+



phosphoenolpyruvate carboxyphosphotransferase (EC 4.1.1.38)

Oxaloacetate + PPi + H_2O

Phosphoenolpyruvate + CO_2

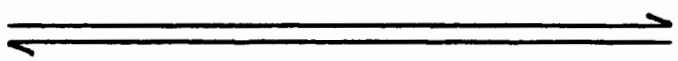


phosphoenolpyruvate carboxylase (EC 4.1.1.31)

Oxaloacetate + Pi

From acetyl CoA, net oxaloacetate synthesis occurs as a result of a functional tricarboxylic acid cycle supplemented by a glyoxylate pathway (Olson, 1959; Barnett and Kornberg, 1960; Dixon et al., 1960), originally described in Pseudomonas (Kornberg and Krebs, 1957).

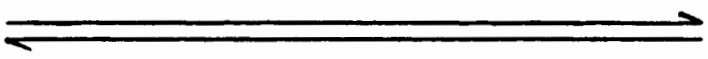
The glyoxylate cycle is made up of the following reactions:

Isocitrate 

isocitrate lyase (EC 4.1.3.1)

Succinate + Glyoxylate

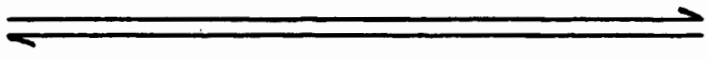
Glyoxylate + Acetyl-CoA + H_2O



malate synthetase (EC 4.1.3.2)

Malate + CoA

Malate + NAD^+



malate dehydrogenase (EC 1.1.1.37)

Oxaloacetate + NADH + H^+

Oxaloacetate + Acetyl - CoA + H_2O

$\rightleftharpoons$

citrate synthase (EC 4.1.3.7)

Citrate + CoA

Citrate $\rightleftharpoons$ Isocitrate

aconitase (EC 4.2.1.3)

Thus,

2 Acetyl - CoA + $2H_2O$ + NAD^+

$\longrightarrow$ Succinate + NADH + H^+ + 2CoA

In aerobic metabolism, therefore, the above reactions are important in explaining a functional tricarboxylic acid cycle, supplementary mechanisms to ensure regeneration of oxaloacetate during yeast growth and a high yield coefficient of ATP (Y_{ATP}).

Since 1929, however, it has been known (Crabtree, 1929) that, under aerobic conditions at high glucose concentrations, the metabolism of cells is fermentative; glucose degradation essentially occurring anoxidatively via the formation of ethanol. This phenomenon, in contradistinction to the Pasteur effect, which is essentially based on the inhibition of fermentation by the presence of oxygen (for review, see Krebs, 1972), has been called the reverse Pasteur effect or the Crabtree effect (De Deken, 1966). This repression of respiration has been shown to allow optimum growth in strains of Saccharomyces (Lagunas, 1976). Thus, as in the yeast Saccharomyces there is a bias towards the type of metabolism which allows the highest possible growth rate, growth only becomes oxidative at the point where the carbon source finally limits the faster fermentative growth (Oura, 1974; Fraenkel, 1982), if oxygen is available.

Brewery fermentations are characterised by high fermentable carbon levels at the beginning of the fermentation when there is only a limited amount of oxygen available to satisfy the yeast strain's requirements (Kirsop, 1974) for unsaturated fatty acid and sterol synthesis (Andreasen and Stier, 1954; Aries and Kirsop, 1978; Haukeli, 1979). Therefore, brewery fermentations are under the regulation of the Crabtree effect essentially exhibiting anaerobic degradation of carbohydrates.

1.2.2 Catabolite Repression and Inhibition

Catabolite repression (Magasanik, 1955) refers to the suppression of the synthesis of cellular enzymes to enable co-ordinated control sequences when one readily utilizable substrate represses the utilization of other substrates. Original (Monod, 1942) and subsequent investigations (see, for example, Beck, 1968) on diauxic growth showed that, in a variety of substrate combinations, the substrate that allows the higher growth rate was metabolized first resulting in a biphasic growth curve. This phenomenon usually refers to glucose or a catabolic transformation product of glucose, such as glucose-6-phosphate, but may equally refer to fructose. Lampen (1973) found that the addition of a high level of fructose (150 mM final concentration) to a suspension of Saccharomyces protoplasts actively synthesizing invertase, halted invertase formation as rapidly as cycloheximide which acts directly on the formation of the polypeptide chain.

It is now generally recognised that the energy flux generated by the carbon source and not simply the presence of the substrate per se is

the primary cause of repression (Barford and Hall, 1979). Although the repression of respiratory enzymes, including repression of certain tricarboxylic acid cycle enzymes, glyoxylate cycle enzymes, and electron transport components (Witt et al., 1966; Haarasilta and Oura, 1975), has come to characterise the Crabtree effect, catabolite repression should be considered as a general mechanism affecting many different metabolic pathways and moreover, as a result of the repression effect, may affect cell structure, particularly at the level of mitochondria (Strittmatter, 1957; Polakis and Bartley, 1965; Beck and von Meyenburg, 1968; Chapman and Bartley, 1968).

As a rule it can be stated that substrates utilized rapidly by constitutive enzyme systems cause catabolite repression of inducible pathways. In terms of the utilization of the specific fermentable mono-, di- and trisaccharides in wort by brewing yeast, catabolite repression is responsible at the initial level for their sequential uptake pattern (see 1.2.5, Pathways into glycolysis). Suppression of transcription of the structural genes for the specific permeases responsible for maltose and maltotriose uptake and the α-glucosidase responsible for hydrolyzing these endogenous di- and trisaccharides to glucose, is under the control of this regulatory mechanism (Görts, 1969; Van Wijk et al., 1969). Hockney and Freeman (1980) recently found that, besides glucose, glucosamine acted as a gratuitous catabolite repressor of maltose utilization in S. cerevisiae when present at a concentration of 1.5 g/l in complete medium with maltose at 10 g/l.

Catabolite repression explains a mechanism where synthesis rather than activity of the enzymes is concerned. Inactivation of the pre-formed enzyme is known as catabolite inhibition. This type of metabolic regulation is particularly prominent with respect to the gluconeogenic enzymes

(Holzer, 1976) in yeast. Glucose-induced inactivation of several other enzymes has been reported: the galactose-metabolizing system (Spiegelman and Reiner, 1947), α -glucoside permease (Robertson and Halvorson, 1957; Görts, 1969), cytoplasmic malate dehydrogenase (Witt et al., 1966), and isopropylmalate synthase (Brain et al., 1975).

Catabolite inhibition has been found to be complimentary to catabolite repression (McGinnis and Paigen, 1969) in explaining the sequential utilization of maltose with respect to maltotriose in brewery fermentations (Ramos et al., 1973; Haboucha and Masschelein, 1976). The distinction between catabolite repression and inhibition is important because it is only catabolite inhibition which can reduce the fermentation speed of maltose and maltotriose to a critical value thus causing "hanging" or "tailing" fermentations.

The mechanism of catabolite inhibition is not understood. McGinnis and Paigen (1973) postulated the transport of a particular sugar into the cell which causes inhibition of the other sugar transport systems. Equally, there may be structural or conformational alterations to the target enzyme in such a way that it becomes vulnerable to intracellular proteolytic degradation (Holzer, 1976). Takeda (1981) speculated on a possible activation - inactivation mechanism for succinate and malate dehydrogenases in S. cerevisiae taking into account a proteinase I which converts the enzyme precursors into active forms and a proteinase II, positively influenced by glucose, which degrades the active enzymes.

The mechanism of catabolite repression has been explained at the level of gene-cyclic AMP interactions (Pastan and Perlman, 1971; Perlman and Pastan, 1971). In S. cerevisiae and S. uvarum (carlsbergensis), van Wijk et al. (1969) showed that, under varying conditions, the lower

the degree of catabolite repression, the higher the concentration of intracellular cyclic AMP.

The reason for decreased levels of cyclic AMP when glucose is being metabolized is thought to be due to a sugar-dependent regulation of adenyl cyclase via phosphorylation-dephosphorylation (Peterkofsky, 1977). Current hypotheses assume that glucose (and fructose) transport into the cell is by way of the phosphoenolpyruvate-dependent phosphotransferase system, other sugars entering by alternative mechanisms. The phosphoenolpyruvate-phosphotransferase system is explained in Figure 1.3. (Saier, 1977) schematically demonstrating how the sugar becomes phosphorylated in transit, during passage through the plasma membrane. Phosphoryl transfer from phosphoenolpyruvate to sugar requires the intermediate participation of a number of enzymes. Thus, phosphate is sequentially transferred down the phosphate transfer chain, first from phosphoenolpyruvate to enzyme I, then to a heat stable protein, HPr, which transfers its phosphoryl moiety to enzyme III. The phosphoryl moiety of enzyme III phosphorylates the sugar via the participation of the specific sugar membrane carrier, enzyme II.

Peterkofsky (1977) has explained the interaction of the phosphoenolpyruvate-phosphotransferase system with adenyl cyclase, as follows. When enzyme I exists in a phosphorylated form, i.e. in the presence of phosphoenolpyruvate and the absence of glucose (or fructose), adenyl cyclase activity is high; no catabolite repression. When enzyme I is dephosphorylated, i.e. in the presence of glucose (or fructose) which becomes phosphorylated, - the phosphoenolpyruvate level drops and adenyl cyclase becomes less active creating catabolite repression.

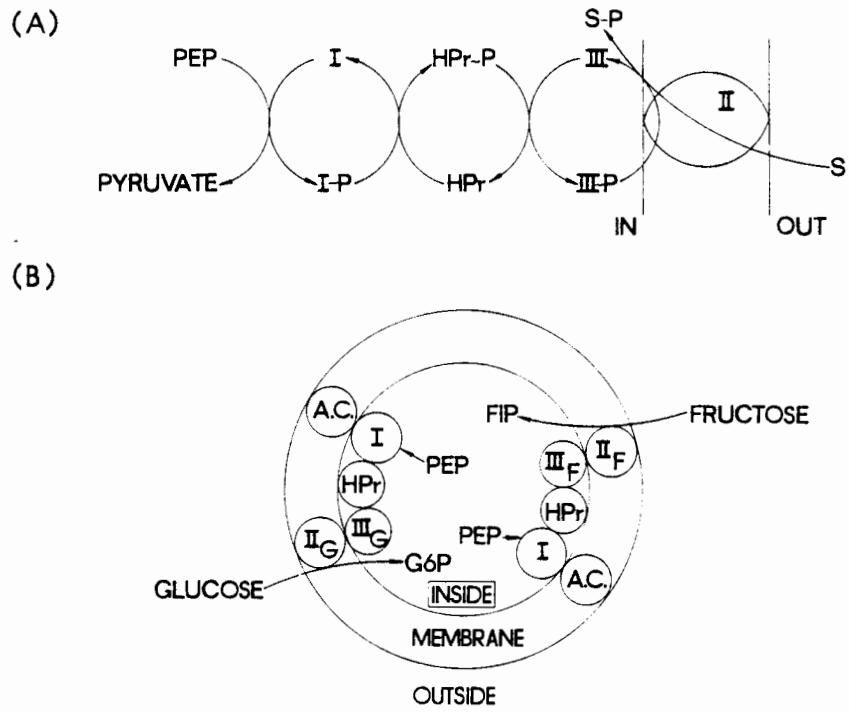


FIGURE 1.3: Proposed mechanisms of the phosphoenolpyruvate-phosphotransferase system in catabolite repression.

A: phosphate transfer chain (Saier, 1977).

B: interaction of the phosphate transfer chain with adenyl cyclase (Peterkofsky, 1977).

In yeast, however, there is a dearth of information in this area compounded by data being collected under incompletely defined physical conditions (Barford and Hall, 1979).

Yeast adenyl cyclase is expected to be inhibited by glucose or one of its early metabolites, because levels of cyclic AMP in yeast are depressed by glucose (Schlanderer and Dellweg, 1974). However, Varimo and Londesborough (1982) studying S. cerevisiae found that glucose did not inhibit adenyl cyclase in situ under conditions similar to those used by Harwood and Peterkofsky (1975) to demonstrate the control of Escherichia coli adenyl cyclase by glucose.

1.2.3 The Tricarboxylic Acid Cycle under Anoxidative, Catabolite Repressed Conditions

The changes that occur in the activities of the tricarboxylic acid cycle when yeast adopts an anoxidative, catabolite repressed form of metabolism are quite well documented. Reductions of activity, for example, are found to occur in citrate synthase, NAD-linked isocitrate dehydrogenase, fumarase, malate dehydrogenase and NAD-linked glutamate dehydrogenase (Polakis and Bartley, 1965; Witt et al., 1966; Chapman and Bartley, 1968; Beck and von Meyenburg, 1968; Mian et al., 1969). Moreover, a fully functional tricarboxylic acid cycle during anaerobic growth would only occur slowly, if at all, because it is doubtful whether yeast shows any succinate dehydrogenase and α-ketoglutarate dehydrogenase activity under anaerobiosis (Chapman and Bartley, 1968). As the tricarboxylic acid cycle products must nevertheless be formed in anaerobic metabolism in order to supply anabolic reactions for the synthesis of cell material

or its components, the oxidising pathway leads from oxaloacetate to 2-oxo-(α-keto) glutarate and the reducing pathway to succinate (Oura, 1973), as shown in Figure 1.2.

Glyoxylate cycle activity in yeast is repressed by glucose (Polakis and Bartley, 1965; Witt et al., 1966; Beck and von Meyenburg, 1968) although this repression can be partially reversed in aerobic, glucose-limited chemostat cultures (Rogers and Stewart, 1973; Oura, 1974). Glucose repression is more severe for isocitrate lyase than for malate synthase or the requisite tricarboxylic acid cycle enzymes (Witt et al., 1966). Moreover, isocitrate lyase and malate synthase activity do not occur in anaerobic yeasts (Beck and von Meyenburg, 1968; Rogers and Stewart, 1973). Thus, during brewing yeast fermentations where the glyoxylate by-pass would be repressed, anaplerotic replenishment of oxaloacetate must be formed in carboxylase reactions. Investigations undertaken by Cazzulo et al. (1968) have shown that phosphoenolpyruvate carboxylase in yeast is susceptible to catabolite repression. Furthermore, Haarasilta (1981) even doubts the occurrence of phosphoenolpyruvate carboxylase in yeast. His attempts to confirm the existence of this enzyme by radiochemical methods were unsuccessful. In contradistinction, Cazzulo et al. (1968) found that the biotinyl protein, pyruvate carboxylase, increased in activity during fermentative growth and would therefore explain the formation of the required oxaloacetate. As phosphoenolpyruvate carboxykinase activity is doubted in the oxaloacetate direction in yeast (Haarasilta, 1981), the pyruvate carboxylase reaction is generally thought to be the only anaplerotic reaction of yeast that incorporates CO_2 into cell constituents and can therefore be considered to be the only anaplerotic reaction occurring under conditions of catabolite repression in brewery fermentations.

Growing S. cerevisiae has been found to obtain 4,8% of its carbon from CO_2 (Liener and Buchanan, 1951) although Oura (1974) has calculated that, theoretically, 6,3% total carbon should be able to be derived from CO_2 when all oxaloacetate regeneration proceeds through pyruvate carboxylase.

Slonimski (1953) reported that S. cerevisiae, when grown under strictly anaerobic conditions, has little, if any, respiratory activity and lacks cytochromes a, a_3, b, c and c_1; and that a normal system of respiratory enzymes cannot be synthesised until the yeast is aerated. However, anaerobically grown yeast cells contain various haematin compounds characterised by α -absorption bands at 557 nm and 580 nm (Ephrussi and Slonimski, 1950) that are not completely blocked even at very high concentrations of glucose (30%) (Reilly and Sherman, 1965). Furthermore, cytochrome P-450 is present in larger amounts in anaerobic cells (Lindenmayer and Smith, 1964). The precise role of the anaerobic haematin compounds is not understood although the involvement of cytochrome P-450 in lipid metabolism in the presence of oxygen has been suggested (Atkins et al., 1972).

1.2.4 Gluconeogenesis

Gluconeogenesis is conceptually the reversal of glycolysis (Figure 1.4). As the biosynthetic pathways of the yeast cell must always be functional in order that cellular integrity may be maintained, irrespective of the environmental conditions, provision is made for metabolic flux to function in this reverse direction using such carbon sources as ethanol, acetate or the glycogenic amino acids, aspartate and glutamate. Certain glycolytic steps, however, are physiologically irreversible. Thus, for gluconeogenesis to occur in biological tissues, specific enzyme steps are

necessary, i.e.

(i) Oxaloacetate + ATP

$\rightleftharpoons$

phosphoenolpyruvate carboxykinase (PEPCK)

Phosphoenolpyruvate + ADP + CO_2

(ii) Fructose - 1,6 - bisphosphate + H_2O

$\longrightarrow$

fructose - 1,6 - bisphosphatase (EC 3.1.3.11)

Fructose - 6 - phosphate + Pi

(iii) Glucose - 6 - phosphate + H_2O

$\longrightarrow$

glucose - 6 - phosphatase (EC 3.1.3.9)

Glucose + Pi

A certain amount of controversy surrounds the enzyme catalyzing the first step of gluconeogenesis, i.e. the decarboxylation of oxaloacetate to phosphoenolpyruvate. According to most authors the phosphoenolpyruvate carboxykinase (PEPCK) activity of yeast grown on gluconeogenic carbon is high, whereas cells grown on glucose have repressed PEPCK activity (Ruiz-Amil et al., 1965; De Torrontegui et al., 1966; Gancedo and Scherzmann, 1976). As mentioned previously, yeast possesses pyruvate carboxylase for anaplerotic regeneration of oxaloacetate. Therefore PEPCK flux is generally thought to be in the phosphoenolpyruvate direction in vivo and to have a purely gluconeogenic role. However, Divjak and Mor (1973) proposed that PEPCK has an anaplerotic role for oxaloacetate replenishment as they could not substantiate glucose repression.

It has been suggested that at a high intracellular ATP : ADP ratio, PEPCK

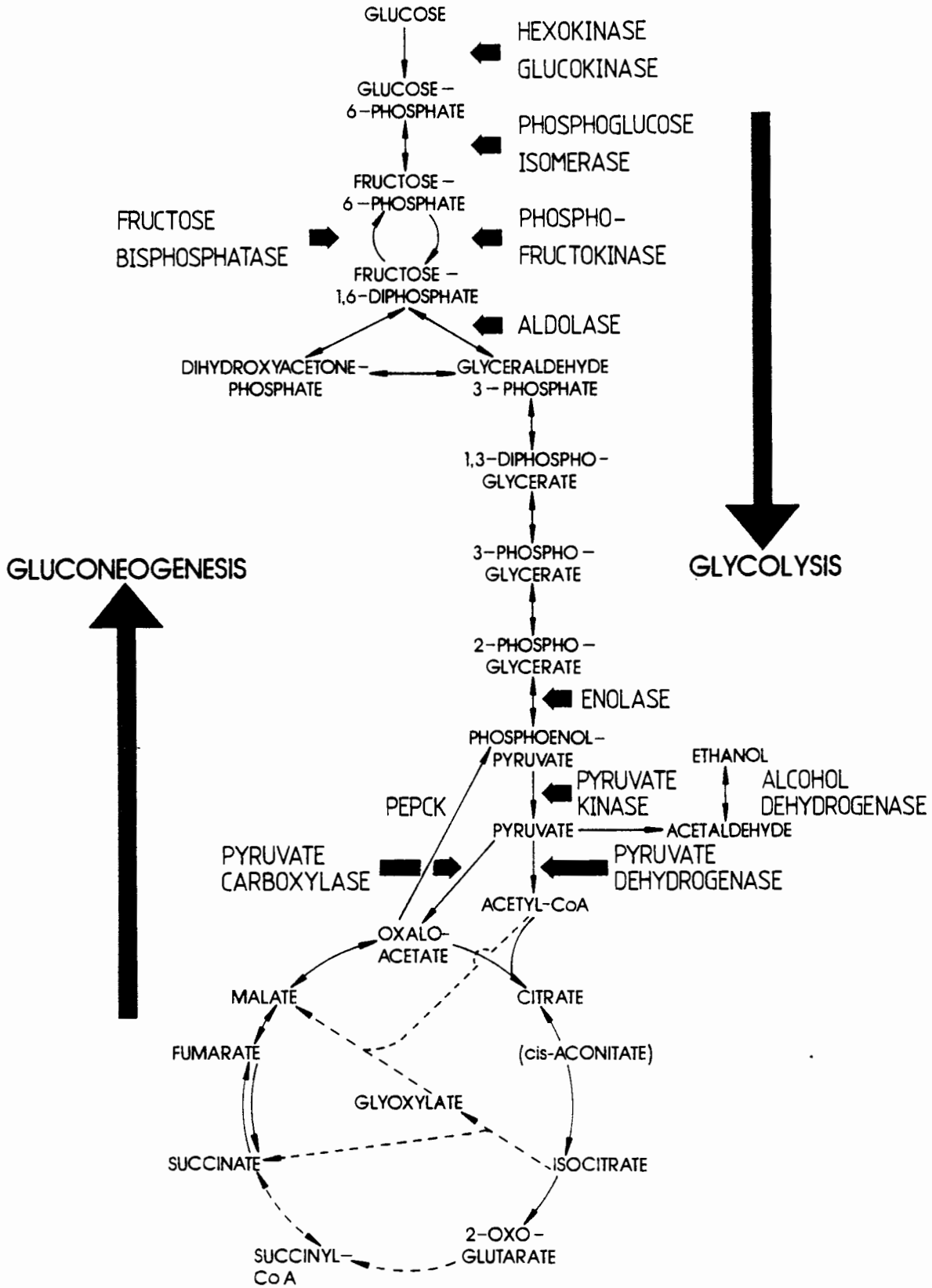


FIGURE 1.4: Major pacemaking enzymes of glycolysis and gluconeogenesis.

would be stimulated, whereas inhibition would set in under conditions of high energy demand, when the ATP : ADP ratio is low (Cannata and de Flombaum, 1974). The K_m of purified PEPCK for oxaloacetate was first reported to be 7,7 mM (Cannata and Stoppani, 1963), but more sensitive analytical measurements later showed a value of 0,6 mM (Gancedo and Scherzmann, 1976). Even this lower K_m is an order of magnitude higher than the average intracellular concentration of oxaloacetate found in yeast, which is less than 50 uM according to Gancedo and Gancedo (1973). This may indicate that oxaloacetate and PEPCK are compartmentalised in yeast. Finally, PEPCK is not only dependent on oxaloacetate as substrate, but also on Mg^{++} GTP. Recent kinetic studies (Schramm et al., 1981) are also investigating the stimulatory interaction of Mn^{++} ions for the enzyme with respect to its two substrates.

From phosphoenolpyruvate until fructose-1,6-bisphosphate, gluconeogenesis in yeast is precisely the reverse of glycolysis (Maitra and Lobo, 1978). Thus, these steps of gluconeogenesis are catalyzed by glycolytic enzymes, which are constitutive and probably located in the cytosol (Lobo et al., 1977). The physiologically irreversible phosphofructokinase reaction of glycolysis is by-passed in yeast by fructose-1,6-bisphosphatase, the activity of which is repressed by glucose (Gancedo et al., 1965; Gancedo et al., 1967), although glucose-6-phosphate is suggested to be the actual repressor of de novo synthesis of fructose-1,6-bisphosphatase (Gancedo et al., 1967). Contrary to this, Foy and Bhattacharjee (1977) have found activity of fructose-1,6-bisphosphatase in a wild type yeast grown in the presence of glycolytic carbon sources.

On balance, however, it would appear from previous research studies,

that glucose, or a catabolic product arising from glucose, controls the degradation of fructose-1,6-bisphosphatase as well as its synthesis. Thus, Gancedo (1971) and Takeda (1981) found that the addition of glucose to yeast cells exhibiting a high level of fructose-1,6-bisphosphatase not only caused catabolite repression of this enzyme but also catabolite inhibition.

Fructose-1,6-bisphosphatase in yeast is inhibited by AMP *in vitro* (Gancedo *et al.*, 1965). This inhibition has also been found in permeabilized cells or in *in situ* conditions, but, because of the high K_i value, Haarasilta (1981) has queried the physiological significance of this inhibition.

Fructose-6-phosphate or glucose-6-phosphate formed from fructose-6-phosphate by phosphoglucose isomerase is the end product of gluconeogenesis in yeast (Haarasilta, 1981). In contrast to some animal cells, yeast does not possess a glucose-6-phosphatase.

The intracellular location of the two specific gluconeogenic enzymes, PEPCK and fructose-1,6-bisphosphatase, is not known. It is generally accepted that glycolysis, and thus the steps of gluconeogenesis that are in common with glycolysis, occurs primarily in the cytosol. Moreover, Haarasilta (1981), following studies on anaplerotic and gluconeogenic enzymes in baker's yeast, proposed that intracellular compartmentalisation provides no special feature for the regulation or functioning of anaplerotic and gluconeogenic pathways.

1.2.5 Regulation of Glycolysis

1.2.5.1 Pathways into Glycolysis for Brewing Yeasts

Brewer's wort contains the sugars, sucrose, glucose, fructose, maltose and maltotriose together with maltotetraose and dextrin material (Harris et al., 1951). Although some degree of overlap may occur, brewing strains of S. cerevisiae and S. uvarum exhibit selective fermentation removing the sugars from the wort in approximately the same sequence of increasing complexity indicated above, except for maltotetraose and other dextrins which are unfermented (Harris et al.,1951; Phillips, 1955).

Sucrose has generally been understood to be hydrolyzed by invertase (β-D-fructofuranosidase, EC 3.2.1.26) outside the cell wall (de la Fuente and Sols, 1962). Santos et al. (1982), however, have recently presented evidence of a non-inducible specific, active transport system for sucrose in S. cerevisiae which is inhibited by maltose. Such transport of the sucrose molecule into the cell could provide a role for cytosolic invertase (Sutton and Lampen, 1962; Meyer and Matile, 1975). Nevertheless, for metabolic purposes, sucrose may be considered as being equivalent to its constituent monosaccharides.

Cellular uptake of glucose and fructose is by way of constitutive facilitated diffusion, and much concern has been directed towards ascertaining whether yeast possesses a phosphoenolpyruvate-phosphotransferase system similar to that in E. coli (Saier, 1977). To date, studies have not revealed such a system (Cooper, 1982). In this regard, there have been studies directed towards establishing whether the cell membrane carrier protein or the

sugar is phosphorylated as it is transported in S. cerevisiae but, thus far, the question remains open (Franzusoff and Cirillo, 1982).

Maltose and maltotriose transport is mediated by inducible active transport systems (Harris and Thompson, 1960; Harris and Thompson, 1961; Harris and Millin, 1963).

Studies conducted during the wort fermentation cycle have demonstrated that yeast cells unable to take up maltotriose may nevertheless utilize maltose. Furthermore, maltotriose negative cells were shown, using cell-free extracts, to be capable of hydrolyzing maltose and maltotriose to glucose. These results are in favour of independent permease systems for the disaccharide and trisaccharide in brewing yeast strains, sharing a common inducible α-glucosidase for hydrolyzing endogenous maltose and maltotriose (Millin, 1963; Stewart et al., 1979). With respect to the energy source of wort disaccharide and trisaccharide transport, there is now evidence to suggest that chemiosmotic phenomena may play a role (Deak, 1978) but, to date, proton symport systems in S. cerevisiae have only been investigated with respect to sucrose transport (Santos et al., 1982).

Thus, entry of the wort sugars into the glycolytic pathway is by way of phosphorylation of glucose and fructose by two hexokinase isoenzymes (hexokinase A or P-I and hexokinase B or P-II) and one glucokinase. Studies have shown that it is unlikely that other enzymes exist for initial glucose and fructose metabolism (Maitra, 1970, 1971; Gancedo et al., 1977; Lobo and Maitra, 1977). The hexokinases are active with both glucose and fructose while the glucokinase will only phosphorylate glucose.

It should be noted that selective monosaccharide fermentation, i.e. preferential utilization of glucose over fructose, has been reported (Gottschalk, 1947; Panchal and Stewart, 1982). It is well known that hexoses and their phosphorylated derivatives exist as anomers (see, for example, Kotyk, 1973), in either the pyranose or furanose form. Investigations by Gottschalk (1947) and Panchal and Stewart (1982) demonstrated that the anomeric form of the hexose would appear to be significant with respect to monosaccharide selectivity. This may be able to be explained at the level of cell membrane carriers. The latter workers also showed that growth conditions were pertinent where preferential uptake of glucose over fructose in sucrose substrate was considerably reduced in non-growth promoting conditions. Equally, competition for the target sites of the hexokinase isoenzymes, or even subsequently as an anomeric phosphorylated derivative for other glycolytic enzymes, may play a role in understanding this phenomenon (Fraenkel, 1982). Finally, it has recently been reported (Muratsubaki and Kasume, 1979) that although the glucokinase of S. cerevisiae is constitutive, the two hexokinase isoenzymes are repressible in gluconeogenesis and that their activity is restored only gradually under conditions which favour glycolysis. Thus, if the yeast strain has been subjected to a situation where the levels of cellular effectors encourage gluconeogenesis, the yeast cell may be severely inhibited towards fructose utilisation when a glycolytic-promoting environment re-occurs.

1.2.5.2 Pacemakers of Glycolysis (Figure 1.4)

1.2.5.2.1 Control at the Level of Phosphofructokinase

Many studies undertaken to elucidate glycolytic control have invariably

cited the importance of the multivalent allosteric properties of phosphofructokinase to mediate glycolytic flux at the level of the production of fructose-1,6-bisphosphate from fructose-6-phosphate (for example, see Betz and Moore, 1967). Thus, in S. uvarum (carlsbergensis), phosphofructokinase has been shown to be the control site of oscillations in the glycolytic chain (Ghosh and Chance, 1964).

The control of the activity of this enzyme has been attributed to a regulation by various effectors and mechanisms and not to varying amounts of this enzyme in the cell.

Mansour and Mansour (1962) showed that phosphofructokinase of the liver fluke can be activated by ATP, fructose-6-phosphate, Mg^{++} and cyclic AMP. Passoneau and Lowry (1962), made similar observations on muscle phosphofructokinase and added fructose-1,6-bisphosphate to the list of activators as well as K^{+} and NH_4^{+} ions. Gevers and Krebs (1966) confirmed the phosphofructokinase-stimulating effect of AMP. It was also shown (Mansour and Mansour, 1962) that ATP in excess to physiological requirements inhibited phosphofructokinase and this observation was further supported by studies on yeast phosphofructokinase by Viñuela et al. (1963). Viñuela et al. (1963) showed that yeast phosphofructokinase is inhibited by ATP competitively with fructose-6-phosphate such that within the normal range of physiological concentrations the activity of the enzyme decreases with an increase of ATP concentration and increases with the square of fructose-6-phosphate concentration. In contrast to its specificity for allosteric inhibition by ATP, Sols et al. (1971) reported that phosphofructokinase has a broad specificity for the nucleoside triphosphates, GTP, CTP, ITP and UTP, which can replace ATP as a phosphate donor.

Investigations by Sols (1968), however, to account for the Pasteur effect in yeast, could not substantiate sufficient changes in ATP levels (or other nucleotides), when passing from anaerobic to aerobic conditions, to account for the phosphofructokinase inhibition observed. Sols' investigations indicated that increased intracellular citrate levels in aerobiosis created inhibition, either independently or co-operatively with ATP, of yeast phosphofructokinase, as had been shown previously for other biological tissues (Parmeggiani and Morgan, 1962; Passonneau and Lowry, 1962; Garland et al., 1963; Williamson, 1965).

The very complicated kinetics associated with phosphofructokinase suggest a variety of binding sites (Goldhammer and Paradies, 1979). Thus, it has been determined that the binding sites for AMP and cyclic AMP are distinct from two ATP sites, which in themselves differ from each other by affinities for either the uncomplexed or complexed (with Mg^{++}) form of ATP (Pettigrew and Frieden, 1979). In the sheep heart or rabbit muscle enzyme there may be a maximum of three binding sites for ATP and it has been shown that citrate increases the affinity of phosphofructokinase for ATP in these tissues (Colombo et al., 1975).

In rabbit muscle phosphofructokinase, citrate acts to depolymerise the active tetrameric enzyme into inactive dimers. Moreover, phosphoenolpyruvate and 3-phosphoglycerate compete with the binding of citrate, the former being a much weaker inhibitor (representative K_i values are 480 uM for phosphoenolpyruvate and 52 uM for 3-phosphoglycerate), yet all three can cause enzyme inhibition with ATP concentrations that are not high enough to cause inhibition alone (Krzanowski and Matschinsky, 1969).

A number of enzymes are controlled by phosphorylation-dephosphorylation

mechanisms, such as, for example, the control enzymes of glycogen metabolism. Reports have indicated that phosphofructokinase may be regulated in this way. Brand and Solling (1974) demonstrated that the rat liver enzyme is activated by phosphorylation by a cyclic AMP-independent protein kinase and inactivated by a phosphatase-catalyzed dephosphorylation. They were able to show that radiolabelled phosphate could be incorporated from ATP upon activation and that this was released from the active enzyme upon inactivation. The matter is confused, however, as high concentrations of fructose-1,6-bisphosphate can activate phosphofructokinase without any covalent modification (Goldhammer and Paradies, 1979).

Another mechanism of phosphofructokinase control may be centred at its interaction with the gluconeogenic enzyme, fructose-1,6-bisphosphatase. The term "futile cycle" or, more appropriately, "substrate cycle" has been used to describe the possible wasteful conversion of ATP to ADP and inorganic phosphate by the simultaneous synthesis of fructose-1,6-bisphosphate from fructose-6-phosphate by phosphofructokinase and its hydrolysis by fructose-1,6-bisphosphatase (Gevers and Krebs, 1966; Hers et al.,1981). The usefulness of such a cycle has been of controversial significance. Newsholme and Gevers (1967) suggested that while the action of substrate cycling is wasteful in terms of energy utilization, the simultaneous operation of all enzymes in such a cycle permits a rapid response to changing physiological conditions and thus facilitates a constant supply of essential biosynthetic precursors. Horecker et al. (1981) proposed that the cycle is more important as a fine-tuning mechanism for glycolysis. Such substrate cycles may serve the purpose of amplifying the responses of pathways to changes in the concentration of an allosteric regulator. Hers et al.(1981) have postulated that the cycle, if opera-

ting, appears to play an important role in the control of gluconeogenesis. In contradistinction to phosphofructokinase being stimulated by AMP, fructose-1,6-bisphosphatase is allosterically inhibited by this effector (Taketa and Pogell, 1965; Gevers and Krebs, 1966). The inhibition is reversible and non-competitive. Thus, changes in the concentration of AMP (or in the ATP/AMP ratio) would be expected to have reciprocal effects on glycolysis and gluconeogenesis and, by consequence, determine both the direction and quantity of flux in the glycolytic pathway. However, it should be noted that the role of AMP *in vivo* remains unclear, since its concentration does not appear to decrease significantly under gluconeogenic conditions (Horecker *et al.*, 1981). Additionally, other workers have reported that allosteric inhibition of fructose-1,6-bisphosphatase by AMP is highly dependent on both the concentration and nature of monovalent cations (Hubert *et al.*, 1970; Black *et al.*, 1972; Hochachka, 1972; Villanueva and Marcus, 1974).

Attention has been given to the AMP-synergistic role of fructose-1,6-bisphosphate as a positive effector of phosphofructokinase and an inhibitor of fructose-1,6-bisphosphatase (Horecker *et al.*, 1981). Substrate inhibition of fructose-1,6-bisphosphatase has been found in rat liver by Taketa and Pogell (1965) but was found to be incomplete at up to mM concentrations of fructose-1,6-bisphosphate.

Recently, Stone and Fromm (1980) have suggested one more mechanism by which AMP can regulate the activity of fructose-1,6-bisphosphatase. They have reported the formation of a complex between AMP and the substrate of the enzyme. The AMP-fructose-1,6-bisphosphate complex is inactive with fructose-1,6-bisphosphatase. Thus, it is possible that AMP can inhibit the activity of this enzyme by complexing with

fructose-1,6-bisphosphate and reducing the amount of substrate available to fructose-1,6-bisphosphatase.

A further possibility for substrate cycling control could be citrate (Eagle and Scopes, 1981) which besides inhibiting phosphofructokinase has been found to be an activator of fructose-1,6-bisphosphatase Datta et al., 1974). Theoretical models to support the above proposals have, however, only considered observations in specific biological tissues which, thus far, have excluded yeast.

The situation has recently been further complicated by the discovery by Van Schaftingen and Hers (1981) of a second phosphofructokinase (6-phosphofructo 2-kinase) which forms fructose-2,6-bisphosphate from fructose-6-phosphate. The activity of phosphofructokinase 2 is increased by AMP and Pi and decreased by phosphoenolpyruvate and citrate in the physiological concentration range. A cyclic AMP-dependent protein kinase may be involved in the inactivation of phosphofructokinase 2 (Van Schaftingen et al., 1981). El-Maghrabi et al. (1982) have suggested that the formation and degradation of fructose-2,6-bisphosphate is as a result of the same enzyme, which they termed 6-phosphofructo 2-kinase/ fructose-2,6- bisphosphatase.

Fructose-2,6-bisposphate is not a direct intermediary metabolite of glycolysis but has been shown to be a potent positive effector of phosphofructokinase I and an inhibitor of fructose-1,6-bisphosphatase (for review, see Hers and Van Schaftingen, 1982).

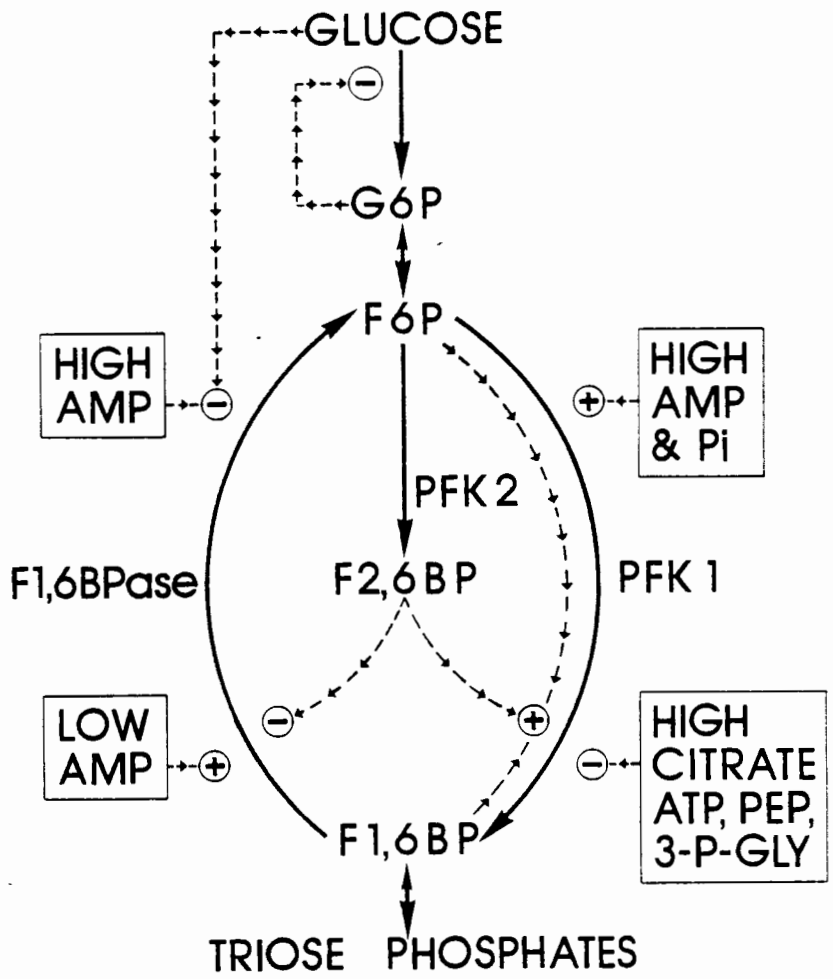


FIGURE 1.5: Interpretation of major effector controls of glycolytic/gluconeogenic flux between fructose-6-phosphate and fructose-1,6-bisphosphate. Feedback control on glucose utilization is also shown.

Fructose-2,6-bisphosphate has been found to be formed in many biological tissues, including S. cerevisiae (Lederer et al., 1981). At concentrations lower than a micromolar, it is capable of relieving inhibition of phosphofructokinase I by ATP and has been found to increase the binding affinity of the enzyme for fructose-6-phosphate and to AMP (Bartrons et al., 1982; Kessler et al., 1982).

The main characteristics of its inhibition over fructose-1,6-bisphosphatase are that: (i) it is much stronger at low rather than at high substrate concentrations for this enzyme; (ii) it is markedly synergistic with the action of AMP; and (iii) it changes the substrate saturation curve from hyperbolic to sigmoidal. Nevertheless, there is good experimental evidence to suggest that this inhibition is incomplete (Pilkis et al., 1981; Hers et al., 1982).

Taking into account the major effector control of phosphofructokinase I and fructose-1,6-bisphosphatase, Figure 1.5 shows a possible interpretation exerted by cellular intermediates at this important glycolytic/gluconeogenic junction.

The figure shows feedback control on glucose utilization by glucose-6-phosphate which would occur if either the activity of phosphofructokinase I was decreased or that of fructose-1,6-bisphosphatase increased. However, in contrast to that of animal tissues, yeast hexokinase is not sensitive to feedback inhibition by glucose-6-phosphate (Sols, 1967) and it has therefore been postulated that control is exerted at the hexose transport stage by glucose-6-phosphate, directly or in concert with a second metabolite.

1.2.5.2.2 Control at the Level of (Fructose Bisphosphate) Aldolase

Horecker et al. (1981) have further considered the dual role of fructose-1,6-bisphosphate as an activator of phosphofructokinase I and inhibitor of fructose-1,6-bisphosphatase.

The intracellular concentration of fructose-1,6-bisphosphate is not only

affected by the activities of phosphofructokinase I and fructose-1,6-bisphosphatase but also by the relative activity of aldolase. Horecker et al. (1981) found that under starvation (gluconeogenic) conditions in rabbit liver, aldolase activity decreased to less than 50% of that found in fed rabbits but fructose-1,6-bisphosphatase activity (and phosphoenolpyruvate carboxykinase) increased.

An increase in the concentration of fructose-1,6-bisphosphate would produce a net flux in the glycolytic direction while a decrease would favour a flux shift towards gluconeogenesis. Therefore, the conclusion that Horecker et al. (1981) drew from their experiments was that under conditions that promoted gluconeogenesis (such as starvation), a high fructose-1,6-bisphosphatase/aldolase activity ratio would cause the steady-state concentration of fructose-1,6-bisphosphate to fall, enhancing flux in the direction of gluconeogenesis.

1.2.5.2.3 Control at the Level of Enolase

Frohlich and Entian (1982) found a possible regulatory mechanism concerning enolase, the enzyme catalyzing the glycolytic/gluconeogenic step between 2-phosphoglycerate and phosphoenolpyruvate. These workers detected three enolase isoenzymes, enolase I, II and III, when studying S. cerevisiae in gluconeogenesis and only two isoenzymes under conditions of glucose repression. Under the latter conditions electrophoretic mobility patterns in polyacrylamide gels showed that enolase I was converted to enolase II.

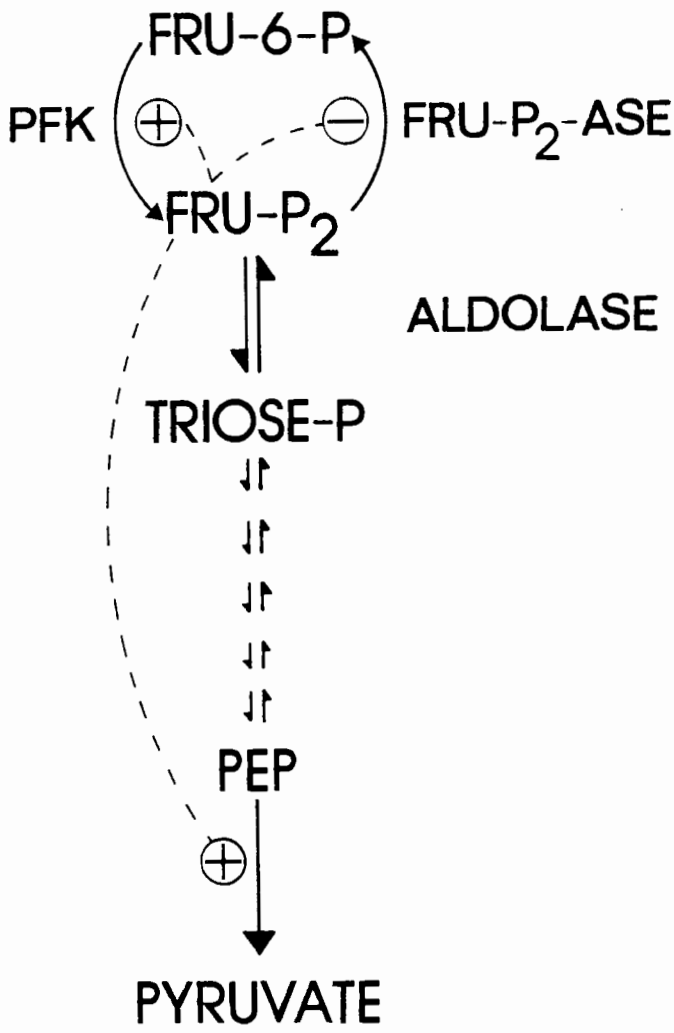


FIGURE 1.6: Role of fructose-1,6-bisphosphate in the regulation of glycolysis and gluconeogenesis.

1.2.5.2.4 Control at the Level of Pyruvate Kinase and Pyruvate Dehydrogenase

In addition to the regulatory properties of fructose-1,6-bisphosphate previously mentioned, it is also a positive effector of yeast pyruvate kinase which would therefore enhance its role in the regulation of glycolysis (Hess et al., 1966; Tanaka et al., 1967; Taylor and Bailey, 1967). Indeed, among glucose-negative mutants the majority of lesions have been found at the level of pyruvate kinase (Lam and Marmur, 1977; Sprague, 1977).

Pyruvate kinase is not needed for gluconeogenesis. Thus, studies in biological tissues other than yeast have been directed at understanding the mode of its inactivation. It is probable that this allosteric enzyme is phosphorylated under gluconeogenic conditions which is mediated by a cyclic AMP-dependent protein kinase (Clark et al., 1981). However, for yeast it is uncertain whether the cyclic AMP-dependent protein kinase acts at this level or whether the concentration of fructose-1,6-bisphosphate is more important.

The pyruvate dehydrogenase reaction is obligatory for the entry of carbohydrates into the tricarboxylic acid cycle. This allosteric enzyme is regulated by the level of ATP and Ca^{++} ions (Linn et al., 1972). ATP causes inhibition as a result of an ATP-dependent phosphorylation by pyruvate dehydrogenase kinase. Phosphorylated pyruvate dehydrogenase may be reactivated in the presence of Mg^{++} and Ca^{++} ions by pyruvate dehydrogenase phosphatase (Denton et al., 1972). In animal tissues the kinase and phosphatase are regulatory subunits of the pyruvate dehydrogenase complex (Linn et al., 1972). In yeast cells, a similar regulatory control remains to be confirmed.

1.2.5.2.5 Control at the Level of Alcohol Dehydrogenase

Chapman and Bartley (1968) observed that the activity of alcohol dehydrogenase increases with the transfer to anaerobic conditions. In contrast, other reports (Hommes, 1966; Witt et al., 1966; Maitra and Lobo, 1971) indicated that yeast alcohol dehydrogenase is repressed by glucose. It is probable that these differing views can be explained by the existence of three alcohol dehydrogenase isoenzymes, two of which are cytoplasmic - one of which is constitutive (ADH I), the other repressible (ADH II); and one which is mitochondrial and repressible (ADM) (Schimpfessel, 1968; Fowler et al., 1972). Although product inhibition studies in both ethanol and acetaldehyde directions have revealed ratelimiting data concerning the pathway (Dickenson and Dickinson, 1978), mutational studies have indicated that ADH II, if derepressed, can facilitate the glycolytic function (Ciriacy, 1979).

1.3 ANCILLARY PATHWAYS

1.3.1 The Glycogenic Route and Carbohydrate Reserves

There has been considerable speculation in recent years concerning the precise role of the yeast storage carbohydrates, glycogen and trehalose. Functions ascribed to these reserve materials, particularly glycogen, have been as a utilizable source of maintenance energy on depletion of fermentable sugars (Chester and Byrne, 1968; Sols et al., 1971), involvement in cell cycle control and sporulation (Lillie and Pringle, 1980), as the source of metabolic energy for the synthesis of essential lipids and respiratory enzymes at the initial stage of fermentation (Chester, 1963;

Quain et al., 1981), and also a role in yeast flocculation (Patel and Ingledew, 1975). Panek (1962) suggested that trehalose may function as an energy reserve for those metabolic steps which precede cell division. Panek and Mattoon (1977) further postulated that trehalose may be the chief source of energy for mitochondrial biogenesis, but as some yeast strains accumulate trehalose after the beginning of ethanol oxidation, such a role could be argued (Costa-Carvalho et al., 1978).

Glycogen has been shown to commence building-up during the yeast growth phase in fermentation, accumulating rapidly with trehalose towards the end of the growth phase and beginning of the stationary phase. Trehalose remains very low in actively dividing yeast (Panek and Mattoon, 1977). Glycogen and trehalose may reach levels of c. 40% and 10% respectively of the dry weight of the brewing yeast cell (Chester, 1963; Sols et al., 1971).

As fermentable carbohydrate is being channelled to these storage materials and thus offering nothing with respect to beer, there is considerable interest in understanding glycogen and trehalose needs of individual brewing strains for specific metabolic purposes and in understanding the mechanisms involved in controlling reserve material. In addition, this interest may, by consequence, extend to the utilization of carbohydrate for cell wall polymers.

1.3.1.1 Synthesis of Glycogen and Trehalose

The generally accepted pathway for the biosynthesis of glycogen and trehalose is shown in Figure 1.7. Trehalose synthesis requires uridine

5'-diphosphate glucose (UDPG) and glucose-6-phosphate whereas glycogen synthesis requires UDPG and an α (1 — 4) polyglucose primer having at least four glucose residues (Leloir and Cardini, 1957). There have been indications, however, to suggest that glucose-6-phosphate may not be an obligatory intermediate and that there may be an alternative pathway leading to UDPG, not involving glucose-6-phosphate (Ryman and Whelan, 1971). Nevertheless, the pivotal role of UDPG and its formation and consumption, is evident.

Chester and Byrne (1968), as a result of investigations using glycogen-deficient mutants suggested that these mutants were unable to replenish the UDPG necessary for glycogen synthesis as easily as the parent yeast. Their observations led these workers to suggest that the level at which UDPG limits glycogen synthesis must vary with different growth conditions. Madsen (1963) felt that the intracellular concentration of UDPG was the most important factor in the control of glycogen metabolism and that a high concentration of UDPG would be expected to increase the rate of glycogen synthesis since it is the substrate for the irreversible reaction catalyzed by glycogen synthetase (UDP-glucose : glycogen α -4-glucosyl transferase) (EC 2.4.1.11) while concomitantly limiting the rate of glycogen degradation by glycogen phosphorylase (α -1,4-glucan : orthophosphate glucosyl transferase) (EC 2.4.1.1). However, Madsen's conclusions, based on studies of Agrobacterium tumefaciens assumed that data gained on specific cellular intermediates measured in independent experiments, was relatable.

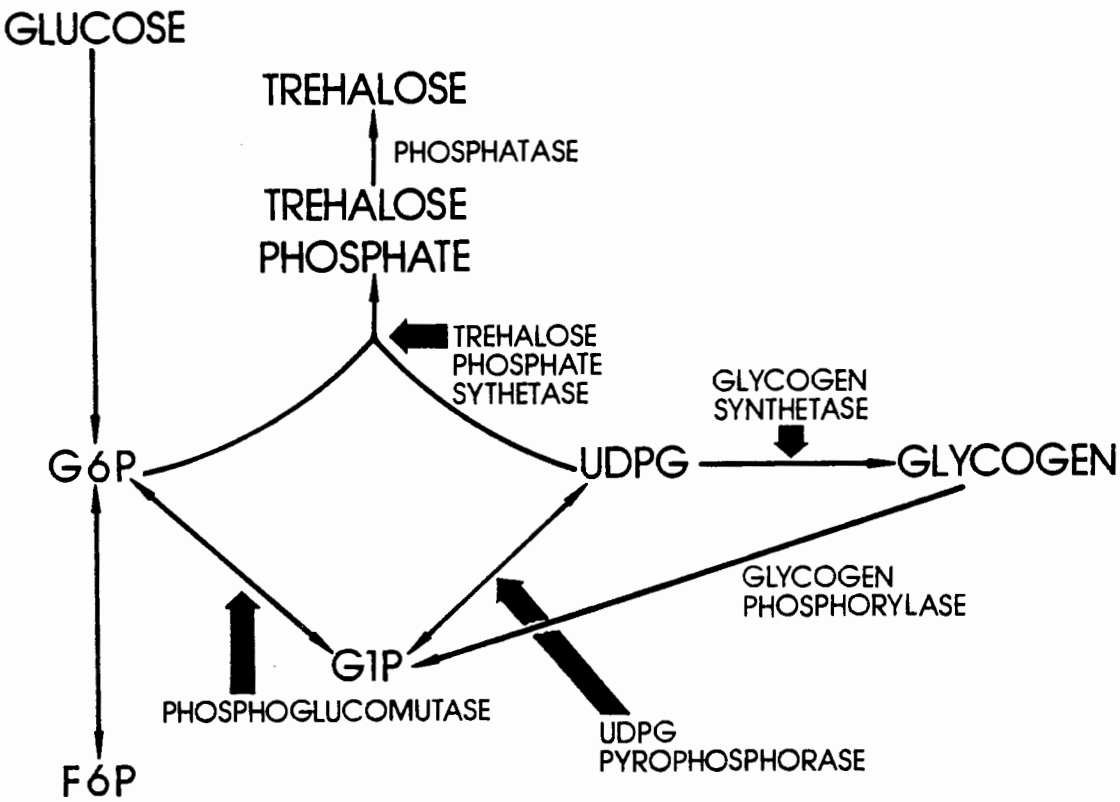


FIGURE 1.7: Pathways to glycogen and trehalose in yeast.

The allosteric properties of yeast glycogen synthetase have been examined in some detail by Rothman-Denes and Cabib (1966). Glucose-6-phosphate was shown to be a positive effector while certain anions such as Cl^- and PO_4^- as well as ATP, ADP and GTP were shown to cause marked inhibition which could be overcome by high levels of glucose-6-phosphate.

It is interesting to note that two interconvertible forms of yeast glycogen synthetase exist, designated D and I (Rothman-Denes and Cabib, 1970). The D form, which is dependent on relatively high concentrations of glucose-6-phosphate for its activity is found in cells during the growth phase whereas the I form predominates in cells isolated from stationary-phase cultures.

The key enzyme in the biosynthesis of trehalose is trehalose phosphate synthetase (UDPG-glucose-6-phosphate transglycosylase) (Cabib and Leloir, 1958) which catalyzes the following irreversible reaction:

UDPG + glucose-6-phosphate

$$\xrightarrow[\text{trehalose phosphate synthetase}]{}$$

UDP + trehalose phosphate

In terms of the metabolic control of this enzyme, and also the phosphatase which catalyzes the reaction from trehalose phosphate to trehalose, there is relatively little that has been published. Sols (1971) indicated that trehalose phosphate synthetase may be inducible via its substrates and there have been indications that, like glycogen synthetase and glycogen phosphorylase, trehalose phosphate synthetase may exist in interconvertible forms (Killick and Wright, 1972).

The energetic state of the yeast cell to produce glycogen and trehalose is of profound importance, as UTP together with glucose-1-phosphate are the substrates for the UDPG-pyrophosphorylase catalyzed reaction to UDPG (Trucco, 1951). To account for a low glycogen content in respiratory deficient mutants, Chester (1968) suggested that this might be due to the yeast cells being unable to produce sufficient ATP for conversion of UDP to UTP.

1.3.1.2 Degradation of Glycogen and Trehalose

As compared with studies on animal tissues, the degradation of glycogen in yeast by glycogen phosphorylase has received little attention. Consequently, unanswered aspects of glycogen degradation include the inability of the yeast enzyme to hydrolyze the (1 - 6) linkages, which may occur as a result of isoamylase activity (Gunja et al., 1961).

Glycogen phosphorylase in yeast occurs, as in animal tissues, in two interconvertible pyridoxal phosphate dependent forms, phosphorylase a and phosphorylase b (Fossett et al., 1971; Lerch and Fischer, 1975).

It is usually accepted that only the a form is able to catalyze the phosphorolysis of glycogen and consequently most of our knowledge of glycogenolysis results from studies of this active enzyme form. We now know, for example, that phosphorylase a has been found to be a potent inhibitor of glycogen synthetase in animal tissues (Van de Werwe, 1981).

However, recent studies on both a and b forms have recently shed some light on differences between effector control. Again studying animal

tissues, Stalmans and Gevers (1981) showed that, at concentrations within the physiological range, glucose, glucose-6-phosphate, ATP and ADP were potentially important inhibitors of phosphorylase b. UDPG within the range of 1-3 mM did inhibit phosphorylase a, but not at all the b form. In contrast, physiological concentrations of AMP together with inhibitors caused a modest inhibition of phosphorylase a but completely suppressed phosphorylase b. In comparing the activating effect of AMP with other nucleoside monophosphates, IMP was found to be a weak activator of phosphorylase b.

With respect to bacterial phosphorylase, Madsen (1961) reported a very effective inhibition (73%) by UDPG at a molar concentration of 8×10^{-3} but this study did not take into account changes to other cellular intermediates.

Unfortunately, there is little information available concerning regulation of the yeast phosphorylases and, to my knowledge, no information available for *in vivo* intermediate changes between glycogenesis and glycogenolysis.

Trehalose degradation in baker's and brewer's yeast is catalyzed by a specific trehalase to provide two molecules of glucose. According to Sols *et al.* (1971) trehalase is inhibited by AMP, and Panek and Mattoon (1977) concluded from observations in *S. cerevisiae* that the level of glucose repression determines the concentration and/or state of activation of the trehalose synthetase/trehalase complex. However, Sols *et al.* (1971) have reported that, although the trehalose level varies during the yeast growth cycle, there are no detectable variations in trehalase activity. Their investigations suggested that cellular com-

partmentalisation may be important in trehalose accumulation and degradation.

1.3.2 Pentose Phosphate Pathway

The pentose phosphate (or hexose monophosphate) pathway was recognised in yeast in 1958 by Nord and Weiss.

The purpose of the pathway, besides the production of pentose-5-phosphate, for biosynthesis of nucleotides and histidine, and erythrose-4-phosphate for biosynthesis of the aromatic amino acids, is to generate reducing power in the cell in the form of NADPH as well as a mechanism for the conversion of pentoses to hexoses (Utter, 1958).

The defined reaction mechanism of the oxidative and non-oxidative pentose 5-phosphate/hexose 6-phosphate inter-conversions have classically been shown to be as schematically outlined in Figure 1.8, now known as the F-type pentose pathway (Horecker and Mehler, 1955).

It has recently been reported (Williams et al., 1978; Williams, 1980), however, that the pathway may be more complex than has been previously assumed. Indeed, carbon-balance studies have indicated that a significant amount of ribose 5-phosphate carbon could not be accounted for in the intermediates assigned to the reaction scheme of Figure 1.8.

The theoretical stocheiometry of non-oxidative pentose metabolism has shown (Wood et al., 1963) that 3 molecules of pentose 5-phosphate will form 2 molecules of hexose monophosphate and 1 molecule of triose phos-

phate. In order to account for this stocheiometry, recent examination of the metabolic evidence for pentose phosphate reactions has revealed, in certain biological tissues, the possible existence of an alternative reaction scheme, shown in Figure 1.9 which is known as the L-type pentose pathway. In this scheme, an interconversion of arabinose 5-phosphate and ribose 5-phosphate has been postulated (Williams, 1978). In addition, the L-type pathway is characterised by the actions of aldolase, pentose 5-phosphate 2'-epimerase and phosphotransferase, and three additional intermediates to arabinose 5-phosphate, namely sedoheptulose 1,7-bisphosphate, glycero-ido-octulose 1,8-bisphosphate and glycero-ido-octulose 8-phosphate. In the figure, it can be seen that arabinose 5-phosphate is utilized to produce hexose 6-phosphate and dihydroxyacetone phosphate (DHAP). Transaldolase has no role in the metabolic flux of the L-type pathway (Williams, 1978).

Precisely whether the F-type and L-type pentose pathways both exist and compliment each other in certain tissues, is unresolved. In yeast there is little information available on pentose metabolism. Studies using baker's yeast have indicated (Wood and Gascon, 1980) that arabinose 5-phosphate may inhibit transketolase and there is doubt as to whether the L-type pathway exists in yeast.

The availability of C^{14}- labelled glucoses enabled procedures to be devised that made it possible to demonstrate that intact yeast cells had the potential to follow the tricarboxylic acid cycle and pentose phosphate pathway simultaneously. Although estimates of flux are sufficient to match the metabolic needs of S. cerevisiae (Lagunas and Gancedo, 1973), there is great disparity as to the maximum levels of flux which are possible (Wang et al., 1956, 1958; Chen, 1959). On balance,

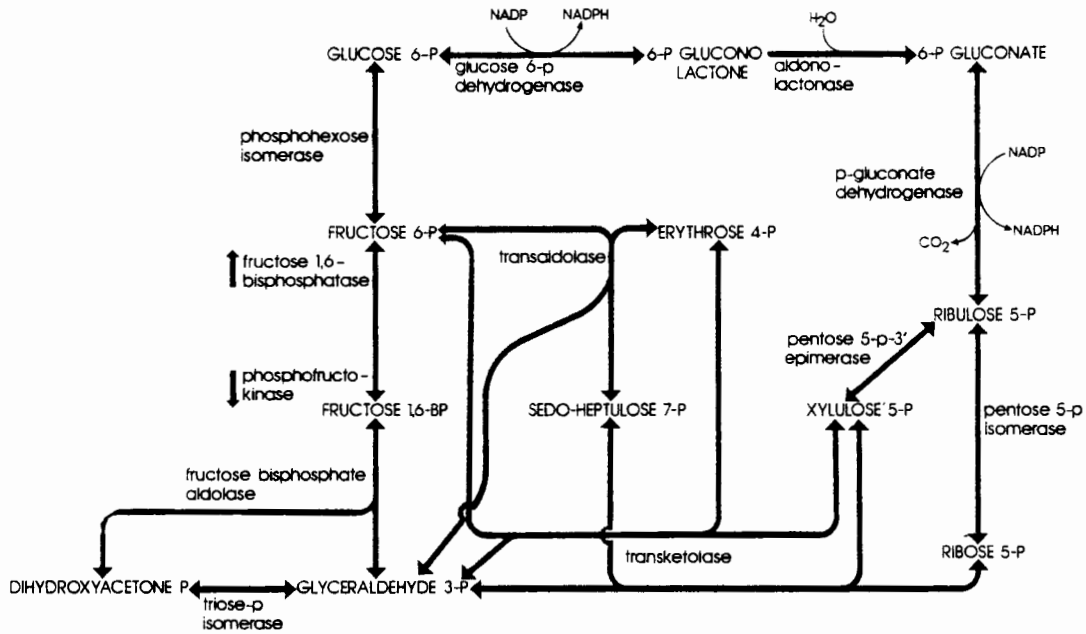


FIGURE 1.8: Reaction sequence of F type pentose pathway.

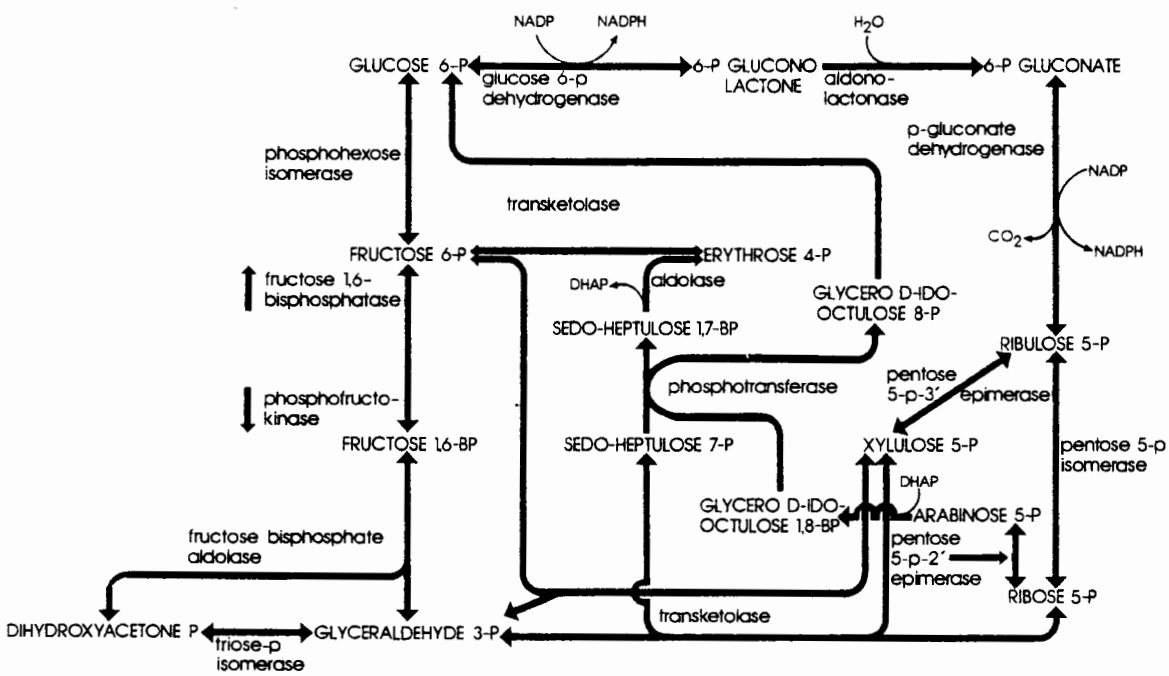


FIGURE 1.9: Reaction sequence of L type pentose pathway.

though, and under aerobic conditions, it would appear that the pentose pathway may account for up to 10% of the sugar metabolised. According to Chapman and Bartley (1968), the activity of glucose-6-phosphate dehydrogenase, the first enzyme on the pentose phosphate pathway, decreases under conditions that cause loss of mitochondria, i.e. on change towards an anaerobic metabolism.

Witt et al. (1966) established that with yeast grown aerobically on glucose in the presence of acetic acid and pyruvic acid, either alone or in various combinations, the activity of glucose-6-phosphate dehydrogenase did not vary. It thus seemed unlikely that it was repressed or induced by glucose.

Indeed, it has been suggested (Osmond and Rees, 1969) that the capacity of the pentose phosphate pathway in yeast is controlled by variations in the amounts of glucose-6-phosphate dehydrogenase. In S. uvarum (carlsbergensis), glucose-6-phosphate dehydrogenase has been shown to be strongly inhibited by NADPH (Kuby et al., 1974), but it would appear that no studies have shown that it regulates this enzyme. Other reported metabolic inhibitors of glucose-6-phosphate dehydrogenase are ATP, acetyl CoA, erythrose-4-phosphate and glyceraldehyde-3-phosphate but the significance of these effectors in vivo may be questionable (Levy, 1979).

1.4 AIM OF THIS STUDY

In S. cerevisiae and S. uvarum (carlsbergensis), the glycolytic pathway may be taken as, essentially, the only route for anaerobic degradation

of carbohydrates to ethanol, and the biosynthesis and energy-producing components of this catabolic process and the tricarboxylic acid cycle are of fundamental interest and of technical importance in brewing and other fields of industrial microbiology.

Regulation of glycolysis has been subject to recurring periods of intensive investigations since the pathway was first discovered. The majority of biochemical studies has centred on the transition from the anaerobic to the aerobic state leading to a suppression of glycolytic flux, and/or examinations of the so-called glucose effect (Crabtree effect) (Petrik et al., 1983). However, a complete picture regarding the interplay of the various cellular effectors to allosteric enzyme control is still far from evident. Genetic information is also limited. Most biochemical investigations and all the regulatory mutants obtained so far have only been used to study certain segments of the whole complex of carbon catabolite repression leading to a rather diffuse pattern of regulation (Montenecourt et al., 1973; Schamhaart et al., 1975; Rytka et al., 1976; Ciriacy and Breitenbach, 1979). Far less fundamental information is available concerning the metabolic inter-relationship between the rate of glycolytic flux and the dependency on yeast growth (Masschelein et al., 1965; Sols et al., 1971; Heyse and Piendl, 1973; Oura, 1973). In addition, documentation of the pattern of synthesis and dissimilation of yeast carbohydrates as a function of growth in fermentation is scarce (Quain et al., 1981).

The present work investigated the relationship between yeast growth, regulation of glycolytic/gluconeogenic flux and accumulation of glycosyl donors for polysaccharide synthesis in brewing yeast (S. uvarum) fermentations.

It was considered that the nature of the glycolytic/gluconeogenic controlling allosteric enzymes, complicated by the variety of intracellular modulators, prevented reproducing in vitro, in vivo enzymatic situations. Moreover, it was further considered that any interpretation of regulatory phenomena demanded that the phases of cellular intermediates and energy surplus and deficiency should be well established. Thus, as part of the overall strategy, it was necessary to develop sufficiently specific and sensitive (picomole range) analytical techniques for the measurement of glycolytic intermediates and cellular effectors to gain an accurate overall picture of intracellular metabolic control.

The following questions were posed:-

1. To what extent may variations in growth affect glycolytic flux and how does this relate to cyclic variations in the physiological state of the brewing yeast cell?

2. What is the importance of anaerobic fermentative growth in relation to cellular effector levels and glycolytic/gluconeogenic regulatory links?

3. What is the relationship of growth and possible substrate cycling at the level of phosphofructokinase I/fructose-1,6-bisphosphatase activity?

4. May gluconeogenesis occur during brewing yeast fermentations? If so, is the intermediate end-point glucose-6-phosphate, as has previously been reported (Haarasilta, 1981)?

5. What is the importance of anaerobic fermentative growth towards cellular effector levels and accumulation of glycosyl donors for reserve material and cell wall polymers?

CHAPTER 2

DEVELOPMENT OF PRINCIPAL EXPERIMENTAL TECHNIQUES

SUMMARY: This metabolic study required that intracellular nucleotides, organic acids, and hexose and triose phosphates be recovered in solution. Measurement techniques using the techniques of isotachophoresis and bioluminescence were developed to achieve the desired specificity and sensitivity (picomole range). Cell permeabilization and metabolite extraction were achieved using a procedure mediated by boiling water. In spite of its simplicity, this procedure appeared more efficient than alternative methods on the basis of recovering higher amounts of nucleotides. Isotachophoresis was used for the quantitative measurement of organic acids, and adenosine, guanosine, uridine, and cytidine, mono-, di-, and triphosphates. To separate nucleotides, discrete intermediate-mobility "spacers" were added to the sample solution, and steady-state mixing techniques were applied. Quantitative measurement of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and triose phosphate was achieved by coupled enzymatic assays and a photobacterial bioluminescence system.

2.1 INTRODUCTION

Although numerous investigations have developed and promoted methods for extraction of cellular nucleotides, phosphate esters and organic acids, no single procedure has emerged as the most efficient. Low nucleotide triphosphate values in previous studies (Chapman et al., 1971) probably reflect insufficient detail being paid to speed of sampling and to the

avoidance of nucleotide triphosphate degradation during sampling or in the extraction procedure. If nucleotide concentrations are to remain unchanged during sampling and processing, enzyme activity must be abolished before the environment of the cells in the substrate changes. Thus, realistic sampling requires conditions which would not affect cellular metabolism. Subsequently, the permeabilizing and extracting agent and procedure must be geared so as not to substantially change the cellular energy charge.

It was therefore considered necessary for this study on brewing yeast metabolism to carefully re-evaluate permeabilization and extraction methods in yeast so that cellular extracts used for the analysis of nucleotides, would best reflect a true physiological value.

There are a variety of methods available for the quantitative determination of cellular nucleotides, phosphate esters and organic acids, such as various chromatographic, radioisotopic and enzymatic techniques. For nucleotides and organic acids, high performance liquid chromatography (HPLC) has met with general approval. However, in order to achieve resolution of these components in biological samples by HPLC, it is often necessary to concentrate cellular extracts by, for example, lyophilization, and/or effect secondary or tertiary treatments to remove interfering contaminants - creating a situation where losses may be incurred (Khym, 1976; Lothrop and Uziel, 1980).

The use of isotachophoresis as an electrophoretic separation technique has gained increasing acceptance during the last few years in various application areas (Hjalmarsson and Baldesten, 1981). This appears to be due to an appreciation of its specific attributes including sensitivity

of measurement in the lower picomole range, small sample requirements, short analysis times and ease of quantification. These potential attributes of isotachophoresis prompted an investigation to establish whether it would be possible to use this technique for direct measurement (without concentration) of nucleotides and organic acids in cellular extracts from yeast.

As a complementary technique to isotachophoresis, firefly bioluminescence offers comparable sensitivity for ATP (Lundin and Thore, 1975), and was used in this study as such. Due to the very small quantities of hexose and triose phosphates in cellular extracts, accurate measurement by spectrophotometry presents difficulties without extract concentration (Entian et al., 1977). Coupled to the need to measure these quantities rapidly, it was decided to modify classical enzymatic methods for measurement of these sugar phosphates by bioluminescence.

2.1.1 Cell Permeabilization and Intracellular Metabolite Extraction

In studying the positive and negative effectors of glycolytic control mechanisms, the most important requisites include instantaneous cell death and rapid lysis, complete nucleotide (and other relevant effector) release with negligible destruction, and complete and irreversible inactivation of enzyme activity. In addition, recovery of the applicable metabolites should be achieved by a single extraction as judged by maximal yields and the inability to recover further levels by a second extraction. Moreover, long term stability of the extracted metabolites (and nucleotides in particular) is desirable as is the inability of associated extraction agents or extracted materials to interfere with the analysis of the

required components.

Some reported agents and procedures for extraction of cellular nucleotides are listed in Appendix F. Although the list is reasonably comprehensive, there is apparently no extraction method that can be regarded as universally acceptable for metabolic studies.

2.1.2 Isotachophoresis

Capillary isotachophoresis has, in recent years, proved to be an excellent tool for quantitative determination of nucleotides (Gower and Woledge, 1977; Eriksson, 1980; Wielders and Muller, 1980; Ryder et al., 1983c, 1984) and organic acids (Baldesten et al., 1978; Kaiser and Hupf, 1979; Ito et al., 1980; Ryder et al., 1983c, 1984).

As isotachophoresis, as a working method, is the latest of the electrophoretic separation techniques (for review, see Hjalmarsson and Baldesten, 1981) and not in common use in most laboratories, the basic principles will be briefly explained in order that the refinements of the technique, as applicable to development work needed for this study, may be appreciated.

Isotachophoresis refers to an electrophoretic technique whereby ion species of the same sign migrate in an electric field into discrete zones in a discontinuous electrolyte system. Thus, a mixture of sample ions is injected at the interface of two electrolytes (Figure 2.1), usually referred to as leading and terminating electrolytes, with an electrophoretic mobility difference appropriate to the ions to be separated, t_o.

Under the influence of a constant-intensity electric field, the different ions arrange themselves into separate consecutive zones in order of their net mobilities, t_1. At equilibrium, these ions travel at a constant velocity and concentration,

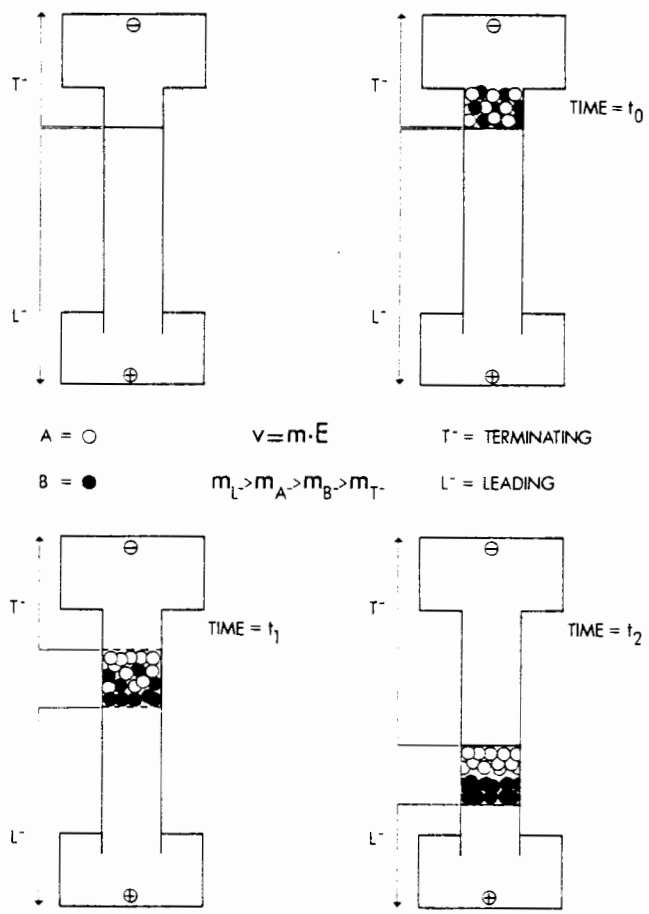


FIGURE 2.1: Electrophoresis in a Discontinuous Electrolyte System - Isotachophoresis.

determined by the intensity of the electric field, so that zone length is directly proportional to the concentration of corresponding ions in the zone, t_2. Because isotachophoretic separations are conducted at a constant current but with a continuous increase in voltage, the temperature of each zone (as well as pH, as for isoelectrofocusing) will rise as a function of the field strength in spite of the general thermostabilization at a constant temperature, usually 15°C. Therefore, in addition to UV-absorbance for detection of zones, thermal detection may play a useful role.

A further advantage of isotachophoresis is that because of the different field strengths between zones any diffusion of different sample ions to other zones is effectively counteracted and as a consequence, results in sharp zone boundaries, hence high resolution. The above factors together contribute to an extremely high sensitivity and reproducibility between samples.

2.1.2.1 Influence of Electrolyte System

According to Kohlrausch (1897) the electrolyte conditions in the leading zone determine all the parameters in successive zones. It follows that the pH in the leading zone will determine the degree of dissociation at the beginning of separation and, therefore, the effective mobility of the following zones (Ornstein, 1964). In principle, the separability of samples by isotachophoresis is determined by the effective mobilities of the sample components, which are closely related to their absolute mobilities and pK_a values and to the pH of the leading electrolyte, pH_L, buffered by an appropriate counter-ion. The pK of the counter-ion

should be such that it ensures optimal buffering capacity in the pH interval within which separation takes place. Mikkers et al. (1979) favoured the use of a low mobility counter-ion which would provide an efficient use of applied power for separation of sample ions.

Becker and Everaets (1972) in studying the separability of nucleotides by isotachophoresis measured thermal step-heights for pHs of leading electrolytes ranging from 3,4 to 7.0. This study showed which pH should be chosen for the leading electrolyte to separate sets of nucleotides, and that, for the analysis of composite mixtures, optimal separation would be achieved around pH 3,9.

Recently, papers have demonstrated successful separation of nucleotides from muscle extracts at pH 3,92 (Gower and Woledge, 1977) and liver extracts at pH 3,89 (Eriksson, 1980) where the composition of the leading electrolyte was 5 mM HCl in 0,5% hydroxypropylmethylcellulose (HPMC) and where the final pH was achieved using β -alanine as counter-ion. The terminating electrolyte in both cases was 5 mM n-caproic acid.

2.1.2.2 Influence of Electroosmosis

Electroosmosis is one of the main electrokinetic disturbances in isotachophoresis (Reijenga et al., 1983). When an electric field is applied to an electrolyte system, an electroosmotic flow, defined as the movement of the liquid with respect to the tubular wall, results. The effect of electroosmosis will be negative with respect to sharpness of the zone boundaries and, therefore, the detection limit of the component. Moreover, the situation in isotachophoresis is complicated as electro-

osmotic flow will differ from zone to zone. In addition, in capillary systems, surface conductance is also important in achieving optimal zone differentiation. Reijenga et al. (1983) have further discussed axial and radial temperature differences particularly with respect to varying capillary diameters.

To overcome disturbances in flow profile in Teflon capillaries and to promote laminar flow, different additives have been used. Of these, Triton X-100 and polyvinylalcohol are least effective (Reijenga et al., 1983) although some success has been provided by the use of methyl cellulose (Gower and Woledge, 1977) and hydroxypropylmethylcellulose (HPMC) (Eriksson, 1980) at a concentration of 0,5% (w/v). However, Woledge and Reilly (1980) reported that 2% (w/v) HPMC in the leading electrolyte provided sharper zones compared with previously suggested concentrations.

2.1.2.3 Separation of Components

As the resolved sample zones are forced to run in immediate contact with each other (termed "steady-state stacking"), this may lead to practical difficulties in detecting the separation that has been achieved.

For UV-absorbing components, separation is evidenced by the differing peak heights of successive components. This may be aided by the presence of non-UV-absorbing impurities, in the analyzed solution, that have mobilities in between those of certain of the nucleotides. For fairly uncomplicated samples, therefore, such as muscle extracts (Gower and Woledge, 1977) quantification of nucleotides may be achieved on this

basis. For more composite cellular extracts (Eriksson, 1980), UV-absorbing components may not be present in sufficient quantities to achieve their maximum peak heights, so identification on this basis and quantification by zone length cannot be realized. In addition, non-UV-absorbing components may be lacking or not present in sufficient concentrations to separate adjacent UV-absorbing zones. In such cases, small quantities of intermediate-mobility non-UV-absorbing components need to be added to the sample solution to force apart consecutive UV-absorbing zones. These intermediate-mobility components, known as "spacers", may be achieved either with a continuous-mobility gradient, such as polyaminopolycarboxylic carrier ampholytes (Binion and Rodkey, 1981), or with discrete spacers (Eriksson, 1980; Oerlemans et al., 1981).

A spacing mobility gradient contains a large number of ions which possess mobilities very close to each other. Commercial applications of mobility gradient spacers in isotachophoresis have mainly centred on protein separations (Delmotte, 1977) using a complex mixture of polyaminopolycarboxylic acids with a great number of pK-values distributed over a certain pH range. Such mixtures of polyaminopolycarboxylic acids are available commercially as Ampholine carrier ampholytes (LKB, Stockholm, Sweden), in pH fractions according to the corresponding pI values of the ampholyte species.

When this mixture is added to the sample in an isotachophoretic system, its components arrange themselves in order of effective mobility. The result is an isotachophoretically moving mobility and pH gradient. The choice of the ampholyte pH range is important in order to obtain the correct mobility gradient and, thereby, optimal separation. This was demonstrated by Hjalmarsson (1975) with respect to the preparative

separation of serum proteins.

A discrete spacer is, in principle, a single compound chosen to have an intermediate mobility to two sample components of interest. The discrete spacer can either be UV-absorbing or non-UV-absorbing. In the latter case, two UV-absorbing components can be spaced apart by the addition of a non-UV-absorbing ion.

Unfortunately, there is a dearth of information concerning the absolute mobilities (m_o) of substances relative to the dissociation constants (pK_a) of the buffers used in the narrow pH range under which nucleotides are isotachophoretically separated. Eriksson (1980) determined a number of discrete spacers for nucleotide separation in liver extracts, but the components separated were relatively few and separation was not achieved at, or near, baseline level.

Wielders and Muller (1980) showed that careful balancing of the effective electrophoretic mobility of a UV-absorbing component with an inert non-UV-absorbing component during isotachophoretic analysis, creating a steady-state mixed zone of both components, made it possible to separate and quantify traces of the UV-absorbing component several orders of magnitude below that of non-mixed zone components. These workers demonstrated this method for the analysis of ADP using acetate as the inert carrier. For the trace analysis of individual nucleotides, therefore, this method proved useful providing that, under specific isotachophoretic conditions, the two components had the same effective mobility.

2.1.3 Bioluminescence

During this study, ATP was determined by isotachophoresis, HPLC and bioluminescence, in order to cross check results. The commercial availability of luciferin-luciferase reagents specific for sensitive measurements of ATP, prompted its use. Although the fundamental aspects of the firefly bioluminescence assay of ATP have remained essentially unchanged since they were first described by Strehler and Totter (1952), available reagents now contain highly purified and stabilized firefly luciferase (De Luca, 1976; Lundin et al., 1976) that provides a steady light signal intensity according to the Michaelis-Menten equation (Figure 2.2). The expression denotes that the light intensity, i.e. the reaction rate of the luciferase reaction, can be maintained constant for long periods of time in response to a given, low concentration of ATP. A pre-requisite is that the rate of ATP consumption during the reaction should be sufficiently low so as to change the ATP concentration only gradually. This is achieved using low activities of luciferase.

ATP determinations, in addition to being extremely sensitive in the low picomole range (Spielmann et al., 1981), have now become relatively easy to conduct with considerable timesaving over alternative methods. Although detailed determinations in yeast cell extracts have been published before (Hysert and Morrison, 1978; Miller et al., 1978), modifications were necessary in order to accurately measure low ATP levels as applicable to this study.

Bacterial bioluminescence also provides a unique system for the quantitative determination of specific metabolites. Luciferases have been studied in several different luminous bacteria including Beneckea and

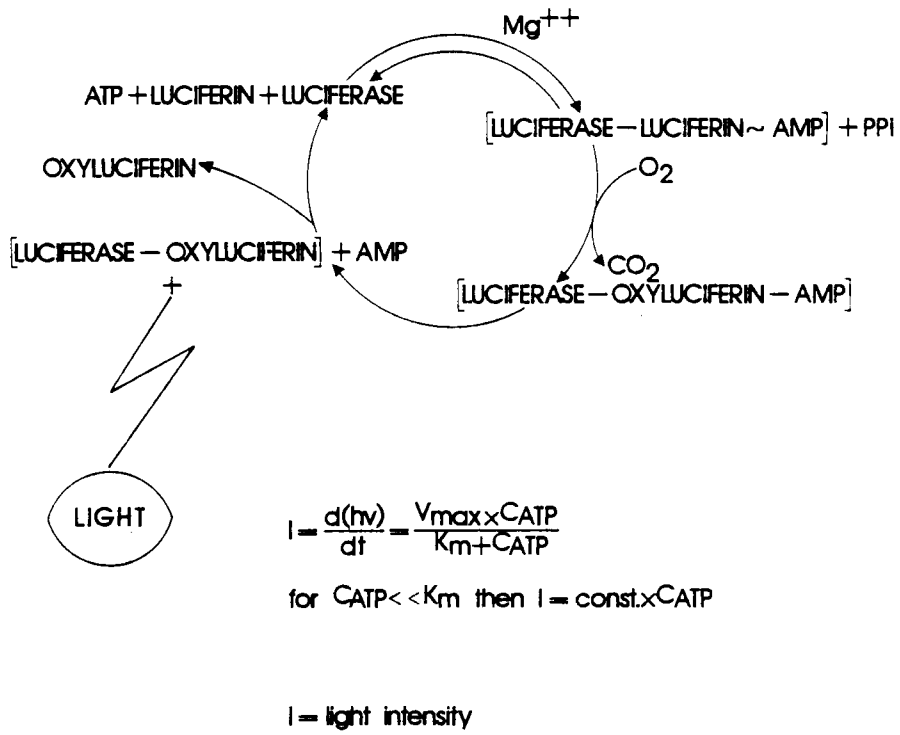


FIGURE 2.2: ATP measurement by bioluminescence using purified, stabilized luciferase to provide a steady light signal intensity.

Photobacterium species (Lavi et al., 1981). Bacterial luciferase requires reduced flavin mononucleotide (reduced FMN) in addition to several other reactants. Under suitable reaction conditions, the rate of light emission is proportional to the concentration of reduced FMN. Commercial preparations of bacterial luciferase also contain NADH : FMN oxidoreductase (dehydrogenase) activity, in addition to luciferase, thereby extending the versatility of the assay to include quantitative determinations of NADH, NADPH and FMN (Stanley, 1978).

Thus, the bioluminescence system from photobacteria is based on a coupled reaction in which two enzymes, FMN-reductase and luciferase

react. NADH : FMN-oxidoreductase or NADPH : FMN-oxidoreductase reduce FMN to $FMNH_2$ in the presence of the reduced pyridine nucleotide. Compared to the previously mentioned bioluminescence method for ATP, the reduced-flavin mononucleotide reacts similarly to luciferin, and, in the presence of oxygen and a long-chain aliphatic aldehyde, results in light emission, with an emission maximum at 495 nm.

To measure glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, and the triose phosphates, microassays were developed incorporating classical enzymatic methods (Entian et al., 1977) coupled to the described bioluminescence system for the measurement of NADH and NADPH.

2.2 MATERIALS AND METHODS

Maintenance of Brewing Yeast Strains (S. uvarum (carlsbergensis))

Media are listed in Appendix A. Specialized chemicals and their sources are listed in Appendix B.

For long term maintenance of S. uvarum, the strains were cultivated and kept (@ 0°C) in M.Y.G.P. medium (Wickerham, 1951). For routine use, the cultures were maintained on wort agar.

2.2.2 Propagation of Brewing Yeast Strains

Propagation procedures are outlined in Appendix C.

2.2.3 Infection Monitoring

All cultures and fermentations were monitored for bacterial and wild yeast infections according to the methods outlined in Appendix D.

2.2.4 Fermentation Conditions

Malt wort at ± 12°P was fermented in tall tubes at 10°C as described by Ryder *et al.* (1980) and Kruger *et al.* (1982). Pitching was undertaken at 6 g/l (centrifuged), the wort always having an initial dissolved oxygen value of 8,5 ppm to meet the brewing yeast strain's oxygen requirements. Two commercial lager strains of *S. uvarum (carlsbergensis)* were employed during the test procedures.

2.2.5 Anaerobic Sampling from Fermentation

For quantitative measurements of intracellular nucleotides, it is essential that *in situ* growth conditions remain constant throughout the sampling period. Transitions in temperature, pH, pressure, moisture and dissolved gases are likely to affect the physico-chemical nature of the sample and thereby directly or indirectly influence the intracellular nucleotide concentrations and their ratios (Karl, 1980).

In order to circumvent metabolic changes in the yeast population during sampling procedures, a glass apparatus was built exclusively for this purpose and is described in Figure 2.3 (Ryder *et al.*, 1983c). The apparatus allowed anaerobic sampling from laboratory fermenters such that

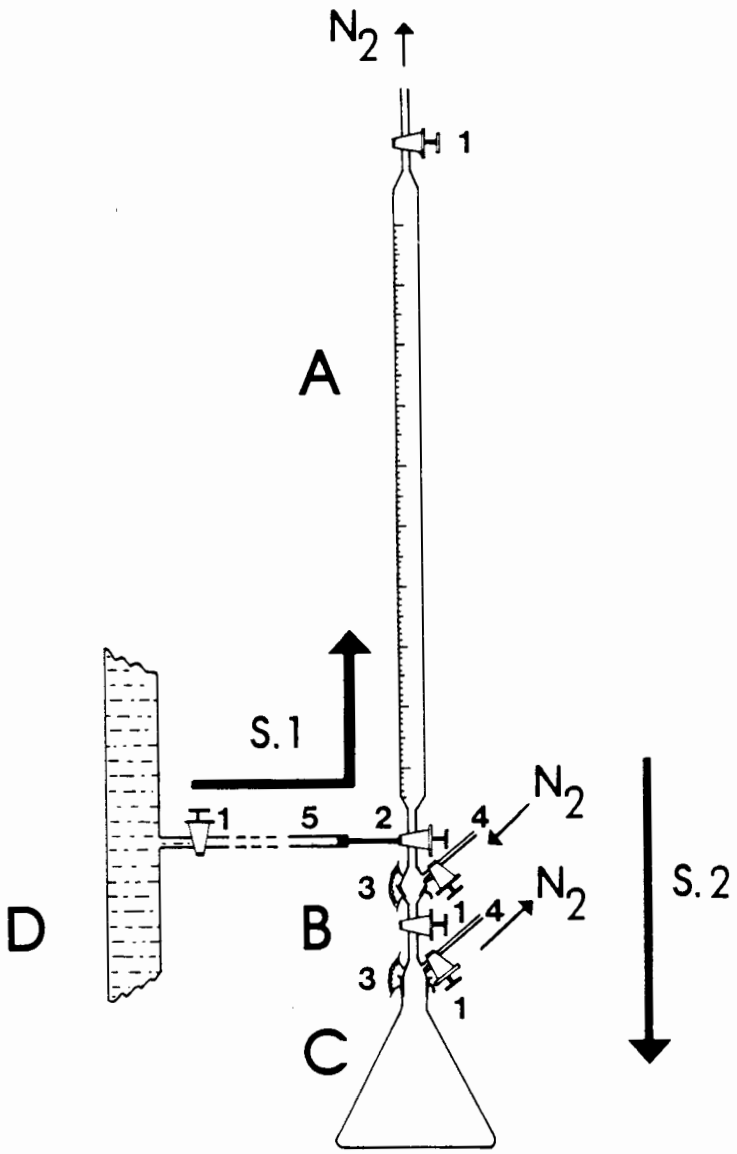


FIGURE 2.3: Anaerobic sampling device. A, 100 - to 200 - ml burette equipped with ground glass connection; B, centre piece equipped with ground glass connection; C, 150- or 300- ml collection flask; D, fermentation tube; 1, 2-mm bore, 2-way tap; 2, 2-mm bore, 3-way tap; 3, connecting spring; 4, 2-mm bore capillary tube; 5, flexible connection to fermentation tube sampling capillary. By regulation of various taps, nitrogen (N_2) at suitable pressure is used to purge sampling device of air. S.1 = initial sampling operation for measurement of volume of yeast suspension based on 100 mg yeast (dry weight). S.2 = secondary sampling operation for collection of yeast suspension from S.1.

varying sample sizes based on a constant yeast dry weight value could be achieved. In addition, the sampling procedure allowed the fermentation temperature to be maintained, i.e. the entire operation was undertaken in a constant temperature environment, and the system allowed equalisation of pressure between fermenter and sampling apparatus so that disturbances in dissolved gas levels would be minimal.

2.2.6 Permeabilization and Extraction Techniques

The following mediating agents were compared according to the respective published methods for yeast cells:

(i) Boiling ethanol (Entian et al., 1977).

(ii) Perchloric acid (Rose and Brockman, 1977; Wild et al., 1978; Heldt et al., 1980).

(iii) Trichloroacetic acid (Bagnara and Finch, 1972; Khym, 1976; Lothrop et al., 1980)

(iv) Formic acid (Klofat et al., 1969).

(v) Sulphuric acid (Forrest and Walker, 1965).

(vi) Boiling acetone (Hysert and Morrison, 1978).

(vii) Ambient acetone (Miller et al., 1978).

(viii) Butanol (Dhople and Hanks, 1973).

(ix) Chloroform (Dhople and Hanks, 1973).

(x) Boiling water (Ryder et al., 1983c).

2.2.7 Isotachophoresis

Isotachophoretic separations were performed using an LKB 2127 Tachophor (LKB, Bromma, Sweden) equipped with a UV detector monitoring at 254 nm,

and a thermal detector. Separations were achieved in a Teflon capillary tube 620 mm long (0,5 mm i.d.), thermostated through Peltier elements at 15 °C.

The leading electrolyte was 5 mM HCl with 19 mM β-alanine as counter ion in 0,5% hydroxypropylmethylcellulose (HPMC). The pH of the leading electrolyte was 3,92. The terminating electrolyte was 5 mM n-caproic acid.

Unless otherwise stated, separations, which required about 25 min, commenced with a current of 150 uA, which was reduced to 50 uA at 25 kV. This accelerated the procedure without changing the final separation. The recorder used was an LKB 2210 two channel recorder (LKB, Bromma, Sweden). Initial recorder speed, for adjustment of baseline, was 2 mm/min until immediately before detection, when it was increased to 2 mm/sec.

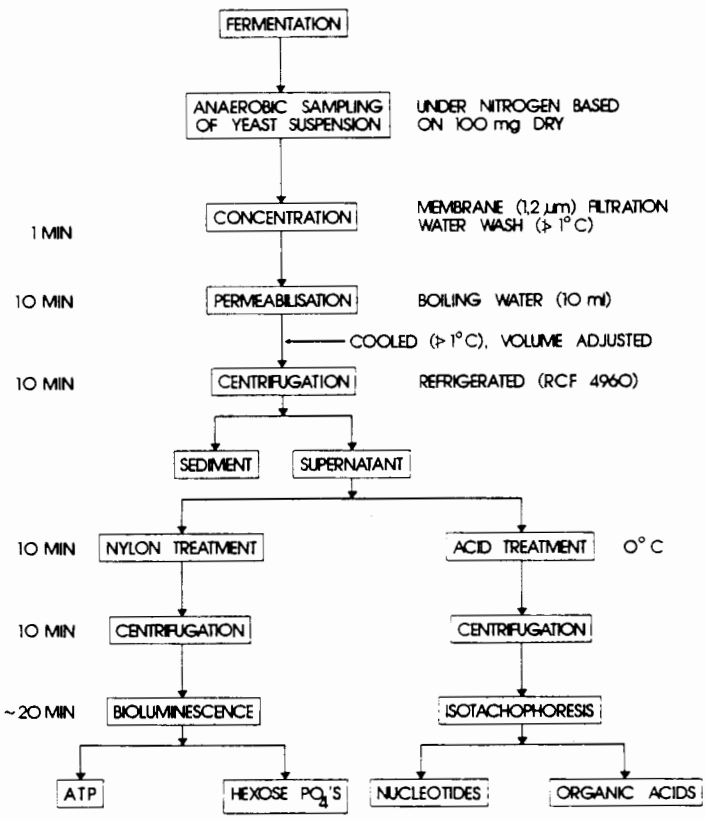


FIGURE 2.4: Mode of operation for cell permeabilization and extraction of intracellular nucleotides, organic acids, and hexose (and triose) phosphates (Ryder et al., 1983 c).

A sample volume of 5 ul was injected at the interface of the leading and terminating electrolytes for isotachophoretic runs for the yeast extracts. Occasionally, 10 ul was used to achieve a better evaluation of certain zones.

The capillary was always stored in 5 mM HCl in 1% Triton X solution. On each day of analysis, the isotachophoretic apparatus was carefully rinsed and prerun with water at 0,05 uA until 25 kV was reached. Subsequently, the instrument was prerun twice using freshly prepared electrolytes. The rationale behind this strategy was that, despite previous overnight rinsing of the capillary a considerable memory effect could be observed for very small quantities of nucleotides and organic acids or components of previously used electrolytes. Adherence of these compounds to the walls of the capillary may affect the duration of an experiment, a loss in boundary sharpness, changes in temperature profile or drift of the UV baseline.

Intermediate runs without sample were made after approximately five or six analyses, for the same purpose. Moreover, in order to circumvent electroosmotic disturbances during routine analysis, the double prerun with electrolytes only was essential. In other words, these preruns facilitated an increase in viscosity near the capillary wall effecting a reduction in electroosmotic changes. Thus, the first run stabilized the system, and the second attained a blank value for the UV- and non-UV-absorbing impurity zones in the electrolytes.

Success of separation also depended on ensuring that the membrane located between the leading electrolyte reservoir and capillary was regularly changed. Blockages created an extreme disturbance to separation.

It was shown that each batch of leading and terminating electrolytes, before use, should be heated in a waterbath to ± 80 °C to eliminate the possibility of air bubbles being transferred to the capillary.

Finally, it was very important to prepare the electrolyte solutions with the utmost care. Impurities in the chemicals used are highlighted as unwanted peaks or non-UV-absorbing zones which would negatively influence separation and interpretation unless taken into account. Each batch of electrolytes was carefully analyzed for impurity zones and correlated to component zones in the samples so that accurate measurement of sample components could be achieved. In addition, it was found essential to change the leading electrolyte in its respective reservoir on a regular basis to prevent a build-up of analyzed components which might contaminate a subsequent run.

2.2.8 Bioluminescence

All assays were performed using a Lumac Biocounter model 2010 (Lumac V.B., Schaesberg, Netherlands) at 25 °C. The bioluminescence reagents were from the same company. All other specialized chemicals and their sources are listed in Appendix B.

2.2.8.1 ATP Assays

Assays to determine ATP concentration in the yeast extracts were conducted by a method which was similar to that used by other workers (Lundin et al., 1976; Lust et al., 1981; Spielmann et al., 1981),

using extracts from other biological material. Suitable dilutions of the extracts (usually 10^{-3}) were made in glycine buffer at pH 7,75. This enabled bioluminescence measurements to be made within the linear range for ATP (Lundin et al., 1976; Lust et al., 1981; Spielmann et al., 1981).

Internal standardization was effected by the addition of ATP at a concentration range of 1×10^{-7} to 1×10^{-8} M/L to duplicate samples of the diluted extracts. Aliquots of 100 ul of the diluted extracts were automatically mixed with 100 ul of the bioluminescence reagents in disposable polystyrene sample cells. The light signal produced was measured over a 10 sec period at 25 °C.

2.2.8.2 Hexose and Triose Phosphate Assays

The strategies (Ryder et al., 1983c), outlined in Figures 2.5 and 2.6, show that the measurement of fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate (F1,6BP) was achieved by enzymatic assays coupled to the measurement of glucose-6-phosphate and triose phosphates, respectively. Glucose-6-phosphate (G6P) was computed by the production of NADPH from NADP in the enzyme mediated redox reaction to 6-phosphogluconate. For triose phosphates, the loss of NADH was measured through oxidation to NAD in the enzymatic reaction to glycerol phosphate.

Each step of the enzymatic assay, including suitable blanks, was made up to a final volume of 750 ul within which was placed one unit each of the respective enzymes for the hexose or triose phosphate metabolite to be determined.

The system was buffered with phosphate buffer (pH 7.0) and included internal standards for each of the hexose phosphates measured. For triose phosphate, the internal standard was dihydroxyacetone phosphate (DAP). Internal standard concentrations ranged from 1×10^{-7} to 1×10^{-6} M/L for G6P and F6P, and 1×10^{-6} to 4×10^{-6} M/L for F1, 6BP and DAP. For the glucose-6-phosphate assay, NADP was added at a concentration of 5×10^{-6} M/L. For the triose phosphate assay, NADH was added at a concentration of 9×10^{-6} M/L.

Following a reaction period of 40 min at ambient temperature, aliquots of 100 ul of the reaction mixture were automatically mixed with 100 ul of the bioluminescence reagents in disposable polystyrene sample cells. The light signal produced was measured over a 60-sec period at 25°C.

2.2.9 High Performance Liquid Chromatography

In order to correlate nucleotide levels against an accepted HPLC reference method (Rose and Brockman, 1977), measurements were conducted using a Perkin-Elmer Series 3 B Liquid Chromatograph (Perkin-Elmer, Norwalk, Connecticut, U.S.A.) equipped with a standard pump module and a Partisil-10 SAX colums of 25 cm x 4.6 mm (Whatman, Clifton, N.J., U.S.A.). Samples of 50-100 ul of the cellular extracts were used, and standard solutions of adenosine, guanosine, cytidine and uridine mono-, di- and triphosphates were analyzed to confirm retention times. Resolution of the nucleotides was achieved at ambient temperature using a linear gradient (40 min) from 4 mM (pH 2.8) to 750 mM (pH 3.7) ammonium dihydrogen phosphate at a flowrate of 2 ml/min. A Perkin-Elmer LC-75 Spectrophotometric Detector was used to detect the eluting nucleotides at 254 nm.

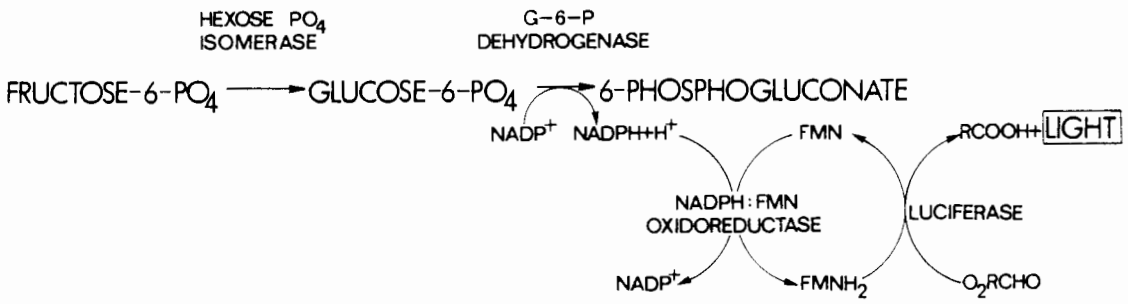


FIGURE 2.5: Strategy for measurement of fructose-6-phosphate and glucose-6-phosphate using a photobacterial system for the evaluation of NADPH.

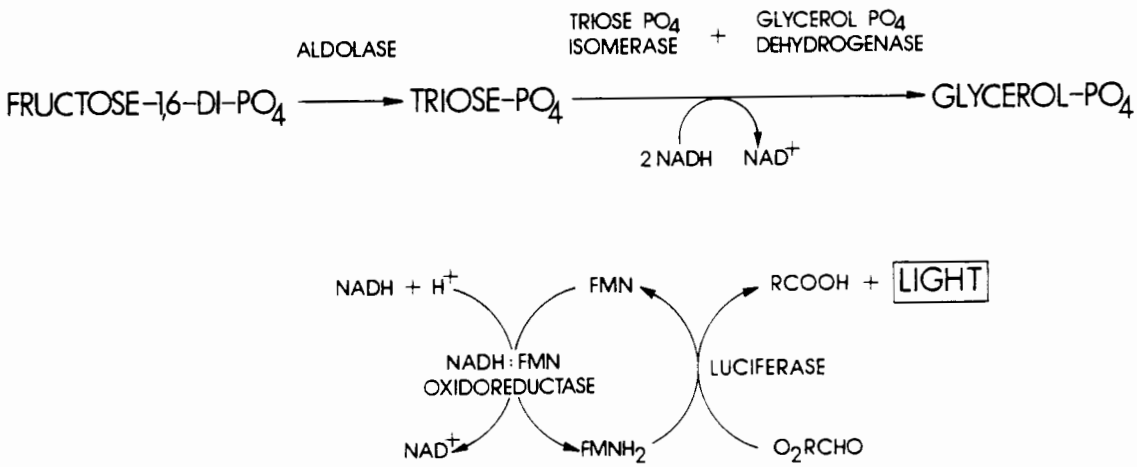


FIGURE 2.6: Strategy for measurement of fructose-1,6-bisphosphate and triose phosphate using a photobacterial system for the evaluation of NADH.

2.3 RESULTS

2.3.1 Cell Permeabilization and Intracellular Metabolite Extraction

If a procedure permits the recovery of only a percentage of a standard solution of nucleotide then it is unlikely to demonstrate more than that percentage from cells. It follows that valid estimations of yeast intracellular nucleotides depend firstly on demonstrating the effects of the extraction procedure on standard nucleotide solutions.

Table 2.1 provides an example of isotachophoretic data obtained by studying the effects of each procedure on standard solutions of commercial preparations of ATP, UTP and GTP.

The results demonstrated that perchloric acid and boiling ethanol treatments were disinctly less favourable for the recovery of ATP than alternative treatments, and repeat analyses confirmed this view. The boiling water and chloroform techniques appeared to permit a better recovery. It was therefore decided to use the boiling water method as the reference method to compare other techniques.

TABLE 2.1: Effect of extraction procedure on nucleotide recovery as compared to standard solutions.

EXTRACTION PROCEDURE	NUCLEOTIDES					
	ATP		UTP		GTP	
	nM	% to STAN-DARD	nM	% to STAN-DARD	nM	% to STAN-DARD
Standard Solution	0,500	-	0,500	-	0,500	-
Perchloric Acid	0,424	84,8	0,460	92,0	0,436	87,2
Trichloro-acetic Acid	0,454	90,8	0,448	89,6	0,440	88,0
Formic Acid	0,442	88,4	0,458	91,6	0,458	91,6
Sulphuric Acid	0,452	90,4	0,455	91,0	0,457	91,5
Acetone (Boiling)	0,476	95,2	0,470	94,0	0,479	95,8
Acetone (Ambient)	0,475	95,0	0,474	94,8	0,478	95,6
Butanol	(0,481)	96,2	(0,483)	96,6	(0,479)	95,8
Chloroform	0,489	97,8	0,487	97,4	0,485	97,0
Ethanol (Boiling)	0,431	86,2	0,451	90,2	0,460	92,0
Water (Boiling)	0,490	98,0	0,492	98,4	0,493	98,6

TABLE 2.2: Effect of extraction procedure (acids) on metabolite levels from yeast populations - exponential growth phase cells - as compared to reference method (all calculations taken to nM/mg dry yeast).

EXTRACTION PROCEDURE	NUCLEOTIDES								ORGANIC ACIDS			
	ATP		UTP		GTP		CTP		ACETIC		GLUTAMIC	
	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF
Reference Method	3,72	-	0,76	-	0,71	-	0,55	-	49,8	-	43,0	-
Water (Boiling)												
Perchloric Acid	2,64	70,9	0,59	77,6	0,58	81,7	0,41	74,5	51,2	102,8	42,1	97,9
Trichloro-acetic Acid	3,35	90,2	0,68	89,5	0,63	88,7	0,50	90,9	-	-	40,6	94,4
Formic Acid	3,05	81,9	0,66	86,8	0,57	80,3	0,44	80,0	-	-	39,8	92,6
Sulphuric Acid	3,05	81,9	0,62	81,6	0,60	84,5	0,46	83,6	50,2	100,8	41,3	96,0

TABLE 2.3: Effect of extraction procedure (solvents) on metabolite levels from yeast populations - exponential growth phase cells - as compared to reference method (all calculations taken to nM/mg dry yeast).

EXTRACTION PROCEDURE	NUCLEOTIDES								ORGANIC ACIDS			
	ATP		UTP		GTP		CTP		ACETIC		GLUTAMIC	
	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF	nM/mg dry yeast	% to REF
Reference Method	3,50	-	0,75	-	0,66	-	0,48	-	55,6	-	47,8	-
Water (Boiling)	2,80	80,0	0,56	74,7	0,59	89,6	0,40	83,3	57,0	102,5	36,2	75,7
Acetone (Boiling)	2,89	82,5	0,60	80,0	0,62	93,9	0,43	89,6	55,8	100,4	31,5	65,9
Acetone (Ambient)	(2,68)	76,6	(0,62)	82,7	(0,54)	81,8	(0,40)	83,3	(45,8)	82,4	(29,6)	61,9
Butanol	3,19	91,1	0,68	90,7	0,60	90,9	0,45	93,7	55,6	100,0	45,3	94,8
Chloroform	2,63	75,0	0,58	77,3	0,58	87,9	0,39	81,2	58,3	104,8	36,8	77,0
Ethanol (Boiling)												

Tables 2.2 and 2.3 show the effects of extraction procedures on yeast metabolite levels taken from S. uvarum fermentations while the yeast was in the exponential growth phase.

Table 2.2 shows that perchloric acid performed less satisfactorily than the alternative acid procedures for the nucleotides while trichloroacetic acid was clearly better. However, in comparing the two tables, the closest to the reference method in nucleotide triphosphate values was the chloroform procedure. Moreover, it was subsequently shown that if similar precautions to the boiling water procedure were observed concerning temperature and speed of sample handling, results between the two procedures were less marked.

TABLE 2.4: Effect of boiling time on nucleotide recovery as compared to standard solutions, using reference method.

PROCEDURE	NUCLEOTIDE							
	ATP		UTP		GTP		CTP	
	nM	% to STAN-DARD	nM	% to STAN-DARD	nM	% to STAN-DARD	nM	% to STAN-DARD
Standard Solution	0,125	-	0,125	-	0,125	-	0,125	-
Boiling time (min)								
2	0,125	100	0,125	100	0,125	100	0,125	100
5	0,125	100	0,125	100	0,125	100	0,125	100
10	0,123	98,4	0,125	100	0,125	100	0,124	99,2
15	0,122	97,6	0,121	96,8	0,124	99,2	0,122	97,6

TABLE 2.5: Effect of boiling time on nucleotide extraction from yeast populations - exponential growth phase cells - using reference method (all calculations taken to nM/mg dry yeast).

BOILING TIME (MIN.)	NUCLEOTIDE			
	ATP nM/mg dry yeast	UTP nM/mg dry yeast	GTP nM/mg dry yeast	CTP nM/mg dry yeast
2	1,80	0,56	0,45	0,35
5	3,06	0,75	0,67	0,51
10	4,02	0,80	0,72	0,60
15	3,86	0,73	0,70	0,51

Table 2.4 shows the effect of boiling time on standard solutions of the nucleotides. After 15 minutes boiling time hydrolysis of the labile triphosphates occurred rapidly. Tables 2.5 to 2.8 show the effects of boiling time on extraction of nucleotides and organic acids from exponential growth phase and stationary phase populations. The tests were conducted to establish the optimal boiling period and to check possible effects of differing cell wall network densities on extraction efficacy.

The results show that the maximum level of nucleotides was achieved after a boiling period of 10 minutes and that $\pm$ 10 minutes were necessary to achieve the maximum value of intracellular organic acids from both growth phases.

TABLE 2.6: Effect of boiling time on nucleotide extraction from yeast populations - late stationary phase cells - using reference method (all calculations taken to nM/mg dry yeast).

BOILING TIME (MIN.)	NUCLEOTIDE			
	ATP nM/mg dry yeast	UTP nM/mg dry yeast	GTP nM/mg dry yeast	CTP nM/mg dry yeast
2	0,78	0,24	0,32	0,12
5	1,53	0,49	0,68	0,32
10	1,74	0,55	0,80	0,39
15	1,70	0,50	0,80	0,34

TABLE 2.7: Effect of boiling time on organic acid extraction from yeast populations - exponential growth phase cells - using reference method (all calculations taken to nM/mg dry yeast).

BOILING TIME (MIN.)	ORGANIC ACID	
	ACETIC nM/mg dry yeast	GLUTAMIC nM/mg dry yeast
2	18,8	24,7
5	40,0	44,0
10	49,6	48,4
15	50,0	48,8

TABLE 2.8: Effect of boiling time on organic acid extraction from yeast populations - late stationary phase cells - using reference method (all calculations taken to nM/mg dry yeast).

BOILING TIME (MIN.)	ORGANIC ACID	
	ACETIC nM/mg dry yeast	GLUTAMIC nM/mg dry yeast
2	20,8	24,5
5	46,3	43,0
10	54,6	48,0
15	55,2	48,0

The effect of pH on nucleotide extraction is shown in Table 2.9. Using untreated double-distilled water, the pHs of the cellular extracts were approximately 6,2 (this altered slightly due to age and condition of the yeast population and associated adsorption of hop resinous material on the yeast cell wall). A range of final extract pHs was achieved between 5,2 and 9,2. pH 9,2 was chosen as the end-point because of observations by Beutler and Baluda (1963) on red blood cells indicating a superior extraction at this pH. The results do not substantiate such a finding for brewing yeast cells.

The final table in this series (Table 2.10) shows the addition of an internal standard to the yeast sample immediately prior to extraction. The results show 2% overall losses.

TABLE 2.9: Effect of pH on nucleotide extraction from yeast populations - exponential growth phase cells - using reference method (all calculations taken to nM/mg dry yeast).

FINAL pH	NUCLEOTIDES			
	ATP nM/mg dry yeast	UTP nM/mg dry yeast	GTP nM/mg dry yeast	CTP nM/mg dry yeast
5,2	3,40	0,62	0,65	0,47
6,2	3,44	0,69	0,65	0,51
7,2	3,36	0,69	0,65	0,53
8,2	3,48	0,69	0,65	0,53
9,2	3,40	0,69	0,65	0,47

TABLE 2.10: Internal standard addition before nucleotide extraction from yeast populations - exponential growth phase cells - using reference method (all calculations taken to nM/mg dry yeast).

PROCEDURE	NUCLEOTIDES			
	ATP nM/mg dry yeast	UTP nM/mg dry yeast	GTP nM/mg dry yeast	CTP nM/mg dry yeast
Without Internal Standard	3,48	0,75	0,66	0,52
With Internal Standard (2,50 nM/mg dry yeast)	5,87 (98,2)	3,21 (98,7)	3,08 (97,5)	0,52 (100)

2.3.2 Isotachophoresis

2.3.2.1 Investigation into the Choice of Electrolytes

For the separation of a mixture of 12 commercially prepared nucleotides at approximately 1 nM each in concentration, a series of separations was conducted between pH 3,7 and pH 4,2. The leading electrolyte was 4 mM HCl in 0,5% HPMC achieving a pH range using glycine and β -alanine as counter-ions. The terminating electrolyte was 5 mM n-caproic acid. All other conditions were as described under Materials and Methods. Optimal separation was unequivocally shown (Figure 2.7) at pH 3,92 using 19 mM β -alanine and this concentration was adopted for all subsequent analyses. The terminating electrolyte must always possess an effective mobility lower than that of the sample ions of interest. As 5 mM n-caproic acid clearly demonstrated this ability for the nucleotides and as previous studies had used this electrolyte successfully, it was decided that further investigations would be unprofitable.

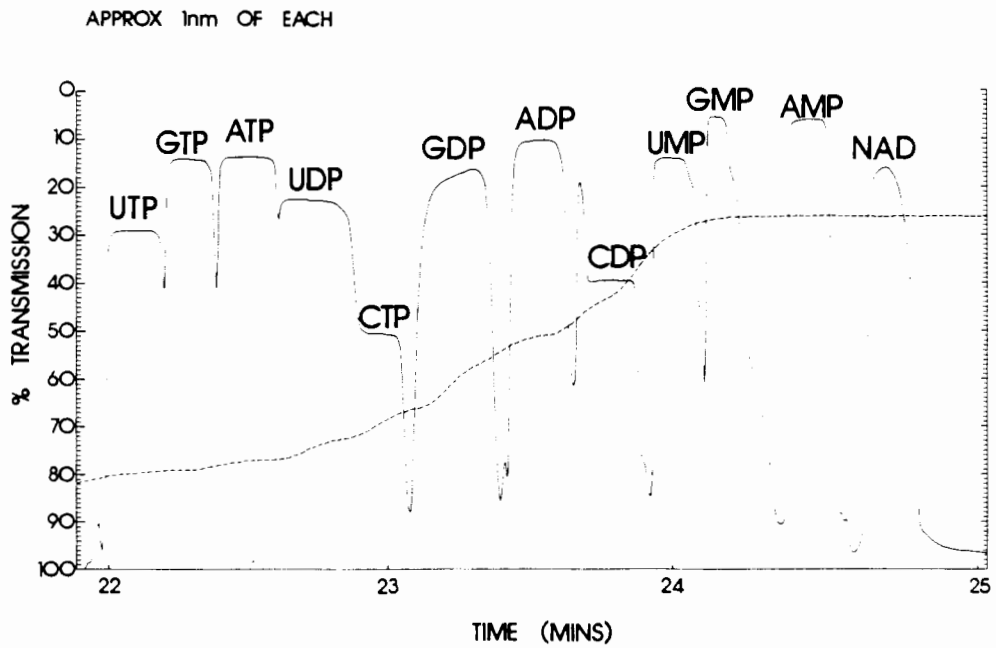


FIGURE 2.7: Separation of a mixture of 12 nucleotides by isotachophoresis.

2.3.2.2 Investigation into the Optimal Concentration of Hydroxypropylmethylcellulose (HPMC)

An investigation was launched to study concentrations of HPMC in the range of 0,2 - 2% (w/v) and additionally to examine HPMC samples of different viscosity potentials. From these investigations, where all other parameters were as described under Materials and Methods, it was shown that 0,5% HPMC was optimal for nucleotide separation, but that the viscosity rating of the product should be 15 000 cps. HPMC from alternative manufacturers other than those mentioned under Appendix B, even with similar viscosity ratings, was not found to be suitable and provided inferior separations. Furthermore, it was interesting that a concentration of 2% (w/v), as described by Woledge and Reilly (1980), was impractical to use, being far too viscous as well as exhibiting inferior separation and contributing to impurity zones.

2.3.2.3 Influence of Capillary Length

The capillary length was found to be crucial for successful separation of nucleotides, particularly in the yeast cellular extracts. In an evaluation study using capillaries of two different sizes, 230 mm and 620 mm, both 0,5 mm i.d., consistently better separation was shown using the 620 mm length.

2.3.2.4 Influence of Working Temperature

Although small variations in working temperature did not unduly affect nucleotide separation and reproducibility, the most consistent separa-

tions were achieved at 15 °C. Apart from some slight influence on total analysis time, temperature was, within reason, found not to be a very critical parameter for nucleotide analysis. Similar results have also been found for protein analysis (Delmotte, 1977).

2.3.2.5 Influence of Current during Analysis

Investigations were undertaken to ascertain whether analysis time could be reduced from initial experiments undertaken at a constant 50 uA and taking $\pm$ 40 minutes, without being detrimental to component separation and quantification. Prolonged experimentation showed that almost imperceptible differences to separation could be achieved by commencing with a current of 150 uA, which was reduced to 50 uA at 25 kV, until detection. If the voltage was allowed to rise to a higher level than this figure, there was a definite danger of bubble formation in the system creating severe problems for separation.

2.3.2.6 Separation of UV-absorbing Components in Yeast Extracts

The very complex nature of the yeast cellular extracts prompted an intense investigation into the application of using spacers for spacing apart adjacent UV-absorbing components. UV-absorbing components were not normally present in these extracts in sufficient quantities to achieve their maximum peak heights. In addition, insufficient non-UV-absorbing components were present to separate most UV-absorbing zones.

It was considered essential for accurate separation and quantification

of components to use a spacer mobility gradient in sufficient quantity to achieve spacing as close to the baseline as possible.

In the first set of experiments, Ampholine carrier ampholytes at pH range 2,5 to 4,0 were added to the sample at the recommended rate of 1% (w/v) (Hjalmarsson, 1977). Unfortunately, this concentration caused total capillary overloading, resulting in a diffuse zonal pattern. Further experiments revealed that a concentration of 0,2% (w/v) facilitated separation over the majority of components but had little effect on separating components with slowest mobilities. Separation of these components could be overcome by using 0,2% (w/v) Ampholine carrier ampholytes at pH range 3,5 to 5,0. Capillary overloading prevented use of 0,2% (w/v) of each type so that finally 0,1% (w/v) of each was used as a compromise in achieving some reasonable separation. Reducing the sample size created a more precise appreciation.

Although reproducible, one drawback to the use of ampholytes was that on reaching steady-state conditions, the range of mobilities was so large that mixed zones of Ampholine and component were apparent, i.e. certain ampholytes had similar mobilities to the UV-absorbing zones. This created rather broad zones. A second disadvantage in their use was that, without overloading the capillary, it was not possible to achieve non-UV-absorbing zones between UV-absorbing components at, or near, baseline level. Therefore, a survey had to be conducted in order to find and, subsequently, optimise discrete spacer ions for this study.

Approximately 140 anionic substances were analyzed consisting of inorganic and organic acids as well as organic phosphates. Investigations demonstrated that it was possible to use a combination of non-UV-absorbing discrete spacers to achieve baseline or near baseline level, for accurate

quantification of UV-absorbing components (Ryder, 1983c). Studies had to be conducted at varying pHs for the leading electrolyte in order to ascertain the purity of a UV-absorbing zone, i.e. slight pH differences caused the spacer to fall on one side of the UV-absorbing component or the other. This manifested a situation where nucleotides, in the initial spacer experiments, were attached to either a slightly faster mobility UV-absorbing compound or a slightly slower one. Curiously, the adenine nucleotides were found to be particularly characteristic of this "jumping nucleotide" phenomenon. Similarly, this evaluation facilitated determination of the purity of the non-UV-absorbing zones already present in the extracts in measurable quantities.

These methods eventually demonstrated the extreme difficulty of separating ATP from an unknown UV-absorbing component in the yeast cellular extracts, the electrophoretic mobility of which, under the conditions described, was very similar to that of ATP. This prompted an investigation to ascertain whether it was possible to separate the two components on a steady-state mixed zone basis.

Investigations eventually highlighted the indispensability of glucose-1,6-bisphosphate for this purpose (see Figure 2.10). The mobility zone of glucose-1,6-bisphosphate, under the described isotachophoretic conditions was found to overlap that of ATP but not the associated unknown UV-absorbing component. Thus, by careful balancing of glucose-1,6-bisphosphate in the sample, ATP during migration, would selectively mix with this component. Under the optimised conditions already discussed, at equilibrium ATP was uniformly dispersed in the non-UV-absorbing carrier and could therefore be quantified.

As a result of the spacer study, Table 2.11 lists suitable spacers in order of mobility as they would occur in a typical isotachophoretic record in relationship to the UV-absorbing components found in the extracts. Spacers given in parentheses are alternatives, but they may overlap the mobility of UV-absorbing zones in the event of small pH differences to the system. UV-absorbing components in parentheses show components that may occur in the same peak as the major compound measured and, although noticeable as shoulders, can only be separated using alternative isotachophoretic conditions, as suitable spacers have not yet been found. Suitable quantities of these spacers will provide resolved zones as shown in Figures 2.8 and 2.9.

TABLE 2.11: A table of suitable "spacers" (in order of mobility) for nucleotide separation and quantification in yeast extracts.

UV-Absorbing[b] Components in Yeast Extracts	Non-UV-Absorbing[b] Spacers
	Perchloric acid
	Hydrochloric acid
Oxaloacetic acid	
	Phosphoenolpyruvic acid
	Phospho-α-D-ribose diphosphate
UTP	
	Formic acid
	Tartronic acid
	Pyruvic acid
Unknown	
	Tartaric acid
(Fumaric acid)	
UDP glucuronic acid	
GTP	
	Malonic acid
	(Chloroacetic acid)
Unknown	
	Dichloroacetic acid
Unknown	
	Glucose-1,6-bisphosphate
ATP	
	Fructose-1,6-bisphosphate
	(Glyoxylic acid) .
UDP	
	Oxo-glutaric acid
	Trichloroacetic acid
Unknown	
	D-Saccharic acid
CTP	
	Phosphoric acid
GDP	
	Isocitric acid
	Citric acid
	Malic acid
Unknown	
	Glycolic acid
UDPG	
	Glyceric acid
	Cyclamic acid
	Phosphocreatine
UDP acetylglucosamine	
(NADPH)	
	α-keto-valeric acid
Unknown	
	Citramalic acid
ADP	
	Lactic acid

TABLE 2.11: (Continued)

UV-Absorbing[b] Components in Yeast Extracts	Non-UV-Absorbing[b] Spacers
Unknown	
	Glycerin phosphoric acid
	(Itaconic acid)
FAD	
	Glucuronic acid
	Dihydroxyacetone phosphate
CDP	
(NADH)	
	Glucose-6-phosphate
	Fructose-6-phosphate
	Aspartic acid
NADP	
	Succinic acid
Unknown	
	Gluconic acid
	(Galacturonic acid)
XMP	
UMP	
	Glutaric acid
IMP	
GMP	
	Adipic acid
Cyclic AMP	
	Glutathione (reduced)
	Glutamic acid
Unknown	
	Acetic acid
	DL-β-Hydroxybutyric acid
Unknown	
	Pimelic acid
AMP	
	Levulinic acid
Unknown	
	Propionic acid
Unknown	
	Butyric acid
	Isovaleric acid
NAD	
	Valeric acid
	(Isobutyric acid)
CMP	
	Caproic acid

[a] Approximately 140 compounds were appraised.

[b] At 254 nm and at the concentrations used. This table is valid only for the electrolyte system and isotachophoretic conditions used in this work.

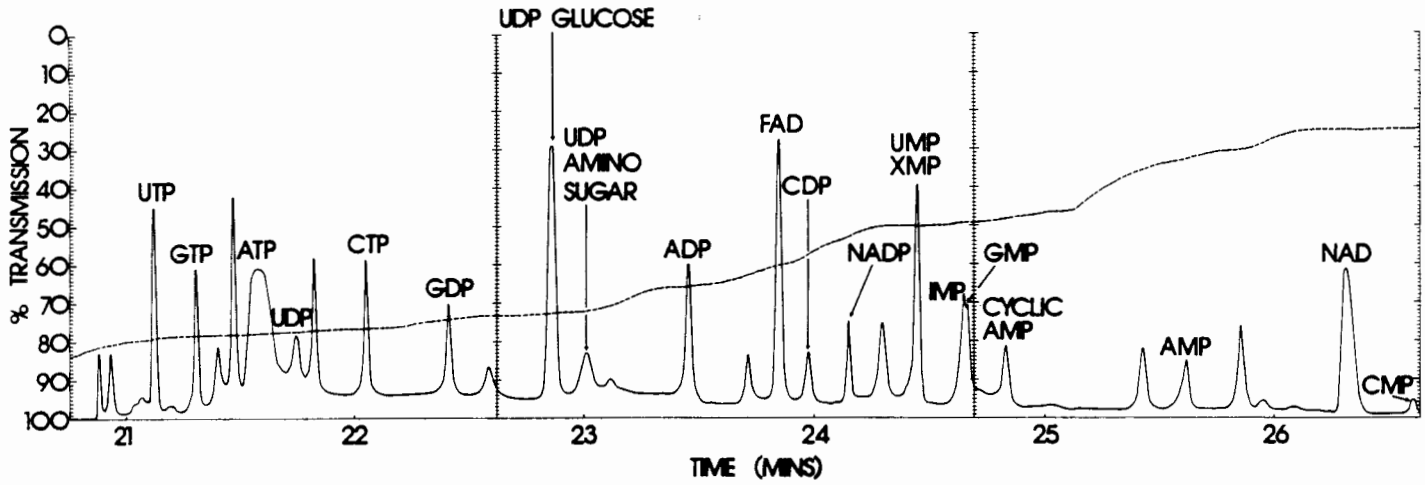


FIGURE 2.8: Separation of nucleotides in yeast extracts by "spacer" technique. Nucleotides highlighted. ——= thermal detection plot.

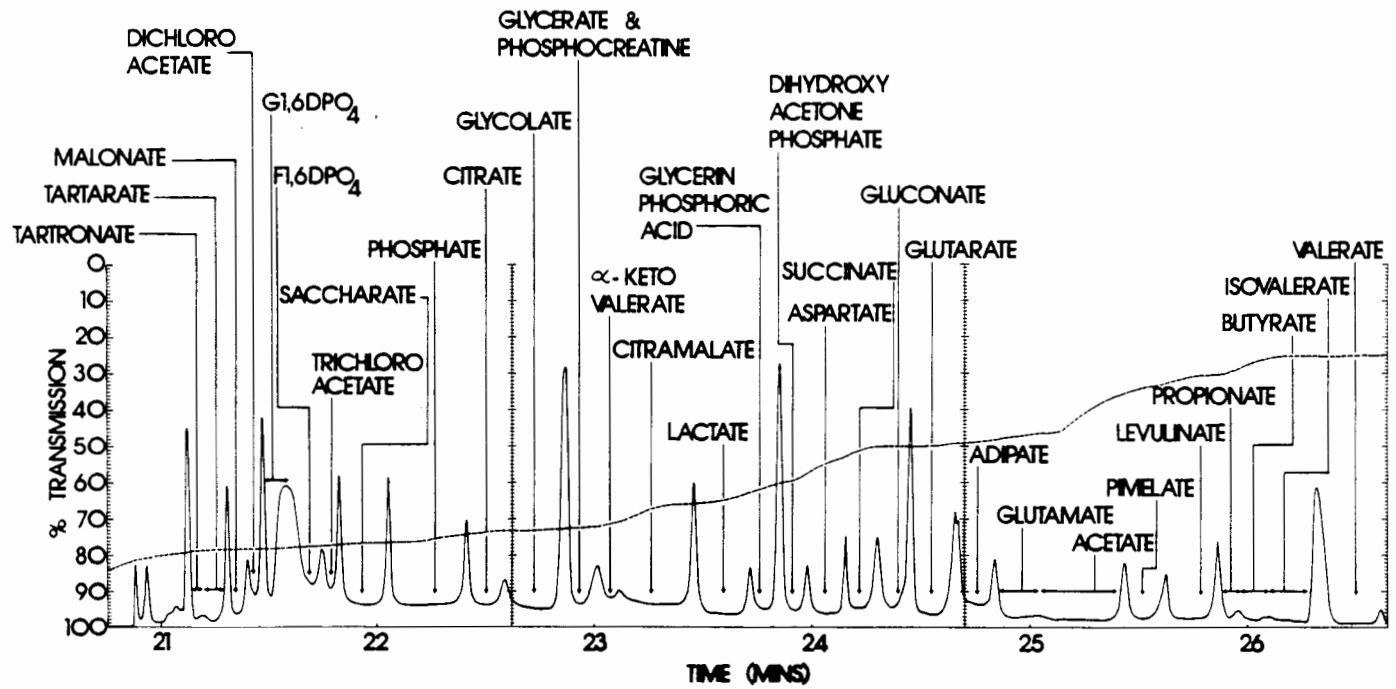


FIGURE 2.9: Separation of nucleotides in yeast extracts by "spacer" technique. Spacers highlighted. ——= thermal detection plot.

2.3.2.7 Use of Spacers for Routine Isotachophoretic Analysis

For optimum resolution of zones, two sets of spacers - primary and secondary - were used. For convenience, stock solutions of these spacers were made by dissolving millimolar quantities, or multiples thereof, of each in 1 ml of double-distilled water. Suitable quantities of each were mixed and further diluted before use (Table 2.12), before being added to the cellular extracts at the rate of 2 ul of spacers per 100 ul of acidified extract.

For routine analysis, four isotachophoretic runs were made for each sample: 1, extract alone (no spacers); 2, extract + primary spacers; 3, extract + primary spacers + glucose-1,6-bisphosphate (G1,6BP) for steady-state mixed zonal separation of ATP. G1,6BP was added to the cellular extract at the rate of 2 ul of a solution of G1,6BP (made by dissolving 10 mg of the crystallized tetracyclohexylammonium salt in 1,5 ml of double-distilled water) to 100 ul of extract; and 4, extract + secondary spacers.

2.3.2.8 Quantification of Components

Isotachophoretic runs were made with and without spacers for routine analysis (Figure 2.10). The latter records provided an evaluation of organic acid and phosphate components, using both the UV- and thermal detectors. Identification of zones was made by standard addition analysis. Purity of all zones in this work was regularly checked by isotachophoretic examination, using alternative electrolytic and pH conditions and sample sizes.

TABLE 2.12: "Spacer" system used in routine isotachophoretic nucleotide analysis[a].

No.	"Spacer" Constituent	Solution Molarity	Volume to Stock Solution (ul)
	1 - 10: Primary Spacers		
1	Formic acid	2,0	100
2	Tartronic acid	1,5	100
3	Tartaric acid	3,0	100
4	Malonic acid	4,5	100
5	Dichloroacetic acid	3,0	100
6	Fructose-1,6-bisphosphate	3,0	100
7	Trichloroacetic acid	3,0	200
8	2-Oxoglutaric acid	1,5	100
9	D-Saccharic acid	1,5	100
10	Gluconate-6-phosphate	0,75	100
	11 - 27: Secondary Spacers		
11	Citric acid	1,0	100
12	Glycolic acid	2,0	100
13	Glyceric acid	1,0	100
14	Citramalic acid	1,0	200
15	Lactic acid	4,0	100
16	Glycerol-3-phosphate	1,0	100
17	Aspartic acid	1,0	100
18	Glucuronic acid	2,0	100
19	Succinic acid	1,0	200
20	Gluconic acid	1,0	100
21	Glutaric acid	2,0	100
22	Adipic acid	1,0	200
23	Pimelic acid	2,0	100
24	Levulinic acid	1,0	100
25	Propionic acid	2,0	100
26	Butyric acid	4,0	100
27	Valeric acid	4,0	100

[a] Primary spacers were diluted 1 vol + 25 vol before use and secondary spacers 1 vol + 5 vol with double-distilled water. Two microlitres of spacers were added to 100 ul of extract immediately before analysis.

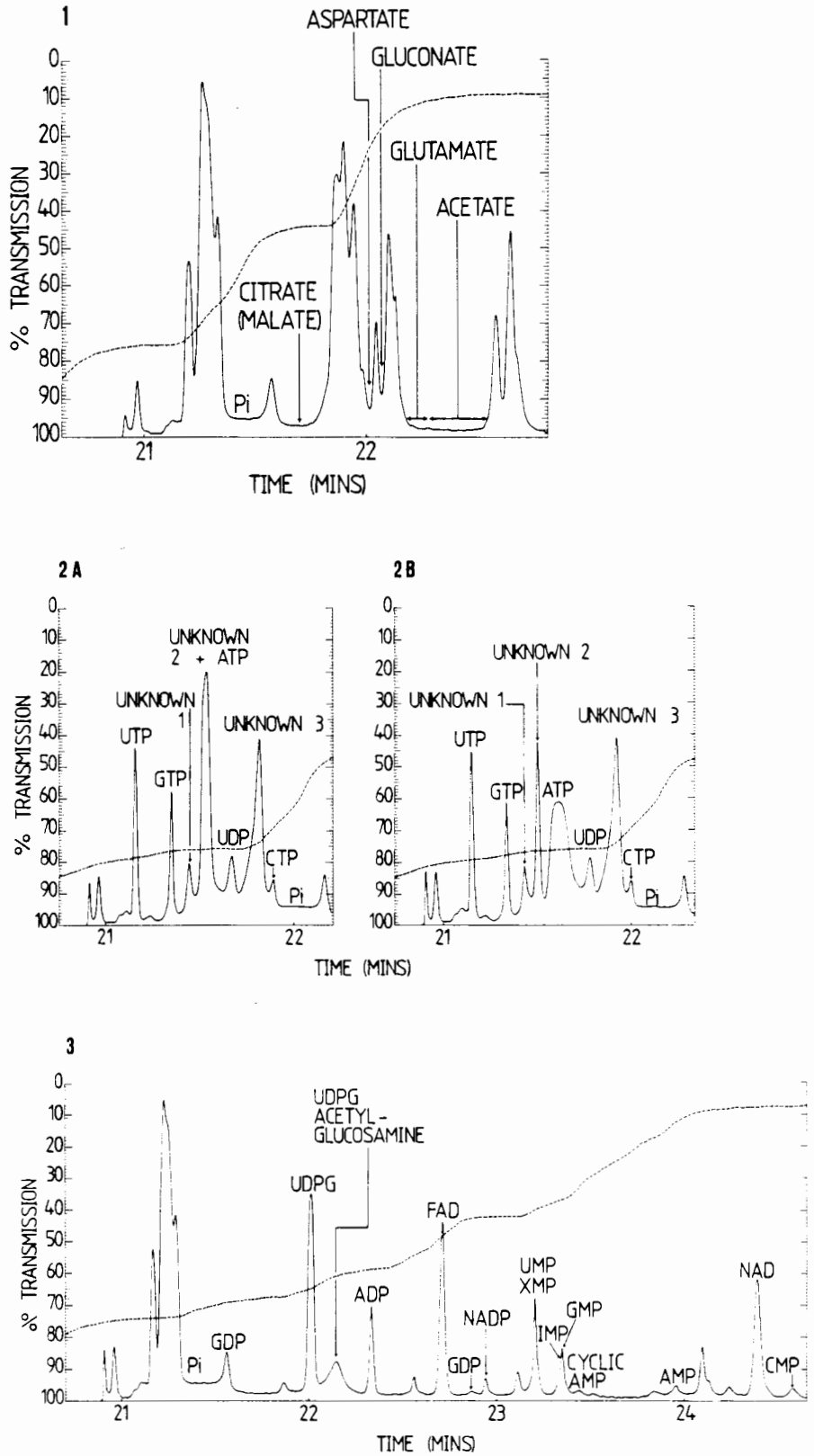


FIGURE 2.10: Mode of operation for routine analysis of yeast extracts by isotachophoresis.

1, extract alone (no spacers) for evaluation of organic acids; 2A, extract + primary spacers; 2B, extract + primary spacers + glucose-1,6-bisphosphate for evaluation of ATP; 3, extract + secondary spacers.

——= thermal detection plot.

Quantification of components was initially found to be complicated by interfering UV-absorbing complexes in the cellular extracts. This was particularly found to be the case with yeast extracts from wort fermentations; hence, it was reasoned that these complexes were probably associated with polyphenols. A number of methods were used in trying to eliminate these complexes including polyacrylamide gel columns (Khym, 1976), borate-impregnated silica cartridges (Lothrop and Uziel, 1980) and neutral alumina mini-columns (El Kouni and Cha, 1981). These methods were found unsatisfactory for this purpose with losses of either the nucleotide or organic acid components. Experimentation finally demonstrated that acidification of the extracts, immediately before analysis, with 1 M HCl (at the rate of 2 ul acid/100 ul extract) and separation of the resulting precipitate by centrifugation at $0^{o}C$, greatly assisted in alleviating this interference without loss of required metabolites.

HPLC analysis of nucleotides (and organic acids) correlated well, as far as the limit of resolution of the method used. Isotachophoresis, however, was found to be 10 times more sensitive.

It was important to ascertain whether the spacers used in this programme, at their respective concentrations, contributed to any degradation of the nucleotides. Additions of spacers were made to "pure" commercial nucleotides as well as standard addition analysis of the commercial reagent to the extract sample with spacers. Adjusting for the slight loss due to dilution, correlation coefficients for 12 nucleotides with and without spacers were above 0,9970.

Calibration curves were made by using increasing concentrations of standards, which included the presence of spacers for the UV-absorbing components. Such a comprehensive exercise has not been previously reported in

the literature and is included as a relevant part of this thesis (Figures 2.11 - 2.23). During this exercise runs were also made with standard addition analysis to the extracts. Correlation coefficients showed agreement as above when compared with the aqueous solution. However, it should be noted that certain of the calibration curves do not pass directly through the origin. There are two reasons for this, viz. interference of impurities in the electrolytes creating slight gains for certain of the nucleotides and slight losses in the system for certain of the organic acids. It was reasoned that these latter slight losses were as a result of either interaction between HPMC and the sample-ions, affinity to the capillary or some slight deterioration due to the electrolytic system.

Reproducibility between routine isotachophoretic runs was considered excellent with a coefficient of variation of $\pm$ 2% for nucleotide and organic acid components.

2.3.2.9 Measurement of Analysis Records

As, in isotachophoresis, zone length is directly proportional to the amount of ions in the zone, quantification in the majority of previous isotachophoretic studies has been simplified to measuring the lengths of the individual zones as they appear on the recorder chart. However, in this work, the extreme sensitivity of this instrument was taken advantage of to appreciate the generally very small amounts of each component present in the analyzed sample. Therefore, a different approach had to be adopted.

It was reasonable to suppose for the UV-absorbing components that the area under the UV absorbance curve, after conversion to optical density, would be proportional to the amount of material. Log-linear conversions and integration were carried out manually, rather than electronically, assuming that the curves had the form of a normal distribution pattern. In this way, it was possible to obtain a straight line relationship for quantification of nucleotides.

The non-UV-absorbing components were measured by the spacing between the bracketing of adjacent UV-absorbing zones. Two strategies were adopted, viz., measurement between the summits or between 1/2 OD max of the adjacent peaks. Both estimations provided linearity but the latter calculations proved superior in enabling appreciation to, or very close to, the origin.

Impurity zones due to impurities in the electrolytes were, as far as was feasible, always measured for each batch of electrolytes and deducted from respective contaminated sample zones.

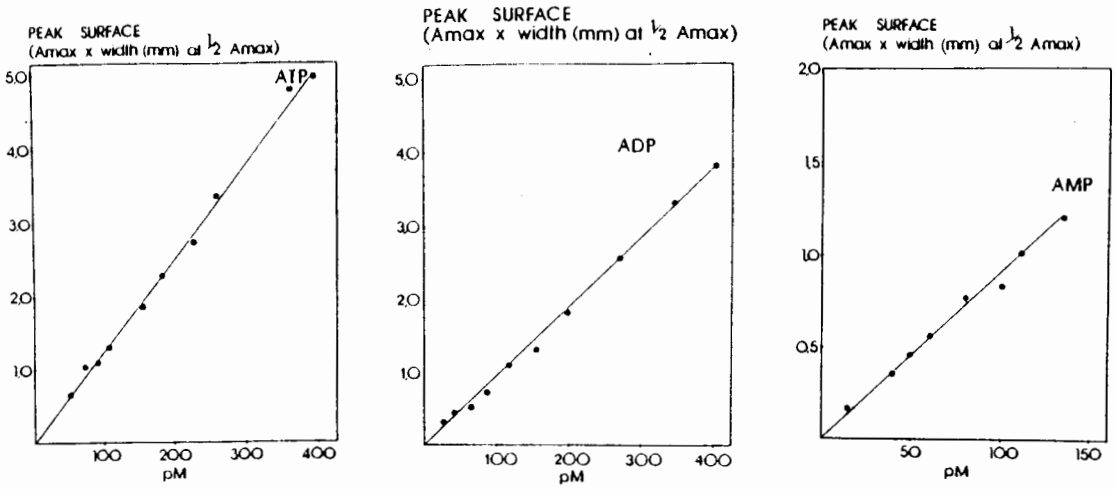


FIGURE 2.11: Linearity of adenine nucleotides in lower picomole range.

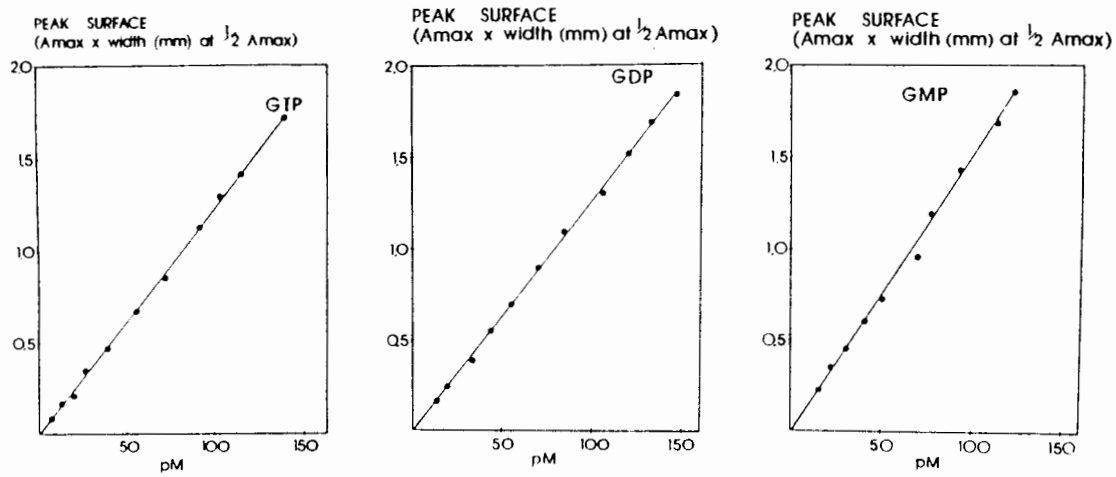


FIGURE 2.12: Linearity of guanosine nucleotides in lower picomole range.

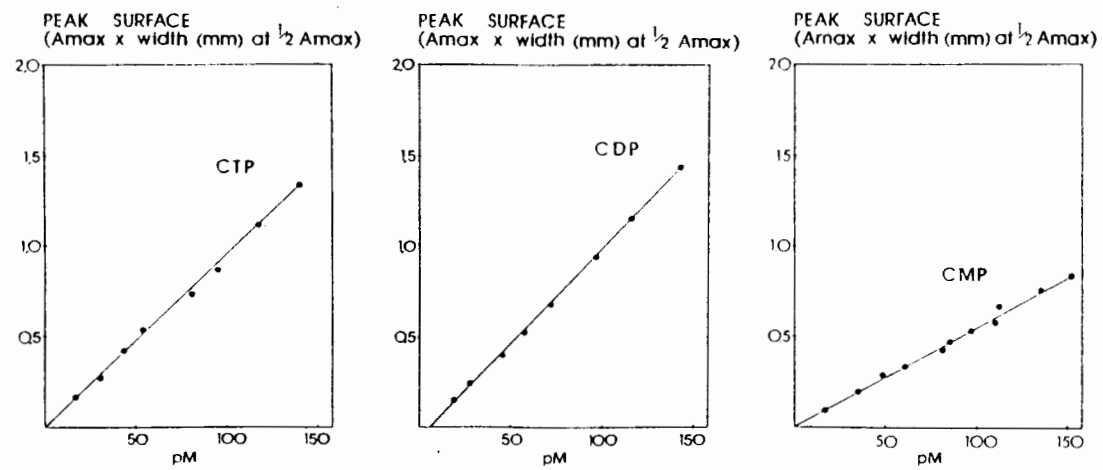


FIGURE 2.13: Linearity of cytidine nucleotides in lower picomole range.

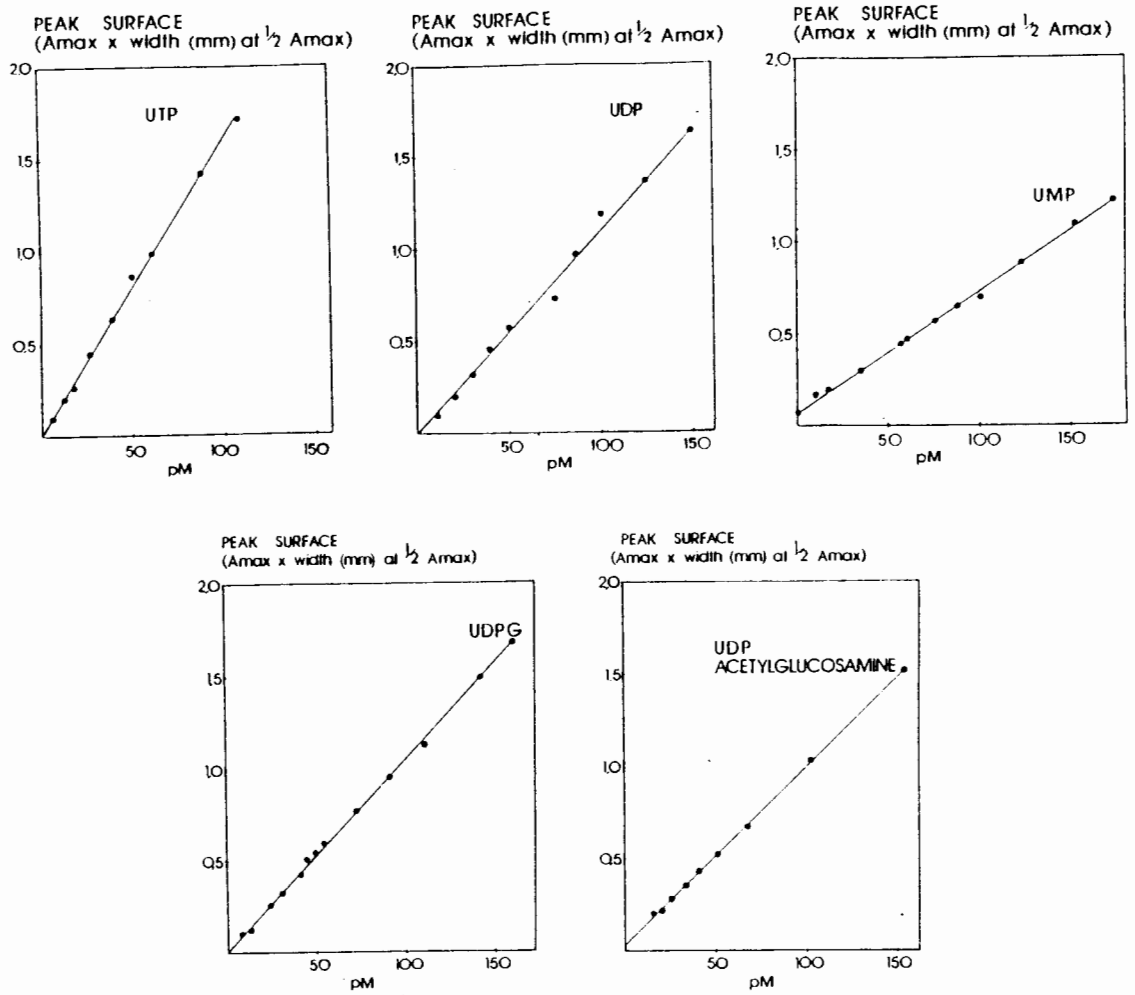


FIGURE 2.14: Linearity of uridine nucleotides in lower picomole range.

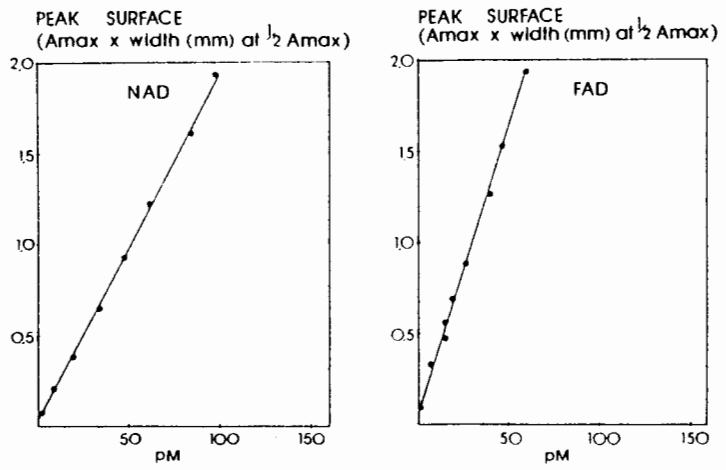


FIGURE 2.15: Linearity of NAD and FAD in lower picomole range.

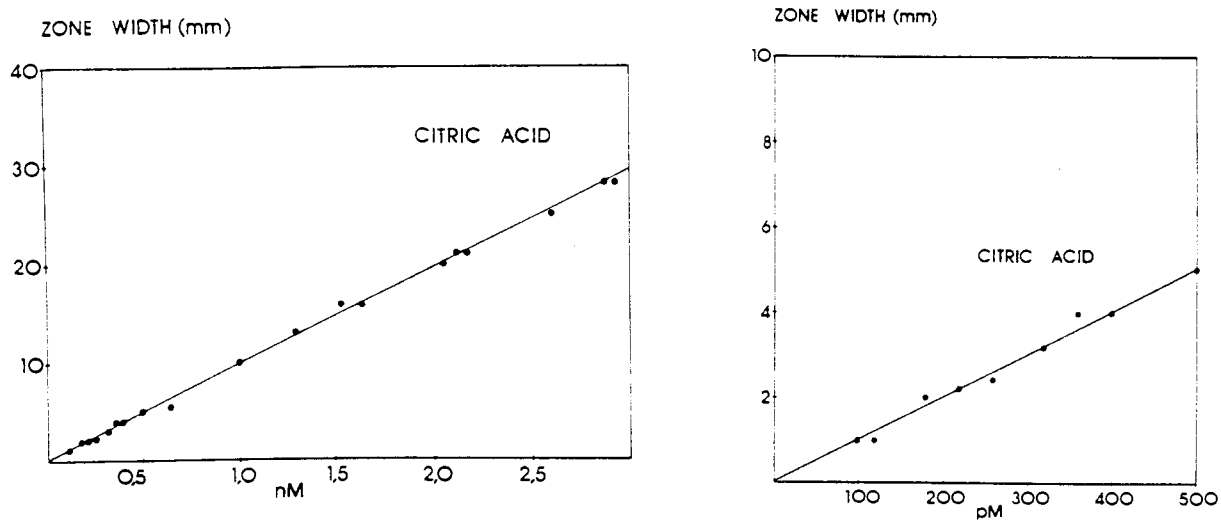


FIGURE 2.16: Linearity of citric acid in lower nanomole and picomole range.

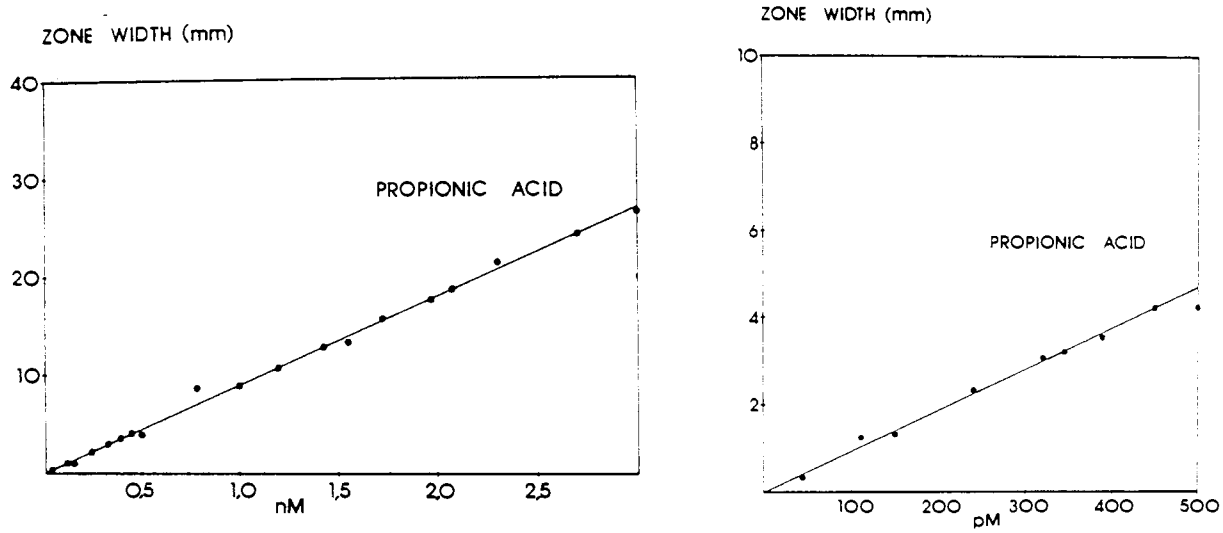


FIGURE 2.17: Linearity of propionic acid in lower nanomole and picomole range.

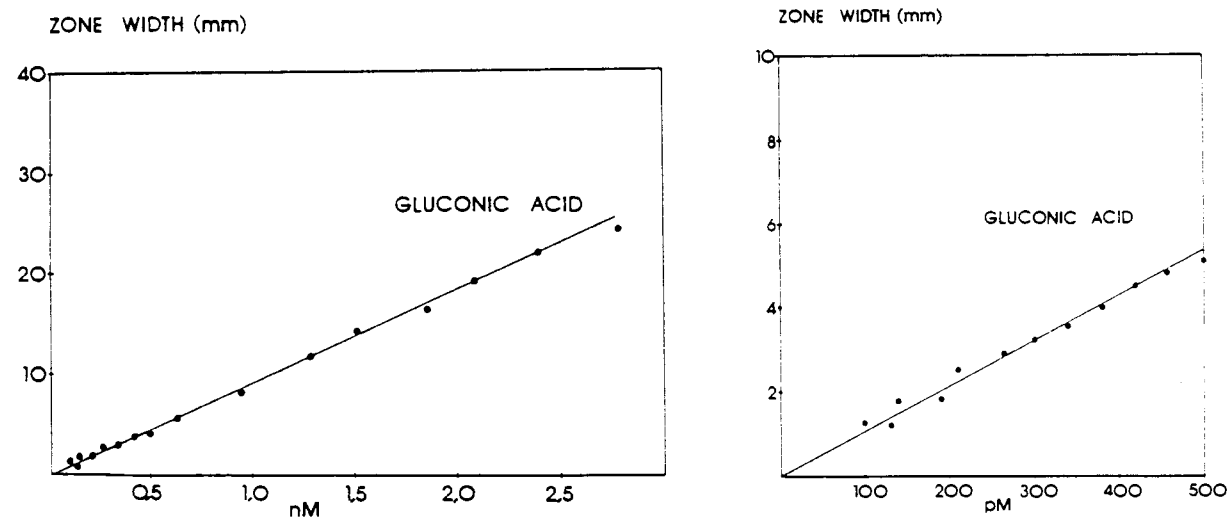


FIGURE 2.18: Linearity of gluconic acid in lower nanomole and picomole range.

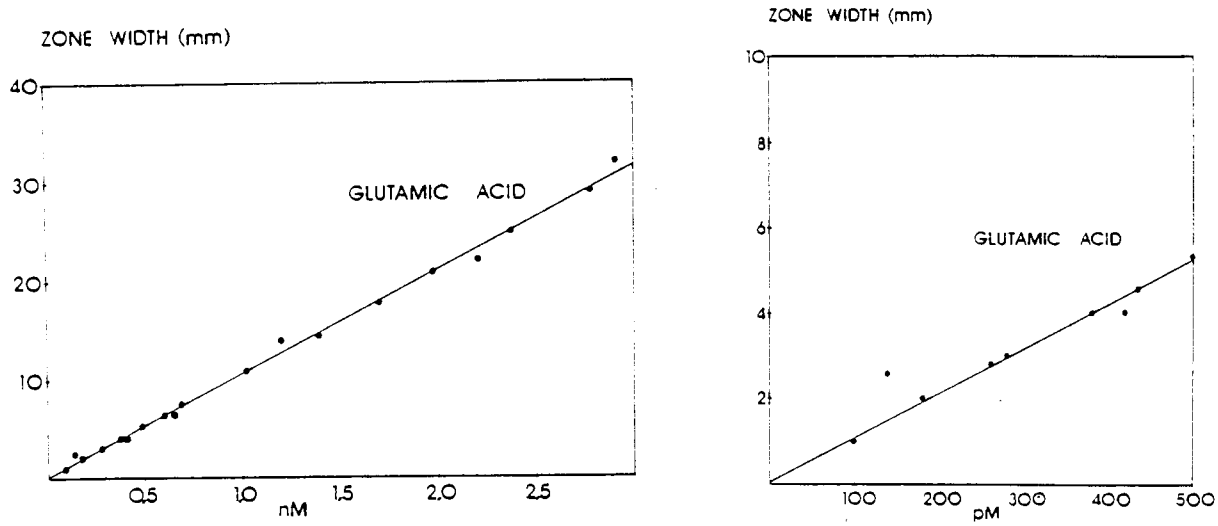


FIGURE 2.19: Linearity of glutamic acid in lower nanomole and picomole range.

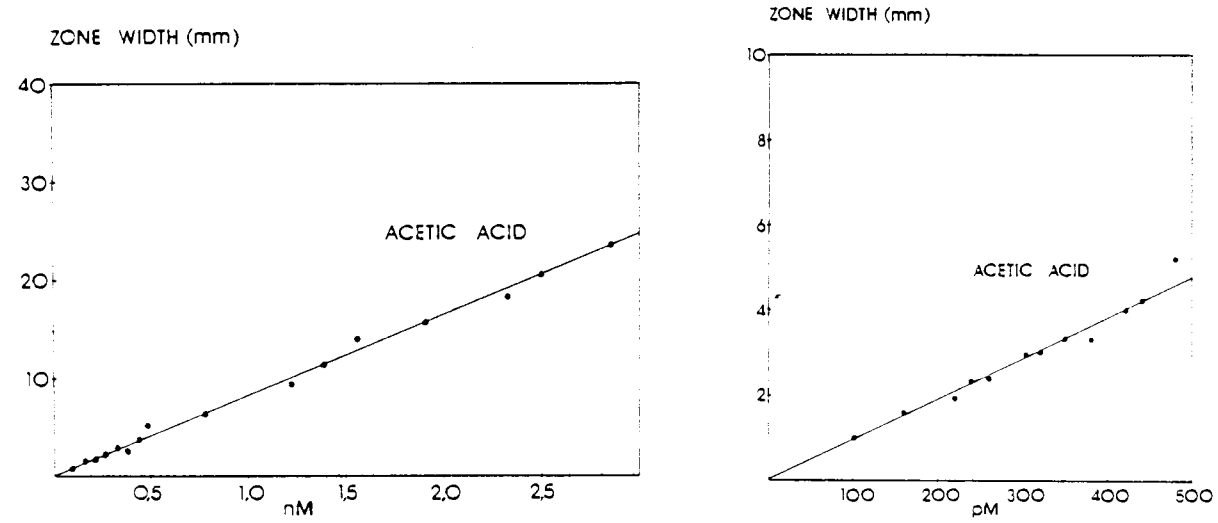


FIGURE 2.20: Linearity of acetic acid in lower nanomole and picomole range.

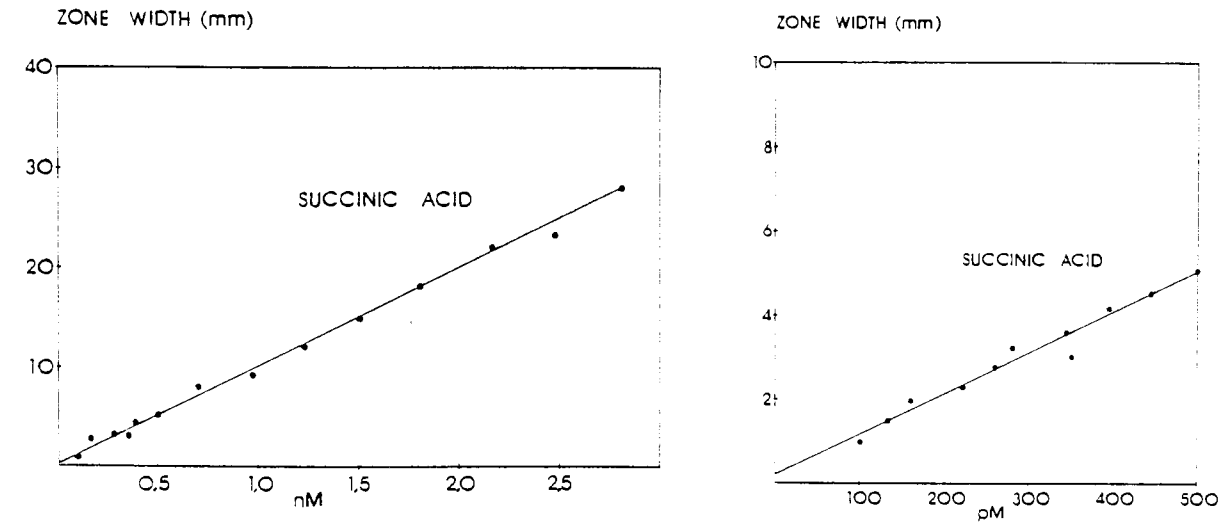


FIGURE 2.21: Linearity of succinic acid in lower nanomole and picomole range.

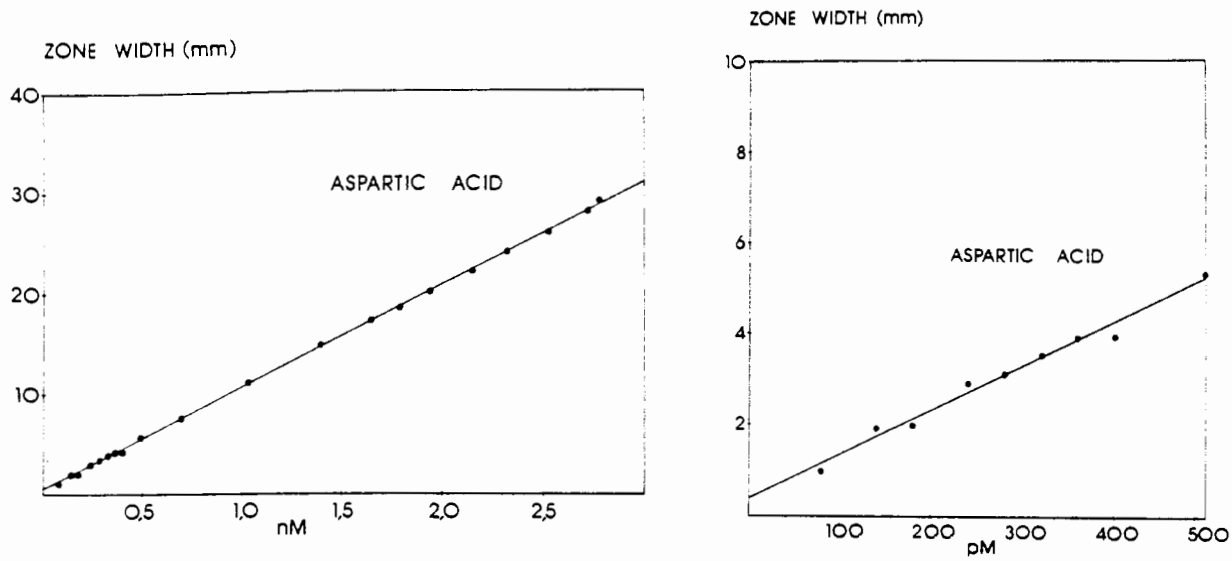


FIGURE 2.22: Linearity of aspartic acid in lower nanomole and picomole range.

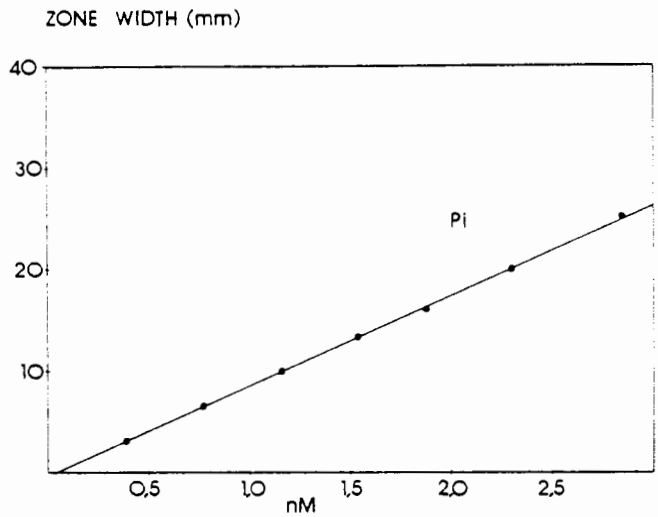


FIGURE 2.23: Linearity of inorganic phosphate in lower nanomole and picomole range.

2.3.3 Bioluminescence

The microassays for ATP and the sugar phosphate metabolites were specific for the components measured when compared with alternative analytical procedures, i.e. isotachophoresis and HPLC for ATP and spectrophotometry (Entian et al., 1977) for the hexose and triose phosphates. For the latter, measurement by bioluminescence increased sensitivity well into the picomole range. However, initial difficulties had to be overcome to achieve this level of sensitivity. These difficulties may be summarised as follows.

2.3.3.1 Inhibition by Complexes in the Cellular Extracts

Specific complexes in the extracts that were capable of interfering with analytical evaluation of the hexose and triose phosphates occasionally required further treatment. Before measurement, these extracts were treated with Polyamide TLC 6 at a rate of .50 mg per 1 ml of extract for 20 minutes at 0 °C followed by separation of the nylon by centrifugation at the same temperature.

2.3.3.2 Inhibition by Buffer System

Several buffers were appraised in the investigations. Particularly for hexose and triose phosphate measurement, imidazol buffer at pH 7,0 (optimal pH for NADH and NADPH measurement) (Karl et al., 1983) created a distinct inhibition for metabolite appreciation so that the linearity range for the compounds was poor. Phosphate buffer at pH 7,0 was finally adopted for this work. Figures 2.24 and 2.25 demonstrate the sensitivity and

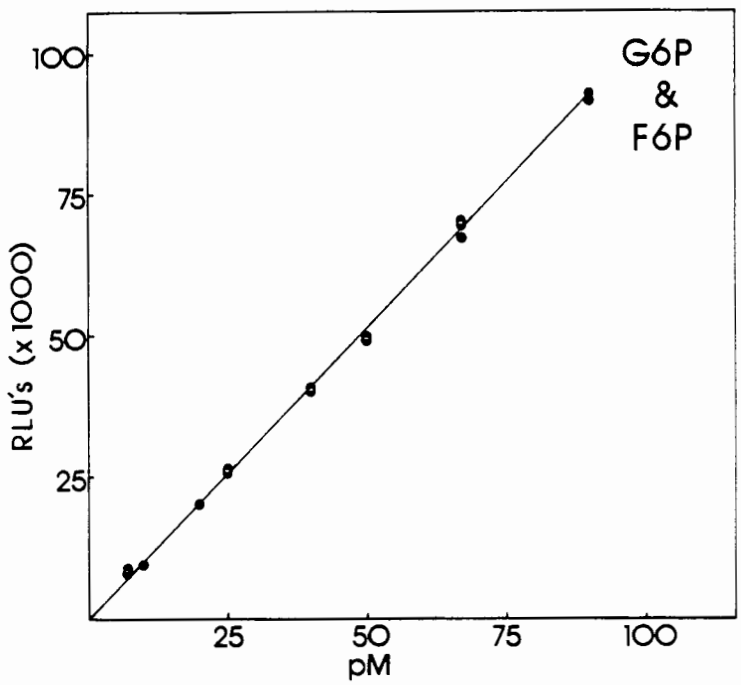


FIGURE 2.24: Linearity of bioluminescence for measurement of glucose-6-phosphate and fructose-6-phosphate following increase of NADPH in enzymatic reaction. RLU= relative light unit.

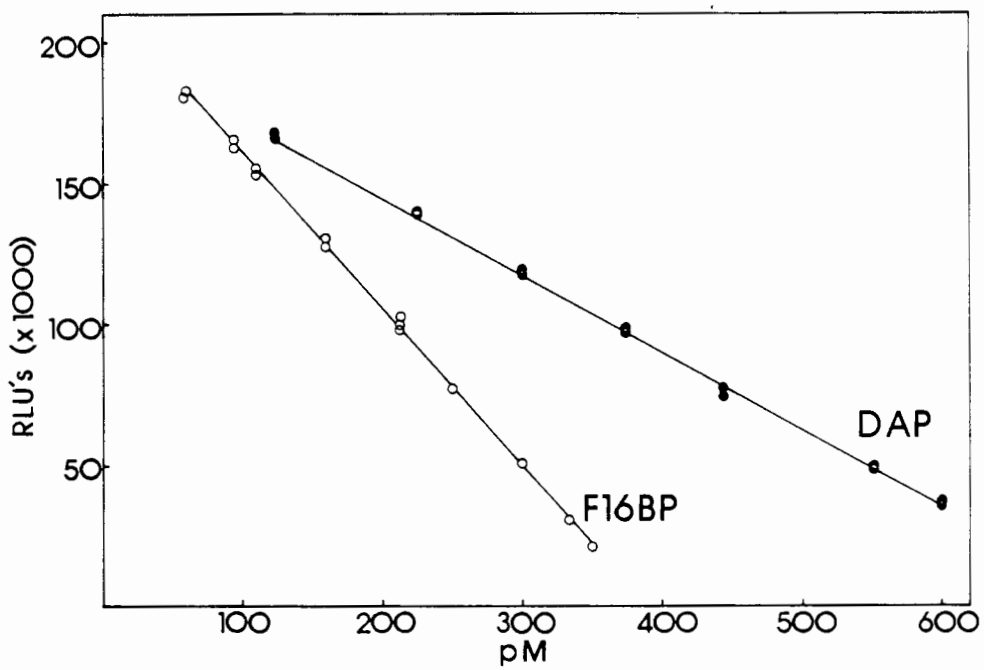


FIGURE 2.25: Linearity of bioluminescence for measurement of fructose-1,6-bisphosphate and dihydroxyacetone phosphate following depreciation of NADH in enzymatic reaction. RLU= relative light unit.

bioluminescence linearity range for the hexose phosphates measured, using this buffer.

2.3.3.3 Inhibition by NADP

For the assay of glucose-6-phosphate and fructose-6-phosphate the addition of NADP at concentrations higher than recommended in Materials and Methods, inhibited the bioluminescence response.

2.3.3.4 Photosensitivity of Reagents

FMN is photosensitive and it was therefore necessary to protect the final FMN-luciferase - aldehyde solution from light. As automatic injection of these reagents was employed, the reagent vial and injection capillary were wrapped in aluminium foil to reduce photodegradation. This strategy also helped to reduce phosphorescence from the reagents which could contribute to high blank values.

2.3.3.5 Time Course for Bioluminescence Response

Samples were counted until bioluminescence achieved maximum intensity. The studies showed that, using the reagents described, a 10 second time course was satisfactory for ATP appreciation and a 60 second time course sufficient for the fructose-6-phosphate, glucose-6-phosphate, fructose-1,6-bisphosphate and triose phosphate studies. The bioluminescence response for the hexose phosphates showed an initial rapid augmentation subsequently followed by a period where the increase was slower, to plateau within one minute. The light signal then declined after approximately two minutes.

2.3.3.6 Reproducibility of Bioluminescence Assays

Experiments were conducted to ascertain the reproducibility of the bioluminescence reactions as well as the initial enzymatic reactions for hexose and triose phosphate evaluation. Successive tests showed that, as for isotachophoresis, the coefficient of variation was usually within $\pm$ 2% and never greater than 3%.

2.4 DISCUSSION

2.4.1 Cell Permeabilization and Intracellular Metabolite Extraction

It has previously been reported that centrifugation has a detrimental effect on ATP levels of E. coli (Cole et al., 1967). Lundin and Thore (1975) showed that centrifugation decreased the amount of adenine nucleotides extracted, by 10 - 50% in E. coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae and led to low energy charge values.

Equally, deleterious effects of membrane filtration on the final ATP concentrations of biological extracts have been highlighted (Jones and Simon, 1977; Karl and Holm-Hansen, 1978; Sutcliffe et al., 1976; Sinclair et al., 1979). Wiebel et al. (1974) found that filtration at 4°C decreased ATP values in S. cerevisiae. Karl and Holm-Hansen (1978) paid particular attention to cell density and to the pressure differential during the sampling procedure. In considering the latter point these workers attributed metabolic stress to be proportional to the vacuum imposed. In contrast, Rudd and Hamilton (1973) were unable to demonstrate any effect of

membrane filtration on ATP levels of a variety of biological samples. In addition, the same ATP level was found following filtration of Myxococcus xanthus (Hanson and Dworkin, 1974) and E. coli (Lowry et al., 1971), where high energy charge values have been observed even after a 10 minute delay imposed by filtration (Franzen and Binkley, 1961).

During the above evaluation programme it was found that by substituting centrifugation by membrane filtration at 0°C (as described for the boiling water extraction procedure), improvements could be made to the extraction procedure. However, particular attention during the membrane procedure was paid to ensuring a minimum of stress by vacuum. Hence, a low-vacuum water device was employed as well as using a porosity of membrane large enough to facilitate ease of flow without creating a risk of cell pull-through. Additionally, care was always taken to immediately break the vacuum on terminating the filtration.

Washing of the yeast cells with ice-cold double-distilled water could easily be achieved speedily using the membrane filtration method. This was found to be important as nucleotide and organic acid levels in the substrate could be significant, particularly in stationary phase populations. Exogenous ATP has previously been found to be important when considering bacterial levels in contaminated foods (Sharpe et al., 1970). This has been reinforced by Chapman et al. (1971), Moses and Sharp (1972) and Miović and Gibson (1973) where adenine nucleotides have occasionally been shown to be excreted into the growth medium.

It should be noted, however, that excessive washing will leach intracellular intermediates, particularly the hexose phosphates, as was demonstrated during the course of this work. Cole et al. (1967) showed that excessive

washing caused a 90% decrease in ATP of E. coli.

There are probably a number of reasons why acids may not be considered optimal metabolite extracting agents (Dhople and Hanks, 1973), although Lundin and Thore (1975) considered that trichloroacetic acid extraction of adenine nucleotides from bacterial cells most accurately reflected physiological levels when compared to a variety of other methods.

A decrease in the penetrability of the cell membrane-wall complex (and subsequent leaching efficiency) may result, particularly when acids are used at concentrations in excess of 1 M (Dhople and Hanks, 1973). In addition, the acidic conditions, as shown in this work, will promote nucleotide hydrolysis. Also, when small quantities of acids are used the only practical method of achieving a reasonably accurate neutralization is to titrate in the presence of a colour indicator. This point is important for detection of ATP by bioluminescence where the optimum pH should be 7,75 (Lundin and Thore, 1975).

Irrespective of pH, the use of solvents in extraction procedures creates quenching problems for measurements by bioluminescence. This was found to be the case, to a high degree, using butanol as an extractive agent because it is difficult to eliminate due to its high boiling point (118°C). Elimination difficulties caused extreme problems for accurate isotachophoretic measurements. Acetone, although recommended by others (Hysert and Morrison, 1978; Miller et al., 1978), for the extraction of adenine nucleotides from yeast cells, was not the ideal agent in this work. It is possible that the higher cell density as used in this study when compared to the lower original recommendation, may have an important bearing on extraction efficiency although this point could not be substantiated by auxiliary experiments at lower cell values.

For the boiling water technique it was found to be critical not to exceed a sample size equivalent to 100 mg dry yeast. Smaller sample sizes (90 mg, 60 mg and 45 mg dry yeast) were found to provide linear metabolite values to those originating from the 100 mg dry yeast sample. Larger sample sizes (150 mg, 200 mg dry yeast) did not reflect this trend.

Boiling water extraction techniques have not been recommended by Beutler and Baluda (1963) for adenosine triphosphate extraction from blood cells. In their studies, ATP was thought to be lost in the denatured cellular coagulum after boiling. This was not found to be the case for the described experiments with yeast cells. This is to say that recovery of the required intermediates was achieved by one extraction; second and third extractions were found to be superfluous.

Lundin and Thore (1975) found it necessary to add 2 mM EDTA to the extraction agents studied for adenine nucleotide release from bacterial cells. The presence of EDTA helped destabilise nucleotide hydrolyzing enzymes, e.g. ATPase, by complexing metal cofactors. Certainly, this is a very worthy safeguard and one which may be essential with specific extraction procedures.

For the boiling water extraction procedure described, EDTA made no difference to nucleotide triphosphate levels providing the outlined conditions were strictly adhered to. Reproducibility investigations using replicate samples showed no difference in nucleotide levels. Moreover, the presence of EDTA in extracts for isotachophoretic runs highlighted a U.V. absorbing complex zone which interfered with measurements for the nucleotide diphosphates. It was therefore finally decided to exclude EDTA from the method parameters.

The majority of deleterious effects of sampling and extraction, generally results in decreased concentrations of the nucleotide triphosphates (Karl, 1980). Although it can never be said with certainty that a given extraction method demonstrates the true physiological cellular value, it follows that a high yield of nucleotide triphosphates should be taken as the main criterion in evaluating the relative extraction efficiencies of various methods and is more likely to reflect the true nucleotide content.

It was concluded that the boiling water extraction method for brewing yeast cells, as described, met the requisites previously outlined for a successful extraction procedure. Degradation of the nucleotides in these extracts, when kept in ice, was found to be less than 3% per day. Deviations from the described procedure resulted in inefficient extraction or in measurable changes to required intermediates.

The choice of an extraction method for a given microorganism will obviously be purpose specific. Therefore, as in this study, the final choice should rest with a comparison of methods. In this context it would appear that existing data on microbial nucleotide pools may be misleading because of incomplete extraction and failure to quantify the deleterious effects of the extracting agent.

2.4.2 Isotachophoresis

It became apparent during this study that isotachophoresis, as described in this text, can provide specific and reproducible analyses of nucleotides and organic acids in yeast cellular extracts and that this technique is capable of achieving sensitivities in the picomole range. Thus, this

method exhibited a sensitivity often greater than alternative techniques and avoided such complications as cycling reactions, as used for fluorimetry. Moreover, if sufficient of the analyzed components were present in the sample size, isotachophoresis could provide an evaluation of nucleotides and organic acids in the same electrophoretic record. Where organic phosphate material occurred in the same zone as an organic acid, and this was found to be the case for glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-bisphosphate, these components were measured by bioluminescence, and deducted from the respective zones. Of course, it is still possible that unidentified non-UV-absorbing material could have been contaminating other organic acid zones. Alternative electrolytic conditions did not reveal such contamination and it was therefore assumed that, if it was occurring, it was at negligible concentrations to make a meaningful difference to the accuracy of the components measured. When two non-UV-absorbing components occurred together in the same area, it was usual to find, as with glutamate and acetate, that slight differences in effective mobility and absorption caused either a slight step height difference in either the thermal detection or UV detection records and/or a noticeable pulse on the UV detection record between the two components caused through the change in conductance.

In addition, and to my knowledge, this is the first reported work capable of tabulating organic acid measurements with respect to the tricarboxylic acid cycle. Previous works have mostly concentrated on foods and beverages (Kaiser and Hupf, 1979; Everaerts and Verheggen, 1974) with some specific biotransformation work by Sollenberg and Baldesten (1977) who quantified the aromatic acids excreted in urine after exposure to styrene, toluene and xylene.

2.4.3 Bioluminescence

Variable inhibition was encountered for the bioluminescence response during the initial stages of this work. However, such inhibition could be reduced by the nylon treatment described without demonstrable loss of hexose and triose phosphates. Nevertheless, it was important to correct for the concentrating effect of the polyamide in order to realise accurate metabolite values. For ATP such nylon treatment was not found to be necessary probably because of the different properties of firefly luciferase as compared to bacterial luciferase (Karl, 1980) but also because of the greater dilution effect of the extracts for ATP determinations.

Analytical interference could also be caused by the extraction agent. Perchloric acid and/or the presence of chelating agents, e.g. EDTA, cause noticeable interference. Certain solvents, e.g. butanol, produce quenching effects and in such cases it is necessary to remove the extractant before the assay (Thore, 1979). Re-use of polystyrene sample cells may also result in interference (Palmisano and Schwartz, 1982).

Irrespective of the type of interference, the degree can only be appreciated by the inclusion of internal standards as described in this text and is a pre-requisite for accurate bioluminescence analysis.

The pH optima and temperature sensitivity of the pyridine nucleotide bacterial luciferase were particularly noted. Maintaining the pH at 7,0 and the temperature at 25°C during the reaction was of extreme importance as was ensuring that all reagents before use were maintained in ice to preserve integrity and/or activity. These precautions were

equally applicable to the firefly luciferase except that the final assay pH was between 7,75 and 8,0 - optimal for this enzyme (Lundin and Thore, 1975).

Phosphorescence, besides being caused by exposure of the final FMN-luciferase-aldehyde solution to intense, especially fluorescent, light may equally be as a result of the polystyrene sample cells used. In this type of analysis, therefore, this should be checked and sample cells exhibiting low or negligible phosphorescence should be used.

In conclusion, the assays for ATP and the hexose and triose phosphates in the yeast cell extracts presented here are sensitive, specific and of relatively simple format. When compared to alternative techniques, these assays show an advantage particularly with respect to measurement in the picomole range. For the hexose and triose phosphates, a further advantage when compared to microfluorometric techniques is the absence of complicated reactions to achieve this sensitivity. Providing that the precautions as highlighted are observed, inhibition or interference difficulties will be minimal. The expense of the commercial reagents used may detract from its viability as a method for routine purposes. Nevertheless, when small amounts of components are available, and considering the speed of measurement by this technique, bioluminescence becomes attractive.

CHAPTER 3

THE RELATIONSHIP OF FERMENTATION CAPACITY AND YEAST GROWTH

SUMMARY: Fermentation capacity of the yeast cell as related to growth and cyclic variations in the physiological state of the cell has been examined. Experiments were conducted using commercial brewing strains and strains which were genetically modified to be incapable of synthesising isoleucine, valine and leucine. Assuming transitional stages, glycolytic flux in brewing yeast fermentations may be delineated into two distinct states in which the yeast cell exists, viz., the growth phase in which the specific fermentation rate for each of the wort fermentable sugars reaches a maximum and the longer stationary phase where growth is terminated and fermentative power progressively declines and maintenance activities take precedence.

Loss of fermenting power of the yeast population may be created by a condition that limits amino acid formation and protein synthesis. In commercial brewing strains this loss may be transitory or, if not corrected, may ultimately lead to yeast degeneration. The potential industrial impact is realised for fermentation systems which may limit yeast growth, eg., continuous systems, use of pressure and, particularly, systems utilizing immobilised cells.

3.1 INTRODUCTION

There are few studies reported in the literature that demonstrate the precise relationship of fermentation rate as a function of yeast growth.

Slonimski's classical work (1953) studied three different anaerobic cultures of a strain of baker's yeast in defined phosphate buffer lacking an assimilable nitrogen source. Under these non-growing conditions, Slonimski observed a sequential loss of fermentative activity for glucose (measured manometrically as $Q_{CO_2}^{N_2}$) to a level of 13% of the initial value after 21 hours. Although the rate of loss of fermentative activity was temperature dependent, all cultures eventually became incapable of fermenting glucose until a complete growth medium was restored. Slonimski, however, did not specify the factor (s) necessary for the yeast to be able to follow a fermentative metabolism in the absence of cellular proliferation.

This transitory loss of fermenting power was further studied by Masschelein et al. (1960) in brewing yeast fermentations. These workers attributed loss of the glucose fermenting system to impoverishment of the intracellular amino acid pool. In nitrogen-starved cells the effects of replenishing the amino acid pool by incubation in a medium containing casein hydrolysate, re-established fermenting power. Ploss and Eschenbecher (1981) supported similar conclusions regarding the dependence of fermentation capacity on the level of the intracellular amino acid pools.

Harris and Millin (1963) and Masschelein et al. (1965) followed this early work by studying the cyclic nature of fermentation capacity during wort fermentations. Harris and Millin (1963) demonstrated fermentation capacity levels for a strain of S. cerevisiae for glucose, maltose and maltotriose with respect to wort gravity during fermentation; Masschelein et al. (1965), also using a strain of S. cerevisiae, correlated the changes in fermentation capacity for glucose and maltose to the yeast growth cycle during fermentation. It was shown for these sugars that maximum

fermentation capacity levels were reached during the growth phase.

Since these specific investigations a re-analysis of the relationship between fermentation capacity and the dependency on yeast growth has not been reported. It was therefore considered prudent, as part of this study, to re-establish such data in order that the extent of this dependency may be fully appreciated.

Brewing strains of S. uvarum (carlsbergensis) were examined under varying growth conditions. In specifically investigating the effect of limiting amino acid metabolism, a commercial brewing strain was used genetically altered in its ability to synthesise the key amino acids, valine, leucine and isoleucine.

3.2 MATERIALS AND METHODS

3.2.1 Maintenance of Brewing Yeast Strains (S. uvarum (carlsbergensis))

Media are listed in Appendix A. Specialized chemicals and their sources are listed in Appendix B.

For long term maintenance of S. uvarum, the strains were cultivated and kept (@ $0^{\circ}C$) in M.Y.G.P. medium (Wickerham, 1951). For routine use, the cultures were maintained on wort agar.

3.2.2 Propagation of Brewing Yeast Strains

Propagation procedures are outlined in Appendix C.

3.2.3 Infection Monitoring

All cultures and fermentations were monitored for bacterial and wild yeast infections according to the methods outlined in Appendix D.

3.2.4 Fermentation Conditions

Malt wort at ± 12 °P was fermented in tall tubes at 11 °C (as designed and correlated to brewery fermentations - see below) as described by Ryder et al. (1980) and Kruger et al. (1982), or in stirred fermentations as described by Masschelein et al. (1960, 1965).

Pitching was undertaken at 6 g/l (centrifuged), the substrate always having an initial dissolved oxygen value of 8,5 ppm to meet the brewing yeast strains' oxygen requirements. Two commercial flocculent lager strains of S. uvarum (carlsbergensis) were mainly employed during the test procedures (strain 2036 from the Alfred Jørgensen Collection, Copenhagen, Denmark and strain 167 from the C.E.R.I.A. Collection, Brussels, Belgium), although several other commercial strains of S. uvarum and S. cerevisiae were used in establishing general trends.

3.2.4.1 Reproducibility of Fermentation Patterns in Tall Tubes

To examine the reproducibility of fermentation patterns, experimental fermentations were carried out at 11°C using brewery worts collected after chilling at a point before aeration. Wort aeration and pitching were conducted according to standard procedures. An example of the patterns obtained is shown in Figure 3.1 where Brewery wort 'A' after pitching with yeast 'a' was fermented in the described laboratory conditions in quadruplicate.

3.2.4.2 Comparative Fermentation Studies between Tall Tube and Brewery Fermentations

A series of tests was run of which some representative results are shown in Figure 3.2.

Wort samples were drawn and treated according to 3.2.4.1 above or after aeration and pitching from the fermenting vessel. Fermentations using these samples were then undertaken at 11°C and the fermentation patterns compared against those of the respective brewery fermentations. For completeness various sizes of fermentation vessels were included in the study, i.e. 330 hl and 2640 hl cylindroconical vessels, and 1 000 hl horizontal fermenters.

The results showed that fermentation velocities and °Plato on the 8th day of fermentation, for each set of experiments, were very similar. Only small differences in gravity during fermentation were apparent and results at the end of fermentation were within 0,1°P. Calculated from a series of 10 comparative studies for each vessel size and type, the correlation

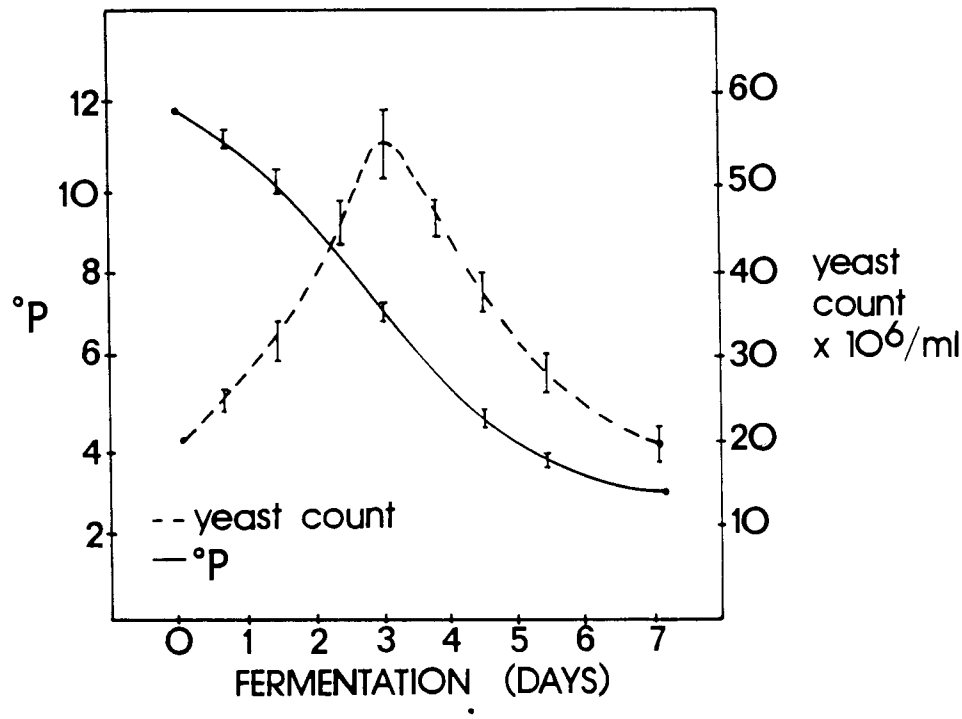


FIGURE 3.1: Reproducibility of fermentation patterns in tall tubes (Kruger et al., 1982).

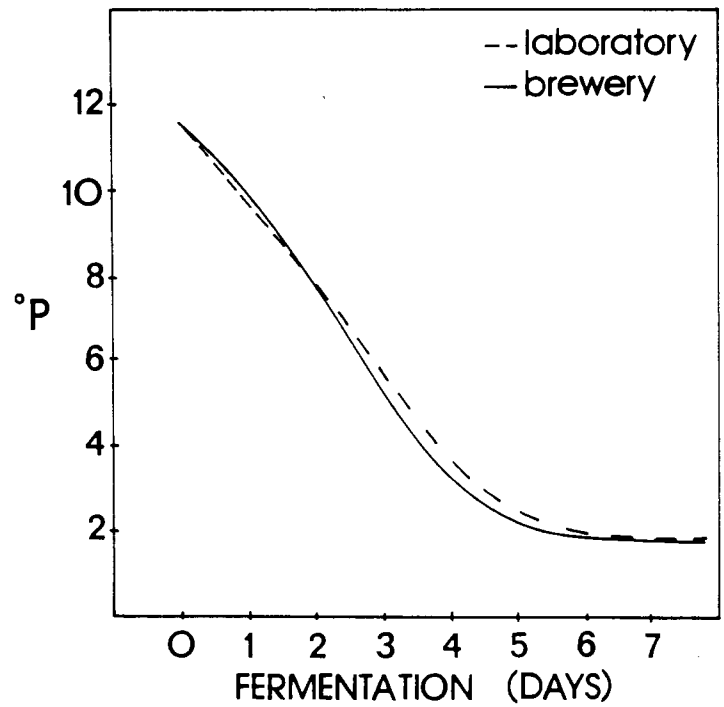


FIGURE 3.2: Comparison between laboratory and brewery fermentations.

coefficient for ^{o}P on the 8th day between brewery and laboratory fermentations was 0,96 with a standard error of 0,07. The correlation coefficient for ^{o}P at 96 hours was 0,97 with a standard error of 0,15.

3.2.5 Fermentation Sampling

As previously described in 2.2.1.5.

3.2.6 Yeasts Counts

Conducted according to Appendix E.

3.2.7 Manometric Measurements

Cells were harvested during the fermentation cycle, washed three times in ice-cold M/15 KH_2PO_4 buffer at pH 4,5 and resuspended in the same buffer to an O.D. of $\pm$ 0,600 for a 1 cm cuvette. All other conditions were as previously described by Masschelein *et al.* (1960) using the manometric method of Warburg (see Umbreit *et al.*, 1951) under anaerobic conditions. Measurement of the fermentation rates of glucose, maltose and maltotriose was at non-limiting concentrations. Fermentation capacity was expressed as $Q^{N_2}_{CO_2}$ being ul of CO_2 evolved per h per mg dry weight of yeast.

3.2.8 Rare Mating

During studies on the genetic control of the formation of vicinal diketones in yeast, Ramos and Masschelein (1977) isolated mutants lacking acetohydroxy acid synthetase or threonine deaminase from a haploid laboratory strain of *S. cerevisiae* by treatment with ethylmethanesulfonate. Subsequent hybridisation studies revealed recombinants lacking both these enzymes (*ivl 1*$^-$, *ivl 2*$^-$).

Due to the non-sporulating or weakly sporulating nature of polyploid or aneuploid brewing yeasts, it was decided for this study to take advantage of the rare mating ability (Pomper *et al.*, 1954) occasionally displayed by such yeasts when crossed with haploid strains. In the present study, the respiratory deficient (RD) - auxotrophic mating method (Gunge and Nakatomi, 1972; Spencer and Spencer, 1977, 1979) was employed in an attempt to cross certain of the described haploid mutants with a marked strain of AJL 2036 in order to obtain hybrids incapable of synthesizing isoleucine, valine and leucine. A single mutant, N41 (*ivl 2*$^-$), *a* mating type, and a double mutant, DM33 (*ivl 1*$^-$, *ivl 2*$^-$), also *a* mating type, were used in these studies (*ivl 1*$^-$: auxotrophic for isoleucine by lacking threonine deaminase; *ivl 2*$^-$: auxotrophic for isoleucine, valine and leucine by lacking acetohydroxy acid synthetase (Figure 3.3). Thus, these genetically marked auxotrophic strains were used to recover the products of direct, rare, mass matings between the auxotrophs and petite mutants of the prototrophic brewing strain.

3.2.8.1 Isolation of RD Mutants from Prototrophic Strain

RD mutants of strain AJL 2036 were induced and isolated on acriflavin medium (Ephrussi, 1953).

3.2.8.2 Growth Conditions of Parental Yeasts

The two marked parental strains were grown for 16 hours at 30 °C under agitated conditions in 20 ml of yeast extract - glucose medium.

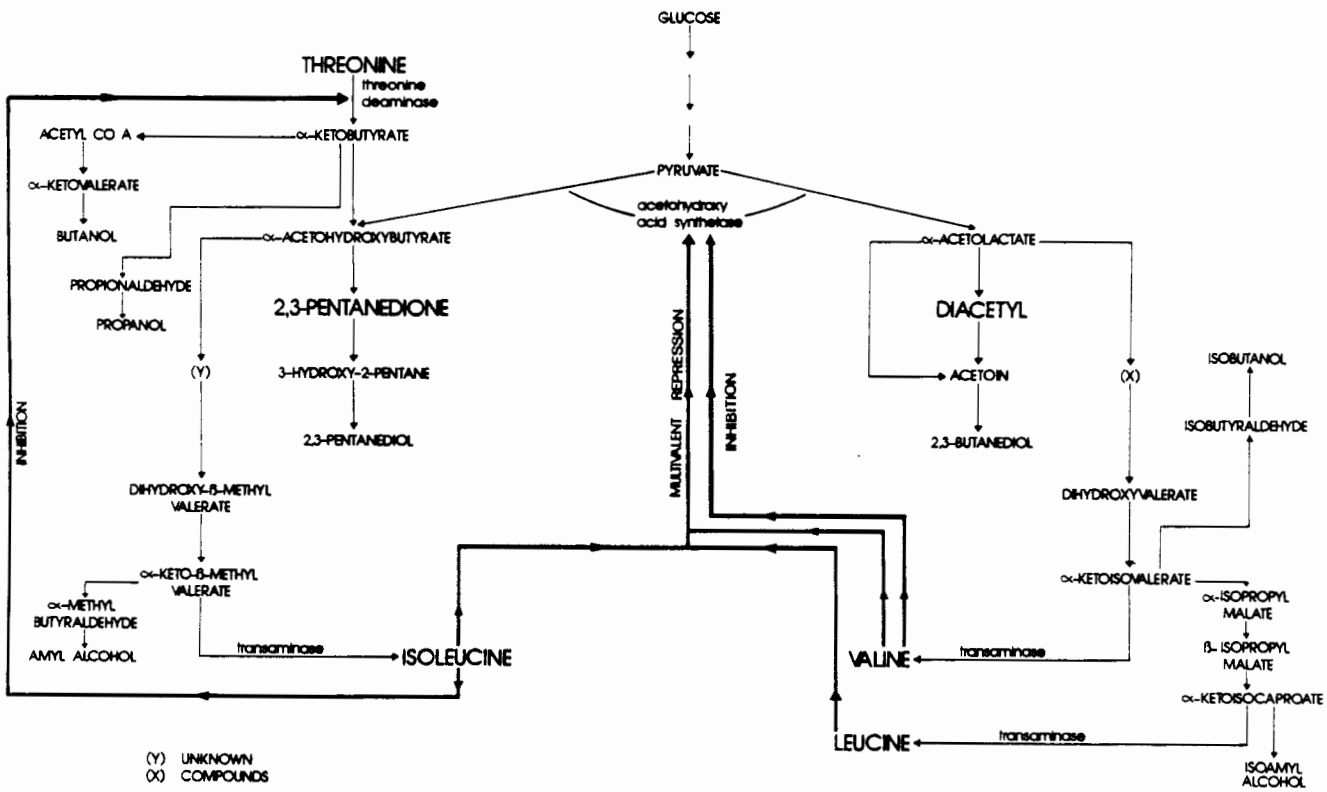


FIGURE 3.3: Regulation of isoleucine, valine and leucine metabolism.

3.2.8.3 Rare Mating Technique

The strains were harvested by centrifugation under aseptic conditions, suitably diluted in sterile double-distilled water and counted. Samples containing 10^8 cells of each strain were mixed and plated on to three plates of yeast extract - glucose agar. Samples containing 10^8 cells of each parent acted as controls.

Following incubation for 16 hours at 30°C each test culture was recovered in 2 ml of sterile double-distilled water and each control culture in 1 ml of sterile double-distilled water. Approximately 10^8 cells of each culture were individually plated on to a selection medium consisting of minimal agar with glycerol as the sole carbon source. This selection medium permitted the development of hybrids only which, after 5 days incubation at 30°C, were replicated in quadruplicate on to yeast extract - glucose agar and subsequently on to acetate agar (Fowell, 1952) and examined microscopically for sporulation after 3 - 10 days at 25°C.

3.2.8.4 Isolation of Ascospores

Mass isolation of ascospores (Emeis, 1965) was achieved by washing the sporulation medium with 1,5 ml of 0,5% Tween and subsequently breaking the ascus walls using glass beads (0,25 mm diameter) in a Braun vibrator for 15 seconds at 0°C. The ascospores were then separated from the cellular debris by centrifugation in the presence of liquid paraffin, the spores layered at the aqueous/organic interface. Single spore clones on yeast extract - glucose medium were then tested for mating type and auxotroph (isoleucine, leucine, valine auxotrophs).

3.2.8.5 Selection of Hybrids on Maltotriose Medium

For the ivl 1⁻, ivl 2⁻ mutants isolated by Ramos and Masschelein (1977), poor fermentation characteristics were found to be displayed for maltotriose. Thus, in order to ensure that the rare recombinants were capable of utilizing maltotriose, a selection was made after growth at 22 °C on a medium containing 0,3% (w/v) maltotriose. Those colonies that utilized maltotriose rapidly, at a similar rate to strain 2036, were tested further for growth and fermentation capacity characteristics.

3.2.8.6 Measurement of Vicinal Diketones

Hybrids devoid of threonine deaminase and acetohydroxy acid synthetase should be unable to produce the acetohydroxy acids leading to the formation of the vicinal diketones, diacetyl and 2,3-pentanedione (Figure 3.3) (Ramos and Masschelein, 1977). Thus, in order to more completely clarify the metabolic lesions of the rare recombinants, gas chromatographic headspace analysis was used for the measurement of these diketones during wort fermentations.

3.3 RESULTS

3.3.1 Fermentation Capacity under Growth-promoting Conditions

Manometric determinations for the wort sugars, glucose, maltose and maltotriose were undertaken during wort fermentations using stirred and tall tube conditions. Figure 3.4 shows an example of the results obtained for strain AJL 2036 in tall tube fermentations and demonstrates that each sugar reached a fermentation capacity maximum during the growth phase,

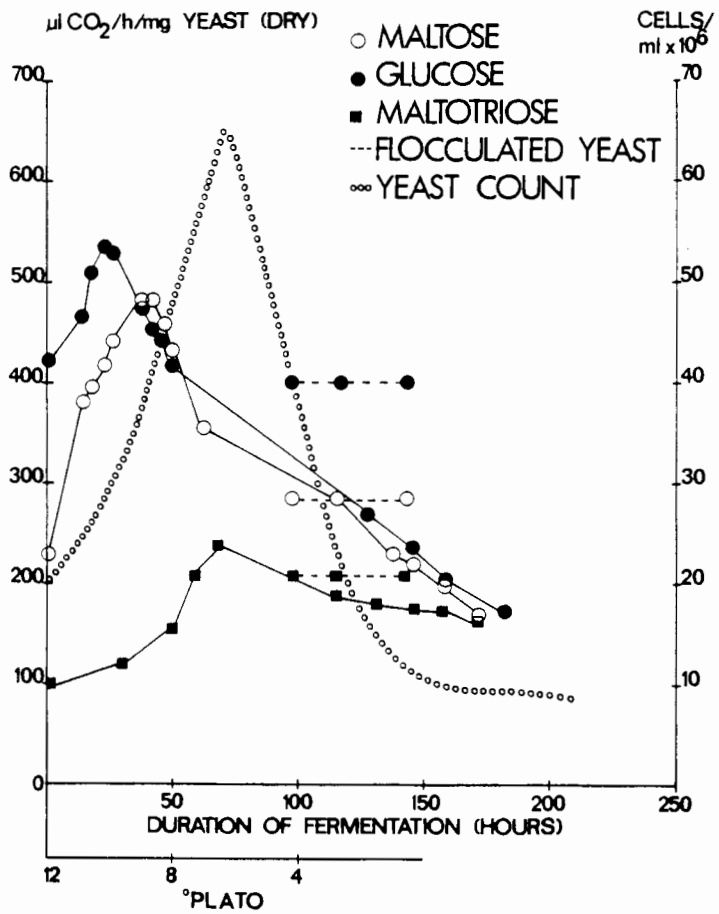


FIGURE 3.4: Fermentation capacity of strain 2036 for glucose, maltose and maltotriose in 12 °P wort at 10 °C. Tall tube fermentation.

though at varying times during this period.

Levels for all three sugars diminished rapidly during cessation of growth as the yeast entered the stationary phase and flocculation commenced. Thus, fermentation capacity for glucose peaked at 10,2°P during the early exponential growth phase. Maltose became maximal at 8,4°P and exhibited a considerably greater fermentation rate than that of maltotriose which subsequently peaked at 6,3°P before diminishing to a maintenance level. Other interesting features concerning this pattern were the fermentation capacity commencement point for glucose which was higher than for maltose and maltotriose, and the staggered peak values for all three wort sugars, particularly maltose and maltotriose, which were dependent upon individual permease systems for their uptake.

Other strains tested were similar with respect to the above trend but showed differing fermentation capacity peak levels for each of the wort sugars and, significantly, the diminution of fermentation capacity was found to be extremely variable from strain to strain.

It is interesting to note that, for all strains examined, the flocculated yeast had a substantially higher fermentation capacity per unit yeast than the yeast remaining in suspension.

The above results prompted further experiments where the yeast was maintained in a growing condition by the judicious addition of further aliquots of wort. Following each addition it can be seen in Figure 3.5 (which highlights the fermentation of maltose) that fermentation capacity per unit yeast could be substantially improved to eventually reach a maximum value which, again, was found to be specific for each

individual yeast strain.

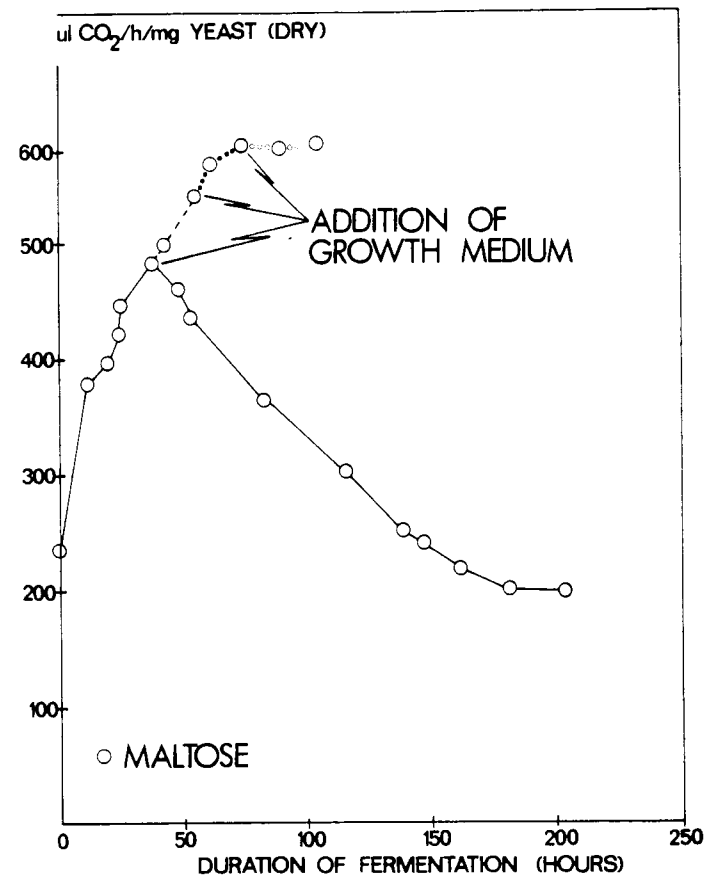


FIGURE 3.5: Fermentation capacity of strain 2036 for maltose in wort demonstrating effect of maintaining in growth condition.

Stimulation of the yeast growth rate by the addition of lipids, in the form of spent grains pressings or as a mixture of oleic acid and ergosterol, to non-aerated wort, increased maximum fermentation capacity per unit yeast for each of the measured wort sugars. Figure 3.6 shows the pattern for maltose fermentation using strain 2036.

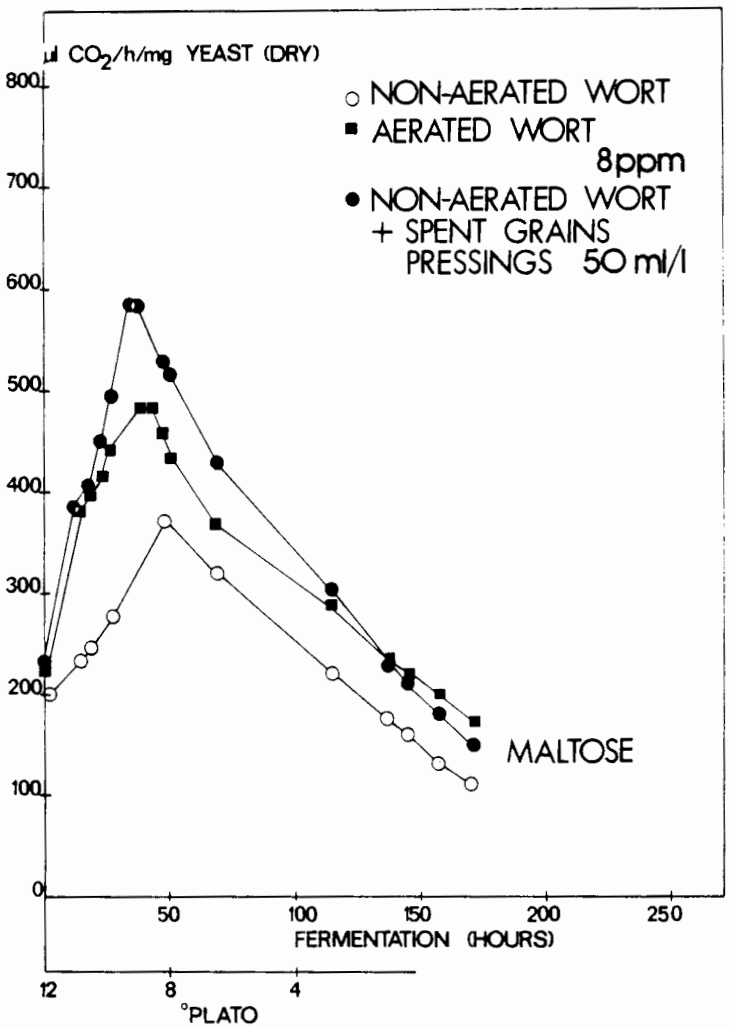


FIGURE 3.6: Effect of addition of spent grains pressings to wort on fermentation capacity of maltose. Tall tube fermentation.

3.3.2 Fermentation Capacity under Non-growth-promoting Conditions

The primary point of action of the anti-fungal agent cycloheximide lies in the sequence of reactions leading to the synthesis of nucleic acid and protein. Previous studies have highlighted its effects on DNA and RNA and cycloheximide has been used extensively as an effective inhibitor of growth by preventing synthesis of new proteins (Greig et al., 1958; Kerridge, 1958; Kelker and Pogo, 1980).

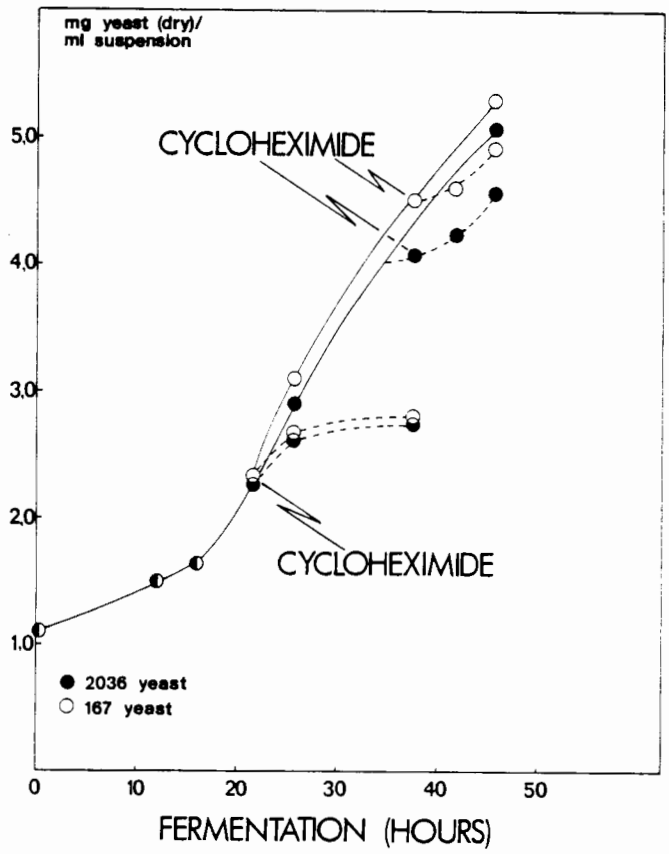


FIGURE 3.7: Effect of addition of cycloheximide on yeast growth in agitated wort fermentation.

To highlight the effect of arresting protein synthesis on fermentation

capacity cycloheximide was added at a level of 10 ppm in malt wort, in stirred fermentations during the exponential phase of growth. The effect on growth is shown in Figure 3.7.

Figures 3.8 and 3.9 show the effect of cycloheximide on fermentation capacity of glucose and maltose. A similar consequence was noted for maltotriose.

Repercussions on gravity drop were as expected with a tailing off following the addition of the anti-fungal agent. It was interesting to note the relatively severe effects on fermentation capacity of maltose where levels of under 150 ul CO_2/h/mg yeast (dry) were rapidly reached compared to the more mild effects on glucose and maltotriose.

A similar trend for fermentation capacity levels, although more gradual, was seen (Figure 3.10) by transfering strain 2036 (transfer A in Figure) during exponential growth in wort to synthetic media, a growth-promoting medium containing an assimilable nitrogen source, $(NH_4)_2SO_4$, and glucose and a non-growth-promoting medium where the nitrogen source was omitted. Interestingly, if the yeast from the non-growth experience was recovered and re-pitched again into a similar medium (transfer B in Figure), the fermentation capacity per unit yeast for glucose rapidly fell to zero. The yeast strain could be maintained for relatively long periods of time ($\pm$ 5 days) in this condition at 15°C with only partial loss of viability as measured by the methylene blue index and slide culture method. (In Figure 3.10 the percentage viability figures are shown as a function of time in parentheses during this period). If this yeast was again transferred to a growth-promoting medium (transfer C in Figure), fermentation capacity only slowly recovered.

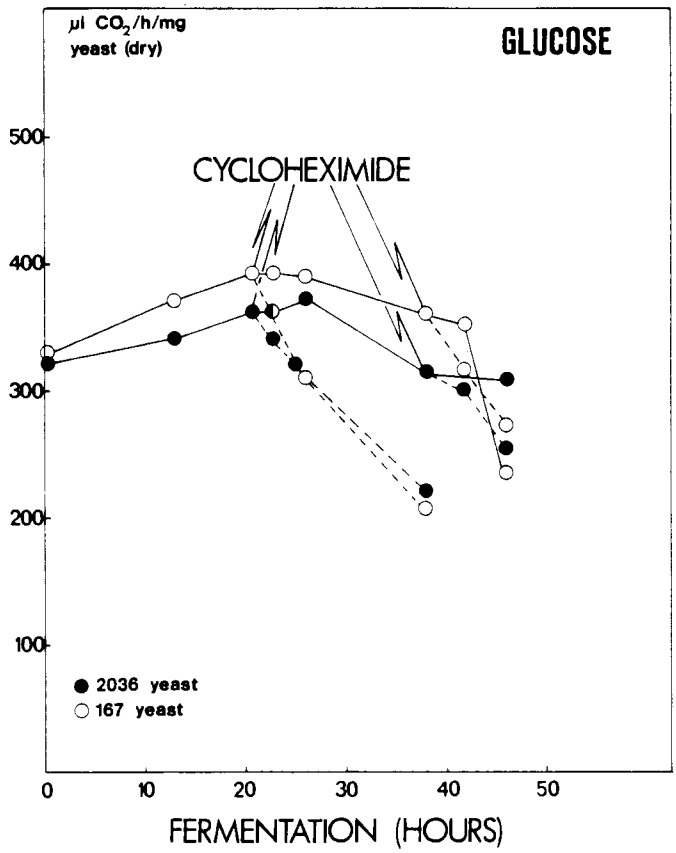


FIGURE 3.8: Effect of additions of cycloheximide on fermentation capacity of glucose in agitated wort fermentation.

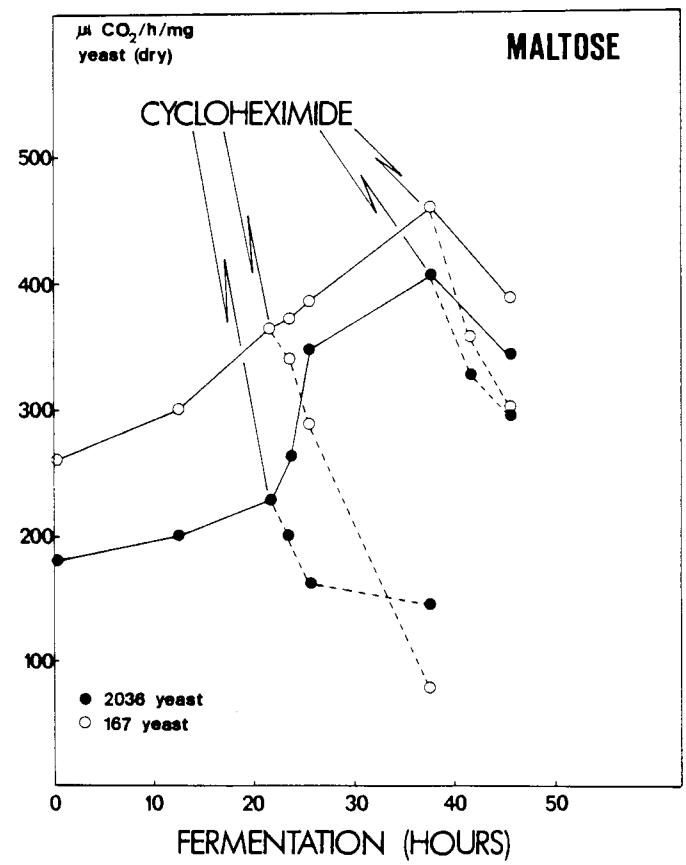


FIGURE 3.9: Effect of additions of cycloheximide on fermentation capacity of maltose in agitated wort fermentation.

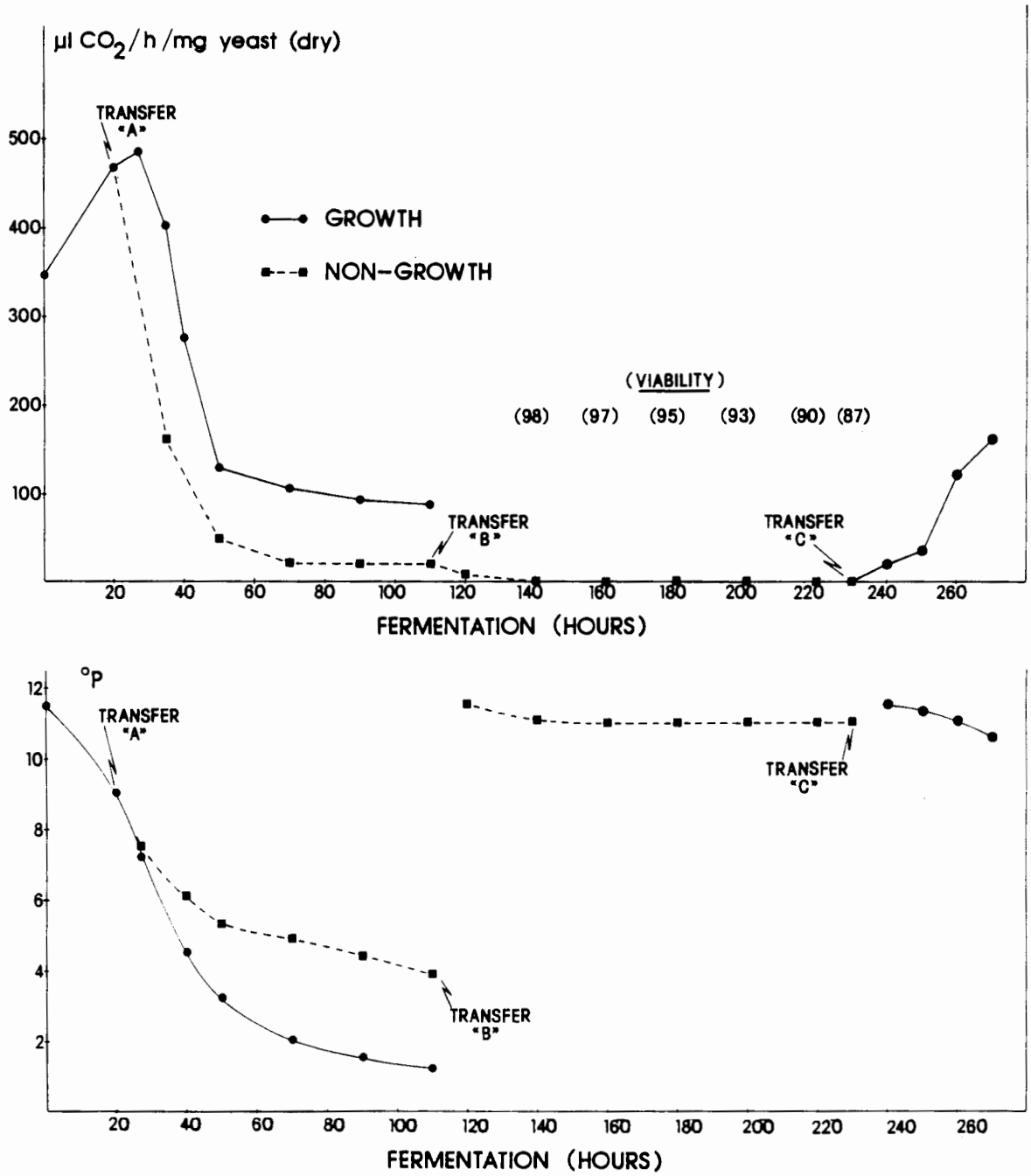


FIGURE 3.10: Effect of transfer of strain 2036 from exponential growth in wort to synthetic growth and non-growth media in agitated fermentation. Fermentation capacity of glucose highlighted. Legend on following page.

FIGURE 3.10: Legend

Transfer "A" = initial transfer of yeast from wort to synthetic growth and non-growth media.

Transfer "B" = transfer of yeast from non-growth medium into similar, fresh medium.

Transfer "C" = transfer of yeast from second non-growth medium into growth medium.

Density drop (^{o}P) after each transfer shown.

Viability % of cells highlighted between Transfers "B" and "C".

3.3.3 Fermentation Capacity of Rare Mating Products

In crossing strain 2036 with strain DM_{33}, 30 rare mating products were isolated of which 50% demonstrated weak sporulation characteristics. Mass isolation of the ascospores of a hybrid designated RM_{43} provided 260 colonies, 42 of which were auxotrophic for isoleucine, valine and leucine and lacked the ability to produce diacetyl and 2,3-pentanedione. These strains were also shown to be a mating type of which 8 utilized maltotriose rapidly. Two of these mutants, designated $RM_{43.9}$ and $RM_{43.12}$ showed aerobic growth characteristics on maltotriose at a similar rate to 2036 (Vanderghinste, 1982).

Fermentations were therefore conducted under anaerobic agitated conditions, as previously described, in wort using strain 2036 and strain $RM_{43.12}$. Strain 2036 attained an attenuation of 70% after 3 days but the hybrid strain $RM_{43.12}$ took 3,5 days to attain an attenuation of 60% (Table 3.1).

TABLE 3.1: $Q^{N_2}_{CO_2}$ for yeast strains 2036 and $RM_{43.12}$ on glucose, maltose and maltotriose during agitated fermentation in wort.

STRAIN	TIME (h)	ATTENUATION (%)	GLUCOSE ul CO_2/h/mg yeast dry	MALTOSE ul CO_2/h/mg yeast dry	MALTOTRIOSE ul CO_2/h/mg yeast dry
2036	15	21	364	331	-
	48	43	186	230	101
	64	64,5	-	-	141
	88	73	-	56	12
$RM_{43.12}$	15	19,5	230	239	-
	48	38	175	176	83
	64	45,5	-	-	70
	88	58	-	68	18

Fermentation capacity figures show that strain $RM_{43.12}$ demonstrated lower values for glucose, maltose and maltotriose as compared to the parental strain. Growth curves undertaken under similar conditions show a much slower growth rate for the hybrid (Figure 3.11).

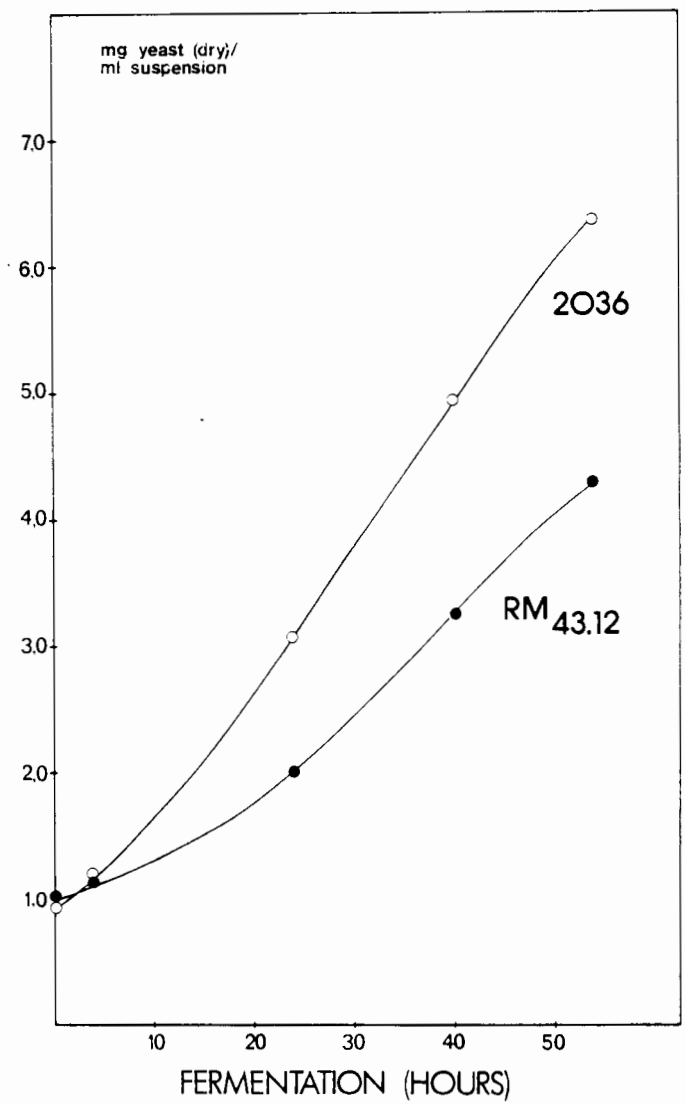


FIGURE 3.11: Growth curves for strains 2036 and $RM_{43.12}$ in agitated fermentation in wort.

Tall tube fermentations indicated a similar trend but with no measurable fermentation capacity figures for maltotriose for the hybrid such that only 64% attenuation was reached after 6 days. Further fermentations undertaken in yeast extract - glucose medium where the sugar was present at 10% w/v demonstrated that the loss of fermentation ability for the mutant was not limited to those sugars mediated by a permease system, i.e. maltose and maltotriose, and was indeed a general phenomenon. Table 3.2 shows the marked difference in fermentation ability for glucose using this medium.

TABLE 3.2: $Q^{N_2}_{CO_2}$ for yeast strains 2036 and $RM_{43.12}$ on glucose during agitated fermentation in yeast extract - glucose (10% w/v) medium.

STRAIN	TIME (h)	ATTENUATION (%)	ul CO_2/h/mg yeast dry
2036	22	15	344
$RM_{43.12}$	22	9	173

Rare mating products, isolated from crossing strain 2036 with strain N41, which were also shown to be ivl$^-$, also demonstrated similar characteristics to the rare mating products already described.

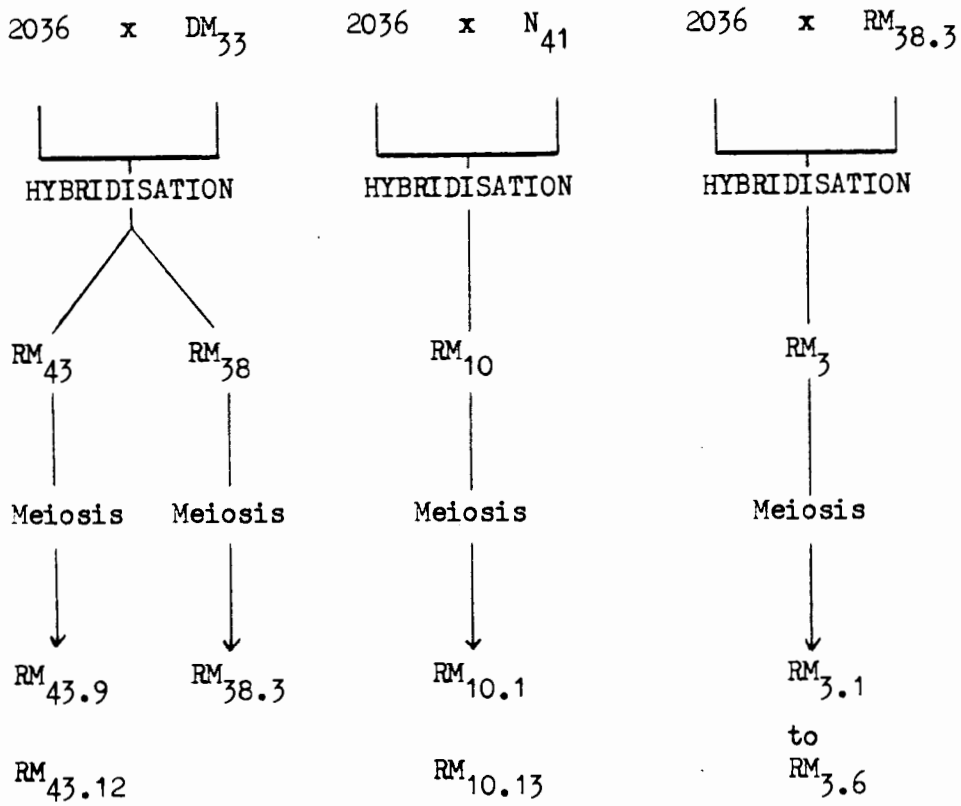


FIGURE 3.12: Origin of hybrids auxotrophic for isoleucine, valine and leucine used in studies as described in this chapter.

3.4 DISCUSSION

It has been shown as an extension of the work of Masschelein et al. (1960, 1965) and Harris and Millin (1963) that, assuming transitional stages, glycolytic flux, in the fermentations described, may be delineated into two distinct states in which the yeast cell exists, viz., the growth phase in which the specific fermentation rate for each of the wort fermentable sugars reaches a maximum and the longer stationary phase where growth is terminated and fermentative power progressively declines and maintenance activities take precedence.

The studies as described in this text show that when the nitrogen requirements of the yeast cell are limited, the fermentative power of the cell diminishes. Under these conditions, reductions in fermentation capacity of the wort sugars may be regarded as a general phenomenon and irrespective of the type of cellular uptake mechanism, i.e. facilitated diffusion for glucose, active transport for maltose and maltotriose, and, thus, independent of catabolite repression and inhibition.

Although the degree will be strain specific, if a growth inhibitory condition persists over a number of yeast generations, this will progressively reduce the fermentation capacity per cell and perpetuate longer lag phases and declining attenuative rates. Strain 2036 was found to be particularly sensitive to these effects when compared to other commercial brewing strains. Thus, "hanging" or "tailing" fermentations are not only caused by insufficient yeast being produced, but also by the negative impact of the growth inhibiting condition on the cell population already present. The cells may be alive, able to maintain their vital functions and integrity but diminishing with respect to their ability to ferment wort sugars. To obtain rapid and efficient attenuations over a generation cycle, it is not only important to obtain adequate cellular multiplication but also to maintain the yeast population with an optimum fermentation capacity per cell; the two parameters not necessarily being the same.

The role of oxygen and lipids with respect to yeast growth has been the subject of many papers in the brewing and biochemical literature. Taylor et al. (1979) and Dillemans and Masschelein (1981) recently demonstrated that the addition of spent grains pressings to wort increased fermentation efficiency. Therefore, it was reasonable to find that stimulation of yeast growth rate by this complex of free and bound lipids or,

equally by additions of oleic acid and ergosterol, increased maximum fermentation capacity per unit yeast for each of the measured wort sugars.

It was observed that flocculated yeast shows higher fermentation capacity values than the yeast remaining in suspension. This tends to indicate that continued contact of yeast in an environment that cannot support growth but where fermentable sugars are still present creates faster degeneration than yeast which is not subjected to this continual stress condition. This latter situation would exist when the yeast has flocculated.

Yeast strains unable to synthesise isoleucine, valine and leucine, and thus restricted in their growth rate due to the sequential pattern of amino acid uptake in brewing yeast strains (Jones and Pierce, 1964; Palmqvist and Ayräpää, 1969), show a concomitant reduction in fermentation capacity and attenuative rate. Vanderghinste (1982) showed that facilitating the specific growth rate of the (ivl$^-$) hybrids by addition of ergosterol and oleic acid assisted the attenuation rate when compared to the brewing strain.

However, because of the sequential amino acid uptake pattern and the necessity to synthesise isoleucine, valine and leucine to achieve a normal growth curve, fermentation capacity figures of the hybrids could not match that of the parent.

These observations would therefore assist in explaining the inefficiency of maltotriose fermentation for the haploid ivl$^-$ mutants previously described by Ramos and Masschelein (1977). Moreover, Ramos and her co-workers (1982) have recently shown that a double rare mating as outlined in Figure 3.12, although providing a certain number of ivl$^-$ hybrids, does not result in improving fermentation capacity values or the final attenuation limit;

again because of reduced growth rate.

It should be noted that all the mutants isolated during this work were of a mating type. These segregants have often been shown to possess a slower fermentation rate than α mating types (Ramos and Masschelein, 1977) suggesting that hybridisation studies using α mating types may be rewarding.

In conclusion, the potential industrial impact is realised for fermentation systems which may limit yeast growth (Ryder et al., 1983a,b,d). In the field of brewing this is particularly the case for certain continuous techniques or where pressure may be used during fermentation.

Considerable potential industrial benefits have recently been conceived for the utilization of immobilized or entrapped cell systems. However, in this technique, growth is usually severely limited. Thus, the information acquired during these experiments prompted their application in achieving an explanation at the metabolic level for the observed changes in fermentation capacity.

CHAPTER 4

THE RELATIONSHIPS OF GLYCOLYTIC INTERMEDIATES TO YEAST GROWTH

SUMMARY: The levels of a range of glycolytic and tricarboxylic acid cycle intermediates and adenosine, guanosine, uridine and cytidine mono-, di-, and triphosphates were measured in S. uvarum under growth-promoting and growth-limiting conditions. Measurements were made using isotachophoresis and bioluminescence. The results have been used to assess the regulatory role of the metabolites in glycolytic flux of S. uvarum.

Experiments indicated that growth-limiting conditions are associated with high ATP, citrate and phosphoenolpyruvate levels and low levels of AMP and fructose-1,6-bisphosphate in agreement with the proposed regulatory mechanisms of phosphofructokinase I, pyruvate kinase, phosphoenolpyruvate carboxykinase and fructose 1,6-bisphosphatase.

The aggregate effect of these changes is in favour of inhibiting phosphofructokinase I and pyruvate kinase activity and orientating the glycolytic pathway in a gluconeogenic direction. Regulation at the phosphofructokinase I/fructose 1,6-bisphosphatase junction creates a build-up of fructose-6-phosphate and subsequently glucose-6-phosphate which, by feedback, would be expected to inhibit glucose utilization.

Measurements showed that the end point of gluconeogenesis in yeast is not glucose-6-phosphate in yeast as was previously thought. Under growth-limiting conditions, the glycogenic pathway was utilized at least as far as the glycogen substrate, uridine diphosphate glucose. Concomitantly, a build-up

of the cell wall precursor, uridine diphosphate acetyl glucosamine, occurred. This indicated that control of glutamine fructose-6-phosphate transaminase by UDP-6-acetyl glucosamine, was incompletely suppressed, at least as far as the yeast strains were concerned.

4.1 INTRODUCTION

The multivalent allosteric properties of phosphofructokinase I have been shown to play a major role in the regulation of glycolytic flux on the transition from aerobic to anaerobic conditions, or vice versa, in biological tissues. It seemed reasonable, therefore, to suppose that this regulatory enzyme may be implicated in the inhibition of glycolysis under non-growth conditions in yeast during fermentation.

Numerous experiments have been reported which show that the postulated changes in the concentrations of the effectors of phosphofructokinase I correlate to the rate of glycolysis or of fermentation (Williamson et al., 1964; Williamson, 1965; Newsholme and Gevers, 1967; Randle et al., 1968; Wu and Davis, 1981).

Attempts to rationalize the regulation of phosphofructokinase I in vivo, based on in vitro results, however, have not been satisfactory. One difficulty is that phosphofructokinase I activity is affected by a variety of intracellular modulators. In many cases, it has been difficult to correlate the sometimes complex allosteric effects observed in in vitro steady state kinetic studies of phosphofructokinase I activity with intracellular metabolic events. Another difficulty has been the determination of only a limited number of glycolytic intermediates in

yeast under specific conditions. A drawback to specific intermediate studies has been the sensitivity of the methods employed and/or the demonstration that the concentration of metabolites does not appear to change significantly to warrant kinetic conclusions. For example, it has been found in liver (Newsholme and Gevers, 1967) that the ATP and citrate concentrations usually remain constant at 3 nM/mg and 0,3 mM/mg respectively, under various nutritional states. Similarly, the amounts of the activators of phosphofructokinase I, such as AMP and fructose-1,6-bisphosphate do not fluctuate. It appears that no systematic determination of glycolytic and tricarboxylic acid cycle intermediates in brewing yeast strains of S. uvarum, under varying growth conditions, has been carried out or reported.

The intractability of understanding the regulation of glycolytic flux under normal lager fermentation conditions and under conditions where yeast growth would be adversely affected, prompted such a systematic determination of cellular intermediates, using the specific picomole range measurement methods already outlined in Chapter 2.

4.2 MATERIALS AND METHODS

4.2.1 General Conditions

Maintenance and propagation of brewing strains of S. uvarum, the strains used, infection monitoring and fermentation conditions and sampling were as previously described in Chapter 3.

4.2.2 Non-proliferating Conditions

Non-proliferating conditions were induced as previously described in Chapter 3, either by the addition of cycloheximide at 10 ppm during exponential growth or by transferring exponentially growing yeast in wort to synthetic media, a growth-promoting medium containing an assimilable nitrogen source, $(NH_4)_2SO_4$, and glucose and a non-growth promoting medium where the nitrogen source was omitted.

4.2.3 Cell Permeabilization and Extraction

Yeast cell permeabilization and extraction were achieved using the boiling water mediated technique as previously described in Chapter 2.

4.2.4 Measurement of Cellular Intermediates

Measurement of cellular intermediates was achieved using the methods described in Chapter 2. Nucleotide and organic acid levels were measured using isotachophoresis; ATP was also measured by bioluminescence. Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate and the triose phosphates were measured using bioluminescence.

During measurement procedures, extracts were kept in ice. When delays in measurement were encountered, extracts were frozen at -20 °C.

4.3 RESULTS

Results are expressed on a concentration basis in accordance with the majority of previous studies concerning cellular intermediates. Lowry et al. (1971) have discussed the importance of this at length, particularly where discrepancies arose from earlier data calculated on a per cell basis, due to cell size changing with growth rate. Their view and that of other workers emphasised that it is the molar concentration of a metabolite rather than the content per cell which must influence enzyme velocity.

The changes which take place in levels of yeast cellular intermediates have been examined at two levels, viz. where protein synthesis has been arrested by the use of cycloheximide and where growth has been terminated as a result of nitrogen limitation. The dual strategy was adopted in order to ensure that cycloheximide was not promoting metabolic changes due to side reactions of the agent itself.

Under control conditions the intermediate concentrations observed essentially agree with those reported in the recent literature, (see Chapter 1 for references). It should be mentioned, however, that the cultural conditions played a major part in the level of intermediates reached in the cellular population. Steady state levels of intermediates in tall tube fermentations where cell growth was relatively slow were always slightly higher than in agitated conditions where the specific growth rate was increased promoting rapid turnover of cellular constituents. As far as could be determined for the yeast strains examined, the steady state pools always fell within a defined range. For example, depending on cultural conditions, the steady-state ATP pool during exponential growth

was found to be poised between 3 - 5 nM of ATP per mg (dry weight) of cell material.

4.3.1 Intermediate Changes when Growth was Arrested by Cycloheximide

The data for agitated fermentations where cycloheximide was added during the exponential phase of growth are presented in Figures 4.1 - 4.11.

4.3.1.1 Nucleotide Levels (Figures 4.1 - 4.5)

Substantial changes were obtained in the level of nucleotides when growth was arrested. Particularly noteworthy were the levels of the adenine nucleotides where ATP and ADP more than double in concentration and AMP decreased to approximately half its value, four hours after growth had terminated, to levels not encountered under normal growth conditions. Following the initial transient peak for ATP and ADP, levels continued to remain in excess to those encountered under control conditions. Since the cumulative effects of the adenosine phosphates on the rate of cellular metabolism is expressed in terms of the adenylate energy charge (EC_A) (Atkinson and Walton, 1967),

$$EC_A = \frac{(ATP) + 1/2(ADP)}{(ATP) + (ADP) + (AMP)}$$

it is of interest to calculate this value under test and control conditions. Curiously, as shown in Figure 4.1, the EC_A remained remarkably steady under the test conditions for both strains for all samples and was found to be between 0,81 and 0,86, which is considered to be within

the usual range for S. cerevisiae (0,80 - 0,90) (Ball and Atkinson, 1975).

Guanosine, uridine and cytidine triphosphates also increased when growth was arrested (Figures 4.2, 4.3 and 4.4). Although fluctuations were observed under test conditions, the GTP/ATP, UTP/ATP, and CTP/ATP ratios remained almost constant under control conditions, i.e. GTP/ATP (0,17 - 0,19); UTP/ATP (0,20 - 0,21); CTP/ATP (0,14 - 0,15). The respective nucleoside diphosphates showed varying patterns under control situations, probably as a result of biosynthetic demand and formation (see discussion). Under growth limitation GDP decreased significantly, UDP increased and CDP remained almost constant. The monophosphates, as far as could be determined considering separation problems, for GMP and UMP, remained at low and fairly constant levels.

Of the cyclic nucleotides, cyclic AMP, although at levels at the limitation of isotachophoresis for the sample size and concentration used decreased to approximately half the control value when growth was arrested and remained relatively constant at this level (Figure 4.1).

UDPG and UDP-N-acetyl glucosamine levels rose as a function of time for approximately 25 hours after growth had been arrested before a very slow but gradual depletion (Figure 4.4). Values reached under test conditions were over 3 x those encountered in the respective controls.

NAD and NADP levels decreased for about 4 hours when moving from a growth to a non-growth condition before remaining at a relatively constant level (Figure 4.5). FAD levels increased slightly during growth inhibition.

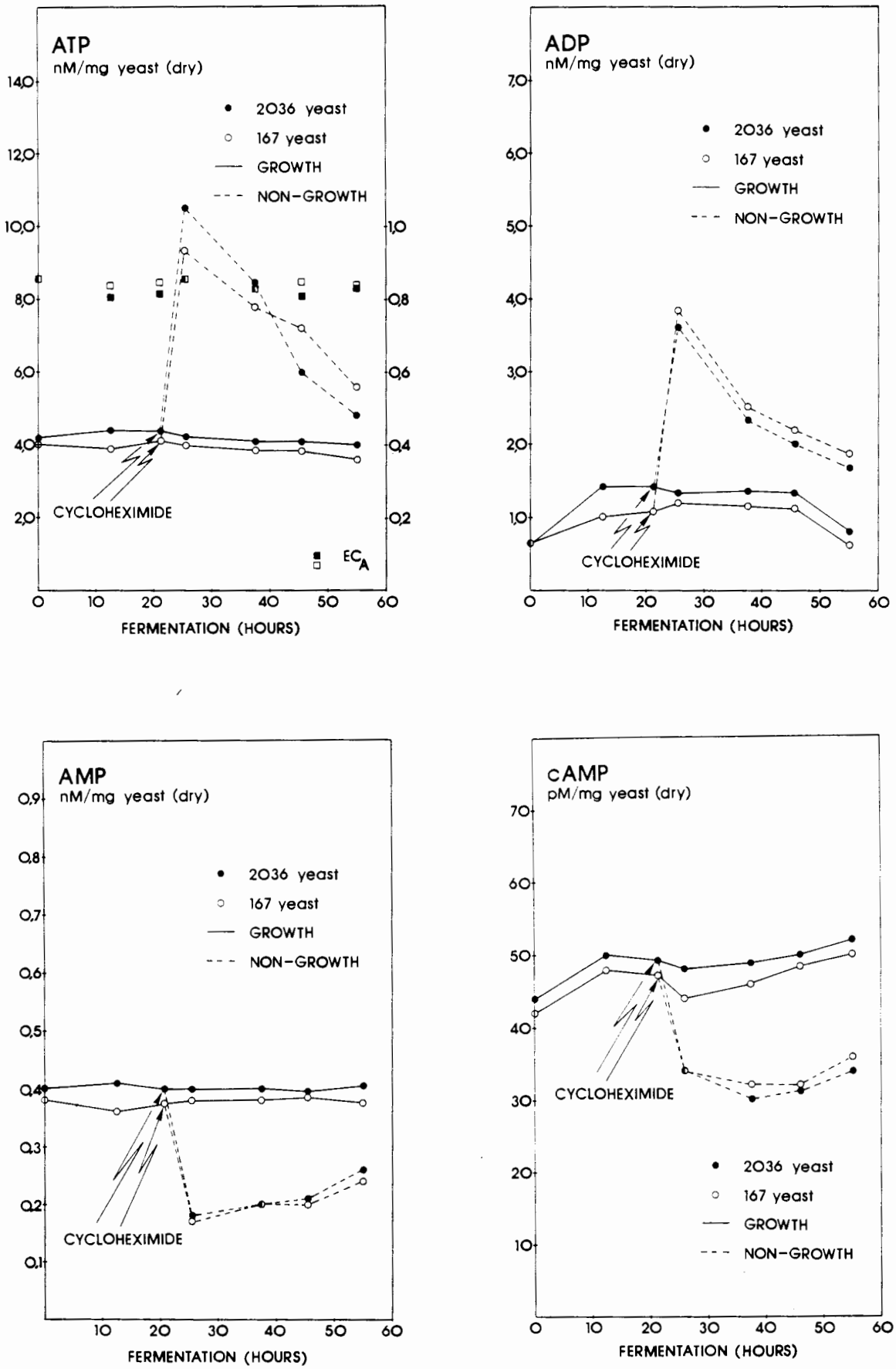


FIGURE 4.1: Adenine nucleotides under growth and non-growth conditions.

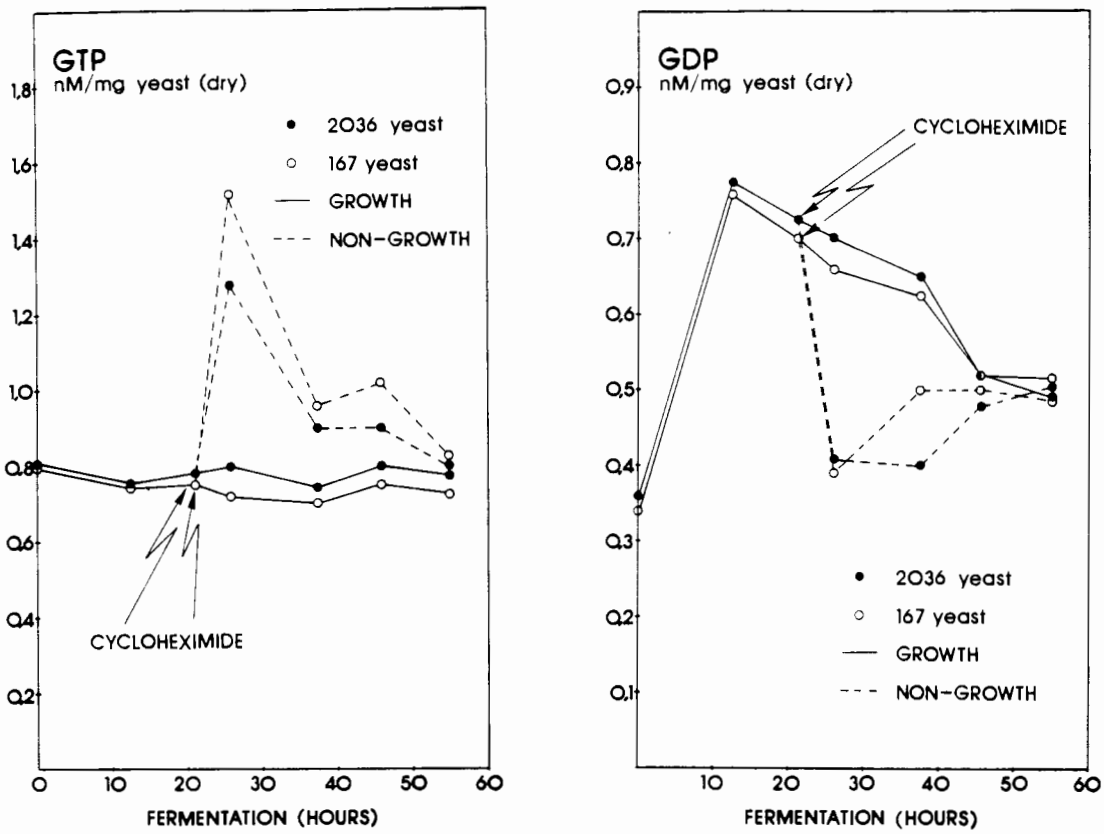


FIGURE 4.2: Guanosine di- and triphosphates under growth and non-growth conditions.

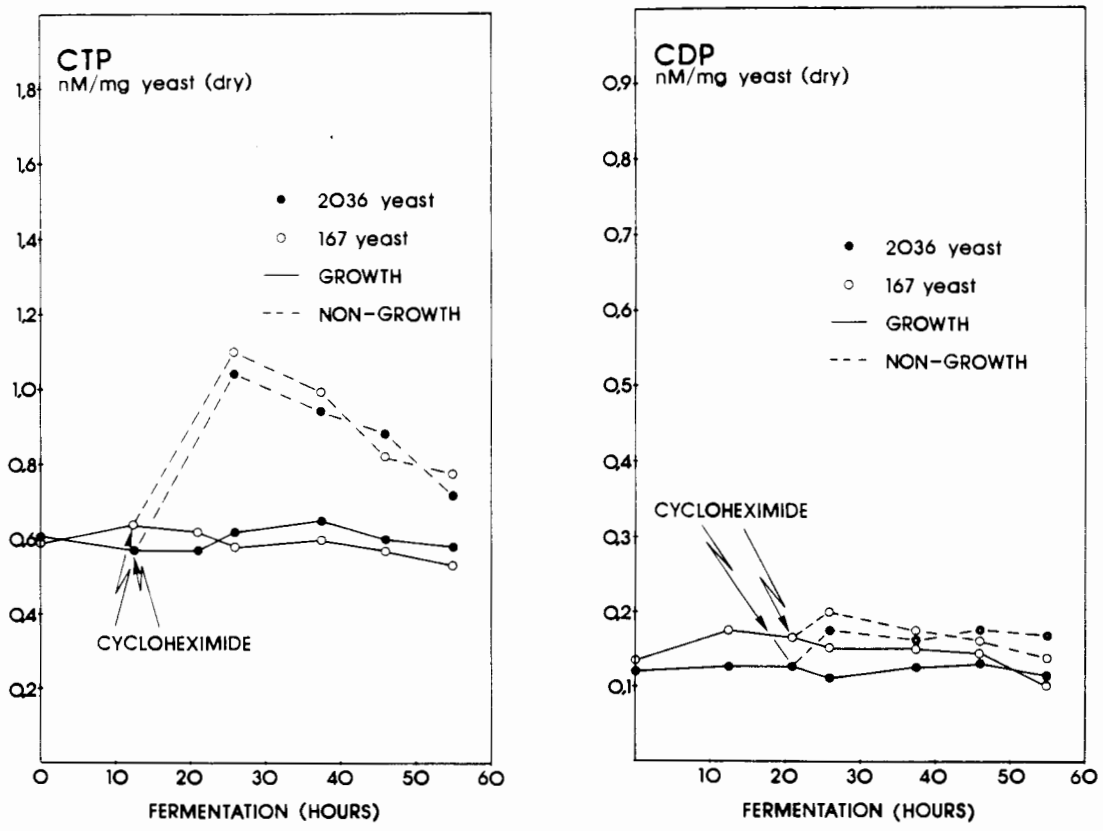


FIGURE 4.3: Cytidine di- and triphosphates under growth and non-growth conditions.

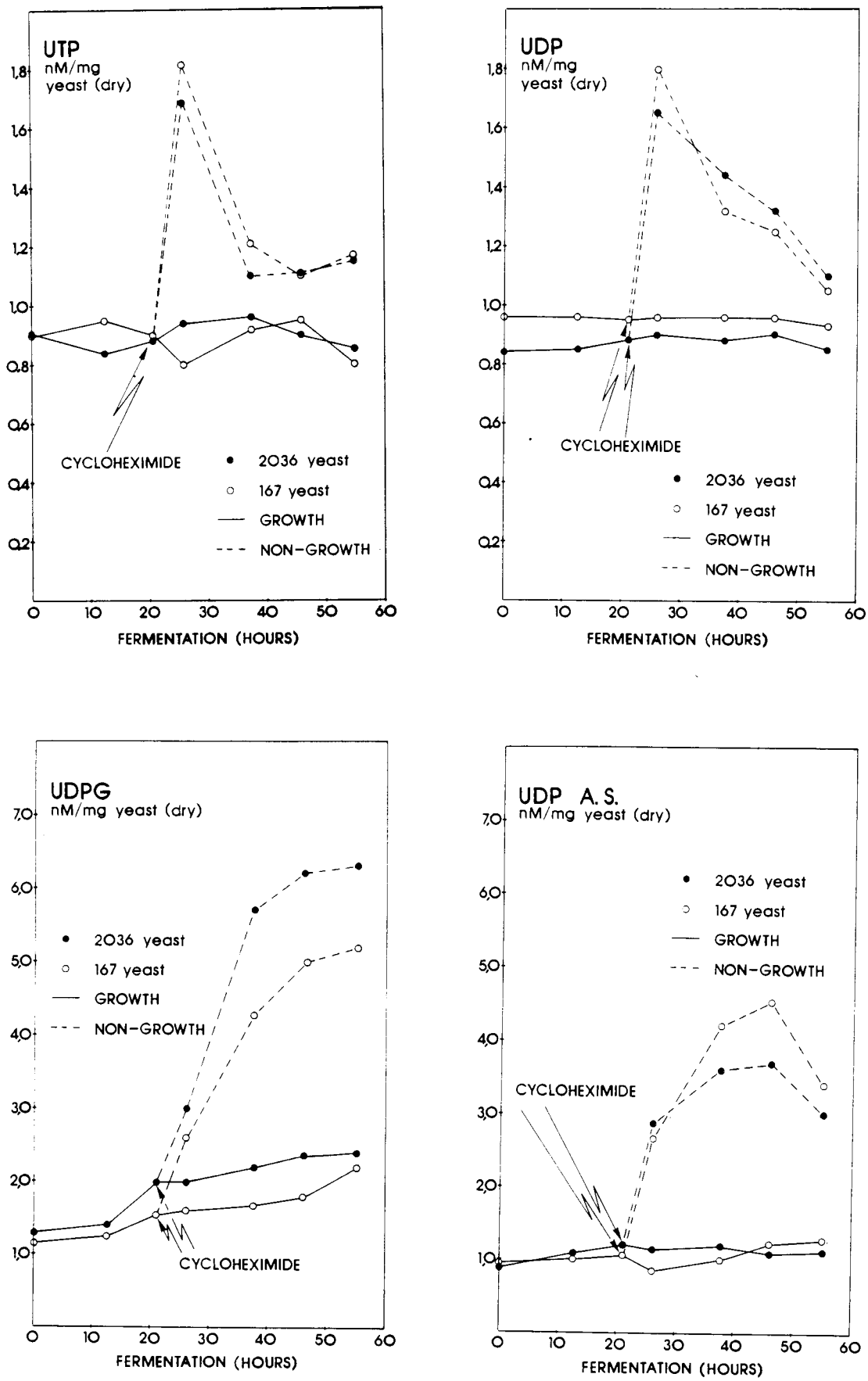


FIGURE 4.4: Uridine nucleotides under growth and non-growth conditions.

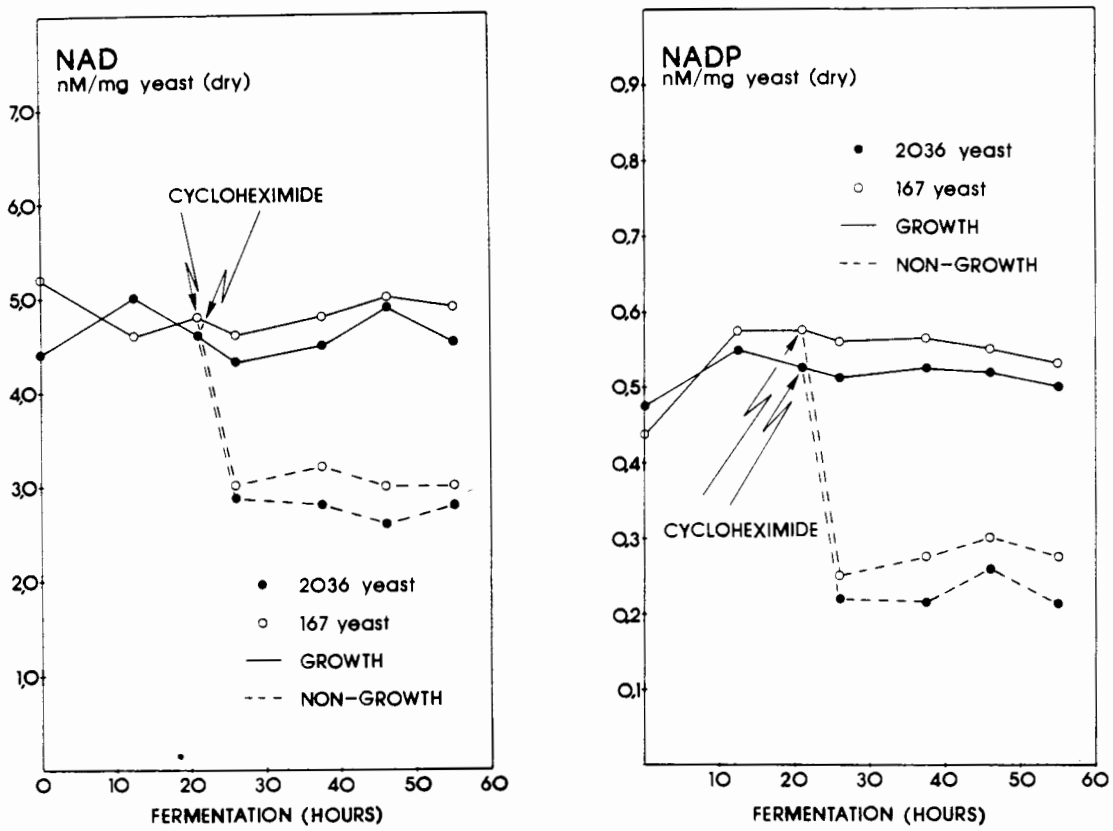


FIGURE 4.5: NAD and NADP under growth and non-growth conditions.

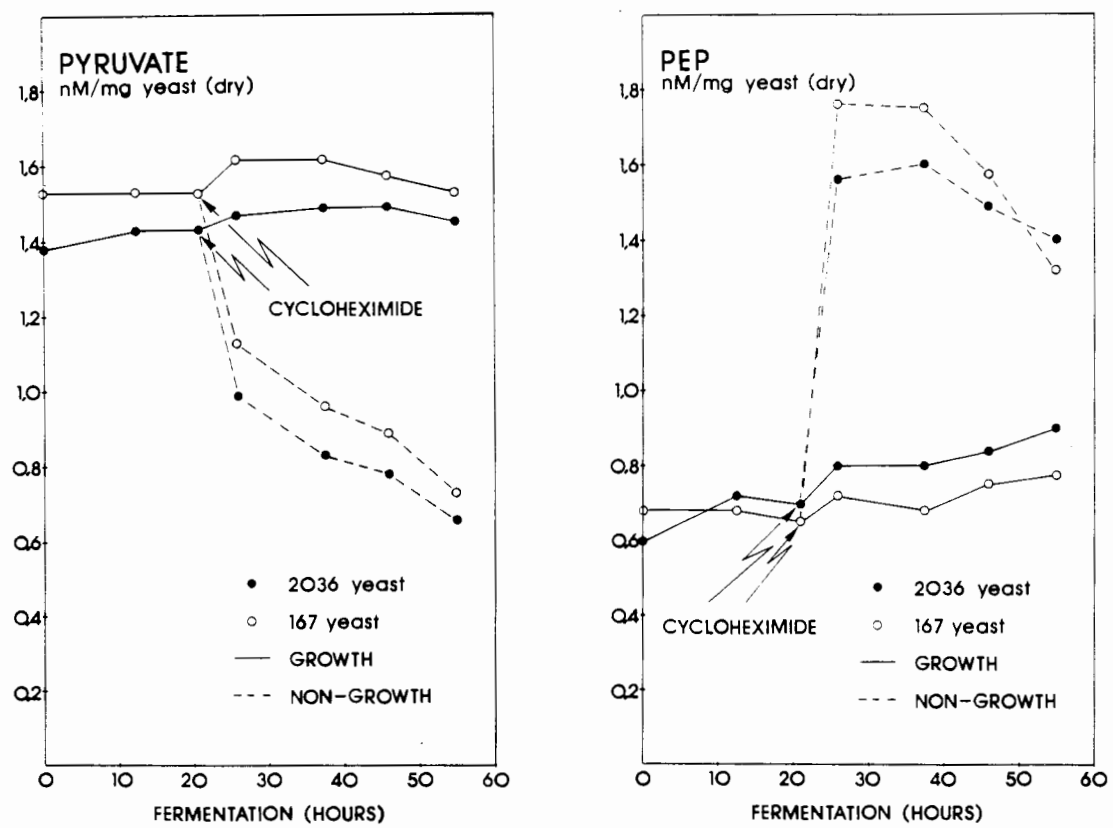


FIGURE 4.6: Pyruvate and phosphenolpyruvate under growth and non-growth conditions.

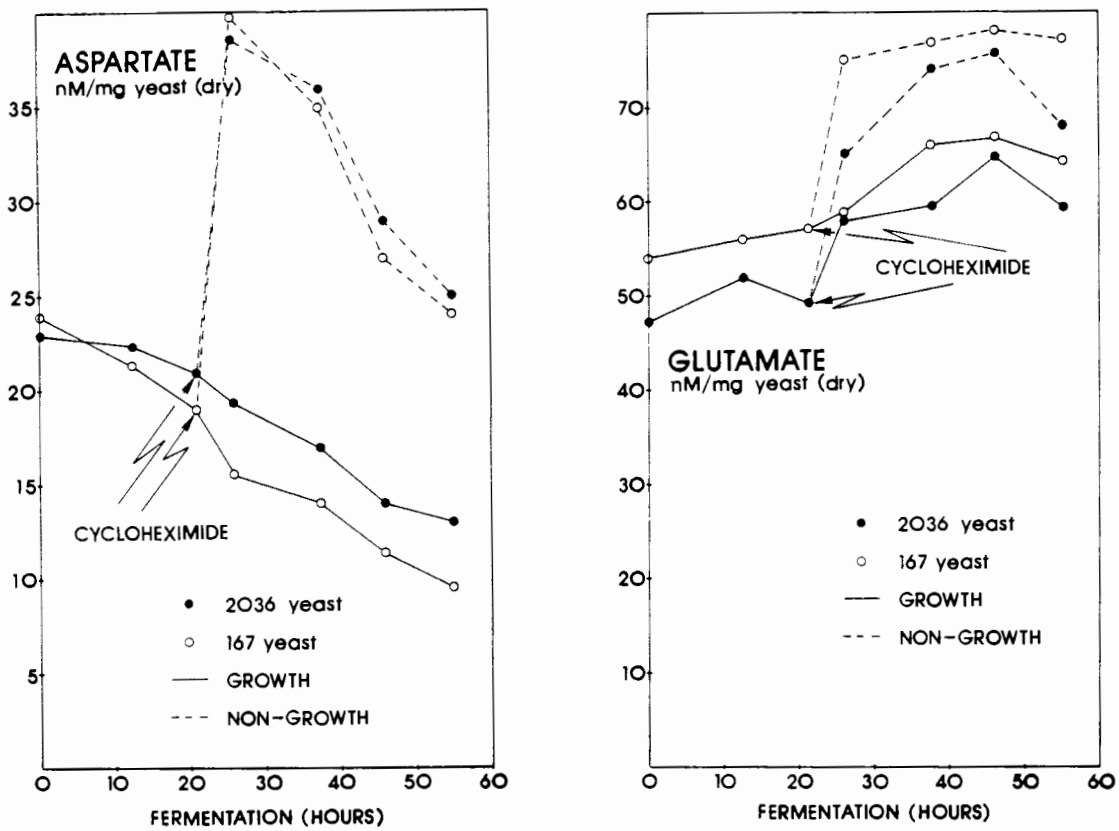


FIGURE 4.7: Aspartate and glutamate under growth and non-growth conditions.

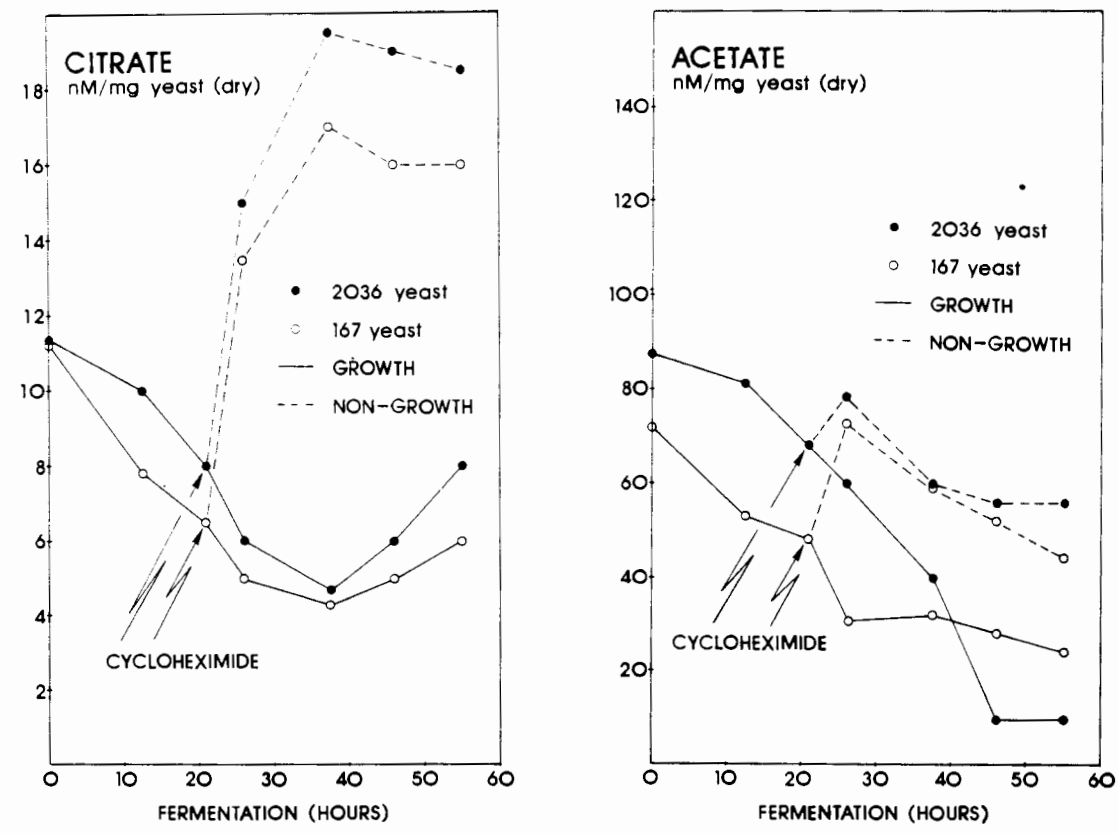


FIGURE 4.8: Citrate and acetate under growth and non growth-conditions.

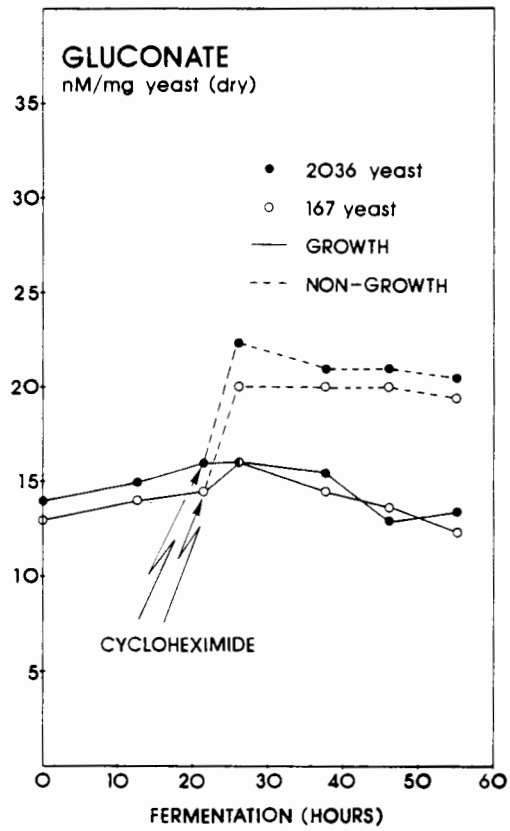


FIGURE 4.9: Gluconate under growth and non-growth conditions.

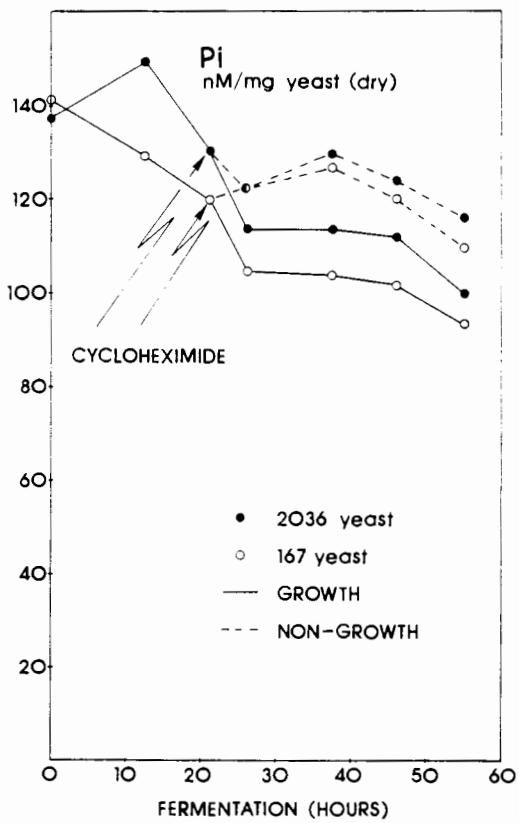


FIGURE 4.10: Inorganic phosphate under growth and non-growth conditions.

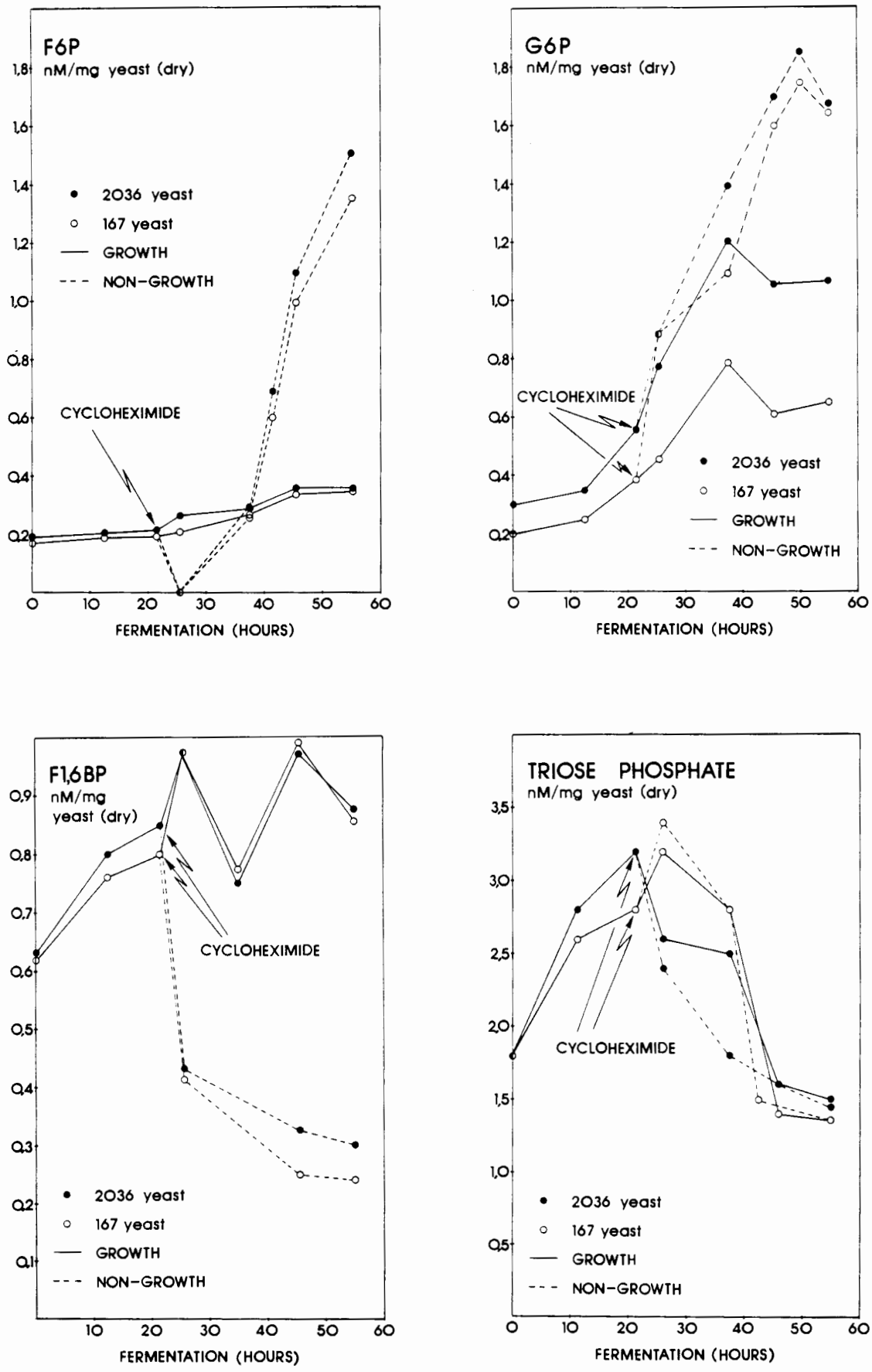


FIGURE 4.11: Hexose and triose phosphates under growth and non-growth conditions.

4.3.1.2 Organic Acid Levels (Figures 4.6 - 4.9)

Phosphoenolpyruvate doubled in concentration when moving from a growth to a non-growth situation while pyruvate levels initially fell by approximately 35 - 40%, before a further, more gradual decline occurred (Figure 4.6).

Acetate (Figure 4.8) and gluconate levels (Figure 4.9) demonstrated notable increases immediately after growth was arrested as did the amino acids, glutamate and aspartate (Figure 4.8). Aspartate almost doubled in concentration four hours after the addition of cycloheximide before subsequently decreasing as a function of time. With respect to glutamate it was interesting to note that increased levels were not accompanied by an increase in α -keto-glutarate. Indeed, although α -keto-glutarate levels were always at relatively low values as seen in the isotachophoretic records (in a mixed zone with fructose 1,6-bisphosphate which had to be initially deducted), these levels showed a decrease under growth inhibition. Succinate was not detectable in measurable concentrations.

In comparison, citrate was shown to increase steadily after the addition of cycloheximide and reached a maximum value at approximately 12 hours.

4.3.1.3 Inorganic Phosphate Levels (Figure 4.10)

The inorganic phosphate level showed an approximate 5% increase on growth inhibition by cycloheximide which was maintained when compared to control values.

4.3.1.4 Phosphate Ester Levels (Figure 4.11)

Fructose-6-phosphate was shown to normally exhibit fairly low levels (± 0,3 nM/mg dry weight) during the growth phase which increased slightly as the yeast entered the stationary phase. The addition of cycloheximide in exponential growth, however, promoted a rapid increase in this metabolite, although an initial decrease was always observed.

Although glucose-6-phosphate levels were similar to those of fructose-6-phosphate at the beginning of fermentation the level increased during the growth phase to reach its highest value as the yeast entered the stationary phase. However, terminating growth by the use of cycloheximide in the exponential phase caused extraordinary increases in the glucose-6-phosphate level.

In comparison to fructose-6-phosphate and glucose-6-phosphate, fructose-1,6-bisphosphate levels fell significantly on growth inhibition to approximately 20% of the control value.

Triose phosphate levels remained relatively undisturbed under test and control situations, although 2036 yeast, under test conditions, showed an initial decline.

Although not shown, an isotachophoretic zone, identified as glucose-1,6-bisphosphate was always present under proliferating conditions. However, when growth was inhibited, this zone was absent.

4.3.2 Intermediate Changes when Growth is Inhibited by Nitrogen Limitation

For the majority of cellular intermediates, measurements from agitated fermentations demonstrated similar values to those already reported for the use of cycloheximide, except that the changes showed an expected delay due to the slower response to the experimental conditions. The notable difference at the 6 hour stage after transfer to non-growth media was a seven fold difference in the citrate-malate zone as compared to the control. Additional isotachophoretic analysis demonstrated that this zone consisted of approximately 10% malate where citrate was at a level of 54,8 nM/mg dry weight of yeast for strain 2036. This value was maintained for approximately 8 hours before a very gradual decrease. Increases for the other organic acids were also noted; the major differences being shown in Table 4.1.

TABLE 4.1: Organic acid levels at the 6 hour stage after transferring strains 2036 and 167 to non-growth media.

	nM/mg yeast (dry)					
	CITRATE	MALATE	ASPARTATE	GLUTAMATE	ACETATE	GLUCONATE
TEST	54,8	5,2	28,4	48,5	73,2	15,6
CONTROL	7,4	N.D.	12,0	33,4	30,8	11,2

N.D. = not detectable

Similar differences between cellular intermediates were encountered during similar experiments conducted on tall tube fermentations.

In all experiments conducted under a non-proliferating framework, a return

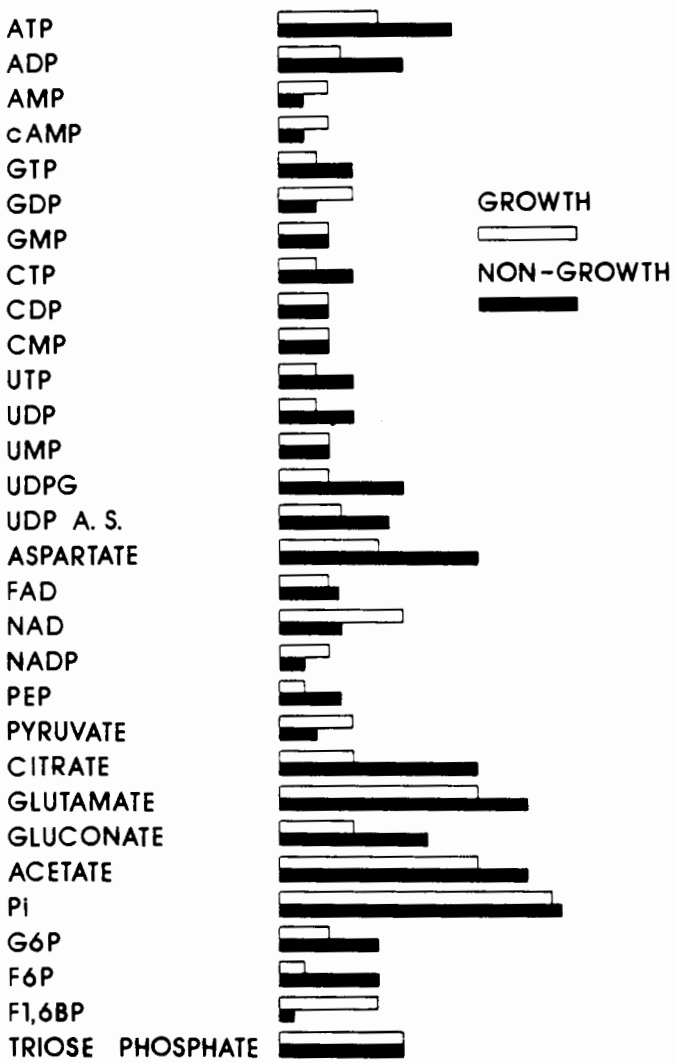


FIGURE 4.12: Schematic summary of cellular intermediates under growth and non-growth conditions.

to active growth caused a rapid reversal of intermediate changes to match those encountered under control conditions.

A schematic summary of the major cellular intermediates measured under growth and non-growth conditions is shown in Figure 4.12.

4.4 DISCUSSION

Under conditions where growth is inhibited or arrested very large changes take place in the steady state levels of yeast cellular intermediates to correlate with a reduction in glycolytic flux, before cessation of enzyme synthesis caused a gradual depletion of all components.

Interpretation of the observed intermediate levels was aided by a consideration of the displacement from equilibrium of the glycolytic reactions. Thus, enzymatic steps far removed from equilibrium are the most probable sites of metabolic control.

In the first instance, the results clearly indicate an inhibition of phosphofructokinase I as shown by the highly disturbed product-substrate relationship creating an increase in the levels of hexose monophosphates and the precipitous fall in fructose-1,6-bisphosphate.

As has previously been reviewed in Chapter 1, phosphofructokinase I in yeast has been found to be sensitive to inhibition by ATP (and GTP).

When protein synthesis is inhibited or arrested, particularly in active fermentation, it follows, as shown, that ATP levels increase in the

cellular environment, as ATP is then not being utilized for such biosynthetic purposes. In addition, it has been reported for Salmonella typhimurium (Smith and Maaløe, 1964) and Anacystis nidulans (Smith, 1979) that the intracellular GTP pool size fluctuates in direct proportion to the demand for biosynthetic energy and that GTP exerts a major influence on the rates of protein synthesis. Therefore, the increased GTP concentration may be expected when growth is inhibited in yeast.

The initial doubling of ATP concentration on growth inhibition was significantly in excess of levels that Viñuela et al. (1963) found to rapidly decrease yeast phosphofructokinase I. Although this inhibition may be counteracted in animal tissues by increasing concentrations of ADP (Passonneau and Lowry, 1964) this was not found to be the case for yeast phosphofructokinase I (Viñuela et al., 1963). However, these latter workers established that inhibition of ATP is competitive with the square of the fructose-6-phosphate concentration and, therefore, the observed rise in fructose-6-phosphate would be expected to have an ameliorating effect on ATP inhibition. The situation is further complicated by the observation that the ATP-sensitive enzyme can convert to a form which is not inhibited by excess ATP (Atzpodien et al., 1970). Thus, this complex picture associated with phosphofructokinase I suggests that continued inhibition of this regulatory enzyme may not only be due to ATP but to an aggregate and sequential effect of the intermediates found.

Based on kinetic and equilibrium binding studies of phosphofructokinase I (Betz and Moore, 1966; Blangy et al., 1968; Krzanowski and Matschinsky, 1969; Goldhammer and Paradies, 1979) relative to the cellular intermediate values reported in this dissertation, the results indicate that initial inhibition of this enzyme under growth inhibition is created by the

extraordinary increase in ATP (which may be assisted by the other nucleoside triphosphates and the initial low level of fructose-6-phosphate) and the diminution of AMP. The data further suggest that citrate plays an important role in the initial regulation of glycolysis, synergistically with ATP at the commencement of growth inhibition and more importantly as the level of citrate rises, particularly as seen under the nitrogen limitation experiments.

The decreased activity of phosphofructokinase I cannot be explained by changes in the inorganic phosphate level as reported by Wu and Davis (1981) for the enzyme in rat tissues. The concentration of inorganic phosphate did not change sufficiently on the transition from growth to non-growth to suggest any inhibitory effect by this component. Whether this type of mechanism is important in yeast has not yet been established experimentally.

In examining the kinetic properties of the muscle enzyme, glucose-1,6-bisphosphate has been shown by Hofer and Pette (1968) to be a significant activator. The observation from the isotachophoretic records, therefore, that the glucose-1,6-bisphosphate zone disappears in non-growth conditions, may be important to yeast phosphofructokinase I inhibition.

Such inhibition created a build-up of fructose-6-phosphate and subsequently glucose-6-phosphate, due to the high activity of phosphoglucose isomerase. The results showed the increased glucose-6-phosphate level to be associated with the amount of glucose utilized, as shown in Chapter 3. Concomitantly, the level of fructose-1,6-bisphosphate decreased, as seen in the results, which would have a further inhibitory effect on phosphofructokinase I. Simultaneously, the ratio of pyruvate to phosphoenolpyruvate indicates decreased activity of pyruvate kinase (and - see later - activation

of phosphoenolpyruvate carboxykinase) further decreasing glycolytic flux. It is proposed that the drop in fructose-1,6-bisphosphate and the levels reached would be responsible for this inhibition. This supports the kinetic studies of pyruvate kinase by Maeba and Sanwal (1968). It should be mentioned that, in all cases examined, a return to proliferating conditions was accompanied by a rise in fructose-1,6-bisphosphate and pyruvate, and a decrease in phosphoenolpyruvate indicating the probable activation of pyruvate kinase by this phosphate ester.

The raised level of phosphoenolpyruvate under non-proliferating conditions would increase inhibition of phosphofructokinase I additively with the increasing concentration of citrate. Wu and Davis (1981) have also shown phosphoenolpyruvate to be synergistic in its ability to enhance the inhibitory effect of ATP. In addition, levels of phosphoenolpyruvate and citrate observed under test conditions would be expected to inhibit phosphofructokinase 2 (Van Schaftingen and Hers, 1981) preventing formation of fructose-2,6-bisphosphate and the associated stimulatory effect of this metabolite towards phosphofructokinase I.

In summary, phosphofructokinase I is indicated as the primary rate controlling enzyme of glycolytic flux under non-proliferating conditions in yeast. Decreased activity at this step suggests an indirect secondary control on pyruvate kinase and glucose utilization.

Whether glucose-6-phosphate accumulation alone is responsible for controlling glucose utilization is open to speculation. Sols (1967) found that supraphysiological levels of glucose-6-phosphate did not inhibit yeast hexokinase but may affect the sugar transport mechanism. Under growth inhibition, the reported raised levels of fructose-6-phosphate and

glucose-6-phosphate may indicate that the sum of these hexose phosphates is more effective in controlling glucose utilization. Alternatively, or in addition, the hexokinases in rat tissues can also be inhibited by raised levels of ADP (Grossbard and Schimke, 1966; England and Randle, 1967). Thus, the combined effects of the high levels of ADP encountered under the experimental conditions and the hexose monophosphates may be responsible for preventing glucose phosphorylation.

Computer simulation of the flux pattern obtained for the intermediate values observed indicated a strong possibility for the occurrence of gluconeogenesis. Among the data presented, there are metabolic changes which correlate with the requirements for gluconeogenesis.

As a result of inhibition of protein synthesis, it would be expected that amino acid pools would increase in size, and indeed, this was seen for the glycogenic amino acid, aspartate, and, where growth was inhibited due to nitrogen depletion, also glutamate. Pyruvate carboxylase, the enzyme that controls the anaplerotic reaction from pyruvate to oxaloacetate, exhibits its highest activity in anaerobic yeast grown on glucose (Haarasilta, 1981). The presence of aspartate, however, at levels encountered under the non-growth conditions, would indicate a decreased pyruvate carboxylase activity. This reduction of activity has been calculated on kinetic parameters demonstrated in anaerobiosis by Haarasilta (1981) to be a $\pm$ 80% reduction. It should be mentioned that although aspartate reduces the amount of oxaloacetate produced by this route, it does not totally block the pathway. In addition, glutamate, under anaerobiosis has also been found to reduce the activity of pyruvate carboxylase, but to a lesser degree than aspartate. Therefore, the two amino acids may act co-operatively in reducing the activity of this enzyme.

The reduction of pyruvate carboxylase activity in the presence of aspartate, a direct precursor of oxaloacetate, would seem a logical consequence of the reduced need for the replenishment of oxaloacetate by pyruvate carboxylase, as the commencement point for anabolic reactions would now be aspartate.

Thus, tricarboxylic acid cycle activity would now occur via the route from aspartate to oxaloacetate and so on, particularly because of the previously discussed reduction in glycolytic flux due to inhibition of phosphofructokinase I and pyruvate kinase.

From the observed intermediates it is proposed that feedback control by glutamate through α-keto-glutarate would result in this intermediate being preferentially channelled to citrate, which, under anaerobic non-growth conditions, would be unlikely to be utilized to any degree. The results do not eliminate the additional formation of citrate from acetate under gluconeogenesis.

It is recalled that the glyoxylate cycle would be non-functional due to inhibition of isocitrate lyase under anaerobic conditions (Oura, 1972), which would additionally be enhanced due to the observed build-up of phosphoenolpyruvate (Hanozet and Guerritore, 1972).

It has been shown (Cannata and de Flombaum, 1974) that phosphoenolpyruvate carboxykinase, catalyzing the first step of gluconeogenesis, would be stimulated at high intracellular ATP and GTP levels.

Calculations have shown that ATP levels reached under non-growth conditions would allow this stimulation (which may be facilitated by a decrease in glucose utilization), which would account for the observed doubling of

phosphoenolpyruvate concentration.

As fructose-1,6-bisphosphatase in yeast is inhibited by AMP and inactivated by glucose (Gancedo, 1971; Lenz and Holzer, 1980), it may be expected that changes in the concentration of AMP or in the ATP/AMP ratio coupled to changes in glucose utilization would have reciprocal effects on glycolysis and gluconeogenesis. Increasing attention has been given to fructose-1,6-bisphosphate at raised concentrations as a negative effector of fructose 1,6-bisphosphatase (Horecker et al., 1981). This would lend credence to the possibility that, under growth inhibitory conditions, when the AMP and fructose-1,6-bisphosphate levels are reduced and the positive effector, citrate, (Datta et al., 1974) is increased, fructose-1,6-bisphosphatase activity may occur fulfilling a gluconeogenic role. Such activity is further supported by the extraordinary increase in fructose-6-phosphate as shown in the results.

Furthermore, it is tempting to speculate on the decrease of cyclic AMP observed under non-growth conditions. Increased cyclic AMP concentrations have been shown to lead to irreversible inactivation of yeast fructose-1,6-bisphosphatase by a cyclic AMP-dependent protein kinase-catalyzed phosphorylation in the presence of ATP (Londesborough, 1982; Purwin et al., 1982). Since ATP is required for the formation of cyclic AMP by adenyl cyclase as well as the phosphorylation of fructose-1,6-bisphosphatase, a depletion of cyclic AMP in the yeast cells offers an interesting aspect of regulation in preventing activation of the protein kinase. Stimulation of adenyl cyclase and/or inhibition of cyclic AMP phosphodiesterase is thought to be initiated by rate limiting levels of glucose and/or its catabolites. This offers speculation as to whether the hexose phosphates at the levels observed may be responsible for a cyclic AMP decline.

The virtually unchanged level of triose phosphates under growth inhibition was unexpected considering the low level of fructose-1,6-bisphosphate. In accounting for these levels cognizance was paid to the contribution not only by any gluconeogenic activity but also by the continued possibility of pentose pathway metabolism. However, the decrease of NADP as shown under non-growth conditions would appear to complement the work conducted by Smith and Buchanan (1979) on human fibroblasts. The conclusions reached by these workers were that under conditions of non-proliferation there would be little cycling of NADP to NADPH, and thus reduced pentose synthesis. Furthermore, a parallel relationship exists for the NAD-linked pyruvate dehydrogenase complex reaction to acetyl CoA. They postulated that reduced acetyl CoA would lead to limited generation of NADP due to reduced lipid synthesis (and utilization of NADPH) and, thus, limited availability of NADP, further regulating the amount of glucose metabolized in the pentose phosphate pathway. Moreover, Fitch and Chaikoff (1960) have previously reported that the activity of glucose-6-phosphate dehydrogenase can fall to one tenth of the maximum value when the requirement for NADPH is reduced. The alternative route to phosphogluconate from gluconate would also appear to be blocked as interpreted from the results due to the substantial build-up of gluconate. Thus, in summarising these observations, the contribution of the pentose pathway to the triose phosphate level would be expected to be minimal.

The unchanged triose phosphate level still does not explain the reason why fructose-1,6-bisphosphate does not build-up, as aldolase functions in both glycolytic and gluconeogenic directions. The ratios of the two intermediates suggest inhibition of aldolase under growth inhibitory conditions. In agreement with the work of Horecker et al. (1981) on rabbit liver

extracts, a decrease in aldolase activity during gluconeogenesis would accentuate the key role of fructose-1,6-bisphosphate as a regulator of both glycolysis and gluconeogenesis.

This work demonstrates that glucose-6-phosphate cannot be the end-point of gluconeogenesis in yeast as reported by Haarasilta (1981) or as a result of phosphofructokinase I inhibition. The isotachophoretic results clearly indicate a particularly striking diversion of the hexose monophosphates to polysaccharide precursors. In this regard, glycogenesis is favoured from glucose-6-phosphate at least as far as uridine diphosphate glucose, the usually accepted route being to glucose-1-phosphate via phosphoglucomutase and to UDPG via UDPG pyrophosphorylase. The isotachophoretic records did not reveal a build-up of UDP glucuronate after this point.

Furthermore, as seen from the results, it is proposed that the build-up of fructose-6-phosphate not only influences a glucose-6-phosphate build-up but contributes towards an increase in UDP amino sugars as observed for UDP acetyl glucosamine. The steady increase in UDP acetyl glucosamine is noteworthy on two accounts. Firstly, because the biochemical literature shows that the first enzyme of the pathway, glutamine fructose-6-phosphate transaminase, is controlled by feedback inhibition by UDP acetyl glucosamine. This may indicate that, at least for the yeast strains examined, this pathway is incompletely suppressed. Secondly, in catalyzing this first reaction, glutamine will convert to glutamate adding to the levels observed in the results.

The continued supply of UTP as energy source for the formation of UDPG and UDP acetyl glucosamine is probably achieved by indirect energy coupling

(Thompson and Atkinson, 1971). A phosphate shuttle catalyzed by nucleotide diphosphate kinase enables the free energy from hydrolysis of the γ-phosphate group of ATP to be reversibly transferred to the diphosphates of other nucleotides.

It is also interesting to note that Thompson and Atkinson (1971) have determined that the activity of nucleoside diphosphate kinase is directly correlated to the adenylate energy charge such that the flow of phosphate into non-adenine nucleotides is curtailed when the EC_A falls below a minimum critical level.

In the majority of experiments conducted the adenylate energy charge remained remarkably constant as previously shown. Thus, the usefulness of this unitless metabolic indicator can be argued under stress situations since fairly large changes in the absolute concentration of the individual adenine nucleotides or in ATP/ADP or ATP/AMP ratios may be disguised if the EC_A only is examined in yeast. Suffice to say that, for any given kinase, especially one that is allosteric, a twofold change in any of the adenine nucleotides, must be of enormous significance.

Furthermore, the proposal that the extent of growth of a micro-organism is proportional to the amount of ATP available to it (Bauchop and Elsden, 1960) would not appear to be unequivocal and needs to be more carefully defined.

The EC_A diminished, however, under prolonged starvation where, as described in Chapter 3, fermentation capacity was transiently lost under non-growth conditions. Although the viability <u>per se</u> showed a relatively gradual decline, the EC_A fell significantly after $\pm$ 3 days starvation and at $\pm$ 5 days starvation, before transferring back into growth conditions, was at

a level of approximately 0,1. The lower level of EC_A, therefore, able to be sustained by brewing strains of S. uvarum was extremely low when compared to bacteria (Atkinson, 1977).

The EC of the other individual sets of nucleotides from the results obtained would not appear to be as stable as that of the adenine nucleotides. Fluctuations are seen probably because of a fundamental cascade regulation between their need for formation of cellular precursors already discussed and their formation through indirect energy coupling from ATP. However, the fairly constant ratios of ATP to the other nucleoside triphosphates are noted and may be an important parameter in maintaining cellular integrity.

The assumption is implied in interpreting these observations that the glycolytic and tricarboxylic acid cycle intermediates in S. uvarum are not compartmentalized. Although the possible existence of metabolic gradients as additional control has to be kept in mind, research to date has provided the almost universal acceptance that glycolysis, and thus the steps of gluconeogenesis that are in common with glycolysis, occurs primarily, if not exclusively, in the cytosol.

CHAPTER 5

THE RELATIONSHIP OF URIDINE-5'-DIPHOSPHO-GLUCOSE AND GLYCOGEN TO YEAST GROWTH

SUMMARY: The fragmentary knowledge concerning the regulation of glycogen synthesis and degradation in yeast prompted an investigation to understand the potential key role of UDPG under varying growth conditions. This investigation showed that proliferating conditions in the presence of glucose allow a steady increase in the level of glycogen following an initial decrease during the lag phase at the beginning of fermentation. Non-proliferating conditions result in an augmented UDPG pool which is not channelled to glycogen. Moreover, under these conditions the level of glycogen decreases to a base level or to a level where formation and consumption are virtually equal.

This work demonstrated that glycogen synthesis and degradation in brewing yeast cells is a highly regulated, physiological process, dependent not only on the availability of UDPG and glycogen as respective substrates. It is proposed that allosteric and covalent regulation of the interconvertible phosphorylated and dephosphorylated forms of glycogen synthetase and phosphorylase is of major significance. In this regard the intracellular level of the adenine nucleotides and glucose-6-phosphate may be expected to play a particularly important role. A regulation model is proposed of glycogen synthesis and degradation when exponential growth is inhibited in S. uvarum. The physiological meaning of the increased UDPG concentration under the described conditions is unclear. It may be that the maintenance of a UDPG pool is a more efficient mechanism for the yeast cell to tenuously support viability and integrity even though, under the test procedures, the cell is ultimately unable to ferment.

5.1 INTRODUCTION

It was reported earlier in this dissertation that uridine disphospho-glucose (UDPG) increases steadily during normal growth and fermentation conditions leading to a slightly augmented UDPG pool as the specific growth rate decreases and the yeast enters the stationary phase.

These results are in accordance with those of Oura and Suomalainen (1967) and Chester and Byrne (1968) who reported that the UDPG concentration was higher in resting than in growing cells of baking and brewing strains of S. cerevisiae. Chester and Byrne (1968) explained these observations by suggesting that as cell division is most active in the early stages of yeast fermentation, UDPG would be used not only for glycogen (and trehalose) synthesis but for other biosynthetic purposes. As a consequence of cellular division slowing down, less UDPG would be needed for ancillary pathways and this would therefore lead to an increase in UDPG and, subsequently, glycogen.

In yeast, as for other biological tissues, glycogen metabolism is controlled by changes in glycogen synthetase and phosphorylase activities.

Chester and Byrne (1968) postulated that the increased UDPG concentration in stationary phase yeast cells may play a role in the control of possible glycogen degradation by exerting a greater inhibitory effect on glycogen phosphorylase. Madsen (1961) had previously shown UDPG to be a potent inhibitor of glycogen phosphorylase in A. tumefaciens. This worker suggested that the cellular UDPG concentration might be the basis for the biological control of glycogen metabolism in unicellular organisms since a high concentration of UDPG, being the substrate of glycogen synthetase,

would be expected to increase the rate of glycogen synthesis besides inhibiting its degradation. Thus, a fall in UDPG concentration as may be expected towards the very end of fermentation and/or in storage conditions would release the inhibition of phosphorylase and allow the breakdown of glycogen for maintenance purposes. Madsen (1963), however, was unable to demonstrate a causal relationship between UDPG and glycogen concentrations by showing that they vary together.

It has been previously shown in this dissertation that growth inhibition during the exponential growth phase of yeast fermentations creates a rapid rise in the cellular concentration of UDPG. As cellular energy cannot be directed towards protein synthesis there is an abundance of ATP and, therefore, by energy coupling, UTP. It would be expected that the accumulation of hexose phosphates enables UDPG pyrophosphorylase in the presence of UTP to create the extraordinary increase in the level of UDPG.

In discussing that the intracellular concentration of UDPG is the most important factor in the control of glycogen metabolism in yeast, it may be argued that the levels of other cellular intermediates may play a supporting or ameliorating role.

A summary of allosteric and covalent regulation of glycogen synthesis and degradation is schematically shown in Figure 5.1.

Enzyme control of glycogen synthetase, or more precisely glycogen synthase (Mc Gilvery, 1970), and phosphorylase is exerted at the level of interconvertible phosphorylated and dephosphorylated forms of these enzymes (Fischer et al., 1971; Larner and Villar-Palasi, 1971). In glycogen metabolism, four systems are known to effect regulation. These are:

(i) the glycogen synthase activating system mediated by a phospho-protein phosphatase (glycogen synthase phosphatase)

(ii) the glycogen synthase inactivating system mediated by protein kinase

(iii) the phosphorylase activating system mediated by phosphorylase kinase and phosphorylase kinase kinase (protein kinase)

(iv) the phosphorylase inactivating system mediated by phosphorylase phosphatase.

Thus, reactions are catalyzed by kinases in one direction and phosphatases in the other direction, with additional allosteric control acting in conjunction with covalent controls. Interconvertible forms of glycogen synthase have been shown to exist in S. cerevisiae (Rothman-Denes and Cabib, 1970).

Glycogen synthase, in the phosphorylated form, is essentially inactive, unless allosterically stimulated by high concentrations of glucose-6-phosphate (D or dependent form of the enzyme). This has been conclusively demonstrated for liver cells (Hizukuri and Larner, 1964), skeletal muscle cells (Rosell Perez and Larner, 1964) and more recently in adenocarcinoma cells (Paris et al., 1983). These workers showed that the effect of glucose-6-phosphate on the phosphorylated form significantly increased the V_{max} of the enzyme in the presence of UDPG (V effect). In comparison, the effect of glucose-6-phosphate on the dephosphorylated form decreased the K_m of the enzyme for UDPG (K effect). Indeed, in the absence of glucose-6-phosphate, De Wulf and Hers (1967) found that the dephosphorylated enzyme had much higher K_m values (range 0.48 - 1.8 mM) than in its presence (range 0.007 - 0.2 mM). The dephosphorylated form of glycogen synthase is therefore known as the I or independent form.

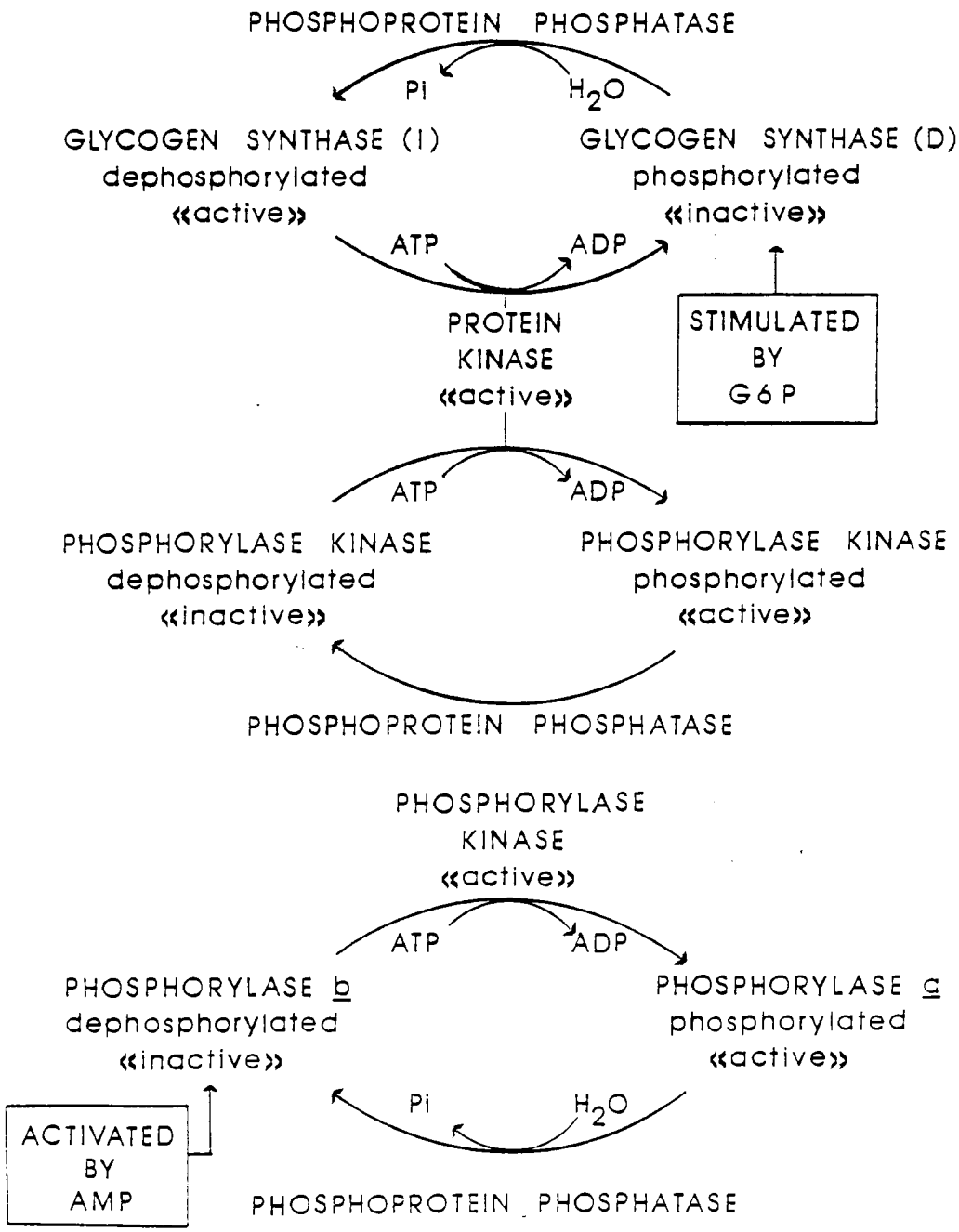


FIGURE 5.1: Summary of allosteric and covalent regulation of glycogen synthesis and degradation in animal tissues.

It is generally considered that the I form is the physiologically active form within the cell (Larner and Villar-Palasi, 1971).

Glycogen synthase phosphatase which catalyses conversion of the D-form of glycogen synthase into the I form is inhibited by increasing concentration of glycogen in skeletal muscle and liver although the kinetics of the inhibition are competitive with the substrate (Danforth, 1965; Hers et al., 1970). Hizukuri and Takeda (1970) have reported that glucose-6-phosphate stimulates spleen synthase phosphatase.

Inhibition of glycogen synthase in skeletal muscle has been shown by raised levels of nucleotides (Rosell Perez and Larner, 1964) and in particular ATP, ADP, UDP and GTP (Piras et al., 1968). This inhibition has been shown to be competitive with UDPG.

For muscle as well as liver cells, stimulation of glycogen synthase by inorganic phosphate (Pi), particularly the D-form, has been demonstrated (Robison et al., 1965; Vardanis, 1967). However, whether Pi could play a regulatory role physiologically has yet to be shown.

In the presence of ATP, the I form of glycogen synthase may be converted into the D form by protein kinase. This was first reported in skeletal muscle by Friedman and Larner (1963) and has been demonstrated in other tissues (Larner and Villar-Palasi, 1971). Protein kinase was shown to be allosterically activated by cyclic AMP (Manchester, 1969) and this same protein was shown by Walsh et al. (1968) to be the catalyst responsible for activating phosphorylase kinase. Therefore, the intracellular cyclic AMP concentration is potentially a mechanism for exerting control on synthase and phosphorylase systems. However, in rat tissues, cyclic AMP

control is disputed (Goldberg et al., 1967; Craig et al., 1969).

With respect to the physiological glycogenolytic direction and in comparison to glycogen synthase, it is the phosphorylated form of the enzyme, phosphorylase a, which is active in glycogen degradation. The dephosphorylated form is inactive unless stimulated by concentrations of AMP (Fischer et al., 1971). Binding of AMP produces a conformational change to the enzyme resulting in activity (Ullman et al., 1964). It is generally accepted that physiologically only the a form is able to catalyze the phosphorylysis of glycogen (Hers and Whitton, 1980).

Phosphorylase b is converted to phosphorylase a through phosphorylation by phosphorylase kinase in the presence of $Mg^{++}ATP$. As shown in Figure 5.1, phosphorylase kinase exists in phosphorylated (active) and dephosphorylated (inactive) forms and may only be activated by the protein kinase in the presence of ATP (Krebs and Fischer, 1956).

In interconverting phosphorylase b and a, the overall reaction is that of an ATPase, viz.:

$$\text{phosphorylase b} + 4\ \text{ATP} \xrightarrow[Mg^{++}\text{kinase}]{} \text{phosphorylase a} + 4\ \text{ADP}$$

$$\text{phosphorylase a} + H_2O \xrightarrow[\text{phosphatase}]{} \text{phosphorylase b} + 4\ \text{Pi}$$

Thus, $4\ \text{ATP} + H_2O \longrightarrow 4\ \text{ADP} + 4\ \text{Pi}$.

Phosphorylase b, activated by AMP, is allosterically inhibited by ATP at the same binding site (Madsen, 1961; Parmeggiani, 1962). ADP and glucose-6-phosphate have also been found to be strong inhibitors of phosphorylase b, but these cellular intermediates -as well as ATP- have no effect on

phosphorylase a (Fischer et al., 1971).

Rabbit muscle phosphorylase a dissociates from a tetramer to a dimer form in the presence of glycogen with concomitant increase in specific activity (Wang and Graves, 1964). In yeast, however, the phosphorylated form of the enzyme exists as a dimer while the inactive b form is tetrameric (Fischer et al., 1971).

Other compounds of possible metabolic significance have also been found to affect the activity of phosphorylase. The affinity of phosphorylase b for AMP is increased by two orders of magnitude by increasing concentrations of glucose-1-phosphate and inorganic phosphate (Pi) (Lowry et al., 1964). Inhibition of phosphorylase by UDPG in rabbit liver cells has been shown to be competitive towards glucose-1-phosphate and Pi (Fletterick and Madsen, 1980).

For yeast cells, however, there is fragmentary knowledge correlating cellular intermediate changes to the synthesis and degradation of glycogen. The intermediate changes shown to occur in brewing yeast strains under varying growth conditions prompted an investigation to understand the relationship of cellular intermediates to glycogen metabolism and whether a potential key role is played by UDPG.

5.2 MATERIALS AND METHODS

5.2.1 General Conditions

Maintenance and propagation of brewing strains of S. uvarum, the strains

used, infection monitoring and fermentation conditions and sampling were as previously described in Chapter 3.

5.2.2 Non-proliferating Conditions

Non-proliferating conditions were induced as previously described in Chapter 3, either by the addition of cycloheximide at 10 ppm during exponential growth or by transferring exponentially growing yeast in wort to synthetic media, a growth-promoting medium containing an assimilable nitrogen source, $(NH_4)_2SO_4$, and glucose and a non-growth promoting medium where the nitrogen source was omitted.

5.2.3 Determination of Glycogen in Yeast

Preliminary studies using the methods of Trevelyan and Harrison (1956), Becker (1978) and Quain (1981) demonstrated that a slightly modified method of Becker (1978) was to be prefered in terms of specificity and yield. The rationale for these comparative studies was to be sure of using a method which was specific and exhaustive.

The modified Becker (1978) method finally used in these studies was as follows. A sample of 100 mg yeast based on dry weight was collected by centrifugation at RCF 4960 for 10 min and suspended in 1 ml of 0,25 M Na_2CO_3. The suspension was incubated in a boiling waterbath for 120 min with regular agitation in vials capped with glass marbles in order to reduce evaporation. After cooling to ambient, the samples were made up to 2 ml total volume using double-distilled water and frozen at -20 °C

until analysed for glycogen in triplicate.

Aliquots of 0,2 ml of the well-mixed suspension were acidified with 0,3 ml of 3 M acetic acid, shifting the pH to 4,8 - 5,2. The volume was then made up to 1 ml using 0,5 ml of 0,2 M Na-acetate buffer at pH 4,8. For enzymatic hydrolysis of the glycogen, 1,4 units of amyloglucosidase were added and the suspension was incubated for 2 hours at 37 °C with regular agitation. Following centrifugation of cell residues at RCF 4960 for 10 min, the liberated glucose was determinated using the glucose oxidase/peroxidase method of Hill and Kessler (1961).

By strictly following the described procedure, no further glucose was produced from the cell residues by continued amyloglucosidase treatment. In addition, the cell residues did not stain with iodine and further glycogen could not be extracted by additional treatment. Thus, it was considered reasonable to suppose that the method was exhaustive.

5.2.4 Measurement of Cellular Intermediates

Measurement of UDPG and other cellular intermediates was achieved using the developed methods as described in Chapter 2. Nucleotides and organic acid levels were measured using isotachophoresis; ATP was additionally quantified using bioluminescence. Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate and the triose phosphates were measured using bioluminescence. For the measurement of cellular intermediates, yeast cell permeabilization and extraction was achieved using the boiling water mediated technique as previously described in Chapter 2.

5.3 RESULTS

The results for UDPG and glycogen are expressed graphically in Figures 5.2 and 5.3 for the agitated fermentations where non-proliferation was achieved using cycloheximide. Although the results of strain 2036 only are shown, strain 167 exhibited comparable values. The nitrogen limitation experiments demonstrated a similar trend. Other cellular intermediate levels were similar to those previously reported in Chapter 4. In particular, and as applicable to the discussion, fructose-6-phosphate, glucose-6-phosphate, citrate, ATP and ADP showed notable increases in non-proliferating conditions while AMP and cAMP showed an approximate 50% reduction.

Under control conditions, glycogen is seen to decrease at the initial stages of fermentation when lipid biosynthesis is most active (Quain et al., 1981) and to begin to accumulate during the period of maximum specific fermentation rate. This accumulation continues into the stationary phase. These results are in accordance with other investigators who found a similar trend for brewing yeast strains (Patel and Ingledew, 1973; Quain et al.,1981). Patel and Ingledew (1973), however, suggested that nitrogen limitation is a pre-requisite for glycogen synthesis. In Figure 5.3 glycogen synthesis starts to occur before nitrogen becomes limiting ($\pm$ 30 hours) in the normal course of fermentation, although, indeed, it continues to occur after nitrogen becomes limiting. Moreover, when growth is terminated by cycloheximide in exponential growth (or when nitrogen is limiting during the same period) the cellular glycogen content is seen to decrease. Thus, it was reasoned that a rational explanation to these somewhat paradoxical observations would only be found at the level of cellular effector control of the enzymes involved in glycogen synthesis and degradation.

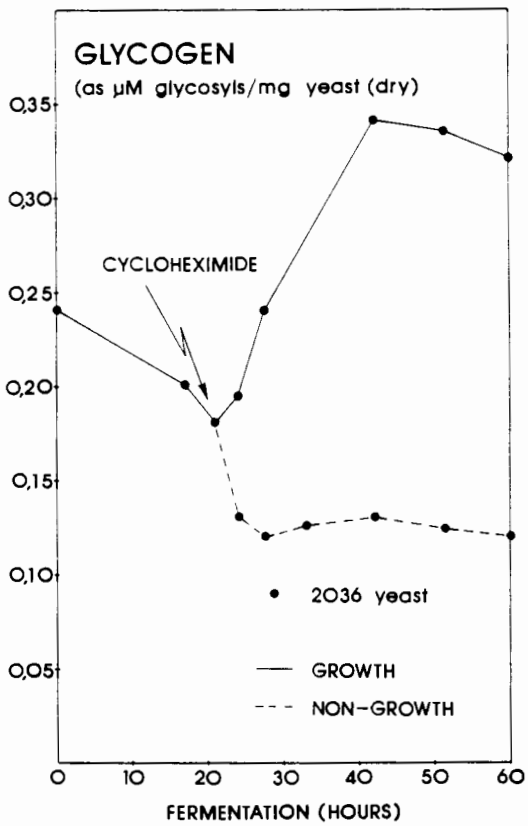


FIGURE 5.2: Glycogen levels in strain 2036 under growth and non-growth conditions.

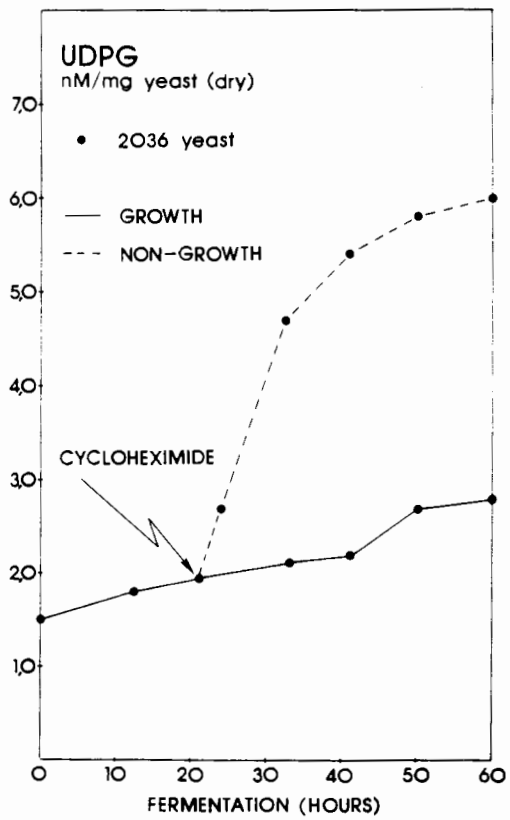


FIGURE 5.3: UDPG levels in strain 2036 under growth and non-growth conditions.

The results for the glycogen substrate, UDPG, showed a gradual increase during normal growth conditions and an extraordinary increase, as was demonstrated earlier in this dissertation, when growth was terminated. Thus, on growth inhibition, a reciprocal relationship was observed for glycogen and UDPG.

5.4 DISCUSSION

As stated earlier, phosphorylase b may be activated either by interaction with AMP or by covalent modification. Glucose-6-phosphate, ATP and ADP counteract this effect and favour the inactive state (Fischer et al., 1971). As previously seen in Chapter 4, and again confirmed at this point, glucose-6-phosphate, ATP and ADP show significant increases when yeast growth is inhibited during the exponential growth phase of fermentation while AMP levels decrease by almost 50%, thus promoting the inactive state of phosphorylase b. Moreover, no ameliorating effect by glucose-1-phosphate and/or Pi would be expected to occur since Pi remains relatively constant under the experimental conditions, with only a slight increase in level - well below its K_m value (Morgan and Parmeggiani, 1964) and glucose-1-phosphate did not demonstrate any increase in level by isotachophoretic measurement.

However, the intermediate results would certainly favour covalent modification of phosphorylase b to phosphorylase a, since the increased ATP level would be able to act as substrate for protein kinase in order to phosphorylate phosphorylase kinase. Active phosphorylase kinase shows high specificity for phosphorylase b (De Lange et al., 1968), and would create a further opportunity for ATP to act as substrate, to phosphory-

late phosphorylase b to phosphorylase a. Since phosphorylase a is active even with increased levels of glucose-6-phosphate, ATP and ADP (Fischer et al., 1971) this would explain the diminution of glycogen as seen for brewing yeast cells under the experimental growth inhibited conditions. It would also partially explain the associated increase of ADP.

AMP is a strong inhibitor of phosphorylase phosphatase so that it may be argued that any decrease in intracellular AMP might promote a phosphorylase a to b futile cycle. However, in muscle cells, the activity of phosphorylase phosphatase appears highly regulated and is switched on and off in an inverse manner to the phosphorylase kinase (Keller and Cori, 1953). Indeed, it has been demonstrated that phosphorylase a only returns to the b form when ATP reaches a low level (Fischer et al., 1971). It might be expected that a similar strict regulation exists for the yeast enzyme.

Phosphorylase kinase and glycogen synthase are equally good substrates for the protein kinase since both are phosphorylated at almost the same rate (Soderling et al., 1970). However, as previously expounded, while phosphorylation activates phosphorylase kinase, it leads to an inhibition (I to D conversion) of glycogen synthase. The inactive state of the D form would be promoted by the raised levels of ADP, UDP and GTP (Piras et al., 1968) as previously shown in Chapter 4. Thus, this situation would explain the build-up of UDPG as a cellular pool and the non-utilisation of this intermediate for glycogen synthesis. Whether the build-up of UDPG could eventually inhibit phosphorylase a activity or whether increased levels of glucose-6-phosphate could eventually facilitate glycogen synthase activity needs further clarification. A summary of proposed regulation of glycogen synthesis and degradation when exponential

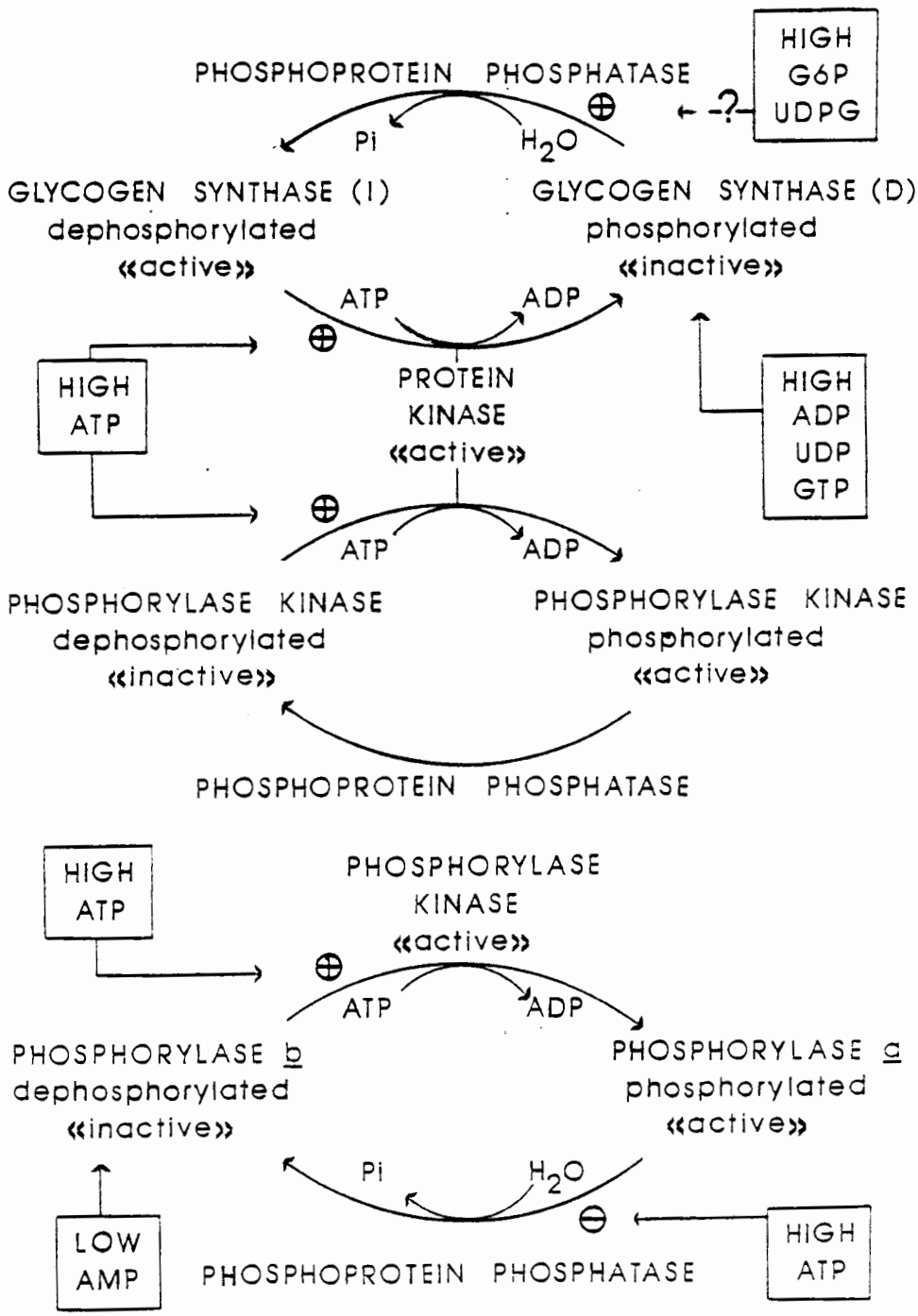


FIGURE 5.4: Summary of proposed regulation of glycogen synthesis and degradation when exponential growth is inhibited in S. uvarum.

growth is inhibited in S. uvarum is outlined in Figure 5.4.

Other questions remain to be answered. In particular the role of cyclic AMP for the activation of the protein kinase in brewing yeast. In animal tissues, activation of protein kinases from several sources occurs through the binding of cyclic AMP to a regulatory subunit of the enzyme, creating its dissociation and enzymatic activity. The levels of intracellular cyclic AMP reported in Chapter 4 when yeast growth is inhibited would not indicate a similar cyclic AMP-dependent role for the protein kinase in S. uvarum. Assimacopoulos-Jeannet et al. (1977) found activation of phosphorylase kinase to be correlated to the presence of low levels of fructose-1,6-bisphosphate. Furthermore, Exton (1979) has found that such a mechanism may be cyclic AMP-independent. Soderling (1977) has proposed that glycogen synthase may be controlled in a similar cyclic AMP-independent manner. Whether a cyclic AMP-independent protein kinase is active in yeast, has yet to be demonstrated.

It has been shown that inhibition of active yeast growth during fermentation leads to the formation of a cellular pool of UDPG as well as a concomitant depletion in glycogen.

The lack of correlation between the rate of glycogen synthesis and UDPG concentration demonstrates that stimulation of synthesis is not simply the result of push mechanism given by glucose-6-phosphate on the entire metabolic chain as has been previously thought for yeast. On the contrary, while the results show that the concentration of UDPG is influenced by a push mechanism, the formation of glycogen from UDPG has been shown in this work to be a pull mechanism by stimulation of the glycogen synthase by an alternative metabolite. The push mechanism to UDPG is

further emphasised by glucose-1-phosphate not building up, even on glycogen degradation in the presence of high glucose-6-phosphate levels, as seen in the isotachophoretic data. While these observations help to further account for the increase in UDPG as a cellular pool under growth inhibition, they do not explain the physiological meaning for the brewing yeast cell. It may be that the maintenance of a UDPG pool is a more efficient mechanism for the yeast cell to tenuously support viability, even though, under the test procedures, the cell is ultimately unable to ferment.

This work demonstrated that glycogen synthesis and degradation in brewing yeast cells is a highly regulated, physiological process, dependent not only on the availability of UDPG and glycogen as respective substrates. It is proposed that allosteric and covalent regulation of the interconvertible phosphorylated and dephosphorylated forms of glycogen synthetase and phosphorylase is of major significance. In this regard the intracellular level of the adenine nucleotides and glucose-6-phosphate would appear to play a significant role in the cascade regulation of the associated kinases and phosphatases.

CHAPTER 6

CONCLUSIONS

The aim of this work was to study and characterise the metabolic inter-relationship between yeast growth, regulation of glycolytic/gluconeogenic flux and accumulation of glycosyl donors for polysaccharide synthesis in brewing yeast (S. uvarum) fermentations.

Loss of fermenting power of a yeast population may be created by a condition that limits amino acid formation and protein synthesis. When the nitrogen requirements of the yeast cell are limited, the specific fermentation rate diminishes for each of the wort fermentable sugars.

It was considered at the outset of this work that the nature of the glycolytic/gluconeogenic controlling allosteric enzymes, often complicated by numerous intracellular modulators, prevented reproducing in vitro, a relatable in vivo enzymatic situation. To gain an accurate overall picture of intracellular glycolytic/gluconeogenic control, it was, therefore, decided to measure the levels of pertinent cellular intermediates and effectors. To this end, development of isotachophoresis and bioluminescence as measurement techniques provided the specificity and sensitivity required to examine a wide range of intracellula nucleotides, organic acids and hexose and triose phosphates.

Under conditions, where protein synthesis was inhibited or arrested during the exponential growth phase of S. uvarum fermentation, very large changes were shown to take place in the steady state levels of cellular intermediates to correlate with a reduction in glycolytic flux and glucose utilization and orientation of the glycolytic pathway in a gluconeogenic direction.

In conclusion, metabolic events were chronicled as follows.
ATP and other nucleoside triphosphate levels increased in the cellular environment as they were then not being utilized for such biosynthetic purposes. Energetically, this situation would become insupportable unless alternative mechanisms for conversion of ATP to ADP and inorganic phosphate were possible. The results suggest that the potential for substrate cycling, and, as a consequence, gluconeogenic flux, increases under conditions where growth is inhibited Clearly, this potential or ability must vary between yeast strains.

The increases observed for ATP (and GTP) favour activation of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase. Gluconeogenic activity from this point is supported by raised levels in phosphoenolpyruvate and decreased levels in pyruvate. Furthermore, the virtually unchanged levels of triose phosphates in relation to the decreased levels of fructose-1,6-bisphosphate provide further evidence of gluconeogenic stimulation.

Inhibition of protein synthesis created increases in the amino acid pool concentration of aspartate, a direct precursor of oxaloacetate. The continued availability of oxaloacetate from aspartate would therefore allow the use of this intermediate as substrate for this first step in gluconeogenesis to form phosphoenolpyruvate. The build-up of aspartate (and glutamate) would be expected to limit the production of oxaloacetate from pyruvate by ameliorating pyruvate carboxylase activity (Haarasilta, 1981). The observed reduced availability of pyruvate would strengthen this limitation.

The observations that effector levels for the allosteric enzymes, phosphfructokinase I and fructose-1,6-bisphosphatase, significantly changed from a growth-promoting to a growth-limiting condition suggest reciprocal regulation of the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle under varying growth conditions and emphasise the critical nature of this glycolytic/gluconeogenic junction.

The results clearly indicated inhibition of phosphofructokinase I as shown by the highly disturbed product-substrate relationship, creating an increase in the levels of hexose monophosphates and the precipitous fall in fructose-1,6-bisphosphate.

The results implied that inhibition of phosphofructokinase I was created by an aggregate and sequential effect of the levels of intermediates found; initially by the extraordinary increase in ATP (which may be assisted by the other nucleoside triphosphates) and further by the decreased level of fructose-1,6-bisphosphate and increased levels of citrate and phosphoenolpyruvate. Concomitant diminution of AMP (and fructose-1,6-bisphosphate) and increased levels of fructose-6-phosphate lent credence towards a distinct propensity for fructose-1,6-bisphosphatase activity enhanced by decreased glucose utilization and hence decreased glucose inactivation. Furthermore, the ratios of fructose-1,6-bisphosphate and the triose phosphates indicated a decreased aldolase activity under growth inhibition which, during gluconeogenesis, would accentuate the key role of fructose-1,6-bisphosphate as a glycolytic/gluconeogenic regulator. Simultaneously, the ratio of pyruvate to phosphoenolpyruvate indicated decreased activity of pyruvate kinase further decreasing glycolytic flux. It is proposed that the drop in fructose-1,6-bisphosphate and the levels reached would be responsible for this inhibition.

The build-up of fructose-6-phosphate not only influenced increased levels of glucose-6-phosphate, but also an increase in the cell wall precursor, UDP acetylglucosamine. No evidence was found, however, under these conditions, that this led to increased cell wall content.

Glucose-6-phosphate is not the end-point of gluconeogenesis and/or as a result of phosphofructokinase I inhibition in S. uvarum. Glycogenesis continued from glucose-6-phosphate as far as uridine diphosphoglucose (UDPG). However, non-

proliferating conditions resulted in an augmented UDPG pool which is not channelled to glycogen. Indeed, under these conditions, the level of glycogen decreased to a base level or to a level where formation and consumption are virtually equal.

Glycogen synthesis and degradation in brewing yeast cells is dependent not only on the availability of UDPG and glycogen as respective substrates. The intracellular levels of the adenine nucleotides and glucose-6-phosphate would appear to be of major significance in allosteric and covalent regulation of the interconvertible phosphorylated and dephosphorylated forms of glycogen synthase and phosphorylase.

Aggregate cellular effector control, as reported in this dissertation, is of vital importance in commercial brewing strains of S. uvarum with respect to orientation of a shift from glycolysis to gluconeogenesis under conditions where growth may be inhibited in the exponential growth phase during active fermentation. The net result is to redirect carbon to glycosyl intermediates and therefore produce an immediate limitation of biosynthetic precursors for protein synthesis. For the yeast strains examined, it may be interpreted that substrate cycling is clearly possible in vivo, particularly at the phosphofructokinase I/fructose-1,6-bisphosphatase junction. Under brewery fermentatior conditions, it is proposed that glycolytic flux may never reach a maximum. There will always be factors, depending upon fermentation parameters, tempering the activity of phosphofructokinase I and ultimately favouring gluconeogenesis. It is speculated that for maximum activity of phosphofructokinase I, when fructose-1,6-bisphosphatase activity is zero, maximum activity of phosphofructokinase 2 must be achieved.

APPENDIX A

MEDIA

Acriflavin Medium

I	Glucose	30,0 g
	$MgSO_4.7H_2O$	0,7 g
	$CaCl_2.2H_2O$	0,4 g
	Agar	20,0 g
	Distilled water	500 ml
II	$(NH_4)_2SO_4$	2,0 g
	KH_2PO_4	1,87 g
	K_2HPO_4	1,1 g
	Yeast extract	0,2 g
	Acriflavin	20,0 g
	Distilled water	500 ml

The two solutions are sterilised separately and then mixed before use.

Amino Acid Media

Solutions of pertinent amino acids were prepared at a concentration of 5 mg/ml and incorporated into minimal medium at 50 ug/ml (see minimal medium).

Glycerol Medium

Glycerol medium is minimum medium where glycerol replaces glucose (see minimum medium).

Lactose Broth

Beef extract (DIFCO)	3,0 g
Peptone (DIFCO)	5,0 g
Lactose	5,0 g
Distilled water	1 000 ml

The pH is adjusted to 6,9 before sterilising.

Lysine Medium (LYS)

Lysine medium	65,0 g
K Lactate 50% (w/v)	10,0 ml
Lactic acid 10% (v/v)	5,0 ml
Distilled water	1 000 ml

The potassium lactate and lactic acid are added after sterilisation of the other medium ingredients.

Malt Extract, Yeast Extract, Glucose, Peptone Medium (MYGP)

Malt extract (DIFCO)	3,0 g
Yeast extract (DIFCO)	3,0 g

Glucose	10,0 g
Peptone (DIFCO)	5,0 g
Distilled water	1 000 ml

The pH is adjusted to 5,5 before sterilisation.

Maltotriose Medium

I	Maltotriose	3,0 g
	$MgSO_4.7H_2O$	0,5 g
	$CaCl_2.2H_2O$	0,5 g
	Agar	30,0 g
	Distilled water	500 ml
II	$(NH_4)_2SO_4$	2,6 g
	KH_2PO_4	1,87 g
	K_2HPO_4	1,1 g
	Yeast extract	1,5 g
	Distilled water	500 ml

The pH of II is adjusted to 6,5 with KOH. The two solutions are sterilised separately and then mixed before utilization.

Minimum Medium

I	Glucose	20,0 g
	$MgSO_4.7H_2O$	0,7 g
	$CaCl_2.2H_2O$	0,4 g

	Agar	30,0 g
	Distilled water	500 ml
II	NaCl	0,5 g
	$(NH_4)_2SO_4$	1,2 g
	KH_2PO_4	1,0 g
	Citric acid	10,5 g
	Distilled water	500 ml

The pH of II is adjusted to 6,1 with KOH. The two solutions are sterilised separately and then mixed before use.

After sterilisation the following solutions are added per litre of medium:

Trace minerals	1 ml
Vitamins	10 ml

Trace mineral solution

Boric acid	500 mg
$CuSO_4.5H_2O$	40 mg
KI	100 mg
$FeCl_3.6H_2O$	5 000 mg
$MnSO_4.H_2O$	400 mg
$ZnSO_4.H_2O$	720 mg
Na molybdate	200 mg
Citric acid	1 000 mg
Distilled water	1 000 ml

Vitamin solution

Na pantothenate	200 mg
Biotin	250 mg
Nicotinic acid	100 mg
Thiamin	100 mg
Riboflavin	100 mg
Inositol	500 mg
p-aminobenzoic acid	50 mg
Pyridoxin	100 mg
Distilled water	1 000 ml

Schwarz Differential Medium (SDM)

Fuchsin - sulphite Mixture

Basic Fuchsin	4,0 g
Na_2SO_3	25,0 g
Dextrin	1,0 g

Medium

Malt extract (DIFCO)	3,0 g
Yeast extract (DIFCO)	3,0 g
Glucose	10,0 g
Peptone (DIFCO)	5,0 g
Fuchsin - Sulphite mixture	3,5 g
Agar (DIFCO)	20,0 g
Distilled water	1 000 ml

Following sterilisation the dispensed medium is stored at 30°C overnight before use.

Sporulation Medium (Fowell)

K acetate	4,0 g
Agar	15,0 g
Distilled water	1 000 ml

The pH is adjusted to 6,5 with diluted acetic acid before sterilisation.

Synthetic Growth and Non-Growth Media

I	Glucose	100,0 g
	$MgSO_4.7H_2O$	0,5 g
	$CaCl_2.2H_2O$	0,5 g
	Distilled water	500 ml
II	KH_2PO_4	2,72 g
	$(NH_4)_2SO_4$	2,6 g
	Distilled water	500 ml

The two solutions are sterilised separately and then mixed before utilization. The pH is adjusted to 5,2 before use with 1 M K_2HPO_4. For non-growth medium $(NH_4)_2SO_4$ is omitted. Media are supplemented with vitamins and trace minerals as described on pages 191-192 or by inclusion of yeast extract at 5,0 g/ 1 000 ml for enriched growth.

Universal Liquid Medium

Peptonised milk (BBL)	15,0 g
Tomato juice broth (DIFCO)	25,0 g

Glucose	10,0	g
Cycloheximide	0,001	g
Distilled water	1 000	ml

The dissolved ingredients are dispensed in 10 ml aliquots into test tubes.

The tubes are capped and sterilised at 121°C for 10 minutes only.

Wallerstein Laboratories' Nutrient Medium (WLN)

WLN Medium (DIFCO)	80,0	g
Distilled water	1 000	ml

The pH is adjusted to 5,5 - 5,8 using diluted hydrochloric acid before sterilising.

Yeast Extract Glucose Medium

I	Glucose	20,0	g
	$MgSO_4.7H_2O$	0,5	g
	$CaCl_2.2H_2O$	0,5	g
	Agar	30,0	g
	Distilled water	500	ml
II	$(NH_4)_2SO_4$	2,6	g
	KH_2PO_4	1,87	g
	K_2HPO_4	1,1	g

Yeast extract	5,0 g
Distilled water	500 ml

The pH of II is adjusted to 6,5 with KOH. The two solutions are sterilised separately and then mixed before utilization.

APPENDIX B

SPECIALISED CHEMICALS

All organic acids and other chemicals used were of analytical grade and were used without further purification. Unless otherwise mentioned, these reagents were obtained from either

E. Merck, D-6100 Darmstadt, West Germany

or

Calbiochem AG, CH-6000 Lucerne S., Switzerland.

All specialised chemicals, as listed below, were obtained from

Boehringer Mannheim GmbH, West Germany

unless otherwise stated.

ADENOSINE-5'-DIPHOSPHATE: crystallized monopotassium salt.

ADENOSINE-5'-MONOPHOSPHATE: crystallized disodium salt.

ADENOSINE-3'(2')-MONOPHOSPHORIC ACID: crystallized free acid.

ADENOSINE-3':5'-MONOPHOSPHORIC ACID, CYCLIC: crystallized free acid.

ADENOSINE-5'-TRIPHOSPHATE: crystallized disodium salt.

ALDOLASE (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13).

AMPHOLINES-LKB, Bromma, Sweden.

AMYLOGLUCOSIDASE (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3).

CYCLOHEXIMIDE.

CYTIDINE-5-DIPHOSPHORIC ACID: crystallized free acid.

CYTIDINE-5'-MONOPHOSPHATE: crystallized disodium salt.

CYTIDINE-2':3'-MONOPHOSPHORIC ACID, CYCLIC: crystallized free acid.

CYTIDINE-5'-TRIPHOSPHATE: disodium salt.

DIHYDROXYACETONE PHOSPHATE: crystallized dicyclohexylammonium salt.

FLAVINE ADENINE DINUCLEOTIDE: disodium salt.

FLAVINE MONONUCLEOTIDE: monosodium salt.

FRUCTOSE-1,6-BISPHOSPHATASE (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11).

FRUCTOSE-1,6-BISPHOSPHATE: crystallized trisodium salt.

FRUCTOSE-1-PHOSPHATE: disodium salt.

FRUCTOSE-6-PHOSPHATE: monobarium salt.

FRUCTOSE-6-PHOSPHATE KINASE (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11).

FUMARASE (L-malate hydro-lyase, EC 4.2.1.2).

GALACTOSE-1-PHOSPHATE: crystallized dipotassium salt.

GALACTOSE-6-PHOSPHATE: monobarium salt.

GLUCONATE-6-PHOSPHATE: crystallized trisodium salt.

GLUCOSE-1,6-BISPHOSPHATE: crystallized tetracyclohexylammonium salt.

GLUCOSE OXIDASE (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4).

GLUCOSE-1-PHOSPHATE: crystallized dipotassium salt.

GLUCOSE-6-PHOSPHATE: disodium salt.

GLUTATHIONE, reduced form: crystallised.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (D-glyceraldehyde-3-phosphate:NAD oxidoreductase: phosphorylating, EC 1.2.1.12).

GLYCERATE-2,3-DIPHOSPHATE: crystallized pentacyclohexylammonium salt.

GLYCERATE-2-PHOSPHATE: crystallized trisodium salt.

GLYCERATE-3-PHOSPHATE: trisodium salt.

L-GLYCEROL-3-PHOSPHATE: crystallized dicyclohexylammonium salt.

GLYCEROL-3-PHOSPHATE DEHYDROGENASE/TRIOSEPHOSPHATE ISOMERASE (glycerol-3-phosphate:NAD 2-oxidoreductase/D-glyceraldehyde-3-phosphate ketol-isomerase, EC 1.1.1.8/EC 5.3.1.1).

GLYCOGEN.

GLYCOLATE-2-PHOSPHATE: crystallized tricyclohexylammonium salt.

GUANOSINE-5'-DIPHOSPHATE: dilithium salt.

GUANOSINE-5'-MONOPHOSPHATE: crystallized disodium salt.

GUANOSINE-2':3'-MONOPHOSPHATE, CYCLIC: monosodium salt.

GUANOSINE-5'-TRIPHOSPHATE: disodium salt.

HYDROXYPROPYLMETHYLCELLULOSE (HPMC), Grade 90 HG-15000 cps - Dow Chemical Company, Midland, Mi., U.S.A.

IMIDAZOLE: crystallized.

INOSINE-5'-TRIPHOSPHATE: disodium salt.

MANNOSE-6-PHOSPHATE: crystallized monobarium salt.

β-NICOTINAMIDE-ADENINE DINUCLEOTIDE: lyophilised free acid.

β-NICOTINAMIDE-ADENINE DINUCLEOTIDE, REDUCED: disodium salt.

β-NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE: disodium salt.

β-NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE, REDUCED: tetrasodium salt.

5'-NUCLEOTIDASE (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) - Sigma Chemical Company, St. Louis, Mo., U.S.A.

PHOSPHATASE, ALKALINE (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1).

PHOSPHOENOLPYRUVATE: crystallized monopotassium salt.

PHOSPHOGLUCOMUTASE (α-D-glucose-1,6-bisphosphate: α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1).

5-PHOSPHO-α-D-RIBOSE-1-DIPHOSPHATE: tetrasodium salt.

POLYAMIDE TL6 - Macherey, Nagel and Company, D-516 Düren, West Germany.

PYRUVATE: crystallized monosodium salt.

URIDINE-5'-DIPHOSPHATE: crystallized dipotassium salt.

URIDINE-5'-DIPHOSPHO-N-ACETYLGLUCOSAMINE: disodium salt.

URIDINE-5'-DIPHOSPHO-GLUCOSE: disodium salt.

URIDINE-5'-MONOPHOSPHATE: crystallized disodium salt.

URIDINE-2':3'-MONOPHOSPHATE, CYCLIC: monosodium salt.

URIDINE-5'-TRIPHOSPHATE: trisodium salt.

APPENDIX C

LABORATORY YEAST PROPAGATION TECHNIQUE

1. Aeration of the Wort

The wort must be vigorously aerated with sterile air or oxygen. The dissolved oxygen content of the wort at each incremental stage should be 8 - 9 ppm.

2. Propagation Procedure

The incremental factor must never exceed 10 times the previous volume of fermented wort. Before transfering at each stage of propagation the previous stage should have attained at least 45% of the apparent attenuation of the fermenting wort.

Stage 1 Working aseptically, remove a loopful of culture yeast from a slope culture on MYGP medium with a sterile loop and inoculate into 10 ml of sterile wort. Allow to stand in the dark at 20°C with frequent agitation.

Stage 2 Transfer the 10 ml culture into 100 ml of sterile wort. Maintain in the dark at 20°C with frequent agitation.

Stage 3 Transfer the 100 ml culture into 1 l of sterile wort. Maintain in the dark at 20°C with frequent agitation.

Stage 4 Transfer the 1 l culture into 10 l of sterile wort. Maintain in the dark at 20°C with frequent agitation.

APPENDIX D

INFECTION MONITORING PROCEDURES

A. Bacteria

1. Inoculation. 10 ml each of universal liquid medium and lactose broth is inoculated with 1 ml of sample.

2. Incubation. At 30°C for 4 days.

3. Interpretation. Microscopically for presence of bacteria.

B. Wild Yeast

1. Inoculation. Use a washed (with physiological saline 3x) suspension of yeast cells (± 10 x 10^6 cells/ml) from the fermention to be monitored. Inoculate 0,2 ml of this suspension on to one plate of lysine medium, 0,2 ml on to one plate of Schwarz differential medium and 0,1 ml of diluted suspension (10x) on to two plates of Wallerstein Laboratories' Nutrient medium. Spread the suspension evenly using a sterile Drigalski spatula.

2. Incubation. At 25°C for 4 - 5 days.

3. Interpretation. According to the table on the following page.

YEAST	LYS (Distinct colonies)	SDM (Distinct colonies)	WLN (Colonies other than culture yeast)
Non-Saccharomyces wild yeast	+	+	+
Saccharomyces wild yeast	-	+	+
Bottom culture yeast (S. uvarum)	-	-	+

APPENDIX E

YEAST COUNTING TECHNIQUES

The haemacytometer and coulter counter methods were used during the course of this work.

A. Yeast Counts: Haemacytometer Method

1. Requirements

Hawksley-Cristallite haemacytometer with improved Neubauer ruling

Haemacytometer coverslip

Capillary pipette

Test tube

10 ml graduated pipette

10% acetic acid (10 ml glacial acetic acid in 100 ml distilled water)

Tally counter

Sample for yeast count.

2. Method

2.1 Mix the sample well and transfer 1 ml to a test tube.

2.2 Add a known amount of 10% acetic acid by pipette so that the final suspension is such that the markings on a pipette can be seen through it. Note the amount of acetic acid added.

2.3 Place the clean, dry haemacytometer coverslip on the

counting chamber, ensuring that there is perfect contact with the supporting ribs, i.e. Newton's rings are visible.

(Note: a normal coverslip cannot be used as its surface has not been ground and may, therefore, be irregular).

2.4 Mix the suspension very well and remove a small quantity with the capillary pipette.

2.5 Blow out a small quantity from the tip where differentiation can occur due to capillary action, and immediately allow a small amount of sample to run under the coverslip so that the chamber is properly filled but not flooded, and without dislodging the coverslip.

2.6 Place the chamber on the microscope stage and examine under low power magnification to ensure that there is an even distribution of cells and that there are not so many cells that counting is difficult.

2.7 Refocus under 400 x magnification and count the number of cells in the four corner squares and the central square (= 5 squares).

(Note: A cell is considered to be inside a square if its edge does not cross the centre line of the triple boundary ruling.

Buds are only counted it they are greater than half the size of the mother cell to which they are attached).

2.8 After counting, immerse the counting chamber and

coverslip in a detergent, followed by thorough rinsing with distilled water and drying with a lens tissue.

3. Calculations

x = amount of acetic acid added in ml

y = number of cells counted in 5 large squares

So if there are y cells in 5 squares there must be 5 x y cells in 25 squares.

The volume under the coverslip is 0,1 cubic mm.

So there are 5y cells in 0,1 cubic mm

or

$5\ y\ x\ 10^4$ cells in 1,0 cubic cm

or

1,0 ml of undiluted suspension

Therefore $5\ y\ x\ 10^4$ in $\frac{1}{(x+1)}$ ml of diluted suspension.

Which can be expressed as:

$$\frac{5}{1} \times y \times 10^4 \times (x+1)$$

or $5\ y\ x\ 10^4\ x\ (x+1)$ = yeast count

This is generally reported in yeast count x 10^6 i.e. yeast count in millions.

B. Yeast Counts: Coulter Counter Method

1. Requirements

1.1 Equipment

Coulter Counter Model Industrial 'D'

Coulter Automatic Diluter III

Ultrasonic Waterbath

Particle-free cuvettes

Sample for yeast count

2 - litre flask

Aluminium foil

Clean, dry membrane filter apparatus

Clean, dry Buchner flask to fit membrane filtration apparatus

Membranes of the following porosities:
12 or 8 microns, 3,0 microns, 1,2 microns, 0,45 microns

Waterbath set at 37°C

Whatman no. 1 filter paper

Vacuum pump

pH meter

1.2 Electrolyte Solution

1,0 g .. .. .. Pepsin (Merck 7190)

1,8 g .. .. .. EDTA

1 000 ml .. .. .. Isoton (Coulter Electronics)

Dilute HCl for adjustment of pH.

1.3 Cleaning Solution

1 000 ml 0,1% Sodium Hypochlorite Solution

1 000 ml Isoton (Coulter Electronics)

2. Methods

2.1 Preparation of Electrolyte Solution

2.1.1 Dissolve 1,0 pepsin in 1 000 ml Isoton in a clean, dust-free glass flask.
(Larger quantities of diluent may be prepared if required. The solution will keep at room temperature for up to four weeks, provided the container is kept closed and free from exposure to dust or contamination).

2.1.2 Adjust the pH of the solution to 4,4 using dilute HCl (no other acid is suitable). The solution may appear slightly milky after adjustment of pH.

2.1.3 Cover the mouth of the flask and place in a waterbath at 37°C for a minimum of 24 hours. A milky precipitate will appear.

2.1.4 Filter the solution throug filter paper (Whatman no. 1 or Selecta no. 080601/2 fluted filter papers) into a clean, dust-free flask.

2.1.5 Pass the filtrate through a clean, dust-free membrane-filter apparatus using a membrane of large porosity (8 - 12 microns) into another clean dust-free flask.

2.1.6 Repeat the membrane filtration with membranes of

decreasing porosity (e.g. 3 microns, 1,2 microns) and finally filter through a 0,45 micron membrane.

2.1.7 The solution is now ready for use. Store in a clean glass flask or bottle, the mouth of which must be kept securely covered with foil, parafilm or a closely-fitting stopper to exclude dust. Keep covered during storage, and when in use.

2.1.8 With each batch of yeast counts a "base-count" using only the above solution is done in exactly the same way (including 15 minutes exposure to ultrasonic) and should not exceed 0,5 million particles/ml.

2.2 Yeast counts

2.2.1 Remove covers from Coulter Counter and diluter, and switch on. Allow 5 minutes for the pump to build up a vacuum.

2.2.2 Press diluter bar once, and then again, to flush. Note: The diluter should be set to give a 1 in 500 dilution (WBC).

2.2.3 Allow sample containing yeast cells to mix on a stirrer for 15-20 seconds.

2.2.4 Remove the beaker of yeast from the stirrer and immediately immerse the white plastic tip of the diluter to a depth of $\pm$ 1-2 cm below the surface of the liquid (not foam). Press the diluter bar momentarily. The diluter will automatically suck up the right amount of fluid.

2.2.5 Remove sample and set aside. Using a tissue, wipe the white plastic diluter tip gently downwards to remove excess sample, at the same time taking care not to blot the sample inside the tip.

2.2.6 Open a particle-free cuvette. Hold in such a way that fluid from both the plastic and the metal tip will flow into it. Depress diluter bar momentarily. The diluted sample will run into the container. The tips of the diluter must not be immersed in the fluid. Hold the container at a suitable angle (i.e. so that the liquid runs down to the side of the container and not straight into it) to avoid splashing.

2.2.7 When fluid flow stops, replace container lid, gently suspend the dilution by inverting the container two or three times, and place in the ultrasonic waterbath.

2.2.8 Switch on waterbath for 15 minutes. NOTE that the level of the water in the waterbath should not be below the level of sample in the container.

2.2.9 Switch off waterbath. (Do not operate waterbath and Coulter simultaneously). Remove container.

2.2.10 Before taking a reading, invert the container gently 4-5 times to resuspend the yeast cells without creating bubbles.

2.2.11 Open container. Lower the platform of the counter, place opened container on it, and allow it to come up again so that the aperture tube and electrode

are immersed in the liquid.

2.2.12 Check, by means of the telescope, that the aperture is clearly visible, rotate the container on the platform until it comes into view.

2.2.13 Open the upper tap. A baseline threshold will appear as a green line on the screen. Press the button to revert the digital reading to close the upper tap.

2.2.14 The instrument will now commence counting. While it is doing so, be on the alert for possible blocking of the aperture detectable by audible irregularities or visible irregularities on the screen.

2.2.15 If a blockage should occur, lower the platform to expose the aperture tube and brush the pink wafer very gently with the brush provided to clean the aperture. Raise the platform to re-immerse the tube and check with the telescope that the aperture is clean.

2.2.16 Repeat from No - 2.2.13

(It is advisable to take two readings per sample to ensure that no undetected blockages have occurred).

NOTE: For every batch of counts done, do one "background" count, i.e. do a count on diluent without yeast from the diluter. Treat in exactly the same way as for other counts.

3. Calculations

The reading on the digital readout, divided by 1 000, minus the reading of the background count, divided by 1 000 is equal to the yeast count x 10^6.

N.B. If the reading is greater than 10,0 x 10^6 it must be corrected on a coincidence chart.

APPENDIX F

SOME REPORTED METHODS FOR EXTRACTION OF CELLULAR NUCLEOTIDES

Aqueous Buffers

TRIS	pH 7.75, 20 mM, 100°C	(Holm-Hansen and Booth 1966)
TRIS-EDTA	pH 7.75, 20 mM - 2 mM, 100°C	(Patterson et al., 1970; Lundin and Thore, 1975)
TRIS-arsenate-EDTA-butanol	pH 7.4, 100 mM - 10 mM - 10 mM - 6%, 100°C	(Chapelle and Levin, 1968; Lundin and Thore, 1975)
Glycine-EDTA	pH 10, 10 mM - 5 mM, 100°C	(Tobin et al., 1978)
Sodium bicarbonate	pH 8.5, 100 mM, 100°C	(Bancroft et al., 1976)
Phosphate	pH 7.7, 65 mM, 100°C	(Bulleid, 1978)
Phosphate-citrate	pH 7.7, 40 mM - 20 mM, 100°C	(Bulleid, 1978)
HEPES	pH 7.5, 25 mM, 100°C	(Cunningham and Wetzel, 1978)
Water	pH 5 to 8.55 M, 100°C	(Beutler and Baluda, 1963; Ryder et al., 1983)

Inorganic Acids

Nitric	0.1 N, 20°C	(Picciolo et al., 1976)
Perchloric	0.4 M, 0°C	(Rose and Brockman, 1977; Wild et al., 1978; Heldt et al., 1980)

Inorganic Acids (Cont'd)		
Perchloric-EDTA	2.3 M - 67 mM, 0°C	(Lundin and Thore, 1975)
Phosphoric-EDTA	1 M - 48 mM, 4°C	(Karl and Craven, 1980)
Sulphuric	0.3 M, Ambient	(Forrest and Walker, 1965)
Sulphuric-charcoal adsorption	0.6 N, 4°C	(Hodson et al., 1976)
Sulphuric-EDTA	0.6 N - 48 mM, 4°C	(Karl and La Rock, 1975)
Sulphuric-ion exchange	0.6 N - Na^+ resin, 4°C	(Lee et al., 1971)
Sulphuric-ion exchange-ethanolamine	0.6 N - Na^+ resin - 5 N, 25°C	(Eiland, 1979)
Sulphuric-oxalic	1 to 2 N - 62 mM, 4°C	(Cunningham and Wetzel, 1978)
Organic Acids		
Acetic	1 M, freeze-thaw	(Nazar et al., 1970)
Formic	pH 3.2 M, 0°	(Klofat et al., 1969; Bagnara and Finch, 1972)
Formic-EDTA	0.46 M - 2 mM, 0°C	(Klofat et al., 1969)
Trichloroacetic	5 to 10% (wt/vol), 0°C	(Bagnara and Finch, 1972; Khym, 1976; Lothrop et al., 1980)
Trichloroacetic-EDTA	0.5 M - 17 mM, 0°C	(Lundin and Thore, 1975)

Organic Acids (Cont'd)

Trichloroacetic-phosphate-paraquat	0.5 M - 0.25 M - 0.1 M, 4°C	(Jenkinson and Oades, 1979)

Organic Solvents

Acetone	90% (vol/vol) 100%	(Clark et al., 1978; Miller, 1978; Hysert and Morrison, 1978)
Acetone-trichloroacetic acid-ether	90% - 10% - 100%	(Clark et al., 1978)
N-Bromosuccinimide-EDTA	10 mM - 20 mM, 25°C	(MacCloed et al., 1969)
Formamide	10%, 25°C	(Knust et al., 1975)
Dichloromethane	90%, 25°C	(Knust et al., 1975)
Methanol	100%, 25°C	(Knust et al., 1975)
Butanol	5%, Ambient	(Dhople and Hanks, 1973)
Butanol-EDTA	25% (vol/vol) - 15 mM, 20°C	(Conklin and MacGregor, 1972)
Butanol octanol	50 to 90% (vol/vol) - 100%	(Sharpe et al., 1970)
Chloroform	23% (vol/vol), 98%	(Dhople and Hanks 1973)
Chloroform-EDTA	100% - 100 mM, 20°C	(Lundin and Thore, 1975)
Chloroform phosphate	pH 11.7, 23% (vol/vol) - 10 mM, 25°C	(Tobin et al., 1978)

Organic Solvents (Cont'd)		
Dimethyl sulfoxide	90% in 50 mM TRIS	(Lee et al., 1971)
Dimethyl sulfoxide–sulfuric acid	90% in 0.1 N H_2SO_4	(Lee et al., 1971)
Ethanol	pH 7, 96%, 78°C	(Entian et al., 1977; Lundin and Thore, 1975)
Inorganic Bases		
Potassium hydroxide–EDTA	10 mM - 2 mM, 100°C	(Lundin and Thore, 1975)

LITERATURE CITED

ANDREASEN, A.J., and T.J.B. STIER. 1954. Anaerobic nutrition of Saccharomyces cerevisiae. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell. Comp. Phys. 43: 271-281.

ARIES, V., and B.H. KIRSOP. 1978. Sterol biosynthesis by strains of Saccharomyces cerevisiae in the presence and absence of dissolved oxygen. J. Inst. Brew. 84: 118-122.

ASSIMACOPOULOS-JEANNET, F.D., P.F. BLACKMORE, and J.H. EXTON. 1977. Studies on α-adrenergic activation of hepatic glucose output. J. Biol. Chem. 252: 2662-2669.

ATKINS, S.D., E.D. PALMER, P.D. ENGLISH, B. MORGAN, M.A. CAWTHORNE, and J. GREEN. 1972. The role of cytochrome P-450 in cholesterol biogenesis and catabolism. Biochem. J. 128: 237-242.

ATKINSON, D.E. 1977. Cellular energy metabolism and its regulation. Academic Press, Inc., New York.

ATKINSON, D.E., and G.M. WALTON. 1967. Adenosine triphosphate conservation in metabolic regulation. J. Biol. Chem. 242: 3239-3241.

ATZPODIEN, W., J.M. GANCEDO, V. HAGMAIER, and H. HOLZER. 1970. Desensitization of yeast phosphofructokinase to ATP-inhibition by a protein fraction from yeast. Eur. J. Biochem. 12: 6-10.

BAGNARA, A.S., and L.R. FINCH. 1972. Quantitative extraction and estimation of intracellular nucleoside triphosphates of Escherichia coli.

Anal. Biochem. 45: 24-34.

BALL, W.J., Jr., and D.E. ATKINSON. 1975. Adenylate energy charge in Saccharomyces cerevisiae during starvation. J. Bacteriol. 121: 975-982.

BANCROFT, K., E.A. PAUL, and W.J. WIEBE. 1976. The extraction and measurement of adenosine triphosphate from marine sediments. Limnol. Oceanogr. 21: 473-480.

BARFORD, J.P. 1979. Microbial growth models. Ph. D. thesis. University of New South Wales, Sidney.

BARFORD, J.P., and R.J. HALL. 1979. Investigation of the significance of a carbon and redox balance to the measurement of gaseous metabolism in Saccharomyces cerevisiae. Biotech. Bioeng. 21: 609-626.

BARFORD, J.P., and R.J. HALL. 1979. An examination of the Crabtree effect in Saccharomyces cerevisiae: The role of respiratory adaptation. J. Gen. Microbiol. 114: 267-275.

BARNETT, J.A., and H.L. KORNBERG. 1960. The utilization by yeasts of acids of the tricarboxylic acid cycle. J. Gen. Microbiol. 23: 65-82.

BARTON-WRIGHT, E.C. 1953. The carbohydrates of wort and the problem of attenuation. Wallerstein Lab. Commun. 16: 5-28.

BARTRONS, R., E. VAN SCHAFTINGEN, S. VISSERS, and H-G. HERS. 1982. The stimulation of yeast phosphofructokinase by fructose-2,6-bisphosphate. FEBS Lett. 143: 137-140.

BAUCHOP, T., and S.R. ELSDEN. 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. 23: 457-469.

BECK, C., and H.K. VON MEYENBURG. 1968. Enzyme pattern and aerobic growth of Saccharomyces cerevisiae under various degrees of glucose limitation. J. Bacteriol. 96: 479-486.

BECKER, J.U. 1978. A method for glycogen determination in whole yeast cells. Anal. Biochem. 86: 56-64.

BECKERS, J.L., and F.M. EVERAETS. 1972. The separation of nucleotides by isotachophoresis. J. Chromatogr. 71: 380-385.

BETZ, A., and C. MOORE. 1967. Fluctuating metabolite levels in yeast cells and extracts, and the control of phosphofructokinase activity in vitro. Arch. Biochem. Biophys. 120: 268-273.

BETZ, A., U. RÖTTGER, and K.H. KREUZBERG. 1975. Allosteric activation and competitive inhibition of yeast phosphofructokinase by D-fructose. Arch. Microbiol. 103: 123-126.

BEUTLER, E., and M.C. BALUDA. 1963. Simplified determination of blood adenosine triphosphate using the firefly system. Blood. 23: 688-698.

BINION, S., and L.S. RODKEY. 1981. Simplified method for synthesizing ampholytes suitable for use in isoelectric focusing of immunoglobulins in agarose gels. Anal. Biochem. 112: 362-366.

BLACK, W.J., A. VAN TOL, J. FERNANDO, and B.L. HORECKER. 1972. Isolation of a highly active fructose diphosphatase from rabbit muscle: Its subunit

structure and activation by monovalent cations. Arch. Biochem. Biophys. 151: 576-590.

BLANGY, D., H. BUC, and J. MONOD. 1968. Kinetics of the allosteric interactions of phosphofructokinase from Escherichia coli. J. Mol. Biol. 31: 13-35.

BRAND, I.A., and H.D. SOLLING. 1974. Rat liver phosphofructokinase. J. Biol. Chem. 249: 7824-7831.

BROWN, H.D., T. SATYANARAYANA, and H.E. UMBARGER. 1975. Biosynthesis of branched-chain amino acids in yeast: Effect of carbon source on leucine biosynthetic enzymes. J. Bacteriol. 121: 959-969.

BULLEID, N.C. 1978. An improved method for the extraction of adenosine triphosphate from marine sediment and seawater. Limnol. Oceanogr. 23: 174-178.

CABIB, E., and L.F. LELOIR. 1958. The biosynthesis of trehalose phosphate. J. Biol. Chem. 231: 259-275.

CANNATA, J.J.B., and M.A.C. DE FLOMBAUM. 1974. Phosphoenolpyruvate carboxykinase from baker's yeast. J. Biol. Chem. 249: 3356-3365.

CANNATA, J.J.B., and A.O.M. STOPPANI. 1963a. Phosphopyruvate carboxylase from baker's yeast. I. Isolation, purification and characterization. J. Biol. Chem. 238: 1196-1207.

CANNATA, J.J.B., and A.O.M. STOPPANI. 1963b. Phosphopyruvate carboxylase from baker's yeast. II. Properties of enzyme. J. Biol. Chem. 238:

1208-1212.

CANNATA, J.J.B., and A.O.M. STOPPANI. 1963c. Catalytic properties of phosphopyruvate carboxylase from baker's yeast. Nature. 200: 573-574.

CAZZULO, J.J., L.M. CLAISSE, and A.O.M. STOPPANI. 1968. Carboxylase levels and carbon dioxide fixation in baker's yeast. J. Bacteriol. 96: 623-628.

CHAPMAN, C., and W. BARTLEY. 1968. The kinetics of enzyme changes in yeast under conditions which cause the loss of mitochondria. Biochem. J. 107: 455-465.

CHAPPELLE, E.W., and G.V. LEVIN. 1968. Use of the firefly bioluminescent reaction for rapid detection and counting of bacteria. Biochem. Med. 2: 41-52.

CHEN, S.L. 1959. Carbohydrate assimilation in actively growing yeast, Saccharomyces cerevisiae. I. Metabolic pathways for ^{14}C glucose utilization by yeast during aerobic fermentation. Biochim. Biophys. Acta. 32: 470-476.

CHESTER, V.E. 1963. The dissimilation of the carbohydrate reserves of a strain of Saccharomyces cerevisiae. Biochem. J. 86: 153-160.

CHESTER, V.E. 1968. Heritable glycogen-storage deficiency in yeast and its induction by ultra-violet light. J. Gen. Microbiol. 51: 49-56.

CHESTER, V.E., and M.J. BYRNE. 1968. Carbohydrate composition and UDP-glucose concentration in a normal yeast and a mutant deficient in glycogen. Arch. Biochem. Biophys. 127: 556-562.

CIRIACY, M. 1978. A yeast mutant with glucose-resistant formation of mitochondrial enzymes. Mol. Gen. Genet. 129: 329-335.

CIRIACY, M. 1979. Isolation and characterization of further cis- and trans-acting regulatory elements involved in the synthesis of glucose-repressible alcohol dehydrogenase (ADH II) in Saccharomyces cerevisiae. Mol. Gen. Genet. 176: 427-432.

CIRIACY, M., and J. BREITENBACH. 1979. Physiological effects of seven different blocks in glycolysis in Saccharomyces cerevisiae. J. Bacteriol. 139: 152-160.

CLARK, J.R., D.I. MESSENGER, K.L. DICKSON, and J. CAIRNS, Jr. 1978. Extraction of ATP from Aufwuchs communities. Limnol. Oceanogr. 23: 1055-1059.

CLARK, M.G., D.P. BLOXHAM, P.C. HOLLAND, and H.A. LARDY. 1973. Examination of the fructose diphosphatase - phosphofructokinase substrate cycle in the flight muscle of Bombus affinis. Biochem. J. 134: 589-597.

CLARK, M.G., N.M. KNEER, A.L. BOSCH, and H.A. LARDY. 1974. The fructose-1,6-diphosphate - phosphofructokinase substrate cycle. J. Biol. Chem. 249: 5695-5703.

CLARK, M.G., S.D. NEVILLE, and D.G. CLARK. 1981. Adrenergic regulation of pyruvate kinase and gluconeogenesis in hepatocytes from phosphorylase kinase-deficient (gsd/gsd) rats. Biochem. Biophys. Res. Commun. 103: 690-697.

CLARK, M.G., C.H. WILLIAMS, W.F. PFEIFER, D.P. BLOXHAM, P.C. HOLLAND, C.A.

TAYLOR, and H.A. LARDY. 1973. Accelerated substrate cycling of fructose-6-phosphate in the muscle of malignant hyperthermic pigs. Nature (London). 245: 99-101.

COLE, H.A., J.W.T. WIMPENNY, and D.E. HUGHES. 1967. The ATP pool in Escherichia coli. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta. 143: 445-453.

COLOMBO, G., P.W. TATE, A.W. GIROTTI, and R.G. KEMP. 1975. Interaction of inhibitors with muscle phosphofructokinase. J. Biol. Chem. 250: 9404-9412.

CONKLIN, A.R., Jr., and A.N. MacGREGOR. 1972. Soil adenosine triphosphate: Extraction recovery and half-life. Bull. Environ. Contam. Toxicol. 7: 296-300.

COOK, A.H. 1969. Yeast performance - its significance and assessment. Proceedings of the European Brewery Convention Congress, Interlaken, p. 225-238. Elsevier, Amsterdam.

COOPER, T.G. 1982. Transport in Saccharomyces cerevisiae, p. 399-409. In J.N. Strathern, E.W. Jones and J.R. Broach (eds.), The molecular biology of the yeast Saccharomyces: Metabolism and gene expression. Cold Spring Harbor Laboratory, New York.

COSTA-CARVALHO, V.L.A., A. PANEK, and J.R. MATTOON. 1978. Effects of carbon source and genetic modifications of phosphoglucomutase in trehalose metabolism in Saccharomyces cerevisiae. FEMS Microbiol. Lett. 4: 221-224.

CRABTREE, H.G. 1929. Observations on the carbohydrate metabolism of tumours. Biochem. J. 23: 536-545.

CRAIG, J.W., T.W. RALL, and J. LARNER. 1969. The influence of insulin and epinephrine on adenosine 3',5'-phosphate and glycogen transferase in muscle. Biochim. Biophys. Acta. 177: 213-219.

CUNNINGHAM, H.W., and R.G. WETZEL. 1978. Fulvic acid interferences on ATP determinations in sediments. Limnol. Oceanogr. 23: 166-173.

CURTIS, N.S., and S. WENHAM. 1958. Some observations on the declining attenuative power of brewery yeast. J. Inst. Brew. 64: 421-428.

DANFORTH, W.H. Glycogen synthetase activity in skeletal muscle. J. Biol. Chem. 240: 588-593.

DATTA, A.G., B. ABRAMS, T. SASAKI, J.W.O. VAN DEN BERG, S. PONTREMOLI, and B.L. HORECKER. 1974. The activation of rabbit muscle, liver and kidney fructose bisphosphatases by histidine and citrate. Arch. Biochem. Biophys. 165: 641-645.

DEAK, T. 1978. On the existence of H^+-symport in yeasts. A comparative study. Arch. Microbiol. 116: 205-211.

DE DEKEN, R.H. 1966. The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44: 149-156.

DE LA FUENTE, G., and A. SOLS. 1962. Transport of sugars in yeast. II: Mechanisms of utilization of disaccharides and related glycosides. Biochim. Biophys. Acta. 56: 49-62.

DE LANGE, R.J., R.G. KEMP, R.D. RILEY, R.A. COOPER, and E.G. KREBS. 1968. Activation of skeletal muscle phosphorylase kinase by adenosine tri-

phosphate and adenosine 3',5'-monophosphate. J. Biol. Chem. 243: 2200-2208.

DELLWEG, H., G. SCHLANDERER, and R. MEGNET. 1973. Catabolit-Repression und cyclisches Adenosin-3',5'-monophosphat in Saccharomyces cerevisiae. Proceedings of the European Brewery Convention Congress, Salzburg, p. 241-253. Elsevier, Amsterdam.

DELMOTTE, P. 1977. Analysis of complex protein mixtures by capillary isotachophoresis - some qualitative and quantitative aspects. Sci. Tools. 24: 33-41.

DE LUCA, M. 1976. Firefly luciferase. Adv. Enzymol. Relat. Areas Mol. Biol. 44: 37-68.

DENTON, R.M., P.J. RANDLE, and B.R. MARTIN. 1972 Stimulation by Ca^{++} of pyruvate dehydrogenase phosphate phosphatase. Biochem. J. 128: 161-163.

DE TORRONTEGUI, G., E. PALACIÁN, and M. LOSADA. 1966. Phosphoenolpyruvate carboxykinase in gluconeogenesis and its repression by hexoses in yeasts. Biochem. Biophys. Res. Commun. 22: 227-231.

DE WULF, H., and H-G. HERS. 1967. The stimulation of glycogen synthesis and of glycogen synthetase in the liver by the administration of glucose. Eur. J. Biochem. 2: 50-56.

DE WULF, H., W. STALMANS, and H-G. HERS. 1970. The effect of glucose and of a treatment by glucocorticoids on the activation in vitro of liver glycogen synthetase. Eur. J. Biochem. 15: 1-8.

DHOPLE, A.M., and J.H. HANKS. 1973. Quantitative extraction of adenosine triphosphate from cultivable and host-grown microbes: Calculation of adenosine triphosphate pools. Appl. Microbiol. 26: 399-403.

DICKENSON, C.J., and F.M. DICKINSON. 1978. Inhibition by ethanol, acetaldehyde and trifluoroethanol of reactions catalysed by yeast and horse liver alcohol dehydrogenases. Biochem. J. 171: 613-627.

DILLEMANS, M., and C.A. MASSCHELEIN. 1981. Influence des lipides libres et liés sur la fermentation du moût. Cerevisia. 4: 195-200.

DIVJAK, S., and J-R. MOR. 1973. On the activity of carbon dioxide fixation in growing yeast. Arch. Microbiol. 94: 191-196.

DIXON, G.H., H.L. KORNBERG, and P. LUND. 1960. Purification and properties of malate synthetase. Biochim. Biophys. Acta. 41: 217-233.

EAGLE, G.R., and R.K. SCOPES. 1981. Control of substrate cycling at fructose phosphates in a reconstituted muscle glycolytic system. Arch. Biochem. Biophys. 208: 593-601.

EILAND, F. 1979. An improved method for determination of adenosine triphosphate (ATP) in soil. Soil. Biol. Biochem. 11: 31-35.

EL KOUNI, M.H., and S. CHA. 1981. A simple radioisotopic assay for nucleoside kinases employing alumina for separation of nucleosides and nucleotides. 111: 67-71.

EL-MAGHRABI, M.R., E. FOX, J. PILKIS, and S.J. PILKIS. 1982. Cyclic AMP-dependent phosphorylation of rat liver 6-phosphofructo 2-kinase/

fructose-2,6-bisphosphatase. Biochem. Biophys. Res. Commun. 106: 794-802.

EMEIS, C.C. 1965. Genetische Untersuchungen und Kulturhefen als Grundlagen zur Züchtung neuer Stämme mit technologisch günstigen Eigenschaffen. Thesis. Technische Universität, Berlin.

ENGLAND, P.J., and P.J. RANDLE. 1967. Effectors of rat-heart hexokinase and the control of rates of glucose phosphorylation in the perfused rat-heart. Biochem. J. 105: 907-920.

ENTIAN, K-D. 1980. A defect in carbon catabolite repression associated with uncontrollable and excessive maltose uptake. Mol. Gen. Genet. 179:1969-1975.

ENTIAN, K-D., F.K. ZIMMERMANN, and I. SCHEEL. 1977. A partial defect in carbon catabolite repression in mutants of Saccharomyces cerevisiae with reduced hexose phosphorylation. Mol. Gen. Genet. 156: 99-105.

EPHRUSSI, B. 1953. Nucleo-cytoplasmic relations in microorganisms. Oxford Univ. Press, London.

EPHRUSSI, B., and P. SLONIMSKI. 1950. La synthèse adaptative des cytochromes chez la levure de boulangerie. Biochim. Biophys. Acta. 6: 256-273.

ERIKSSON, G. 1980. A method for measuring nucleotides in the liver of rat with isotachophoresis. Anal. Biochem. 109: 239-246.

ESSIEN, D., and D.L. PYLE. 1983. Energy conservation in ethanol production by fermentation. Proc. Biochem. 18: 31-37.

EVERAERTS, F.M., and Th. P.E.M. VERHEGGEN. 1974. Isotachophoresis. Applications in the biochemical field. J. Chromatogr. 91: 837-851.

EXTON, J.H. 1979. Mechanisms involved in effects of catecholamines on liver carbohydrate metabolism. Biochem. Pharmacol. 28: 2237-2240.

FANG, M., and R.A. BUTOW. 1970. Nucleotide reversal of mitochondrial repression in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 41: 1579-1583.

FELIX, H. 1982. Permeabilized cells. Anal. Biochem. 120: 211-234.

FISCHER, E.H., L.M.G. HEILMEYER, Jr., and R.H. HASCHKE. 1971. Phosphorylase and the control of glycogen degradation. Curr. Top. Regul. 4: 211-251.

FITCH, W.M., and I.L. CHAIKOFF. 1960. Extent and pattern of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem. 235: 554-557.

FLETTERICK, R.J., and N.B. MADSEN. 1980. The structures and related functions of phosphorylase a. Annu. Rev. Biochem. 49: 31-61.

FORREST, W.W., and D.J. WALKER. 1965. Synthesis of reserve materials for endogenous metabolism in Streptococcus faecalis. J. Bacteriol. 89: 1448-1452.

FOSSETT, M., L.W. MUIR, L.D. NIELSEN, and E.H. FISCHER. 1971. Purification and properties of yeast glycogen phosphorylase a and b. Biochemistry. 10: 4105-4110.

FOWELL, R.R. 1952. Sodium acetate agar as a sporulation medium for yeast. Nature. 170: 578.

FOWLER, P.W., A.J.L. BALL, and D.E. GRIFFITHS. 1972. The control of alcohol dehydrogenase isoenzyme synthesis in Saccharomyces cerevisiae. Can. J. Biochem. 50: 35-43.

FOY, J.J., and J.K. BHATTACHARJEE. 1977. Gluconeogenesis in Saccharomyces cerevisiae: determination of fructose-1,6-bisphosphatase activity in cells grown in the presence of glycolytic carbon sources. J. Bacteriol. 129: 978-982.

FRAENKEL, D.G. 1982. Carbohydrate metabolism, p. 1-37. In J.N. Strathern, E.W. Jones and J.R. Broach (eds.), The molecular biology of the yeast Saccharomyces: Metabolism and gene expression. Cold Spring Harbor Laboratory, New York.

FRANZEN, J.S., and S.B. BINKLEY. 1961. Comparison of the acid-soluble nucleotides in Escherichia coli at different growth rates. J. Biol. Chem. 236: 515- 519.

FRANZUSOFF, A., and V.P. CIRILLO. 1982. Uptake and phosphorylation of 2-deoxy-D-glucose by wild-type and single-kinase strains of Saccharomyces cerevisiae. Biochim. Biophys. Acta. 688: 295-300.

FRIEDMAN, D.L., and J. LARNER. 1963. Studies on UDPG-α-glucan transglucosylase. III. Interconversion of two forms of muscle UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochemistry. 2: 669-675.

FROHLICH, K-U., and K-D. ENTIAN. 1982. Regulation of gluconeogenesis in the yeast Saccharomyces cerevisiae. Evidence for conversion of enolase isoenzymes. FEBS Lett. 139: 164-166.

FUNAYAMA, S., J. MOLANO, and C. GANCEDO. 1979. Purification and properties of a D-fructose 1,6-bisphosphatase from Saccharomyces cerevisiae. Arch. Biochem. Biophys. 197: 170-177.

GANCEDO, C. 1971. Inactivation of fructose-1,6-bisphosphatase by glucose in yeast. J. Bacteriol. 107: 401-405.

GANCEDO, C., J.M. GANCEDO, and A. SOLS. 1967. Metabolite repression of fructose-1,6-disphosphatase in yeast. Biochem. Biophys. Res. Commun. 26: 528-531.

GANCEDO, C., M.L. SALAS, A. GINER, and A. SOLS. 1965. Reciprocal effects of carbon sources on the levels of an AMP-sensitive fructose-1,6-diphosphatase and phosphofructokinase in yeast. Biochem. Biophys. Res. Commun. 20: 15-20.

GANCEDO, C., and K. SCHERZMANN. 1976. Inactivation by glucose of phosphoenolpyruvate carboxykinase from Saccharomyces cerevisiae. Arch. Microbiol. 109: 221-225.

GANCEDO, J.M., D. CLIFTON, and D.G. FRAENKEL. 1977. Yeast hexokinase mutants. J. Biol. Chem. 252: 4443-4444.

GANCEDO, J.M., and C. GANCEDO. 1973. Concentration of intermediary metabolites in yeasts. Biochimie. 55: 205-211.

GANCEDO, J.M., M.J. MAZÓN, and C. GANCEDO. 1982. Kinetic differences between two interconvertible forms of fructose-1,6-bisphosphatase from Saccharomyces cerevisiae. Arch. Biochem. Biophys. 218: 478-482.

GARLAND, P.B., P.J. RANDLE, and E.A. NEWSHOLME. 1963. Citrate as an intermediary in the inhibition of phosphofructokinase in rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes and starvation. Nature. 200: 169-170.

GEVERS, W., and H.A. KREBS. 1966. The effects of adenine nucleotides on carbohydrate metabolism in pigeon-liver homogenates. Biochem. J. 98: 720-735.

GHOSH, A., and B. CHANCE. 1964. Oscillations of glycolytic intermediates in yeast cells. Biochem. Biophys. Res. Commun. 16: 174-181.

GOLDBERG, N.D., C. VILLAR-PALASI, H. SASKO, and J. LARNER. 1967. Effects of insulin treatment on muscle 3',5'-cyclic adenylate levels in vivo and in vitro. Biochim. Biophys. Acta. 148: 665-672.

GOLDHAMMER, A.R., and H.H. PARADIES. 1979. Phosphofructokinase: structure and function. Curr. Top. Cell. Regul. 15: 109-154.

GORTS, C.P.M. 1969. Effect of glucose on the activity and the kinetics of the maltose-uptake system and of α-glucosidase in Saccharomyces cerevisiae. Biochim. Biophys. Acta. 184: 299-305.

GOTTSCHALK, A. 1947. The mechanism of "selective fermentation" by yeast. Wallerstein Lab. Commun. 10: 109-117.

GOWER, D.C., and R.C. WOLEDGE. 1977. The use of isotachophoresis for analysis of muscle extracts. Sci. Tools. 24: 17-21.

GREIG, M.E., R.A. WALK, and A.J. GIBBONS. 1958. Effect of Actidione (cycloheximide) on yeast fermentation. J. Bacteriol. 75: 489-491.

GROSSBARD, L., and R.T. SCHIMKE. 1966. Multiple hexokinases of rat tissues. J. Biol. Chem. 241: 3546-3550.

GUNGE, N., and Y. NAKATOMI. 1972. Genetic mechanisms of rare matings of the yeast Saccharomyces cerevisiae heterozygous for mating type. Genetics. 70: 41-58.

GUNJA, Z.H., D.J. MANNERS, and KHIN MAUNG. 1961. Studies on carbohydrate-metabolizing enzymes. Biochem. J. 81: 392-398.

HAARASILTA, S. 1981. On the regulation of anaplerotic and gluconeogenic enzymes in baker's yeast (Saccharomyces cerevisiae). Ph.D. thesis, University of Helsinki, Helsinki.

HAARASILTA, S., and E. OURA. 1975. On the activity of anaplerotic and gluconeogenic enzymes during the growth process of baker's yeast. The biphasic growth. Eur. J. Biochem. 52: 1-7.

HABOUCHA, J., and C.A. MASSCHELEIN. 1976. Répression et inhibition catabolique des enzymes intervenant dans la fermentation du maltotriose chez S. cerevisiae et S. carlsbergensis. Cerevisia. 1: 17-30.

HALVORSEN, H.O., S. WINDERMAN, and J. GORMAN. 1963. Comparison of the α-glucosidases of Saccharomyces produced in response to five non-

allelic maltose genes. Biochim. Biophys. Acta. 67: 42-53.

HAN, P., G. HAN, H. McBAY, and J. JOHNSON. 1982. Adenosine 5'-monophosphate removing system in fructose-1,6-bisphosphatase assay mixture: a new approach. Anal. Biochem. 122: 269-273.

HANOZET, G.M., and A. GUERRITORE. 1972. Role of phosphoenolpyruvate and 6-phosphogluconate in the short-time control of yeast isocitrate lyase. Arch. Biochem. Biophys. 149: 127-135.

HANSON, C.W., and M. DWORKIN. 1974. Intracellular and extracellular nucleotides and related compounds during the development of Myxococcus xanthus. J. Bacteriol. 118: 486-496.

HARRIS, G., E.C. BARTON-WRIGHT, and N.S. CURTIS. 1951. Carbohydrate composition of wort and some aspects of the biochemistry of fermentation. J. Inst. Brew. 57: 264-280.

HARRIS, G., and D.J. MILLIN. 1963. Sequential induction of maltose permease and maltase systems in Saccharomyces cerevisiae. Biochem. J. 88: 89-95.

HARRIS, G., and D.J. MILLIN. 1963. Protein formation including enzyme synthesis in yeast. Proceedings of the European Brewery Convention Congres: Brussels, p. 400-411. Elsevier, Amsterdam.

HARRIS, G., and C.C. THOMPSON. 1960. Uptake of nutrients by yeasts. II. Maltotriose permease and the utilization of maltotriose by yeasts. J. Inst. Brew. 66: 293-297.

HARRIS, G., and C.C. THOMPSON. 1961. The uptake of nutrients by yeasts. III.

The maltose permease of a brewing yeast. Biochim. Biophys. Acta. 52: 176-183.

HARWOOD, J.P., and A. PETERKOFSKY. 1975. Glucose-sensitive adenylate cyclase in toluene treated cells of Escherichia coli B. J. Biol. Chem. 250: 4656-4662.

HAUKELI, A.D. 1979. Yeast growth and metabolic changes during brewery fermentation. Proceedings of the European Brewery Convention Congress, Berlin, p. 461-473. IRL Press, London.

HELDT, H.W., A.R. PORTIS, R. McC. LILLEY, A. MOSBACH, and C.J. CHON. 1980. Assay of nucleotides and other phosphate-containing compounds in isolated chloroplasts by ion-exchange chromatography. Anal. Biochem. 101: 278-287.

HEMS, D.A., and P.D. WHITTON. 1980. Control of hepatic glycogenolysis. Physiol. Rev. 60: 1-50.

HERS, H-G., H. DE WULF, W. STALMANS, and G. VAN DEN BERGHE. 1970. The control of glycogen synthesis in the liver. Adv. Enzyme Regul. 8: 171-190.

HERS, H-G, L. HUE, and E. VAN SCHAFTINGEN. 1981. The fructose-6-phosphate/ fructose-1,6-bisphosphate cycle. Curr. Top. Cell. Reg. 18: 199-210.

HERS, H-G, L. HUE, and E. VAN SCHAFTINGEN. 1982. Fructose-2,6-bisphosphate. Trends in Biochem. Sci. 7: 329-331.

HERS, H-G, and E. VAN SCHAFTINGEN. 1982. Fructose-2,6-bisphosphate, 2 years after its discovery. Biochem. J. 206: 1-12.

HESS, B., R. HAECKEL, and K. BRAND. 1966. FDP-activation of yeast pyruvate kinase. Biochem. Biophys. Res. Commun. 24: 824-831.

HEYSE, K.U., and A. PIENDL. 1973. Influence of pitching rate on enzyme pattern of yeast. M.B.A.A., Tech. Quart. 10: 190-198.

HILL, J.B., and G. KESSLER. 1961. An automated determination of glucose utilizing a glucose oxidase-peroxidase system. J. Lab. Clin. Med. 57: 970-980.

HIROKAWA, T., M. NISHINO, N. AOKI, Y. KISO, Y. SAWAMOTO, T. YAGI, and J. AKIYAMA. 1983. Table of isotachophoretic indices. J. Chromatogr. 271: D1-D106.

HIZUKURI, S., and J. LARNER. 1964. Studies on UDPG: α-1,4-glucan α-4-glucosyl-transferase. VII. Conversion of the enzyme from glucose-6-phosphate-dependent to independent form in liver. Biochemistry. 3: 1783-1788.

HIZUKURI, S., and Y. TAKEDA. 1970. Activation of the D to I form conversion of glycogen synthetase by glucose-6-phosphate in bovine spleen. Biochim. Biophys. Acta. 212: 179-181.

HJALMARSSON, S-G. 1975. Preparative isotachophoresis - the effect of using Ampholine of different pH ranges as spacer ions in the fractionation of serum proteins. Sci. Tools. 22: 35-38.

HJALMARSSON, S-G, and A. BALDESTON. 1981. Capillary isotachophoresis, p. 261-352. In B. Campbell (ed.), CRC Critical Reviews in Analytical Chemistry. Vol. 13. CRC Press, Florida.

HOCHACHKA, P.W. 1972. The functional significance of variants of fructose-1,6-diphosphatase in the gill and hypodermis of a marine crustacean. Biochem. J. 127: 781-793.

HOCKNEY, R.C., and R.F. FREEMAN. 1980. Gratuitous catabolite repression by glucosamine of maltose utilization in Saccharomyces cerevisiae. J. Gen. Microbiol. 121: 479-482.

HODSON, R.E., O. HOLM-HANSEN, and F. AZAM. 1976. Improved methodology for ATP determination in marine environments. Mar. Biol. 34: 143-149.

HOFER, H.W., and D. PETTE. 1968. Wirkungen und Wechselwirkungen von Substraten und Effektoren an der Phosphofructokinase des Kanichen - Skeletmuskels. Hoppe-Seyler's Z. Physiol. Chem. 349: 1378-1392.

HOLM-HANSEN, O., and C.R. BOOTH. 1966. The measurement of adenosine tri-phosphate in the ocean and its ecological significance. Limnol. Oceangr. 11: 510-519.

HOLZER, H. 1976. Catabolite inactivation in yeast. Trends Biochem. Sci. 1: 178-184.

HOMMES, F.A. 1966. Effect of glucose on the level of glycolytic enzymes in yeast. Arch. Biochem. Biophys. 114: 231-233.

HORECKER, B.L., J.S. MacGREGOR, V.N. SINGH, E. MELLONI, and S. PONTREMOLI. 1981. Aldolase and fructose bisphosphatase: key enzymes in the control of gluconeogenesis and glycolysis. Curr. Top. Cell. Regul. 18: 181-197.

HORECKER, B.L., and A.H. MEHLER. 1955. Carbohydrate metabolism. Ann. Rev. Biochem. 24: 207-275.

HUBERT, E., J. VILLANUEVA, A.M. GONZALEZ, and F. MARCUS. 1970. Univalent cation activation of fructose-1,6-diphosphatase. Arch. Biochem. Biophys. 138: 590-597.

HUE, L., and H-G. HERS. 1974. On the use of $[^{3}H,^{14}C]$ labelled glucose in the study of the so-called "futile cycles" in liver and muscle. Biochem. Biophys. Res. Commun. 58: 532-539.

HYSERT, D.W., and N.M. MORRISON. 1978. Studies on ATP, ADP, and AMP concentrations in yeast and beer. J. Am. Soc. Brew. Chem. 36: 160-167.

ITO, Y., M. TOYODA, H. SUZUKI, and M. IWAIDA. 1980. Isotachophoretic determination of ethylenediaminetetraacetate in salad dressing, mayonnaise and margarine. Jap. Assoc. Off. Anal. Chem. 63: 1219-1223.

JENKINSON, D.S., and J.M. OADES. 1979. A method for measuring adenosine triphosphate in soil. Soil. Biol. Biochem. 11: 193-199.

JOHNSON, R.A., J-G. HARDMAN, A.E. BROADUS, and E.W. SUTHERLAND. 1970. Analysis of adenosine 3',5'-monophosphate with luciferase luminescence. Anal. Biochem. 35: 91-97.

JONES, J.G., and B.M. SIMON. 1977. Increased sensitivity in the measurement of ATP in fresh-water samples with a comment on the adverse effect of membrane filtration. Freshwater Biol. 7: 253-260.

JONES, M., and J.S. PIERCE. 1964. Absorption of amino acids from wort by yeasts. J. Inst. Brew. 70: 307-315.

KAISER, K-P., and H. HUPF. 1979. Isotachophorese in der Lebensmittel-

analytik. 1. Teil: Prinzip der Isotachophorese. Nachweis und Bestimmung von Säuren. Dtsch. Lebensm. Rundsch. 75: 300-304.

KAISER, K-P., and H. HUPF. 1979. Isotachophorese in der Lebensmittelanalytik. 2. Teil: Untersuchungen von Lebensmitteln. Dtsch. Lebensm. Rundsch. 75: 346-349.

KARADSHEH, N.S., and K. UYEDA. 1977. Studies on structure of human erythrocyt phosphofructokinase. J. Biol. Chem. 249: 3515-3524.

KARL, D.M. 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol. Rev. 44: 739-796.

KARL, D.M., and D.B. CRAVEN. 1980. Effects of alkaline phosphatase activity on nucleotide measurements in aquatic microbial communities. Appl. Environ. Microbiol. 40: 549-561.

KARL, D.M., J.A. HAUGSNESS, L. CAMPBELL, and O. HOLM-HANSEN. 1978. Adenine nucleotide extraction from multicellular organisms and beach sand: ATP recovery, energy charge ratios and determination of carbon/ATP ratios. J. Exp. Mar. Biol. Ecol. 34: 163-181.

KARL, D.M., and O. HOLM-HANSEN. 1977. Adenylate energy charge measurements in natural seawater and sediment samples, p. 141-169. In G.A. Borun (ed.), Second Bi-Annual ATP Methodology Symposium. SAI Technology Co., San Diego, California.

KARL, D.M., and O. HOLM-HANSEN. 1978. Methodology and measurement of adenylate energy charge ratios in environmental samples. Mar. Biol. 48: 185-197.

KARL, D.M., and P.A. LaROCK. 1975. Adenosine triphosphate measurements in soil and marine sediments. J. Fish. Res. Board Can. 32: 599-607.

KARP, M.T., R.P. RAUNIO, and T.N-E. LÖVGREN. 1983. Simultaneous extraction and combined bioluminescent assay of NAD^+ and NADH. Anal. Biochem. 128: 175-180.

KELKER, H.C., and A.O. POGO. 1980. The stringent and relaxed phenomena in Saccharomyces cerevisiae. J. Biol. Chem. 255: 1526-1535.

KELLER, P.J., and G.T. CORI. 1953. Enzymic conversion of phosphorylase a to phosphorylase b. Biochim. Biophys. Acta. 12: 235-238.

KERRIDGE, D. 1958. The effect of Actidione and other anti-fungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. J. Gen. Microbiol. 19: 497-506.

KESSLER, R., K. NISSLER, W. SCHELLENBERGER, and E. HOFMANN. 1982. Fructose-2,6-bisphosphate increases the binding affinity of yeast phosphofructokinase to AMP. Biochem. Biophys. Res. Commun. 107: 506-510.

KHAN, N.A., and N.R. EATON. 1971. Genetic control of maltose formation in yeast. I. Strains producing high and low basal levels of enzyme. Mol. Gen. Genet. 112: 317-322.

KHYM, J.X. 1976. Separation of nucleic acid components on polyacrylamide gel columns. Anal. Biochem. 71: 231-242.

KHYM, J.X., J.W. BYNUM, and E. VOLKIN. 1977. The co-use of retention time and bandwidth measurements in evaluations of nucleotide pools by ion-exchange chromatography. Anal. Biochem. 67: 446-463.

KILLICK, K.A., and B.E. WRIGHT. 1972. Trehalose synthesis during differentiation in Dictyostelium discoideum. J. Biol. Chem. 247: 2967-2969.

KIRSOP, B.H. 1974. Oxygen in brewery fermentation. J. Inst. Brew. 80: 252-259.

KIRSOP, B.H. 1978. Fermentation: From wort to beer. Proceedings of the European Brewery Convention, Fermentation and Storage Symposium, Zoeterwoude, p. 3-16. Brauwelt-Verlag, Nürnberg.

KLOFAT, W., G. PICCIOLO, E.W. CHAPELLE, and E. FREESE. 1969. Production of adenosine triphosphate in normal cells and sporulation mutants of Bacillus subtilis. J. Biol. Chem. 244: 3270-3276.

KNUST, E.A., E.W. CHAPELLE, and G.L. PICCIOLO. 1975. A comparison of certain extracting agents for extraction of adenosine triphosphate (ATP) from microorganisms for use in the firefly luciferase ATP assay, p. 27-34. In E.W. Chapelle and G.L. Picciolo (ed.), Analytical applications of bioluminescence and chemiluminescence. NASA SP-388. National Aeronautics and Space Administration, Washington, D.C.

KOHLRAUSCH, F. 1897. Über Konzentrations - Verschielungen durch electrolyse im innern von Lösungen un Lösungsgemischen. Ann. Physik. 62: 209-217.

KORNBERG, H.L. 1966. Anaplerotic sequences and their role in metabolism. Essays Biochem. 2: 1-31.

KORNBERG, H.L., and H.A. KREBS. 1957. Synthesis of cell constituents from C_2 - units by a modified tricarboxylic acid cycle. Nature. 179: 988-991.

KOTYK, A. 1973. Molecular aspects of nonelectrolyte transport in yeasts. Proceedings of the Third International Specialized Symposium on Yeasts, Helsinki, p. 103-127. Print Oy, Helsinki.

KREBS, E.G., and E.H. FISCHER. 1956. The phosphorylase b to a converting enzyme of rabbit skeletal muscle. Biochim. Biophys. Acta. 20: 150-157.

KREBS, H.A. 1972. The Pasteur effect and the relations between respiration and fermentation. Essays Biochem. 8: 1-34.

KRUGER, L., D.S. RYDER, C. ALCOCK, and J.P. MURRAY. 1982. Malt quality: prediction of malt fermentability. M.B.A.A. Tech. Quart. 19: 45-51.

KRZANOWSKI, J., and F.M. MATSCHINSKY. 1969. Regulation of phosphofructokinase by phosphocreatine and phosphorylated glycolytic intermediates. Biochem. Biophys. Res. Commun. 34: 816-823.

KUBY, S.A., J.T. WU, and R.N. ROY. 1974. Glucose-6-phosphate dehydrogenase from brewer's yeast (Zwischenferment). Arch. Biochem. Biophys. 165: 153-178.

LAGUNAS, R. 1976. Energy metabolism of Saccharomyces cerevisiae. Discrepancy between ATP balance and known metabolic functions. Biochim. Biophys. Acta. 440: 661-667.

LAGUNAS, R., and J-M. GANCEDO. 1973. Reduced pyridinenucleotides balance in glucose-growing Saccharomyces cerevisiae. Eur. J. Biochem. 37: 90-94.

LAM, K.B., and J. MARMUR. 1977. Isolation and characterization of Saccharomyces cerevisiae glycolysis mutants. J. Bacteriol. 130: 746-751.

LAMPEN, J.O., S-C. KUO, and P. LIRAS. 1973. Synthesis of external glycoprotein enzymes by yeast. Proceedings of the Third International Specialized Symposium on Yeasts, Helsinki, p. 129-147. Print Oy, Helsinki.

LARNER, J., and C. VILLAR-PALASI. 1971. Glycogen synthase and its control. Curr. Top. Cell. Regul. 3: 195-236.

LAVI, J.T., T.N-E. LÖVGREN, and R.P. RAUNIO. 1981. Comparison of luminous bacteria and their bioluminescence-linked enzyme activities. FEMS Microbiol. Lett. 11: 197-199.

LEDERER, B., S. VISSER, E. VAN SCHAFTINGEN, and H-G. HERS. 1981. Fructose-2,6-bisphosphate in yeast. Biochem. Biophys. Res. Commun. 103: 1281-1287.

LEE, C.C., R.F. HARRIS, J.D.H. WILLIAMS, D.E. ARMSTRONG, and J.K. SYERS. 1971. Adenosine triphosphate in lake sediments. I. Determination. Soil Sci. Soc. Am. Proc. 35: 82-86.

LELOIR, L.F., and C.E. CARDINI. 1957. Biosynthesis of glycogen from uridine diphosphate glucose. J. Am. Chem. Soc. 79: 6340-6345.

LENZ, A-G., and H. HOLZER. 1980. Rapid reversible inactivation of fructose-1,6-bisphosphatase in Saccharomyces cerevisiae by glucose. FEBS Lett. 109: 271-274.

LERCH, K., and E.H. FISCHER. 1975. Amino acid sequence of two functional sites in yeast glycogen phosphorylase. Biochemistry. 14: 2009-2014.

LEVY, R.H. 1979. Glucose-6-phosphate dehydrogenase. Adv. Enzymol. 48: 97-192.

LIENER, I.E., and D.L. BUCHANAN. 1951. The fixation of carbon dioxide by growing and non-growing yeast. J. Bacteriol. 61: 527-534.

LILLIE, S.H., and J.R. PRINGLE. 1980. Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation. J. Bacteriol. 143: 1384-1389.

LINDENMAYER, A., and L. SMITH. 1964. Cytochromes and other pigments of baker's yeast grown aerobically and anaerobically. Biochim. Biophys. Acta. 93: 445-461.

LINN, T.C., J.W. PELLEY, F.H. PETTIT, F. HUCHO, D.D. RANDALL, and L.J. REED. 1972. Purification and properties of the component enzymes of the pyruvate dehydrogenase complexes from bovine kidney and heart. Arch. Biochem. Biophys. 148: 327-342.

LOBO, Z., and P.K. MAITRA. 1977. Physiological role of glucose-phosphorylating enzymes in Saccharomyces cerevisiae. Arch. Biochem. Biophys. 182: 639-645.

LONDESBOROUGH, J. 1982. Cyclic nucleotide-dependent inactivation of yeast fructose 1,6-bisphosphatase by ATP. FEBS Lett. 144: 269-272.

LOTHROP, C.C., and M. UZIEL. 1980. Rapid group separations of nucleotides and related compounds on silica columns. Anal. Biochem. 109: 160-166.

LOWRY, O.H., J. CARTER, J.B. WARD, and L. GLASER. 1971. The effect of carbon and nitrogen sources on the level of metabolic intermediates in Escherichia coli. J. Biol. Chem. 246: 6511-6521.

LOWRY, O.H., J.V. PASSONNEAU, F.X. HASSELBERGER, and D.W. SCHULZ. 1964. Effect of Ischemia on known substrates and cofactors of the glycolytic pathway in brain. J. Biol. Chem. 239: 18-30.

LUNDIN, A., A. RICKARDSSON, and A. THORE. 1976. Continuous monitoring of ATP-converting reactions by purified firefly luciferase. Anal. Biochem. 75: 611-620.

LUNDIN, A., and A. THORE. 1975. Analytical information obtainable by evaluation of the time course of firefly bioluminescence in the assay of ATP. Anal. Biochem. 66: 47-63.

LUNDIN, A., and A. THORE. 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. 30: 713-721.

LUST, W.D., G.K. FEUSSNER, E.K. BARBEHENN, and J.V. PASSONNEAU. 1981. The enzymatic measurement of adenine nucleotides and P-creatine in picomole amounts. Anal. Biochem. 110: 128-266.

MacLEOD, N.H., E.W. CHAPPELLE, and A.M. CRAWFORD. 1969. ATP assay of terrestrial soils: A test of an exobiological experiment. Nature, (London). 223: 267-268.

MADSEN, N.B. 1961. The inhibition of glycogen phosphorylase by uridine diphosphate glucose. Biochem. Biophys. Res. Commun. 6: 310-312.

MADSEN, N.B. 1963. The biological control of glycogen metabolism in Agrobacterium tumefaciens. Can. J. Biochem. Physiol. 41: 561-571.

MAEBA, P., and B.D. SANWAL. 1968. The regulation of pyruvate kinase of Escherichia coli by fructose diphosphate and adenylic acid. J. Biol. Chem. 243: 448-450.

MAGASANIK, B. 1955. The metabolic control of histidine assimilation and dissimilation in Aerobacter aerogenes. J. Biol. Chem. 213: 557-569.

MAITRA, P.K. 1970. A glucokinase from Saccharomyces cerevisiae. J. Biol. Chem. 245: 2423-2431.

MAITRA, P.K. 1971. Glucose and fructose metabolism in a phosphoglucoisomeraseless mutant of Saccharomyces cerevisiae. J. Bacteriol. 107: 759-769.

MAITRA, P.K., and Z. LOBO. 1971. Kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. 246: 475-488.

MAITRA, P.K., and Z. LOBO. 1978. Reversal of glycolysis in yeast. Arch. Biochem. Biophys. 185: 535-543.

MANANDHAR, M.S.P., and K. VAN DYKE. 1974. Guanosine triphosphate analysis: PEI-thin layer purification and luciferin-luciferase liquid scintillation quantification. Anal. Biochem. 60: 122-129.

MANCHESTER, K.L. 1969. A comment on the role of magnesium in the control of muscle glycogen synthetase kinase. FEBS Lett. 5: 279-284.

MANCHESTER, K.L. 1980. Influence of extraction procedure on size of free amino acid pool of heart muscles. Anal. Biochem. 102: 300-303.

MANNERS, D.J. 1971. The structure and biosynthesis of storage carbohydrates in yeast, p. 419-439. In A.H. Rose and J.S. Harrison (eds.), The Yeasts, Vol. 2. Academic Press, London and New York.

MANSOUR, T.E., and J.M. MANSOUR. 1962. Effects of serotonin (5-hydroxytryptamine) and adenosine 3,5-phosphate on phosphofructokinase from the liver fluke, Fasciola hepatica. J. Biol. Chem. 237: 629-634.

MASSCHELEIN, C.A., J. DUPONT, C. JEUNEHOMME, and A. DEVREUX. 1960. Degeneration and reversible loss of fermenting power by yeast. J. Inst. Brew. 66: 502-508.

MASSCHELEIN, C.A., H. JENARD, C. JEUNEHOMME-RAMOS, and A. DEVREUX. 1965. Métabolisme fermentaire et synthèse des volatils au cours de la fermentation. Proceedings of the European Brewery Convention Congress, Stockholm, p. 209-222. Elsevier, Amsterdam.

Mc GILVERY, R.W. 1970. Biochemistry: A functional approach. Saunders, Philadelphia, Pennsylvania.

McGINNIS, J.F., and K. PAIGEN. 1969. Catabolite inhibition: A general phenomenon in the control of carbohydrate utilization. J. Bacteriol. 100: 902-913.

McGINNIS, J.F., and K. PAIGEN. 1973. Site of catabolite inhibition of carbohydrate metabolism. J. Bacteriol. 114: 885-887.

MEYER, J., and Ph. MATILE. 1975. Subcellular distribution of yeast invertase isoenzymes. Arch. Microbiol. 103: 51-55.

MIAN, F.A., and Z. FENCL. 1971. Growth and physiology of a yeast cultivated in batch and continuous culture systems. Folia microbiologica. 16: 249-259.

MIKKER, F.E.P., F.M. EVERAETS, and J.A. PEEK. 1979. Isotachophoresis: the concepts of resolution, load capacity and separation efficiency. II. Experimental evaluation. J. Chromatogr. 168: 317-332.

MILLER, L.F., M.S. MABEE, H.S. GRESS, and N.O. JANGAARD. 1978. An ATP bioluminescence method for the quantification of viable yeast for fermenter pitching. J. Am. Soc. Brew. Chem. 36: 59-62.

MILLIN, D.J. 1963. Variations in α-glucosidase and α-glucoside permease systems of yeast. J. Inst. Brew. 69: 389-393.

MOLANO, J., and C. GANCEDO. 1974. Specific inactivation of fructose-1,6-bisphosphatase from Saccharomyces cerevisiae by a yeast protease. Eur. J. Biochem. 44: 213-217.

MONOD, J. 1942. La croissance des cultures bactériennes, p. 1-30. Hermann, Paris.

MONTENECOURT, B.C., S-C. KUO, and J.O. LAMPEN. 1973. Saccharomyces mutants with invertase formation resistant to repression by hexoses. J. Bacteriol. 114: 223-228.

MORGAN, H.E., and A. PARMEGGIANI. 1964. Regulation of glycogenolysis in muscle. J. Biol. Chem. 239: 2440-2445.

MÜLLER, D., and H. HOLZER. 1981. Regulation of fructose-1,6-bisphosphatase in yeast by phosphorylation/dephosphorylation. Biochem. Biophys. Res. Commun. 103: 926-933.

MURATSUBAKI, H., and T. KATSUME. 1979. Distribution of hexokinase isoenzymes depending on a carbon source in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 86: 1030-1036.

NADKARNI, M., Z. LOBO, and P.K. MAITRA. 1982. Particulate phosphofructokinase of yeast: physiological studies. FEBS Lett. 147: 251-255.

NAZAR, R.M., H.G. LAWFORD, and J.T.F. WONG. 1970. An improved procedure for the extraction and analysis of cellular nucleotides. Anal. Biochem. 35: 305-313.

NEWSHOLME, E.A., and W. GEVERS. 1967. Control of glycolysis and gluconeogenesis in liver and kidney cortex. Vitamins and Hormones. 25: 1-87.

NORD, F.F., and S. WEISS. 1958. The hexose monophosphate pathway in yeast. p. 323-339. In A.H. Cook (ed.), The Chemistry and Biology of Yeasts. Academic Press, New York.

OERLEMANS, F., C. DE BRUYN, F. MIKKERS, Th. VERHEGGEN, and F. EVERAERTS. 1981. Isotachophoresis of urinary purines and pyrimidines. The use of spacers and enzymes for identification. J. Chromatogr. 225: 369-379.

OGUR, M., and R. ST. JOHN. 1956. A differential and diagnostic plating method for population studies of respiration deficiency in yeast. J. Bacteriol. 72: 500-504.

OLSON, J.A. 1959. The purification and properties of yeast isocitrate lyase. J. Biol. Chem. 234: 5-10.

ORNSTEIN, L. 1964. Disc electrophoresis - I. Background and theory. Ann. N.Y. Acad. Sci. 121: 321-349.

OSMOND, C.B., and T. REES. 1969. Control of the pentose phosphate pathway in yeast. Biochim. Biophys. Acta. 184: 35-42.

OURA, E. 1973. Some aspects of the growth process of baker's yeast. Proceedings of the Third International Specialized Symposium on Yeasts, Helsinki, p. 215-230. Print Oy, Helsinki.

OURA, E. 1974. Effect of aeration intensity on the biochemical composition of baker's yeast. II. Activities of the oxidative enzymes. Biotechnol. Bioeng. 16: 1213-1225.

OURA, E. 1974. CO_2-fixation, theoretical background. Alkon Keskuslaboratoris, report no. 6270. State Alcohol Monopoly, Helsinki.

OURA, E., and H. SUOMALAINEN. 1967. Free nucleotides in resting and metabolising baker's yeast. J. Inst. Brew. 73: 370-376.

PALMISANO, J., and J.H. SCHWARTZ. 1982. Microassays for glucose-6-phosphate and 6-phosphogluconate based on bioluminescent techniques. Anal. Biochem. 126: 409-413.

PALMQVIST, U., and T. ÄYRÄPÄÄ. 1969. Uptake of amino acids in bottom fermentations. J. Inst. Brew. 75: 181-190.

PANCHAL, C.J., and G.G. STEWART. 1982. The influence of media conditions on the utilization of monosaccharides by a strain of Saccharomyces uvarum (carlsbergensis). J. Inst. Brew. 88: 86-89.

PANEK, A. 1962. Synthesis of trehalose by baker's yeast (Saccharomyces cerevisiae). Arch. Biochem. Biophys. 98: 349-355.

PANEK, A., and J.R. MATTOON. 1977. Regulation of energy metabolism in Saccharomyces cerevisiae. Relationship between catabolite repression, trehalose synthesis, and mitochondrial development. Arch. Biochem. Biophys. 183: 306-316.

PARIS, H., B. TERRAIN, V. VIALLARD, M. ROUSSET, A. ZWEIBAUM, and J.C. MURAT. 1983. Activity of glycogen metabolizing enzymes in glucose deprived HT 29 adenocarcinoma cell-line. Biochem. Biophys. Res. Commun. 110: 371-377

PARMEGGIANI, A., and H.E. MORGAN. 1962. Effect of adenine nucleotides and inorganic phosphate on muscle phosphorylase activity. Biochem. Biophys. Res. Commun. 9: 252-261.

PARRY, M.J., and D.G. WALKER. 1966. Purification and properties of adenosine 5'-triphosphate-D-glucose 6-phosphotransferase from rat liver. Biochem. J. 99: 266-274.

PASSONNEAU, J.V., and O.H. LOWRY. 1962. Phosphofructokinase and the Pasteur effect. Biochem. Biophys. Res. Commun. 7: 10-15.

PASSONNEAU, J.V., and O.H. LOWRY. 1964. The role of phosphofructokinase in metabolic regulation. Adv. Enzyme Regul. 2: 265-274.

PASTAN, I., and R.L. PERLMAN. 1971. Cyclic AMP in metabolism. Nature. 229: 5-7.

PASTEUR, L. 1861. Expériences et vues nouvelles sur la nature des fermentations. C.R. Acad. Sci. 52: 1260-1264.

PATEL, G.B., and W.M. INGLEDEW. 1973. Internal carbohydrates of Saccharomyces carlsbergensis during commercial lager brewing. J. Inst. Brew. 79: 392-396.

PATEL, G.B., and W.M. INGLEDEW. 1975. The relationship of acid-soluble glycogen to yeast flocculation. Can. J. Microbiol. 21: 1608-1613.

PATEL, G.B., and W.M. INGLEDEW. 1975. Glycogen - a physiological determinant of yeast flocculation? Can. J. Microbiol. 21: 1614-1621.

PATTERSON, J.W., P.L. BREZONIK, and H.D. PUTNAM. 1970. Measurement and significance of adenosine triphosphate in activated sludge. Environ. Sci. Technol. 4: 560-575.

PERLMAN, R.L., and I. PASTAN. 1971. The role of cyclic AMP in bacteria. Curr. Top. Cell. Regul. 3: 117-134.

PETERKOFSKY, A. 1977. Regulation of Escherichia coli adenylate cyclase by phosphorylation-dephosphorylation. Trends in Biochem. Sci. 2: 12-14.

PETRIK, M., O. KÄPPELI, and A. FIECHTER. 1983. An expanded concept for the glucose effect in the yeast Saccharomyces uvarum: involvement of short- and long-term regulation. J. Gen. Microbiol. 129: 43-49.

PETTIGREW, D.W., and C.J. FRIEDEN. 1979. Binding and regulatory ligands to rabbit muscle phosphofructokinase. J. Biol. Chem. 254: 1887-1895.

PHILLIPS, A.W. 1955. Utilization by yeasts of the carbohydrates of wort. J. Inst. Brew. 61: 122-126.

PICCIOLO, G.L., E.W. CHAPPELLE, J.W. DEMING, M.A. McGARRY, D.A. NIBLEY, H. OKREND, and R.R. THOMAS. 1976. Applications of luminescent systems to infectious disease methodology. X-726-76-212. Goddard Space Flight Center, Greenbelt, Maryland.

PILKIS, S.J., M.R. EL-MAGHRABI, M.M. McGRANE, J. PILKIS, and T.H. CLAUS. 1981. The role of fructose-2,6-bisphosphate in regulation of fructose-1,6-bisphosphatase. J. Biol. Chem. 256: 11489-11495.

PIRAS, R., L.B. ROTHMAN, and E. CABIB. 1968. Regulation of muscle glycogen synthetase by metabolites. Differential effects on the I and D forms. Biochemistry. 7: 56-66.

PLOSS, M., and F. ESCHENBECHER. 1981. Zur Abhängigkeit der Gärleistung von verschiedenen Parametern. Proceedings of the European Brewery Convention Congress, Copenhagen, p. 587-598. IRL Press, London.

POLAKIS, E.S., and W. BARTLEY. 1965. Changes in enzyme activities of Saccharomyces cerevisiae during growth on different carbon sources. Biochem. J. 97: 298-302.

POMPER, S., K.M. DANIELS, and D.W. McKEE. 1954. Genetic analysis of polyploid yeast. Genetics. 39: 343-355.

PURWIN, C., F. LEIDIG, and H. HOLZER. 1982. Cyclic AMP-dependent phosphorylation of fructose-1,6-bisphosphatase in yeast. Biochem. Biophys. Res. Commun. 107: 1482-1489.

QUAIN, D.E. 1981. The determination of glycogen in yeasts. J. Inst. Brew. 87: 289-291.

QUAIN, D.E., P.A. THURSTON, and R.S. TUBB. 1981. The structural and storage carbohydrates of Saccharomyces cerevisiae: Changes during fermentation of wort and a role for glycogen catabolism in lipid biosynthesis. J. Inst. Brew. 87: 108-111.

RAMOS-JEUNEHOMME, C. 1982. Personal communication. C.E.R.I.A. Brussels.

RAMOS-JEUNEHOMME, C., J. HABOUCHA, A. DEVREUX, and C.A. MASSCHELEIN. 1973. Inhibition catabolique chez les levures et conséquences industrielles. Proceedings of the European Brewery Convention Congress, Salzburg, p. 111-124. Elsevier, Amsterdam.

RAMOS-JEUNEHOMME, C., and C.A. MASSCHELEIN. 1977. Contrôle génétique de la formation des dicétones vicinales chez Saccharomyces cerevisiae. Proceedings of the European Brewery Convention Congress, Amsterdam, p. 267-283. Elsevier, Amsterdam.

RANDLE, P.J., R.M. DENTON, and P.J. ENGLAND. 1968. Citrate as a metabolic regulator in muscle and adipose tissue, p. 87-1034. In T.W. Goodwin (ed.), The Metabolic Roles of Citrate, Biochemical Society Symposium Vol. 27. John Wiley and Sons, New York.

REIJENGA, J.C., G.V.A. ABEN, Th. P.E.M. VERHEGGEN, and F.M. EVERAETS. 1983. Effect of electroosmosis on detection in isotachophoresis. J. Chromatogr. 260: 241-254.

REILLY, C., and F. SHERMAN. 1965. Glucose repression of cytochrome a synthesis in cytochrome deficient mutants of yeast. Biochim. Biophys. Acta. 95: 640-651.

ROBERTSON, J.J., and R.O. HALVORSON. 1957. The components of maltozymase in yeast, and their behaviour during deadaptation. J. Bacteriol. 73: 186-198.

ROBISON, G.A., R.W. BUTCHER, I. ØYE, H.E. MORGAN, and E.W. SUTHERLAND. 1965. The effect of epinephrine on adenosine 3',5'-phosphate levels in the isolated perfused rat heart. Mol. Pharmacol. 1: 168-177.

ROGERS, P.J., and P.R. STEWART. 1973. Mitochondrial and peroxisomal contributions to the energy metabolism of Saccharomyces cerevisiae in continuous culture. J. Gen. Microbiol. 79: 205-217.

ROGNSTAD, R. 1981. Effects of glucose and of lactate on phosphofructokinase flux during gluconeogenesis. Biochim. Biophys. Acta. 676: 373-378.

ROGNSTAD, R., and J. KATZ. 1976. Effects of hormones and of ethanol on

the fructose-6-P/fructose-1,6-P_2 futile cycle during gluconeogenesis in the liver. Arch. Biochem. Biophys. 177: 337-345.

ROSE, L.M., and R.W. BROCKMAN. 1977. Analysis by high pressure liquid chromatography of 9-B-D-arabinofuranosyladenine 5'-triphosphate levels in murine leukaemia cells. J. Chromatogr. 133: 335-343.

ROSELL PEREZ, M., and J. LARNER. 1964. Studies on UDPG: α -1,4-glucan α -4-glucosyltransferase. VI. Specificity and structural requirements for the activator of the D form of the dog muscle enzyme. Biochemistry. 3: 773-778.

ROTHMAN-DENES, L.B., and E. CABIB. 1966. Regulatory properties of yeast glycogen synthetase. Biochem. Biophys. Res. Commun. 25: 644-650.

ROTHMAN-DENES, L.B., and E. CABIB. 1970. Two forms of yeast glycogen synthetase and their role in glycogen accumulation. Proc. Natl. Acad. Sci. 66: 967-972.

ROTHMAN-DENES, L.B., and E. CABIB. 1971. Glucose-6-phosphate dependent and independent forms of yeast glycogen synthetase. Their properties and interconversions. Biochemistry. 10: 1236-1242.

RUDD, J.W.M., and R.D. HAMILTON. 1973. Measurement of adenosine triphosphate (ATP) in two Precambrian shield lakes of north western Ontario. J. Fish. Res. Board. Can. 30: 1537-1546.

RUIZ-AMIL, M., G. de TORRONTEGUI, E. PALACIÁN, L. CATALINA, and M. LOSADA. 1965. Properties and function of yeast pyruvate carboxylase. J. Biol. Chem. 240: 3485-3492.

RYDER, D.S., C. ALCOCK, and L. KRUGER. 1980. Local malt/quality aspects. Progress report on fermentability tests. South African Breweries Ltd., Central Laboratory report no. 19/80, p. 2-260.

RYDER, D.S., M. CASTIAU, and C.A. MASSCHELEIN. 1983a. Metabolic regulation of glycolytic activity during brewing yeast fermentations. Proceedings of the European Brewery Convention Congress, London, p. 185-192. IRL Press, London.

RYDER, D.S., and C.A. MASSCHELEIN. 1983b. Aspects of metabolic regulatory systems and physiological limitations with a view to the improvement of brewing yeast performance. Proceedings of the European Brewery Convention Biotechnology Symposium, Nutfield, U.K., p. 2-29. Brauwelt-Verlag, Nürnberg.

RYDER, D.S., D.R. WOODS, M. CASTIAU, and C.A. MASSCHELEIN. 1983c. Glycolytic flux in lager yeast fermentations: Application of Isotachophoresis and Bioluminescence for measurement of cellular intermediates. J. Am. Soc. Brew. Chem. 41: 125-132.

RYDER, D.S., D.R. WOODS, M. CASTIAU, and C.A. MASSCHELEIN. 1984. Application of Isotachophoresis for measurement of glycolytic intermediates in Saccharomyces uvarum fermentations. Anal. Biochem., in press.

RYDER, D.S., D.R. WOODS, J.P. MURRAY, and C.A. MASSCHELEIN. 1983d. Some practical implications of yeast growth and yeast performance. M.B.A.A., Tech. Quart. 20: 9-21.

RYMAN, B.E., and W.J. WHELAN. 1971. New aspects of glycogen metabolism. Adv. Enzymol. 34: 285-443.

RYTKA, J., A. SLEDZIEWSKI, J. LITWINSKA, and T. BILINSKI. 1976. Haemoprotein formation in yeast. II. Isolation of catalase regulatory mutants. Mol. Gen. Genet. 145: 27-42.

SAIER, M.H. 1977. Bacterial phosphoenolpyruvate: Sugar phosphotransferase systems: Structural, functional, and evolutionary interrelationships. Bacteriol. Rev. 41: 856-871.

SANTOS, E., L. RODRIGUEZ, M.V. ELORZA, and R. SENTANDREU. 1982. Uptake of sucrose by Saccharomyces cerevisiae. Arch. Biochem. Biophys. 216: 652-660.

SCHAMHART, D.J.H., A.M.A. TEN BERGE, and K.W. VAN DE POO. 1975. A spontaneous catabolite repression insensitive mutant in Saccharomyces. J. Bacteriol. 121: 247-260.

SCHARFF, R., and I.G. WOOL. 1963. Accumulation of amino acids in muscle of perfused rat heart. Biochem. J. 97: 257-271.

SCHIMPFESSEL, L. 1968. Présence et régulation de la synthèse de deux alcool déshydrogénases chez la levure Saccharomyces cerevisiae. Biochim. Biophys. Acta. 151: 317-322.

SCHLANDERER, G., and H. DELLWEG. 1974. Cyclic AMP and catabolite repression in yeasts. Eur. J. Biochem. 49: 305-316.

SCHRAMM, V.L., F.A. FULLIN, and M.D. ZIMMERMAN. 1981. Kinetic studies of the interaction of substrates, Mn^{++}, and Mg^{++} with the Mn^{++}-sensitive and -insensitive forms of phosphoenolpyruvate carboxykinase. J. Biol. Chem. 256: 10803-10808.

SHARPE, A.N., M.N. WOODROW, and A.K. JACKSON. 1970. Adenosinetriphosphate (ATP) levels in foods contaminated by bacteria. J. Appl. Bacteriol. 33: 758-767.

SHERMAN, F., and B. EPHRUSSI. 1962. The relationship between respiratory deficiency and suppressiveness in yeast as determined with segregational mutants. Genetics. 47: 695-700.

SINCLAIR, M., E. KEIGHAN, and J. JONES. 1979. ATP as a measure of living phytoplankton carbon in estuaries. J. Fish. Res. Board. Can. 36: 180-186.

SLONIMSKI, P. 1953. La formation des enzymes respiratoires chez la levure. Actualités Biochimiques, No. 17, p. 140-157. Masson, Paris.

SMITH, M.L., and J.M. BUCHANAN. 1979. Nucleotide and pentose synthesis after serum-stimulation of resting 3T6 fibroblasts. J. Cell. Physiol. 101: 293-310.

SMITH, R.C., and O. MAALØE. 1964. Effect of growth rate on the acid-soluble nucleotide composition of Salmonella typhimurium. Biochim. Biophys. Acta. 86: 229-234.

SMITH, R.J. 1979. Increasing guanosine 3'-diphosphate 5'-diphosphate concentration with decreasing growth rate in Anacystis nidulans. J. Gen. Microbiol. 113: 403-405.

SODERLING, T.R., J.P. HICKENBOTTOM, E.M. REIMANN, F.L. HUNKELER, D.A. WALSH, and E.G. KREBS. 1970. Inactivation of glycogen synthetase and activation of phosphorylase kinase by muscle adenosine 3',5'-monophosphate-dependent protein. J. Biol. Chem. 245: 6317-6328.

SODERLING, T.R., M.F. JETT, N.J. HUTSON, and B.S. KHATRA. 1977. Regulation of glycogen synthase. J. Biol. Chem. 252: 7517-7524.

SOLLENBERG, J., and A. BALDESTEN. 1977. Isotachophoretic analysis of mandelic acid, phenylglyoxylic acid, hippuric acid and methylhiphuric acid in urine after occupational exposure to styrene, toluene and/or xylene. J. Chromatogr. 132: 469-478.

SOLS, A. 1967. Regulation of carbohydrate transport and metabolism in yeast, p. 47-66. In A.K. Mills and H.A. Krebs (eds.), Aspects of Yeast Metabolism. Blackwell Scientific, Oxford.

SOLS, A. 1968. Phosphorylation and glycolysis, p. 53-87. In F. Dickens, P.J. Randle and W.J. Whelan (eds.), Carbohydrate metabolism and its disorders, Vol. I. Academic Press, London and New York.

SOLS, A., C. GANCEDO, and G. DE LA FUENTE. 1971. Energy yielding metabolism in yeasts, p. 271-307. In A.H. Rose and J.S. Harrison, The Yeasts, Vol. 2. Academic Press, New York and London.

SPENCER, J.F.T., and M.D. SPENCER. 1977. Hybridisation of non-sporulating and weakly sporulating strains of brewer's and distiller's yeast. J. Inst. Brew. 83: 287-289.

SPENCER, J.F.T., and M.D. SPENCER. 1979. Aberrations in sexual behaviour in some brewing and other industrial yeasts. Mol. Gen. Genet. 170: 277-281.

SPIEGELMAN, S., and J.M. REINER. 1947. The formation and stabilization of an adaptive enzyme in the absence of its substrate. J. Gen. Physiol. 31: 175-193.

SPIELMANN, H., U. JACOB-MULLER, and P. SCHULZ. 1981. Simple assay of 0,1 - 1,0 pmol of ATP, ADP and AMP in single somatic cells using purified luciferin luciferase. Anal. Biochem. 113: 172-178.

SPRAGUE, G.F. 1977. Isolation and characterization of a Saccharomyces cerevisiae mutant deficient in pyruvate kinase activity. J. Bacteriol. 130: 232-236.

STALMANS, W., and G. GEVERS. 1981. The catalytic activity of phosphorylase b in the liver. Biochem. J. 200: 327-336.

STANLEY, P.E. 1978. Quantification of picomole amounts of NADH, NADPH, and FMN using bacterial luciferase. Methods Enzymol. 57: 215-222.

STEWART, G.G., J. ERRATT, I. GARRISON, T. GORING, and I. HANCOCK. 1979. Studies on the utilization of wort carbohydrates by brewer's yeast strains. M.B.A.A., Tech. Quart. 16: 1-7.

STONE, S.R., and H.J. FROMM. 1980. Studies on the mechanism of adenosine 5'-monophosphate inhibition of bovine liver fructose-1,6-bisphosphatase. Biochemistry. 19: 620-625.

STREHLER, B.L., and J.R. TOTTER. 1952. Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. Arch. Biochem. Biophys. 40: 28-41.

STRITTMATTER, C.A. 1957. Adaptive variation in the level of oxidative activity in Saccharomyces cerevisiae. J. Gen. Microbiol. 16: 169-183.

SUTCLIFFE, W.H., Jnr., E.A. ORR, and O. HOLM-HANSEN. 1976. Difficulties with ATP measurement in inshore waters. Limnol. Oceanogr. 21: 145-149.

SUTTON, D.D., and J.O. LAMPEN. 1962. Localization of sucrose and maltose fermenting systems in Saccharomyces cerevisiae. Biochim. Biophys. Acta. 56: 303-312.

TAKEDA, M. 1981. Glucose-induced inactivation of mitochondrial enzymes in the yeast Saccharomyces cerevisiae. Biochem. J. 198: 281-287.

TAKETA, K., and B.M. POGELL. 1965. Allosteric inhibition of rat liver fructose-1,6-diphosphatase by adenosine 5'-monophosphate. J. Biol. Chem. 240: 651-662.

TANAKA, T., F. SUE, and H. MORIMURA. 1967. Feed-forward activation and feed-back inhibition of pyruvate kinase type L of rat liver. Biochem. Biophys. Res. Commun. 29: 444-449.

TAYLOR, C.B., and E. BAILEY. 1967. Activation of liver pyruvate kinase by fructose-1,6-diphosphate. Biochem. J. 102: 32c-33c.

TAYLOR, G.T., P.A. THURSTON, and B.H. KIRSOP. 1979. The influence of lipids derived from malt spent grains on yeast metabolism and fermentation. J. Inst. Brew. 85: 219-227.

TEN BERGE, A.M.A., G. ZOUTEWELLE, and K.W. VAN DE POLL. 1973. Regulation of maltose fermentation in Saccharomyces carlsbergensis. I. The function of the gene MAL6 as recognised by mal6 mutants. Mol. Gen. Genet. 123: 233-246.

THOMPSON, F.M., and D.E. ATKINSON. 1971. Response of nucleoside diphosphate kinase to the adenylate energy charge. Biochem. Biophys. Res. Commun. 45: 1581-1585.

THORE, A. 1979. Technical aspects of the bioluminescent firefly luciferase assay of ATP. Sci. Tools. 26: 30-34.

THORN, J.A. 1971. Yeast autolysis and its effect on beer. Brew. Dig. 46: 110-113.

TOBIN, R.S., J.F. RYAN, and B.K. AFGHAN. 1978. An improved method for the determination of adenosine triphosphate in environmental samples. Water Res. 12: 783-792.

TREVELYAN, W.E., and J.S. HARRISON. 1956. Studies on yeast metabolism. 7. Yeast carbohydrate fractions. Biochem. J. 63: 23-33.

TRUCCO, R.E. 1951. Enzymatic synthesis of uridine diphosphate glucose. Arch. Biochem. 34: 482-487.

ULLMANN, A., P.R. VAGELOS, and J. MONOD. 1964. The effect of 5'adenylic acid upon the association between bromothymol blue and muscle phosphorylase b. Biochem. Biophys. Res. Commun. 17: 86-92.

UMBREIT, W.W., R.H. BURRIS, and J.F. STAUFFER. 1951. Manometric techniques and tissue metabolism. Burgess Publ. Co., Minneapolis.

UTTER, M.F. 1958. Carbohydrate metabolism. Annu. Rev. Biochem. 27: 245-284.

VAN DE POLL, K.W., A. KERKENAAR, and D.H. J. SCHAMHART. 1974. Isolation of a regulatory mutant of fructose-1,6-diphosphatase in Saccharomyces carlsbergensis. J. Bacteriol. 117: 965-970.

VANDERGHINSTE, G. 1982. Caractérisation industrielle d'hybrides dépourvus d'acétohydroxy acide synthase obtenus par conjugaison rare. Mémoire. I.I.F.-I.M.C., C.E.R.I.A., Brussels.

VAN DE WERWE, G. 1981. Inhibition of liver glycogen synthase by calcium: new evidence for an interaction between synthase activation and phosphorylase a. Biochem. Biophys. Res. Commun. 102: 1323-1329.

VAN SCHAFTINGEN, E., D.R. DAVIES, and H-G. HERS. 1981. Inactivation of phosphofructokinase 2 by cyclic AMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 103: 362-368.

VAN SCHAFTINGEN, E., and H-G. HERS. 1981. Phosphofructokinase 2. The enzyme that forms fructose-2,6-bisphosphate from fructose-6-phosphate and ATP. Biochem. Biophys. Res. Commun. 101: 1078-1084.

VAN WIJK, R., and T.M. KONHIJN. 1971. Cyclic 3',5'-AMP in Saccharomyces carlsbergensis under various conditions of catabolite repression. FEBS Lett. 13: 184-186.

VAN WIJK, R., J. OUWEHAND, T. VAN DEN BOS, and V.V. KONINGSBERGER. 1969. Induction and catabolite repression of α-glucosidase synthesis in protoplasts of Saccharomyces carlsbergensis. Biochim. Biophys. Acta. 186: 178-191.

VARDANIS, A. 1967. Glycogen synthetase of bee larvae. J. Biol. Chem. 242: 2306-2311.

VARIMO, K., and J. LONDESBOROUGH. 1982. Adenylate cyclase activity in permeabilised yeast. FEBS Lett. 142: 285-288.

VILLANUEVA, J., and F. MARCUS. 1974. Fish liver fructose-1,6-diphosphatase. J. Biol. Chem. 249: 745-749.

VIÑUELA, E., M.L. SALAS, and A. SOLS. 1963. End product inhibition of yeast phosphofructokinase by ATP. Biochem. Biophys. Res. Commun. 12: 140-145.

WALSH, D.A., J.P. PERKINS, and E.G. KREBS. 1968. An adenosine 3',5'-monophosphate dependent protein kinase from rabbit skeletal muscle. J. Biol. Chem. 234: 3763-3765.

WANG, C.H., and D.J. GRAVES. 1964. The relationship of the dissociation to the catalytic activity of glycogen phosphorylase a. Biochemistry 3: 1437-1445.

WANG, C.H., C.T. GREGG, I.A. FORBUSCH, B.E. CHRISTENSEN, and V.H. CHELDELIN. 1956. Carbohydrate metabolism in baker's yeast. I. Time course study of glucose utilization. J. Am. Chem. Soc. 78: 1869-1875.

WANG, C.H., I. STERN, C.M. GILMOUR, S. KLUNGSOYR, D.J. REED, J.J. BIALY, and V.H. CHELDELIN. 1958. Comparative study of glucose catabolism by the radio-respirometric method. J. Bacteriol. 76: 207-211.

WICKERHAM, L.J. 1951. Technical bulletin No. 1029. U.S. Dept. Agric., Washington D.C.

WIEBEL, K.E., J-R. MOR, and A. FIECHTER. 1974. Rapid sampling of yeast cells and automated assays of adenylate, citrate, pyruvate and glucose-6-phosphate pools. Anal. Biochem. 58: 208-216.

WIELDERS, J.P.M., and J.L.M. MULLER. 1980. The determination of ADP and ATP in picomole amounts by steady-state mixed zones in isotachophoresis.

Anal. Biochem. 103: 386-393.

WILD, J.R., R. DAVID, L. SCHRONK, and A.R. HANKS. 1978. Developmental changes in purine nucleotide pools of Dictyostelium discoideum. Texas J. Sci. 31: 9-17.

WILDENRADT, H.L., and M.J. LEWIS. 1970. Production of hydrogen sulphide by yeast. J. Am. Soc. Brew. Chem. 28: 113-122.

WILLIAMS, J.F. 1980. A critical examination of the evidence for the reactions of the pentose pathway in animal tissues. Trends in Biochem. Sci. 5: 315-319.

WILLIAMS, J.F., P.F. BLACKMORE, and M.G. CLARK. 1978. New reactions sequences for the non-oxidative pentose phosphate pathway. Biochem. J. 176: 257-282.

WILLIAMSON, J.R. 1965. Glycolytic control mechanisms. J. Biol. Chem. 240: 2308-2321.

WILLIAMSON, J.R., E.A. JONES, and G.F. AZZONE. 1964. Metabolic control in perfused rat heart during fluoracetate poisoning. Bioch. Biophys. Res. Commun. 17: 696-702.

WITT, I., R. KRONAU, and H. HOLZER. 1966. Repression von Alkoholdehydrogenase, Malatdehydrogenase, Isocitratlyase und Malatsynthase in Hefe durch Glucose. Biochim. Biophys. Acta. 118: 522-537.

WOOD, T., and A. GASCON. 1980. A lack of reactivity of D-arabinose-5-phosphate with enzymes of the pentose phosphate pathway. Arch. Biochem. Biophys. 203: 727-733.

WOOD, H.G., J. KATZ, and B.R. LANDAU. 1963. Estimation of pathways of carbohydrate metabolism. Biochem. Z. 338: 809-847.

WU, T-F.L., and E.J. DAVIS. 1981. Regulation of glycolytic flux in an energetically controlled cell-free system: the effects of adenine nucleotide ratios, inorganic phosphate, pH, and citrate. Arch. Biochem. Biophys. 209: 85-99.

ZIMMERMANN, F.K., and N.R. EATON. 1974. Genetics of induction and catabolite repression of maltose synthesis in Saccharomyces cerevisiae. Mol. Gen. Genet. 134: 261-272.

ZIMMERMANN, F.K., N.A. KHAN, and N.R. EATON. 1973. Identification of new genes involved in disaccharide fermentation in yeast. Mol. Gen. Genet. 132: 29-37.