FEMS Microbiology Reviews 32 (1986) 199–224
Published by Elsevier

FER 00014

Redox balances in the metabolism of sugars by yeasts

(NAD(H); NADP(H); glucose metabolism; xylose fermentation; ethanol; Crabtree effect; Custers effect)

Johannes P. van Dijken and W. Alexander Scheffers *

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Received 4 October 1985
Revision received and accepted 7 January 1986

1. SUMMARY

The central role of the redox couples NAD^+/NADH and $NADP^+$/NADPH in the metabolism of sugars by yeasts is discussed in relation to energy metabolism and product formation. Besides their physical compartmentation in cytosol and mitochondria, the two coenzyme systems are separated by chemical compartmentation as a consequence of the absence of transhydrogenase activity. This has considerable consequences for the redox balances of both coenzyme systems and hence for sugar metabolism in yeasts.

As examples, the competition between respiration and fermentation of glucose, the Crabtree effect, the Custers effect, adaptation to anaerobiosis, the activities of the hexose monophosphate pathway, and the fermentation of xylose in yeast are discussed.

2. INTRODUCTION

The catabolism of sugars by microorganisms is accomplished by a variety of metabolic pathways. Yeasts, as a group, are more homogeneous with respect to sugar catabolism than are bacteria. All yeasts described so far are able to grow on glucose. Invariably, the major portion of this sugar is catabolised via the Embden-Meyerhof pathway; respiration proceeds only with oxygen as the terminal electron acceptor, and if fermentation occurs, ethanol is the major end product. Despite these similarities, however, many differences may be observed between different yeasts, especially with respect to the ability to utilise various sugars and the regulation of respiration and fermentation.

Fundamental knowledge of the physiology of yeasts is a prerequisite for the successful use of these organisms. This holds both for improvements in existing applications, such as production of ethanol and baker's yeast, and for the development of new processes. For example, commercial exploitation of yeasts as hosts for the expression of heterologous DNA requires research efforts in the field of physiology, since gene expression is governed by environmental conditions.

In this article an attempt will be made to illustrate the usefulness of physiological studies as a basis for the exploitation of yeasts. Emphasis is placed on the formation and consumption of redox equivalents as a key to understanding the partitioning of the carbon flow over anabolic and catabolic pathways.

* To whom correspondence should be addressed.

2.1. The redox couples $NAD^+/NADH$ and $NADP^+/NADPH$

In the metabolism of sugars by yeasts the nicotinamide adenine dinucleotides NAD(H) and NADP(H) play separate and distinct roles. NADH may be regarded as a predominantly catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes (Fig. 1). Under conditions of oxygen depletion, NADH generated in glycolysis can be re-oxidised in the conversion of pyruvate to ethanol and CO_2. In the presence of oxygen many yeasts do not form ethanol and NADH, generated during catabolism, is re-oxidized with oxygen. Since catabolic and anabolic pathways share the initial reactions of sugar metabolism, NADH is also formed during the assimilation of sugars to cell material. The formation of NADH during assimilation (Fig. 1) is even higher than is anticipated on the basis of a comparison of the reduction levels of sugar and biomass. This is due to the fact that the NADH produced during the formation of intermediates of glycolysis and TCA cycle is not the principal reductant for the conversion of these intermediates to the building blocks of cell polymers.

Most anabolic reductive reactions require NADPH rather than NADH. Since transhydrogenase ($NADH + NADP^+ \leftrightharpoons NAD^+ + NADPH$) is absent [1] in yeasts (section 4.3), the overall process of assimilation leads to the production of a considerable surplus of NADH [2,3]:

$$\begin{aligned} &737\ C_6H_{12}O_6 + 680\ NH_3 + 6\ H_2SO_4 \\ &\quad + 931\ NADPH + 1349\ NAD^+ \\ &\rightarrow 1000\ C_4H_{7.32}O_{2.24}N_{0.68}S_{0.006}\ (100\ g\ cells) \\ &+ 424\ CO_2 + 931\ NADP^+ + 1349\ NADH \\ &+ 1358\ H_2O + 418\ H^+ \end{aligned} \quad (1)$$

From this equation it can be seen that the formation of 100 g biomass is associated with the production of 1349 mmol NADH, although the net production of reducing equivalents is only 418.

The specific requirement for NADPH in the assimilation of sugars to cell material, in combination with the absence of transhydrogenase activity, necessitates the conversion of part of the sugar exclusively for the purpose of generating reducing power in the form of NADPH (Fig. 1). This is accomplished in the oxidative steps of the hexose monophosphate pathway [4]. Thus, in the overall process of aerobic growth and biomass formation, two separate flows of reducing equivalents can be distinguished: production of NADH for the purpose of ATP formation and production of NADPH for reductive processes in the cell's anabolism, mainly in the synthesis of amino acids and fatty acids. A similar scheme holds for anaerobic growth. In this case, however, NADH plays no direct role in ATP formation and is reoxidised in the final reaction of the alcoholic fermentation.

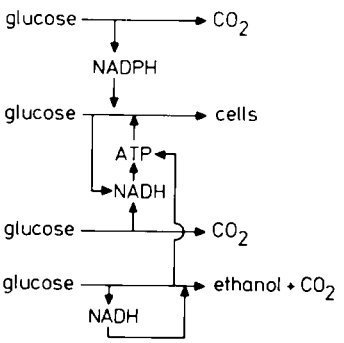

Fig. 1. Schematic representation of redox and ATP flows in assimilation and dissimilation of glucose.

2.2. Redox balances and product formation

Apart from ethanol, yeasts may excrete a variety of metabolic products. These include polyalcohols (glycerol, erythritol, arabinitol, xylitol, ribitol), monocarboxylic acids (mainly acetic and pyruvic acid), dicarboxylic and tricarboxylic acids (succinic, citric, and isocitric acid). Generally, the metabolic basis for the formation of these products and the fate of reducing power during their synthesis is poorly understood. Excretion of metabolites can occur under either aerobic or anaerobic conditions, and is dependent on the particular species and on environmental conditions.

Incidentally, a considerable part of the carbon source may be converted to a single product that accumulates intracellularly. A typical example is the formation of fat by oleagenous yeasts. The results of Evans et al. [5] have revealed that lipid accumulation is the result of a very complex series

of reactions occurring in different cell compartments. The production of fat in quantities approaching 70–80% of the cell dry weight requires the formation of excessive amounts of NADPH. It is not yet clear what benefit the cell might derive from such an extreme bias. However, a clear example of product formation in yeasts for which a metabolic rationale can be put forward is the formation of ethanol and glycerol during anaerobic fermentation of sugars. Formation of both compounds is essentially required to maintain the redox balance. The formation of ethanol ensures reoxidation of the NADH formed in the oxidation of glyceraldehyde 3-phosphate, whereas production of glycerol originates from excess NADH generated in the assimilation of sugars to biomass. As can be seen from Eqn. 1, the formation of 100 g biomass should, under anaerobic conditions, be associated with the formation of 1349 mmol glycerol, according to:

$$1\ \text{glucose} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}^+ \rightarrow 2\ \text{glycerol} + 2\ \text{ADP} + 2\ \text{P}_i + 2\ \text{NAD}^+ \quad (2)$$

The energetically expensive formation of glycerol may thus be considered to represent a redox valve. Frequently, a higher glycerol concentration than expected on the basis of Eqns. 1 and 2 is observed, especially in the early stages of batch cultivation of *Saccharomyces cerevisiae* [6]. This may be explained by the formation of products more oxidised than glucose, such as acetic acid. Acetate formation by yeasts, which can also occur under anaerobic conditions (see section 3.2) is frequently neglected, although it has an important effect on the redox balance. Formation of a certain amount of acetic acid requires the production of double that amount of glycerol, according to:

$$1\ \text{glucose} + 4\ \text{NAD}^+ \rightarrow 2\ \text{acetic acid} + 2\ \text{CO}_2 + 4\ \text{NADH} + 4\ \text{H}^+ \quad (3)$$

$$2\ \text{glucose} + 4\ \text{NADH} + 4\ \text{H}^+ \rightarrow 4\ \text{glycerol} + 4\ \text{NAD}^+ \quad (4)$$

$$3\ \text{glucose} \rightarrow 4\ \text{glycerol} + 2\ \text{acetic acid} + 2\ \text{CO}_2 \quad (5)$$

It has long been known that glycerol is an important by-product of anaerobic alcoholic fermentation in yeasts. During beer and wine fermentation and in the raising of dough for bread, considerable amounts of glycerol are usually formed. The ability of *Saccharomyces* yeasts to produce glycerol has even been industrially exploited. During the first World War, the glycerol required for production of explosives was obtained on a large scale by way of the so-called 'Abfangverfahren'. This process employed the addition of bisulphite to cultures of fermenting yeast. In this way acetaldehyde is trapped, so that this intermediate can no longer serve as an electron acceptor in the re-oxidation of NADH. In order to restore its redox balance, the yeast is thus forced to produce glycerol [7,8].

Glycerol formation may also occur under strictly aerobic conditions, namely in osmotolerant yeasts such as *Zygosaccharomyces bailii* [9,10]. In this case the metabolic basis for glycerol formation is as yet obscure.

2.3. Compartmentation of redox reactions

In eukaryotic organisms redox reactions may occur in various cell compartments. These are surrounded by membranes impermeable to nicotinamide adenine dinucleotides. As a result, each compartment must maintain a delicate balance between formation and consumption of reducing equivalents. This holds for both nicotinamide nucleotide systems, since the reducing power contained in NADH cannot be directly transferred to $NADP^+$, or vice versa, due to the absence of transhydrogenase activity. 'Redox communication' between different compartments is only possible via shuttling of oxidised and reduced metabolites over the bordering membrane. In contrast to mammalian cells, yeasts have not been investigated in detail with respect to the metabolic implications of the compartmentation of redox reactions in cytosol, mitochondria and peroxisomes. Studies on the location of the various isoenzymes involved in redox shuttling are complicated by difficulties in obtaining intact organelles from yeasts. This is due to the necessity of cell wall removal by lytic enzymes, a procedure which may damage subcellular organelles [11].

Most (assimilatory) processes involving NADPH as a reductant are located in the cytosol.

Significantly, the enzyme systems producing this reducing power, i.e., the hexose monophosphate pathway and $NADP^+$-linked isocitrate dehydrogenase (Fig. 2) are located in the same compartment. Since citrate is produced intramitochondrially, export of citrate or isocitrate from the mitochondria to the cytosol is required for the production of NADPH by isocitrate oxidation. At present it is not known to what extent the cytosolic $NADP^+$-linked isocitrate dehydrogenase con-

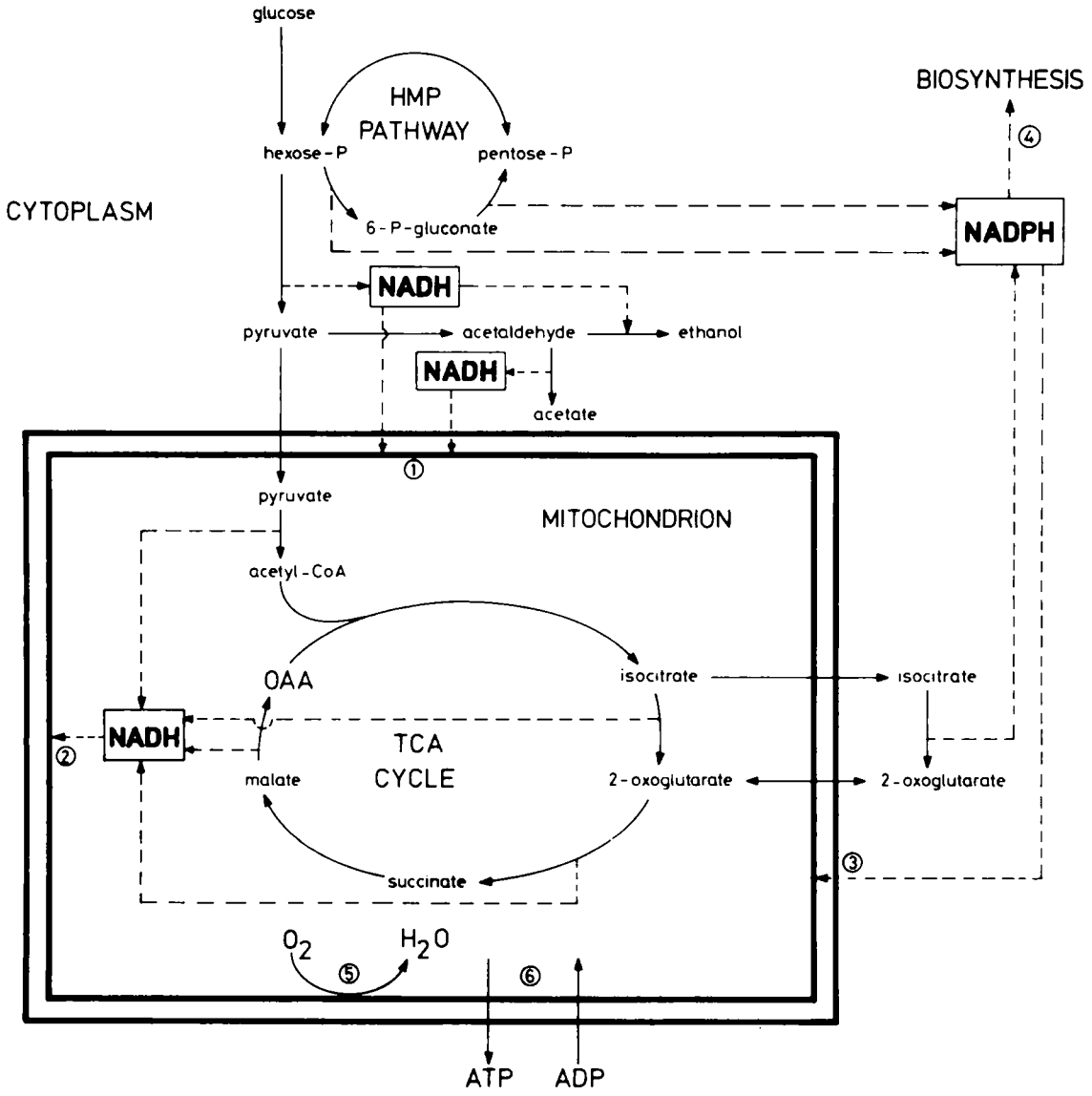

Fig. 2. Schematic representation of the flows of reduced nicotinamide nucleotides in glucose metabolism and their subcellular location. (1) Oxidation of cytoplasmic NADH initiated by NADH dehydrogenase at the outer surface of the inner mitochondrial membrane. (2) Oxidation of NADH generated within the mitochondrion by NADH dehydrogenase located at the inner surface of the inner membrane. (3) Oxidation of NADPH by a dehydrogenase at the outer surface of the inner membrane. (4) Utilisation of NADPH in assimilatory processes (i.e. formation of nucleic acids, fatty acids and amino acids). (5) Reduction of oxygen by cytochrome oxidases. (6) Export of ATP by the ADP/ATP translocator.

tributes to the synthesis of NADPH, as the enzyme also has a function in the generation of 2-oxoglutarate, the precursor of glutamate, in the cytoplasm.

The fate of NADH in intermediary metabolism is much more complicated than that of NADPH. NADH is generated both in the cytosol (during glycolysis) and in the mitochondria (via TCA-cycle enzymes) (Fig. 2). In mammalian cells, various shuttle mechanisms are involved in the transport of reducing equivalents between mitochondria and cytosol [12]. Fungal and plant mitochondria, however, differ from the mammalian organelle in being able to oxidise exogenous NAD(P)H directly [13,14]. This can be accomplished via one or more NAD(P)H dehydrogenases located at the outer surface of the inner membrane [15]. Oxidation of exogenous NAD(P)H by yeast mitochondria is subject to respiratory control [16]. When intact mitochondria of these organisms are disrupted, additional NADH oxidase activity is unmasked. This is a consequence of the presence of another NAD(P)H dehydrogenase, located on the inner surface of the inner membrane (Fig. 2). This enzyme is functional in the re-oxidation of NADH produced by the TCA-cycle enzymes. Although a role for shuttle systems in the mitochondrial oxidation of cytoplasmic reducing equivalents cannot be excluded, it is relevant that the activity of the external (rotenon-insensitive) oxidase is high enough to be of physiological significance [16].

Knowledge of the subcellular location of the key reactions of glucose metabolism in yeasts is still incomplete, and it is not yet clear whether in different yeasts the same reactions are located in the same compartments. For example, in *Candida utilis,* as in mammalian cells, pyruvate carboxylase (EC 6.4.1.1) has been found to be mitochondrial [5]. In *S. cerevisiae,* on the other hand, the enzyme has been reported to be cytosolic in nature [17]. It is obvious that such differences in location may have important consequences for the regulation of metabolic processes.

It is beyond the scope of this article to discuss the compartmentation of redox reactions during growth on non-sugar substrates. It must be realised, however, that the nature of subcellular compartmentation of metabolism is very much dependent on the growth substrate. During growth of yeasts on acetate, for example, a net efflux of reducing equivalents from the mitochondria to the cytosol must occur for the purpose of gluconeogenesis. In addition, the possible involvement in acetate metabolism of yet other compartments such as glyoxysomes with their associated shuttle systems further complicates the understanding of subcellular events. It must be concluded that more work is required on the location of various enzyme systems before the subcellular events in sugar metabolism by yeasts can be adequately described.

3. THE NAD^+/NADH BALANCE

Yeasts can be divided into three groups with respect to their fermentative properties (Table 1). As discussed below, the occurrence of alcoholic fermentation in facultatively fermentative yeasts is by no means restricted to anaerobic conditions. Furthermore, it is also not coupled to the ability to grow under these conditions: many yeasts can rapidly ferment certain sugars to ethanol but are unable to grow at the expense of these sugars in the absence of oxygen. Even *S. cerevisiae* is auxotrophic for certain growth factors under anaerobic conditions. It has long been known that prolonged anaerobic growth of this yeast is dependent on the medium composition. Cochin, a pupil of Pasteur, first demonstrated that malt extract sustained growth for only a limited number of generations, whereas in yeast extract-containing media, unlimited serial transfer under anaerobic conditions was possible (Fig. 3). Andreasen and Stier [18,19] identified the growth-promoting factors for *S. cerevisiae* with respect to anaerobic cultivation. They found that prolonged anaerobic growth is possible in mineral media supplemented with various vitamins, provided that sterols and unsaturated fatty acids are present. The synthesis of these components requires some oxygenase-catalysed reactions, and this explains the well-known stimulatory effect of traces of oxygen on anaerobic growth of *S. cerevisiae.* The work of Schatzmann [20] indicates that compounds needed for anaerobic growth of *S. cerevisiae* also include nicotinic acid.

Table 1

Classification of yeasts on the basis of their fermentative capacities

Class	Example
Obligately fermentative [a]	*Candida pintolopesii*
Facultatively fermentative	
Crabtree-positive	*Saccharomyces cerevisiae*
Crabtree-negative	*Candida utilis*
Non-fermentative [b]	*Rhodotorula rubra*

[a] Naturally occurring respiratory-deficient yeasts.

[b] Many yeasts presently considered as non-fermentative may belong to the group of Crabtree-negative, facultatively fermentative yeasts.

The subcellular events during anaerobic growth of *S. cerevisiae* are still poorly understood. It is known that part of the assimilatory processes required for cell synthesis are located within the mitochondria [11], and hence transport of certain intermediates over the mitochondrial membrane remains a necessity under anaerobic conditions. This raises the problem of energising these transport processes in the absence of electron transfer. The results of Šubík et al. [21] and Gbelská et al. [22] strongly suggest that under anaerobic conditions transport processes and other energy-requiring reactions in mitochondria are energised by the import of cytoplasmic ATP via reversal of adenosine nucleotide translocation. Anaerobic growth of *S. cerevisiae* was shown to be arrested in the presence of bongkrekic acid, a specific inhibitor of the ATP/ADP translocator of the inner mitochondrial membrane. This inhibition could not be relieved by addition of a variety of growth factors.

So far, it is unclear why in a variety of yeasts the role of mitochondria in anabolic reactions is even more important than in *S. cerevisiae*. Various Crabtree-negative yeasts such as *C. utilis* cannot grow anaerobically, despite their ability to perform rapid alcoholic fermentation, in the same media which allow rapid anaerobic growth of *Saccharomyces* species. In this type of yeast the petite mutation [23,24] seems to be lethal [25].

Even when an extract of aerobically grown *C. utilis* is present, this yeast fails to grow on glucose for more than two or three generations in the absence of oxygen. The same phenomenon was observed in a variety of other Crabtree-negative yeasts such as *Candida shehatae, Candida tenuis, Pachysolen tannophilus, Pichia segobiensis* and *Pichia stipitis* (van Dijken et al., unpublished results). The molecular basis for the correlation be-

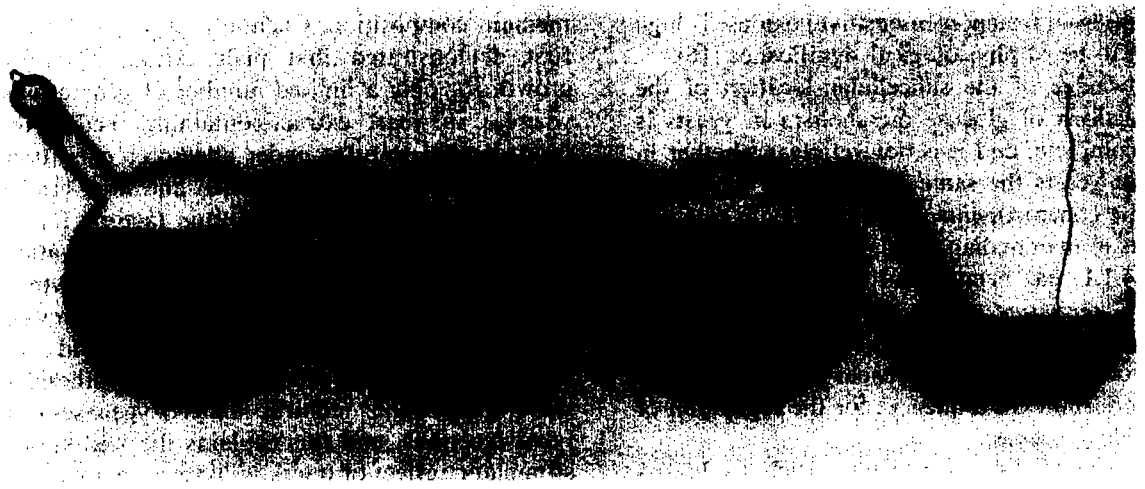

Fig. 3. The Cochin apparatus. Photograph of a drawing by Beijerinck from the collection of the Laboratory of Microbiology at Delft. Beijerinck and his successor Kluyver used the apparatus in classroom experiments to demonstrate that under strict anaerobiosis *S. cerevisiae* does not grow after three serial transfers in malt extract, in contrast to a medium with glucose and yeast extract. By pulling the wire through a bowl with mercury the yeast is transferred from one compartment to the next.

tween the lethality of the petite mutation and the absence of a Crabtree effect [26,27] remains to be elucidated.

Approximately one-third of the 439 species of yeasts listed by Barnett et al. [28] are classified as non-fermentative. The ability of yeasts to perform alcoholic fermentation with certain sugars is routinely tested in complex media using Durham tubes and static incubation. Absence of visible gas production under the test conditions is generally taken as a criterion for the inability to ferment the sugar in question. However, a recent study in our institute has revealed that this test is rather insensitive and not a good measure for fermentative ability. When ethanol formation rather than (visible) CO_2 production was followed, many yeasts hitherto regarded as non-fermentative were found to perform a slow but significant alcoholic fermentation. However, slow fermentation under the test conditions is not necessarily indicative of poor fermentative capacity. Indeed, when such yeasts are grown under oxygen limitation, for example in shake-flask culture, alcoholic fermentation may proceed at rates approaching those observed in typical fermentative yeasts (Figs. 4–7).

These results make clear that all species which at present are classified as non-fermentative should be retested under conditions of oxygen limitation before it can be concluded that they are truly non-fermentative. Apparently, the onset of alcoholic fermentation in these organisms is related to a delicate balance of the flow of NADH between fermentation and respiration.

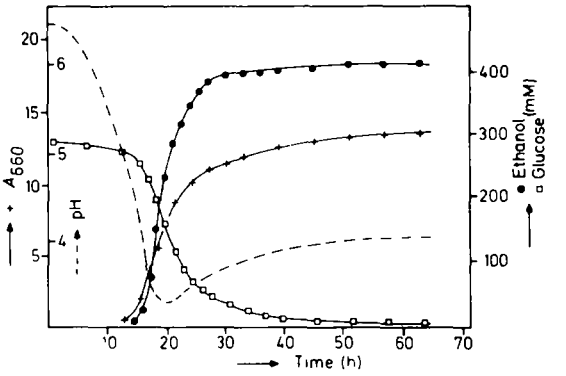

Fig. 5. Growth, sugar consumption and ethanol production by *C. utilis* CBS621 during growth in shake flasks on a medium containing 5% glucose and 1% yeast extract (J.P. van Dijken et al., unpublished results).

3.1. Competition for reducing equivalents between fermentation and respiration

Facultatively fermentative yeasts differ in their response to environmental conditions with respect to alcoholic fermentation. As pointed out above, in many yeasts alcoholic fermentation occurs under conditions of oxygen-limitation. However, in certain organisms a Crabtree effect operates (i.e.,

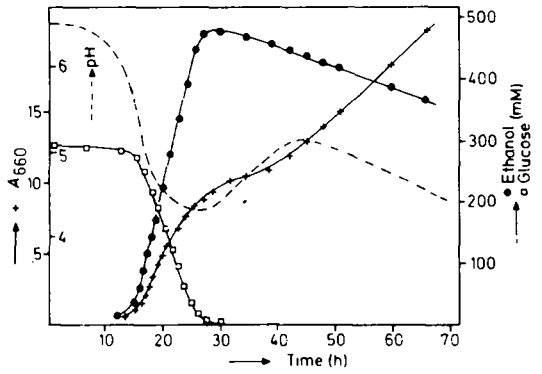

Fig. 4. Growth, sugar consumption and ethanol production by *S. cerevisiae* CBS8066 during growth in shake flasks on a medium containing 5% glucose and 1% yeast extract (J.P. van Dijken et al., unpublished results).

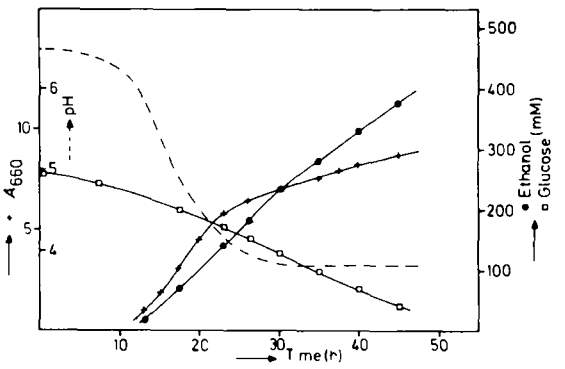

Fig. 6. Growth, sugar consumption and ethanol production by *Candida silvae* CBS5498 during growth in shake flasks on a medium containing 5% glucose and 1% yeast extract (J.P. van Dijken et al., unpublished results).

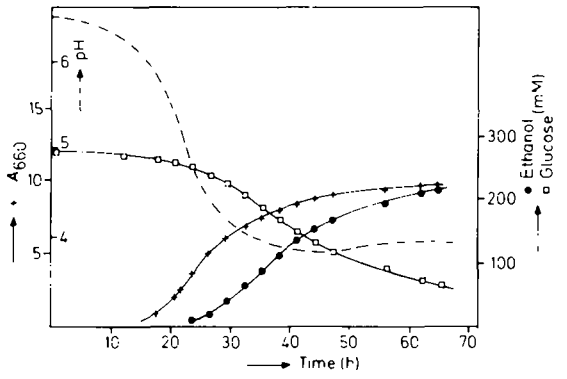

Fig. 7. Growth, sugar consumption and ethanol production by *Hansenula nonfermentans* CBS5764 during growth in shake flasks on a medium containing 5% glucose and 1% yeast extract (J.P. van Dijken et al., unpublished results).

alcoholic fermentation occurs in the presence of excess sugar under strictly aerobic conditions). Those Crabtree-positive yeasts which possess the ability to grow on ethanol show diauxic growth in batch cultures on glucose (Fig. 4). In the first phase, growth occurs at the expense of glucose with concurrent production of ethanol. In this phase the synthesis of enzymes involved in oxidative metabolism is repressed. After exhaustion of glucose, growth resumes after an adaptation phase, and ethanol is respired [6]. Although this is a well-established phenomenon, it is frequently neglected in genetic and biochemical studies on the regulation of respiration in *S. cerevisiae*. Cultures growing in the presence of high concentrations (5–10%) of glucose are often designated as glucose-repressed, whereas cultures grown at lower sugar concentrations (0.1–1.0%) and harvested after the same incubation time are designated as derepressed glucose-grown cells. The differences in respiratory activity between the two types of cultures must, however, be explained on the basis of the fact that with high sugar concentrations, alcoholic fermentation of glucose still continues at the time of harvesting, whereas at that time the culture with low initial glucose has already entered the phase of ethanol consumption, with the concomitant increase in respiratory activity. In other words, glucose-fermenting cells are compared with ethanol-oxidising cells, and thus the observed effects of the glucose concentration on the properties of the cells are indirect. Apart from this, shake-flask cultures are frequently used in studies on respiratory phenomena in *S. cerevisiae*. Such cultures become intrinsically oxygen-limited, and under these conditions alcoholic fermentation is triggered anyway, even in Crabtree-negative yeasts (Figs. 4–7).

When adequate precautions are taken with respect to sufficient oxygen supply, a clear difference is observed between Crabtree-positive and Crabtree-negative yeasts during growth on glucose. In batch cultures of Crabtree-positive yeasts, glucose is first fermented to ethanol, and only after glucose is depleted is the ethanol produced in the first phase oxidised to CO_2. Crabtree-negative yeasts such as *C. utilis*, on the other hand, do not produce any ethanol under these conditions. It is important to note in this respect that measurement of a discrete dissolved oxygen concentration in the culture does not always guarantee sufficient aeration. This is especially true at high cell densities when a high oxygen transfer rate is required. We have observed in cultures of *C. utilis* that formation of ethanol did occur, despite an apparently sufficient oxygen supply as indicated by the oxygen electrode. Depending on the geometry of the fermenter, (semi-)anaerobic pockets, away from the electrode, may occur due to imperfect mixing. With equipment able to sustain strict aerobiosis (i.e., dissolved oxygen tensions in excess of 20% of air saturation) throughout the fermenter, batch cultures of *C. utilis* produced no ethanol. Thus, as a result of imperfect mixing, the organism may temporarily encounter oxygen tensions below the critical dissolved-oxygen tension [29–31] and, as a consequence, respiration may be impeded and fermentation triggered.

With respect to glucose respiration in batch cultures, yeasts of the genus *Brettanomyces* exhibit a behaviour intermediate between that of *S. cerevisiae* and *C. utilis*. *Brettanomyces* spp. can be classified as Crabtree-positive yeasts since they exhibit aerobic alcoholic fermentation in the presence of excess glucose. However, in addition to ethanol these yeasts also produce acetic acid, and a more complex pattern of substrate consumption and metabolite production is observed [32]. In the

first phase, glucose is converted to ethanol and acetic acid. Afterwards, most of the ethanol is also oxidised to acetic acid. Finally, in the third phase, the acetic acid produced in the previous phases is oxidised to CO_2 and water (Fig. 8).

Aerobic glucose-limited chemostat cultures also reveal marked differences with respect to ethanol formation in different yeasts. Chemostat cultures of *C. utilis* do not produce ethanol at all and the cell yield remains constant at approx. 0.5 g cells/g glucose up to the critical dilution rate (Fig. 9). In aerobic glucose-limited chemostat cultures of *S. cerevisiae,* on the other hand, ethanol formation becomes apparent above a certain dilution rate, the value of which is strain-dependent (Fig. 10). Above this dilution rate the cell yield decreases. In similar cultures of the yeast *Brettanomyces intermedius* (Fig. 11) three stages can be distinguished. At low dilution rates the catabolism of glucose is by respiration to CO_2. At a certain dilution rate, acetic acid formation becomes apparent, but ethanol is not detectable. At slightly higher dilution rates, both acetic acid and ethanol are produced. We observed the same pattern of metabolite production in *Brettanomyces lambicus,* which had been reported to produce acetic acid at all dilution rates [33].

Although in aerobic glucose-limited chemostat cultures of *S. cerevisiae* alcoholic fermentation is absent at low dilution rates, the capacity to perform alcoholic fermentation is nevertheless present. When such cultures are pulsed with glucose,

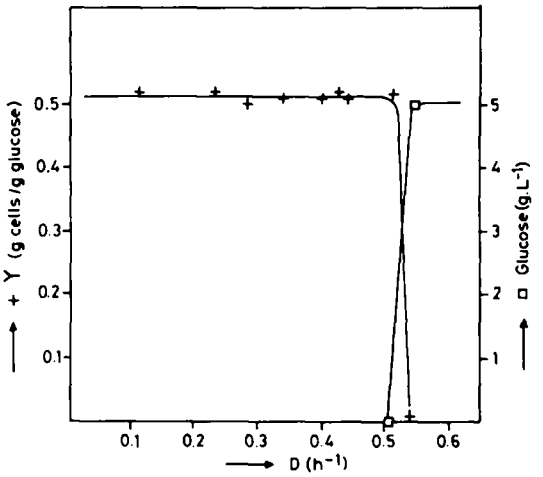

Fig. 9. Cell yield of *C. utilis* CBS621 in aerobic glucose-limited chemostat cultures on a mineral medium. At all dilution rates the concentration of ethanol was below the detection limit (J.P. van Dijken et al., unpublished results).

the sugar is rapidly converted to ethanol (Fig. 12). This ethanol production is instantaneous [34] although some acceleration occurs (Fig. 12). Besides ethanol, acetic acid is produced in significant quantities. This acid continues to accumulate after glucose is exhausted and oxidation of ethanol goes on. The extent to which acetic acid formation occurs in these experiments depends on the dilution rate. At higher dilution rates (compare Figs. 12 and 13) acid production is considerable, notably in the phase of ethanol consumption, when the alcohol is almost quantitatively converted to acetic acid. In this respect *S. cerevisiae* has some similar-

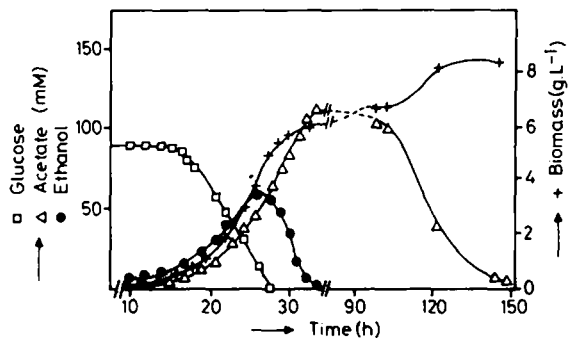

Fig. 8. Growth, glucose consumption and metabolite production in an aerobic batch culture of *B. intermedius* CBS1943. The organism was grown on a medium with 1.5% glucose and 1% yeast extract at pH 5.5 (see Wijsman et al., [32]).

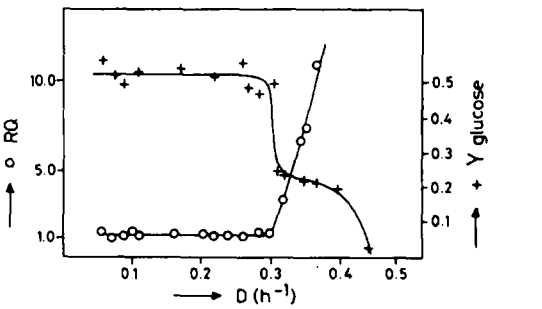

Fig. 10. Cell yield and respiratory quotient (RQ) of *S. cerevisiae* CBS8066 in aerobic glucose-limited chemostat cultures on a mineral medium. Above a dilution rate of 0.3 h^{-1} ethanol production sets in (J.P. van Dijken et al., unpublished results).

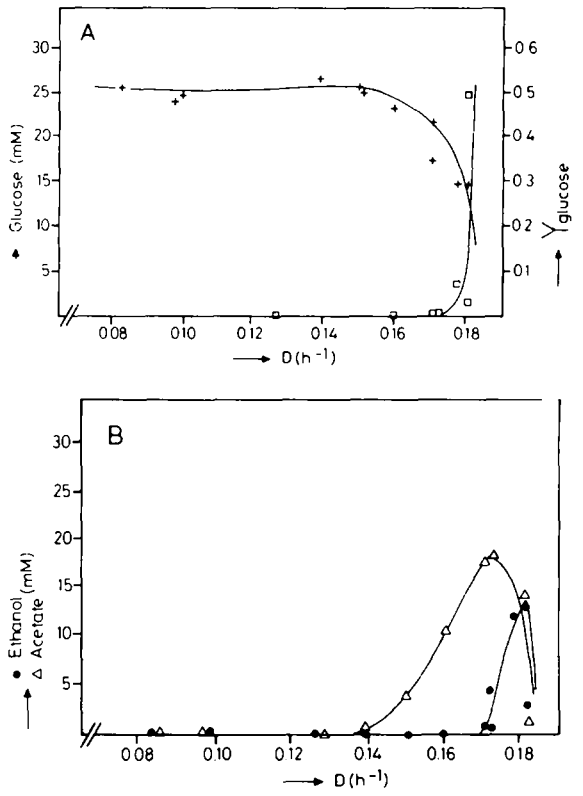

Fig. 11. Cell yield and product formation in aerobic glucose-limited chemostat cultures of *B. intermedius* CBS1943. (A) Cell yields as a function of D. (B) Concentrations of ethanol and acetic acid in steady-state cultures. Acetate formation starts at $D = 0.14\ h^{-1}$, whereas ethanol formation is absent upto $D = 0.17\ h^{-1}$.

ity to *B. intermedius*. When excess glucose is added to a non-fermenting glucose-limited culture of *B. intermedius*, three phases can also be distinguished. Glucose is first converted to acetic acid and ethanol. After depletion of glucose, ethanol is oxidised to acetic acid, and finally the acetic acid is consumed (Fig. 14).

The biochemical basis for the production of ethanol by Crabtree-positive yeasts such as *S. cerevisiae* under strictly aerobic conditions has been intensively studied, but is still far from being resolved. Originally it was thought that this ethanol production was solely due to an impediment in respiration as a consequence of the repression of the synthesis of certain respiratory enzymes. In fact, the Crabtree effect in yeasts is frequently

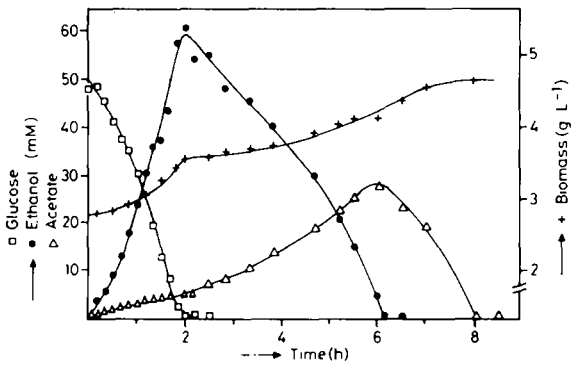

Fig. 12. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *S. cerevisiae* CBS8066, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the dissolved oxygen tension was kept at 50% air saturation. Note that after the depletion of glucose some of the ethanol is further oxidised to acetic acid. Finally, after depletion of ethanol, oxidation of acetate goes on (J.P. van Dijken et al., unpublished results).

defined on the basis of occurrence of alcoholic fermentation due to repression of respiration [6]. However, although repression of the synthesis of respiratory enzymes is likely to contribute to ethanol production in batch cultures, it is probably not the only basis for this phenomenon in glucose-limited chemostat cultures. Recent studies

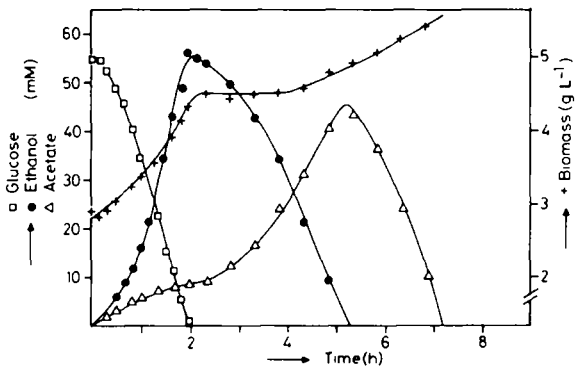

Fig. 13. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *S. cerevisiae* CBS8066, pregrown aerobically in a chemostat at $D = 0.2\ h^{-1}$ under glucose limitation. During the experiment the dissolved oxygen tension was kept at 50% air saturation. Note that, as compared to the experiment depicted in Fig. 12, cells pregrown at $D = 0.2\ h^{-1}$ exhibit a more pronounced oxidation of ethanol to acetic acid (J.P. van Dijken et al., unpublished results).

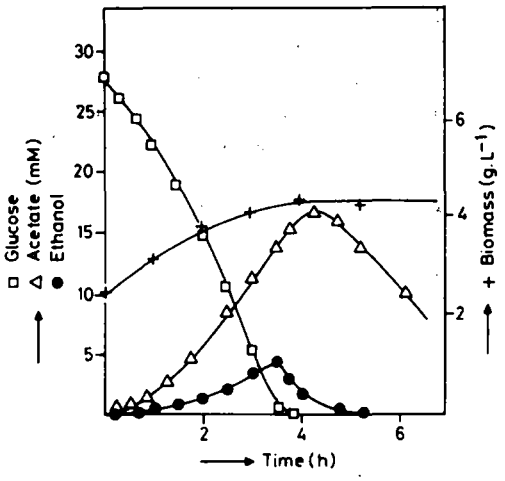

Fig. 14. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *B. intermedius* CBS1943, pregrown aerobically at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the dissolved oxygen tension was kept at 50% air saturation. As compared to *S. cerevisiae* (Figs. 12 and 13) direct conversion of glucose to acetic acid by *B. intermedius* is much stronger (J.P. van Dijken et al., unpublished results).

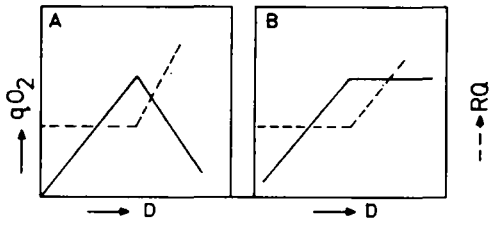

Fig. 15. Schematic representation of the relationships between respiratory quotient (RQ) and specific rate of oxygen consumption ($q\ O_2$), and the dilution rate in glucose-limited chemostat cultures of *S. cerevisiae*. (A) Repression of the synthesis of respiratory enzymes above a certain dilution rate results in a decrease of the specific rate of oxygen consumption. (B) Oxygen utilisation persists above the dilution rate where alcoholic fermentation sets in. This situation may be achieved by careful adaptation of the yeast to higher dilution rates. Small disturbances lead to a situation as depicted in Fig. 15A. Recovery from repression of respiration may take 100 generations or more [35].

[35,36] have made clear that above a certain dilution rate the transition from respiratory to fermentative catabolism may not be accompanied by decreased rates of oxygen consumption through repression of respiratory enzymes. In contrast to the response seen after a sudden single-step increase in dilution rate, careful stepwise increase may result in establishing steady-state cultures up to μ_{max} which perform alcoholic fermentation at a constant, unaffected rate of oxygen consumption (Fig. 15). Thus, via careful adaptation, a situation may be reached where the specific rate of oxygen consumption is not decreased but remains constant and independent of the dilution rate. Apparently, above a certain dilution rate, the cells cannot further increase their respiratory capacity. Any imbalance in metabolism, however, results in repression of synthesis of respiratory enzymes; the culture can only recover from such disturbances after many generations [35]. These phenomena demand reconsideration of the definition of the Crabtree effect in yeasts. Ethanol formation in Crabtree-positive yeasts under strictly aerobic conditions is apparently due to the inability of these organisms to increase the rate of respiration above a certain value. This critical value, above which alcoholic fermentation occurs, is dependent on the strain and the culture conditions. In our opinion, the Crabtree effect in yeasts can better be defined in purely phenomenological terms, namely as the 'occurrence of alcoholic fermentation under strictly aerobic conditions'. This definition also covers ethanol production observed after pulsing glucose-limited chemostat cultures at low dilution rates. In that case, ethanol production cannot be explained by effects at the level of enzyme synthesis, since the appearance of ethanol is immediate. The occurrence of alcoholic fermentation at high dilution rates and after glucose pulsing of cultures at low dilution rates have been named the long-term and short-term Crabtree effects, respectively [37]. Both phenomena may have a common basis. All available evidence indicates a bottleneck in the respiratory system [38,39]. However, it is not yet possible to attribute these effects to particular enzymes. Competition between respiration and fermentation may be seen in terms of carbon flows. For example, a limited capacity of the mitochondrial transport system for pyruvate or insufficient activities of the pyruvate dehydrogenase complex and the TCA-cycle enzymes might be involved. Triggering of fermentation may also be interpreted in terms of redox metabolism. In this respect, the capacity of the external and inter-

nal NADH dehydrogenases and other components of the respiratory chain may play a crucial role. The activity of the respiratory chain is under respiratory control which further complicates an analysis of the relative kinetics of respiration and fermentation.

In our opinion the most promising approach to solving the metabolic basis of the aerobic alcoholic fermentation is a broad comparative study with various yeasts. Such studies must include an analysis of the kinetics of growth and metabolite production under well-defined environmental conditions, measurement of enzyme levels, kinetic studies with purified enzymes, as well as investigations on the subcellular location of possible bottleneck reactions. Different Crabtree-positive yeasts not only show marked differences in the extent of aerobic alcoholic fermentation, but the question should also be addressed why Crabtree-negative yeasts such as *C. utilis* do not perform alcoholic fermentation unless grown under oxygen limitation.

The instantaneous formation of ethanol after pulsing glucose-limited chemostat cultures of *S. cerevisiae* with glucose does not seem to be caused by limitation at the level of the respiratory chain. This is evident from comparative studies of mitochondria isolated from *S. cerevisiae* and *C. utilis* growing at the same dilution rate. Oxidation of NADH by mitochondria from both organisms proceeded at a similar rate. This was found for intact mitochondria (external NADH oxidase) as well as for sonicated mitochondria (external plus internal NADH oxidase) (H. van Urk et al., unpublished results). Furthermore, after adaptation, *S. cerevisiae* may exhibit oxygen consumption rates significantly higher than that occurring at the dilution rate at which ethanol production in glucose-limited chemostat cultures is switched on. For example, after a glucose pulse, *S. cerevisiae* CBS8066 oxidises ethanol to acetic acid with associated qO_2 values approaching 10 mmol $O_2 \cdot$ (g cells)$^{-1} \cdot h^{-1}$ as compared to a value of 7 at the 'critical' dilution rate. qO_2 Values of 10 and more are also observed when *S. cerevisiae* is grown carbon-limited on mixtures of glucose and formate as an additional energy source (P.M. Bruinenberg et al., unpublished results).

Although the capacity of the respiratory chain itself may not be the limiting factor for the complete oxidation of glucose to CO_2 under conditions of balanced growth, it seems very likely that the occurrence of aerobic alcoholic fermentation finds its explanation in a limited capacity of the aerobic catabolic pathway. In this respect reactions of the TCA cycle must be considered. A limited capacity for the oxidation of C_2 compounds, produced via pyruvate dehydrogenase (EC 1.2.4.1) or pyruvate decarboxylase (EC 4.1.1.1) may be involved. This would explain the formation of acetic acid under certain conditions, despite high rates of oxygen consumption. A limited oxidation capacity of mitochondria for pyruvate in *S. cerevisiae* seems unlikely: the rate of pyruvate oxidation by mitochondria of *S. cerevisiae* is similar to that of mitochondria of *C. utilis,* a yeast which does not perform aerobic alcoholic fermentation (H. van Urk et al., unpublished results).

Another parameter, which of course is important in the distribution of the carbon and redox flows between fermentation and respiration, is the activity of pyruvate decarboxylase, a key enzyme at the branching point of fermentation and respiration. Absence of this enzyme would preclude ethanol formation. Although the regulation of pyruvate decarboxylase in Crabtree-negative yeasts is somewhat different from that in *S. cerevisiae,* absence of aerobic alcoholic fermentation in Crabtree-negative yeasts is probably not exclusively caused by a limited fermentation capacity. In aerobic glucose-limited chemostat cultures of *C. utilis* the activity of this enzyme is 0.1 μmol $\cdot$ min^{-1} (mg protein)$^{-1}$ as compared to 0.4 μmol $\cdot$ min^{-1} (mg protein)$^{-1}$ in such cultures of *S. cerevisiae* (J.P. van Dijken et al., unpublished results). The presence of pyruvate decarboxylase therefore apparently is not the only determinant for ethanol formation after glucose pulsing of carbon-limited cultures of *C. utilis.*

3.2. Adaptation to anaerobiosis

Not only the sugar concentration, but also the dissolved oxygen concentration is a critical parameter in the distribution of NADH between the respiratory and the fermentative pathway in yeasts. Although under oxygen limitation often only

qualitative differences are observed between facultatively fermentative yeasts with respect to ethanol formation (Figs. 4–7), transfer to strict anaerobiosis reveals other striking differences. When aerobic steady-state cultures of *C. utilis* are made anaerobic and then pulsed with glucose, a long lag period preceeds the anaerobic formation of ethanol (Fig. 16). In a similar pulse experiment with *S. cerevisiae*, an immediate and rapid ethanol formation is apparent (Fig. 17). As pointed out above the slow adaptation of *C. utilis* to anaerobiosis is not solely due to insufficient levels of pyruvate decarboxylase, since steady-state levels of this enzyme are only four-fold lower than in *S. cerevisiae*. Rather, the formation of acetate and pyruvate by *C. utilis* after anaerobic pulsing (Fig. 16) indicates that alcoholic fermentation may be impeded at the level of redox reactions.

Absence of alcoholic fermentation in *S. cerevisiae* during sugar-limited growth at low dilution rates, and the immediate occurrence of ethanol production after the introduction of anaerobiosis, are well-known factors in the production and functioning of baker's yeast. During large-scale cultivation of the organism in fed-batch cultures, aerobic alcoholic fermentation is suppressed by imposing sugar limitation. However, the potential fermentation capacity of the cells becomes apparent in the anaerobic fermentation of the sugars in the dough.

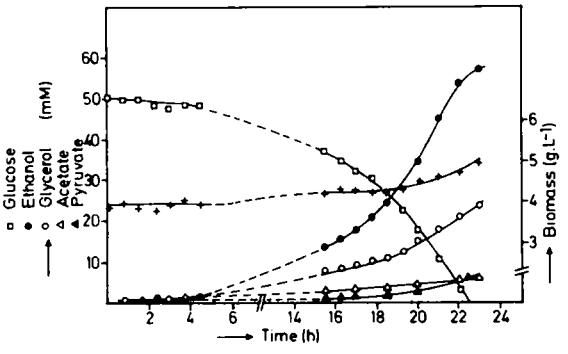

Fig. 16. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *C. utilis* CBS621, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the culture was kept anaerobic by flushing with oxygen-free nitrogen. The simultaneous introduction of excess glucose and anaerobiosis does not result in immediate alcoholic fermentation (J.P. van Dijken et al., unpublished results).

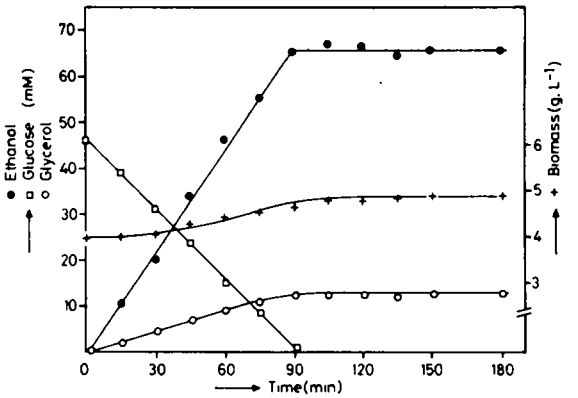

Fig. 17. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *S. cerevisiae* CBS8066, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the culture was kept anaerobic by flushing with oxygen-free nitrogen. The simultaneous introduction of excess glucose and anaerobiosis results in an immediate alcoholic fermentation (J.P. van Dijken et al., unpublished results).

Alcoholic fermentation after a pulse of glucose to chemostat cultures of *S. cerevisiae* proceeds more rapidly under anaerobic than under aerobic conditions (compare Figs. 12 and 17). This phenomenon is known as the Pasteur effect: inhibition of glycolysis in the presence of oxygen [6]. The occurrence of the Pasteur effect is strongly dependent on the physiological status of the cells and is particularly evident in non-growing cells [40].

Present views on the modulation of glycolysis in yeasts by oxygen emphasise a major role for inorganic phosphate. Lagunas and Gancedo [41] have shown that under conditions which enhance the rate of glycolysis, the intracellular level of inorganic phosphate is increased. The concentration of other possible effectors of glycolysis, such as fructose-2,6-bisphosphate, did not correlate with the rate of ethanol formation. Inorganic phosphate is a strong allosteric activator of phosphofructokinase (EC 2.7.1.11) and activation of glycolysis is thought to be effected, at least partly, via this enzyme [41]. Little attention has so far been paid to the crucial T-junction of fermentation and respiration occurring at the level of

pyruvate. At first sight, an activating role for phosphate in alcoholic fermentation seems incompatible with the effects of phosphate on pyruvate decarboxylase. Boiteux and Hess [42] have shown that inorganic phosphate is an inhibitor of pyruvate decarboxylase. Inhibition of the enzyme by physiological concentrations of phosphate is competitive with pyruvate so that the action of phosphate at the level of phosphofructokinase is counteracted. Interestingly, the degree of inhibition of pyruvate decarboxylase by phosphate is higher in Crabtree-positive *S. cerevisiae* than in Crabtree-negative *C. utilis* (Table 2). The physiological significance of these differences in phosphate inhibition of pyruvate decarboxylase remains to be established.

Even Crabtree-positive yeasts do not react uniformly to the introduction of anaerobiosis. When aerobic glucose-limited chemostat cultures of *Brettanomyces intermedius* are made anaerobic and pulsed with glucose, a long lag phase occurs before alcoholic fermentation finally commences (Fig. 18). This contrasts with the behaviour of the same organism under aerobic conditions, where acetic acid and ethanol are formed after pulsing glucose-limited cells (Fig. 14). The negligible metabolic activity in *B. intermedius* after a sudden transfer from aerobiosis to anaerobiosis thus contrasts to the behaviour of *S. cerevisiae* which even exhibits a Pasteur effect (stimulation of alcoholic fermentation under anaerobiosis). This behaviour of *B. intermedius* is known as the Custers effect:

Table 2

Effect of phosphate on the K_m (mM) of pyruvate decarboxylases (EC 4.1.1.1) for pyruvate in *Saccharomyces cerevisiae* CBS8066 and *Candida utilis* CBS621

In both yeasts the inhibition by phosphate was competitive with respect to pyruvate (J.P. van Dijken et al., unpublished results).

Phosphate concentration (mM)	Enzyme source	
	S. cerevisiae	*C. utilis*
0	3.5	3.7
10	7.7	4.5
50	25.0	7.0
100	48.0	11.0

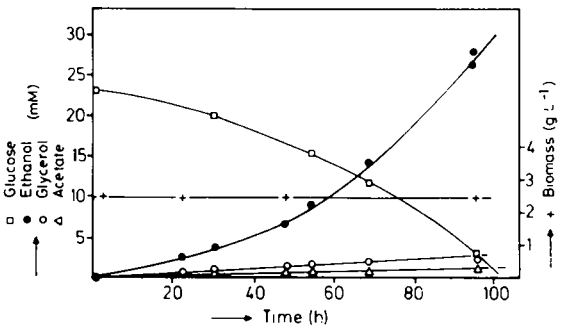

Fig. 18. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *B. intermedius* CBS1943, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the culture was kept anaerobic by flushing with ultra-pure nitrogen. After the simultaneous introduction of excess glucose and anaerobiosis alcoholic fermentation is extremely slow as compared to that in *S. cerevisiae* (Fig. 17). From the fermentation balance it can be deduced that, apart from ethanol, glycerol, acetic acid, and another product (as yet unidentified) are formed (J.P. van Dijken et al., unpublished results).

inhibition of alcoholic fermentation in the absence of oxygen [43]. This (transient) phenomenon is characteristic for all members of the genera *Brettanomyces, Dekkera* and *Eeniella* [44–46]. These yeasts have a strong tendency to form acetic acid. Under anaerobic conditions, formation of acetic acid from glucose leads to overproduction of NADH:

$$1\ \text{glucose} + 4\ \text{NAD}^+ \rightarrow 2\ \text{acetic acid} + 2\ \text{CO}_2 + 4\ \text{NADH} + 4\ \text{H}^+ \quad (6)$$

Formation of acetic acid thus necessitates the formation of reduced products when oxygen is absent. Ethanol formation cannot compensate the production of excess NADH, since the conversion of glucose to ethanol is redox-neutral. That a disturbance of the redox balance is indeed the primary cause of the Custers effect is readily recognised from the effects of exogenous hydrogen acceptors such as acetoin. Acetoin can be reduced in *B. intermedius* by a constitutive butanediol dehydrogenase, according to:

$$\text{acetoin} + \text{NADH} + \text{H}^+ \rightarrow \text{2,3-butanediol} + \text{NAD}^+ \quad (7)$$

When acetoin is added to cultures of *B. inter-*

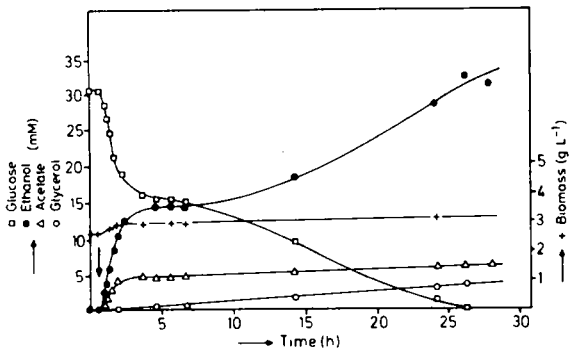

Fig. 19. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *B. intermedius* CBS1943, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the culture was kept anaerobic by flushing with oxygen-free nitrogen. After the simultaneous introduction of excess glucose and anaerobiosis alcoholic fermentation is negligible (see also Fig. 18). However, addition of 10 mM acetoin (indicated by the arrow) results in an immediate and rapid alcoholic fermentation which halts when the acetoin has completely been reduced to 2,3-butanediol (data not shown). This is due to the production of stoichiometric amounts of acetic acid. After a lag phase production of ethanol, but not of acetic acid resumes (J.P. van Dijken et al., unpublished results).

medius which have undergone a shift to anaerobiosis, immediate formation of ethanol and acetic acid is observed (Fig. 19). The rate of product formation is even higher than under strictly aerobic conditions (compare Figs. 19 and 14). This effect of acetoin is observed in all species that display the Custers effect [43]. These yeasts are apparently unable to compensate, via the formation of reduced products such as glycerol, for the overproduction of redox equivalents under anaerobic conditions. When acetoin is exhausted, the culture again becomes metabolically inactive. After some time, however, ethanol formation, accompanied by glycerol production, sets in (Fig. 19). Therefore, the sudden inhibition of glucose metabolism after the introduction of anaerobiosis is due to two factors: a strong tendency to produce acetic acid and the inability to remove, via glycerol production, the excess of redox equivalents generated during this process [10].

The phenomena discussed above clearly illustrate that, under the same environmental conditions, different yeasts show different responses with respect to the occurrence of alcoholic fermentation. However, it must be stressed that even with a single organism the occurrence of the various phenomena (Pasteur effect, Crabtree effect and Custers effect) very much depends on the history of the cells. This, as mentioned above, has clearly been demonstrated for the Pasteur effect [40]. A further example may serve to illustrate the importance of the conditions under which a yeast has been precultured with respect to the phenomenology of alcoholic fermentation.

When aerobic glucose-limited chemostat cultures of *C. utilis* are pulsed with excess glucose, the sugar is converted to CO_2 and biomass (Fig. 20). Aerobic alcoholic fermentation (i.e., a Crabtree effect) which occurs characteristically under similar conditions in *S. cerevisiae* (Figs. 12 and 13) and *B. intermedius* (Fig. 14) is absent in *C. utilis*. However, when this yeast is grown under oxygen limitation on glucose in chemostat culture, a different behaviour is observed. Under steady-state conditions ethanol is produced, due to limited oxygen supply (Fig. 21). When such a culture is pulsed with excess glucose under aerobic conditions, alcoholic fermentation continues, even at an enhanced rate. Thus, when precultivated under oxygen limitation, the Crabtree-negative yeast *C. utilis* may exhibit, although transiently, a Crabtree effect (Fig. 21). This example clearly illustrates the necessity of comparative studies: the Crabtree ef-

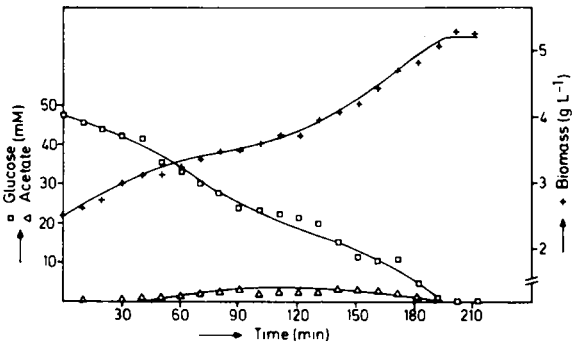

Fig. 20. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *C. utilis* CBS621, pregrown aerobically in a chemostat at $D = 0.1\ h^{-1}$ under glucose limitation. During the experiment the dissolved oxygen tension was kept at 50% air saturation. Glucose is almost completely converted to CO_2 and biomass. Minor amounts of acetic acid are formed, but ethanol production is absent (J.P. van Dijken et al., unpublished results).

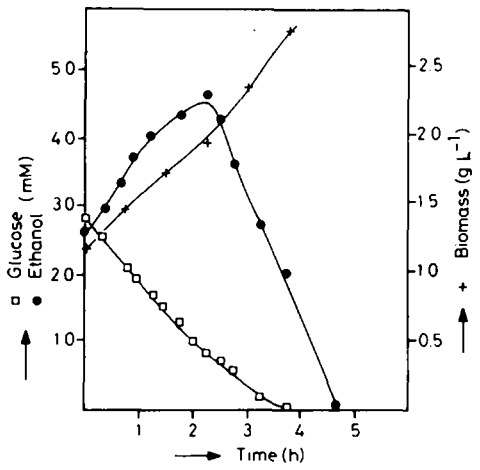

Fig. 21. Growth, glucose consumption and product formation after the addition of excess glucose to a culture of *C. utilis* CBS621, pregrown oxygen-limited at $D = 0.1\ h^{-1}$ with glucose as carbon and energy source. Simultaneously with the addition of glucose also excess oxygen was introduced and this situation (50% air saturation) was maintained throughout the experiment. In the steady-state situation glucose was completely consumed and partially converted to ethanol. It can be calculated that, after introduction of excess glucose, the rate of alcoholic fermentation increases, despite the presence of excess oxygen (J.P. van Dijken et al., unpublished results).

fect in *C. utilis*, pregrown under oxygen limitation, may have a basis similar to that encountered in *S. cerevisiae*.

4. THE $NADP^+$/NADPH BALANCE

Whereas NADH is a reducing equivalent produced and consumed mainly in catabolic reactions, NADPH must be regarded primarily as an anabolic reductant. The regulation of the redox balance with respect to formation and consumption of NADPH is of obvious importance in intermediary sugar metabolism. Bruinenberg et al. [3,47] pointed out that formation of NADPH in *C. utilis* (and probably in other yeasts as well) must occur via central metabolic routes, since transhydrogenase is not detectable in this yeast. During growth on sugars, NADPH may be produced in the hexose monophosphate pathway and the $NADP^+$-linked isocitrate dehydrogenase reaction [3]. If NADPH production via these pathways were to exceed its consumption in the synthesis of amino acids, fatty acids and nucleic acids, additional NADPH-consuming mechanisms would be necessary. Under aerobic conditions oxidation of NADPH by the mitochondria could be involved, whereas under anaerobic conditions formation of products via NADPH-linked reductions are required. The evidence for the absence of transhydrogenase in yeasts and its implications for the metabolism of sugars are discussed below.

4.1. The formation of NADPH and its consumption in anabolic processes

The requirements for NADPH in biosynthetic processes are dependent on the carbon and nitrogen source for growth. In *C. utilis* CBS621 assimilatory nitrate reduction proceeds via NADPH-linked nitrate and nitrite reductases, according to:

$$NO_3^- + 4\ NADPH + 6\ H^+ \rightarrow NH_4^+ + 3\ H_2O + 4\ NADP^+ \quad (8)$$

Thus, whereas during growth of this yeast the formation of 100 g of cell material requires 931 mmol of NADPH (Eqn. 1), during growth with nitrate an additional 680×4 NADPH have to be formed for the reduction of nitrate to the level of ammonia:

$$\begin{aligned} 737\ C_6H_{12}O_6 &+ 680\ HNO_3 + 6\ H_2SO_4 \\ &+ 3659\ NADPH + 1349\ NAD^+ + 2302\ H^+ \\ \rightarrow\ &1000\ C_4H_{7.32}O_{2.24}N_{0.68}S_{0.006}\ (100\ g\ cells) \\ &+ 424\ CO_2 + 3659\ NADP^+ + 1349\ NADH \\ &+ 3398\ H_2O \end{aligned} \quad (9)$$

Since the NADPH for assimilation must be produced via the hexose monophosphate pathway and the $NADP^+$-linked isocitrate dehydrogenase, it is to be expected that the carbon flow over these pathways will be, at least partially, dependent on the NADPH requirement of the assimilation process and will be manifested in the activities of these key enzymes in relation to the carbon and nitrogen source used for growth. Indeed, during growth of *C. utilis* on glucose with nitrate all enzymes of the HMP pathway are significantly higher than during growth with ammonia as the nitrogen source (Table 3) [47]. The activity of $NADP^+$-linked isocitrate dehydrogenase is, how-

Table 3

Activities of HMP pathway enzymes and $NADP^+$-linked isocitrate dehydrogenase in *Candida utilis* CBS621, grown in chemostat cultures under carbon and energy limitation at $D = 0.1\ h^{-1}$ on glucose or xylose, with ammonium or nitrate as the nitrogen source

Data from P.M. Bruinenberg et al., J. Gen. Microbiol. 129 (1983) 965–971.

Enzyme	Glucose		Xylose	
	Ammonium	Nitrate	Ammonium	Nitrate
Glucose-6-Phosphate dehydrogenase	647	1784	1480	2270
6-Phosphogluconate dehydrogenase	298	590	561	722
Transaldolase	102	379	302	456
Transketolase	136	310	248	441
Fructose-1,6-bisphosphatase	55	48	38	65
Isocitrate dehydrogenase	350	494	475	516

ever, not significantly affected by the nitrogen source. This does not necessarily mean that the NAD^+-linked isocitrate dehydrogenase is unimportant for the production of NADPH, since activity measurements in vitro only provide circumstantial information on the flow of carbon. A more direct approach to establishing the relative contributions of the HMP pathway and the $NADP^+$-linked isocitrate dehydrogenase reaction is an estimation of the required flows of carbon via these reactions, on the basis of a theoretical assessment of the NADPH requirement for assimilation [2]. Such calculations show that $NADP^+$-linked isocitrate dehydrogenase alone cannot produce sufficient amounts of NADPH, especially not during growth with nitrate [3]. The following example may serve to illustrate this conclusion. The cell yield of *C. utilis* during growth with nitrate is 0.43 g cells (g glucose)$^{-1}$. Thus, from 1292 mmol glucose, 100 g of cell material is synthesised during carbon-limited growth in chemostat culture. Part of the glucose is required for assimilation (i.e., 737 mmol, Eqn. 1). It follows that from a net amount of 1292 − 737 = 555 mmol glucose, all the NADPH required for assimilation (i.e., 3651 mmol) must be produced. If $NADP^+$-linked isocitrate dehydrogenase were the only source of NADPH, no more than 2 NADPH can be generated for every glucose molecule catabolised, and thus only 1110 NADPH could be produced. This example demonstrates the necessity of an alternative pathway with a high yield of NADPH per mol glucose catabolised. Thus, not only enzymic analyses but also theoretical considerations point to the HMP pathway as an important route for NADPH formation. Only via this pathway can sufficient NADPH be formed to fulfil the requirements for biosynthesis during growth on glucose.

The stoicheiometry of NADPH production via the HMP route depends on the mode of operation of this pathway. When there is no net recycling of the products of this pathway, the following set of equations applies with respect to NADPH production:

$$3\ \text{glucose-6-P} + 6\ NADP^+ \rightarrow 3\ \text{pentose-P} + 3\ CO_2 + 6\ NADPH + 6\ H^+ \quad (10)$$

$$3\ \text{pentose-P} \rightarrow 2\ \text{hexose-P} + 1\ \text{triose-P} \quad (11)$$

$$2\ \text{hexose-P} + 1\ \text{triose-P} + 5\ NADP^+ \rightarrow 15\ CO_2 + 5\ NADPH + 5\ H^+ \quad \text{(via glycolysis and TCA cycle)} \quad (12)$$

Via these reactions, 11/3 mol NADPH is formed per mol of glucose. During growth with nitrate, however, at least 3651/555 = 6.6 mol NADPH per mol glucose catabolised must be produced. Therefore adequate production of NADPH via the HMP pathway and TCA cycle requires recycling of the products of the HMP pathway (Fig. 22).

The NADPH requirement for assimilation and thus the flow of carbon over the NADPH-producing pathways is also strongly dependent on the carbon source for growth. This is due to the fact that the extent to which NADPH production and

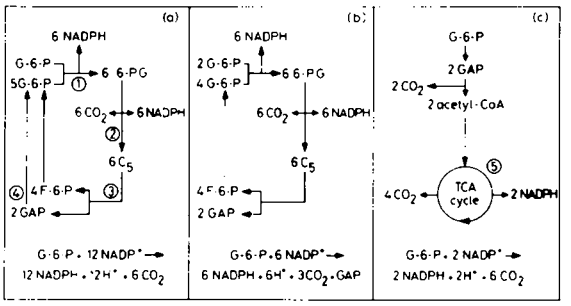

Fig. 22. Generation of NADPH by the HMP pathway and the tricarboxylic acid cycle in yeasts in the case that: (a) pentose phosphates are rearranged to hexose phosphates via transaldolase/transketolase, fructose 1,6-bisphosphate aldolase and fructose 1,6-bisphosphatase; (b) pentose phosphates are rearranged to hexose phosphates during repression of fructose 1,6-biphosphatase; (c) NADPH is only generated via the $NADP^+$-dependent isocitrate dehydrogenase. G-6-P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; F-6-P, fructose 6-phosphate; C_5, pentose phosphate; GAP, glyceraldehyde 3-phosphate. The enzymes catalysing key reactions in these routes have been indicated by numbers (circled): 1, glucose 6-phosphate dehydrogenase; 2, 6-phosphogluconate dehydrogenase; 3, transaldolase and transketolase; 4, fructose 1,6-bisphosphate aldolase and fructose 1,6-bisphosphatase; 5, $NADP^+$-linked isocitrate dehydrogenase (From [3]).

consumption occur during conversion of the growth substrate to intermediates of glycolysis and TCA cycle is variable. During growth of yeasts on glucose, formation of triose phosphates via glycolysis does neither require nor produce NADPH. During growth on xylose, however, at least one NADPH is required for the formation of triose phosphates as a result of the involvement of NADPH-linked xylose reductase in the metabolism of xylose by *C. utilis* [48]. The importance of the HMP pathway as a source of NADPH is again apparent from the levels of its enzymes during growth on xylose. All activities of HMP pathway enzymes are higher in xylose-grown cells than in glucose-grown cells, and are even further increased during growth with nitrate as the nitrogen source (Table 3).

4.2. Dissimilation of NADPH?

Although the function of NADPH as a reductant in biosynthetic processes is now well-established, its function in intermediary metabolism has been debated during the first part of this century. Indeed, NAD^+ was first discovered as one of the agents stimulating in vitro glucose fermentation in yeasts [49] and in muscles [50], but nevertheless the elucidation of the chemical structure of $NADP^+$ [51,52] preceded that of the cozymase (NAD^+). Warburg and coworkers demonstrated that $NADP^+$ was the hydrogen-transferring agent of the reaction catalyzed by the 'Zwischenferment' (i.e., glucose 6-phosphate dehydrogenase). Early schemes of glucose respiration in yeasts implied a major role for the Zwischenferment. The reducing equivalents of this reaction (NADPH) were thought to be the substrate for oxygen-linked respiration via 'old yellow enzyme' [51,52] or 'new yellow enzyme' [53]. In those days, the function of NAD(H) was thought to be restricted to fermentative processes. Only after the work of Lehninger [54] did it become clear that NADH, and not NADPH, is the primary hydrogen donor for the mitochondrial electron transport chain.

Although the distinction between NADH as a catabolic and NADPH as an anabolic reductant holds in general, it is certainly not absolute. In a variety of microorganisms, notably in certain bacteria, NADPH has been shown to be a substrate for the respiratory chain. Also in yeasts NADPH may be re-oxidised to $NADP^+$ with oxygen as the terminal electron acceptor. This may occur in reactions catalysed by mixed-function oxidases (via cytochrome P450, yellow

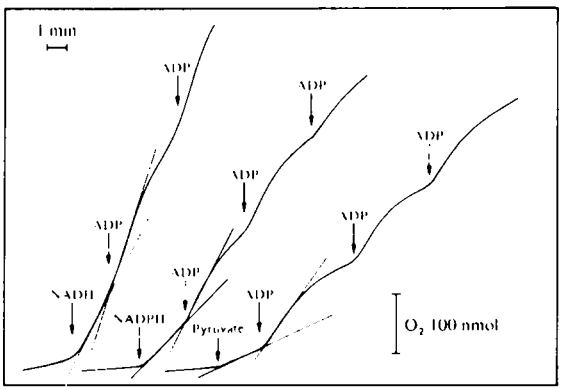

Fig. 23. Recorder tracings of oxygen consumption with NADH, NADPH or pyruvate by isolated mitochondria from *C. utilis* CBS621. Respiratory control is observed after the addition of ADP (From [16]).

enzymes) [55,56]. In addition, NADPH may be oxidised via the mitochondrial electron transport chain. Oxidation of cytoplasmic NADPH by intact mitochondria of the yeast *C. utilis* is subject to respiratory control (Fig. 23) [16]. The ability of yeast mitochondria to oxidise exogenous NADPH with concurrent ATP production indicates that under aerobic conditions overproduction of NADPH (i.e., production in amounts exceeding the requirement for biosynthesis) by the (cytoplasmic) enzymes of the HMP pathway may occur in vivo. It was noticed as early as 1965 by Tempest and Herbert [57] that cells of *C. utilis* grown in xylose-limited chemostat cultures had a much higher glucose-dependent rate of oxygen consumption than glucose-grown cells. They concluded, in our opinion correctly, that this was due to higher levels of HMP-pathway enzymes in *C. utilis* during growth on xylose (Table 3), thus pointing to the possibility of catabolic re-oxidation of NADPH.

4.3. Evidence for the absence of transhydrogenase in yeasts

As mentioned above, transhydrogenase activity ($NADH + NADP^+ \rightarrow NAD^+ + NADPH$) could not be detected in cell-free extracts of *C. utilis* [47]. In vivo studies have confirmed the absence of transhydrogenase activity. This was indicated for instance by the finding that the cell yield on glucose, even in the presence of excess NADH, remains dependent on the NADPH requirement for biosynthesis. If transhydrogenase were absent one would expect the cell yield of a yeast growing on glucose in the presence of excess formate to be lower with nitrate than with ammonium as the nitrogen source. Oxidation of formate to CO_2 by NAD^+-linked formate dehydrogenase would only replace the portion of glucose required for ATP formation via NADH oxidation, but not the part required for NADPH production (Fig. 1). Studies with chemostat cultures of *C. utilis* growing on mixtures of glucose and formate confirm this expectation [58]. During growth with ammonia as the nitrogen source a maximum cell yield of 0.69 g cells (g glucose)$^{-1}$ is obtained, whereas in the presence of nitrate as a nitrogen source cell yields in the presence of excess formate did not increase above 0.56 g cells (g glucose)$^{-1}$ (Figs. 24 and 25). These results strongly support the assumption that the redox equivalents, generated by formate oxidation (i.e., NADH) cannot be used for the production of NADPH.

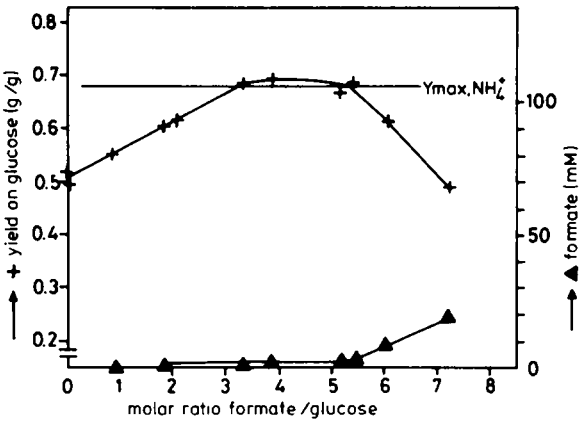

Fig. 24. Cell yields and residual formate concentrations in aerobic chemostat cultures of *C. utilis* CBS621, growing on mixtures of glucose and formate, with ammonium as the nitrogen source. The maximum growth yield is obtained at a formate: glucose ratio of 3.3 when the culture becomes carbon-limited. Y_{max}, NH_4^+ = maximum theoretical yield on glucose with ammonium as the nitrogen source (From [58]).

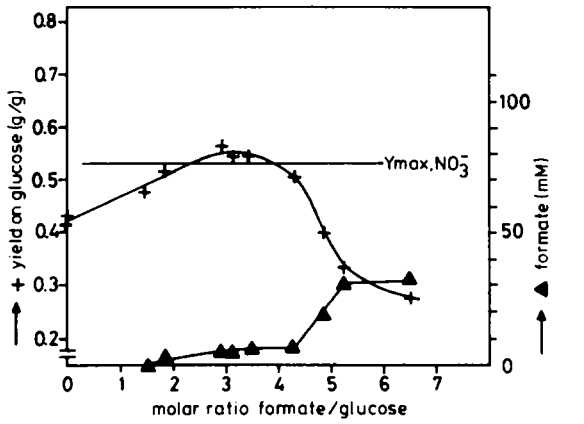

Fig. 25. Cell yields and residual formate concentrations in aerobic chemostat cultures of *C. utilis* CBS621 growing on mixtures of glucose and formate with nitrate as the nitrogen source. The maximum growth yield is lower than with ammonium as the nitrogen source and is obtained at a formate: glucose ratio of 2.9. Y_{max}, NO_3^- = maximum theoretical yield on glucose with nitrate as the nitrogen source (From [58]).

4.4. Activity of the HMP pathway in yeasts

The absence of transhydrogenase activity in yeasts has an important bearing on the activity of the NADPH-producing pathways. For balanced growth, the rate of NADPH production must at least equal its rate of consumption in assimilatory processes. If such a balance were maintained, then the amount of sugar catabolised via the HMP pathway should be approximately the same for different yeasts of the same biomass composition.

In the literature, widely diverging values have been reported for HMP pathway activities in yeasts as calculated from the results of radiorespirometric studies. For *S. cerevisiae,* estimates of the contribution of the HMP pathway to glucose metabolism range from 2.5% [59] to 34% [60]. In *Candida utilis,* values between 30% [61] and 72% [60] have been reported, whereas in *Rhodosporidium toruloides* (formerly *Rhodotorula gracilis*), between 20 and 80% of the glucose may be metabolised via this pathway [62,63]. It is difficult to compare these values, since different growth conditions and different calculation methods have been used in these studies. P.M. Bruinenberg et al. (unpublished results), using radiorespirometry, performed a steady-state analysis of the activities of these three yeasts growing in chemostat cultures. Their data (Table 4) indicate that the contribution of the HMP pathway to glucose metabolism is similar in these three yeasts when grown glucose-limited at the same dilution rate.

Table 4

Fraction of radioactivity, recovered as $^{14}CO_2$ during continuous substrate feeding to glucose-limited cultures of yeasts grown at $D = 0.1\ h^{-1}$ with ammonium as the nitrogen source

The percentages of glucose metabolism via the HMP pathway were calculated according to Katz and Wood [65]. Cell yields are expressed as g dry wt. (g glucose)$^{-1}$. Data from P.M. Bruinenberg et al., unpublished results.

	Saccharomyces cerevisiae	*Candida utilis*	*Rhodosporidium toruloides*
[U-^{14}C]Glucose	0.33	0.40	0.31
[1-^{14}C]Glucose	0.37	0.33	0.37
[6-^{14}C]Glucose	0.21	0.18	0.13
Contribution of HMP pathway to glucose metabolism (%)	8	7	11
Cell yield	0.51	0.51	0.50

The high values of the HMP pathway's contribution to glucose metabolism reported by various authors may in part be explained by the calculation methods used. With the formula of Wang and Krackov [64], which does not take recycling of hexose phosphates into account, the values are approximately twice those obtained with the formula of Katz and Wood [65], which covers possible recycling. Different values obtained for a single yeast probably also result from differences in growth conditions. Radiorespirometric studies are usually performed with batch cultures, in which different organisms have different growth rates. Studies with chemostat cultures are rare [60,66] and even when these have been used, the cells taken were subjected to transient-state analysis. Mian et al. [60], for example, studied washed cell suspensions in the absence of ammonium. Since ammonium is an allosteric activator of phosphofructokinase [67,68], its absence may lead to metabolism of a higher proportion of the glucose via the HMP pathway. Furthermore, as pointed out in a previous paragraph, pulsing of yeast cells with glucose may lead to a drastic rearrangement of metabolism, the nature and extent of which is dependent on the yeast species. Therefore the conclusion is justified that comparative radiorespirometric studies can only adequately be performed with steady-state cultures, fed with different specifically labelled glucose substrates from the reservoir, and not by pulse analysis.

In vivo analysis of HMP pathway activities in steady-state cultures of *C. utilis* (Table 5) confirm the conclusion from the enzymic analyses (Table 3) that the flow of carbon via this pathway is dependent on cultivation conditions. When nitrate serves as the nitrogen source for *C. utilis,* the flow of carbon over the HMP pathway is increased three-fold as compared to growth with ammonium. When excess formate is present as an additional source of NADH, the ratio of CO_2 from [1-^{14}C]glucose to CO_2 from [6-^{14}C]glucose drastically increases (P.M. Bruinenberg et al., unpublished results). This is due to the sparing effect of formate on glucose catabolism via the TCA cycle. However, formate does not replace the por-

Table 5

Fraction of radioactivity, recovered as $^{14}CO_2$ during continuous substrate feeding to glucose-limited cultures of *Candida utilis* CBS621 grown at $D = 0.1$ h^{-1} with ammonium or nitrate as the nitrogen source, or with additional formate in the medium with ammonium as the nitrogen source

The percentages of glucose metabolism via the HMP pathway were calculated according to Katz and Wood [65]. Cell yields are expressed as g dry wt. (g glucose)$^{-1}$. Data from P.M. Bruinenberg et al., unpublished results.

	Glucose		Glucose + formate
	Ammonium	Nitrate	Ammonium
[U-^{14}C]Glucose	0.40	0.47	0.23
[1-^{14}C]Glucose	0.33	0.59	0.32
[6-^{14}C]Glucose	0.18	0.24	0.06
Contribution of HMP pathway to glucose metabolism (%)	7	21	11
Cell yield	0.51	0.43	0.69

tion of glucose metabolized via the HMP pathway, which remains active for the production of NADPH. These results (Table 5) provide additional evidence that the NADH produced during formate oxidation cannot serve as a source of NADPH via transhydrogenase.

The flow of glucose via the HMP pathway, required to produce the NADPH for biosynthesis, cannot be precisely evaluated theoretically. This is due to the unknown contribution of $NADP^+$-linked isocitrate dehydrogenase to the overall NADPH production. When, however, NADPH formation via this reaction is neglected, theoretical minimum values of HMP pathway activities are calculated which are close to the values obtained from radiorespirometric studies (P.M. Bruinenberg et al., unpublished results). This indicates that dissimilation of NADPH via the mitochondrial electron transport chain is not very important in quantitative terms. Rather, the amount of NADPH produced in the HMP pathway seems to be adjusted to the amount required for biosynthesis. The balance of NADPH production and consumption is thus primarily maintained at the production level, via adjustments of enzyme levels (Table 3) and modulation of enzyme activities. The latter control mechanism is probably accomplished via inhibition of glucose 6-phosphate dehydrogenase by NADPH [69,70].

5. INTERCONNECTIONS BETWEEN THE NAD(H) AND NADP(H) BALANCES

Under aerobic conditions yeasts may balance any 'overproduction' of redox equivalents by mitochondrial respiration. Under anaerobic conditions, on the other hand, a redox balance must be obtained for both nicotinamide adenine dinucleotide coenzyme systems separately via product formation. For example, production of NADPH in amounts exceeding the requirements of assimilation can only be compensated via NADPH-linked reductions. Similarly, overproduction of NADH can only be balanced by NADH-consuming reactions. Production and consumption of NADPH and NADH are thus entirely separated processes, though NAD(H) and NADP(H)-linked reactions may be tightly coupled in a single metabolic pathway. This holds, for example, for the metabolism of xylose in yeasts (Fig. 26). In *C. utilis* and many other yeasts the metabolism of xylose proceeds via an initial reduction to xylitol with an NADPH-linked xylose reductase. Subsequently xylitol is converted to xylulose by an NAD^+-linked xylitol dehydrogenase. Thus, as a consequence of the absence of transhydrogenase, the reduction equivalents produced in the second reaction cannot be used for the initial reduction step. The metabolic implications of this mode of xylose metabolism are discussed below.

Of the 439 species listed by Barnett et al. [28], 291 are known as facultatively fermentative with respect to metabolism of glucose. Of all yeasts, 317 possess the ability to grow on xylose aerobically and 193 species share both properties. Significantly, only very few yeasts have been found to ferment xylose anaerobically [71]. This phenomenon has been called a Kluyver effect [72] and is defined as the inability of a facultatively fermentative yeast to metabolise a certain sugar anaerobically although the sugar is rapidly metabolised aerobically. This is a general phenomenon observed for many sugars in a large variety of yeasts [72]. Apparently these yeasts cannot combine their

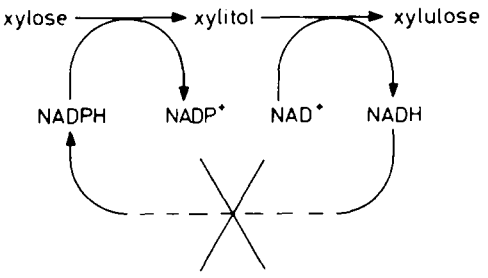

Fig. 26. Initial reactions of xylose metabolism in yeasts. Due to the absence of transhydrogenase activity, the NADH produced in the conversion of xylitol to xylulose cannot be used for the NADPH-linked reduction of D-xylose to xylitol.

fermentative capacities with anaerobic conversion of such sugars. In many cases the metabolic basis for this incapacity is not known. For xylose, however, it has become clear that a deregulation of the redox balance prevents anaerobic alcoholic fermentation of the sugar.

When aerobic batch cultures of *C. utilis* growing on xylose as a carbon and energy source are shifted to anaerobic conditions, growth and xylose consumption are arrested (Fig. 27). This is not due to the absence of the key enzymes of the fermentative route (i.e., pyruvate decarboxylase and alcohol dehydrogenase). When glucose is added to anaerobic xylose-grown *C. utilis,* the hexose is rapidly fermented to ethanol [48]. The metabolic inactivity of *C. utilis* towards xylose under anaerobic conditions apparently resides in the initial steps of xylose metabolism. When xylose isomerase is added to such cultures, the blockade in xylose metabolism is relieved (Fig. 27). Apparently, xylulose can be fermented when provided extracellularly via xylose isomerase, but cannot be formed intracellularly via xylose reductase and xylitol dehydrogenase.

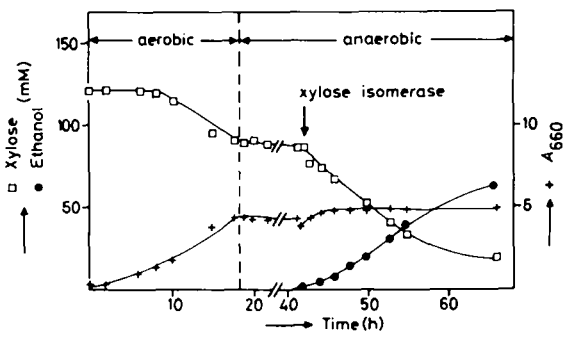

Fig. 27. Effect of anaerobiosis on xylose metabolism in batch cultures of *Candida utilis* CBS621. Addition of xylose isomerase results in alcoholic fermentation of xylose (From [48]).

Only a few yeasts are capable of anaerobic alcoholic fermentation of xylose [71]. Interestingly, in all xylose-fermenting yeasts investigated to date, the reduction of xylose to xylitol may proceed with both NADPH and NADH. In *Pichia stipitis* the ratio between the NADH- and NADPH-linked xylose reductase activities was independent of the cultivation conditions and both activities co-purified to a homogenous preparation [73]. In *Pachysolen tannophilus,* on the other hand, the ratio between the two activities varied. In contrast to *P. stipitis* which possesses only one xylose reductase, active with both NADH and NADPH, *P. tannophilus* contains two xylose reductases. One enzyme is specific for NADPH and the other reacts with both NADPH and NADH [74]. The presence of NADPH- and NADH-linked xylose reductase activities, whether or not catalysed by a single enzyme, in the few yeasts capable of anaerobic xylose fermentation provides a clue to their exceptional behaviour.

In *Candida utilis,* metabolism of xylose via NADPH-linked xylose reductase would lead to an overproduction of NADH under anaerobic conditions (Fig. 28). This would explain the organism's

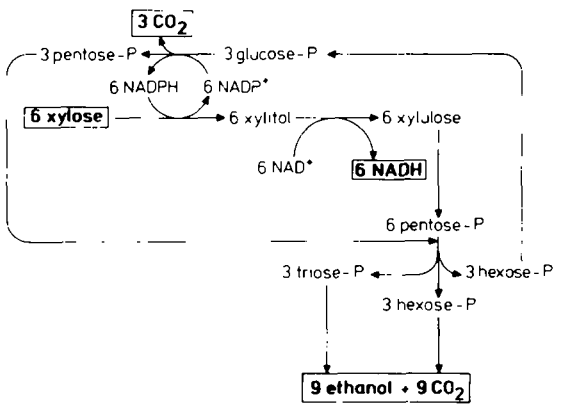

Fig. 28. Schematic representation of xylose metabolism via NADPH-linked xylose reductase in the case that alcoholic fermentation would occur. The scheme makes clear that this would lead to a discrepancy between production and consumption of NADH in the overall conversion of xylose to ethanol (From [48]).

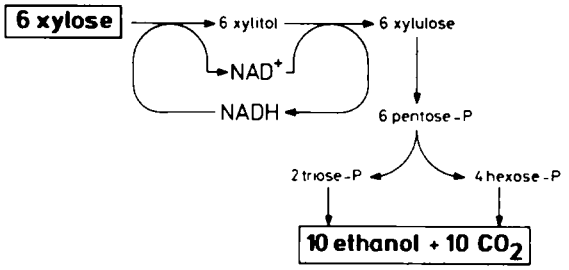

Fig. 29. Schematic representation of xylose metabolism via NADH-linked xylose reductase. Formation of ethanol can proceed with a closed redox balance (From P.M. Bruinenberg et al. (1984) Appl. Microbiol. Biotechnol. 19, 256–269).

inability to ferment xylose in contrast to its ability to ferment xylulose, since with this latter substrate overproduction of NADH does not occur. In organisms like *Pichia stipitis* the NADH produced by xylitol dehydrogenase can be used for the initial reduction of xylose, overproduction of NADH need not occur (Fig. 29), and a relatively rapid anaerobic xylose fermentation is possible (Fig. 30).

The metabolic schemes depicted in Figs. 28 and 29 are based on the formation of triose and hexose phosphates via transaldolase and transketolase reactions. Recent work of Ratledge and coworkers has provided evidence in favour of another route of pentose phosphate metabolism, namely via phosphoketolase. During aerobic growth of a variety of yeasts on xylose, induction of phos-

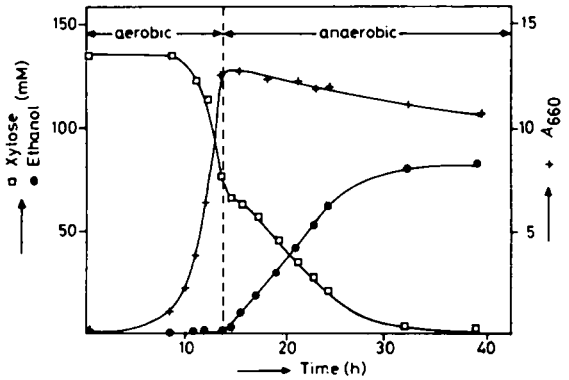

Fig. 30. Shift from aerobic to anaerobic conditions in a batch culture of *Pichia stipitis* CBS5773 with D-xylose as the substrate. In contrast to *C. utilis*, *P. stipitis* exhibits alcoholic fermentation under anaerobic conditions (From P.M. Bruinenberg et al. (1984) Appl. Microbiol. Biotechnol. 19, 256–269).

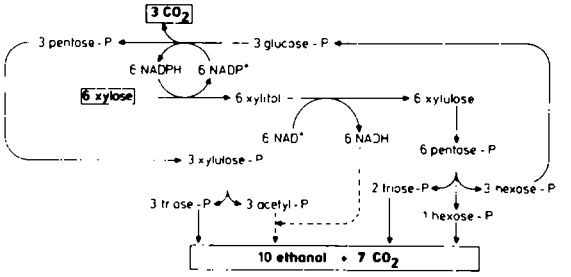

Fig. 31. Schematic representation of xylose metabolism, indicating the consequences of the involvement of NADPH-linked xylose reductase in combination with phosphoketolase (Eqn. 13). Formation of ethanol under anaerobic conditions with a closed redox balance is only possible in case acetyl phosphate is converted to ethanol via NADH-linked enzymes.

phoketolase activity was observed [63,75]. This enzyme catalyzes the direct conversion of xylulose 5-phosphate to triose phosphate:

$$\text{xylulose 5-P} + P_i \rightarrow \text{glyceraldehyde 3-P} + \text{acetyl-P} \quad (13)$$

A scheme based on the functioning of phosphoketolase in xylose metabolism is shown in Fig. 31. From this figure it is clear that metabolism of xylose via NADPH-linked xylose reductase and phosphoketolase still requires the functioning of transaldolase and transketolase to provide the HMP pathway with hexose phosphates for the formation of NADPH. However, in this case, metabolism of xylose via an NADPH-linked reductase is no longer incompatible with anaerobic

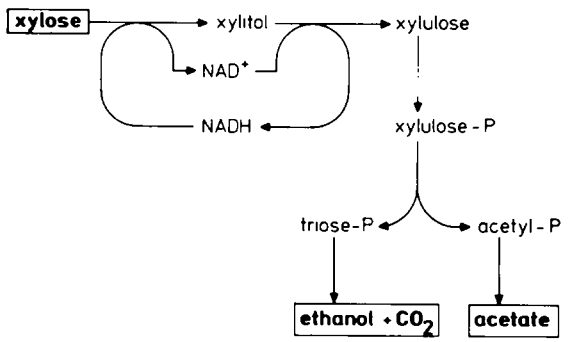

Fig. 32. Schematic representation of alcoholic fermentation of xylose via NADH-linked xylose reductase and phosphoketolase. Under anaerobic conditions a closed redox balance is only obtained when equal amounts of ethanol and acetic acid are formed.

alcoholic fermentation. The NADH produced in the conversion of xylitol to xylulose could be used for the reduction of acetyl phosphate to ethanol. Such a scheme, on the other hand, does not explain the marked differences in behaviour of *C. utilis* towards xylose and xylulose under anaerobic conditions. Moreover, in the case that xylose metabolism proceeds via NADH-linked xylose reductase the involvement of phosphoketolase would lead to a shortage of NADH unless ethanol and acetic acid are produced in stoichiometric amounts (Fig. 32). However, experiments with *P. stipitis* (Fig. 30) show that the major product of xylose fermentation is ethanol, and acetic acid is only produced in minor quantities. It can thus be concluded that although phosphoketolase is most likely a key enzyme in aerobic metabolism of xylose in yeasts [63], under anaerobic conditions this enzyme is probably by-passed.

6. CONCLUDING REMARKS

In this review, we have illustrated the crucial role of redox balances in sugar metabolism by yeasts. The absence of transhydrogenase activity in yeasts implies that redox equivalents cannot be exchanged between the two nicotinamide adenine dinucleotide systems, NAD(H) and NADP(H). This may be regarded as 'chemical compartmentation'. Also, mitochondria and other organelles in the yeast cell, being separated from the cytosol by membranes impermeable to the coenzymes, must each maintain their own redox balances for both coenzyme systems.

This is of significance for the formation of biomass and fermentation products, as exemplified by experiments on glucose metabolism in some model organisms under various environmental conditions. Moreover, also the interrelations between the NAD(H) and NADP(H) balances in yeasts may have important consequences, as is illustrated by studies on alcoholic fermentation of xylose.

In view of the complexities of redox balances in yeasts, further studies are needed, especially regarding the metabolism of other sugars. The fundamental insights anticipated might lead to improvements in current processes, as well as to the development of novel applications of a variety of yeasts.

ACKNOWLEDGEMENTS

We are indebted to Dr. P.M. Bruinenberg, G.B. Houweling-Tan, R. Jonker, E. van den Bosch, M.R. Wijsman, C. Verduyn, J. Kempers, J.J. Meijer, H. van Urk, J.J. Hermans, G.J. Breedveld, G.W. Waslander and P.E.J. Groenewegen for contributing unpublished results.

REFERENCES

[1] Lagunas, R. and Gancedo, J.M. (1973) Reduced pyridine-nucleotides balance in glucose-growing *Saccharomyces cerevisiae*. Eur. J. Biochem. 37, 90–94.
[2] Oura, E. (1972) The effect of aeration on the growth energetics and biochemical composition of baker's yeast. Ph.D. Thesis, University of Helsinki.
[3] Bruinenberg, P.M., van Dijken, J.P. and Scheffers, W.A. (1983) A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. 129, 953–964.
[4] Horecker, B.L. (1965) Pathways of carbohydrate metabolism and their physiological significance. J. Chem. Education 42, 244–253.
[5] Evans, C.T., Scragg, A.H. and Ratledge, C. (1983) A comparative study of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts. Eur. J. Biochem. 130, 195–204.
[6] Fiechter, A., Fuhrmann, G.F. and Käppeli, O. (1981) Regulation of glucose metabolism in growing yeast cells. Adv. Microb. Physiol. 22, 123–183.
[7] Neuberg, C. and Reinfurth, E. (1918) Natürliche und erzwungene Glycerinbildung bei der alkoholischen Gärung. Biochem. Z. 92, 234–266.
[8] Holzer, H., Bernhardt, W. and Schneider, S. (1963) Zur Glycerinbildung in Bäckerhefe. Biochem. Z. 336, 495–509.
[9] Nickerson, W.J. and Carroll, W.R. (1945) On the metabolism of *Zygosaccharomyces*. Arch. Biochem. 7, 257–271.
[10] Scheffers, W.A., van Dijken, J.P., Kaytan, G., Kloosterman, M., Wijsman, M.R. and van Kleeff, B.H.A. (1982) Effect of oxygen on growth, alcohol, acetic acid, and glycerol production by the yeasts *Brettanomyces intermedius* and *Zygosaccharomyces bailii*, in Fünftes Symposium Technische Mikrobiologie (Dellweg, H., Ed.) pp. 214–220. Institut für Gärungsgewerbe, Berlin.
[11] Perlman, P.S. and Mahler, H.R. (1970) Intracellular localization of enzymes in yeast. Arch. Biochem. Biophys. 136, 245–259.

[12] Dawson, A.G. (1979) Oxidation of cytosolic NADH formed during aerobic metabolism in mammalian cells. Trends Biochem. Sci. 4, 171–176.

[13] Schwitzguébel, J.P. and Palmer, J.M. (1981) Properties of mitochondria isolated from *Neurospora crassa* grown with acetate. FEMS Mirobiol. Lett. 11, 273–277.

[14] Palmer, J.M. and Møller, I.M. (1982) Regulation of NAD(P)H dehydrogenases in plant mitochondria. Trends Biochem. Sci. 7, 258–261.

[15] Møller, I.M. and Palmer, J.M. (1981) The inhibition of exogenous NAD(P)H oxidation in plant mitochondria by chelators and mersalyl as a function of pH. Physiol. Plant. 53, 413–420.

[16] Bruinenberg, P.M., van Dijken, J.P., Kuenen, J.G. and Scheffers, W.A. (1985) Oxidation of NADH and NADPH by mitochondria from the yeast *Candida utilis*. J. Gen. Microbiol. 131, 1043–1051.

[17] Haarasilta, S. and Taskinen, L. (1977) Location of three key enzymes of gluconeogenesis in baker's yeast. Arch. Microbiol. 113, 159–161.

[18] Andreasen, A.A. and Stier, T.J.B. (1953) Anaerobic nutrition of *Saccharomyces cerevisiae*, I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. 41, 23–36.

[19] Andreasen, A.A. and Stier, T.J.B. (1954) Anaerobic nutrition of *Saccharomyces cerevisiae*, II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell Comp. Physiol. 43, 271–281.

[20] Schatzmann, H. (1975) Anaerobes Wachstum von *Saccharomyces cerevisiae*. Ph.D. Thesis, ETH Zürich.

[21] Šubík, J., Kolarov, J. and Kováč, L. (1972) Obligatory requirement of intramitochondrial ATP for normal functioning of the eucaryotic cell. Biochem. Biophys. Res. Commun. 49, 192–198.

[22] Gbelská, Y., Šubík, J., Svoboda, A., Goffeau, A. and Kováč, L. (1983) Intramitochondrial ATP and cell functions: yeast cells depleted of intramitochondrial ATP lose the ability to grow and multiply. Eur. J. Biochem. 130, 281–286.

[23] Ephrussi, B. (1953) Nucleo-cytoplasmic Relations in Microorganisms. Clarendon Press, Oxford.

[24] Ephrussi, B., Slonimski, P.P., Yotsuyanagi, Y. and Tavlitzki, J. (1956) Variations physiologiques et cytologiques de la levure au cours du cycle de la croissance aérobie. Compt. Rend. Trav. Lab. Carlsberg, Sér. Physiol. 26, 87–102.

[25] Bulder, C.J.E.A. (1963) On respiratory deficiency in yeasts. Ph.D. Thesis, Delft University of Technology, Delft, The Netherlands.

[26] De Deken, R.H. (1966) The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44, 149–156.

[27] De Deken, R.H. (1966) The Crabtree effect and its relation to the petite mutation. J. Gen. Microbiol. 44, 157–165.

[28] Barnett, J.A., Payne, R.W. and Yarrow, D. (1979) A Guide to Identifying and Classifying Yeasts. Cambridge University Press, Cambridge.

[29] Moss, F.J., Rickard, P.A.D., Beech, G.A. and Bush, F.E. (1969) The response by microorganisms to steady state growth in controlled concentrations of oxygen and glucose. I. *Candida utilis*. Biotechnol. Bioeng. 11, 561–580.

[30] Harrison, D.E.F. (1972) Physiological effects of dissolved oxygen tension and redox potential on growing populations of micro-organisms. J. Appl. Chem. Biotechnol. 22, 417–440.

[31] Harrison, D.E.F. (1973) Growth, oxygen and respiration. CRC Crit. Rev. Microbiol. 2, 185–228.

[32] Wijsman, M.R., van Dijken, J.P., van Kleeff, B.H.A. and Scheffers, W.A. (1984) Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect). Antonie van Leeuwenhoek 50, 183–192.

[33] Wöhrer, W., Forstenlehner, L. and Röhr, M. (1981) Evaluation of the Crabtree effect in different yeasts grown in batch and continuous culture, in Current Developments in Yeast Research (Stewart, G.G. and Russell, I., Eds.) pp. 405–410. Pergamon Press, Toronto.

[34] Verduyn, C., Zomerdijk, T.P.L., van Dijken, J.P. and Scheffers, W.A. (1984) Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. Appl. Microbiol. Biotechnol. 19, 181–185.

[35] Barford, J.P. and Hall, R.J. (1979) An examination of the Crabtree-effect in *Saccharomyces cerevisiae*: the role of respiratory adaptation. J. Gen. Microbiol. 114, 267–275.

[36] Barford, J.P., Jeffery, P.M. and Hall, R.J. (1981) The Crabtree-effect in *Saccharomyces cerevisiae* —primary control mechanism or transient? in Current Developments in Yeast Research (Stewart, G.G. and Russell, I., Eds.) pp. 255–260. Pergamon Press, Toronto.

[37] Petrik, M., Käppeli, O. and Fiechter, A. (1983) An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short- and long-term regulation. J. Gen. Microbiol. 129, 43–49.

[38] Rieger, M., Käppeli, O. and Fiechter, A. (1983) The role of a limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae*. J. Gen. Microbiol. 129, 653–661.

[39] Käppeli, O., Arreguin, M. and Rieger, M. (1985) The respirative breakdown of glucose by *Saccharomyces cerevisiae*: an assessment of a physiological state. J. Gen. Microbiol. 131, 1411–1416.

[40] Lagunas, R., Dominguez, C., Busturia, A. and Sáez, M.J. (1982) Mechanisms of appearance of the Pasteur effect in *Saccharomyces cerevisiae*: inactivation of sugar transport systems. J. Bacteriol. 152, 19–25.

[41] Lagunas, R. and Gancedo, C. (1983) Role of phosphate in the regulation of the Pasteur effect in *Saccharomyces cerevisiae*. Eur. J. Biochem. 137, 479–483.

[42] Boiteux, A. and Hess, B. (1970) Allosteric properties of yeast pyruvate decarboxylase. FEBS Lett. 9, 293–296.

[43] Scheffers, W.A. (1966) Stimulation of fermentation in yeasts by acetoin and oxygen. Nature 210, 533–534.

[44] Scheffers, W.A. (1961) On the inhibition of alcoholic

fermentation in *Brettanomyces* yeasts under anaerobic conditions. Experientia 17, 40–42.

[45] Wikén, T., Scheffers, W.A. and Verhaar, A.J.M. (1961) On the existence of a negative Pasteur effect in yeasts classified in the genus *Brettanomyces* Kufferath et Van Laer. Antonie van Leeuwenhoek 27, 401–433.

[46] Scheffers, W.A. and Wikén, T.O. (1969) The Custers effect (negative Pasteur effect) as a diagnostic criterion for the genus *Brettanomyces*. Antonie van Leeuwenhoek 35, Suppl. Yeast Symp. A31–A32.

[47] Bruinenberg, P.M., van Dijken, J.P. and Scheffers, W.A. (1983) An enzymic analysis of NADPH production and consumption in *Candida utilis*. J. Gen. Microbiol. 129, 965–971.

[48] Bruinenberg, P.M., de Bot, P.H.M., van Dijken, J.P. and Scheffers, W.A. (1983) The role of redox balances in the anaerobic fermentation of xylose by yeasts. Eur. J. Appl. Microbiol. Biotechnol. 18, 287–292.

[49] Harden, A. and Young, W.J. (1906) The alcoholic ferment of yeast-juice, Part II. The coferment of yeast-juice. Proc. R. Soc. Lond. Ser. B 78, 369–375.

[50] Meyerhof, O. (1918) Über das Vorkommen des Coferments der alkoholischen Hefegärung im Muskelgewebe und seine Bedeutung im Atmungsmechanismus. Z. Physiol. Chem. 101, 165–175.

[51] Warburg, O. and Christian, W. (1932) Über ein neues Oxydationsferment und sein Absorptionsspektrum. Biochem. Z. 254, 438–458.

[52] Warburg, O. and Christian, W. (1936) Pyridine, der wasserstoffübertragende Bestandteil von Gärungsfermenten (Pyridin-Nucleotide). Biochem. Z. 287, 291–328.

[53] Haas, E., Horecker, B.L. and Hogness, T.R. (1940) The enzymatic reduction of cytochrome *c*. Cytochrome *c* reductase. J. Biol. Chem. 136, 747–774.

[54] Lehninger, A.L. (1951) Oxidative phosphorylation in diphosphopyridine nucleotide-linked systems, in Phosphorus Metabolism. A Symposium on the Role of Phosphorus in the Metabolism of Plants and Animals, Vol. I (McElroy, W.D. and Glass, B., Eds.), pp. 344–366. The Johns Hopkins Press, Baltimore.

[55] Kärenlampi, S.O., Marin, E. and Hänninen, O.O.P. (1981) Effect of carbon source on the accumulation of cytochrome P-450 in the yeast *Saccharomyces cerevisiae*. Biochem. J. 194, 407–413.

[56] Trinn, M., Käppeli, O. and Fiechter, A. (1982) Occurrence of cytochrome P450 in continuous cultures of *Saccharomyces cerevisiae*. Eur. J. Appl. Microbiol. Biotechnol. 15, 64–68.

[57] Tempest, D.W. and Herbert, D. (1965) Effect of dilution rate and growth-limiting substrate on the metabolic activity of *Torula utilis* cultures. J. Gen. Microbiol. 41, 143–150.

[58] Bruinenberg, P.M., Jonker, R., van Dijken, J.P. and Scheffers, W.A. (1985) Utilization of formate as an additional energy source by glucose-limited chemostat cultures of *Candida utilis* CBS621 and *Saccharomyces cerevisiae* CBS8066. Arch. Microbiol. 142, 302–306.

[59] Gancedo, J.M. and Lagunas, R. (1973) Contribution of the pentose-phosphate pathway to glucose metabolism in *Saccharomyces cerevisiae*: a critical analysis of the use of labelled glucose. Plant Sci. Lett. 1, 193–200.

[60] Mian, F.A., Fencl, Z., Prokop, A., Mohagheghi, A. and Fazeli, A. (1974) Effect of growth rate on the glucose metabolism of yeast grown in continuous culture. Radiorespirometric studies. Folia Microbiol. (Prague) 19, 191–198.

[61] Blumenthal, H.J., Lewis, K.F. and Weinhouse, S. (1954) An estimation of pathways of glucose catabolism in yeast. J. Am. Chem. Soc. 76, 6093–6097.

[62] Höfer, M. (1968) Estimation of pathways of glucose catabolism in *Rhodotorula gracilis*. Folia Microbiol. (Prague) 13, 373–378.

[63] Evans, C.T. and Ratledge, C. (1984) Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: the key to efficient xylose metabolism. Arch. Microbiol. 139, 48–52.

[64] Wang, C.H. and Krackov, J.K. (1962) The catabolic fate of glucose in *Bacillus subtilis*. J. Biol. Chem. 237, 3614–3622.

[65] Katz, J. and Wood, H.G. (1963) The use of $C^{14}O_2$ yields from glucose-1 and -6-C^{14} for the evaluation of the pathways of glucose metabolism. J. Biol. Chem. 238, 517–523.

[66] Dawson, P.S.S., Okada, W. and Steinhauer, L.P. (1976) Some comparative observations on the relative contributions of alternate pathways in the metabolism of glucose by *Candida utilis*. Can. J. Microbiol. 22, 996–1001.

[67] Bloxham, D.P. and Lardy, H.A. (1973) Phosphofructokinase, in The Enzymes, Vol. 8, 3rd ed. (Boyer, P.D., Ed.) pp. 239–278. Academic Press, New York and London.

[68] Mavis, R.D. and Stellwagen, E. (1970) The role of cations in yeast phosphofructokinase catalysis. J. Biol. Chem. 245, 674–680.

[69] Bonsignore, A. and De Flora, A. (1972) Regulatory properties of glucose-6-phosphate dehydrogenase. Curr. Top. Cell. Regul. 6, 21–62.

[70] Levy, H.R. (1979) Glucose-6-phosphate dehydrogenases. Adv. Enzymol. 48, 97–192.

[71] Toivola, A., Yarrow, D., van den Bosch, E., van Dijken, J.P. and Scheffers, W.A. (1984) Alcoholic fermentation of D-xylose by yeasts. Appl. Environm. Microbiol. 47, 1221–1223.

[72] Sims, A.P. and Barnett, J.A. (1978) The requirement of oxygen for the utilization of maltose, cellobiose and D-galactose by certain anaerobically fermenting yeasts (Kluyver effect). J. Gen. Microbiol. 106, 277–288.

[73] Verduyn, C., van Kleef, R., Frank, J., Schreuder, H., van Dijken, J.P. and Scheffers, W.A. (1985) Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. Biochem. J. 226, 669–677.

[74] Verduyn, C., Frank, J., van Dijken, J.P. and Scheffers, W.A. (1985) Multiple forms of xylose reductase in *Pachysolen tannophilus* CBS 4044. FEMS Microbiol. Lett. 30, 313–317.

[75] Radledge, C. and Holdsworth, J.E. (1985) Properties of a pentulose-5-phosphate phosphoketolase from yeasts grown on xylose. Appl. Microbiol. Biotechnol. 22, 217–221.