TRANSPORT OF SUGARS IN YEASTS

II. MECHANISMS OF UTILIZATION OF DISACCHARIDES AND RELATED GLYCOSIDES*

GERTRUDIS DE LA FUENTE AND ALBERTO SOLS**
Department of Enzymology, Instituto G. Marañón, C.S.I.C., Madrid (Spain)

(Received May 24th, 1961)

SUMMARY

1. The outstanding ability of some yeasts for the fermentation of certain disaccharides and the recent identification of specific transport systems for the uptake of hexoses prompted the investigation of the likely involvement of transport processes in the fermentation of oligosaccharides.

2. The kinetics and specificity of the fermentation of the main types of glycosides by intact yeasts and of their hydrolysis by cell-free extracts have been studied.

3. Extracellular trapping of hexoses can interfere with the fermentation of β-fructosides by *S. cerevisiae* and with that of α-galactosides by *S. carlsbergensis*. This observation added to the fact that the pH–activity curve and substrate specificity are the same for fermentation and hydrolysis, demonstrates that the first step in the fermentation of sucrose, melibiose, and their analogues is a hydrolysis outside the membrane, followed by transport of the liberated hexoses.

4. On the contrary, the utilization of maltose by *S. cerevisiae* and that of lactose by *S. fragilis* show substrate specificity and pH–activity curves markedly different *in vivo* and *in vitro*. Maltose and lactose fermentation by intact yeasts can be faster than that of their constituent hexoses and was not affected by the addition of a system for the trapping of extracellular hexoses. The combined evidence strongly suggests that specific transport systems are the first step in the fermentation of maltose and lactose, followed by splitting by intracellular enzymes.

INTRODUCTION

Some yeasts can ferment sugars at very high rates. This makes the study of the problem of transport across the yeast membrane particularly important.

CONWAY AND DOWNEY[1] showed that baker's yeast is virtually impermeable to sugars. ROTHSTEIN *et al.*[2] obtained evidence of an active process in the membrane

Abbreviation: ONPGal, *o*-nitrophenyl-β-galactopyranoside.

* Preliminary reports of this work were presented at the IV Intern. Congress of Biochemistry (Vienna, 1958) and at the Symposium on Membrane Transport and Metabolism (Prague, 1960). Although a number was not assigned to it when it was published, ref. 3 should be considered to be the number one of this series.

** Postal address: Dr. A. SOLS, Instituto G. Marañón, Velazquez 138, Madrid 6 (Spain).

involved in the uptake of fermentable hexoses. SOLS[3] identified this process as a stereospecific transport prior to the phosphorylation step.

Yeasts are well known for their ability to ferment certain oligosaccharides as fast or even faster than any hexose. There has been a long standing controversy between the so-called "indirect fermentation" theory involving hydrolysis by glycosidases as the first step and the "direct fermentation" theory of a utilization independent of the glycosidases. The subject was reviewed in the late forties by LEIBOWITZ AND HESTRIN[4,5] and GOTTSCHALK[6]. These reviews show a confusing picture of contradictory results. Farsightedly, GOTTSCHALK pointed out that the lack of appreciation of the permeability problem was likely to be a major factor of misunderstanding.

Following the identification of a specific system for the transport of glucose, fructose and mannose in yeast[3], we approached the question of the fermentation of oligosaccharides starting from the hypothesis that an oligosaccharide to be fermented by intact yeast ought to be either transported across the membrane or split outside the same, with subsequent transport of the liberated hexoses. Evidence for a maltose transport was reported by ROBERTSON AND HALVORSON[7] while this work was in progress. The present paper gives conclusive evidence that both mechanisms do in fact occur in yeasts: specific transport systems are the first step in the fermentation of maltose and lactose, while the fermentation of sucrose and melibiose are normally strictly indirect.

MATERIALS AND METHODS

Yeasts

Saccharomyces cerevisiae PM-1, was isolated from a commercial baker's yeast (Danubio). *S. cerevisiae* JF-168, *S. fragilis* 189-KS and *Hansenula anomala* 59-1, were obtained from the Instituto Jaime Ferrán. Three strains of *S. carlsbergensis*: 303-49 (α-galactosidase gen), 223-16 and 186-17 (α-glucosidase gen M_2) were kindly supplied by Prof. O. WINGE.

The yeasts were usually grown for 48 h aerobically at 25° in stationary Roux bottles, in a medium of the following composition: 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7\ H_2O$, 2.0 g $(NH_4)_2SO_4$, 10.0 g peptone (Difco), and 30 g of the appropriate sugar, in 1 l of tap water, adjusting the pH to 5.0. 25-ml Erlenmeyer flasks of medium were inoculated from nutrient agar slants and allowed to grow overnight. 1-ml aliquots of these cultures were used to inoculate the Roux bottles. The cells were harvested on the centrifuge and washed three times with water at low speed. Yeast concentration in cell suspensions was determined turbidimetrically by reference to a calibration curve with pressed baker's yeast.

Fermentation assays

Washed cells were suspended in water and starved for 2 h at room temperature with occasional shaking to decrease endogeneous metabolism. Fermentation rates were estimated in a conventional Warburg manometric apparatus, at 30°, with N_2 as the gaseous phase, in 0.05 *M* potassium phosphate buffer pH 3.5, unless stated otherwise.

Cell free extracts

Pellets of cells obtained after 5 min centrifugation at about 3000 × *g* were mixed

with 3 times their weight of alumina (Alcoa A-301) and ground vigorously in a cooled mortar for 4–5 min. After dilution with 10 volumes of cold 0.005 *M* EDTA, pH 6.8, and centrifugation at 3000 × *g* for 5 min, the opalescent supernatant was used for enzymic assays.

Assay of glycosidase activity

Glycosidase activity was usually measured by estimation of the free glucose formed after a suitable period of incubation of a glycoside with a cell free extract and a buffer. Liberated glucose was estimated by a glucose oxidase photometric method[8]. Hydrolysis of ONPGal was measured by colorimetric estimation of the liberated *o*-nitrophenol. When none of these methods was applicable, the hydrolysis was measured by estimation of the increase in reducing sugar[9].

Relative efficiencies

In the quantitative evaluation of hydrolysis and fermentation the usual parameters of V_{max} and K_m were not determinable in some cases. The coefficient introduced by SOLS AND CRANE[10] is used as an index of relative efficiencies in conditions of first order kinetics, which are the only observable ones in cases of low affinity or solubility.

Sugars and other biochemicals

Glucono-γ-lactone was obtained from Dr. G. A. LEVVY; isomaltose from Dr. OKADA; maltotriose from Dr. A. M. MICHELSON; phenyl-α-glucoside from Dr. H. HALVORSON; glucono-δ-lactone from Chas Pfizer & Co.; ONPGal from Sigma; other sugars were from Pfanstiehl. Maltose, fructose, galactose and methyl-α-glucoside were recrystallized from ethanol. When it was necessary to use a maltose virtually free of glucose, the maltose was treated with an α-glucosidase-less yeast.

Yeast hexokinase was prepared as previously described[11]. For experiments in which the absence of contaminating invertase activity was essential, a similar preparation was obtained from the β-fructosidase-less, α-glucosidase-less strain 303-49 of *S. carlsbergensis*. An hexokinase–galactokinase preparation was obtained by the same procedure from galactose grown *S. fragilis*.

The glucose oxidase reagent ("Glucostat" and its constituent reagents) and ATP were kindly supplied by the Worthington Biochemical Corp. and the Sigma Chemical Co. respectively.

EXPERIMENTAL AND DISCUSSION

Sucrose and β-fructosides

S. cerevisiae is very rich in β-fructosidase. There was considerable evidence that the enzyme is located in the cell surface, as indicated by the fact that the pH-activity curve *in vivo* is the same as *in vitro* (see ref. 6), by the inhibition by uranyl ions *in vivo*[12] and by its association to polysaccharides[13]. In *S. fragilis*, in which the ability to ferment sucrose is adaptive, the β-fructosidase content had been found to be parallel to the ability to ferment sucrose and raffinose[14].

Rates of hydrolysis and fermentation

The fermentation of sucrose is equal to or smaller than that of glucose in different

yeasts. In the first case, the addition of a second yeast, unable to attack sucrose, enables an estimation of the possible excess of hydrolysing capability over fermentation capability of the yeast under examination. We have observed invertase/fermentation ratios of about 50–100 in *S. cerevisiae*, 7 in *S. fragilis*, and 0.7 in *H. anomala*. The order of magnitude of the invertase/fermentation ratio ought to be taken into account for the planning of the experiments described below. The fact that there is about hundred times excess of invertase in baker's yeast can account for the results of MYRBÄCK AND WILLSTAEDT[13] of the inactivation of β-fructosidase by acid treatment without major loss of the ability to ferment sucrose, since the inactivation in their experiment was only 99%

Extracellular trapping of hexoses

If the fermentation of a glycoside starts by hydrolysis outside the membrane, the addition of a hexose-trapping system can be expected to interfere with fermentation. Hexokinase plus ATP–Mg can fulfil the requirements of efficiency to convert glucose and fructose into products virtually unfermentable by intact yeast[2]. Preliminary trials showed that fermentation of glucose can be virtually stopped by the addition of hexokinase and ATP–Mg in excess over the glucose present. The quickness of the effect depends on the relative amounts of hexokinase and glucose. With *S. cerevisiae* and sucrose, the system becomes flooded with excess of liberated hexose so rapidly that attempts to interfere with the actual fermentation of sucrose would be meaningless. Therefore, two different approaches were followed. In one, *H. anomala*, in which sucrose hydrolysis is smaller than the maximal fermentation ability, and *S. fragilis*, with only a moderate excess of invertase, were used. In the other one, raffinose was used instead of sucrose with *S. cerevisiae*. β-Fructosidase splits the fructosidic linkage of raffinose much less efficiently than that of sucrose (the V_{max} with raffinose is about half of that with sucrose, and the K_m about 15 times greater). At moderate concentrations of raffinose, the rate of hexose liberation by the invertase of *S. cerevisiae*, although still exceeding the fermentation ability, goes down to controllable levels. These experiments were carried out at pH 5.5, at which the hexokinase has about half its maximal activity whereas the invertase is still within its optimal range. The results were positive in the three cases, as shown in Fig. 1. The fact that an enzyme added to the medium can successfully compete with the yeast for the hexose residues of sucrose and raffinose to the extent of near suppression of fermentation gives unequivocal evidence of a splitting to free hexose outside the membrane. This strictly indirect fermentation is the common mechanism for sucrose fermentation by yeasts. An alternative mechanism of sucrose fermentation as an α-glucoside will be described below.

The addition of a glucose oxidase–peroxidase–chromogen reagent enables a direct visualization of the trapping of the glucose moiety of sucrose. KEILIN AND HARTREE[15] had used glucose oxidase for the manometric estimation of invertase activity by intact yeast, interpreting as "overflow" the glucose measured in this way. Nevertheless, since the affinity of invertase for sucrose is rather small, it is possible by drastic lowering of the sucrose concentration to decrease the rate of glucose formation below the maximal rate of hexose utilization. Under these conditions the nearly stoichiometric recovery of glucose from the sucrose utilized in the experiment shown in Fig. 2 cannot be interpreted as overflow, since the added glucose oxidase

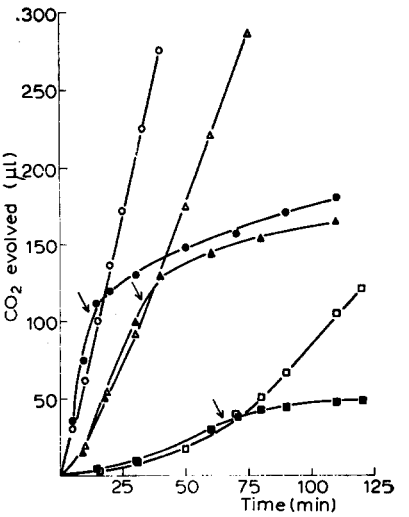

Fig. 1. Interference with the fermentation of β-fructosides by extracellular trapping of hexoses. Warburg vessels contained in the main compartment, in a total volume of 3.0 ml, 200 µmoles potassium phosphate buffer pH 5.5, 12 units of invertase-free hexokinase, and 150 µmoles sugar and yeast as follows: O—O, ●—●, sucrose and 30 mg of *H. anomala*; △—△, ▲—▲, sucrose and 13 mg of *S. fragilis*; □—□, ■—■, raffinose and 6 mg of *S. cerevisiae*. At the times indicated by arrows 15 µmoles ATP–Mg in 0.1 ml were tipped in from a side arm to the second vessel of each series.

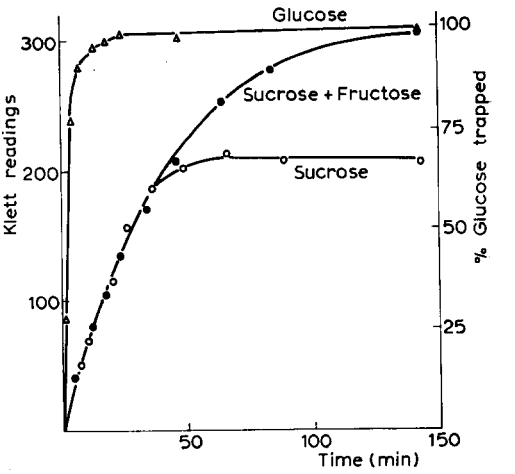

Fig. 2. Trapping of the glucose moiety of sucrose during the utilization of the latter by intact baker's yeast. Photometer tubes were prepared containing 2.0 mg of *S. cerevisiae* PM-1, 2 ml of Glucostat containing 10 times the standard amount of glucose oxidase and 0.8 ml of citrate phosphate buffer, pH 5.5 (see ref. 16), and sugar(s) as indicated below, in a total volume of 2.3 ml. O—O, 0.2 µmole sucrose; ●—●, 0.2 µmole sucrose + 100 µmoles fructose; △—△, 0.2 µmole glucose. The increase in absorbancy at 420 mµ was followed against a blank without sugar.

could never successfully compete with the no longer limiting glucolytic machinery of the yeast, if the sucrose splitting had occurred inside the yeast membrane.

A direct confirmation of the localization of the β-fructosidase in the cell wall has been obtained by FRIIS AND OTTOLENGHI[17] and by SUTTON, MARINI AND LAMPEN[18] who have observed that it tends to disappear in protoplast preparations. WICKERHAM[19] has observed a secretion of invertase in certain yeast cultures. There is a possibility that this phenomenon might be related to the mechanism by which the enzyme reaches its place in the cell wall.

Inulin fermentation

Inulin, the β-fructoside used by CONWAY AND DOWNEY in the characterization of an "outer metabolic region" in yeasts[1] appeared as a potentially important tool for a further discrimination of the localization of the β-fructosidase within the cell wall.

At 2% concentration, inulin was fermented by *S. cerevisiae* PM-1 at a rate 1/350 of that of fructose, after the utilization of an easily fermentable impurity amounting to approx. 5%.

The quantitative relationship between the ability of intact yeast to split sucrose and to ferment inulin has been compared with the ability of a soluble preparation of β-fructosidase[11] from the same yeast to split sucrose and inulin. In the conditions indicated in Fig. 3, the ratios of activity sucrose/inulin for intact yeast and the

purified enzyme preparation were 3100 and 2600 respectively. This result strongly suggests that the enzyme is located so peripherally in the cell wall that it is essentially as freely accessible to inulin as it is to sucrose.

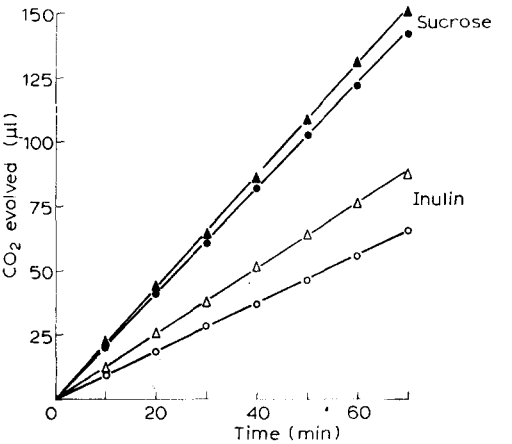

Fig. 3. Relationship between the abilities of intact *S. cerevisiae* and a soluble preparation of β-fructosidase obtained from the same yeast to split sucrose and inulin. Warburg vessels contained in a total volume of 0.8 ml, 100 µmoles phosphate buffer pH 5.5, 20 mg *S. carlsbergensis* 303–49, and *S. cerevisiae* or β-fructosidase preparation as stated below. 8 mg inulin or 20 µmoles sucrose, in 0.2 ml, were added from the side arm. The mixtures containing inulin were incubated for 30 min before starting to measure CO_2 evolution, to eliminate the easily fermentable impurity accompanying the inulin. ○—○, inulin and 200 mg *S. cerevisiae*; ●—●, sucrose and 0.067 mg *S. cerevisiae*; △—△, inulin and 0.6 ml of a β-fructosidase preparation[11]; ▲—▲, sucrose and 0.6 ml of a 1/3000 dilution of the same preparation.

The above conclusion involves the assumption that the enzyme responsible for the hydrolysis of inulin in yeasts is actually the β-fructosidase whose main substrate is sucrose. The ability to ferment sucrose has been shown to be a Mendelian character linked with the ability to split β-fructosides[20]. We have examined three β-fructosidase-less strains (303-49, 186-17 and 223-16) and found that none of them could ferment inulin. *S. cerevisiae* can grow, although slowly, on inulin as the source of carbon and energy. Growth on inulin did not increase the ability to ferment inulin. Probably the arguments raising the suggestion of an "inulase" in yeast[21] could be interpreted as due to kinetic problems involved in the comparison between the main substrate and a very poor substrate[22].

Maltose and related α-glucosides

Maltose fermentation by yeasts has been extensively studied and discussed (see refs. 4, 5, 6, 23). The evidence accumulated against the indirect fermentation theory appeared to be overwhelming. On the other hand, maltase activity is always present whenever a yeast is actually able to ferment maltose. Recent hypotheses involving phosphorolysis had been borrowed from the discovery in other micro-organisms of enzymes which have not actually been found in yeasts. Since the identification of a mechanism for the transport of hexoses[3], the most likely explanation of the maltose fermentation puzzle seemed to be a sequence of maltose transport followed by intracellular hydrolysis. The combined evidence presented below fully supports this hypothesis.

pH effects on maltose fermentation and maltase activity

The pH optimum for maltose fermentation by intact yeast was known to be very different from that for maltose hydrolysis by cell-free preparations (see ref. 4). We have studied the effect of the pH on both the maximal rate and the Michaelis constant *in vivo* and *in vitro*. The results in Table I show that the major factor for the

TABLE I

pH EFFECTS ON MALTOSE FERMENTATION BY INTACT YEAST AND HYDROLYSIS BY EXTRACTS

S. cerevisiae PM-1, grown on maltose. Anaerobic fermentation was measured manometrically. Hydrolysis was estimated with the glucose oxidase method. Potassium phosphate buffers, approximately 0.05 *M*, were used. Maximal rates are referred to μmoles of hexose liberated or fermented $\left(\frac{\mu\text{moles } CO_2}{2}\right)$/min/g yeast at 30°.

pH	*Fermentation*			*Hydrolysis*		
	V_{max}	K_m (moles/l)	*Relative efficiency*	V_{max}	K_m (moles/l)	*Relative efficiency*
3.5	6.0	0.002	1.0	—*		< 0.005
5.0	3.3	0.004	0.3	15	0.1	0.03
6.0	2.5	0.01	0.08	45	0.03	0.3
7.0	> 0.15**			50	0.01	1.0
8.2				*ca.* 45	*ca.* 0.3	0.03

* No hydrolysis was detected at 0.1 *M* substrate concentration.
** Rate observed at 0.1 *M* substrate concentration.

marked difference between intact yeast and maltase lies in an opposite effect on the aparent affinities.

The fact that, within the pH range at which maltose is fermented by intact yeast the maltase *in vitro* is virtually inactive, has been frequently interpreted as indicating that the latter is not involved in maltose fermentation. It is important to emphasize that there is more than a marked difference in pH optima. The optimum for maltase activity is close to the average pH prevailing in the cytoplasm. It is the pH optimum for fermentation which is markedly different from that of the cytoplasm. This fact suggests a step in maltose fermentation on or outside the cytoplasmic membrane. It could either liberate glucose or transport the maltose beyond the cell membrane and thus make it available for an intracellular maltase independent of the external pH. The evidence presented below makes the first possibility, a splitting outside the membrane, highly unlikely.

Adaptation and crypticity

Maltose fermentation is inducible in *S. cerevisiae*. Adaptation to maltose fermentation is known to be facilitated by small amounts of a fermentable hexose[23,24]. The conclusion that the availability of an external source of energy facilitates the induction process is supported by the fact that the induction facilitating effect of a given dose of glucose is strongly related to the amount of yeast involved, as shown in Fig. 4.

The maltase activity of cell free extracts of 5 strains of *S. cerevisiae* has been found to be 70–140 μmoles maltose hydrolyzed/min/g of yeast, which is several times over the minimum that would be required to account for the rate of maltose fermentation by the corresponding intact yeasts. Extracts of the same strains grown on glucose had only about 1 μmole/min/g of apparent maltase activity. Nevertheless, it was known that maltase containing yeasts may be unable to ferment maltose without a lag. While this work was in progress, ROBERTSON AND HALVORSON[7] discovered that

in the course of deadaptation a component of the "maltozymase" system is lost before the maltase itself, giving rise to a transient state of crypticity. They suggested, on the basis of an apparent accumulation of methyl-α-glucoside, that the more labile component involved in maltose fermentation was a "permease". Following this line we have observed that "readaptation" of a yeast in cryptic state is easier than adaptation *de novo*, but it also involves protein synthesis, since it is inhibited by *o*-fluorophenylalanine and the inhibition is reversed by excess of phenylalanine (Fig. 5).

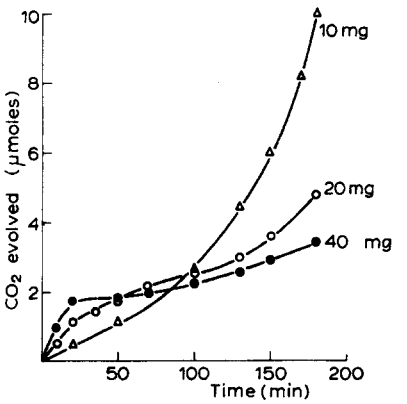

Fig. 4. Effect of a constant amount of glucose on the adaptation to maltose of different amounts of baker's yeast. Warburg vessels contained in the main compartment yeast as indicated in 1 ml of 0.05 *M* potassium phosphate buffer pH 3.5. At zero time, 0.2 ml of a solution containing 100 μmoles of maltose and 1 μmole of glucose were tipped in from a side arm.

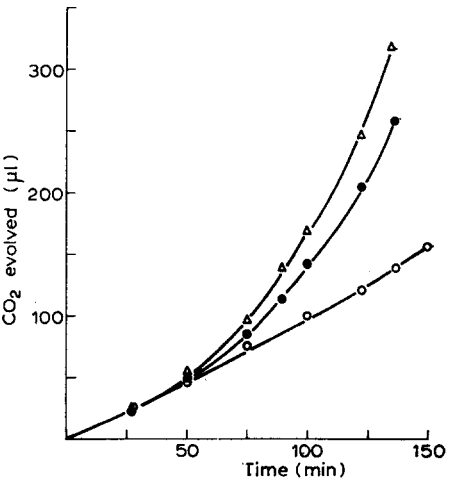

Fig. 5. Inhibition by fluorophenylalanine of the adaptation to maltose of a yeast in cryptic state and its reversal by phenylalanine. Warburg vessels contained in the main compartment, in a total volume of 1.3 ml, 5 mg of baker's yeast in cryptic state, 75 μmoles potassium buffer pH 3.5 and additions as follows: O—O, 12 μmoles L-*o*-fluorophenylalanine; ●—●, 12 μmoles L-*o*-fluorophenylalanine plus 30 μmoles DL-phenylalanine; △—△, no additions. A side arm contained 0.2 ml of a solution containing 100 μmoles maltose and 0.5 μmole glucose, to be tipped in at zero time, after 0.5 h contact of the yeast with the amino acids.

Relationship between the rates of fermentation of maltose and glucose

It has been reported that maltose fermentation by yeasts is frequently faster than that of glucose. We have compared in identical conditions the fermentation rates of maltose and glucose by 7 strains of *Saccharomyces* grown on maltose. In all these cases the rates of maltose fermentation at pH 3.5 were significantly higher than those of glucose fermentation, the ratios maltose/glucose ranging from 1.1 to 2.0. This fact is consistent with the hypothesis of a transport of maltose without previous hydrolysis.

Attempts to interfere with the fermentation of maltose by the addition to the medium of hexokinase–ATP–Mg have consistently failed, in contrast with the results obtained with sucrose. The inability of extracellular hexokinase to interfere with the fermentation of maltose strongly supports the hypothesis of a maltose transport.

During the fermentation of maltose by PM-1 at a rate twice that of glucose fermentation no more than a trace of free glucose in the medium was detected with the sensitive glucose oxidase method. Accordingly, there is no significant overflow despite the fact that in the fully adapted yeast maltase is in large excess over fermentation capability.

Substrate specificity in vitro and in vivo

Substrate specificity in the hydrolysis of α-glucosides is a rather complicated problem. There are several types of α-glucosidases. To those having as the main substrate maltose and trehalose, has recently been added a third one acting on isomaltose[25,26]. The usual concept of structurally related analogues may become quite confusing when considering other oligosaccharides having α-glucosidic terminal linkages and hetero α-glucosides. In many cases it would be nonsense to attempt to define *a priori* which is related to which.

In the discrimination of a specific transport prior to enzymic transformation, the search for differences in substrate specificity *in vitro* and *in vivo* is a major approach. Of course, there can be transport without any difference in substrate specificity, just as two enzymes having different action may have the same specificity pattern. Nevertheless, in the case of glycosides if a transport is involved it is quite likely that significant differences would exist, since the marked tolerance of glycosidases with respect to the size of the aglycon can hardly be expected to be matched by a transport system.

It was not our purpose to study the substrate specificity of a yeast maltase. What might be relevant here was to study the total α-glucosidase activity of the cell free extract of a maltose grown yeast on maltose and other α-glucosides, in order to compare the efficiencies of the hydrolysis *in vitro* with the efficiencies of the fermentation by the intact yeast. The observations were carried out at the optimal ranges for maltose, namely, at pH 7.0 for hydrolysis by homogenates and pH 3.5 for fermentation by intact yeast. Phosphate buffer was used throughout, after preliminary trials showed that glucose liberation from maltose was not increased by the addition of phosphate. Fig. 6 illustrates the experimental procedures and shows that the fermentation of maltose and turanose by intact yeast follows Michaelis-Menten kinetics.

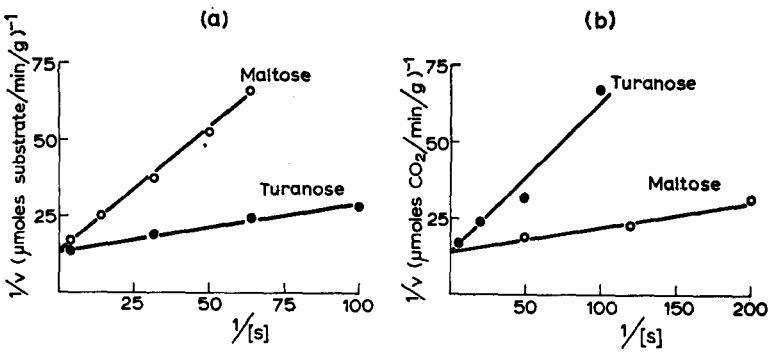

Fig. 6. Effect of the concentration of maltose and turanose on the rates of hydrolysis and fermentation. *S. cerevisiae* JF-163 grown on maltose. (a) Hydrolysis at pH 7 by cell free extracts estimated by the glucose oxidase method. (b) Anaerobic fermentation at pH 3.5 by intact yeast estimated manometrically.

TABLE II

SUBSTRATE SPECIFICITY FOR α-GLUCOSIDES

Conditions as in Table I.

Compound	*Fermentation by intact yeast, pH 3.5*			*Hydrolysis by cell free extract, pH 7.0*		
	V_{max}	K_m (moles/l)	*Relative efficiency*	V_{max}	K_m (moles/l)	*Relative efficiency*
Substrates:						
Maltose	33	0.002	1.0	200	0.01	1.0
Turanose	30	0.05	0.04	250	0.005	2.6
Maltotriose	$>1.3^{***}$			40	0.02	0.1
Melezitose	*ca.* 0.3^{*}			10	0.08	0.005
Methyl-α-D-glucoside	$>0.7^{*}$		*ca.* 0.0002	11	0.02	0.03
Phenyl-α-D-glucoside	$<0.07^{**}$		<0.0001	470	0.002	12.0
Isomaltose	*ca.* 0.1^{***}			15	0.04	0.02
Inhibitors:						
Glucono-γ-lactone		>0.02		—	0.0004	
Glucono-δ-lactone		>0.02		—	0.003	

* Rate observed at 0.2 *M* substrate concentration.
** Rate observed at 0.04 *M* substrate concentration.
*** Rate observed at 0.07 *M* substrate concentration.

The results with *S. cerevisiae* PM-1 are summarized in Table II. From these observations the following conclusions can be drawn: (a) Every fermentation rate can be accounted for by a greater V_{max} of hydrolysis by extracts. (b) The fermentation ability of the intact yeast may be very poor despite high efficiency for hydrolysis by its homogenate. (c) The ratio of apparent affinities for two related substrates *in vitro* and *in vivo* may be inverted. (d) Certain strong competitive inhibitors of the glycosidase activity *in vitro* do not inhibit maltose fermentation *in vivo*.

The first conclusion supports the hypothesis of splitting by α-glucosidase as a step in the fermentation of maltose and related α-glucosides (related with respect to suitability for the enzyme(s)). The other three suggest that in the fermentation of maltose by intact yeast there is a stereospecific step prior to the splitting. In this respect, the inability to ferment phenyl-α-glucoside is a very striking contrast. And the inversion of apparent affinities for turanose and maltose from *in vitro* to *in vivo* is a very safe enzymic criterion, since no physicochemical effect either of diffusion or of physical state of the hydrolase could account for it.

Our results show that methyl α-glucoside is a very poor maltose analogue for the maltase of *S. cerevisiae*. TERUI, OKADA AND OSHIMA[26] have shown that it is a good analogue of isomaltose for another inducible α-glucosidase. Their work and that of BURGER, HEJMOVÁ AND KLEINZELLER[27] indicate a transport of methyl-α-glucoside not related to that of maltose.

It has been shown above that fermentation of sucrose in *Saccharomyces* is initiated by an extracellular β-fructosidase. The possibility remained of sucrose being also fermentable as an α-glucoside. This has been found to be the case in the β-fructosidase-less *S. carlsbergensis* 223–16. This strain, when adapted to maltose can ferment sucrose, although with much smaller efficiency (V_{max} approx. 1/3 and K_m approx. 20 times those of maltose). This observation explains previous reports that

the addition of certain sugars could induce a slow sucrose fermentation in primarily sucrose-negative yeasts (see ref. 20). The fermentation of sucrose by invertase-less, maltase-containing *S. globosus* adapted to sucrose observed by OPARIN *et al.*[28] probably involved the induced formation of a transport mechanism in a cryptic yeast. On the other hand, the fact that the haploid yeast hybrid studied by AVIGAD[29] did not ferment sucrose even after adaptation to maltose, suggests that the efficiency of the maltose transport system with sucrose may be insignificant in some yeasts.

Maltose transport

The results of the approaches described above are consistent with the transport of maltose as the first step in maltose fermentation, although by itself each is open to at least one alternative hypothesis.

Nevertheless, when considered together, these results have more than an additive weight. There seems to be no alternative not involving a maltose transport that could fit in with all of the above observations. The pattern of specificity and the lack of glucose overflow suggest that the transport is the conditioning step and probably the rate limiting one in maltose fermentation.

Lactose and β-galactosides

The fermentation of lactose by *S. fragilis* and certain other yeasts has been extensively studied. The evidence in favor and against the involvement of β-galactosidase in the fermentation of lactose shows many resemblances to the case of α-glucosidase and maltose fermentation[4–6]. DAVIES[30] has recently emphasized the need for disruption of the cells for full estimation of the β-galactosidase content in adapted *S. fragilis*.

Rates of fermentation and hydrolysis

It has been reported that the fermentation of lactose by yeasts can be considerably faster than that of glucose, galactose or a mixture of both. We have observed in *S. fragilis* that the relative rates of fermentation at pH 3.5, optimum for lactose fermentation, are markedly influenced not only by the conditions of growth but also by the duration of the experiment. The initial rate of fermentation of glucose and galactose may in fact be considerably lower than that of lactose although later on they tend to increase, approaching the lactose rate. The fact that lactose can be fermented at a faster rate than its constituent hexoses rules out the possibility of splitting outside the membrane as the sole mechanism of lactose utilization by intact yeast.

In cell free extracts of *S. fragilis* grown on either lactose or galactose we have found a β-galactosidase activity about ten times greater than that necessary to account for the rate of fermentation of lactose.

After anaerobic fermentation of excess lactose, at pH 3.5, for 2 h, the glucose detectable in the medium with glucose oxidase was less than 1 % of that corresponding to the fermented lactose, despite the existence of an excess of β-galactosidase in cell free extracts.

Substrate specificity in vitro and in vivo

S. fragilis tends to flocculate during the fermentation of lactose, especially at high concentrations of the latter, giving rise to erratic results. As long as flocculation

is avoided, the fermentation of lactose and ONPGal follows Michaelis kinetics. A comparative study of the effect of substrate concentration on hydrolysis by cell free extracts and on fermentation by intact cells, has been carried out. The pH was 7.8 for hydrolysis and 3.5 for fermentation, which are within the respective optimal ranges. The results are summarized in Table III. The inversion of efficiencies with lactose and ONPGal from *in vitro* to *in vivo* and the inability of galactono-γ-lactone to inhibit *in vivo*, despite its high affinity as competitive inhibitor of the β-galactosidase, suggest the existence of a stereospecific step prior to hydrolysis *in vivo*. As in the case of maltose, the alternative possibility that such results were due to non involvement of the β-galactosidase in lactose fermentation would be now an unwarranted speculation.

TABLE III

SUBSTRATE SPECIFICITY FOR β-GALACTOSIDES

S. fragilis grown on galactose. ONPGal hydrolysis was evaluated by estimation of the liberated *o*-nitrophenol. Other conditions as in Table I.

Compound	*Fermentation by intact yeast, pH 3.5*			*Hydrolysis by cell free extract, pH 7.8*		
	V_{max}	K_m (moles/l)	*Relative efficiency*	V_{max}	K_m (moles/l)	*Relative efficiency*
Substrates:						
Lactose	24	0.002	1.0	140	0.02	1.0
ONPGal	20	0.025	0.07	70	0.0016	6.3
Inhibitor:						
Galactono-γ-lactone	—	>0.2		—	0.0013	

Effects of pH

As emphasized in the case of maltose, the low pH optimal for fermentation by intact yeast cannot be expected to influence the activity of an intracellular enzyme. Nevertheless, when we tried to measure the percentage of residual activity for lactose fermentation at pH 7.8, very variable results were obtained. Even more so, the liberation of *o*-nitrophenol from ONPGal at the alkaline pH was frequently found to be considerably high. We have found that this variability is related to the very fact that *S. fragilis* is fragile. The excess of β-galactosidase over fermentation capability makes it possible that a small percentage of damaged cells may contribute enough hydrolytic capacity in the neighbourhood of pH 7 to supply the undamaged ones with fermentable hexose. In the case of ONPGal, the fact that the affinity of the β-galactosidase for it is very good while that of intact yeast is rather poor (Table III), tends to increase the effect of damaged cells. The following observations on the kinetics of *o*-nitrophenol liberation from ONPGal at pH 7.8 by the presumedly intact yeast support this interpretation: (a) the K_m goes down to the value of that of the free β-galactosidase, and (b) it becomes sensitive to competitive inhibition by galactonolactone, like is the free enzyme. Moreover, the ability of a given population to ferment lactose or liberate *o*-nitrophenol from ONPGal at pH 7.8 can be markedly decreased either by careful washing on the centrifuge or by allowing the yeast to stand at pH 3.5 for 1 h at 30° prior to the test. The latter treatment inactivates any β-galactosidase not protected by an intact cell membrane. In fresh, thoroughly washed populations of *S. fragilis*,

both the fermentation of lactose and the liberation of *o*-nitrophenol from ONPGal are at pH 7.8 only about 5% or less of those at pH 3.5.

These results indicate that a stereospecific transport is an essential requirement for the utilization of β-galactosides by truly intact *S. fragilis*. Recently, it has been shown that *S. cerevisiae* is impermeable to lactose[27,31]

Melibiose and α-galactosides

Melibiose is fermentable by some "bottom" brewing yeasts. Genetic studies have given evidence that yeasts able to ferment melibiose have an α-galactosidase which can also split the α-galactosidic linkage of raffinose[20].

The ability to ferment melibiose is inducible. Growth on galactose gives full adaptation to melibiose fermentation. Within the pH range optimal for melibiose fermentation, adapted *S. carlsbergensis* ferments melibiose at the same rate as glucose. We have found at pH 3.5 a maximal hydrolysis by intact yeast 7 times greater than the maximal fermentation rate, using an auxiliary yeast to ferment the excess of liberated hexose.

The hydrolysis of melibiose by cell free extracts and its fermentation by intact yeast are optimal in the acid range. With extracts we have found that the pH optimum for melibiose hydrolysis is 3.5–4.0. Above this range there is a marked decrease in the apparent affinity for the substrate. The fact that there is an excess of melibiase accounts for the apparently broader pH range for melibiose fermentation.

The above observations suggest extracellular hydrolysis as in the case of sucrose. A comparison of the specificities for the splitting of α-galactosides by extracts and for their fermentation by intact yeast (Table IV) showed a definite parallelism with a series of compounds with increasing size of the aglycon. This parallelism supports the hypothesis of α-galactosidase being located outside the permeability membrane.

As emphasized above, the interference with fermentation of a glycoside by extracellular trapping of hexoses gives virtually inescapable evidence of splitting outside the membrane. Such has been found to be the case in the fermentation of melibiose by *S. carlsbergensis*, using the experimental approach described for β-fructosides except for the use of a preparation containing galactokinase in addition to hexokinase.

TABLE IV

SUBSTRATE SPECIFICITY FOR α-GALACTOSIDES

S. carlsbergensis 303-49 grown on galactose. All activities were measured at pH 3.5. Hydrolysis of the non-reducing oligosaccharides was evaluated by estimation of the increase in reducing sugar. Maximal hydrolysis by intact yeast was estimated manometrically with excess of an auxiliary yeast (*S. carlsbergensis* 223-16, grown on galactose). Other conditions as in Table I.

Compound	*Hydrolysis by cell free extracts*			*Intact yeast*		
				Fermentation	*Hydrolysis*	
	V_{max}	K_m (moles/l)	*Relative efficiency*	V_{max}	V_{max}	*Relative efficiency*
Melibiose	330	0.002	1.0	23	200	1.0
Raffinose	100	0.2	0.003			0.005
Stachiose			0.003			0.005
Verbascose			0.003			0.005
Glucose				23		
Galactose				10		

CONCLUSIONS

The combined evidence presented above clearly indicates that two kinds of mechanisms are involved in the fermentation of oligosaccharides by yeasts. The utilization of β-fructosides by *S. cerevisiae* and that of α-galactosides by *S. carlsbergensis*, involve hydrolysis outside the membrane followed by transport of the liberated hexoses. The utilization of maltose and certain other α-glucosides by *S. cerevisiae* and that of β-galactosides by *S. fragilis*, involve transport of the oligosaccharides themselves, to be split inside the membrane. The transport systems and the splitting enzymes are inducible and at least in part independent of each other. The case of *S. cerevisiae* with respect to sucrose and maltose shows that both kinds of mechanisms may coexist in a given yeast.

The long standing controversy on "direct" *versus* "indirect" fermentation of oligosaccharides resolves itself into transport before or after cleavage to monosaccharides.

ACKNOWLEDGEMENTS

This work was supported in part by the Lilly Research Laboratories and the Fundación Juan March.

The authors are indebted to Dr. G. TEJERINA (Instituto Jaime Ferrán, Madrid) for help in the handling of the yeast cultures.

REFERENCES

1 E. J. CONWAY AND M. DOWNEY, *Biochem. J.*, 47 (1950) 347.
2 A. ROTHSTEIN, *Symposia Soc. Exptl, Biol.*, 8 (1954) 165.
3 A. SOLS, *Biochim. Biophys. Acta*, 20 (1956) 62.
4 J. LEIBOWITZ AND S. HESTRIN, *Advances in Enzymol.*, Vol. 5, Interscience Publishers, Inc., New York, 1945, p. 87.
5 S. HESTRIN, *Wallerstein Labs. Communs.*, 11 (1948) 193; 12 (1949) 45.
6 A. GOTTSCHALK, *Wallerstein Labs. Communs.*, 12 (1949) 55.
7 J. J. ROBERTSON AND H. O. HALVORSON, *J. Bacteriol.*, 73 (1957) 186.
8 A. SOLS AND G. DE LA FUENTE, *Methods in Medical Research*, Vol. 9, 1961.
9 M. SOMOGYI, *J. Biol. Chem.*, 195 (1952) 19.
10 A. SOLS AND R. K. CRANE, *J. Biol. Chem.*, 210 (1954) 581.
11 A. SOLS, G. DE LA FUENTE, C. VILLAR-PALASI AND C. ASENSIO, *Biochim. Biophys. Acta*, 30 (1958) 92.
12 D. J. DEMIS, A. ROTHSTEIN AND R. MEIER, *Arch. Biochem. Biophys.*, 48 (1954) 55.
13 K. MYRBÄCK AND E. WILLSTAEDT, *Arkiv Kemi*, 8 (1955) 367.
14 R. DAVIES, *Biochem. J.*, 55 (1953) 484.
15 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 42 (1948) 230.
16 T. C. MCILVAINE, *J. Biol. Chem.*, 49 (1921) 183.
17 J. FRIIS AND P. OTTOLENGHI, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, 31 (1959) 259.
18 D. SUTTON, F. MARINI AND J. O. LAMPEN, *Bacteriol. Proc.*, (1960) 160.
19 L. J. WICKERHAM, *Arch. Biochem. Biophys.*, 76 (1958) 439.
20 O. WINGE AND C. ROBERTS, *Compt. rend. trav. lab. Carlsberg, Sér. physiol.*, 25 (1950-1957) 419.
21 M. ADAMS, N. K. RICHTMYER AND C. S. HUDSON, *J. Am. Chem. Soc.*, 65 (1943) 1369.
22 G. DE LA FUENTE AND M. SALAS, to be published.
23 H. SUOMALAINEN, E. AXELSON AND E. OURA, *Biochim. Biophys. Acta*, 20 (1956) 319.
24 S. SPIEGELMAN, J. R. REINER AND R. COHNBERG, *J. Gen. Physiol.*, 31 (1948) 27.
25 J. LARNER AND C. M. MCNICKLE, *J. Am. Chem. Soc.*, 76 (1954) 4747.
26 G. TERUI, H. OKADA AND Y. OSHIMA, *Technol. Repts. Osaka Univ.*, 9 (1959) 237.
27 M. BURGER, L. HEJMOVÁ AND A. KLEINZELLER, *Biochem. J.*, 71 (1959) 233.
28 A. I. OPARIN, N. S. GELMAN AND I. G. ZHUKOVA, *Biokhimiya*, 20 (1955) 571.
29 G. AVIGAD, *Biochim. Biophys. Acta*, 40 (1960) 124.
30 A. DAVIES, *J. Gen. Microbiol.*, 14 (1956) 425.
31 V. P. CIRILLO, *Symposium on Membrane Transport and Metabolism*, Prague, 1961, p. 343.