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Microcalorimetric Study of Glucose Permeation in Microbial Cells[1]

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A microcalorimetric method for measuring the influence of extracellular glucose concentration on the rate of catabolism is described. This method has been applied to anaerobically growing cultures of *Zymomonas mobilis* and of a respiratory-deficient ("petite") mutant of *Saccharomyces cerevisiae* (strain YFa). The Michaelian kinetics recorded with both organisms were apparently related to glucose transport. With *Z. mobilis*, it was found that, in the range of glucose concentrations at which this organism was growing exponentially, cell activity was limited by the maximal rate of the catabolic enzymes; at lower concentrations, glucose transport was the rate controlling step. The metabolic activity of yeast always depended on external glucose concentration; when this was lowered under a threshold, a change of kinetics took place. The microcalorimetric method described seems to be widely applicable to kinetic studies of the permeation of metabolizable substrates in microorganisms.

We previously reported some applications of microcalorimetry to the study of microbial growth (5, 22). In the present paper, a new microcalorimetric technique is described which permits the easy determination of the influence of the external concentration of a metabolizable substrate on the metabolic activity of growing cells.

The studies have been performed on anaerobic cultures growing under conditions where glucose was the energy source and the limiting growth factor. One of the organisms utilized, *Zymomonas mobilis*, ferments glucose via the Entner-Doudoroff pathway (13) with a simple overall metabolic balance (6): 1 glucose $\rightarrow$ 1.7 ethyl alcohol + 1.7 CO_2 + 0.3 lactate.

The other organism is a respiratory-deficient mutant of *Saccharomyces cerevisiae* which, being purely fermentative, has the experimental advantage of excluding any possible interference from incidental respiration at the expense of oxygen contamination of the cultures.

MATERIALS AND METHODS

Organisms and culture media. Z. mobilis (NCIB 8938) was grown in a complex medium of the following composition: yeast extract (Difco), 4 g; peptone (Difco), 4 g; tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (*p*H 6.8), 0.025 M; glucose (separately sterilized), 0.5 to 2 g; and distilled water to 1,000 ml.

The strain of yeast studied (*S. cerevisiae* YFa) is a respiratory-deficient ("petite") mutant kindly provided by P. Slonimsky. It was verified that, when grown in the presence of air, this strain has no respiratory activity and does not display any Pasteur effect. The same complex culture medium was utilized for this organism, except that 0.05 M phosphate buffer (*p*H 5.82) was substituted for Tris-maleate.

Microcalorimetric methods. The microcalorimetric experiments were performed with a Tian-Calvet differential isothermic calorimeter (8) whose reaction vessels had a working volume of 10 ml. The apparatus directly records the rate of heat evolution (dQ/dt) with a maximal sensitivity of 1 mm of galvanometric deviation for 2 μw and a response time of 300 sec. The quantities of heat evolved (Q) were determined by graphical integration of the experimental curves.

The experiments were done anaerobically under argon in a closed Pyrex vessel containing 10 ml of liquid culture medium with a gas phase of 1.5 ml. A syringe attachment allowed the introduction of the inoculum (0.1 ml) after temperature equilibration for 1 hr in the apparatus.

Because of the small quantities of glucose and the *p*H value utilized in our growth experiments with *Z. mobilis*, all the CO_2 evolved during the fermentation remained in aqueous solution. With *S. cerevisiae* also, it may be considered that under our experimental conditions CO_2 was dissolved.

In the case of yeast, it was found important to avoid the sedimentation of cells during the experiments. This was achieved by use of a stirring device

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adapted from that previously described by Leydet (19). The slight quantity of heat evolved by this attachment is constant and is marked on the thermograms by a strictly horizontal shift from the base line. Stirring proved to be unnecessary with *Z. mobilis* because its motility kept the suspension homogenous throughout the culture.

Analytical methods. Glucose was estimated by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Microbial densities were measured turbidimetrically at 475 mμ, with a Jean & Constant spectrophotometer.

RESULTS

Thermograms and growth yields of glucose-limited cultures. Figure 1 shows two examples of thermograms recorded with anaerobic cultures of *Z. mobilis* and of *S. cerevisiae* YFa growing in complex media where glucose concentration was the limiting growth factor. From these microcalorimetric curves giving the rate of heat evolution (dQ/dt) versus time, the quantities of heat evolved (Q) were determined by graphical integration (Fig. 2).

With both organisms, the thermograms show a short initial break due to the introduction of the inoculum. After a lag phase, the length of which depends on the age and the amount of the inoculated cells, the microcalorimetric curve assumes an exponential shape. During this period, the slope of the corresponding straight lines drawn by semilog conversion of the experimental thermograms is identical to the exponential growth

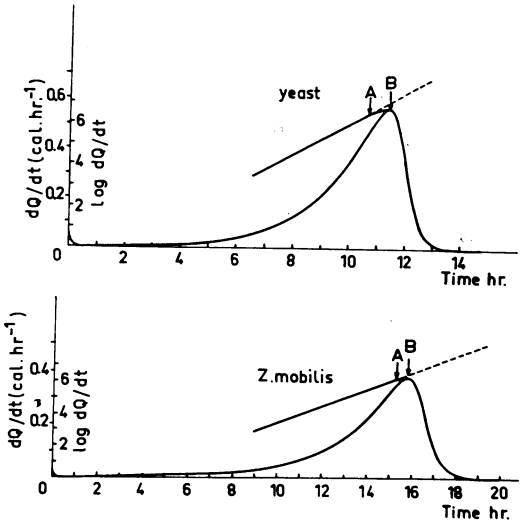

FIG. 1. *Thermograms of Saccharomyces cerevisiae and of Zymomonas mobilis growing anaerobically in glucose limited media. The straight lines correspond to semilog transformation of the experimental curves.*

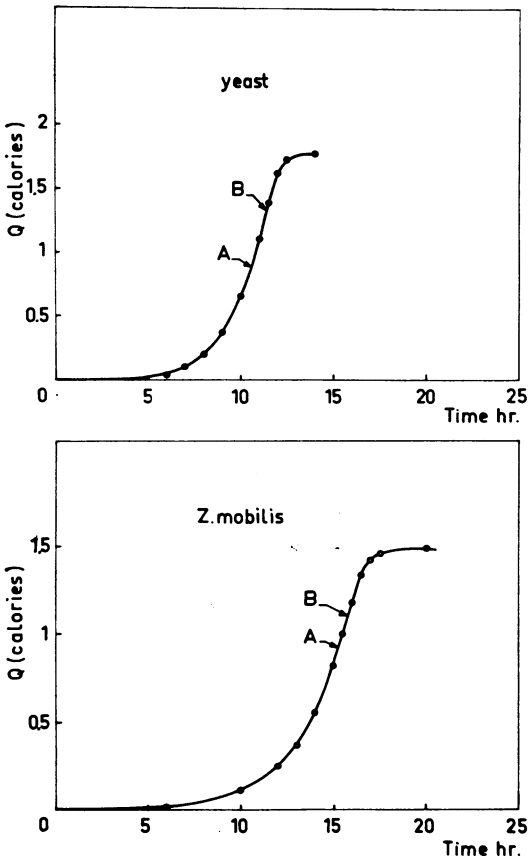

FIG. 2. *Quantities of heat determined by graphical integration of the experimental thermograms shown in Fig. 1.*

rate determined by conventional turbidimetric measurements with the same organism growing under the same conditions.

In a series of experiments, it was observed that at point B the residual glucose concentration was 0.4 mg/ml in the case of *Z. mobilis*, and 0.23 mg/ml for *S. cerevisiae*, no matter what the initial glucose concentration was. This shows that the decrease of dQ/dt at point B is due to a limitation of the catabolic activity by glucose concentration and not to the accumulation of some fermentation product.

From point B, the thermograms come back slowly to the base line, which is reached when glucose is completely exhausted. In the course of this last phase, the growth yields by reference to glucose remain constant and are identical to the values determined on exponentially growing cultures (5). The fact that the microcalorimetric curves come back to the zero line after glucose exhaustion indicates that, under physiological conditions where growth is limited by the energy

source (glucose), both organisms have no detectable "endogenous" activity. In addition, this fact also demonstrates that glucose is the only constituent of the complex media which is catabolized and utilized as an energy source by *Z. mobilis* and by *S. cerevisiae*.

The molecular growth yields (Y_G) and enthalpy changes (K) per mole of glucose consumed are reported in Table 1.

In the case of *Z. mobilis*, the quantities of cells produced and of heat evolved (Q) are linearly proportional to the quantity of glucose metabolized (Fig. 3), and the corresponding straight lines start from the origin of the graphs, confirming the fact that glucose is the only constituent of the complex medium utilized as an energy source for growth. It should be noted that, on the buffered culture medium employed for these experiments, the growth yield (Y_G) of *Z. mobilis* averages 6.5 g of cells (dry weight) per mole of glucose and, for still undetermined reasons, is consistently lower than the value (Y_G = 8.3) reported by Bauchop and Elsden (4) and confirmed by us (22) for the same organism cultivated on a nonbuffered medium of otherwise similar composition.

The experimental enthalpy change for *Z. mobilis* fermentation has been determined in a series of 27 experiments, and the average value (K) has been found to be -32.00 ± 1.32 kcal per mole, with $P = 0.01$. This value should be corrected for glucose assimilation. Previous experiments (6) done with uniformly ^{14}C-labeled glucose have shown that, when *Z. mobilis* is grown in complex medium, 48% of the cellular carbon is derived from glucose. Furthermore, it is known that cellular carbon is at the same oxidation level as carbohydrates (2) and represents 50% of the dry weight (20). From these data and from the average Y_G value (6.5), it may be calculated that the quantity of glucose which is assimilated corresponds to only 2.2% of the consumed glucose. The corresponding value of the experimental enthalpy change corrected for glucose assimilation (K_c) is thus -32.71 kcal per mole.

TABLE 1. *Molecular growth yields and enthalpy changes for the fermentation of glucose by Zymomonas mobilis and by Saccharomyces cerevisiae*

Determination	*Z. mobilis*	*S. cerevisiae*
Molecular growth yield (Y_G)[a]	6.5	22.7
Enthalpy change (kcal/mole)		
Experimental (K)	−32.00 (±1.32)	−28.5
Corrected for glucose assimilation (K_c)	−32.71	−30.70
Calculated	−32.22	−33.05[b]

[a] Milligrams (dry weight) of cells produced per mole of glucose consumed.

[b] For purely ethanolic fermentation.

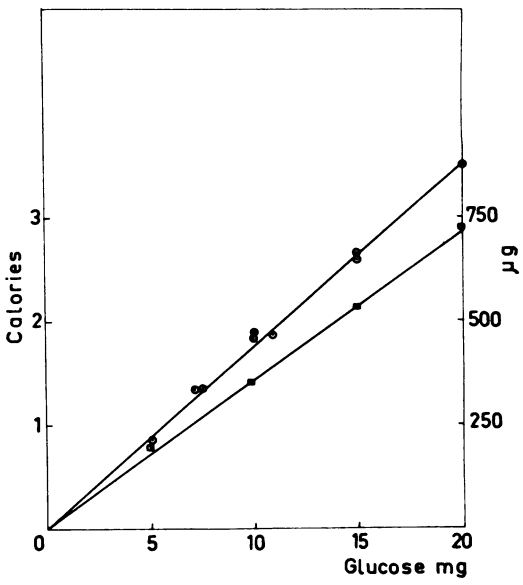

FIG. 3. *Heat evolved (⊙) and dry weight of cells produced (⊡) as a function of the amount of glucose consumed by cultures of Zymomonas mobilis in complex glucose-medium.*

The theoretical enthalpy change for *Z. mobilis* fermentation has been calculated from the standard enthalpies of formation given by Battley (3) for aqueous glucose, ethyl alcohol, and CO_2, i.e., −302.03, −68.85, and −98.69 kcal per mole, respectively. With regard to aqueous lactic acid, calculation has been made from the standard enthalpy of formation (−164.02 kcal/mole) reported by Saville and Gundry (21) and from the heat of neutralization (−0.85 kcal/mole) directly measured by Forrest et al. (12). The theoretical figure so obtained (−32.22 kcal/mole) is practically identical to K_c, showing that, as has been already discussed elsewhere (5, 22), under our experimental conditions of growth, the enthalpy incorporated into covalent bonds of biological macromolecules is negligible compared to the enthalpy of catabolic processes. In other words, under these conditions the corrected experimental enthalpy change (K_c) is practically equal to the enthalpy change corresponding to the "nonconservative reaction" as defined by Battley (3).

The average growth yield of anaerobic yeast was found to be 22.7 g of cells per mole of glucose, in close agreement with the value (Y_G = 21.0)

reported by Bauchop and Elsden (4). In the case of *S. cerevisiae*, the experimental enthalpy change (K) averages -28.5 kcal per mole of glucose. The assimilation of glucose by this organism has not been experimentally determined. However, assuming the same figure as for *Z. mobilis*, the enthalpy change corrected for glucose assimilation (K_c) would be -30.7 kcal per mole. This is consistently lower than the theoretical value (-33.05 kcal.) calculated by Battley (3) for pure ethanolic fermentation, and the difference could be due to the formation of some minor metabolic products other than ethyl alcohol and CO_2.

Microcalorimetric determination of cellular activity and of glucose concentration. The cellular rate of catabolic activity (A_c), i.e., the quantity of substrate metabolized per unit of cells and of time, is given by the relation:

$$A_c = dS_f/dt \cdot \frac{1}{m} \quad (1)$$

where S_f is the amount of substrate metabolized by the culture and m is the biomass.

A_c can be determined from the thermogram itself, provided that, as is the case with the organisms and growth conditions previously described, the growth yield (Y_G) and the experimental enthalpy change per mole of substrate consumed (K) are known and constant throughout the culture. When these conditions are fulfilled, the quantity of heat evolved at any time (Q_t) is directly proportional to K and to the amount of substrate metabolized (S_f):

$$Q_t = KS_f \quad (2)$$

and the rate at which the substrate is consumed by the culture (dS_f/dt) is thus proportional to the rate of heat evolution (dQ/dt):

$$dS_f/dt = \frac{1}{K} dQ/dt \quad (3)$$

On the other hand, Y_G being constant, the biomass is directly proportional to the amount of substrate consumed:

$$m = Y_G \cdot S_f \quad (4)$$

Combining relations 2 and 4, it becomes:

$$m = \frac{1}{K} Y_G \cdot Q_t \quad (5)$$

and substituting for m and for dS_f/dt in relation 1:

$$A_c = dQ/dt \cdot \frac{1}{Q_t} \cdot \frac{1}{Y_G} \quad (6)$$

Glucose concentration at any time t can also be calculated from the microcalorimetric curve. The total quantity of substrate initially introduced in the culture is directly proportional to the total quantity of heat (Q_T) evolved by the culture at the end of growth when glucose is completely exhausted and the thermogram has returned to the base line. Thus, Q_t being the quantity of heat evolved at time t and v being the volume of the culture, the residual concentration of glucose at any time t is given by the relation:

$$(S)_t = \frac{1}{v} \cdot \frac{Q_T - Q_t}{K} \quad (7)$$

Influence of glucose concentration of the catabolic activity of yeast and of Z. mobilis. The experimental thermograms recorded with *Z. mobilis* and *S. cerevisiae* have been analyzed by the method described above. The variations of A_c with the external concentration of glucose in the medium are represented in Fig. 4 and the corresponding double reciprocal plots in Fig. 5 and 6.

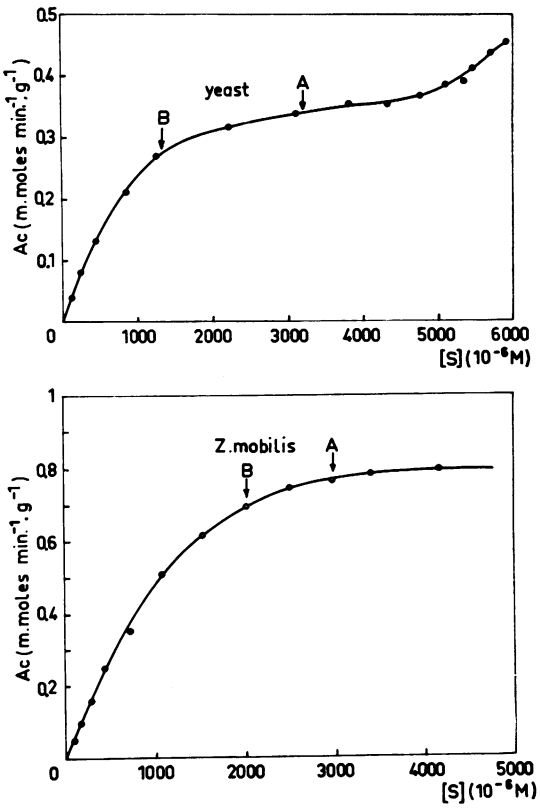

FIG. 4. *Cellular rates of catabolic activity (A_c) versus extracellular glucose concentration (S).*

In the case of *Z. mobilis*, it is clear from examination of the double reciprocal plots (Fig. 6) that before point A of the thermograms, i.e., when the organism is growing exponentially, A_c does not depend on glucose concentration and has a maximum constant value of about 816 μmoles per g per min. At point A of the microcalorimetric curve, glucose concentration is 3.0 mM (0.54 mg per ml) and point B corresponds to 2.03 mM glucose (0.37 mg/ml). Between A and B, the double reciprocal plot assumes a curved form, and slightly after point B the experimental values forms a straight line (Fig. 5) from which an apparent Michaelis constant can be accurately determined (K_m = 3.15 mM). Extrapolation of this straight line to the ordinate axis corresponds to a theoretical maximal A_c (V_{max} = 1,818 μmoles of glucose per g per min) which is about twice the experimental maximal value recorded during the log phase of growth.

In the case of yeast, A_c always depends on glucose concentration, and the kinetics are more complex (Fig. 6). During the log phase, the apparent Michaelis constant (K_m^I) is 3.2 mM and the extrapolated V_{max} is 597 μmoles per g per min. Points A and B occur at glucose concentrations 3.2 mM (0.58 mg/ml) and 1.33 mM (0.24 mg/ml), respectively. In the range of lower glucose concentrations, the double reciprocal plot becomes a straight line and extrapolates to the same V_{max} as for the log phase, but corresponds to a significantly different Michaelis constant (K_m^{II} = 1.88 mM). Between A and B, the double reciprocal plot obviously corresponds to a phase of transition from the first to the second kinetics.

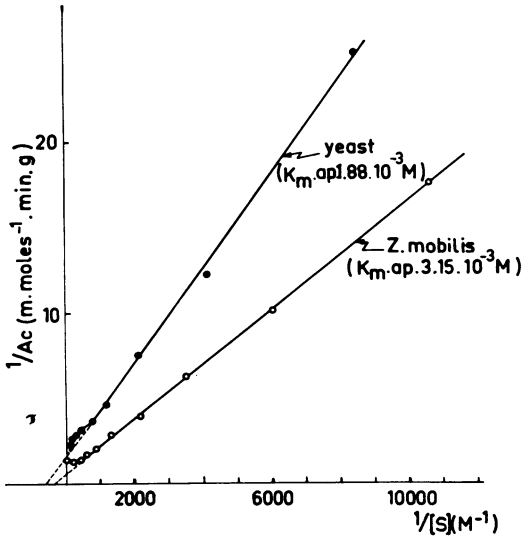

FIG. 5. *Lineweaver-Burk plots of the curves represented in Fig. 4.*

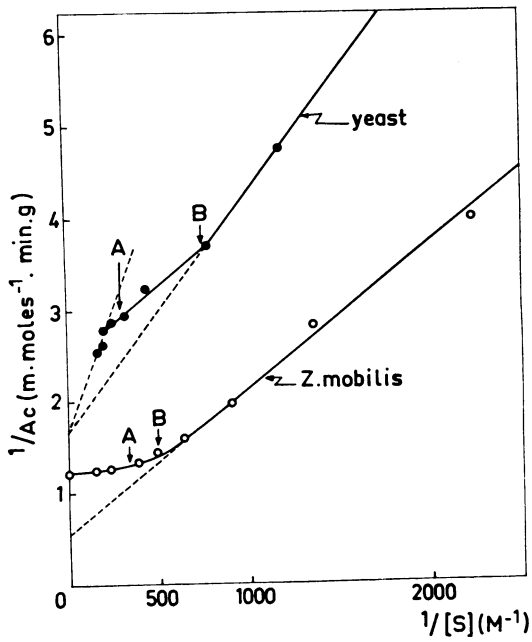

FIG. 6. *Magnification of the initial parts of Fig. 5. For the sake of clarity, only a few experimental points are reported.*

In Table 2, these results are shown in comparison with other data from the literature concerning the properties of microbial permeases.

DISCUSSION

From theoretical considerations on multienzyme systems, it has been demonstrated by Higgins (14) that, under conditions where the concentration of the initial substrate is the limiting factor of cell activity, the overall rate of metabolism is controlled by the first enzyme of the sequence. Therefore, the kinetics we have determined for *S. cerevisiae* are related to the enzyme-like system of glucose transport demonstrated in this organism by Burger et al. (7). This system, characterized by Cirillo (9) as a "carrier-mediated facilitated diffusion," does not accumulate glucose against a concentration gradient and does not require an energy supply. Its kinetics have been extensively studied by means of indirect methods based on competition for transport between glucose and a nonmetabolizable sugar (25).

The reproducible change of kinetics revealed by our new microcalorimetric method (Fig. 6) has not been reported by other investigators, probably because the observed transition occurs at a relatively low glucose concentration (1.33 mM),

TABLE 2. *Comparison of the kinetics determined by microcalorimetry and literature data on some microbial transport systems*

Organism[a]	Substrate	Physiological conditions	K_m (M)	V_{max} (μmoles per g per min)
Zymomonas mobilis	Glucose	Log phase	—	816
Z. mobilis	Glucose	Retardation phase	3.15×10^{-3}	1,818
Saccharomyces cerevisiae	Glucose	Log phase	3.2×10^{-3}	597
S. cerevisiae	Glucose	Retardation phase	1.88×10^{-3}	597
Bakers' yeast (1)	Glucose	Growth	—	~400
Escherichia coli (17)	Lactose	Resting cells	7.0×10^{-5}	158
E. coli (24)	Maltose	Resting cells	?	20
E. coli (16)	Galactose	Resting cells	7.0×10^{-6}	—
S. cerevisiae (26)	Glucose	Resting cells	3.0×10^{-3}	?

[a] Numbers in parentheses refer to reference numbers in the Literature Cited section.

i.e., under experimental conditions not easily amenable to the usual methods of study.

From measurements of D-arabinose counter-transport, Wilkins (26) recently obtained experimental evidence that, in a range of glucose concentrations extending from 2 to 100 mM, glucose transport across the membrane of aerobic yeast is a symmetrical process. Thus, according to this author, the kinetics we have recorded for yeast growing exponentially at glucose concentrations higher than 1.33 mM should be the overall kinetics of a symmetrical transport.

The change of kinetics observed at lower glucose concentrations suggests the existence in yeast of two processes governing sugar transport and utilization: one limiting the catabolism at low external concentrations and the other limiting at high external concentrations. The possibility that these processes are mediated by two distinct transport systems has to be considered, but it seems difficult to reconcile with the fact that for both kinetics the V_{max} values are apparently identical.

Another possibility is that glucose transport is mediated by a single system, the affinity of which is modified when the external concentration of glucose drops below 1.33 mM. In this regard, it has been reported by Hoffee et al. (15) that the kinetics of α-methylglucose (α-MG) accumulation in *Salmonella typhimurium* and *Escherichia coli* depend on the availability of an exogenous energy supply. Both the steady state level of intracellular α-MG and the apparent affinity of the cells for this glucose analogue were found to be lower in the presence than in the absence of a metabolizable substrate. From these data, it was concluded that two energy-requiring processes are mediated by the glucose permease, and that the second one does not take place in starved cells. This is consistent with the situation observed by us in yeast where the apparent K_m for glucose is greater at high than at low extracellular concentration of the energy source.

Alternatively, the double reciprocal plots on which two straight lines converge to the same V_{max} (Fig. 6) might be interpreted by the assumption that, in exponentially growing yeast, the transport system is transiently and competitively inhibited by some intermediary product of glucose metabolism. Sols et al. (23) have suggested that glucose transport in yeast is inhibited by intracellular accumulation of glucose-6-phosphate. In such a case, one could expect the depletion of glucose and consequently of the glucose-6-phosphate pool to relieve the inhibition of the transport system.

Further experimentation will be necessary to check these various possibilities. But, whatever the explanation for the observed kinetics, it is noteworthy that the apparent Michaelis constants and V_{max} we have determined microcalorimetrically are close to the values previously reported by Wilkins (36), by Azam and Kotyk (1), and by Kotyk and Kleinzeller (18) for glucose uptake by yeast.

A system of active permeation or of facilitated diffusion of glucose has not been directly demonstrated in *Z. mobilis*. However, several indirect arguments convincingly suggest that the kinetics determined with this organism belong to a system of mediated transport. According to Fick's law, the rate of passive diffusion of a substrate should be a linear function of the concentration gradient across the membrane. Thus, the Michaelis kinetics recorded with *Z. mobilis* exclude the possibility that the rate-controlling step of cell activity is a process of passive diffusion, and they obviously correspond to the saturation of an active factor which could be either a transport system or the first enzyme of the catabolic sequence.

The initial step of glucose fermentation by *Z.*

mobilis is catalyzed by a hexokinase (13). This enzyme has not been purified and its kinetic properties are not known, but they can be expected to to be similar to those of other bacterial hexokinases (10) whose K_m values are closely related and at least 30 times lower than the apparent Michaelis constant determined by our microcalorimetric technique.

Another indirect argument in favor of a transport system being responsible for the observed kinetics is provided by literature data on sugar permeability of bacteria and especially of the permeaseless mutants of *E. coli*. It seems most unlikely that glucose could penetrate *Z. mobilis* by a process of passive diffusion without being the rate-limiting step of catabolic activity.

It is interesting to compare our data with the kinetics of sugar permeation reported in the literature for other bacteria and sugars. As shown in Table 2, the glucose transport system in *Z. mobilis* is characterized by an apparent affinity ($1/K_m$) considerably lower than the corresponding values reported by Kepes and Cohen (17) for the β-galactoside-permease of *E. coli*, and by Horecker et al. (16) for the galactose-permease of the same organism.

Some preliminary experiments have been performed to determined by our microcalorimetric method the influence of glucose concentration on the catabolic activity of anerobically growing *E. coli*. An accurate determination of the kinetics from these thermograms was hindered by a transient accumulation of intermediary fermentation products which interfered with the analysis of the curves. Nevertheless, it was obvious that the thermograms of *E. coli* come back to the base line much more rapidly and sharply than do those obtained with *Z. mobilis* and *S. cerevisiae*. Thus, in *E. coli* the affinity of glucose permease for its natural substrate seems to be high and approximately similar to the affinities reported in the literature for the β-galactoside- and galactose-permeases of this organism. It must be noted that the low affinities of the glucose transport system in *Z. mobilis* and *S. cerevisiae* are physiologically compensated by high maximal rates (V_{max}) of glucose penetration (Table 2).

In exponentially growing cultures of *Z. mobilis*, A_c does not depend on the external concentration of glucose, and its experimental value is about half the theoretical V_{max} estimated by extrapolation of the double reciprocal plots (Fig. 6). Thus, it is clear that, in the range of concentrations corresponding to the log phase of growth, A_c is not limited by glucose permeation and is controlled by the maximal activity of the endocellular enzymes. In this regard, *Z. mobilis* differs from *E. coli*, in which, according to Kepes and Cohen (17), Wiesmeyer and Cohn (24), and Horecker et al. (16), the rates of lactose, maltose, and galactose utilization are always limited by the corresponding permeases.

Kinetics similar to those of *Z. mobilis* have been reported by Ferroluzzi-Ames (11) for a histidineless mutant of *S. typhimurium* growing aerobically on glucose in the presence of increasing concentrations of histidine. It was found that the rate of histidine uptake and incorporation depends on the concentration of this amino acid only below a threshold (1.5×10^{-7} M). At higher concentrations, the histidine-permease system is no longer the rate-controlling factor, and the Lineweaver-Burk plots behave similarly to what has been observed by us during the log phase of *Z. mobilis* cultures.

No evidence has been found for an intracellular accumulation of glucose or of glycogen in exponentially growing *Z. mobilis* (6). Therefore, as V_{max} of the glucose transport system is about twofold larger than the maximal rate of glucose utilization by the cells, an excess of glucose entry most probably occurs in exponentially growing cells and has to be compensated by an outflow.

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