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**LA THÈSE A ÉTÉ
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THE EFFECT OF ACETATE AND ETHANOL
ON THE GLUCOSE METABOLISM OF
SCHIZOSACCHAROMYCES POMBE

by

Antonio Javier Aveledo-Padron B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Chemistry

Carleton University

Ottawa, Ontario

September, 1986

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Metabolism of Schizosaccharomyces pombe "

submitted by Antonio Javier Aveledo Padron in partial
fulfillment of the requirements for the degree of Master
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ABSTRACT

The fission yeast Sch. pombe is capable of growing on ethanol if glucose is present in the medium. The yeast exhibited a diauxic growth pattern. No growth on the alcohol occurs in the absence of the sugar. The yeast cannot utilize acetate for growth. However, it consumes the acid and glucose concurrently, increasing the biomass yield. Whereas the organic acid is utilized anabolically, the alcohol is used catabolically. Acetate utilization is not affected by metabolic inhibitors.

The presence of either acetate or ethanol in the medium gave rise to toxic effects in the cultures. The yeast was more susceptible to the acid. The presence of acetate in chemostat cultures, decreased the specific rates of glucose utilization and ethanol production. The organic acid and the alcohol induce changes in the protein profile of the fission yeast grown in chemostat cultures. In both cases the yeasts ability to metabolize either compound was increased. Acetate and ethanol also induced changes in the density of the cells. The alcohol induced morphological changes in batch culture cells, during the exponential phase..

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This work is dedicated to my family.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
D	Dilution rate
Disc	Discontinuous
DNA	Desoxyribonucleic acid
EDTA	Ethylene diaminetetracetate
EMM2	Edinburgh minimal medium No. 2
FAD	Flavine adenine dinucleotide
FADH	Reduced form of FAD
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
MEB	Malt extract broth
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form NADP
NCYC	National collection of yeast cultures
PFK	Phosphofructokinase
RNA	Ribonucleic acid
S	Saccharmyces
Sch	Schizosaccharomyces
SDS	Sodium dodecyl sulphate.

TCA

Trichloroacetic acid

YEPD

Yeast extract, peptone, dextrose

INTRODUCTION

The fission yeast Schizosaccharomyces pombe (Sch.pombe) was first isolated from an East African millet beer called "pombe" in Swahili (1,2). The cells of this organism (in exponential growth) are rounded cylinders with a diameter of about 3.5 μm and a length which varies from 5 to 20 μm depending upon growth conditions, stage in the cell cycle, stage in the growth of the culture and the strain (1).

Schizosaccharomyces pombe has been used extensively in the wine industry for deacidification of grape musts (3,4,5,6). This yeast has the ability of decomposing malate to ethanol and carbon dioxide under anaerobic conditions or oxidizing it completely under aerobic conditions (62). The organism is capable of functioning at very low pH (pH optimum for malic acid fermentation is 2.8). Most of the malic acid is utilized during the sugar fermentation (62).

The Schizosaccharomyces species belongs to the same family as Saccharomyces, the Saccharomycetaceae (62). For the purposes of this work, both species will be assumed to be very similar physiologically, the main differences are that the absence of the glyoxylate cycle (8) in the

fission yeast and the inability of this organism to grow in the absence of oxygen (115).

The fission yeast is not a very proficient organism with respect to the amount of substrates it can grow on (1,2). This yeast can grow on glucose, sucrose, maltose, xylulose, fructose, cellobiose and glycerol (1,2). Sch. pombe cannot grow on pyruvate, succinate, lactate, citrate, acetate, tartrate, acetaldehyde, ethanol, mannitol, peptone, tryptone, lactose, trehalose, galactose, xylose, L or D arabinose and others (1,2). The yeast also needs biotin, pantothenic acid, inositol (91) and niacin (1). It can use either ammonium sulphate or asparagine as sole source of nitrogen. Growth of the fission yeast is stimulated by glutamic acid, aspartic acid, adenine and uracil (91). The requirements for the trace elements in Sch. pombe are uncertain (1).

All yeast are capable of utilizing D-glucose (7) which is catabolized via the glycolytic pathway (Fig. 1) to pyruvate, giving rise to the following overall reaction (7) :

Glucose as a substrate for growth has to provide three major requirements : (i) the metabolic intermediates

Fig. 1. The glycolytic pathway (8).

for biosynthesis, (ii) ATP and (iii) reducing power (87). The first step in this process is the uptake of the sugar from the medium. This is accomplished by facilitated diffusion in *S. cerevisiae* (8). In this process it may be that the carrier-substrate complex undergoes diffusion, either translational or rotational, in response to a gradient of the complex within the plasma membrane (8). In *Sch. pombe* two glucose-binding proteins and one mannose-binding protein have been isolated from the plasmalemma of the yeast (88).

Once glucose enters the cell, it is phosphorylated to glucose-6-phosphate by a constitutive hexokinase. The phosphorylated sugar is kept in equilibrium with fructose-6-phosphate by glucose isomerase. This reaction is followed by the phosphorylation of fructose-6-phosphate by phosphofructokinase to fructose-1,6-diphosphate by (87). If glucose-6-phosphate starts to accumulate glucose uptake is inhibited (89). Hexokinase from yeasts is not inhibited by glucose-6-phosphate (90).

Phosphofructokinase (PFK) catalyses the first committed step in glycolysis and is the site for regulation in this pathway (87, 89, 90). This enzyme is subjected to a very rigid regulation (89, 90), it gives a hyperbolic saturation curve for ATP followed by a steep inhibition curve as the ATP concentration is increased (90). In the

presence of an activator, such as AMP, the catalytic part of the curve is not changed, while the inhibitory curve reaches almost a plateau after maximal activity (90). The saturation curve for fructose-6-phosphate at pH 6.9 is sigmoidal. Inhibitors will increase the sigmoidicity of the curve, while activators will decrease it (90). In other words, an activator will decrease the apparent K_m while an inhibitor will increase it. In yeasts the ammonium ion can also activate PFK by counteracting the ATP inhibition, while citrate acts as an inhibitor when present in physiological concentrations (87).

The fructose-1,6-diphosphate is cleaved into dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate by aldolase (90). This enzyme, in yeasts, is stimulated by K^+ and NH_4^+ . It contains Zn^{+2} and is inhibited by chelating agents such as EDTA (87). Triosephosphate isomerase catalyses the interconversion of the two products of fructose-1,6-diphosphate cleavage. The equilibrium is displaced in favor of dihydroxyacetone-phosphate, but glyceraldehyde-3-phosphate is removed by glyceraldehyde-3-phosphate dehydrogenase (90). This enzyme phosphorylates and oxidizes the intermediate to form 1,3 phosphoglycerate. This transformation drives the equilibrium in favor of glyceraldehyde-3-phosphate and produces NADH (87).

1,3-phosphoglycerate is cleaved to 3-phosphoglycerate producing ATP in the process. This reaction is catalysed by phosphoglycerate kinase (87). The next reaction in the pathway is the conversion of 3-phosphoglycerate to 2-phosphoglycerate by phosphoglycerate mutase. Catalytic amounts of 2,3-diphosphoglycerate are needed in yeasts, compound acting as a specific cofactor (87). The 2-phosphoglycerate is dehydrated to phosphoenolpyruvate by enolase. This intermediate is a high energy compound which is cleaved to pyruvate and ATP by pyruvate kinase (87).

Pyruvate kinase is regulated by two different mechanisms in yeasts (87). First the amount of the enzyme present depends on the substrate used for growth and second, its activity depends on the presence of fructose-1,6-diphosphate (87). This enzyme is also activated by K^+ and NH_4^+ (89).

Under anaerobic conditions yeast converts pyruvate into ethanol by the following sequence:

The sequence of reactions described above gives rise to the following overall equation for glucose

fermentation:

The fate of the glucose carbon varies according to the species of yeast (7,12). Other products of fermentation may be glycerol, acetic acid and lactic acid (71).

Under aerobic conditions, pyruvate is converted into acetyl coenzyme A, which drives the tricarboxylic acid cycle (Fig. 2), in which the carbohydrate is oxidized into carbon dioxide and water (7,12).

• Yeasts can also oxidize D-glucose via the pentose phosphate cycle, the use of this pathway is believed to be regulated by the supply of NADP^+ (92). In this pathway, glucose is phosphorylated to D-glucose-6-phosphate and then oxidized to 6-phospho-gluconate. This reaction is followed by a decarboxylation to ribulose-5-phosphate, which enters the cycle (Fig. 3). The overall reaction results in the complete oxidation of glucose :

In *Saccharomyces cerevisiae*, 12% of the D-glucose is catabolized by the pentose phosphate cycle, the rest by glycolysis (7). Gancedo and Lagunas (92) put the figure as low as 2.5% .

D-glucose is capable of catabolic repression in many

Fig. 2. The tricarboxylic acid cycle (7).

Fig. 3. The pentose-phosphate cycle (7).

yeasts (8,9,36) and other microorganisms (37). In yeasts, the glucose or Crabtree effect operates during the exponential growth on glucose. The net effect is the degradation of glucose via fermentation, leading to the accumulation of ethanol (9). The magnitude of the effect varies with the hexose concentration as well as with the species of the organism (9,38). In the case of Saccharomyces cerevisiae, 3% (w/v) of D-glucose is needed for complete repression (9). In other organisms such as Sch. pombe, the concentration needed is much smaller (8,9); this particular yeast is very sensitive to D-glucose.

The effect of the sugar is to repress the respiratory pathways, the increase in glucose concentration bringing about a decrease in cytochromes a', b and c (39). D-Glucose also inhibits several enzymes such as: NAD^+ and NADP^+ isocitrate dehydrogenase (40), citrate synthetase (40), isocitrate lyase (40,41), malate synthetase (40,41), NAD^+ and NADP^+ glutamate dehydrogenase (40), succinate dehydrogenase (42), malate dehydrogenase (43) and alcohol dehydrogenase (41) (just to mention a few).

Another response of the Crabtree effect is the existence of specific growth patterns in the batch cultures (8). Yeasts can be divided into three separate groups . The first group (Type 1) or the glucose-

insensitive strains, which are characterized by exponential growth until the limiting medium component is exhausted and does not accumulate a metabolic intermediate (Fig. 4). Type 2 pattern is observed with glucose-sensitive organisms under conditions of maximum oxygen supply. Glucose is utilized during the first exponential phase; due to the repression, ethanol is formed as an intermediate and serves as the carbon source during the second growth phase which starts after the release from glucose repression (Fig. 4). This growth pattern is characteristic of organisms such as Saccharomyces cerevisiae. Type 3 represents the behaviour of a glucose-sensitive yeast strain growing on glucose but not on ethanol; only one exponential growth phase is obtained. Ethanol accumulated during the first phase is completely oxidized but no changes in biomass occurs. The fission yeast Sch. pombe is reported to follow this type of pattern (mono-auxie) (8,10). The inability of this organism to grow on ethanol has been attributed to the absence of the glyoxylate shunt in this yeast (8) (see below). It should be noted that glucose is not the only effector that may cause this type of metabolic behaviour. Oxygen may also interfere in some cases (8).

In chemostat cultures, growth patterns also occur (Fig. 4). Plots of biomass (x) and substrate concentration (s) against dilution rate (D) show that the growth

Fig. 4. Growth patterns of various yeast types in batch and chemostat cultures (8). The figures represent changes in biomass (X) and substrate concentration (S) versus time (batch cultures) or dilution rate (chemostat cultures).

TYPE 1
Candida sp.

TYPE 2
Saccharomyces sp.

TYPE 3
Schizosaccharomyces sp.

patterns are a function of dilution rate (see below). Section I represents a regulatory state of decreasing biomass yield; no glucose repression is observed, no ethanol is formed and the respiration quotient is less than unity. As the dilution rate is increased (Section II), derepression is still observed. The biomass change yield is unaffected but the respiration is decreased (respiration quotient = 1). At Section III, glucose repression begins and ethanol starts to accumulate. The biomass yield decreases and respiration is lowered (respiration quotient > 1) under this situation (8).

The accumulated ethanol can be utilized by the yeast cells in the following sequence of reactions (7,11) :

The resulting acetate can be "fed" into the tricarboxylic acid cycle or the glyoxylate cycle via acetyl CoA (11,12).

The synthesis of acetyl CoA from pyruvate is catalysed by the pyruvate dehydrogenase complex (87). This complex transforms pyruvate to acetyl CoA. The first step is the activation of pyruvate by protein-bound thiamine pyrophosphate, resulting in the formation of hydroxyethyl-

carboxylthiamine pyrophosphate. This compound is decarboxylated to form acetyl-dihydrolipoate. This compound reacts with CoA to form acetyl CoA (87). Another source of acetyl CoA is the reaction catalysed by acetyl CoA synthetase (98). This enzyme converts acetate to acetyl CoA. The reaction involves the phosphorylation of acetate and the condensation with CoA to form the product (98).

The acetyl CoA enters the TCA cycle by condensing with oxaloacetate to form citrate. This reaction is catalysed by citrate synthetase (87). This reaction is favored because the free energy of hydrolysis is -8.8 Kcal/mole (87). The citrate is converted to isocitrate by aconitase, which is then oxidized to α -ketoglutarate by isocitrate dehydrogenase. This reaction involves the formation of NADH and the production of CO_2 (see Fig.2). Yeasts have two isocitrate dehydrogenases (87). One is cytosolic and is NAD^+ dependent. The second is mitochondrial and is NADP^+ linked. The mitochondrial enzyme requires AMP as an allosteric activator (87).

The next reaction in the cycle is carried out by an enzyme complex. The reaction involves the conversion of α -ketoglutarate to succinyl CoA, producing NADH^+ and CO_2 in the process. The enzyme responsible for this reaction is α -ketoglutarate dehydrogenase complex (99). The succinyl

CoA is cleaved to succinate by succinyl CoA synthetase. This reaction is coupled with the phosphorylation of GDP to GTP which harnesses the energy evolved in the hydrolysis (99). The succinate is converted to fumarate by succinate dehydrogenase, this reaction involves the reduction of FAD to FADH (87). Fumarate is dehydrated to malate by fumarase, and the product is then oxidised to oxaloacetate by malate dehydrogenase with the generation of NADH (99).

The net result of a full turn of the TCA cycle is the synthesis of one energy-rich phosphate bond, the production of one protein-bound FADH, three molecules of NADH and two molecules of CO_2 .

The TCA cycle is regulated at several points (99). The first irreversible step in the cycle is the conversion of pyruvate to acetyl CoA. The pyruvate dehydrogenase complex (PDC) is regulated in three ways: (i) Product inhibition by acetyl CoA and NADH as well as activation by CoA and NAD^+ . (ii) Feedback regulation by nucleotides; PDC is inhibited by GTP and activated by AMP. (iii) Regulation by covalent modification (99). A second point of regulation in the cycle is the condensation reaction which is inhibited by ATP. The formation of α -ketoglutarate is allosterically stimulated by ADP, which enhances the affinity for NAD^+ and Mg^{2+} . The binding of NAD^+ , Mg^{2+} and

ADP is mutually cooperative (99). The next regulation point is found at the formation of succinyl CoA; the reaction is regulated by succinyl CoA and NADH via feedback control(99).

The energy trapped in NADH is transferred to ATP by the electron transport chain (87). In the genus *Saccharomyces* only sites II and III are coupled to phosphorylation(100); therefore only two molecules of ATP instead of three are produced for each molecule of NADH oxidized.

The presence of ethanol in the medium also affects the microorganisms in other ways (13,14,15). The cell membranes are known to be highly permeable to ethanol (44,45). Since the uptake of ethanol is very fast, the yeast cells studied achieved 60% of the final level in only 10 seconds irrespective of the concentration of ethanol used. Metabolic inhibitors had no effect on uptake (44) and it was concluded that simple (nonsaturable) diffusion was the sole transport mode for ethanol (44,45).

The toxic effects of ethanol in yeasts are varied. It affects growth and fermentation (17). The effect on growth is stronger than on fermentation. Concentrations below 1% (w/v) had no effect on either growth or fermentation. However, any increase led to a gradual inhibition of growth which was completely arrested when a

concentration of 12% (w/v) was reached. In the case of fermentation, at 12% (w/v) ethanol, the yeasts were still fermenting at 25% of the control rate (17). It has been shown that the inhibitory effect of ethanol is more severe if the alcohol is produced by the cell rather than added to the medium (46). This has led several workers to postulate that the toxicity is due to high intracellular ethanol concentration (20,47). Several enzymes such as hexokinase (48) and alcohol dehydrogenase (47,48) have been reported to be inhibited *in vivo* with increasing ethanol concentration. However, *in vitro* studies have shown that glycolytic enzymes are stable in the presence of high concentration of ethanol. The idea of protein denaturation as main cause for ethanol toxicity was ruled out by the work of Desari et al. (21). They showed that the high intracellular concentrations detected were artifacts.

Another target of ethanol toxicity can be found in the different transport systems such as that for glucose (50) and maltose (51). In both cases the effect is believed to be due to changes in the lipid composition of the plasma membrane (50,57). These changes may also explain the decrease in the thermal resistance of the cells (53) and the increase in cell death (52,54) in the presence of the alcohol. Ethanol is also known to dissipate the proton motive force across the plasma

membrane (16).

It has been observed that tolerance to ethanol is affected by the plasma membrane composition and the presence of active mitochondria and oxidative metabolism (55). However, the action of ethanol on yeast is still not completely understood (14,15).

The second product of glucose fermentation, acetate, is metabolized by several yeasts. The pathway of acetate oxidation in these organisms has been studied extensively. Krebs et al. (56) noted that the tricarboxylic acid cycle could not be the main mechanism for acetate oxidation. [^{14}C]-Acetate was incorporated into C_4 dicarboxylic acids but not into ketoglutarate (56) in Baker's yeast. Further work with *Saccharomyces cerevisiae* showed that different oxidative patterns occur depending on the stage of the growth cycle at which the cells are harvested (57). Cells collected during glucose oxidation were not capable of oxidizing acetate. However, late logarithmic or stationary phase cells oxidized glucose, ethanol and acetate rapidly. The elucidation of the anapleurotic glyoxylate cycle provided the clue for acetate oxidation (24,25,58).

When ^{14}C -acetate is added to the medium in which aerobic microbes are growing, the isotope is taken up by

the cells with great rapidity and appears in all cellular constituents (80). The TCA cycle cannot supply, by itself, the need of the growing cells for energy. At the same time it cannot provide the precursors of cell constituents. If a cell is going to grow on 2-carbon compounds a replenishing or anapleurotic pathway is needed to restore the the TCA cycle intermediates used for biosynthesis (80).

The glyoxylate cycle is present in all microorganisms capable of utilizing acetate as the sole carbon source for growth (12). The operation of the cycle is as follows:

The first reaction is catalysed by isocitrate lyase. It involves the cleavage of isocitrate to glyoxylate and succinate. The enzyme requires the addition of Mg^{2+} and a sulfhydryl compound, such as cysteine or glutathione, for maximal activity (80). The second reaction involves the condensation of glyoxylate with acetyl CoA to give malate, and is catalysed by malate synthetase (80). In yeasts this enzyme has a pH optimum of 8.5 and the K_m for glyoxylate and acetyl CoA is $9.3 \times 10^{-5} \text{M}$ and less than $1 \times 10^{-5} \text{M}$ respectively (80). The enzyme requires Mg^{2+} for

maximal activity and is specific for glyoxylate (80).

The pathway is controlled at the level of isocitrate lyase. This enzyme is regulated via feed-back control by succinate and oxaloacetate (80). The amount of enzyme present is also controlled. It is abundant only if the cells are growing on acetate (80). Oxaloacetate also inhibits malate synthetase (80).

The overall reaction provides the means of forming two units of C_4 -dicarboxylic acid from acetate and isocitrate. In cells metabolising acetate, the reactant, isocitrate must be reformed from one of the C_4 -dicarboxylic acid units produced in this reaction via TCA cycle. This pathway bypasses the steps in the TCA cycle which produce CO_2 and leads to an increase in the organic acid carbon of the system (80). The two enzymes involved in the cycle along with other enzymes such as citrate synthetase, aconitase hydratase, fumarate hydratase, malate dehydrogenase and fructose diphosphatase are stimulated by the presence of acetate in Baker's yeast (25,58). Cells grown on glucose undergo an adaptation period (see above) due to catabolite repression. The limiting activity for acetate utilization has been reported to be that of isocitrate lyase, which increases during this period (25,58).

The fate of acetate when used as the sole carbon source in sporulating *Saccharomyces cerevisiae* is the production of intermediates for biosynthesis (59). Acetate carbon was found in glutamate, trehalose and lipids. The pattern changes in vegetatively growing cells (in complex medium). No acetate was incorporated into trehalose and only low amounts were detected in lipids. The levels of acetate carbon in the glutamate pool also decreased, most of the acetate being simply respired (59).

Fatty acids are known to be toxic to yeasts (19,22,23). Acetate is known to inhibit phosphate uptake in *S. cerevisiae* (19). The inhibition is concentration dependent and is active under aerobic and anaerobic conditions (19). The effect is pH dependent, increasing with decreasing pH. The fatty acid was observed to inhibit fermentation at high concentrations but enhanced it at low concentrations. In *S. cerevisiae*, *Candida utilis* and *Candida lipolytica*, acetate concentrations over 0.1 M caused a lowering of growth rate, cell division and phosphate uptake (22). Work with *Candida utilis* (60) showed that cells grown on a nitrogen-limited chemostat in the presence of excess acetate resulted in a decrease in maximum steady state growth and a highly inefficient utilization of the energy generated by oxidative metabolism. The effect of pH was drastic. If the pH of the culture was decreased from 7 to 4.8 (this increases the

amount of protonated acetate which is capable of permeating through lipid membranes (61)), growth was stopped and caused the culture to wash out of the chemostat (see below). The drop in pH to 4.6 led to a complete inhibition of respiration (60). The target of acetate toxicity has been proposed to be the plasma membrane (23) due to the effect on anion transport. The undissociated fatty acid would accumulate within the plasma membrane phase. This could explain the interference with the energy generating process observed above (60).

The effect of ethanol and acetate on Sch. pombe has not been documented, but the fission yeast is classified as being unable to utilize ethanol or acetate for growth (1,2,26) and unable to produce acetic acid from ethanol (26). The yeast has been observed to oxidize acetate very slowly when present as lone carbon source in complex media (27). In the case of ethanol, the literature available shows that ethanol is utilized in endogenous respiration. When the yeast is grown in batch culture using 2% glucose as carbon source in semi-synthetic media (10), it is known to inhibit xylulose fermentation (63). Concentrations as low as 0.1% have been observed to be toxic under carbon limitation in chemostat cultures (31). However, observations made by C.S. Tsai (personal communication) showed that Sch. pombe in carbon limited continuous culture was capable of using both glucose

(intended carbon source) and acetate (buffer) at the same time, giving rise to mixed substrate utilization. It was further observed that the amount of acetate consumed was dependent upon pH and dilution rate of the culture. These observations suggest that under special conditions the microorganism would be capable of utilizing a substrate which does not sustain growth along with one that does.

One of the main characteristics of microorganisms is their ability to vary their metabolism according to environmental pressures (12). In order to study these changes, two different systems have been developed: one is a closed system known as batch culture, the second an open system known as continuous culture or chemostat (28).

In a batch culture, the microbial cells are inoculated into a nutrient medium and allowed to grow. The growth goes through three phases - the first is a "lag" period during which the cells increase in mass and then start to divide. At this point they enter the second phase known as the exponential growth phase. During this phase, the cells are actively uptaking nutrients and excreting waste, changing the environment constantly. When the nutrients are exhausted, the cells enter the stationary phase. This is characterized by an inability of the cells to grow in the now nutrient-deficient and toxic medium (28).

A batch culture during the exponential growth phase can be described by the following equation:

$$\frac{1}{x} \frac{dx}{dt} = \mu = \frac{d \ln x}{dt} = \frac{\ln 2}{t_d} \quad (I)$$

where x is the initial concentration of organisms, t_d is the culture doubling time and μ is the specific growth-rate constant. Both μ and t_d are influenced by the environment; the dependence of μ on substrate concentration (5) can be represented by a Michaelis-Menten type equation,

$$\mu = \mu_{\max} \cdot \frac{s}{K_s + s} \quad (II)$$

where $\mu_{\max}$ is the maximum value of μ and K_s is a saturation constant (28).

In a continuous culture, it can be said that the cells remain in "exponential growth phase" indefinitely. One of the main features of the continuous culture system is the medium which is always low in the concentration of one of the essential nutrients. This medium is added continuously to the culture at a fixed rate, the volume of the culture (V) however is kept constant by providing the vessel with a sidearm. The growth rate of this culture will be dependent upon the rate of medium addition (F) and particularly upon the dilution rate (D); which is defined as the number of culture volumes of medium passing through

the growth vessel per unit time (F/V) (29).

The net change in concentration of the organism with time can be described by :

$$dx/dt = \mu x - Dx \quad (III)$$

If steady state is attained, μ will be equal to D and dx/dt will be zero. In a chemostat, conditions are set so that the specific growth rate (μ) and the dilution rate (d) can be equal and invariant with time, therefore

$$D = \mu = \mu_{\max} \cdot \frac{s}{K_s + s} = \frac{\ln 2}{t_d} \quad (IV)$$

The dependence of the growth rate on the rate of supply of growth-limiting nutrient make it proportional to the dilution rate (29).

Microorganisms become a structural and functional unit whose characteristics are a direct response to their microenvironment (93). The properties of any microbial cell are ultimately determined by the characteristics of the genome. However, microbes generally express only that part of their genome that enables them to become adjusted to a certain set of conditions (30,93). Therefore, microbial populations grown under different conditions, differ not only in properties expressed quantitatively (population kinetics, growth rates, etc.) but also in the

sum of their physiological properties (94).

Two extreme situations are represented by the conditions which exist in the exponential phase of a batch culture (nutrient excess) and in a chemostat being operated at a subcritical D (nutrient limitation) (30). Phenotypic variations occur both in chemostat and batch culture. In batch culture, during exponential phase, growth conditions change constantly. These changes are not reflected in the growth rate (which remains constant). However, physiologically the growing organism undergoes changes in response to the environment (30). The precise factors inducing phenotypic variations in batch cultured cells generally are not easy to identify due to the highly dynamic, continuously transient situation. In chemostat cultures, the system operates in steady state (see above). By changing dilution rates, limiting substrates, substrate concentrations, etc., it is possible to attain different culture conditions. This allows the study of phenotypic variations induced by the culture conditions in a steady state situation (94).

Batch cultures can be used to study the general changes undergone by the organism under a particular set of conditions. This system will not provide information on the specific changes that occurred in the organism during the exponential phase. The use of a chemostat allows the

study of critical sites in the metabolic sequence followed by the microbes during the exponential phase of the batch culture (94). The system allows the discovery and study of the triggering mechanisms that lead to the phenotypic changes observed in the culture. The chemostat can be used to set the culture conditions required to induce the changes in metabolism of the microbe. The system can be "fine tuned" to maximize the effect of the variation under study, be it biomass production, metabolite accumulation or substrate utilization (94). Once the conditions are set, then questions such as : What is the actual process of change? what mechanism will trigger the variations and how are they regulated ? can be studied.

In this work the medium used is carbon-limited, this type of limitation is characterized by high efficiency in carbon utilization with a minimum of it going to extracellular products (30). The organism growing under these conditions would tend to depress its catabolic enzymes and restrict their anabolic pathways to a minimum level which would allow growth (30).

The fission yeast has been studied in continuous cultures by several workers (31,32,33,34,35). They have concentrated on the study of cell division, population models, change in protein and nucleic acid content and cell morphology. This research has shown that cell division

in the fission yeast is affected by the type of nutrient limitation used (35). Carbon limited conditions stimulated conjugation in these cells. The biomass yield was dependent on dilution rate and the population of the cells were observed to be nonhomogeneous, both morphologically and physiologically (33). The largest morphological variations were observed at low D (33,34,35). Protein, DNA and RNA synthesis were also affected by D when carbon limitation was used (32). All the specific macromolecular synthesis rates decreased with decreasing D, the ethanol yield also decreased in the process while the biomass yield increased. However, no work involving the study of the effect of either acetate or ethanol on the metabolism of the yeast has been performed.

In this work, *Schizosaccharomyces pombe* will be studied in both batch and continuous culture. The fission yeast is known to be unable to grow on ethanol or acetate (see above); however, it is capable of consuming the organic acid along with glucose in carbon-limited chemostat cultures. The object of this work is to investigate how is acetate utilized by the yeast and the effect of this compound on glucose metabolism. The ability of *Sch. pombe* to utilize ethanol will also be investigated along with the effect of the alcohol on the glucose metabolism of the yeast.

MATERIALS AND METHODS

A - General Section

1 - Growth Media

The Malt Extract Broth was obtained from Oxoid, the Bacto Peptone, yeast extract and Agar were obtained from Difco.

The following components of the Edinburgh Minimal Media No. 2 (EMM2) were obtained from Fisher : potassium chloride, magnesium chloride heptahydrate, citric acid monohydrate, copper sulphate pentahydrate and ferric chloride hexahydrate. From Anachemia, ammonium chloride, dextrose, sodium acetate, calcium chloride, sodium sulphate, magnesium sulphate monohydrate, zinc sulphate pentahydrate and potassium phosphate dibasic were used. The following vitamins were purchased from Sigma: nicotinic acid, myo-inositol, D-biotin and D-pantothenic acid (hemi-calcium salt). Sodium molybdate was obtained from the General Chemical Division, potassium iodate and boric acid from BDH Chemicals, absolute ethanol from Consolidated Alcohols Ltd. and sodium phosphate monobasic monohydrate from J.T. Baker.

2- Chemicals.

The following products were purchased from Anachemia Chemicals: glacial acetic acid, nitric acid, ammonium persulphate, ammonium sulphate, glycerol and sodium formate. From Sigma, the following compounds were obtained: N,N,N',N', tetramethylethylenediamine (TEMED), N,N' methylene-bis-acrylamide, acrylamide and tris(hydroxymethyl)aminomethane (Tris). Phosphoric acid, n-propanol, Scinti Verse I, formaldehyde solution and phenol reagent solution (2N) were purchased from Fisher. Isoamyl alcohol, sodium azide, potassium dichromate (technical), sodium dodecyl sulphate (SDS) (specially pure) were obtained from BDH Chemicals. Potassium sodium tartrate and silver nitrate were obtained from Mallinckrodt Chemical Works; imidazole and trichloroacetic acid from J.T. Baker, calcofluor from American Cyanamid Company (provided by Dr. B.F. Johnson) and 2,4-dinitrophenol (technical) from Eastman Organic Chemicals. [1-¹⁴C]-Ethanol, D-[1-¹⁴C]-glucose and [1-¹⁴C] acetate were obtained from New England Nuclear. Unless otherwise noted, all chemicals were reagent grade.

3 - Organism and its Maintenance

The fission yeast Schizosaccharomyces pombe N.C.Y.C.

132 S₂-2 was used throughout the research project. The

strain was kept on YEPD slants for long term storage at 4°C. The cultures were propagated every two months by growing them at 30°C for 72 hours. The yeast was also kept through sequential transfers using 10 ml of 2% (w/v) Malt Extract Broth in 20 ml universal containers kept tightly capped. Every transfer consisted of 0.1 ml of the "old" culture into 10 ml of fresh medium, the cultures were incubated at 30°C in either Psycrotherm, G27 Gyrotherm bath or a G24 Environmental shaker, all from New Brunswick Scientific Co. The transfers were performed every 72 hours.

4 - Preparation of YEPD slants:

In 1000 ml of deionized water, 10 g of yeast extract were added along with 20 g of Bacto Peptone and 20 g of glucose. The mixture was heated on low heat until everything dissolved. To this solution, 20 g of agar were added and the temperature increased to boiling point to obtain a homogenous solution. Approximately 15 ml of the solution was then dispensed into 20 ml universal containers and loosely capped. The containers were autoclaved using an Amsco autoclave at 121°C for 20 minutes and allowed to solidify at an angle, at room temperature. The containers were then tightly capped and stored at room temperature until used.

5 - Preparation of 2% Malt Extract Broth.

To prepare a 1000 ml solution, 20 g of Malt Extract Broth was dissolved in warm water and 10 ml of the solution transferred into 20 ml universal containers. These containers were then autoclaved at 121°C for 20 minutes and stored at room temperature.

6 - Culture Medium

Throughout the project, the Edinburgh Minimal Media No. 2 (EMM2) designed by J.M. Mitchison (1) was used. Glucose and acetate concentrations were varied depending on the experiment and in some cases, ethanol was used in place of acetate.

The medium was composed of the following compounds: 0.2% (w/v) glucose, 93.5 mM NH_4Cl , 2.5 mM NaH_2PO_4 , 16.7 mM CH_3COONa , 13.4 mM KCl , 2.5 mM MgCl_2 , 70.4 μM Na_2SO_4 , 90.1 μM CaCl_2 , 55.5 μM inositol, 81.2 μM niacin, 4.2 μM panthothenic acid, 0.04 μM biotin, and the following trace compounds: 8.1 μM H_3BO_3 , 2.4 μM MnSO_4 , 1.4 μM ZnSO_4 , 0.7 μM FeCl_3 , 0.8 μM Na_2MoO_4 , 0.6 μM KI , 0.16 μM CuSO_4 , 4.8 μM citric acid. The chemostat culture medium was sterilized by filtration through a 0.2 μm Ultipore DFA ARP filter from Pall Corporation. For smaller volumes, 0.2 micron Sybaron/Nalge disposable filter units (Nalgene) were used.

7 - Glucose Determination

The calorimetric enzymatic determination of glucose from Sigma (113) was used. The procedure is based on the enzymatic reaction of glucose with glucose oxidase to produce gluconic acid and peroxide, which is reduced by peroxidase in the presence of o-dianisidine which, when oxidized, produces a chromophore with the absorption maximum at 450 nm.

One preweighed capsule containing 500 International units of glucose oxidase (*Aspergillus niger*), 100 Purpurogallin units of peroxidase (horseradish) and buffer salts were dissolved in 100 ml of deionized water. To this solution, 1.6 ml of 2.5 mg/ml of o-dianisidine dihydrochloride was added. This preparation was stable for up to 1 month when stored at 0-5°C (solution A).

A standard curve was constructed ranging from 0 to 0.10 mg/ml of glucose. The assay was performed by mixing 0.5 ml of sample (cell free) with 5.0 ml of solution A and allowing them to react for 60 minutes at room temperature. The optical density of the preparation was determined at 450 nm using a Coleman Jr. spectrophotometer.

8 - Ethanol and Acetate Determination

The analysis was carried out by gas chromatography

using a GOW/MAC GC series 750, equipped with a Hewlett-Packard 3390 integrator. A 6" X 1/4" coil column packed with 10% FFAP, 1% H_3PO_4 , 100/200 mesh Chromosorb (W/AW), from Chromatographic Specialties was used.

This method is based on that described by Ackman (114) The following conditions were used for the analysis:

a) Ethanol

The column temperature was set at 80°C while the injection temperature was 140°C. The flow rates for H_2 , He gas and air were 40, 60 and 300 ml/min. respectively. As an internal standard, 0.1% (w/v) n-propanol was used.

b) Acetate

The column temperature was set at 150°C while the injection temperature was 225°C. The flow rates for H_2 , He gas and air were 55, 45 and 300 ml/min respectively. He gas was passed over 98% distilled formic acid before entering the gas chromatograph. The acid was contained in a 100 ml bottle equipped with two side arms through which the gas flowed. As an internal standard, 0.1% isoamyl alcohol was used. The column was calibrated with 0.1% (w/v) ethanol and 0.1% (w/v) acetic acid.

The cell-free medium was prepared for analysis by mixing 0.5 ml of it with the proper internal standard. Of this preparation, 2 μ l was injected into the gas chromatographer using a Hamilton 10 μ l microsyringe (No.

701-N). The samples were analyzed in triplicate.

9.- Dry Weight Determination

Two different procedures were utilized for the determination of the dry weight of Sch. pombe. In both cases, 10 ml or 20 ml of culture were used depending upon the cell density of the sample.

a) Method 1

In this procedure, 25 mm nitrocellulose filters with a pore size of 0.45 microns (Schleicher and Schnell Inc.) were dried overnight at 80°C and their dry weight determined using a Sartorius analytical balance. These filters were kept in a dessicator until used.

The dry weight of the cells were determined by filtering 10 ml of the culture through a sampling manifold from Millipore and washing the cells with three aliquots of 5 ml of deionized water to remove any remnant of the culture medium. The filters were allowed to dry for 24 hours at 80°C before determining their weight. For every sample, two determinations were performed.

b) Method 2

In this procedure, metal trays were used instead of filters. They were given the same treatment as described

above and their weight determined using a Mettler AE160 electronic analytical balance. The cells in a 10 ml sample of culture medium were centrifuged down using a Sorval RC2-B or an International HT centrifuge at 12000Xg for 3 minutes. The pellet was suspended in 5 ml of deionized water and the procedure repeated. The washing was performed twice. The cells were then transferred quantitatively to the trays and allowed to dry at 80°C for 24 hours before weighing. Two determinations were carried out per sample.

10 - Total Cell Count

The cell count was performed microscopically using a Reichert Zetopan phase contrast microscope or a Wild Light transmitted microscope at 40X. In both cases, an improved Neubauer counting chamber from Arthur H. Thomas Company was used. If dilutions were needed, they were performed using a 0.9% (w/v) NaCl solution. A minimum of 500 cells were counted and all counts were performed twice.

The sample to be counted was vortexed to make sure all the cells were suspended and a drop placed in the chamber; time was allowed for the cells to settle before proceeding with the count.

11 - Protein Determination

The Lowry method for protein determination was used

(105). A solution containing 2% (w/v) of sodium potassium tartrate, 0.01% (w/v) NaCO_3 and 0.005% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was prepared (Solution A) by mixing 49 ml of 2% (w/v) sodium potassium tartrate with 0.5 ml. NaCO_3 in 0.5 N NaOH and 0.5 ml. of 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. The assay was performed by mixing 1 ml of the sample with 5 ml of A and incubating at room temperature for 10 minutes. After this period, 0.5 ml of 1N phenol reagent was added and the solution mixed vigorously. This was followed by a 30 minute incubation at room temperature. The optical density at 750 nm was then determined using a Beckman 25 spectrophotometer. A standard curve ranging from 0 to 170 $\mu\text{g/ml}$ of protein was constructed using bovine serum albumin.

12 - Preparation of Cell-Free Extracts.

The cells were extracted following the method described by Williamson et al. (101). The process was carried out using an Eaton press (102) (kindly provided by Dr. B.F. Johnson) cooled to approximately -60°C with dry ice. The cells, which had been stored at -20°C in 0.1M phosphate buffer (pH 7.5) and 15% (v/v) glycerol, were thawed and transferred to the pre-cooled cylinder. The preparation was allowed 5 minutes to solidify before inserting the pre-cooled plunger. The press was assembled and a pressure of 5000 to 8000 p.s.i. was applied to the

piston by a hydraulic press. The crushed material was thawed at 4°C and centrifuged at 12000Xg for 20 min in a Sorval RC2-B centrifuge to remove intact cells and debris. The breakage procedure is reported to be about 80% efficient and takes only 10 minutes to perform once the press has been pre-cooled.

13 - SDS Disc-Polyacrylamide Gel Electrophoresis

The method is a variation of that described by Laemmli (103). The stacking gel contained 5% (w/v) acrylamide in 0.125 M Tris-HCl (pH 6.8) with 0.1% (w/v) SDS. The separation gel contained 11% (w/v) acrylamide in 0.375 M Tris-HCl (pH 8.8) and 0.1% (w/v) SDS. The gels were prepared from a stock solution of 30% (w/v) of acrylamide and 0.8% (w/v) of N,N'-bis-methylene acrylamide. The polymerization was carried out by adding 0.025% (v/v) of TEMED and 0.08% (w/v) ammonium persulphate. The electrode buffer contained 25mM Tris-HCl (pH 8.3), 0.192 M glycine and 0.1% (w/v) SDS. The sample buffer contained 62.5mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol and 5% (w/v) 2-mercaptoethanol.

The samples were prepared by heating them for 3 minutes at 90°C. 25 µg of protein, as determined by the Lowry method. The electrophoresis was performed at 4°C, with a current of 25 mA.

Standards from the MW-SDS-70 kit (Sigma) were run along with the samples. These standards were: bovine albumin (66,000), ovalbumin (45,000), trypsinogen (24,000), β -lactoglobulin (18,400) and lysozyme (14,300). The standards were prepared as described above and 1 μ g of each protein standard was run.

The electrophoresis was performed in cooperation with K. Mitton.

14 - Silver Staining of Gels.

The method described by Nielson and Brown was used with a small modification (104). The gels were fixed in 10% (w/v) TCA for 30 minutes, followed by 3 washes with 10% (w/v) ethanol and 5% (w/v) acetic acid for 10 minutes each time. The slab was then oxidized with 3.4 mM sodium dichromate in 0.0032N nitric acid for 6 minutes and washed 3 times with deionized water for 5 minutes each time. Next, the gel was reacted with 0.012 M silver nitrate for 20 minutes, after which it was washed once with deionized water for 30 seconds. Up to this point, the procedure was carried out at 30°C with gentle shaking, using a G76 water bath shaker. The development of the gel was carried out at 40°C using 0.28 M sodium carbonate and 0.0185% (w/v) formaldehyde. The first wash was performed for 30 seconds;

the second and subsequent washings were carried out for 5 minutes each time until the desired results were obtained. The development was stopped with 5% (w/v) acetic acid.

The background of the gel could be reduced by storing the slab in 5% (w/v) acetic acid for 3 to 5 days at room temperature.

The experimental procedure has been divided into batch and continuous culture sections. For each section, a general description of the apparatus used is given as well as sterilization procedures. This section is followed by a detailed description of the experiments performed.

B - Continuous Culture Section

Two different apparatus were used in this project.

15 - Apparatus A

This apparatus was composed of a jacketed fermenter jar with an overflow side-arm located so as to maintain a volume of 500 ml (Pegasus Industrial Specialists). Agitation and aeration of the cultures was accomplished by using a Vibro Mixer (Chemopac) with a hollow stainless-steel shaft through which sterile air was passed. The air was saturated with water and sterilized through a 0.2 micron Ultipor DFA ARP filter (Pall Corp.). The temperature was controlled by water circulation through

the jacket of the vessel using a Haake D1-G water circulator set at 30°C.

The pH of the culture was controlled by a control system from New Brunswick Scientific Co., model pH 40, with an Ingold autoclavable electrode through the addition of sterile 1N KOH. Only teflon fittings (Swagelok) and silicone tubing (Cole-Parmer) was used. This chemostat was sampled through a tee connector in the waste line.

The medium was stored in a 20l Pyrex carboy and fed through a Harvard variable pump. The waste was collected in a 15l carboy (Nalgene).

16 - Apparatus B

This setup was purchased from the Virtis Company Inc. It was composed of a Omni-culture benchtop chemostat which was used for agitation control. The original vessel was replaced by a jacketed vessel (as described above) with a working volume of 520 ml (manufactured by M. Vandenhoff). Mixing was achieved by a 75 mm. long stirring bar. The temperature was controlled by a Haake water circulator. The air was provided by a Hagen air pump, the air was humidified and filtered through a 0.2 micron air filter (Nalgene) and fed to the culture by a scintered glass sparger.

The fresh medium addition was controlled by a variable speed peristaltic pump (Cole-Parmer). The waste collection and the sampling was performed as previously described. The pH was controlled by a pH control module, model 6715-1180 from Virtis Company Inc., using an Ingold autoclavable electrode. Sterile 1N KOH was used to titrate the medium. The flow rate of the fresh medium was calculated in both setups by stopping the flow system from the reservoir and monitoring the consumption of the medium in a biurette connected "in series". All the components in contact with the culture and medium were autoclaved for 15 minutes at 121°C. All experiments were performed at 30°C, pH 5.0 and an aeration rate of 400 ml/min.

In most cases the chemostat was inoculated using 10 ml of an overnight MEB culture. The culture was allowed to grow as a batch culture overnight and started at a dilution rate (D) greater than 0.1 h^{-1} . A minimum of 10 doubling times (t_d) were allowed for steady state to be achieved and sampling was performed for about 3 to 5 days before conditions were changed.

The collected samples were centrifuged to remove all cells and the supernatant stored at -20°C until analyzed. The biomass was monitored by recording the absorbance at 660 nm. using either a Coleman Jr. spectrophotometer or a Gilford 240 spectrophotometer. In some experiments, cell

counts and dry weight determination were performed instead, as described above.

17 - Continuous Culture of Sch. pombe growing on glucose as lone carbon source.

a) Growth using 0.2% (w/v) glucose.

The experiment was performed using apparatus A. The culture was grown at D ranging from 0.08 to 0.19 h⁻¹. The absorbance at 660 nm. was monitored and the samples were analyzed for glucose, acetate and ethanol.

18 - Effect of Acetate on a Chemostat of Sch. pombe.

a) Effect of a fixed concentration of acetate at different dilution rates.

This experiment was performed using apparatus A. The medium contained 0.2% (w/v) glucose, 0.06% (w/v) acetate. The absorbance at 660 nm. and the concentrations of glucose, ethanol and acetate were monitored for values ranging from 0.09 to 0.18 h⁻¹.

b) Effect of Increasing Acetate Concentration at Fixed Dilution Rate.

The concentration of acetate varied from 0% to 0.175% (w/v) using different cultures for each condition to

minimize selection for a mutant. Apparatus A was used throughout the experiment. The absorbance at 660 nm. was monitored and the residual concentrations of glucose, ethanol and acetate determined.

The experiment described above was repeated using apparatus B and fixing, D at 0.15 h^{-1} . The acetate concentration was varied from 0% to 0.25% (w/v) and residual acetate, ethanol and glucose were monitored along with the total cell count and dry weight. Samples of cells were collected and kept frozen for further analysis.

19 - Effect of Ethanol on the Growth of a *Sch. pombe* Chemostat.

a) Growth in the presence of 0.03% (w/v) Ethanol.

Apparatus A was utilized for this experiment, the medium contained 0.2% (w/v) glucose and 0.03% (w/v) ethanol. The culture was grown using dilution rates ranging from 0.08 to 0.23. The residual concentrations of glucose and acetate were monitored and the cell count determined. Two cultures were used for this experiment.

b) Effect of Increasing Ethanol Concentration at Fixed Dilution Rate.

The D was fixed at 0.15 h^{-1} using apparatus B. The medium contained 0.2% (w/v) glucose and the ethanol

concentrations were from 0.3% to 0.6% (w/v). A "fresh" chemostat was used for each set of conditions in order to minimize the danger of mutant selection (109).

C - Batch Culture Section.

A wide variety of vessels was used, from 2000 ml to 50 ml Erlenmeyer flasks, fitted with foam plugs. The exact sizes are described below.

In general, all vessels were autoclaved prior to the addition of the medium. The vessels ranging from 50 to 2000 ml, were autoclaved for 15 minutes at 121°C. Unless otherwise specified, the inoculum was grown in the experimental medium. The cells used to start the inoculum were grown overnight in Malt Extract Broth using the same procedure described for periodic transfers. The inoculum was cultivated under the same conditions to be used in the experiment.

All the experiments described were performed at least twice.

20 - Growth of Sch. pombe on Media containing Glucose as Lone Carbon Source.

The cultures were grown on 600 ml of medium containing 0.2% (w/v) glucose, using a 2l Erlenmeyer flask as a vessel. The experiment was performed in a psycrotherm at

30°C, with a mixing rate of 200 r.p.m.. The inoculum was grown in 60 ml of medium in 250 ml Erlenmeyer flasks. The cultures were inoculated with the full 60 ml of culture.

The cultures were sampled every two hours during the exponential phase, after which they were sampled twice a day for a total of five days. The samples were analyzed for ethanol, glucose and acetate content and cell counts were performed.

21 - Effect of Acetate Concentration on Sch. pombe Growing in Batch Culture.

a) Growth Curve in the presence of 0.2% (w/v) acetate.

The yeast was grown in 600 ml of medium containing 0.2% glucose and 0.2% (w/v) acetate, using a 2000 ml Erlenmeyer flask with a foam plug as a vessel. The inoculum was grown using 100 ml of the same medium in a 500 ml Erlenmeyer flask, which was inoculated with 10 ml of an overnight malt extract broth culture. The inoculum was grown overnight in a psychrotherm at 30°C and mixing at 200 r.p.m..

The culture was started using 60 ml of the inoculum and grown under the conditions previously described. The medium was sampled every two hours and the concentration of ethanol, acetate and glucose were determined. Cell

counts were performed as described above. A duplicate culture was run and sampled at the same time.

b) Effect of Initial Acetate Concentration on Biomass.

The cultures were grown in 50 ml Erlenmeyer flasks containing 20 ml of medium. The following concentrations were tested : 0, 0.2, 0.3 and 0.4% (w/v) acetate.

The inoculum for each concentration was grown using the appropriate acetate concentration. The inoculum was started as described above and grown in a G-24 Environmental Shaker at 30 °C and mixing at 150 r.p.m. The cultures were inoculated with 2 ml of the overnight-grown inoculum and incubated for 4 days at the conditions previously described. At the end of this period, the concentration of acetate was determined, as well as the dry weight of the culture (Method A). The absorbance of the culture at 660 nm was measured using a Beckman DB spectrophotometer.

c) Effect of Initial Glucose and Acetate Concentration on Acetate Consumption.

Three concentrations of glucose (0%, 0.1% and 0.2% (w/v)) and four concentrations of acetate (0.1%, 0.2%, 0.4% and 0.8% (w/v)) were tested.

The cultures were grown in 250 ml Erlenmeyer flasks containing 20 ml of medium. The inoculum was grown on 0.2% (w/v) glucose and 0.06% (w/v) acetate. The cells were incubated as described above. The cultures were inoculated with 2 ml of the overnight-grown culture and incubated under the same conditions for 8 days. At the end of this period, the cultures were stopped, their absorbance at 660 nm. read and the residual acetate concentration determined.

d) [1-¹⁴C]- Acetate Uptake.

The experiment was performed using 0.2% (w/v) glucose and 0.2% (w/v) acetate. The [1-¹⁴C]-acetate solution was prepared by dissolving 50 μ Ci of sodium acetate (specific activity of 718 μ Ci/mg) in 1 ml of 10 mg/ml acetic acid.

The experimental medium contained 50 nCi/ml of ¹⁴C-acetate, which was added prior to filter sterilization. The cultures were grown in 10 ml of medium using 125 ml Erlenmeyer flasks with foam plugs as vessels. The inoculum was grown in the medium to be tested using the same volume and vessel size described above. The cells were grown overnight in a G-76 Gyrotherm (New Brunswick Scientific Co.) at 30°C, with constant shaking at 150 r.p.m..

The cultures were started with 1 ml of the inoculum

and incubated as described above. The cultures were sampled by filtering the cultures through a 0.2 micron Millipore filter and washed twice with 5 ml 0.9% (w/v) NaCl. The filter was placed in a scintillation vial containing 5 ml of Scinti Verse I and counted using a Beckman LS 120 scintillation counter for 20 minutes. The medium samples were analysed by dissolving 50 μ l into 5 ml of Scinti Verse I and counted as mentioned above.

e) The Effect of Azide and 2,4-Dinitrophenol on [14 C]-Acetate Uptake and Utilization.

The medium and condition described for the acetate uptake experiments (21d) were used. The cells were grown for 12h before adding the filter sterilized metabolic inhibitor to the culture. The final concentration of the inhibitor in the medium was 0.1 mM. An equal volume of sterile deionized water was added to the control culture. The growth was monitored on parallel "cold" cultures. The sampling was conducted as described above.

f) Mutant Detection.

To test the yeast strain used for a mutant capable of utilizing acetate as lone carbon source, a culture was set up as described above (21a) using 0.2% glucose and 0.2% acetate. The same medium volume and vessel size as

previously described in 18a was used. When the acetate utilization phase begun, 1 ml of this culture was transferred to a 50 ml Erlenmeyer flask containing 10 ml of the medium with 0.2% (w/v) of acetate as the lone carbon source and incubated under the same conditions. The culture was sampled for three days. The same procedure was followed for the detection of a mutant capable of consuming ethanol. Conditions described in 19a were used.

22 - Effect of Ethanol Concentration on the Growth of Sch. pombe.

a) Growth of Sch. pombe in the Presence of Ethanol.

This experiment was performed as described for acetate (21a) but using either 0.5% (w/v) or 1.0% (w/v) of ethanol with no acetate present in the medium. The medium volume and the vessels used were the same as above. The cultures were run in duplicate and sampled every two hours for the first ten hours. The samples were analyzed for glucose, ethanol and acetate content and the cell count determined.

b) Uptake of [1-¹⁴C]-Ethanol.

This experiment was performed using 0.2% (w/v) glucose and 0.5% (w/v) ethanol.

The [1-¹⁴C]-ethanol solution was prepared by the same procedure used for [1-¹⁴C]-acetate except that cold

(-20°C) absolute ethanol was used. The final solution contained 0.5 $\mu\text{Ci/ml}$ with a specific activity of 111.2 $\mu\text{Ci/ml}$ of ethanol. The inoculum was grown overnight in the experimental medium and 1 ml was used to inoculate the cultures.

The cultures were grown in 10 ml of medium containing 2.5 nCi/ml of labelled ethanol and a 125 ml Erlenmeyer flask was used as a vessel.

The cultures were sampled by centrifuging the 10 ml of culture at 3500g for 3 minutes. The pellet was washed three times with 0.9% (w/v) NaCl and suspended in 1 ml of the salt solution, which was then filtered through a 0.2 micron filter and counted as previously described. Medium samples were analysed as described in section 21d.

23 - Effect of Acetate and Ethanol on the Density of the Cells and Their Morphology.

a) Cell Density:

Cells were grown in medium containing 0.2% (w/v) glucose alone or with either 0.2% (w/v) acetate or 0.5% (w/v) ethanol. The cultures were grown in 250 ml of medium using a 1000 ml Erlenmeyer flask as a vessel. The inoculum was grown in the experimental medium using 25 ml of the medium in a 125 ml Erlenmeyer flask. The cultures

were grown at 30°C and mixed at 150 r.p.m. in a G25 Environmental Shaker.

The cultures were sampled every two hours during the exponential phase and the cell count and dry weight of the sample was performed. The dry weight was determined using Method 2. A total of four counts were performed per sample and the dry weight determination was carried out in duplicate. Duplicates of each culture were run and assayed at the same time.

b) Cell Morphology

Using the same medium described above, cells were grown in 10 ml of the medium in 50 ml Erlenmeyer flasks using a Psycrotherm (New Brunswick Scientific Co.) at 30°C and at a rate of mixing of 200 r.p.m. The inoculum was grown in the experimental medium and 1 ml was used to inoculate the culture. The cultures were grown for six hours and stopped. The cells were photographed using a Reichart Zetopan phase contrast microscope, equipped with a Nikon UFX II Microflex controller. The film used was Kodak technical pan film 2415.

24 - The Effect of Acetate and Ethanol on the Utilization of [U-¹⁴C]-Glucose.

The same condition used in sections 20 (scaled down

to the volumes of 10 ml) 21d, and 22b were used in this experiment. The final concentration of the uniformly labelled glucose in the medium was 50 μ Ci/ml, which was added prior to filter sterilization. The sampling and analysis was performed as previously described.

23 - Chemical Extraction of Cells Grown on 14 C-labelled Ethanol Glucose and Acetate.

The method was described by Sutherland and Wilkinson (71). The cells were grown in 100 ml cultures as described above (18d,19b) for 24 hours. The growth was monitored by counting a duplicated "cold" culture.

At 24 hours, the culture was centrifuged at 12000g for 1 minute in an International HT Model centrifuge at room temperature. The pellets of approximately 160 mg wet weight, were resuspended in 1 ml saline and transferred into a 15 ml test-tube. The suspension was centrifuged again as described and the washing repeated again. The "washed" pellet was then suspended in cold (4° C) 10% TCA and the test-tube placed in an ice-water bath. The cells were kept suspended by constant stirring for 30 minutes, using a magnetic stirring bar. The cells were centrifuged down at 10,000g for 5 minutes in a refrigerated Sorval RC2-B centrifuge (4° C). The supernatant was collected and the pellet washed with 2 ml cold TCA for 5 minutes. The

cells were centrifuged down as described and the second supernatant was pooled with the first supernatant collected. This fraction contained low molecular weight compounds such as polyphosphates, free amino-acids and coenzymes.

To the pellet, 1 ml of methanol was added. The mixture was refluxed for 5 minutes and allowed to cool to room temperature. To this mixture, 2 ml of chloroform was added and the cells were extracted at room temperature for 20 minutes, with constant stirring. The residue was centrifuged and re-extracted with 2 ml chloroform/methanol (2:1, v/v). The two supernatants were combined. This fraction was composed of the microbial lipids.

The nucleic acids were extracted by suspending the pellet in 1 ml of 5% (w/v) TCA at 90°C for 30 minutes. The residue was collected by centrifugation at 5000g for 15 minutes. This fraction contains nucleic acids, the insoluble polyphosphate fraction and some polysaccharides.

The pellet was suspended in 1.5 ml distilled water and warmed to 65°C. An equal volume of 90% (w/v) aqueous phenol at the same temperature was added and mixed vigorously for 5 minutes. The mixture was then placed in an ice bath for 2 minutes and centrifuged at 3000g for 30 minutes at 0°C. The upper aqueous layer contains certain

polysaccharides, especially glycogen; the phenol layer contains proteins, while the residue on the interphase contains protein, and cell wall debris. The phenol layer was labelled the "protein" fraction, the aqueous layer was named to be the polysaccharide fraction, and the interphase was labelled the "cell wall" fraction.

26 - The Production of $^{14}\text{CO}_2$ by Cells Growing in the Presence of $[1-^{14}\text{C}]\text{-Ethanol}$, $[\text{U-}^{14}\text{C}]\text{-Glucose}$ or $[1-^{14}\text{C}]\text{-Acetate}$.

The medium used was the same described for the uptake experiments (21d, 22b, 24). The cultures were grown in 125 ml Erlenmeyer flasks which were adapted with a side-arm. The vessels contained 10 ml of medium. Water-saturated air was pumped using a Hagen Elite 802 air pump. The flow was fixed at 10 ml/min and filtered through a 0.2 micron disposable filter (Nalgen). The air entered the flask and mixed with the atmosphere. The air exited the flask and was bubbled through 10 ml of 4N KOH at a rate of approximately one bubble per second. The culture was inoculated as described above. The test-tubes containing base were changed every 12 hours for 5 days. The samples were analysed as described in section 21d.

RESULTS

To investigate the ability of *Sch. pombe* to utilize both acetate and ethanol in batch culture, the cells were grown in a carbon-limited medium (EMM2) normally used for chemostats. This medium contains only 10% of the carbon source normally used in batch culture.

The fission yeast was grown on glucose as the lone carbon source consuming the sugar to produce ethanol (Fig. 5). The cells went through an initial lag phase of 2 to 3 hours, followed by an exponential phase, which lasted 8 to 9 hours. This phase came to an end as the glucose was exhausted. The culture became stationary (inter-logarithmic phase) for 6 hours, after which a second growth phase started. During this phase, the cells grew slowly as ethanol was consumed. This second growth phase lasted 6 to 8 hours. The culture became stationary again and remained unchanged as acetate was consumed (Fig. 5). When glucose and acetate were present in the medium, a similar diauxic pattern was observed (Fig. 6). However, there were two main differences: first, acetate was consumed along with glucose for 6 to 12 hours and second, the length of the inter-logarithmic phase (i.e. after the glucose was exhausted) was extended to approximately 12 hours. The growth pattern however did not vary. There was

Fig. 5. Growth of *Sch. pombe* in batch culture on EMM2 medium containing 0.2% (W/V) glucose. Unless otherwise stated, conditions for all batch cultures were: 30°C, pH 5.0 and mixed at 150 r.p.m.. The figure represents changes in glucose (○), ethanol (□) and acetate (△) concentrations. The changes in total cell count (●) are also shown.

Fig.6. The growth of *Sch. pombe* in batch culture on EMM2 containing 0.2% (W/V) glucose and 0.2% (W/V) acetate. Conditions and symbols as in figure 5.

Fig. 7. Growth of the fission yeast in batch culture on EMM2 containing 0.2% (W/V) glucose and 0.5% (W/V) ethanol. Conditions and symbols in figure 5.

no increase in biomass during acetate utilization. If the cells were grown in the presence of ethanol, the diauxic pattern was again observed (Fig. 7). The alcohol however, extended the duration of the inter-logarithmic phase to 48 hours. The growth phase on ethanol lasted 24 hours after which it levelled off even though the alcohol concentration was still high (over 7 mg/ml). The ethanol utilization continued with little change in biomass. Acetate accumulated in the medium as ethanol was consumed. The results showed that *Sch. pombe* could utilize ethanol for growth and that it was oxidized to acetate. The acid accumulated in the medium and was consumed after the exhaustion of ethanol, with no further increase in biomass. This pattern was observed with media containing glucose alone or with either ethanol or acetate.

The presence of either ethanol or acetate affected the cell. This can be noticed by the prolongation in the length of the inter-logarithmic phase (after glucose consumption). To improve the understanding of the effect of these two metabolic products, the yeast was grown under different acetate and ethanol concentrations. The yeast could not grow on media containing acetate as the lone carbon source regardless of its concentration (Table 1). The extent of growth was dependent on the sugar concentration; however, the amount of biomass obtained

increased two-fold if acetate was present (Tables 1 & 2). An increase in acetate concentration past 0.2% (w/v) decreased the biomass yield (Table 1) (all concentration values given in % refer to % (W/V)). The extent of growth did not drop below the control (0% acetate). Acetate utilization was decreased as its initial concentration was increased (Table 2).

The initial ethanol concentration affected the cell yield (Table 3). The yield decreased 17.1% when the alcohol concentration was increased from 0 to 1.0%. The addition of 0.5% ethanol decreased the yield by 5.5%. The ability of the cells to metabolize ethanol increased considerably when the initial ethanol concentration was incremented. The specific rate of consumption increased from 0.46 to 1.01 $\mu\text{moles/day}/10^6$ cells in the presence of 1.0% ethanol. No increment was observed for the initial concentration of 0.5%. The consumption rate decreased slightly under these conditions. The organism could not grow on ethanol alone; the presence of D-glucose was required for growth and the subsequent utilization of the alcohol. The presence of ethanol or acetate in the medium affected the rate of glucose utilization (Table 4). When the initial acetate concentration was 0.3% the glucose consumption rate decreased by 23%. In the presence of ethanol the glucose utilization rate also decreased. At 0.5% ethanol the rate dropped by 12%. If the ethanol

TABLE 1

Ability of *Sch. pombe* to grow at different concentrations of glucose and acetate. The cultures were incubated for one week at 30°C, 150 r.p.m. and an initial pH of 5. This experiment was performed twice using duplicate cultures. The data presented is the average of four assays of the same sample. The error for the data is 10%.

Initial [Glucose]	[Acetate]	A _{660nm}
g (W / V)		
0	0.1	0
	0.2	0
	0.4	0
	0.8	0
0.1	0.1	0.081
	0.2	0.076
	0.4	0.061
	0.8	0.071
0.2	0.1	0.222
	0.2	0.319
	0.4	0.177
	0.8	0.168

TABLE 2 •

Effect of initial acetate concentration on a batch culture of Sch. pombe grown for four days at 30°C, 150 r.p.m. and an initial pH of 5.0. This experiment was performed twice. The error in the acetate determination was 10%, and for the dry weight it was 20%.

[Acetate]	A _{660nm}	Dry Weight	Y _B *	Acetate Consumed
% (W/V)		mg/ml		mg
0	0.086	2.9	1.5	—
0.2	0.125	4.6	2.3	33.8
0.3	0.122	4.2	2.1	21.0
0.4	0.125	4.6	2.3	12.1

* Y_B: Biomass yield, mg of dry weight produced per mg of glucose consumed.

TABLE 3

Effect of initial ethanol concentration on batch cultures of *Sch. pombe* incubated at 30°C for 5 days at 200 r.p.m.. The experiment was performed twice. The error in the ethanol determination was 10%.

Initial Ethanol Concentration	Y_C *	Decrease in Y_C	Specific Rate of Ethanol Consumption ***
% (W/V)		%	**
0	1.64	0	0.46
0.5	1.55	5.5	0.33
1.0	1.36	17.1	1.01

* Y_C : Cell yield, 10^9 cells produced per mmole of glucose consumed.

** μ moles/ day/ 10^6 cells.

*** Corrected for evaporation.

TABLE 4

Effect of initial acetate and ethanol concentration on the rate of glucose consumption in batch cultures grown at 30°C, 150 r.p.m. and an initial pH 5.0. The error in glucose determination was 15%.

Initial Acetate Concentration	Initial Ethanol Concentration	Glucose Consumption Rate
% (W / V)		mmole/h/l
0	0	0.94
0.2	0	1.00
0.3	0	0.72
0	0.5	0.83
0	1.0	0.72

concentration was doubled, the decrease was equivalent to 23% of the control rate.

To rule out the possibility of having a spontaneous mutation that would allow the "new yeast" to grow on ethanol or acetate as lone carbon source cannot. In order to test for this phenomenon, cultures growing in the presence of glucose and acetate or glucose and ethanol were sampled and used to inoculate media containing either acetate or ethanol as lone carbon source. In neither case was growth detected after three days of incubation, irrespective of the phase of growth at which the culture was sampled.

The morphology of the cells grown in the presence of glucose and acetate or glucose alone appeared very similar (Plates 1 & 2). However, cells grown in the presence of ethanol (Plate 3) appear darker under phase contrast microscopy.

The data obtained indicated that both metabolic products are capable of inducing changes in the cell. To monitor their uptake and subsequent utilization, $[1-^{14}\text{C}]$ -acetate and $[1-^{14}\text{C}]$ -ethanol were used. When $[1-^{14}\text{C}]$ -acetate was present, the uptake of the organic acid occurred during the first 24 hours, during both glucose and ethanol utilization phases. The radio-labelled compound concentration in the cells decreased with time as

soon as ethanol was exhausted. The decrease continued for 48 hours after which it levelled off. During this phase, acetate in the medium was being consumed by the cells (Fig. 8). If [1- 14 C]-ethanol was present in the medium, the alcohol did not start to accumulate in the cells until the culture was 6 hours old. The cells at this point are in exponential phase and growing very rapidly and the glucose concentration had dropped by about 50% during this period of time (Fig. 7). The yeast continued to take up the radio-labelled alcohol for the duration of the experiment (Fig. 9).

Ethanol is known to diffuse through the cell membrane without any obstacles. In the case of acetate the situation is not clear (80). Therefore, the effect of two metabolic inhibitors, azide and 2,4-dinitrophenol (2,4-DNP) on [1- 14 C]-acetate uptake was started.

2,4-Dinitrophenol is an uncoupler of oxidative phosphorylation (64), while azide is an inhibitor which acts on several "targets", one of which is cytochrome a_3 of the electron transport chain (65,66). The addition of 2,4-DNP to a culture entering inter-logarithmic phase did not affect the diauxic pattern observed before. The growth in the presence of the uncoupler could not be distinguished from the control (Fig. 10). The uptake of [1- 14 C]-acetate was also unaffected by the presence of the

Plate 1. *Schizosaccharomyces pombe* cells during exponential phase, growing on glucose alone medium : Magnification= 3600X.

Plate 2. Fission yeast cells growing on glucose and acetate medium during the exponential phase. Magnification= 3600X.

Plate 3. Fission yeast cells during the exponential phase,
grown on medium which contained glucose and ethanol.
Magnification= 3600X.

Fig. 8. Uptake of $[1-^{14}\text{C}]$ - acetate by the fission yeast in batch culture. The medium contained 0.2% (W/V) glucose and 0.2% (W/V) acetate. The changes in glucose (O) and acetate (Δ) concentration, as well as the labelled acetate uptake ($\blacktriangle$) are shown.

2

MICROCOPY RESOLUTION TEST CHART
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Fig. 9. Uptake of $[1-^{14}\text{C}]$ - ethanol by the fission yeast in batch culture. The medium contained 0.2% (W/V) glucose and 0.5% (W/V) ethanol. The uptake of the labelled alcohol by the cells (■) and the changes in glucose (O) and ethanol (□) concentrations are shown.

dinitrophenol (Fig. 11). However, there was a faster utilization of the labelled acetate in the medium and of the labelled stored carbon in the cell. If azide was added to the culture, it did not undergo a second growth phase (Fig. 12) and [1- 14 C]-acetate carbon storage was stopped (Fig. 13). The fatty acid utilization was not affected initially, as shown by the decrease in the medium concentration of [1- 14 C]-acetate. However, after 60 hours, acetate utilization stopped and an increase in radioactivity was observed in the medium. This coincided with a decrease in the labelled carbon stored in the cell. These observations suggest that the cells might have become "leaky". No decrease in cell count was observed which discards cell lysis as a possibility.

To elucidate the fate of acetate and the alcohol in cells, 24 hour cells, grown in the presence of either radiolabelled intermediate were chemically extracted. The yeast cultivated in the presence of [1- 14 C]-acetate had most of the extracted label in the cell wall fraction and in the protein fraction. The lipid, small molecular weights and polysaccharide fractions contained a small amount of label (Table 5). Cells grown in media containing [1- 14 C]-ethanol exhibited a similar pattern to that described for acetate-grown cells. However, the amount of 14 C-ethanol carbon incorporated into macromolecules was very low (Table 5) and no label was observed for the

Fig. 10. Effect of 2,4-DNP on the growth of *Sch. pombe* in batch culture. Control (○), 2,4-DNP (●). Arrow indicates addition of inhibitor. Error = 25%.

Fig. 11. Effect of 2,4-DNP on the uptake ($\circ$ $\bullet$) and utilization (∇ $\blacktriangledown$) of $[1-^{14}\text{C}]$ - acetate by the fission yeast in batch culture. Control ($\circ$ ∇), inhibitor ($\bullet$ $\blacktriangledown$). Arrow indicates addition of inhibitor. Error for ^{14}C counts=25%.

Fig. 12. Effect of azide on the growth of the fission yeast in batch culture. Control (○), azide (●). Arrow indicates addition of inhibitor.

Fig. 13. The effect of azide on the uptake ($\circ$ $\bullet$) and utilization (Δ $\blacktriangle$) of [1- 14 C] - acetate by the fission yeast in batch culture. Control (Δ $\circ$), azide ($\blacktriangle$ $\bullet$). Arrow indicates addition of inhibitor.

nucleic acids or polysaccharide fractions.

To examine whether the presence of acetate or ethanol would affect the utilization of glucose, cells grown on [U- 14 C]-glucose in the presence of either ethanol or acetate for 24 hours were extracted (Table 5). The fractions which contained most of the U- 14 C-glucose carbon were the small molecular weight, nucleic acids, polysaccharide, cell wall and protein fractions. The addition of acetate to the medium decreased the amount of label in the protein and lipid fractions but had little effect on any other. The addition of ethanol led to a decrease in the small molecular weight, lipid and polysaccharide fractions. The results obtained with the chemical extraction of the yeast cells indicated that ethanol and acetate can be used (to a certain extent) as substrates for the manufacture of biomacromolecules. It was of interest therefore, to investigate the effect of acetate and ethanol on the uptake and subsequent utilization of the glucose carbon.

Batch cultures of *Sch. pombe* grown on [U- 14 C]-glucose alone or in the presence of either acetate or ethanol showed no difference in the uptake of the labelled sugar during the first six hours (Fig. 14). The cultures containing either ethanol or acetate started utilizing the stored [U- 14 C]-glucose by eight hours. The control culture

TABLE 5

Chemical extraction of cells grown in the presence of [U- 14 C]-glucose, [1- 14 C]-ethanol or [1- 14 C]-acetate, using EMM2 containing either 0.2% (w/v) D-glucose alone or with 0.5% (w/v) ethanol or 0.2% (w/v) acetate. The cultures were grown at 30°C, with an initial pH of 5.0 and mixed at 150 r.p.m.. The experiment was performed three times using duplicate cultures. The data presented has a % error of 20%, and represents the average of one set of duplicate cultures.

Fraction	*	A	E	G	GA	GE
pmole incorporated 14 C-substrate/ μ l fraction						
small molecular weight		1.2	0.1	11.1	9.6	4.9
lipids		0.7	0.1	2.4	1.1	0.6
nucleic acids		1.4	0.0	13.8	14.2	10.5
polysaccharides		0.2	0.0	2.1	1.7	1.2
cell wall debris		14.4	1.6	34.4	72.3	31.6
protein		23.2	1.7	36.3	42.3	31.6

- * A = [1- 14 C]-acetate , in glucose and acetate medium
 E = [1- 14 C]-ethanol , in glucose and ethanol medium
 G = [U- 14 C]-glucose , in glucose alone medium
 GA = [U- 14 C]-glucose , in glucose and acetate medium
 GE = [U- 14 C]-glucose , in glucose and ethanol medium

did not exhibit this behaviour until it was twelve hours old. The level of radioactivity levelled off by twenty-eight hours in the three cultures. It should be noted that the culture containing ethanol (Fig. 14) levelled off much faster (12 hours) than the other two cultures (glucose alone and glucose and acetate . There is no difference in the radioactivity remaining in the medium (Fig. 15). In the three cases, it increased with time until the cultures were 8.5 hours old. The accumulation phase is followed by the consumption of the labelled compound(s) in the medium, leading to a decrease in the radioactivity. The initial increase is attributed to the production of ^{14}C -ethanol and other metabolic products.

Both acetate and ethanol can be oxidized completely to carbon dioxide. The rate of evolution of $^{14}\text{CO}_2$ from cultures grown in the presence of either labelled ethanol or acetate was monitored. Cells grown in the presence of $[1-^{14}\text{C}]$ -acetate oxidized part of the organic acid during D-glucose consumption (Fig. 16) and stored the rest. The rate decreased during ethanol utilization phase but increased again during the utilization of acetate indicating that it is being oxidized along with the stored labelled carbon. In the case of $[1-^{14}\text{C}]$ -ethanol utilization, $^{14}\text{CO}_2$ was produced in two phases. The first occurred during glucose utilization, the second during

Fig. 14. Uptake of [U- 14 C] - glucose by *Sch. pombe* in batch culture. The cells were grown on medium containing either glucose alone ($\bullet$), glucose and acetate (Δ) or glucose and ethanol ($\square$).

the growth phase on ethanol (Fig. 17) which indicates that part of the alcohol is being fully oxidized to CO_2 and the rest to acetate. The rate of production of $^{14}\text{CO}_2$ from D-[U- ^{14}C]-glucose as lone carbon source, exhibited two peaks. The first phase occurred during the utilization of the sugar. The second coincided with the decrease in the stored label and the consumption of the ^{14}C -labelled metabolic product(s) (^{14}C -ethanol for example) accumulated during the aerobic fermentation (Fig. 18). The same pattern described above was observed in the presence of ethanol (Fig. 19) and acetate (Fig. 20).

To investigate the effect of both glucose fermentation products on the metabolism of the fission yeast, carbon-limited chemostat cultures were run under different conditions. The changes with dilution rate of the concentrations of glucose, ethanol, acetate, and the cell density (monitored spectrophotometrically at 660 nm) of a chemostat culture grown in the presence of glucose alone can be seen in figure 23. At low dilution rates, the steady state concentration of glucose was very low (about 20 $\mu\text{g/ml}$). As the dilution rate increased over 0.12 h^{-1} , the sugar was observed to accumulate in the medium. The ethanol concentration rose when D was increased above 0.10 h^{-1} . No acetate was detected until D was above 0.16 h^{-1} . The biomass in the culture was observed to decrease with increasing D.

Fig. 16. $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ - acetate by the fission yeast. The cells were grown on glucose and acetate medium. $^{14}\text{CO}_2$ production rate ($\blacksquare$), $[1-^{14}\text{C}]$ -acetate concentration ($\diamond$) and uptake by the cells ($\bullet$) are shown.

Fig. 17. $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ -ethanol by the fission yeast in batch culture. The cells were grown on glucose and ethanol medium. The uptake of the labelled alcohol (●) and the $^{14}\text{CO}_2$ production rate (■) are shown.

Fig. 18. $^{14}\text{CO}_2$ production by fission yeast grown on ^{14}C -labelled glucose. Cells grown on glucose alone. $^{14}\text{CO}_2$ production rate (■) and [U- ^{14}C]-glucose uptake (●) are shown.

Fig. 19. $^{14}\text{CO}_2$ production from ^{14}C -labelled glucose in the presence of ethanol. Fission yeast cells grown on glucose and ethanol medium. For symbols see figure 18.

Fig. 20. $^{14}\text{CO}_2$ production from ^{14}C -labelled glucose in the presence of acetate. Fission yeast cells grown on glucose and acetate. For symbols see figure 18.

If acetate was present in the inflowing medium, the yeast consumed both D-glucose and acetate (Fig. 23). The organic acid accumulated rapidly as the values of D increased above 0.09 h^{-1} . Below this value, no acetate was detected. The steady state acetate concentration remained below the feed concentration throughout the experiment. The amount of biomass remained almost unchanged and only started to decrease at D equal to or above 0.16 h^{-1} . The ethanol and residual glucose concentration built up with increasing D . When ethanol and D-glucose were used in the medium, *Sch. pombe* was observed to utilize both concurrently (Fig. 23). The ethanol started to accumulate over feed concentration when D was greater than or equal to 0.08 h^{-1} . However if the D was increased to 0.23 h^{-1} the concentration dropped. There was also a drastic drop in biomass between 0.08 h^{-1} and 0.14 h^{-1} , after which it decreased gradually with increasing D . No acetate was detected at any stage. The glucose concentration increased considerably when the D was incremented over 0.2 h^{-1} (Fig. 23).

The effects of acetate and ethanol on the D-glucose consumption rate are shown in figure 24. There is no change in the increase of the rate with dilution rate if 0.06% acetate was present in the feed; the data is similar to the control (glucose alone). The rate increases rapidly between 0.08 h^{-1} and 0.16 h^{-1} after which it levels off.

Fig. 21. Carbon-limited chemostat culture of *Sch. pombe*. Unless otherwise stated, conditions were: 0.2% (W/V) glucose, pH 5.0, 30°C, 400 ml/min air. [Ethanol] ($\blacktriangle$), [glucose] ($\bullet$), [acetate] ($\blacksquare$) and biomass ($\square$) in the medium are shown.

Fig. 22. Chemostat culture of the fission yeast under carbon-limitation, in the presence of 0.06% (W/V) acetate. For symbols, see figure 21.

Fig. 23. Carbon-limited chemostat culture of Sch. pombe in the presence of 0.03% (W/V) ethanol. For symbols see figure 21.

If acetate was replaced with ethanol the rate increases linearly between 0.08 h^{-1} and 0.19 h^{-1} , a further increase did not change the consumption rate. The change in ethanol production rate with D was affected differently by ethanol and acetate (Fig. 25). The presence of the organic acid caused a decrease in the rate over the range tested. The presence of the alcohol led to a linear increase in the rate between 0.08 and 0.19 h^{-1} . At 0.23 h^{-1} however, a decrease was observed. This decrease coincides with the lack of change in the glucose consumption described above (Fig. 24). In the three cases ethanol production starts between 0.08 h^{-1} and 0.09 h^{-1} under the chemostat conditions used.

The effect of acetate on Sch. pombe was investigated further by carrying out studies at fixed D and increasing the concentration of acetate in the inflowing medium. The patterns observed at 0.12 h^{-1} and 0.15 h^{-1} are similar. At D of 0.12 h^{-1} (Fig. 26), there is a decrease in the residual concentration of glucose, as the acetate concentration was increased. The ethanol concentration decreased initially when the acetate concentration was increased from 0 to 0.05%. However, a further increment led to an increase in the steady state concentration of ethanol. The alcohol concentration levelled off as the level of acetate went over 0.1%. Any further increase above 0.15% led to a decrease in ethanol levels. The

Fig. 24. Effect of acetate (Δ) and ethanol($\square$) on the glucose consumption rate of chemostat grown cells. Control ($\bullet$).

Fig. 25. Effect of acetate (Δ) and ethanol ($\square$) on the ethanol production rate of chemostat grown cells. Control ($\bullet$).

increment in acetate concentration led to an increase in the amount of biomass obtained.

If the dilution rate was increased to 0.15 h^{-1} , the same pattern was observed again (Fig. 27). However, the levels of ethanol remained below those of the control when the acetate was increased from 0 to 0.2%. At this concentration, the ethanol concentration was the same as the control (0.4 mg/ml). Any further increase depressed the residual ethanol concentration. Any addition of acetate to the inflowing medium led to an increase in biomass. However, if the concentration went above 0.05%, the yield decreased slightly. The rates of glucose consumption and ethanol production (Fig. 28) changed if acetate was present. The net effect of acetate concentration on the glucose consumption rate of the culture was to increase it. The magnitude of the change was greater at a higher dilution rate ($D = 0.15 \text{ h}^{-1}$). The rate of ethanol production was also affected by acetate. At a D of 0.12 h^{-1} , the addition of a low concentration of acetate (0.05%) led to a decrease in ethanol production. However, if the amount was increased further (to 0.1% or above) the rate of production increased. Any further increment past 0.2% caused the rate to decrease. However, if D was increased to 0.15 h^{-1} , the rate of ethanol production decreased at low concentrations of acetate. On the other hand, the rate never went above the control. A

Fig. 26. Effect of increasing acetate concentration on a carbon-limited chemostat culture at 0.12 h^{-1} . [Acetate] (■), [ethanol] (▲), [glucose] (●), biomass (◇) in the culture are shown.

Fig. 27. Effect of increasing acetate concentration on a carbon-limited chemostat of *Sch. pombe* at 0.15 h^{-1} . [Acetate] (■), [ethanol] (▲), [glucose] (●), total cell count (□) and dry weight (▽) in the culture are shown.

better indication of the effect of acetate concentration on the fission yeast is given by the use of specific rates. These rates can only be calculated for the chemostat grown at 0.15 h^{-1} , since the total cell count data is not available for the other chemostat. The effect of acetate concentration on the specific rate of consumption of glucose and acetate as well as the specific rate of ethanol production can be seen on figure 29. As the concentration of the organic acid increased the specific rate of ethanol production was depressed, and the same was observed for glucose consumption. The specific rate of acetate consumption increased with acetate concentration until it reached 0.25%, but at this point the rate dropped. The results indicate that acetate is depressing the ability of the cells to consume glucose and produce ethanol. The increments observed in figure 28 are due to the increase in biomass caused by the consumption of acetate. The presence of the organic acid stimulated its consumption by the fission yeast.

The yield obtained for ethanol (Fig. 30) mirrored the results obtained for the rates of ethanol production. The maximum biomass yield was obtained at 0.05% (w/v) acetate and any increase in acetate decreased the yield (Fig. 31). The effect of acetate on the density of the cells was dependent on the concentration (Fig. 31). The cells were

Fig. 28. Effect of acetate concentration on ethanol production ($\bullet$ $\circ$) and glucose consumption (Δ $\blacktriangle$) rate of chemostat cultures. 0.12 h^{-1} ($\circ$ Δ), 0.15 h^{-1} ($\blacktriangle$ $\bullet$).

Fig. 29. Effect of acetate concentration on the specific rates of: ethanol production (Δ), acetate ($\square$) and glucose consumption ($\circ$). The chemostat culture was grown at 0.15 h^{-1} .

heavier than the control at 0.05%. As the concentration increased, the density decreased. At 0.2% acetate, the cells were lighter than the control. However, a slight increase in the concentration of acetate from 0.2% to 0.25% led to a drastic increase in the density of the cells.

The effect of ethanol concentration on a chemostat at fixed D (0.15 h^{-1}) was investigated. Only two concentrations were used. The presence of ethanol did not affect the cell count (Table 6). However, the dry weight of the culture increased indicating an increase in the density of the cells with respect to the control. The biomass yield was similar to that obtained for the control. The cells grown under these conditions consumed the ethanol; the ability of these organisms to utilize ethanol increased at higher concentrations, showing a 3.6-fold increase in the specific rate (Table 6). The specific rate of glucose utilization was decreased in the presence of ethanol. However, the extent of the depression was less than that caused by the increase in acetate concentration (Table 6 & Fig. 29). It is interesting to note that the specific rate of acetate production did not change with increasing ethanol concentration, suggesting that a larger amount of ethanol is being oxidized to carbon dioxide (Table 6).

The electrophoresis of the cell-free extracts from

Fig. 30. Effect of acetate concentration on the ethanol yield of chemostat cultures grown at 0.12 h^{-1} (O) and 0.15 h^{-1} (●).

Fig. 31. The effect of acetate concentration on the dry weight (■) and biomass yield (●) of a carbon-limited chemostat of *Sch. pombe*. The cells were grown at 0.15 h^{-1} .

TABLE 6

Effect of increasing ethanol concentration on a Chemostat culture of *Sch. pombe* growing on EMM2 containing 0.2% (W/V) D-glucose, at 0.15 h^{-1} , 30°C , pH 5.0 and an aeration rate of 400 ml/min. The data was obtained from four different chemostat runs. The error in the determination of glucose, ethanol and acetate was 10%. The other data has an error of 20%.

	Initial Ethanol Concentration			
	0	0.3	0.6	
Total Cell Count	13.30	11.08	12.45	10^6 cells/ml
Dry Weight	0.230	0.265	0.310	mg/ml
[Glucose]	0.064	0.076	0.146	mg/ml
[Acetate]	0.235	0.236	0.212	mg/ml
[Ethanol]	0.420	0.539	2.042	mg/ml
Density of Cells	0.173	0.236	0.212	$\text{mg}/10^7\text{ cells}$
Biomass Yield	0.119	0.138	0.159	mg/mg
Specific Rate of:				
Glucose Consumption	6.9	6.0	5.2	$\mu\text{moles/h/mg}$
Acetate Production	1.3	2.2	1.7	$\mu\text{moles/h/mg}$
Ethanol Consumption*	5.8	9.8	34.8	$\mu\text{moles/h/mg}$

* Corrected for loss due to evaporation.

Sch. pombe grown at different acetate (plate 4 & table 7) and ethanol (plate 5 & table 8) concentration exhibit changes in their protein patterns. In both cases the bands that varied could be grouped into three general groups. The first group is composed of the bands whose intensity increased with either ethanol or acetate concentration. The second group represents those bands whose intensity decreased as the concentration of either acetate or ethanol rose. The third group is formed by the bands whose intensity peaked at low concentrations of the substrates.

PLate 4. Effect of acetate concentration on the protein profile of the fission yeast. Cells grown in a carbon-limited chemostat culture at 0.15 h^{-1} .

BAND NUMBER

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

ACETATE CONCENTRATION (% W / V)

0 0.05 0.10 0.20 0.25

66000
45000
24000
15400
14300
MOLECULAR WEIGHT

TABLE 7

Changes in protein patterns of cell-free extracts of Sch. pombe grown in a chemostat culture at fixed dilution rates increasing acetate concentration. The cells were grown on EMM2 at 30°C, 0.15 h⁻¹, pH 5.0, and an aeration rate of 400 ml/min. The data was derived from plate 4. The photograph was scanned visually.

Band Type	Band #	Mobility	Molecular Weight
			10 ³
Increase in intensity with [Acetate]	3	0.208	56.0
	10	0.685	18.0
	11	0.759	15.5
	12	0.780	15.0
Decrease in Intensity with [Acetate]	2	0.113	>70.0
	6	0.393	35.0
	7	0.435	29.0
	9	0.625	19.5
	13	0.810	15.0
Intensity Peaks at 0.05 or 0.1% Acetate	1	0.080	>70.0
	4	0.310	41.0
	5	0.363	35.5
	8	0.476	26.0

Plate 5. Effect of ethanol concentration on the protein profile of the fission yeast. The cells were grown in a carbon-limited chemostat culture at 0.15 h^{-1} .

ETHANOL CONCENTRATION (% W/V)

TABLE 8

Changes in protein patterns of cell-free extracts from Sch. pombe grown in a chemostat culture at fixed dilution rate and increasing ethanol concentration. The cells were grown on EMM2, at 30°C, 0.15 h⁻¹, pH 5.0 and an aeration rate of 400 ml/min. The data was derived from plate 5. The photograph was scanned visually.

Type of Band	Band #	Mobility	Molecular Weight
			10 ³
<hr/>			
Increase in Intensity with [Ethanol]	1	0.087	>70.0
	2	0.151	>70.0
	5	0.307	46.5
	6	0.344	42.5
Decrease in Intensity with [Ethanol]	7	0.427	35.5
	8	0.537	26.5
	9	0.573	24.0
	10	0.587	23.5
	13	0.743	16.5
	14	0.748	16.5
	15	0.826	15.3
Intensity Peaks at 0.3% Ethanol	3	0.171	>70.0
	11	0.624	22.5
	12	0.665	21.0
<hr/>			

DISCUSSION

The literature indicates that *Sch. pombe* does not grow on either ethanol or acetate (1,2,10,26). It also states that ethanol is not converted to acetate (26). The growth curves obtained however, show that if the fission yeast is grown on EMM2 containing a low concentration of glucose, a diauxic growth pattern results. The sequence is identical to that exhibited by *S. cerevisiae* (8). If glucose is used as lone carbon source, aerobic fermentation results in the production of ethanol. The yeast then grows on ethanol producing acetate in the process. No growth was observed during acetate utilization.

The sequence of events is explained by the operation of the Crabtree effect, described in the Introduction. The growth on glucose leads to the inhibition of the respiratory metabolism, resulting in aerobic fermentation. Once glucose is exhausted, the cell stops growing and enters stationary phase. During this period, derepression of respiratory metabolism occurs, which allows the consumption of ethanol via the TCA cycle. Some of the alcohol is oxidized to carbon dioxide; however, most of the ethanol goes to acetate. This is contrary to Barnett's observations (26). The acetate is then utilized but no

growth occurs. This indicates that the carbon is not being "fixed" by the cells; therefore the organic acid must be oxidized to carbon dioxide (see below). This lack of growth on acetate has been attributed to the lack of the glyoxylate cycle in this organism (8). The glyoxylate cycle is operational in all organisms capable of using the organic acid for growth (80). Preliminary results (K. Mitton, personal communication) have not detected either isocitrate lyase or malate synthetase in cell free extracts from yeast grown in the presence of acetate.

The presence of acetate in the growth medium gave rise to the same diauxic pattern. The interesting feature of this combination was the utilization of acetate while growing on glucose. This behaviour is similar to that observed with malic acid, which is consumed during glucose utilization in grape musts (69). The highest rate of malic acid consumption was observed at the beginning of aerobic fermentation. This observation coincides with acetate utilization during the first hours of the batch culture. The concentration of the acid decreases about 50% before increasing again due to oxidation of ethanol (see below). The amount of cells (monitored spectrophotometrically at 660 nm) obtained was higher in the presence of acetate than in the presence of glucose alone, an indication that acetate is being incorporated into biomass. The effect of acetate is concentration dependent. Concentrations below

or equal to 0.2% (w/v) have no negative effect on the growth of the yeast. Maximum utilization of acetate is obtained at this concentration (Table 2). The increase in acetate depresses the growth of the yeast, as well as the acetate consumption. At 0.8%, no change in the medium acetate concentration was observed.

The increase obtained in the biomass yield was not affected by acetate concentrations past 0.2%. The same yield was obtained even if the concentration was doubled. However, the amount of acetate consumed decreased by 36% in the process. This indicates that only part of the consumed acetate goes to biomass production.

Acetate concentration over 0.2% decreased the glucose consumption rate (Table 4). The depression of the rate was observed at 0.3% (50mM) acetate; however, no change was encountered at 0.2% (33mM) acetate. These observations agree with the data obtained for cell growth and acetate utilization. Concentrations above 0.2% acetate have a negative effect on *Sch. pombe*. In *S. cerevisiae*, the anaerobic glucose fermentation rate decreased 81% if the acetate concentration was incremented from 17 mM to 68 mM (72). Under aerobic conditions, a concentration of 50 mM acetate causes a 84% decrease in the rate of glucose utilization (73). Under the conditions tested, the fission yeast appears less sensitive to acetate than *S.*

cerevisiae. However, the experimental work cited was performed using 1% (72) and 2.5% (73) glucose. In order to make a comparison, the experiments should be repeated at these glucose concentrations. It is known that cells grown at lower concentrations of glucose are physiologically different (particularly in their ability to consume acetate) from those grown at higher concentrations (38,42).

The replacement of acetate with ethanol affected the ability of the cells to grow on glucose (Table 3). The cell yield decreased under the conditions studied. The effect of ethanol on growth is well known. In S. cerevisiae the alcohol inhibits growth before it affects fermentation (17,18). Concentrations of 1.0% are reported not to affect S. cerevisiae (17). However, Sch. pombe exhibits clear signs of ethanol inhibition at this concentration (13), and in chemostat cultures concentrations as low as 0.1% have shown adverse effects (31). However, the fission yeast is known to tolerate ethanol concentrations as high as 7% and to produce wine containing 15% ethanol (69). The literature indicates that the response of the yeast will depend on the culture conditions used (13,31,69). The alcohol also decreased the rate of glucose utilization (Table 4). This outcome was expected since ethanol is known to act on glucose metabolism via noncompetitive inhibition (63,70).

The yeasts growing in the presence of 1.0% ethanol consumed the alcohol at faster rates than did the control cells or those growing at a lower ethanol concentration (Table 3). It is known that growth conditions which favour the development of oxidative respiration, stimulate the increase of alcohol dehydrogenase activity in Baker's yeast (74). The specific activity of total alcohol dehydrogenase was higher in yeast grown on ethanol than those grown on glucose, and also correlated with an increase in respiration (74). The fission yeast has been reported to have only one alcohol dehydrogenase (108); however, K. Mitton (personal communication) has detected two bands in polyacrylamide gel electrophoresis with alcohol dehydrogenase activity. These preliminary results suggest that the yeast possesses a second isozyme as that described for Baker's yeast (74) (see below).

The uptake of 1- ^{14}C -acetate occurs during glucose and ethanol utilization. The labelled carbon is stored during these two growth phases. Some of the organic acid is oxidized during glucose consumption, as shown by the increase in the specific rate of production of $^{14}\text{CO}_2$ and the uptake of the labelled acetate from the medium. The second peak of $^{14}\text{CO}_2$ production occurs, as expected, during the oxidation of the accumulated acetate. The start of the acetate oxidation phase is accompanied by a decrease in the amount of stored labelled carbon in the

cell. The data suggests that the yeast utilizes the acetate in two different ways, depending on the stage of the culture. The first stage involves the utilization of the organic acid in the manufacture of a "storage compound" which is later utilized (see below). In the second stage the acetate is used in respiration.

Useful information on the fate of acetate is obtained from the extraction of cell components. The label was found in all the fractions (Table 6). The fractions with highest amount of radioactivity contained polysaccharides, protein and mucopeptides which can be manufactured from the TCA cycle intermediates (12). Accumulation of acetate carbon by Sch. pombe occurs under two different metabolic states, aerobic fermentation during growth on glucose and oxidative metabolism during ethanol utilization (8). During aerobic fermentation the activity of the TCA cycle is repressed (25,56,58) along with the enzymes involved in gluconeogenesis (76) and oxidative metabolism (39,40,41,42) (see introduction). However, during ethanol utilization all these enzymes are derepressed (8,42). The cells that were extracted had undergone these two phases. As observed above, the acetate utilization was different under these two phases. It is possible that the extraction results reflect the events that occurred during growth on ethanol. If this is the case, the labelled macromolecules might derive from the utilization of

acetate ~~via~~ TCA cycle and gluconeogenesis. However, this leaves one problem unsolved. What is the fate of acetate during glucose utilization? It can be assumed that the activity of the TCA cycle at this point is low (8,41,42) (however, it is active enough to allow the oxidation of some of the acetate consumed during the exponential phase). Therefore the stored acetate could be channelled into lipid synthesis.

The amount of label extracted in the lipid fraction was low. However, acetate is involved in fatty acid synthesis via acetyl CoA (107). The enzyme complex (fatty acid synthetase) that catalyses fatty acid synthesis requires acetyl CoA as a cofactor as well as for the condensation reaction (107). This enzyme is present under both aerobic and anaerobic conditions in yeasts (106,107). The total lipid content in yeasts is reported to increase during the exponential growth on glucose (106). When the culture reaches stationary phase, the lipid content of the cells decreases. Yeasts are known to accumulate lipids as a potential source of energy and carbon (87). Therefore, the possibility that acetate is being used for fatty acid synthesis cannot be ruled out. The acetate utilization pattern agrees with this hypothesis and would explain the low amount of label obtained in the lipid fraction. The model of acetate utilization proposed would have the acetate carbon being used in fatty acid synthesis during

growth on glucose. Once glucose is exhausted, the yeast would consume its energy reserves decreasing the amount of lipid and other storage material (see below) in the cells. The cells would then "switch on" their oxidative metabolism and acetate would go mostly into the TCA cycle and gluconeogenesis increasing the amount of label in the other fractions. When the acetate oxidation phase is reached the amount of label should decrease in all the fractions due to the turn over of the macromolecules. To confirm this model, cells should be grown in the presence of labelled acetate and chemically extracted at the different stages mentioned above. The experiment could also be performed by adding the labelled acetate to the cultures at different times, allowing the cells to grow and extracting them.

Acetate utilization was affected by 2,4-DNP. The amount of labelled acetate stored decreased in the presence of the uncoupler. The concentration of 2,4-DNP (0.1 mM) used has been observed to stimulate the production of ethanol in *Sch. pombe* by interfering with the oxidative metabolism of the yeast (82). This concentration has also been observed to increase the rate of respiration and inhibit ATP-inorganic phosphate exchange reactions in isolated mitochondria from yeasts (84). Higher concentrations suppress the $K^+ - H^+$ exchange in

S. cerevisiae leaving fermentation intact (83).

The net effect of 2,4-DNP on yeasts growing on glucose is to increase the rate of catabolism (85). The results show that the uptake of the labelled acetate continued during the consumption of ethanol. However, the stored carbon and the 1- ^{14}C -acetate concentration in the medium decreased much faster in the presence of the uncoupler. If the rate of catabolism of the fission yeast has increased in the presence of 2,4-DNP, then the observations can be explained in terms of redirection of the acetate from synthesis to oxidation. This model can be tested by monitoring the rate of $^{14}\text{CO}_2$ production from labelled acetate in the presence and absence of the uncoupler.

The addition of azide to the culture stopped the growth of the fission yeast on ethanol. Azide inhibits respiration in Baker's yeast at 0.1mM (85). However, it allows the consumption of glucose by aerobic fermentation and increases the rate of catabolism in the organism (85). Oxidative metabolism is needed for ethanol utilization. The inhibition of oxidative metabolism arrests the growth of the yeast. However, the cells continued to consume acetate without storing it. This indicates that the acetate is being used in the TCA cycle and its carbon being "lost" in the form of CO_2 . This observation can be confirmed by the same type of experiment described for 2,4-DNP. The large increase in

radioactivity was observed as the culture aged in the presence of the inhibitor. This is believed to be due to the leakage of labelled intermediates from dead cells.

The results indicate that the uptake of acetate is not an active process. The uncoupler decreases the amount of ATP being produced but did not reduce drastically the amount of acetate taken up by the cells. Azide stopped the storage of the labelled organic acid but did not stop its consumption. Acetate consumption by *Sch. pombe* in chemostat cultures increases when the pH is dropped from 6.0 to 4.0 (C.S. Tsai, personal communication) suggesting that the protonated form of acetate diffuses into the cell. The literature consulted supports this model (19,22,23,80) .

The uptake of ^{14}C -ethanol shows that the labelled carbon was not fixed by the cells in the first six hours of the culture. During this period the ratio between radioactivity and the number of cells remains unchanged. After 6h, the ratio increased indicating the initiation of the ethanol carbon fixation. The residual glucose concentration at this point has decreased by about 50%. This change manifested by the yeast might be due to the decrease in Crabtree effect. The low sugar concentration would allow the increase in activities of the oxidative metabolism enzymes (8,40,42).

The rate of production of $^{14}\text{CO}_2$ from the labelled ethanol shows two periods during which the rates peak. The first period coincides with glucose utilization and the second, with ethanol consumption. The peak observed at 12h coincides with a sharp increase in the amount of radioactivity stored in the cells. The results indicate that the fixation of ethanol by the fission yeast requires the operation of the oxidative pathways. In contrast with acetate, no ethanol carbon was accumulated during the initial growth on the sugar. These observations agree with those made for Baker's yeast (75,76). During aerobic fermentation, the ethanol produced by *S. cerevisiae* could be oxidized further to acetaldehyde or acetate (75). Haarasilta and Oura (76) observed that ethanol utilization did not begin until the activities of hexose diphosphatase, isocitrate lyase and phosphoenolpyruvate increased. This change occurred as the glucose concentration decreased and the cells entered the inter-logarithmic phase. This study showed that ethanol utilization requires the operation of gluconeogenesis and the glyoxylate cycle as well as the TCA cycle. The "early" utilization of ethanol in *Sch. pombe* can be explained in terms of the derepression of the TCA enzymes as the glucose concentration decreased (8). The sugar and the alcohol would be utilized concurrently. However, a question arises when the growth on ethanol occurs. How

does the yeast grow on ethanol without an anapleurotic cycle?

Ethanol is converted into acetaldehyde and then to acetyl CoA, which would enter the TCA cycle (see introduction). If no anapleurotic cycle is operating in the cells, the ethanol carbon would be burned in respiration and no growth should occur. This is the same problem faced by the yeast when it "tries" to grow on acetate.

The EMM2 medium was designed to allow growth of the yeast on 1 to 2% glucose (1). In other words, there are enough nutrients in the carbon-limited medium to allow the yeast to grow on a glucose concentration tenfold higher than that used in this work. However, when ethanol is present in concentrations of 0.5% or higher, the yeast consumes only a small amount of the alcohol before it enters stationary phase. This behaviour indicates that the culture is running out of a compound it needs for growth. As stated above, this medium is capable of sustaining a heavier growth, the limiting factor is the carbon source concentration. When the growth on ethanol occurs, the residual concentration of glucose is very low (10 to 30 ug/ml). This glucose is still available to the cell; therefore, it could be providing the C_4 needed to replenish the TCA cycle intermediates and allow growth.

The glucose would be oxidized to pyruvate which could then be carboxylated to oxaloacetate (12). The growth on ethanol would last as long as enough sugar was available to keep the cycle running. As the glucose concentration decreases further, more ethanol will be utilized in respiration and growth slows down. The same fate awaits the acetate accumulated in the medium.

This model would explain why no growth is observed if the yeast is transferred into fresh glucose-free medium containing either ethanol or acetate as the lone carbon source. There is no residual glucose concentration and therefore, no growth occurs. The model can be tested by transferring the cells into medium containing similar residual concentrations of the hexose and monitoring glucose and ethanol consumption along with changes in cell number. Another possibility is that *Sch. pombe* has a glyoxylate cycle which has not been detected. This is not considered likely because, if a cell can use ethanol via the glyoxylate cycle it would also be able to consume acetate the same way. This would result on growth on both substrates, which is not the case. A third possible model would have ethanol going to acetaldehyde, which would then be carboxylated to pyruvate and could then be converted to oxaloacetate by pyruvate carboxykinase (12). However, no reaction leading to the carboxylation of acetaldehyde is known to this author. The actual mechanism

of growth on ethanol remains an open question at this point.

The chemical extraction of the cells cultivated in the presence of the labelled alcohol provided fractions with low amounts of radioactivity. The fractions with the highest concentration of label contained cell wall polysaccharides, and proteins. A small amount of label was detected in the lipid and small molecular weight fractions. The small amount of ethanol going into macromolecules indicate that the alcohol is being used catabolically. As in the case of acetate (see discussion above), ethanol may also be used in two different ways depending on the stage at which the organism happens to be, ie. aerobic fermentation or oxidative metabolism. The utilization patterns in these two phases have to be investigated to determine the fate of ethanol.

The information obtained so far, indicates that ethanol and acetate are used differently in the first 24h of the culture. The alcohol is used mostly in catabolism while the organic acid is used first anabolically (during glucose and ethanol consumption) and then catabolically, during the acetate oxidation phase.

The presence of the alcohol or acetate affected the utilization of U- ^{14}C -glucose. If Sch. pombe was grown in the presence of glucose alone, the uptake of the labelled

growth on glucose. Once glucose is exhausted, the yeast would consume its energy reserves decreasing the amount of lipid and other storage material (see below) in the cells. The cells would then "switch on" their oxidative metabolism and acetate would go mostly into the TCA cycle and gluconeogenesis increasing the amount of label in the other fractions. When the acetate oxidation phase is reached the amount of label should decrease in all the fractions due to the turn over of the macromolecules. To confirm this model, cells should be grown in the presence of labelled acetate and chemically extracted at the different stages mentioned above. The experiment could also be performed by adding the labelled acetate to the cultures at different times, allowing the cells to grow and extracting them.

Acetate utilization was affected by 2,4-DNP. The amount of labelled acetate stored decreased in the presence of the uncoupler. The concentration of 2,4-DNP (0.1 mM) used has been observed to stimulate the production of ethanol in *Sch. pombe* by interfering with the oxidative metabolism of the yeast (82). This concentration has also been observed to increase the rate of respiration and inhibit ATP-inorganic phosphate exchange reactions in isolated mitochondria from yeasts (84). Higher concentrations suppress the K^+ - H^+ exchange in

sugar continued for 9h. After this period, the amount of accumulated label decreased. The production rate of $^{14}\text{CO}_2$ in this culture displayed two phases which coincided with the exponential phase and the decrease in the stored label in the cells respectively. This decrease is believed to be due to the utilization of the energy reserves such as lipids, glycogen and trehalose (79,113).

If either acetate or ethanol was present in the culture medium, the uptake of the label stopped before it did in the control. The stored glucose carbon decreased as observed before giving rise to the same pattern of $^{14}\text{CO}_2$ production as the control. The decrease in the utilization of glucose may be due to toxic effects of both ethanol and acetate. The fission yeast was sensitive to the toxic effects of ethanol at the concentrations used (see table 4 and (31)). The same applies to acetate. A concentration of 6 mg/ml is toxic to *C. utilis*, which can grow on the acid (22). *Sch. pombe* was more susceptible to the toxic effects of acetate which decreases the consumption rate of glucose at a concentration in excess of 0.2% (Table 4).

The carbon provided by U- ^{14}C -glucose appeared, as expected, in all the fractions extracted. The presence of acetate changed the amount of label in several fractions. The amounts of labelled lipids and polysaccharides

decreased. This observation can be interpreted in three ways. Either acetate carbon enters into the synthesis of these molecules or the presence of acetate increases the utilization of these fractions during the inter-logarithmic phase. The third possibility, is that both processes are occurring concurrently. The three options are feasible. Acetate can be used as a precursor for lipids and polysaccharides via acetyl CoA. On the other hand, the acid affected the utilization of glucose. Acetate is known to increase the rate of respiration in yeasts (22,23) which could account for a faster consumption of the energy reserves. The presence of acetate increased the amount of labelled glucose carbon in the cell wall fraction. This increase maybe due to funnelling of the glucose to the synthesis of these macromolecules, while the carbon provided by acetate would go to the synthesis of the others.

Ethanol decreased the amount of labelled glucose carbon in the small-molecular weight and lipid fraction. These fractions appeared labelled when 1- ^{14}C -ethanol was used. However, a decrease of 50% of the amount of label cannot be explained in terms of ethanol carbon being used instead of glucose alone, especially in the case of the small molecular weight fraction. The amounts of nucleic acids and polysacchacarides labelled also decreased. This decreases may be due, as discussed above, to the increase

in the utilization of the stored energy reserves. The fission yeast has been observed to use RNA as storage material (B. Johnson, personal communication). The nucleic acid fraction was not labelled when 1- ^{14}C -ethanol was present in the growth medium.

The data obtained support the conclusion that acetate is being used to provide carbon for biosynthesis, while ethanol is being used for respiration. As suggested above, the chemical extractions should be repeated using cells in the aerobic fermentation phase, to allow a better understanding of how the three substrates are being used by the cell.

There was no morphological difference between the cells grown in the presence of acetate and the control. The "ethanol" cells under a phase microscope appear to be different from the control; the cytoplasm appears much darker in these cells. The significance of this observation is not known. The effect of acetate and ethanol on the utilization of glucose in *Sch. pombe* are more clearly observed in continuous culture.

Growth on glucose alone shows that ethanol starts to accumulate at a dilution rate (D) of 0.08 h^{-1} . The ethanol concentration is low, but as soon as the residual concentration of glucose increases, so does the ethanol. This is a good example of how the Crabtree effect operates

(see introduction), since the catabolite repression increased with the rise in glucose concentration (8). Acetate production is not detected until the dilution rate is increased to 0.19 h^{-1} . At this point, the yeast has part of its oxidative metabolism repressed (8,68), as shown by the accumulation of ethanol, but is still capable of oxidizing the alcohol to CO_2 .

If 0.06% acetate was added to the inflowing medium, it was consumed completely at 0.09 h^{-1} . Ethanol was accumulated and a residual concentration of glucose was present. As the dilution rate was increased, the acetate and ethanol accumulated, but the levels of glucose increased very little. The cell yield decreased with D , but, the change was less severe than in the presence of glucose alone. The data indicates that acetate and glucose are being used concurrently. The low concentration of glucose present would allow the derepression of the oxidative metabolism enzymes, while the presence of acetate could stimulate the synthesis of the enzymes involved in the metabolism as observed in *S. cerevisiae* (25,58,80). Acetate is also known to increase the respiration in *C. utilis* grown on the acid as lone carbon source in chemostat cultures (60). As acetate is increased, the rate of the oxidative metabolism in the fission yeast is incremented and more glucose would go into the TCA cycle. Then a decrease in the ethanol

production rate should be observed. This phenomenon was observed when acetate was present. The rate of ethanol production of the culture increased at a much slower rate with D in the presence of the organic acid than in the control. The slow decrease in cell population shows that acetate is providing carbon for growth. The number of cells decreases when glucose starts to accumulate. Little change in the cell population is observed before this point even though the residual acetate concentration started to increase at a lower dilution rate. From this observation it can be deduced that glucose, as expected, is the main substrate for growth.

The substitution of acetate for 0.03% ethanol in the inflowing medium showed that the fission yeast was also capable of consuming ethanol at low dilution rates (0.08 h^{-1}). No ethanol was accumulated at 0.08 h^{-1} . However, ethanol was detected at D above or equal to 0.14 h^{-1} . This variation in the culture signals the beginning of catabolite repression by glucose due to a 3-fold increase in the sugars residual concentration. No acetate was detected in the culture regardless of the D used indicating that the alcohol was being oxidized to CO_2 . The rate of increase in ethanol production rate with D was greater than in the control. At 0.23 h^{-1} the production rate decreased along with the glucose consumption rate. The increase in the rate of ethanol production in the

presence of the alcohol was not expected. Since the glucose utilization rate increased at a slower rate than the control when the D was varied. This shows that the alcohol is interfering with the consumption of the sugar by the yeast. The rate of ethanol production will depend on the ratio between oxidative and fermentive glucose utilization (68). The greater the extent of the Crabtree effect, the higher the amount of ethanol produced. The rate of glucose consumption increases at a slower rate with increasing D, this means that the residual glucose concentration at a given D will be higher than in the control. Therefore, the repression of the oxidative metabolism would be greater in the presence of ethanol, leading to an increase in the production of the alcohol. To confirm this model, the experiment should be repeated and the activity of those enzymes inhibited by glucose (39,40,41) should be monitored and compared to the changes in the control (glucose alone).

A better understanding of the effect of acetate concentration on the fission yeast is obtained by growing the organism at fixed D and increasing the concentration of the organic acid. The responses observed under these conditions should be a reaction to the changes in acetate levels (see Introduction).

The effect of increasing acetate concentration is

dependent on the D used. This means that it depends on the ratio between the oxidative and the fermentative metabolism operating in the yeast. At a low dilution rate (0.12 h^{-1}) the increase in acetate concentration from 0 to 0.1% caused an increase in the rate of glucose consumption and in the rate of ethanol production of the culture. The amount of cells produced also increased over this period. The data support the observations made with the batch culture, in other words acetate is being used as an auxiliary carbon source. More glucose is going to ethanol, but at the same time more biomass is being produced. However, if the concentration was increased to 0.06% the ethanol production dropped but the glucose consumption increased. This suggests that the increase in the concentration of acetate is inducing changes in the metabolism of the cell. The initial increase (0.06%) seems to have increased the oxidative metabolism of the yeast (drop in ethanol production with increasing glucose consumption). However, if the concentration was raised to 0.1%, more glucose was converted to ethanol. This suggests that the acetate must be supplying more carbon to the oxidative pathways, or is inhibiting their operation. If the former is occurring, it would require the induction of the enzymes involved in the metabolism of acid (see below). The cells growing at 0.06% acetate may not yet contain these enzymes. The rate of acetate consumption

increased with acetate concentration suggesting that acetate-metabolizing enzymes might be induced in the process (see below).

The ethanol yield depended on the change in the rate of ethanol production with D . However, if the acetate concentration was increased to 0.2% both the yield and the rate of ethanol production of the culture decreased. This phenomenon may be due the onset of inhibitory effects of acetate, one of which is the increase in the rate of respiration (60).

If the D was increased from 0.12 to 0.15 h^{-1} the results obtained are different. For this chemostat the specific rate data will be used in the discussion. This data was not available for the prior example discussed. The specific rate of ethanol production never goes over that of the control and the specific rate of glucose consumption decreased as the concentration of acetate was increased in the inflowing medium. The cells growing at a D equal to 0.15 h^{-1} are doing so at a higher residual concentrations of glucose and acetate than those growing at 0.12 h^{-1} . The data indicates that under these conditions, acetate is interfering with glucose utilization. The yeasts show a drastic increase in the utilization rate of acetate when the organic acid concentration was increased from 0.05 to 0.20%. This

increase coincides with an increment in the biomass yield. The rise in biomass with the decrease in glucose utilization demonstrates that acetate is being used anabolically. The behaviour of the culture at 0.05% suggests again that the utilization of acetate may induce a particular "set" of enzymes. At this concentration the cells "behaved" in the same way as the culture growing at the lower D and similar acetate concentration (see discussion below).

The density of the cells was also affected by the increase in the concentration of the organic acid. At 0.05 and 0.25% the density of the cells was high and the values were lower at 0.1 and 0.2% acetate. However the cell density never went below that obtained for the control. The change in density may be due to either a change in the size of the cell or to a change in the amount of energy reserves stored, or both. The results indicate that the cell is undergoing changes; however, no information is available to help pin point the nature of the variation. Experiments which would measure the volume of the cells (see 110) as well as their glycogen content (see 111) would help clarify the nature of the variation.

Another change that occurred in the yeasts as the acetate concentration was increased, was a change in the protein profile of the cells obtained by SDS-Disc

electrophoresis. The molecular weight of most of the protein bands that underwent changes in intensity was between 15.5×10^3 and 35.5×10^3 . None of the bands can be identified, but they can be divided into three major groups. The first group is composed by those bands whose intensity increases with acetate concentration, the second group is formed by those whose intensity decreases with the addition of the acid, the final group is that of the bands whose peak intensity occurs between 0.05 and 0.10% acetate. These results suggests that the cells are undergoing changes in their protein composition as a response to acetate concentration. These proteins may be involved in the metabolism of the cell or from part of the cell structure. To determine what enzymes might be affected by the addition of the acid the activity of several enzymes should be monitored. Gossling and Duggan (25) observed that in Baker's yeast, acetate increased the activity of several enzymes, particularly isocitrate lyase and fructose 1,6 diphosphatase. The fission yeast has not exhibited any glyoxylate cycle activity, so enzymes involved in gluconeogenesis should be checked along with those TCA cycle enzymes known to be inhibited by glucose, and those involved in lipid synthesis.

The effect of ethanol concentration on *Sch. pombe* provides some evidence supporting the idea of enzyme induction by the alcohol. The specific rate of ethanol

consumption increased as the ethanol concentration was increased. This indicates that the alcohol is stimulating its consumption by the yeast. In *S. cerevisiae* the maximum oxidative ability occurs when the mitochondrial alcohol dehydrogenase is induced (74). The alcohol may be inducing a similar system in the fission yeast. As mentioned above a possible second isozyme of alcohol dehydrogenase has been detected (K. Mitton, personal communication).

The specific rate of glucose consumption remained unchanged in the presence of 0.3% ethanol but decreased slightly at 0.6% ethanol. This contrasts with the decrease caused by the addition of acetate. The concentrations of alcohol used are higher than those reported (31) to give rise to inhibition in *Sch. pombe*. The difference in results may be due to the fact that the ethanol produced by the yeast is more toxic to it than that added to the medium (15). The physiology responsible for this phenomenon is not understood (13,14,15). The fact that the yeast tolerates ethanol better than acetate agrees with similar observations made with Baker's yeast (22,23).

Although the glucose consumption rate does not change significantly in the presence of ethanol, the biomass yield increases. This indicates that some of the alcohol is being fixed by the yeast. The increase in yield is small and does not mimic the increase in the ethanol

consumption rate ; therefore it can be assumed that most of the alcohol is being utilized in catabolism. The specific rate of acetate production remains unchanged suggesting that the alcohol is being oxidized to carbon dioxide. The addition of ethanol also caused a change in the protein pattern of the cells. As discussed above, further work is required to determine the significance of these changes, in particular the presence of an isozyme of alcohol dehydrogenase.

The chemostat culture data for both ethanol and acetate suggest that their effect on *Sch. pombe* will depend on the phase at which the culture is, ie. aerobic fermentation, oxidative metabolism or somewhere in between. Cells growing at low dilution rate are similar to those in the late exponential phase in batch culture or early inter-logarithmic phase. These cells show signs of the Crabtree effect (ethanol accumulation) but have their oxidative enzymes derepressed (the extent of derepression being dependent on D) (68). If the dilution rate is increased the cells start to physiologically resemble those in the exponential phase and the effect of catabolite regulation by glucose is accentuated (68). Acetate and ethanol are used differently by the cell. However, the response of the yeast will depend on both, the concentration of the substrate and the dilution rate at which it is growing. For example, 0.20% acetate caused

a decrease in the cell yield of the culture when D was 0.12 h^{-1} . If the D was raised to 0.15 h^{-1} , the acetate concentration had to be increased to 0.25% before the same effect was observed. The difference in the response indicates that the cells at the two different D are not identical. The nature of the variation cannot be determined without looking into the physiology of the yeast under these conditions.

SUMMARY AND CONCLUSION

The batch and chemostat culture results confirm the notion that Sch. pombe can utilize acetate and ethanol. In batch culture the yeast could use the alcohol for growth as long as it had gone through glucose utilization. The growth on ethanol is believed to be dependent on the presence of residual glucose concentration in the culture medium. Fission yeast could not grow on either ethanol or acetate alone. The organic acid could not support growth, even if glucose was originally present. However, it was consumed along with glucose increasing the biomass yield. The alcohol is used catabolically by the yeast while the acetate carbon was used in anabolism. In chemostat cultures, acetate, ethanol and glucose were consumed concurrently giving rise to mixed-substrate utilization. The effects of acetate and ethanol on the fission yeast were varied. Acetate was toxic to the cells at concentrations above 0.2%. In a chemostat culture at 0.15 h^{-1} , the presence of acetate in the medium decreased glucose utilization and ethanol production by the yeast. The alcohol was less toxic than acetate. Concentrations above 0.3% were required to elicit toxic effects in the fission yeast. Ethanol and acetate also caused physical changes in the cells, the nature of the which remains to

be elucidated. Both compounds induced changes in the protein composition of the cells. The increase in concentration of either substrate increased the ability of the yeast to consume it. The data suggest that the enzymes involved in the metabolism of ethanol and acetate are induced by their presence in the growth medium. Acetate uptake was not affected by the presence of metabolic inhibitors indicating that the process is not active. However, the utilization of the acid was affected, particularly with azide.

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