Some Factors Which Affect Amino Acid Uptake by
Saccharomyces carlsbergensis

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When fully grown cells of Saccharomyces carlsbergensis were suspended in a solution of glucose and labeled amino acids, there was a lag phase before rapid uptake of certain amino acids. During this lag, significant amounts of sugar were utilized. The lag phase varied in length, depending upon the amino acid under study, but could be shortened by aeration of the cells and eliminated by their preincubation in glucose solution. Divalent metal ions, especially Ca2+ added during the early stages of the lag phase, increased the length of the lag, an effect that could be reversed by washing with ethylenediaminetetraacetate, but amino acids which normally showed little or no lag before uptake were insensitive to Ca2+. The rate of uptake of amino acids or of sugar was essentially unaffected by Ca2+, whereas 2,4-dinitrophenol caused an overall decrease in the rate of uptake of all amino acids tested. The relevance of these observations to commercial brewing practice is shown.

Most work on the uptake of amino acids by microorganisms (e.g., 6, 9, 11, 17, 27, 28) has been conducted with growing cells or with cells harvested in the exponential phase of growth. Little information is available concerning amino acid uptake by yeast in the stationary phase. Such cells are used as inocula by the fermented beverage industries, and slow uptake of many amino acids is a characteristic of the early fermentation stage. The vigor of yeast performance in the brewery and the genesis of satisfactory flavor in the product have frequently been related to the efficiency of amino acid utilization (10, 12, 15, 16, 19, 23). This paper describes some factors which affect amino acid uptake by a brewers' yeast in the presence of sugar: (i) the previous history of the cell, especially age and aeration; (ii) the amino acid under study; (iii) the presence of divalent ions especially calcium; and (iv) the temperature and the sugar supplied.

Lewis and Stephanopoulos (18) have reported a lag phase before the uptake of some amino acids by mature yeast. Shieh and Hedrick (26) have shown a lag in L-glutamate uptake by Hansenula subpelliculosa and Candida utilis requires a similar adaptive lag before the uptake of uric acid (24, 25). An effect of Ca2+ on the lag phase has not been reported and is examined here.

MATERIALS AND METHODS

Test organism. A flocculent commercial brewers' strain of Saccharomyces carlsbergensis (UCD 67-80) was used. It was grown in the following medium (per cent, w/v): glucose, 5; peptone (Difco), 2; yeast autolysate (Albimi Laboratories, Inc., Flushing, N.Y.), 0.5; KH2PO4, 0.05; MgSO4, 0.03; adjusted to pH 5.0 with phosphoric acid. The yeast was grown in a 250-ml Erlenmeyer flask containing 100 ml of this medium, transferred after 2 days into 1,400 ml of the same medium, and grown for 3 days or the time cited at 23 to 25 C with occasional stirring. The cells were harvested by centrifugation and washed three times with glass-distilled water.

Measurement of uptake. Uptake experiments were performed at 30 C in the presence of 5% (w/v) glucose (unless otherwise stated) with 10 mg (dry weight) of yeast per ml. After inoculation and before each sample was taken, the cell suspension was mixed with a stream of nitrogen gas.

Uniformly labeled 14C-L-amino acids, obtained from International Chemical and Nuclear Corp., City of Industry, Calif., were used. Approximately 0.01 μCi of tracer per ml was used in each experiment to give 20,000 counts per min per ml. Each sample of the test suspensions, taken at suitable time intervals, was centrifuged, and radioactivity was measured in a Nuclear Chicago Unilux II liquid scintillation counter. For measurement, 1.0 ml of the sample was mixed with 9.0 ml of scintillation liquid composed of 7.0 g of 2,5-diphenyloxazole per liter, 0.3 g of scintillation grade 1,4-bis-(5 phenoxazoyl)-benzene per liter (both from Packard Instrument Co., La Grange, Ill.), and 100 g of naphthalene per liter in reagent.

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grade dioxane. The tracer amino acid is reported directly in counts per minute per milliliter. The addition of unlabeled \( \mathrm{L} \)-amino acid to the system did not alter the pattern of results reported here but, depending on the amount of unlabeled material added, served to extend the length of time necessary to achieve a given uptake of radioactivity.

**Chemicals.** Calcium, magnesium, and zinc were added as chloride salts, analytical reagent grade, obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

**RESULTS**

**Uptake of amino acids.** When washed yeast cells, harvested from the stationary phase of growth, were suspended in a medium containing glucose and \(^{14} \mathrm{C}\)-amino acid, net uptake patterns could be established for the amino acids (shown representatively in Fig. 1). Alanine, glycine, tyrosine, valine, and glutamic acid showed the longest period of initial lag (up to 60 min). Leucine and phenylalanine showed a shorter lag (about 15 to 25 min), whereas arginine, lysine, and serine showed little or no lag before uptake. These differences could not be detected in young (24 hr) yeast cells (Fig. 2), and there was no effect of \( \mathrm{Ca}^{2+} \) on uptake. With increasing age of the cells, the lag phase in alanine uptake, for example, became quite pronounced, whereas lysine uptake was unaffected by cell age.

**Effect of sugar source.** Uptake of glucose, followed either by \(^{14} \mathrm{C}\)-glucose or by the colorimetric method of Somogyi-Nelson (29), was linear in all cases when an excess of sugar was present. Glucose uptake was reproducibly about 0.7 mg per hr per mg (dry weight) of yeast, showed no lag, and was unaffected by metal ions.

The extent of lysine uptake responded more or less linearly to the amount of glucose available (Fig. 3), but alanine was not taken up until more than 0.6% sugar had been provided. When \( \mathrm{Ca}^{2+} \) (10\(^{-3}\) M) was also present (not shown), sugar in excess of 1% (w/v) was necessary to support the uptake.

Glucose, fructose, and sucrose supported similar uptake rates of the amino acids, with similar lengths of lag phase (Table 1). Maltose, the major fermentable sugar of brewers' wort, supported slower uptake and a prolonged lag phase, a condition that was ameliorated by adaptation of the cells to maltose fermentation. Galactose, even with adaptation, supported slower uptake than the other sugars, whereas lactose and sorbose were not utilized by this yeast.

**Effect of preexposure to glucose.** When a limiting amount of glucose was exhausted, uptake of alanine or lysine ceased (Fig. 4).

Addition of glucose (after 90 min) reinitiated the uptake of the amino acid (alanine) with a greatly reduced lag phase. However, if the cells were starved for an extended period of time before the second addition of glucose (after 6 hr), the lag phase in alanine uptake reappeared. Labeled alanine added to cells preincubated for 90 min in an excess of glucose was taken up without a lag. If these cells were washed before amino acid addition, they did not take up any amino acid (e.g., lysine) until sugar had been supplied.

**Effect of temperature.** S. carlsbergensis strain 67-80 has a maximum growth temperature of about 34°C and grows optimally on a complex medium in the range 24 to 30°C. Within this optimum range, the effect of temperature on amino acids uptake was small (Table 2). At temperatures below the optimum range, the rate of uptake was slower and the length of the lag phase increased. At low temperatures, the uptake lag was sensitive to low levels (10\(^{-6}\) M) of \( \mathrm{Ca}^{2+} \).

**Effect of aeration.** Incubation of the cells with glucose in the oxygen or nitrogen atmosphere did not affect the rates of amino acid uptake. However, a pretreatment (30 min) of the yeast with oxygen shortened the lag phase although it
had little effect on rate of uptake (Table 3). More prolonged yeast oxygenation (6 hr) promoted amino acid uptake in the absence of exogenous sugar.

**Effect of metal ions.** Zinc or magnesium ions increased the length of the lag phase (Table 4), but, when Zn\(^{2+}\) and Mg\(^{2+}\) were supplied, no further increase in the length of the lag was detected. Calcium ions were significantly more effective than either Zn\(^{2+}\) or Mg\(^{2+}\). Calcium and zinc ions, or calcium and magnesium ions, caused a further extension of the lag phase. In each treatment, except Ca\(^{2+}\) and Mg\(^{2+}\), the ion(s) had its main effect on the length of the lag phase and a relatively minor effect on the subsequent uptake rate.

If an amino acid was taken up without a lag, or if the lag phase was eliminated by preincubation with glucose, the uptake was insensitive to these metal ions (e.g., lysine; Fig. 5). Alanine uptake, which had a considerable adaptive lag time, was sensitive to Ca\(^{2+}\). Leucine values were between these extremes in duration of lag phase and in Ca\(^{2+}\)-sensitivity.

Maximum extension of the lag phase was achieved when Ca\(^{2+}\) was present at the initiation of the experiment (Table 5), and the effect on the lag phase was lost if addition of Ca\(^{2+}\) was delayed for 30 min or longer after the initiation of the experiment. When the lag phase was completed, the subsequent rate of alanine uptake was little affected by the presence of Ca\(^{2+}\).

The length of the lag phase in alanine uptake, for example, was dependent on the concentration of Ca\(^{2+}\) present (Table 6). Increasing concentration of Ca\(^{2+}\) caused an increase in the duration of the lag phase, although there was a slowing of the rate of alanine uptake with the highest concentration of Ca\(^{2+}\) tested.

**Effect of preexposure to calcium.** Yeast was preexposed to glucose solution (5%, w/v) or to glucose solution (5%, w/v) containing calcium ions (10\(^{-2}\) M). After 1 hr, the cells were washed...
and glucose and $^{14}$C-alanine were added. The cells preexposed to glucose and Ca$^{2+}$ showed a longer lag phase than those which had been preexposed only to glucose (Table 7). If cells preexposed to glucose (0.1%, w/v) or to glucose (0.1%, w/v) with Ca$^{2+}$ ($10^{-2}$ M) were treated, similarly, relatively little of the lag phase was eliminated. However, when these cells were washed in water and then in ethylenediaminetetraacetic acid (EDTA) solution, the effect of calcium was essentially reversed.

**Effect of metabolic inhibitors.** 2,4-Dinitrophenol (10$^{-5}$ to 10$^{-4}$ M) caused an overall slowing of amino acid uptake (Fig 6) but did not have a marked effect on the length of the lag phase. The effects of 2,4-dinitrophenol and Ca$^{2+}$ in this system are evidently dissimilar (Fig. 5 and 6).

**DISCUSSION**

In anaerobically grown brewers' yeast cells, there are some sites for amino acid transport (e.g., those for lysine) that are stable and are unaffected by the age of the cell. The energy derived from the sugar is immediately applied to the transport of these amino acids even in mature cells. However, there was no evidence of accumulation of an active metabolite utilizable in transport (24–26), as an exogenous source of energy was always necessary for uptake (Fig. 4) by anaerobic cells. Other sites (e.g., for the transport of alanine) are unstable and degenerate as the cell approaches the stationary phase of growth. These sites can be regenerated in the presence of a source of energy, during which phase little uptake is evident, and then can be eliminated by starvation (Fig. 4). Since this regeneration takes place without a specific inducer, i.e., an amino acid, it may be a normal part of maintenance and repair necessary in stationary-phase cells to reinitiate active growth.

The effect of temperature on the rate of amino acid uptake and on the length of the lag phase in uptake is probably directly related to the rate of generation of energy from sugar (9). This is broadly supported by the data for sugar utilization (Table 2), by the effect of 2,4-dinitrophenol (Fig. 6), and by the minor effect of temperature in the optimum temperature range for growth. The effect of yeast oxygenation on amino acid uptake (Table 3) may also be related to the efficiency of energy generation, possibly through the oxidation of reduced cofactors in the anaero-

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**TABLE 2. Effect of temperature on amino acid uptake by mature yeast cells**

| Temp. (°C) | Glucose uptake (mg per hr per mg of yeast) | Rate of uptake of $^{14}$C-lysine (counts/min) | Length of lag phase in $^{14}$C-alanine uptake (min)$^a$ | Sugar utilized to end of lag (mg/mg of yeast) |
|------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
|            |                                               |                                               | No Ca$^{2+}$                                  | With Ca$^{2+}$ ($10^{-6}$ M)                  |
| 30         | 0.72                                          | 170                                           | 55                                            | 0.66                                          |
| 28         | 0.70                                          | 170                                           | 60                                            | 0.70                                          |
| 25         | 0.70                                          | 175                                           | 60                                            | 0.70                                          |
| 21         | 0.58                                          | 140                                           | 80                                            | 0.77                                          |
| 17         | 0.45                                          | 110                                           | 110                                           | 0.82                                          |
| 14         | 0.33                                          | 80                                            | 130                                           | 0.72                                          |
| 10         | 0.22                                          | 50                                            | 180                                           | 0.66                                          |

$^a$ Time required to take up 5% of the $^{14}$C-alanine.
TABLE 3. Effect of aeration on amino acid uptake by mature yeast cells

| Treatment                     | Rate of 14C-lysine uptake (counts/min) | Length of lag in 14C-alanine uptake (min) |
|-------------------------------|----------------------------------------|------------------------------------------|
|                               | Glucose (5%) present | Glucose (5%) absent | Glucose (5%) present | Glucose (5%) absent |
| Yeast sparged (30 min) with   |                           |                           |                           |                           |
| Air                           | 170                      | 0                        | 40                        | >300                      |
| Oxygen                        | 170                      | 0                        | 40                        | >300                      |
| Nitrogen                      | 165                      | 0                        | 65                        | >300                      |
| Yeast sparged (6 hr) with     |                           |                           |                           |                           |
| Air                           | 175                      | 120                      | 2                         | 2                         |
| Oxygen                        | 175                      | 125                      | 2                         | 2                         |
| Nitrogen                      | 170                      | 0                        | 55                        | >300                      |

* Time required to take up 5% of the 14C-alanine.

TABLE 4. Effect of some divalent metal ions on the length of the lag phase before 14C-alanine uptake by mature yeast

| Metal ion                   | Length of lag phase (min) |
|-----------------------------|---------------------------|
| None                        | 60                        |
| Ca2+ (10^-2 M)              | 150                       |
| Mg2+ (10^-2 M)              | 105                       |
| Zn2+ (10^-2 M)              | 105                       |
| Zn2+ and Mg2+ (each 10^-4 M)| 165                       |
| Ca2+ (10^-4 M) and Zn2+ (10^-2 M) | 195b                      |

* Time required to take up 5% of the 14C-alanine.

TABLE 5. Effect of time of addition of Ca2+ on the length of the lag before uptake of 14C-alanine by mature yeast

| Time of addition of Ca2+ (10^-2 M) after initiation of the expt (min) | Length of lag phase (min) |
|------------------------------------------------------------------------|---------------------------|
| 0                                                                      | 195                       |
| 5                                                                      | 150                       |
| 10                                                                     | 120                       |
| 15                                                                     | 95                        |
| 20                                                                     | 75                        |
| 30 or no Ca2+                                                          | 60                        |

* Time required to take up 5% of the 14C-alanine.

TABLE 6. Effect of calcium ion concentration on the length of the lag phase before uptake of 14C-alanine by mature yeast

| Ca2+ concn (m) | Length of lag phase (min) |
|----------------|---------------------------|
| 10^-6          | 60                        |
| 10^-4          | 80                        |
| 10^-2          | 120                       |
| 10^-1          | 150                       |
| 10^-1          | 200                       |
| 10^-1          | 300                       |

* Time required to take up 5% of the 14C-alanine.

† Subsequent rate of uptake was appreciably slowed in this case.

TABLE 7. Effect of preexposure on the length of the lag phase before uptake of alanine by mature yeast

| Preexposure (60 min) to | Length of lag phase (min) |
|------------------------|---------------------------|
| Water                  | 55                        |
| Water + Ca2+ (10^-3 M) | 60                        |
| Glucose (5%, w/v)      | 10                        |
| Glucose (5%, w/v) + Ca2+ (10^-3 M) | 50                  |
| Glucose (0.1%, w/v)    | 50                        |
| Glucose (0.1%, w/v) + Ca2+ (10^-2 M) | 150               |
| Glucose (0.1%, w/v), EDTA wash | 50                  |
| Glucose (0.1%, w/v) + Ca2+ (10^-2 M), EDTA wash | 60                 |

* Time to take up 5% of the 14C-alanine, measured after completion of preexposure, washing with water or saline or with water-EDTA (10^-4 M) water, and supplying the cells with glucose and 14C-alanine.

Fig. 5. Effect of calcium ions on the uptake of labeled lysine, leucine, or alanine by mature yeast. Symbols: O, no calcium added; ■, Ca2+ (10^-3 M) added; ●, Ca2+ (10^-3 M) added;

bically grown cells, or, more clearly in the case of the yeast oxygenated for 6 hr, through the mobilization of glycogen reserves (5).

The lag phase preceding the uptake of certain amino acids was markedly lengthened by Ca2+.
The mechanism of action of Ca\(^{2+}\) is not clear. Since Ca\(^{2+}\) behaved differently from 2,4-dinitrophenol (Fig. 5, 6) which interfered with the application of energy at the membrane where transport is to occur (22), it seems unlikely that Ca\(^{2+}\) acts primarily through the energy requirements of transport. Given the well-established association of divalent metal ions with membrane structure (1, 2, 4) and function (3, 8, 13, 20), an effect of Ca\(^{2+}\) at specific sites in the cytoplasmic membrane seems possible. The ready reversal of the effect of Ca\(^{2+}\) by washing with EDTA (Table 7) which can remove ions only from the surface region of the intact cell, including the cytoplasmic membrane (7, 13, 14), indicates that the effective calcium ions are bound in the cell surface; this association was too strong to be reversed by washing with water or saline, although such washing can remove Ca\(^{2+}\) bound to the cell wall (13). Moreover, Ca\(^{2+}\) that was effective in extending the lag phase was bound in the presence but not in the absence of glucose, which suggests a specific or directed binding. This is also supported by the distinctly greater effectiveness of Ca\(^{2+}\) than Mg\(^{2+}\) (Table 4) when the physical properties of the two ions would indicate almost identical effects.

Although some amino acids (e.g., lysine) show little uptake lag in a yeast (brewery) fermentation, most amino acids are taken up after a lag of about 20 hr. Our work shows that this long lag is a result of commercial conditions of yeast handling and fermentation: the use of starved (washed and stored) stationary-phase cells with a fermentation medium (wort) that is cold (8 to 12°C), relatively low in oxygen, and which contains Ca\(^{2+}\) (about 10\(^{-3}\) M; reference 21).

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