NET UTILIZATION OF FREE AMINO ACIDS DURING THE INDUCED SYNTHESIS OF MALTOZYMASE IN YEAST[1]

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Previous investigations with yeast have examined the effect of variations in the availability of the internal free amino acids on the capacity of resting cells to synthesize maltase. Control over the free amino acid pool as a source of nitrogen for protein synthesis was achieved by the use of analogues of amino acids (Halvorson and Spiegelman, 1952) and by nitrogen starvation and replenishment procedures (Halvorson and Spiegelman, 1953).

It was found that interference with the utilizability of the free amino acids or the absence of an adequate supply resulted in inhibition of enzyme synthesis. These data as well as those obtained with bacteria (Pinsky and Stokes, 1952; Monod *et al.*, 1952; Ushiba and Magasanik, 1952) lead to the conclusion that the primary pathway of induced enzyme formation involves synthesis of new enzyme from free amino acids.

Halvorson and Spiegelman (1952) were able to exhibit a striking correlation between enzyme forming capacity and the level of the internal free amino acid pool. From the viewpoint of these results it seemed possible that the free amino acid pool of cells actively synthesizing a new enzyme might well be demonstrably different from the pool in cells held under identical conditions but not subject to the induction of a new enzyme. It was of interest therefore to study the reverse of the problem thus far considered and examine the effect of enzyme synthesis on the free amino acid pool.

A simple comparison of the free amino acid pools under these two conditions, however, is complicated by the observations of Roine (1947) which were confirmed amply in the investigations of Halvorson and Spiegelman (1952). Resting yeast cells deplete their free amino acid pools when metabolizing either exogenous or endogenous substrates in the absence of an external source of nitrogen. The extent and rate of amino acid incorporation from the pool during a nitrogen-free incubation of this sort will be influenced by the energy available during the incubation. Any attempt at determining the effect of induction on the free amino acid pool, therefore, to be interpretable, must ensure that the new enzyme being formed is not increasing or otherwise modifying the amount of energy available to the cells. This would be a difficult situation to control if the agent acting as the inductor were also metabolizable. The possibility of circumventing this difficulty with yeast came with the finding (Spiegelman, 1948) that α-methyl-glucoside is active as an inductor in the synthesis of maltozymase under conditions in which it is not utilizable. An analogous situation was uncovered by Monod *et al.* (1951) who found that melibiose, though inactive as a substrate for the cells, was effective in the induction of β-galactosidase in *Escherichia coli*.

It is the purpose of the present paper to describe experiments using the α-methyl-glucoside-maltozymase system to examine the effect of induced synthesis of enzyme on the quantitative composition of the free amino acid pool. The data exhibit the existence of active incorporation from the free amino acid pool as a consequence of the formation of maltozymase in yeast.

METHODS AND MATERIALS

Strain employed and conditions of growth. The yeast strain used is a diploid representative of *Saccharomyces cerevisiae* (strain K). It was grown in a medium prepared by adding the following to one liter of water: glucose, 40 g; Difco bactopeptone, 5 g; Difco yeast extract, 2.5 g; ammonium sulfate, 2 g; calcium chloride, 0.25 g; magnesium sulfate, 9.25 g; and 60 per cent sodium

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lactate, 6 ml. Log phase cells were employed invariably, and these were prepared by inoculating 500 ml of the complete medium with 0.2 ml of a 24 hr culture. The resulting suspension was allowed to incubate unagitated at 30 C for 12 hr.

Preparation of cells. Cells were harvested immediately prior to an experiment by centrifugation and washed twice with cold water. Standard cellular suspensions containing 2.84 mg dry cells per ml were prepared with the aid of a Klett-Summerson photoelectric colorimeter previously calibrated for this purpose. Two suspending media were used. One was M/10 succinate M/10 phosphate adjusted to pH 4.5. The other was Burkholder's (1943) synthetic medium modified by the omission of the asparagine, ammonium sulfate, and carbohydrate and by the addition of 5.9 g of succinic acid per liter to increase the buffering capacity at pH 4.5.

Measurement of enzymatic activity. Enzymatic activities were determined manometrically by the rate of anaerobic CO_2 evolution from 2 per cent maltose at 30 C using the standard Warburg apparatus. Prior to determination the cells were exposed to ultraviolet irradiation to prevent enzyme synthesis during the course of the assay. The results obtained agree well with direct enzyme assays on dried cellular preparations (Halvorson and Spiegelman, 1952). Merck's maltose was purified further by recrystallization from 50 per cent alcohol.

Collection and analysis of free amino acid pools. The free amino acid pools were collected by the methods devised by Gale (1947). Their components were analyzed by the use of specific amino acid decarboxylases (Gale, 1945; Umbreit and Gunsalus, 1945), microbiological analysis (Henderson *et al.*, 1948), and paper strip chromatography (McFarren, 1951). The details of the applications of these methods to yeast have been described previously (Halvorson and Spiegelman, 1952).

EXPERIMENTAL RESULTS

Changes in the free amino acid pool during the induced synthesis of maltozymase in unstarved cells. Despite the existence of evidence indicating that active incorporation of amino acids is a necessary concomitant of enzyme formation, it is important nevertheless to recognize that it is not evident *a priori* how the free amino acid pool level will respond to the induced synthesis of an enzyme under a particular set of conditions. The difficulty in making a prediction stems in large part from observations made on the process of enzymatic synthesis itself. Enzymes once induced will disappear in certain cases if the cells containing them are allowed to metabolize in the absence of inductor. However, Spiegelman and Reiner (1947) and Sussman and Spiegelman (1950) demonstrated that agents such as NaN_3 and Na_2HAsO_4 which inhibit enzymatic synthesis in yeast also were capable of preventing the disappearance of the induced enzymes which usually attends the removal of their inductors. Fowler (1951) observed a similar situation with 2-4-dinitrophenol in the case of a fermentative enzyme in *E. coli*. This demonstration that one and the same agent can prevent both the appearance and the disappearance of an enzyme receives a likely interpretation in terms of a coupling between the synthesis and breakdown of protein. The response of the free amino acid pool to induction of enzymatic synthesis therefore may depend upon the relative rates of the synthetic and degradative reactions which may result from the formation of the enzyme being studied.

In view of these considerations experiments were undertaken to examine the free amino acid pool during induction under a variety of conditions. In principle two diverse experimental conditions were examined. One was a minimal one so far as the synthesis of new enzyme was concerned, and the other was the optimal achievable under the conditions of the experiment. The former was obtained by running the induction in cells suspended in phosphate-succinate buffer, and the second, by suspending the cells in modified Burkholder's synthetic medium containing all the cofactors necessary for optimal growth except nitrogen.

The induction of the maltozymase was achieved by means of α-methyl-glucoside, glucose being added as a utilizable source of energy in addition to that provided by the endogenous metabolism. Control cells received the same amount of glucose, and hence both sets had equal amounts of energy available during the incubation period. Details and results are summarized in table 1.

In view of the purpose of the experiment it was necessary to adjust the times of incubation so that comparable amounts of enzyme would be synthesized by the cells being induced under the two conditions. Thus, the induction was allowed to proceed for 166 min in the phosphate-succinate suspension and for 122 min in the N-free syn-

thetic medium. This difference in time is reflected in the levels of glutamic acid found at the termination of the incubations, being higher in the shorter induction.

Consider first the experiment carried out under the minimal conditions. It will be noted in table 1 that cells suspended in phosphate-succinate medium and incubated in the presence of inductor possessed a slightly larger pool at the end of the incubation than those held for the same period without inductor. That this difference is significant is indicated from a statistical analysis of the data by means of the Student t-test which yielded a value of $P < 0.01$. Furthermore, this difference is consistent since virtually identical results were obtained in another independent experiment performed under the same conditions. In this repetition four experimentals and four noninduced controls were run. The glutamic acid (in μM per 100 mg dry weight of cells) found in the induced cells was 11.7 ± 0.2 whereas the comparable controls contained 10.5 ± 0.3, yielding a P value of 0.015. Turning now to the experiment carried out in the nitrogen-free synthetic medium (table 1) we note that the pool levels of induced and noninduced cells do not differ significantly. It would appear then that if the induction is carried out under conditions which are more favorable for enzymatic synthesis, the mechanism which resulted in the accumulation of free amino acid during the phosphate-succinate induction no longer operates.

TABLE 1

Comparison of free glutamic acid content following induced synthesis of maltase in phosphate-succinate buffer and nitrogen-free synthetic medium

Six separate flasks were used for each induction condition. Each flask contained 100 ml suspension of washed log phase cells adjusted to a density of 2.56 mg dry weight of cells per ml. One group was suspended in phosphate-succinate buffer at pH 4.5 and the other in the nitrogen-free synthetic medium at the same pH. In both cases the control (noninduced) cells received 5.5 ml of 6.6 per cent glucose and the experimentals received, in addition, 14 ml of 30 per cent α-methyl-glucoside. The flasks were shaken aerobically at 30 C for the indicated periods, the times being adjusted so that comparable amounts of enzyme were synthesized in the two experimental groups. At the end of the incubation period 5 ml aliquots were irradiated, washed, and assayed for enzymatic activity ($Q^N_{CO_2}$). The remaining cells in each sample were centrifuged, washed, and free amino acid pools prepared which were then analyzed for their glutamic acid contents by the decarboxylase method. The results are reported in terms of μM of glutamic acid per 100 mg dry weight. Averages ±2 standard deviations of the mean are given. The P values represent the probability of obtaining the indicated pair of average values by chance as calculated by the Student t-test.

CONDITIONS	INDUCTOR ABSENT		INDUCTOR PRESENT	
	$Q^N_{CO_2}$	μM Glutamic acid	$Q^N_{CO_2}$	μM Glutamic acid
Phosphate-succinate buffer incubation for 166 min	13	10.8 10.5 10.6 10.1 10.8 10.7	103	11.7 11.6 11.4 11.6 12.0 11.8
	Average $P < 0.01$	10.6 ± 0.21		11.7 ± 0.17
Nitrogen-free synthetic medium incubation for 122 min	8	15.8 16.2 14.8 15.4 16.5 15.3	99	15.2 15.0 15.8 16.3 15.1 16.1
	Average $P = 0.62$	15.8 ± 0.60		15.6 ± 0.45

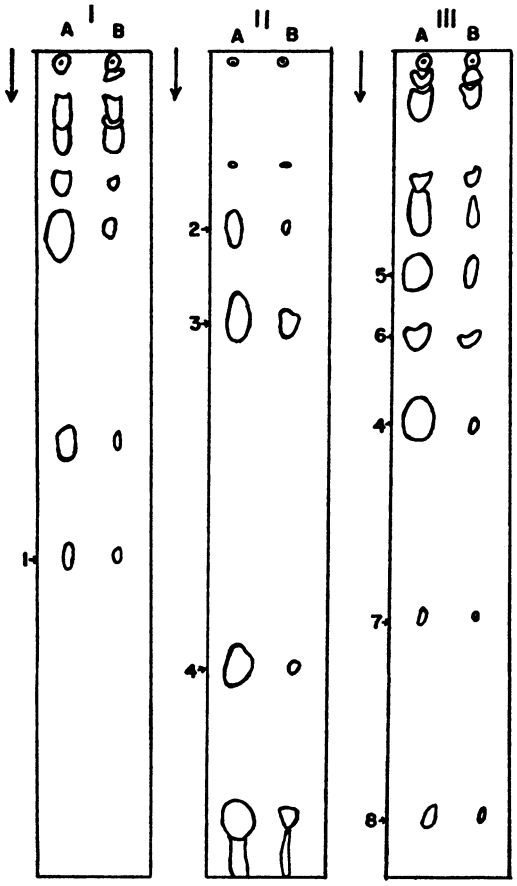

Figure 1. Replenishment of the free amino acid pools in N starved cells by exposure to external $(NH_4)Cl$. The (B) columns represent pools obtained from cells subjected to 120 min starvation, and the (A) columns, pools from such cells subsequent to a 15 min replenishment procedure. Strips I, II, III refer to the three solvent systems employed, being in order, o-cresol buffered at pH 6.2 (24 hr), phenol buffered at 12.0 (24 hr), and m-cresol buffered at pH 8.4 (48 hr). The numbers indicating the spots identify the following amino acids: (1) phenylalanine, (2) aspartic acid, (3) glutamic acid, (4) glutamine, (5) lysine, (6) arginine, (7) histidine, and (8) valine.

One cannot at present interpret such findings with certainty. They are consistent, however, with the supposition that enzyme synthesis is coupled with the breakdown of preexisting protein which results in a partial replenishment of the free amino acid pool. A slight relative depression of protein synthesis in the minimal conditions then would explain the difference in behavior between the minimal and the optimal induction of enzyme. Both the interpretation and the findings are consistent with the data and conclusions derived from the inhibitor experiments (Spiegelman and Reiner, 1947; Sussman and Spiegelman, 1950; Fowler, 1951). Of greater immediate interest, however, is the fact that these results strongly suggest that *marked* net changes in the free amino acid pool levels as a consequence of induced enzyme synthesis will be observable, if they exist, only by suppressing the pool replenishing mechanism.

In principle one method is to deplete the cells of those components which might be utilized as a source for replenishing the pool. Earlier studies suggested the means of attaining the desired experimental conditions. Whether a cell is being induced to synthesize a new enzyme or not, a net flow from the free amino acid pool occurs during active metabolism in a nitrogen-free medium (Roine, 1947; Halvorson and Spiegelman, 1952, 1953).

Such nitrogen starved cells, lacking as they do a free amino acid pool, virtually are incapable of synthesizing enzyme. This capacity, however, can be restored by replenishing the free amino acid pool by means of a short exposure to an exogenous nitrogen source (Halvorson and Spiegelman, 1953). It was shown by Roine (1947) and confirmed by the present authors that the replenishment is virtually complete before a significant portion of the newly formed pool is used for protein synthesis. Thus, one could obtain in principle cells containing available amino acids without replacing to any extent the complex nitrogenous components which might serve as pool replenishers. Experiments were undertaken to study the effect of enzymatic synthesis on such replenished pools.

Properties of nitrogen starved and replenished cells. It was found that the prolonged 12 hr starvation employed in a previous study (Halvorson and Spiegelman, 1953) was not convenient for the purposes of the present investigation. The following procedure was adopted as a routine procedure. Log phase cells, prepared as described under methods, were harvested and washed twice with cold H_2O. They were suspended then in nitrogen-free synthetic medium containing 3 per cent glucose and incubated with aeration for 80 min. Ammonium chloride, equivalent to 400 per

cent of the cellular nitrogen, then was added and the incubation continued for 15 min.

That this treatment serves to replenish the depletion of the free amino acid pool which occurs during the 80 min starvation may be seen from the results of chromatographic analysis of the free amino acid pools as diagrammed in figure 1.

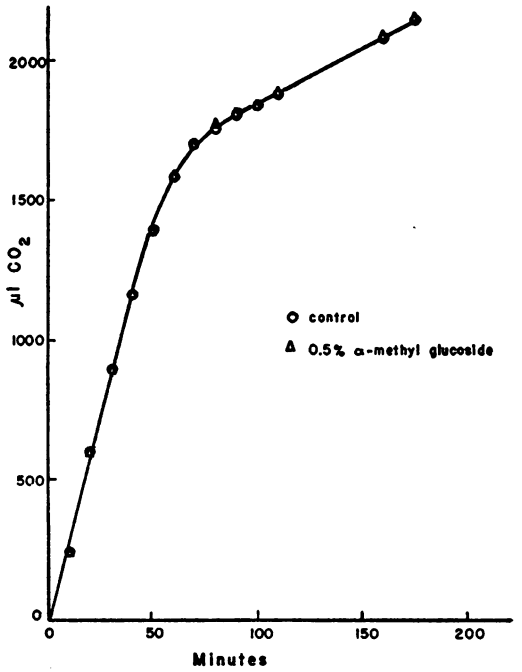

Figure 2. A test of the ability of nitrogen starved and replenished cells to metabolize α-methyl-glucoside. Both sets of cells were suspended in Warburg flasks at a density of 2.84 mg dry weight per ml in the nitrogen-free synthetic medium adjusted to 4.5. After equilibration 8 mg of glucose were tipped from the side arm into the controls and the experimentals received 8 mg + 10 mg of α-methyl-glucoside. The bend observed in the curves represents exhaustion of the glucose and the onset of endogenous respiration.

Columns B represent the components found in the cells after the 80 min nitrogen starvation, and columns A, the analysis subsequent to the 15 min replenishment. Comparison of the two indicates that the replenishment procedure restores virtually all components. The size and intensity of the spots in the A columns are indistinguishable from those obtained in the analysis of unstarved log phase cells (Halvorson and

TABLE 2

Free glutamic acid and arginine depletion in N replenished cells as a consequence of the induced synthesis of enzyme

Cells were nitrogen starved for eighty minutes in the synthetic medium, replenished with NH_4Cl for 15 minutes, centrifuged, washed, and resuspended in buffer. Equal aliquots were placed in 10 flasks containing 0.3 per cent glucose with or without 0.5 per cent α-methyl-glucoside. Following aerobic incubation at 30 C for 140 minutes, 5 ml samples were removed, irradiated, and assayed anaerobically for enzymatic activity ($Q^N_{CO_2}$). Free amino acid extracts were prepared from the remaining cells. Glutamic acid and arginine were assayed manometrically by the decarboxylase method. Averages ±2 standard deviations of the mean are reported. Comparison of the average values between the induced and control cells by the Student t-test yields $P < 0.01$ for both glutamic acid and arginine.

FLASKS	$Q^N_{CO_2}$	µM/100 MG DRY CELL	
		Glutamic acid	Arginine
Control			
1	30	19.6	3.2
		20.4	3.2
2	24	17.9	2.4
		17.2	2.4
3	27	17.2	2.6
		18.0	2.9
4	28.8	18.9	2.8
		19.6	3.0
5	24.6	17.3	2.6
		17.5	2.6
Average	26.9 ± 2.3	18.6 ± 2.1	2.9 ± 0.6
0.5 per cent α-Methyl-glucoside			
1	116	10.3	1.7
		10.1	1.7
2	116	10.9	1.9
		10.8	2.3
3	105	9.9	1.8
		9.9	1.6
4	110	11.1	1.9
		11.5	1.8
5	118	12.0	2.0
		11.8	1.9
Average	113 ± 5.2	10.8 ± 1.7	1.8 ± 0.4

Spiegelman, 1952). A quantitative analysis of the glutamic acid contents by the decarboxylase method confirmed the conclusion that the 15 min nitrogen replenishment is sufficient to restore the free amino acid pool to the log phase level.

Before employing the starved and replenished cells it was necessary to ascertain whether the treatment modified in any way the inability of the cells to utilize α-methyl-glucoside under the conditions of induction.

TABLE 3

Free amino acid depletion as a consequence of the induced synthesis of maltozymase

Two control (3 and 4) and two induced (4 and 5) extracts from the experiment detailed in table 2 were analyzed microbiologically for their amino acid content. Five separate tubes were used for each assay and the results averaged. The results are reported as μM per 100 mg dry weight of cells. Depletion due to synthesis is reported as (average of induced/average of controls) × 100.

AMINO ACID	μM/100 MG DRY CELLS				% Depletion
	Control cells		Induced cells		
	(3)	(4)	(4)	(5)	
Leucine	1.42	1.42	0.90	0.94	35
Valine	3.06	2.92	1.55	1.46	48
Phenylalanine	0.69	0.69	0.41	0.41	41
Tryptophan	0.088	0.096	0.041	0.048	47
Glutamic acid	19.7	21.4	11.5	10.3	47
Aspartic acid	2.72	2.32	1.23	1.27	50
Lysine	7.4	6.75	4.9	3.45	42
Isoleucine	1.17	1.08	0.48	0.48	56
Proline	1.00	1.09	0.67	0.65	42
Tyrosine	0.47	0.49	0.24	0.19	54
Methionine	0.26	0.26	0.18	0.16	35
Threonine	1.87	1.86	1.47	1.50	21
Arginine	2.68	2.30	1.60	1.59	35
Average					42.5

To test this point nitrogen starved and replenished cells were prepared according to the procedure described, and the effect of 0.5 per cent α-methyl-glucoside on the aerobic recovery of CO_2 and O_2 uptake from 8 mg of glucose was examined. The results of such an experiment are shown in figure 2. It is evident that α-methyl-glucoside does not contribute detectably to the aerobic metabolism of such cells. Any effect of α-methyl-glucoside induced maltozymase synthesis on the movement of the free amino acids cannot be attributed consequently to metabolism of the inductor.

Behavior of free amino acids during induction of maltozymase in nitrogen starved and replenished cells. Cells were nitrogen starved for 80 minutes and replenished for 15 min as described. These were washed and then suspended in nitrogen-free synthetic medium containing 0.3 per cent glucose with and without α-methyl-glucoside at a level of 0.5 per cent. Five separate controls without inductor and five experimentals with inductor were included.

The flasks were incubated aerobically while shaking at 30 C for 140 min, subsequent to which enzymatic assays and analyses of free amino acid pool contents were carried out in duplicate. The enzymatic levels achieved as well as the glutamic and arginine contents as determined by the decarboxylase procedure are summarized in table 2. The glutamic acid level in the cells induced to synthesize enzyme is 58 per cent of that found in the controls and the arginine is 62 per cent of the control level. It would appear then that enzymatic synthesis results in a marked net increase in the utilization of the free amino acids.

The generality of this conclusion was checked by examining other components of the free amino acid pool. The composition of two control and two experimental pool extracts obtained in the experiment of table 2 was analyzed in duplicate microbiologically for 13 constituents. The results are summarized in table 3. It is clear that the cells induced to form enzyme used more of every component analyzed. In general, the increased utilization which attended the synthesis of the maltozymase is quantitatively similar for all constituents.

DISCUSSION

It is evidently possible to exhibit experimentally an increased utilization of the free amino acid pool as a concomitant of the induced synthesis of enzyme. The fact that this phenomenon is observable only in nitrogen starved and replenished cells is a finding of no little interest. There are undoubtedly a number of explanations which can be offered to explain this observation. At the present time the authors are inclined to accept as the most fruitful working hypothesis the one used more or less explicitly in the design of the experiments recorded here.

The available data suggest the existence of a pool replenishing mechanism involving the break-

down of preexisting protein and which is coupled to protein synthesis. In unstarved cells the replenishing mechanism would be expected to function, and hence effects of enzyme formation on the free amino acid pool level would be observed only if there is a resultant unbalance between utilization and degradation. A situation of this sort appeared to have obtained in the "minimal" induction described in table 1. In the course of a nitrogen starvation those proteins which tend to degrade easily will do so and contribute their amino acids to the pool. As the starvation progresses, the continual recycling of the amino acids from the proteins through the pool and back would lead to their eventual accumulation in the least labile proteins, and in the ones therefore least capable of furnishing free amino acids to the pool. One would expect thereby to eliminate the pool restoring mechanism. Upon replenishing the pools of such cells by a short exposure to external nitrogen a system would be provided which would permit the detection of any increased utilization as the result of forming a new enzyme.

Information obtained in previous investigations is consistent with the above interpretation of the present data. The sensitivity of enzyme forming capacity to pool depletion in starved-replenished cells was found to depend upon the length and extent of the starvation to which the cells were subjected prior to restoring their pools to log phase levels (Halvorson and Spiegelman, 1953). The enzyme forming capacity of cells starved for only a brief period was more resistant to a second starvation than those subjected to prolonged nitrogen starvation. Furthermore, the correlation between pool level and enzyme forming capacity in nitrogen replenished cells increased with the duration of the initial starvation. That these observations cannot be ascribed to an amino acid independent transformation of complex precursor into active enzyme in unstarved or briefly starved cells is shown by their sensitivity to amino acid analogues (Halvorson and Spiegelman, 1952). The data suggested that the "free amino acid" pool was not an exhaustive measure of the available supply of amino acids for the synthesis of proteins in unstarved cells or those starved for a short period. This additional source was eliminated, however, by a prolonged starvation.

The fact that it is possible to demonstrate a net increase in free amino acid utilization as a result of induced enzymatic synthesis in starved-replenished cells implies that induction adds to the over-all rate of protein synthesis. Evidently the noninduced control cells are not synthesizing protein at maximal capacity. This in turn would suggest that at least some of the amino acids employed in the synthesis of the new enzyme are not obtained at the expense of other protein synthesizing systems. It remains to be seen whether this situation obtains in unstarved cells. This is a question which is one of extreme relevance to the nature of the interaction observed between enzyme forming systems in bacteria (Monod, 1942) and yeast (Spiegelman and Dunn, 1947). The use of cells containing isotopically labeled free amino acid pools will make an experimental analysis possible.

In conclusion we may state that the data presented offer the most direct evidence thus far available in support of the conclusion that induced enzymes are synthesized from free amino acids.

SUMMARY

The effect of induced synthesis of enzyme on the composition of the free amino acid pool of yeast was studied. The formation of maltozymase induced by α-methyl-glucoside, a nonutilizable analogue, was the system employed. It was possible to demonstrate a net increase in the utilization of the internal free amino acids as a result of the induction of enzymatic synthesis in resting cells suspended in a nitrogen free medium. The data offer further evidence in support of the conclusion that induced enzymes are synthesized from free amino acids.

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