SELECTIVE FERMENTATION AND PHOSPHORYLATION OF SUGARS BY SAUTERNES YEAST*

by

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Some yeasts from Sauternes wines ferment fructose faster than glucose from the mixture of both sugars[1]. If tested separately, the rate of glucose fermentation, always smaller than that of fructose, decreases with the decrease in sugar concentration more than does that of fructose[2].

In the early forties it was established that baker's yeast has a common phosphorylating enzyme for the three fermentable hexoses glucose, fructose and mannose[3]. Then GOTTSCHALK[4] observed that dried and ground Sauternes yeast fermented glucose preferentially to fructose, in contrast to the intact yeast. He attributed the behavior of living Sauternes yeast to a preferential permeability of its membrane for fructose.

Later it was found that enzymes specific for the phosphorylation of fructose do occur in certain tissues[5,6]. It seemed possible, therefore, that there existed in Sauternes yeast, in addition to hexokinase, some kind of fructokinase that had not survived the drying procedure or whose activity was not demonstrable under the conditions of GOTTSCHALK's experiments.

On the other hand, recent work has shown that the fermentation of glucose by baker's yeast can be inhibited by some structural analogs. CRAMER AND WOODWARD found that 2-deoxyglucose (2DG) is a very strong competitive inhibitor[7]. MITCHELL and co-workers found that glucosone is a strong inhibitor of fermentation[8] and of yeast hexokinase[9]. Glucosamine also inhibits fermentation[10]. Recently, BLAKLEY AND BOYER[11] found that 6-deoxy-6-fluoroglucose competitively inhibits the fermentation of glucose and fructose while it has only slight inhibitory effect on yeast hexokinase.

This paper is concerned with the competition between sugars for phosphorylation by fresh homogenates of Sauternes yeast and for fermentation by intact yeast. It has been found that phosphorylation of fructose and glucose is carried out by a single enzyme. Sauternes yeast hexokinase is similar to that of baker's yeast except for a somewhat higher relative maximal rate of fructose phosphorylation. In intact yeast there is competition between fermentable sugars, and fermentation is competitively inhibited by certain structural analogs. The results indicate the existence in Sauternes yeast, prior to the phosphorylation step, of a transferring agent also common to fructose and glucose but with a greater affinity for fructose.

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MATERIALS AND METHODS

A pure culture of a strain of Sauternes yeast was obtained from the collection of the Pasteur Institute. The yeast was grown in a medium containing (g/l): $(NH_4)_2SO_4$, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 0.1; yeast extract (Difco), 1; and fructose, 50. The pH was adjusted to 5.4. The inoculated flasks were maintained at 28° with shaking; after 2–4 days the yeast was harvested and washed by centrifugation*.

Sedoheptulose was prepared by acid treatment of sedoheptulosan monohydrate kindly supplied by Dr. N. K. RICHTMYER. A sample of 6-deoxy-6-fluoroglucose was kindly supplied by Dr. P. D. BOYER. Glucose was obtained from the Pfanstiehl Chemical Co. The other sugars were obtained as described in a previous paper[12]. Adenosinetriphosphate was obtained as the disodium salt from the Pabst Laboratories. Fructose-1-P was a gift of Dr. R. K. CRANE. Fructose-6-P was obtained from the Schwartz Laboratories.

Utilization of sugars was tested by incubation at 30° in open test tubes of a suspension of washed yeast, usually 2%, with a sugar or a mixture of two sugars. The contents of the tubes were mixed every 5–10 minutes to prevent settling of the yeast. After appropiate intervals of time, aliquots were analysed for reducing sugar[13], ketose[14] or for both ketose and aldose[15] as appropriate.

To follow the fermentation of fructose, fructose alone or plus a non-fermentable sugar in a volume of 2.8 ml was placed in the main compartment of a Warburg flask and 0.2 ml of a 10% suspension of washed yeast was placed in the side arm. The rate of fermentation at 28° in an atmosphere of 95% nitrogen and 5% CO_2 was determined by the conventional procedure**.

Homogenates were prepared by grinding the yeast with 3 times its weight of Alumina A-301 (Aluminum Company of America) in a mortar for about 5 minutes, mixing thoroughly with 20 volumes of ice-cold 0.005*M* ethylenediaminetetraacetic acid neutralized to pH 7.0, and centrifuging for 5 minutes at 6,000 × *g*. The cell-free homogenates were used shortly after preparation or were stored at —20° until use.

Phosphorylation of sugars was studied by (1) sugar disappearance, (2) labile phosphorus disappearance, or (3) the photometric indicator method, as previously described[12] except that sodium fluoride (0.05*M*) was present in all cases. In the experiments carried out by the first two methods the pH was 8.0 (0.05*M* tris(hydroxymethyl)aminomethane as buffer) except when indicated otherwise.

RESULTS

Selective utilization of fructose

Cultures of the Sauternes strain were tested for selective utilization of fructose. There was always a faster utilization of fructose than of glucose from initially equimolar mixtures of both sugars. In a typical experiment shown in Fig. 1, a fructose/glucose utilization ratio of 1.8 was obtained.

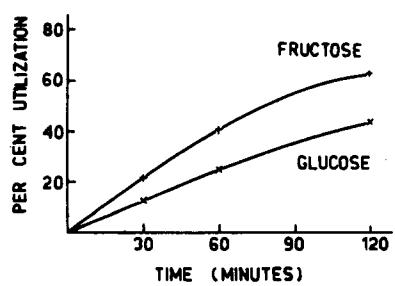

Fig. 1. Selective utilization of fructose by Sauternes yeast. Fructose and glucose were initially $2 \cdot 10^{-2}M$ each. Aliquots were analysed for ketose and aldose.

Utilization of other sugars

The strain of Sauternes yeast used in these experiments utilized mannose at a rate somewhat smaller than that of fructose. Apparently it can also utilize sedoheptulose after adaptation. A similar finding has been recently reported for *Saccharomyces cerevisiae*[16].

* Cultures were carried out in the Instituto de Farmacología Española by Dr. J. MARTÍNEZ MATAS, to whom I am deeply indebted. I wish also to thank Dr. E. FEDUCHI for some small scale cultures carried out in the Instituto Jaime Ferrán de Microbiología.

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Competitive inhibition of the fermentation of fructose in intact yeast

2DG inhibits the utilization of both glucose and fructose by Sauternes yeast. At equimolar concentrations of fermentable sugar and 2DG the inhibition of fructose utilization was 0.6 times that of glucose (Fig. 2). A 10 times molar excess of 2DG gave an inhibition of fructose utilization of 84% (Table I). The effect of a series of concentrations of 2DG on the fermentation of fructose is shown in Fig. 3. The results indicate that the inhibition approaches 100% with increasing concentrations of 2DG. The ratio of apparent affinities of the intact Sauternes yeast for fructose and 2DG was 1.0. The inhibition is competitive, since the order of magnitude of the inhibition in different experiments depends on the ratio of concentrations and not on the absolute concentration of 2DG.

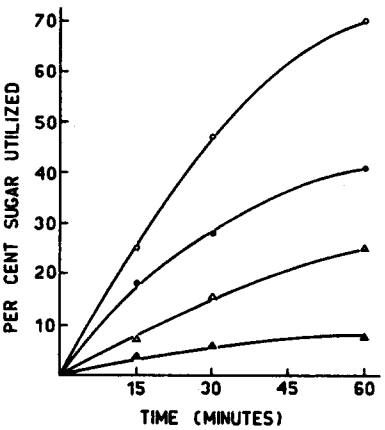

Fig. 2. Effect of 2DG on the utilization of fructose by living yeast. 50 mg yeast in a total volume of 1 ml; each sugar at $3 \cdot 10^{-2} M$. ○ fructose; ● fructose plus 2DG; △ glucose; ▲ glucose plus 2DG.

Some other sugars were also tested as presumptive competitive inhibitors. Fermentation of fructose was not affected by a 20 times molar excess of either arabinose or L-sorbose. 6-Deoxy-6-fluoroglucose can inhibit the utilization of fructose. The results in Table I would correspond to a ratio of apparent affinities for fructose: 6-deoxy-6-fluoroglucose of *ca.* 1:0.15.

TABLE I

INHIBITION OF FRUCTOSE UTILIZATION BY 2DG AND 6-DEOXY-6-FLUOROGLUCOSE

	Yeast mg	*Volume* ml	*Incubation* minutes	*Fructose utilized* μM
Fructose, 7.5 μM	10	0.25	30	1.75
Fructose, 7.5 μM	10	0.25	60	3.42
Fructose, 7.5 μM, 2DG, 75 μM	10	0.25	60	0.58
Fructose, 7.5 μM, 2DG, 75 μM	10	0.25	120	1.00
Fructose, 4 μM	5	0.125	90	1.60
Fructose, 4 μM, 6FG, 2 μM	5	0.125	90	1.44
Fructose, 4 μM, 6FG, 5 μM	5	0.125	90	1.24

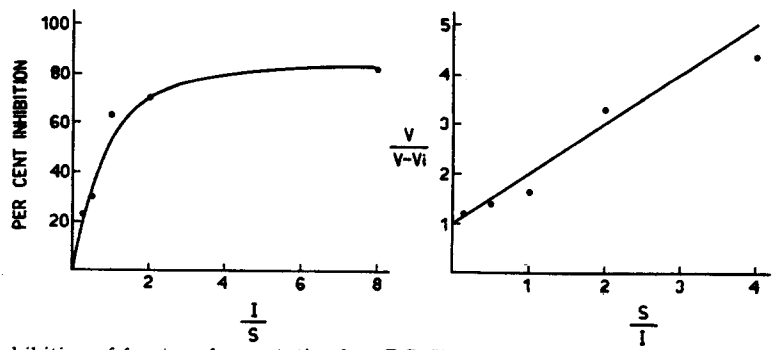

Fig. 3. Inhibition of fructose fermentation by 2DG. The concentration of fructose was $1 \cdot 10^{-2} M$.

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Phosphorylation of sugars by cell-free homogenates

Fresh homogenates phosphorylated glucose, fructose and mannose, but not L-sorbose, when tested by the sugar disappearance method. The specific activity (with glucose as substrate) was similar (10 μM/mg protein/15 minutes at 30°) to that of baker's yeast homogenates[17]. Glucose and N-acetylglucosamine (NAGA) strongly inhibited the phosphorylation of fructose, while fructose inhibited the phosphorylation of glucose only slightly (Table II). The observed relative rates, as well as the competition between fructose and glucose, were of the same order of magnitude as those with baker's yeast[5,17] except for a phosphorylation of fructose *ca.* 50% faster than the maximal rate with baker's yeast hexokinase or homogenates. Phosphorylation of fructose as measured by ketose disappearance was over 95% inhibited by a 5 times molar excess of glucose. Lowering the pH to 6.0 (phosphate-acetate buffer) decreased the activity *ca.* 45% without apparent change in the selective phosphorylation of glucose from the equimolar mixture of glucose and fructose.

TABLE II

PHOSPHORYLATION OF SUGARS BY SAUTERNES YEAST HOMOGENATE

The initial concentration of each sugar was $1 \cdot 10^{-2} M$. Rates were measured by the sugar disappearance method. Incubation times were chosen for 20–40% utilization of glucose, fructose and mannose.

Sugar	*Relative rates*	
Glucose	1.00	
Fructose	2.20*	
L-Sorbose	< 0.05*	
Mannose	0.65	
Fructose, glucose	0.40*	0.80**
Fructose, NAGA	0.90*	

* ketose disappearance.
** total sugar disappearance minus ketose disappearance.

Relative rates of phosphorylation at $2 \cdot 10^{-2} M$ substrate concentration were directly observed with the photometric indicator method (Fig. 4). The results were: glucose 1.00,

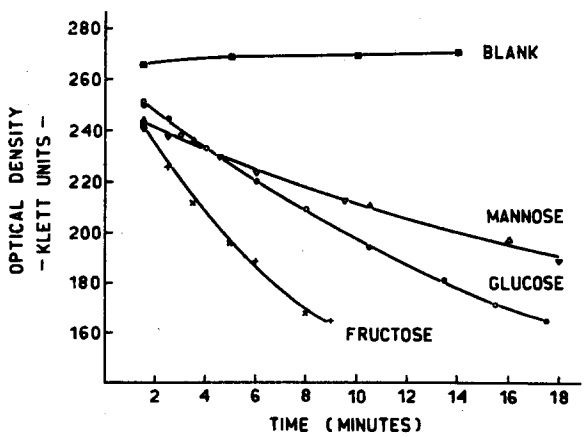

Fig. 4. Phosphorylation of fructose, glucose and mannose by Sauternes yeast homogenate. Photometric indicator method.

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fructose 2.30, mannose 0.60; L-sorbose, mannoheptulose and NAGA <0.02. Under the same conditions an homogenate of baker's yeast (Danubio) gave a rate 1.3 times that of glucose with fructose.

The effect of the concentration of substrate on the rate of phosphorylation of fructose, fructose in the presence of NAGA, and glucose, was tested with the labile phosphorus disappearance method. The results indicated a Michaelis constant, K_m, for fructose of *ca.* $2 \cdot 10^{-3} M$ and a K_i NAGA (with respect to fructose) of *ca.* $1.3 \cdot 10^{-3} M$; the affinity for glucose was too great to be measured under these conditions, the results indicating a K_m smaller than $5 \cdot 10^{-4} M$. The photometric indicator method was used for the accurate estimation of the Michaelis constants because of its much greater sensitivity. The experiment presented in Fig. 5 gave the following values: K_m fructose $2.6 \cdot 10^{-3} M$, K_m glucose $2.5 \cdot 10^{-4} M$, K_i NAGA (with respect to fructose) $1.4 \cdot 10^{-3} M$; in addition an approximate value of $1.7 \cdot 10^{-3} M$ was obtained for K_i NAGA with respect to glucose. The ratio V_{max} fructose/V_{max} glucose calculated from the results of this experiment was 2.6. The phosphorylation coefficient for fructose taking glucose as unity[12] is then 0.25.

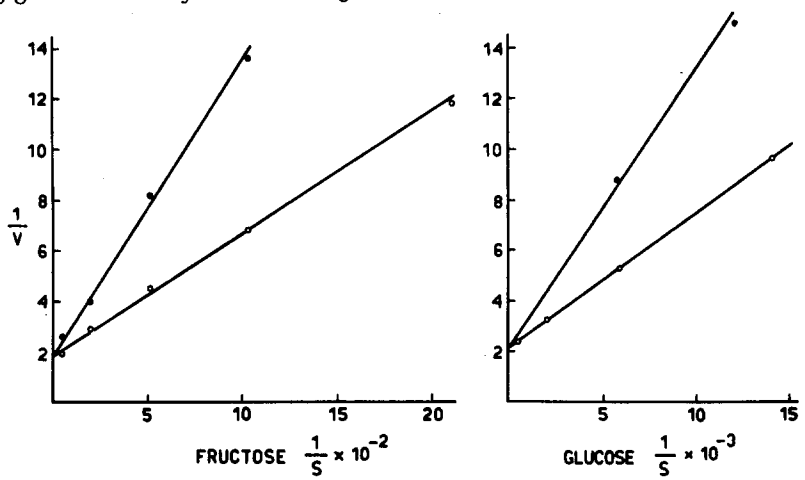

Fig. 5. The effect of the concentration of fructose and glucose on their phosphorylation rates and the inhibition by NAGA. ○ substrate alone; ● substrate plus $2 \cdot 10^{-3} M$ NAGA. Average substrate concentrations are plotted.

Action of homogenates on fructose phosphates

Incubation of fructose-6-P with Sauternes yeast homogenates caused a decrease of total ketose rapidly approaching the equilibrium point catalyzed by phosphoglucose isomerase. Under the same conditions ($1 \cdot 10^{-2} M$, pH *ca.* 7.0) no change was observed with fructose-1-P as substrate, even when incubation was allowed to proceed some 50 times longer (1 hour) than that required for measurable activity on fructose-6-P.

DISCUSSION

The results indicate that phosphorylation of sugars in Sauternes yeast is essentially similar to that in baker's yeast, except for the fact that with Sauternes yeast hexokinase the ratio V_{max} fructose/V_{max} glucose is significantly greater than with baker's

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yeast[5,17] and other well characterized hexokinases[18]. The increase in relative maximal phosphorylation rate without appreciable change in relative apparent affinities increases the phosphorylation coefficient (see [12]) of fructose with respect to baker's yeast hexokinase. But since the phosphorylation coefficient of fructose is still smaller than that of glucose, hexokinase alone could not account for the selective fermentation of fructose by intact Sauternes yeast. These results are essentially in agreement with the indirect evidence obtained by GOTTSCHALK[4].

The possibility of Sauternes yeast having in addition to an hexokinase common for glucose and fructose some other enzyme specific for the latter is very slight because: (a) fructose phosphorylation by homogenates apparently can be completely inhibited by excess glucose or NAGA; (b) the effect of the concentration of fructose on its phosphorylation rate gives no indication of any second enzyme with greater affinity; (c) quantitatively, the hexokinase activity in Sauternes yeast is of the same order of magnitude as in baker's yeast; (d) the apparent inability of the homogenates to metabolize fructose-1-P (reaction product of several, although not all[19], fructokinases); and (e) no activity whatsoever has been detected with L-sorbose (a substrate for several, although not all[19], fructokinases).

On the other hand, competitive inhibition of fructose uptake by 2DG indicates a transferring agent prior to the hexokinase but also common to fructose and glucose. The relative affinities for glucose and 2DG resemble those of baker's yeast[7]; but its affinity for fructose is considerably greater in Sauternes yeast than in baker's yeast. It would appear that both Sauternes and baker's yeast have a transferring agent responsible for the active uptake of sugars (see ROTHSTEIN[20]) and an hexokinase for their phosphorylation. Lack of permeability to sugars[21] would make possible the existence of hexokinase inhibitors unable to inhibit fermentation by intact cells. Such seems to be the case for NAGA, which does not inhibit fermentation by baker's yeast[10] although it strongly inhibits its hexokinase[17]. The existence of compounds like 2DG that apparently can combine with the transferring agent without being transferred inside the cell seems to be fortunate for further research on its nature. If 2DG were transferred inside the cell it would, in addition, compete with the fermentable sugars at the hexokinase level[22]. An active transfer not primarily dependent on hexokinase makes it easier to understand the apparently divergent findings of ROTHSTEIN[20] and of DERRICK, MILLER AND SEVAG[23]. CRAMER AND WOODWARD[7] and BLAKLEY AND BOYER[11] have already postulated the probable existence of an specific transport mechanism in baker's yeast.

Selective fermentation of fructose by wine yeasts may be rather common. PEYNAUD has recently identified at least 4 species with this property[24]. There may be a broad spectrum of selectivities among yeasts.

Research in related fields, in conjunction with the above considerations, points to the possibility of hitherto unidentified transferring agents for sugars being a basic cellular mechanism (intestinal mucosa[25], ascites tumor cells[26], diaphragm[27,28] and heart[29] muscle). But lack of inhibition of the intestinal absorption of glucose by 2DG[30] suggests that transferring agents with marked differences in structural requirements may exist in different tissues. In the case of the intestinal mucosa, the configuration of the aldoses at C-2 seems to be critical for affinity, while in the yeasts a number of changes (2-deoxy, 2-deoxy-2-amino, 2-dehydro) are compatible with high affinity, although they affect the efficiency.

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SUMMARY

The phosphorylation of sugars by a strain of Sauternes yeast that selectively ferments fructose has been studied in fresh homogenates.

Fructose and glucose are phosphorylated by a single enzyme. The hexokinase of Sauternes yeast can phosphorylate fructose faster than that of baker's yeast. But its phosphorylation coefficient for fructose is smaller than that for glucose.

2-Deoxyglucose competitively inhibits the utilization of fructose as well as that of glucose by intact yeast. The apparent affinity for glucose is smaller than that for fructose and 2-deoxyglucose.

The results are interpreted as indicating that in Sauternes yeast there is a transferring agent common to fructose and glucose and with a greater affinity for the former. This transferring agent must be prior to the phosphorylation step.

The possible general occurrence of transferring agents for sugars is discussed.

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