

Microcalorimetric Determination of the Affinity of *Saccharomyces cerevisiae* for Some Carbohydrate Growth Substrates

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A microcalorimetric method using a Tian-Calvet apparatus was utilized for measuring the K_m and V_{max} of intact yeast cells for some carbohydrate substrates. These values were obtained for fructose, galactose, and maltose. The heats of ethanolic fermentations were also directly determined, and the results were very close to those obtained by using theoretical calculations and standard values.

Many applications of microcalorimetry to the study of microbial growth have been reported in recent years (1, 2, 12). More recently, however, we described a microcalorimetric technique that permits the determination of the affinity of microbial cells for glucose under conditions in which glucose was the limiting energy source (5; J. P. Belaich, Thesis, Aix-Marseille Univ., Marseille, 1967; M. Murgier, Thesis, Aix-Marseille Univ., Marseille, 1970).

The purpose of the present paper is to report experiments carried out with the same strain of *Saccharomyces cerevisiae* under the same conditions of growth as previously cited (5), but with fructose, galactose, and maltose as the limiting energy sources.

MATERIALS AND METHODS

Organism and culture medium. The strain of yeast studied is a respiratory-deficient "petite colonie" YFa mutant kindly provided by P. Slonimsky, the physiological properties of which are described elsewhere (5).

The culture medium utilized contained (per liter) 0.5 g of Bacto-peptone (Difco) and 0.5 g of Yeast Extract (Difco), and was buffered by a solution of monopotassium (KH_2PO_4) and disodium (Na_2HPO_4) phosphates (0.06 M) to final pH 5.8. The carbohydrates were separately sterilized in distilled water and pipetted into the medium at a suitable concentration (between 1.5 and 2 g).

Apparatus for the microcalorimetric method. The microcalorimeter used was a Tian-Calvet apparatus, the properties of which are described in a monograph of Calvet and Prat (8). This apparatus records directly a heat flux dq/dt versus time, with a maximal sensitivity of 1 mm of galvanometric deviation for $2 \mu w$ and a response time of 300 sec. The quantities of heat evolved (Q) were determined by graphic integration of the thermograms. The experiments were done anaerobically under an argon gas phase in a closed Pyrex vessel con-

taining 10 ml of liquid culture medium. After temperature equilibration, the duration of which was 1 to 3 hr, the inoculum (0.1 ml of culture) was introduced into the medium by means of a syringe attachment. A stainless-steel stirring device having a reciprocating movement with a frequency of 100 Hertz was utilized to avoid sedimentation of the cells during growth. This device was described by Belaich and Sari (3).

Microcalorimetric calculation method. The method of calculation was described in detail in a previous paper (5). We report here only the principal equations. When a microorganism is growing in the Pyrex vessel of the apparatus, it is easy to determine the cellular rate of catabolic activity (Ac), i.e., the quantity of energy substrate metabolized per unit of cells and of time and the energy substrate concentration $[S_i]$ at any time, t , from the data (dq/dt) given by the apparatus and $Q_t = \int_0^t (dq/dt)dt$. The equations that permit us to calculate Ac and $[S_i]$ are:

$$Ac = dq/dt \times (1/Q_t) \times (1/Y_i) \quad (1)$$

$$[S_i] = (1/v) \times (Q_r - Q_t)/K \quad (2)$$

where Y_i , v , K , and Q_r are, respectively, the molecular growth yield of the organism for the energy substrate (i) studied, the volume of the culture, the experimental heat evolved per mole of fermented energy substrate, and the total heat evolved by the culture between the beginning and the end of growth.

The relationship between Ac and $[S_i]$ is hyperbolic (5), and thus it is possible to plot $1/Ac$ versus $1/[S_i]$ and estimate a K_m and a V_{max} of the studied microorganisms for different energy substrates.

Standard values and data utilized for determining the enthalpies of the fermentation reactions. It is possible from the experimental value K to estimate the enthalpy of the fermentation reaction (Kc) carried out by the organism studied, and to compare Kc with the theoretical value (K_t) obtained from the calculation. For this purpose, it is necessary to know the percentage of the cellular carbon that is derived from the energy substrate and incorporated into the cellular material. We have

not made direct determinations of this percentage in the case of *S. cerevisiae*, and we have taken the value determined by one of us in *Zymomonas mobilis* growing in the same culture condition (4). This value, obtained with uniformly labeled ^{14}C -glucose, is equal to 48%. In other words, when *S. cerevisiae* grows in a complex medium, we assume that 48% of the cellular carbon is derived from the energy substrate growth. From this data and from the molecular growth yield Y_1 , it is possible (5) to calculate the quantity of substrate that is truly fermented, i.e., transformed into fermentation products. Table 1 shows the standard values that permit the calculation of the theoretical enthalpy change associated with the different fermentations studied here.

Analytical method. Ethanol was determined by the microdiffusion technique of Conway (10).

The determination of the molecular growth yield was done by direct weighing. For this purpose, 0.5 liter of the medium containing 1 g of energy substrate was incubated in a water bath at the same temperature as that of the calorimeter and was centrifuged after total growth. The cells were harvested and washed three times with distilled water, and the last centrifugation pellet was desiccated at 120 C and weighed after complete desiccation.

All the experiments were carried out anaerobically at 30 C. Anaerobiosis was achieved by extensive flushing with pure argon gas.

RESULTS

Determination of data necessary for thermogram analysis. Figure 1 shows three thermograms of *S. cerevisiae* growing in a complex medium where the fructose, galactose, or maltose concentration was the limiting growth factor.

From four experiments on each substrate, we determined the experimental heat quantities (K) evolved per mole of energy substrate fermented; these values are reported in Table 2. The enthalpy of each fermentation reaction (K_c) was calculated by assuming, as explained above, that 48% of the cellular carbon is derived from the energy substrate. These values are also reported in Table 2. Table 3 shows the results obtained from a series of control experiments which establish that the carbohydrate fermentation performed by our strain of yeast is purely ethanolic. From these results and from the data of Table 1,

TABLE 1. Standard values for calculation of the theoretical enthalpy changes

Substance	Standard enthalpy of formation (Kcal/mole)	Reference or method of calculation
Fructose	-302.38	Clarke and Stegeman (9) + heat of solution (Maggie, 16)
Galactose	-307.33	Clarke and Stegeman (9) + heat of solution (Hendricks, 13)
Monohydrate maltose ..	-587.68	Clarke and Stegeman (9) + heat of solution (Maggie, 16)
Ethanol aq.	-68.85	Rossini et al. (17)
CO_2 aq.	-98.69	Rossini et al. (17)

we calculated (Table 2) the theoretical enthalpy change K_i corresponding to the three ethanolic fermentation reactions studied.

It should be noted that corrections due to CO_2 are negligible. Indeed, the carbohydrate concentrations are so low that all the CO_2 evolved during the fermentation reactions remains dissolved in the culture medium. Moreover, from the data given by Roughton (18), it may be estimated that, at the pH of our culture, 0.28 mole of HCO_3^- is formed during the fermentation of 1 mole of hexose. The heat corresponding to this carbonate formation calculated from the data of Roughton (18) and from the heat of ionization of the second hydrogen of phosphoric acid [+0.85 Kcal per mole; Forrest et al. (12)] is equal to -0.81 Kcal per mole of hexose fermentation, that is, negligible compared with the heat observed during the fermentation reaction (Table 2).

The molecular growth yields with respect to the growth of this strain of *S. cerevisiae* on fructose, galactose, and maltose were measured as described above. The mean of three experiments with each substrate is: Y (fructose) = 26 g/mole; Y (galactose) = 26 g/mole; and Y (maltose) = 40 g/mole.

Results of the thermogram analysis. In all cases, A_c and $[S_i]$ were obtained from equations (1) and (2).

Figure 2 shows the double reciprocal plots ($1/A_c$ versus $1/[S]$) obtained when fructose is the limiting energy substrate. From this figure, the apparent Michaelis constant and the maximum rate of fructose utilization by whole cells are, respectively, 4×10^{-3} M and 551 $\mu\text{moles per min per g}$. In this case, the cellular rate of catabolic activity, i.e., the rate of fructose fermentation, is a hyperbolic function of fructose concentration in all the ranges of fructose concentrations examined.

Figure 3 shows the parameters cited previously for galactose. In this case, A_c is a hyperbolic function of the galactose concentration only when the sugar concentration is lower than 1.2 mM. For concentrations higher than 1.2 mM, A_c becomes practically independent of the galactose concentration. This result will be discussed further. From Fig. 3, the K_m and V_{\max} values of the cells for galactose can be extrapolated from the linear part of the curve and are, respectively, 3.2×10^{-3} M and 550 $\mu\text{moles per min per g}$. It should be noted that the extrapolation of the straight part of the curve gives the theoretical V_{\max} . The experimental $V_{\max}(V_{me})$, i.e., the rate of galactose fermentation when the galactose concentration is higher than 1.2 mM, is equal to approximately 180 $\mu\text{moles per min per g}$.

In the case of maltose, A_c versus $[S]$ is reported in Fig. 4. This curve seems to have charac-

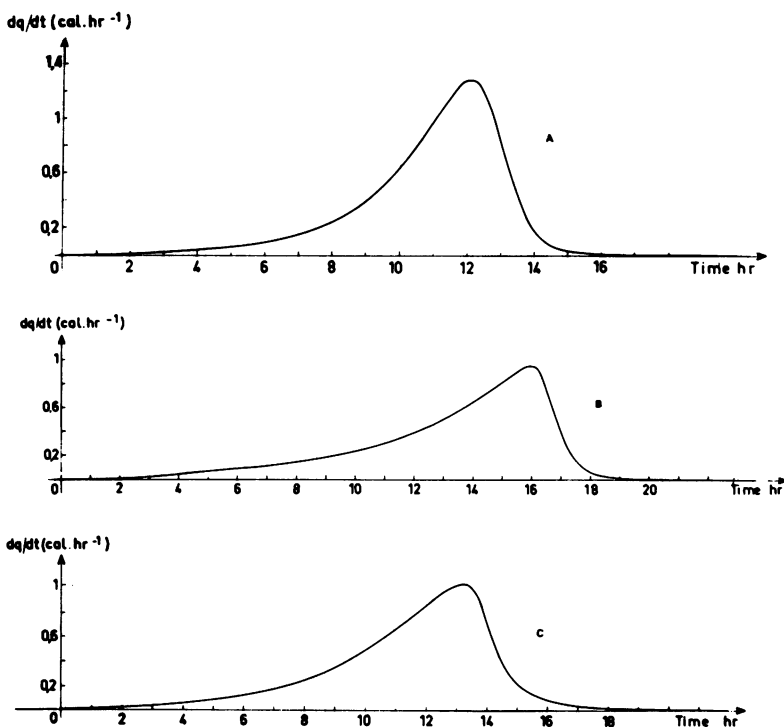


FIG. 1. Thermograms of *Saccharomyces cerevisiae* growth limited by energy source. A, Growth on fructose; B, growth on galactose; C, growth on maltose.

TABLE 2. Comparison between experimental enthalpy and theoretical enthalpy changes during the carbohydrate fermentation (Kcal/mole)

Substrate	Experimental enthalpy (K)	Experimental corrected enthalpy (Kc)	Theoretical enthalpy (Kt)
Fructose	-29.67	-32.22	-34.6
Galactose	-29.75	-32.71	-31.0
Maltose	-60	-64.26	-85.48

TABLE 3. Formation of ethyl alcohol from different substrates

Energy substrate	Fructose ^a	Galactose ^a	Mono-hydrate maltose ^b
Experimental results (moles of ethyl alcohol/mole of substrate)	1.86	1.84	3.65
Corrected values ^c	2.03	2.01	3.91
Means with a confidence value of 99%	2.03 ± 0.13	2.01 ± 0.21	3.91 ± 0.24

^a Averages of six experiments.

^b Averages of seven experiments.

^c Values obtained after correction for the incorporation of energy substrate into cellular carbon.

teristic allosteric kinetics. $\log A_c/A_{c_{max}} - A_c$ versus $\log [S]$ is illustrated in Fig. 5, and the

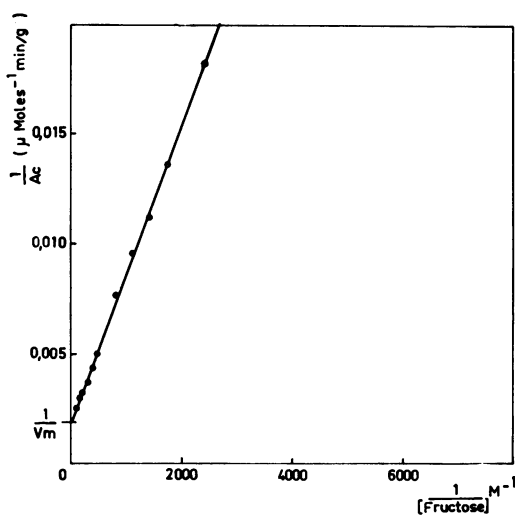


FIG. 2. Lineweaver-Burk plots of the kinetics obtained in the case of fructose.

slope of the straight line obtained (n) is equal to 1.6. The V_{max} calculated from a Lineweaver-Burk plot of the data in Fig. 4 is equal to 200 μ moles per min per g.

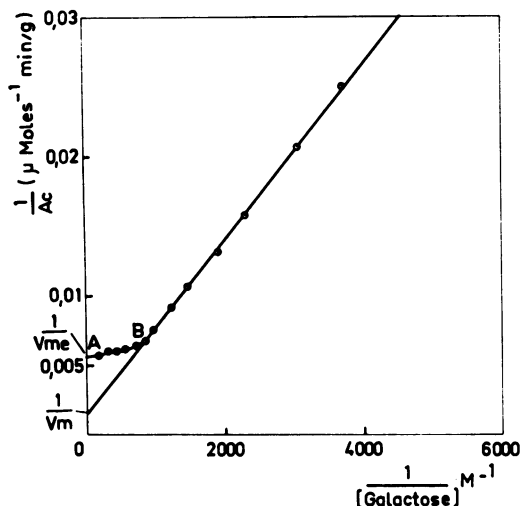


FIG. 3. Lineweaver-Burk plots of the kinetics obtained in the case of galactose. AB: range of galactose concentrations where A_c is independent.

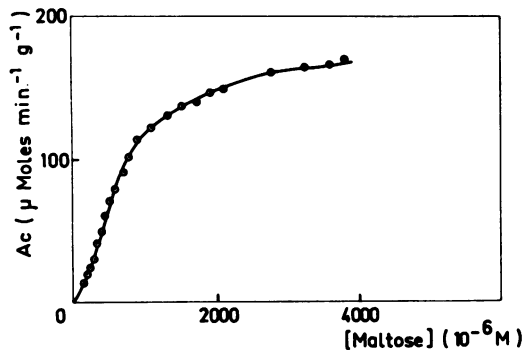


FIG. 4. A_c versus maltose concentration.

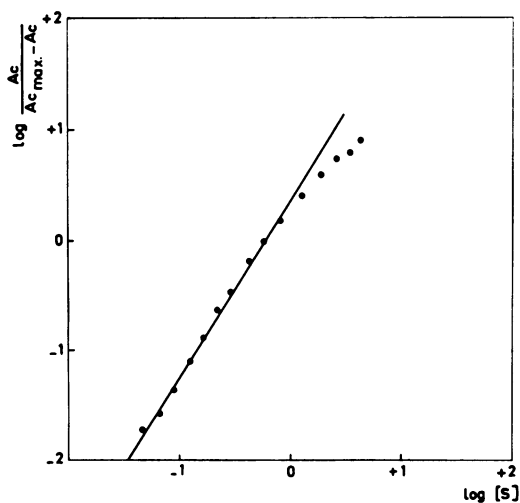


FIG. 5. $\log(Ac)/(A_{c_{\max}} - A_c)$ versus \log maltose concentration.

DISCUSSION

We previously discussed the significance of K_m and V_{\max} values determined by the microcalorimetric method that we utilized (5). When the concentration of the first substrate controls the overall rate of a linear multienzyme system, the kinetics of the whole chain is controlled by the first enzyme (14).

Thus, in cases in which the extracellular concentration of the energy substrate is the controlling factor of the cellular rate of catabolic activity, i.e., when the catabolic activity is dependent on the external energy substrate concentration, the V_{\max} and the K_m that we measured are the kinetic parameters of the first step of the chain, i.e., of the "permease" system.

It is now interesting to compare the results of the experiments that form the subject of this paper with the results that we previously described (5) and the results obtained by other authors concerning the kinetic parameters of the permeation systems of yeasts. All of the data useful for the discussion are reported in Table 4.

The K_m of intact yeast cells, i.e., of nonpoisoned cells, for glucose, fructose, and galactose determined by numerous investigations with different methods (6, 19, 20, 22-24) are of the same magnitude as ours (5 and this paper), with the exception of the results of Scharff and Kremer (19), who reported a five times higher value for the K_m of yeast for glucose and fructose. From our results and those of Sols (20) and Blackley and Boyer (6), it seems that yeasts have a higher affinity for glucose than for fructose and galactose. Although our results concerning the Michaelis constants are in agreement with most of the literature data, the same is not true for the V_{\max} values. Our V_{\max} values are, in general, between 10 and 50 times higher than other published values (19, 22, 23). We think that the low V_{\max} values obtained by numerous investigators are due to various treatments of the cells before kinetic determinations.

The fact that our measurements were made without preliminary stressful treatment, with cells in good physiological growth condition, leads us to think that our values are the true physiological V_{\max} . Moreover, it is obviously impossible that the observed V_{\max} of the different permeation systems are 10 or 50-fold lower than the maximum cellular rate of catabolic activity. The V_{\max} values that we have measured for glucose, fructose, and galactose are very similar. For glucose and fructose, this fact is not surprising and confirms the results and the hypothesis of Sols (20) and of Scharff and Kremer (19), that the entry of glucose and of fructose is mediated by the same transport system. It is well known that the galac-

TABLE 4. Kinetic parameters of permease yeast systems for various substrates in different culture conditions

References	Substrates	Physiological conditions	K_m (10^{-3} M)	V_{max} (μ mole per min per g)
Blackley and Boyer (6)	Glucose Fructose	Resting cells	1.8 5	
Sols (20)	Glucose Fructose	Resting cells	1 5	
Scharff and Kremer (19)	Glucose Fructose	Resting cells	5.3 26	46.6 60
Van Stevenink and Rothstein (23)	Glucose		6	33
Wilkins and Cirillo (24)	Glucose		2.8	
Van Stevenink and Dawson (22)	Galactose	Non-induced Induced pH 4.5 Induced pH 7.5	653 4.9 4.7	6 23 9.6
Belaich et al. (5)	Glucose		1.88	597
This paper	Fructose Galactose		4 3.2	550 550

tose permeation system is specific and different from that of glucose, and the similarity of V_{max} in the case of galactose must be considered a coincidence.

The sensitivity of our method also permitted us to observe interesting kinetic changes that always occur when the substrate concentration reaches some definite value. We have already reported an inhibition phenomenon of glucose permeation which is always present in the beginning of the logarithmic growth phase, as demonstrated by Fig. 6, where we have plotted Ac versus $[S]$ for three growths on different initial glucose concen-

trations. In the beginning of growth, i.e., for the higher values of $[S]$, Ac was markedly depressed in the three cases. The fact that this inhibition is specific for glucose and does not occur with fructose is in good agreement with the hypothesis proposed by Sols et al. (21) and accepted by us (5), that glucose-6-phosphate is an inhibitor of glucose transport since, in the case of fructose fermentation by yeast, it can be assumed that the internal glucose-6-phosphate pool is lower than in the case of glucose fermentation. Indeed, Ac is equal to 0.24 mmole per min per g when the glucose concentration becomes inhibitory, that is, at

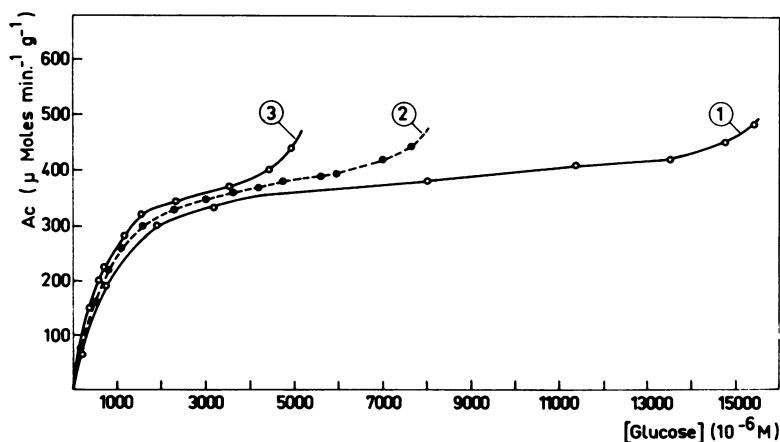


FIG. 6. Ac versus glucose concentration. 1, Initial glucose concentration, 3 g/liter; 2, initial glucose concentration, 1.5 g/liter; 3, initial glucose concentration, 1 g/liter.

1.3 mM (5); in the case of fructose fermentation with 1.3 mM fructose, A_c is only 0.14 mmole per min per g, or about one-half of the value obtained with glucose. This means that, for a hexose concentration of 1.3 mM, all the internal concentrations of the intermediary metabolites are lower in the case of fructose than in the case of glucose metabolism and, more precisely, that the fructose-6-phosphate pool and consequently the glucose-6-phosphate pool are lower in the case of fructose fermentation than in glucose fermentation.

The kinetics observed in the case of galactose fermentation shows unambiguously that the galactose permeation system is the controlling step of the fermentation when the galactose external concentration is lower than 1.2 mM. For the higher galactose concentrations, the cellular rate of catabolic activity is controlled by another system, the nature of which is easy to predict. Indeed, the chain of galactose fermentation differs from the chain of glucose fermentation by only four enzymes: galactokinase, uridyl transferase, epimerase, and phosphoglucosmutase. It is probably one of these four enzymes that is the limiting factor when the external galactose concentration is higher than 1.2 mM. Kinetics of the same type have been reported by Belaich (Thesis, Aix-Marseille Univ., Marseille, 1967) and by Belaich et al. (5) in the case of the glucose fermentation of *Z. mobilis* and by Ferroluzzi-Ames (11) studying histidine transport of *S. typhimurium*. These types of kinetics show that the transport potential of the cells is, in these cases, greater than the substrate utilization potential; more precisely, in the case of galactose fermentation by yeast, the permissible V_{max} , i.e., the extrapolated V_{max} (550 μ moles per min per g) is threefold higher than the experimental V_{max} (180 μ moles per min per g). The results are in agreement with the results of Bürger et al. (7) and explain the accumulation of free galactose into the yeast cells, an accumulation that has not been reported in the case of glucose and fructose.

The kinetics observed in the case of maltose fermentation (Fig. 4 and 5) suggest that the maltose permeation system is an allosteric one. Indeed, the Lineweaver double reciprocal plot and the value of the n coefficient of the Hill equation are very characteristic of allosteric kinetics. The V_{max} observed in this case is approximately 2.5-fold lower than the V_{max} of the hexose fermentation. This fact is interesting in the sense that it permits one to imagine a relationship between the energetic potential of the substrate molecule (i.e., the molecular growth yield) and the maximum rate of substrate permeation.

From a thermochemical point of view, Table 2 shows that the experimental corrected enthalpies

(K_c) of the galactose and fructose ethanolic fermentation reactions are very close to those of (K_t), which can be estimated from thermodynamic standard values. In the case of maltose fermentation, a discrepancy of 21 Kcal/mole appears between K_c and K_t . This result is probably due to an erroneous standard enthalpy of maltose formation given by Clarke and Stegeman (9). Indeed, if we estimate K_t of the maltose fermentation reaction from the K_t of the glucose ethanolic fermentation reaction (30.7 Kcal/mole; reference 3) and from the enthalpy of hydrolysis of maltose into two glucose molecules (-3 Kcal/mole; reference 15), the theoretical enthalpy of the maltose fermentation reaction becomes equal to -64.30 Kcal/mole. The latter is very close to the K_c that we measured. These thermochemical data are in agreement with those previously expressed and discussed by Forrest et al. (12), Belaich (Thesis, Aix-Marseille Univ., Marseille, 1967), and Belaich et al. (5), and confirm that, with the observed quantity of the energy substrate incorporated into the cellular carbon when a carbohydrate is the energy source, the heat evolved by microbial growth is practically equal to the enthalpy of the catabolic reaction.

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