A Continuous Fermentation Technique for Studying the Kinetics of Sugar Uptake by Baker's Yeast

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1. Solutions of glucose, maltose or other sugars were pumped at controlled rates into a yeast suspension and the extracellular sugar concentration was determined. The technique was especially suitable for studying the kinetics of fermentation at low rates of sugar utilization. At high rates the fermentation system was unstable. 2. Glucose fermentation was fitted by a model in which a diffusion barrier with first-order kinetics is interposed between the environment and the site of hexokinase. 3. Aerobic conditions affected the fermentation enzyme system but not the diffusion mechanism. 4. The kinetics of maltose fermentation at low rates approximated to those of the hydrolysis of maltose by the enzyme maltase, as studied in suspensions of broken cells.

Many investigators have determined the rate at which yeast metabolizes sugar, and expressed their results as a function of the extracellular concentration of substrate. Such determinations often form part of a study of the mechanism whereby sugar is transported into and out of the cell (for references, see Hokin & Hokin, 1963; Wilkins & Cirillo, 1965).

The procedure generally adopted involves the addition of a known amount of sugar to a suspension of yeast, after which the concentration of unchanged sugar, or the amount of carbon dioxide evolved, is determined at intervals. Metabolic rate is calculated from the slope of a plot of the analytical data against time. This plot is rarely strictly linear. When the concentration of sugar is low, the rate of metabolism decreases progressively as the substrate is consumed, and extrapolation to zero time is necessary. Even with excess of sugar, the rate is not constant. Hopkins & Roberts (1935*a,b*), for example, found that the maximum rate of carbon dioxide production by yeast increased rapidly at first, remained steady for some time and then declined as the fermentation progressed (cf. Trevelyan & Harrison, 1954). The calculation of rate is therefore somewhat uncertain.

Fermentation is most readily followed by manometric techniques. However, measurement of the rate at which sugar is consumed is preferable, because it gives direct information about the initial reaction of the multi-enzyme fermentation system. Here, operations such as sampling and separating yeast cells, which precede analysis for residual sugar, add to the error. Difficulty is encountered in evaluating high as well as low rates of sugar uptake, since, if the initial concentration of

substrate is high, the proportion fermented in a given time is small and the error of analysis correspondingly severe.

I decided to investigate the relation of rate to concentration by a procedure in which rate is made the independent variable. This was fixed by the continuous addition of sugar solution at a known rate to a suspension of yeast. At intervals the concentration of sugar established in the medium was determined.

METHODS

Yeast. In most cases, commercial pressed baker's yeast was used. The different batches are identified, where appropriate, by an index number (K1-6). The yeast was suspended in water, sodium succinate buffer (50 mM, pH 5.3) or a medium containing buffer together with 10 mM-KCl and 2.5 mM-MgSO₄. The pH of buffered media changed by less than 0.1 unit after fermentation for 2 hr. There was very little extracellular carbohydrate in the yeast suspensions (0.02 mM, as glucose). This was reduced in some cases by centrifuging the yeast (twice) after it had been suspended in 10 times its volume of buffer.

A few samples of the same strain of yeast were propagated in the laboratory by a continuous technique. The synthetic medium contained 75 or 150 mM-glucose (for yeast batches GG1 and GG2 respectively), or 37.5 mM-maltose (for yeast batches MG1 and MG2), 30 mM assimilable nitrogen (as ammonia and L-asparagine), 2.5 mM-phosphate, other inorganic supplements and vitamins. The pH was maintained at 4.5 with a succinate buffer.

Determination of maltase. The activity of the enzyme maltase (α -D-glucoside glucohydrolase, EC 3.2.1.20) was determined on samples of yeast the cells of which had been disintegrated by shaking suspensions with glass beads

(Harrison & Trevelyan, 1963). The initial rate of hydrolysis of maltose at 30° in 25 mM-phosphate buffer, pH 6.9, was measured at four substrate concentrations (6.25, 12.5, 25 and 50 mM-maltose).

Fermentation technique. A 2 l. Pyrex 'tall form' beaker, containing either 10 or 20 g. of yeast (weight of pressed yeast, at 27% dry matter) suspended in 1 l. of medium, was clamped in a bath of water maintained at 30°. The actual temperature of the suspension, after equilibration, was 29.8°. The suspension was stirred mechanically, and a stream of gas from a sintered-glass diffuser was passed through it. Air or nitrogen was supplied (0.4–4.0 l./min.) in different experiments. Foam was not excessive when the flow was below 1 l./min.

After temperature equilibration, a solution of sugar (glucose, fructose, mannose, sucrose or maltose, 50–450 mM, in the appropriate medium) was fed at a constant rate to the suspension of yeast. In some experiments, sugar solution was added at zero time in an amount calculated to bring the initial concentration of sugar close to the expected steady-state value. The pump (D.C.L. Micro Pump, manufactured by F. A. Hughes and Co. Ltd., Great Burgh, Epsom, Surrey) made 85 strokes/min., and by adjusting the length of stroke the delivery rate could be set within the range 0–3 ml./min. After each of 27 experiments, the pump was checked by measuring the time taken to deliver 50 ml., the figures being reproducible to $\pm 1\%$. Within the usual experimental period of 2 hr., the volume delivered in successive intervals of 10 min., was reproducible to $\pm 1\%$ (pump set to 1 ml./min.).

At intervals of 10 min., 10 ml. of cell-free medium was withdrawn by applying suction to a porosity 4 sintered-glass filter-stick partially immersed in the yeast suspension, an operation which took 1 min. The total amount of yeast in the suspension was thus unaltered by the sampling procedure. In most cases, the volume of sugar solution supplied was exactly balanced by the volume of medium withdrawn, so that the volume of the yeast suspension was maintained at 1000–1010 ml.

Determination of sugar concentration. Glucose, fructose, mannose and maltose were estimated colorimetrically with the anthrone reagent. Colour was developed at the optimum temperature for each sugar, and with the appropriate pair of reagents both fructose and glucose could be determined in a solution containing both sugars (Trevelyan & Harrison, 1956).

Sugars were determined in addition by a copper-reduction method (Nelson, 1944).

RESULTS

Calculation of rate of sugar uptake in the steady state. This situation is illustrated by curve A, Fig. 1.

If the yeast suspension received P ml./min. of a solution containing S mM-sugar, the total sugar supplied in dt min. was $SPdt$ μ moles. The amount metabolized was $QYdt$, where Q is the rate of sugar uptake in μ moles/g. of yeast, and Y the weight of yeast in grams. If the extracellular concentration of sugar established in a suspension of total volume V ml. was c mM, then, provided that the volume of the yeast cells was small compared with V , the total

Fig. 1. Extracellular concentration of glucose during anaerobic fermentation. Yeast K1, 1% (w/v), in water. Pump rate 1 ml./min. Curve A, glucose 200 mM, feed stopped at 125 min. Curve B, glucose 350 mM. Curve C, glucose 400 mM.

amount of sugar in the suspension was Vc μ moles, and in dt min. this changed by $d(Vc) = Vdc + c dV = Vdc + Pcdt$ μ moles (since $dV = Pdt$). Hence

$$V \frac{dc}{dt} + Pc + QY = SP \quad (1)$$

$$Q = \frac{P}{Y}(S - c) - \frac{V}{Y} \frac{dc}{dt} \quad (2)$$

In the steady state, $dc/dt = 0$, and the equation reduces to

$$Q = \frac{P}{Y}(S - c_s) \quad (3)$$

where c_s denotes the steady-state concentration of sugar.

In curve A, Fig. 1, c_s was small (2.60 mM) compared with S (200 mM) and therefore errors in the estimation of c had little effect on the calculated value of Q . Between 40 and 110 min., dc/dt was only 0 ± 0.001 μ mole/ml./min., equivalent to an uncertainty of ± 0.1 μ mole/min./g. in the value of 19.4 calculated for Q . However, since dc/dt had to be multiplied by the factor V/Y , which was rather large (100) it is evident that any drift in c would have had a pronounced effect on the calculated value of Q . In a more concentrated suspension of yeast, V/Y would have been smaller, but then the neglect of the cell volume would have been less justifiable; moreover, diffusion effects (see Hopkins & Roberts, 1935a), and an increase in concentration of alcohol and other end products, would have tended to introduce additional error.

Approach to the steady state. When not too close to its maximum value, Q is approximately proportional

to c . With the substitution $Q = kc$, equation (1) may be integrated to give

$$c = c_0(1 - e^{-mt}) \quad (4)$$

where $m = SP/c_0V$. The larger m is, the more rapidly steady-state conditions are established.

This expression, with $m = 0.076$, fitted curve A, Fig. 1, fairly well. It was calculated that c should have been within 1% of the steady-state value after 60 min. In practice, this condition was reached in 40 min.

For a given value of the rate of sugar uptake, c_0 was found to increase in the order glucose < fructose < mannose. The time taken to achieve the steady state was correspondingly increased.

An increase in the concentration of yeast should, in theory, have the advantage of decreasing the time taken to reach the steady state (but see the discussion on this point above).

Non-steady-state conditions. When sugar was supplied at a rate greater than the maximum rate of

consumption ($Q_{\max}$) the yeast suspension could sustain, the extracellular concentration of sugar increased progressively with time, as illustrated by curves B and C, Fig. 1. Here dc/dt became constant after a certain period of time, and a value for $Q_{\max}$ could be obtained using equation (2).

In general a different method of calculation was preferred. Let the concentration of sugar in the medium be c_t at time t and c_{t+10} 10 min. later. Then in the 10 min. interval the sugar metabolized is $10SP - \{(V_t + 10P)c_{t+10} - V_t c_t\}$, from which the mean value of Q during the interval is readily calculated.

This method of calculation, unlike that based on equation (2), can be applied when the volume of suspension is not kept constant by balancing the volume withdrawn for analysis against the volume pumped in.

Relation between rate of sugar uptake and concentration of sugar during anaerobic fermentation of glucose. Nine experiments were performed at intervals of one day, with the results shown in Fig. 2. A plot of rate of glucose uptake against extracellular concentration, derived from the steady-state portions of the curves of Fig. 2, is given in Fig. 3.

Samples of yeast were taken daily for these experiments from a master block stored at 4°. To

Fig. 2. Extracellular concentration of glucose during anaerobic fermentation. Yeast K2, 1% (w/v), in succinate-KCl-MgSO₄ medium. Gas phase, nitrogen (1 l./min.); 0-450 mm-glucose supplied at 1 ml./min. At zero time glucose was added to give initial concentrations indicated by filled-in circles. The steady-state rate in μ moles of glucose/min./g. of yeast (fresh wt.) is marked on each curve.

Fig. 3. Relation between steady-state concentration of glucose and rate of utilization. Yeast K2. O, Data from Fig. 2. —, Plot of $c = \frac{0.25Q}{40.0 - Q} + \frac{Q}{13.0}$ (see the Discussion section).

check that deterioration during storage had not significantly affected the results of Fig. 2, a modified experimental technique was adopted. Yeast concentration was increased to 2% (w/v) to shorten the pre-steady-state period. At intervals of 50 min. the pump rate was altered, so that in an experimental period of 200 min. values of the steady-state concentration were obtained corresponding to four values of the rate Q . Pump rates were 0.5, 1, 1.5 and 2 ml./min. in that order (ascending step method), or in the reverse order (descending step method). Within each 50 min. period, samples were withdrawn for analysis at 10 min. intervals. Results are given in Table 1.

In the ascending step method, a constant extracellular glucose concentration was not observed at the highest rate studied (Q 20 μ moles of glucose/min./g.), owing to the instability of the fermentation system (see below). This difficulty was not encountered with the descending step method. However, in this procedure mannann was liberated from the yeast cells in the later stages of the fermentation, and interfered with determinations made by the anthrone technique. Determinations of reducing sugar were not affected.

With these reservations, the results of Table 1

show that the steady-state concentration was the same whether approached from higher or from lower values.

Instability of the fermentation system. Fig. 2 shows that as the rate of metabolism was increased, a steady state was maintained for progressively shorter periods of time. The subsequent alteration in the characteristics of the fermentation, which caused the extracellular concentration of glucose to increase, may have been due to the accumulation of ethanol or other toxic products, or to changes in the yeast cell itself resulting from biosynthetic activity. Because of this, the method is more suitable for the study of low rates of fermentation, say up to half the maximum rate.

Fermentation of fructose and of mannose. This was studied by the step procedure (cf. Table 1). The value of k in the relation $Q = kc$, which applied at low rates of fermentation, was found to be 1.58/min./g. of yeast for mannose, 3.35 for fructose, and 10.2 for glucose.

Fermentation of a mixture of glucose and fructose. In the single case examined (Fig. 4), no interference of either sugar with the utilization of the other could be detected. In the steady state, the mean extracellular concentrations were 0.52 mm-glucose and

Table 1. *Step procedure for investigating the relationship between rate of uptake and extracellular concentration of glucose*

Nitrogen atmosphere, 2% (w/v) washed baker's yeast K4 in succinate buffer, pH 5.3. Ascending method: 200 mm-glucose supplied at 0.5 ml./min. from 0 to 51 min., at 1 ml./min. from 51 to 101 min., at 1.5 ml./min. from 101 to 151 min., and at 2 ml./min. from 151 to 200 min. Descending method: pump rates in reverse order to ascending method. *A*, Glucose estimated by anthrone procedure. *R*, Glucose estimated by reducing-sugar method.

Rate of glucose uptake in steady state (μ moles/min./g. of yeast)	Ascending method			Descending method		
	Time (min.)	Concn. of glucose (mm)		Time (min.)	Concn. of glucose (mm)	
		<i>A</i>	<i>R</i>		<i>A</i>	<i>R</i>
4.9	20	0.56		170	0.61	0.48
	30	0.49	0.53	180	0.61	0.48
	40	0.48	0.50	190	0.62	0.49
	50	0.48	0.49	200	0.62	0.48
	70	0.96		120	1.04	
9.9	80	0.96	0.97	130	1.01	
	90	0.95	0.96	140	1.05	0.91
	100	0.94	0.95	150	1.02	0.90
	120	1.60		70	1.72	
15.0	130	1.59	1.60	80	1.68	
	140	1.59	1.59	90	1.65	1.63
	150	1.60	1.61	100	1.64	1.63
	170	2.14		20	2.37	
20.0	180	2.26	2.29	30	2.33	
	190	2.36	2.37	40	2.34	2.37
	200	2.41	2.40	50	2.30	2.31

Fig. 4. Extracellular concentrations of sugar during fermentation of a mixture of glucose and fructose. Yeast K4, 2% (w/v), in succinate buffer, pH 5.3. Solution (100 mm-glucose+100 mm-fructose) was supplied at 1 ml./min. ●, Concentration of fructose; ○, concentration of glucose+fructose. Feed was stopped at 100 min.

Fig. 5. Extracellular concentrations of maltose during fermentation by commercial yeast. Yeast K5, 2% (w/v), in succinate buffer, pH 5.3. Pump rate 1 ml./min. ○, 25 mm-maltose supplied. ●, 50 mm-maltose supplied. Figures attached to the curves show the steady-state rate of fermentation (μ moles of maltose/min./g. of yeast).

1.43 mm-fructose, as compared with 0.49 mm-glucose and 1.47 mm-fructose calculated from the velocity constants given in the last paragraph.

Fermentation of sucrose. The invertase activity of the yeast was so high that the same results were obtained as when a mixture of glucose and fructose was supplied.

Fermentation of maltose. The only extracellular sugar detected during fermentation was maltose itself (cf. Suomalainen, Axelson & Oura, 1956). Its extracellular concentration changed in a complicated way as the fermentation progressed (Fig. 5). Evidently some adaptive process occurred, related either to the synthesis or activation of the enzyme maltase itself, or to a transport mechanism. The ratio rate/concentration increased about fivefold in the first 90 min.

Maltose fermentation was further investigated with 2% (w/v) suspensions of yeast which had been grown by a continuous process on synthetic media with either maltose or glucose as carbohydrate source. When yeast had been propagated on a medium containing 150 mm-glucose, a steady-state concentration of maltose of 1.77 mm was observed between 30 and 120 min. when maltose was fermented at 2.36μ moles/min./g. of yeast. With other samples of yeast, a true steady state was not established, the extracellular concentration slowly

increasing with time. When glucose was fermented, the reverse situation was found. With the yeast grown on excess of glucose a steady state was not set up until 90 min., whereas with other samples the extracellular concentration reached a constant value after 20–30 min. The addition of 0.1 mm-sodium azide (with the intention of inhibiting biosynthetic processes) was without observable effect.

The rate of increase of extracellular sugar concentration in those fermentations where a steady state was not established (during the period of observation) was small, about 1%/min., taking the 30 min. value as basis. Calculations were based on the 30 min. concentration, the rate of sugar uptake being found from equation (2). Results for the fermentation of maltose by maltose-grown yeast are given in Table 2. With other samples of yeast fermentation was investigated at only one (low) rate (Table 3).

For comparison, the rate of hydrolysis of maltose by suspensions of broken cells was expressed in terms of a maximum rate and a Michaelis constant, as found by plotting the reciprocal of rate against the reciprocal of concentrations (Table 3).

Glucose uptake by aerated suspensions of yeast. The relation between rate of glucose uptake and extracellular concentration was investigated over a

limited range with an aerated suspension of commercial yeast (Fig. 6). The concentration-time curves were of complex shape. The hump in them may have been connected with cell division, which was observed microscopically to take place at about the same time.

When the rate was varied between 0 and $20 \mu\text{moles/min./g.}$ of yeast, the steady-state concentration established between 20 and 50 min. varied approximately linearly with rate. The mean value of Q/c_s was 10.4, nearly the same as found for anaerobic fermentation in the lower range. From a comparison of Fig. 2 and Fig. 6, it is evident that

aeration lowered the maximum rate of glucose uptake the yeast could sustain.

In other experiments the rate at which glucose was supplied was set at a value which resulted in distinctly different extracellular concentrations of glucose in aerated and non-aerated suspensions, and the effect studied of a change in the gas phase during the course of metabolism. Response was almost immediate (Fig. 7).

Table 2. Relation between rate of uptake and extracellular concentration of maltose during anaerobic fermentation by maltose-grown yeast

Yeast, 2% (w/v), MG2 in succinate buffer, pH 5.3. Ascending step method (see protocol of Table 1). Maltose concentration given is that established after 30 min. at each rate.

Rate of maltose uptake ($\mu\text{moles/min./g.}$ of yeast)	Extracellular concn. (mm)
2.13	0.201
4.53	0.458
6.99	0.826
9.05	1.24
10.47	2.12

Fig. 6. Extracellular concentrations of glucose in an aerobic system. Yeast K3. Air, 11./min. Other details were as given in Fig. 2.

Table 3. Comparison between kinetics of maltose fermentation at low rates and kinetics of hydrolysis of maltose by suspensions of broken yeast cells

Fermentation anaerobic, 2% (w/v) yeast in succinate buffer, pH 5.3. Azide, when added, was 0.1 mm-Na₃N. Maltose hydrolysis, in 25 mm-phosphate, pH 6.9. Q , Rate of uptake in fermentation as $\mu\text{moles/min./g.}$ c , Extracellular concentration during fermentation (mm) after 30 min. or in steady-state, denoted by (s). V , Rate of hydrolysis of maltose as $\mu\text{moles/min./g.}$ K_m , Michaelis constant (mm).

Yeast	Fermentation				Hydrolysis of maltose by broken cells		
	Glucose		Maltose		$V_{\text{max.}}$	K_m	V/c
	c	Q/c	c	Q/c			
Commercial K5			0.64(s)	3.9			
Commercial K6					61	21	2.5
Maltose-grown							
MG1	1.30(s)	7.6	0.32	14.9			
MG2			0.46	9.9	154	15	10.0
MG2+azide			0.37	12.6			
Glucose-grown							
GG1*+azide	0.62(s)	14.7	0.79	5.8	77	22	3.4
GG2†+azide	1.05(s)‡	9.6	1.77(s)	1.33	21	14	1.29

* Rate of uptake from 25 mm-maltose $20.3 \mu\text{moles/min./g.}$

† Rate of uptake from 25 mm-maltose $6.4 \mu\text{moles/min./g.}$, and from 50 mm-glucose $34.7 \mu\text{moles/min./g.}$

‡ Steady state attained only after 90 min.

Fig. 7. Effect of change of gas phase on the extracellular concentration of glucose. Yeast, 1% (w/v), in succinate-KCl-MgSO₄ medium. Glucose was supplied at 19.7 μ moles/min./g. of yeast. Curve A: ●, gas phase 0.4l. of nitrogen/min.; ○, 4.0l. of air/min.; yeast K1. Curve B: ●, gas phase 2l. of nitrogen/min.; ○, 2l. of air/min.; yeast K2.

DISCUSSION

Many investigators, of whom Dawson (1932) was the first, have summarized their findings on the relation between the rate of fermentation by baker's yeast and the sugar concentration by fitting their data to a Michaelis-Menten equation. In the temperature range 10–35°, I found that K_m 2.9 $\pm$ 0.3 mm applied to the rate of production of carbon dioxide from glucose (unpublished work).

It is one of the merits of the continuous-fermentation technique that it shows clearly that the situation is much more complex than this. Although data obtained with rates of glucose utilization between 10 and 35 μ moles/min./g. of yeast (Fig. 3) were described accurately by the equation

$$Q/c = 15.01 - 0.2023 Q$$

(a transformation of the Michaelis equation), this did not fit results at 5.0 and 8.3 μ moles/min./g. Moreover, the maximum rate predicted by the equation (74.2 μ moles/min./g.) was impossibly high. Owing to the instability of the fermentation system, it was difficult to assign a value for the actual maxi-

mum rate to the data of Fig. 2 and Fig. 3, but calculations on the non-steady-state upper curve of Fig. 2 suggested a figure of 40.0 μ moles/min./g. of yeast.

It is a reasonable assumption that maximum rate of glucose utilization was reached when the concentration of glucose at the site of hexokinase, c' , was sufficient to saturate the enzyme. The combination of glucose with hexokinase *in vitro* appears to have a Michaelis constant of about 0.25 mm (see e.g. Trayser & Colowick, 1961). One would therefore expect 90% of the maximum rate with an internal glucose concentration of 2.5 mm. This rate (Fig. 2) corresponded to an external concentration of 4–5 mm-glucose, the higher figure providing evidence for the existence of some sort of diffusion barrier.

Values of c' were calculated assuming a maximum rate of 40.0 μ moles/min./g. of yeast and K_m 0.25 mm. It was then found that the rate Q was approximately proportional to the concentration gradient ($c - c'$), the mean value of $Q/(c - c')$ being 13.0 ± 1.1 . In other words, the experimental data fitted an equation of the type

$$c = \frac{K_m Q}{Q_{\max} - Q} + \frac{Q}{k}$$

which is a transformation of one suggested by Best (1955).

The kinetics of the hypothetical transport mechanism are the same as those applying to simple diffusion, but this does not preclude a linkage with metabolism. Such a linkage could be indirect, as well as direct (active transport). For example, as yeast is surrounded by an elastic cell wall, volume changes may accompany the exchange of metabolites between the cell and the surrounding medium, leading to stretching or contraction of the cell membrane which may influence its resistance to diffusion.

In aerobic fermentation, the diffusion mechanism appears to be relatively unaffected by oxygen, which, however, limits the maximum rate of glucose utilization.

Maltose fermentation differs from glucose fermentation in that the first known enzyme in the fermentation sequence, maltase, has a relatively high Michaelis constant, 15–20 mm (Table 3). The data on maltose fermentation by maltose-grown yeast (Table 2) at rates below 12 μ moles/min./g. of yeast fitted a Michaelis equation with K_m 12 mm. The maximum rate found by extrapolation was 222 μ moles/min./g. These values were of the same order as those found for the hydrolysis of maltose by the enzyme in suspensions of broken yeast cells (Table 3). With other samples of yeast, the ratio Q/c was roughly what was predicted from the amount and characteristics of the enzyme.

In these cases the diffusion barrier was not rate-

limiting, although it may be in other circumstances (for discussion see Cook, 1963). Possibly the formation of a maltose permease system may involve the transport of the α -glucosidase to a location in the cell membrane.

The kinetics of fermentation may depend on the genetic constitution of the yeast strain, and on the way the organism has been propagated. The extent to which the mechanisms discussed here are generally applicable is therefore an open question.

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