A STUDY OF THE PROPERTIES OF THE FREE AMINO ACID POOL AND ENZYME SYNTHESIS IN YEAST*

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Yeasts possess an unusual ability to alter their enzyme patterns in the absence of an external nitrogen source. This phenomenon is most dramatically exhibited by the induced synthesis of enzymes. In such inductions the nitrogen employed by the cell in fabricating the new enzyme molecule must be derived from some preexisting nitrogenous compounds in the cell. The investigations of Taylor (1) have provided a reasonable explanation for the apparent independence of the yeast enzyme-synthesizing mechanism. In a survey of three yeast types, Taylor demonstrated the presence of detectable quantities of free arginine, glutamic acid, histidine, lysine, and tyrosine in the internal environments of these cells. The Gram-positive bacteria were found to possess primarily glutamic acid and lysine, while the Gram-negative bacteria surveyed were devoid of detectable quantities of these amino acids.

Thus it would appear that an internal supply of free amino acids would enable these yeasts to support enzyme synthesis in the absence of exogenous nitrogen. Several lines of evidence support the conclusion that the primary pathway of induced enzyme synthesis in non-growing cells involves the utilization of a large proportion of the components of this free amino acid pool. Amino acid analogues not only inhibit growth and suppress enzyme synthesis in a parallel manner, but also inhibit net incorporation from the free amino acid pool into the protein fraction (2, 3). Complete and specific reversal was achieved by the addition of the corresponding homologous amino acid. No evidence for a direct amino acid–independent transformation of a complex precursor into active enzyme was obtained. In studies employing cells in which pool levels were modified by a series of nitrogen starvations and replenishments, a strong correlation was established between the enzyme-synthesizing capacity and the availability of the internal amino acids for protein synthesis (4). A net utilization of the internal free amino acids also was demonstrated as a result of the induction of enzyme synthesis in resting cells (5).

These observations possess considerable implications for a rational examination of the problem of the enzyme precursor in yeasts. It soon became obvious

that to properly analyze this problem, an adequate understanding of the behavior of the free amino acid pool, of the factors controlling its size and availability, and of an experimental control over its composition would be necessary. The present paper represents an examination of the properties and behavior of the pools in yeast and a comparison of these pools with those found in other organisms.

Methods and Materials

Unless otherwise specified, a diploid representative of *Saccharomyces cerevisiae* (strain K) was used in this study. It was grown in a nutrient medium prepared by adding the following to 1 liter of water: glucose, 40 gm.; Difco bactopeptone, 5 gm.; Difco yeast extract, 2.5 gm.; ammonium sulfate, 2 gm.; calcium chloride, 0.25 gm.; magnesium sulfate, 9.25 gm.; and 60 per cent sodium lactate, 6 ml. Log phase cells were employed, and these were prepared by inoculating 500 ml. of the nutrient medium with 0.2 ml. of a 24 hour culture. The resulting suspension was allowed to incubate unagitated at 30°C. for 12 hours. In some experiments a synthetic medium was used. A modified Burkholder (6) synthetic medium was prepared by omission of the asparagine and by the addition of 5.9 gm. of succinic acid per liter to increase the buffering capacity at pH 4.5.

Cells were harvested by centrifugation immediately prior to the experiment 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. Unless otherwise stated, the cells were suspended in a nitrogen- and carbohydrate-free, modified Burkholder's synthetic medium.

Enzymatic activities were determined manometrically by the rate of aerobic or anaerobic CO_2 evolution from 3 per cent maltose at 30°C. using standard Warburg apparatus. Anaerobic conditions were established by flushing with nitrogen. The conditions of induction and the measurement of enzyme synthesis were the same as those previously described (2).

The free amino acid pools were collected by the methods devised by Gale (7). Their components were analyzed by the use of specific decarboxylases (8, 9), paper strip chromatography (10), and Kjeldahl nitrogen (11). The details of the applications of these methods to yeast have been described previously (2).

Crystalline samples of amino acids, purines, and pyrimidines were obtained from the Nutritional Biochemical Corporation. These were dissolved in buffer and adjusted to pH 4.5 with HCl or NaOH. Merck's maltose was purified further by recrystallization from 50 per cent alcohol.

Experimental Results

1. Pool Composition.—Employing specific amino acid decarboxylases, Taylor (1) was able to demonstrate detectable levels of arginine, glutamic acid, histidine, lysine, and tyrosine in the free amino acid pools of three strains of yeast. Other investigators have confirmed these results and extended the analysis to include sixteen amino acids as components of this pool (4, 12–14). In the

strain K of *S. cerevisiae* that was studied extensively in this investigation, the major components are glutamic acid, alanine, aspartic acid, serine, and lysine. As will be noted later, the levels of serine and lysine are dependent upon the conditions of growth of the culture and in certain cases may be present only in trace amounts.

In order to collect further information on the extent of the internal pool of amino acids in yeasts, a survey was made of a variety of strains of yeasts. The pools which were collected by boiling harvested, log phase cells (15) were analyzed by the paper chromatographic method of McFarren (10). The results obtained are shown in Table I. It is apparent from this table that qualitatively

TABLE I

Free Amino Acid Pool Composition in Various Yeast Strains as Determined by Paper Chromatography

Strain	1*	2	3	4	5	6	7	8	9	10	11	12
P. strain α (haploid)	+++	++	++	+++	++	++	++	+	+	++	+	−
R. 427 a "	++	++	+	++	+++	++	++	tr.	tr.	++	+	+
" " α "	+++	++	+	+++	+++	++	++	+	+	++	−	−
" *cerevisiae sex* (haploid)	++	++	++	+++	++	++	+++	+	+	++	+	−
" " (strain K)	+++	++	++	+++	+++	++	+++	+	+	++	+	−
"*carlsbergensis* (Y-379)	+++	++	++	++	+++	++	+++	+	+	−	−	−
" " (Y-1005)	+++	+	++	++	+++	+	+++	+	+	+	−	−
" *fragilis* (VN)	+++	++	++	+++	+++	+	++	+	+	+	−	−
" *chevalieri*	+++	++	++	+++	+++	++	++	+	+	+	+	−
" *ludwigii*	+++	++	tr.	++	+++	++	+++	+	tr.	+	−	−
" *italicus* (Y-1434)	++	+	+	++	++	++	++	tr.	+	−	+	−
C₁ α *methyl* +	+++	++	++	+++	+++	++	++	+	+	+	+	−

* Amino Acids

1. Alanine	5. Glutamic acid	9. Threonine
2. Serine	6. Aspartic acid	10. Arginine/valine
3. Glycine	7. Glutamine/alanine	11. Tyrosine
4. Leucine	8. Lysine	12. Hydroxyproline

the compositions of the various pools are reasonably constant. Only arginine plus valine, tyrosine, and hydroxyproline levels appear to vary extensively among the strains studied. In all these cases alanine, leucine, glutamic acid, and glutamine plus alanine appear to represent the major components of the pool. Similar results have been reported by Bair and Rouser (14).

A quantitative analysis of the pool and total cell nitrogen and also of the free glutamic acid pool contents of these cells is seen in Table II. Although the free glutamic acid nitrogen may vary between 9 and 28.6 per cent of the pool nitrogen, the pool nitrogen ranges only between 5 and 12 per cent of the total protein nitrogen of these cells. The data of this table clearly illustrate that the free glutamic acid analysis does not represent an accurate estimate of the size of the pool among a variety of strains. In the case of the haploid R427 for example, although only 5 per cent of the total nitrogen is present in the

pool, free glutamic acid comprises 28.6 per cent of the nitrogen of this pool. As will be shown here and elsewhere (4, 5, 16), free pool glutamic acid represents a fairly close index of behavior of the other pool components of *S. cerevisiae* (strain K) under various physiological conditions. It is also interesting to note from Tables I and II that the haploid stocks contain a consistently higher content of free glutamic acid. These findings may be related to the lower levels of RNA and DNA reported in haploids by Ogur *et al.* (17).

2. Effect of Growth Medium on Pool Composition.—Employing procedures previously described, pools were collected from log phase cells grown either in

TABLE II

Survey of Free Amino Acid Pools in a Variety of Yeast Strains

Cells were grown in complete medium and harvested at the end of the log phase. Pools were analyzed for glutamic acid (GA) and total nitrogen content.

Strain	GA/100 mg. dry cells		Total mg. pool N/100 mg. dry cells	Per cent pool N as GA
	μM	mg. N		
P. strain α (haploid)	23.5	0.225	1.13	19.9
R. 427 a "	23.8	0.228	1.342	17.0
" " α "	21.1	0.210	0.713	28.6
S. cerevisiae sex α (haploid)	18.2	0.174	1.92	9.05
" " (strain K)	13.8	0.132	0.505	26.1
" carlsbergensis (Y-379)	10.2	0.096	0.781	12.3
" " (Y-1005)	10.3	0.097	1.038	9.35
" fragilis (VN)	11.2	0.107	1.13	9.5
" " (Y-1342)	12.3	0.118	1.24	9.5
" chevalieri	13.2	0.126	1.24	10.2
" ludwigii	14.1	0.135	0.921	14.6
" italicus (Y-1434)	16.4	0.157	1.67	9.4
" cerevisiae (V.N.)	22.6	0.216	2.20	9.8
C_1 α methyl +	13.2	0.126	1.24	10.2

broth or synthetic medium employing glucose or a triose as an energy source. The results of paper chromatographic analysis of these pools are shown in Table III.

The results presented in this table demonstrate that with glucose as the major energy source, relatively little difference is observed in the pools between cells grown in complete or synthetic medium. Taylor has reported similar results with pools obtained from a Dutch top yeast grown in a medium either deficient or free of amino acids (18). From the results of table 3, however, several of the amino acids almost disappear from pools of cells grown on either pyruvate, lactate, or glycerol. Of particular interest is the almost complete absence of serine and glycine from such cells. Since it has been shown that serine and glycine are precursors for porphyrin biosynthesis (19–21), the lower

levels of these two amino acids in triose-grown cells may be a consequence of a greater demand for porphyrin synthesis in such cells that are known to contain primarily an aerobic metabolism.

Another example of varying the pool composition by modifying the growth medium was recently reported by Nagi (22). He also found that when yeasts are grown in a medium containing hypertonic levels of NaCl, a number of the components of the pool disappear. Only glutamic acid, alanine, histidine, and small amounts of aspartic acid are found in such cells.

3. Effect of the Age of the Culture on the Pool Content.—Taylor has reported that the levels of lysine, glutamic acid, and arginine in pools of a Dutch top

TABLE III

Composition of the Free Amino Acid Pool of Strain K Grown on Different Carbon Sources as Determined by Paper Chromatography

Cells were harvested near the end of the logarithmic phase. The synthetic medium employed was Burkholder's (6) modified by omission of asparagine.

Amino acid	Glucose broth	Glucose synthetic medium	Pyruvate synthetic medium	Lactate synthetic medium	Glycerine synthetic medium
Aspartic acid	++	+	+++	++	+
Glutamic acid	+++	++	+++	+++	+++
Serine	++	++	tr.	tr.	tr.
Glycine	++	+	tr.	tr.	tr.
Glutamine + alanine	+++	++	+++	++	+++
Tyrosine	++	+	++	tr.	tr.
Threonine	+	+	+	tr.	tr.
Lysine	+	+	+	tr.	tr.
Valine	++	+	++	+	+
Leucine	++	+	++	+	+

yeast grown in an amino acid–rich medium are influenced by the age of the culture (18). Early log phase cultures possess a considerably higher level of these amino acids. In order to study the influence of age on the synthesis of the components of this pool, uncomplicated by amino acid assimilation, a similar study was undertaken using *S. cerevisiae* (strain K) grown in synthetic medium. For purposes of comparison, the behavior of a lysine-deficient strain, grown in a limiting concentration of lysine was also examined. The results, shown in Fig. 1, indicate that the lysine contents of pools from strain K are relatively constant throughout the growth curve. On the other hand, the internal concentration of lysine in the mutant (X16-3.4) rises rapidly and approaches a maximum at 25 hours. The rapid drop in pool lysine corresponds with its disappearance from the external medium. Free glutamic acid, however, shows a slight rise at 20 hours in both strains, and then remains relatively constant until the cells go into the stationary phase.

4. Depletion of the Pool.—

(a) *Effect of the Energy Source.*—One of the most successful methods for influencing both pool levels and pool composition was the employment of nitro-
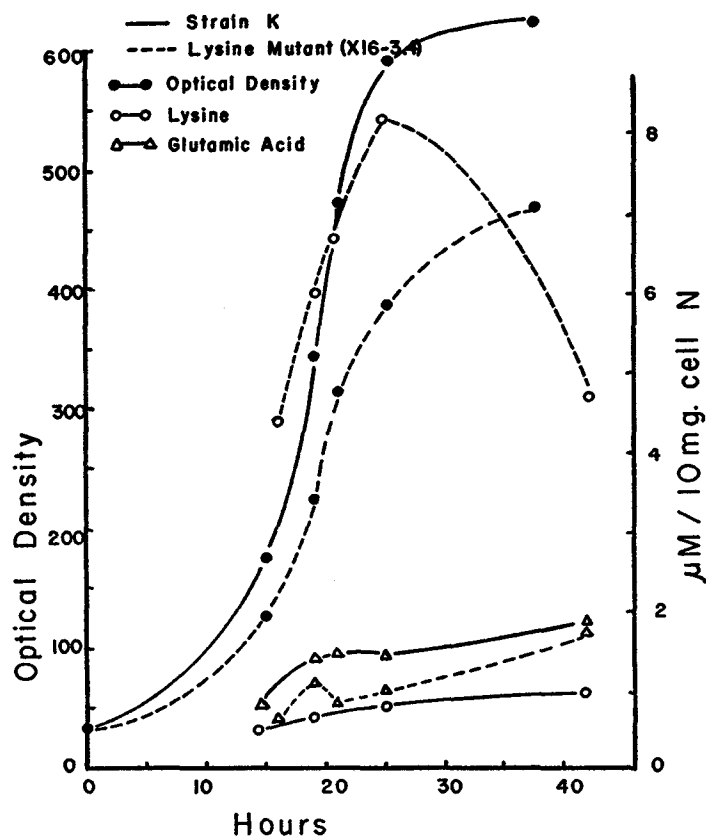

Fig. 1. The effect of age of the culture on the pool content. Strain K was grown in synthetic medium, and the lysine-deficient mutant (X16-3.4) in synthetic medium containing 2 mg./ml. of L-lysine. The cultures were aerated at 30°C. on a rotary shaker. At intervals, aliquots were withdrawn for measurements of optical density in a Klett-Summerson photoelectric colorimeter or analysis of pool levels of lysine and glutamic acid by the decarboxylase procedure.

gen starvation and replenishment (4). The levels of all the recognized pool components can be readily lowered by exposing cells to nitrogen-free medium containing glucose. Such depletions of glutamic acid, arginine, and lysine are shown in Fig. 2. These results further illustrate the validity of using the glutamic acid levels as an index of the behavior of the other components of the pool in this organism.

The substitution of glucose by other energy sources during the pool deple-
tion of glucose broth–grown cells was found to markedly influence the nitrogen
depletion procedure. This is illustrated by the data of Fig. 3. Here cells were
prepared in nitrogen-free medium and the resulting suspensions subjected to
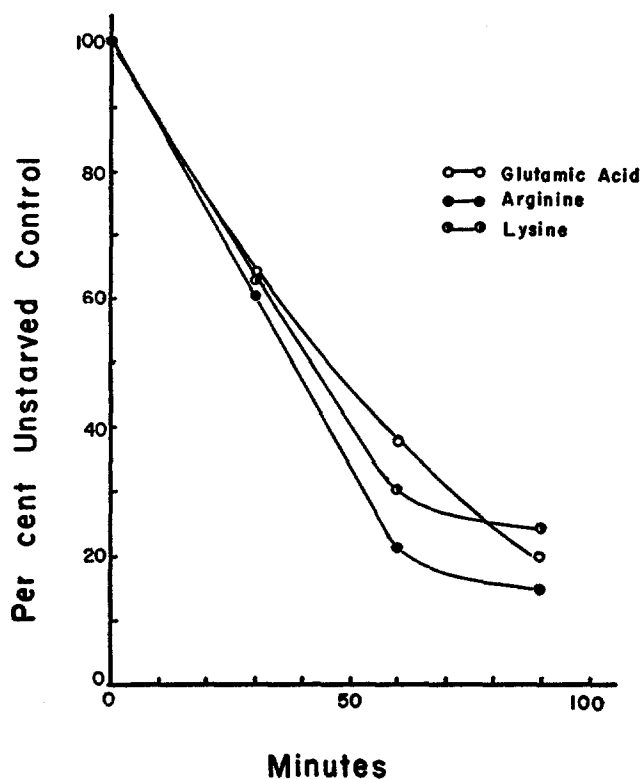

Minutes

FIG. 2. The depletion of the free amino acid pool during glucose metabolism. Log
phase, washed cells were suspended in N and carbohydrate-free synthetic medium
containing 3 per cent glucose. The suspension was aerated on a rotary shaker at
30°C.; and at various intervals, aliquots were removed. After washing the cells, free
amino acid pools were prepared and assayed by the use of specific decarboxylases.

starvation procedures in the presence of 3 per cent glucose (A) and 3 per cent
ethyl alcohol (B) as the energy sources. It is evident that ethyl alcohol (B) is
not depleting the pool, but rather a consistent rise is observed above the initial
pool levels. A paper chromatographic analysis of these pools revealed that all
components were rising in a similar manner. That the ethyl alcohol does not
of itself prevent the incorporation of the free amino acids into protein, is dem-
onstrated by the control experiment (C) of fig. 3 in which the ability of glucose

to deplete the pool was examined in the presence of 3 per cent glucose and 3 per cent ethyl alcohol. The depletion in the presence of glucose (A) is not influenced by the presence of ethyl alcohol.

Alcohol oxidation is not the only condition resulting in pool rises, as illustrated by the data of Table IV. Glucose-grown cells were exposed to their endogenous reserves, glucose and pyruvate. At various intervals the free glutamic acid levels were measured. Although glucose reduced the pool levels,
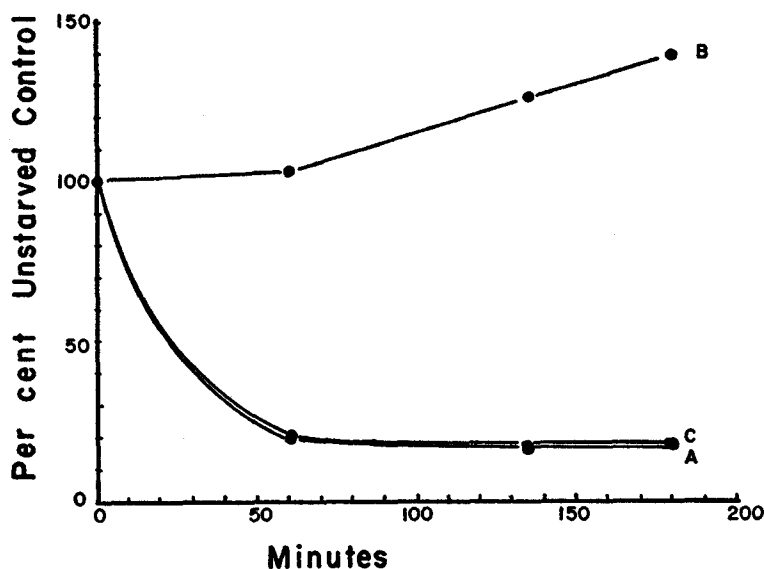

Fig. 3. The effect of the energy source on pool depletion. Log phase, washed cells were suspended in N and carbohydrate-free synthetic medium containing (A) 3 per cent glucose, (B) 3 per cent ethyl alcohol, and (C) 3 per cent glucose and 3 per cent ethyl alcohol. After intervals of aeration at 30°C. on a rotary shaker, aliquots were removed and the free glutamic acid levels determined by the decarboxylase procedure.

both the endogenous respiration and pyruvate led to a rise rather than a fall in pool contents.

The above results represent a contradiction, since the strain K of *S. cerevisiae* can grow in synthetic medium with either ethyl alcohol or pyruvate as a source of carbon and energy. It is thus obvious that these compounds serve as carbon sources for amino acids and provide energy for their incorporation into protein. The most reasonable explanation seemed that the glucose-grown cells were not adapted to the adequate utilization of these compounds. To test this possibility, the effect of prior growth on trioses was examined for their ability to support pool depletion. Cells were grown in the various media indicated and then subjected to "starvation" in the presence of various substrates

in nitrogen-free synthetic medium. The pool depletion of glutamic acid and the Q_{O_2} and Q_{CO_2} of the various cells on these substrates were determined. The results obtained are shown in Table V.

TABLE IV

Comparison of Glucose, Endogenous, and Pyruvic Acid as Energy Sources for Pool Depletion

Conditions are similar to those described in Fig. 3. Numbers express glutamic acid content of pools expressed as percentages of unstarved controls.

Duration of starvation	Substrate		
	Glucose	Endogenous	Pyruvate
min.			
70	21	135	118
140	21	—	136
210	21.2	165	185

TABLE V

The Effect of Prior History on the Ability of Various Carbon Sources to Effect Depletion of Free Amino Acid Pools

Cells (strain K) were grown on the media indicated and subjected to depletions in nitrogen-free synthetic media with indicated carbon compounds as sole energy sources. The numbers represent the glutamic acid content of the free pools as percentages of that found in the unstarved controls. The right-hand side lists the corresponding rates of respiration and fermentation achieved on the substrates indicated.

Growth and medium	Substrate during 80 min. starvation						Endogenous		Glucose		Lactate		Pyruvate		Ethyl alcohol	
	Endogenous	Glucose	Glycerol	Pyruvate	Lactate	Ethyl alcohol	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}
Glucose complete (standing)	183	29	167	147	173	—	23	21	344	47	30	28	24	21	17	38
Glucose complete (shaking)	118	17	116	161	156	100	20	20	115	71	35	32	45	28	16	53
Synthetic pyruvate	73	13	—	85	71	48	—	—	—	—	—	—	—	—	—	—
" lactate	—	—	—	50	46	—	7	6	266	100	74	70	81	69	43	88
" glycerol	—	10	51	—	—	—	10	9	138	86	49	47	88	63	44	92

It is evident from Table V, that glucose is an effective energy source for pool depletion independent of the prior history of the culture. However, with glucose-grown cells the endogenous or other substrates led to considerable rises in the pool levels. In the case of cells grown in pyruvate, lactate, or glycerol consistent depressions in pool levels are observed as a result of the metabolism of these compounds in nitrogen-free medium. Thus, prior history profoundly influences the process of depletion of the pool with a particular energy source.

An examination of Table V suggests that in part the answer may lie in the rate of energy supply. In glucose-grown cultures the respiration and fermentation of pyruvate are not noticeably above the endogenous levels. On the other hand, in either lactate or pyruvate-grown cells they are considerably elevated, and in such cells pyruvate effects a pool depletion. The results obtained with ethyl alcohol, however, indicate that this is not the entire answer. Cells which are grown in aerated, dextrose-complete medium possess a Q_{O2} of 53.5 on ethyl alcohol. Nevertheless, this compound shows no capacity to deplete the pool. Correspondingly, glucose-grown cells which have a Q_{O_2} of 47 on glucose undergo extensive pool depletion in the presence of glucose.

TABLE VI

The Effect of Anaerobic Conditions and Various Inhibitors on the Aerobic Depletion of the Free Amino Acid Pool

The conditions are the same as those described in Fig. 2. The cells were starved in the suspending medium indicated containing 3 per cent glucose for 70 minutes. The numbers represent the glutamic acid content of the free pools as percentages of that found in the unstarved controls.

Incubation conditions	Suspending medium	Glutamic acid
Anaerobic	N-free synthetic medium	24
Aerobic	" " "	18
" 10^{-4}M azide	" " "	83
" 3×10^{-5}" "	" " "	62
" 10^{-4}" DNP	" " "	49
" 3×10^{-5}" "	" " "	18
" 10^{-2}" arsenate	M/15 phthalate buffer pH 4.5	110
" 10^{-3}" "	" " " " "	104
" 10^{-4}" "	" " " " "	100

In order to further test the energy dependency of the depletion process, starvation experiments were conducted in the usual manner with glucose anaerobically or aerobically in the presence of various inhibitors. The results recorded in Table VI indicate that pool starvation proceeds almost as well under anaerobic conditions as aerobically. Arsenate, azide, and DNP at levels in which they act as uncoupling agents (23–25) inhibit the depletion of the pool. It is thus clear that the process requires energy which can be obtained either aerobically or anaerobically from glucose.

(b) *Other Factors Influencing Pool Depletion.*—The ability of exogenous amino acids to influence synergistically amino acid assimilation (26) suggests that they might influence the incorporation of the pool components into cellular proteins. An examination of three of these—glycine, leucine, and arginine—with this process is shown in Table VII. Exogenous leucine and arginine lead

to a rise in free glutamic acid, probably through transamination (27). Glycine, however, has little effect on the incorporation normally observed. As will be noted in the succeeding section, these and similar results are related to the ability of the added amino acids to act as nitrogen sources for glutamic acid synthesis.

Ben-Ishe and Spiegelman (28) have recently observed that both protein synthesis and enzyme synthesis require the presence of a complete nucleotide pool in yeast. When this pool of nucleotides was depleted, either by the use of purine or pyrimidine mutants or under appropriate starvation conditions in the wild type, the rate of incorporation of pool components into proteins was markedly retarded. As in the case of *S. aureus* reported by Gale (26), the addition of purines and pyrimidines increases the rate of incorporation of pool components into protein in these cells. These results suggested an explanation

TABLE VII

The Behavior of the Free Amino Acid Pool to Depletion in the Presence of Exogenous Amino Acids

The conditions are the same as those described in Fig. 2. The amino acids were adjusted to pH 4.5 and added at a concentration of 0.067 M.

Amino acid	μM GA/100 mg. dry cells		
	Unstarved	Starved 70 min.	Unstarved control
			per cent
None	21.6	2.5	10.5
Glycine		3.6	16.5
Leucine		32.2	153
Arginine		67.0	320

for the residual 10 to 20 per cent pool level existing after prolonged nitrogen starvation. If nucleotide starvation occurred during the pool depletion period, then the elimination of one or more of the components of the nucleotide pool might preclude further amino acid pool starvation. That such is not the case is seen by the data of Table VIII, in which it can be seen that a complete mixture of purines and pyrimidines did not further reduce the residual pool level remaining after 120 minutes of nitrogen starvation.

In view of the importance of pyridoxal phosphate in reactions involving amino acids, it might be imagined that it is involved in the depletion process. Attempts to inhibit the pool incorporation by a metabolic antagonist of vitamin B_6, desoxypyridoxine, have consistently been negative. These results may merely reflect the inability to reverse the high level of vitamin B_6 known to be present in yeast and would only be definitive in the case of pyridoxal-deficient mutants grown in limiting concentrations of the vitamin.

The presence of a number of amino acid analogues (2, 3) has been demonstrated to prevent the disappearance of the pool during glucose metabolism.

The inhibitions are competitively reversed by their homologous amino acids. One interesting feature to emerge from these studies in yeast, which appears in contrast to the results observed by Gale and Folkes (29), is the fact that the presence of the analogue inhibits not only the net utilization of its homologue from the pool but also the net utilization of all the recognized components of the pool. The absence of an accumulation of peptides in pools in the presence of amino acid analogues led to the conclusion that the first stable intermediate in protein synthesis was sufficiently complex to demand the simultaneous utilization of a large proportion of the components of the pool. These differences between bacterial and yeast cells may reflect the extent of exchange reactions existing between the pool and proteins in these organisms.

TABLE VIII

Pool Depletion in the Presence of Purines and Pyrimidines

Log phase cells were prepared as previously described and distributed into three 40 ml. aliquots. Flasks 1 and 2 were used to prepare control pools of unstarved and starved cells. 2.5 mg. each of adenine, xanthine, hypoxanthine, guanine, thymine, cytosine, and uracil was added to the third flask, and the pH was adjusted to 4.5. Glucose, at a level of 3 per cent, was added to flasks 2 and 3. These flasks were then aerated at 30°C. for 120 minutes and pools prepared and assayed for glutamic acid by the decarboxylase procedure.

Flask	Treatment	μM GA/100 mg. dry cells	Per cent control
1	Unstarved control	19.20	100
2	Starved control	1.55	7.8
3	Starved with purines and pyrimidines	1.52	7.75

Gale and his coworkers (26) have found in bacteria that numerous antibiotics are powerful inhibitors of the assimilation and incorporation of amino acids into protein. The common antibiotics are, however, generally ineffective in yeasts. The only one thus far reported to inhibit the pool depletion is azaserine, o-diazoacetyl-L-serine (30).

5. *Replenishment of the Pool.*—(a) *Replenishment with Casein Digest and NH₄Cl.*—The restoration of the pool levels could be achieved readily by exposing nitrogen-starved cells to a medium containing a nitrogen source and glucose. The quantitative effects on the replenishment of eleven of the amino acids in the pool are shown in Table IX. These results demonstrate the possibility of varying the composition and extent of the replenishment by modifying both the nitrogen source employed and the extent of the replenishment procedure. In the case of casein digest, virtually all the components are restored to levels equal to or greater than the original unstarved levels. However, short exposure to NH₄Cl does not restore all the components (*e.g.*, methionine, threonine, arginine, proline, and lysine) to normal levels. Yemm and Folkes

have reported similar observations in *Torulopsis utilis* (31). It should be noted here that a more extensive exposure to NH₄Cl does restore all components to normal levels. In any case, such devices permit one to examine the effects of varying the pool levels on enzyme synthesis both during the exhaustion of the pool and its replenishment.

(b) *Replenishment with Individual Amino Acids.*—It was previously observed (4) when nitrogen-starved cells were employed, that the restoration of enzyme-synthesizing capacity upon exposure to exogenous NH₃ could be re-

TABLE IX

A Comparison of Free Amino Acid Pool Components of Unstarved, Starved, and Replenished Cells

Starvation was carried out by incubation in a nitrogen-free, 6 per cent glucose, synthetic medium for 12 hours at 30°C. Replenishment was accomplished by 15 minute incubation in the same medium containing either 1 per cent NH₄Cl or 0.5 per cent enzymatic digest of casein. The free amino acid pools were analyzed microbiologically.

Amino acid	μM/100 mg. dry cells			
	Unstarved	Starved	Replenished (casein digest)	Replenished NH₄Cl
Valine	1.57	0.68	2.36	1.40
Glutamic acid	10.0	3.10	15.6	10.9
Aspartic "	3.6	0.62	3.30	2.70
Histidine	0.97	0.15	0.43	0.21
Lysine	4.3	1.78	7.9	1.76
Isoleucine	0.64	0.35	1.26	0.51
Proline	0.92	0.15	0.83	0.26
Serine	3.67	1.9	6.9	3.0
Methionine	0.60	0.13	0.54	0.14
Threonine	2.20	0.64	1.20	0.82
Arginine	1.20	0.36	2.07	0.58

lated to the increase in pool glutamic acid. In order to extend this analysis and also gain an insight into the metabolic reactions involved in the synthesis of other members of the pool, replenishment experiments were conducted with individual amino acids. The effectiveness of the replenishment procedure is shown in Table X in which the abilities to support induced maltozymase and free glutamic acid synthesis were determined as percentages of an NH₃ replenishment. Chromatograms were also prepared of such replenished cells and the results are summarized in Table XI.

It is evident that in all cases the added amino acid was found in large amounts in the pool. In general, the ability to restore the glutamic acid content was accompanied by a corresponding increase in other pool components and also a restoration of induced enzyme-synthesizing capacity. Argi-

TABLE X

Pool Replenishment Employing Individual Amino Acids as Sources of Nitrogen

12 hour nitrogen-starved cells were exposed aerobically to a synthetic medium containing 0.067 M amino acids. Pools were collected from cells exposed to this medium containing 3 per cent glucose for 60 minutes. The maltase-synthesizing capacity was determined after incubating the cells in 3 per cent maltose and the various amino acids for 165 minutes. The numbers represent percentage replenishment or enzyme synthesis based on that achieved with 1 per cent $(NH_4)_2SO_4$ for the same periods of time.

Amino acid	Per cent pool re-plenishment	Per cent enzyme synthesis	Amino acid	Per cent pool replenish-ment	Per cent enzyme synthesis
Aspartic acid	56	11	Phenylalanine	18	7
Arginine	46	52	Tyrosine	18	28
Leucine	43	38	Valine	17	47
Serine	37	55	Lysine	15	7
Isoleucine	35	31	Cysteine	12	19
Alanine	27	55	OH-proline	10	27
Methionine	24	28	Histidine	5	12
Threonine	21	52	Glycine	4	7
Tryptophan	20	17	Control	4	7

TABLE XI

Chromatographic Analysis of Pool Replenishment Employing Individual Amino Acids as Sources of Nitrogen

The replenished pools were obtained from the experiments described in Table X. These were analyzed chromatographically by the method of McFarren (10). In each case the amino acid replenished was found in ++++ levels in the pools.

Amino acid replenished	Glutamic acid	Aspartic acid	Alanine /glu-tamine	Valine	Arginine	Isoleu-cine/ norleu-cine	Serine	Tyro-sine	Glycine
Aspartic acid	++++	++++	+++	++		++	++	++	
Arginine	++	+	+	+	++++				
Leucine			+						
Serine	+	+	++	+			++++		
Methionine	++		+						
Threonine	++	+	++	+					
Phenylalanine	++	+	++	+	+	+			
Valine	++	+	+	++++			+		+
Lysine	++	+	++	±	±	±	++	+	
OH-proline	++		++						+
Histidine	±	±	±						
Glycine				+					++++

nine, serine, isoleucine, and leucine were the best sources of nitrogen for both glutamic acid and enzyme synthesis. Several exceptions are noted however. Thus, the 60 minute replenishment with aspartic acid, although it increased the amounts of glutamic acid, alanine, valine, and others, failed to influence

the levels of arginine and threonine. Lysine had a similar failure with respect to valine, arginine, and isoleucine. These results may explain the inability of these amino acids to influence enzyme synthesis in spite of their ability to elevate the glutamic acid levels in the pool.

On the other hand, both glycine and histidine are neutral sources of nitrogen. The inertness of glycine was demonstrated previously, since it had no detectable influence on the ease with which a free amino acid pool could be depleted. Leucine and arginine, which prevented pool depletion, are, however, good sources of nitrogen for the synthesis of the components of the free amino acid pool. These results illustrate not only the usefulness of glycine in tracer experiments but also illustrate the methodology of examining transamination reactions *in vivo* in a manner similar to that employed in studying pathways of amino acid biosynthesis in bacteria (32).

(c) *Internal Replenishment Mechanism.*—In previous investigations (4, 5) it was observed that a close relationship existed between free amino acid pool levels and enzyme-forming capacities in cells which had first been nitrogen-starved for 12 hours and then exposed for brief periods to exogenous nitrogen to replenish their free amino acid pools. During a second nitrogen starvation of the same cells, the pool levels and enzyme-synthesizing abilities were abolished in a parallel manner within a 60 minute period. An examination of cells which had been starved for shorter intervals indicated that under certain conditions enzyme synthesis is independent of pool size. These results, shown in Fig. 4, indicate that although a nitrogen starvation of 90 minutes reduces the pool content 90 per cent, its minimal level, the enzyme-synthesizing capacity is decreased only 37 per cent. With such cells, nitrogen starvation had to be continued for 400 minutes to eliminate enzyme synthesis.

In partially starved cells the inhibition of enzyme synthesis by *p*-fluorophenylalanine, (compare curves A and B, Fig. 4), indicates the role of the free amino acid pool in this process. This inhibition can be reversed by phenylalanine in a manner similar to that observed previously in unstarved cells (2).

The observations recorded in the earlier sections indicate that pool levels are not a total measure of the available amino acids. It was observed that under conditions of insufficient energy supply, the pool would rise in some cases over 80 per cent. Paper strip chromatograms demonstrated a parallel rise in each of its components. This increase in pool components could be prevented by energy inhibitors such as arsenate, DNP, and azide.

Some recent experiments involving irradiated cells (33) may provide a possible insight into the nature of the labile nitrogen precursor which contributes to the pool. A consistent rise in pool levels was observed in cells which were irradiated and then incubated in nitrogen-free, synthetic medium containing glucose. A typical experiment is shown in Fig. 5. Yeast cells were irradiated at various intervals during the course of a normal nitrogen starvation proce-

dure. Unstarved cells show only a slight rise in free glutamic acid after irradiation. However, cells which were nitrogen-starved for 30 to 70 minutes show a considerable elevation in free glutamic acid after such treatment. The extent
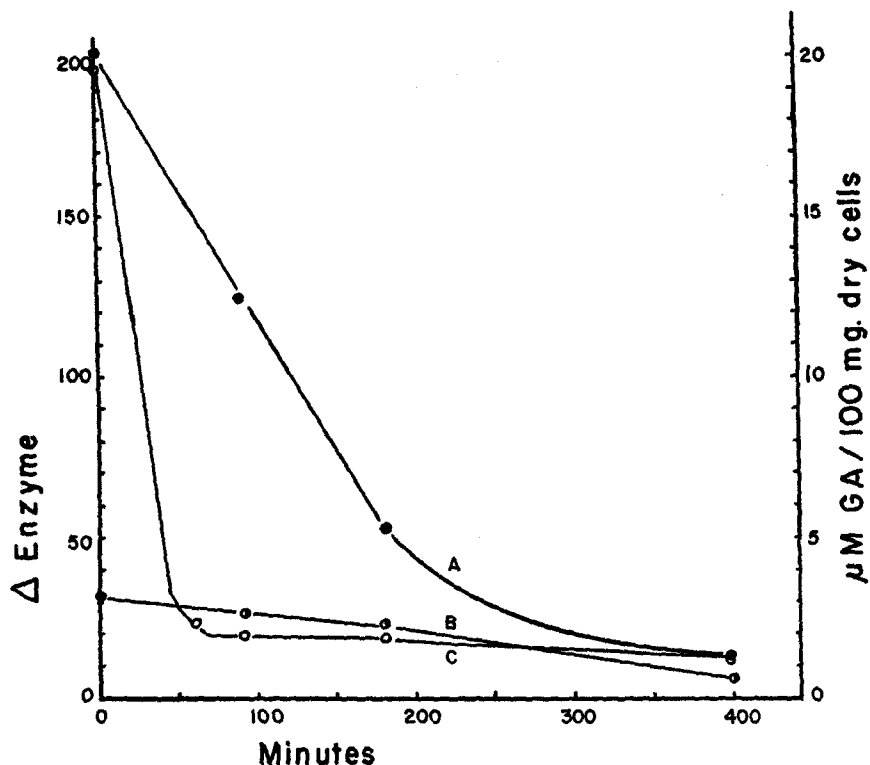

FIG. 4. The effect of nitrogen starvation on the pool level and the enzyme-synthesizing capacity. Log phase cells were nitrogen-starved using glucose as an energy source. At intervals, two aliquots were removed. The first was washed and resuspended in synthetic medium containing 3 per cent maltose. The maximum maltase activity was determined as previously described in the absence (A) and presence (B) of 0.01 M p-fluorophenylalanine. The second aliquot was used to determine the pool level of free glutamic acid (C).

of this rise increases in the early stages of the starvation procedure, and is somewhat reduced following prolonged nitrogen starvation periods. A paper strip chromatographic analysis of the pool which accumulates after irradiation indicated that it involved all the free amino acid components.

(d) *Amino Acid Uptake.*—At present, little information is available on the interactions between exogenous amino acids and the process of internal replenishment indicated in the previous section. It therefore seemed advisable

in studies on the properties of amino acid assimilation to employ cells in which nitrogen starvation was prolonged so that complications arising from internal replenishment were largely or entirely eliminated. Another problem in such investigations arises from the rapidity of other reactions in which these pool components can be involved, of which the incorporation of the pool components into protein and the transamination reactions are quantitatively the most
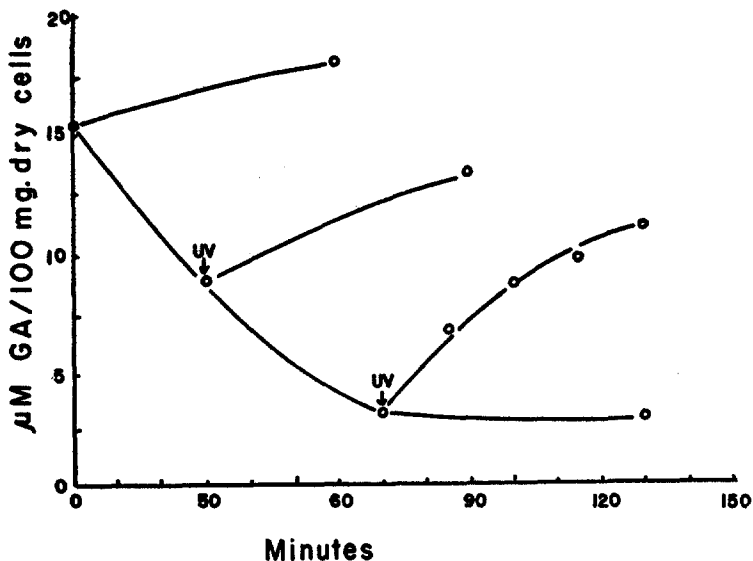

Minutes

FIG. 5. The rise in pool levels following irradiation. Log phase cells were nitrogen-starved as previously described. At 0, 50, and 70 minutes, 50 ml. aliquots were removed, resuspended in distilled water, and irradiated for 150 seconds in open Petri dishes (5.5 inches in diameter) placed at a distance of 15 cm. from a low pressure, 15 watt General Electric germicidal bulb (2537 A). After irradiation, the cells were resuspended in buffer and the nitrogen starvation procedure continued. The points represent analysis of the free glutamic acid by the decarboxylase procedure.

important. Roine (12) has shown that when low nitrogen yeast cells are exposed to exogenous nitrogen, within 20 minutes there is a rapid increase in soluble pool N. Experiments in our laboratory have also demonstrated that there was neither a significant loss of the assimilated amino acid through transamination nor the appearance of other free amino acids within a 10 to 15 minute period. Thus, a 5 to 10 minute exposure to an exogenous amino acid was adopted as a measure of the assimilatory process.

The effect of external concentration on the rate of entry of amino acids was studied employing the above procedures. The results obtained from decarboxylase analysis of free arginine, glutamic acid, and lysine are shown in Fig.

6. In all cases the rate of entry increased rapidly at first as the external concentrations of the amino acid were increased but was constant for high external
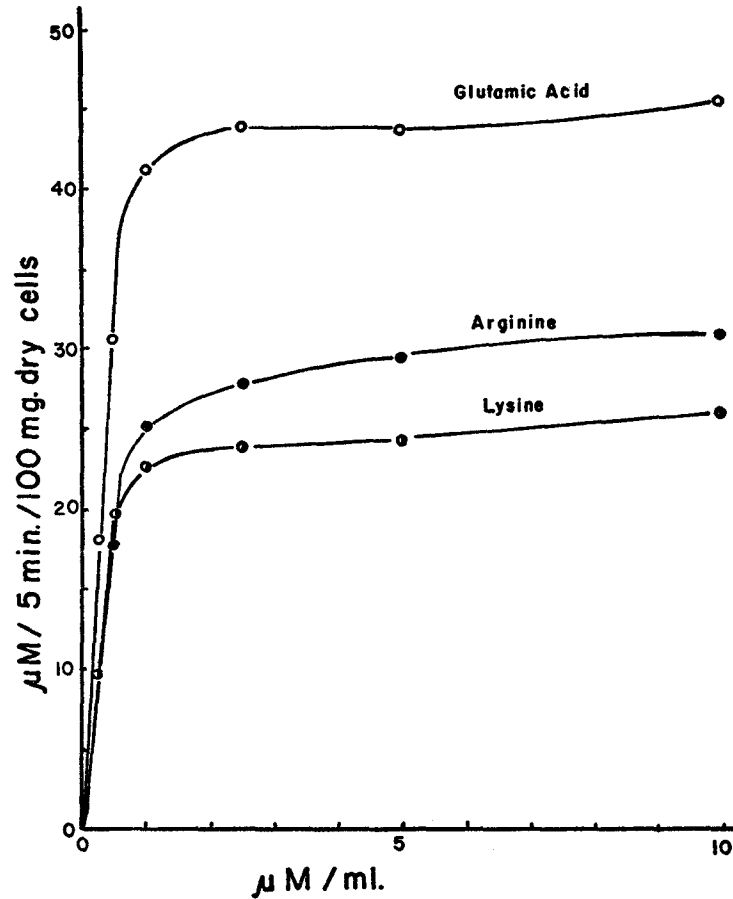

FIG. 6. The assimilation of amino acids by prolonged nitrogen-starved cells. 12 hour nitrogen-starved cells were exposed to varying concentrations of glutamic acid, arginine, and lysine for 5 minutes in tubes which were aerated at 30°C. After the cells had been washed twice with distilled water, pools were prepared and assayed by the decarboxylase procedure. The points plotted represent the values obtained, corrected for the endogenous levels in long starved cells.

concentrations. The half-maximal rate for each of the three amino acids was approximately the same. The similarity of each of the curves in Fig. 6 suggested that a similar mechanism may be involved for each of the amino acids. It was interesting to note that lysine uptake does not appear to be a simple diffusion process as occurs in Gram-positive bacteria (26). Bigger (27) has observed similar results in unstarved yeast cells.

As shown in Fig. 7, the effect of temperature on the rate of uptake of glutamic acid, arginine, and lysine in prolonged nitrogen starvation in yeast was that which one would expect for an enzymatic reaction. Although the uptake
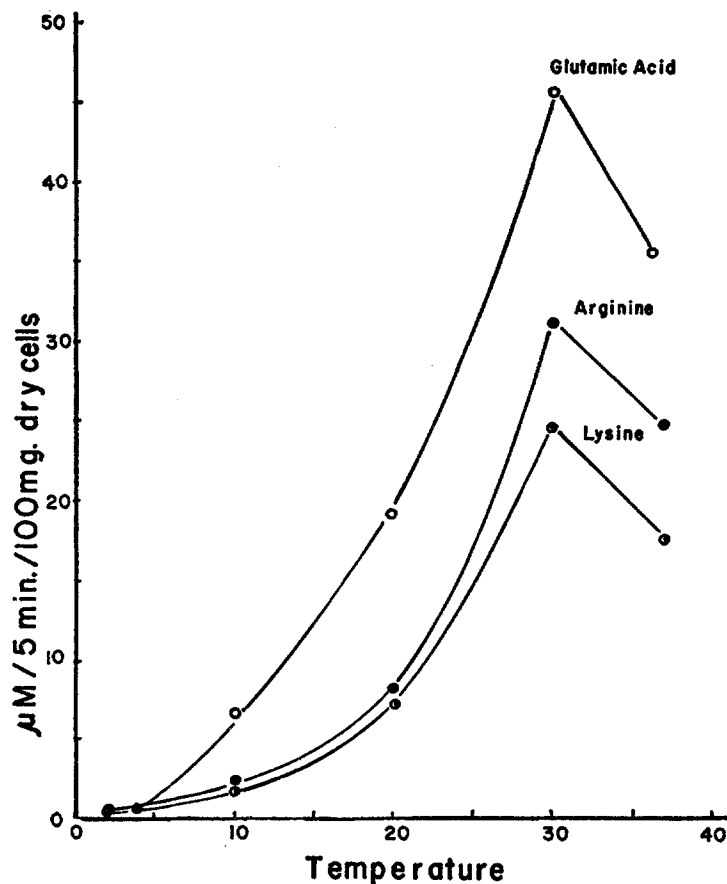

FIG. 7. The effect of temperature on the assimilation of amino acids by cells after prolonged nitrogen starvation. The conditions are the same as those described in Fig. 6. The amino acids were added at a level of 10 μM/ml. The values represent net uptake of amino acid.

was insignificant at 1°, it rose rapidly with elevated temperatures. At 37° the rate was decreased in a manner analogous to the effect of temperature on the growth of these organisms. The temperature coefficients over the range 20 to 30° for glutamic acid, arginine, and lysine were 2.5, 3.6, and 3.4 respectively.

Glutamic acid and lysine uptake by unstarved yeasts (18) requires simultaneous glucose metabolism. The effect of azide, arsenate, and DNP that was

obtained on the accumulation process is shown in Table XII. It is evident from these and other results that the accumulation of amino acids by yeasts is an energy-requiring process. The assimilation of NH_3 was also shown to be inhibited by DNP (31). This energy dependence was further substantiated by the fact that the age of the culture and the pH for optimal uptake were similar to those providing optimal conditions for glucose metabolism. The observation by Taylor that the presence of other amino acids inhibited glutamic acid uptake (18), and the reported loss of both sodium and potassium from yeast during lysine uptake (34) suggest that the accumulation process is perhaps as complicated as that observed in bacteria (26) and mammalian cells (35).

TABLE XII

The Effect of Various Inhibitors on the Uptake of Amino Acids by Long Starved Cells

12 hour nitrogen-starved cells were exposed aerobically for 5 minutes to a medium containing 3 per cent glucose and 10 μ m/ml. of amino acids in the presence and absence of various inhibitors. Nitrogen-free synthetic medium was used for the control, DNP, and azide experiments and m/15 phthalate buffer adjusted to pH 4.5 for the arsenate experiments. Pools were then examined for arginine, lysine, and glutamic acid by the decarboxylase method. The figures represent the per cent net increase of the pool levels compared to the control values.

Amino acid	DNP*	Azide*	Arsenate‡	
			Aerobic	Anaerobic
Glutamic acid	6.3	22	97	110
Lysine	6.1	34	94.5	90
Arginine	5.7	38	92.5	107

* 2×10^{-4} M.

‡ 2×10^{-2} M.

The extent of accumulation of glutamic acid, arginine, and lysine by long starved cells was estimated by determining the concentration gradient established across the cell wall. 40 ml. suspensions of cells were exposed for 10 minutes to 10 μM of amino acid. The cells were then harvested by centrifugation and washed three times with water. This procedure was sufficient to completely remove the exogenous amino acids. The supernates were concentrated *in vacuo*, and analyzed, as were the pool contents, by the decarboxylase assay. The total cellular volume was estimated by centrifuging the cells for 20 minutes at 10,000 R.P.M. in a Servall centrifuge. Although this admittedly gives a maximal approximation of cellular volume, the results shown in Table XIII serve to illustrate the internal concentration process. Preliminary experiments suggested that long nitrogen starved cells accumulate amino acids to a higher extent than do unstarved cells.

DISCUSSION

Comparison with Other Organisms.—A wide variety of microorganisms have been observed to accumulate considerable quantities of an internal supply of

TABLE XIII

The Internal Concentration of Amino Acids by Nitrogen-Starved Cells

See text for details.

Amino acid	Cells			Supernate			Ratio molarity in cells/super-nate
	μM	Volume	Molarity	μM	Vol-ume	Molarity	
Glutamic acid	7.0	0.48	14.8×10^{-3}	2.8	40	0.07×10^{-3}	211
Arginine	9.1	0.48	18.9×10^{-3}	0.77	40	0.0192×10^{-3}	985
Lysine	8.8	0.48	18.3×10^{-3}	0.90	40	0.0225×10^{-3}	815

TABLE XIV

Comparison of Free Amino Acid Pools in Various Microorganisms

Property	*Saccharomyces cerevisiae*	*Staphylococcus aureus*	*Streptococcus fecalis*
1. Pool synthesis and maintenance			
N requirements	NH_4	Amino acids	Amino acids
Changes following N starvation	85 per cent lost	None	60 per cent lost
Internal replenishment	+	?	?
2. Amino acid assimilation			
Energy requirement	Glucose	Glucose	Glucose
Inhibitors: DNP*	+	+	−
Arsenate	+	+	−
Azide	+	+	−
Other amino acids	+	+	+
Salt changes: Na*	Uptake	−	−
K	"	Uptake	Uptake
Temperature coefficient: Lysine	3.4	1.4	1.4
Glutamic acid	2.5	2.7	2.4
Maximal concentration gradients	200–900	200–600	100–300
Release of peptides	−	+	?

* Glutamic acid.

amino acids. The role of these pools in biosynthetic reactions and their physiological control appear to vary among the various organisms. Some of these differences, reported here and elsewhere (26, 34), are summarized in Table XIV.

The present information suggests that bacteria and yeast may differ fundamentally in the role that the free amino acid pool plays in protein synthesis.

The net synthesis of cellular protein in Gram-positive bacteria can be eliminated by either failing to provide a complete mixture of amino acids or by adding a competitive antagonist of phenylalanine *p*-chlorophenylalanine (29), to an otherwise complete medium. As expected, this antagonist inhibited the incorporation of labelled phenylalanine into protein. The failure, however, of *p*-chlorophenylalanine to prevent the incorporation of labelled glutamic acid into the protein of the cell was an observation of considerable interest. These findings made it possible experimentally to separate incorporation due to net protein synthesis from that attributable to exchange reactions. The fact that such differences cannot be detected between the net incorporation of various pool components in yeast in the presence of amino acid antagonists (2, 3, 36) suggests that such exchange reactions may be more restricted in yeasts than in bacteria.

Internal Pool Replenishment.—One of the outstanding features to emerge from the studies in yeast was the existence of an internal mechanism which can, under certain conditions, replenish the free amino acid pool. This is a point of importance, for a number of reasons. In the first place, it tells us that the free amino acid pool, as analyzed in a freshly harvested cell, need not, and probably does not, constitute the sole available internal supply of amino acids in yeast. A paper strip chromatographic analysis of the rising pools during internal replenishment indicated that the material involved all the amino acids. This fact, as well as others which we shall subsequently mention, constitutes an important difference in the behavior of yeast as compared with *E. coli* which lack an amino acid pool and in which no such breakdown of preexistent protein has as yet been reported. An examination of enzyme-forming capacity indicated that the internal replenishment mechanism can be eliminated upon prolonged nitrogen starvation. If such cells are replenished by a short exposure to exogenous nitrogen, a close parallel is observed between pool levels and enzyme-synthesizing capacity upon subsequent starvation.

Evidently in freshly harvested cells the enzyme-synthesizing capacity does not, for a while, parallel the degree of free pool depletion in the course of nitrogen starvation. It would thus seem possible that preexisting protein components might well be stimulated to break down to their constituent amino acids and replenish the pool by an internal device. The loss of these labile components on prolonged starvation could easily explain why such cells, subject to a short replenishment, are strikingly sensitive to a subsequent starvation. A loss of this kind would be an inevitable consequence of prolonged nitrogen starvation. As the starvation process proceeds, the most labile proteins will break down easily and contribute to the free amino acid pool. A prolongation of nitrogen starvation which continually recycles material from the pool to protein, will tend to trap the amino acids in the least labile proteins—those least capable of supplying free amino acids to the pool.

Protein Synthesis in Escherichia coli and Yeast.—The recent experiments of

Cohn and Hogness (see discussion in reference 37) and Rotman and Spiegelman (38) have provided information on the problem of protein turnover in *E. coli*. These workers investigated the induced biosynthesis of β-galactosidase in growing cells of *E. coli*. All the proteins existing prior to the moment of induction were heavily and uniformly labelled. When the β-galactosidase was subsequently isolated from these cells suspended in unlabelled medium, no detectable label was found in the purified enzyme. Had the proteins broken down to their constituent amino acids during the induction period, they would have appeared in the enzyme synthesized. Therefore, it would seem that within the limits of detection, protein synthesis in growing cells of *E. coli* is virtually irreversible. Cohn and Hogness have further shown that the β-galactosidase itself does not turn over in either the presence or absence of the inducer.

It is obvious that these results in *E. coli* differ in some respects from those observed in yeast, for in the latter there are components in the cell which can break down to their constituent amino acids. A parallel situation may, however, exist between the virtually irreversible protein synthesis in *E. coli* and the more stable enzymes or proteins previously noted in yeast. These results further illustrate the necessity of examining individual protein components rather than total cell protein in studies on the problem of protein turnover in yeast.

The investigations of Virtanen and his group (39–41) have suggested a methodology for the problem of identifying stable yeast proteins. These workers examined the effect of lowering the nitrogen content on the enzyme patterns of bacterial cells. They distinguished two classes of enzymes; the indispensable enzymes, which retain their activity although the nitrogen content is lowered, and dispensable enzymes, which lose most or all their activity when the nitrogen content is decreased.

Catalase and protease are examples of the former group and lactase and saccharase of the latter group. Although it is clear that a loss of enzyme activity need not signify protein degradation, this first group presents promising material in which to search for stable proteins. It is hoped that such an approach, coupled with an examination of the ultimate fate of pools which have been replenished with labelled amino acids, may yield valuable information on the nature of the stable proteins.

SUMMARY

A study has been made of the distribution and properties of the free amino acid pool in yeast. The depletion of the pool was found to depend upon the

energy source used, conditions of growth, and the nature of the exogenous nitrogen source. Pool levels could be restored either by an internal replenish ment mechanism or by various nitrogen sources. In the absence of internal replenishment a strong positive correlation was established between the ability of nitrogen compounds to support free glutamic acid synthesis and enzyme-synthesizing capacity. Amino acid assimilation by nitrogen-starved yeast was studied and compared with that in other organisms. The significance of these results for the problem of enzyme and protein synthesis in yeast is discussed.

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