TRACER STUDIES OF NITROGEN ASSIMILATION IN YEAST

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The work of Caspersson (1) and others on the intracellular distribution of nucleic acids in cells has led to the concept that ribonucleic acids are intimately associated with the synthesis of protein. Furthermore, some evidence has been accumulated indicating that not only the concentration of ribopolynucleotide but also the metabolic activity increases during active protein synthesis. Thus tracer studies with radioactive phosphorus have indicated a more rapid turnover of the phosphate component of the polynucleotide when protein was being laid down (2–4). In the present report we have used N^{15} as a tracer to observe the formation of protein and nucleic acid purines in yeast. This organism was selected because of its rapid growth rate, because the state of its nitrogen metabolism could be readily controlled (5), and because ultraviolet microscopy had indicated the importance of nucleic acid in growth (6, 7).

EXPERIMENTAL

Yeast.—The yeast used in these experiments was a strain of *Torulopsis utilis* which had been adapted to growth on ethanol as described by Sperber (5). Samples of approximately 50 gm. of pressed yeast were grown in 8 liter stainless steel Kluyver flasks in a medium made up as indicated in Table I. After growth, the suspension was run through a separator and the sedimented yeast washed repeatedly in a centrifuge. Such yeast has a nitrogen content of approximately 7 per cent of the dry weight.

By omitting either the carbon or the nitrogen from the medium it is possible to prepare yeast with nitrogen contents as high as 9 per cent (high N yeast) or as low as 4.5 per cent (low N yeast). Sperber (5) has shown that high N yeast which has grown in excess ammonia contains many buds in various stages of development; active division begins immediately upon addition of a carbon source to the medium. Low N yeast, however, is ripe and contains very few cells in bud. As a result there is a lag period of 4 to 5 hours after addition of ammonia before division begins. Both these yeasts respire actively on endogenous substrate—high N yeast, 3.5 μl. O_2 consumed/hr./mg. fresh yeast; and low N yeast, 5.5 μl./hr./mg. (5).

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$N^{15}H_4^+$ Turnover in High N Yeast.—High N yeast was used as a control to study nitrogen turnover in cells that were actively respiring but were neither laying down protein nor dividing. Schoenheimer and coworkers have shown (8) that for mammals in nitrogen balance protein is in a "dynamic state," being continuously degraded and resynthesized. We maintained high N yeast in N^{15} ammonium sulfate to determine whether this microorganism, when in "nitrogen balance" also existed in a "dynamic state" as concerns nitrogen metabolism.

47 gm. of fresh high N yeast (9.8 per cent N) were suspended in the medium noted in Table I from which the alcohol was omitted. The ammonium sulfate concentration was 0.06 M with 28.5 atom per cent excess of N^{15}. After aeration in a Kluyver flask at 30° for 3.7 hours, the yeast was centrifuged down, washed twice with water, then with alcohol and ether, and dried.

In this and the other experiments described in this paper the dry yeast was fractionated into total protein and nucleic acid purine portions. This was done as follows: *Fragmentation:* To break the cell walls, the dry yeast was suspended in alcohol and mechanically vibrated with glass beads (50 cycles/sec. with 1 mm. amplitude for 36 hours (9)). The crushed material was washed with ether and dried. *Removal of phospholipids*: The fragmented yeast was boiled for 2.5 hours with an ethanol-ether mixture (3:1). Approximately 200 ml. solvent were used per 5 gm. yeast. This extracted yeast was washed with ether and air-dried. *Removal of acid-soluble nitrogen:* 10 gm. of solvent-extracted yeast were stirred for 1 hour in the cold room with 150 ml. 0.05 M HCl and then centrifuged for 30 minutes. This extraction was repeated 4 times. *Preparation of protein free of nucleotide:* The acid-washed yeast was extracted successively with 200 ml. 0.1 N NaOH, 150 ml. 0.1 N NaOH, and 75 ml. 0.2 per cent Duponol. The clear brown solution was neutralized with concentrated HCl and then 0.2 volume of 30 per cent trichloroacetic acid was added. The heavy suspension was kept at 90° for 10 minutes to hydrolyze and extract the nucleic acid (10). After cooling and centrifuging, the precipitate was resuspended in 250 ml. 5 per cent trichloroacetic acid and again heated to 90° for 10 minutes. A third extraction was carried out without heating. The extracts were combined as the nucleic acid fraction; the residue—the protein fraction—was washed 3 times with alcohol, twice with ether, and dried. *Separation of nucleic acid purines:* Concentrated HCl was added to the trichloroacetic acid extract to make the solution 1 M in HCl, and the purines liberated by refluxing for 1 hour. Then the pH was adjusted to 8–9 with NH_4OH and refluxing continued for 15 minutes to destroy the trichloroacetate. After aerat-

TABLE I

Composition of Medium for Yeast Growth

Tap water, *liter*	2.
KH_2PO_4, *gm*	4.5
$CaCl_2$, *gm*	0.4
$MgCl_2$, *gm*	0.4
C_2H_5OH, *gm*	30
$(NH_4)_2SO_4$, *gm*	9

ing to remove $CHCl_3$, the hot solution was filtered and chilled. Upon addition of a large excess of concentrated NH_4OH and 1 M $AgNO_3$ a white, flocculent precipitate of the silver salts of the purines settled out. Guanine and adenine were separated and purified as described by Levene (11), except that the adenine was not precipitated with picrate but was in part precipitated with β-naphthalene sulfonic acid and recrystallized and in part directly sublimed *in vacuo* at 210–220°. Ultraviolet absorption spectra indicated adenine was free of guanine and *viceversa*.

The fractions taken for analysis were converted to nitrogen gas as described by Rittenberg (12) and the N^{15} content determined in a 60° Nier type mass spectrometer. The results of these measurements are summarized in Table II.

TABLE II

$N^{15}H_4^+$ Assimilation by High N Yeast

Fraction analyzed	Atom per cent excess N^{15}
NH_4^+ in medium	28.5
Total yeast	0.12
Protein	0.04
Guanine	0.01
Adenine	0.01

There was no detectable fixation in the nucleic acid purines, a trace in the protein, and a small amount in the total yeast. Thus one must conclude that in this yeast in which no net assimilation of nitrogen occurs, there may be a small exchange of NH_3 with the "acid-soluble nitrogen," but synthesis of protein and nucleic acid purine takes place to a negligible extent.

$N^{15}H_4^+$ Turnover in Low N Yeast.—When ammonia is added to low N yeast there is a lag period during which nitrogen is actively assimilated but no growth takes place. $N^{15}H_4^+$ was administered to such yeast in order to trace its distribution in yeast which was synthesizing protein but not dividing.

50 gm. fresh low N yeast (6.3 per cent N) were suspended in 2000 ml. medium containing no carbon but 12.1 mM of $N^{15}H_4^+$ (15.6 atom per cent excess N^{15}). After 1 hour at 30° ice was added and the yeast centrifuged off and washed thoroughly with water. During this incubation, the nitrogen content rose to 8.0 per cent. After washing the yeast with cold 0.05 M HCl, purine analyses were carried out by the method of Graff and Maculla (13), and it was found that the content of purine N remained constant at 0.65 per cent of the dry weight. The experiment was repeated with larger amounts of yeast (24.1 gm. dry weight). The medium contained 24 mM of $N^{15}H_4^+$ (31.9 atom per cent excess N^{15}), and during 1 hour of incubation the N content of the yeast rose from 6.55 to 7.34 per cent. Again the purine content remained constant at 0.61 per cent of the dry weight. Only traces of NH_3 were left in the medium after incubation.

The yeast was fractionated as described in the foregoing section, and mass spectrometer analyses are summarized in Table III. In these two experiments 95 to 99 per cent of the NH_3 disappeared from the medium. Yet the N^{15} excess in the medium remained essentially constant indicating either the almost total absence of deaminative degradation processes, or a relatively slow rate of egress for NH_3 formed within the yeast cell. Despite the great assimilation of nitrogen there was no detectable increase in nucleic acid purine. In fact, after N assimilation the ratio of purine N to total N was markedly reduced. However, even though there was no net synthesis of polynucleotide purine, the turnover was rapid as indicated by the high N^{15} content.

TABLE III

$N^{15}H_4^+$ Assimilation by Low N Yeast

Fraction analyzed	Atom per cent excess N^{15}	
	I	II
NH_4^+ in medium		
Before incubation	15.6	31.9
After incubation	—	30.2
Total yeast	1.11	—
Protein	0.44	0.88
Guanine	0.32	0.73
Adenine	—	0.36

$N^{15}H_4^+$ Turnover in Dividing Yeast.—The preceding section indicated that turnover of nucleic acids was associated with nitrogen assimilation. Some further information on the course of protein synthesis in *Torulopsis* was obtained by using yeast which had been previously grown in $N^{15}H_4^+$ and measuring the dilution of N^{15} during subsequent growth in ordinary NH_4^+.

30 gm. fresh yeast were suspended in 4 liters of medium containing 28 mM of $N^{15}H_4^+$ (12.2 atom per cent excess N^{15}). Ethanol and $(N^{15}H_4)_2SO_4$ were added continuously during growth. After 5 hours (total N added was 65 mM), the suspension was run through a separator and the yeast washed thoroughly with water. One-half of the yeast (I) was removed for N^{15} analyses, and the other half resuspended in medium where the N^{15} had been replaced with ordinary N. After 45 minutes, ice was added to stop growth, and the yeast (II) washed and dried. The extent of growth during this period is indicated in Table IV.

In addition to the fractionations indicated in the previous sections of this paper, several amino acids were isolated from the protein fraction after hydrolysis with 20 per cent HCl. Dicarboxylic amino acids were prepared because of their central position in amination and transamination processes (14, 15). Basic amino acids were sought because basic proteins have been reported (1)

to play an important rôle in protein synthesis. The protein hydrolysate was separated into acid, neutral, and basic fractions by the electrolytic method of Sperber (16). Glutamic acid hydrochloride was precipitated from the concentrated acid fraction with strong HCl. Aspartic acid was obtained from the mother liquor as the copper salt. Arginine was precipitated from the basic fraction with flavianic acid. The latter was subsequently removed by electrolysis. The results of mass spectrometer analyses are summarized in Table V.

TABLE IV

Growth of N^{15} Yeast in Ordinary NH_4^+

Fraction	I	II
Dry weight, *gm*	5.49	6.58
Total N, *percent*	9.20	9.18
Protein N, *percent*	5.58	5.50
Nucleic acid purine N, *percent*	0.76	0.77

TABLE V

Distribution on N^{15} in Dividing Yeast

Fraction analyzed	Atom per cent excess N^{15}		Dilution
	I	II	
			per cent
Yeast	5.62	4.98	11.5
Protein	5.58	5.33	3.5
Guanine (nucleic acid)	4.53	4.68	−3.3
Glutamic acid	5.54	4.53	18.3
Aspartic acid	5.73	4.93	13.9
Arginine	4.22	4.35	−3.0

The values in Table V would indicate that most of the ordinary nitrogen had been incorporated in the acid-soluble fraction, the turnover in protein having been only a fraction of that expected from the growth of the yeast. The great dilutions in the glutamate and aspartate fractions indicate that these acids play prime rôles in the fixation of ammonia. This agrees with the work of Roine (15) who has shown that ammonia taken up by *Torulopsis* goes first to the glutamic acid and glutamine of the acid-soluble nitrogen.

In contrast to the dilutions of the N^{15} in the acid amino acids, the basic amino acid, arginine, remained essentially unchanged in N^{15} content as did the nucleic acid guanine. Thus the nitrogenous precursors of these molecules must have been preformed during growth in N^{15} and not significantly diluted during the short period of growth in ordinary nitrogen.

It might be pointed out that a comparison of Tables IV and V reveals that the *amount* of N^{15} in the yeast has remained constant during growth in ordinary N. This confirms the observation on low N yeast that NH_3 does not pass from the yeast cell into the medium.

DISCUSSION

The work of Schoenheimer and coworkers (8) has indicated the existence of an ammonia pool in a dynamic "equilibrium" with protein nitrogen. Thus even structural protein was reported to undergo continuous degradation and resynthesis. From our results, such a dynamic state between cell and medium seems absent from yeast. Once ammonia is incorporated in the yeast cell it is held permanently. Further work is necessary to determine whether this is caused by relatively irreversible NH_3 fixation or by a low permeability of the cell wall for NH_3 within the cell.

The rapid appearance of ammonia N in the dicarboxylic acids indicates the prime rôle these amino acids play in the fixation of ammonia. The transfer of N to other amino acids is a relatively slow process as indicated by the fact that yeast grown for 5 hours in N^{15} had not reached equilibrium with respect to N^{15} distribution and that the N^{15} of arginine was not diluted by subsequent growth in ordinary N. Further work is necessary to elucidate the exact pathways of NH_3 utilization in yeast.

The evidence that nucleic acids play a rôle in protein synthesis has been based upon observations indicating an increased content of ribopolynucleotide in cells which are laying down protein. We have not confirmed these observations, but rather have noted that during the rapid ammonia assimilation in low N yeast the nucleic acid content remains constant and thus the ratio of nucleic acid N to protein N actually decreases. (We have used purine which is not extracted with cold, dilute acid as a measure of nucleic acid content.) When low N yeast is placed in a medium containing ammonia but no carbon source, it rapidly incoporates N and synthesizes new protein. During the initial part of this process there is no increase in polynucleotide purine content but there is a very rapid turnover as indicated by the appearance of N^{15} in the purines. Thus the data are compatible with the concept that turnover of polynucleotide is associated with the processes of ammonia utilization in yeast.

SUMMARY

By using N^{15} as a tracer the assimilation of ammonia by the yeast, *Torulopsis utilis*, has been studied. It has been shown that:

1. There was no measurable incorporation of N in the protein or polynucleotide purine of carbon-starved yeast.

2. When ammonia is added to nitrogen-starved yeast there is a long lag period before division begins during which the yeast rapidly synthesizes pro-

tein, this process being accompanied by a turnover of polynucleotide purine. There was no significant dilution of the $N^{15}H_4^+$ of the medium by ordinary NH_4^+.

3. When yeast containing N^{15} is allowed to divide and grow in ordinary ammonia, the total amount of N^{15} in the yeast remains constant. The dicarboxylic amino acids are most diluted, while arginine and nucleic acid guanine are not diluted at all.

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