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MEMBRANE TRANSPORT AS CONTROLLING PACEMAKER OF GLYCOLYSIS IN *SACCHAROMYCES CARLSBERGENSIS*

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SUMMARY

The frequency of anaerobic NADH oscillations in *Saccharomyces carlsbergensis* following addition of fructose is 1.5–2.0 times slower than that following addition of glucose. Since catabolism of the two sugars differs in the sugar phosphorylation pathway only, the reason for the observed alternation of the frequency must be sought among these reactions. It has been found that:

1. The equilibrium of hexose phosphates, as catalized by phosphoglucose isomerase, is nearly independent of the added hexose.

2. The yield of ethanol, the end product of anaerobic glycolysis in yeast, depends on the rate of sugar consumption rather than on the kind of assimilated sugar.

3. The two hexoses stimulate equally the utilisation of storage material.

4. The half-saturation constant of fructose consumption is 10 times higher than that of glucose consumption.

5. Intracellular fructose concentration may reach a level 10 times higher than that of glucose.

6. Analysis of the kinetic data points to membrane transport as the rate limiting step of glycolytic flux.

Application of the crossover theorem showed that the passage of sugar molecules across the cell membrane is affected by regulatory alterations of the flux as well.

Glucose 6-phosphate is discussed as a possible feed-back inhibitor of membrane transport.

INTRODUCTION

The addition of glucose to a cell suspension of *Saccharomyces carlsbergensis* causes, after transition to anaerobiosis, characteristic oscillations in the redox state of nicotinamideadenine nucleotides and in glycolytic intermediates. We observed that these oscillations occur not only following the addition of glucose but also when other sugars, *e.g.* fructose and mannose are fermented. Fructose, however, causes a 1.5–2 times longer oscillation period than glucose does. It is assumed that the oscillations arise from modulations of the activity of rate-limiting enzymes, *i.e.* pacemakers, in glycolysis with phosphofructokinase (EC 2.7.1.11) as the primary oscillator[1–3]. However, once produced at the phosphofructokinase step, the oscillations

are transferred to other reactions by means of the fluctuating levels of metabolites and adenine nucleotides. The secondary oscillating reactions by their kinetic properties affect, in turn, the primary oscillator, *i.e.* the phosphofructokinase reaction. Since this pacemaker enzyme is also responsible for the Pasteur effect, the oscillations are regarded as a consequence of the Pasteur regulation. Thus, the difference in frequencies of the glucose- and fructose-induced oscillations points to another site of control prior to the phosphofructokinase reaction. Sols[4] and Kotyk and Kleinzeller[5] also claimed that the Pasteur effect is not explained by the allosteric properties of this enzyme solely. They have postulated an additional regulating step prior to this reaction.

Following fructose 6-phosphate, fructose and glucose are degraded along a common pathway. The only steps of their catabolism that differ are illustrated in scheme 1 (see p. 594). They include: (1) transport across the cytoplasmic membrane; (2) phosphorylation by hexokinase (EC 2.7.1.1); (3) isomerisation by phosphoglucose isomerase (EC 5.3.1.9); (4) pathway to the polyglucoside storage; (5) entrance from the polyglucoside storage via glucose 6-phosphate into glycolysis.

In this paper an attempt is made to answer the following questions: (1) Is the flux into glycolysis independent of the kind of hexose that is metabolized? (2) Do glucose and fructose have different influences on the degradation of storage material? (3) Is the membrane or the hexokinase reaction pacemaker of sugar consumption? (4) Can these reactions be regulated?

MATERIALS AND METHODS

Cultivation of yeast cells. Yeast cells (*Saccharomyces carlsbergensis*, ATCC 9080) were cultivated and aerated as described by Betz and Chance[6]. The aerated cells were suspended in 50 mM KH_2PO_4 buffer (pH 4.5) to yield a 10% (w/v) suspension. This suspension was used throughout all experiments which were carried out at 25 °C. The oscillations were recorded by monitoring the redox state of the nicotinamide–adenine dinucleotides[7]. In some experiments the oxygen tension was measured by means of a Clark-type electrode.

Sugar consumption. Samples of the yeast suspension were withdrawn at intervals from 5 to 60 s and the metabolism terminated by addition of 5% $HClO_4$ (final concentration). After centrifugation an aliquot of the supernatant was neutralized and diluted with a mixture of a calculated amount of KOH and 50 mM triethanolamine, so that the resulting sugar concentration amounted to about 1 mM.

Determination of intracellular sugar. Samples were withdrawn from the incubation mixture and vacuum filtered through membrane filters (Selectronfilter, 1.2 μm pore size, Schleicher und Schüll, Dassel, Germany), washed twice with ice-cold water and extracted 10 min with boiling water in a water bath. To shorten the lenghth of time between successive withdrawals of samples, eight Schott filter adaptors were connected in series to which vacuum could be applied individually by means of magnetic valves. This arrangement allowed samples to be taken at intervals of 25 s. Since the whole procedure was carried out in a refrigerated room (4 °C), the loss of sugar during this time is minimal[5].

CO_2 production from storage material. In order to measure the participation of storage material in cell metabolism during fermentation of added hexose, the yeast

cells were grown on D-[^{14}C]glucose to label all sources of endogenous metabolism. CO_2 production was recorded by conventional manometry[8] in Warburg flasks with two side arms. Each flask contained 0.8 ml 50 mM KH_2PO_4 and 1 ml 5 % yeast suspension. The reaction was started by the addition of 0.2 ml hexose into the main compartment from one side arm and terminated by adding 0.1 ml 70 % $HClO_4$ from the other. Anaerobic conditions were induced by blowing pure nitrogen through the flasks for 20 min. Two blanks were obtained by terminating the reaction immediately after flushing with nitrogen. The $^{14}CO_2$ evolved was trapped in 0.075 ml phenylethylamine in the central well. Its radioactivity was counted in a Packard Tricarb scintillation spectrometer, Model 3002. The values were corrected for quenching by means of the external standard.

Hexoses, hexose phosphates and ethanol were determined enzymically following the methods of Slein[9] and Bonnichsen[10], respectively, with minor modifications.

All sugars and other chemicals (of analytical reagent grade) were purchased from E. Merck, Darmstadt, Germany. Enzymes and co-substrates were obtained from Boehringer, Mannheim, Germany. Labelled D-glucose was a product of the Radiochemical Center, Amersham, England.

RESULTS

The equilibrium between glucose 6-phosphate and fructose 6-phosphate

There are two pathways competing for sugar taken up: glycolysis and the storage pathway. Phosphorylation of fructose results in fructose 6-phosphate, which as substrate of phosphofructokinase can be directly degraded further along the glycolytic pathway. Glucose, on the other hand, is phosphorylated to glucose 6-phosphate which can be used as precursor of the storage pathway. The hexose 6-phosphates are converted into one another by phosphoglucose isomerase. *In vivo*, there is only a very slight deviation in the ratio of the Fru-6-*P*/Glc-6-*P* concentrations, regardless whether glycolysis uses glucose or fructose as substrate. The value of the equilibrium constant of the phosphoglucose isomerase (K_{PGI}) in a closed system *in vitro* is[11]:

$$K_{PGI} = [\text{Fru-6-}P]/[\text{Glc-6-}P] = 0.33 \ (30\,^{\circ}\text{C})$$

The mean value *in vivo* of the equilibrium constant of this reaction after addition of glucose was measured to be

$$K_{PGI} = 0.2640 \pm 0.0007$$

and after fructose addition

$$K_{PGI} = 0.3310 \pm 0.0020$$

(mean values of 8 and 12 determinations, respectively, $\pm$ standard error). These constants were determined in samples withdrawn during oscillatory glycolysis. They do not change even if the two metabolites are oscillating. This means that the activity of phosphoglucose isomerase is sufficiently high to always maintain the ratio of the hexose phosphates close to equilibrium as observed for glucose metabolism by Betz and Chance[6].

Due to the high activity of phosphoglucose isomerase, all metabolic routes at the beginning of glycolysis are equally accessible to glucose and fructose.

Correlation of sugar consumption and ethanol production

Betz and Hinrichs[12] reported the storage of 40–45 % of glucose consumed into an insoluble polysaccharide. Only 50 % entered the glycolytic pathway and appeared as ethanol. Even if, as shown above, the two sugars can indiscriminately enter all metabolic routes, it remains to be shown whether indeed equal amounts of glucose and fructose undergo glycolytic degradation. Fig. 1 demonstrates that there is no proportionality between the yield of ethanol and flux of hexose into the cell. With increasing sugar consumption, ethanol production passes through a maximum (at 10–15 μmoles sugar/min per g yeast), with values for yield ranging from 40–70 %. However, there is no difference in ethanol yield, regardless of whether glucose or fructose is catabolized.

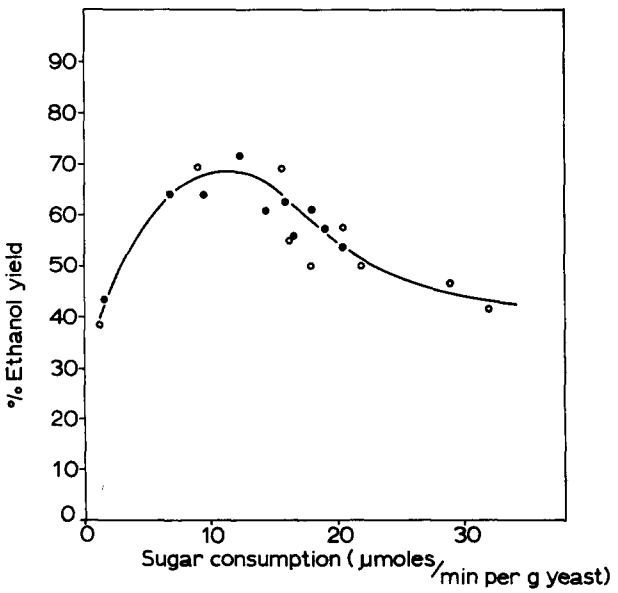


Fig. 1. % Yield of ethanol correlated to hexose consumption rates. Yeast cells were incubated anaerobically at increasing hexose concentrations. Samples were withdrawn at intervals and assayed for hexose and ethanol concentration. The ethanol yield was calculated from hexose consumption and ethanol production by assuming that 1 mole of hexose gives rise to 2 moles of ethanol. Values were obtained from four different yeast batches. ○, glucose added; ●, fructose added.

Degradation of storage material during fermentation of added hexoses

Storage material can enter glycolysis via glucose 6-phosphate. Recently, von Klitzing[13] demonstrated that during continuous feeding of *S. carlsbergensis* a large amount (up to 100 %) of the ethanol comes from storage material. Iscaki[14] reported a similar phenomenon in *Saccharomyces cerevisiae*. However, in both cases the rate of sugar consumption was only one-tenth to one-hundredth of that observed in yeast cells with optimal substrate supply.

In order to be able to estimate the amount of CO_2 from storage material, the

cells were grown on uniformly ^{14}C-labelled D-glucose. The specific activity of the endogenous $^{14}CO_2$ was determined by measuring the volume of CO_2 evolved under aerobic conditions in the absence of any utilizible substrate, and its radioactivity. *S. carlsbergensis*, like other yeast species, uses its storage material in fermentation only so slowly that it is impossible to record CO_2 production manometrically[15]. Therefore the specific radioactivity of $^{14}CO_2$ had to be determined with endogenously respiring yeast, which produces a measurable amount of CO_2. Table I compares CO_2 production under anaerobic and aerobic conditions and shows the enormous difference between them.

TABLE I

$^{14}CO_2$ PRODUCTION FROM STORAGE MATERIAL WITHOUT ADDITION OF SUGARS

$^{14}CO_2$ from cells grown on labelled glucose was trapped and counted for radioactivity. Simultaneously the volume of the CO_2 was determined. Since endogenous CO_2 production is too small to be determined volumetrically under anaerobic conditions, endogenous respiration was used for the determination of $^{14}CO_2$-specific radioactivity. For further details see Materials and Methods.

Anaerobic		*Aerobic*	
CO_2 production, calculated mean (μmoles/h per g yeast)*	*dpm/h per g yeast*	*CO_2 production measured (μmoles/h per g yeast)*	*dpm/h per g yeast*
0.702	174	15.5	3090
	123		3390

* This value was calculated from the specific radioactivity as determined by endogenous respiration, 210 dpm/μmole CO_2, and from the $^{14}CO_2$ trapped during endogenous fermentation.

TABLE II

$^{14}CO_2$ EVOLVED FROM STORAGE MATERIAL ANAEROBICALLY AFTER ADDITION OF EITHER GLUCOSE OR FRUCTOSE

Experimental conditions are described in legend to Table I. The values given are means of two determinations. The specific radioactivity amounted to 210 dpm/μmole CO_2.

Sugar	*CO_2 evolved (μmoles/h per g)*	*dpm/h per g*	*CO_2 from storage material* (μmoles/h per g)*	*% $^{14}CO_2$ from storage material*
Glucose	1992	5160	24.6	1.1
Fructose	1914	4340	20.6	1.2

* These values were calculated from the $^{14}CO_2$ radioactivity trapped and the specific radioactivity determined in Table I.

When cells are supplied with glucose or fructose anaerobically, the production of endogenous $^{14}CO_2$ increases 25-fold in comparison with endogenous fermentation without added hexoses. However, even the 25-fold stimulated endogenous fermentation represents only approximately 1 % of total CO_2 evolved (Table II). The degradation of storage material proceeds continuously throughout the experiment, as

TABLE III

KINETICS OF $^{14}CO_2$ PRODUCTION FROM STORAGE MATERIAL DURING FERMENTATION OF ADDED GLUCOSE

Experimental conditions are given in the legend to Table I. The reaction was terminated at given intervals and duplicate samples counted for trapped $^{14}CO_2$. The mean values are given.

Time (min)	*CO_2 evolved*		*$^{14}CO_2$ from storage material* (μmoles)*	*% CO_2 from storage material*
	μmoles	*dpm*		
2	54.8	256	1.16	2.1
10	274	1280	5.9	2.1
40	1092	3080	14.14	1.3

* Calculated as in Table II, specific radioactivity 218 dpm/μmole CO_2.

shown in Table III. The difference in the utilisation of storage material between yeast suspensions supplied with saturating concentrations of sugars and those supplied with very small quantities by the infusion technique[13] is obvious. In the first case only 1% of CO_2 evolved comes from storage material, whereas it might be up to 100% in the latter. Most important, however, is the fact that production of $^{14}CO_2$ from storage material remains constant, regardless of whether fructose or glucose is fermented (Table II).

Dependence of sugar consumption on extracellular sugar concentration ($[S_0]$)

Since there is no difference in the distribution of a sugar in various metabolic pathways after it has been phosphorylated, alterations in the parameters of the oscillations must be caused by a difference in membrane passage or in the hexokinase reaction. One of these two steps must be rate limiting in the sequence of glycolytic reactions. Moreover, it should be capable of being regulated. Metabolizing yeast cells exhibit different metabolic fluxes depending on the extracellular sugar concentration. This dependence resembles substrate saturation curves of an enzymic reaction

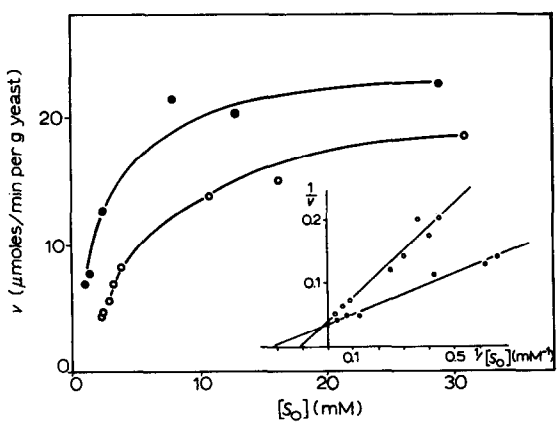


Fig. 2. Dependence of hexose consumption on extracellular hexose concentration $[S_0]$. Yeast cells were incubated anaerobically with varied glucose (●—●) and fructose (○—○) concentrations. Sugar consumption was measured as described in Materials and Methods.

(Fig. 2). A half-saturation constant of sugar consumption corresponding to the Michaelis constant has been calculated for three separate yeast batches (Table IV). However, it is impossible to conclude from the dependence of the flux on $[S_0]$ whether the rate-limiting step is the membrane transport or the intracellular hexokinase reaction.

TABLE IV

HALF-SATURATION CONSTANTS OF GLUCOSE ($K_{glucose}$) AND FRUCTOSE ($K_{fructose}$) CONSUMPTION FOR THREE DIFFERENT YEAST BATCHES

For experimental conditions see legend to Fig. 3. Values were obtained by using the double reciprocal plot of Lineweaver and Burk[27].

Expt	$K_{fructose}$ (*mM*)	$K_{glucose}$ (*mM*)
1	7.7	4
2	10.0	0.6
3	7.0	0.7

Intracellular sugar concentration ($[S_i]$)

According to the crossover theorem by Chance *et al.*[16], the pacemaker reaction in a metabolic sequence can be recognized if following inhibition of such a reaction its substrate concentration increases and its product decreases. The opposite effect is observed after activation of the specific reaction. The intracellular sugar is the "product" of the transport step and simultaneously the substrate for hexokinase. By considering the alteration of the flux and the resulting sugar level in the cell, it is possible to find out which reaction is rate limiting. Fig. 3 shows, for both glucose and fructose, the dependence of $[S_i]$ on $[S_0]$. There are considerable differences between the two hexoses. The intracellular glucose concentration rises up to 10 mM $[S_0]$ and then remains fairly constant. In contrast, the intracellular fructose concentration increases with increasing $[S_0]$ up to 100 mM, eventually reaching saturation. Here $[S_i]$ of fructose exceeds $[S_i]$ of glucose by a factor of 10. Very important is the fact that neither of the two sugars reaches an intracellular concentration that even approaches the diffusion equilibrium. On the contrary, with increasing $[S_0]$ it diverges still more from equilibrium (Fig. 4).

From these results it is possible to decide which of the two reactions in question, transport or phosphorylation, actually limits the flux. The intracellular sugar level, following addition of glucose, is very low and remains nearly constant over the wide range from 10 to 100 mM $[S_0]$. At least in this range which we tested the membrane limits the flux. Hexokinase is sufficiently active to phosphorylate immediately all sugar molecules taken up. Therefore, the intracellular level remains low. From experiments with glucose and mannose in *S. cerevisiae* Sols[4] draws the same conclusion.

Because of the higher intracellular fructose level, at first glance hexokinase seems to be the rate-limiting step in fructose fermentation. However, more detailed

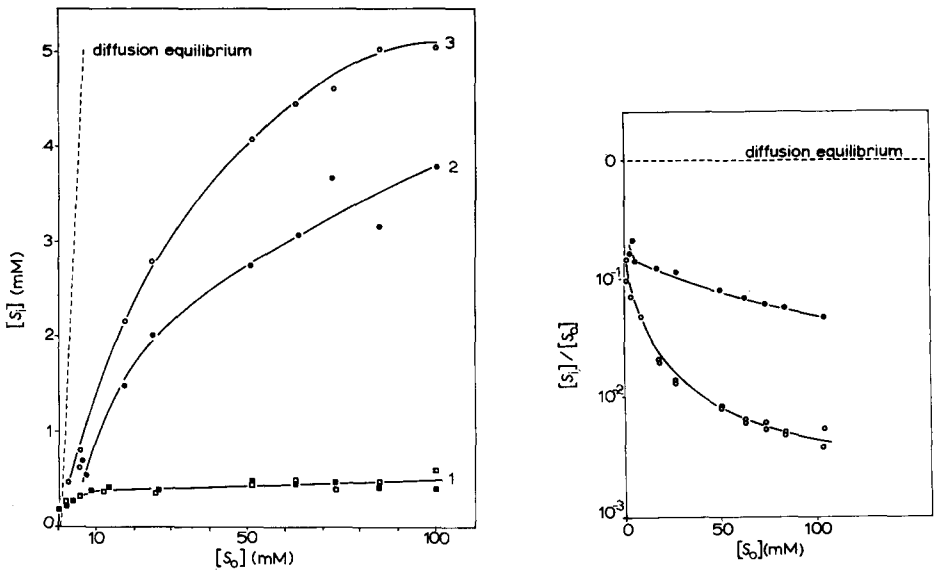


Fig. 3. Dependence of the intracellular hexose concentration $[S_i]$ on the extracellular hexose concentration $[S_0]$ anaerobically and aerobically. Yeast cells were incubated with increasing concentrations of hexose. From each run four samples were withdrawn, two simultaneously in the presence of oxygen and two after transition to anaerobiosis, when hexose consumption under the two given conditions had reached its steady value. Oxygen tension was recorded by a Clark-type electrode. One sample of each pair was injected into $HClO_4$ for determination of the total sugar concentration, the other was assayed for intracellular sugar as described in Materials and Methods. $[S_i]$ is given in mmoles/l cell water, assuming a cell water content of 66 % of yeast fresh weight. Fructose: ○—○, anaerobically; ●—●, aerobically. Glucose: □—□, anaerobically; ■—■, aerobically.

Fig. 4. Dependence of the concentration gradient $[S_i]/[S_0]$ on $[S_0]$ under anaerobic conditions. Experimental values are taken from Fig. 3, Curves 1 and 3 (○—○, glucose; ●—●, fructose).

analysis of the $[S_i]/[S_0]$ ratio for fructose, as $[S_0]$ increases, points again to the membrane as the rate-limiting step in the sugar phosphorylation pathway. In yeast cells hexoses are transported by facilitated diffusion. The rate can be described by the following equation[17], for the case where $[S_0] \gg [S_i]$:

$$v_T = c_t \cdot D' \frac{[S_0]}{[S_0] + K_{CS}} \quad (1)$$

(v_T is rate of transport; c_t, total concentration of carrier molecules at the outer surface of the membrane; D', rate constant of diffusion through the membrane; and K_{CS}, dissociation constant of the substrate–carrier complex). The sugar taken up is phosphorylated at a rate determined by the Michaelis–Menten equation:

$$v_{HK} = E_t \cdot k \frac{[S_i]}{[S_i] + K_m} \quad (2)$$

(v_{HK} is rate of the hexokinase reaction; E_t, total hexokinase concentration; k, rate constant of the breakdown of enzyme–substrate complex; K_m, Michaelis constant). $[S_i]$ is governed by both equations[1,2]. Under all conditions of Fig. 4, v_T equals v_{HK}. If the capacity of the hexokinase reaction were lower or comparable to that of the

transport step, *i.e.* if hexokinase were the pacemaker, then S_i should approach the diffusion equilibrium at least in that concentration range where hexokinase is saturated. However, the opposite results have been found experimentally (see Fig. 4).

There is, of course, another possibility: the transport system is saturated prior to hexokinase saturation. In this case the membrane would only be rate limiting at higher $[S_0]$. This means that at certain low concentrations of extracellular fructose the capacity of transport exceeds that of hexokinase. As a result thereof the $[S_i]$ must show at least a trend towards the diffusion equilibrium. This could be excluded by the following experiment. The highest alteration of the flux (Fig. 2) is caused by external fructose concentration in the range from 0 to 30 mM $[S_0]$. Therefore, neither the transport system nor the hexokinase can be saturated in this concentration range. The corresponding $[S_i]$ values of fructose are shown in Fig. 5. At first $[S_i]$ increases nearly linearly, but then the slope decreases with higher external concentration. Again, there was no trend towards the diffusion equilibrium.

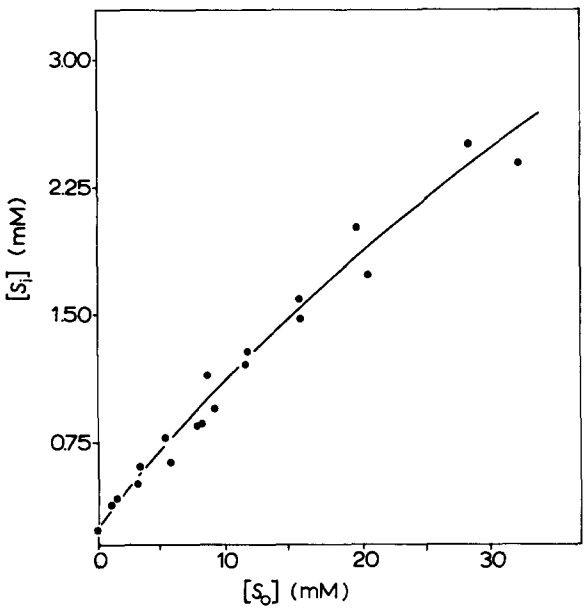


Fig. 5. Dependence of intracellular fructose concentration $[S_i]$ on extracellular fructose concentration $[S_0]$ in the range of highest flux alteration, anaerobically. For experimental conditions see legend to Fig. 3.

These results allow only two explatations: (1) The membrane is rate limiting over the whole range of $[S_0]$ tested. (2) Hexokinase and transport are so tightly coupled that sugar uptake never exceeds the phosphorylating capacity of hexokinase.

In the experiments previously described, the metabolic flux was altered by changing the extracellular sugar concentration. However, the cell is also capable of regulating its sugar consumption in response to a particular changing environment. How far hexokinase or the membrane participate in this regulation, will be shown in further experiments. After transition to anaerobiosis the sugar consumption in a cell suspension fluctuates[18]. Fig. 6 shows an example, where both the intracellular

and the extracellular sugar concentrations were measured at intervals. If hexokinase or one of the glycolytic reactions were the pacemaker of the metabolic flux, the intracellular sugar concentration should be higher at the beginning of the experiment, where the flux is very low. However, $[S_i]$ of fructose decreases nearly in parallel with the external concentration, independent of the variations in flux rates.

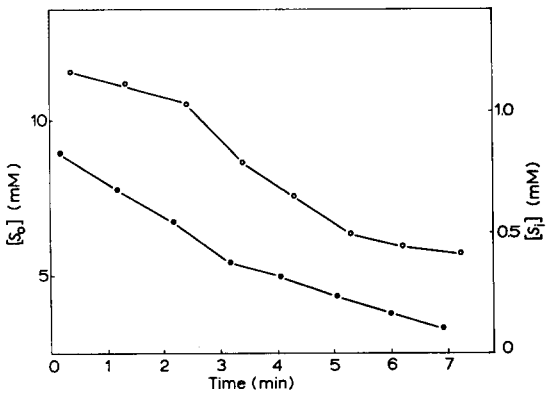


Fig. 6. Alterations of metabolic flux and intracellular fructose concentration, anaerobically. Experimental conditions are given in legend to Fig. 3. Note the different scales for $[S_i]$ (●—●) and $[S_0]$ (○—○).

Aerobic yeast consumes less glucose and fructose than anaerobic (Pasteur effect). However, the aerobic intracellular fructose concentration is only two-thirds to three-quarters that of the anaerobic (Curves 1 and 2 of Fig. 3). Consequently, the lower flux must be adjusted at the membrane. Otherwise one would have to expect a higher intracellular concentration under aerobic conditions.

During oscillatory glycolysis the metabolic flux pulsates, causing alternatively an accumulation and dissipation of metabolites corresponding to 10–15 % of the total flux during one cycle[18]. Therefore, the activities of regulatory enzymes must be regulated in the same range. However, this slight modulation causes oscillations in nearly all metabolites, e.g. in the concentration of glucose 6-phosphate which varies with an amplitude up to 50 % of its mean concentration during fermentation of fructose[19].

Assuming that the transport across the membrane remains constant, the intracellular sugar concentration should oscillate with the same frequency as other glycolytic intermediates do. But as can be seen in Fig. 7, in a typical experiment, $[S_i]$ of fructose does not change throughout the oscillation, except for the first NADH maximum where $[S_i]$ reaches a significantly lower level. From other experiments it is known that the flux during the first NADH maximum is lower than that during the following cycles. Thus, the lower $[S_i]$ during the first NADH maximum points again to membrane transport as the flux-controlling reaction.

During the next NADH cycles, $[S_i]$ of fructose remains constant. If one assumes only a 10 % activation and inhibition of hexokinase alone, the $[S_i]$ of fructose should oscillate between 3.7 and 2.3 μmoles/g yeast. This value is calculated assuming an average flux of 20 μmoles/g yeast per min, a constant transport rate, a period length of 40 s and simplification of the sinusoidal wave form to a triangular oscillation.

Such fluctuations of the intracellular fructose should have been detectable with the method applied.

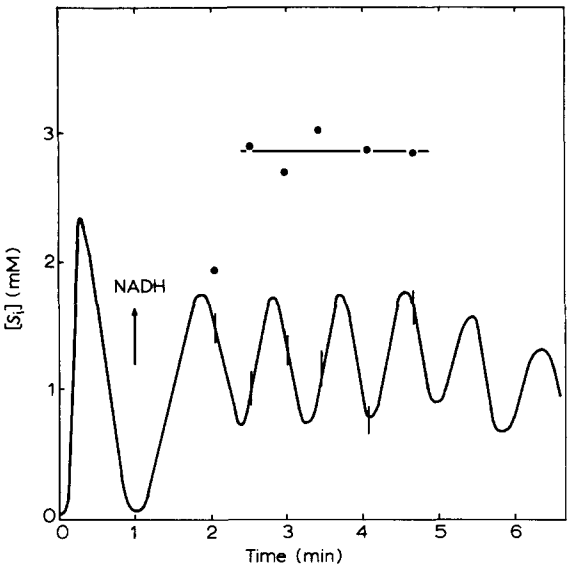
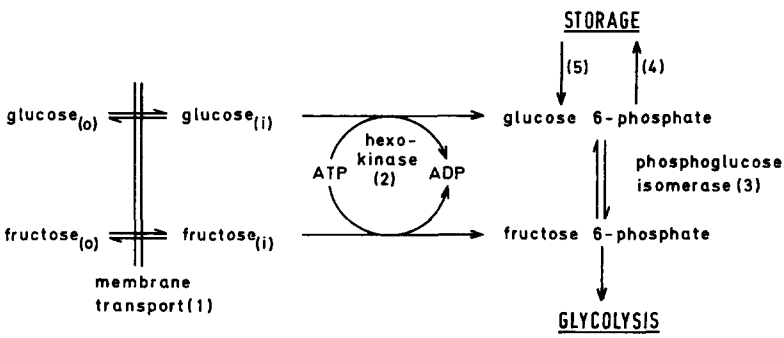


Fig. 7. Intracellular fructose concentration during oscillatory glycolysis. The oscillation of reduced nicotinamide–adenine dinucleotides was monitored fluorimetrically (in arbitrary units). Samples were withdrawn at different times during oscillation and assayed for intracellular fructose as described in Materials and Methods. Initial fructose concentration: 40 mM.

DISCUSSION

The period length of the NADH oscillation after addition of glucose and fructose can be influenced only by one of the reactions shown in Scheme 1. The experiments presented in this paper have shown that only membrane transport or hexokinase can be responsible for the different period lengths. Two possibilities are to be considered: (1) the flux is limited by membrane transport alone, or (2) membrane transport is tightly coupled to hexokinase activity, so that the sugar transported is



Scheme 1. The first reactions of glucose and fructose metabolism. Numbers in parentheses correspond to the reactions described in the Introduction.

immediately phosphorylated*. Two experiments favour the latter possibility. In Fig. 5, the intracellular sugar concentration increases nearly linearly with the external concentration, indicating a coupling of the two processes. The apparent coupling might find further support in the experiment of Fig. 7. The intracellular fructose concentration remains constant, although the glycolytic flux pulsates. If membrane transport were the only process affected, the sugar level in the cells should also oscillate. However, it remains constant. This can only be the case when uptake of the sugar and its removal by the hexokinase reaction are coupled. This view finds further support in the fact that hexokinase is the only glycolytic enzyme which is bound in some cells to a particulate fraction, which may consist of membrane particles[21].

In this respect, there is obviously no essential difference between glucose and fructose. The higher intracellular fructose concentration can be readily explained. The ratio of the Michaelis constant of hexokinase for fructose to that for glucose is known to be 10:1 (ref. 22). In the saturation range of sugar consumption, at $[S_0]$ of about 100 mM for fructose and glucose, the intracellular fructose and glucose concentrations display again the same ratio of 10:1 (Fig. 3). The maximum rate of consumption of the two sugars is nearly the same. Thus, due to its lower affinity for hexokinase, fructose has to reach a 10 times higher $[S_i]$ than glucose to yield the same flux rates as attained with glucose.

All these results, especially the steady deviation of $[S_i]$ from diffusion equilibrium, support the conclusion that membrane transport, in some way coupled with the hexokinase reaction, controls sugar consumption in *S. carlsbergensis* cells. In this connection the data of Racker[21] with ascites tumor cells could supply further support for this conclusion. The cell-free extract consumes as much as three times more glucose than intact cells and displays no indication of Pasteur effect. Kleinzeller and Kotyk[23] have also mentioned the possibility of membrane transport being the rate-limiting step of glycolysis in connection with the Pasteur effect in yeast cells.

As a possible coupling factor between the phosphorylation step and transport, glucose 6-phosphate may be considered. It could function as a feedback inhibitor of membrane transport. Azam and Kotyk[24] have been able to show that efflux of D-xylose from yeast cells was inhibited by high intracellular concentrations of this metabolite. This pentose is transported by the same carrier as D-glucose and D-fructose. The authors concluded that glucose 6-phosphate acts as a moderator of facilated diffusion across the *S. cerevisiae* cell membrane.

The inhibition of sugar transport by glucose 6-phosphate could also account for the pulsating sugar consumption, observed by Betz[18] during oscillatory glycolysis, which is in phase with fructose 1,6-diphosphate synthesis. Because the phase angle between glucose 6-phosphate and fructose 1,6-diphosphate oscillation is 180° (ref. 25), the rate of sugar consumption proceeds parallel with the synthesis of fructose 1,6-diphosphate.

We could not find any significant difference in sugar assimilation between glucose and fructose, with the exception of the hexokinase reaction and membrane

* There is another possibility, namely that fructose is degraded along a completely different pathway, as, for instance, in human liver[20]. However, there is no indication for such a pathway in *S. carlsbergensis*.

transport. Therefore, the results of this communication suggest very strongly that the decreased frequency in fructose-induced anaerobic glycolytic oscillations is caused by the different transport kinetics of fructose, as compared with glucose. It will be shown elsewhere (J. U. Becker, manuscript in preparation) that fructose catabolism considerably changes the concentration of phosphofructokinase effectors. Therefore, we suggest the coupling between the permeability properties of the cell membrane and the phosphofructokinase reaction, as the primary oscillator, to be of great importance for the frequency and eventually for the initiation of glycolytic oscillations.

In this connection it should be mentioned (see, for instance, the review of Hess and Boiteux[26]) that yeast extracts do not oscillate after a single addition of hexose. However, they do oscillate if the hexoses are added by an injection technique or if trehalose or maltose are administered. In the former case the influence of the cell membrane is avoided, in the latter case the cell membrane is simulated by either the continuous limiting flow of added hexose or by enzymic breakdown of the added disaccharides.

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REFERENCES

1 A. Gosh and B. Chance, *Biochem. Biophys. Res. Commun.*, 16 (1964) 174.
2 J. Higgins, *Proc. Natl. Acad. Sci. U.S.A.*, 51 (1964) 989.
3 E. Sel'kov, *Eur. J. Biochem.*, 4 (1968) 79.
4 A. Sols, *Symp. on Some Aspects of Yeast Metabolism, Dublin, 1965*, Blackwell Sci. Publ., Oxford, 1966, p. 47.
5 A. Kotyk and A. Kleinzeller, *Biochim. Biophys. Acta*, 135 (1967) 106.
6 A. Betz and B. Chance, *Arch. Biochem. Biophys.*, 109 (1965) 585.
7 B. Chance and V. Lagallais, *Inst. Electr. Electron. Eng. Trans. Biomed. Eng.*, 10 (1963) 40.
8 W. W. Umbreit, R. H. Burris and J. P. Stauffer, *Manometric Techniques*, Burgess Publ. Co., Minneapolis, Minn., 1957, p. 274.
9 M. W. Slein, in H.-U. Bergmeier, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, Bergstrasse, 1st edn, 1962, p. 117.
10 R. Bonnichsen, in H.-U. Bergmeier, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, Bergstrasse, 1st edn, 1962, p. 285.
11 S. E. Kahana, O. H. Lowry, D. W. Schulz, J. V. Passeneau and E. J. Crawford, *J. Biol. Chem.*, 235 (1960) 2178.
12 A. Betz and R. Hinrichs, *Eur. J. Biochem.*, 5 (1968) 154.
13 L. von Klitzing, *Arch. Mikrobiol.*, 72 (1970) 106.
14 M. Iscaki, *C. R. Acad. Sci. Paris, Sér. D*, 268 (1969) 3043.
15 E. A. Dawes and D. W. Ribbons, *Annu. Rev. Microbiol.*, 16 (1962) 241.
16 B. Chance, J. Higgins, W. Holmes and C. M. Connelly, *Nature*, 182 (1958) 1190.
17 A. Kotyk and K. Janacek, *Cell Membrane Transport*, Plenum Press, New York, 1970, p. 63.
18 A. Betz, *Physiol. Plant.*, 19 (1966) 1049.
19 J. U. Becker, Ph. D. Thesis, Bonn, 1971, p. 46.
20 F. Heinz, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 399.
21 E. Racker, *Mechanisms in Bioenergetics*, Academic Press, New York, 1965, pp. 211–240.
22 M. R. McDonald, in S. P. Colowick, N. O. Kaplan, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 296.

23 A. Kleinzeller and A. Kotyk, in *Méchanismes de Régulation des Activités Cellulaires chez les Microorganismes*, C.N.R.S., Paris, 1965, pp. 371–377.
24 F. Azam and A. Kotyk, *FEBS Lett.*, 2 (1969) 333.
25 A. Betz and E. Sel'kov, *FEBS Lett.*, 3 (1969) 5.
26 B. Hess, A. Boiteux, *Annu. Rev. Biochem.*, 40 (1971) 237.
27 H. Lineweaver and D. Burk, in M. Dixon and E. C. Webb, *Enzymes*, Academic Press, New York, 1964, p. 69.