Crabtree Effect

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CRABTREE EFFECT

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INTRODUCTION

Historical Considerations

With the advent of Warburg manometric procedures it became possible to have a more quantitative picture of cellular respiration (1). Warburg observed that malignant tissue, as well as certain yeast strains have the dual capacity to metabolize carbohydrates through oxidative (respiration) and fermentative (glycolysis[1]) processes (2). One characteristic of malignant cells was that even in aerobic conditions both metabolic routes coexisted. Moreover, the ratio of aerobic glycolysis/respiration of tumor tissues was found to be around three, whereas it was small or zero for normal tissues. This generalization however suffered exceptions and finally Crabtree (3) established that the enhanced ratio was apparently quite a common feature shared by several fast growing tissues, malignant or not. He also noted that "the glycolytic activity of tumor cells exerts a checking effect on their respiration" (4). In fact, in a number of different malignant tissues he demonstrated a decrease of oxygen consumption following the addition of glucose. The consequence of the Crabtree effect, viewed in a contemporary context is that many highly proliferative cells, when fueled by glucose generate almost all their ATP via glycolysis despite abundant oxygenation and a normal complement of fully functional mitochondria (5). In addition, it was noted that a similar phenomenon is present in some yeast strains (6). For example, the baker's yeast *S. cerevisiae* grown under anaerobic conditions obtains its energy only through fermentation of glucose into ethanol. In the presence of oxygen, respiration occurs. However, alcoholic fermentation becomes operative even under aerobiosis if glucose concentration exceeds a threshold value, depending on the strain (7). The phenomenon of overflow metabolism, leading to the formation of organic acids by certain anaerobic facultative bacteria when glucose uptake exceeds a critical threshold value may also be related to the Crabtree effect (8).

More than 78 years has elapsed since the description of the Crabtree effect. Many attempts have since been made to explain the mechanism of this phenomenon. However, numerous questions persist about the precise underlying mechanisms governing the effect. Over the last 20 years research on the Crabtree effect has mainly concentrated on yeasts, one reason being the economic importance of these organisms. Nevertheless, research on mammalian tumor tissues is still continuing because of possible therapeutic benefits that could be afforded by control of the Crabtree effect.

It is the purpose of this contribution to present and discuss recent advances in the domain, with the hope that it could stimulate further research.

Toward a Neutral Definition

In the literature, there are numerous definitions of the Crabtree effect. They are generally built on a respiratory behavior modified by glucose. In the Pasteur effect, the addition of glucose to a culture generally increases the specific respiratory rate, which may seem "intuitively true," as one expects a metabolism increase to result from nutrient intake. In the Crabtree effect, sometimes called *counter Pasteur effect*, one observes a reduction of the specific respiratory rate, which evokes an inhibition of respiration by glucose. This vision of the phenomenon in fact already suggests an explanation of the phenomenon, conjecturing the inhibition of the respiratory chains. It was probably the most common assumption until, say, 1990, and it remains a prevailing definition in many texts treating of the Crabtree effect.

This is explained not only historically (see following section) but also because this view was defended and argumented, especially by Sonnleitner and Käpelli in 1986 (9). There is no doubt that this article largely contributed to consolidate this univocal explanation of the Crabtree effect.

Over the past few years, however, other assumptions have appeared, which more or less severely question this principal explanation [e.g. since Van Urk *et al*. (10)].

Of course, in the Crabtree effect, one indeed observes a limitation of the specific oxygen consumption rate following nutrient addition (mainly glucose), but does that necessarily imply the limitation of the respiratory chains?

The respiratory capacity of an aerobic cell can be defined as the maximum quantity of oxygen which can be "treated" in the metabolic processes. This capacity is not infinite and presents thus a maximum $\max(q_{o_2})$. Obviously, this maximum value is not always reached and depends on the culture conditions (or other elements, like the uncoupling compounds). The most probable "metabolic configuration" to observe $\max(q_{o_2})$ is undoubtedly that in which the metabolism is purely oxidative, as in Fig. 1.

[1]In the historical context, glycolysis referred respectively to lactic acid and ethanol formation by mammalian tissues and yeasts.

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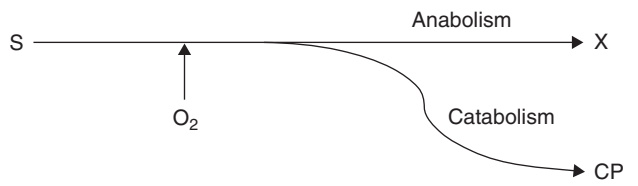

Figure 1. Purely oxidative production of biomass. Only two general pathways are involved: anabolism and catabolism. S, substrate; X, biomass; CP, catabolic products (including CO_2, H_2O, and energy).

Two sets of metabolic ways are used: the anabolic pathway, which leads to the production of biomass, and the catabolic way, which consists in producing the energy necessary to the biosynthesis [in the absence of energy spilling, i.e. energy not used for growth (11)] and which releases carbon dioxide and water in the medium. Under optimum culture conditions, specific respiration will be maximum and $q_{o_2} \equiv \max(q_{o_2})$. The yield coefficient of this culture can be defined compared to the limiting substrate, S, in the following way in Equation 1:

$$Y_{X,S} = \frac{X}{S_c} \tag{1}$$

where S_C is the substrate consumed; it is thus the quantity of biomass formed per unit of substrate consumed.

Now let us regard a process that is not purely oxidative, as in Fig. 2.

The sole difference with the preceding situation lies in the existence of a "bypass" which makes it possible for a part of the metabolism to be anaerobic (and thus to use other electrons acceptors that oxygen, if necessary). Such a situation is well described by Zamora (12) who underlines that, both in acetic bacteria and in yeasts, the metabolic pentose pathway is used in parallel with glycolysis in some situations. The pentose pathway is in that case used only for growth and not for energy production. If the yield coefficient of this configuration is close to the preceding situation, the same quantity of substrate will be used to form the same quantity of biomass. On the other hand, the specific oxygen consumption will be more or less decreased, according to the value of the flow of substrate passing through the anaerobic pathway. As a consequence $q_{o_2} < \max(q_{o_2})$. This result however, does not imply that the respiratory capacity is affected but only that the respiratory regime is lower than its maximal potential value. To summarize, in a respirofermentative metabolism like that of Fig. 2, in the absence of dissimilation, the total yield of the anabolism can remain practically constant with a decreased aerobic regime. It is thus necessary to distinguish between respiratory capacity $\max(q_{o_2})$, and respiratory regime, q_{o_2}.

Figure 2 represents a situation intended to illustrate the difference between respiratory capacity and regime. To represent the Crabtree effect in a simplified way, it is necessary to add an additional pathway leading to dissimilation (Fig. 3).

In this case, there not only exists a respiratory regime decrease but also a drop of the yield due to the fact that part of the substrate is excreted as fermentation compounds.

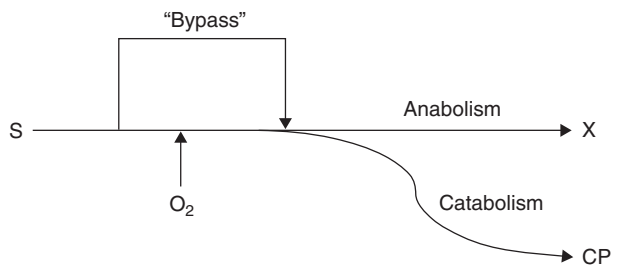

Figure 2. Hypothetic respirofermentative biomass production without dissimilation. The oxygen represents only about 14% of the biomass, coming mainly from the substrate (and not from dissolved oxygen). The biomass yield $Y_{X,S}$ may thus be very close to the purely oxidative process above (Fig. 1), if the catabolic pathway is comparable. In such a situation, oxygen consumption may be reduced, due to the bypass.

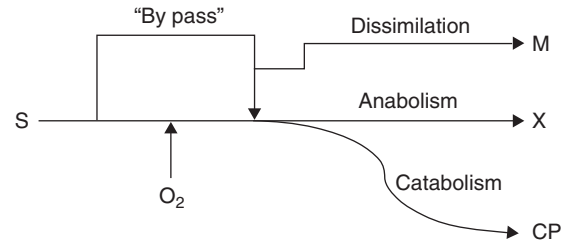

Figure 3. Respirofermentative biomass production with dissimilation [M dissimilation of fermentative product(s)]. The biomass yield, $Y_{X,S}$ is necessarily reduced because dissimilation of (transformed) substrate. In such a situation, oxygen consumption may also be reduced.

With an aim of avoiding a definition of the Crabtree effect already containing an explanatory assumption, on the basis of the above reasoning, we propose the following definition:

> "Crabtree Effect: dissimilation of fermentation compound(s) by a cell presenting a respirofermentative metabolism and accompanied by a respiratory regime decrease occurring in a particular specific growth rate range."

(This definition, in some sense, is poorer than others but it escapes the errors or unconfirmed assumptions about the mechanism involved.)

It is still advisable to specify that

1. the Crabtree effect can be observed in permanent mode ("long term Crabtree effect") or in transitory mode ("short term Crabtree effect");
2. the yield concept was not included in the definition because of characteristics in bacterial Crabtree effect (see this section) and because the study of this parameter is far from being systematic and complete and still necessitates many studies before leading to decisive conclusions.

A last remark is perhaps important before leaving this general section. The authors usually distinguish between Crabtree-positive or Crabtree-negative cells. However, it

is not rare to encounter in the literature, some "contradictions" concerning the possibilities of certain cells to present a Crabtree effect. Such cells can be regarded as Crabtree-positive by one author and Crabtree-negative by another. In a chemostat, under certain conditions (low specific growth rate), a yeast will be Crabtree-negative (in the sense of presenting a purely respiratory metabolism) and will be Crabtree-positive at a higher specific growth rate. These observations raise the question of the "fully intrinsic" or "partially intrinsic" properties of a cell to be Crabtree-positive or Crabtree-negative. From our viewpoint, we need more data and experiments to seriously deal with this problem.

EUKARYOTES

Crabtree Effect in Mammalian Cells

Research on the Molecular Mechanism of the Crabtree Effect in Mammalian Cells. Possible Therapeutic Consequences. The best phenomenological description of the Crabtree effect in mammalian cells is that addition of millimolar concentrations of glucose to the culture medium of different fast growing cells and tissues results in a decrease in the cell respiration rate (4). The phenomenon was observed in malignant cells, for example, tumoral pancreatic islet cells (13), Ehrlich ascites tumor (14) and hepatoma cells (15). Yet, it was also disclosed in different non-malignant, however fast growing cells and tissues, including for example, pig platelets (16), mammalian embryos at a very early development stage (17), proliferating thymocytes (18), intestinal mucosa (19), and spermatozoa (20). Some tissues, for example, thymocytes are characterized by a non-proliferating resting state where the Crabtree effect is absent and only becomes operative when thymocytes shift toward a proliferating state (18). Hence, the Crabtree effect appears to be a characteristic feature of fast growing cells.

Many attempts have been made to explain the molecular mechanism underlying the Crabtree phenomenon in mammalian cells [for an early review see (19)]. A competition between respiration and glycolysis for precursors of adenosine triphosphate (ATP), that is, adenosine diphosphate (ADP) and inorganic phosphate was advocated by different authors, in particular, in ascites cells (21–24). Data supporting this hypothesis were based on dynamic measurements of chemical events, namely cyclic variations in the ADP and ATP pools after addition of glucose to the cells. Given the fact that the ATP/ADP ratio and the phosphorylation potential is a metabolic link between glycolysis and oxidative phosphorylation, the changes observed in these parameters following glucose addition suggests that these factors may be related to the Crabtree effect. This hypothesis was however questioned for several reasons. One of the main ones was that the analogous deoxyglucose is phosphorylated in the cytoplasm, but not further metabolized and therefore does not manufacture ATP. As a matter of fact, deoxyglucose phosphate produced an even stronger decrease of respiration than glucose itself (25). Moreover, phosphate transport into the cells was shown to be non-limiting (25). Other explanations of the Crabtree effect were proposed: (i) inhibition of respiratory enzymes by a shift of intracellular pH following glucose addition (26), (ii) change of permeability of inner mitochondrial membrane (27), (iii) occurrence of specific and highly regulated key glycolytic isoenzymes (28), (iv) location of enzyme systems unique for rapidly growing tumors, for example, a membrane-bound hexokinase (29), and (v) redistribution of Ca^{2+} in tumor cells (30). Use of monitoring of Ca^{2+} with fluorescent probes in intact Ehrlich ascites tumor and Zajdela hepatoma cells has shown that glucose and deoxyglucose elicited the release of Ca^{2+} from endoplasmic reticulum stores and an increase in the cytosolic Ca^{2+} concentration. A parallelly enhanced uptake of Ca^{2+} by mitochondria was observed, leading to strong inhibition of the F_1F_0-ATP synthase.

A more recent contribution emphasized on a possible multisite control of the Crabtree effect in tumor cells (15). Working with hepatoma cells, these authors showed that the addition of glucose or fructose lowered intracellular Pi (40%) and ATP (53%) concentrations and decreased cytosolic pH from 7.2 to 6.8. In parallel, a 30% increase of AMP content was noted as well as an increase in glucose 6-P, fructose 6-P, and fructose 1,6-bisphosphate (15, 13, and 50 times respectively). In contrast to the results obtained by Wojtczak *et al*. (30), no modification in cytosolic Ca^{2+} was found. The authors proposed that the triggering event in the Crabtree effect is a glucose activation of membrane-bound hexokinase. A subsequent activation of phosphofructokinase-1 and pyruvate kinase is afforded by increase in fructose 1, 6-bisphosphate, followed by stimulation of the glycolytic flux, leading to a decrease in Pi content and accumulation of phosphorylated hexoses. A decrease of Pi and acidification of the cytosol may in turn affect oxidative phosphorylation at different levels: (i) inhibition of α-ketoglutarate dehydrogenase, ATP-synthase, and Pi-dependent glutaminase, three enzymes using Pi as cofactor and (ii) inhibition of the highly pH dependent cytochrome bc_1. Therefore, it would seem that the Crabtree effect results from several small changes induced by glucose and fructose and affecting enzymes of the oxidative phosphorylation. An intriguing question is: what could be the selective advantage of the Crabtree effect for proliferating cells? A basic explanation is that because many tumors and fast growing tissues are hypoxic compared to normal tissues, sufficient energy for their survival and growth should be provided by higher glucose metabolism rate than in normal tissues (31). An interesting suggestion is that the Crabtree effect could also be beneficial for tumor cells, as the consequence of a glucose-dependent accumulation of essential metabolic intermediates, such as serine, fructose 1, 6-bisphosphate, glycerol 3-phosphate and phosphoribosyl-pyrophosphate, which can activate mitogenesis of the tumor cells (32).

Modulation of the Crabtree effect has been suggested as a therapeutic strategy to eradicate malignant cells. Oral or intravenous administration of glucose, while not affecting the pH of normal tissues, results in acute acidification of murine and human tumors, due to the production of lactic acid as a consequence of the Crabtree effect (33). The therapeutic benefit should be that acute acidification sensitizes tumors to various physical and chemical agents

such as heat (hyperthermia) (34), alkylating (35) and platinium drugs (36). Hypoxia in tumor cells is an important factor of radioresistance (37). Hyperbaric oxygen (HBO) and normobaric carbogen (95% oxygen, 5% carbon dioxide) can however increase oxygen delivery to tumors. Burd *et al*. (38,39) have shown that in human melanoma cells, acute acidification and enhanced oxygenation can result from exposition to hyperglycemia combined with the mitochondrial respiration inhibitor norepinephrine analog meta-iodo-benzylguanidine (MIGB). Such cells may become more radiation sensitive (40). Secomb *et al*. (41) have shown that this type of approach should be more effective when combined with breathing of hyperoxic gases.

Another possible therapeutic way could be to increase the tumor cells' susceptibility to mitochondrial toxicants by circumventing the Crabtree effect and forcing the malignant cells to respire. Drug-induced mitochondrial toxicity is displayed by several members of important drug classes, including anticancer agents (42). Marroquin *et al*. (5), working with hepG2 cells, have shown that replacing glucose with galactose, a substrate that can be only respired, increased the susceptibility to mitochondrial toxicants. Other recent advances have also pinpointed important role for mitochondria in cell metabolism, more specifically concerning regulation of cell death pathways. Most of the tumor cells are resistant to apoptosis and it was questioned whether such resistance was related to particular properties of mitochondria in cancer cells, from those of mitochondria in non-malignant cells (43). This scenario was in essence already suggested by Otto Warburg, who put forward the hypothesis that a decrease in mitochondrial energy metabolism might lead to the development of cancer (see section titled "Historical Considerations"). Several strategies were proposed to overcome apoptotic resistance of malignant cells and increase their susceptibility to anticancer treatments. From these investigations, it can be concluded that a successful coordinated attack on cancer cells should be based on the concerted modulation of cellular energy metabolism, mitochondrial stability, and other mechanisms involved in the characteristic resistance of cancer cells to apoptosis.

In conclusion, research efforts are to be continued in the future to fully decipher the underlying mechanisms of the Crabtree effect in cancer cells. This would, in our opinion, be of particular importance for improving and/or imagining new approaches in tumor therapy, based on a unique metabolic feature.

The Yeasts (Fungi)

Among the fungi, the yeasts are without doubt the most used and the most studied organisms in industry. In spite of an increasing interest for the so-called *nonconventional* yeasts (44), *S. cerevisiae* remains currently the conventional microorganism of predilection. It is this reality that will give to this species the largest part in the following sections. Figure 4 depicts a schematic view of the glycolytic

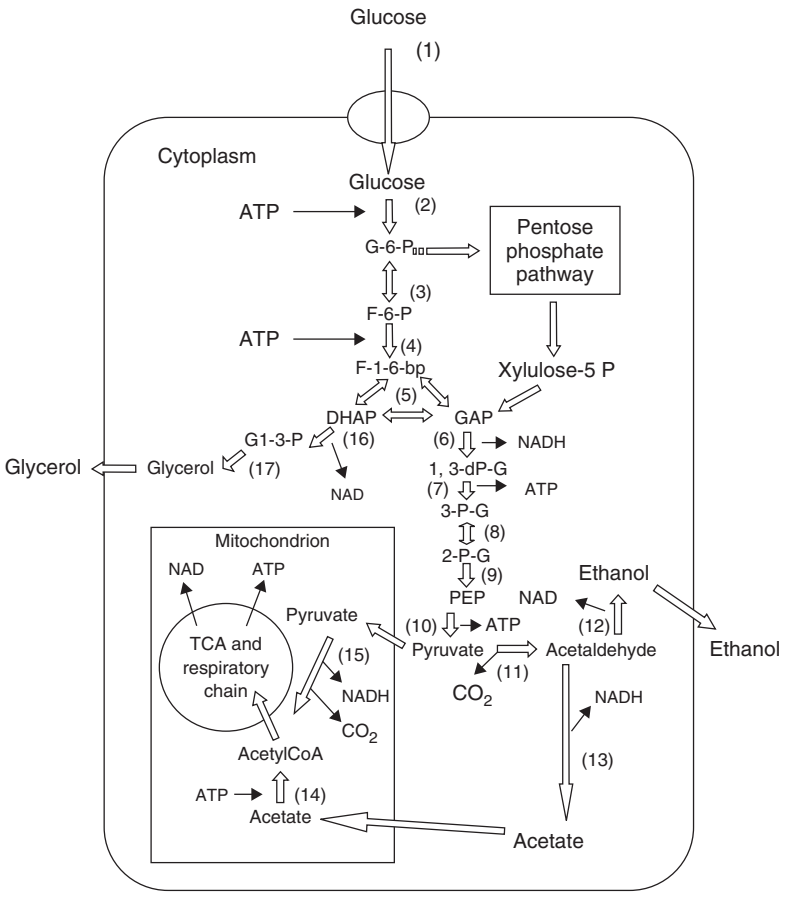

Figure 4. A schematic view of the yeast glycolytic pathway. Intermediates: G-6-P, glucose 6 phosphate; F-6-P, fructose 6 P; F-1,6-bP, fructose 1,6 bis phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3 phosphate; 1,3-dP-G, 3diphosphoglycerate; 3-P-G, 3 phosphoglycerate; 2-P-G, 2 phosphoglycerate; PEP, phosphoenol pyruvate; Gl -3-P, glycerol 3 phosphate. Enzymes: (1) glucose transporter; (2) hexokinase; (3) phosphoglucose isomerase; (4) phosphofructokinase; (5) aldolase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) phosphoglycero mutase; (9) enolase; (10) pyruvate kinase; (11) pyruvate decarboxylase; (12) ethanol dehydrogenase; (13) aldehyde dehydrogenase; (14) acetyl CoA synthetase; (15) pyruvate dehydrogenase; (16) glycerol 3-phosphate dehydrogenase; (17) glycerol-3-phosphatase.

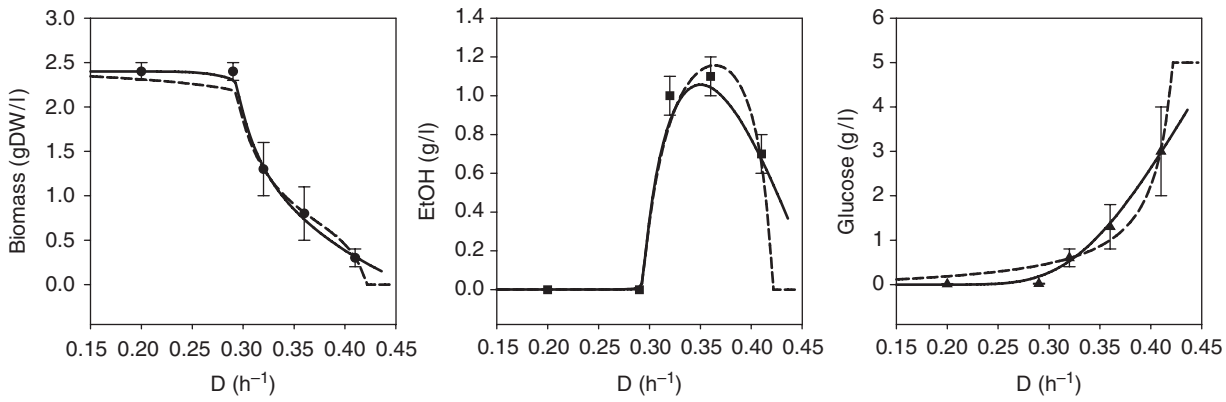

Figure 5. Steady states values of a culture of *Saccharomyces cerevisiae* in a chemostat. The points are the experimental values (from (47)) and the lines are obtained by simulations (48,49) via the PDS model. Full and dashed lines represent simulations carried out by two different methods of evaluation of residual glucose.
(Note: The PDS model will be described further in this section. It was chosen because of the very efficient way it represents the Crabtree effect in several situations, including in bacteria consortia described in a following section).

pathway in the yeast *S. cerevisiae*. The principal fermentation product is ethanol followed by glycerol. Acetic acid may be also produced in small amounts by the yeast in some metabolic circumstances.

Pure Strains. From the point of view of the Crabtree effect, it is usual to classify yeasts as "Crabtree-positive" and "Crabtree-negative". According to the definition that we propose (see above), the Crabtree effect is probably not a rigorously intrinsic property of the organism: dissimilation of fermentative products in aerobic conditions depends, roughly speaking, on the culture conditions (as the growth rate; we think that it is not impossible to find other parameters, such as perhaps, medium composition). Nevertheless, some yeasts never presented Crabtree effect under the conditions of culture studied to date and could thus be indeed unable to present this effect. In other cases, some articles are in contradiction and one presents such strain as positive, the other as negative. This is the case, for example, of *Candida albicans*, which is declared Crabtree-negative by Piškur *et al*. (45) and is associated to some phenomenon similar to the Crabtree effect by Land *et al*. (46).

The purpose is not to initiate a polemic on the classification of yeasts into positive and negative but to draw the attention to the fact that under the same conditions of culture, a yeast strain can be either positive or negative. If the production process is changed, it is prudent to remain attentive and to control if this property is preserved or not.

Figure 5 depicts the general aspect of the behavior of a Crabtree-Positive yeast cultivated in a chemostat at steady state.

The continuous culture is not the more exploited industrial process (it is even rather rare), but this method of culture makes it possible to properly illustrate the problems associated with the Crabtree effect in production.

For the record, dilution rate is defined as, $D = Q/V$ a: Q being the volumic flow of the substrate inlet [units: volume/time] and V represents the working ("true") volume of the bioreactor. The units of D is thus the inverse of a time [t^{-1}] [and the inverse of D, called the *residence time*, τ, is expressed in time (t)]. In a chemostat, the specific growth rate (μ) in the steady state is equal to the dilution rate, thus $\mu = D$.

Figure 5 clearly shows, in a *C*-limited culture of *S. cerevisiae*, that for $D < 0.3$/h approximately, the biomass remains constant, the concentration in glucose is almost zero and there is no ethanol production. At these growth rates, the metabolism is purely oxidative and there is no excretion of fermentation products. In this zone, the specific oxygen uptake rate (OUR) is proportional to the growth rate (Fig. 6).

Beyond $D = 0.3$/h, the specific oxygen uptake rate, qO_2, reaches a plateau and ethanol appears in the medium. The alcohol concentration passes toward a maximum around $D \sim 0.35$/h. Above the critical value of $D = 0.3$/h, the metabolism becomes respirofermentative. At high growth rates, Rieger *et al*. (47) highlight a reassimilation of ethanol by yeast. Thus, there would be cometabolism of glucose and ethanol. Let us mention that $qO_2 = f(D)$ is independent of the limiting substrate concentration inlet for this culture of *S. cerevisiae*. This observation is not general in the Crabtree effect and, in particular, is not observed for bacteria consortia described in section titled "Consortia".

From an industrial production viewpoint, two main applications emerge:

- biomass production (baker's yeast or recombinant proteins);
- ethanol production.

In the first case, Fig. 7 shows the profile of the curve of biomass yield coefficient [quantity of yeast per substrate unit (glucose, molasse, etc.)] as a function of D:

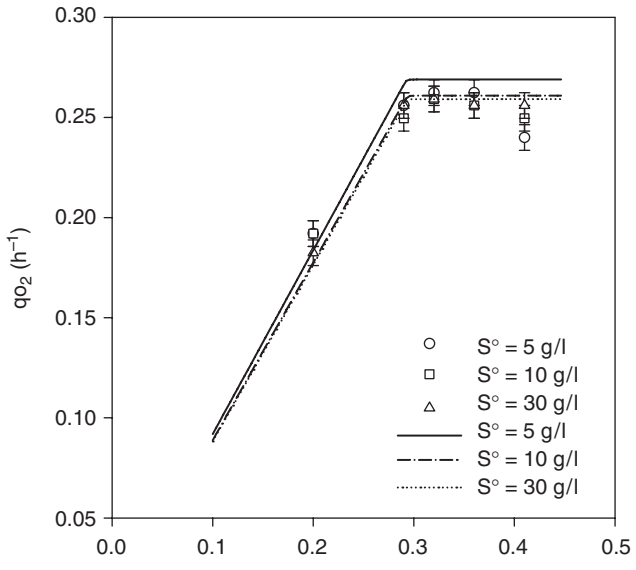

Figure 6. Profile of the specific O_2 consumption rate for three feed concentrations.
Method: Lines represent simulations via the PDS model. Points are the experimental data [from (47)]: S^0=**5 gGLU/L**: open circle, S^0=**10 gGLU/L**: open square, S^0=**30 gGLU/L**: open triangle. [Data reproduced from Rieger *et al*. (1983), with permission; (47).]
Note: Figures 6 and 7 are presented as they appear in the original publication (48,50). The three curves were presented to show the coherence of the model and its ability to describe different situations (various feed concentrations) without changes neither in equations (structural stability) nor in parametric estimation (numerical convergence).

In this figure, it is obvious that the biomass yield and thus also recombinant proteins, for example, drastically decreases as soon as the critical dilution rate is reached, which is contrary to the expectation of the producer. The Crabtree effect thus imposes to work at low rates of growth, which is also unfavorable. One in general thus makes use of aerobic cultures in fed-batch (51,52), which makes it possible to control the concentration in glucose to avoid, or better control, the Crabtree effect. (The culture in batch does not solve either the problem because almost no control on growth is possible and the biomass yields are low.)

In the second case, the production of ethanol in chemostat, in Fig. 5 shows that the maximum of ethanol concentration also corresponds to high residual glucose in the medium. According to the ethanol purity degree wanted, it will thus be necessary to carry out purification processes that slow down the production and increase the production costs.

Considering the preceding remarks, it appears obvious that the Crabtree effect can be an extremely embarrassing phenomenon in production: the Levurist (baker's yeast) wishing to avoid ethanol; the producers of alcohol preferring to limit the biomass, to increase the glucose yield. The metabolic switch of yeasts is thus a process that requires to be controlled as precisely and effectively as possible. It is therefore important to understand the Crabtree effect validly to find solutions that make it possible to control it as far as possible.

Unfortunately, we must begin this analysis by accepting that, according to the very large majority of the authors, there is no complete explanation concerning the Crabtree effect. However, two principal theses (perhaps non-exclusive?) are most frequently met in the literature:

- that of the glucose inhibition of respiration;
- those which privilege the nutrients transport phenomena.

While we are on the subject, let us note that the Crabtree effect in *S. cerevisiae* also occurs with fructose, mannose, and galactose (53), which makes this phenomenon broader than a simple "glucose effect".

It is obvious, that if one looks for a strategy aiming at reducing the Crabtree effect in a production process, the actions to be undertaken will be different according to the chosen assumption. It is impossible to review all the representations of the Crabtree effect. We will thus examine three different concepts. The goal is not to make a fundamental approach, but to provide plausible assumptions for producers faced with the problem to control a metabolic switch.

The Respiratory Model of Sonnleitner and Kapëlli. The motivation of Sonnleitner and Käppeli is based on the control of the exploitation of microorganisms, which undoubtedly contributed to the success of their model (9). According to Nielsen and Villadsen (54), it was the more accepted

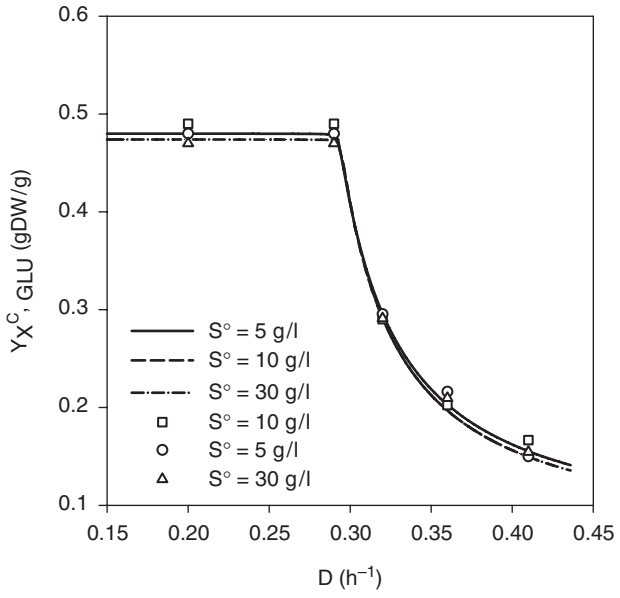

Figure 7. Profile of the yield coefficient for three feed concentrations.
Method: See Fig. 6
Comments: The three curves are confounded and the dispersion of the results tends to increase with D for $D > D_c$.
(Data reproduced from Rieger *et al*. (1983), with permission; (47).)

model in the 1990s and it still is quite often quoted in more recent articles.

The aim here is not only to detail the model, but also to explain its principle and to highlight its qualities and also the uncertainty which remains about its relevance.

The authors consider three stoechiometric equations (Eqs 2,3, and 4 represented below in a simplified form):

1. Oxidative metabolism of glucose (S) to form the biomass (X) in the presence of oxygen:

$$S + aO_2 \rightarrow bX + cCO_2 + dH_2O \tag{2}$$

2. Reductive (fermentative) metabolism of glucose to form biomass and ethanol (E):

$$S \rightarrow gX + hCO_2 + iH_2O + jE \tag{3}$$

3. Oxidative metabolism of ethanol to form biomass in the presence of oxygen:

$$E + kO_2 \rightarrow lX + mCO_2 + nH_2O \tag{4}$$

One of the originalities of the model was to consider that these three equations are simultaneous within the cell, and non-sequential, as in other models. These three equations are obviously expressed in terms of moles. The stoichiometry of the biomass is classically defined (47) and almost does not vary with the dilution rate.

The total reactions rate (Eqs 2–4) can be given starting from partial flow rates. When considering only the partial rates, which are of interest, and using the more compact notation of (54) as in Equations 5 and 6, one defines:

$$r_S = k_S \frac{S}{K_S + S} \tag{5}$$

which represents the glucose flow and

$$r_{o,\max} = k_o \frac{o_2}{k_o + o_2} \tag{6}$$

which represents the maximum oxidative metabolism rate (i.e. the maximum rate that the cell can reach in pathway(s) using oxygen).

To simplify, let us note by r_{FE} the specific fermentation rate, leading to the production of ethanol.

We will not discuss the coherence of these partial rates, although they can be of doubtful soundness. More of interest to us is the way the authors account for the metabolic switch. In algorithmic form, the "mechanism" is represented on the following process chart:

The computer program constantly compares the flow of glucose r_S and the maximum rate of the oxidative metabolism, $r_{O,max}$/a (a is the stoechiometric coefficient, which makes the comparison correct).

If the glucose flow does not exceed the maximum oxidative capacity, then the rate of the oxidative metabolism, r_{OX} is maximum and the fermentative metabolism is zero: $r_{FE} = 0$.

If the flow of glucose exceeds the maximum oxidative capacity, then the rate of the oxidative metabolism, r_{OX} is equal to the glucose flow and the specific fermentative rate is equal to the difference between the current oxidative rate and its maximum value.

The mechanism is thus based on an overflow of the respiratory metabolism (or bottleneck), which ensures the transition between the purely respiratory mode (oxidative) and the respirofermentative mode (oxydoreductive).

The model of Sonnleitner and Käpelli is a well-performing model with regard to this experiment. But does it necessarily follow that this calculation proves the assumption of a limited respiratory capacity? Nielsen and Villadsen (54) quote Van Urk *et al*. (55) who suggest that the bottleneck might be at the level of the intermediates of the tricarboxylic acid (TCA) cycle for the amino-acids formation. Intriguingly, Nielsen and Villadsen (54) conclude that "In a simple model for growth and production formation of *S. cerevisiae*, it is in any case not necessary to know exactly where the bottleneck is positioned (p. 179)." It seems, on the contrary, that from a practical point of view, the exact knowledge of the transition mechanism is necessary to manage the manufacturing processes.

Thus, we conclude that the model of Sonnleitner and Käpelli is a good phenomenological model that may be used, for example, in process monitoring, but that its biological interpretation is indeterminate. In addition, the fact that it is necessary "to integrate the answer in the program" (Fig. 8) seems an "ad hoc" solution, which gives very poor information (if any) on the subjacent mechanism. If the assumption of the limitation of the respiratory capacity of *S. cerevisiae* proves to be false, all the tracks aiming at improving the processes by mitigating this disadvantage would be useless.

Assumptions Based on the Substrate Transport (and Uptake). It is perhaps van Urk *et al*. in 1989, (10) who have mainly interrogated the assumption of the respiratory limitation. For these authors, the essential difference between Crabtree-positive and negative yeasts is due to their capacity to control their glucose transport system. For the positive ones, the glucose excess, combined with a facilitated diffusion transport, does not make it possible for the cell to control the substrate uptake, which would lead to an aerobic fermentation. For the negative ones, this overflow phenomenon could be avoided thanks to a transport mechanism based on a system of proton (H^+) symport. The same year, Postma *et al*. (56), concluded that

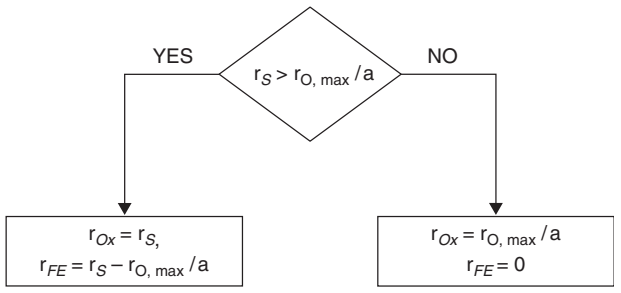

Figure 8. Algorithmic representation of the respirofermentative switch according to (9).

the limitation of the respiratory capacity was not the main cause of alcoholic aerobic fermentation in *S. cerevisiae*. They even noted an increase in respiration by decoupling, due to the secretion of acid (acetate) by the cell. This result was confirmed by Verduyn *et al*. (57) by adding benzoate in the culture medium of an aerobic chemostat. This result had already been announced by van Dijken (58). Since 1989, many other studies have pointed in the same direction. For example, Otterstedt *et al*. (59) have generated a S. *cerevisiae* strain in which glucose uptake is dependent on a chimeric hexose transporter mediating reduced sugar uptake. This strain shows a fully respiratory metabolism also at high glucose levels as seen for aerobic organisms, and switches to fermentation only when oxygen is lacking. However, none of these authors have taken the step to definitively eliminate the respiratory assumption (see also in section titled "Studies on Metabolic Fluxes and Their Consequences for Unraveling the Mechanism of the Crabtree Effect"). Some have even allowed a multifactorial causality, such as, for some yeasts, transport associated with respiratory enzymes regulation; these authors however exclude the priority of the respiratory role in *S. cerevisiae* (58). A study motivated by the optimization of the production of baker's yeast showed as indirect evidence that the reduction of the glucose transport capacity leads to the decrease of the fermentative capacity of the yeast (60).

As the assumption on the respiratory capacity was weakened or even disappeared, another explanation was required to explain the Crabtree effect. One of them is the repression of the catabolism by the glucose, which incorporates the Crabtree effect in the more general "glucose effect." Aon and Cortassa are undoubtedly a good example of this "school" (61,62). They studied the Crabtree effect in a rather exhaustive way, under various culture conditions, using metabolic engineering methods (63). The assumption of "the glucose effect" and catabolites repression was however questioned by Sierkstra *et al*. (64) as galactose is not a repressive carbon source. This result excludes any possible link between the Crabtree effect and the glucose effect. This diverging point of view is likely to still be subject to controversy in the future. For the authors, the possible causes of the Crabtree effect are then reduced to three possibilities: growth rate, glycolytic flow, and metabolism overflow.

As was the case of the respiratory effect (Sonnleitner and Käpelli), there have been several recent attempts at clarifying this situation with the aid of mathematical models. Among these, one may quote, in 2000, the model of Hanegraaf *et al*. (65). This model rests on an assumption of nutrient transport and avoids the pitfall of Sonnleitner and Käpelli by avoiding the problem of the metabolic switch (Fig. 8), which is supposed to be inexistent. However, by examining Figs. 5–7, it appears clearly that there indeed exists a discontinuity in the curve of the stationary states as a function of the dilution rates for the various variables. To anyone who practiced this kind of culture, it is manifest that it is impossible to work exactly at this critical dilution rate, D_c: the data produced are either before or behind the critical point. It is probable that a sharp switch indeed exists and that is highlighted by Aon and Cortassa (66) (for whom the central parameter is the specific glucose transport rate, q_{GLU}, which moreover depends on the nitrogen source).

Obviously, many points still remain unclear in the explanation of the Crabtree effect, but the tendency of the last few years tends to ascribe it more to a transport substrate phenomenon than to the respiratory properties of yeasts, even if one cannot completely eliminate multifactorial assumptions. Just in the same way as Sonnleitner and Käpelli's model brought some useful explanations, we would like to present a model that supports the assumption of the nutrient transport and solves some pitfalls left unresolved in the preceding models.

The model is built starting from the approach of the dispersed polyphasic systems (PDS), which consists in writing the mass balances for each compound in each phase of a system (48–50,67). The model is a little long to establish, so we will simply give the main concepts.

The basic assumption rests on the fact that the substrate transport from outside (matric phase) to inside the cell (cellular phase) uses several substrate transporters, which is a well established fact for a great number of strains (53,65,68,69). The most frequent case presents two transporters, one with a low affinity, the other, with a high affinity for the substrate.

Under these conditions, the specific rate of substrate transport/metabolisation (glucose here) inside the cell takes the following form as given in Equation 7:

$$q_{\mathrm{GLU}} = q_{\mathrm{GLU}}(h) + q_{\mathrm{GLU}}(l) = f(\tilde{C}_{\mathrm{GLU}}, X, \lambda) \tag{7}$$

where h symbolizes high affinity and l low affinity; $\tilde{C}_{\mathrm{GLU}}$ represents the intracellular concentration in glucose and λ a set of parameters.

The function $f(\cdot)$ is given by two hyperbolic equations (of which one is linearized according to the affinity value):

$$f(\cdot) = V^{o}_{\mathrm{GLU}} \frac{\tilde{C}_{\mathrm{GLU}}}{K_{\mathrm{GLU}} X + \tilde{C}_{\mathrm{GLU}}} + \tilde{C}_{\mathrm{GLU}} k_o / X \tag{8}$$

K_{GLU} is the "affinity" for the substrate and k_0 a constant; X is the total biomass in the chemostat.

In a steady state, while solving Equation 8, the concentration in glucose inside the cell takes the simplified form (if high affinity tends toward infinity):

$$\tilde{C}_{\mathrm{GLU}} = \frac{-a + |a|}{b} \tag{9}$$

where a is a combination of the variables of (Eq. 8).

This non-negative function is zero when a is positive and takes a finite positive value when a is negative (Eq. 9). It is characteristic of this function "to jump" abruptly from a zero value toward a positive value with a which represents the discontinuity observed in the Crabtree effect (Figs 5–7); the lines are the result of simulation by PDS model).

It is easy then to calculate in an explicit way the value of the critical dilution rate, D_c, when affinity tends toward infinity:

$$D_c \approx V^{o}_{\mathrm{GLU}} \times Y_M \tag{10}$$

Y_M is a constant calculated as a yield coefficient in the $D < D_c$ zone (Eq. 10). (Without energy spilling, this value indeed corresponds roughly to the yield coefficient at the low dilution rates).

The two main advantages of this model are as follows:

1. to give a discontinuous representation of the phenomenon without "ad hoc" assumptions, simply by the representation of the abrupt activation of the high affinity branch (i.e. the pathway showing a great affinity for the substrate; see Equation 7) beyond the critical dilution rate;
2. to give an interpretable numerical approximation of the value of this critical dilution rate. It is then possible to know which factor to act on to improve this value.

Thus, for a constant transport/metabolisation rate, a strain with high yield coefficient will have a higher critical dilution rate (favorable thus to the levurists). The maximal specific transport/metabolisation rate of the high affinity pathway acts in the same direction, for a same yield coefficient. According to the needs, one may either seek to reduce these values (favoring ethanol production) or to reinforce them (favoring biomass production).

This approach makes it possible to formulate an assumption for the difference between Crabtree-positive and negative yeasts. If the critical dilution rate is lower than wash-out specific rate (value for which all the biomass is eliminated from the chemostat), $D_c < D_W$, the yeast will be Crabtree-positive; on the contrary, the biomass will be completely removed from the bioreactor before the respirofermentative transition does occur: the possible Crabtree effect will thus not be observable. The article by Franzblau and Sinclair (70) confirms this fact remarkably, though under a light dissolved oxygen limitation. They were able to make *Candida utilis* positive, while it is normally considered as Crabtree-negative yeast. This capacity to induce the Crabtree effect is, according to the authors, applicable to all Crabtree-negative yeasts, except for those, which are strictly anaerobic. The phenomenon of induction seems related to the glucose transport rate and is inhibited when transport becomes impossible.

In addition to the relation (Eq. 10), which is of undeniable practical interest, the model makes it possible to calculate the principal intracellular specific flows (Fig. 9).

It is also possible to calculate the stoechiometric coefficients of the total reaction for various dilution rates and for various substrate concentrations at the inlet of the chemostat (Table 1). The simplicity of this model, its absence of ad hoc formulation as well as the great amount of practical information it allows, strongly pleads in favor of an explanation of the Crabtree effect primarily based on transport. One cannot however, definitively eliminate other assumptions, including a multifactorial approach, especially with regard to non-*Saccharomyces* yeasts.

Studies on Metabolic Fluxes and their Consequences for Unraveling the Mechanism of the Crabtree Effect. The metabolic shift between oxidative and fermentative growth in *S. cerevisiae* was recently studied by a new ^{13}C labeling method using Matrix-Assisted Laser Desorption/Ionization T (MALDI-T) of mass spectrometry. The Crabtree effect was investigated by comparative metabolic flux analysis of yeast growing under purely oxidative, respirofermentative, and predominantly fermentative conditions (71,72). It was shown by these authors that

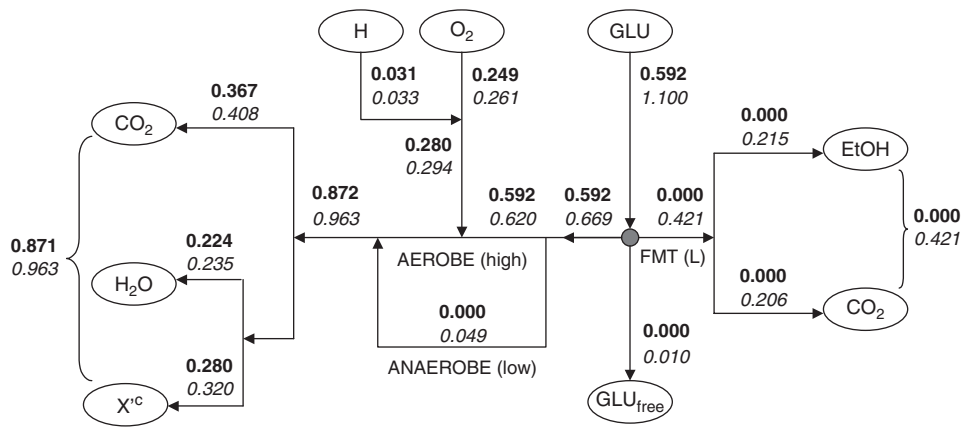

Figure 9. Quantification of the main specific metabolic flux within the cellular phase. This diagram shows numerical values (in h^{-1}) corresponding to the composition of the principal metabolic pathways taken into account by the model. The values were calculated before and after the critical value $D_c = 0.3$/h. The higher value (bold) corresponds to $D = 0.28$/h; the lower value (italic) to $D = 0.32$/h. It is noted that specific ANAEROBE (LOW) fermentation [FMT(L)] rates, as well as the free substrate (adsorbed and/or intracellular) pathway are zero before the critical value of 0.3/h. The conservation of the specific rates can be checked on the whole (sum of the entries = sum of the exits) or partially (brackets). To calculate metabolic flows, it suffices to multiply the specific rates values by the biomass corresponding to the dilution rate considered (after (48) and (49)).

Table 1. Comparison of the Stoechiometric Coefficients

$C_6H_{12}O_6+c_1.O_2+c_2.0.15.NH_3 \rightarrow c_2.CH_{1.79}O_{0.57}N_{0.15}+c_3.C_2H_60+c_4.CO_2+c_5.H_2O$

D (h^{-1})	S^0 (g/L)	Greek Symbols	c1	c2	c3	c4	$c4_{resp}$	$c4_{fer}$	c5	MB (%)
	5	α	2.53	3.30	0.00	2.70	—	—	3.79	
		ν	2.48	3.46	0.00	2.55	2.55	0	3.92	−0.4
0.2	10	α	2.52	3.31	0.00	2.69	—	—	3.78	—
		ν	2.37	3.41	0.00	2.54	2.54	0	3.79	−0.3
	30	α	2.45	3.38	0.00	2.62	—	—	3.74	—
		ν	2.35	3.41	0.00	2.54	2.54	0	3.76	−0.2
	5	α	2.41	3.42	0.00	2.58	—	—	3.71	—
		ν	2.47	3.44	0.00	2.55	2.55	0	3.91	−0.4
0.29	10	α	2.37	3.46	0.00	2.54	—	—	3.68	—
		ν	2.36	3.40	0.00	2.54	2.54	0	3.77	−0.3
	30	α	2.41	3.42	0.00	2.58	—	—	3.71	—
		ν	2.34	3.40	0.00	2.53	2.53	0	3.75	−0.3
	5	α	1.33	2.04	0.84	2.28	—	—	2.11	—
		ν	1.40	2.13	0.76	2.29	1.52	0.77	2.21	0.6
0.32	10	α	1.34	2.08	0.83	2.20	—	—	2.12	—
		ν	1.33	2.09	0.77	2.28	1.51	0.77	2.13	0.7
	30	α	1.33	2.05	0.84	2.27	—	—	2.11	—
		ν	1.32	2.09	0.77	2.28	1.51	0.77	2.12	0.7
	5	α	0.87	1.50	1.18	2.13	—	—	1.45	—
		ν	0.85	1.46	1.13	2.14	1.01	1.13	1.35	2.0
0.36	10	α	0.80	1.41	1.24	2.11	—	—	1.34	—
		ν	0.80	1.42	1.14	2.13	0.99	1.14	1.28	2.1
	30	α	0.80	1.41	1.24	2.11	—	—	1.34	—
		ν	0.80	1.42	1.14	2.13	0.99	1.14	1.27	2.1
	5	α	0.59	1.26	1.35	2.50	—	—	1.01	—
		ν	0.57	1.12	1.30	2.04	0.74	1.30	0.91	3.7
0.41	10	α	0.55	1.13	1.42	2.03	—	—	0.98	—
		ν	0.53	1.08	1.32	2.03	0.72	1.32	0.86	3.8
	30	α	0.54	1.09	1.44	2.03	—	—	0.95	—
		ν	0.53	1.08	1.32	2.03	0.72	1.32	0.85	3.8

[a] Comparison of the stoechiometric coefficients obtained by Rieger and Käpelli (47), α and those generated by the PDS model, ν, for various glucose concentrations, S^0 (g/L), at the inlet of the chemostat and various dilution rates, D (h^{-1}). An advantage of the model is to be able to discriminate the CO_2 coefficients resulting from respiration and fermentation. A systematic relative error in the mass balance (MB%), lower than 4%, probably corresponds to the absence of nitrogen flows. Nitrogen approximately accounts for 8% of the biomass, a value that is compatible with this assumption.

the metabolic shift from oxidative to fermentative growth was accompanied by elaborate changes of carbon flux throughout the whole central metabolism (Fig. 4).

- Flux redirection from the pentose phosphate pathway (PPP) toward glycolysis.
- Increased flux through pyruvate carboxylase, the fermentative pathways and malic enzyme.
- Flux decrease through the Krebs cycle.
- Partial relocation of alanine biosynthesis from the mitochondrion to the cytosol.

Moreover, the presence of a pyruvate dehydrogenase by-pass was shown in all the physiological regimes investigated. In purely oxidative conditions, the by-pass was essentially provided via pyruvate decarboxylase, acetaldehyde dehydrogenase, acetyl-CoA synthase and transport of acetyl CoA into the mitochondrion. During fermentative growth, this route was however saturated most probably due to limited enzyme capacity. Under these conditions, a high carbon flux was assured through a pathway involving pyruvate carboxylase, the oxaloacetate transporter and malic enzyme. The PPP alone was sufficient to supply nicotinamide adenine dinucleotide phosphate (NADPH) for biosyntheses during oxidative growth. In contrast only 60% of the required NADPH was provided by PPP during fermentative growth. The authors concluded that, in order to overcome limited capacity of pyruvate dehydrogenase, yeast has different metabolic bypasses to channel carbon into the mitochondrion. This involves conversion of cytosolic pyruvate either into acetyl CoA or oxaloacetate followed by intercompartimental transport of these metabolites. Under oxidative regime, mainly the NAD specific isoforms of acetyl CoA dehydrogenase and isocitrate dehydrogenase catalyzed the corresponding reactions in the yeast. Under fermentative conditions, sources other than the PPP (e.g. NADPH specific acetaldehyde dehydrogenase or isocitrate dehydrogenase) may become of importance.

Nielsen and collaborators (Vemuri *et al*.) (73) have recently shown that increasing NADH oxidation reduces the Crabtree effect in *S. cerevisiae*. Studies were conducted in chemostat using two strains where respectively an heterologous alternative NADH oxidase (NADH: ubiquinone oxidoreductase) and a non-respiratory water forming oxidase were introduced. A reduced aerobic ethanol formation was observed when the alternative oxidase was introduced. In contrast, increasing the nonrespiratory NADH oxidase reduced aerobic glycerol formation. The metabolic response to elevated alternative oxidase predominantly occurred in the mitochondrion, whereas the nonrespiratory oxidase affected genes coding for cytosolic enzymes. Taken together, these results indicated that the Crabtree effect in yeast may be a consequence of limited respiratory capacity.

In another recent contribution, Feria—Gervasio *et al*. (74) have evaluated the implication of the acetyl CoA-derived carbon transport from cytosol to the mitochondrion at the onset of the transition from respiratory to fermentative metabolism in *S. cerevisiae*. Their strategy consisted in introducing aerobic glucose-limited chemostat during a local perturbation by the addition of oleic acid, a cosubstrate known to stimulate enzymes implicated in the acetyl CoA transport between different cell compartments, and to evaluate the consequences of such an addition on the metabolic transition. Feeding the culture with oleic acid led to a delay in the onset of the metabolic shift, a 33% decrease in ethanol production and a redirection of the carbon flux toward biomass production. These data which could have practical implications, showed a modulation of the carbon distribution among respiration and fermentation, in favor of a decrease in the short-term Crabtree effect by oleic acid.

Associations or Consortia. The processes utilizing associations or yeast consortia appear very seldom in the literature (and undoubtedly also in practice); that is also true, in a general way, for the other forms of fungi

Nevertheless, such types of combination could be of practical interest, especially since a renewed interest for "nonconventional" yeasts appeared (44).

We will quote an example where positive or negative Crabtree behavior could occur or not, according to whether the yeast is used in association or consortium.

In an article of 1983, Moulin *et al*. (75) described a manner of producing yeast starting from whey, a by-product of the milk industry which is a nuisance from the environmental point of view. They used an "association" of three yeasts in continuous culture, and mentioned the stability of the association as compared to the variation of a series of parameters (temperature, pH, dilution rate), which leads us to consider this combination in terms of consortium, as it is more coherent from the point of view of its behavior than a simple association, which underlies a kind of independence of the species. The authors strongly insist on the absence of Crabtree effect, which confers on the strains used as a considerable advantage for the production of biomass. They quote a French cheese industry which in 1958, produced 800 tons of food yeasts and which was able to increase its production 10-fold in 1983. The interest of the process is thus obvious from a commercial and environmental point of view.

The consortium described here is composed of three species, in constant ratio: *Kluyveromyces fragilis* ($2.5\ 10^9$ cells/mL), *Torulopsis sphaerica* ($0.25\ 10^9$ cells/mL), and *Torulopsis bovina* ($0.0025\ 10^9$ cells/mL). *K. fragilis* is thus the very dominant species in the consortium. If it had presented a positive Crabtree effect, it could not have been unperceived.

Moreover, Nor *et al*. (76) reported an evident positive Crabtree effect presented by *K. fragilis* in a fed-batch culture on lactose.

Three major differences exist between the two situations:

- the mode of culture (chemostat vs fed-batch), which can influence the growth rate and the emergence of the Crabtree effect;
- the culture medium (whey vs lactose);
- the involved species (consortium vs pure strain).

The articles do not make it possible to favor an option above another to explain the change in the behavior of *K. fragilis*.

The third assumption is the most interesting and motivating: can a positive Crabtree strain be inhibited and behave as a Crabtree-negative one within a consortium? The answer obviously remains open, but it cannot be discarded, as will appear in section titled "Consortia."

The interest of such a speculation is obvious: a process which is not realizable with a pure strain because of a Crabtree effect (positive or negative) could be made possible if there exists a possibility of carrying it out with a consortium (or even an association).

The example of the cheese dairy (75) suggests that the profit could be doubled by simultaneously increasing the yield of the process beyond the loss due to the Crabtree effect.

Evolutionary Considerations. Several strains of *S. cerevisiae* are characterized by the presence of the Crabtree effect. Yet, how unique is this property among yeasts in the *Saccharomyces* clade? This question was addressed recently by Merico *et al*. (77) using the results of genome sequencing, a recent approach that provided data about phylogenetic relationships among yeasts. In their contribution, the authors have analyzed over 40 yeasts that reflect over 150 millions of years of evolutionary history for their ability to ferment, grow in the absence of oxygen and generate "petites" mutants. A great majority of the isolates exhibited significant fermentation ability, suggesting that this trait was already an intrinsic property of a progenitor strain. Moreover, they have found that, in general, lineages that underwent whole-genome duplication exhibited the Crabtree effect, fermentative lifestyle and capacity to generate petites mutants. There also existed some representatives of pre-genome duplication lineages who exhibited also these traits, but a majority of the studied species were petite-negative, and showed a reduced Crabtree effect and a reduced ability to grow in the absence

of oxygen. The ability to accumulate ethanol in the presence of oxygen, gradual independence from oxygen and/or ability to generate petites strains were developed later in several lineages. As a conclusion, it seems that these traits were combined and perfected only in the ancestor lineage that underwent whole-genome duplication and led to modern *S. cerevisiae* strains.

PROKARYOTES

Pure Strains

The acidogenesis of some bacteria, including in aerobic media, has been known since the years 1940–1950 (78). This phenomenon, initially known as "*acetate switch*", was the subject of fundamental studies during a few years and then lost importance over time, but today there is a revival of interest for industrial production reasons (see further text).

In 1967, however, Mustea *et al*. (79) made the correlation between this phenomenon and the Crabtree effect observed in cancer cells. Their motivation was to develop a less expensive and more rapid process to test anti-tumor agents by using bacteria. (It does not seem that this approach led to significant results.) They, thus, called the phenomenon *bacterial Crabtree effect* and evaluated it quantitatively in the following way as in Equation 11:

$$\text{Crabtree Effect } (\%) = \frac{q_{o_2}(o) - q_{o_2}(\text{GLU})}{q_{o_2}(o)} \times 100 \qquad (11)$$

where $q_{o_2}(o)$ is the specific respiration rate without glucose and $q_{o_2}(\text{GLU})$, the specific respiration rate with glucose.

It is obvious that it is a phenomenological relation, but which implicitly contains respiration as a dominating factor. Nevertheless, the observation that all the bacteria they studied do not show a Crabtree effect (such as *Micrococcus* and *Serratia*) brought them to declare that, probably, the appearance of the Crabtree effect is not related exclusively to the respiratory metabolism.

Wolfe (78) schematically represents the acetate switch as follows (Fig. 10):

The acetate switch is defined at the maximum of acetic acid production, when the dissimilation of the acid equalizes its assimilation. When the phenomenon occurs in aerobic condition, the dissimilation is called *Crabtree effect*. This effect ceases when growth finishes its exponential phase, that is, when the growth rate begins to decrease. However, a peak of acetyl coenzyme A (not secreted) precedes the Crabtree effect and takes place at still low growth rates. This behavior is different from what is observed in the yeasts Crabtree effect. Another main difference compared to yeasts relates to the biomass. Figure 11 represents some graphs showing various behaviors in aerobic batch for two strains of *E. coli E. coli* [MG1655 (closed symbols) and MG1655ΔPsdh ::P*tet* (open symbols)] (80)

It is noted well that the Crabtree effect (dissimilation phase) takes place in all the cases, but not the acetate switch, which takes place only for the weak concentrations in glucose. What is surprising, however, is the absence

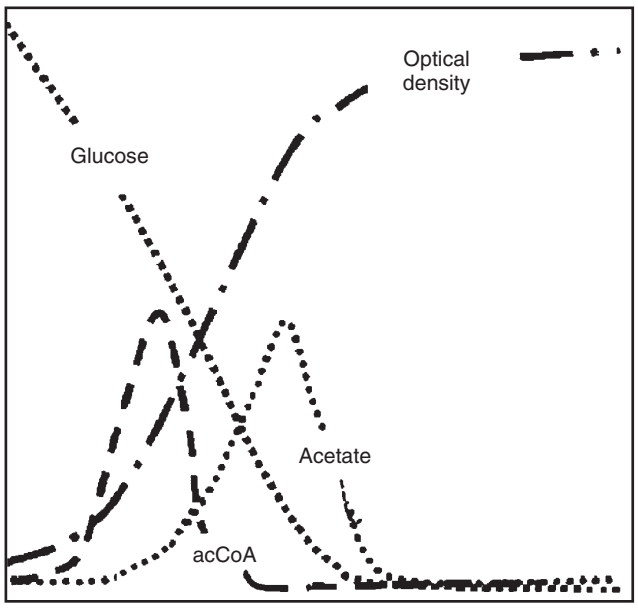

Figure 10. Evolution during time of glucose, acetate and acetyl Coenzyme A in a bacterium cultivated in batch (after (78)).

of biomass drop, which should result mainly from the dissimilation when acetate is not taken up by the cell. On the contrary, even if the total effect depends on the composition of the medium, the biomasses of the two strains are practically identical in all the experiments. This poses a problem either on the level of the mass balance, or on the level of the biomass measurement at 600 nm. However, this last assumption does not seem very plausible, because of the coherence of the different profiles appearing on Fig. 11.

These observations lead to suppose that the Crabtree effect in yeasts and bacteria is different from a metabolic point of view.

From a practical point of view, it should be noted that acetate is not the only metabolite excreted during the bacterial Crabtree effect. So, Wolfe (78) mentions a production of acetoïn by *Bacillus subtilis* in aerobic conditions, as well as other compounds. It also reviews a set of explanatory assumptions, which include the acetate switch in general and not only the Crabtree effect. These assumptions fall outside the scope of this article. Veit *et al*. (81) also quote the production of acetoïn, as well as acetone, lactate, polyhydroxybutyrate and ethanol.

It is necessary to keep in mind that two assumptions are to be rejected: all the mechanisms explaining the Crabtree effect through the mitochondrias are to be rejected, as these organoids are not present in prokaryotes; the explanation calling upon the proton motive force (81), (80) is also suspect when the excreted metabolites are not proton donors such as ethanol and 2, 3 butanediol (78).

The general appearance conditions of the Crabtree effect in bacteria (raised growth rate, or high glucose concentrations) can have negative consequences on industrial processes of production in batch and fed-batch. Luli and Strohl (82) recall that these conditions are prevalent in the fed-batch cultures, where the continuous addition

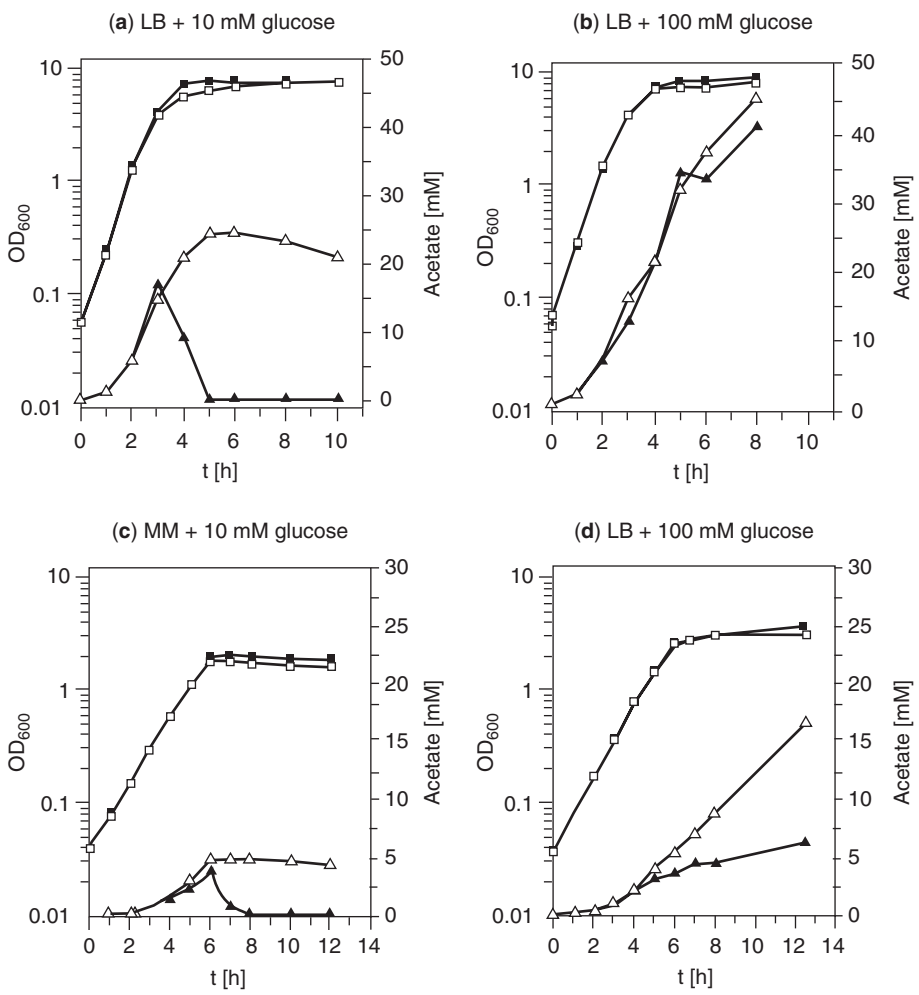

Figure 11. Evolution during time of biomass (□ or ■ OD_{600}) **and of acetate** (△ or ▲) for two strains of *E. coli* cultivated in aerobic batch on two different mediums (LB or MM) more or less supplemented in glucose (after (81)).

of substrate makes it possible to maintain a high growth rate. They report that the acidogenesis (especially due to acetic acid) is the most important limiting factor for obtaining high biomass densities. Moreover, they indicate that the Crabtree effect is also the cause of the reduction in the production of recombinant proteins. They also incriminate the decoupling between anabolism and catabolism for the limitations of fermentations, rDNA, final concentration in cells, cellular yield, genetic stability, and products stability.

Thus, as was the case for yeasts, the bacterial Crabtree effect is of great importance from the point of view of the industrial production of many compounds (recombinant metabolites, proteins etc.). The control of the Crabtree effect, once again, calls for the comprehension of the subjacent metabolic machinery. The explanation of the Crabtree effect is far from being known and it is probable that the phenomenon rests on several distinct mechanisms. It is perhaps important to note that three major assumptions merit a particular attention (81):

- a too high glucose uptake;
- the limitation of the tricarboxylic cycle capacity;
- the limitation of the respiratory potential.

These explanations are not very different from the major assumptions concerning yeasts; as the manifestations of the Crabtree effect sometimes differ significantly in eukaryotes and prokaryotes, it is not impossible that some fundamental mechanisms are still to be highlighted in one or both group(s). It is also likely that a single mechanism cannot give an explanation of the Crabtree effect in general.

Consortia

Associations of bacteria in the form of consortium are extremely current and some have often been studied. For example, the herbivore rumen (83), the dental plaque (84), the biofilms (85,86) or the flocs (87). It is generally admitted that a consortium is able to carry out metabolic tasks that an isolated bacterium could not realize (85,88).

To our knowledge, there exists only one published example, which highlighted a respirofermentative phenomenon within a consortium (89,90). A mathematical modeling of this experiment has shown that the algorithm previously described for *S. cerevisiae* (the PDS model) applies without modification for this metabolic switch

in the consortium (8). Parallelism between the two phenomena is so striking that the authors described it as a Crabtree effect.

Flocs of wastewater treatment plants were cultivated in a chemostat during 2 years in a medium known as *synthetic sewage feed* (91), amended by a little quantity of glucose and in aerobic conditions ($pO_2 > 40\%$). The consortium maintained itself in a stable composition in spite of the absence of sterile conditions (89). The respirofermentative transition arises at a dilution rate slightly higher than $D = 2/h$ (Fig. 12)

It should be noted that several differences exist between this example and that of *S. cerevisiae*. Without developing a comparative discussion here, the two phenomena undoubtedly correspond to the same "law" of transition, but the metabolic mechanisms are undoubtedly different. In particular, there exists a constant butyrate production rate (Fig. 13) for all dilution rates in the same aerobic conditions.

The practical interest of the discovery of this Crabtree effect within a consortium is double because it highlights the following:

1. It is possible to produce metabolites under absolutely not sterile conditions, while keeping a production either constant (butyrate), or variable in various steady states and to optimize the production (acetate, with a maximum toward $D \sim 0.6/h$).
2. The Crabtree effect would make it possible to produce metabolites that some isolated bacteria would not be able to produce alone.

This way to use consortia with production objectives, with or without Crabtree effect, is obviously still rather speculative and requires validating, as well as finding products with higher added value and the need for developing control techniques of the process. Nevertheless, it could

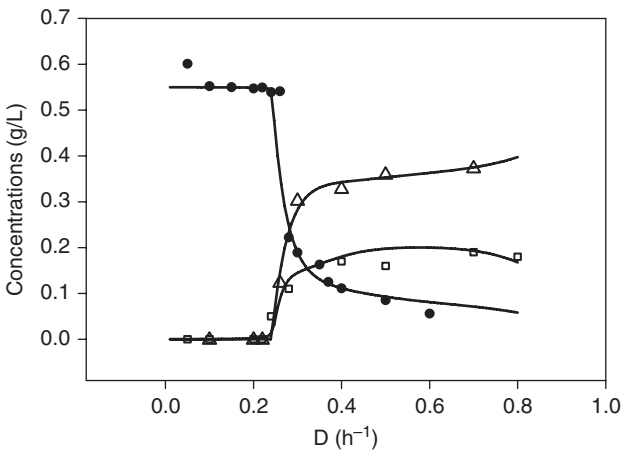

Figure 12. Biomass, residual glucose and lactic acid as a function of the dilution rate. The figure shows the experimental steady states concentrations of the biomass (●), the residual glucose (△) and the lactic acid (□) according to dilution rate. The full lines are the result of the residual glucose fitting of or the biomass and lactic acid simulation by the PDS model (after (8)).

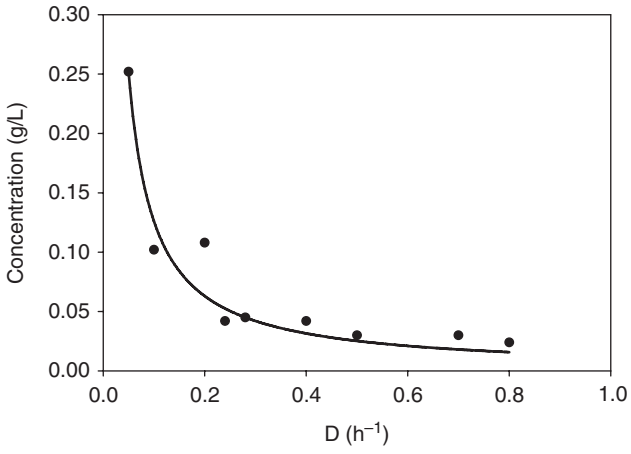

Figure 13. Butyric acid as a function of the dilution rate. The full circles show the experimental values and the full line fitting with the data. The parametric estimation gives a constant production rate of 0.012 g BUT/(L.h) (after (8)).

be that in activated sludge wastewater treatment plants, where the role of the volatile fatty-acids can be important, the description of the Crabtree effect can explain some paradoxes, in particular in the filamentous bacteria bulking. The bulking is a major accident in a wastewater treatment plant: floating biomass appears, escapes final settler and is rejected into the natural environment (river, lake, etc.).

From an industrial point of view, the production of compounds in continuous system, under biomass stability conditions and the absence of sterilization requirements would obviously be a significant improvement.

DISCUSSION

After this overview of the Crabtree effect it seems obvious, that this phenomenon is still far from being understood and has not even been entirely studied. Presently, it thus appears that no systematic methodology can be provided to improve a production process, which would be hindered by a Crabtree effect, especially if this methodology has to deal with every situation (bacteria, yeasts or mammalian cells).

Usually, the central paradigm was the improvement of the respiratory system.

Respiration: It seems that the option "improvement of the respiratory capacity" only is not any more a promising option. It can however be included in a multifactorial strategy. In some situations, especially for nonconventional yeasts, oxygen pressure in the culture medium is a factor to be taken into account.

In view of what precedes, it seems however possible to draw attention to a series of strategies able to generate working hypotheses in order to obtain solutions. Here are some:

- *Transport:* The option "modification of the transport capacities" seems to be a more promising way. Action on the transporters and/or the cellular permeability is thus a possibility not to be neglected.
- *Strain:* The properties of the strain (Crabtree-positive or negative) obviously take a great place and are classically one of the earliest factors taken into account; perhaps however, it is judicious to explore the association of a strain with one or several others and to make use of properties which are associated with "**Association** or **Consortia."** There is certainly a less traditional strategy but undoubtedly a promising one in order to improve production or to produce in a different way.
- *Medium:* The medium is also a traditional manner to seek solutions while trying to change the proportions or the "conventional" qualities of substrates. More "exotic" compounds can however have interesting effects whereas they are not, in general, considered as usual substrates [the oleate addition (51)].
- *Cultivation Technique:* This subject has not really been tackled in this article. The batch (often in lab research) and the fed-batch (especially in production) are the two methods most often used. But many studies which we considered above rest on continuous cultures and mainly on the chemostat. This mode of culture is believed to present great disadvantages in production [instability of the strains, less stability of the genetically modified organisms (GMO), higher infection risks, etc.]. The economic benefit of the reduction of the "idle periods" between two batches is however a considerable advantage. A few industrialists (levurists) have already adopted this mode of production, as far as we know, successfully. It is possible that some prejudices are attached to this mode of production, which, according to us, deserves to be developed.

As a final point, we would like to present an example that results from mathematical modeling. For *S. cerevisiae* in continuous culture, it was demonstrated (48,50) that the critical dilution rate corresponding to the respiratory/respirofermentative transition is given by (see Eq. 10):

$$D_c = V_S^o \times Y_{X^c,S}$$

where $Y_{X^c,S}$ is the global yield compared to the limiting substrate and $V_S^o$the maximum transport/metabolism rate of the substrate. This relation is "observable" if, and only if, the critical dilution rate is lesser than the wash-out dilution rate corresponding to the complete elimination of the biomass from the chemostat ($D_c < D_W$).

For the levurists, this D_c should be as high as possible; for the production of alcohol; on the contrary, this value should be as low as possible. It appears obviously that the optimization strategies are very different and strongly related to the production. The relation above describes the procedure to use in order to optimize the critical value but especially reveals that this procedure is multifactorial. Thus, V_S^o is a specific characteristic of the strain (linked to transport and associated metabolism), whereas $Y_{X^c,S}$ is a state variable depending both on the strain and on the substrate (culture medium). The yield dependence seems rather obvious, but the transport/metabolism rate is a more unexpected factor. In addition, it is conceivable that the two variables are interdependent. In this case, optimization is likely to be only a compromise between the two variables rather than an "independent" maximization of the two variables.

Similar results show how it is difficult to find an improvement of the process by basic empirical developments, and how a "methodological guide," such as the above relation, may be useful. Even the methods using an experimental plan are less reliable and more expensive (in time and money) than the use of a simple algebraic equation.

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LIST OF FURTHER READING

So far as we know, there is no book entirely dedicated to the Crabtree Effect. We refer to books where the subject is directly discussed or where general considerations could be of some interest.

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Abstract: The Crabtree effect is still little known and relates as well to prokaryotic cells (bacteria) as to eukaryotes (fungi and mammals). In this article we havetwo major aims: (i) to treat the Crabtree effect as exhaustively as possible (e.g. by not restricting ourselves to *Saccharomyces cerevisiae*) and (ii) to adopt a presentation tailored to the philosophy of the encyclopedia.
This means that the malignant cells Crabtree effect was treated in a more fundamental and biochemical way because these cells, as such, are less subject to industrial cultivation or production processes.
The other two categories (fungi and bacteria) were treated in a more physiological way, so as to provide a maximum of proposals for the optimization of a culture and/or production process.
In particular, for yeasts, we tried to make the point as precisely as possible and attempted to decide between the various assumptions explaining the metabolism involved. Indeed, a bad interpretation of this process can lead to an inadequate approach to research into practical solutions for a production problem. We are conscious of the slightly polemical aspect of this section, but that reflects the actual state of the art in this field.
Relating to bacteria, the literature is undeniably limited and the results remain basically experimental. The polemic on the possible mechanisms of the Crabtree effect is thus quasi non-existent: either because it is not considered or because it is extrapolated starting from the yeast problems. We also adopted a novel, and perhaps promising approach by considering the use of association or consortia of microorganisms to produce high value products at low costs. Except in very exceptional cases, this approach will probably require numerous fundamental R&D efforts.
We came to the conclusion that we cannot put forward a "universal" and ultimate mechanism. Our discussion is then based on the main parameters that have to be taken into consideration to act in a practical way on the Crabtree effect (in a positive or negative way). It did not appear achievable, in the current state of our knowledge, to provide a simple "recipe" to resolve this kind of problems.

Keywords: cellular respiration; glycolysis; anaerobic facultative bacteria; mammalian tumor tissues; metabolic configuration; metabolic switch;