Energy Requirement for L-Glutamate Uptake and Utilization by *Hansenula subpelliculosa* Cells

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ABSTRACT

SHIEH, K. Z. (Illinois Institute of Technology, Chicago), AND L. R. HEDRICK. Energy requirement for L-glutamate uptake and utilization by *Hansenula subpelliculosa* cells. J. Bacteriol. 92:1638-1644, 1966.—Cells of the yeast *Hansenula subpelliculosa* require an energy source for the uptake of glutamate. A lag period of 20 to 40 min was required after the addition of glucose to the cells before glutamate uptake was initiated. When cells were preincubated in glucose, and washed with distilled water prior to the addition of glutamate, there was no lag period. Preincubation in glucose and glutamate lowered both the rate and the total uptake of glutamate as compared with cells preincubated in glucose alone. This is attributed to the partial utilization of the glucose-metabolite by glutamate or to the partial saturation of binding sites by glutamate during the preincubation period. Transport of glutamate by these yeast cells appears to be via a carrier, where energy is required for the binding of the amino acid to nonspecific binding sites. In addition to total uptake, some aspects of the C^{14} -glutamate utilization were measured. Of the total uptake, 58% was metabolized and converted to CO_2 , 25.2% remained in the soluble pool, and 16.8% was incorporated into trichloroacetic acid-insoluble products. When the available energy source was depleted, the processes of uptake, metabolism, and incorporation ceased, even though there was an ample supply of glutamate present within the cells. Removal of cells from glutamate and addition of glucose reinitiated the incorporation of glutamate into proteins and other trichloroacetic acid-insoluble compounds. Therefore, an additional energy source is required with this species of yeast for glutamate uptake, for the priming of mechanisms required for its metabolism, and for its incorporation.

Cells of the yeast *Hansenula subpelliculosa* strain Y 1683 can readily use L-proline as a sole source of carbon, but they cannot use L-glutamic acid in this way. These cells may be further characterized by absence of an available, stored carbohydrate under growth conditions. This fact has been verified many times by "nitrogen control flasks" in which no growth occurred when cells were incubated with either L-glutamate or ammonium sulfate as a nitrogen source in the salts-vitamin fluid. Furthermore, respiration studies with this strain have never showed evidence of an endogenous reserve, in Warburg flasks with either ammonium sulfate or L-glutamate as substrates.

To determine the reasons for the differences in the utilization of proline and glutamate, which have a common metabolic pathway, we have investigated the uptake and utilization of L-glutamate in *H. subpelliculosa*.

Several groups of workers have reported upon the uptake of amino acids by yeasts, usually for the species *Saccharomyces cerevisiae* (7, 8, 10; A. A. Eddy, Biochem. J. 88:34P, 1963; A. Eddy and K. J. Indge, Biochem. J. 85:15P, 86:35P, 1962).

Eddy and co-workers showed that the uptake of glycine, aspartic acid, and glutamic acid was related to a stimulation of K^+ ion entrance into the cells. These ions were in some way associated with the binding of the amino acids with a proposed carrier model for amino acid transport in the yeast. Britten and McClure (2) proposed a carrier model for the transport of amino acids into *Escherichia coli*. Halpern and Lupo (5) and Vender et al. (15) reported upon the transport and metabolism of L-glutamate in *E. coli* mutants. Wild-type *E. coli* cells cannot use glutamate as a sole carbon source, but certain mutants can. Halpern and Lupo isolated mutants which had an

increased permeability for glutamate, but there was no difference in the metabolic capacity of mutant and parent. Vender et al. reported upon mutants in which the primary difference from the parent was a loss of ability to form glutamic decarboxylase, and this was associated with the utilization of glutamate as a sole source of carbon. Pichler (11) gave evidence supporting the hypothesis of "in-membrane" protein synthesis without the need for equilibration with the intracellular L-glutamate pool in Ehrlich ascites-tumor cells.

MATERIALS AND METHODS

Cells of *H. subpelliculosa* NRRL Y 1683 slow strain (8) were grown in 1 liter of defined medium for 24 hr at 28°C in 2-liter conical flasks on a rotary shaker. The cells were harvested by centrifugation; the packed cells were washed twice in 50 ml of distilled water and were used within 2 hr. For the C^{14} studies, a working suspension of 4% cells (this corresponded to 6.5 mg of dry cell weight per ml) was prepared in the defined-salts solution. The defined medium contained the salts and vitamins of Wickerham (16) plus glucose (10 g/liter) and $(NH_4)_2SO_4$ (5 g/liter). The determination of the intercellular volume was according to the method of Conway and Downey (3). This volume was found to be 19% and was considered in the measuring of the uptake of glutamate by the cells. Glucose was determined by the modified anthrone method of Ecker and Lockhart (4). Nonlabeled L-glutamate was measured by the specific decarboxylase-manometric technique of Seidman and Blish (14).

Uniformly labeled L-glutamic acid- C^{14} , 74.1 μ g containing 50.4 μ c, was added to 150 ml of defined basal salt solution containing 2.0 mg of nonlabeled L-glutamate per ml. This solution was stored in a refrigerator at 4°C until needed. Three 50-ml Erlenmeyer flasks were prepared as follows: one containing 5.0 ml of C^{14} -glutamate solution and 5.0 ml of yeast suspension; a second containing 5.0 ml of labeled glutamate solution, 5.0 ml of yeast suspension, and 10 mg of glucose; and a third containing 5.0 ml of C^{14} -glutamate and 5.0 ml of cells which had been preincubated for 2 hr with 1% glucose and washed twice in distilled water. At specific time intervals, 0.20 ml of a particular flask content was filtered through membrane filters (diameter, 25 mm; pore size, 0.45 μ). The filters were washed twice with 1-ml portions of cold distilled water and were air-dried. Other 0.20-ml portions were transferred to small tubes containing an equal volume of 10% trichloroacetic acid. After 30 min at room temperature, the samples were filtered through membrane filters as described above. The radioactivity in the cells and the trichloroacetic acid-precipitable fraction, as collected on the filters, was measured by liquid scintillation counting with a Beckman spectrometer. The C^{14} counting efficiency was 82%.

For some experiments, cells were removed from the incubation mixture after glutamate incorporation had ceased; these cells were washed twice in cold distilled water and were added to 0.0125 M phosphate

buffer (pH 6.0) containing 1% glucose to reinitiate the incorporation of glutamate carbon.

The relative specific activity is the ratio of the specific activity of the cell-glutamate suspension at a given time to the specific activity at time zero. The relative change in specific activity corresponds with the utilization of glutamate and the release of CO_2 (1). Carbon dioxide production from C^{14} -glutamate was also determined by trapping the CO_2 with 0.4 ml of hyamine hydroxide (10X) in the center well of a Warburg flask, according to the method of Rosenberg et al. (13). This hyamine solution was added to the scintillation mixture and counted. The values obtained by the two methods were in close agreement.

Incubation and preincubation. For incubation with glutamate, 3 g (wet weight) of packed cells was mixed with 100 ml of 0.0125 M phosphate buffer (pH 6.0) containing 0.1% L-glutamic acid; glucose, if added, was usually at a concentration of 1%. The yeast suspensions were shaken on a rotary shaker at 28°C for different time intervals. Samples of 3 ml were withdrawn and placed in a centrifuge tube for removal of the cells, which were then washed twice with distilled water. Cells which were on the shaker for 2 hr accumulated 1.5 mg of soluble carbohydrates per 10 mg of dry cell weight; this corresponds to about 0.75 mg/ml of 3% cell suspension. These were the conditions for most of the experiments with nonlabeled glutamate; for labeled glutamate, a 2% suspension of cells was used. Glucose-preincubated cells were those shaken with 1% glucose for 2 hr; glucose-glutamate-preincubated cells were those which were incubated with 1% glucose and 0.1% glutamate for 2 hr.

Sources of materials. L-Glutamic acid- C^{14} was secured from Volk Radiochemical Co., Chicago, Ill.; hyamine hydroxide was purchased from Packard Instrument Co., La Grange, Ill.; the specific glutamate decarboxylase and L-glutamic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The yeast culture was from L. J. Wickerham, Northern Regional Research Laboratory, Peoria, Ill.

RESULTS

An inducible lag period of 20 to 40 min was required for the uptake of L-glutamic acid with glucose as an energy source; in the absence of an energy source, essentially no glutamate was taken up. The slight initial decrease in the external glutamate concentration in the absence of glucose may be accounted for by dilution with 0.6 ml of intercellular fluid when 3 g of packed cells was added to the mixture. There was no evidence of diffusion of glutamate into the cells. If cells were preincubated in glucose prior to adding the glutamate, the uptake of amino acid from the medium proceeded at once without addition of glucose to the medium (Fig. 1). Glutamate uptake was measured for cells preincubated in glucose alone or in glucose-glutamate to determine whether the glucose-glutamate incubation would eliminate the lag pe-

FIG. 1. Uptake of L-glutamic acid by cells of *Hansenula subpelliculosa*. Cells (3%, wet weight) were suspended in 0.0125 M phosphate buffer (pH 6.0) containing 0.1% L-glutamic acid. Other ingredients were added as indicated. Incubation temperature was 28 C. Vertical axis expresses glutamate concentration remaining in suspending medium. Symbols: ▲, glucose 1% plus 0.001 M sodium azide or 0.001 M 2,4-dinitrophenol; ○, cells without addition of glucose to glutamate-phosphate medium; ■, cells to which glucose was added in 1% concentration at time zero; ●, cells preincubated in 1% glucose for 2 hr, washed in distilled water twice, and then added to the glutamate-phosphate medium without additional glucose.

riod or reduce its length. As indicated in Fig. 2, the rate of uptake of glutamate for cells preincubated in glucose-glutamate was slower than for cells preincubated only in glucose. Furthermore, the removal of glutamate from the medium by cells with prior exposure to the amino acid ceased after only 30% of the glutamic acid had been taken up, whereas cells with no prior exposure to the acid in the incubation period removed about 90% of the amino acid from the medium. The kinetics of uptake for cells not preincubated in glucose gave a ratio of 2 μ moles of glucose required for the uptake of 1 μ mole of glutamate (Fig. 3 and 4). The addition of energy-uncoupling agents such as 0.001 M sodium azide or 2,4-dinitrophenol completely inhibited the uptake of glutamate (Fig. 1).

Results of kinetic studies with radioactive L-glutamic acid paralleled those obtained with the unlabeled amino acid. With labeled glutamate, in addition to uptake, the quantities of glutamate

carbon utilized for energy and incorporated into trichloroacetic acid-insoluble protein and of other macromolecules were also determined. Such results for nonpreincubated cells are given in Fig. 5; those for glucose-preincubated cells are presented in Fig. 6. In the former series, a 20-min lag was required prior to the initiation of uptake; 40 min was required prior to the initiation of a change in the relative specific activity, and 60 min was required prior to rapid incorporation. In the glucose-preincubated cells, there was no lag for any of these three parameters. After 120 to 140 min, all three activities ceased; this corresponded to the time when glucose was depleted. About 17% of the glutamate carbon taken up by the cells was incorporated into "protein," 25% was in the trichloroacetic acid-soluble pool, and 58% was utilized in energy as determined by the change in relative specific activity and release of CO_2 . This latter value was confirmed by direct recovery of CO_2 in a Warburg vessel by hyamine hydroxide.

The cessation of metabolic activity was not due to an absence of glutamate within the cells. As

FIG. 2. Uptake of L-glutamic acid by *Hansenula subpelliculosa* cells preincubated in glucose only or in glucose and L-glutamic acid. Cells (3%, wet weight) were suspended in 0.0125 M phosphate buffer (pH 6.0) containing 0.1% L-glutamic acid. Vertical axis expresses glutamate concentration remaining in suspending medium. Symbols: ■, control, cells not preincubated in glucose; ▲, cells incubated for 2 hr in 0.1% L-glutamic acid and 1% glucose, washed twice in distilled water, and suspended in glutamate-phosphate buffer medium; ●, cells preincubated in 1% glucose for 2 hr, washed twice, and suspended in glutamate-phosphate buffer medium.

indicated in Fig. 7, the glutamate concentration for twice-washed cells in the amino acid pool after growth in glucose and ammonia sulfate medium was 7.2 μ moles per 100 mg (dry weight). When these cells were incubated with 1% glucose for 3 hr in 0.0125 M phosphate buffer (pH 6.0), about 70% of the pool glutamate remained; however, when the cells were incubated with 2% glucose, only about 40% of the glutamate remained. The above information was secured by measuring the glutamate with a specific decarboxylase. Similar results were obtained by paper chromatography and autoradiography of hot-water extracts of the cells. Furthermore, in the incorporation experiments, when small amounts of glucose were added to incubating mixture with glutamate in which incorporation had ceased, there was an immediate initiation of uptake and incorporation of the glutamate- C^{14} (Fig. 5). The results in Fig. 5 and 6 were obtained while the yeast cells were in the suspending medium containing labeled glutamate. If cells were removed from the mixture after the metabolic activities had ceased, were washed twice with cold distilled water, and were added to a medium with glucose, but no glutamate,

FIG. 3. Uptake of L-glutamate by *Hansenula subpelliculosa* cells in different glucose concentrations. Cells were not preincubated in glucose and were suspended at 3% concentration (wet weight) in 0.0125 M phosphate buffer (pH 6.0) containing 0.1% glutamic acid at zero-time. Symbols for solid lines: $\square$, no glucose added to medium; $\circ$, 0.5 mg/ml of glucose in medium; $\triangle$, 1.0 mg/ml of glucose in medium; $\blacksquare$, 1.5 mg/ml of glucose in medium; $\bullet$, 2.0 mg/ml of glucose in medium. Symbols for broken lines: residual glucose concentrations for corresponding zero-time concentrations as indicated above.

FIG. 4. Uptake of L-glutamic acid by cells of *Hansenula subpelliculosa* in relation to glucose consumed. Cells in a concentration of 3% were suspended in 0.0125 M phosphate buffer (pH 6.0) containing 0.1% glutamic acid and varying glucose concentrations at time zero.

mate, incorporation of stored glutamate was initiated (Fig. 8). The above observations indicate that L-glutamate, even though present within the cells, was not metabolized unless an additional energy source was available.

DISCUSSION

Although growing cells of this strain stored no utilizable carbon as a source of energy, nongrowing cells, when exposed to rather high glucose concentrations for relatively short periods of time, accumulated soluble carbohydrates. This was the basis for the activity of glucose-preincubated cells. During the preincubation period, glucose was absorbed very quickly, but an additional 20 to 40 min lapsed before development of the required active metabolite which was consumed during the uptake of glutamate. The exact nature of this metabolite is unknown, but it may be guanosine triphosphate, adenosine triphosphate, or other energy rich compounds. Regardless of the composition of the metabolite, it is not induced by an external source of L-glutamate, since the addition of the amino acid to the preincubation mixture with

FIG. 5. Uptake, utilization, and incorporation of L-glutamic acid- C^{14} by nonpreincubated cells of *Hansenula subpelliculosa*. Cells (2%, wet weight) were suspended in 10 ml of basal salts (pH 6.0) containing 0.1% glucose and L-glutamic acid (25 μ C/mole, 6.7 μ moles/ml). Symbols: ▲, change in relative specific activity which indicates utilization of glutamate as energy and conversion to CO_2 ; ■, amount of C^{14} in the yeast cells; ●, C^{14} incorporation into protein and other cold trichloroacetic acid precipitation compounds; X, no glucose added to medium; at ↓, more glucose was added to the incubation mixture.

FIG. 6. Uptake, utilization, and incorporation of glutamate- C^{14} by preincubated cells of *Hansenula subpelliculosa*. Glucose-preincubated cells at a concentration of 2% (wet weight) (see Materials and Methods) were suspended in 10 ml of basal salts solution (pH 6.0) which contained 6.7 μ moles/ml of L-glutamate (25 μ C/mole). Symbols: ▲, relative specific activity; ■, C^{14} within the cells; ●, incorporation into protein and other cold trichloroacetic acid-insoluble compounds.

FIG. 7. Rates of decrease of L-glutamate pool of *Hansenula subpelliculosa* cells in different glucose concentrations. A 3% suspension of cells (wet weight) which had been incubated with 0.1% L-glutamic acid and 1% glucose in 0.0125 M phosphate buffer (pH 6.0) for 4 hr was then centrifuged and washed twice with distilled water and suspended in buffer with differing glucose concentrations. Symbols: ●, incubated with 1% glucose; ■, incubated with 2% glucose.

FIG. 8. Requirement of glucose for incorporation of C^{14} -glutamate after entrance into cells of *Hansenula subpelliculosa*. Cells were incubated in basal salts solution (pH 6.0) containing C^{14} -glutamate and glucose (same activity and concentration as in Fig. 5) for 240 min until the glucose was entirely depleted and incorporation had ceased. Cells were then filtered through a 25-mm membrane filter and washed twice with 1 ml of glutamate solution (5 mg/ml). The cells were then resuspended in a flask with 10 ml of basal salts only or in a flask with 10 ml of basal salts and 0.2% glucose. Symbols: ●, cells in flask with no added glucose; ▲, cells in flask with added glucose.

glucose reduced the later uptake of glutamate by cells freed from the preincubation mixture. When the metabolite was completely utilized, glutamate uptake ceased. This was not due to a nitrogen deficiency, because amino acids with available nitrogen (Fig. 7) were never completely depleted from the amino acid pools. Furthermore, for cells of this species grown in glucose and ammonia, the C-N assimilation ratio was 40:1. Therefore, when glutamate carbon was used with the aid of another energy source, there was an excess of glutamate nitrogen, because the C-N ratio in this amino acid is about 4:1.

Many aspects of the uptake of uric acid by *Candida utilis* as reported by Quetsch and Danforth (12) are analogous to the uptake of glutamate by *H. subpelliculosa*. A lag time of 25 to 30 min was required for the initiation of uric acid uptake when the purine and glucose were both added to the test system with *C. utilis* cells at zero-time. Preincubation of the cells for 1 hr with 5% glucose at 30 C completely eliminated the lag period. The amount of uptake for both uric acid and glutamate was proportional to the quantity of glucose supplied; after the depletion of energy source, uptake promptly ceased. The ability to accumulate uric acid developed in the absence of an exogenous substrate, a fact also true for glutamate. However, the addition of the purine "inducer" during preincubation slightly increased the uptake of purine. In contrast to this, the uptake of glutamate was significantly decreased when the substrate was added to the preincubation mixture with glucose (Fig. 2).

The explanation for this reduction in glutamate uptake will require more extensive experiments, but two possible reasons are the reduction of available metabolite by the presence of glutamate during the preincubation period and partial elimination of the available binding sites on the yeast cell membranes. With purine uptake by *C. utilis*, the addition of ammonia with glucose to the preincubation mixture decreased the uptake of purine. This reduction was attributed to a decrease in the quantity of the metabolite when ammonia was utilized (12).

The hypothesis for the method of either glutamate or purine uptake must be different from that of an inducible permease, because such systems once induced are stable. Eddy (Biochem. J. **88**:34P, 1963) proposed for the yeast *S. cerevisiae* that the mechanism for transport of amino acids was via a carrier, in which energy was required for the coupling of the amino acid to binding sites. Our results are compatible with his proposal.

In Fig. 5 it is apparent that a change in the rel-

ative specific activity of the C^{14} -glutamate mixture required an additional 20-min lag period, as compared with the time needed to initiate glutamate uptake. This longer time requirement must be related to the establishment and maintenance of mechanisms for the utilization of glutamate. One possible explanation for the time requirement is that needed for the formation of amino group acceptors in transamination reactions; a less plausible idea, is the time required for the biosynthesis of transaminases. *H. subpelliculosa* cells have enzymes for the utilization of oxaloacetate and pyruvate in such reactions.

With L-proline as a sole source of carbon and nitrogen, glucose or a similar type of energy supply was not required either for the uptake or for the metabolism of this amino acid. For some reason, glutamate utilization is not self-generating in the absence of another energy source. At least two energy factors are involved in the utilization of glutamate by this yeast: one is associated with the active transport of the amino acid into the cell; the other is related to its metabolism after entrance. A determination of some of the factors related to the requirement of an added energy source for the utilization of L-glutamate by this organism is the subject of continued investigations.

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