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Differential Sensitivities of the Two Malate Dehydrogenases and the Maltose Permease to the Effect of Glucose in *Saccharomyces carlsbergensis*

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In *Saccharomyces carlsbergensis* the two malate dehydrogenase activities, which are localized in different compartments of the cell, were found to differ in their response to glucose. The cytoplasmic malate dehydrogenase activity appears to be sensitive to inactivation by very low concentrations of glucose. The mitochondrial malate dehydrogenase activity is only repressed at a higher glucose concentration. Maltose permease is also sensitive to inactivation by glucose. Conditions were found such that the maltose permease was present while the cytoplasmic malate dehydrogenase was inactivated. The different sensitivities of the two malate dehydrogenases and maltose permease to the effect of glucose may explain the preferential use of glucose, maltose, and products of glucose metabolism (2- and 3-carbon skeletons) as carbon sources for growth in the order as mentioned.

Yeast cells are able to grow on maltose or alcohol after an adaptation period in which an increase of the activities of some enzymes, which are specifically required for utilization of these substrates, occurs. Thus, the presence of maltose permease activity and α-glucosidase is needed for maltose consumption, whereas growth on products of glucose metabolism (for instance alcohol or acetate) requires the presence of enzymes of the citric acid cycle, the respiratory chain, and the glyoxylate cycle (gluconeogenic) enzymes. The increase in these enzymes not only depends on the presence of the growth substrate but is inhibited in the presence of other carbon sources such as glucose or fructose, which might be used preferentially (9, 20-22, 30).

Two mechanisms have been described to account for the inhibitory effect of glucose on enzyme induction: "catabolite repression" and "inactivation repression" (7). Catabolite repression has been described for α-glucosidase, a number of citric acid cycle enzymes, and some enzymes of the respiratory chain (9, 20, 28, 30). Inactivation repression has been described for the maltose permease (10, 23), isocitrate lyase (2; C. P. M. Görts, Ph.D. thesis, Univ. of Utrecht, 1970), cytoplasmic malate dehydrogenase (7, 27), and fructose 1,6-diphosphatase (8). Thus, adaptation to maltose and alcohol is regulated both by means of inactivation repression and catabolite repression. In a medium containing both maltose and alcohol the resulting type of metabolism suggests that adaptation of the cells to ferment maltose is far more significant than the consumption of alcohol and its use via the gluconeogenic pathway. Although in this system the choice between glycolysis and gluconeogenesis may be related to the known controls of pacemaker enzymes (such as phosphofructokinase and fructose diphosphatase), one may also consider that this is caused by differential sensitivities of the various enzymes to the "glucose effect."

To study the latter possibility, the maltose uptake system, which is concerned with maltose metabolism, and the two malate dehydrogenase activities, which are reported to be concerned in citric acid cycle activity and gluconeogenesis (5), were examined for their sensitivity to inhibition by glucose. Protoplasts prepared from logarithmically growing cells were used in most of these studies since they have the dual advantage of an immediate and complete disruption by lysis and allow the dif-

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ferent phenomena such as induction, catabolite repression, and inactivation repression to be studied in a 4-hr period.

The results of this study clearly show that some enzymes differ in their sensitivity to regulation by "inactivation repression." It is suggested that "inactivation repression" and "catabolite repression" together can regulate the sequence of preference for growth on substrates such as glucose, maltose, and alcohol.

MATERIALS AND METHODS

Chemicals. All reagents were analytical grade. Nicotinamide adenine dinucleotide (NAD), glucose, maltose, and galactose were purchased from BDH; reduced NAD (NADH) and oxaloacetic acid from Koch-Light; L-malic acid, nitrobluetetrazoliumchloride, and phenazine methosulfate from Sigma; acrylamide from Servo, Germany; *N,N'*-methylenebisacrylamide and *N,N,N'*-tetramethyl-ethylenediamine from Fluka, Swiss; Casamino Acids from Difco; glucose reagent from Clinton Laboratories; ^{14}C-maltose and ^{14}C-amino acid mixture from Radiochemicals (England).

Yeast culturing and the preparation of protoplasts. *Saccharomyces carlsbergensis* (strain 74, National Collection of Type Cultures, London) was cultured in nutrient broth with 2% glucose as described by De Kloet et al. (13) and collected in the early exponential phase of growth. Protoplasts were prepared by a 20-min incubation with snail digestive juice (Industr. Biol. Française, Gennevilliers) as described by Eddy and Williamson (6). The protoplasts were used, unless stated otherwise, at a concentration equivalent to approximately 4 mg of cell dry weight per ml of incubation medium.

Cell disruption. Studies on whole cells were performed after growth of the cells as described by van Wijk (30). Cell disruption was produced by sonic treatment using an MSE 100 w ultrasonic disintegrator. The protein yield after centrifugation for 10 min at 3,000 × *g* is approximately 0.25 mg of protein per mg of cell dry weight. This is the same as that obtained with earlier methods (31). Maximal yields of protein were obtained after sonic treatment for 10 min, except with stationary-phase cells or cells which were fully adapted to acetate or alcohol. The latter cells gave maximal yields of protein after 30 min of sonic treatment.

Conditions of incubation. Malate dehydrogenase (MDH) was derepressed by incubating protoplasts at 30 C under aerobic conditions in a medium which contained 0.01 M potassium-sodium phosphate buffer, *p*H 6.2, 12% mannitol, 1% Casamino Acids, and 0.3% glucose, unless mentioned otherwise. Maltose permease and α-glucosidase were induced with the same conditions except for the extra addition of 0.3% maltose.

Sampling and determination of enzyme activities. For the determination of total MDH activity and α-glucosidase activity, samples of the incubation medium were rapidly cooled and centrifuged, and the protoplasts were lysed in 0.05 M sodium-potassium phosphate buffer, *p*H 6.8. The lysate was centrifuged for 10 min at 3,000 × *g*, and the supernatant fraction was used for determination of enzyme activities. MDH was determined at 25 C by the method of Vary et al. (26). α-Glucosidase activity was determined at 30 C by the method of Halvorson and Ellias (11). Enzyme activities are expressed as international units, i.e., 1 unit is equal to the conversion of 1 nmole of substrate per min.

For the determination of maltose permease, a 10-ml sample was centrifuged for 2 min at 3,000 × *g* at room temperature, and the sediment was washed two times with 10 ml of medium containing 0.1 M sodium acetate, *p*H 4.8, as a buffer. Subsequently, the protoplasts were resuspended in 1 ml of this medium and preincubated for 2 min at 30 C. Then 0.5 μCi ^{14}C-maltose (8.8×10^{-4} M) was added and 5 min later a 0.1-ml sample of this incubation mixture was placed on a wet membrane filter (HA, 0.45 μm, Millipore) and washed with ice-cold 12% mannitol. The radioactivity on the filter was determined in a 2,5-diphenyloxazole (PPO)-1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) solution in toluene (1 liter of toluene containing 5 g of PPO and 0.1 g of POPOP) with the aid of a Nuclear-Chicago Mark I liquid scintillation counter. Maltose permease activity is expressed in nanomoles of maltose uptake per milligram dry weight of cells.

For the determination of mitochondrial malate dehydrogenase activity, mitochondria were isolated by the procedure described by Duell et al. (4). The mitochondria were lysed in 0.01 M sodium-potassium phosphate buffer, *p*H 6.8, with 0.1% Triton X-100 and centrifuged for 15 min at 40,000 × *g*.

Protein was determined by the method of Lowry et al. (16) after precipitation with 5% trichloroacetic acid. Bovine serum albumin was used as a standard.

Determination of glucose in the incubation medium. After centrifugation of the incubation medium, glucose in the supernatant was determined with the glucose oxidase-peroxidase reaction as described by Clinton Laboratories.

Determination of overall protein synthesis. Overall protein synthesis was measured as the incorporation of ^{14}C-amino acids in hot trichloroacetic acid-precipitable material as described by Mans and Novelli (17). The radioactivity was determined in a PPO-POPOP solution in toluene (1 liter of toluene containing 4 g of PPO and 0.05 g of POPOP).

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was carried out according to Mauer (19) in gel system 1 a with a Canalco apparatus. The gels were stained as described by Atzpodien et al. (1).

RESULTS

Effect of glucose on MDH activity. Protoplasts of *S. carlsbergensis* incubated aerobically with a high concentration of glucose are unable to derepress maximally the formation of MDH until the concentration of that carbon source has been reduced by utilization to a certain level. An illustrative experiment, in which

the concentration of the carbon source was varied, is shown in Fig. 1. Although there is an early slow derepression, the maximal rate of synthesis does not occur for some time after complete consumption of glucose. Separately it was determined that alcohol was formed during the period of glucose consumption. However, this is not responsible for the time of increase, as the extra addition of alcohol and acetate did not show any stimulating effect.

Anaerobiosis completely eliminates the maximal increase in MDH activity. Under these conditions, using 0.15% glucose as the initial concentration, glucose had disappeared at 60 min, i.e., at approximately the same time as it disappears under aerobic conditions (Fig. 1). Figure 2 shows that the elimination of the increase in MDH is due to the cessation of protein synthesis under anaerobic conditions because the fermentation products formed (alcohol) cannot be used for energy.

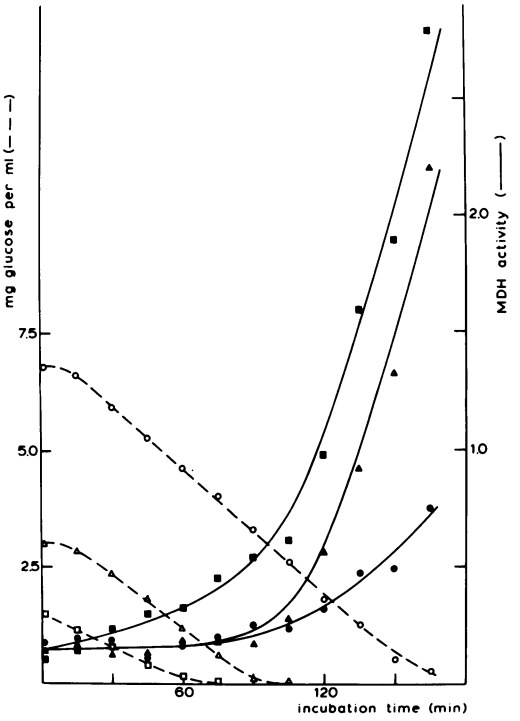

FIG. 1. *Effect of various glucose concentrations on the increase of malate dehydrogenase (MDH) activity in protoplasts of S. carlsbergensis. Solid curves represent MDH at the initial glucose concentration of 0.15% (■), 0.3% (▲), and 0.6% (●). Broken curves show the course of glucose in the medium when initial concentrations were present of 0.15% (□), 0.3% (△), and 0.6% (○). Further conditions as described in Materials and Methods.*

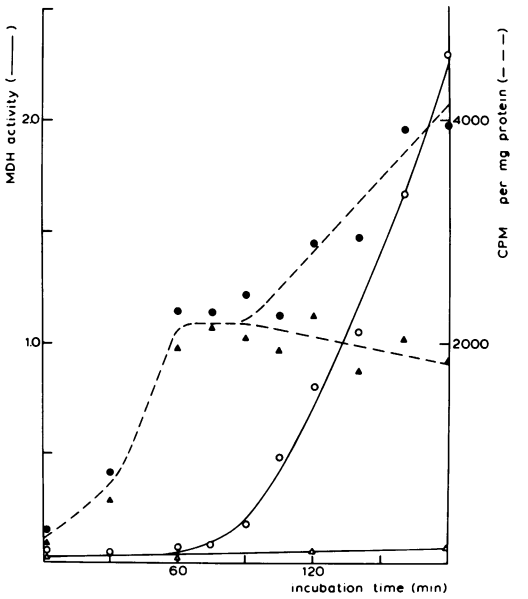

FIG. 2. *Increase of malate dehydrogenase (MDH) activity and incorporation of ^{14}C-amino acids in protoplasts of S. carlsbergensis under aerobic and anaerobic conditions. MDH, aerobic (○); MDH, anaerobic (△); ^{14}C-amino acid incorporation, aerobic (●); ^{14}C-amino acid incorporation, anaerobic (▲). Experimental conditions as described in Materials and Methods. Initial glucose concentration was 0.15%.*

The effect of glucose on derepressed MDH synthesis is shown in Fig. 3. Addition of glucose to a culture that is synthesizing MDH at a maximal rate is almost immediately followed by a decrease of the MDH activity. As is shown in the same figure, the degree to which MDH can be inactivated appears to change during incubation. Glucose addition at an early phase of derepression, i.e., during the period of slow derepression, is not followed by inactivation.

We tested the possibility that the MDH which can be inactivated by glucose and which is present after exhaustion of the glucose from the medium is not present earlier because of its high sensitivity to glucose. The results shown in Fig. 4 favor this explanation since even the lowest concentration of glucose tested, i.e. 0.05%, already affects MDH strongly when added during the period of maximal rate of synthesis. The effect of lower concentrations of glucose could not be tested in an accurate manner because these amounts were utilized too rapidly.

The differences in sensitivity to glucose inactivation for the MDH activity formed during glucose consumption and the MDH activity

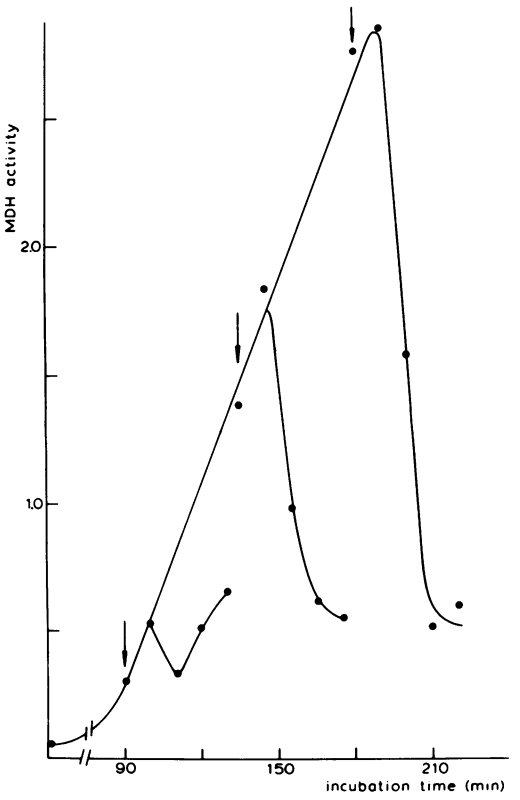

FIG. 3. *Effect of glucose on malate dehydrogenase (MDH) activity in protoplasts of S. carlsbergensis, when added to a final concentration of 2% after various periods of incubation. Experimental conditions as described in Materials and Methods.*

formed after the glucose is exhausted were also studied in connection with the two MDH activities which were found to differ in intracellular localization in *S. carlsbergensis*. Figure 5 shows the time course of MDH activity in the mitochondrial fraction (Fig. 5B) and in the remaining supernatant fraction (Fig. 5A) during derepression and during subsequent addition of glucose. These data show that an increase in specific MDH activity occurs in the mitochondrial fraction almost immediately after the start of the experiment. In contrast, the MDH activity in the supernatant fraction shows a distinct lag period. Moreover, a difference was observed in the effect of glucose on both enzyme activities: whereas MDH in the mitochondrial fraction is not sensitive to glucose inactivation, the MDH in the supernatant fraction is almost completely inactivated after 30 min.

The MDH activities in the two subcellular compartments appear to be due to different MDH isoenzymes. Evidence in support of this suggestion came from acrylamide gel electrophoresis experiments. The electrophoretic pattern of the cytoplasmic MDH under derepressed and repressed conditions and of the mitochondrial MDH, as shown in Fig. 6, show clearly distinct patterns. The presence of more than one band in the mitochondrial fraction is different from the results obtained with *S. cerevisiae* (1) and will require further study.

Effect of glucose on maltose permease. In Fig. 7 the maltose-induced increase of maltose permease is presented. Earlier work from this laboratory has shown that the synthesis of maltose permease and of α-glucosidase occur coincident upon induction by maltose (14). Moreover, it was observed that the increase of both activities is strongly dependent on the concentration of glucose in the medium and that it starts when the glucose concentration is lowered to approximately 0.12% (29). In contrast to α-glucosidase, which is completely

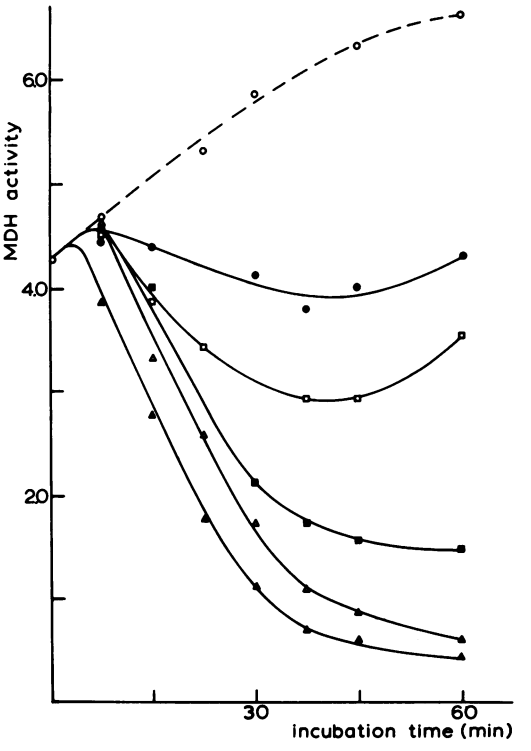

FIG. 4. *Effect of various glucose concentrations on malate dehydrogenase (MDH) activity in protoplasts of S. carlsbergensis when added after 180 min of incubation. No addition (○), 0.05% (●), 0.1% (□), 0.2% (■), 0.5% (△), 1% (▲). Experimental conditions as described in Materials and Methods.*

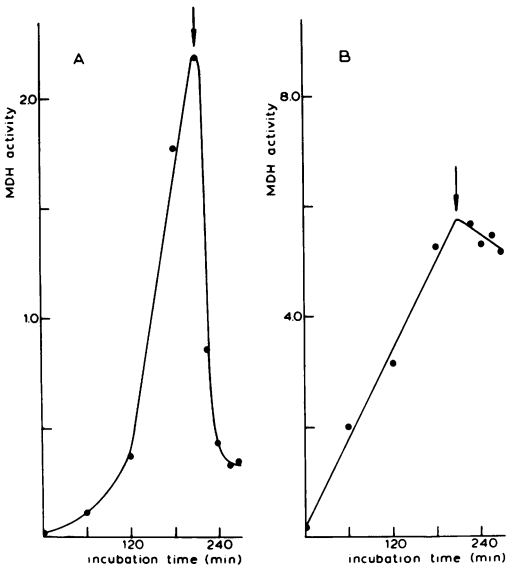

FIG. 5. *Increase in malate dehydrogenase (MDH) in the mitochondrial fraction (A) and in the remaining supernatant fluid (B) of protoplasts of S. carlsbergensis. Specific enzyme activity is the activity per milligram of protein in that fraction. During the incubation, the amount of protein in each fraction remained approximately constant. Further experimental conditions as described in Materials and Methods.*

regulated by catabolite repression, the maltose permease of yeast has been reported to be sensitive to inactivation repression (10, 23).

The sensitivity of maltose permease to inactivation by glucose was measured in an experiment similar to that presented in Fig. 4 for MDH. Figure 7 shows that inactivation of the maltose permease requires a significantly higher glucose concentration than with MDH, namely 0.12%.

Thus, it appears that the glucose concentration at which maltose permease starts increasing is similar to the concentration at which catabolite repression of α-glucosidase is released. This means that during glucose consumption eventually a concentration is reached at which, when maltose is present, adaptation to maltose consumption occurs. The consequence of this adaptation for the derepression of cytoplasmic MDH was studied in protoplasts and in growing cells and will be discussed in the following section.

Effect of maltose on MDH activity. In some previous experiments cells were incubated in a medium containing 0.3% glucose plus 0.3% maltose as the carbon source (see Fig. 7). Under these conditions no MDH was found which could be inactivated. This suggests that adaptation to maltose inhibits the increase in cytoplasmic MDH. A better indication of this inhibition can be obtained by using a lower glucose concentration, namely 0.1%. At this concentration, which is lower than that at which catabolite repression is released, α-glucosidase starts to increase (after a lag period of 40 min which is independent of glucose concentration) at a slow rate and reaches its maximal rate only at 90 min (29). With the initial glucose concentration used, the glucose was consumed within 60 min. As shown in Fig. 8, under these conditions of incubation, MDH is formed very rapidly after initiation of the experiment. The part formed after 60 min, i.e., after exhaustion of glucose, appears to be similar to the part that was inactivated when α-glucosidase was formed at its maximal rate.

More data on MDH activities in the presence of maltose came from studies on growing cells. Table 1 shows the different MDH activities present in cells which were grown on different carbon sources, such as glucose (4%), maltose (4%), galactose (4%), and products of glucose metabolism (0.1% glucose). Moreover, the effect of glucose on the various MDH activities was determined by incubation of the cells in a glucose medium afterwards. From the data presented in Table 1 it is clear that the higher amounts of MDH present in cells grown on maltose cannot be inactivated by glucose. This was also found in cells grown in a galactose-containing medium. The slight decrease after glucose addition in maltose- and galactose-grown cells might be expected as a consequence of repression of synthesis and di-

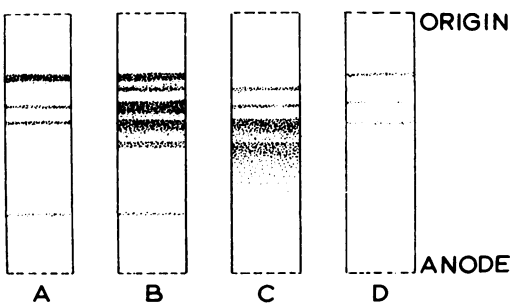

FIG. 6. *Acrylamide gel electrophoresis of different extracts. (A) Derepressed protoplasts stained without substrate; (B) the same with L-malate as substrate; (C) mitochondria from derepressed protoplasts. All staining is substrate-specific; (D) repressed protoplasts with the L-malate as substrate. For electrophoresis and staining of the malate dehydrogenase, see Materials and Methods. Electrophoresis was performed for 80 min with 3.5 ma per gel and ± 7 v/cm (diameter of gel, 0.5 cm²).*

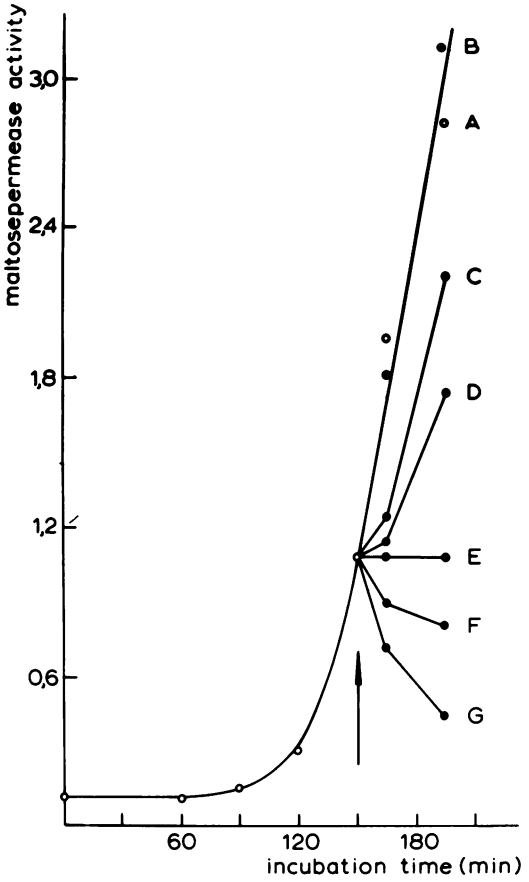

FIG. 7. *Increase of maltose permease activity and the effect of various glucose concentrations on this activity in protoplasts of S. carlsbergensis when added after 150 min of incubation. (A) No extra glucose added; (B) 0.03%; (C) 0.06%; (D) 0.09%; (E) 0.12%; (F) 0.23%; (G) 1%. Further experimental conditions were described in Materials and Methods. Maltose permease activity is expressed as nanomoles of uptake of maltose per milligram dry weight.*

lution by further growth. The higher amount of MDH observed in cells which started growing in the medium containing 0.1% glucose appears to be of the type which can be inactivated.

DISCUSSION

A number of comparative studies have been published on the type of metabolism during aerobic growth of yeast cells in media containing different carbon sources, for instance glucose, maltose, galactose, or products of glucose metabolism. The adaptation of glucose-grown cells to maltose or galactose requires the formation of a permease activity and one or more enzymes which convert the sugar into glucose or a glycolytic intermediate. A number of studies showed that although glycolysis remains active under these conditions, this adaptation coincides with an enhancement of oxidative respiration (9, 20, 24, 30). Although not a prerequisite for growth, the enhanced respiration may play a role in the rate of growth as growth is inhibited under anaerobic conditions (24).

The adaptation to growth on two- or three-carbon skeletons absolutely requires an active citric acid cycle, oxidative phosphorylation, and gluconeogenesis.

From these data, we would expect a different regulation of the activities of the maltose consumption system, the respiratory system, and the gluconeogenic enzymes under the various conditions. Theoretically this difference in regulation can be explained in terms of influence on existing enzyme activity, or in terms of differences in existing enzyme activities, or both. The control of glycolysis and gluconeogensis is related in most theories to the known control of pacemaker enzymes such as phosphofructokinase and fructose diphosphatase resulting from their sensitivity to adenine nucleotides and glycolytic intermediates (12,

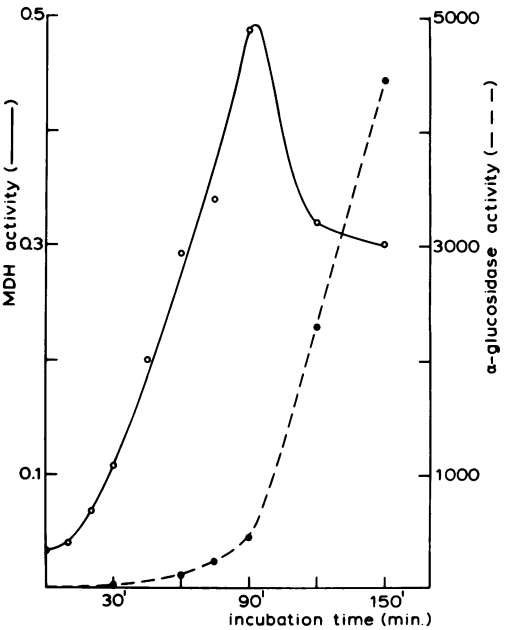

FIG. 8. *Increase and inactivation of malate dehydrogenase (MDH) (○) and increase of α-glucosidase activity (●) in protoplasts of S. carlsbergensis which were incubated in a medium containing 0.1% glucose and 3% maltose as carbon source. Further experimental data as described in Materials and Methods.*

18). However, the results of Chapman and Bartley (3) have made it unlikely that such a control occurs in yeast. Comparative studies of the enzyme patterns in *S. carlsbergensis* and *S. cerevisiae* indicate that regulation by differences in existing activities of some enzymes probably plays a role in determining the occurence of gluconeogenesis although concomitant regulation by effector concentrations may exist too.

Thus, data from *S. cerevisiae* and *S. carlsbergensis* showed that during growth on media containing galactose or maltose as the carbon source, the adaptation on these sugars coincided partly with a derepression of a number of citric acid cycle enzymes and a repression of isocitrate lyase and malate synthase (20). This paper deals with a comparable study on the MDH activity in cultures of *S. carlsbergensis* growing in the presence of different carbon sources.

The existence of two MDH activities was presented by Witt et al. (27). They pointed out that the cytoplasmic form is inhibited by glucose, whereas the enzyme in the mitochondria, which is presumably a part of the citric acid cycle, was suggested to be insensitive, although no data were presented. Other laboratories report that mitochondrial MDH (25) or both cytoplasmic and mitochondrial MDH (15, 26) are repressed by glucose. Our data also show a repression by glucose of both cytoplasmic and mitochondrial levels of this enzyme, although there is a clear difference in their sensitivity. The cytoplasmic form behaves like the glyoxylate (gluconeogenic) enzymes and shows no derepression during growth on galactose or maltose, whereas the mitochondrial enzyme is already derepressed, as has been found for other citric acid cycle enzymes.

With the use of protoplasts in short-term experiments it has been shown that during glucose consumption the formation of the mitochondrial MDH and maltose permease occurs earlier than the formation of cytoplasmic MDH. Both the maltose permease and the cytoplasmic MDH were sensitive to inactivation repression by glucose. The appearance of these enzymes at different times can be explained as a consequence of the release of inactivation repression at different times.

In this paper the term maltose permease is used. Theoretically the maltose uptake system as we measured is not a priori the activity of the maltose permease. The possibility exists that the α-glucosidase activity is the limiting step in this process. However, under our conditions this is not the case, since it was found that the maltose uptake system has an optimal *p*H of 4.8, which differs strongly from that of α-glucosidase and which is similar to the optimal *p*H for the uptake of the nonmetabolized compound α-thioethyl D-glucopyranoside (14). Moreover, the inactivation of the maltose uptake system cannot be due to α-glucosidase since the latter enzyme is only subject to repression (32). The inactivation is also not due to effects on glycolysis as we determined manometrically and which was also shown by Görts (10).

TABLE 1. *Malate dehydrogenase (MDH) activity in cells of Saccharomyces carlsbergensis after growth on media containing different carbon sources*[a]

Carbon source for growth	After 12 hr of growth		After 90 min in presence of glucose	
	OD	MDH/10^7 cells (IU)	OD	MDH/10^7 cells (IU)
Glucose (40 mg/ml)	0.50	0.01	0.70	0.01
Maltose (40 mg/ml)	0.92	0.06	1.10	0.05
Galactose (40 mg/ml)	0.54	0.07	0.80	0.05
Glucose (1 mg/ml)	0.32	0.27	0.48	0.05

[a] After 12 hr of growth, 20 mg of glucose per ml of medium was added and MDH was determined again 90 min later. With the use of previous data (32) the average MDH activity per cell was calculated. The optical density (OD) of the culture was determined with a Kipp colorimeter at 590 nm. Cells were disrupted sonically for 30 min.

The differential sensitivities of the maltose permease and the cytoplasmic malate dehydrogenase to inactivation repression by glucose may be due to a differential sensitivity for repression of their synthesis by glucose or to a differential sensitivity to inactivation by glucose, or both. This problem is now under further study in order to clarify further the regulatory roles of glucose or its catabolite(s) on the various enzymes of carbohydrate metabolism.

ACKNOWLEDGMENTS

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